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REVIEW

IMPORTANT BIOACTIVE PROPERTIES OF OMEGA-3 FATTY ACIDS

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ABSTRACT

Good health has been linked with healthy diet. N-3 fatty acids are required for proper functioning of many physiological systems. There is a large body of evidence documenting the effects of polyunsaturated fatty acids with the first double bond at the third position from methyl-terminal on health benefits. Scientific evidence is accumulating to substantiate the role omega-3 fatty acids play in conditions such as cardiovascular disease, certain cancers and other diseases. The availability of n-3 fatty acids to various tissues is of major importance to health and depends on dietary intake for both normal development and in the prevention and management of chronic diseases. In this review we will summarize the biological properties of omega-3 fatty acids.

- Keywords: omega-3 fatty acids, polyunsaturated fatty acid, biological properties, health -

INTRODUCTION

Long-chain polyunsaturated fatty acid (PUFA) with the first double bond at the third position from the methyl-terminal (so called omega-3 fatty acids or n-3 fatty acids) can be found in plants and fish. N-3 fatty acids refer to a group of three fats called alpha-linolenic acid (ALA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Because these essential fatty acids cannot be synthesized in the human body, they must be derived from dietary sources. They are required for the structure of cell membranes and, because they are unsaturated, they help keep membranes flexible. The peculiar properties of the DHA molecule make it a critical component of nerve and retinal cells. Flaxseed, hemp, canola and walnuts are generally rich sources of the ALA. Fish provide varying amounts of n-3 fatty acids in the form of DHA and EPA. The n-3 PUFA are obtained predominately from fish, seafood, meat and eggs (MEYER *et al.*, 2003) and in recent years from enriched food products such as bread, milk and margarine. The role played by essential fatty acids in the human body has been the subject of volumes of international research in recent years. The results indicate that n-3 fatty acids may be of value in the treatment of various medical conditions (UAUY and VALENZUELA, 2000). Research has been carried out in animal models, tissue cultures and human beings. A present study was to produce pork with enhanced nutritive value for humans, in terms of fatty acid profile. When fish oil was included in the diet, higher levels of EPA, DPA and DHA were measured in the subcutaneous fat (MOREL *et al.*, 2013). The positive effects of essential fatty acids are attributed to their ability to reduce inflammation (SIMOPOULOS, 2002). Many studies show that fish oil with high content of n-3 polyunsaturated fatty acids (PUFAs) plays an important role in human health and disease. But the effects of fish oil with high content of PUFAs on gut microbiota, which are also known play a significant role in several human diseases, is not clear. YU *et al.* (2014) evaluated the effects of fish oil with high content of n-3 PUFAs on gut microbiota. They found that fish oil treatment resulted in a decrease in *Helicobacter, Uncultured bacterium clone WD2_aaf07d12* (GenBank: EU511712.1), *Clostridiales bacterium, Sphingomonadales bacterium and Pseudomonas species Firmicutes*, and several uncultured bacteria. Fish oil with a high content of n-3 PUFAs are capable of producing significant changes in the gut microbiota that may, at least in part, explain the health benefits or injury induced by fish oil use.

In recent years, the number of studies describing the health-promoting benefits of n-3 fatty acids has increased. Some of the reported activities attributed to the n-3 fatty acids include improving serum cholesterol profiles, stabilizing arrhythmias, reducing inflammation, improving insulin sensitivity in patients with Type 2 diabetes, and enhancing the immune response. In this paper, we will discuss diverse effects of an essential component of a healthy diet, long chain n-3 PUFA. Because modern diets are relatively deficient in this special type of fat, there is a great potential for improving many aspects of health by adding it to the diet.

Cardiovascular disease

Cardiovascular disease (CVD) includes all diseases of the blood vessels and circulatory system such as coronary heart disease (CHD), ischemic heart disease (IHD), myocardial infarction (MI) and stroke. CVD is the leading cause of death in Canada and the United States. Since the original epidemiological observations of low CVD mortality in populations with high consumption of fish there has been continuous interest of scientific communities in the possibility of lowering CVD risk by n-3 fatty acids. Both EPA and DHA play a role in modification of lipid and lipoprotein metabolism. N-3 fatty acids have synergistic and additive effects on plasma lipids when co-administered with statins (DAVIDSON *et al.*, 2007). Increased triglyceride levels are seen among individuals with HIV who receive antiretroviral therapy. A study reported the results of a research that examined the effects of a controlled dietary study supplemented with 4 g of fish oil daily to reduce triglyceride levels in HIV (CAPILI and ANASTASI, 2013). Another practical implication of such studies is the proven safety of n-3 fatty acids add on therapy to statins. Both DHA and EPA have profound effect on platelet function. Not only membrane stabilizing effect but also competition of n-3 fatty acids for cyklooxygenase activity with arachidonic acid, which lowers production of platelet activating eicosanoids play an important role (LARSON *et al.*, 2008). In a study in diabetic patients Woodman and co-workers demonstrated administration of highly purified EPA/DHA was associated with a decrease in platelet aggregability by 30 % (WOODMAN *et al.*, 2003). The platelet effects seem to be mediated mostly by EPA (DIN *et al.*, 2008). OLIVEIRA *et al.*(2014) suggested that fish oil upregulate the expression of the cholesteryl ester transfer protein (cetp) gene.

Another possibly cardioprotective action of n-3 fatty acids can be modulation of immune response and anti-inflammatory properties. As demonstrated *in vitro*, DHA lowers cytoadhesive molecules expression on endothelial cells and monocytes (MORI and BEILIN, 2004). Similarly, levels of interleukin 6, interleukin 1β and tissue necrosis factor α decrease after EPA/DHA administration (BHATNAGAR and DURRINGTON, 2003). Consumption of n-3 fatty acids of 8 g/d

was associated with a significant reduction of inflammatory marker levels in patients with severe heart failure (MEHRA *et al.*, 2006).

Some studies have shown that n-3 fatty acids may increase the susceptibility of LDL to oxidation (SORENSEN *et al.*, 1998), whereas others have not (HIGDON *et al.*, 2001). It therefore remains to be established whether LDL oxidative status in vivo is affected by omega-3 fatty acids and, if so, whether this has any adverse clinical implications

Cancer

Cancer is another disease that has generated great interest in evaluating the usefulness of n-3 fatty acids. Cancer and its treatments are associated with significant long-term side effects such as cachexia, cognitive impairment, distress, pain and fatigue that all warrant supportive care. Many experimental studies have shown the role played by n-3 fatty acids in suppressing the development of most cancer processes, including breast, colon, prostate, liver and pancreatic cancers (SIMOPOULOS, 2009). In addition, there is evidence that EPA and DHA exert a potent antiangiogenic effect, inhibiting the production of some of the main angiogenic mediators (SPENCER *et al.*, 2009). This explains the great interest in establishing fatty acid ingestion in adequate proportions.

EPA has been suggested to play a protective role in hormone-related cancers, particularly breast and prostate cancers. In animal experiments, EPA and DHA have consistently inhibited the proliferation of malignant breast and prostate cancers; however, epidemiological studies examining the role of n-3 fatty acids in cancer have not been consistent (TERRY *et al.*, 2003). Fish oils can have a benefit in reversing cancer-related cachexia by decreasing the protein degradation in cachectic muscle (TISDALE, 2003), suggesting that there may be a potential place for n-3 fatty acids in cancer therapy as well as in prevention. XUE *et al.* (2014) suggested that DHA exerted its anticancer activity through down-regulation of Wnt/β-catenin signaling. Thus, it should call for further studies to assess the effectiveness of fish oil as a dietary supplement in the prevention and treatment of breast cancer.

In the last decade, many clinical trials examining the effects of n-3 fatty acids supplementation on cancer cachexia have been conducted (RIES *et al.*, 2012). Some randomized controlled trials confirmed these results, especially at dosages of 1.5–2 g EPA-enriched enteral liquid formula administrated for at least 8 weeks in gastrointestinal or pancreatic cancers (RYAN *et al.*, 2009). Clinical studies are emerging to support providing long chain n-3 fatty acids to prevent muscle loss, minimize side effects and improve chemotherapy response in patients with cancer (MURPHY et al., 2013). A research suggests that marine n-3 fatty acid may increase appetite (SIGNE *et al.*, 2013). This finding would be potentially beneficial for patients with compromised nutritional status.

Bone

The n-3 and n-6 polyunsaturated fatty acids are the immediate precursors to a number of important mediators of immunity, inflammation and bone function, with products of n-6 generally thought to promote inflammation and favour bone resorption. Western diets generally provide a 10 to 20-fold deficit in n-3 PUFAs compared with n-6, and this is thought to have contributed to the marked rise in incidence of disorders of modern human societies, such as heart disease and osteoporosis. Osteoporosis is a disease in which bone mass is low and the risk of bone fractures is high. Regarding bone diseases, studies in animals have shown that the ingestion of n-3 fatty acids could influence bone formation and resorption (POULSEN, 2007). ALA may help prevent bone loss and osteoporosis by blocking the production of tumor necrosis factor α, which promotes bone resorption and inhibits bone formation (BOYCE *et al.*, 2005). When bone metabolism was measured in the volunteers, the high-ALA diet reduced bone resorption without reducing bone formation (ZHAO *et al.*, 2007). The decrease in bone resorption may have been due to a decrease in the dietary n-6/n-3 ratio as a result of the high-ALA diet. TARLTON *et al.* (2013) found that there was a significant 40–60% reduction in keel bone breakage rate, and a corresponding reduction in breakage severity in the n-3 supplemented hens. The biomechanical and biochemical evidence suggests that increased bone turnover has enhanced the bone mechanical properties, and that this may suggest potential benefits for human osteoporosis.

However, some studies found no effect of n-3 fatty acids consumption on measures of bone formation and resorption among postmenopausal women (DODIN *et al.*, 2005; BROOKS *et al.*, 2004). These results suggest that the benefit of n-3 fatty acids on bone metabolism is not sufficient to overcome the bone remodeling that occurs during menopause.

Emotional distress

Most newly diagnosed and recurrent cancer patients presented with a significant level of mental distress. Commonly, distress involves anxiety and depression. A study reported that the antidepressant protective role of n-3 fatty acids might be exerted through production of eicosanoids that are able to reduce the excessive pro-inflammatory cytokine production in depressed patients (KIECOLT-GLASER *et al.*, 2007). Animal studies in which rats were fed an n-3 fatty acids rich diet indicated

a better habituation to chronic restraint stress as they showed less stress-induced weight loss, compared to both control and n-3 deficient rats (HENNEBELLE *et al.*, 2012). Several clinical trials addressing non-cancer populations have also suggested beneficial effects of n-3 fatty acids on anxiety and depression (LUCAS *et al.*, 2009; ARBABI *et al.*, 2014). However, no trials were found exploring the potential benefits of plant-derived EPA. Similar positive results were found by TAJALIZADEKHOOB *et al.* (2011) in elderly patients. Although the potential benefits of n-3 fatty acids on emotional distress per se have not been examined as a primary endpoint in cancer patients, one trial did study depressive symptoms in lung cancer patients. The trial reported an inverse association between n-3 fatty acids intake, as well as serum n-3 fatty acids and minor depression (KOBAYAKAWA *et al.*, 2005). Though controversy exists as to whether EPA, DHA or both are responsible for the efficacy of n-3 fatty acids in depression, results from a randomized controlled trials suggested EPA to be a more effective fatty acid component in the treatment of mild to moderate depression (MOZAFFARI-KHOSRAVI *et al.*, 2013).

There is also evidence that the pathophysiology of major depression is influenced by changes in fatty acid intake. In 2009, DINAN *et al.* evaluated the levels of arachidonic acid, IL-6 and TNF α in depressed responders and non-responders to antidepressive treatment. There were significant differences in the EPA and arachidonic acid ratio between controls and responders versus non-responders. One research reinforces these observations in major depression and bipolar disorder, and low DHA levels may even predict suicidal behaviour (MCNAMARA *et al.*, 2008). It has been shown that DHA content in brain tissue is decreased in patients with neuronal alterations, as in Alzheimer's disease.

Obesity

Obesity, a chronic low-grade inflammatory condition, is considered to be a metabolic disorder, whose prevalence is increasing dramatically in most developed countries over the last 20 years. Obesity is associated with an increased risk of CVD, type 2 diabetes and a number of cancers. One study suggests that at baseline men with high fish consumption were less likely to be overweight, however no data about association of n-3 fatty acids intake and changes in BMI (body mass index) were provided in this 12-year follow-up cohort (HE *et al.*, 2002). The effects of a combination of n-3 fatty acids and a dietary energy restriction in obese volunteers were also examined. PARRA *et al.* (2008) showed that satiety was increased after consumption of the n-3 fatty acids-enriched meals. Researches have demonstrated that diabetes induces learning and memory deficits (JIA *et al.*, 2014). The results suggested that the principle mechanisms involved in the antidiabetic and neuroprotective effect of fish oil were its antioxidant, anti-inflammatory and anti-apoptosis potential, supporting a potential role for fish oil as an adjuvant therapy for the prevention and treatment of diabetic complications. Dietary fish oil showed better tissue preservation that was supported by histopathological observations (JANGALE *et al.*, 2013). Thus, the diet proved to be beneficial in preventing tissue injury and alleviating diabetic insults in the livers of diabetic rats. A recent research explored insulin signaling in the newborn rat heart. A diet rich in fish oil improves cardiac Akt-related signaling in the offspring of diabetic rats (NASU-KAWAHARADA *et al.*, 2013).

HILL *et al.* (2007) found that exercise and n-3 fatty acids supplementation resulted in a significant reduction of body fat. However, MUNRO and GARG (2012) described that dietary supplementation with n-3 fatty acids did not promote additional weight loss when combined with a very-low-energy diet for 4 weeks. The most possible cause for the disparities in these results may be related to phenotypical characteristics of the subjects included in the study. Indeed, mechanisms underlying this differential response in body weight in obese humans remain a challenging point still to be addressed in future. It should be stated that the effects of n-3 fatty acids on fat mass and weight regulation might be difficult to address due to important differences in how the studies were designed as well as the inclusion criteria and source for n-3 fatty acids supplementation. Therefore, effectiveness of the n-3 fatty acids supplementation might be related to dietary and exercise patterns and gender aspects might also be relevant.

However, the effects of these fatty acids on insulin sensitivity remain controversial (KABIR *et al.*, 2007; NAVAS-CARRETERO *et al.*, 2009; HIRABARA *et aal.*, 2013), the fact is that insulin resistance is usually linked to other pathological conditions such as hypertrigliceridemia, overweight and cardiovascular diseases and might be difficult to study on itself. Thus, further studies are needed to evaluate this aspect of n-3 fatty acids in insulinemia management.

Nutritional recommendations for the consumption of n-3 fatty acids

As we have seen, there are a number of pathologies in which the n-3 fatty acids play an important role, thus reflecting the importance of ensuring their adequate dietary intake. Recommendations for dietary intakes of n-3 PUFA vary considerably from the consumption of two fish meals a week to EPA plus DHA intakes of 500 mg/d (KRIS-ETHERTON *et al.*, 2002) and the Japanese recommend consumption of n-3 PUFA of 1.6 g/d (SUGANO, 1996). An approximate estimation of the consumption of n-3 fatty acids

in Europe is 0.1-0.5 g/d. These datas are high in comparison to the estimated intake of DHA and EPA in the United States (0.1-0.2 g/d), but low in comparison with the data corresponding to Japan (up to 2 g/d), where fish is one of the most commonly consumed foods (CARRERO *et al.*, 2005). In Spain, a study carried out by the Ministry of Agriculture, Fisheries and Food, showed that the fact that the Spanish population consumes levels of n-3 close to the recommended level (1.52 g/d). However, the percentage of energy contributed by EPA+DHA with respect to total energy in the diet was lower than the recommended value (0.5%). ALA, the precursor of n-3 fatty acids, can be converted to long-chain n-3 PUFA. The minimum intake of n-3 PUFA needed for beneficial effects depends on the intake of other fatty acids. Dietary amounts of linoleic acid (LA) as well as the ratio of LA to ALA appear to be important for the metabolism of ALA to n-3 PUFA. Indu and GHAFOORUNISSA (1992) showed that a ratio of 4 is appropriate for conversion. This ratio is also consistent with a study by DE LORGERIL *et al.* (1994).

On comparing the recommendations for n-3 consumption of the different organizations and the existing consumption data, the results show that the consumption of n-3 fatty acids is generally low. The recommendations of the American Heart Association (AHA) are that adults should consume fish at least twice a week. Likewise, patients with coronary disease should consume 1 g of EPA+DHA daily; while patients with hypertriglyceridemia should consume 2-4 g/d of EPA+DHA (BAGGA *et al.*, 2002). Taking the above into account, the recommended amounts must be adjusted according to the specific needs for the different diseases, and other important dietary factors. In a sense, an increase in n-3 fatty acids in the diet may be regarded as important. From the above we can deduce that a considerable increase in fish consumption is required.

Maternal levels of n-3 fatty acids during pregnancy determine the levels present in the developing infant. The n-3 fatty acids DHA is critical in supporting infant growth and development, and DHA levels in newborns are correlated with birth weight and head circumference. It has been suggested that women and their infants may benefit if the mother is supplemented with DHA during pregnancy. Breast milk contains about 0.5-2.0% ALA and about 0.1-0.4% DHA (INNIS, 2000). ALA constitutes 75-80% of the total n-3 fatty acids in breast milk (SILVA *et al.*, 2005), supporting a role for ALA in the growth and development of infants.

The diets of Western countries have contained increasingly larger amounts of LA. Essential fatty acids also have antibacterial actions and are found in breast milk (Das, 2006). Indeed, breast milk is rich in LA and contains more of ALA than of any other omega-3 fatty acid (SILVA *et al.*, 2005).

CONCLUSIONS

Our message is that omega-3 fats can contribute to a longer and healthier life. Most studies indicate that the consumption of n-3 fatty acids should be more than that presently found in the general population, with a view to improving general health and reducing the risk of disease. However, further studies are needed to confirm and consolidate this idea. The future study offers the opportunity to clarify the underlying molecular mechanisms and elucidate the observed differences between different ethnic groups and genders in developing population-specific dietary recommendations.

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PAPER

WINEGRAPE BERRY SKIN THICKNESS DETERMINATION: COMPARISON BETWEEN HISTOLOGICAL OBSERVATION AND TEXTURE ANALYSIS DETERMINATION

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ABSTRACT

We analyzed the relation between the assessment of grape berry skin thickness by means of histology sections and instrumental mechanical properties measurements. Berry skin of *Vitis vinifera* L. cultivar Corvina vineyards from Valpolicella Valpantena zone (Verona, Italy) were tested, evidencing a strong correlation between the two thickness determination methods. The middle or equatorial berry skin portion was found to be the less variable in instrumental skin thickness determination. In addition, unlike other studies, no correlation between the skin thickness and cell layers number was found.

- Keywords: grape skin, cell layers, thickness, histology, mechanical properties -

INTRODUCTION

The skin or exocarp forms the grape's dermal system: depending on thickness and berry size, it accounts for between 5 and 18 % of the fresh weight of ripe berries (OJEDA *et al.*, 2002). The skin is composed of epidermis, covered with a waxy cuticle, and a underlying outer hypodermis (CONSIDINE and KNOX, 1979).

During grape berry development from fecundation to ripening the skin thickness varies consistently (COOMBE and McCARTHY, 2000). Immediately after the fruit set until the véraison, when berry weight increases, epidermis and hypodermis cells expands in the tangential direction, increasing their area by 15% and 33%, respectively. After the *véraison*, the mesocarp cells continue their expansion, and the hypodermal and epidermal cells lose size, acting inversely in comparison with mesocarp cells (SCHLOSSER *et al.*, 2008). Thus, the skin become thinner in the berry during ripening: the relative thickness of the skin decreases from one-eighth to one-hundredth of the total berry diameter between fruit set and maturity stages (KELLER, 2010).

The skin thickness is one of the most important grape skin morphological characteristics affecting the gas exchange regulation, the berry susceptibility to fungal diseases and the resistance to mechanical injuries (ROSENQUIST and MORRISON, 1989; KÖK and ÇELIK, 2004). The skin thickness varies depending on variety (MUGANU *et al.*, 2011; GIACOSA *et al.*, 2012) and clone (ROLLE *et al.*, 2012a), confirming that this parameter is genetically influenced: this could be useful to further understand some different varietal characteristics, such as the susceptibility to fungal diseases or the aptitude to the post-harvest dehydration process. Furthermore, the skin thickness seems to be related with the environmental conditions: in the alpine area cv. Nebbiolo berries with similar sugar content showed a generally thickest skin than in the hill side (ROLLE *et al.*, 2012b). This highlights that the skin thickness is very sensitive to the climate and the bunch microclimate conditions (PORRO *et al.*, 2008; MUGANU *et al.*, 2011), although a direct relation with water regimes in cv. Muscat blanc in open field conditions was not found (GIORDANO *et al.*, 2013).

Since epidermis and hypodermis cells contain chloroplasts and phenolic-rich vacuoles (KELLER, 2010), skin berry properties (break force and thickness) can aid in the assessment of the phenolic content during the ripening. In particular, the skin thickness represents a useful indicator to predict anthocyanin extractability, and thinner skins seems to be characterized by higher anthocyanin extractability (RÍO SEGADE *et al.*, 2011a). So, thickness can be useful to support the choice of the harvest data and to rationalize maceration and winemaking processes, thus allowing winemakers to best exploit the grape potential reached in the vineyard. Berry skin thickness assessment can be obtained with histological observation or instrumental methods, i.e. texture analysis (LETAIEF *et al.*, 2008a; ROLLE *et al.*, 2012c). The instrumental skin thickness measurement permits to minimize the sample treatment without using reagents or special procedures, speeding up the analysis process.

The aim of this study was to compare the two cited skin thickness measurement methods (histology and texture analysis) among several vineyards, in order to assess differences between the two techniques and also to investigate the relationship between the thickness and the cell layer number of the analyzed samples. A preliminary test on the influence of the sampling berry skin portion on the instrumental skin thickness determination was also carried out.

MATERIALS AND METHODS

Grape Samples

Grapes were collected from four vineyards located in the "Valpolicella Valpantena" denomination of origin, just to the north of Verona, Italy (45°29'22"N, 11°0'49"E). The vineyards were fifteen years old, planted with Corvina (clone ISV-13) grafted on Kober 5BB. The vines were trained with simple Guyot and the rows were oriented North to South. The number of the vineyards analyzed was considered to be sufficient for this kind of study, following previous studies involving berry skin thickness variation analysis which considered from 3 to 7 vineyards (RÍO SEGADE *et al.* 2011b; ROLLE *et al.*, 2012b). Each vineyard was analyzed in duplicate (two subsamples) using this random sampling schema: each subsample was obtained by sampling fifteen bunches (one per chosen grapevine). From each bunch, twenty intact berries were selected, then the 300 resulting berries were used for the following analysis.

Histology

For the histological characterization, ten berries from each subsample were randomly chosen, and the protocol by BOZZOLA and RUSSELL (1998) for histological observation followed. A berry section was cut from each berry and immediately fixed in 2.5% glutaraldehyde (Ted Pella Inc., Redding, CA, USA) diluted with 0.1 mol/L sodium cacodylate buffer solution at pH 7.4 overnight at 4 °C. Then, they were incubated for 1 hour at 4 °C in osmium tetroxide 1 % in 0.1 mol/L sodium cacodylate buffer, and then water washed three times. After that, the samples were dehydrated in a graded ethanol series and embedded in an epoxy resin (Sigma-Aldrich, St. Louis, MO, USA). Semithin sections (1 μm) were obtained with an Ultrotome V (LKB)

ultramicrotome, counterstained with toluidine blue 1 % and observed with a Leica DMR optical microscope equipped with a camera (Leica DFC 480). Measurements of skin thickness and cell skin number were made using an imaging software (ImageJ 1.38; Wayne Rasband; National Institutes of Health, USA) considering the Epidermis and Hypodermis.

Instrumental skin thickness

A TA.XTplus Universal Testing Machine (Stable Micro Systems, Godalming, UK) was used, operating in the following conditions (LETAIEF *et al.*, 2008a): 5 kg load cell, P/2 2-mm cylindrical flat probe, HDP/90 platform, test speed 0.2 mm/s, data rate 500 points per second, data acquisition and integration using the Exponent software from the same manufacturer. All the analysis were done at 20±2 °C.

The probe was calibrated by force and distance before each session, the latter to define the starting point 1 mm above the platform. A pulp-free clean portion of the peeled skin sample was then placed on the HDP/90 platform base, letting it adhere on the platform surface, and a 0.2 mm/s compression movement was applied by the probe. The berry skin thickness (Sp_{sk}) instrumental parameter was defined as the distance (in μm) between the point when the probe touched the skin sample and the platform base. In correctly defining the touch point, it was necessary to include a 0.05 N instrumental trigger to avoid the "tail" effect (LETAIEF *et al.*, 2008a), as described in Fig. 1.

In order to assess the variability of the instrumental measurements influenced by the analysis position and intra-berry, a preliminary test was carried out. Ten berries were randomly taken from all the previously-formed subsamples: these berries were analyzed in fifteen different spots each, equally distributed in the top (close to the peduncle), equatorial (middle), and bottom side of the berry. Instrumental berry skin thickness (Sp_{sk}) values were then calculated, the results normalized based on the equatorial position, and the relative standard deviation (%RSD) calculated for the three spots and for the intra-berry measurements variation in the same spot.

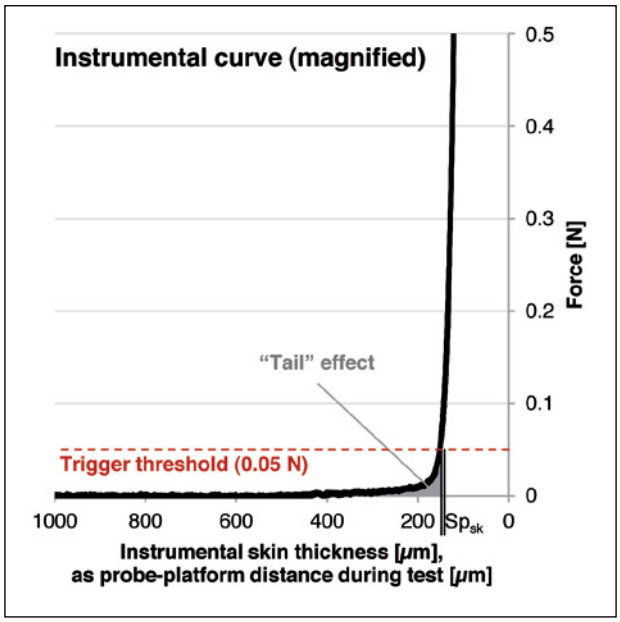


Fig. 1 - Typical force-distance curve of berry skin thickness mechanical test, magnified in the y-axis in order to highlight the "tail" effect influence and the need of a trigger threshold.

For the instrumental skin thickness determination, ten berries from each subsample were randomly chosen. The berries were singularly treated, they were peeled and a skin portion from the equatorial berry position analyzed using the aforementioned method, with the thickness calculated as Sp_{sk} (μm).

Statistical analysis

Statistical analysis was performed using the statistical software package IBM SPSS Statistics (IBM Corporation, Armonk, NY, US). The Tukey-b test at $p < 0.05$ was used in order to establish statistical differences by one-way analysis of variance (ANOVA).

RESULTS AND CONCLUSIONS

The instrumental skin thickness preliminary test results were shown in Table 1. Given 100 the equatorial position skin thickness average,

Table 1 - Comparison between instrumental mechanical skin thickness evaluation on top, equatorial and bottom positions of the berry, and mean variation of intra-berry measurements.

Berry analysis position	Normalized Sp_{sk} [a]	%RSD	Average intra-berry %RSD
Top	78.3[a]	20.1	14.84
Equatorial	100.0[b]	9.9	9.09
Bottom	102.3[b]	17.2	14.07

Instrumental berry skin thickness (Sp_{sk}) data is expressed as normalized result ($n = 50$) with respect to the equatorial side (given as 100). For each measurement the relative standard deviation (%RSD) is reported.
Intra-berry %RSD calculated as average of the ten %RSD values found analyzing each berry in 5 different spots.
[a]Sp_{sk} normalized result values are significantly different at $p < 0.001$. Different letters in Sp_{sk} normalized results mean significance at $p < 0.05$ (Tukey-b test) among berry analysis position.

the other measures were normalized accordingly, and the relative standard deviation (%RSD) calculated. The bottom section gave similar results compared to the equatorial one, however the relative standard deviation measured is about 75 % higher than that of the latter position. The berry top skin section (close to the peduncle) showed lower Sp_{sk} values in relation to the other two sections considered, but the higher relative standard deviation of the group.

Regarding the intra-berry variation, the equatorial position was found the less variable, that means several measurements of the skin thickness on the same berry gave the more similar results when done on the equatorial position, with respect on measurements done only on the top position, or only on the bottom position.

The difference in the berry skin mechanical behavior induced by the analysis position was also found by LETAIEF *et al.* (2008b), which found significantly different berry skin break force and energy values depending on the puncture position when testing berries of Cabernet sauvignon, Pinot noir and Nebbiolo cultivars. The berry skin break force and energy values in the top position (labeled A3) were found the lower ones in most cases, as found for the instrumental thickness parameter in the present study. This can lead to the hypothesis of a link between these skins mechanical parameters, however no evidence of a meaningful correlation between skin break force and thickness analyzed on the same position (berry lateral side) was found in a previous study conducted on grapes from several cv. Mencía vineyards (RÍO SEGADE *et al.*, 2011a).

Fig. 2 shows a picture of the berry skin section taken with the optical microscope: in the picture there were reported the different tissues that form the skin, Epidermis and Hypodermis. The outermost two-three cell layers were considered to be the Epidermis, while the seven to nine cell layers immediately below the Epidermis were considered to be the Hypodermis (HARDIE *et al.*, 2008). Immediately below to these layers there were polygonal shape cells that were considered to be the Mesocarp (SCHLOSSER *et al.*, 2008).

The data collected with the histological method is reported in Table 2. The measured skin cell layers number ranged between 8 to 11, with the average vineyard values resulted from 8.87 to 9.65, in agreement with the values reported for other varieties (CONSIDINE and KNOX, 1979; MUGANU *et al.*, 2011). The cell layers number showed significant differences between vineyards: A and C showed smaller values (8.87 and 8.95 respectively) compared with B (9.65), while D showed an intermediate average cell number (9.23). Also the skin thickness measured from the histological samples showed significant differences between vineyards. The vineyard A showed the thickest skin (173 μm) while the vineyard D showed the thinnest one (152 μm), with B

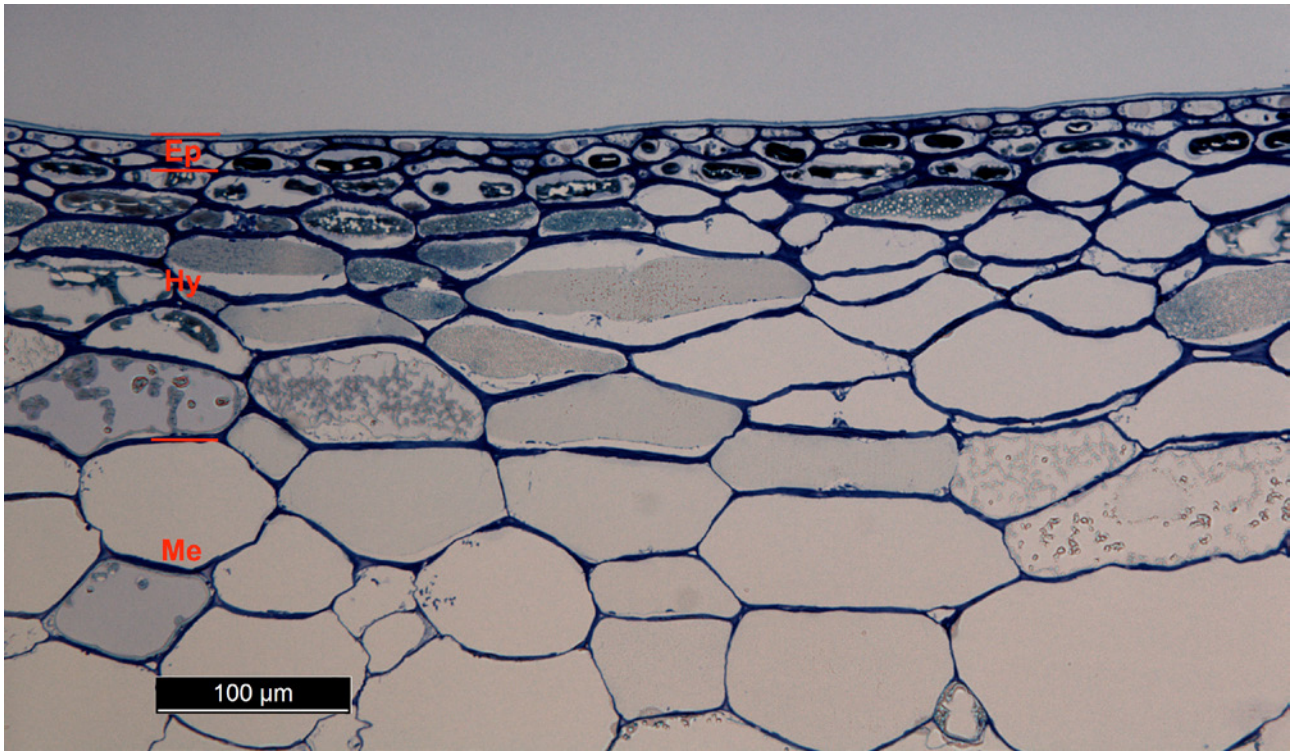


Fig. 2 - Picture of traverse section of Corvina berry skin at maturity taken with Leica DMR optical microscope. In the picture there are indicated the Epidermis (Ep), Hypodermis (Hy), and Mesocarp (Me).

Table 2 - Skin cell layers number and thickness evaluated by histology, and skin thickness by instrumental mechanical properties technique, of the analyzed vineyards (two groups per vineyard).

Vineyard	Cell layers number [by histology]	Thickness [by histology, µm]	Thickness [mechanical as Sp_{sk}, µm]	*Sign*[b]
A	8.87±0.69[a]	173±5[c]	176±8[c]	ns
B	9.65±0.65[b]	163±5[b]	164±8[b]	ns
C	8.95±0.72[a]	161±5[b]	160±7[b]	ns
D	9.23±0.92[ab]	152±5[a]	153±5[a]	ns
Sign[a]	**	***	***	

Data is expressed as average±standard deviation (n = 20). Different letters means significance at $p < 0.05$ (Tukey-b test) among vineyards.

[a]: ** and *** means significance among vineyards at $p < 0.01$ and 0.001, respectively.

[b]: ns means not significant differences between thickness determinations (in a same vineyard sample).

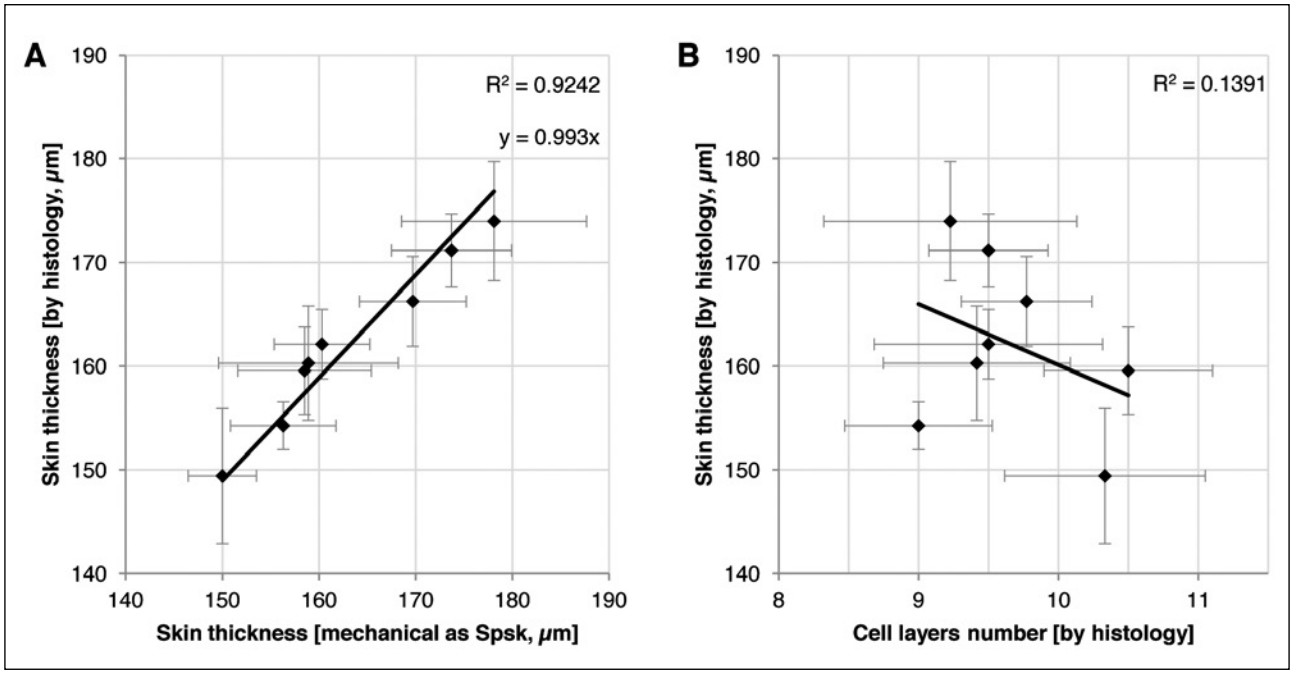


Fig. 3 - Correlation between skin thickness measured by means of instrumental mechanical properties technique and histology, and between histological cell layers number and skin thickness. Each point represents a berry group (n = 10, two groups per vineyard).

and C showing intermediate values (163 and 161 µm, respectively). The skin thickness estimated with the texture analysis equipment (as Sp_{sk}) showed similar values with respect to those measured on the histological sections. Indeed, there was not statistical difference between the values measured using the two different techniques. This was confirmed by the high correlation (R^2 = 0.9242) found between the values recorded with the two methods, as shown in the correlation graph in Fig. 3A.

The obtained values highlighted that there was no correlation (R^2 = 0.1391) between the skin thickness and the cell layers number, both analyzed by histology (Fig. 3B). This means that the different skin thickness recorded between cv. Corvina vineyards was due essentially to the different Epidermis and Hypodermis cells size. Some authors reported that the skin thickness is strictly dependent on the different number of cell layers (CONSIDINE and KNOX, 1979; ROUDOT, 2006; HARDIE *et al.*, 2008; MUGANU *et al.*, 2011) but they based their observation on different grape varieties with respect to the present study.

We can conclude that the comparison between histological observation and texture analysis determination confirmed that the instrumental skin thickness technique by texture analysis give similar values of skin thickness in relation to those obtained by histology. The use of the texture analysis method can speed up the analysis process, minimizing the sample treatment.

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PAPER

METABOLOMICS STUDY OF CEREAL GRAINS REVEALS THE DISCRIMINATIVE METABOLIC MARKERS ASSOCIATED WITH ANATOMICAL COMPARTMENTS

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ABSTRACT

This study used NMR-based metabolomics to compare the metabolic profile of different anatomical compartments of cereal grains i.e. bran and endosperm in order to gain further insights into their possible role in the beneficial health effects of whole grain products (WG). Polar water-soluble metabolites in 64 bran and endosperm, samples from rye and wheat were observed using 600 MHz NMR. Bran samples had higher contents of 12 metabolites than endosperm samples. A comparative approach revealed higher contents of azelaic acid and sebacic acid in bran than in endosperm. In a pilot study, the consumption of WG rye bread (485 g) caused NMR signals in 24h urine corresponding to azelaic acid. The relatively high abundance, anatomical specificity, pattern of metabolism, urinary excretion in human, antibacterial, and anticancer activities suggest further studying of azelaic acid when exposure to WG or beneficial effects of WG are investigated.

- Keywords: Metabolomics, NMR, wholegrain, rye, wheat, metabolites, biomarkers -

INTRODUCTION

Epidemiological studies have consistently shown that intake of whole grain (WG) can protect against the development of chronic diseases (SLAVIN *et al.* 2001), e.g. type 2 diabetes (T2D) (DE MUNTER *et al.* 2007, MURTAUGH *et al.* 2003), cardiovascular disease (CVD) (FLINT *et al.* 2009; JACOBS *et al.* 2007; MELLEN *et al.* 2008), and certain cancers (CHAN *et al.* 2007, HAAS *et al.* 2009; LARSSON *et al.* 2005; SCHATZKIN *et al.* 2008). The American Association of Cereal Chemists provided the following scientific and botanical definition of WG in 1999: "whole grain shall consist of the intact, ground, cracked or flaked caryopsis, whose principal anatomical component-the starchy endosperm, germ and bran-are present in the same relative proportion as they exist in the intact caryopsis" (International 1999). Whole grains are a rich source of fiber and bioactive compounds, including tocopherols, B vitamins, minerals, phenolic acids, and phytoestrogens (FARDET, 2010). It is generally recognized that the synergistic action of compounds mainly present in the bran and germ fractions of cereals accounts for the protective effects of WG products (FARDET, 2010; LIU, 2007). Recently, the composition and the diversity of bioactive compounds in different anatomical components of cereal grains have been systematically investigated in a large number of different species and varieties within the HEALTHGRAIN project (NYSTROM *et al.* 2008; SHEWRY *et al.* 2010; WARD *et al.* 2008). However, that project screened the cereal samples for bioactive compounds already documented in cereals using a targeted approach, and made no comparison of the untagged profile of the metabolites in different compartments of cereal grains.

Metabolomics is an untargeted approach in which the profile of metabolites in a biospecimen is measured using high-throughput analytical methods, e.g. NMR and mass spectrometry (LENZ and WILSON, 2007; NICHOLSON and WILSON 2003). We have used this approach previously to examine the complex physiological/biochemical effects of WG rye products in humans (MOAZZAMI *et al.* 2012; MOAZZAMI *et al.* 2014; MOAZZAMI *et al.* 2011). The aim of the present study was to search for the discriminative metabolites in the two major anatomical compartments in cereal grain, endosperm and bran, using an untargeted NMR-based metabolomics approach and with the emphasis on wheat and rye to gain further insights into their possible role in the beneficial health effects of whole grain products. NMR analysis can potentially provide characteristic structural data, which can be used for elucidation and eventual identification of unknown compounds found to discriminate between the metabolic profiles of bran and endosperm in cereals.

MATERIALS AND METHODS

Serial sample collection and extraction

A total of 64 cereal samples, comprising 18 wheat endosperm, 24 wheat bran, 8 rye endosperm, and 14 rye bran were obtained from the HEALTHGRAIN (WARD *et al.* 2008) project or from a local market. The endosperm and bran samples originated from HEALTHGRAIN projects were from the same grain sample material and therefore were matched (Wheat samples n = 18; and rye samples n = 8). The HEALTHGRAIN project rye varieties (and populations) included potugaise-3, potugaise-6, Haute Loire, Grandrieu, Nikita, Rekrut, Dankowskie-Zlote, and Lovaszpatonai-1. The details about rye varieties are given in NYSTROM *et al.* (2008). The HEALTHGRAIN project wheat varieties included Disponent, Herzog, Tommi, Campari, Tremie, San Pastore, Gloria, Spartanka, Avalon, Claire, Malacca, Maris Huntsman, Rialto, Riband, Obriy, CF99105, Chinese-Spring, and Cadenza. The details about wheat varieties are given by SHEWRY *et al.* (2010). All rye and wheat varieties were grown in the field at Martonvasar, Hungary, in 2005. Full details of the site including soil type, mineral composition, and weather condition has been given by SHEWRY *et al.* (2010).

All samples were milled, and 0.5 g milled material was extracted in 5 mL Milli-Q water for 18 h. The samples were then centrifuged (5 min-1500 g), and 2 mL supernatant was extracted, mixed with 8 mL ethanol and centrifuged (15 min-1,500 g) in order to precipitate the soluble viscose polymers. A 5 mL portion of the ethanol supernatant was dried using an evacuated centrifuge (Savant, SVC 100H, Savant Instrument INC, NJ) and dissolved in phosphate buffer (280 μL, 0.25 mol/L, pH 7.0), D2O (40 μL), and sodium-3-(trimethylsilyl)-2,2,3,3-tetradeuteriopropionate solution (TSP, 30 μL, 23.2 mmol/L) (Cambridge Isotope Laboratories, Andover, MA). The mixture was then used for 1H NMR analysis. An internal standard was added to the mixture in order to ensure semi-quantitative measurements of metabolites captured by 1H NMR. For 2D NMR analysis the mixture was freeze-dried and dissolved in D2O before analysis.

Human experiment and the preparation of urine sample for NMR analysis

In a pilot study, a male subject (age 35; BMI = 23.4) consumed refined wheat bread 485 g for 6 days (breakfast 2 portions, lunch 1 portion, dinner 1 portion). On day six, 24-hour urine was collected. On day seven, he substituted the 485 g refined wheat bread with 485 g of whole grain rye bread and the urine was collected for 24 hours. During the seven days of experiment, any other cereal products were avoided. The choice of consuming refined wheat bread *vs* whole grain rye bread was made to

replicate the condition of previous human interventions in which refined wheat bread was used as the control diet (MOAZZAMI *et al.* 2011; MOAZZAMI *et al.* 2012; BONDIA-PONS *et al.* 2013). The refined wheat bread was prepared from commercial refined wheat flour and whole grain rye bread was prepared from commercial whole grain rye flour. The whole trial was repeated twice, in two different times. This study complied with the Helsinki Declaration, as revised in 1983. The urine samples were kept in -80°C freezers before analysis. The urine samples (500 μL) were mixed with phosphate buffer (250 μL, 0.25 M, pH 7.0) containing 5 mmol/L sodium-3-(trimethylsilyl)-2,2,3,3-tetradeuteriopropionate (TSP) (Cambridge Isotope Laboratories, Andover, MA) as an internal standard. Resulting solutions were centrifuged to remove particulate matter. The supernatant was then transferred into 5-mm NMR tubes for 1H NMR analysis. For 2D-NMR analysis, 600 μL of the supernatant was freeze-dried and dissolved in 600 μL D_2O before 2D-NMR analysis.

NMR measurements and the identification of signals

The 1H NMR analyses (cereal extracts and human urine) were performed on a Bruker spectrometer operating at 600 MHz (Karlsruhe, Germany). 1H NMR spectra were obtained using zgesgp pulse sequence (Bruker Spectrospin Ltd.) at 25°C with 128 scans and 65,536 data points over a spectral width of 17942.58 Hz. Acquisition time was 1.82 s and relaxation delay was 4.0 s. The NMR signals which were found discriminating between different anatomical compartments were identified primarily using the NMR Suite 7.1 library (ChenomX Inc, Edmonton, Canada), Human Metabolome Data Base and Biological Magnetic Resonance Data Bank. In the event of multiplicity, the identity was confirmed with 2D NMR. In human experiment, the identity of phytochemical in the urine originating from the cereals in the diet was also confirmed using 2D-NMR. Phase-sensitive TOCSY and COSY with presaturation (2k × 512 experiments) were performed with 32 scans and a spectral width of 7195 Hz for both F1 and F2. The mixing time for TOCSY was 80 ms. HSQC was performed using 32 scans and a spectral width of 7211 Hz and 250002 Hz for proton and carbon, respectively. All cereal extracts and urine samples were reconstituted in D2O before 2D NMR analysis.

The 1H NMR spectra data (cereal extracts) were processed using Bruker Topspin 1.3 software and were Fourier-transformed after multiplication by a line broadening of 0.3 Hz and referenced to TSP at 0.0 ppm. Spectral phase and baseline were corrected manually. Each spectrum was integrated using Amix 3.7.3 (Bruker BioSpin GmbH, Rheinstetten) into 0.01 ppm integral regions (buckets) between 0.5-10 ppm, in which area between 4.60-5.18 ppm containing residual water was removed. Each spectral region was then normalized to the intensity of internal standard (TSP).

Statistical analysis

Principal component analysis (PCA) and orthogonal partial least squares-discriminant analysis (OPLS-DA) were performed using SIMCA-P+ 12.0.1 software (UMETRICS, Umeå, Sweden) after centering and pareto-scaling of the data as previously described (MOAZZAMI *et al.* 2011). The presence of outliers was investigated using PCA-Hotelling T2 Ellipse (95% CI) and the normality of multivariate data was investigated using the normal probability plot of the PCA model. Variable influences on projection (VIP) values of the OPLS-DA model were used to determine the most important discriminative NMR bucket (signals). NMR buckets (signals) with VIP > 1 for which the corresponding jack-knife-based confidence intervals were not close to or including zero were considered discriminative. The significance of OPLS-DA model was tested using cross-validated ANOVA (CV-ANOVA), which assesses the reliability of OPLS models (CV-ANOVA $p<0.05$ means the OPLS-DA model is reliable) (ERIKSSON *et al.* 2008).

The absolute concentrations of metabolites with corresponding NMR signals that were found to be discriminative in OPLS-DA were calculated from the NMR spectra using the NMR Suite 7.1 profiler (ChenomX Inc, Edmonton, Canada) and internal standard after correction for overlapping signals. The absolute concentrations of the discriminative metabolites were further investigated using ANOVA in the case of normal distribution, and the Mann-Whitney test when the distribution was skewed (Anderson-Darling test, $p<0.05$).

RESULTS AND DISCUSSION

PCA model was fitted using NMR spectral data (buckets) obtained for the bran and endosperm extracts. Three outliers were identified and excluded from the data set based on PCA-Hotelling T2 Ellipse (95% CI). The first and the second component explained 67.4% and 18.1% of spectral variation (R2X) respectively (figure not shown). An OPLS-DA model was fitted including three predictive and six orthogonal components. The first, second, and third predictive components explained 61%, 15.2%, and 1.0% of spectral variation respectively (model parameter: R2Y=0.937; Q2Y=0.876; Cross-validated ANOVA p-value = 2.98 × 10-38) (Fig. 1).

The first component in each model basically separated the bran samples obtained from rye

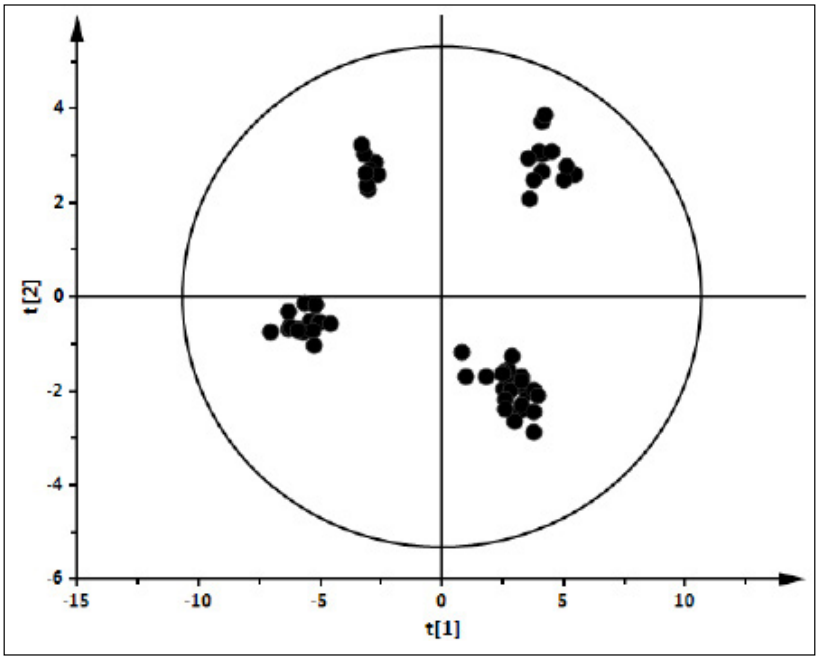


Fig. 1 - OPLS-DA separated different anatomical compartments of cereal grains based on their profile of metabolites measured using NMR. The model parameters were as follow: R2Y=0.937; Q2Y=0.876. Cross-validated ANOVA p-value = 2.98 × 10-38. Score t[1] (component 1) and score t[2] (component 2) are new variables summarizing the X-variables (the intensity of NMR signals corresponding to metabolites).

and wheat from the endosperm samples (Fig. 1, Table 1). The second component separated rye samples (both endosperm and bran samples) from wheat samples (Fig. 1; Table 2). Bran samples contained a higher content of 12 metabolites and four unknown signals than endosperm samples (Table 1), and their contents contributed to composing the first predictive component of the OPLS-DA model. The content of eight metabolites and five unknown signals changed along the second predictive component of the OPLS-DA model (Table 2) separating wheat samples i.e. both anatomical compartments from rye samples. The concentrations of all eight metabolites were found higher in wheat compared with rye. The metabolic signature of wheat and rye samples acquired from the local market did not deviate from those acquired from HEALTHGRAIN project as all sample tightly accumulated in their corresponding species-compartment cluster (Fig. 1). The absolute concentrations of metabolites that were found to differ between different anatomical compartments and species were calculated from NMR spectra and further investigated using ANOVA or the Mann-Whitney test. A total of 12 metabolites were found to differ between different species and different anatomical compartments in the same species, e.g. bran compared *vs* endosperm (Table 3; Fig. 2).

Table 1 - Discriminative metabolites along the first predictive component of the OPLS-DA model (n = 64)[1,2].

Metabolite	NMR signal (ppm)[3]	VIP (Confidence interval)[4]
Azelaic acid & sebacic acid[5]	1.304 ; 1.530 ; 2.179	2.6 (0.67) ; 1.6 (0.41) ; 1.6 (0.40)
Acetate	1.928	1.3 (0.26)
Alanine	1.494	1.1 (0.08)
Betaine	3.269	6.9 (0.59)
Choline	3.206; 4.085	4.4 (0.46); 2.3 (0.24)
Citrate	2.545	1.4 (0.13)
Isoleucine	0.942	1.0 (0.18)
Leucine	0.964	1.4 (0.24)
Malate	2.663	1.1 (0.31)
Maltose	3.280; 3.727; 3.997	1.4 (0.24); 4.4 (0.49)
Succinate	2.412	1.3 (0.16)
Unknown signals[6]	3.778; 4.054; 4.155; 4.020	2.3 (1.07); 2.3 (0.18); 2.2 (0.23); 4.6 (0.32)

[1]OPLS-DA Score scatter plot: the first component separated the bran samples (right) from the endosperm samples (left). All metabolites present in higher concentrations in bran. The model parameters for three predictive component fitted were as follow: R2Y=0.937; Q2Y=0.876. Cross-validated ANOVA p-value = 2.98 × 10-38; [2]Wheat endosperm (n = 18), wheat bran (n = 24), rye endosperm (n = 8), and rye bran (n = 14); [3]One NMR signal from the corresponding spectral bucket with the highest VIP values was reported when several buckets covered a distinct NMR signal; [4]NMR signals with VIP > 1 for which the corresponding jack-knife-based confidence intervals were not close to or including zero were considered discriminative; [5]Concentration equivalent of azelaic acid; [6]Unknow signals are located in sugar region.

Table 2 - Discriminative metabolites along the second predictive component of the OPLS-DA model (n = 64)[1,2].

Metabolite	Loading[3]	NMR signal (ppm)[4]	VIP (Confidence interval)[5]
Azelaic acid & sebacate[6]	-	1.304; 1.545; 2.179	3.3 (0.34); 2.1 (0.25); 1.9 (0.20)
Betaine	-	3.269; 3.904	5.2 (0.84); 3.1 (0.39)
Choline	-	3.206; 4.085	3.5 (0.42); 1.9 (0.15)
Citrate	-	2.545	1.0 (0.18)
Leucine	-	0.964	1.1 (0.16)
Maltose	-	3.278; 3.727; 5.257	6.1 (0.92); 3.2 (0.49); 1.0 (0.78)
Succinate	-	2.414	1.1 (0.23)
Unknown signals[7]	+	3.778; 4.054; 4.155; 3.915; 4.043	3.7 (0.75); 1.7 (0.25); 1.6 (0.21); 2.4 (0.46); 2.7 (0.32)

[1]OPLS-DA Score scatter plot: the second component separated the wheat samples (below) from the rye samples (above). The model parameters for three predictive component fitted were as follow: R2Y=0.937; Q2Y=0.876. Cross-validated ANOVA p-value = 2.98 × 10-38; [2]Wheat endosperm (n = 18), wheat bran (n = 24), rye endosperm (n = 8), and rye bran (n = 14); [3]Loadings: (+): higher concentration in rye samples. (-): higher concentration in wheat samples; [4]One NMR signal from the corresponding spectral bucket with the highest VIP values was reported when several buckets covered a distinct NMR signal; [5]NMR signals with VIP > 1 for which the corresponding jack-knife-based confidence intervals were not close to or including zero were considered discriminative; [6]Concentration equivalent of azelaic acid; [7]Unknow signals are located in sugar region.

Table 3 - Absolute concentrations of metabolites (μmol/g) found to be discriminative along the first and second predictive components[1].

	Concentration μmol/g (mean ± SD)			
Metabolite	1 : Rye endosperm	2 : Rye bran	3 : Wheat endosperm	4 : Wheat bran
Azelaic acid & sebacic acid	0.68 ± 0.21[a]	1.70 ± 0.27[a]	0.70 ± 0.16[a]	4.32 ± 1.25[b]
Acetate	1.19 ± 0.26[a]	3.98 ± 4.08[b]	0.73 ± 0.32[c]	2.70 ± 0.91[d]
Alanine	0.40 ± 0.07[a]	1.81 ± 0.62[b]	0.38 ± 0.13[a]	1.25 ± 0.47
Betaine	10.52 ± 2.80[a]	28.23 ± 6.77[b]	3.70 ± 2.13[c]	34.53 ± 9.79[d]
Choline	0.78 ± 0.13[a]	6.70 ± 1.09[b]	1.12 ± 0.25[c]	6.91 ± 1.18[d]
Citrate	0.55 ± 0.05[a]	5.25 ± 1.55[b]	0.62 ± 0.29[c]	4.93 ± 1.72[b]
Isoleucine	0.15 ± 0.03[a]	0.58 ± 0.21[b]	0.16 ± 0.03[a]	0.48 ± 0.13[b]
Leucine	0.37 ± 0.10[a]	1.52 ± 0.48[b]	0.35 ± 0.08[a]	1.40 ± 0.35[b]
Malate	6.04 ± 0.85[a]	6.22 ± 3.21[b]	7.43 ± 2.73[a]	10.24 ± 5.47[a]
Maltose	17.22 ± 0.182[a]	24.35 ± 8.46[b]	0.86 ± 1.52[c]	21.14 ± 7.27[b]
Succinate	0.54 ± 0.08[a]	1.34 ± 0.70[b]	0.43 ± 0.15[a]	1.43 ± 0.47[a]

[1]ANOVA was performed for betaine, succinate, citrate, alanine, leucine, isoleucine, and maltose. Mann-Whitney test was performed for malate, acetate, and choline. Metabolite means followed by different letters are significantly different (p<0.05). (Mean ± SD).

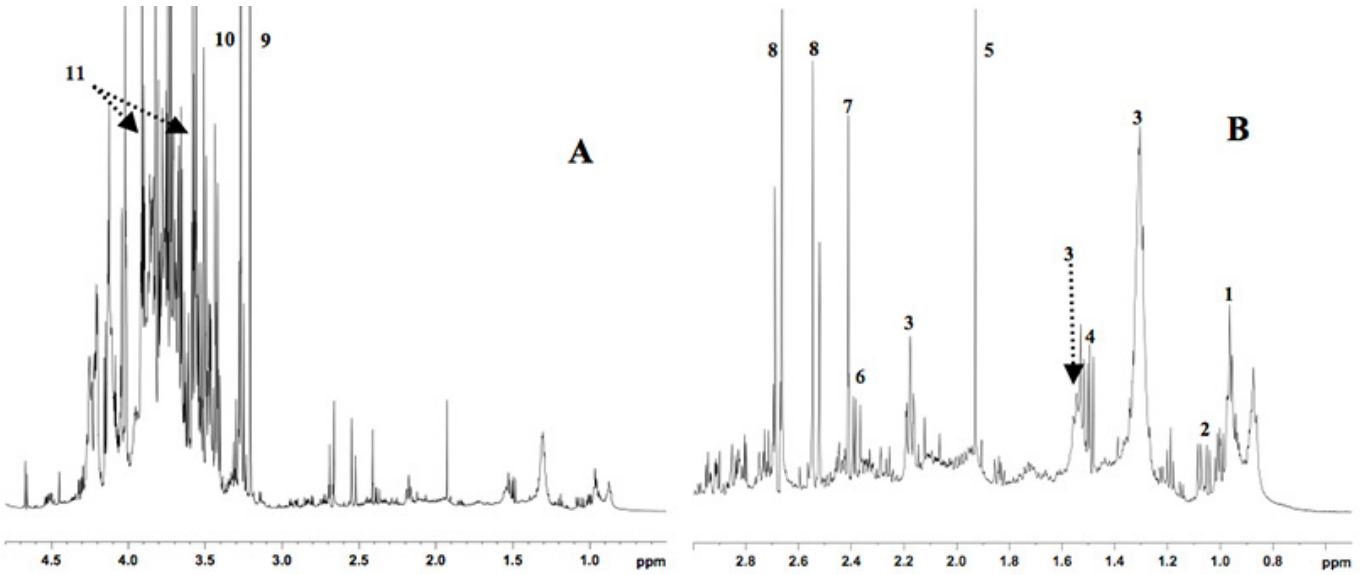


Fig. 2 - (A) A typical 1H NMR spectrum from rye bran polar extract and (B) magnified region 0.5 - 3.0 ppm. Annotated metabolites: Leucine (1), isoleucine (2), Azelaic acid and sebacic acid (3), alanine (4), acetate (5), malate (6), succinate (7), citrate (8), choline (9), betaine (10) and sugar region (11).

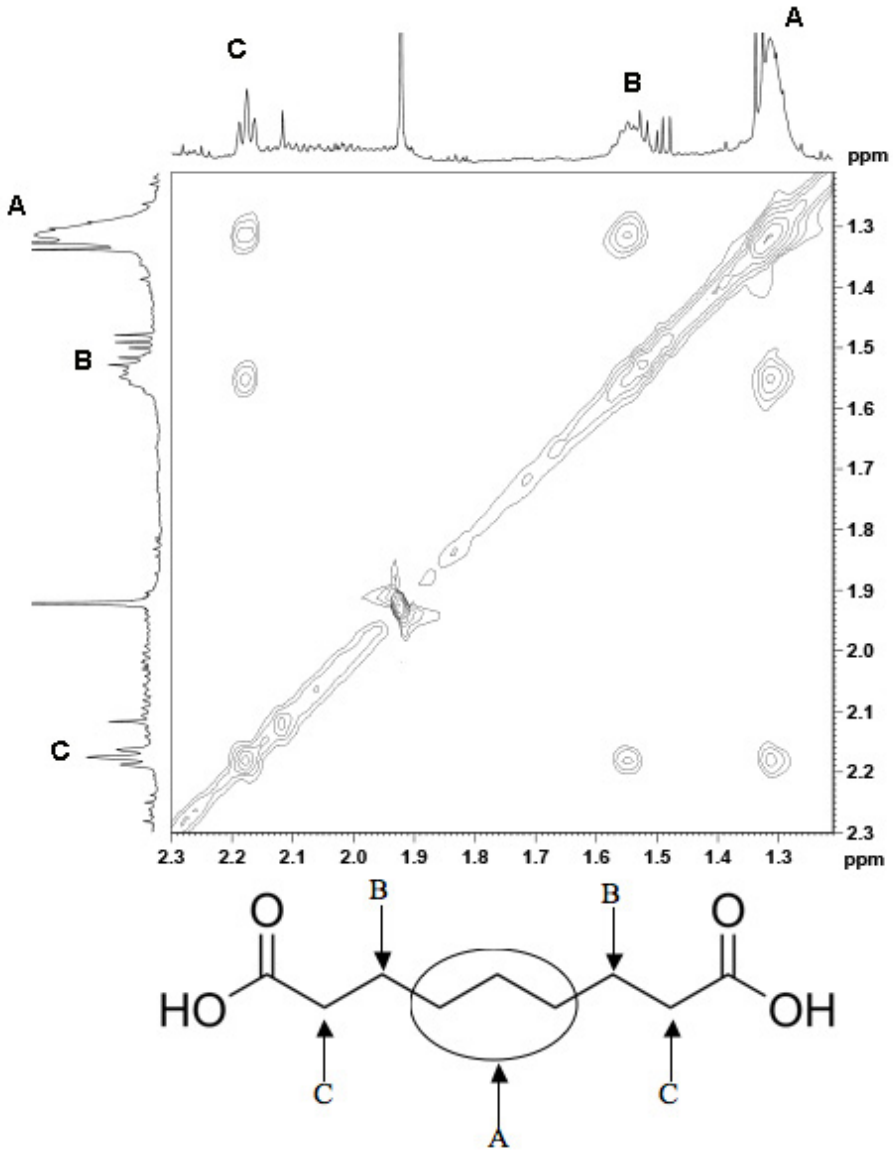


Fig. 3 - TOCSY NMR spectrum of typical rye bran polar extract presenting coupling between a multiple at 1.304 ppm (A), a multiple at 1.545 ppm (B), and a triplet at 2.179 ppm (C), which belong to azelaic acid and sebacic acid, and the assignment of the corresponding -CH2- groups on Azelaic acid molecule.

Multivariate statistical analysis (OPLS-DA model) also included signals discriminating between bran and endosperm, which appeared as a multiplet at 1.304 ppm, a multiplet at 1.545 ppm, and a triplet at 2.179 ppm (Fig. 2; Table 1). Using 2D NMR and spiking with authentic standard, these signals were assigned to two saturated, straight-chain dicarboxylic acids, namely azelaic acid (C9H16O4) and sebacic acid (C10H18O4). TOCSY NMR indicated that these signals were in the same spin system (Fig. 3). COSY NMR also confirmed coupling between (-CH2-) signals at 1.304 ppm and 1.545 ppm, and between (-CH2-) signals at 1.545 ppm and 2.179 ppm. No coupling to a CH3 group was observed on the TOCSY and COSY spectra, confirming dicarboxylic structure. The carbon chemical shifts were assigned from coupling to the corresponding hydrogen in HSQC NMR. There was a cross-peak between protons at 1.304 ppm and carbon at 31.484 ppm, between protons at 1.545 ppm and carbon at 28.926 ppm, and between protons at 2.179 ppm and carbon at 40.735 ppm. After applying new processing consisting of lining broadening (-1) and Gaussian broadening (0.6), the triplet at 2.179 appeared to be two overlapping triplets from azelaic acid and sebacic acid, the chemical shifts of which deviated from each other by 1.54 Hz. The identity of azelaic acid was further confirmed using authentic standard. The molar concentration of total dicarboxylic acids (azelaic + sebacic acid) was then calculated and expressed as equivalent to azelaic acid (Table 3).

No signal corresponding to azelaic acid was detectable in the 24h urine of a male subject af-

ter the consumption of refined wheat bread (485 g). However, after the consumption of whole grain rye bread (485 g), NMR signals corresponding to azelaic acid with similar coupling pattern as azelaic acid in the bran extract were detected in 24h urine (Fig. 4).

The concentrations of azelaic acid and sebacic acid were found to be higher in wheat and rye bran compared with the corresponding endosperm. In addition, wheat bran had higher dicarboxylic acid contents than rye bran (Table 3). To our knowledge this is the first study to report comparative differences in these dicarboxylic acids in different anatomical compartments of wheat and rye. In humans, 60 and 17 % of the administered dosage of azelaic acid and sebacic acid, respectively, are excreted in the urine within the first 12 hours (PASSI *et al.* 1983). It has been suggested that the dicarboxylic acids are to some extent subjected to β-oxidation, since dicarboxylic acids found in serum and urine possess 2, 4, or 6 carbon atoms shorter than the corresponding administered dicarboxylic acids (PASSI, NAZZARO-PORRO, PICARDO, MINGRONE and FASELLA 1983). Recently BONDIA-PONS *et al.* using metabolomics approach have shown that azelaic acid beside alkylresorcinols metabolites and enterolactone are the most discriminate metabolites, and are found in higher concentration urine after the intervention with whole grain rye bread compared with refined wheat bread (BONDIA-PONS *et al.* 2013). The present study showed that the main source of azelaic acid detected in the urine is bran, and that azelaic acid is found in both wheat and rye.

The relatively high abundance, anatomical specificity and localization in bran, pattern of metabolism, and previous findings regarding the identification of azelaic acids as discriminative metabolite in the urine after whole grain *vs* refined grain consumption (BONDIA-PONS *et al.* 2013) suggest that these dicarboxylic acids can be further investigated as biomarkers of exposure to WG products. Further studies are needed to investigate the correlation between azelaic acid concentration and alkylresorcinols concentration, which are validated biomarkers of WG intake (Ross 2012) in different biofluids after the intake of whole grain products. Little is known about the possible metabolic effects of the dicarboxylic acids in mammals. However, azelaic acid is known for its antibacterial (YU and VAN

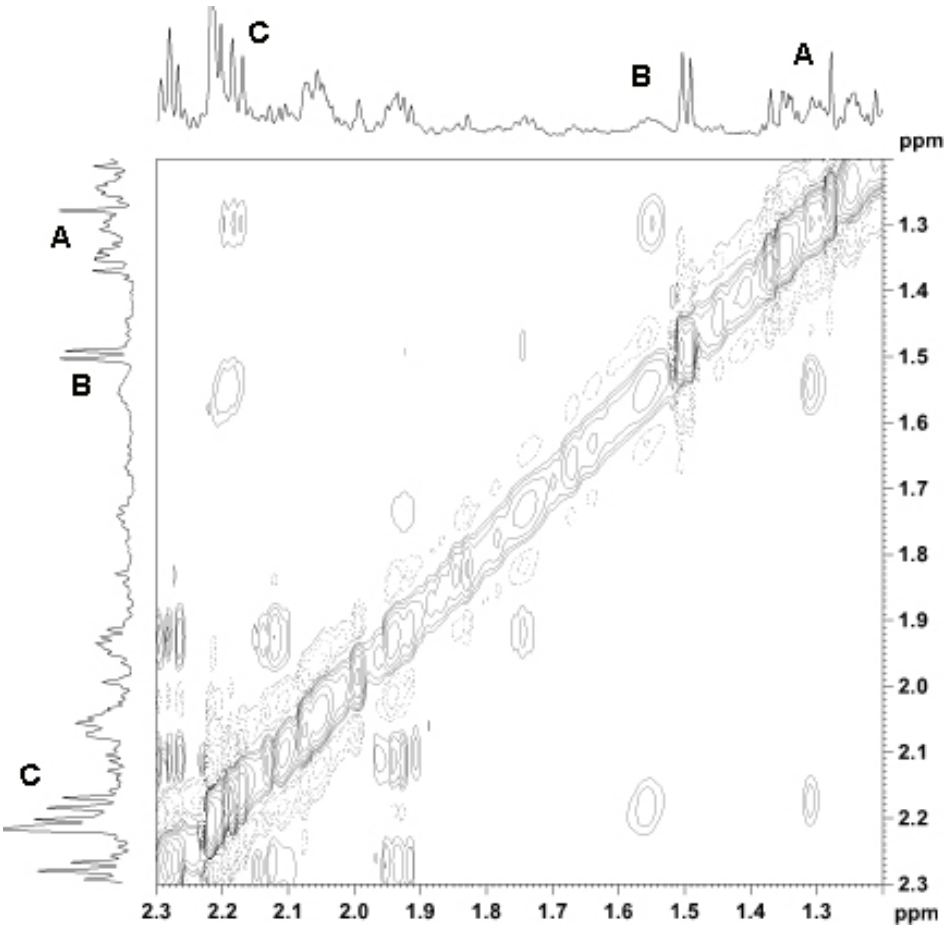


Fig. 4 - TOCSY NMR spectrum of 24 hour urine of a 35 year old man after the consumption of 485 g whole grain rye bread. The pattern of coupling between signals at 1.304 ppm (A), at 1.545 ppm (B), and at 2.179 ppm (C) was similar to that observed in rye bran polar extract.

SCOTT 2004) and anticancer activities (MANOSROI *et al.* 2007), which might contribute to the benefits attributed to WG intake.

Three amino acids i.e. alanine, isoleucine, and leucine, were also present in higher concentrations in bran than in endosperm. These amino acids gave rise to sharp and distinct NMR signals, distinguishing them from the amino acids in proteins, which possess broad NMR signals. In addition, higher contents of succinate, citrate, and malate were observed in bran than in endosperm. These metabolites are associated with the citric acid cycle and central carbon metabolism. The higher levels of amino acids and citric acid may indicate higher metabolic activities in bran compared with endosperm.

Consistent with previous studies, we observed higher levels of betaine and choline in bran than in endosperm (BRUCE *et al.* 2010). In addition, wheat bran had higher levels of betaine than rye bran. Circulating betaine is reported to be increased postprandially in animal models (BERTRAM *et al.* 2009; YDE *et al.* 2012) and in fasting plasma of humans after a 6-8 week intervention with WG rye products (MOAZZAMI *et al.* 2011; MOAZZAMI *et al.* 2012). Betaine acts as a methyl donor in the betaine-homocysteine methyl transferase reaction (BHMT-R), which converts homocysteine and betaine to methionine and N,N-dimethylglycine (DELGADO-REYES and GARROW 2005). Recently, in two separate human studies, we observed an increase in BHMT-R, as indicated by higher N,N-dimethylglycine levels (MOAZZAMI *et al.* 2011; MOAZZAMI *et al.* 2012) and lower homocysteine levels (MOAZZAMI *et al.* 2011), after a 6-8 week intervention with WG rye products compared with refined wheat products, which highlights the metabolic effects of betaine located in the bran of cereals.

In the present study, we used NMR-based metabolomics as an untargeted approach to gain further insights into the metabolic profile of different anatomical compartments of cereal grains. NMR profiling covers metabolites with μmol/g concentration. NMR also proved useful for identification and structural determination of unknown metabolites associated with different anatomical compartments.

ABBREVIATIONS

BHMT, betaine homocysteine methyl transferase; BHMT-R, betaine-homocysteine methyl transferase reaction; CVD, cardiovascular disease; OPLS-DA, orthogonal partial least squares-discriminant analysis; PCA, principal component analysis; T2D, type 2 diabetes; WG, whole grains.

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M.C. and A.A.M. wrote the manuscript. M.C. performed the NMR analysis and metabolomics data analysis. A.G. performed the optimization of extraction method. A.A.M. had primary responsibility for the final content. All authors read and approved the final manuscript and there is no conflict of interest.

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PAPER

INFLUENCE OF SEASON AND FARMING LOCATION ON THE QUALITY PARAMETERS OF SEA BASS (*DICENTRARCHUS LABRAX*) AND SEA BREAM (*SPARUS AURATA*)

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ABSTRACT

The study determined chemical composition of sea brass (*Dicentrarchus labrax*) and sea bream (*Sparus aurata*) farmed in Adriatic Sea, together with variation caused by seasonal variations and farming location. Samples were collected from four different fish farms at three times: June 2012, October 2012 and January 2013. The presented results clearly show seasonal variations of moisture and fat content in the edible part of the fish, while the farming location was proven not to have any significant impact ($p>0.05$). Fatty acid composition was significantly influenced both by the season and the farming location ($p<0.05$). The resulting n-3/n-6 ratios were lower than those reported in other studies, which can be attributed to differences in diet the fish were fed on. Seasonal variations and farming location did not affect fish mineral composition, but mutual differences between the two species were significant.

- Keywords: Sea bass, Sea bream, Quality parameters, Season, Farming location -

INTRODUCTION

European sea bass (*Dicentrarchus labrax*) and sea bream (*Sparus aurata*) are some of the most important finfish species farmed in the Mediterranean region (GRIGORAKIS *et al*., 2002; ALASALVAR *et al*., 2002; KYRANA and LOUGOVOIS, 2002; FUENTES *et al*., 2010). Fish as human seafood is widely consumed because of its high nutritional value, i.e. high protein content, low saturated fatty acid content and high n-3 fatty acid content. Studies have reported that the consumption of only one fatty fish meal per day can result in an approximate n-3 fatty acid intake of 900 mg/day (EPA and DHA), sufficient to have a protective effect on cardiovascular system (KRIS-ETHERTON *et al*., 2002). Furthermore, fish is a rich source of minerals (e.g. sodium, calcium, iron, magnesium, copper, zinc and selenium) and vitamins (e.g. vitamin A, E and D) (FAO/WHO, 2002; CAPELLI *et al*., 2008; CUSTÓDIO *et al*., 2011).

Organoleptic properties and nutritional value are two sets of characteristics that, together with freshness, are accountable for fish quality. Both of them strongly depend on the chemical composition of fish and are affected by many factors including intrinsic fish characteristics (such as species, age, sex, etc.), environmental factors (seasonal changes in temperature, salinity, etc.) and feeding regimen (composition of the feed in use, feeding ratio, etc.) (PIRINI *et al*., 2000; CORDIER *et al*., 2002; GRIGORAKIS *et al*., 2004; GRIGORAKIS, 2007).

The quality of farmed fish strongly depends on factors involved into the production processes (PERIAGO *et al*., 2005). Studies have also shown that, like most Mediterranean fish, farmed fish spawns between December and March, correspondent to the peak sex steroid levels in their plasma. This period is featured by ovarian growth and a substantial reduction in food intake (CORDIER *et al*., 2002; CERDÀ *et al*., 1995). Feeding on commercial nutritive products directly influences growth rates and meat quality, especially its lipid content and composition that can both be modified by diet (IZQUIERDO *et al*., 2005). At the same time, an intense production of cultured fish has raised concerns over the quality of such fish in comparison to the wild one (ALASALVAR *et al*., 2002). An advantage of farmed fish over a wild-caught is that the first is produced and harvested under controlled conditions, so that hazards associated with fish consumption get to be reduced.

A number of previous studies dealing with physico-chemical and organoleptic properties of wild and cultivated sea bream and sea bass have drawn attention to differences in fish quality, especially to the differences in lipid content and saturated and unsaturated fatty acid composition (GRIGORAKIS *et al*., 2002; ALASALVAR *et al*., 2002; FUENTES *et al*., 2010; CUSTÓDIO *et al*., 2011; PERIAGO *et al*., 2005; SAGLIK *et al*., 2003; ORBAN *et al*., 2003). However, no data on the quality of these two species cultivated in the area of the Eastern Adriatic coast under our study, nor data on possible differences in fish meat composition between the two, arising from farming locations or seasonal variations, have been published insofar. Therefore, the aim of this study was to evaluate the influence of season and geographical location on quality parameters of farmed sea bass and sea bream by virtue of analysing their chemical parameters, mineral and fatty acid composition.

MATERIALS AND METHODS

Growth conditions and fish sampling

Three individual samples of market size fish containing ten specimens of sea bass with mean body weight 342.77±86.90 g (92.92-473.18 g) and sea bream with mean body weight 294.70± 49.46 g (184.85-351.35 g) were collected in June 2012, October 2012 and January 2013 from four different commercial marine fish farms. Two of these farms (1 & 2) are situated in the northern part of the Adriatic coast (Istria), while the remaining two (3 & 4) are situated in the mid-eastern coast (Dalmatia). Despite of their common location on the eastern Adriatic coast, each of these sites has its particularities. Site 1 is situated in a very long bay protected from the influence of an open sea, unlike Site 2, which is situated at the entrance of the bay and is exposed to currents. At both sites, the temperature of the sea water fluctuates from 6°C in the winter to 2°C in the summer due to water shallowness. The remaining two sites are situated in the mid-coast, on the islands exposed to currents and waves, so that the sea water temperature fluctuates less strikingly. Its lowest temperature registered in the winter is about 12°C, while its highest temperature registered in the summer is about 25°C. Fry is referred to on-growing originated from different commercial hatcheries and was cultivated in floating net cages of different size and shape for 22 to 28 months, the cultivation density thereby ranging from 5 to 12 kg per cubic meter. The fry was fed on a commercially available fish feed, with a daily ratio of 4.0-1.5 % of body weight for juveniles and 1.5-0.6% of body weight for premarket size. Daily ratio was dependent on the sea temperature, photoperiod and oxygen saturation according to manufacturer's recommendations. All samples of sea bass and sea bream were fed on a same commercial feed manufactured by international feed mills. According to the manufacturer's declaration, the feed on which the sea bass was fed contained 40% of proteins, 24% of fat, 3% of fibres, 6.3% of ash and 0.9% of phosphorus from fish meal, as well as soybean meal, corn gluten, wheat and

wheat by-products. The composition of the feed on which the sea bream was fed was very similar, with slight differences in the share of proteins (43%) and fat (16%).

The fish was killed by virtue of immersing into ice/water slurry and transported to the laboratory on ice. Before eviscerating and filleting the edible part of the fish for analysis, body weight and body length were measured to the closest g (184.85 to 473.18 g) and cm (23.9 to 31.7 cm).

Analysis of compositional parameters

Ten fillets per individual sample were homogenized separately using Grindomix GM200 (Retch, Germany), so as to obtain a homogeneous sample for the determination of chemical parameters. For the sake of analysis, the entire meat portion was employed as edible. The sea bass and sea bream samples were analysed using standard analytical methods: ISO 1442:1997 (moisture), ISO 936:1998 (ash), ISO 937:1978 (crude protein) and ISO 3496:1994 (hydroxyproline/collagen). Sodium and calcium content were determined by using an in-house-validated titration methods described by TRAJKOVIĆ *et al.* (1983). For the determination of sodium, 2 g of the sample were homogenized with sand and 3 mL of water, transferred into a 100 mL-volumetric flask, stirred and placed into a water bath at 100 °C for 15 min. After cooling, the mixture was made up to volume with water and filtered. An aliquot (25 mL) of the filtrate was transferred into an Erlenmeyer flask containing a few drops of K_2CrO_4 (62 g/100 mL of water) as indicator and titrated with 0.1 M-$AgNO_3$ until a persistent reddish colour was obtained. Sodium content was calculated based on the volume of titration reagent used and its concentration. For the determination of calcium, the sample was heated in a furnace at 550 °C up to white ashes were obtained. After cooling, ashes were transferred into a 250 mL-glass, into which 40 mL of HCl (30%), 60 mL of water and few drops of HNO_3 (65%) were added. The solution was boiled for 30 min, cooled, and transferred into a 250 mL-volumetric flask and filled with water up to the mark. An aliquot of the filtered solution (100 mL) was transferred into a 250 mL-glass into which 1 mL of citric acid (300 g/L) and 5 mL of ammonium chloride solution (50 g/L) were added and made up to 100 mL with water. The solution was shortly boiled; then, 10 drops of bromocresol green solution and 30 mL of hot ammonium oxalate solution (4,5 g/100 mL of water) were added. The solution was neutralized by the addition of ammonia (25%) with constant steering up to pH 4.4-4.6, when the colour of the solution turned light blue. The solution was then left in a dark place for 30 min and filtered. After addition of 80 mL of H_2SO_4 (20%), the solution was heated to 80 °C till all precipitates were dissolved and then titrated with $KMnO_4$ (0.1 N) till the pink colour of the solution remained stable for 1 min. Calcium content was calculated based on the expenditure of titration reagent and its concentration.

For the determination of phosphorus, ISO 13730:1996 spectrophotometric method was employed. All chemicals used for the analyses were of an analytical grade.

Lipid analysis

Total lipid contents were determined gravimetrically after extraction in a Soxhlet apparatus according to the AOAC method 948.22:2000. Homogenized samples (5 g) had been extracted with petrol ether for six hours. The solvent was evaporated to dryness in a heated oven at 105°C, following which total lipid content was calculated.

For fatty acid analyses, extracted triacylglycerols were converted into corresponding fatty acid methyl esters (FAME) by trans-esterification with methanolic solution of potassium hydroxide. Approximately 60 mg of the sample was weighed into a test tube equipped with a glass stopper, and dissolved in 4 mL of isooctane. After that, 200 μL of potassium hydroxide solution in methanol (2 mol/L) was added; the reaction was carried out at the room temperature, enhanced by vigorous shaking (2 x 30 s). The solution was neutralized by adding 1 g of sodium hydrogen sulphate monohydrate and transferred into a 2 mL vial. GC analyses were performed on CP-3800 (Varian, Palo Alto, USA) using split/splitless injector and flame-ionisation detector. Capillary column DB 23ms 60 m x 0.25 mm, film thickness 0.25 μm, was used, the temperature thereby being first set at 60°C, then risen up to 210°C at the rate of 4 °C/min, and then rested at 210°C for 15 minutes. Helium was used as a carrier gas at a flow rate of 1 mL/min. The temperature of the split/splitless injector was 250 °C; the same applies to the flame-ionisation detector, while the split ratio equalled to 1:20. The samples were injected manually (1.0 μL). The detector flow rates were as follows: hydrogen 30 mL/min, air 300 mL/min, and detector makeup gas was helium with flow rate of 27 mL/min. A detailed description of the employed method and its suitability for the given purpose was published elsewhere (PETROVIĆ *et al.*, 2010).

Statistical analysis

The statistical significance of difference between samples was tested by analysis of variance (ANOVA) using Statistica Ver. 10.0 Software (STATSOFT INC. 1984-2011, USA). A p-value of 0.05 was considered statistical significance (p=0.05).

RESULTS AND DISCUSSION

Mean values and standard deviations of chemical parameters determined in farmed sea bass and sea bream sampled at different Adriatic coast sites and at three different times, are shown in Table 1 and Table 2, respectively. A significantly higher total fat content was observed for sea bream in comparison to sea bass, with a significant rise in total fat content in both species sampled in October in comparison to those sampled in June and January. Results of our study also showed the same proportion of fat and moisture in both species, with fat content in range from 3.2 to 12.3% in sea bass and 4.2 to 15.0% in sea bream, dependent on, and highly varying according to, the farming season. Our results respective to the seasonal variations of seam beam's fat content are in agreement with the results of CARDINAL *et al.* (2011).

Previous data have shown the approximate composition of sea bass to be 70.71% of moisture, 20.35% of protein, 6.10% of total fat and 1.66% of ash (ERKAN and ÖZDEN, 2007) or, according to another source, 76.72% of moisture, 19.43% of protein, 4.81 % of total fat and 1.23% of ash (KYRANA and LOUGOVOIS, 2002); the aforementioned data are similar to the results of this study obtained for the sea bass sampled in June and January. In 2001, HUIDOBRO *et al.* reported the chemical composition of the Spanish sea bream to be 71.83% of moisture, 22.31% of protein, 5.28% of total fat and 1.27% of ash, whereas ALASALVAR *et al.* (2002) gave the approximate composition of 74.74% of moisture, 18.80% of protein, 6.53% of fat and 1.53% of ash. These results are also comparable to the values obtained in this study for the sea bream sampled in June.

As fish is included in the category of ectothermic poikilotherms, the content of fat in a certain period of the year can be explained by physiological process of fat stock saving or spending. High values of fat obtained in October for all sampling sites and in both species, indicate the preparation of fish bodies for the winter period that comes after a long period of intense feeding. Reduction of fat content in the winter mirrors the spending of fat consequent to the diet minimized due to the low sea temperature and slow fish metabolism. In early summer, when a change in the environmental conditions occurs, primarily in terms of temperature rises, fish metabolism is accelerated, and the energy taken from the food is used for fish growth, which, as seen in both this and earlier studies (JAMES, 1995), resulted in a lower fat content.

The analysis of variance (ANOVA) revealed statistically significant differences ($p<0.05$) in moisture, fat and crude protein content of the sea bass, and statistically significant differences in moisture and fat content in the sea bream dependent on the sampling season (Ta-

Table 1 - Mean chemical composition (±SD) of the sea bass.

	June 2012			October 2012			January 2013		
Parameter	**Farm 1**	**Farm 2**	**Farm 3**	**Farm 1**	**Farm 2**	**Farm 3**	**Farm 1**	**Farm 2**	**Farm 3**
Moisture (%)	73.2±2.4	74.0±1.8	75.5±2.3	67.5±1.6	72.0±2.6	68.8±2.28	70.9±2.87	72.4±2.01	72.6±1.85
Ash (%)	1.16±0.13	1.24±0.15	1.42±0.07	1.32±0.12	1.40±0.09	1.27±0.10	1.62±0.11	1.44±0.06	1.40±0.09
Fat (%)	6.6±0.3	3.7±0.4	3.2±0.2	12.3±1.6	8.0±1.1	11.9±0.9	6.0±0.6	5.5±0.4	5.1±0.2
Crude protein (%)	20.64±0.82	21.50±0.71	22.52±1.21	19.30±1.18	19.09±0.98	18.89±1.02	21.47±1.22	20.51±0.95	20.82±1.03
Hydroxyproline (%)	0.059±0.007	0.064±0.005	0.059±0.010	0.061±0.008	0.059±0.005	0.060±0.004	0.061±0.007	0.065±0.006	0.064±0.008
Collagen (%)	0.47±0.06	0.51±0.04	0.47±0.08	0.49±0.06	0.47±0.04	0.48±0.03	0.49±0.06	0.52±0.05	0.51±0.06
Ca (mg/kg)	701±12.1	685±11.4	731±9.2	754±12.3	813±17.1	662±18.4	723±20.5	802±21.8	725±24.3
P (mg/kg)	3665±33.6	3913±28.5	3843±17.5	3751±30.1	3656±26.5	3721±25.3	3616±41.3	3856±34.5	3589±29.4
Na (mg/kg)	626±7.5	621±9.3	568±7.3	515±6.6	711±10.4	625±8.6	638±7.1	558±6.9	738±8.9

Table 2 - Mean chemical composition (±SD) of sea bream.

	June 2012			October 2012			January 2013		
Parameter	**Farm 1**	**Farm 2**	**Farm 4**	**Farm 1**	**Farm 2**	**Farm 4**	**Farm 1**	**Farm 2**	**Farm 4**
Moisture (%)	72.7±2.1	71.1±1.7	74.5±1.3	66.2±1.8	64.3±2.0	66.5±1.8	67.2±1.7	68.3±2.1	66.3±2.4
Ash (%)	1.33±0.14	1.34±0.09	1.33±0.17	1.32±0.08	1.61±0.13	1.36±0.07	1.71±0.09	1.32±0.11	1.41±0.06
Fat (%)	6.9±0.15	7.4±0.22	4.2±0.17	13.5±0.26	15.0±0.34	14.2±0.28	10.9±0.17	11.3±0.29	11.6±0.22
Crude protein (%)	21.06±1.09	20.32±1.15	21.73±1.08	19.42±1.15	19.65±1.66	18.76±1.09	20.39±1.23	19.14±1.31	20.76±0.92
Hydroxyproline (%)	0.069±0.008	0.070±0.012	0.063±0.008	0.071±0.006	0.073±0.010	0.070±0.011	0.061±0.009	0.062±0.007	0.065±0.006
Collagen (%)	0.55±0.06	0.56±0.10	0.50±0.06	0.57±0.05	0.58±0.08	0.56±0.09	0.49±0.07	0.50±0.06	0.52±0.05
Ca (mg/kg)	281±5.6	255±8.7	246±9.1	268±7.4	303±5.6	241±3.8	269±4.5	307±5.2	261±3.9
P (mg/kg)	3551±19.6	3456±25.8	3503±22.7	3321±18.4	3410±25.7	3545±28.8	3412±16.3	3478±23.4	3388±17.6
Na (mg/kg)	312±4.7	365±3.4	311±6.2	268±2.5	338±3.8	341±4.5	283±5.1	298±3.9	319±4.8

Table 3 - Statistical analyses (ANOVA) of chemical parameters witnessed on various farming locations and during different farming seasons.

Parameter of analysis	Sea bass (p values)		Sea bream (p values)	
	Season	Location	Season	Location
Moisture	0.026*	0.193	0.008*	0.623
Ash	0.243	0.998	0.615	0.833
Fat	0.012*	0.207	0.002*	0.460
Crude protein	0.031*	0.814	0.090	0.485
Hydroxyproline	0.250	0.466	0.053	0.647
Collagen	0.250	0.534	0.059	0.609
Ca	0.583	0.431	0.558	0.154
P	0.472	0.442	0.512	0.785
Na	0.867	0.793	0.430	0.170

*significantly different ($p<0.05$).

ble 3). Farming location had no significant effect ($p>0.05$) on chemical parameters and mineral composition of either of the two. DEL COCO *et al.* (2009) studied the difference between the nutritional value of sea bream produced within three different farming systems and the wild fish. Their results showed a significant difference in protein, lipid and cholesterol content between the fish grown within different farming systems. Among fatty acids only oleic acid varied significantly.

Fatty acid composition, expressed as mean values and standard deviations obtained for sea bass and sea bream samples is shown in Table 4 and Table 5. The most represented fatty acid in both analysed species was oleic acid (C18:1 n-9, OA), followed by linoleic acid (C18:2 n-6, LA) and palmitic acid (C16:0, PA). STROBEL *et al.* (2012) have recently reviewed studies that

Table 4 - Fatty acid composition (%) of the sea bass[1].

	June			October			January		
Fatty acid	Farm 1	Farm 2	Farm 3	Farm 1	Farm 2	Farm 3	Farm 1	Farm 2	Farm 3
C14:0	3.53±0.24	2.56±0.16	3.71±0.21	2.92±0.18	3.34±0.09	2.85±0.10	4.05±0.36	2.51±0.15	3.84±0.16
C15:0	0.35±0.01	0.30±0.02	0.47±0.17	0.30±0.11	0.43±0.01	0.30±0.01	0.37±0.03	0.25±0.01	0.35±0.05
C16:0	16.97±0.12	15.49±0.35	16.61±0.39	15.11±0.38	18.23±0.35	15.60±0.68	16.37±0.27	15.74±0.42	16.74±0.44
C16:1n-7	4.45±0.06	3.77±0.14	5.28±0.29	3.64±0.19	3.63±0.06	3.77±0.14	4.30±0.15	3.31±0.41	4.97±0.26
C17:0	0.55±0.02	0.52±0.11	0.65±0.09	0.53±0.05	0.67±0.02	0.55±0.02	0.55±0.04	0.43±0.03	0.37±0.02
C17:1n-7	0.12±0.03	0.17±0.01	0.32±0.09	0.18±0.01	0.14±0.01	0.17±0.01	0.18±0.03	0.16±0.01	0.17±0.02
C18:0	3.55±0.27	3.86±0.22	3.87±0.11	3.64±0.11	5.07±0.22	3.82±0.25	2.94±0.04	4.12±0.20	3.96±0.18
C18:1n-9	25.53±0.37	26.70±0.19	26.68±0.23	30.31±0.47	31.84±0.54	31.16±0.21	24.38±0.33	29.50±0.58	21.86±0.36
C18:1n-7	2.59±0.14	2.36±0.06	2.66±0.23	2.54±0.08	2.71±0.09	2.61±0.10	3.07±0.22	1.92±0.32	2.76±0.12
C18:2n-6	18.16±0.37	25.81±0.34	20.92±0.29	22.30±1.19	16.01±0.34	21.66±0.91	16.49±0.57	23.83±0.69	21.49±0.28
C18:3n-6	0.08±0.01	0.10±0.02	0.28±0.01	0.19±0.07	0.21±0.04	0.18±0.01	0.22±0.03	0.22±0.01	0.20±0.02
C18:3n-3	2.69±0.11	3.22±0.14	3.21±0.23	5.61±0.19	2.71±0.04	4.42±0.68	3.04±0.11	4.49±0.71	2.67±0.18
C18:4n-3	0.90±0.03	0.57±0.05	0.76±0.02	0.56±0.02	0.46±0.01	0.51±0.07	1.23±0.02	0.53±0.03	0.88±0.23
C20:0	0.31±0.02	0.28±0.02	0.31±0.02	0.31±0.03	0.49±0.02	0.30±0.02	0.16±0.02	0.28±0.02	0.16±0.05
C20:1n-9	3.75±0.28	1.93±0.19	1.48±0.14	2.17±0.35	2.24±0.38	2.09±0.11	5.27±0.32	2.54±0.40	1.75±0.08
C20:2n-6	1.13±0.27	0.95±0.03	0.87±0.08	0.73±0.17	0.25±0.01	0.76±0.03	0.68±0.02	0.76±0.08	0.82±0.20
C20:3n-6	ND	ND	0.08±0.03	0.08±0.04	0.06±0.00	0.08±0.01	0.05±0.03	0.08±0.02	0.18±0.01
C20:4n-6	0.33±0.02	0.33±0.01	0.33±0.02	0.30±0.03	0.58±0.02	0.24±0.01	0.35±0.07	0.28±0.06	0.44±0.02
C20:3n-3	ND	ND	0.09±0.02	0.15±0.06	0.10±0.01	0.11±0.04	0.16±0.02	0.12±0.02	0.19±0.02
C20:4n-3	0.46±0.02	0.42±0.02	0.43±0.13	0.30±0.01	0.44±0.04	0.29±0.01	0.48±0.02	0.29±0.02	0.37±0.02
C22:0	ND	0.09±0.02	ND	0.15±0.01	0.29±0.12	0.16±0.02	0.07±0.01	0.16±0.01	0.32±0.03
C20:5n-3	3.46±0.90	3.64±0.54	2.88±0.14	2.61±0.35	2.18±0.15	2.72±0.15	3.78±0.44	2.55±0.27	6.65±0.29
C22:1n-11	3.24±0.15	1.04±0.09	0.86±0.39	1.03±0.05	1.57±0.23	0.93±0.14	3.53±0.32	1.16±0.42	0.60±0.06
C22:1n-9	0.38±0.02	0.20±0.01	0.39±0.02	0.26±0.03	0.46±0.01	0.24±0.03	0.45±0.02	0.26±0.03	0.29±0.10
C22:5n-3	0.92±0.14	0.80±0.22	1.81±0.16	0.71±0.16	0.71±0.05	0.71±0.02	1.00±0.32	0.70±0.21	1.70±0.14
C24:1n-9	0.41±0.03	0.24±0.02	0.40±0.08	0.20±0.12	0.71±0.02	0.21±0.03	0.23±0.05	0.17±0.01	0.13±0.03
C22:6n-3	6.14±0.21	4.65±0.34	4.66±0.22	3.21±0.55	4.49±0.72	3.58±0.53	6.58±0.23	3.62±0.42	6.15±0.20
SFA	25.26±0.19	23.09±0.64	25.61±0.88	22.96±0.29	28.52±0.64	23.56±0.97	24.52±0.32	23.49±0.48	25.70±0.97
MUFA	40.46±0.38	36.41±0.26	38.07±0.24	40.35±0.72	43.28±0.47	41.18±0.24	41.41±0.29	39.03±0.47	32.58±0.34
PUFA	34.28±0.57	40.50±0.74	36.32±0.75	36.69±0.91	28.20±0.68	35.25±1.21	34.06±0.41	37.48±0.58	41.72±0.62
Total n-3	14.58±0.40	13.30±0.11	13.85±0.70	13.14±0.62	11.09±0.29	12.34±1.02	16.27±0.37	12.30±0.20	18.60±0.54
Total n-6	19.70±0.37	27.20±0.48	22.47±0.35	23.55±0.35	17.11±0.48	22.91±0.76	17.79±0.25	25.18±0.21	23.13±0.18
Total n-3/total n-6	0.74±0.01	0.49±0.01	0.62±0.03	0.56±0.03	0.65±0.04	0.54±0.04	0.91±0.02	0.49±0.01	0.80±0.04
HH	2.87±0.01	3.70±0.09	3.02±0.13	3.46±0.03	2.71±0.09	3.60±0.14	2.82±0.01	3.63±0.02	3.01±0.04

[1]Each value represents the mean value ± standard deviation of three samples of the extracted fat per group, analysed twice (n=6). ND – not detected, detection limit 0.05%

Abbreviations: SFA, saturated fatty acids, MUFA, monounsaturated fatty acids, PUFA, polyunsaturated fatty acids,

HH, hypocholesterolaemic/hypercholesterolaemic ratio = (C18:1 n-9+ C18:2 n-6+C20:4 n-6 + C18:3 n-3+C20:5n-3+ C22:5 n-3+ C22:6n-3)/(C14:0+C16:0)

Table 5 - Fatty acid composition (%) of the sea bream[1].

	June			October			January		
Fatty acid	**Farm 1**	**Farm 2**	**Farm 4**	**Farm 1**	**Farm 2**	**Farm 4**	**Farm 1**	**Farm 2**	**Farm 4**
C14:0	3.67±0.13	4.29±0.16	3.09±0.10	5.41±0.30	2.75±0.11	2.95±0.30	2.71±0.34	3.32±0.44	2.77±0.45
C15:0	0.30±0.02	0.43±0.03	0.38±0.04	0.38±0.05	0.24±0.03	0.27±0.06	0.24±0.02	0.26±0.01	0.23±0.01
C16:0	15.87±0.31	17.99±0.26	15.37±0.44	20.77±0.11	13.77±0.24	14.55±0.51	14.09±0.42	15.25±0.24	15.00±0.42
C16:1n-7	4.61±0.16	5.35±0.27	4.59±0.33	6.19±0.16	4.41±0.17	4.43±0.22	3.65±0.24	4.85±0.23	3.99±0.16
C17:0	0.40±0.02	0.58±0.02	0.68±0.04	0.49±0.04	0.44±0.02	0.46±0.02	0.42±0.14	0.57±0.04	0.41±0.03
C17:1n-7	0.06±0.01	0.20±0.02	0.29±0.04	0.11±0.01	0.21±0.02	0.20±0.03	0.21±0.03	0.32±0.02	0.20±0.02
C18:0	3.92±0.27	4.55±0.29	3.46±0.21	3.94±0.17	3.28±0.19	3.40±0.12	3.67±0.13	4.07±0.54	3.29±0.27
C18:1n-9	25.74±0.37	27.11±0.34	26.51±0.27	30.68±0.30	28.02±0.34	30.02±0.91	27.79±0.32	25.27±0.46	28.44±0.73
C18:1n-7	2.50±0.04	2.67±0.15	2.42±0.12	3.15±0.12	2.32±0.05	2.46±0.17	2.01±0.20	2.97±0.57	2.44±0.53
C18:2n-6	22.96±0.38	22.61±0.28	24.93±0.27	10.92±0.18	24.20±0.32	22.46±0.44	27.32±0.46	25.19±0.62	25.80±0.19
C18:3n-6	0.22±0.01	0.05±0.01	0.26±0.01	0.09±0.01	0.21±0.02	0.23±0.08	0.23±0.01	0.21±0.05	0.30±0.02
C18:3n-3	2.90±0.15	2.09±0.15	3.41±0.17	1.43±0.15	4.51±0.25	5.21±0.54	5.37±0.10	3.01±0.07	5.34±0.07
C18:4n-3	0.76±0.10	0.46±0.01	0.60±0.04	0.45±0.02	0.57±0.03	0.59±0.02	0.52±0.11	0.63±0.11	0.51±0.03
C20:0	0.31±0.01	0.41±0.02	0.28±0.03	0.31±0.02	0.38±0.04	0.35±0.02	0.27±0.03	0.23±0.02	0.21±0.01
C20:1n-9	2.86±0.28	1.85±0.24	1.97±0.10	5.00±0.20	1.74±0.14	1.72±0.36	1.54±0.23	1.67±0.31	1.59±0.38
C20:2n-6	0.67±0.07	0.61±0.02	0.80±0.05	0.14±0.03	0.76±0.21	0.64±0.22	0.66±0.02	0.59±0.12	0.65±0.05
C20:3n-6	0.26±0.07	0.07±0.02	0.08±0.03	0.11±0.01	0.24±0.10	0.19±0.08	0.22±0.02	0.22±0.02	0.21±0.01
C20:4n-6	0.28±0.02	0.32±0.11	0.38±0.03	0.14±0.02	0.25±0.11	0.23±0.04	0.22±0.03	0.33±0.01	0.21±0.01
C20:3n-3	0.20±0.02	0.40±0.12	0.06±0.01	0.11±0.03	0.31±0.17	0.26±0.02	0.28±0.05	0.16±0.01	0.27±0.02
C20:4n-3	0.52±0.06	0.48±0.15	0.42±0.11	0.32±0.02	0.35±0.03	0.33±0.01	0.47±0.02	0.49±0.02	0.47±0.02
C22:0	ND	0.11±0.02	0.07±0.01	0.15±0.01	0.19±0.05	0.16±0.04	0.20±0.02	0.16±0.02	0.16±0.00
C20:5n-3	2.04±0.26	1.48±0.15	3.46±0.38	0.79±0.26	2.45±0.02	2.15±0.40	1.96±0.22	2.96±0.43	1.97±0.09
C22:1n-11	2.81±0.15	1.40±0.12	1.00±0.03	5.64±0.19	1.04±0.31	1.04±0.08	0.70±0.13	0.76±0.14	0.72±0.04
C22:1n-9	0.55±0.03	0.43±0.02	0.19±0.02	0.81±0.32	0.41±0.09	0.39±0.02	0.32±0.02	0.29±0.04	0.31±0.01
C22:5n-3	1.49±0.27	1.07±0.16	0.82±0.11	0.60±0.07	2.01±0.26	1.45±0.43	1.36±0.31	2.04±0.43	1.34±0.41
C24:1n-9	0.54±0.04	0.49±0.11	0.14±0.04	0.65±0.13	0.36±0.11	0.37±0.04	0.32±0.02	0.28±0.02	0.14±0.03
C22:6n-3	3.58±0.28	2.48±0.23	4.34±0.40	0.94±0.18	4.56±0.23	3.47±0.47	3.19±0.33	3.89±0.32	3.04±0.32
SFA	24.52±0.21	28.42±0.28	23.34±0.34	31.46±0.15	21.06±0.48	22.14±0.52	21.60±0.88	23.86±0.29	22.08±0.64
MUFA	39.61±0.29	39.51±0.47	37.11±0.24	52.33±0.29	38.50±0.47	40.64±1.04	36.52±0.34	36.40±0.38	37.76±0.46
PUFA	35.87±0.41	32.07±0.31	39.56±1.21	16.21±0.34	40.44±0.24	37.22±0.56	41.87±0.75	39.73±0.57	40.16±0.58
Total n-3	11.49±0.37	8.46±0.20	13.11±0.65	4.64±0.31	14.77±0.29	13.46±0.69	13.13±0.70	13.18±0.40	12.94±0.49
Total n-6	24.39±0.25	23.61±0.21	26.45±0.48	11.57±0.19	25.67±0.21	23.76±0.87	28.74±0.25	26.55±0.37	27.22±0.48
Totak n-3/total n-6	0.47±0.01	0.36±0.01	0.50±0.04	0.40±0.01	0.58±0.01	0.57±0.02	0.46±0.03	0.50±0.01	0.48±0.01
HH	3.08±0.03	2.63±0.08	3.54±0.09	1.84±0.03	4.00±0.08	3.77±0.08	4.04±0.02	3.42±0.02	3.78±0.02

[1]Each value represents the mean value ± standard deviation of three samples of the extracted fat per group, analysed twice (n=6). ND – not detected, detection limit 0.05%

Abbreviations: SFA, saturated fatty acids, MUFA, monounsaturated fatty acids, PUFA, polyunsaturated fatty acids,

HH, hypocholesterolaemic/hypercholesterolaemic ratio = (C18:1 n-9+ C18:2 n-6+C20:4 n-6 + C18:3 n-3+C20:5n-3+ C22:5 n-3+ C22:6n-3)/(C14:0+C16:0)

confirmed an increase in fatty acids with 18C such as OA, LA and ALA in farmed fish, gained through the use of vegetable oils in their feed. The concentration of long- chain polyunsaturated acids – eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) - in total fatty acids was in range of 2.18-6.65% for EPA and 3.21-6.58% for DHA in sea bass samples, and in the range of 0.79-1.48% for EPA and 0.94-3-19% for DHA in sea bream samples. These results are due to the feeding strategy, since it is well known that fatty acid composition in fish meat reflects dietary fatty acid profile. Over the last decades, fish nutrition research has devoted continued effort to the development of sustainable feeds that can provide long-chain n-3 fatty acid levels adequate for human nutrition (IZQUIERDO *et al.*, 2005; KRIS-ETHERTON *et al.*, 2003).

It has been suggested that "n-3/n-6 ratio" represents a reliable index for inter-species comparisons of relative nutritional values (PIGGOT and TUCKER, 1990). According to SARGENT (1997), the optimum n-3/n-6 PUFA ratio should be 1:5 (0.2). Generally fish have this ratio more favourable as also evident from the results shown in Tables 4 and 5. However, our results showed lower n-3/n-6 ratios in comparison to comparative studies of farmed and wild sea bass and sea bream (ALASALVAR *et al.*, 2002; FUENTES *et al.*, 2010; PERIAGO *et al.*, 2005; SAGLIK *et al.*, 2003). ORBAN *et al.* (2003) studied lipid quality of wild fish and fish farmed in North Adriatic, and also obtained n-3/n-6 ratio higher than ours. Similar or even lower n-3/n-6 ratios were presented by CARDINAL *et al.* (2011), who studied seasonal variations of physico-chemical and sensory characteristics of sea bream coming from the market. These differences in n-3/n-6

ratios are caused by the differences in formulation of diets used in fish farms. It is obvious that farms that were compared to the wild fish have better adapted their fish meals. However, the recent research conducted by SOFI and co-workers (2013) confirmed that the intake of fish with similar EPA+DHA content but different n-3/n-6 ratio has different effects on lipid, inflammatory and haemoreological parameters of healthy subjects.

As it is formally recommended to humans to take 0.3 to 0.5 g of n-3 fatty acids (EPA + DHA) per day (PRATOOMYOTET *et al.*, 2010), consumers´ weekly needs should be satisfied with the consumption of approximately 600 g of sea bass or sea bream. Consumers with coronary heart disease should be encouraged to increase their daily consumption to even 200 g of sea bass or sea bream.

Due to the known effects of specific fatty acids on cholesterol metabolism, one of the indicators of nutritional quality may also be the ratio between hypocholesterolaemic and hypercholesterolaemic fatty acids (HH) (SANTOS-SILVA *et al.*, 2002; TESTI *et al.*, 2006). Generally, sea bream had higher HH index due to the lowest share of saturated fatty acids. The highest HH value, i.e. the most desirable one (4.04), was found in the sea bream sampled in January.

The influence of season and location on fatty acid composition of both fish species is presented in Table 6. Statistical analysis showed the influence of these parameters on the sea bass to be stronger than on the sea bream. As for the sea bass, significant difference ($p<0.05$) in the content of almost all n-3 fatty acids was shown, resulting in differences in both total n-3 fatty acid content and n-3/n-6 ratio. Although diet is the main factor that affects n-3 and n-6 PUFA content in fish, location, species, season and environmental conditions may also play a role (HOSSAIN, 2011). CORDIER *et al.* (2002) also reported differences in n-3/n-6 ratio, especially the difference in EPA over AA ratio. That is in accordance with our results, although within the frame of our study arachidonic acid ($p=0.012$) was influenced by the season, and not by the farming location. EPA over AA ratio is considered to be an important parameter, since dietary intake of n-3 PUFAs helps replacing, at least to a point, n-6 fatty acids in cell membranes, most importantly in platelet, erythrocyte and neutrophil cell membranes (SIMOPOULOS, 2002). CARDINAL *et al.* (2011) also reported significant season-dependent differences in fatty acid content, but this was attributed to the rearing conditions, feed formulation included, which were actually not controlled within their study. Significant variations in some fatty acids present in fish meat, especially variations in linoleic acid (C18:2 n-6) ($p=0.013$), possibly arise as a consequence of different plant contents present in the diets selected as common feed by various farms.

Muscle protein content may be less important than the fat one, but proteins, especially those interacting with water, contribute to organoleptic quality of the fish meat (ZAYAS, 1997). From the nutritional point of view, fish proteins are important since, according to the recent FAO data, fish accounts for 15.7% of the global population's animal protein intake and 6.1% of the entire protein intake (FAO, 2012). In this study, crude protein content varied in range from 18.89% to 22.52% for sea bass, and 18.76% to 21.73% for sea bream, in accordance with the proximate values obtained in earlier studies, summarized in the review of GRIGORAKIS (2007).

Depending on the species, collagen and hydroxyproline fish meat contents vary in range from 0.28% to 0.79% and 30 to 98 mg/100 g, respectively (MORRISEY and FOX, 1981). Values

Table 6 - Statistical analyses of fatty acid profile (ANOVA) of the sea bass and sea bream.

	Sea bass (p values)		Sea bream (p values)	
Fatty acid	**Season**	**Location**	**Season**	**Location**
C14:0	0.715	0.184	0.521	0.315
C15:0	0.556	0.392	0.216	0.947
C16:0	0.258	0.463	0.672	0.473
C16:1n-7	0.841	0.476	0.344	0.278
C17:0	0.244	0.719	0.408	0.859
C17:1n-7	0.909	0.597	0.665	0.523
C18:0	0.225	0.196	0.538	0.514
C18:1n-9	0.069	0.497	0.132	0.778
C18:1n-7	0.565	0.238	0.795	0.171
C18:2n-6	0.210	0.013*	0.200	0.310
C18:3n-6	0.183	0.220	0.610	0.683
C18:3n-3	0.714	0.528	0.262	0.151
C18:4n-3	0.043*	0.106	0.764	0.596
C20:0	0.089	0.419	0.083	0.477
C20:1n-9	0.052	0.001*	0.380	0.216
C20:2n-6	0.406	0.859	0.538	0.333
C20:3n-6	0.417	0.095	0.650	0.932
C20:4n-6	0.012*	0.153	0.242	0.913
C20:3n-3	0.004*	0.015*	0.722	0.133
C20:4n-3	0.053	0.407	0.001	0.563
C22:0	0.243	0.433	0.027	0.255
C20:5n-3	0.120	0.328	0.762	0.717
C22:1n-11	0.153	0.002*	0.329	0.200
C22:1n-9	0.983	0.090	0.257	0.209
C22:5n-3	0.009*	0.009*	0.681	0.868
C24:1n-9	0.117	0.112	0.311	0.313
C22:6n-3	0.041*	0.310	0.892	0.800
SFA	0.301	0.624	0.627	0.437
MUFA	0.153	0.915	0.246	0.431
PUFA	0.066	0.135	0.420	0.419
Total n-3	0.033*	0.193	0.736	0.549
Total n-6	0.326	0.014*	0.415	0.560
Total n-3/total n-6	0.038*	0.092	0.442	0.439

*significantly different ($p<0.05$).

obtained in this study for both fish types are within this range. Other studies reported lower collagen values in farmed fish in comparison to the wild one, which was presumed to be related to the swimming behaviour (SATO *et al.*, 1986), as well as to other factors such as a higher number of muscle fibres which predestines for greater collagen content (SIKORSKI *et al.*, 1984).

The main functions of fish minerals include skeleton structuring, maintenance of colloidal system and regulation of acid-base equilibrium; in addition, these minerals also represent the important hormone, enzyme and enzyme activator constituents (BELITZ and GROSCH, 2001). Literature data have demonstrated that the origin of fish and their feeding pattern did not have any effect on mineral composition, except for that on calcium content (FUENTES *et al.*, 2010).

Calcium (Ca) and phosphorus (P) are necessary for maintaining optimal bones development, being a higher intake of both minerals required during childhood and growing ages so as to prevent rickets and osteomalacia (ERKAN and ÖZDEN, 2007). The content of these minerals determined in sea bass of this study, ranged from 662 to 813 mg/kg and from 3589 to 3913 mg/kg, respectively, while that in sea bream from 241 to 281 mg/kg and from 3321 to 3551 mg/kg, respectively. As reported earlier by ERKAN and ÖZDEN (2007), mean contents of these minerals in sea bass were also significantly higher ($p<0.05$) than those determined in sea bream. Furthermore, obtained results for Ca content in sea bream are in a good agreement with results published by ORBAN *et al.* (2003) whose results ranged from 220 to 230 mg/kg.

As for Ca, sodium (Na) content found in sea bass was significantly higher than in sea bream. Results obtained for sea bass ranged from 515 to 738 mg/kg, while for sea bream ranged from 268 to 365 mg/kg. Literature have reported sea bream Na contents ranging from 280 to 370 mg/kg and sea bass Na contents of 773±1.8 mg/kg (ORBAN *et al.*, 2003). Other sources reported Na content about 289±1.6 mg/kg in sea bream (ERKAN and ÖZDEN, 2007), which is quite similar to the data obtained within this study.

CONCLUSION

Results of our study clearly show seasonal variations of moisture and fat content in both fish species. As for sea bass, differences in fatty acid composition were shown, while in sea bream these differences were not observed. The n-3/n-6 ratios were lower than those previously reported for farmed and wild fish. Fish nutritional value is related to n-3 fatty acids, and is heavily dependent on the production process. Since fish is promoted as a good source of n-3 fatty acids, efforts should be made to properly tailor lipid quality, basically relying on dietary manipulation so as to fit fat deposition and fatty acid profile. Seasonal and location changes did not affect mineral composition, but mutual difference between the two species under study was significant.

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PAPER

ASSESSMENT OF TOTAL POLAR MATERIALS IN FRYING FATS FROM CZECH RESTAURANTS

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ABSTRACT

Deep-frying is commonly used as convenient technique for the preparation of foods. The frying oils and fats are absorbed by fried food and become a part of diet. The content of total polar materials was determined in frying oils and fats in 46 restaurants from South Moravia and the Olomouc regions. Twenty-eight samples were found with total polar materials with limit of rejection over 24%. The highest total polar materials values were observed in cooking fat; the lowest one was in vegetable shortening oil. This conclusion corresponds with frying temperatures, which were highest in cooking fat.

Keywords: deep-frying, fats, oils, restaurant, total polar materials

INTRODUCTION

Fried foods are consumed worldwide with increasing popularity since their unique sensory properties, such as colour, flavour, texture and palatability, are highly appreciated by consumers. Oils and fats are used as means of heat transfer from the fryer to the food. The quality of oils and fats during the frying process has a major influence on the quality of the final product (ANDRIKOPOULOS *et al.*, 2003). Although many studies have dealt with the mechanism and products of fat deterioration under laboratory conditions, relatively little attention has been paid to changes that occur in cooking oils and fats during use in restaurants and other establishments.

In relation to frying operations of fried foods, most of them are conducted at elevated temperatures (160°-195°C) in presence of air, metal container and moisture, resulting in both thermal and oxidative disintegration of the oil (BANSAL *et al.*, 2010b). The thermal treatment of the cooking oils results in oxidative and hydrolytic reactions i.e. hydrolysis, cyclisation or polymerisation. These chemical and physical changes take place and lead to the formation of numerous decomposition products (volatile and non-volatile compounds) (FRITSCH, 1981; FRIEDMAN, 2000).

Furthermore, the extent and nature of these decomposition products are affected by the food being fried, the type of the fat used as well as by the choice of the fryer design and the operating conditions (temperature, oxygen exposure, heating time, turnover rate) (AL KAHTANI, 1991). Regarding the majority of the non-volatile by-products, they are categorized as the total polar materials (TPM). The TPM constituents include dimeric fatty acids, triglyceride monohydroperoxides, polymerized triglycerides (PTG), cyclic fatty acid monomers and aldehydic triglycerides (MÁRQUEZ-RUIZ *et al.*, 1998; GERTZ, 2000).

During the reactions mentioned above, the functional, sensory and nutritional qualities of frying fats are changed and may reach a point where it is no longer possible to prepare high quality fried foods and the frying fat will have to be discarded (VAHČIČ and HRUŠKAR, 1999). The discarding point of the oil that is used repeatedly for frying of food is very closely related to the health issues. The compounds formed during deep-frying e.g. enzyme inhibitors, vitamin destroyers, lipid oxidation products, gastrointestinal irritants and/or potential mutagens are harmful to human health and can therefore become a chemical and physical hazard (SORIANO *et al.*, 2002). There are many studies examining how the quality of frying oils and fats, which are consumed in a diet, influences the health of live animals, especially the rats. Many surveys dealing with the effect of these oils on the growth, liver size, cholesterol or phospholipids in rats showed the relevant negative changes of mentioned parameters by rats fed with oils containing the higher percentage of TPM.

However, no epidemiological or public health investigations have directly proved the effect of abused frying oils on the healthy persons (BILLEK, 2000; BANSAL *et al.*, 2010b). Therefore, it is important to observe the quality of frying oils and fats in view of the fact that they are absorbed by frying food and so become a part of our diet. The uptake of absorbed oil in food ranges in percentage from 4% to 14% of the total weight, depending on the food and the type of frying medium (ANDRIKOPOULOS *et al.*, 2003).

Nevertheless, it is necessary to test the oil quality and establish the cut-off point at which the oil should be discarded in order to protect public health. Since there are health and safety issues related to the reuse of frying oils, several countries have established relevant laws, regulations or recommendations regarding the further use or the discarding of such oils. These countries have set limits for parameters such as frying temperature, acid value, smoke point, polar compounds and polymers (BANSAL *et al.*, 2010b).

In general, the percentage (%) of TPM in the cooking oil has been shown to be almost identical to the one present in the oil absorbed by the food. Thus, by measuring TPM % in frying oil, the direct content of TPM in the fried food could be reflected. Moreover, most European countries have established the limits for the rejection and replacement of cooking oil in restaurants as the content of TPM% where its maximum values range from 24 to 27% (CALDWELL *et al.*, 2001). The limit 24% for TPM was recommended as the most appropriate for rejection while the limit of 20% TPM (GERTZ, 2000) has been recommended for the replenishment of the oil or fat. The concentration of TPM is so rapidly becoming the most widely accepted parameter for the determination of used frying oil quality (DOBARGANES *et al.*, 2000; GERTZ, 2000).

As to the present study, the TPM% of six different types of cooking oils and fats from 46 restaurants in South Moravia and the Olomouc regions are reported indicating the quality status of these oils and fats used under current catering practice.

MATERIALS AND METHODS

Samples

The quality of frying oils and fats was examined during one month in 46 restaurants and fast-food outlets of various types from South Moravia and the Olomouc regions, in the Czech Republic. The measurements were performed in daily frying operations of these restaurants and were analysed repeatedly for the relevance of results. In total were evaluated 46 samples of

Table 1 - Oils and fats used for deep-frying in 46 selected restaurants and fast-food outlets in South Moravia and Olomouc region, Czech Republic.

Producer (trademark)	Type of oil or fat	n
A	Canola oil	2
B	Canola oil	2
C	Canola oil	6
D	Canola oil	2
E	Canola oil	4
F	Canola oil	2
G	Canola oil	2
H	Canola oil with palmolein	4
I	Canola oil with palmolein	2
J	Palm oil	2
K	Palm oil	2
L	Palm oil	4
M	Sunflower oil	2
N	Sunflower oil	2
O	Vegetable cooking fat	2
P	Vegetable shortening oil	2
Q	Vegetable shortening oil	4
Total		46

oils and fats that were divided according to raw materials. The types of oils and fats used for frying are summarized in Table 1. The most of restaurants used only one type of frying fat during measurements.

Methods

The amount of TPM was determined by using TESTO 270 (Testo Inc., Germany). This instrument has to provide the content of TPM in percentage with accuracy +/- 2% TPM. The samples were analysed by inserting the sensor into oil heated to frying temperature and reading the temperature and the TPM content in percentage from the display after about 30 s. The sensor was calibrated with the calibration oil supplied by the manufacturer before analysing the frying oils. The equipment was cleaned with warm water and neutral detergent and dried well between the measurements. Each test was performed in three times. The limit value for the replacement of frying oils and fats was established on 24% for TPM according to German regulations (BANSAL *et al.*, 2010b).

Statistical analysis

The data obtained were statistically analysed by the analysis of variance (ANOVA) and Tukey's multiple range test for comparison of means. Other functions were calculated using the Unistat, v. 5.1 statistical package and Office Excel® Microsoft 2010.

RESULTS AND DISCUSSION

The mean values, the medians and the range of the parameter studied (total polar materials) for oils and fats used for frying from 46 restaurants are shown in Table 2. The mean values for TPM were determined below 20% in all types of oils and fats and therefore did not reach the limit for rejection. The minimum of TPM observed was about 5% in canola oil and vegetable shortening oil at the 1st day of frying, while the maximum value increased to 33% in vegetable cooking fat after 9 days of frying. The analogous wide range was also found by ANDRIKOPOULOS *et al.* (2003), who mentioned in their papers that the minimum of TPM is about 3% and maximum reaches the level of 40%.

Total polar materials reflect the total level of breakdown products from the frying process. The amount and character of these products are affected by some frying parameters such as fat and food composition, frying conditions (temperature, oxygen exposure, heating time, turnover rate) and the design and material of frying equipment (AL-KAHTANI, 1991; VAHČIČ and HRUŠKAR, 1999).

The reported cases of oils and fats that exceed the TPM limit for rejection (24%) are presented in Table 3. Approximately 60% of all samples (12 samples of canola oil, 6 samples of canola oil with palm olein, 4 samples of palm oil, 4 samples of sunflower oil and 2 samples of vegetable cooking fat) were found over the TPM limit for rejection. In case of canola oil with palm oil, sunflower oil and vegetable cooking fat was found out, that all the average values for TPM were above the limit for rejection. The values above 24% TPM were measured already after 6 days of frying by vegetable cooking fat, 7 days by canola oil with palm olein, palm oil and 9 days by sunflower oil.

Table 2 - The content of TPM in examined frying oils and fats in percentage.

	Canola oil	Canola oil with palmolein	Palm oil	Sunflower oil	Vegetable cooking fat	Vegetable shortening oil
n	138	50	66	34	18	28
Mean	15.3[a]	18.1[b]	17.9[b]	14.9[a]	19.6[c]	12.3[d]
SD	±6.08	±7.47	±8.41	±7.22	±8.47	±6.06
Minimum	4.5	5.5	6.3	5.0	8.2	5.0
Median	16.0	20.4	15.0	11.8	22.5	10.7
Maximum	26.6	30.8	31.8	27.1	32.5	24.0

n - number of the measurements from the 1st day of frying time to day of used oil replacement.

Table 3 - The summary of examined oil and fat samples that exceed TPM limit for rejection.

Type of oil or fat	n	TPM < 24%	TPM > 24%
Canola oil	20	8	12
Canola oil with palmolein	6		6
Palm oil	8	4	4
Sunflower oil	4		4
Vegetable cooking fat	2		2
Vegetable shortening oil	6	6	
Total	46	18	28

On the other hand, the average content of total polar materials in the samples of vegetable shortening oil was observed below this limit. In the study related to frying oils and fats from 63 restaurants in Athens, Greece were determined only (17%) of all samples over the TPM limit for rejection. The most samples above this limit were observed in vegetable cooking fat (40%) and then in sunflower oil (30%) and palm oil (18%) (ANDRIKOPOULOS *et al.*, 2003). Approximately (41%) of frying oils used in the restaurants in Zagreb, Croatia reached the oil discard level. However, there was no information about the type of frying oils and fats (VAHČIČ and HRUŠKAR, 1999).

The extent of oxidative degradation in frying oils and fats can be reliably determined using the content of total polar materials. The results of this study showed that the content of TPM increased with frying time (Fig. 1). The initial values of the TPM were below 10%. At the end of frying time (the 9th of frying) the content of TPM reached above 24%, which is oil discard level set in many European countries. These values were observed after 5 days of frying at the earliest.

After 6 up to 9 days of deep frying, the final TPM levels were: "19,8"% in vegetable shortening oil, "23,1"% in canola oil, "25,8"% in sunflower oil, "28,8"% in canola oil with palm olein, "31,8"% in palm oil and "32,5"% in vegetable cooking fat (Fig. 1).

The maximum content of TPM in frying oil was accepted as 24%. From this point of view the percentage (%) of TPM in determined oils would be ranged in ascending sequence: vegetable shortening oil > canola oil > sunflower oil > canola oil with palm olein > palm oil > vegetable cooking fat. In the other study the highest amount of TPM in frying oils was established at 27% and was found out that sunflower oil reached lower value for %TPM than palm oil (XU *et al.*, 1999).

There are many factors that impact the amount of total polar content in frying oils and fats. For example the fatty acid composition of oil has marked effects on its frying performance as well as on its physical and chemical behaviour (BRINKMANN, 2000). The formation of polar compounds during repeated frying operations has been shown to increase with the degree of oil unsaturation, both during repeated frying and during the heating of oils (TAKEOKA *et al.*, 1997; ROMERO *et al.*, 1998).

The next significant parameter that influences the formation of polar compounds in heated oils is the ratio of the surface oil area to, oil volume in the fryer. The specific surface also plays an important role in behaviour of oils during frying, as the overall deterioration is an oxidation process rather than an interaction with frying foods according to BRACOO *et al.*, (1981). The differences in temperatures do not cause significant changes in frying oils (JORGE *et al.*, 1996). The

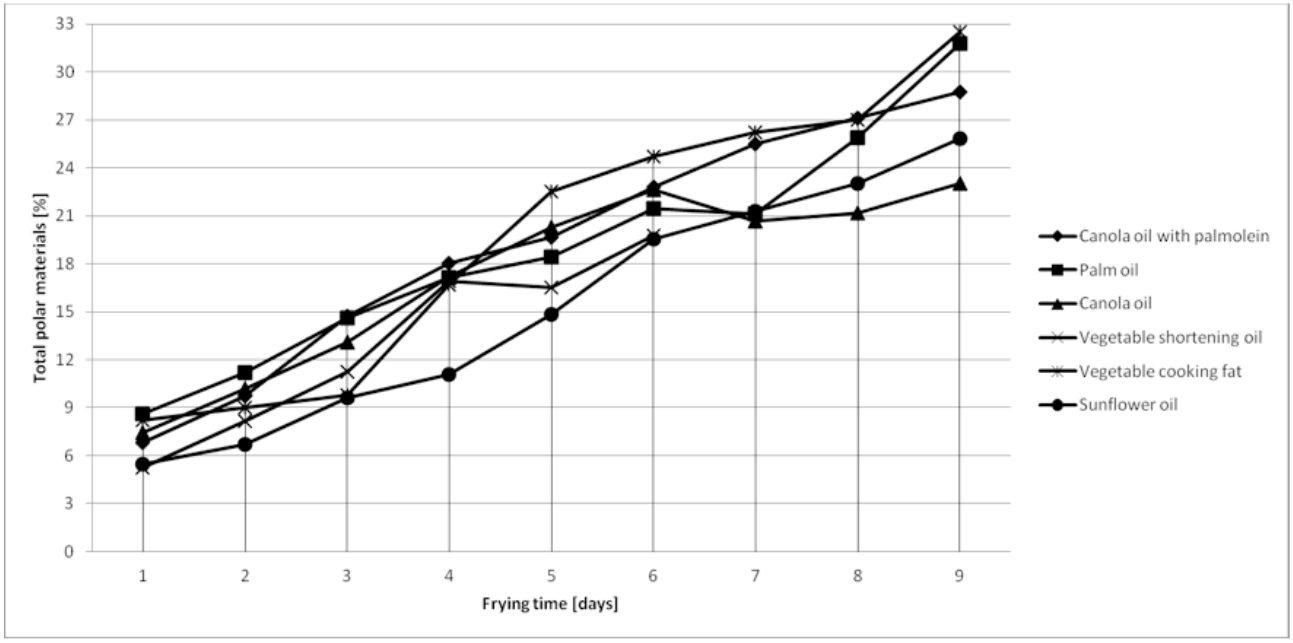


Fig. 1 - The content of total polar materials during frying in examined types of oils and fats

Table 4 - The temperature of examined frying oils and fats during deep-frying [°C].

	Canola oil	Canola oil with palmolein	Palm oil	Sunflower oil	Vegetable cooking fat	Vegetable shortening oil
n	138	50	66	34	18	28
Mean	154.6[a]	158.5[a]	167.3[b]	136.8[c]	173.6[d]	124.1[e]
SD	±42.54	±38.62	±57.71	±45.18	±32.26	±27.29
Minimum	156.4	162.1	147.0	152.6	161.8	94.0
Median	171.0	176.2	166.0	165.4	168.3	132.5
Maximum	186.3	189.5	173.5	174.6	180.5	161.2

n - number of the measurements from the 1st day of frying time to day of used oil replacement.

oil alteration depended on the frying procedure mainly as a result of different surface area to volume ratios and specific areas because pan-frying caused more marked changes than deep-frying on all the parameters studied including the content of total polar materials (ANDRIKOPOULOS *et al.*, 2002).

The next important factor is the temperature which should be in the range of 160 - 180°C for frying operations (SORIANO *et al.*, 2002). In this research the highest temperatures were observed for vegetable cooking fat (173.6°C) while the lowest temperatures were examined for using vegetable shortening oil (124.1°C) (Table 4). Similar conclusion was found also by ALADEDUNYE and PRZYBYLSKI (2009), who mentioned in their paper that the extent of oxidative deterioration, as measured by the TPM formation, was faster during frying at 215°C compared to 185°C. SORIANO *et al.*, (2002) recommended the continuous heating as the intermittent heating is much deleterious due to an increased rate of oil breakdown. Their team was concerned with the daily oil turnover too which should be ranged between 15 to 25 weight per cent in food service kitchens. This suggestion was determined with regard to much longer turnover periods influenced by fluctuations in the demand for fried foods. In our study only 40% of restaurants replenished the frying oils and fats. The replenishment of frying medium was made between the 4th and the 7th day of using. The average volume of replenished oil was 1.5litres. Our finding about catering practice in examined restaurants showed, too, that oils and fats were replaced after 9 days of using while the content of TPM reached the oil discard level already on the sixth day (Fig. 1).

CONCLUSION

Among 46 restaurants, the samples of oils and fats over the rejection limit comprise a relatively high part of samples examined (60% regarding TPM). There was observed higher content of total polar materials with increasing frying temperature. The catering practice in Czech restaurants as such has some lacks, like the turnover rate or the late replacement of used oils and fats, too. From all the findings of the presented study it appears, that it is necessary to survey the conditions of the usage of frying oils and fats more in detail. In addition, more frequent controls and the application of strict regulations by food authorities are important as well.

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PAPER

EXTRA VIRGIN OLIVE OIL STORED IN DIFFERENT CONDITIONS: FOCUS ON DIGLYCERIDES

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ABSTRACT

The effects of storage conditions of extra virgin olive oil (EVOO) on the isomerization of diglycerides (DGs) have been investigated. Aliquots of EVOO were stored for 14 months under four different conditions: at 20°C in darkness and in light, at 4-6°C in light and at 20°C in light with argon in the headspace. Samples were analysed bimonthly: 12 DGs with C34 and C36 (1,2 and 1,3 isomers) were tentatively identified and quantified by GC-FID. After 14 months, a clear tendency towards a decrease of 1,2-DGs and a significant increase of 1,3-DGs during storage was observed for all samples. 1,2-DGs were always predominant compared to 1,3-DGs and, for both types, C36 DGs were prevalent compared to C34 DGs. Overall, EVOO stored at 4-6°C in light showed the highest preservation of 1,2-DGs.

- Keywords: Extra Virgin Olive Oil, Diglycerides,1,2/1,3-DGs ratio, GC, Storage conditions -

INTRODUCTION

Extra virgin olive oil (EVOO) is fresh olive (*Olea europaea L.*) juice obtained by mechanical and physical processes (Lozano-Sanchez *et al.*, 2012), and it is well known as one of the major components of the diet of Mediterranean countries. EVOOs consist of triglycerides as the main components (about 98%) and other minor components including diglycerides, free fatty acids, squalenes, sterols, phospholipids, phenolics and different volatile compounds (BOSKOU, 1996). Some of these minor components, in addition to a high content of mono-unsaturated fatty acids, play a major role in keeping EVOO more stable against oxidation during storage compared to other vegetable oils (BENDINI *et al.*, 2009a). Elimination of air in the head space, either by fully filling the EVOO bottles or by its replacement with inert conditioning gas, has been found to add marked improvement in terms of oxidation quality, stability, shelf life and slow down the oxidation process of EVOO (URDA- ROMACHO, 2009; GIOVACCHINO *et al.*, 2002).

Newly produced EVOO contains a low concentration of diglycerides (DGs) (1-3%), which are formed as intermediate products of the incomplete biosynthesis of triglycerides (SPYROS *et al.*, 2004) and partial hydrolysis of triglycerides. During storage many changes may occur in DG composition due to isomerisation of 1,2-DGs, the predominant form in fresh EVOO, to 1,3-DGs (SACCHI *et al.*, 1991). The effects of storage temperature and exposure to light during different periods of time on the quality of EVOO have been investigated by different authors (VELASCO and DOBARGANES, 2002; MENDEZ and FALQUE, 2007), while other studies have assessed the amount of DGs as an indicative parameter of the freshness of EVOO. CATALANO *et al.* (1994) investigated DGs isomerisation occurring in EVOO stored in darkness, at room temperature and at 4°C. In particular, the results revealed that the 1,2-DGs remained less than 1.5 % after one year of storage for all samples analysed, while about 10% and 45% of the samples stored at room temperature and at 4°C, respectively, contained less than 0.4% 1,3-DGs. Furthermore, PÉREZ-CAMINO *et al.* (2001) studied the evolution of the two DG isomer classes in oils obtained from olives of different qualities stored at different temperatures, concluding that triacylglycerol hydrolysis and DG isomerisation depended not only on the value of free acidity, but also on the storage temperature. In addition, the 1,3/1,2-DG ratio was a useful parameter for assessing the genuineness of EVOOs with low free acidity during early storage stages.

Another interesting study was carried out by SPYROS *et al.*, (2004), assessing olive oil through investigation of 1,2 and 1,3-DG isomerisation during 18 months of storage at room temperature, at 5°C with light and in darkness. The result of the isomerisation process was mainly dependent on the initial quality parameters of the oil, and in particular the free acidity. Another study based on the evaluation of olive oil quality in relation to storage conditions through the analysis of DG isomerisation was carried out by COSSIGNANI *et al.* (2007) on samples produced from different olive cultivars stored at 15°C and at 30°C in darkness for 12 months. The results showed important differences in the percentage of each individual DG and in the ratio among classes; in particular, samples analysed at time zero exhibited the highest percentage of 1,2-DGs and the lowest of 1,3-DGs, whereas samples stored at 30°C showed the highest content of 1,3-DGs suggesting that temperature plays an important role in the isomerisation process. More recently, a study carried out by CAPONIO *et al.* (2013) investigated the effects of storage of EVOO in green glass bottles in light and darkness for 24 months, providing evidence that the degree of isomerisation was affected by the initial hydrolysis level of the oil and by the storage time, although other storage conditions did not show any effect. Overall, these results suggest that the content of DGs and the ratio between isomers might be considered as possible markers to establish the freshness state of an EVOO alongside with other quality parameters defined by official regulations (EU Reg. 61/2011).

Therefore, the main aim of this study was to investigate the isomerisation processes related to diacylglycerols, and in particular the amounts of 1,2- and 1,3-DGs and relative C34 and C36 sub-classes as well as the 1,2/1,3-DG ratio in EVOO during storage under different conditions for 14 months. The purpose was to investigate how these compounds were influenced by different variables such as temperature, light and headspace gases.

MATERIALS AND METHODS

Samples

EVOO samples used in this study were produced from olives of the Arbequina cultivar (Coop. Sant Bartomeu, Soller, Spain) using an industrial plant working with a three-phase decanter. Once in the laboratory, the EVOO was poured into 250 mL transparent glass bottles. The headspace in each bottle was about 2 mL. The bottles were hermetically sealed and divided into four batches. The first batch was stored in darkness inside a thermostatic chamber at 20°C (Cond. 1); the second batch was stored at 20°C under diffuse light (600 Lux for 12 h/day 11 W; 595 lm; 6400°K) simulating the conditions of a supermarket shelf (Cond. 2); the

third batch was stored in a refrigerated chamber at 4-6°C with diffuse light (Cond. 3); finally, the fourth batch was stored with argon in the headspace of bottles at 20°C with diffuse light (Cond. 4). Samples were analysed in triplicate after 2, 4, 6, 8, 10, 12 and 14 months of storage after production.

Basic chemical analysis

Free acidity, peroxide value and UV absorption (K_{232}, K_{270}) were determined according to the official methods described in EEC Reg. 2568/91 for all samples at the initial period of storage (2 months) and after the end of storage simulation (14 months).

Gas chromatographic (GC) determination of diglycerides

The silylated samples were prepared according to a previous work (SWEELEY *et al.*, 1963) and DGs were determined according to a modified version of the method suggested by SERANI *et al.*, (2001) using a GC Carlo Erba MFC500 with a Rtx-65TG (Restek, Bellefonte, PA) fused silica capillary column (30 m length x 0.25 mm i.d. x 0.10 μm f.t.) coated with 35 % dimethyl-65 % diphenylpolysiloxane. The oven temperature was programmed from 250 to 320°C at a rate of 2°C min^{-1} and then increased to 365°C at a rate of 5°C min^{-1}. The final temperature was maintained for 21 min. The injector and FID temperatures were both set at 360°C. Helium was used as carrier gas at a pressure of 130 kPa. The split ratio was 1:70. Identification of DGs was carried out by comparing peak retention times and GC traces with those of DG standards and chromatograms reported in the literature (SERANI *et al.*, 2001; BENDINI *et al.*, 2009b). The results, expressed as mg of each DG per 100 mg of oil, were quantified with respect to dilaurin, added as internal standard (0.5 mL of a solution 2 mg mL^{-1} of dilaurin dissolved in chloroform, added to 100 mg of oil).

Statistical analysis

The software XLSTAT 7.5.2 version (Addinsoft, USA) was used to elaborate the data by analysis of variance (ANOVA, Fisher LSD, $p < 0.05$).

RESULTS AND DISCUSSION

The free acidity, peroxide values and extinction coefficients (K_{232} and K_{270}), shown in Table 1, indicated that at the end of the storage period all samples were within the accepted limits established by EU regulations for the EVOO category (EU Reg. 61/2011).

Fig. 1 shows a comparison between the gas chromatography traces of DG fractions of EVOO stored for 2 and 14 months in dark at 20°C. Twelve different DGs were tentatively identified and quantified as 1,2 and 1,3 isomers with 34 or 36 carbon atoms (C34, C36). Only a co-elution was present (peak 11) between 1,3 isomers of the oleic-linoleic and linoleic-linoleic couples. The peaks numbered from 1 to 6 (Fig. 1) were relative to C34 DGs whereas from 7 to 11 belonged to C36, and palmitic-oleic (PO) and oleic-oleic (OO) were the most abundant DGs for the two classes, respectively. Observing the GC traces (Fig. 1), it is also possible to note that the 1,2 isomers eluted before the 1,3 ones for both groups with 34 and 36 carbon atoms.

Fig. 2 illustrates the evolution of 1,2/1,3-DG ratios, and Tables 2-5 highlight the trends of 1,2-DGs (C34, C36) and 1,3-DGs (C34, C36) for EVOOs stored under the four different experimental conditions. For the samples kept at 20°C in darkness (Cond. 1), a rapid and significant decrease was observed in the 1,2/1,3-DG ratio for the first 8 months; this ratio continued to decrease slowly until the end of storage period (Fig. 2). A similar trend was also seen for the 1,2-DGs C34 and C36 under the same condition (Table 2), and the rapid decrease continued for up to 8 months. At the end of storage period, total 1,2-DG remained about 60 % (data not shown) of

Table 1 - Results for free acidity (FA, g of oleic acid per 100 g of oil), peroxide values (PV, Meq O_2 Kg^{-1}) and extinction coefficient at 232 and 270 nm (K_{232}, K_{270}) at time zero and after 14 months of storage under the four different conditions (Cond. 1 - 4)*.
* Cond. 1, stored at 20°C in dark, Cond. 2, stored at 20 °C in light, Cond. 3, stored at 4-6°C in light, Cond. 4 stored at 20°C in light with argon in the headspace.
Different letters (a-e) represent significant differences among mean values for a same parameter during the storage time (from 2 to 14 months). Different letters (x-z) indicate significant differences among the four storage conditions after 2 and 14 months of storage.

	2 months of storage				14 months of storage			
	FA	PV	K232	K270	FA	PV	K232	K270
Cond. 1	0.15 ± 0.01 b,x	11.63 ± 1.29 a,xy	2.11 ± 0.03 b,x	0.10 ± 0.00 b,z	0.20 ± 0.01 a,x	12.74 ± 0.55 a,y	2.34 ± 0.02 a,x	0.15 ± 0.01 a,y
Cond. 2	0.15 ± 0.01 b,x	14.00 ± 0.04 a,x	2.00 ± 0.09 b,xy	0.17 ± 0.01 b,x	0.20 ± 0.01 a,x	14.74 ± 1.02 a,xy	2.19 ± 0.07 a,y	0.18± 0.01 a,x
Cond. 3	0.15 ± 0.01 b,x	10.59 ± 0.01 b,y	1.94 ± 0.12 b, y	0.13 ± 0.00 b,y	0.17 ± 0.01 a,y	15.47 ± 0.80 a,x	2.19 ± 0.06 a,y	0.14 ± 0.00 a,y
Cond. 4	0.16 ± 0.01 b,x	14.00 ± 0.15 a,z	2.15 ± 0.04 b,x	0.17 ± 0.00 b,x	0.20 ± 0.01 a,x	14.70 ± 0.40 a,xy	2.24 ± 0.03 a,xy	0.18 ± 0.01 a,x

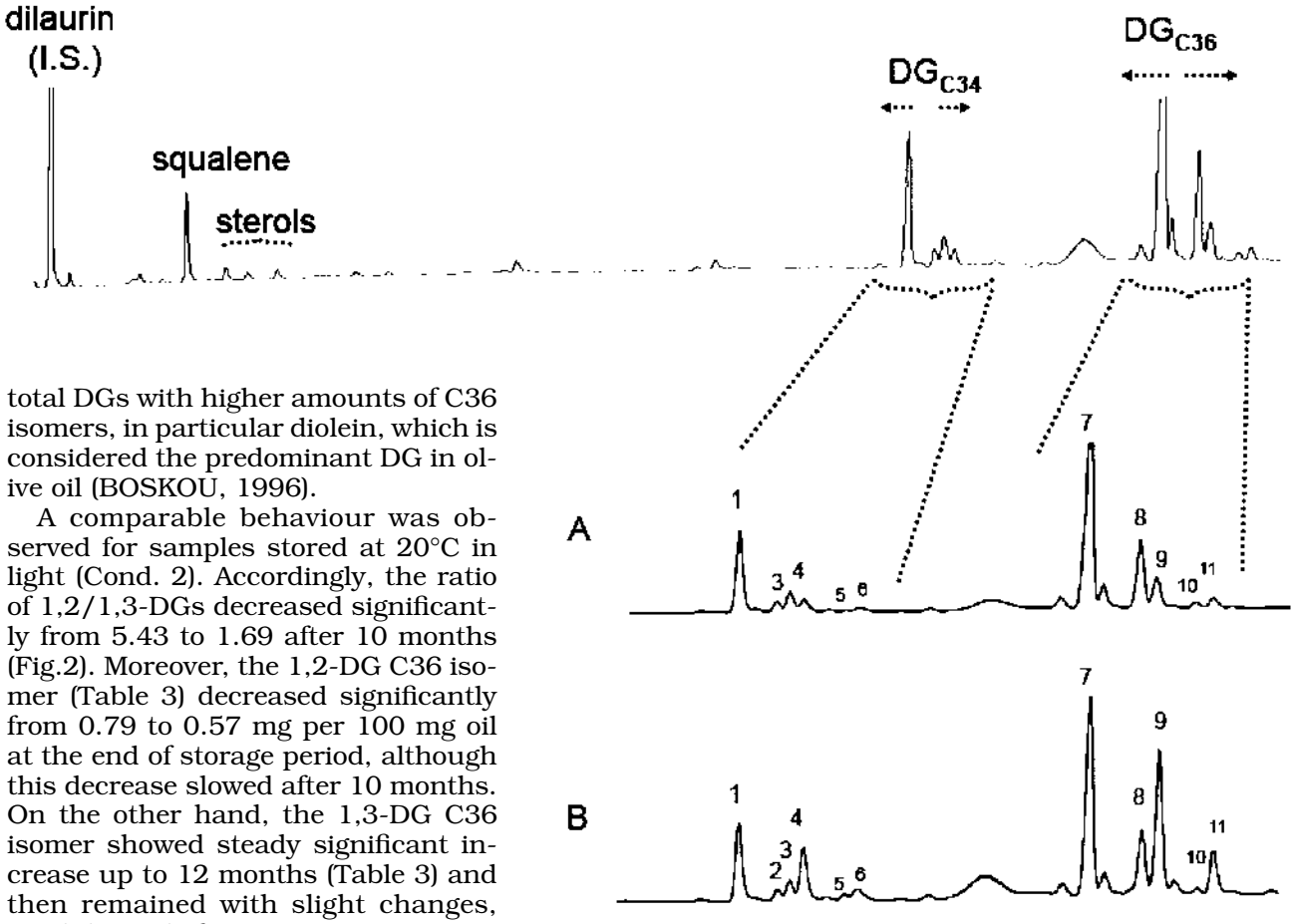


Fig. 1 - Example of full chromatogram of the EVOO sample at 20°C in dark. A) GC tracing of the diglyceride fraction of EVOO stored for 2 months at condition 1; B) GC trace of the diglyceride fraction of EVOO stored for 14 months at condition 1. 1, 1,2-PO; 2, 1,2-PoO; 3, 1,2-PL; 4, 1,3-PO; 5, 1,3-PoO; 6, 1,3-PL; 7, 1,2-OO; 8, 1,2-OL; 9, 1,3-OO; 10, 1,2-LL; 11, 13-OL + 1,3-LL. P = palmitic acid; Po = palmitoleic acid; O = oleic acid; L = linoleic acid.

total DGs with higher amounts of C36 isomers, in particular diolein, which is considered the predominant DG in olive oil (BOSKOU, 1996).

A comparable behaviour was observed for samples stored at 20°C in light (Cond. 2). Accordingly, the ratio of 1,2/1,3-DGs decreased significantly from 5.43 to 1.69 after 10 months (Fig.2). Moreover, the 1,2-DG C36 isomer (Table 3) decreased significantly from 0.79 to 0.57 mg per 100 mg oil at the end of storage period, although this decrease slowed after 10 months. On the other hand, the 1,3-DG C36 isomer showed steady significant increase up to 12 months (Table 3) and then remained with slight changes, until the end of storage. However, 1,3-DG C34 isomers showed a significant slight change toward increases, after 6 months of storage, reaching about 0.14 mg per 100 mg sample after 14 months of storage (Table 2).

The results for samples stored at low

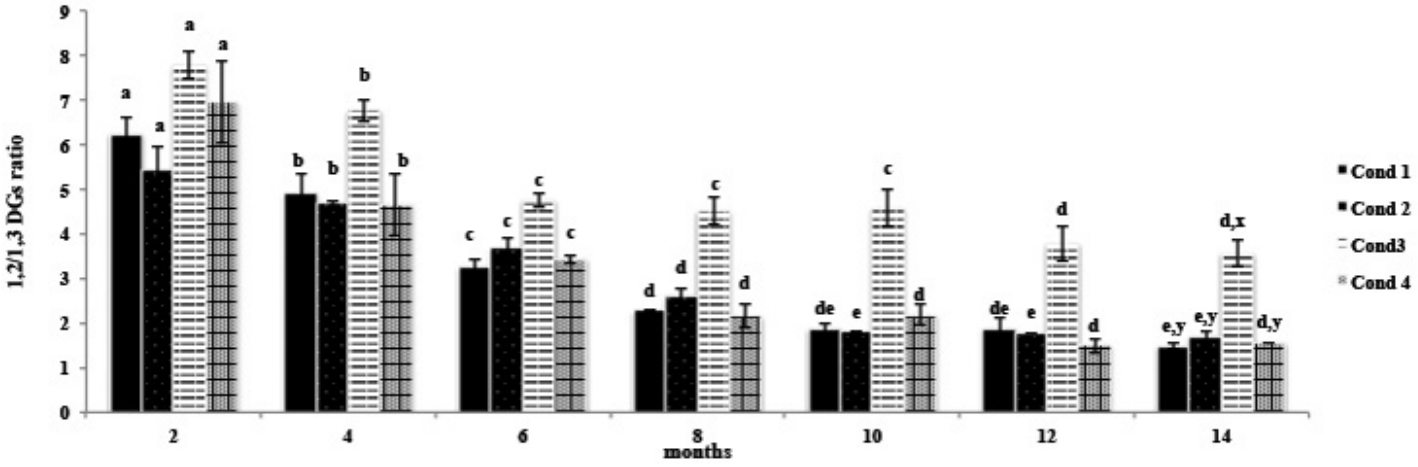


Fig. 2 - Trends of 1,2/1,3 DGs during the EVOO storage of 14 months at the four different conditions (Cond 1-4)*. The concentration of DGs was calculated as mg dilaurin per 100 mg of oil. Different letters (a-e) represent significant differences among mean values for a same condition during the storage time (from 2 to 14 months). Different letters (x-z) indicate significant differences among the four storage conditions after 14 months.
* Cond. 1, stored at 20°C in dark, Cond. 2, stored at 20°C in light, Cond. 3, stored at 4-6°C in light, Cond. 4 stored at 20°C in light with argon in the headspace.

Table 2 - Evolution of 1,2 and 1,3 isomers of C34 and C36 diglycerides during the EVOO storage of 14 months under condition 1 (at 20°C in dark). The concentration of DGs was calculated as mg dilaurin per 100 mg of oil. Different letters (a-e) represent significant differences among mean values for a same isomer during the storage time (from 2 to 14 months). Different letters (x-z) indicate significant differences among the four storage conditions after 14 months of storage.

	Cond. 1			
Months of oil storage	1,3 C34- DGs	1,3 C36- DGs	1,2 C34- DGs	1,2 C36- DGs
2	0.09 ± 0.01 f	0.19 ± 0.03 e	0.48 ± 0.06 a	1.25 ± 0.14 a
4	0.11 ± 0.01 e	0.25 ± 0.02 de	0.47 ± 0.05 a	1.27 ± 0.16 a
6	0.13 ± 0.01 de	0.26 ± 0.01 d	0.38 ± 0.05 b	0.89 ± 0.07 b
8	0.13 ± 0.00 cd	0.33 ± 0.01 c	0.28 ± 0.01 c	0.77 ± 0.02 b
10	0.15 ± 0.00 bc	0.40 ± 0.07 b	0.28 ± 0.01 c	0.75 ± 0.05 b
12	0.16 ± 0.01 b	0.37 ± 0.03 bc	0.27 ± 0.01 c	0.74 ± 0.10 b
14	0.19 ± 0.02 a,x	0.49 ± 0.02 a,x	0.27 ± 0.02 c,yz	0.73 ± 0.05 b,y

temperature (4-6°C) (Cond. 3) showed that, at the end of the storage period, the 1,2/1,3-DG ratio remained about 2 times higher than the values for EVOO samples stored at 20°C (Fig. 2). Furthermore, the 1,2-DGs isomers C36 and C34 showed a significant decrease from 2 to 14 months (Table 4).

Regarding the samples stored with argon in the headspace (Cond. 4), the 1,2/1,3-DGs ratio decreased significantly during the first 8 months of storage, and minor changes were detected up to the end of storage (Fig. 2). Similarly, 1,2-DGs for both C36 and C34 classes decreased after 14 months of storage compared to the initial value, with a fluctuation trend (Table 5), while 1,3-DG C36 isomers showed a significant increase throughout the entire storage period.

By comparing the different conditions, after 2 months of storage the highest 1,2/1,3-DG ratio corresponded to the sample stored at low temperature (4-6°C), followed by the sample stored under light at 20°C with argon in the headspace

Table 3 - Evolution of 1,2 and 1,3 isomers of C34 and C36 diglycerides during the EVOO storage of 14 months under condition 2 (at 20 °C in light). The concentration of DGs was calculated as mg dilaurin per 100 mg of oil. Different letters (a-e) represent significant differences among mean values for a same isomer during the storage time (from 2 to 14 months). Different letters (x-z) indicate significant differences among the four storage conditions after 14 months of storage.

	Cond. 2			
Months of oil storage	1,3 C34- DGs	1,3 C36- DGs	1,2 C34- DGs	1,2 C36- DGs
2	0.06 ± 0.00 e	0.15 ± 0.01 e	0.35 ± 0.01 ab	0.79 ± 0.15 cd
4	0.09 ± 0.02 d	0.21 ± 0.02 d	0.38 ± 0.05 a	1.06 ± 0.14 a
6	0.12 ± 0.02 cd	0.25 ± 0.02 d	0.37 ± 0.02 a	0.98 ± 0.05 ab
8	0.15 ± 0.01 ab	0.30 ± 0.01 c	0.32 ± 0.01 b	0.85 ± 0.07 bc
10	0.15 ± 0.01 a	0.36 ± 0.01ab	0.22 ± 0.01 c	0.69 ± 0.02 de
12	0.13 ± 0.00 bc	0.39 ± 0.00 a	0.23 ± 0.00 c	0.68 ± 0.00 de
14	0.14 ± 0.01 ab,y	0.32 ± 0.06 bc,y	0.21 ± 0.01 c,z	0.57 ± 0.04 e,z

Table 4 - Evolution of 1,2 and 1,3 isomers of C34 and C36 diglycerides during the EVOO storage of 14 months under condition 3 (at 4-6°C in light). The concentration of DGs was calculated as mg dilaurin per 100 mg of oil. Different letters (a-e) represent significant differences among mean values for a same isomer during the storage time (from 2 to 14 months). Different letters (x-z) indicate significant differences among the four storage conditions after 14 months of storage.

	Cond. 3			
Months of oil storage	1,3 C34- DGs	1,3 C36- DGs	1,2 C34- DGs	1,2 C36- DGs
2	0.08 ± 0.01 c	0.14 ± 0.01 d	0.58 ± 0.08 a	1.09 ± 0.18 ab
4	0.08 ± 0.00 c	0.17 ± 0.01 c	0.46 ± 0.02 b	1.25 ± 0.06 a
6	0.12 ± 0.01 ab	0.18 ± 0.01 c	0.41 ± 0.02 bcd	1.01 ± 0.11 b
8	0.12 ± 0.01 ab	0.16 ± 0.01 cd	0.34 ± 0.04 d	0.9 ± 0.12 b
10	0.10 ± 0.03 bc	0.22 ± 0.00 b	0.38 ± 0.01 cd	1.08 ± 0.03 ab
12	0.14 ± 0.02 a	0.25 ± 0.02 b	0.45 ± 0.02 bc	1.03 ± 0.17 b
14	0.13 ± 0.01 a,y	0.28 ± 0.03 a,y	0.39 ± 0.04 cd,x	1.07 ± 0.03 ab,x

Table 5 - Evolution of 1,2 and 1,3 isomers of C34 and C36 diglycerides during the EVOO storage of 14 months under condition 4 (at 20 °C in light with argon in the headspace). The concentration of DGs was calculated as mg dilaurin per 100 mg of oil. Different letters (a-e) represent significant differences among mean values for a same isomer during the storage time (from 2 to 14 months). Different letters (x-z) indicate significant differences among the four storage conditions after 14 months of storage.

	Cond. 4			
Months of oil storage	1,3 C34- DGs	1,3 C36- DGs	1,2 C34- DGs	1,2 C36- DGs
2	0.07 ± 0.00 c	0.14 ± 0.02 d	0.41 ± 0.08 a	1.07 ± 0.18 ab
4	0.07 ± 0.01 c	0.18 ± 0.01 d	0.32 ± 0.02 bc	0.82 ± 0.13 cd
6	0.14 ± 0.00 b	0.31 ± 0.06 c	0.46 ± 0.05 a	1.09 ± 0.11 a
8	0.15 ± 0.01 b	0.37 ± 0.04 bc	0.29 ± 0.01 c	0.82 ± 0.02 d
10	0.21 ± 0.01 a	0.46 ± 0.04 ab	0.38 ± 0.01 ab	1.06 ± 0.21 abc
12	0.17 ± 0.01 b	0.48 ± 0.06 a	0.25 ± 0.01 c	0.70 ± 0.05 d
14	0.21 ± 0.04 a,x	0.53 ± 0.10a,x	0.31 ± 0.06 bc,y	0.84 ± 0.15 bcd,y

(Fig. 2). Moreover, during the first 4 months, when EVOOs were stored at 20°C under light without headspace modification (Cond. 2), the sample exhibited a lower ratio than the respective sample stored in darkness (Cond. 1). The results also highlighted the positive effect of using inert gas in the head space. The total 1,2-DGs remained after 14 months (data not shown) of storage was about 1.5 times higher, in comparison with their presence in EVOO stored under the same conditions, but with air in the head space. The findings are in accordance with SPYROS *et al.* (2004), suggesting that the length of storage time plays an important role in isomerisation changes of DGs, which is accelerated by temperature.

The formation of oxidation products by photo-oxidation was confirmed by the high values of K_{270} obtained for samples stored under diffuse light, especially for those stored at 20°C after 14 months of storage (Table 1). It should be noted that, at the end of storage period, all the samples remained within EVOO category parameters. As expected, free acidity (Table 1), which is considered to be the main driving factor affecting DG isomerisation (PÉREZ-CAMINO *et al.*, 2001), showed only a minor increase after 14 months of storage.

The results of this study showed that the isomerisation of DGs in EVOOs depends not only on the length of storage, but also on the temperature of storage. This finding is in agreement with the studies of PÉREZ-CAMINO *et al.* (2001) and COSSIGNANI *et al.* (2007). Moreover, the results showed that after 14 months of storage at 20°C (Cond. 1, 2 and 4) there were slight but not significant differences in the 1,2/1,3 ratio among samples stored under diffuse light (Cond. 2 and 4) and for those stored in darkness (Cond. 1), in spite of the fact that light exposure has an adverse effect on the oxidation of EVOO (significantly higher K_{270} values were found for samples stored under diffuse light). This result is in agreement with considerations noted by AFANEH *et al.* (2013).

CONCLUSION

The results of this study confirmed that the isomerisation of DGs in EVOO depends not only on the length of storage, but also on the temperature. By comparing the different conditions, it was found that after 10-14 months of storage the 1,2/1,3-DG ratio remained higher for samples stored at low temperature (4-6°C). Moreover, the presence of argon gas in the headspace of the sample was not sufficient to protect it from DG isomerisation when the EVOO was exposed to light.

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PAPER

EFFECTS OF ALGINATE EDIBLE COATING ON QUALITY AND ANTIOXIDANT PROPERTIES IN SWEET CHERRY DURING POSTHARVEST STORAGE

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ABSTRACT

Two sweet cherry (*Prunus avium* L.) cultivars ("Big Lory" and "Grace Star"), were treated with 1%, 3% and 5% sodium alginate as an edible coating before storage. Analytical determinations were made after 7, 14 and 21 days of storage at 4°C. Cherries were analyzed for the following quality parameters: firmness, weight loss, titratable acidity, soluble solid content, external color, anthocyanin content, phenolic content and total antioxidant capacity. Alginate treatment at 3% delayed changes in most of the ripening parameters, weight and acidity losses, softening and color changes. However, the soluble solids content was not affected by the alginate fruit coating. In terms of the antioxidant properties, no significant results were obtained with the use of the alginate coating. The results of this study suggest that alginate treatments at 1% and 3% could be used as natural postharvest treatments in cherry cultivars with the aim of delaying the postharvest ripening process and maintaining fruit quality.

- Keywords: sweet cherry, quality, shelf life, edible coating -

INTRODUCTION

Sweet cherry is one of the most appreciated fruits by the consumer due to its precocity and quality. Among the factors determining consumer acceptability, soluble solids, skin color and acidity are the most important (DÍAZ-MULA *et al.*, 2012). Color is an indicator of quality and ripening of fresh sweet cherry and depends on the accumulation and profile of anthocyanins (CRISOSTO *et al.*, 2003). The antioxidant properties of sweet cherry are associated with the ascorbic acid and polyphenolic content (CHAOVANALIKIT and WROLSTAD, 2004; SERRANO *et al.*, 2005). Sweet cherries deteriorate rapidly after harvest and, in some cases, do not reach consumers with the optimal organoleptic quality. Both the fruit and the stem consist largely of air and water, and the water is lost very quickly. The main causes of sweet cherry deterioration are weight loss, loss of acidity, softening, color changes, surface pitting and stem browning, along with changes in the soluble solids content (BERNALTE *et al.*, 2003). Adequate postharvest technologies combined with cold storage are fundamental. Several pre- and postharvest technologies have been used to control cherry decay. In this sense, the use of an edible coating could be a new technological alternative to the use of modified atmosphere packaging to maintain fruit quality during storage, especially in minimally processed cherries (commercialized without the steam) (CAMPOS *et al.*, 2011). Edible coatings are traditionally used to improve fruits appearance and conservation. Coatings on products create a semipermeable barrier to external elements that can reduce moisture loss, solute migration, respiration and oxidative reactions and retard the natural physiological ripening process (VARGAS *et al.*, 2008). Maintenance of fruit quality has been achieved by the utilization of different coatings such as chitosan in peach (LI and YU, 2001) and nectarines (CHIABRANDO and GIACALONE, 2013), pectin coating in melon (FERRARI *et al.*, 2013), alginate in apple (OLIVAS *et al.*, 2007; ROJAS-GRAU *et al.*, 2007; CHIABRANDO and GIACALONE, 2012), and hydroxypropylmethylcellulose and whey protein in plum (NAVARRO-TARAZAGA *et al.*, 2008; REINOSO *et al.*, 2008). In particular, alginate is a hydrophilic biopolymer that has a coating function because of its unique colloidal properties, which have allowed its use as a thickening agent, in forming suspensions and gels, and for stabilizing emulsions (ACEVEDO *et al.*, 2012). Sodium alginate has been effective in maintaining postharvest quality in tomato (ZAPATA *et al.*, 2008) and peach (MAFTOONAZAD *et al.*, 2008). In sweet cherry, some effects on fruit quality have been obtained with edible coatings based on chitosan (ROMANAZZI *et al.*, 2003), sodium alginate (DIAZ-MULA *et al.*, 2012), Semperfresh™ (YAMAN and BAYINDIRLI, 2001) and with the use of aloe vera gel (RAVANFAR *et al.*, 2012).

Thus, the aim of this study was to analyze the effect of sodium alginate applied as an edible coating at three concentrations (1%, 3% and 5% w/v) on the quality properties and antioxidant activity in Grace Star and Big Lory sweet cherry cultivars during storage at 4°C.

MATERIALS AND METHODS

Plant material

Sweet cherry (*Prunus avium* L. cv "Grace Star" and "Big Lory") fruits were harvested from a commercial plot. Fruits were picked at commercial maturity stage, with a score of 4 on the color chart from the Centre Technique Interprofessionel de Fruits et Légumes (CTIFL, Paris). Fruits were transported immediately to the laboratory, and only whole and unwounded fruits were selected for the experiment. The following treatments were used: 0% (control), 1%, 3% and 5% (w/v) alginate coating. Sodium alginate (Sigma-Aldrich Co., Steinhein, Germany) was prepared according to previous reports (DIAZ-MULA *et al.* 2012; CHIABRANDO and GIACALONE, 2013), dissolved in hot water (45°C) with continuous shaking until the solution became clear. After cooling to 20°C, glycerol at 20% v/v was added as a plasticizer. Fruits were dipped twice in the fresh coating solution for 1 min to assure the uniformity of the coating of the whole surface. After, fruits were dried for 30 min under an air-flow heater at 25°C. Control fruits were dipped in distilled water. After drying, 30 cherries were placed in polypropylene (PP) punnets, weighed, and stored in a controlled chamber at 4°C and relative humidity of 90-95%. Six punnets for each treatment were prepared, and after 7, 14 and 21 days of cold storage, two punnets per treatment were taken at random and used for the analysis.

Quality properties measurements and weight loss

Quality measurements were determined at day 7, 14 and 21 of storage. Total acidity (meq/l), pH and the percentage of soluble solids (°Brix) were measured according to official methods (AOAC, 1995). The total soluble solids content (TSS) was determined using the juice from five cherries at 20°C. Three replicates were used for each treatment. Titratable acidity (TA) was determined by titration with 0.1 N NaOH up to pH 8.1, using 10 ml of diluted juice in distilled H_2O. Three replicates were used for each treatment.

For fruit firmness measurements, a hand-held Shore Durometer (T.R. Turoni, Italy) was used and 30 fruits (replicates) per treatment were an-

alyzed (KAPPEL *et al.*, 1996). Weight loss was determined in each punnet by the percentage weight loss with respect to day 0.

Color measurements

The color of coated cherries was measured at day 7, 14 and 21 of storage, individually for each fruit (30 for each treatment). The surface color was analyzed with a tri-stimulus CR-400 Chroma Meter (Konica Minolta Sensing) with D75 illumination and observation angle of 10° calibrated with a standard white plate (Y = 94.00, x = 0.3158, y = 0.3322, L* = 97.79, a* = -0.43, b* = + 2.25). Two readings of L* (lightness), b* (yellow chromaticity), and a* (green chromaticity) coordinates were recorded for each cherry. Numerical values of a* and b* parameters were employed to calculate the hue angle ($h° = \tan^{-1}(b^*/a^*)^2$) and chroma ($C = (a^{*2} + b^{*2})^{0.5}$). The reported values are the mean ± SD of 60 determinations.

Anthocyanin content, phenolic content and Total Antioxidant Capacity

The anthocyanin content, phenolic content and total antioxidant capacity were measured at day 0 and after 21 days of storage. For determination of the anthocyanin content, phenolic content and total antioxidant capacity, extracts were prepared by weighing 10 g of fresh cherries into a centrifuge tube, adding methanol (25 ml) and homogenizing the sample for 1 min. Extractions were performed under reduced light conditions. Tubes were centrifuged (3000 rpm for 15 min) and the clear supernatant fluid collected and stored at -26°C. For identification and quantification, extraction was performed as three replicates. The anthocyanin content was quantified according to the pH differential method of CHENG and BREEN (1991). Anthocyanins were estimated by their difference in absorbance at 515 and at 700 nm in buffer at pH 1.0 and at pH 4.5, where A = $(A515 - A700)_{pH1.0}$ - $(A515 - A700)_{pH4.5}$. Results are expressed as mg of cyanidin-3- glucoside (C3G) per 100 g of fresh cherries.

Total phenolics were determined with Folin-Ciocalteu reagent following the method of SLINKARD and SINGLETON (1977), using gallic acid as the standard. Absorption was measured at 765 nm. Results are expressed as mg gallic acid equivalents (GAE) per 100 g of fresh cherries.

The antioxidant activity was determined using a ferric reducing antioxidant power (FRAP) assay, following the methods of PELLEGRINI *et al.* (2003) with some modifications. The antioxidant capacity of the diluted cherry extract was determined by its ability to reduce ferric iron to ferrous iron in a solution of TPTZ prepared in sodium acetate at pH 3.6. Results are expressed as mmol Fe^{2+}/kg of fresh cherries.

Statistical analysis

The basic experimental design consisted of four coating treatments, each having three replicates. For each parameter evaluated, two punnets containing 30 fruits each were considered a replicate and all determinations were performed in triplicate. Data were analyzed by analysis of variance using statistical procedures in STATISTICA ver. 6.0 (Statsoft Inc., Tulsa, OK, USA). The sources of variance were the coating treatments. Tukey's test HSP (honestly significant differences) was used to determine significant differences among treatment means. Mean values were considered significantly different at $p \leq 0.05$. The mean values were calculated and reported as the mean ± SD (n = 3).

RESULTS AND DISCUSSION

Quality properties measurements and weight loss

Texture is a major factor defining the quality of fruit and strongly influences acceptability by consumers. The firmness values of cherries decreased, demonstrating texture softening during storage for both coated cultivars and control fruits, as shown in Table 1.

In cv Big Lory, the alginate coatings had a beneficial effect on fruit firmness. Retention of firmness can be explained by retarded degradation of the components responsible for the structural rigidity the fruit, primarily insoluble pectin and proto-pectin. The fruit firmness of cv Big Lory at harvest was 67.24 N; after 14 days of storage, this value increased and then decreased at the end of storage at 4°C, reaching a final value of 41.41 N for the control and 57.08 N, 53 N and 48.72 N for the 1%, 3% and 5% coatings, respectively (Table 1). By the end of the storage period, all the coating treatments gave rise to fruit with greater flesh firmness than the untreated fruit ($P < 0.05$) (Table 1). In addition, significant differences were noted between the alginate coating treatments: higher values for flesh firmness were found for fruit coated with 1% and 3% alginate. The beneficial effect of the alginate concentration on firmness has also been reported for strawberry (HERNÁNDEZ-MUÑOZA *et al.*, 2008), peach, Japanese pear, kiwifruit (DU *et al.*, 1997) and citrus (CHIEN *et al.*, 2007). The coating of fruits can be expected to modify the internal gas composition of fruits, especially reducing the oxygen concentration and elevating the carbon dioxide concentration, which might explain the delayed textural changes in the coated fruits.

In cv Grace Star, fruits tended to be less firm than cv Big Lory. At harvest, fruit firmness was 44.47 N; after 7 days of storage, this value increased and then decreased during storage at 4°C, reaching a final value of 32.87 N for the

Table 1 - Changes in the quality parameters of coated cherries (cv Big Lory and Grace Star) stored at 4°C for 21 days. Different letters in the same column indicate significant differences ($p \leq 0.05$). Column without letters have no significant differences.

Cv Big Lory Quality parameter	Treatments	Values at storage days 0	7	14	21
Firmness (N)	control	67.24	65.80	80.60 b	41.41 b
	sodium alginate (1%)	67.24	65.06	85.26 a	57.08 a
	sodium alginate (3%)	67.24	64.80	84.31 a	53.00 a
	sodium alginate (5%)	67.24	66.01	84.05 a	48.72 ab
Total soluble solids content (°Brix)	control	16.43	15.67 a	14.85 b	14.37 b
	sodium alginate (1%)	16.43	16.97 a	14.37 b	16.17 a
	sodium alginate (3%)	16.43	13.83 b	14.47 b	16.00 a
	sodium alginate (5%)	16.43	15.47 a	16.93 a	14.23 b
Titratable acidity (meq/l)	control	69.27	46.81 b	46.2 b	41.77 a
	sodium alginate (1%)	69.27	51.49 a	41.41 b	40.46 a
	sodium alginate (3%)	69.27	42.31 c	42.23 b	43.92 a
	sodium alginate (5%)	69.27	47.79 b	48.25 a	31.56 b
Cv Grace Star Quality parameter	**Treatments**	**0**	**7**	**14**	**21**
Firmness (N)	control	44.47	77.06 b	54.96 a	32.87 b
	sodium alginate (1%)	44.47	78.83 b	56.18 a	37.4 a
	sodium alginate (3%)	44.47	69.47 c	49.97 b	29.97 b
	sodium alginate (5%)	44.47	82.01 a	53.76 a	36.15 a
Total soluble solids content (°Brix)	control	17.87	18.16	18.3	17.17
	sodium alginate (1%)	17.87	18.27	17.03	16.13
	sodium alginate (3%)	17.87	18.3	18.5	17.97
	sodium alginate (5%)	17.87	17.43	18.27	17.7
Titratable acidity (meq/l)	control	127.6	113.59	113.24 b	105.01 b
	sodium alginate (1%)	127.6	112.48	113.95 b	101.53 b
	sodium alginate (3%)	127.6	112.6	123.36 a	114.82 a
	sodium alginate (5%)	127.6	110.91	113.03 b	116.43 a

control and 37.4 N, 29.97 N and 36.15 N for the 1%, 3% and 5% coatings, respectively (Table 1). Fruit softening was delayed in the 1% and 5% alginate-treated cherries, while the control and 3% alginate-treated cherries exhibited a significantly higher reduction in firmness (Table 1). For this parameter, the alginate concentration of 1%, according to the results obtained in cv Big Lory, was more effective than 3% and 5% alginate in reducing softening, especially at the last sampling date. The effects of edible coatings on decreasing softening have been also found in sweet cherry coated with Semperfresh™ (YAMAN and BAYINDIRLI, 2002) and with aloe vera gel (MARTÍNEZ-ROMERO *et al.*, 2006). According to the results obtained for cv Big Lory, a 1% alginate edible coating significantly slowed down the softening process compared to the other coated treatments.

In cv Big Lory, the TSS at harvest was 16.43° Brix, which decreased slightly during storage. At the end of storage, the 1% and 3% alginate coatings had a significant effect on TSS (Table 1), with significantly higher values compared with the control and 5% alginate coating. In this case, the 1% and 3% alginate coatings delayed the degenerative processes of the treated fruits. Grace Star showed higher TSS values compared to the cv Big Lory at harvest and during storage with similar values in all the samples. No significant differences in TSS values were found in relation to specific treatments, in accordance with the results of YAMAN and BAYOINDIRLI (2002).

A decrease in total acidity is typical during postharvest storage of fleshy fruit and has been attributed to the use of organic acids as substrates for respiratory metabolism (VALERO and SERRANO, 2010). In cv Big Lory, TA values decreased from 69.27 meq/l at harvest to 41.77 meq/l in control fruits after 21 days at 4°C, and 40.46 meq/l, 43.92 meq/l and 31.56 meq/l in cherries coated with 1%, 3% and 5% alginate, respectively (Table 1). Only samples coated with 5% alginate showed significantly lower TA values. In this case, the use of the coating did not limit the degradation of organic acids. In cv Grace Star, the storage period led to a similar drop in the acidity of all samples, including the control. However, at the end of storage, acidity losses were significantly higher in the 1% algi-

nate coated cherries and control, since after 21 days of storage was ≈ 20% and ≈ 18% respectively (Table 1) respect to ≈ 9% in the 3% and 5% coated fruits. A decline in acidity demonstrates advanced maturation, thus the coating on the fruits contributed to delaying fruit maturation/ripening.

In cv Big Lory, weight loss increased during storage, reaching values of 7.35% in control fruits after 21 days of cold storage and 8.15%, 7.4%, 8.25% in fruits coated with alginate at 1%, 3% and 5%, respectively, without significant differences between treatments. In cv Grace Star, after 21 days of storage, weight loss ranged from ≈10% in the control and 1% alginate coated cherries, to ≈ 12% in the 3% and 5% alginate coated cherries. Greater losses were found in Grace Star compared to Big Lory, probably due to the larger size of the fruits of this cultivar. In both cultivars, no significant reduction of weight loss was detected in cherries treated with the coatings (data not shown). The use of an edible coating did not lead to a general reduction in weight loss, as expected.

Color measurements

It is accepted that the most important quality parameters determining sweet cherry visual quality and acceptability by consumers are a bright red color and firmness (Crisosto *et al.*, 2003). Hue angle is an indicator of ripeness and it is expressed as $\tan^{-1}$ $(b^*/a^*)^2$. At harvest, Big Lory cherries had a red bright color with a hue angle of 24.44. During storage, an increase in hue angle was observed in all samples, in particular the coated cherries (Table 2) with final values of 27.43, 27.56 and 27.65 in cherries coated with alginate at 1%, 3% and 5%, respectively, similar to that found in other sweet cherry cultivars (SERRANO *et al.*, 2009). At the end of storage, the hue angle was significantly higher for all the coated fruits than in the controls (Table 2).

A decrease in hue angle could indicate the senescence process of sweet cherry, which is considered detrimental. In Big Lory, the coating treatments maintained the typical bright red color of recently harvested fruits with high

Table 2 - Changes in color parameters of coated cherries (cv Big Lory and Grace Star) stored at 4°C for 21 days. Different letters in the same column indicate significant differences ($p \leq 0.05$). Column without letters have no significant differences.

		Values at storage days			
Cv Big Lory **Color parameter**	**Treatments**	**0**	**7**	**14**	**21**
Chroma	control	33.88	27.91 b	31.66 b	26.26 b
	sodium alginate (1%)	33.88	33.76 a	34.56 a	31.56 a
	sodium alginate (3%)	33.88	33.89 a	30.96 b	33.34 a
	sodium alginate (5%)	33.88	33.78 a	28.72 b	30.46 a
Hue angle	control	24.44	23.39 b	26.32	24.56 b
	sodium alginate (1%)	24.44	26.03 a	26.64	27.43 a
	sodium alginate (3%)	24.44	25.09 a	25.47	27.56 a
	sodium alginate (5%)	24.44	25.53 a	24.85	27.65 a
Lightness	control	33.01	30.23 a	28.33 a	26.23 b
	sodium alginate (1%)	33.01	30.66 a	29.64 a	26.57 b
	sodium alginate (3%)	33.01	32.09 a	28.32 a	27.2 a
	sodium alginate (5%)	33.01	27.73 b	27.87 b	26.74 b
Cv Grace Star **Color parameter**	**Treatments**	**0**	**7**	**14**	**21**
Chroma	control	22.48	16.01 b	19.79 b	16.66 b
	sodium alginate (1%)	22.48	19.98 a	21.11a	18.57 a
	sodium alginate (3%)	22.48	15.62 b	18.46 b	16.87 b
	sodium alginate (5%)	22.48	21.50 a	19.20 b	17.92 a
Hue angle	control	21.92	22.2	26.38 a	27.08
	sodium alginate (1%)	21.92	22.28	25.81 a	25.72
	sodium alginate (3%)	21.92	22.05	26.77 a	26.55
	sodium alginate (5%)	21.92	22.1	23.46 b	26.64
Lightness	control	23.81	24.77	18.39 b	21.16
	sodium alginate (1%)	23.81	25.56	21.68 a	22.66
	sodium alginate (3%)	23.81	24.9	20.17 b	22.33
	sodium alginate (5%)	23.81	24.11	22.18 a	21.88

hue angle values during postharvest storage and even after 21 days of cold storage. In this case, the use of an alginate coating contributed to maintaining the original color of the cherries. The same trend was also observed in cv Grace Star, where hue angle values increased during storage, but without significant differences between treatments, in agreement with the results of YAMAN and BAYOINDIRLI (2002) with the Semperfresh™ coating, but in disagreement with the results of cv Big Lory, where the use of the alginate coating contributed to maintaining the original color of the cherries.

A decrease in L (lightness) is an indicator of fruit darkening. During storage, Big Lory darkened slightly as evidenced by decreasing values of L for control and all treated cherries (Table 2). By the end of the storage period, L decreased by around 21% for control fruit, by around 19% for fruit coated with 1% and 5% alginate and by 17% for fruit coated with 3% alginate. This result confirms that the alginate coating exert significant effects in maintaining the original color of Big Lory cherries. In Grace Star, the lightness values of the cherries increased after 7 days of storage and then showed a decreasing trend until the end of cold storage. After 21 days of storage at 4°C, there were no significant ($P \leq 0.05$) differences in lightness values between the treated samples and the control (Table 2). We may therefore conclude that the use of an alginate coating on Grace Star sweet cherries did not significantly alter, but rather improved the skin color or its evolution during storage at 4°C.

The changes in the chroma values (C) of Big Lory cherries during storage are presented in Table 2. Fruit developed less vivid coloration, as evidenced by lower values of C in cherry samples during storage. The reduction in C values was significantly greater for uncoated fruit, and significant differences were found between control and coated cherries ($P \leq 0.05$). Regarding the coated fruits, significant differences were found among samples treated with different concentrations of alginate, since 3% coated cherries showed higher C values. Chroma was reduced by around 30% for control and 10% for coated cherries.

In cv Grace Star, the main color changes were observed in the C values, which diminished during cold storage at 4°C, in particular in the control and 3% coated fruits. The values at 21 days were 25% and 24% lower, respectively, than those found at day 0 (Table 2).

Anthocyanin content, phenolic content and Total Antioxidant Capacity

Anthocyanins are responsible for the red color in sweet cherry (GARDINER *et al.*, 1993) and are beneficial to human health. In cv Big Lory, the anthocyanin content at harvest was 28.5 mg (C3G) and decreased significantly during storage (Table 3), in agreement with BERNALDE *et al.* (2003). After 21 days of storage, significantly lower levels were found in 5% alginate coated fruits with mean values of 18.12 mg (C3G).

In cv Grace Star, during post-harvest storage, anthocyanin significantly increased. Thus, the cherries became darker during storage as ripening progressed. Anthocyanin values showed that 5% alginate treatment delayed the ripening process, with significantly lower anthocyanin accumulation during storage compared with the other treatments (Table 3). Cherries coated with 1% and 3% alginate showed the greatest anthocya-

Table 3 - Values of anthocyanin contents, phenolic contents and total antioxidant capacity of coated cherries (cv Big Lory and Grace Star) at harvest and at the end after 21 days of storage. Different letters in the same column indicate significant differences ($p \leq 0.05$).

	Storage (days)	Anthocyanins (mg cyanidin-3- glucoside (C3G) per 100 g)	Polyphenols (mg gallic acid equivalents (GAE) per 100 g)	Antioxidant activity (mmol Fe^{2+}/kg)
cv Big Lory Treatments				
harvest	0	28.5	57.49	13.64
control	21	15.11 b	42.17 a	12.62 a
sodium alginate (1%)	21	15.14 b	41.41 a	12.00 a
sodium alginate (3%)	21	14.32 b	35.68 b	10.92 b
sodium alginate (5%)	21	18.12 a	39.88 ab	12.91 a
cv Grace Star Treatments				
harvest	0	53.85	194	15.33
control	21	64.65 b	156.56 a	15.17 a
sodium alginate (1%)	21	85.96 a	140.44 a	15.76 a
sodium alginate (3%)	21	76.15 a	128.22 b	15.07 a
sodium alginate (5%)	21	58.15 c	93.83 c	15.12 a

nin accumulation after cold storage. Post-harvest increases in anthocyanin have been previously reported for cherries and for other small red fruits like raspberries, plums and strawberries (WANG and STRETCH, 2001; SERRANO *et al.*, 2009; DÍAZ-MULA *et al.*, 2012). Anthocyanin accumulation during storage is attributed to normal sweet cherry ripening. WONG *et al.* (1992) suggested that the edible coating film forms a gas barrier, probably due to the dense structure of the film, so a possible modification of the internal atmosphere in coated samples due to film application could explain this behavior; this seemed to delay anthocyanin synthesis and/or degradation.

Polyphenols are important non-color compounds present in sweet cherry at harvest and during storage. These compounds not only contributed to the flavor but may also influence fruit color (MAZZA and BROUILLARD, 1990). The total polyphenol content decreased in both cultivars after 21 days of storage at 4°C and total polyphenol contents were significantly different between treatments (Table 3). In Big Lory, more pronounced changes were observed for the 3% and 5% coated samples, resulting in 37% and 30% losses, respectively (Table 3). In Grace Star, there was a 19-51% loss, depending on the treatment (Table 3). In the control and 1% alginate coated fruit, the decrease in the polyphenol content was significantly lower. The polyphenol content in the 5% coated fruit decreased during storage, and the value at 21 days of storage was 51% lower than that found at 0 days.

No changes in antioxidant capacity were observed during cold storage (Table 3). In particular, in Big Lory samples, the total antioxidant capacity at harvest was 13.69 mmol Fe^{2+}/kg and at the end of storage period this was 12, 10.92 and 12.91 mmol Fe^{2+}/Kg in cherries coated with alginate at 1%, 3% and 5%, respectively. Similarly, in Grace Star, the antioxidant activity remained stable during storage without differences between treatments (Table 3).

CONCLUSIONS

Alginate treatments can be used as a natural postharvest treatment in sweet cherry cultivars with the aim of delaying the postharvest ripening process and maintaining fruit quality. Alginate treatment at 1% and 3% was effective in delaying weight and acidity losses, softening and color changes in the cultivars Big Lory and Grace Star. In terms of the antioxidant properties, no significant results were obtained using the alginate coating. The results of this study suggest that alginate treatments at 1% and 3% can be used as natural postharvest treatments to improve cherry quality after harvest.

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PAPER

HOW SENSORY AND HEDONIC QUALITY ATTRIBUTES AFFECT FRESH RED MEAT CONSUMPTION DECISION OF TURKISH CONSUMERS?

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ABSTRACT

The aim of the study is to explore how the sensory and hedonic quality attributes of the fresh red meat affect its consumption preference and amounts of Turkish consumers. The data obtained from 385 households in Erzurum were used for *Principal Component (PCA)*, *K-means Cluster* and *Multiple Regression/Correlation (MRC) Analyses.* The results of the study highlighted considerably that the sensory quality attributes on their consumption preference had a much bigger effect than the hedonic ones for each cluster. However, its price and their income accepted as the important indicators of the hedonic ones, but a much lower impact on all clusters.

- Keywords: Fresh red meat consumption, Principal Component, K-Means Cluster and Multiple Regression Analyses, Sensory and hedonic quality attributes -

INTRODUCTION

In recent years, the increases of the red meat prices due to the contractions in the meat supply covering the production, processing and marketing of the meat and the meat products obliged the political units of the government to import live red meat materials, and thus they have provided stabilization at some levels for the meat supply and price (YAVUZ *et al.*, 2013). However, the origins of the meat sources among the consumers consuming the fresh red meat have been considered as an important decision factor. A few portion of them, therefore, exhibited the buying attitude and behaviors within a moderate consumption trend of those preferred the meat sources imported while the others formed the purchase models with a positive motivation in the red meat consumption preferences based on the national meat sources.

There was a string relationship between the origin of the red meat sources as an indicator of the consumers' purchase decisions and both the sensory quality including in its intrinsic quality attributes and the hedonic quality consisting of its extrinsic quality attributes (BERNUES *et al.*, 2003). The strong motivational effects of their individual, demographic, socioeconomic, psychological and health characteristics, on the other hand, are among the primary preference factors, as well (TOPCU, 2012).

Turkish consumers' purchase decisions based on the food safety and quality of the fresh red meat have changed considerably due to its confidential and sensory quality attributes affected directly by microbiological insecurity (dioxin effect, BSE and salmonella diseases) of foreign-origined red meat sources, and by the contamination risks with various pollutants (antibiotic and hormones) in the last years (TOPCU, 2015; TOPCU and UZUNDUMLU, 2012). They have also concerned about deterioration of their health conditions with the meat consumption of the imported animals being exposed to genetic manipulations, and then about inheriting to the future generations of the negative phenomenon (TOPCU, 2012).

In addition to imported live animal sources, presence of unhealthy fresh red meats penetrating to domestic markets uncontrollably and illegally, unknown origin, exposing to some additives at levels threatening human health, and the pollution, contamination and microbiological insecurity of the manufacturing meats in the livestock farms and meat processing facilities under unhygienic conditions have caused the consumers to exhibit more sensitive purchase attitude and behaviours by considering the sensory quality attributes (YAVUZ *et al.*, 2013).

On the other hand, not only digestive and coronary heart diseases caused by obesity based on the passive lifestyles revealed the communication and information age but also chronic illnesses resulting from nutrition with the intensive protein diets consisting of the red meats and the impacts of the life circle of the mature people have gradually reduced the consumers' red meat consumption tendencies, and thus nowadays their purchase decisions towards the red meats have been changing habitually (REALINI *et al.*, 2013; TOPCU, 2012).

The analyses of the customers reviews/assessments about the intrinsic quality attributes having a direct relationship between the sensory quality and confidential attributes (food quality measured by the chemical and microbiological tests) of the red meats such as the structural and visual characteristics (REALINI *et al.*, 2013; McCARTY *et al.*, 2003) are of a much more importance. On the other hand, they also focus on the extrinsic quality attributes correlating with the hedonic quality attributes such as the actual product image, the cost to customer, the origin of the meat source, disposable income (TROY and KERRY, 2010; BURNUES *et al.*, 2003; McCARTY *et al.*, 2003), and reflecting the consumers' individual characteristics and determining their purchase decisions about the fresh red meat. Therefore, eliminating the factors affecting negatively their consumption satisfaction and loyalty accepted at the focal point of the production, consumption and marketing activities, determining the main attributes accelerating the consumption trends of the homogenous target consumers masses, and then designing the marketing tactic and strategies for them could provide the important advantages in terms of the efficiency of the production resources and the maximization of the expected utilities on all market dynamics.

The diet meeting the main requirements of the people by providing the metabolic energy needed for biological organisms consists of the plant and animal foods, and thus they must be consumed to sustain a healthy and balanced life of the people at an adequate level (TOPCU and UZUNDUMLU, 2012). In particular, the animal-derived proteins such as the meat, milk, eggs, cheese being of a sufficient level for the essential amino acids are also suitable in terms of digestion. About 75-80% of high-quality animal-derived proteins are transformed into the body proteins. In contrast, plant-derived proteins classified as low-quality protein have an insufficient level of some essential amino acids, and it is benefited from only 40% of their proteins since their digestion is fairly difficult (TOPCU, 2012).

A person must consume 70 gr proteins per day for an adequate and balanced diet; therefore, it must be at least half of animal origin protein (SEKER *et al.*, 2011). The consumption amounts of the animal origin foodstuffs per capita are very low in Turkey, but that of the plant-derived foods is greater than that of the developed countries (KARKACIER, 2000). If people fed with cereal-based food products give more weight into

the animal origin ones in Turkey, and they gain the proper eating habits, it could be contributed significantly to the improvement of the life quality of the community.

Today, the consumption levels of the animal-origin products are considered as a development indicator of the countries, and thus as their socioeconomic structures improves, the consumption amount of the protein foods increases in contrast to reduce that of the carbohydrate foods (YAYLAK *et al.*, 2010). As a result of all this, the meat includes an important part of people's daily diets in USA, EU and other developed countries, and it meets 15%, 40% and 20% of the energy, protein and fat requirements per day, respectively, but its consumption fluctuates significantly in various regions of the world (DANIEL *et al.*, 2011). For example, while the annual meat consumption amounts per capita in 2011 were calculated as 142, 125, 82 and 80 kg in Austria, USA, Germany and UK, representing the leader countries in the meat production and consumption, respectively, it was only 12 kg in Turkey. According to the data obtained from the research area (Erzurum), on the other hand, the annual meat consumption amount per capita calculated as 17.8 kg in 2008, 12.5 and 11.5 kg in 2011 and 2012 were found (TOPCU and UZUNDUMLU, 2012).

The substitution of the imported red meats instead of the red meat derived from the animal husbandry based on a quality pastures and a wide variety flora having a significant impact on the red meat quality caused the consumers' preferences shift to other meat groups in the research area, and their red meat consumption trends decreased significantly by influencing negatively its sensory and hedonic quality attributes (TOPCU and UZUNDUMLU, 2012).

As stated above, the annual red meat consumption amount per capita in the research area and Turkey was about 5-8 times less than that in developed countries, but this difference continued to increase steadily, in the last five years. Especially, there has been an increasing trend towards the poultry meat consumption due to the dissatisfactions of not only the sensory quality attributes at the meat production, manufacture and retail levels based on the imported meat materials but also the hedonic quality attributes pointing the actual product images. However, it has been experienced the significant changes such as the region origin and the types of the red meat sources in the consumption preferences of the consumers obtained from the red meats about 58, 34 and 36% of the total meat consumption in the developed countries, Turkey and the research area, respectively (McAFEE *et al.*, 2010; LICHTENSTEIN *et al.*, 2006).

The red meat consumption amounts at much low levels in Turkey and the study area could result from not only the sensory and hedonic quality attributes of the fresh red meat but also the socioeconomic and individual attitudes of the consumers and the confidence to the manufacturer, marketer and retailers (REALINI *et al.*, 2013; TROY and KERRY, 2010; McCARTY *et al.*, 2003). However, the supply amount of the fresh red meat in the research area is higher than its demand amount due to its high sensory quality attributes resulting from the advantages of the organic cattle fattening farming based on the quality pastures and rich flora varieties under the agroecological and topographical properties at the high mountainous areas of the region (TOPCU and UZUNDUMLU, 2012). The consumers, therefore, considering the advantages of both the hedonic quality attributes reflecting the images of the core and actual products focused on the numerous factors of the marketing mix and higher sensory quality attributes have preferred and consumed intensively them. As a result, the consumers residing in the research region and migrating from the research area to the different regions of Turkey have not only maintained their demands for the red meats with the region of the origin to provide much higher main utility but also contributed to the regional/rural development through the improvement of the farmer families' life qualities.

On the other hand, the inefficient policies implemented to meet the fresh meat deficit without effect on the rural/regional developments and the sensory quality attributes affecting negatively the consumer satisfaction with the penetration of the imported red meat to domestic markets caused by the supply contraction of the red meat have led the consumers with dissatisfactions to reflect the different purchase decisions, in the last decade. All these individual responses of the consumers about the fresh red meat and their purchase decisions have emerged as the acceptable results of the relationships between the sensory and hedonic quality attributes.

This study, therefore, was designed to reach all the objectives mentioned above. In this scope, the main aims of the study are to determine how the sensory and hedonic quality attributes affect the fresh red meat consumption decisions of Turkish consumers; and then to construct the target homogenous consumer segments based on their consumption frequencies, and finally to analyze the effectiveness of the factors effecting on their consumption amounts.

MATERIAL AND METHODS

Material

The preliminary data used in this study were obtained from a survey consisting of Turkish consumers' attitude and behaviors with respect to the fresh red meat consumption de-

cisions conducted in Erzurum,[1] Turkey. In order to determine the sample size (selected statistically from the local household consumers through simple random sampling method) population minimizing sample bias and representing the main population correctly, the city center was divided into three districts covering Aziziye, Palandoken and Yakutiye districts with 6.562, 30.022 and 44.075 households at the west, south and north-east parts of Erzurum, respectively (ANONYMOUS, 2013).

METHODS

Method used in determination of the sample size

In order to calculate the sample size for each district, the following formula was used (TOPCU *et al.*, 2010).

$$n = \frac{Z^2 * p * (1 - p)}{c^2} = 385$$

Where, n = sample size, Z = z value (1.96 for 95% confidence level), p = percentage making a choice (0.5 used for sample size needed), c = confidence interval (used 0.05 = ±5). Then, based on the population of each district, the weighted sample size and distribution of the surveys for each district were determined proportionally. A total of 385 surveys were distributed with 52, 136 and 197 questionnaires allocated to the Aziziye, Palandoken and Yakutiye Districts, respectively.

Methods used in the preparation of the questionnaires

Participants of the survey were asked to respond to each statement indicating the significance level of the fresh red meat attributes for them. A liker-type scale was used (where 1 refers to the least important and 5 refer to the most important attribute). Table 1 reported that 35 attributes of the fresh red meat consisted of 17 sensory quality attributes and 18 hedonic quality ones (TOPCU, 2015; TOPCU and UZUNDUMLU, 2012). Of five numeric variables referring to the consumers' socioeconomic characteristics, on the other hand, two referred to their monthly fresh red meat consumption amount and purchase frequency and three included in their monthly income (₺), the price per kg of the fresh red meat (₺/kg), and the share of its expenditure within total food one.

Methods used in the statistics analyses

After editing and coding, the primary data were first used in *Principal Component Analysis (PCA)*[2] to determine the main factors related to the product attitudes influencing on the consumers' fresh red meat purchase patterns. *PCA* is a data reduction technique that reduces the number of variables used in an analysis by creating new variables (called factors) that combine redundancy in the data (SPSS 15.0, 2006). The first step in *PCA* is to determine the number of relevant factors. This was conducted by *PCA* using the varimax rotation method *(VRM)*[3]. *PCA* was used initially to identify underlying aspects explaining a correlation among a set of the food product attributes. The purpose of *PCA* was to identify those attributes accounting for a relatively large proportion of the variance in the sample.

In the second and final step of the statistical analyses, the main factors obtained from *PCA* were used for *k-means cluster and multiple regression/correlation MRC Analyses,* respectively. In the second step, according to the fresh red meat consumption frequencies, therefore, the target consumers were separated to three homogeneous clusters including in light users (2-3 times per month), medium users (2-3 times per 15 days) and heavy users (2-3 times per week) (TOPCU, 2012), and then the main factors were allocated to the homogeneous consumer clusters based on the meat consumption frequencies of the target consumers by *k-means cluster analysis.*

In the final step, *MRC analysis* was used for the measurements of the efficiency of the main factors and socioeconomic variables (independent variables) effecting on the consumers' meat consumption amounts (dependent variable). In the *MRC* model taken into consideration the variables related to the main factors obtained from the *PCA* and some socioeconomic characteristics of the consumers, therefore, it was tried to measure the relationships between monthly consumption amount of the fresh red meat per household as a dependent variable and the main factors including in their sensory and hedonic quality attributes as long with the consumers' socioeconomic characteristics called as the independent variables.

In order to test whether or not the normal distribution of the main factors' group scores de-

[1] Erzurum (39°45'N latitude and 41°15'E longitude) is located in the north-east region of Turkey and is on the Silk Road. Erzurum is one of the biggest provinces in the region with a total population 778.195, and the city centre population is 509.474.

[2] A factor extraction method used to form uncorrelated linear combinations of the observed variables. The first component has the maximum variance. Successive components explain progressively smaller portions of the variance and are all uncorrelated with each other. PCA is used to obtain the initial factor solution. It can be used when there is a single correlation matrix.

[3] This method is an orthogonal rotation method that minimizes the number of variables that have high loading on each factor. It simplifies the interpretation of the factors.

rived from *PCA* and socioeconomic variables collected from the consumers exhibited, was applied the various transformations techniques, and finally logarithmic transformation one providing the closest distribution to the normal was chosen. On the other hand, the parameter coefficients were estimated by using *ordinary least squares (OLS).* Individual and group significance of these coefficients were tested using *t* and *F* tests, respectively. In order to evaluate whether to be any econometrical problem among the variables, it was tested the overall multicollinarity and auto-correlation problems by considering *the variance-inflating factor (VIF)* and *Durbin-Watson d statistics*, respectively. Multicollinearity among variables was detected by calculating *(VIF)* (GUJARATI, 2005; SPSS 15.0, 2006).

MRC model could be written as following equation:

$$FMCONS = f(AROMA, MICSAF, CRERIS, VISQLY, IMAG, NUTVAL, COSCON, MESDUR, ORGLOC, FORORG, MEPRC, DISINC, e_i)$$

Dependent Variable

FMCONS:	*Monthly fresh red meat consumption amount (kg)*

Independent Variables

AROMA :	*Aroma of the fresh red meat (sensory quality attribute)*
MICSAF :	*Microbial safety (associated with sensory quality attributes)*
CRERIS :	*Chemical residue-related risk (associated with sensory quality attributes)*
VISQLY :	*Visible quality (sensory quality attribute)*
IMAG :	*Accual fresh meat image (hedonic quality attribute)*
NUTVAL :	*Nutritional value (associated with sensory quality attributes)*
COSCON:	*Cost to the consumer (hedonic quality attribute)*
MESDUR:	*Meat sources and its durability (associated with hedonic quality attribute)*
ORGLOC:	*Local-origined organic meat willingness (hedonic quality attribute)*
FORORG:	*Foreign-origined meat willingness (hedonic quality attribute)*
MEPRC :	*The price of the meat ($) (hedonic quality attribute)*
DISINC :	*The disposable income (hedonic quality attribute)*

All analyses were run in sequence with the SPSS 15.0 statistical software. These techniques were used widely to analyze the meat attributes playing the important roles on the consumers' consumption preference and amounts in marketing studies (TOPCU and UZUNDUMLU, 2012; KRYSTALLIS *et al.*, 2007; DIEZ *et al.*, 2006; OLIVER *et al.*, 2006; VERBEKE and VACKIER, 2004; BERNUES *et al.*, 2003).

RESULTS AND DISCUSSION

Results of PCA related to the sensory and hedonic quality attributes of fresh red meat

Kaiser Normalization (KMO) which compares partial correlation coefficients with observed ones including in the sensory and hedonic quality attributes effecting on the fresh red meat consumption preferences of the Turkish consumers was calculated as 0.85, and this means that the data set for the *PCA* was at a perfect level since the test score was greater than 0.5 (Table 1). On the other hand, the *chi-square value* calculated for *Bartlett's test of Sphericity statistics* of their main factors with respect to the consumers' meat consumption preferences was calculated as 5060.68 *(p: 0.000),* and thus *the unit matrix hypothesis* was rejected ($p<0.01$). The *PCA* using *varimax rotation method* grouped the thirty-seven variables related to the sensory and hedonic quality attributes for fresh red meat into the ten main factors with Eigen-values greater than 1, and the main factors explained the 71% of the total variance (Table 1).

As shown in Table 1, the results of the study showed that *aroma (F1)* and *visible quality (F4)* affecting the fresh red meat consumption preferences of Turkish consumers by explaining about 29.6 and 5.3% of total variance constituted directly the first and second sensory quality factors, respectively. By having an important impact on the quality attributes of *F1* and *F4;* moreover, *microbial safety (F2)* and *chemical residue-related risk (F3)* associated much closely with the sensory quality attributes of the fresh red meat were interpreted as the second and third sensory quality factors indicating about 7.5 and 7.1% of total variance. The same main factors for the sensory quality attributes based on the various similar variables taken into consideration by TOPCU and UZUNDUMLU (2012), KERGOAT *et al.* (2010), TROY and KERRY (2010), AASLYNG *et al.* (2007), KRYSTALLIS *et al.* (2007) were reported in their previous researches, as well.

On the other hand, the results of the present study indicated that *actual product image (F5); nutritional value (F6), cost to the consumer (F7) and meat sources and durability (F8) of the fresh meat; local-origined organic meat willingness (F9)*

Table 1 - The measurement results of *PCA* with regard to the sensory and hedonic quality attributes based on the fresh red meats consumption decisions of Turkish consumers.

Factos interpretions and the variables	**Factor loadings***									
	F1	**F2**	**F3**	**F4**	**F5**	**F6**	**F7**	**F8**	**F9**	**F10**
AROMA *(F1: AROMA)*										
Flavour	**0.841**	0.078	0.121	0.127	0.032	0.127	0.102	0.039	0.015	0.029
Succulence	**0.831**	0.076	0.061	0.138	0.047	0.076	0.014	-0.007	-0.035	-0.014
Smell/odour	**0.750**	0.083	0.115	0.083	0.205	0.051	0.026	0.165	-0.035	-0.038
Juiciness	**0.705**	-0.003	0.086	0.146	0.017	0.178	0.054	0.120	0.099	0.277
Visible fat	**0.530**	0.251	-0.021	0.070	-0.014	0.246	0.217	0.068	0.355	-0.333
MICROBIAL SAFETY *(F2: MICSAF)*										
Dioxin scare	0.079	**0.880**	0.192	0.088	0.103	0.071	0.037	0.008	0.112	-0.019
Salmonella disease	0.088	**0.875**	0.096	0.120	0.096	0.162	0.053	0.040	0.049	0.006
BSE	0.081	**0.802**	0.133	0.151	-0.004	0.130	0.119	0.050	0.044	0.011
Trustworthiness	0.090	**0.666**	0.073	0.119	0.209	0.262	0.163	0.122	-0.188	-0.242
CHEMICAL RESIDUE-RELATED RISK *(F3: CRERIS)*										
Meat manufacturing methods	0.139	0.161	**0.698**	0.090	0.178	0.108	0.087	0.086	0.059	-0.013
Antibiotic residues	0.128	0.352	**0.687**	0.022	0.220	0.172	0.010	0.064	-0.111	0.193
Hormone residues	0.017	0.449	**0.603**	0.153	0.092	0.148	0.065	0.156	-0.163	0.122
VISIBLE QUALITY *(F4: VISQLY)*										
Freshness of the meat	0.346	0.164	0.010	**0.789**	0.028	0.155	0.085	-0.038	0.032	0.039
Meat part obtained from the animal	0.038	-0.007	0.077	**0.772**	0.084	0.091	0.098	0.211	0.013	0.080
Natural structure of the meat	0.197	0.103	0.138	**0.762**	0.161	0.164	0.074	-0.015	0.064	0.065
The meat colour	0.104	0.234	0.093	**0.740**	0.149	0.121	0.119	0.154	0.001	-0.078
Tenderness	0.140	0.053	0.342	**0.527**	0.077	-0.012	0.210	0.231	0.237	0.244
ACCUAL FRESH MEAT IMAGE *(F5: IMAG)*										
Brand name	0.179	-0.020	0.239	0.113	**0.769**	-0.006	0.041	0.021	0.145	0.016
Package	0.029	0.142	0.012	0.130	**0.672**	0.133	0.306	0.274	-0.006	0.080
Advertisement	-0.066	0.002	0.141	0.181	**0.647**	0.055	0.152	0.251	0.398	-0.114
Free of additives/natural product	0.264	0.144	0.190	0.091	**0.627**	0.193	0.173	-0.044	-0.030	0.005
Labelling	0.134	0.134	0.156	0.068	**0.605**	0.151	0.474	0.131	-0.151	0.055
NUTRITIONAL VALUE *(F6: NUTVAL)*										
Brand name	0.112	0.111	0.200	0.216	0.153	**0.832**	0.000	0.091	0.070	0.072
Package	0.206	0.153	0.230	0.183	0.115	**0.820**	0.056	0.097	0.114	0.109
Advertisement	0.248	0.254	0.147	0.139	0.072	**0.764**	0.168	0.018	-0.064	0.021
Organic product	0.172	0.029	0.188	0.100	0.175	**0.754**	0.171	0.095	0.064	-0.044
COST TO THE *CONSUMERS (F7: COSCON)*										
Discounts in the meat prices	-0.019	0.136	0.220	0.158	0.143	-0.031	**0.707**	0.065	0.295	0.006
The initial price of the meat	0.120	0.114	0.040	0.069	0.253	0.098	**0.702**	-0.030	-0.103	-0.029
Outlets/sales points of the meat	0.187	-0.034	0.097	0.146	0.154	0.087	**0.693**	0.238	0.196	0.041
WILLINGNESS TO BUY MEAT SOURCES AND ITS DURABILITY *(F8: MESDUR)*										
Beef	0.180	0.068	0.144	0.212	0.002	0.064	0.113	**0.777**	0.030	-0.037
Sheep and goad meats	0.052	0.024	0.036	0.026	0.253	0.046	-0.017	**0.747**	0.221	0.049
The shelf life of the meats	0.330	0.050	0.245	0.063	0.105	0.104	0.327	**0.551**	-0.128	-0.024
LOCAL-ORIGINED ORGANIC MEAT *(F9: ORGLOC)*										
Willingness to buy local-orig. meat	0.301	-0.055	0.159	0.124	0.094	0.309	0.020	-0.007	**0.593**	0.127
Willingness to buy organic meat	0.068	0.192	0.076	0.222	0.136	0.239	0.137	0.235	**0.582**	0.130
WILLINGNESS TO BUY FOREIGN-ORIGINED MEAT *(F10: FORORG)*										
Willingness to buy foreign-orig. meat	-0.055	0.145	-0.019	0.029	0.107	0.063	0.118	0.113	0.062	**0.803**
Eiqen-values	*10.655*	*2.689*	*2.554*	*1.914*	*1.545*	*1.452*	*1.233*	*1.173*	*1.119*	*1.034*
Share of explained variance (%)	***29.598***	***7.468***	***7.095***	***5.318***	***4.292***	***4.033***	***3.426***	***3.263***	***3.109***	***2.872***
Cumulative share of that (%)	***29.598***	***37.066***	***44.160***	***49.478***	***53.763***	***57.803***	***61.229***	***64.492***	***67.600***	***70.472***
KMO (Kaiser-Meyer-Olkin) statistic	***0.848***									
Bartlett's test of Sphericity										
										[Chi - square (^{2}, *df*:630):5060.68](p:0.000)

* Bold numbers indicated the largest loading for each variable.

and foreign-origined meat willingness (F10) constructing the hedonic quality attributes of the fresh red meat consisted of about 4.3, 4.0, 3.4, 3.3, 3.1 and 2.9% of total variance, respectively (Table 1). When the obtained main factors related to the hedonic quality attributes in the present study were compared with the others presented by the results of the previous researches; while the *F5, F6 and F7* factors with respect to the hedonic quality were supported directly with the results of the studies conducted by FURNOLS *et al.* (2011), KRYSTALLIS *et al.* (2007), AUDEBERT *et al.* (2006), BERNUES *et al.* (2003) and VERBEKE and VACKIER (2004), the *F8, F9 and F10* factors focused on the attributes of the hedonic quality were of the strong relationships with the results of those presented by FURNOLS *et al.* (2011), NAPOLITANO *et al.* (2010), BERNUES *et al.* (2003), McCARTY *et al.* (2003).

As a result of all these, the sensory quality attributes explaining about 70% of the main factors effecting on the fresh red meat consumption preferences of Turkish consumers were of a much bigger influence than the hedonic quality ones.

Results of cluster analysis related to the sensory and hedonic quality attributes of fresh red meat

The sensory and hedonic quality attributes derived from the *PCA* and effecting on the fresh red meat purchasing patterns of Turkish consumers were separated into three homogeneous consumers segments through *k-means cluster* according to their red meat purchasing frequencies including the light, medium and heavy users. The main factors reflecting Turkish consumers' attitude and behaviors impacting on each homogeneous consumers segment were given in Table 2.

The results of the study showed that *the heavy users of the fresh red meat (C1)* preferred its consumption according to *the aroma and visible quality* being the main components of the sensory quality by taking into consideration *the local-origined organic meat willingness* accepted as an indicator of the hedonic quality attributes. Therefore, the homogeneous consumers in the *C1* determined their purchase attitude and behaviors by considering the sensory quality attributes of the red meat delivered from the animal materials fattened under the advantages of the superior agroecological characteristics of the study area. The fresh red meat presentations positioned at the retailer shelves by highlighting their core benefits, and causing no changes on the sensory quality attributes of them with the designation of the local-origined meat under the generic brands for the consumers in *C1* could have an important impact on their purchase patterns.

The results of the study also indicated that *the medium users of the fresh red meat (C2)* focused on the purchase patterns constructed by a combination of the factors affecting the sensory quality including in *the microbial safety* and *chemical residue-related risk* associated with willingness to obtain the salutary meats minimizing the negative effects on human healthy at the feeding and nourishment levels of the livestocks and the manufacture levels of the red meats. They, moreover, paid a major attention to the hedonic quality related to *the nutritional value* and *the cost of the ownership* minimizing their expenditures by being provided easily and

Table 2 - Final cluster centres and the number of cases in each cluster.

Main factors	Clusters*		
	Heavy users (C1)**	Medium users (C2)**	Light users (C3)**
AROMA (F1)	**0.465**	-0.122	-0.127
MICSAF (F2)	-0.751	**0.423**	-0.174
CRERIS (F3)	-0.641	**0.342**	0.073
VISQLY (F4)	**0.156**	-0.241	0.076
IMAG (F5)	-0.263	-0.034	**0.333**
NUTVAL (F6)	-0.955	**0.328**	**0.215**
COSCON (F7)	-0.042	**0.079**	-0.009
MESDUR (F8)	0.117	-0.890	**0.473**
ORGLOC (F9)	**0.330**	-0.423	0.108
FORORG (F10)	-0.078	-0.320	**0.222**
Disposable income *(DISINC)*	**3.570**	**3.280**	**2.980**
The prices of the meats *(MEPRC)*	**1.340**	**1.330**	**1.330**
Number of total cases in each cluster ***	81	193	111
% of total cases in each cluster	21%	50%	29%

* Bold numbers indicate the largest final cluster centre scores for each factor.

** According to F statistics, the final cluster center scors were found very importance ($p<0.01$).

*** The total number of the cases (n): 385.

comfortably to the consumer of *C2*. The implementations of differentiated actual product strategies based on the price discriminations under own brands of the manufacturers of the red meat with food safety that there was no negative impact on their health, therefore, could also affect positively their purchasing motivation by emphasizing the core benefits of the red meat for *C2*.

The results of the study, furthermore, explained that *the light users of the fresh red meat (C3)* tended to buy the fresh red meat with the hedonic quality attributes positioned to the focus point of the purchase attitude psychology alienated under *the actual fresh meat image* with *the foreign-origined meat willingness*, *its resources and durability*. They, thus, preferred the national or global brands exhibiting the actual fresh red meat image by turning to durable foreign-origined meat sources with long shelf life. It could be implemented the marketing tactic and strategies focused on the designs of the actual fresh red meat image under the name of the national and global brands for the consumers in *C3*.

Results of MRC analysis related to the sensory and hedonic quality attributes of fresh red meat

The results of the statistical tests in Table 3 reported that the *VIF* values with 1.06 and 2.47 indicating the scores between 1.00 and 2.50 determining the acceptable reference range and 1.85 *Durbin-Watson d statistics* positioned between d_u (1.96) and $4\text{-}d_u$ (1.68) referred that there were no the econometrics problems for *multicollinarity* and *auto-correlation* in the *MRC* model (GUJARATI, 2005; KALAYCI, 2005). According to the results diagnosing the econometrics problems, the data sets could be used directly for the *MRC* model.

The determination statistics, *OLS* estimates of the parameter coefficients and other statistic measurements such as *F* and *t*, collinearity and correlation matrix scores were given in Table 3. The results of the *MRC* analysis indicated that the determination coefficient *(R^2)* and adjusted *(adj.) R^2* was calculated as 0.51 and 0.50 in the *MRC* model, and thus all the independent variables explained the least 50% of the dependent variable. Rejecting the null hypothesis, furthermore, referring that there was no the relationships between the dependent variable and all the independent variables by making the parameter coefficients of them equal to zero, *F-statistic* was calculated as 6898 *(p:0.000)*. On the other hand, the partial regression coefficients of all the independent variables taking into consideration *t-statistics*, except for those that of *the microbial safety* (*MICSAF*), *the cost to the consumers* (*COSCON*), *the meat sources and its durability (MESDUR)* and *the foreign-origined meat willingness (FORORG)*, were statistically found to be meaningful ($t_{c(df;12;0.01/0.05)}$); and then the signs of their coefficients including in *the chemical residue-related safety (CRERIS)* and *the price of the meats (MEPRC)* with negative and the others with positive ones at the levels *(p:0.01* and *0.05*) were also found consistent with the economic theories.

The results of the *MRC* analysis also highlighted that *the visible quality* (*VISQLY*), *the aroma (AROMA)*, *the nutritional value (NUTVAL)*, *the accual fresh meat image (IMAG)*, *MEPRC*, *the local-origined organic meat willingness (ORGLOC)*, *CRERIS*, *the disposable income (DISINC)* were of very important impacts on the fresh red meat

Table 3 - The measurement results of the *MRC* analysis and some statistic tests.

n: 385	R^2: 0.51			Adj.R^2: 0.50		$F_{c(12;361)}$: 6.898*		DW d_c: 1.85	
Variables	**MRC model**			**Collinearity statistics**			**Correlations**		
	β:Coefficients[a]	**Std. error**	**t_c-value**	**p-value**	**Tolerance**	**VIF**	**Zero-order**	**Partial**	**Part**
Constant	1.478	0.563	2.623	0.009*	-	-	-	-	-
LOGAROMA	0.484	0.189	2.556	0.011*	0.374	2.472	0.027	0.133	0.121
LOGMICSAF	0.023	0.093	0.244	0.807	0.550	1.818	0.037	0.013	0.012
LOGCRERIS	-0.161	0.086	-1.868	0.053**	0.559	1.788	0.102	-0.098	-0.088
LOGVISQLY	0.553	0.194	2.844	0.005*	0.487	2.483	0.093	0.148	0.135
LOGIMAJ	0.245	0.086	2.845	0.005*	0.461	2.167	0.115	0.148	0.135
LOGNUTVAL	0.370	0.136	2.716	0.007*	0.413	2.418	0.035	0.142	0.129
LOGCOSCON	-0.107	0.084	-1.277	0.202	0.603	1.658	0.016	-0.067	-0.060
LOGMESDUR	0.008	0.081	0.095	0.924	0.682	1.465	0.022	0.005	0.005
LOGORGLOC	0.210	0.109	1.920	0.056**	0.428	2.338	0.078	0.101	0.091
LOGFORORG	0.004	0.026	0.156	0.876	0.778	1.285	-0.019	0.008	0.007
LOGMEPRC	-0.240	0.091	-2.633	0.009*	0.947	1.056	-0.140	-0.137	-0.125
LOGDISINC	0.106	0.072	3.791	0.001*	0.886	1.129	0.133	0.078	0.070

[a] The coefficients consisted of the standardized coefficients.
* (p<0.01) **(p<0.05).

consumption amounts of Turkish consumers ($p<0.01$ and 0.05). In relation to the profiles of the cluster segments based on the underlying dimensions of the sensory and hedonic quality attributes emerged from *PCA*, the satisfaction of *VISQLY* and *AROMA* identifying the sensory quality attributes of the fresh red meat supported by *ORGLOC* of Turkish consumers in *C1* increased considerably very high effects on their meat consumption amounts with 0.55 ($p<0.01$), 0.48 ($p<0.01$) and 0.21 ($p<0.01$) rates, respectively.

When considered the credence quality characteristics assigned to the sensory quality attributes affecting the fresh red meat consumption amounts of Turkish consumers in *C2*, there was an adverse relationship between *CRERIS* (β: -0.16 $p<0.05$) and its consumption amounts, but not *NUTVAL* (β: 0.37 $p<0.01$). As minimized its *CRERIS,* and improved its *NUTVAL,* therefore, it could be increased those at a lower level than all the others. *IMAG* (β: 0.24 $p<0.01$) standing for the hedonic quality attributes and *NUTVAL* (β: 0.37 $p<0.01$) symbozing the sensory ones, on the other hand, had a moderate impact on those of them in *C3.* Overall, the average agreement of all the cluster members in relation to *the price of the meat (MEPRC)* exhibiting an adverse relation with its consumption amounts occurred at a moderate level (β: -0.24 $p<0.01$), but *the disposable income (DISINC)* having a linear relation with those substantiated at lower levels (β: 0.11 $p<0.01$) than the others.

The results of the present study confirmed briefly that the sensory quality attributes, especially *VISQLY, AROMA, NUTVAL*, on the fresh red meat consumption amounts had a much higher impact than the hedonic quality attributes such as *IMAG, MEPRC, DISINC*. In particular, the results emphasized that the pleasure of Turkish consumers provided from its consumption were under not only the effects of the numerous post-purchasing criterion (aroma, nutritional value, microbial safety, etc.) but also the impacts of the various pre-purchasing processes based on the visual quality cues (colour, freshness, natural structure, specific animal parts and meat outlets, etc.) impacting on their purchase decisions.

The results of the present study with regard to the sensory and hedonic quality attributes of the fresh red meat are in line with the results of the previous studies reported that the consumers paid particular much more attention to the sensory quality attributes of the red meat inspected visually in a pre-purchasing process (colour, freshness, natural structure, fat content, specific animal parts and meat outlets) (REALINI *et al.*, 2013; TROY and KERRY, 2010; AASLYNG *et al.*, 2007; VERBEKE and VACKIER, 2004), and then to its credence attributes perceived such as nutritional value (vitamin and protein content), microbial freeness (salmonella, dioxins, BSE) (KRYSTALLIS *et al.*, 2007; VERBEKE and VACKIER, 2004) and chemical freeness (hormones, antibiotics) (KRYSTALLIS *et al.*, 2007; VERBEKE and VACKIER, 2004) rather than the hedonic ones (meat image, price, income, country of origin) (FURNOLS *et al.*, 2011; NAPOLITANO *et al.*, 2010; KRYSTALLIS *et al.*, 2007; AUDEBERT *et al.*, 2006; OLIVER *et al.*, 2006; VERBEKE and VACKIER, 2004; BERNUES *et al.*, 2003).

CONCLUSIONS

The results of the study confirmed clearly that *the heavy users* in *C1* attributed a much more importance to the sensory quality attributes based on willingness to buy the local-origined organic meat endorsing the aroma as an indicator of the post-purchasing satisfaction followed by the fresh red meat consumption and the visual quality considered in the pre-purchasing process of it than the others. On the other hand, *the medium and light users* in *C2* and *C3* paid also a more attention to the sensory quality attributes including in the credence attributes such as the microbial and chemical freeness and the nutritional value of it than the members of *C1* as considered the efficiency scores of the sensory and hedonic quality attribute variables effecting on its consumption amounts of Turkish consumers in *MRC* model.

Consequently, the sensory quality attributes had a much bigger impact on its consumption decision and amounts of Turkish consumers than the hedonic quality attributes in the overall agreements with regard to the importance of all the factors. Therefore, the fresh red meat consumption amount in Turkey maintaining at a very low level of the annual fresh red meat consumption amount per capita (about 12 kg) when compared with the leading countries in the meat consumption could be increased meaningfully by the innovative red meat and meat products designed through the product and price differentiations under the production, manufacturing and marketing tactic and strategies taking into account first its core benefit based on the sensory quality attributes such as the visible quality, aroma and credence attribute, and then its actual benefit focused on the hedonic quality attributes (the country of the origin, its actual image and price and their disposable income) for each clusters.

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PAPER

NEW NATIONAL CONVERSION LINE FOR BACTOSCAN FC IN ITALY: A STEP FORWARD

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ABSTRACT

To improve the reproducibility of flow cytometry technique for total bacterial count in milk, a conversion from instrumental results (impulses/μL) to the reference method resultes (cfu/mL) is needed. In 2008 in Italy, a project for a common conversion line for Bactoscan FC was initiated. In this paper we report on the second phase of the project focusing on the statistical procedure used to evaluate the validity of the data. The new conversion line, representative of national milk (2,732 valid samples from 29 labs) obtained from both rounds of the study is: Log_{10} (cfu mL^{-1}) = Log_{10} (IBC μL^{-1}) x 0.939 + 2.559, with $S_{y:x}$ = 0.282 with an application range up to 70,000 IBC μL^{-1}.

- Keywords: Bactoscan FC, conversion line, cow milk, total bacterial count -

INTRODUCTION

Regulation (EC) 1664 (EC 1664:2006) established that the reference method for determining total bacterial count at 30°C in raw milk is EN ISO 4833 (ISO 4833:2003), however the use of alternative methods is acceptable when they are validated against the reference method in accordance with the protocol set out in EN/ISO standard 16140 (ISO 16140:2003) or other similar internationally-accepted protocols. In the case of milk, ISO 21187 (ISO 21187:2004) and ISO 16297 (ISO 16297:2013) are examples of other such protocols.

EN ISO 4833 instructs that colonies grown in defined conditions must be counted after 72 h of incubation at 30°C whereas flow cytometry instruments count free cells independently from their physiological status or their capability to develop into a colony. The counts are obtained from electrical impulses (derived by the fluorescence of bacterial DNA and RNA stained by fluorochrome ethidium bromide) and must be converted into cfu mL^{-1} equivalents, as this is the regulatory unit of measure. This conversion (when calculated by a single laboratory) is the main reason for the low reproducibility of the alternative method in spite of its otherwise better repeatability, rapidity and cost effectiveness compared to the reference method and this could have major consequences both from economic and food safety points of view. Currently the flow-cell automatic instruments for total bacterial count are indispensable to the centralized and specialized laboratories in charge of large numbers of milk samples per day. For this reason, at the end of 2008, the Reference Centre for Bovine Milk Quality of IZSLER launched a project for a "common conversion line" for Bactoscan FC (Foss, DK), the most commonly used instrument in Italy. The result of that study (BOLZONI and MARCOLINI, 2010) was adopted on a voluntary basis by several laboratories in our country in the last few years. In 2012, with the coordination of the Italian National Reference Laboratory (NRL) for milk (Istituto Superiore di Sanità), a second round of the project was developed with the objectives to: verify the results of the first round of the project; study a wider range of milk contamination levels; derive a conversion formula that is more tailored to Italian milk, meaning a single, mandatory conversion formula to be applied at the national level; propose a statistical model to evaluate the reliability of the raw data.

MATERIALS AND METHODS

The study involved 29 laboratories from all over Italy. The number of samples analyzed from each laboratory and for the different levels of contamination was determined on the basis of their previous participation or not in the first round of the project in 2008 (Table 1).

The protocol adopted in 2008 (BOLZONI and MARCOLINI, 2010) was adopted again in 2012 with the intention of producing comparable data. Participating laboratories, during the period from January to June 2012, selected samples of cow bulk tank milk (refrigerated and without preservatives) from those submitted for daily analytical activity. The instrument's calibration status was checked through an inter-laboratory trial using lyophilized milk samples at 3 different contamination levels that were shipped to participants (data not shown). Considering that ISO/TS 19036 (ISO 19036, 2006) estimates that the standard deviation for aerobic mesophilic flora in milk ($S_R = 0.12$) is affected more by operative conditions ($S_{cond} = 0.09$) than by the initial suspension ($S_{IS} = 0.04$), it was decided that the reference method would be performed using 2 plates per dilution with one series of dilutions. In each laboratory, immediately before analysis, each sample was mixed as stated in ISO 6887-5 (ISO 6887-5, 2010), tested in duplicate by the Bactoscan FC and immediately analyzed by the reference method. A single series of at least 3 decimal dilutions was pre-

Table 1 - Selection of samples – percentage of samples and respective ranges of impulses required from each lab.

Range Impulses (IBC µL-1)	% samples analyzed (from 10 to 50 samples)[A]	% samples analyzed (from 50 to 100 samples)[B]
0-20	3	3
21-100	30	10
100-1,000	30	10
1,000-5,000	25	10
5,000-10,000	4	30
10,000-50,000	4	27
50,000-99,999	4	10

A: Laboratories WITH participation in the project prior to 2009.
B: Laboratories WITHOUT participation in the project prior to 2009.

pared with quarter-strength Ringer's solution (the level of dilution was established on the basis of the previous instrumental results); 1 mL of each dilution was dispensed in each of 2 plates of milk-PCA medium and then incubated at 30°C ± 1°C for 3 days. Each participating lab contributed their data on the Bactoscan FC double counts in "impulses" (IBC μL^{-1}) and colonies counts from the two plates of each dilution to a database. After the relevant controls of raw data (see: point "d" in the "selection of results" section below) as indicated by ISO 7218 (ISO 7218:2010; ISO 14461-2:2005) and the additional controls (see: points "e" and "f"), the linear mixed effect model (LME) was applied to produce the regression line of the data from the "valid samples". The statistical evaluation of the results is described in the following section. The software "Procedure R 2.15" and Excel 2010 (Microsoft Corp., Redmond, WA) were used.

RESULTS AND DISCUSSION

Range of measurement and linearity

The ratio between observed values (O.V.) and expected values (E.V.) in impulses μL^{-1} (IBC μL^{-1}) from serial dilutions of *ad hoc* heavily contaminated milk samples was taken as an indicator of linearity of the instrumental signal response. The ratio O.V./E.V. ~ 1 (Fig. 1) suggests the acceptable instrumental linearity continues up to 50,000 IBC μL^{-1}, which is well above the producer's declared limit of 30,000 IBC μL^{-1} and confirms our previous evaluation (BOLZONI *et al.* 2000, BOLZONI *et al.,* 2001).

Since one of the aims of the work was to evaluate whether a broader range of instrumental measures could be accepted without affecting the conversion line, values > 30,000 IBC μL^{-1} were also considered. Ratio O.V./E.V. = 0.9 was adopted as an arbitrary lower limit of acceptability of the linearity indicator (equivalent to 3 standard deviations from the mean of the ratios obtained). These considerations allowed us to accept 70,000 IBC μL^{-1} as the upper limit for the range of application of the conversion line. We would like to note that samples with IBC μl^{-1} > 30,000 (approximately > 4,000,000 cfu mL^{-1}) are rather unusual in Italy.

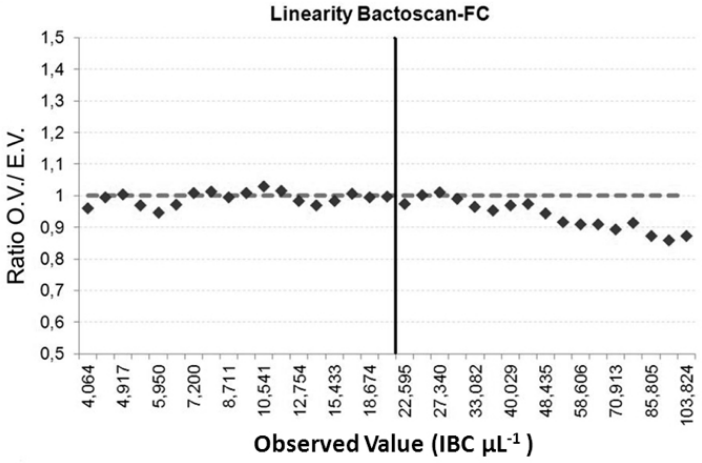


Fig. 1 - Bactoscan FC linearity: the relationship between the observed values and the ratio of the observed value to the expected value.

Selection of results

Of the 1,827 total milk samples analyzed, which is equivalent to more than 10,000 analytical results produced by 29 participating laboratories, the selection process for valid data led to the rejection of 499 (27%) samples due to the following factors:

a) *Unreliability* – 19 samples were eliminated for absence of correspondence between the instrumental results and the reference method results or errors in the report transmission results.

b) *Out of range of measurement* – 65 samples were eliminated because their values were outside the established range of linearity (12 samples lower than 10 IBC μL^{-1} l and 53 higher than 70,000 IBC μL^{-1}).

c) *Instrumental repeatability* – 31 samples were eliminated because the difference between replicates exceeded the repeatability limit of the Bactoscan FC: Critical Log Difference between replicates > 2.83 S_r (P 95%). Additionally 12 samples were eliminated because they exceeded the instrumental reproducibility limit (S_R).

d) *Maximum - minimum numbers of colonies on the plates and proportionality between dilutions* – plates outside the range 10 - 324 colonies were not considered for the count (ISO 7218:2007). The G^2 factor test, which compares the relationship between pairs of plates and dilutions, led to the elimination of 179 samples.

e) *Sub-dispersion of reference method results* - no laboratories were eliminated on this basis (which compares the relationship between observed and expected values on plates) but the frequency of sub-dispersed samples was one criterion used for the selection of laboratories described in point f.

f) *Single laboratory performance evaluation* – the effect of each individual laboratory on the extrapolation of the final regression line was considered on the basis of the following factors:

- excessive or insufficient dispersion of the individual lab's regression line;
- high frequency of sub-dispersed results from the reference method;
- high frequency of eliminated results from the G^2 factor test.

The dispersion of data around single-lab regression lines is reported in Table 2 as $S_{y:x}$. Giv-

Table 2 - Dispersion of the conversion line for individual laboratories (S y:x).

Lab Code	Samples (n)	Intercept	Slope	Sy:x
40	50	2.1184	1.0309	0.0139
27	98	2.9025	0.7797	0.0930
14	42	2.4432	0.9911	0.1455
38	36	2.2563	1.0279	0.1577
31	93	2.3363	1.0408	0.2517
35	52	2.1976	1.0711	0.2556
41	16	2.1718	1.0859	0.2594
1	40	2.1280	0.9966	0.2676
39	88	2.6219	0.8914	0.2766
15	26	2.5538	1.0119	0.3086
11	26	2.5408	0.9711	0.3118
23	98	2.6394	0.9257	0.3223
24	50	2.4829	0.9593	0.3291
6	68	2.2774	1.0927	0.3365
28	54	3.5260	0.6413	0.3546
37	79	3.6620	0.5508	0.3707
22	76	2.7561	0.8592	0.3756
26	55	2.1806	0.9484	0.3766
7	22	2.4747	1.0033	0.3830
29	24	2.7238	0.9293	0.3893
33	89	3.0733	0.6950	0.4104
34	103	2.1782	1.2029	0.4145
25	97	2.8959	0.7690	0.4286
30	36	2.8759	0.7964	0.4379
8	34	2.6250	0.9531	0.4410
32	29	3.1796	0.6974	0.4567
9	30	3.0099	0.8643	0.6225
36	32	2.5325	0.6897	0.6386
21	110	3.1939	0.8881	0.8504

en $S_{y:x} < 0.40$ is a criterion for acceptability (listed as a "tentative value" in ISO 16297:2013), nine of twenty-nine labs were over range. Of the nine, six were considered borderline and only laboratories 21, 36 and 9 were eliminated for over-dispersion. Furthermore laboratory 40 was eliminated for sub-dispersion, which suggested their results were not completely reliable. Two laboratories exhibited a high frequency of eliminated samples by the G^2 factor test (> 50% of samples); in the first case we decided to eliminate all results (Lab 36, which had already been eliminated for high dispersion as mentioned above), whereas in the second case (Lab 28) we decided to preserve the remaining "valid results" considering the very low value of dispersion of its regression line (0.3546 $S_{y:x}$).

Evaluation of the regression line

The LME model was applied to produce the regression line of the selected 1,388 valid samples. Multi-step selection of outliers (residual standard deviation > 2.58) was preliminarily applied (ISO 21187:2004). In synthesis, after a 3-step sequential elaboration, 65 outliers were eliminated, narrowing the number of valid results to 1,323 and improving the $S_{y:x}$ value from 0.3547 to 0.2781. After the third step, no significant improvement in the level of estimation could be obtained so no further elimination of data was considered appropriate.

The following conversion equation was calculated from the 1,323 residual samples (characteristics of the conversion equation are reported in Table 3):

$$\text{Log}_{10}(\text{cfu mL}^{-1}) = \text{Log}_{10}(\text{IBC } \mu\text{L}^{-1}) \times 0.946 + 2.569$$

Fig. 2 shows the conversion line from 2012 alongside the conversion line from 2009 (black dashed line) (6), calculated by:

$$\text{Log}_{10}(\text{cfu mL}^{-1}) = \text{Log}_{10}(\text{IBC } \mu\text{L}^{-1}) \times 0.911 + 2.599$$

The conversion line from 2012 is very similar to the line from 2009 although differences are seen at high and very high contamina-

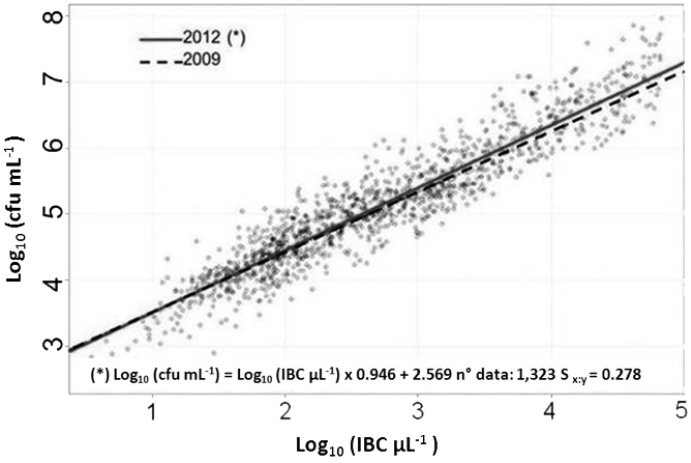


Fig. 2 - Distribution of data from the 2012 conversion line compared with the 2009 conversion line.

Table 3 - Characterization of the conversion line from 2012.

Parameters	Coefficient	St. error	T	Sig	Low	High
Intercept	2.569	0.038	67.57	0.000	2.493	2.645
Slope	0.946	0.009	106.91	0.000	0.928	0.964
Number of samples = 1,323; S y:x = 0.278.						

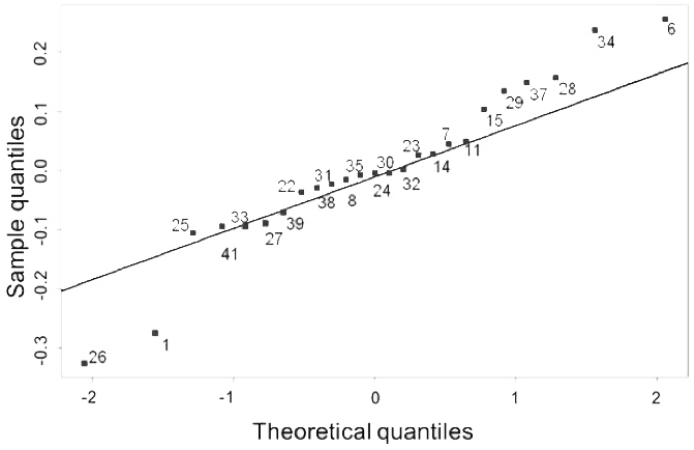


Fig. 3 - Q-Q plot of random effects from each laboratory in the Linear Mixed Effect Model

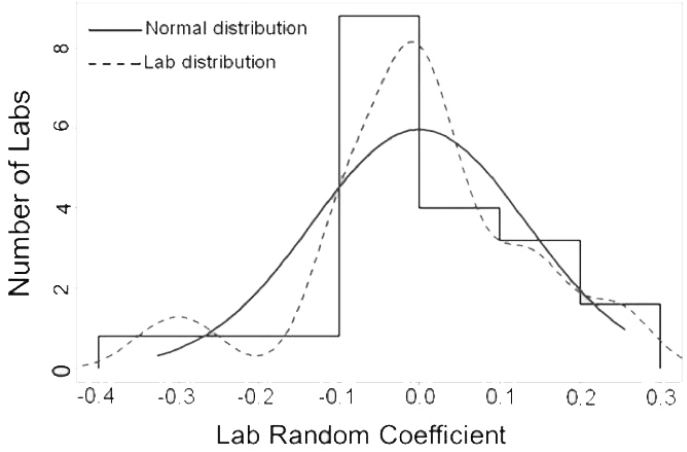


Fig. 4 - Distribution of random effect coefficients from the labs compared with a normal distribution (2012 conversion line).

tion levels as a consequence of the extension of the measurement field in the second round of the project.

In Figs. 3 and 4, the distribution of random effects in the LME for data from individual laboratories is presented. Statistically four labs were found to be apparently different from the others: numbers 6 and 34 overestimated their counts while numbers 1 and 26 underestimated their counts. No factors affecting this distribution could be identified (e.g. bacterial flora, sample characteristics, or systematic bias in reference method execution), so the data from these labs were kept in the regression line calculation.

New national conversion line

Considering that the same procedure and the same statistical evaluation were used in both rounds of the project, we considered it not only possible but also appropriate to pool the valid results from 2009 and 2012 and to run a new mixed statistical evaluation. Taking a step back before the respective outliers were excluded, a new multi-step selection was performed on the 1,474 valid results from 2009 combined with the 1,388 from 2012. The total elimination of 130 samples at the third step of selection led to no further increase in estimation (Table 4).

The final regression line was computed from 2,732 samples and it is represented by the equation:

$$\text{Log}_{10}(\text{cfu mL}^{-1}) = \text{Log}_{10}(\text{IBC } \mu\text{L}^{-1}) \times 0.939 + 2.559$$

The characteristics of the combined regression line are reported in Table 5 and Fig. 5.

Table 4 - Multi-step selection of outliers on 2009 and 2012 aggregated data.

Step No.	Samples (n)	S y:x	Intercept	Slope	Min Std Residual	Max Std Residual
1	2,862	0.3533	2.591	0.921	4.503	-5.798
2	2,793	0.3048	2.575	0.931	2.989	-3.103
3	2,752	0.2886	2.565	0.937	2.707	-2.691
4	2,732	0.2821	2.559	0.939	2.651	-2.645
5	2,724	0.2796	2.558	0.939	2.660	-2.597
6	2,718	0.2778	2.557	0.939	2.620	-2.590

Table 5 - Characterization of the new national conversion line (2009 and 2012 pooled results).

Parameters	Coefficient	St. error	T	Sig	Low	High
Intercept	2.559	0.032	80.77	0.000	2.496	2.622
Slope	0.939	0.006	150.38	0.000	0.927	0.952
Number of samples = 2,732; S y:x = 0.282						

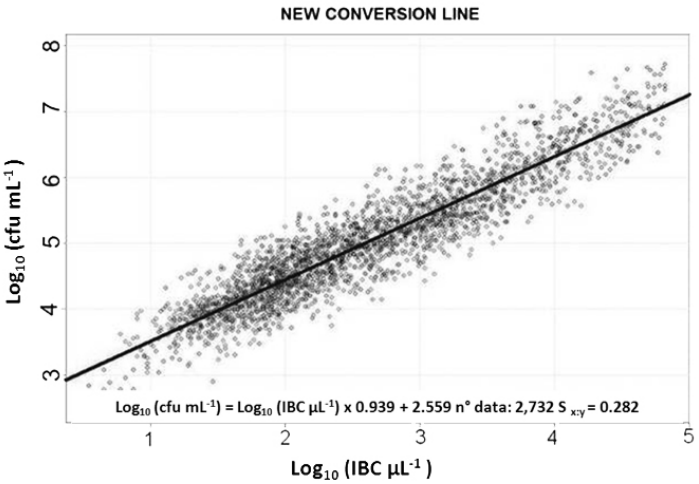


Fig. 5 - The new national conversion line (computed from 2009 and 2012 aggregated data).

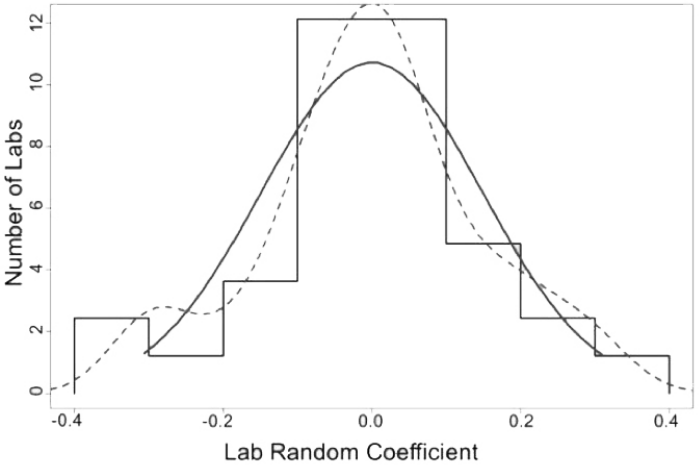


Fig. 6 - Distribution of random effect coefficients from labs compared with a normal distribution (new conversion line).

The distribution of random effects in the LME for individual laboratory data is presented in Fig. 6.

CONCLUSIONS

During the first round, in 2008, the main focus was on milk samples with total bacterial counts around 100,000 cfu mL^{-1}, the European Legal Limit for compliance (Reg. EC 853:2004). Contrastingly, during the second round, laboratories were invited to include milk samples with high to very high levels of bacterial contamination in order to test the instrumental response to bacteria levels outside of the linear range indicated by the Bactoscan FC producers. In routine situations the submission of very highly contaminated samples is a rare occurrence, however they should nonetheless be analyzed and their results should be entered into the geometric mean of the last three months, as per the calculation system defined by Reg. EC 853:2004.

The present project led to the creation of a conversion relationship between impulse μl^{-1} and cfu $mL^{-1}$for the enumeration of the total bacterial counts in Italian raw cow milk using a Bactoscan FC. In summary the conversion line incorporates the following points:

- the conversion relationship was constructed according to ISO 21187:2004;
- the level of accuracy obtained was satisfactory ($S_{y:x}$ = 0.282 $\log_{10}$);
- the number of samples was representative of Italian milk production variability;
- 80% of all Italian laboratories involved in milk control by routine method joined the project.

The new conversion line appeared robust and representative of milk quality and variety in Italy, with a range of application up to 70,000 IBC μL^{-1}. It was ultimately validated and adopted as the national conversion line in Italy. This is an important advance for both the industry and public hygiene because the use of a unique conversion line should significantly improve the reproducibility of the bacterial count results obtained by Bactoscan FC in Italy. In addition, the use of the conversion line for highly-contaminated samples is a further contribution to improve analytical harmonization. Data quality control was focused on the evaluation of data entry quality and consequently the accuracy and robustness of the elaborated conversion line. This was done by checking the raw data (agreement between pairs of plates, and proportionality between successive dilutions).

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PAPER

INACTIVATION OF *LISTERIA MONOCYTOGENES* ATCC 7644 ON TOMATOES USING SODIUM DODECYL SULPHATE, LEVULINIC ACID AND SODIUM HYPOCHLORITE SOLUTION

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ABSTRACT

The human pathogen *Listeria monocytogenes* poses a serious threat to public health. A study was carried out to evaluate the effectiveness of four sanitizers, used individually or combined, against *L. monocytogenes* ATCC 7644. The contact times for bacteria and sanitizer were varied to 1, 3 and 5 minutes. Levulinic acid, sodium dodecyl sulphate (SDS), sodium hypochlorite solution (chlorine) and a combination of SDS and levulinic acid (mixture) were tested. Results revealed that 0.5% levulinic acid, when used individually, is capable of reducing the surviving colonies by 3.63 log CFU/mL, 4.05 log CFU/mL, 6.71 log CFU/mL after exposure for 1, 3 and 5 minutes respectively. SDS resulted in an 8 log CFU/mL reduction after 1, 3 and 5 minutes. A combination of 0.5% levulinic acid and 0.05% SDS caused a 3.69 log CFU /mL reduction, 4.4 log CFU/mL reduction, 7.97 log CFU/mL reduction for 1, 3 and 5 minutes respectively. Chlorine was the least effective with 2.93 log CFU/mL reduction, 3.16 log CFU/ mL reduction and 4.53 log CFU/ mL reduction respectively. When stored for up to 72 hours at 4°C, the surviving colonies remained viable and decreased in number significantly $P < 0.05 = 0.001$. The titratable acidity of samples treated with levulinic acid and samples treated with SDS/Lev mixture was lowered significantly compared to the control sample. No significant differences were noted in these same parameters for samples treated with chlorine or SDS. The application of SDS in the fresh produce industry as a sanitizing agent may be successful in eradicating or reducing the viability of *L. monocytogenes* on fresh produce, thereby replacing the routine chlorine washing.

- Keywords: food safety, Listeria monocytogenes, fresh produce, food borne illnesses, tomatoes, food borne pathogens -

INTRODUCTION

The increase in fresh produce consumption has caused a rapid evolution in the fresh produce industry (JOHNSTON *et al.*, 2005). This, coupled with recommendations to eat minimally processed foods, has led to an increase in the consumption of fresh fruits and vegetables among consumers (BERGER *et al.*, 2010). The consumption of minimally processed foods and fresh produce has also been encouraged among the immune compromised populations, such as those affected by HIV/AIDS, children and pregnant women (BERGER *et al.*, 2010; GANDHI and CHIKINDAS, 2007). Consumer demands and habits have also shifted, with many consumers in the busy world preferring to eat ready-to-eat foods and eating from salad bars (OMS-OLIU *et al.*, 2010; BERDEGUÉ *et al.*, 2005).

A variety of fresh produce such as lettuce cantaloupes, peppers, tomatoes, herbs and green leafy vegetables, among others, have been linked to food borne illnesses associated with either Salmonella, *Escherichia coli* O157:H7 or *Listeria monocytogenes* contamination (TAUXE *et al.*, 2010). Contamination of fresh produce by these pathogens occurs by various means. IJABADENIYI *et al.* (2011a) cited irrigation water as major pre-harvest source of contamination of fresh produce. Other factors as cited by JOHNSTON *et al.* (2005) include use of biocides as fertilizer, poor worker hygiene and poor sanitation.

L. monocytogenes among other food borne pathogens have been implicated as a public health threat (VELUSAMY *et al.*, 2010) and are estimated to cause about 1,600 incidents of illness, more than 1400 hospitalisations and about 250 deaths per year in United states (KYLE, 2012). These pathogens are responsible for food borne Listeriosis. They can grow in the soil, drains and on food preparation surfaces (GÁLVEZ *et al.*, 2010; PAN *et al.*, 2006; DJORDJEVIC *et al.*, 2002). They have been largely associated with dairy products, but recent research has also shown their increasing association with fresh produce (GANDHI and CHIKINDAS, 2007) including tomatoes.

Tomatoes are widely consumed and can be eaten raw, partially cooked or can be processed into other products. They are a very rich source of carotenoids, folate, vitamin C, mineral elements and phenolic compounds (FRUSCIANTE *et al.*, 2007). Of major importance are the antioxidants (carotenoids). Epidemiological research has shown that the antioxidants are capable of preventing chances of cancers and cardio vascular diseases (LEONARDI *et al.*, 2000). Tomatoes also provide a dietary source of soluble and insoluble fibres such as pectin, hemicellulose, and cellulose. Due to their nutritional value, they form an important part of the human diet. The elimination of food borne pathogens that can contaminate tomatoes is essential for preventing food borne illnesses that may be associated with the consumption of tomatoes.

Many methods are being used to try and eliminate the food borne pathogens. Use of phage or phage products in food production has been considered as a novel method for bio-control of pathogens in fresh and ready-to-eat food products (HAGENS and LOESSNER, 2010), but the cost associated with their use is very high. Other methods include bacteriocin-activated films high-hydrostatic pressure, high-pressure homogenization, in-package pasteurization, food irradiation, pulsed electric fields, or pulsed light and electrolyzed water (GÁLVEZ *et al.*, 2010). Sanitizers such as carvacrol, vanillin, peroxyacetic acid, hydrogen peroxide, N-acetyl-l-cysteine and citrox among others have also been tried (ABADIAS *et al.*, 2011). Sanitizers affect cell components, for example proteins, DNA, RNA and cell wall constituents through physicochemical interactions or chemical reactions. They cause irreversible damage to these structures and a loss of cell contents, thereby rendering the bacteria inactive or dead (CERF *et al.*, 2010).

The action of sanitizers is governed by contact time (exposure time), pH and temperature, among other factors. Some researchers conclude that sanitizers are not effective in eradicating food borne pathogens when used individually, although a combination of agents increases the sanitizer ability (SAGONG *et al.*, 2011; ZHAO *et al.*, 2009). Recent studies have also shown that if not used properly, sanitizers can be detrimental to the quality of fresh produce (SALGADO *et al.*, 2013; GUAN *et al.*, 2010). With regard to tomatoes, pH and acidity are the most important determinants of tomato quality (ANTHON *et al.*, 2011), hence the interaction of tomatoes with sanitizers during washing should be monitored. The study was performed to evaluate the effectiveness of SDS, chlorine and levulinic acid in reducing the viability of *L. monocytogenes* on tomatoes and the effect of these sanitizers on pH, titratable acidity and total soluble solids.

MATERIALS AND METHODS

Fresh produce

Tomatoes were purchased from a local supermarket on three separate occasions in Durban, South Africa. On the day of purchase the tomatoes were washed in running water. The tomatoes were then washed in 70% alcohol (IJABADENIYI *et al.*, 2011a). Prior to subjection to different sanitizer treatments, the tomatoes were tested for the presence of *L. monocytogenes*.

Bacterial strains

Listeria monocytogenes ATCC 7644 (Merck, South Africa) was used for this study. The

strain was cultured in Fraser broth for 24 hours at 37°C and stored at 4°C (IJABADENIYI *et al.*, 2011a). Prior to each experiment, a fresh culture was prepared from the stock culture by sub-culturing in Fraser broth for 24 hours at 37°C, an 8 log cfu/mL culture of *L. monocytogenes*, using McFarland Standards (JI *et al.*, 2010).

Chemicals and chemical treatments

Sodium dodecyl sulphate (SDS), levulinic acid, sodium hypochlorite solution, all purchased from Merck, South Africa, were tested, individually or combined with varying contact times (1, 3 and 5 minutes) for their effect on *L. monocytogenes* ATCC 7644 in tomatoes. The chemicals were used as follows;

1% SDS individually

0.5% Levulinic acid individually

200 ppm Sodium hypochlorite solution individually and 0.5% levulinic acid/0.05% SDS combined and termed mixture.

Inoculation of bacterial strains into tomatoes

The method of ZHAO *et al.* (2009) was followed. A 25 g sample of tomatoes was cut into approximately 5 cm long pieces in the lamina flow hood. The samples were submerged into bacterial suspension (108 cfu/mL, 50 mL of bacterial solution into 950 mL of distilled water) for 60 seconds and then air dried for 20 minutes in the lamina flow hood. The samples were then suspended into 500 mL test solution and agitated by a magnetic stirrer at 100 rpm for 1, 3 and 5 minutes. Following treatment, the individual samples were placed in double zipper bags containing 25 mL of phosphate buffered saline and pummelled for one minute. The suspension was serially diluted (1:10) in 0.1% buffered peptone water and enumerated for *L. monocytogenes* ATCC 7644.

Enumeration of *L. monocytogenes*

A method by Taormina and BEUCHAT (2001) was followed. Populations of *L. monocytogenes* ATCC 7644 were determined by surface plating serially diluted samples; 0.1 mL in duplicates on Listeria Selective Agar (Oxford formulation; Oxoid Ltd, Wade Road, Basingstoke, Hants UK). Plates were incubated for 24 hours at 37°C, after which colonies were counted.

Preparation of samples for Scanning Electron Microscopy

Untreated samples and samples subjected to chlorine, levulinic and SDS/Lev were used for SEM viewing. A method used by Ijabadeniyi *et al.* (2011b) was followed with a few modifications, according to the requirements of University of KwaZulu Natal microscopy unit. Pieces of tomatoes inoculated with *L. monocytogenes* ATCC 7644 and subjected to different treatments were cut into small pieces of 2 x 2 mm using a sterile blade. Primary fixation was carried out in 2.5% glutaraldehyde for 12 hours, followed by rinsing three times in phosphate buffer (0.1 M, pH 7.0). Post fixation was done using 0.5% Osmium tetroxide for one hour. Fixed samples were dehydrated in graded ethanol (30%, 50%, 75% and 100%) each for 5 minutes. The samples were then dried in a critical point dryer with carbon dioxide as a transition gas. The samples were mounted on specimen stubs and coated with gold palladium. The samples were then analysed using Desmond Clarence scanning electron microscopy.

Analysis of tomato physicochemical properties

Preparation of samples: The method of ZHAO *et al.* (2009) was followed for sample preparation, except that the tomato was further homogenised into slurry. A 25 g sample of tomatoes was cut into approximately 5 cm long pieces. The samples were then suspended into 500 mL test solutions as follows:

25 grams of tomatoes + 500 mL de-ionised water (control)

25 grams of tomatoes + 500 mL 1% SDS

25 grams of tomatoes + 500 mL of 0.5% levulinic acid

25 grams of tomatoes + 500 mL of 200 ppm sodium hypochlorite solution

25 grams of tomatoes + 500 mL of 0.5% levulinic acid/0.05% SDS (mixture)

The samples were agitated by a magnetic stirrer at 100 rpm for 1, 3 and 5 minutes (contact times). After each contact time was achieved, samples were immediately drained and the tomatoes were homogenized to form a slurry using Waring Commercial Laboratory blender. The slurry was used to test for pH, titratable acidity and total soluble solids immediately.

Determination of pH

The determination of pH was done on freshly made tomato paste using the Thermo Scientific Orion 2star pH meter. The electrodes were rinsed with distilled water in between samples.

Determination of Titratable Acidity

For estimating titratable acidity, the slurry was filtered using Whatman syringe filters. A 100 mL of the filtrate was titrated by adding 0.1N sodium hydroxide until a pH of 8.1 was attained. The volume of the sodium hydroxide added to the solution was multiplied by a correction factor of 0.064 to estimate titratable acidity as a

percentage of citric acid (CHEEMA *et al.*, 2014; TURHAN and SENIZ, 2009).

Determination of Soluble Solids Content

TSS is an index of soluble solids concentration in fruit. For an estimation of soluble solids content, 1.5 mL tomato slurry was centrifuged at 10,000 rpm (15 min, 25°C), and the supernatant was filtered through Whatman nonsterile syringe filters (0.45 μm). The filtered tomato serum (40 μL) was measured using a digital refractometer ATAGO (ATAGO, USA Inc. Kirkland, WA, USA). Measurements were taken once for each sample, and 70% ethanol was used to clean in between samples. The refraction index was expressed as percent soluble solids in°Brix (Wilkerson *et al.*, 2013; Javanmardi and Kubota, 2006).

Data analysis

Three trials were conducted for each experiment. Analysis of the data was performed using SPSS version 21 (IBM Statistics). Analysis of variance was conducted with repeated measures and Greenhouse Geisser correction to study the effect of contact time on the survival of *L. monocytogenes*, ATCC 7644 and the effect of each sanitizer on the survival of *L. monocytogenes* ATCC 7644 at varied time intervals (0, 24, 48 and 72 hours). The number of surviving colonies was plotted against contact time (1, 3 and 5 minutes) and also against time interval (0, 24, 48 and 72 hours). Log reductions for each contact time and sanitizer were also calculated and is presented in a table. Pair wise comparison with Bonferroni adjustment was used to determine any significant difference between subjects. To analyse results for physicochemical properties, ANOVA was used to assess if there was a significant difference in pH, total soluble solids and titratable acidity of treated and untreated tomato samples.

RESULTS

Effect of storage time, sanitizer treatments and contact time on the survival of *L. monocytogenes* ATCC 7644

The treatment of *L. monocytogenes* with sanitizers resulted in a decrease in the populations of bacteria. All the sanitizers tested had the ability to reduce the surviving colonies, with varying degree of effectiveness. Among the sanitizers tested, sodium hypochlorite solution was the least effective, with the highest counts of surviving colonies. The next in the list is levulinic acid, then a mixture of SDS and levulinic (termed mixture), with SDS the most effective of them all. The results of repeated measures (CASTRO *et al.*) with Greenhouse-Geisser correction showed that there was a significant difference at 5% level between effectiveness of sanitizers used, [$F(3, 9) = 63.00$; $P < 0.05 = 0.01$]. The surviving colonies were reduced progressively as storage time increased from 0 hours to 72 hours. The means of surviving colonies are shown in Table 1.

Marginal means for each sanitizer's contact time were also plotted in Fig. 1 for 1, 3 and 5 minutes. As shown in the figure, sodium hy-

Table 1 - Mean 1 count of *L. monocytogenes* ATCC 7644 after treatment with different sanitizers at different contact times and storage times.

Contact times		Time intervals			
		0 Hours	24 Hours	48 Hours	72 Hours
Chlorine	1 minute	[a] 5.36±0.02	[a] 5.14±0.03	[a] 5.02±0.03	[a] 4.75±0.04
	3 minutes	[a] 5.06±0.03	[a] 5.06±0.03	[a] 4.78±0.05	[a] 4.45±0.04
	5 minutes	[b] 4.17±0.09	[b] 3.77±0.09	[b] 3.33±0.10	[b] 2.60±0.09
SDS/Lev	1 minute	[c] 4.60±0.01	[c] 4.59 ±0.02	[c] 4.27±0.08	[c] 4.01±0.06
	3 minutes	[c] 4.35±0.05	[c] 4.24 ±0.06	[c] 3.39±0.36	[c] 2.53±0.08
	5 minutes	[d] 1.33±0.15	[d] 1.40±0.03	[d] 0.56±0.09	[d] 0.00
Levulinic	1 minute	[e] 4.68±0.03	[e] 4.60±0.02	[e] 4.15±0.14	[e] 4.06±0.11
	3 minutes	[e] 4.68±0.03	[e] 4.34±0.09	[e] 4.12±0.10	[e] 2.60±0.30
	5 minutes	[f] 3.17±0.07	[f] 2.06±0.04	[f] 1.50±0.10	[f] 0.43±0.20
SDS	1 minute	[g] 0.00	[g] 0.00	[g] 0.00	[g] 0.00
	3 minutes	[g] 0.00	[g] 0.00	[g] 0.00	[g] 0.00
	5 minutes	[g] 0.00	[g] 0.00	[g] 0.00	[g] 0.00

Mean counts ±Standard Deviation (Log 10 CFU /mL).
[1]Means followed by different letters in the same column are significantly different.

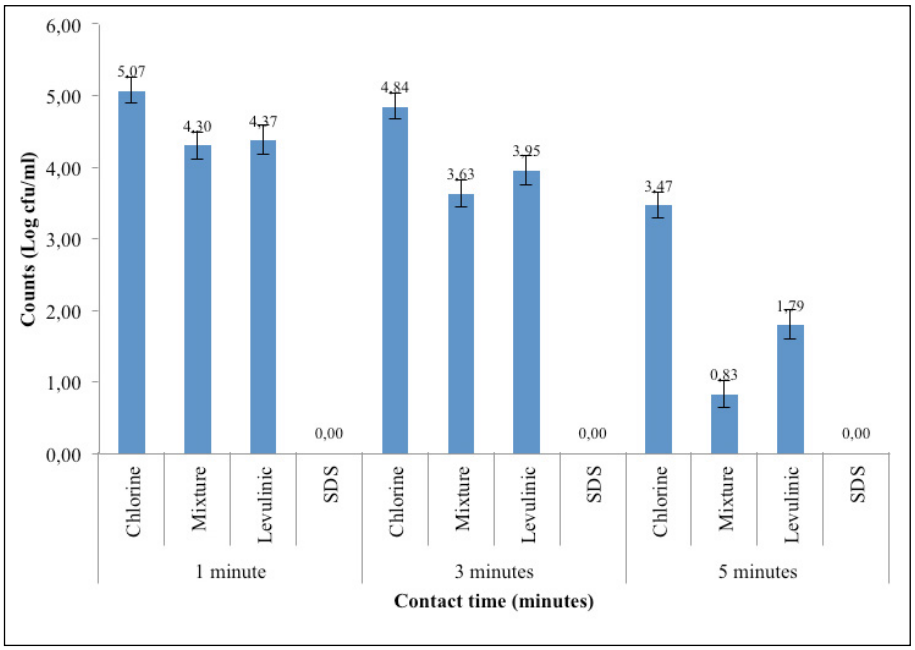


Fig. 1 - Means of surviving colonies of *L. monocytogenes* ATCC 7644; based on marginal means. The highest means associated with chlorine show that it was least effective.

pochlorite has the highest mean values, meaning that the highest number of surviving colonies was observed after exposure to this sanitizer compared to other solutions. Sodium chloride was thus not very efficient in reducing survival of the pathogen in this particular study.

Increasing the contact time (1, 3 and 5 minutes) significantly reduced the surviving colonies for all sanitizers tested at 5% level; [F (3, 180) = 30.70; P< 0.001]. However, the results of ANOVA with Green House Geisser correction showed that the reduction for 1 minute and 3 minutes of treatment were not significantly different (P = 0.16). This shows that increasing the contact time of each of the sanitizer to 3 minutes did not make much difference to the surviving colonies.

Overall log reductions

When exposed for 1 minute to 200 ppm chlorine, *L. monocytogenes* were inactivated by 2.93 log CFU/mL. A log reduction of 3.16 log CFU/mL and 4.53 log CFU/mL was achieved after increasing contact time to 3 minutes and 5 minutes respectively. A mixture of 0.5% levulinic acid and 0.05% SDS (mixture) reduced the surviving colonies to 3.69 log CFU/mL, 4.4 log CFU/mL and log 7.97 CFU/mL after exposure for 1 minute, 3 minutes and 5 minutes, respectively. Using 0.5% levulinic acid resulted in log reductions of 3.63 log CFU/mL, 4.05 log CFU/mL and 6.71 CFU/mL after exposure for 1 minute, 3 minutes and 5 minutes. The overall log reductions are presented in Table 2.

Observations of specimens using a scanning electron microscope

Samples of tomatoes treated with sodium hypochlorite solution, levulinic acid and mixture were viewed under SEM to verify the existence of colonies even after exposure to sanitizers. The results for this current work showed that there were surviving colonies after exposure to sodium chlorite solution, levulinic acid and a mixture. However, viewing samples treated with the above sanitizers did not clearly show the remains of surviving colonies. The SEM images did not show the presence of an abundance of bacteria on the surfaces. It is possible that the bacteria that were inoculated on the surfaces could have been washed out during the sample preparation procedure. The procedure used for sample preparation might not be suitable in this specific case. Bacteria might also have migrated into other hidden sections of the pictures due to the irregularities of the topography.

Table 2 - Log reductions (CFU/ mL) for chlorine, mixture, levulinic acid and SDS at 1, 3 and 5 minutes.

	Overall log reduction		
Sanitizer	**1 minute**	**3 minutes**	**5 minutes**
Chlorine	2.93	3.16	4.53
Mixture	3.69	4.40	7.17
Levulinic	3.63	4.05	6.71
SDS	8.00	8.00	8.00

Titratable acidity, pH and total soluble solids of tomato samples

Table 3 presents the results of the TA, pH and TSS. The TA of tomato samples treated with levulinic acid and SDS /Lev mixture was significantly different from the control ($P< 0.05$). The TA for levulinic acid treated tomatoes was 2.78%, 2.81%, and 2.81%; while the TA for mixture treated tomatoes was 3.81%, 3.73%, 3.74% for 1, 3 and 5 minutes respectively. The pH for levulinic acid and mixture treated tomato samples was relatively lower than the pH of the control sample, as shown in the table. There was no significant difference between the TA and pH of the mixture and levulinic acid treated samples. The TA for tomato samples treated with SDS was 0.16 and for samples treated with chlorine was 0.15%, 0.14% and 0.14%. These results did not vary significantly from the control. The pH for the SDS and chlorine treated samples were also slightly different from the control sample, as shown in the table. TSS for levulinic acid treated samples were reduced significantly to 3.20% brix for 1, 3 and 5 minutes, while the TSS for mixture treated samples was reduced to 3.24, 3.26, 3.24% brix respectively. Though the TSS for SDS treated and chlorine treated samples were reduced, the effect was not significant according to the findings of this study. Contact time was varied from 1 minute to 5 minutes; but there were no significant changes in theses parameters from 1 minute to 5 minutes.

DISCUSSION

The food manufacturing industry depends on the use of sanitizers for reducing the risk associated with food borne pathogens. Many sanitizers have been tried, but to date food borne pathogens are still a problem in the food and fresh produce industry. Some researchers have suggested that this is due to development of resistance by the bacteria with repeated exposure to sanitizers (MANI-LÓPEZ *et al.*, 2012; RIAZI and MATTHEWS, 2011).

Most fruits and vegetable units resort to chlorine based sanitizers because they are cheaper and have a long standing credibility with reducing surviving bacteria. However, this is proved not to be the case in this current research, as well as other previous research. Findings from this study show that though chlorine has been widely used for washing produce and sanitising food surfaces, it is not really capable of killing all food borne pathogens. This is shown by high mean counts associated with chlorine as presented in the results above. Chlorine washing has also been tried on *Escherichia coli* O157:H7 and Salmonella, but the reports on that work also shows that chlorine is not effective against food borne pathogens (KESKINEN *et al.*, 2009b). Other researchers also agree that chlorine cannot reduce food borne pathogens effectively (IJABADENIYI *et al.*, 2011b; ALLENDE *et al.*, 2009; MAHMOUD *et al.*, 2007).

Several research projects are under way to try

Table 3 - Effects of levulinic acid, chlorine, SDS/Lev mixture and SDS on physicochemical properties of tomatoes.

Tomato treatment	Contact times	pH of sample	Titratable acidity (% citric acid)	Total soluble solids (%Brix)
Distilled water	1 minute	4.77 a	0.16 a	4.90 a
	3 minutes	4.78 a	0.14 a	4.90 a
	5 minutes	4.78 a	0.16 a	4.90 a
Levulinic acid	1 minute	3.61 b	2.78 b	3.20 b
	3 minutes	3.67 b	2.81 b	3.20 b
	5 minutes	3.69 b	2.81 b	3.20 b
Mixture (SDS/Lev)	1 minute	3.81 b	2.76 b	3.24 b
	3 minutes	3.73 b	2.78 b	3.26 b
	5 minutes	3.74 b	2.78 b	3.24 b
Chlorine	1 minute	5.09 a	0.15 a	4.60 a
	3 minutes	5.17 a	0.14 a	4.63 a
	5 minutes	5.20 a	0.14 a	4.61 a
SDS	1 minute	4.68 a	0.16 b	4.65 b
	3 minutes	4.88 a	0.16 b	4.61 b
	5 minutes	4.87 a	0.16 b	4.63 b

Each value represents the mean of three trials. For each parameter, the values significantly different at $P \leq 0.05$ are indicated by different letters. Samples treated in distilled water were used as control. Chlorine = Sodium hypochlorite solution.

to find other alternative sanitizers because of the challenges that are associated with chlorine (KESKINEN *et al.*, 2009a). Some researchers point out that its pH sensitivity affects its effectiveness (ZHAO *et al.*, 2009). Another challenge is that it diminishes quickly upon contact with organic matter and hence leads to reduced effectiveness (NEAL *et al.*, 2012). Other concerns raised include the environmental and health risks associated with the formation of carcinogenic halogenated disinfection by-products such as trihalomethanes (GIL *et al.*, 2009; KIM *et al.*, 2009). For these reasons chlorine has not been gainfully useful in the fresh produce industry in recent years. Though it has been a long standing sanitizer in the food industry, other sanitizers that have been shown to be more effective than chlorine; through this research and previous research can be employed for the betterment of microbiologically quality of fresh produce.

Levulinic acid is applied in the food manufacturing industry as a food additive. It has been designated as a generally safe additive to food by the Food and Drug Administration (FDA)(ZHAO *et al.*, 2009). Levulinic acid disrupts the membrane structure of bacteria due to its polarity, thereby exposing cell constituencies and lethality (Thompson, 2007). Levulinic acid can be used over a wide pH and temperature range (SAGONG *et al.*, 2011). In this particular study, levulinic acid showed mean counts that were much lower than those of chlorine. With these findings, it can be concluded that levulinic acid at 0.5% can perform better than a 200ppm sodium hypochlorite solution against *L. monocytogenes* ATCC 7644. Other researchers also tried levulinic acid in their work with related findings. THOMPSON *et al.* (2008) concluded that it was effective in inhibiting outgrowth of *L. monocytogenes* in ready-to-eat meat products. Other studies using lactic acid, acetic acid and levulinic acid on meat revealed that though levulinic acid is effective, it does not provide as effective decontamination as lactic acid, nor as much residual protection as acetic acid (CARPENTER *et al.*, 2011). Levulinic acid shows potential in the fresh produce industry, and therefore further research can be pursued on the most usable concentrations and most applicable pathogens. Its detrimental effects on quality should be taken into consideration as well.

Sodium dodecyl sulphate is generally regarded as a safe (GRAS) food additive (LU and WU, 2012). In this study, using 1% SDS alone resulted in 8 log CFU/mL reduction of *L. monocytogenes.* SDS has amphilic properties (12 carbon chain attached to sulphate group) and its anti-microbial effectiveness increases when pH is decreased, it has the ability to denature cell proteins and damage cells membranes irreversibly (ZHAO *et al.*, 2009). The action of SDS was much better than that of levulinic acid in this particular study when they were used individually. This is because levulinic acid has a shorter carbon chain (5 carbons and a hydroxyl group), which makes it a weak acid, therefore its effectiveness is less than SDS. Extra care must be taken if SDS is employed in fresh produce as it was established during this study that very low concentrations of 1% can have a very large impact on survival of pathogens.

A combination of 0.05% SDS and 0.5% levulinic acid was also used in this study. Findings show that this mixture achieved better results as compared to levulinic acid alone. Many researchers have reported on the advantages of mixing SDS and levulinic acid. The findings of ZHAO *et al.* (2009) show an increased antimicrobial activity by the combination of SDS and levulinic acid against Salmonella and E. coli O157: H7. Gurtler and Jin (2012) found that a combination of 2% acetic acid, lactic acid and levulinic acid reduce Salmonella on tomatoes. ORTEGA *et al.* (2011) reported that a combination of levulinic acid and SDS was highly effective against E. coli when exposure times were increased to 30 and 60 minutes. On the contrary, GUAN *et al.* (2010) reported that a combination of these had no commercial value as they have detrimental effects on the quality of fresh produce. Combining sanitizers has shown to have a positive contribution in the food market. This has potential for implementation in the fresh produce industry. The implementation of a combination of sanitizers can be tried together with an assessment of their effects on sensory qualities.

Increasing exposure time significantly decreased the surviving colonies of *L. monocytogenes.* In this particular study, a greater fall in surviving colonies was achieved at 5 minutes exposure time. This is evidence that the longer the bacteria are exposed to chemicals, the greater the chances of reducing their survival. PARK *et al.* (2011) also reported that log reductions increase with increasing contact times. Other writers indicate that an exposure time of 3 minutes is effective against food pathogens (MATTSON *et al.*, 2011). DING *et al.* (2011) and Møretrø *et al.* (2012) also reported that the effectiveness of a sanitizer depends on treatment time. Other writers also note that a significant decrease in the bacterial counts occurs in the first minute and the subsequent decrease after one minute is not significant (STEBBINS *et al.*, 2011; TIRPANALAN *et al.*, 2011). From the reports written by other writers and from this research, it can be said that contact time is one of the factors that should be monitored when using sanitizers. Insufficient contact time will lead to high survival after treatment, while extended contact time may lead to damage in the sensory qualities of fresh produce.

The bacteria where further stored for a period of 72 hours at 4 0 C. During this storage period *L. monocytogenes* survived up to 72 hours after being treated with sanitizers, except in

SDS. Survival of pathogens after a storage period of 72 hours is also reported by IJABADENIYI *et al.* (2011b). Sufficient exposure of pathogens to sanitizers is paramount to reduce surviving colonies, as some have the ability to recover even after being treated with sanitizer.

For sanitizers to be effectively used on tomatoes, they should cause negligible changes to pH and titratable acidity of the tomatoes, the major determinants of tomato quality (ANTHON *et al.*, 2011). In this research it was revealed that sanitizers can alter the physicochemical attributes of fresh tomatoes if the sanitizers come into contact with the sub surfaces, thereby affecting the final sensory quality of tomatoes. Other recent studies also point out that sanitizers can affect sensory qualities of fresh produce to some extent (PÉREZ-GREGORIO *et al.*, 2011). In this study, major effects were noted on the pH, TA and TSS with levulinic acid and mixture. Changes effected by SDS and chlorine were not significant. These findings pronounce SDS as a better sanitizer to replace the routine chlorine washing as it causes minimal changes to quality.

In previous studies, SDS was tested together with organic acids and hydrogen peroxide on blue berries and no significant difference was detected in pH and total anthocyanin value between untreated and treated blueberries (LI and WU, 2013). In another study using Iceberg and Romane lettuce, chlorine had high quality scores for Romane lettuce, but caused quality deterioration on Iceberg lettuce. A combination of SDS and Tsunami did not show any effect on sensory attributes of Iceberg lettuce either (SALGADO *et al.*, 2013).

Though levulinic acid did not have favourable results in this particular study, previous studies report that using levulinic acid caused no sensory changes in turkey meat and pork sausages (VASAVADA *et al.*, 2003). A combination of SDS and levulinic did not give favourable results in this study. Other studies also report that SDS used in combination with other sanitizers such as levulinic acid are of low commercial value compared to chlorine, since they cause detrimental effects to sensory attributes (GUAN *et al.*, 2010).

Total soluble solids were reduced for all treatments with levulinic acid having the highest reductions followed by SDS/Lev mixture. This could have been attributed to leaching of contents into treatment solutions as a larger surface area of the subsurface area of tomatoes was exposed. Leaching of materials is also reported by ALEGRIA *et al.* (2009). Though previous studies also report that longer contact times result in deterioration of sensory characteristics (Rico *et al.*, 2007), there was no significant difference for all attributes tested in relation to contact time in this particular study. Contact of sanitizers with sub-surfaces of fresh produce should be minimised to prevent unnecessary damage to sensory quality attributes.

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CONCLUSION

This works confirms that use of sanitizers in food processing at shorter contact time of 1 minute may not eradicate food borne pathogens. SDS alone is capable of destroying *L. monocytogenes,* causing no detrimental effect to sensory attributes of tomatoes. It is also important to consider exposure time to increase the effectiveness of sanitizers. Sanitizers can have detrimental effects on the sensory attributes of fresh produce; hence careful consideration is required when selecting sanitizers for particular produce. Further studies are required to validate the application of levulinic acid and SDS as sanitizers in food processing as well as their efficacy.

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PAPER

PHYSICOCHEMICAL, MICROBIAL AND SENSORY QUALITY OF FRESH-CUT RED BEETROOTS IN RELATION TO SANITIZATION METHOD AND STORAGE DURATION

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ABSTRACT

Effects of sanitization and storage on fresh-cut beetroots (*Beta vulgaris* L.) were evaluated following sanitation – peeling - cutting (SPC), peeling – sanitation – cutting (PSC) and peeling – cutting – sanitation (PCS) methods with (Cl), or without (TW), 100 ppm chlorine solution, then packaged in polyethylene bag and stored at 5°C for up to 14 days. Chroma values of fresh-cut beetroots significantly declined whereas whiteness index and titratable acidity values increased, however, texture and total soluble solid contents showed no significant variation. Betalain contents decreased gradually and total phenol content showed inconsistence trend. PCS-Cl treated samples accounted for higher betalains decline and received lower visual quality scores despite its lower total aerobic bacterial count. Minimum microbial population was observed in PSC-Cl methods along with higher levels of betalain contents. Considering pigment retention, microbial and visual qualities, beetroots sanitized with chlorine water following PSC method was the best processing way for fresh-cut beetroots and therefore, PSC-Cl treatment could commercially be used for processing of fresh-cut beetroots.

- Keywords: betalain, microbial population, quality, red beetroot, sanitation, total phenolic -

INTRODUCTION

Beet is a root vegetable of the Chenopodiaceae family whose edible part is its tuberous root. Its purple-red color is due to the presence of betalain pigments. These pigments are similar to anthocyanins and flavonoids, which were wrongly termed in the old literature as anthocyanins containing nitrogen. Betalains are water-soluble, vacuolar pigments and are found only in ten families of the *Centrospermae* group and are divided into two classes: red betacyanin and yellow betaxanthin (FENENA, 1995). In addition to their color, betalains possess several desirable biological activities including antioxidant, anti-inflammatory, hepatoprotective, and antitumor properties (ESCRIBANO *et al.*, 1998; WINKLER *et al.*, 2005). Although some studies have indicated their potential as antioxidant pigments, betalains have not been much explored because of their relative scarceness in nature. The bioavailability of betalains was reported to be high in humans, and they remain stable in the gastrointestinal tract without any significant loss in antioxidative properties, which increases their value as health food additives (FRANK *et al.*, 2005; PAVLOV *et al.,* 2005). Among other compounds with antioxidant properties, phenolics are believed to act as antioxidant, anti-carcinogenic, anti-microbial, anti-mutagenic and anti-inflammatory, as well as in the reduction of cardiovascular diseases (YANG *et al.*, 2001; KIM *et al.*, 2003; VALI *et al.*, 2007). Because of these nutritional importances of plant phenolics, there has been an increasing interest in the evaluation of their changes with postharvest treatment (CHAUDRY *et al.*, 1998; LEWIS *et al.*, 1999). However, literatures are scanty on phenolic content of plant foods, especially of roots vegetables, which are important constituents of diets in many countries. Storage and processing can reduce the content of phenolic compounds as some of them are easily oxidized, while others are more stable. Processing in the form of simple peeling of fruit and vegetables can remove a major portion of the phenols, as the concentrations of these substances are often higher in the outer than the inner parts (MANACH *et al.*, 2004). Pigment distribution in beetroots, on the other hand, also varied substantially among the outer, middle and center tissues, the former contained much higher pigments than the later tissues (GAERTNER and GOLDMAN, 2005).

Beet was traditionally used as a vegetable boiled in stews, baked in tarts, roasted as a whole and cut into salads. This vegetable has recently been using as a minimally processed food in many countries. However, the main technological problems of processing fresh-cut beet roots are the significant discoloration and dehydration of the minimally processed material. Washing and rinsing operations carried out after slicing have favored the loss of betacyanin and betaxanthin, since these pigments are soluble in water (NILSON, 1970). Moreover, minimally processed produces are more perishable than their whole counterparts, due to undergoing severe physical stresses especially during peeling and cutting procedures. Minimal processing comprises selection, washing, peeling and cutting procedures that are aimed at producing a fresh and convenient product to prepare and consume (BURNS, 1995). The quality of fresh-cut produce depends on many factors, such as the state of the original plant (variety, plant part and maturation stage), harvesting date and storage, environmental factors and processing techniques (MOURE *et al.*, 2001). However, rapid quality deterioration and shorter shelf-life are major problems facing the industry for maintaining the quality and safety of fresh-cut produces. Several studies have shown that fresh-cut produces are particularly susceptible to microbial growth owing to the removal of plant protective tissues and the release of cellular fluids from cutting (CARLIN *et al.*, 1989; HEARD, 2002). The cut slices of beetroot become vulnerable to microbial attack, moisture loss and dehydration because of their large surface area. To ensure the microbial safety, use of proper sanitizing agents along the processing line is an important step in fresh-cut produce processing. Chlorine has been used for sanitation purposes in food processing industry for several decades and perhaps the most widely used sanitizer in food industry. It is inexpensive, convenient to use and works against many food borne pathogens. Liquid chlorine and hypochlorite generally used in the 50 to 200 mg L^{-1} concentration range with a contact time of 1 to 2 min. ADAMS *et al.* (1989) reported that washing lettuce leaves with 100 mg L^{-1} free chlorine can reduce population of aerobic mesophiles by more than 98% as compared to 92% reduction in tap water without chlorine.

The use of beetroots as a fresh-cut produce is relatively new, especially in Asian countries. The information on various quality parameters of fresh-cut beetroots processed with different sanitization methods is limited. Hence, the objectives of this study were to find out the best processing and sanitation way for maintaining some physicochemical, microbial and sensory qualities, especially pigment and phenolic contents of fresh-cut beetroots during storage at 5°C.

MATERIALS AND METHODS

Preparation of sample

Beetroots (*Beta vulgaris* L. var. Udan) samples were harvested from a commercial farm in Jeju island, Republic of Korea, and were transported to our laboratory within two days. Samples were thoroughly washed in running tap water and subjected to different ways of sani-

tation treatments such as sanitation – peeling - cutting (SPC), peeling – sanitation – cutting (PSC) and peeling – cutting – sanitation (PCS) with (Cl), or without (TW), 100 ppm chlorinated solution. Chlorinated solution was prepared with NaOCl (100 ppm free chlorine, pH 7.0) as a standard industrial disinfection treatment for fresh-cut produces. After peeling out, beetroot samples were cut into small pieces (ca. 4 × 5 × 1 cm) excluding the top and bottom most portions. Sanitation treatments lasted for 2 min. To remove the excess surface water, all sanitized samples were spread over a sieve like plastic tray (40 × 50 cm) previously washed and disinfected with the same sanitizing solution and allowed at room temperature. Fresh-cut beetroots sample of about 300 g were packaged in 80 μm nylon polyethylene bag (25 × 20 cm) and thermally sealed. Four replicates of each bag per treatment and storage duration (0, 3, 7, 10 and 14 days of storage) were prepared and stored in a dark cold room at 5°C. On each evaluation day, outer and inner tissues were separated from the fresh-cut red beetroots slices discarding the middle portion and were stored at -80°C until needed for biochemical analyses.

Color and texture measurement

Using a chromameter (Minolta CR-400, Minolta, Osaka, Japan), color readings were taken from the middle portion of both sides of sliced red beetroots on each evaluation day. Three pieces of sliced beetroot from each pouch were randomly selected and six readings (from both sides of each piece) were taken from each replicate and a total of 24 readings were averaged from each treatment on the measurement day. The chromameter was calibrated using the manufacturer's standard white plate (Y 93.5, x 0.3155, y 0.3320). Color changes were quantified in the L^*, a^*, b^* color space. L^* refers to the lightness and ranges from black = 0 to white = 100. A negative value of a^* indicates green, while a positive number indicates red color. Positive and negative b^* indicate yellow and blue color, respectively. The color values were further converted into Chroma value (OZTURK *et al.*, 2009) and 'Whiteness Index' (WI) (BOLIN and HUXSOLL, 1991) and were computed by the formulae:

$$\text{Chroma} = (a^{*2} + b^{*2})^{1/2};$$
$$\text{WI} = 100 - [(100 - L^*)^2 + a^{*2} + b^{*2}]^{1/2}$$

The texture of fresh-cut red beetroot was measure in term of force required to make a puncture hole horizontally on the sliced beetroots. The test was conducted through destructive puncture test performance using a texture analyzer (TA Plus, Lloyd Instruments, Ametek Inc., UK) following the method of HAMPSHIRE *et al.* (1987). Each sliced beetroots sample was placed horizontally on the stationary platform of the analyzer for the test. Tests were carried out on the middle part of each sliced beetroots with a 4 mm diameter stainless steel cylindrical probe. The movement of the probe was adjusted to 5, 2 and 10 mm/s as the pre-test, test and post-test speed, respectively. With a running load cell of 100 N, the probe was attached to a creep meter equipped with the software (NEXYGEN™MT v 4.5, Lloyd Instruments, Ametek Inc., UK) for automatic analysis using a computer. The maximum amount of force (N) needed to make a puncture hole on the sliced beetroot was recorded. Five pieces of sliced beetroots from each pouch were tested and a total of 20 data from four replicates were averaged from each treatment. All measurements were taken at room temperature (20°±2°C).

Determination of total soluble solid, pH, and titratable acidity

Total soluble solid (TSS), pH and titratable acidity (TA) were measured according to the AOAC (1980) procedures. On each evaluation day, about 5 pieces of sliced beetroot from each bag were cut into small pieces and wrapped with 2 layers of cotton cloth and placed in a Juice maker (Fru-X80, GooJung Chromatech Inc., Korea) attached to an air supplier (EvaA–0300) of the same company. Then pressure was created by the air supplier to obtain a homogenized solution of beetroots sample. TSS of the resultant cleared juice was measured in terms of °Brix using a refractometer (PAL–1, Atago Co. Ltd, Tokyo, Japan). The pH was determined using a pH meter (D-55122, Schott Instruments GmbH, Germany) with a glass electrode. Titratable acidity was measured by potentiometric titration with 0.1 N NaOH up to pH 8.2 using 10 mL juice and the results were expressed as percentage of citric acid.

Determination of betalains (betacyanin and betaxanthin) and total phenolic contents

The methodology used for the determination of betacyanin and betaxanthin were adopted from VON ELBE (2001). Five gram of previously frozen samples from the outer and inner parts of sliced beetroots were macerated separately in 15 mL distilled water using an Ultra-Turrax tissue homogenizer (T 25 B, Ika Works Sdn. Bhd, Malaysia) at a moderate speed for about 1 min. The homogenate was transferred to a volumetric flask filtered through Whatman no. 1 filter paper placed on a glass funnel. The filter cake was washed several times with distilled water until the extract became colorless. The extract volume was adjusted with distilled water. The resulting beetroots juice or tissue extract was diluted with 0.05 M phosphate buffer, pH 6.5 such that the absorbance of beetroots juice at

538 nm was in between 0.4 and 0.5 AU. Finally, the absorbance of beetroots juice was measured at 476, 538 and 600 nm with an UV-VIS recording spectrophotometer (DU 650, Beckman Coulter™, USA) and 0.05 M phosphate buffer, pH 6.5 was used as the blank. Values of betacyanin and betaxanthin amounts were obtained through the equation:

$$x = 1.095 \times (a - c),\ y = b - z - x/3.1,\ z = a - x$$

where a = sample reading at 538 nm; b = sample reading at 476 nm; c = sample reading at 600 nm; x = betacyanin absorption; y = betaxanthin absorption; z = impurities absorption (VON ELBE, 2001).

Total phenolic compound was determined based on the method described by SINGLETON and ROSSI (1965) with few modifications. Five gram previously frozen sample from the outer and inner parts of fresh-cut red beetroots slices were separately homogenized with 80% ethanol using an Ultra-Turrax tissue homogenizer (T 25 B, Ika Works Sdn. Bhd, Malaysia) at a moderate speed for about 1 min. The homogenate was incubated at 60°C water bath for 30 min and centrifuged at 15,000 × *g* for 15 min at 20°C and then the supernatant was collected in a volumetric flask. The homogenized tissue was re-extracted with 80% ethanol at the same way and the resulting supernatants were mixed together and carefully made known volume with 80% ethanol. For the determination, Folin-Ciocalteu reagent was diluted with distilled water to make 1 N phenol reagent. In a test tube, 1 mL supernatant was diluted with 8 mL distilled water and 1 mL of 1 N phenol reagent was added followed by mixing. After 5 min, 1 mL 15% sodium carbonate solution was added, mixed well and allowed the mixture at room temperature (~20°C) for 2 h. The absorbance was read at 725 nm using an UV-VIS recording spectrophotometer (DU 650, Beckman Coulter™, USA). The concentration of total phenol was calculated using standard curve of gallic acid and expressed as gallic acid equivalents in mg 100 g^{-1} fresh weight.

Monitoring of microbial population

Microbiological counts were carried out on every sampling day including washing day. Fresh-cut beetroots pouches were aseptically opened using a sterilized scissors dipped in 95% ethanol and then ignited in the flame of a Bunsen burner. Twenty grams beetroots sample was weighed out from each pouch and placed in a stomacher bag (Masher-bag P-LTS, BAC-cT®, NBT, Japan). The beetroots sample was diluted 1:9 in double distilled autoclaved water and homogenized in a stomacher (Seward Stomacher 400C, Brinkmann, USA) for 1 min at 230 rpm. A 10-fold serial dilution was also made from the homogenate and 1 mL of homogenate solution was inoculated onto total aerobic bacterial (TAB) count Petrifilm™ (3M Microbiology Products, St. Paul, Minn., USA). The plates were then incubated at 35°C for 48 h and the developing colonies (about 25 – 250) were counted with the assistance of a 3M microbial colony counter (same company as of plate) and reported as colony forming units (CFU) per gram of sample. Similarly, 1 mL homogenate was plated onto 3M Petrifilm™ yeast and mold (YM) count plates (same company) and incubated for 72 h at 25°C. After incubation, yeast and mold colonies were counted manually according to the instruction guide of the company. Colonization data were then converted to log CFU per gram of fresh sample.

Sensory evaluation

The sensory analysis of fresh-cut beetroots sample was carried out by an 8-member (aged 24 - 48) expert panel. The members of the panel were trained to recognize and score off-odor and overall visual quality of fresh-cut beetroots prior to the test. Off-odor was evaluated immediately after opening the packages and scored on a 5-point scale in which 0 = none, 1 = slight, 2 = moderate, 3 = strong, and 4 = extremely strong (LOPEZ-GALVEZ *et al.*, 1997); a score of 3 was considered non-acceptable. Overall visual quality was evaluated by using 9-point scale (9 = excellent, 7 = good, 5 = fair, 3 = poor and 1 = unusable) (GONZALEZ-AGUILAR *et al.*, 1999). A score of 6 was considered as the limit of marketability.

Statistical analysis

The experiment was conducted with four replications per treatment. Statistical analyses of the data were carried out using SAS software (SAS Institute, Cary, NC, USA). The level of significance was calculated from the *F* value of ANOVA. Mean comparison was achieved by Duncan's multiple range test. Prior to the final experiment, two preliminary experiments were conducted with limited replications that resulted similar trend.

RESULTS AND DISCUSSION

Texture, total soluble solids, pH and titratable acidity

The effects of different washing methods and storage duration on the textural properties of fresh-cut red beetroots are presented in Table 1. The forces were almost similar for all the sanitization methods, except for an insignificant ($P>0.05$) lower value was recorded in PSC-Cl treatment on washing day. However, the values were slightly increased to an

Table 1 - Changes in some physicochemical qualities of fresh-cut redbeet root during storage at 5°C after processed with different sanitization methods.

Parameter/storage day	Sanitization method					
	SPC-TW	SPC-Cl	PSC-TW	PSC-Cl	PCS-TW	PCS-Cl
Texture (N)						
0	52.58aA	52.22aA	49.92aA	47.61aA	51.13aA	52.49aA
7	52.83aA	53.80aA	51.40aA	49.51aA	52.47aA	53.85aA
14	53.43aA	54.97aA	53.39aA	50.36aA	54.29aA	55.36aA
°Brix						
0	8.50aA	8.67aA	7.83aA	8.70aA	8.33aA	8.23aA
7	8.23aA	8.60aA	8.37aA	8.03aA	8.57aA	8.53aA
14	7.90aA	8.67aA	8.03aA	8.27aA	8.43aA	8.37aA
pH						
0	6.28aA	6.25aAB	6.24bAB	6.28aA	6.22aAB	6.16cB
7	6.42aAB	6.38aBC	6.38aBC	6.30aC	6.35aBC	6.50aA
14	6.26aA	6.28aA	6.21bA	6.30aA	6.21aA	6.36bA
TA (% citric acid)						
0	0.10bA	0.10bA	0.10bA	0.12aA	0.09bA	0.09bA
7	0.09bAB	0.08bB	0.08bB	0.10aAB	0.11bA	0.10bAB
14	0.17aA	0.15aA	0.15aA	0.16aA	0.18aA	0.14aA

SPC = sanitation - peeling - cutting, PSC = peeling - sanitation - cutting, PCS = peeling - cutting - sanitation, Cl = sanitation with 100 ppm chlorinated water, TW = sanitation with tap water.
Values represent the mean of four replicates. Means under the same heading in each column or row with different small or capital letters, respectively are significantly different according to the Duncan test ($P<0.05$).

insignificant level at the end of 14 days storage. PCS-Cl treated sample exhibited the highest value among the treatments at the end of storage. Maintaining texture of fresh-cut produces is one the main criteria which is affected by morphology, cell turgor, cells wall-middle lamella structure, water content, biochemical components and also by the genetic background of plant species (HARKER *et al.*, 1997). Peeling and cutting of vegetable exposes the interior tissues and drastically increase the rate of evaporation of water. However, we observed less than 1% moisture loss of the original weight at the end of storage (data not shown). Apart from water loss, the slight increase in texture value during storage of fresh-cut beetroots in our study might be due to the lignification of cells that rendered harder tissues and as a result of wound healing at cut surfaces. Packaging fresh-cut produces with suitable polyethylene film and selecting appropriate storage temperature have shown to preserve quality. The insignificant increases in texture of fresh-cut beetroots indicate that our packaging film and storage temperature were appropriate.

There was no significant difference ($P>0.05$) found in total soluble solid (TSS) content in fresh-cut beetroots regardless of sanitization treatment and storage duration, whereas slight variations were observed in pH and titratable acidity (TA) values among the treatments and different storage durations (Table 1). These reflect that although TSS was maintained throughout the storage, TA values increased significantly at the end of the storage that resulted smaller decline in pH values. The lowest TSS (7.83°Brix) was found in PSC-TW treated sample on washing day while the highest value (8.70°Brix) was recorded in PSC-Cl treated sample on the same day. It was reported that TSS of fresh-cut beetroots are varied depending on the cut type (KLUGE *et al.*, 2006), sanitization period (VITTI *et al.*, 2011) and storage temperature (VITTI *et al.*, 2005). Although our TSS data were little higher than that of VITTI *et al.* (2011) and VITTI *et al.* (2005) studies with fresh-cut beetroots, we found both similar amount and changing pattern of TSS during storage as reported by KLUGE *et al.* (2006). The differences in TSS in this study and other studies might be due to the differences in soil condition, cultivar and sowing time (FELLER and FINK, 2004). The pH and TA values ranges from 6.16 to 6.50 and 0.08 to 0.18, respectively among the processing methods and throughout the storage (Table 1). In general, pH values slightly increased in the middle of storage and decreased later to their initial values. TA values expressed as percentage of citric acid, on the other hand, remained unchanged until the middle of storage and then increased about 1.5 fold at the end of storage to their initial levels. Among the treatments, PCS-Cl treated sam-

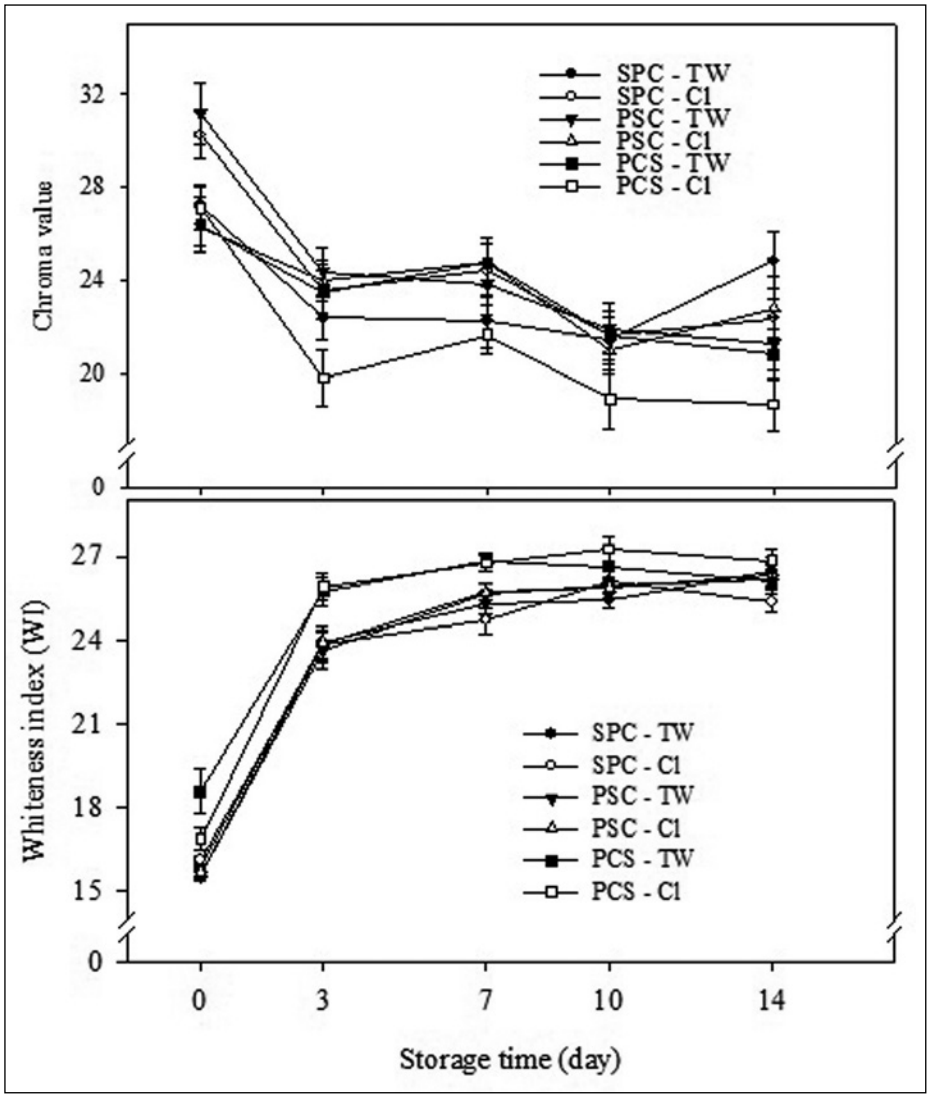


Fig. 1 - Changes in chroma value (above), whiteness index (below) of fresh-cut red beetroot after processing with different sanitation methods and during storage at 5°C. Legends: SPC = sanitation–peeling-cutting, PSC = peeling–sanitation–cutting, PCS = peeling–cutting–sanitation, Cl = sanitation with 100 ppm chlorinated water, TW = sanitation with tap water.

ple showed the lowest values of pH and TA on washing day and 14 days of storage, respectively. In agreement with our results, LOPEZ OSORNIO and CHAVES (1998) found no variation in pH values over a 7-day storage period of raw grated beetroots at 4°C and packaged in trays wrapped with polyvinylchloride film. However, they found steadily increased values of TA throughout the storage. Again, PILON *et al.* (2006) reported that the contents of titratable acidity were not affected by the storage period in minimally processed carrot while the pH values decreased at the beginning of storage and increased thereafter to their initial levels at the end of 3-week storage period. The smaller range of changes in citric acid content during storage of fresh-cut beetroots might be accounted for lower respiratory activity, which is the indicator of retardation of overall metabolic activities.

Color parameters

Produce color is one of the most important quality factors that directly affect consumers' choice. In this study, the effects of different sanitization methods on the color parameters of fresh-cut beetroots were measured in term of chroma value as well as whiteness index and presented in Fig. 1. Among the treatments, SPC-Cl and PSC-TW showed significantly higher (P<0.05) chroma value on washing day. However, chroma values declined gradually in all treatments when storage progressed except few fluctuations on 7 and 14 days of storage. There was a significant (P<0.05) decline in chroma value in PCS-Cl treatment when storage progressed exhibiting the lowest values of chroma throughout the storage among the treatments. This result implies that sanitation after cutting yielded higher loss of pigment in beetroots slices there-

by decreased the intensity of color. Between the other two sanitation treatments using chlorine water, PSC-Cl showed no significant decline in chroma value until the end of storage except on 10-day while SPC-Cl showed gradual decrease until 10-day followed by a slight increase at the end of storage (Fig. 1). The decrease in color is a consequence of loss of betalains, the main pigments of red beetroots (VON ELBE, 2001). Therefore, we found significant variation and decrease in betalain contents both on washing day and subsequent storage of fresh-cut beetroots in this study (Table 2 and later discussion). In consistence with our result, gradual decrease in color index of fresh-cut beetroots was reported previously (VITTI *et al.*, 2005; KLUGE *et al.*, 2006; VITTI *et al.*, 2011). Since chroma represents color saturation, which varies from dull (low value) to vivid color (high value), we used chroma value as an indicator of freshness and purity of the color of beetroots slices. However, LOPEZ OSORNIO and CHAVES (1998) found significant increase in chroma values during storage of grated beetroots. This might be attributed to the differences in processing, subsequent packaging and storage condition of beetroots in the present study with their study. Whiteness index (WI) on the other hand, sharply increased on the third day of storage in all samples (Fig. 1). Both of the sanitized and water washed samples of peeling – cutting - sanitation (PCS) method exhibited significantly (P<0.05) higher values of WI from the washing day to 10 days of storage. This result also indicates that higher pigment loss was occurred when beetroots samples were subjected to sanitize after cutting. Other two methods showed similar values of WI both on processing day and throughout the storage. At the end of the storage, WI values reached nearly similar level for all samples. Although WI was first measured for lignin formation on the surface of fresh-cut carrot slices (BOLIN and HUXSOLL, 1991), LOPEZ OSORNIO and CHAVES (1998) reported that the whitish substances in grated beetroots are a protective lignin biochemically synthesized after peeling. It was also reported that WI is the most sensitive and easily measured indicator of sensory quality of fresh-cut carrot.

Betalains (betacyanin and betaxanthin) content

Significantly higher betalain contents were found in the outer tissues than in the inner tissues of fresh-cut beetroots slices (Table 2). Beetroots samples processed with peeling – cutting - sanitation (PCS) method exhibited significantly (P<0.05) lower amount of betalain (betacyanin and betaxanthin) contents on washing day compared to other methods. In general, sanitation – peeling – cutting (SPC) method ensured higher amount of betalain contents than other methods whereas the betalain contents

Table 2 - Changes in betalain (betacyanin and betaxanthin) content in the outer and inner tissues of fresh-cut red beetroots processed with different sanitization methods and during storage at 5°C.

Treatment	Betalain content/storage day													
	Betacyanin (mg 100g^{-1} FW)					Betaxanthin (mg 100g^{-1} FW)					Total betalain (mg 100g^{-1} FW)				
	0	3	7	10	14	0	3	7	10	14	0	3	7	10	14
Outer tissue															
SPC-TW	74.65aA	65.48aAB	55.79aBC	54.57aBC	49.41aC	60.54aA	54.26aA	51.64aB	47.33aBC	42.95aC	135.19aA	119.74aAB	107.43aBC	101.90aBC	92.36aC
SPC-Cl	72.85abA	62.08aAB	60.37aAB	59.70aAB	51.91aB	56.30aA	54.53aA	51.15aAB	49.12aAB	44.13aB	129.15abA	116.61aAB	111.52aAB	108.82aAB	96.04aB
PSC-TW	65.35abA	55.80aA	53.31aA	52.49aA	49.03aA	52.50aA	51.21aA	47.35aA	41.62aA	42.49aA	117.85abA	107.01aA	100.66aA	94.11aA	91.52aA
PSC-Cl	66.72abA	55.50aA	53.36aA	54.96aA	54.95aA	50.91aA	45.60aA	45.49aA	43.42aA	41.88aA	117.63abA	101.10aA	98.85aA	98.38aA	96.83aA
PCS-TW	58.06bA	53.75aA	52.47aA	49.13aA	47.92aA	46.46aA	41.78aA	39.78aA	44.37aA	39.74aA	104.52bA	95.53aA	92.25aA	93.50aA	87.66aA
PCS-Cl	57.65bA	51.52aA	48.42aA	45.02aA	42.80aA	47.87aA	43.86aA	45.17aA	40.34aA	39.25aA	105.52bA	95.38aA	93.59aA	85.36aA	82.05aA
Inner tissue															
SPC-TW	37.45abA	34.18aAB	34.78aAB	30.65aAB	25.38aB	29.82abA	28.31aA	28.60aA	23.74aAB	18.72aB	67.27abA	62.49aA	63.38aA	54.39aAB	44.10aB
SPC-Cl	39.88aA	31.61aAB	32.46abAB	29.43aB	29.24aB	32.30aA	23.91aBC	26.88aAB	21.41aBC	18.08aC	72.18aA	55.52aB	59.34abAB	50.84aB	47.32aB
PSC-TW	30.88bcA	30.53aA	30.89abA	26.03aA	25.27aA	25.31abA	22.80aA	24.09aA	19.34aA	18.93aA	56.19bcA	53.33aA	54.98abA	45.37aA	44.20aA
PSC-Cl	28.50cA	27.71aA	31.93abA	26.76aA	28.15aA	25.93abA	21.19aAB	21.04aAB	20.76aAB	18.35aB	54.43bcA	48.90aA	52.97abA	47.52aA	46.50aA
PCS-TW	28.06cA	26.88 aA	24.03bA	24.72aA	25.29aA	22.19bA	20.33aA	21.52aA	19.68aA	17.96aA	50.25cA	47.21aA	45.55bA	44.40aA	43.25aA
PCS-Cl	25.48cA	25.79 aA	24.39abA	26.18aA	23.37aA	22.17bA	22.98aA	21.75aA	19.76aA	18.45aA	47.65cA	48.77aA	46.14abA	45.94aA	41.82aA

SPC = sanitation – peeling - cutting, PSC = peeling – sanitation – cutting, PCS = peeling – cutting – sanitation, Cl = sanitation with 100 ppm chlorinated water, TW = sanitation with tap water.
Values represent the mean of four replicates. Means under the same heading in each column or row with different small or capital letters, respectively are significantly different according to the Duncan test (P<0.05).

of peeling – sanitation – cutting (PSC) methods were in between the betalain contents levels of SPC and PCS methods. However, betalain contents gradually decreased when storage progressed in all washing/sanitation treatments. The rate of betalain decline during storage was higher in SPC sanitization method compared to other two methods thereby significant (P<0.05) declines were observed in all components of both tissues at the end of storage (Table 2). The highest (34.4%) decline of total betalain was found in both of SPC-TW and SPC-Cl treated samples of inner tissues while the lowest (12.2%) decline was observed in PCS-Cl treated sample of same portion of sliced beetroots. Overall, the decline in betacyanin was little higher than betaxanthin in the outer tissues whereas opposite trend was found in the inner tissues. At the end of the storage, total betalain content was almost similar in SPC-TW and PSC-TW treated samples as well as in SPC-Cl and PSC-Cl treated samples of both tissues (Table 2). Samples of PCS treatments showed the lowest amount of total betalain contents at the end of storage in both tissues. It appears that although the declining rate of betalain was lower in PCS method than other methods, the total amount of betalain content was comparatively lower than other methods at the end of storage probably due to the higher loss of betalain in this method on washing day. In this study, sanitization and washing favor larger pigment losses of beetroots slices owing to their exposure to water or sanitized solution and therefore, we observed significant variation in betalain contents on washing day among the processing/sanitization methods. Similar to our results, the variations and decreases in betalains were also found in few studies (VITTI *et al.*, 2005; KLUGE *et al.*, 2006; VITTI *et al.*, 2011). In contrast our pigment decline rate, LOPEZ OSORNIO and CHAVES (1998) found about 40-50% decreases in betalain amount in grated beetroot after 7 days of storage at 0°C, whereas at 4°C the decreases were greater. However, these authors did not measure the pigment contents at different tissues of beetroots, which we did in the present study. Betalains accumulate in cell vacuoles of the leaves, flowers and fruits of the plants that synthesize them, mainly in epidermal and/or subepidermal tissues (JACKMAN and SMITH, 1996). Moreover, it was reported that the total phenolic contents and the main betacyanin present in red beetroots distributed mostly towards the outer parts of the root and decreasing in the order peel, crown and flesh (KUJALA *et al.*, 2000). This localized distribution of betalains was also found in our study. However, due to large number of samples, we did not use the middle part of beetroots tissues and only the outer and inner tissues were used for biochemical measurement. Nevertheless, we assume that our fresh-cut beetroots slices were larger in size than that of grated beetroot which might prevented higher decline of pigments compared to that of LOPEZ OSORNIO and CHAVES (1998) findings. NILSON (1973) observed the contents of betacyanin and betaxanthin is cultivar dependent and the levels of these pigments are around 45 to 210 and 20 to 140 mg 100 g^{-1}, respectively.

Total phenolic contents

Total phenolic contents of sliced beetroots (Fig. 2) followed similar distribution trend at different tissues that we found in betalain contents and is supported by the results of KUJALA *et al.* (2000). On the washing day, total phenolic contents also followed similar pattern as we observed for betalains contents among the treatments. The amount of total phenolic contents in the outer and inner tissues of beetroots samples ranged from 102.4 to 116.4 mg GAE 100 g^{-1} FW and 52.1 to 80.0 mg GAE 100 g^{-1} FW, respectively on washing day (Fig. 2). Higher amount of total phenolic content was observed in SPC method followed by PSC and PCS methods. However, we did not find any specific trend in total phenolic content of sliced beetroots during storage at low temperature. In the outer tissue, total phenolic contents declined on the third day of storage and then slightly increased the content or keep constant throughout the storage. At the end of the storage, total phenolic contents of this portion slightly increased (P>0.05) than the levels observed on washing day. PCS-TW treated sample showed the lowest amount of total phenolic contents both on washing day and throughout the storage. At the end of storage, SPC-TW treated sample showed the highest amount of total phenolic content (118.1 mg GAE 100 g^{-1} FW) followed by PSC-Cl treated sample (116.8 mg GAE 100 g^{-1} FW). Variations in total phenolic contents were also observed in the inner tissues on washing day and the contents either increased or decreased or maintained its level after fluctuating in between the storage. Although the highest amount of total phenol (79.9 mg GAE 100 g^{-1} FW) was determined in SPC-TW treated inner tissues' sample on washing day, PSC-Cl treated sample retained the highest amount (71.3 mg GAE 100 g^{-1} FW) among the treatments at the end of storage (Fig. 2). KUJALA *et al.* (2000) observed decreased amount of total phenolic content until 63 days and the amount remained almost unchanged until 196 days of storage of raw beetroots. The effects of different storage temperatures on the total phenolic content in plants have been studied and, like our study, both increases and decreases in phenolic contents have been reported. For example, LEWIS *et al.* (1999) observed an increase in total phenolic contents of colored potato tubers during storage at 4°C, whereas CORDENUNSI *et al.* (2005) found that total phenolic contents of strawberry remained

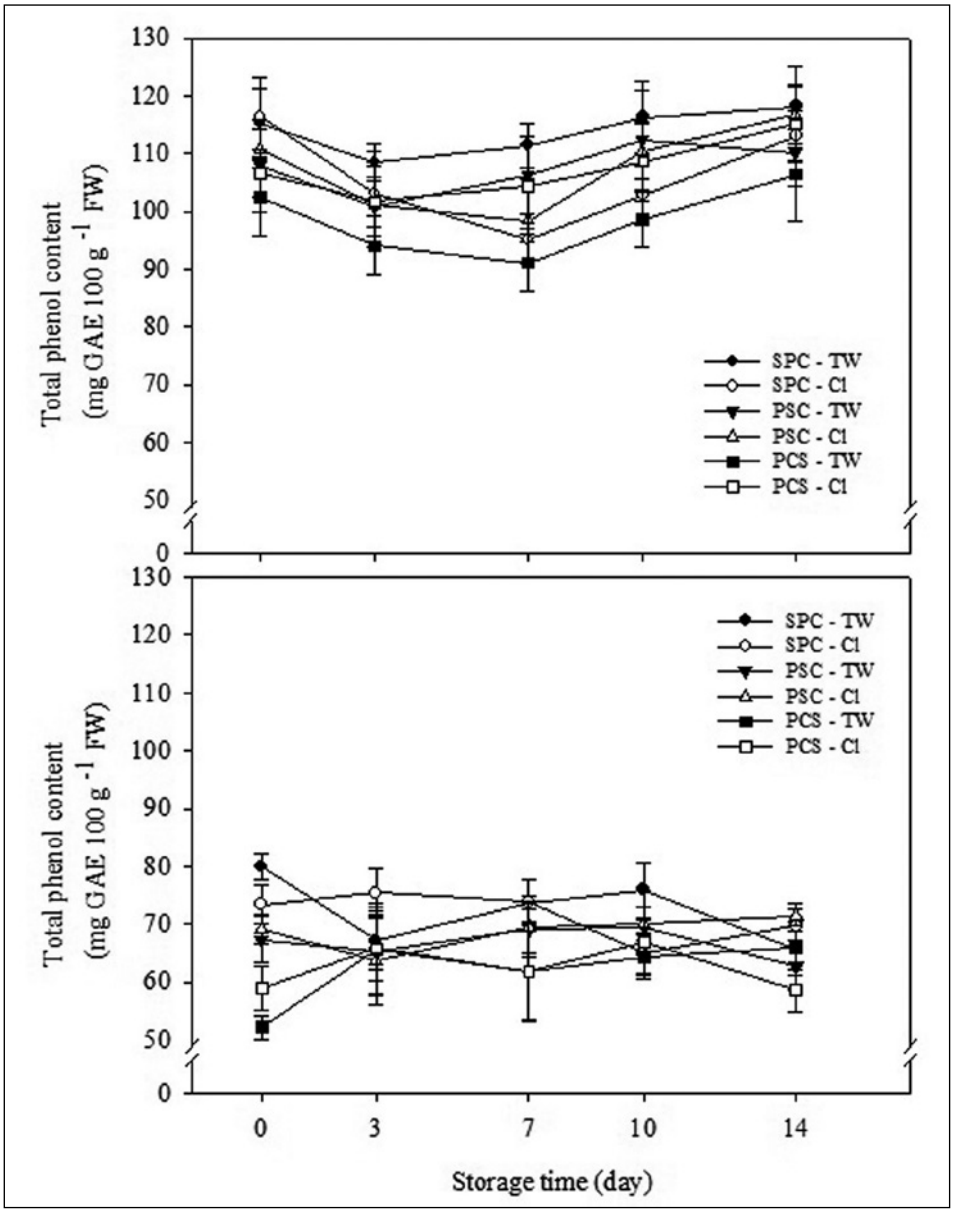


Fig. 2 - Total phenol content of outer (above) and inner (below) tissues of fresh-cut red beetroots after processing with different sanitation methods and during storage at 5°C. Legends: same as shown in Fig. 1.

constant or even slightly decreased during storage at different temperatures.

Microbial quality

Figure 3 shows the changes in total aerobic bacteria (TAB) count and yeast and mold (YM) count of fresh-cut red beetroots on washing day and during successive storage at low temperature. Significant variation ($P<0.05$) was found in TAB among the washing treatments both on washing day and throughout the storage. The range of TAB was found 2.6 log CFU g^{-1} in PSC-Cl to 3.4 log CFU g^{-1} in SPC-TW treated samples on washing day. This result indicates that sanitation of beetroots with 100 ppm chlorinated water ensured a significant reduction in TAB both in PSC and PCS methods. In order to reduce the microbial population, chlorine solution has been used as sanitizer in many fresh-cut vegetables including beetroots (LOPEZ OSORNIO and CHAVES, 1997; VITTI *et al.*, 2011) and sweet potatoes (ERTURK and PICHA, 2006). However, studies on microbial safety or monitoring of microbial population in fresh cut beetroots are still limited as compared to other fresh produces. Since beetroots contain several bioactive compounds, its use as fresh-cut produce is promising where microbial safety should be addressed and ensured. Beetroots washed with tap water either before or after peeling had nearly similar number of TAB which indicates the necessity of sanitation of fresh-cut beetroots with appropriate sanitizer. Sanitation with chlorinated water be-

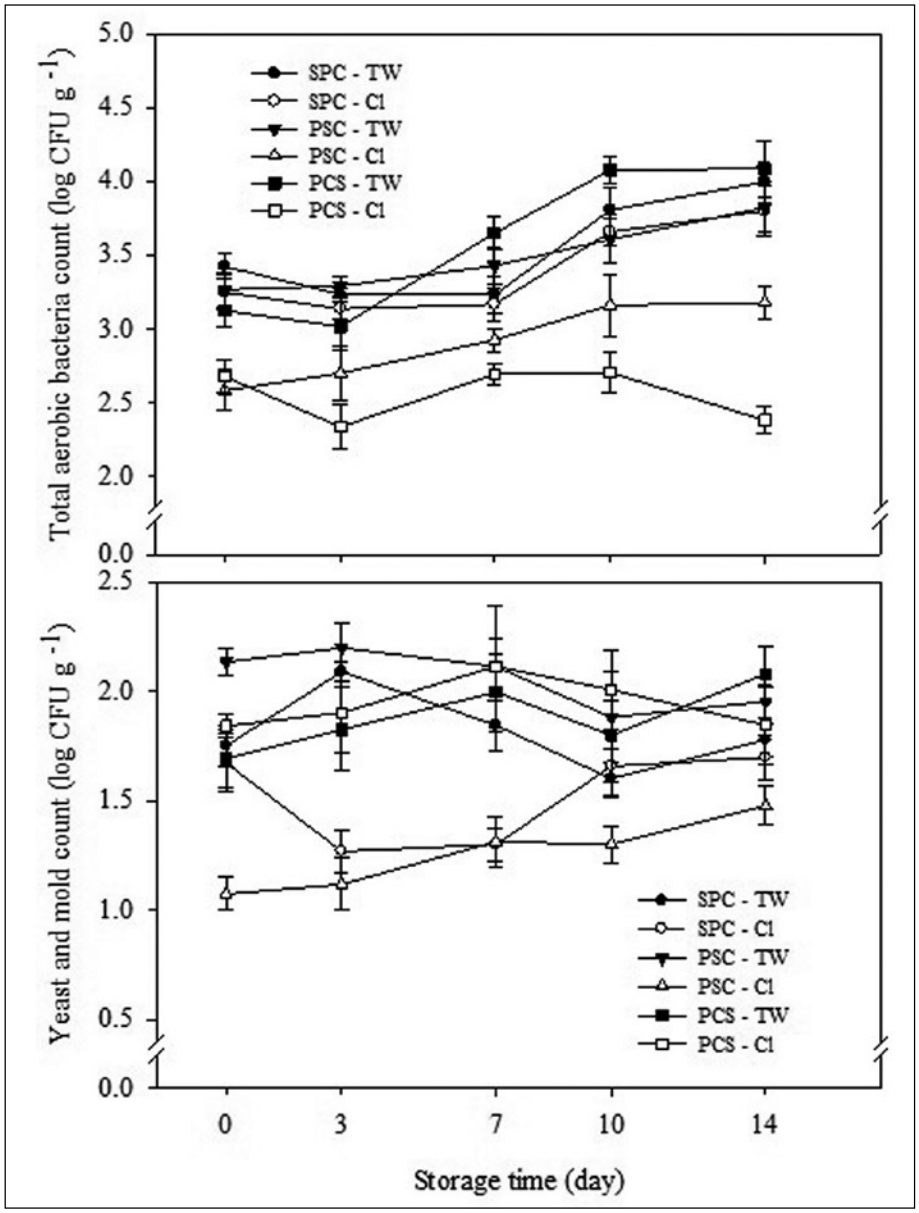


Fig. 3 - Effects of different sanitation methods on total aerobic bacteria (TAB) count and yeast and mold (YM) count of fresh-cut red beetroots during storage at 5°C. Legends: same as shown in Fig. 1.

fore peeling showed almost no effect in reducing TAB as compared to after peeling or after slicing sanitation. ERTURK and PICHA (2006) reported that chlorination of sweet potatoes before slicing could not ensure acceptable microbiological quality of fresh-cut sweet potatoes. However, in our study we found significant reduction in TAB when beetroot samples were sanitized after peeling but before slicing though the numbers of TAB were significantly lower in after slicing sanitation throughout the storage (Fig. 3). LOPEZ OSORNIO and CHAVES (1997) found significant reduction in yeast and total aerobic count in grated beetroots after washing treatment with chlorinated water. The number of TAB gradually increased until the end of storage with few fluctuations. Among the treatments, PCS-Cl treated sample showed the lowest numbers of TAB throughout the storage followed by PSC-Cl treated sample (Fig. 3). However, we found higher losses of betalain (Table 1), lower level of total phenol contents especially in inner tissues (Fig. 2) as well as lower visual quality score (later discussion) in PCS-Cl treated samples which limited the potentiality of this treatment. All tap water washed samples regardless of processing methods showed similar pattern of changes in TAB. Sanitation of beetroot with 100 ppm chlorine water following SPC method exhibited similar number of TAB until 7 days of storage and the number increased thereafter to reach the similar number with PSC-TW treatment.

Yeast and mold (YM) count, on the other hand, showed different patterns of changes that we ob-

served in TAB (Fig. 3). Among the treatments, PSC-Cl treatment ensured the lowest number of YM count on the washing day (1.1 log CFU g^{-1}) and throughout the storage. This result indicates that about 1.1 log CFU g^{-1} reduction in YM count was occurred in PSC-Cl treatment on the washing day as compared to PSC-TW treatment in which the highest YM count (2.2 log CFU g^{-1}) was observed on that day. Unlike TAB count, there was no remarkable increase found in YM count when storage progressed. It appeared that survival and growth pattern of aerobic bacteria as well as yeast and mold are different in fresh cut beetroots. The decontamination effect of chlorinated water depends on dipping time, concentration of active chlorine, water temperature and the type of produce used (ERTURK and PICHA, 2006; VITTI *et al.*, 2011). In agreement with our results, ERTURK and PICHA (2006) found no significant reduction in yeast and mold count in fresh-cut sweet potato among control, chlorination of peeled whole roots and chlorination after slicing the roots regardless of water temperatures and concentration of chlorine, except for a significant reduction in chlorination after slicing at 20°C using 200 ppm chlorine solution. However, these authors found significant reduction in initial mesophilic population using both 100 and 200 ppm chlorine water washing of peeled whole sweet potato roots. LOPEZ OSORNIO and CHAVES (1997), on the other hand, found significant decrease in the initial yeast counts in grated beetroots after washing in 252 ppm active chlorine solution at 8°C. These findings may suggest that higher concentration of active chlorine solution might be effective in reducing yeast and mold count in root vegetables. However, higher concentration of chlorine and longer dipping time caused a substantial decline in pigment contents in beetroots (VITTI *et al.*, 2011). Our YM count result suggests that higher surface area exposed to washing treatment were more susceptible to YM contamination and thereby unlikely TAB count, we found significantly (P<0.05) higher levels of YM count in PCS-Cl treatment (Fig. 3). Among the three sanitation methods used in our study, peeling-sanitation-cutting (PSC) was the best method for reducing YM count both on washing day and throughout the storage.

Sensory quality

Visual quality of sliced beetroot decreased gradually when storage time elapsed (Fig. 4). However, we did not notice off-odor in any sample until the end of storage (data not shown). Although all samples retained marketable limit until 7 days of storage, samples processed only with SPC and PSC methods retained their marketable limit which was set at 6 in a 9-point scale until 10 days of storage. In a previous study, VITTI *et al.* (2005) also reported that minimally processed beetroots could be stored until 10 days at low temperature and the produce quality drastically reduced corresponding with the increases in storage temperatures. Due to

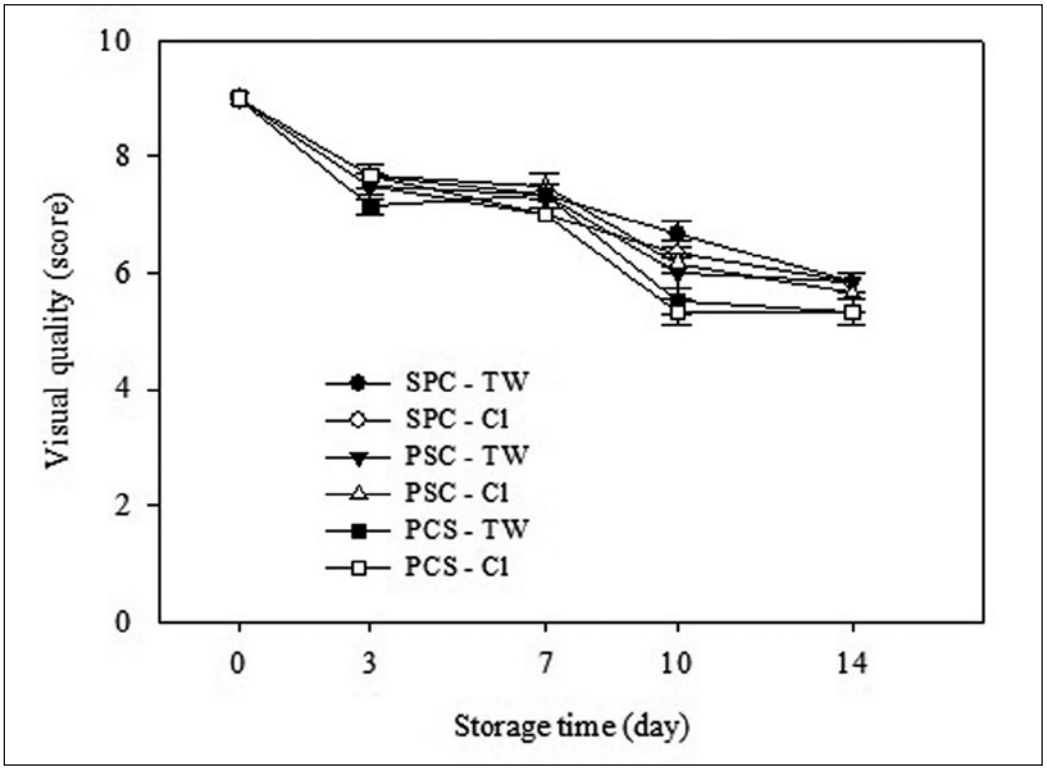


Fig. 4 - Visual quality scores of fresh-cut red beetroots during storage at 5°C after processing with different sanitation methods. Legends: same as shown in Fig. 1.

the earlier development of whitish substances on the surface of beetroots slices, samples processed with PCS method received lower visual quality scores from 7 days of storage. Moreover, we found higher losses of betalain and as a consequence higher values of whiteness index in samples treated with this method (Table 2 and Fig. 1). However, the chlorine treated sample of PCS methods showed the lowest number of TAB among the treatments (Fig. 3). This result indicates that maintenance of visual quality of fresh-cut beetroots may not depend on microbial load at least until certain limit. In general, consumer can only assess the sensory appearance. Hence, there is an increasing demand for the development of improved methods that guarantee a high produce quality and safety until the end of shelf life, especially for fresh-cut produces. At the end of storage, samples of all treatments exhibited the lower values of marketable limit. Overall our results indicate that fresh-cut beetroots processed with SPC and PSC methods could be marketable until 10 days of storage at low temperature whereas samples processed with PCS could have only 7 days of marketable life.

CONCLUSIONS

Significantly lower values of chroma and higher values of whiteness index were found in samples processed with peeling – cutting –sanitation (PCS) method, especially in PCS-Cl treatment. Although PCS-Cl treatment showed the lowest values of TAB throughout the storage, higher betalain decline along with lower level of total phenol and visual quality score limited the storability of beetroots in this treatment only until 7 days. Beetroots processed with SPC method exhibited the highest amount of betalain and total phenol contents, but showed higher number of microbial population. Samples of PSC method exhibited intermediate levels of betalain and PSC-Cl treatment ensured minimum number of microbial population. Based on measured parameters, we conclude that PSC would be the most suitable method for processing of fresh-cut beetroots and PSC-Cl would be the best treatment for ensuring minimum number of microbial population and maintaining betalain and total phenolic contents. Therefore, PSC-Cl treatment could commercially be use for processing and storing of fresh-cut beetroots at low temperature.

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PAPER

CONSUMER PERCEPTIONS OF NANOTECHNOLOGY APPLICATIONS IN ITALIAN WINE

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ABSTRACT

This paper examines Italian consumer acceptance of nanotechnology applications in wine production, surveying wine consumers from the Abruzzo Region. Conjoint and *post-hoc* segmentation analysis establishes how consumers value different wine product attributes and place them within the context of applications of nanotechnology. Consumers appear relatively unfamiliar with nanotechnology applications, both generally and specifically to food. Although, an overall rejection of the concept of "nano wine" is evident, low acceptance scores disguise a somewhat more open attitude to specific applications of the technology. In particular, consumers appear more receptive to applications that enhance certain wine attributes. Practical implications are discussed.

- Keywords: conjoint analysis, consumer acceptance, consumer segmentation, nanotechnology, purchase intention, wine -

1. INTRODUCTION

1.1. Nanotechnology applications in food and wine

Nanotechnology is the science that studies the manipulation of matter at atomic and molecular scales; a nanometre refers to one-billionth of a metre. Nanotechnology is perceived to offer many potential benefits (MURA *et al.*, 2014), such as producing healthier foods without compromising taste (WEISS *et al.*, 2006). Applications in food packaging and food contact material include microfilms that incorporate nanomaterials to improve packaging properties, e.g. flexibility and moisture stability, and "smart packaging" that incorporates nano-sensors that detect pathogens and contaminants in food (SORRENTINO *et al.*, 2007; CHAUDHRY *et al.*, 2008).

OBERDÖRSTER *et al.* (2005) argue that the properties of materials at the nanoscale can differ considerably from conventional materials. Therefore, nanotechnology-based foods have generated significant debate, particularly about potential associated risks (CHAUDHRY *et al.*, 2008; SIEGRIST *et al.*, 2008). Specifically, concerns have been expressed regarding potential negative impacts of certain nanoparticles on the health of humans, animals and the environment (KUZMA and VERHAGE, 2006). Furthermore, the FAO/WHO (2009) argues that when the size of particles decreases, this increases the surface-to-volume ratio and therefore, creates new properties, potentially resulting in altered toxicity profiles.

To date, a limited number of "nanofoods" appear to have been made available on the market (SIEGRIST *et al.*, 2008). That said, it is difficult to truly establish the extent of the application of nanotechnology in food and beverage production at present across international markets, as there is currently no legal requirement to declare the use of such ingredients on product labels. Nevertheless, there is some indication of nanotechnology being applied within the food domain (MOMIN *et al.*, 2013; DURÁN and MARCATO, 2013).

Focusing on the wine sector, nanotechnology could potentially be applied at the following stages of production: grape-growing, wine making and packaging. Specifically, nano-compounds could improve grape growth when added to pesticides and fertilizers to increase soil fertility and crop production (ALLIANZ AG and OECD, 2005). Furthermore, nanoparticle-based pesticides could be more easily absorbed by plants than conventional pesticides, or could equally be programmed to be released more gradually over time, thereby optimising their usage (*Ibid*). Hypothetically, yet plausibly, nanotechnology could be applied during wine making to alter the characteristics of the wine including its taste, flavour or other product characteristics, including the calorie or alcohol content of the wine (ALLIANZ AG And OECD, 2005; WEISS *et al.*, 2006; DURÁN and MARCATO, 2013).

Other possible applications of nanotechnology during wine production include the use of nanotechnology-based devices and materials for nano-filtration and water treatment (MOMIN *et al.*, 2013). Nanotechnology-based devices could also potentially improve surveillance systems and the tracking of products as they move through the supply chain (WEISS *et al.*, 2006), thereby enhancing authenticity measures. Finally, pertaining to wine bottling, nanotechnology could be used to produce bottle caps that more effectively regulate gas exchange with the outside environment (DURÁN and MARCATO, 2013).

1.2. Consumer acceptance of nanofoods

It is important to understand public perceptions of nanofoods (SIEGRIST *et al.*, 2008). However, these may be difficult to measure at present, as opinions may not yet have formed, given low levels of public awareness of nanotechnology (FELL *et al.*, 2009; SIEGRIST, 2010). GASKELL *et al.* (2010) found that approximately ½ of EU-27 citizens (46%) and just over $^1/_3$ of Italian citizens (37%) were aware of nanotechnology. Gaskell and colleagues also found that a significant minority (40%) of EU-27 citizens is likely to be unsure about their feeling towards applications of nanotechnology and that awareness generally resulted in more positive views regarding its safety. However, as more information becomes available through mass media, public attitudes will become more solidified (DUDO *et al.*, 2010). Although several studies have found the impact of awareness on attitudes towards novel food technologies to be mixed (FELL *et al.*, 2009; SACCHETTI *et al.*, 2009); KAHAN *et al.* (2007) found a positive relationship between awareness of nanotechnology in general and the belief that associated benefits outweigh potential risks.

Attitudes towards and, in turn, willingness to buy nanofoods may be influenced by general values, for example risk sensitivity and attitudes towards nature, the environment, science and technology (RONTELTAP *et al.*, 2007; FELL *et al.*, 2009; STAMPFLI *et al.*, 2010). For example, numerous studies suggest that the dichotomy between nature and technology is important in determining receptivity (ROZIN, 2005; SIEGRIST *et al.*, 2008). In addition to naturalness, other product characteristics, including taste and price may impact consumer acceptance (FELL *et al.*, 2009). Willingness to buy nanofoods is also strongly influenced by risk and benefit perceptions (STAMPFLI *et al.*, 2010). Personal belief in the ability to control exposure to the technology may also influence acceptance (SIEGRIST *et al.*, 2008).

Consumers use intrinsic and extrinsic cues to form opinions regarding objective and subjective product quality (VEALE *et al.*, 2006). Grunert (2005), among others, notes that subjective hedonic characteristics, e.g. taste and pleasure, are important determinants of purchase and consumption decisions. This is particularly evident in the case of wine (OLSEN *et al.*, 2007). Wine purchase decisions are based on a complex array of factors including region of origin, grape variety and price (ATKIN *et al.*, 2006; LOCKSHIN *et al.*, 2006), in addition to other aspects including health and authenticity characteristics (CHIODO *et al.*, 2011; BARREIRO-HURLÉ *et al.*, 2008). That said, given the hedonistic nature of wine, certain health characteristics may not have the same prevalence for wine as they do for other food products. Furthermore, a greater focus by consumers on environmental aspects of wine production and distribution systems is emerging (REMAUD *et al.*, 2008).

Elsewhere, CARDELLO *et al.* (2007) and VON SCHOMBERG and DAVIES (2010) describe how the public may have concerns about novel food technologies, including nanofoods. These concerns, if not addressed, can lead to consumers rejecting these technologies and searching the supermarket shelves for products claiming to be "nano-free" (KUZMA and VERHAGE, 2006). Some of the applications of nanotechnology in wine production outlined may be negatively perceived by consumers, due to perceptions of unnaturalness and tampering with winemaking traditions. Potential concerns may also emerge in terms of the unknown health and environmental consequences of applying nanotechnology in wine production, as indicated in various studies (e.g. KUZMA and VERHAGE, 2006; CHAUDHRY *et al.*, 2008).

Nonetheless, potential associated benefits may be positively perceived. These includes benefits to: 1) consumers, for example improving the wine's health characteristics (WEISS *et al.*, 2006) by, for instance, reducing its calorie or alcohol content; 2) industry, for example improving production processes, such as the bottling process (DURÁN and MARCATO, 2013); and, 3) the environment, for example decreasing the use of pesticides during grape cultivation (ALLIANZ AG and OECD, 2005). In turn, this may lead to nanotechnology application to wine being acceptable to consumers and adopted by industry.

Following these considerations, the aim of this study was to understand the impact of the application of nanotechnology in wine production on consumers' wine purchase intention. Possible consumer reactions towards nanotechnology application to wine and varying determinants of consumer acceptance were explored, as well as the homogeneity of consumers' responses.

2. MATERIALS AND METHODS

2.1. Overview

The study involved wine consumers from the Abruzzo Region of Italy completing a face-to-face administered questionnaire. An overall profile of respondents and also profiles using an *a-priori* segmentation variable (frequency of wine consumption) is presented. Following this, consumers' preferences are analysed using a Conjoint Analysis (CA) approach. The influence of production methods (conventionally produced versus produced using nanotechnology) and product attributes (e.g. associated with health and naturalness) on product preference are examined. Within this study, conventional methods refer to production practices currently in place which comply with present PDO production regulations. "Produced using nanotechnology" refers to the use of nanotechnology in any one or more phases of the production chain, e.g. during the cultivation of grapes or packaging of wine. Conjoint and *post-hoc* segmentation analysis establishes how respondents value different wine attributes and place them within the context of the application of nanotechnology. Both the *a-priori* and *post hoc* segments are profiled based on importance placed on different wine attributes, perceptions of different applications of nanotechnology to wine and demographic variables.

The wine used within the experiment was "Montepulciano d'Abruzzo DOC", the predominant PDO wine in the Abruzzo Region and one of the largest wine denominations in Italy.

Data collection was completed in October-December 2011. In total, 221 wine consumers completed the survey. No incentive was offered to respondents to complete the questionnaire. Similarly to VERDÜ JOVER *et al.* (2004), sample stratification was based on previous studies carried out which included a similar number of study items. The sample of wine consumers is representative of the regional population in terms of age and gender, based on demographic data provided by the Italian National Institute of Statistics and referred to the same period (ISTAT, 2014), as follows: 6% of women and 5.7% of men aged 18-24 years; 10% of women and 10.3% of men aged 25-34 years; 12.2% of women and 12.2% of men aged 35-44 years; 11.8% of women and 11.4% of men aged 45-54 years; and, 10.3% of women and 9.8% of men aged 55-64 years.

2.2. Questionnaire

Respondents were screened to ensure: 1) they did not work in the agro-food sector; 2) purchased or consumed wine at least once a month on average; 3) were between the ages of 18 and 64; and, 4) were either an Italian citizen or had

been living in Italy for at least five years. The questionnaire, presented in Italian, posed questions regarding frequency of wine consumption and habits; attitudes towards wine production and wine purchasing/consumption habits; factors that influence choice of wines; and, awareness of nanotechnology and its applications in food and beverage production.

Low levels of public awareness of nanotechnology, as previously outlined, presented a clear challenge in terms of deciding whether to present prior information about the technology to respondents. Consequently, in designing this experiment, we looked to those who have examined consumers' appraisals of novel food technologies in the past and best practice in terms of an appropriate CA approach (e.g. SIEGRIST *et al.*, 2009; SCHNETTLER *et al.*, 2012).

An underlying principle of conjoint analysis research is that it should be as realistic, reasonable and understandable as is feasibly possible (COX *et al.*, 2008). Thus, similar to SIEGRIST *et al.* (2009), our study was conducted in terms of a "virtual market", i.e. what consumers would do if they were informed (via a label) that a product is produced using nanotechnology and had some prior awareness of the concept of nanotechnology. Therefore, following the aforementioned general questions, in the context of ensuring a minimal level of awareness of nanotechnology among respondents in advance of completing the CA experiment, a brief (neutral) definition of nanotechnology and its potential food applications (Appendix 1) was presented. The definition provided is similar in content and structure to that which was included in SIEGRIST *et al.*'s (2009) study.

Following the provision of this definition, the 10 wine labels (based on the conjoint analysis profiles generated - see section 2.3) were presented for scoring. Attitudes towards the use of nanotechnology in wine production were then measured. Specifically, questions were posed regarding attitudes towards the use of nanotechnology in wine production in general and attitudes towards different applications in wine production for a variety of purposes. Finally, demographic information was gathered. All statements and associated scales are summarised in Table 1.

2.3. Conjoint analysis

Conjoint analysis (CA), a market research approach used to support product and service design, has been widely applied to consider the impact of different product attributes on food and beverage purchase decisions (MAKOKHA *et al.*, 2006; SZOLNOKI *et al.*, 2010). CA assumes that consumers are able to evaluate a range of products/services along key dimensions, called factors (attributes) and involves constructing a series of different product profiles (concepts) that represent a possible product or service. In the case of this research, the CA experiment involved different combinations of information about wine that may (or may not) be modified using nanotechnology, i.e. different profiles. The aim of this approach is to estimate the importance of each factor (product attribute) presented to consumers.

For categorical product attributes, the utility function consists of part-worth estimates for each level of the attribute. Market simulation models use this information to predict how each respondent would choose among alternative products. Therefore, CA enables an understanding of how people make choices between products or services across different combinations of levels and attributes. The CA method has several advantages, including the possibility to measure consumer preferences for each attribute level using more realistic decision models (SCHAUPP, 2005). Using CA, the researcher can answer questions such as what product attributes are important/unimportant to the consumer.

CA has previously been applied to explore consumer perceptions of the application of specific novel food technologies (e.g. ARES and GAMBARO, 2007; BECH-LARSEN and GRUNERT, 2003; CARDELLO *et al.*, 2007; COX *et al.*, 2008; HAILU *et al.*, 2009; SCHNETTLER *et al.*, 2012; ANNUNZIATA and VECCHIO, 2013), including nanotechnolo-

Appendix 1: Definition of nanotechnology presented to respondents in advance of conjoint analysis experiment (English version)

"New and advanced technologies with applications in food are constantly being developed. Nanotechnology is one such technology, which deals with nanoparticles (particles that are 100 nanometres or less in dimension). A nanometre is one-billionth of a metre. A sheet of paper is about 100,000 nanometres thick. Some nanoparticles are naturally occurring, for instance, it is nano-size particles that make milk appear white. Materials can possess new properties at this nanoscale and this technology makes interesting innovations possible in food.

Nanotechnology, potentially, has widespread applications in food, including uses in food products, processing and packaging. It can be used to make food products with additional benefits such as better availability of vitamins or longer shelf-life without altering the taste, appearance or texture of food. However, possible consequences or risks of using nanotechnology for humans and the environment are largely unknown.

On the one hand, additional benefits may enhance our health and improve products. On the other hand, the use of nanotechnology in food stuffs may be associated with potential risks".

Table 1.

Type of Questions	Question or Statements Posed/ Attributes Listed	Scales	Source
Frequency of consumption.	How often do you consume wine on average?	4-point frequency scales *(1 is "everyday" and 4 is "at least once a month")*	Developed by researchers.
Wine consumption habits.	I always drink the same variety of wine. I always drink wine produced in my region. I always drink wine from the same territory.	7-point Likert scales *(1 is "disagree strongly" and 7 is "agree strongly")*	Developed by researchers and adapted from Seghieri et al. (2007).
Attitudes towards wine production and wine purchasing/ consumption habits.	Wine is an important part of Italians' culture. I am proud of Italian tradition in wine production. I spend a lot of time deciding which bottle of wine to purchase.	7-point Likert scales *(1 is "disagree strongly" and 7 is "agree strongly")*	Developed by researchers.
Attributes influencing wine choice. How important are each of the following when selecting wine?	Region of production; brand; type of cork; price; age of the wine; grape variety; packaging; territory of origin; alcohol content; sulphite content; and, calorie content.	7-point importance scales *(1 is "extremely important" and 7 is "extremely unimportant")*	Developed by researchers and adapted from and Gil & Sánchez (1997) and Atkins & Johnson (2010).
Awareness of nanotechnology.	Have you ever heard of nanotechnology? Have you ever heard of nanotechnology being used in food and beverage production?	Yes/No	Developed by researchers.
Nanotechnology acceptance.	I do not want nanotechnology to be applied in wine production. I consider the use of nanotechnology in wine production to be acceptable. I would be happy to consume wine produced using nanotechnology.	7-point Likert scales *(1 is "disagree strongly" and 7 is "agree strongly")*	Developed by researchers.
Acceptance of nanotechnology applications in wine production. *How acceptable do you consider it to use nanotechnology to:*	Produce lower calorie wine. Produce lower alcohol content wine. Modify the colour of wine. Modify the structure and properties of the cork. Enhance the taste of wine. Reduce the amount of pesticides used when growing the grape. Produce less expensive wine. Enhance authenticity.	7-point acceptance scales *(1 is "extremely unacceptable" and 7 is "extremely acceptable")*	Developed by researchers.

Overview of questionnaire statements and associated scales.

gy (SIEGRIST *et al.*, 2009), and associated product attributes. Furthermore, various CA studies have explored preferences for different wine attributes (e.g. GIL and SÁNCHEZ, 1997; ATKIN *et al.*, 2006; MARTÍNEZ-CARRASCO *et al.*, 2006) including, for instance, price, origin and grape variety/vintage.

Bearing in mind the attributes examined across these CA studies, within this work, a full profile conjoint analysis was applied in order to determine consumers' preference (purchase intention) for the following wine attributes: price, method of production and benefits. The conjoint experiment was generated using SPSS 19. Product profiles were presented as wine labels with different information included on each label (Appendix 2 includes an example of one of the labels). The text included in each wine label was presented in Italian.

Appendix 2: Example of a wine label used in the conjoint experiment (English and Italian versions of Profile 8).



Similar to O' CONNOR *et al.* (2005), SORENSON and BOGUE (2006) and SIEGRIST *et al.* (2008), a ten-point purchase intention rating scale was used to measure purchase preference. Assigning a score from 1 to 10, based on willingness to purchase the product, emulated a real-life wine purchase situation. A rating, rather than ranking, scale was considered most suitable as the former *"avoid[s] validity and reliability problems as a consequence of the large number of concepts presented to respondents for evaluation"* (SORENSON and BOGUE, 2006: 705)

The wine attributes that varied across the profiles are outlined in Table 2.

In order to make the conjoint labels presented were as realistic as is feasibly possible (COX *et al.*, 2008; SIEGRIST *et al.*, 2009), the labels included additional standardised information. This approach is not novel, as several other CA studies (e.g. LABOISSIÈRE *et al.*, 2007) have included additional attributes in their experiments, which were not then included in the CA plan. Each of the labels contained the following standardised information:

- Name of the producer: *"Azienda Agricola La Collina"*
- Designation of origin: *"Montepulciano d'Abruzzo DOC"*
- Product description: *"This red wine is ideal to serve with roast meat. Serve at 18-20°C"*

The product attributes (e.g. price) that varied were the specific focus of consideration. In terms of the variable attributes, the selected price levels (€5.99 and €11.99) are reflective of two different price segments; premium and super-premium wines, as recommended by HEIJBROEK (2003). Furthermore, they are representative of the price points for several brands of Montepulciano d'Abruzzo wine currently offered in Italian supermarkets.

Where the wine was not produced using nanotechnology, i.e. was produced using conventional methods, the method of production was not stated on the label. In many conjoint studies applied to food labelling (e.g. SILAYOI and SPEECE, 2007; COX *et al.*, 2008), the level "absence of information" or "no claim" is included for certain attributes. This results in various degrees of information being included on the different product labels (i.e. for some of the product profiles). This lack of information for certain attributes is reflective of real life purchase situations. In comparison, when produced us-

Table 2.

Attribute	Level 1	Level 2	Level 3	Level 4
Price	€5.99	€11.99		
Method of Production	Conventionally produced *(Method of production not stated on label)*	Produced using nanotechnology *(stated on label)*		
Benefits	Lower sulphite levels *(Sulphite information excluded from label)*	Lower calorie content	Lower alcohol content (9% instead of 12.5%)	No claim on label

Attributes (and levels) that varied across wine profiles.

ing nanotechnology, it was explicitly stated on the wine label.

Within this conjoint experiment, if the wine had a sulphite level lower than 10 mg/l (the limit established from the Regulation (EU) No 1169/2011 for omitting the indication of the presence of sulphites from the label), sulphite information was not included on the label. Thus, in keep with our research goals, this attribute level best resembles market place situations.

Therefore, how the "benefit" attribute levels were presented is based on what is practical, relevant and realistic within the marketplace (GIL and SÁNCHEZ, 1997). Furthermore, the approach used for the "benefit" attribute levels is similar to that of other published CA studies in the context of the inclusion of a "no claim" or "no information" level (e.g. DELIZA *et al.*, 2003; KRYSTALLIS and NESS, 2005).

The rating task was carried out applying the full-profile conjoint analysis method using SPSS 19.0. This software calculated the utility values for each level of each factor. CA is useful in evaluating purchase intentions (SÁNCHEZ and GILL, 1998). An "average importance" value was also calculated for each factor that reflects the relative range of utility values for the levels within each factor (CARDELLO *et al.*, 2007).

When adopting the full-profile method, the number of possible profiles can increase rapidly due to the various combinations of factors and levels. The design must be balanced with a sufficient rotation of the factors and number of profiles in order to maintain the overall significance of the experiment. Therefore, a fractional factorial design (orthogonal array) was used which presented a suitable fraction of all possible combinations of the factor levels. Table 3 summarises the 10 profiles generated in SPSS 19; two holdouts were included to ensure the validity of the test.

In the results section, an overall profile of respondents is presented as well as profiling using an *a-priori* segmentation variable (frequency of wine consumption). Following this, perspectives on nanotechnology are considered. Conjoint and *post-hoc* segmentation analysis establishes how respondents value different wine product attributes. The influence of production methods (conventionally produced versus produced using nanotechnology) and other product attributes (e.g. associated with health and naturalness) on product preference are examined.

3. RESULTS

3.1 Consumers' behaviours and attitudes to wine

Fifteen percent, 33% and 24% of respondents indicated that they had a daily, weekly or fortnightly wine consumption habit respectively. The remaining 28% were relatively infrequent consumers, with consumption levels at around once monthly. Respondents reported that they do not always drink the same varieties of wine ($\bar{x}$ = 4.69; S.D. = 1.63), drink wine from their region ($\bar{x}$ = 4.01; S.D. = 1.91) or drink wine from the same territory ($\bar{x}$ = 4.07; S.D. = 1.94). Generally, participants indicated that they spend

Table 3.

Profile	Price	Method of Production	Benefits	
1	€ 5.99	Conventionally produced *(Method of production not stated on label)*	Lower sulphite content *(Sulphite information excluded from label)*	Design
2	€ 11.99	Produced using nanotechnology	Lower sulphite content *(Sulphite information excluded from label)*	Design
3	€ 5.99	Conventionally produced *(Method of production not stated on label)*	Lower calorie content	Design
4	€ 5.99	Produced using nanotechnology	No claim on label	Design
5	€ 5.99	Produced using nanotechnology	Lower alcohol content	Design
6	€ 11.99	Conventionally produced *(Method of production not stated on label)*	No claim on label	Design
7	€ 11.99	Conventionally produced *(Method of production not stated on label)*	Lower alcohol content	Design
8	€ 11.99	Produced using nanotechnology	Lower calorie content	Design
9	€ 5.99	Produced using nanotechnology	Lower sulphite content *(Sulphite information excluded from label)*	Holdout
10	€ 11.99	Produced using nanotechnology	Lower alcohol content	Holdout

List of profiles used in conjoint analysis experiment (fractional factorial design).

some time selecting which wine to purchase ($\bar{x}$ = 4.55; S.D. = 1.50).

The general sentiment of the sample to Italian wine was very positive, which was reflected in their view that wine forms an important part of Italian culture ($\bar{x}$ = 5.84; S.D. = 1.52) and in their expression of pride in Italian wine tradition ($\bar{x}$ = 5.97; S.D. = 1.28). When selecting wine, price, region of production and grape variety were among the most important selection attributes (Fig. 1). A paired sample t-test highlighted that as an information cue, price was significantly more important than all other cues ($p < 0.001$).

Using frequency of consumption as an *a-priori* segmentation variable, we observed significant differences in wine behaviour patterns. One-way ANOVA analysis ($p \leq 0.002$) highlights that everyday consumers were more likely to drink wine from a variety of territories when compared to the fortnightly and monthly consumers. While the patterns of the daily and weekly consumers were similar, the weekly consumers ($p \leq 0.001$) were also more likely to spend time engaging in the selection of wine than their fortnightly or monthly counterparts. In addition to this analysis, evidence of differences in the importance of quality attributes in the selection of wine was also apparent. Levels of importance for territory of origin ($p = 0.03$), cork ($p < 0.01$), price ($p < 0.01$), age ($p < 0.01$), variety ($p = 0.018$) and packaging ($p < 0.01$) varied across the segments (ANOVA analysis with *post hoc* Bonferroni). The everyday consumers placed more importance on territory of origin ($p \leq 0.031$) and variety ($p < 0.02$) than the fortnightly consumers; while the weekly consumers placed more importance on the age ($p < 0.01$) and less importance on the price ($p \leq 0.03$) than the fortnightly and monthly consumers. Interestingly, irrespective of their consumption level, all held similar sentiments towards Italian wine.

To assess the relative importance of each attribute for each segment, a paired sample t-test was applied. This indicated that it was only in the case of fortnightly and monthly consumers that price was significantly more important than other key information cues. In the case of both everyday and weekly consumers, no significant differences were identified in terms of price, region, grape variety and territory of origin. This suggests that frequent consumers of wine rely equally on a greater variety of information cues in their selection of wine.

The segments differed significantly ($\chi^2 = 9.46$; $p = 0.024$) based on gender, with males being more likely to be daily drinkers, accounting for 71% of the everyday category. In comparison, 62% of the monthly category was female. There were no significant differences with regard to age and frequency of consumption.

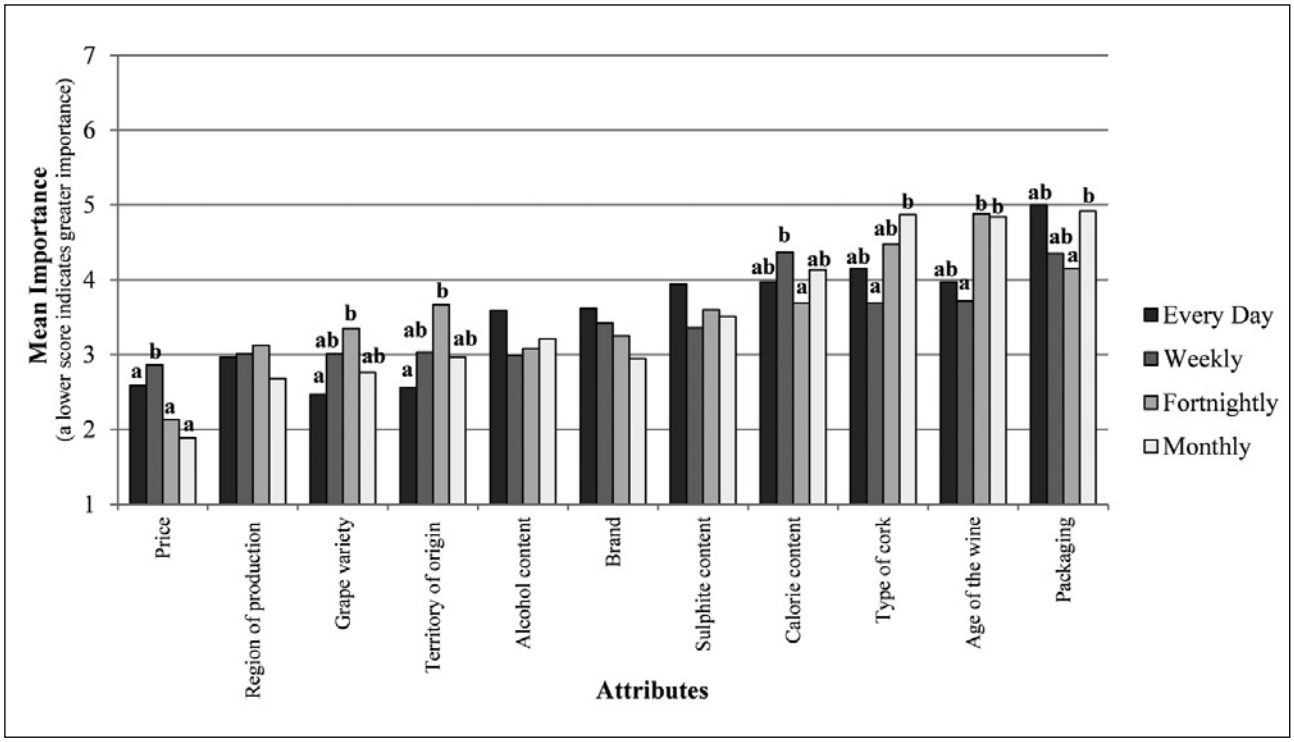


Fig. 1 - Mean importance of attributes influencing wine purchase decisions by frequency of consumption.

Note: Scale from 1 to 7, where 1 is extremely important, 7 is extremely unimportant and 4 is neither important nor unimportant. Letters above the bars reflect significant differences between frequencies of consumption groups at the 95% confidence level.

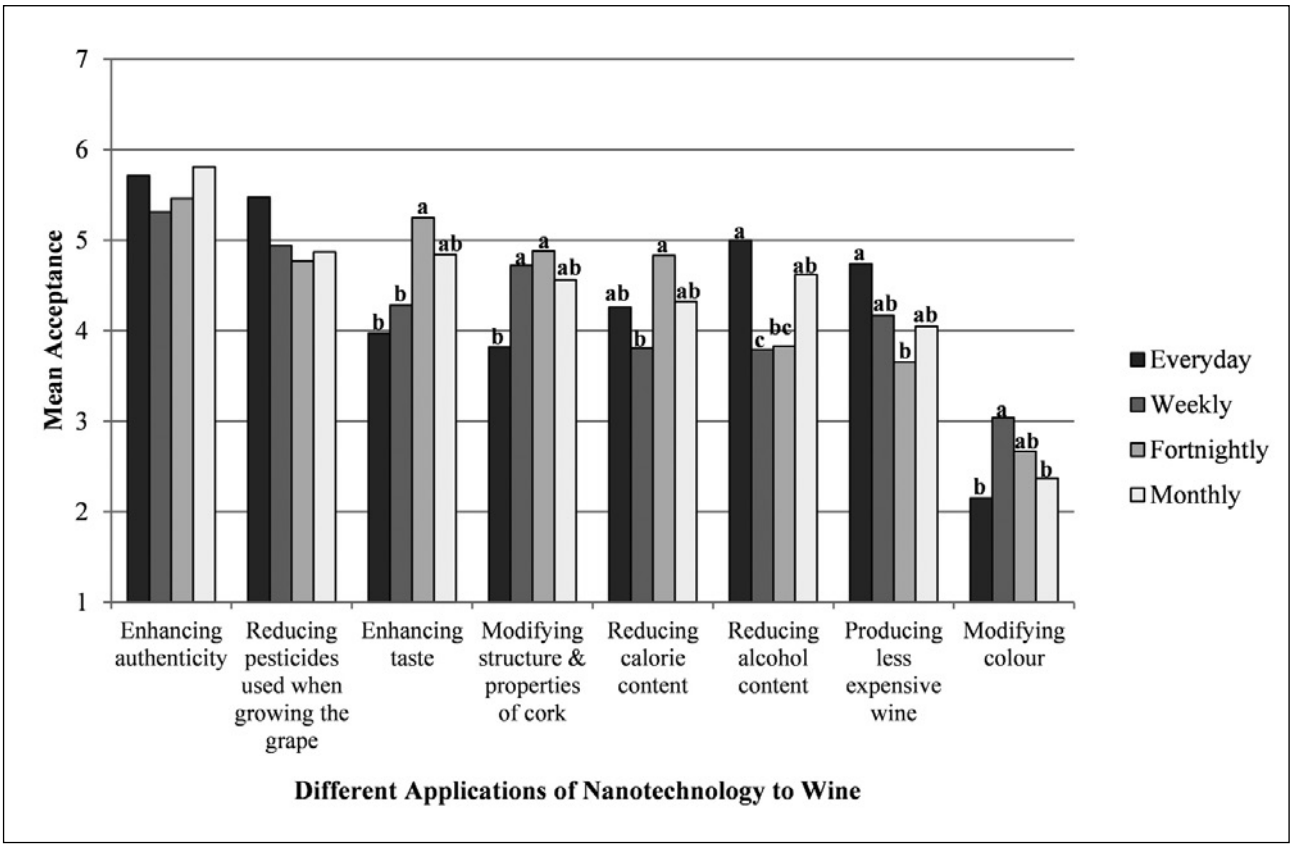


Fig. 2 - Acceptance of applying nanotechnology to obtain the following benefits by frequency of consumption.

Note: Scale from 1 to 7, where 1 is extremely unacceptable, 7 is extremely acceptable and 4 is neither acceptable or unacceptable. Letters above the bars reflect significant differences between frequencies of consumption groups at the 95% confidence level.

To provide a rich account of consumer acceptance of adopting nanotechnology in wine production, the next section explores respondents' awareness of and perspectives on nanotechnology. It examines acceptance of nanotechnology applications in wine, both generally and specifically, at the segment level based on frequency of consumption.

3.2. Awareness and attitude towards nanotechnology

The majority of the sample was unaware of nanotechnology applications in general (58%). This lack of awareness increased considerably for food applications (84%). To get an initial indication of attitudes towards nanotechnology, following the provision of information on this technology, respondents were asked about their level of acceptance of the use of nanotechnology in wine production using three statements (Table 1). Unidimensionality of this measure was assured on the basis of principal axis factor analysis, with 84% of variation explained by a single factor and factor loadings ranging from 0.51 to 0.86. Reliability of the measure was also good (α = 0.936). An overall acceptance score was calculated based on a mean score for the three statements. Widespread acceptance of nanotechnology in wine production is unlikely ($\bar{x}$ = 3.06; S.D. = 1.75) and was not significantly different across consumption levels.

To further understand levels of acceptance, an examination of potential applications of nanotechnology that offer specific benefits was undertaken. Although the applications presented are hypothetical at present, they may become a reality in the future. This suggested that certain applications are more acceptable than others, as summarised in Fig. 2. Enhancing the authenticity of wine, relates to improving the traceability and safety of the wine and ensuring the preservation of product characteristics linked to its origins. This enhancement was considered the most acceptable application, followed by reducing the use of pesticides and enhancing sensory characteristics. Paired sample t-tests highlight that the application of nanotechnology to enhance the authenticity of wine was significantly more acceptable ($p < 0.001$) than its applications for other purposes. However, this disguised differences across consumption levels. While the monthly consumers displayed the same pattern as the overall sample, the everyday and weekly consumers considered applications to reduce the

usage of pesticides equally as acceptable as applications to enhance authenticity.

Enhancing taste was the third most acceptable application and was significantly less acceptable than authenticity improvements. That said, in the case of the fortnightly group, taste along with modifying cork and reducing calories were judged as equally as acceptable as authenticity improvements. Furthermore, taste benefits were significantly less acceptable than benefits such as price and reduced alcohol content for everyday consumers. ANOVA analysis confirmed that the everyday and weekly consumers were significantly less accepting ($p \leq 0.01$) of taste benefits when compared with the fortnightly consumers. Finally, colour modification was the least acceptable application across all consumption levels.

ANOVA analysis highlights that the everyday consumers were significantly more accepting of low alcohol benefits when compared to weekly ($p = 0.008$) or fortnightly ($p = 0.019$) consumers. Furthermore, they were significantly less accepting of modifications to the cork and colour in comparison to the fortnightly ($p = 0.018$) and weekly ($p = 0.013$) consumers respectively. The weekly consumers were significantly more accepting of modifying colour than the monthly ($p = 0.031$) and were significantly less accepting of reducing calories than the fortnightly ($p = 0.008$) consumers. No other significant differences in acceptance were noted across the segments.

The following section further explores wine preferences, presenting the findings of the conjoint experiment which involved wine products based on combination of attributes, one of which was "produced using nanotechnology". This conjoint analysis therefore provides additional insights into varying levels of acceptance of applications of nanotechnology in wine production across the sample. *Post-hoc* segmentation analysis enables further understanding of how different consumer value different wine attributes and place them within the context of the application of nanotechnology.

3.3. Conjoint and post-hoc segmentation analysis

The conjoint analysis suggests that, across the sample, price was the most important factor influencing wine preference (47.8%) with a preference for lower priced wine (utility = 1.08) being evident (Table 4). Method of production (35%) was the second most important attribute. In this case, conventionally produced wine (utility =0.79) was preferred over wine produced using nanotechnology (utility = -0.79). Benefits (17.2%) were the least important factor influencing preference. Benefits with positive utility values were lower sulphite levels (0.4) and lower calorie content (0.21). In fact, the negative utility of applying nanotechnology (-0.79) may be traded-off against, for example, the positive utility of a lower price (1.08) coupled with either lower sulphite levels (0.4) or lower calorie content (0.21). In terms of the other benefits offered, a negative utility for lower alcohol content (-0.23) indicates that consumers disliked this suggested benefit.

Table 4.

% of Sample	Total Sample (n = 221) 100%	Price Sensitive (n = 131) 59.3%	Traditionalist (n = 46) 20.8%	Indifferent (n = 44) 19.9%
Intercept	4.77	4.96	4.40	4.62
Price				
€5.99	1.08	1.61	0.49	0.13
€11.99	-1.08	-1.61	-0.49	-0.13
Relative importance (%)	47.80	50.78	20.40	18.44
Method of Production				
Conventionally produced	0.79	0.90	1.07	0.19
Produced using Nanotechnology	-0.79	-0.90	-1.07	-0.19
Relative importance (%)	34.99	28.37	43.95	27.46
Benefits				
Lower sulphite levels	0.40	0.12	1.14	0.46
Lower calorie content	0.21	0.66	-0.59	-0.29
Lower alcohol content	-0.23	-0.11	-0.52	-0.27
No claim on label	-0.38	-0.67	-0.03	0.11
Relative importance (%)	17.20	20.85	35.65	54.10
R of Pearson	0.99	0.99	0.96	0.90

Utility values of levels in the conjoint experiment and cluster segment.

Table 5.

Price						
Construct	**Level**	**Total (%)**	**Sensitive (%)**	**Traditionalist (%)**	**Indifferent (%)**	**χ^2 (P-value)**
Gender	Male	50	40	61	66	11.40 (<0.001)
	Female	50	60	39	34	
	Total	100	100	100	100	
Age	18-24	12	8	0	34	108.1 (< 0.001)
	25-34	20	22	0	36	
	35-44	25	33	4	21	
	45-54	23	24	35	9	
	55-64	20	13	61	0	
	Total	100	100	100	100	
Frequency of consumption	Everyday	15	11	28	16	62.92 (<0.001)
	Weekly	33	16	52	61	
	Fortnightly	24	32	9	14	
	Monthly	28	41	11	9	
	Total	100	100	100	100	

Segment characteristics.

The "ideal" profile (i.e. the profile respondents were most willing to purchase) was Profile 1, with the following characteristics: €5.99, conventionally produced *(method of production not stated on label)* and lower sulphite content *(sulphite information excluded from label).* The least preferred profile is hypothetical and was not presented in the profiles that respondents scored. This hypothetical profile did not include any proposed benefits, was priced at €11.99 and produced using nanotechnology.

To identify different consumer segments based on product attribute utility scores derived from the conjoint experiment, "*K-MEANS cluster analysis*" was employed across two to five clusters. Each of these was evaluated and three clusters were identified as best representing the data.

Of these three segments, the first and largest segment (59.3% of respondents), labelled "*price sensitive*"; price (50.8%) was the most important product attribute, followed by method of production (28.4% of importance) and subjective benefits (20.8% of importance). Low priced (1.61), conventionally produced (0.9), lower calorie (0.66) and lower sulphite (0.12) wine offered the greatest positive utilities.

The second segment (20.8% of respondents), labelled "*traditionalist*", placed most importance on method of production (43.9%), followed by subjective benefits (35.6%) and price (20.4%). They displayed a strong negative utility for nanotechnology produced wine (-1.07) relative to conventionally produced wine. This negative utility may not be traded-off by the positive utility of a lower price (0.49). The only benefit offering a positive utility was lower sulphite levels (1.14).

Finally, the third segment (19.9% of respondents), labelled "*indifferent*", considered benefits to be the most important attribute (54.1%), followed by method of production (27.5%) and then price, which they considered to be the least important attribute (18.4%). However, not all proposed benefits offered utility; the benefit of interest for this segment was low sulphite levels (0.46), with no other benefits offering utility.

Compared to the other segments, the "*price sensitive*" included significantly (χ^2 = 11.395; p = 0.003) more females (60%) than males (40%) and were among the least frequent consumers of wine, with 73% of them consuming wine, at most, once fortnightly (Table 5) compared to 19% and 23% for the "*traditionalist*" and "*indifferent*" segments respectively (χ^2 = 108.092; p < 0.001). Furthermore, the consumers belonging to the first segment were more inclined to always purchase the same variety of wine (p ≤ 0.001), from the same territory (p ≤ 0.034) when compared to the consumers of the other two segments.

ANOVA analysis with *post hoc* Bonferroni suggests that, in comparison to the two other segments, the *price sensitive* placed greater importance on price and region and less importance on age and packaging when selecting wine (p ≤ 0.022). They were also the most open to appli-

Table 6.

Please indicate how acceptable you consider it to use nanotechnology to:	Sample *Mean* (St. Dev.)	Price Sensitive *Mean* (St. Dev.)	Traditionalist *Mean* (St. Dev.)	Indifferent *Mean* (St. Dev.)
Enhancing the authenticity wine.	5.55 (1.58)	5.65 (1.81)	5.24 (1.32)	5.57 (0.97)
Reducing the amount of pesticides used when growing the grape.	4.96 (1.87)	4.81 (2.09)	4.98 (1.39)	5.41 (1.53)
Enhancing the taste of wine.	4.62 (1.72)	4.89 (1.76)	3.91 (1.66)	4.57 (1.47)
Modifying the structure and properties of the cork.	4.57 (1.62)	4.63 (1.85)	4.37 (1.10)	4.64 (1.33)
Reducing the calorie content of wine.	4.26 (1.75)	4.57 (1.89)	3.35 (1.21)	4.30 (1.47)
Reducing the alcohol content of wine	4.22 (1.84)	4.50 (2.05)	3.70 (1.41)	3.95 (1.38)
Producing less expensive wine.	4.10 (1.70)	4.08 (1.72)	3.85 (1.75)	4.43 (1.56)
Modifying the colour of wine.	2.62 (1.42)	2.29 (1.35)	2.91 (1.26)	3.32 (1.49)

Acceptance of applications of nanotechnology by segment.

cations of nanotechnology that reduced calorie content and the least open to those to modify colour ($p \leq 0.026$) (Table 6). They placed greater importance on alcohol and calorie content of wine and were more receptive to applications that reduce alcohol content and enhance taste than the *traditionalist* ($p \leq 0.032$). However, the *price sensitive* had the lowest overall acceptance score ($\bar{x} = 2.83$; S.D. = 1.87), which was significantly lower ($p < 0.01$) than that of the *indifferent* segment ($\bar{x} = 3.75$; S.D. = 1.73)

The *traditionalist* segment was older, in fact 90% were 45 years or over; this compares to 9% and 37% for the *indifferent* and *price sensitive* segments respectively. They also represented the most frequent consumers of wine, with almost 30% consuming wine everyday and almost 80% of the segment consuming wine at least weekly. The *traditionalists* were the most different to the *price sensitive* in their perspectives on wine and were less interested in changes to the current characteristics of wine, as indicated by their lower receptivity to many of the suggested benefits associated with the application of nanotechnology. However, no significant difference in overall acceptance ($\bar{x} = 3.08$; S.D. = 1.31) was evident between the *traditionalist* and two other segments.

The *indifferent* segment included predominately younger respondents; 70% were 36 or younger. Furthermore, males (66%) were disproportionately represented within the segment. They were also quite frequent consumers of wine, with 77% consuming wine at least weekly.

The findings illustrate that utility scores offer an effective means of dividing the market and establishing different perspectives on wine attributes across the *post hoc* segments. Each segment displayed a negative utility for applying nanotechnology. However, the extent of such negative attitudes (utilities) and the relative importance placed on applying nanotechnology in comparison to the other attributes (i.e. price and benefits) varied across the segments.

4. DISCUSSION AND CONCLUSIONS

In this paper, we sought to understand consumer acceptance of nanotechnology within a product category that is strongly embedded in Italian culture. The analysis of consumers indicates that tradition continues to be important in choice decisions in the wine category; however, price plays a more important role in wine choice. These factors combined with region of production and grape variety are key choice attributes.

Based on the findings, Italians are relatively unfamiliar with applications of nanotechnology, both generally and specifically to food. As suggested by others (e.g. FELL *et al.*, 2009), we observed a cautious response to the concept of nanotechnology. Indeed, within the sample, there was an overall rejection of the concept of "nano wine". However, low acceptance scores disguised a somewhat more open attitude to specific applications of this technology. It is clear that for many, acceptance is considered on a case by case basis, and the bundle of benefits offered by a product is central to evaluations of the associated technology. Acceptance of the technology increases when the specific application satisfies an unfulfilled need. Thus, while the concept of the technology results in a reluctant response, this changes when more concrete product examples of personal relevance are considered.

Within this study, consumers were most receptive to applications that result in improved authenticity and reduced use of pesticides. The findings therefore concur with the views of Bruhn (2007) and Siegrist (2008) that if an objective of a communication is to successfully market and sell novel food technology products, including nanotechnology-based foods and beverages, attention should be given to communicating explicit, tangible benefits of relevance to consumers.

The conjoint analysis results suggest that, across the sample, price was the most signifi-

cant factor influencing wine preference followed by method of production; with consumers displaying a preference for conventionally produced rather than "nano" wine. Given the significance of price, it is not surprising that some consumers may be willing to purchase "nano wine" if it is priced lower than its conventional counterpart and additional benefits are offered.

This work implies that segmentation is a useful platform for exploring consumer acceptance of nanotechnology application in wine production. For example, while the *price sensitive*, *traditionalist* and *indifferent* segments all displayed negative utility for nanotechnology, the extent of such negative attitudes (utilities) could be traded-off against a lower price and the enhancement of other product characteristics which were valued by particular segments (e.g. lower sulphite levels). However, the extent of "trading-off" between these attributes clearly depended on the segment in question. In addition, the *a-priori* and *post hoc* segmentation analysis demonstrates that variation exists in how groups of individuals evaluate and consume wine. In particular, significant variation was evident in wine behaviour patterns and the importance placed on different wine attributes (i.e. region of origin, cork, price, age, variety and packaging) and was also apparent in consumers' evaluations of the different nanotechnology applications.

Heterogeneity in behaviour across consumer segments, in addition to variation in terms of the importance placed on wine product attributes have been highlighted in several other studies. Empirical evidence supports the finding of this work that frequent consumers of wine rely on a greater variety of information cues in their wine selection. Specifically, ATKIN and JOHNSON (2010) found that core consumers (i.e. those who drink wine at least once a week) draw more heavily on place-of-origin cues than infrequent consumers. Elsewhere, PERROUTY *et al.* (2006) found that perceived expert consumers make use of a greater number of attributes, particularly region, brand, variety and price when evaluating wine products compared to perceived non-expert (novice) consumers. The former also evaluate relationships between attributes more deeply than novices.

Within this study, although authenticity improvements were considered the most acceptable application of nanotechnology to wine overall, the segments were not homogenous in their assessments of the other applications presented. For example, the more frequent wine consumers considered applications to reduce the use of pesticides to be as acceptable as those that enhance authenticity. Furthermore, the conjoint and *post-hoc* segmentation analysis illustrate how, although the *price sensitive* segment had the lowest overall acceptance score, they were more responsive than the *indifferent* and *traditionalist* segments to applications that reduce calorie content. This finding, once again, demonstrates that acceptance is lower at the conceptual/abstract level than the product attributes level, thereby illustrating the merits of segmenting the population.

Both segmentation approaches can guide approaches to targeting different consumer groups. In particular, insights from the utility based segmentation may be useful in designing and developing a "nano wine" that is targeted at the most suitable market segments. Based on the findings, a *traditionalist* segment would be an inappropriate target market for "nano wine", given the high importance this cautious group places on conventional production methods. Conversely, considering optimum commercialisation and marketing strategies for "nano wine", producers and distributors may be interested in offering a competitively priced "nano wine" that has reduced sulphite levels to an *indifferent* segment that frequently consume wine and could therefore be a profitable target market. Furthermore, another strategy might be to offer a competitively priced "nano wine" with reduced calorie content to *price sensitive* consumers.

The emerging positive reactions towards applications that enhance wine authenticity align with the connotations of wine being a "natural" product, strongly associated with heritage, origin and region, as ROMANO and NATILLI (2009) have previously argued. This "natural" perception of wine is particularly evident in the case of traditional wine producing and consuming countries including Italy, where PDO and PGI wines are prevalent. Building on this research, marketers should recognise the influence of perceived "naturalness" on wine preferences and develop communication strategies around emphasising how nanotechnology can, in fact, enhance "natural" qualities of wine, e.g. improve authenticity and lower sulphite levels, rather than tamper with its "natural" properties.

To sum, although the application of nanotechnology is not generally positively perceived in wine production, low measures of overall acceptance may conceal greater acceptance of specific applications which enhance valued wine attributes.

Finally, we recognise the potential limitations of this study. Specifically, while this work is in keeping with the approach of SIEGRIST *et al.* (2009) and SCHNETTLER *et al.* (2012), we are cognisant that the provision of information about one of the product attributes, i.e. nanotechnology, may be viewed by some as a departure from traditional CA approaches. Equally, within this work, we acknowledge the argument previously made by Siegrist *et al.* (2009) in their conjoint study that provision of a different description of nanotechnology, may have more positively or negatively impacted responses to the application of nanotechnology within the CA experiment.

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PAPER

INVESTIGATION ON MICROBIOLOGY OF OLIVE OIL EXTRACTION PROCESS

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ABSTRACT

Several batches of approx. 200 kg olives from *Frantoio* and *Moraiolo* cultivars were processed in an oil mill at two dates of harvesting. Samples were collected in several steps of extraction process for sensory, chemical and microbial analyses.

All extracted olive oil from the second olive harvesting date was affected by sensory defects and hence classified as being "non-extra virgin". A distinction between extra virgin olive oil and non-extra virgin olive oil obtained from both harvesting dates was explained by the volatile compounds content of olive oil samples and by yeast and mould counts collected at different processing steps.

- Keywords: moulds, sensory defects, virgin olive oil, volatile compounds, yeasts -

INTRODUCTION

The absence of sensory defects is necessary for olive oil to be marketed as "extra virgin" in the EU.

Extra virgin olive oil (EVOO) is characterized by pleasant sensory notes. They are mainly originated by aldehydes, esters, alcohols and ketones, which are responsible for oil sensory attributes such as "green" and "fruity" (APARICIO and MORALES, 1998; MORALES *et al.*, 2005, BENDINI *et al.*, 2012). However, several phenomena can alter the initial pleasant flavour, giving rise to unpleasant sensory notes.

The current olive oil regulations (EU Reg. 1348/2013) classify the most frequent sensory defects into four groups as follows: "fusty", "musty", "winey–vinegary", and "rancid".

Storage of olive fruits in piles before being processed is a cause of sensory alterations in EVOO. Olive transpiration during storage is known to increase pile temperature, enabling microbial cells to grow and to affect the chemical composition of olives (MORALES *et al.*, 2005). Both biogenesis of volatile compounds and transformation phenomena of phenolic compounds can be significantly influenced by microbial contamination of olives. Effects of olive microbiota on oil characteristics are considered even greater than time-temperature conditions of malaxation (VICHI *et al.*, 2011).

Oil quality may be affected by microorganisms, according to their metabolic activities. During olive crushing, microorganisms might migrate into oil through both solid particles of olive fruit and micro-drops of vegetation water (CIAFARDINI and ZULLO, 2002). Some microorganisms do not survive a long time, but others may persist and become a typical microbiota of olive oil. For example, yeasts may remain metabolically active during olive oil storage and thus modify olive oil characteristics (ZULLO *et al.*, 2010).

Enzymatic activities of yeasts and moulds isolated from either olives or EVOO have been reported to include β-glucosidase, β-glucanase, polyphenoloxidases, peroxidase and, in some cases, lipase and cellulase activities (CIAFARDINI and ZULLO, 2002; CIAFARDINI *et al.*, 2006; ZULLO and CIAFARDINI, 2008; ROMO-SANCHEZ *et al.*, 2010). Enzymes such as β-glucosidase are known to improve oil quality by increasing phenolic compound extractability, while others such as lipase, polyphenoloxidases and peroxidase are known to cause detrimental effects (PALOMARES *et al.*, 2003; ROMO-SANCHEZ *et al.*, 2010; VICHI *et al.*, 2011; MIGLIORINI *et al.*, 2012). *Penicillium* and *Fusarium* spp. isolates have been shown to produce amounts of exogenous lipoxygenase (FAKAS *et al.*, 2010) that, together with endogenous lipoxygenase, is the key enzyme of LOX pathway (ANGEROSA *et al.*, 2004).

Extraction process control should include monitoring activities on microbial contamination in olives and EVOO, as associated with sensory and chemical analyses. The study of the microbial populations occurring at different steps of EVOO extraction process, as well as their role in affecting oil characteristics, appears to be increasingly useful.

The aim of this work was to investigate both microbial ecology throughout olive oil processing and a possible relationship between EVOO volatile compound content and microbial contamination.

MATERIALS AND METHODS

Experimental design

During 2011 crop season, several batches of approx. 200 kg olives from *Frantoio* and *Moraiolo* cultivars were processed in an oil mill (Azienda Agricola Buonamici, Fiesole, Florence, Italy).

Plant for oil extraction (TEM, Florence, Italy) consisted of a cleaning and water washing system, an olive grinding cutter crusher (mod. FR350), a controlled-temperature vertical axis malaxation equipment (500 kg capacity) (mod. V500), a "decanter" (two-step mod. D1500) with 1500 kg/h maximum capacity and a cardboard filter press (15 μm cut-off). Plastic residue or "alperujo" from decanter was subjected to separation by centrifugation of stone fragments to obtain destoned pomace (Fig. 1).

Olives were processed within 12 h from harvest at two dates (HD): November 16, 2011 (HD1) and November 23, 2011 (HD2). Oil extraction trials were carried out in quadruple.

Olives were crushed at 2,500 rpm (crusher holes 6.5 mm in diameter); malaxation was carried out at half capacity under vacuum (residual pressure of 20 kPa) at 22 ± 1°C for a mean time of 15 min to work under low oxidative stress impact conditions; decanter worked with a screw conveyor rotating at a slower speed than that of the bowl.

Samples were collected in several steps of extraction process for sensory, chemical and microbial analyses, as shown in Fig. 1.

Chemical analyses

Olives

A homogeneous olive sample was crushed with a laboratory crusher, and resulting olive paste was used for chemical analyses.

The water content (g kg^{-1} of dry matter) was measured on olive paste by gravimetric method (CHERUBINI *et al.*, 2009).

The total sugar content was determined by the UNI 22608 method, modified as described in a previous study (CHERUBINI *et al.*, 2009). Results for sugar content obtained from the equipment (Compact Titrator, Crison, Modena, Italy) were expressed as g kg^{-1} of dry matter.

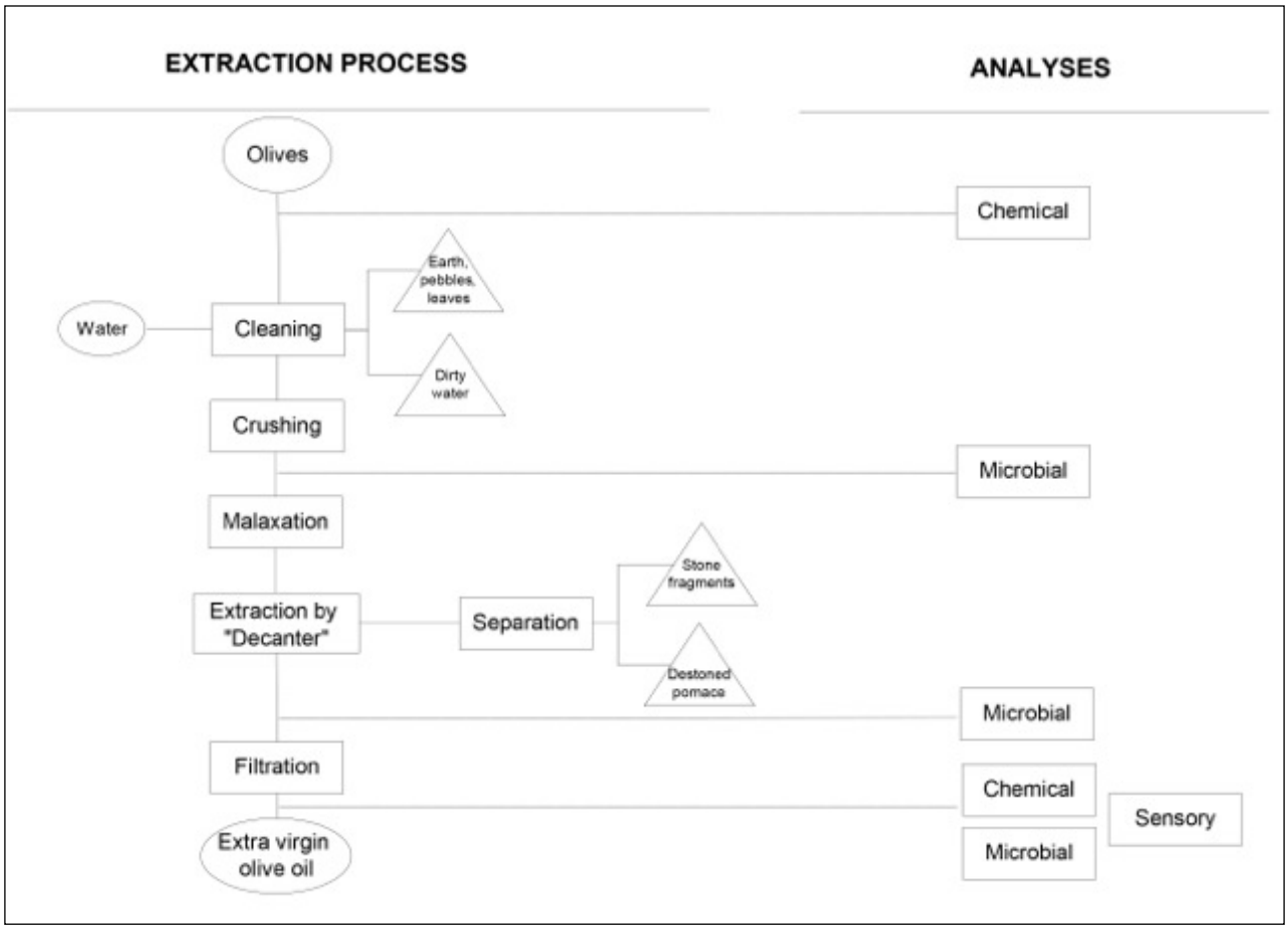


Fig. 1 - Overview of extraction process and analyses carried out.

The total oil content was determined with hexane in an automatic Randall extractor (mod. 148, Velp Scientifica, Milan, Italy), following the analytical technique described in a previous study (CHERUBINI *et al.*, 2009). Results were expressed as g kg^{-1} of dry matter.

The total phenolic compounds content was determined by weighing 4 g crushed olives and adding 80 mL Methanol:Water (60:40) solution; two series of stirring for 30 min and centrifugation at 4,000 rpm for 15 min were performed, and the supernatant was collected. The phenolic extract was adjusted to volume of 200 mL by Methanol:Water (60:40) and placed in the freezer for 2 h. After thawing, the phenolic extract was filtered. One mL filtered extract, 5 mL Folin Ciocalteu reagent, and 20 mL sodium carbonate were placed into a 100 mL flask and adjusted to volume with distilled water. Sixty minutes were waited for colour development; after one hour, UV reading (UV/VIS, Varian model Cary 1E, The Netherlands) was performed at 765 nm wavelength. The total phenolic compounds content was expressed as mg kg^{-1} of dry matter on a calibration curve.

Olive oil

Acidity (% oleic acid), peroxide value (meq O_2 kg^{-1}) and spectroscopic indices were measured according to EU official method (EC Reg. 1989/2003).

Extraction, identification and determination of phenolic compounds were performed in agreement with IOC Official Method (IOC, 2009) by an HPLC equipment consisting of a Hewlett Packard 1200 diode-array detector system and a Hewlett Packard model 1200 autosampler (Agilent Technologies, Santa Clara, California, USA). Secoiridoids, lignans, flavonoids and phenolic acids were quantified in $mg_{tyrosol}kg_{oil}^{-1}$. The total phenolic compounds content ($mg_{tyrosol}kg_{oil}^{-1}$) was determined using the sum of the peak areas of phenols recorded at 280 nm.

The tocopherol content was determined according to ISO 9936:2006 (ISO, 2006) using a Hewlett Packard mod. 1050 liquid chromatograph with quaternary pump and fluorescence detector, provided with Hewlett Packard mod.1100 autosampler (Agilent Technologies, Santa Clara, California, USA). Quantitative analysis was carried out using the external standard method. Results were expressed as mg of total tocopherols per kg of oil.

The volatile compound content was determined according to the literature (VICHI *et al.*, 2003), using HS-SPME-GC-MS technique (solid phase microextraction of the head space, cou-

pled with a gas chromatograph with a mass spectrometer as a detector). Analysis was performed using the Trace CG instrument combined with a Trace DSQ Thermo Finnigan instrument (Fisher Scientific SAS, Illkirch, France). Quantitative analysis was performed using 4-methyl-2-pentanol as an internal standard. Results were expressed as mg of volatile compound per kg of oil.

Sensory analyses

Sensory evaluation of olive oil was performed by a panel test according to the EU official method (EU Reg. 1348/2013). Samples were analyzed by a panel of professional tasters (8 tasters and a panel leader) of CCIAA (Chamber of Commerce, Industry, Handcraft and Agriculture) of Florence. The panel has been recognized by MIPAAF (Ministry of Agricultural Policies, Food and Forestry) since 2002. Intensity of both sensory defects and "fruity", "bitter" and "pungent" attributes was assessed and expressed as the median of tasters score on a scale range from 0 to 10.

Microbiological analysis

Paste and oil samples from each batch were sterilely withdrawn and then transported to the laboratory under refrigerated conditions (4°C). Ten g of olive paste or 10 mL of unfiltered oil were transferred into 90 mL of sterile saline and homogenized for 10 min with a Stomacher Lab Blender 400 (Seward Ltd, Worthing, West Sussex, UK) and a magnetic stirrer, respectively. After decimal dilutions, 100 µL suspension was plated on specific growth media for cell enumeration in triplicate using the spread plating technique. Yeasts were counted on MYPG agar (ZULLO and CIAFARDINI, 2008) integrated with ampicillin and sodium propionate in order to inhibit growth of bacteria and moulds, respectively (ROMO-SANCHEZ, 2010). The plates were incubated at 30°C for 48-72 h. Moulds were counted on MYPG agar without inhibitors (KAWAI *et al.*, 1994) after incubation at 30°C for 24-48 h. Finally, total mesophilic microorganisms were counted on Plate Count Agar (Oxoid Ltd, Basingstoke, Hampshire, UK) after incubation at 30°C for 48 h.

Filtered oil samples (100 mL) were microfiltered through nitrocellulose filters with a porosity of 0.45 µm (Minisart NML-Sartorius, Göttingen, Germany), which was able to retain yeasts and moulds. Then the nitrocellulose filters containing the microorganisms were washed with 10 mL saline and placed onto the specific media described above.

Data processing

Chemical, sensory and microbiological determinations were processed according to one-way ANOVA followed by Tukey's test (significance level: $p = 0.05$).

Principal Component Analysis (PCA) was used to classify samples by Statistica 7.0 software package (Stasoft GmbH, Hamburg, Germany). Correlation studies between microbial cell density and the volatile compounds content of oil samples were carried out by calculating both Pearson and Spearman rank correlation coefficients (significance level: $\alpha = 0.05$).

RESULTS

Characteristics of olive and olive oil samples

Chemical characteristics of processed olives are given in Table 1. They show a slight increase in olive ripening level between the two dates of harvesting. As reported in the literature (RYAN *et al.*, 2002; SERVILI *et al.*, 2004; CHERUBINI *et al.*, 2009), a significant decrease in phenolic compounds content occurred, and a decrease in sugar content, even if significant only for *Frantoio* cultivar, was also observed. No significant variations were measured in both water and olive oil contents during the harvesting interval.

Sensory and chemical characteristics of extracted olive oil are given in Table 2, while their volatile compounds content is reported in Table 3. Samples are encoded in relation to olive cultivar, harvesting date and batch.

Table 2 shows that all olive oil samples extracted from olives of the first harvesting date were extra virgin. They had much lower values

Table 1 - Olive characteristics on two harvesting dates (HD1 and HD2). SD: standard deviation; different letters in the same row indicate significant differences ($p < 0.05$) for the same cultivar; dm: dry matter.

	Frantoio Cultivar				*Moraiolo* Cultivar			
	HD1		HD2		HD1		HD2	
	Mean value	SD	Mean value	SD	Mean value	SD	Mean value	SD
Phenolic Compounds (mg/kg dm)	33000 [a]	2285	26000 [b]	1811	37000 [a]	2579	30000 [b]	2097
Sugar Content (g/kg dm)	75 [a]	5	54 [b]	4	77 [a]	5	69 [a]	5
Water Content (g/kg)	391 [b]	20	437 [a]	22	411 [a]	21	430 [a]	22
Oil Content (g/kg dm)	440 [a]	31	460 [a]	32	500 [a]	35	450 [a]	31

Table 2 - Chemical and sensory characteristics of extracted olive oil.

		Frantoio cultivar								*Moraiolo* cultivar							
		HD1				HD2				HD1				HD2			
	Batch code [1]	F1a	F1b	F1c	Mean value ± SD	F2a	F2b	F2c	F2d	Mean value ± SD	M1a	M1b	M1c	M1d	Mean value ± SD	M2a	M2b	M2c	M2d	Mean value ± SD
EU legal characteristics	Free acidity (% oleic acid)	0.20	0.20	0.22	**0.21^{a}**±0.01	0.19	0.19	0.23	0.24	**0.21^{a}**±0.03	0.20	0.22	0.22	0.22	**0.22^{b}**±0.01	0.25	0.25	0.27	0.27	**0.26^{b}**±0.01
	Peroxide value (meq O_2/kg oil)	3.6	3.6	3.5	**3.8^{b}**±0.1	6.2	4.9	5.3	4.6	**5.2^{a}**±0.7	3.9	4.0	4.3	4.6	**4.2^{b}**±0.3	5.3	4.5	5.2	4.9	**5.0^{a}**±0.4
	K_{232}	1.76	1.71	1.74	**1.74^{a}**±0.03	1.77	1.80	1.87	1.85	**1.82^{a}**±0.05	1.80	1.74	1.82	1.80	**1.79^{a}**±0.03	1.79	1.86	1.79	1.83	**1.82^{a}**±0.03
	K_{270}	0.15	0.15	0.16	**0.15^{b}**±0.01	0.19	0.19	0.23	0.24	**0.21^{a}**±0.03	0.17	0.16	0.17	0.17	**0.17^{b}**±0.01	0.15	0.17	0.17	0.17	**0.17^{b}**±0.01
	ΔK	0.00	0.00	0.00	**0.00^{a}**±0.00	0.00	0.00	0.00	0.00	**0.00^{a}**±0.00	0.00	0.00	0.00	0.00	**0.00^{a}**±0.00	0.00	0.00	0.00	0.00	**0.00^{a}**±0.00
	P.A.: Fruity* (0-10)	3.5	3.4	4.1	**3.7^{a}**±0.4	n.d.	2.9	3.3	4.0	**3.4^{a}**±0.6	3.8	3.9	4.4	3.7	**4.0^{a}**±0.3	2.9	3.2	3.4	4.1	**3.4^{a}**±0.5
	P.A.: Bitter* (0-10)	2.6	3.6	3.6	**3.3^{a}**±0.6	n.d	3.6	3.3	5.1	**4.0^{a}**±1.0	2.6	3.6	4.6	3.7	**3.6^{a}**±0.8	3.8	3.5	3.5	3.5	**3.6^{a}**±0.1
	P.A.: Pungent* (0-10)	3.7	4.5	4.9	**4.4^{a}**±0.6	4.1	4.1	4.2	5.0	**4.4^{a}**±0.4	4.0	4.2	4.7	4.6	**4.4^{a}**±0.3	4.9	5.8	5.6	5.6	**5.5^{a}**±0.4
	N.A.: Rancid* (0-10)	0.0	0.0	0.0	**0.0^{b}**±0.0	0.0	1.9	1.6	1.6	**1.3^{a}**±0.9	0.0	0.0	0.0	0.0	**0.0^{b}**±0	1.2	1.4	1.4	1.1	**1.3^{a}**±0.2
	N.A.: Fusty* (0-10)	0.0	0.0	0.0	**0.0^{b}**±0.0	2.1	1.6	1.3	1.0	**1.5^{a}**±0.5	0.0	0.0	0.0	0.0	**0.0^{b}**±0	1.1	1.2	0.8	1.0	**1.0^{a}**±0.2
	N.A.: Winey-Vinegary* (0-10)	0.0	0.0	0.0	**0.0^{b}**±0.0	1.7	1.7	0.6	1.0	**1.3^{a}**±0.5	0.0	0.0	0.0	0.0	**0.0^{b}**±0	1.2	1.4	1.4	1.1	**1.3^{a}**±0.2
Biocompounds (mg/kg)	Total phenol content	720	640	720	**690^{a}**±46	560	610	700	690	**640^{a}**±68	690	670	730	720	**700^{a}**±30	570	700	610	700	**640^{a}**±65
	Oleuropein	39	32	32	**34^{a}**±4	30	40	50	50	**40^{a}**±11	40	50	70	60	**55^{a}**±11	40	50	60	60	**50^{a}**±10
	3,4 DHPEA-EDA [2]	230	170	170	**190^{a}**±32	130	130	150	140	**140^{a}**±10	153	142	152	157	**151^{a}**±6	100	140	100	110	**110^{b}**±19
	p-HPEA-EDA [3]	92	84	84	**83^{a}**±4	110	90	60	50	**80^{a}**±27	67	59	46	47	**55^{a}**±10	40	38	34	26	**34^{b}**±6
	3,4 DHPEA-EA [4]	31	29	26	**29^{a}**±2	41	38	58	51	**47^{a}**±9	26	36	33	33	**32^{b}**±4	48	48	40	49	**46^{a}**±4
	pHPEA-EA [5]	8.4	8.1	8.0	**8.1^{a}**±0.2	8.0	8.0	19.0	18.0	**13^{a}**±6	8.6	8.2	7.7	7.4	**8.0^{a}**±0.5	17	15	11	12	**14^{b}**±2
	3,4 DHPEA [6]	0.5	0.5	0.7	**0.6^{a}**±0.1	0.4	0.6	0.8	0.7	**0.6^{a}**±0.2	0.8	0.6	0.7	0.6	**0.7^{a}**±0.1	0.6	0.7	1.2	0.9	**0.9^{a}**±0.3
	Tocopherols	290	250	240	**260^{a}**±25	292	292	287	288	**290^{a}**±3	249	256	252	257	**254^{a}**±4	280	280	250	250	**270^{a}**±17
	Fruity volatile compounds [7] (mg/kg)	13.4	14.0	13.4	**13.6^{a}**±0.4	9.8	10.2	10.9	10.5	**10.3^{b}**±0.4	13.6	13.4	13.1	14.2	**13.6^{a}**±0.5	11.7	11.5	13.0	10.9	**11.8^{b}**±0.9

HD: harvesting date; SD: standard deviation; ^{a,b} different letters in the same row indicate significant differences ($p < 0.05$) for the same cultivar; n.d.: not determined; *median of the tasters score; P.A. and N.A.: Positive and Negative Attributes.
(1) F: *Frantoio*; M: *Moraiolo*; 1: First harvesting date; 2: Second harvesting date; a, b, c, d: olive batches.
(2) Dialdehydic form of decarboxymethyl oleuropein aglycone; (3) Dialdehydic form of decarboxymethyl ligstroside aglycone; (4) Oleuropein aglycone; (5) Ligstroside aglycone; (6) Hydroxytyrosol; (7) Sum of the underlined volatile compound contents in Table 3.

than EU legal chemical limits, no sensory defects and a value of "fruity" attribute with a medium intensity of perception, as reported in EU Reg. 1348/2013.

Conversely, all olive oil samples extracted from olives of the second harvesting date were not extra virgin, as they had significant sensory defects. Despite this, they were in compliance with all legally established (EU Reg. 1348/2013) chemical characteristics and "fruity" attribute.

As a result of malaxation operating conditions at low oxidative stress impact, olive oil resulted in high phenolic compounds content and a phenolic profile characterized by slightly degraded phenolic compounds (SERVILI *et al.*, 2004; GOMEZ-RICO *et al.*, 2009). The total phenolic compound content was approx. 670 mg/kg; the dialdehydic form of decarboxymethyl oleuropein aglycone (3,4-DHPEA-EDA) was the most abundant phenolic compound, and its content was approx. 150 mg/kg; low (approx. 0.7 mg/kg) hydroxytirosol content (3,4-DHPEA) was found. No significant differences were observed between samples at the two harvesting dates; the medium intensity of "bitter" and "pungent" attribute perception can be explained by phenolic compounds values (EU Reg. 1348/2013).

Volatile compounds content of olive oil samples were subdivided into chemical classes, as reported in Table 3. Compounds that have been shown (KALUA *et al.*, 2007; DI GIACINTO *et al.*, 2010; APARICIO *et al.*, 2012) to be significantly related to oil defects are reported. Underlined volatile compounds are intermediate of LOX pathway and they are considered (DI GIACINTO *et al.*, 2010; KOTTI *et al.*, 2011; APARICIO *et al.*, 2012) to be responsible for olive oil "fruity" positive attribute.

A sum of underlined compound contents is reported in Table 2 as "Fruity volatile compounds"; "fruity" attribute, measured by panel test, can be explained by these values.

Table 3 - Volatile compounds content of extracted olive oil. HD: harvesting date; n.d. not determined.

A. Class of esters, acids and hydrocarbons

	HD	Batch code	Methyl acetate (mg/Kg)	Ethyl acetate (mg/Kg)	Butyl acetate (mg/Kg)	Cis-3-hexenyl acetate (mg/Kg)	Trans-2-hexenyl acetate (mg/Kg)	Butyric acid (mg/Kg)	Pentanoic acid (mg/Kg)	Hexanoic acid (mg/Kg)	Octanoic acid (mg/Kg)	Heptan (mg/Kg)	Octan (mg/Kg)
Frantoio													
	1	**F1a**	0.036	0.019	0.002	0.144	0.003	nd	0.010	0.274	0.070	0.007	0.048
		F1b	0.022	0.017	0.002	0.131	0.002	nd	0.013	0.333	0.089	0.006	0.043
		F1c	0.015	0.020	0.002	0.134	0.001	nd	0.011	0.255	0.047	0.004	0.038
	2	**F2a**	0.015	0.042	0.002	0.070	nd	0.011	0.007	0.210	0.121	0.005	0.053
		F2b	0.007	0.035	0.002	0.409	nd	0.010	0.008	0.243	0.135	0.002	0.039
		F2c	0.008	0.027	0.001	0.614	nd	0.012	0.013	0.237	0.170	0.003	0.036
		F2d	0.006	0.023	0.001	0.619	nd	0.010	0.010	0.235	0.166	0.003	0.036
Moraiolo													
	1	**M1a**	0.005	0.018	0.001	0.747	0.003	nd	0.004	0.236	0.095	0.003	0.032
		M1b	0.005	0.016	0.002	1.070	0.027	nd	0.007	0.259	0.083	0.005	0.031
		M1c	0.006	0.019	0.003	1.173	0.004	nd	0.008	0.277	0.065	0.003	0.033
		M1d	0.005	0.015	0.001	0.480	0.006	nd	0.004	0.214	0.065	0.005	0.028
	2	**M2a**	0.006	0.021	0.001	1.032	nd	0.013	0.012	0.277	0.185	0.003	0.035
		M2b	0.005	0.020	0.002	0.858	nd	0.013	0.011	0.261	0.160	0.004	0.040
		M2c	0.008	0.022	0.001	0.927	nd	0.012	0.008	0.201	0.119	0.002	0.036
		M2d	0.006	0.023	0.001	0.701	nd	0.014	0.015	0.262	0.171	0.003	0.037

B. Class of aldehydes

	HD	Batch code	Valeraldheyde (mg/Kg)	Hexanal (mg/Kg)	Trans-2-Pentenal (mg/Kg)	Cis-3-Hexenal (mg/Kg)	Heptanal (mg/Kg)	Trans-2-Hexenal (mg/Kg)	Octanal (mg/Kg)	Trans-2-Heptenal (mg/Kg)	2.4 Hexadienal (mg/Kg)	Trans-2-Octanal (mg/Kg)	Benzaldehyde (mg/Kg)	Trans-2-Nonenal (mg/Kg)	Trans-2-Decenal (mg/Kg)
Frantoio															
	1	**F1a**	0.128	0.586	0.031	1.421	0.025	10.090	0.131	0.038	0.292	0.015	0.031	0.190	0.229
		F1b	0.106	0.644	0.035	1.822	0.026	10.217	0.159	0.033	0.313	0.010	0.031	0.196	0.232
		F1c	0.087	0.596	0.030	1.569	0.025	9.963	0.134	0.024	0.270	0.010	0.030	0.185	0.237
	2	**F2a**	0.094	0.481	0.023	1.085	0.017	7.331	0.063	0.042	0.198	0.010	0.030	nd	0.132
		F2b	0.079	0.504	0.028	1.084	0.017	6.938	0.066	0.034	0.189	0.015	0.032	nd	0.125
		F2c	0.070	0.547	0.035	1.157	0.023	6.785	0.090	0.031	0.177	0.014	0.033	nd	0.078
		F2d	0.071	0.535	0.035	1.050	0.022	6.545	0.103	0.030	0.172	0.016	0.034	nd	0.092
Moraiolo															
	1	**M1a**	0.092	0.556	0.036	1.742	0.028	8.750	0.148	0.013	0.271	0.019	0.028	0.187	0.203
		M1b	0.079	0.443	0.036	2.016	0.022	7.684	0.137	0.009	0.262	0.016	0.029	0.197	0.18
		M1c	0.100	0.489	0.042	1.937	0.029	7.371	0.152	0.020	0.246	0.014	0.031	0.194	0.204
		M1d	0.087	0.563	0.032	2.045	0.025	9.435	0.117	0.023	0.276	0.019	0.028	0.202	0.180
	2	**M2a**	0.053	0.538	0.039	1.320	0.027	6.595	0.117	0.024	0.206	0.012	0.036	nd	0.081
		M2b	0.044	0.474	0.043	1.803	0.022	6.172	0.093	0.021	0.229	0.009	0.035	nd	0.058
		M2c	0.042	0.416	0.045	4.051	0.015	5.121	0.072	0.010	0.348	0.022	0.034	nd	0.059
		M2d	0.070	0.585	0.038	1.123	0.025	6.607	0.126	0.036	0.192	0.018	0.039	nd	0.099

C. Class of alcohols, ketones and phenols

	HD	Batch code	1-Penten-3-ol (mg/Kg)	2-Heptanol (mg/Kg)	Pentanol (mg/Kg)	1-Octen-3-ol (mg/Kg)	Trans-3-Hexenol (mg/kg)	Cis-3-Hexenol (mg/Kg)	Cis-2-Pentenol (mg/Kg)	2-Butanone (mg/Kg)	2-Octanone (mg/Kg)	1-Octen-3-one (mg/Kg)	1-Penten-3-one (mg/kg)	6-methyl-5-Hepten-2-one (mg/Kg)	Guaiacol (mg/Kg)	Phenol (mg/Kg)	Ethyl-guaiacol (mg/Kg)	4-Ethyl-phenol (mg/Kg)
Frantoio	1	F1a	0.446	nd	0.005	0.002	0.004	0.242	0.337	0.433	0.003	0.001	0.433	0.004	0.005	0.261	0.131	nd
		F1b	0.454	nd	0.005	0.002	0.004	0.260	0.358	0.477	0.004	0.001	0.477	0.005	0.005	0.275	0.124	nd
		F1c	0.475	nd	0.004	0.002	0.003	0.200	0.353	0.484	0.004	0.001	0.484	0.004	0.008	0.253	0.158	0.080
	2	F2a	0.403	0.225	0.004	0.005	0.004	0.089	0.288	0.360	0.014	0.003	0.360	nd	0.002	0.198	nd	nd
		F2b	0.491	0.294	0.004	0.007	0.005	0.259	0.310	0.481	0.012	0.002	0.481	nd	0.004	0.200	nd	nd
		F2c	0.597	0.351	0.005	0.008	0.006	0.431	0.382	0.708	0.010	0.001	0.708	nd	0.005	0.193	nd	nd
		F2d	0.582	0.346	0.005	0.010	0.007	0.454	0.376	0.712	0.035	0.004	0.712	nd	0.006	0.203	nd	nd
Moraiolo	1	M1a	0.554	nd	0.006	0.002	0.006	0.521	0.428	0.586	0.008	0.002	0.586	0.004	0.004	0.245	nd	nd
		M1b	0.569	nd	0.006	0.002	0.009	0.695	0.435	0.590	0.004	0.002	0.590	0.002	0.004	0.251	0.117	nd
		M1c	0.584	nd	0.005	0.002	0.008	0.709	0.444	0.606	0.006	0.002	0.606	0.004	0.003	0.352	0.114	0.067
		M1d	0.571	nd	0.006	0.002	0.006	0.346	0.447	0.533	0.005	0.001	0.533	0.003	0.004	0.238	nd	nd
	2	M2a	0.658	0.375	0.005	0.009	0.008	0.714	0.420	0.715	0.009	0.002	0.715	nd	0.006	0.208	nd	nd
		M2b	0.575	0.369	0.005	0.007	0.005	0.745	0.412	0.711	0.003	0.002	0.711	nd	0.004	0.210	nd	nd
		M2c	0.603	0.361	0.004	0.006	0.006	0.853	0.402	0.720	0.003	0.002	0.720	nd	0.002	0.196	nd	nd
		M2d	0.636	0.381	0.006	0.012	0.007	0.509	0.394	0.753	0.011	0.004	0.753	nd	0.006	0.205	nd	nd

A multidimensional map of all samples related to volatile compounds was obtained by PCA. The relevant sample loading and score plots are reported in Fig. 2. The model explained 60% of data variability along the first (Factor 1) and second (Factor 2) principal components.

A comparison between the score plot and the loading plot showed that olive oil samples extracted from olives of the second harvesting date were all positioned on the left side of the plot. They were characterized by high values of benzaldehyde, 2-butanone, butyric acid, 2-heptanol, octanoic acid, 1-octen-3-ol, 1-octen-3-one and 2-octanone.

All these compounds are related to olive oil defects: These compounds have been associated with "musty", "winey–vinegary" and "fusty" defects by some literature data (KALUA *et al.*, 2007; APARICIO *et al.*, 2012), whereas they have been associated with "rancid" defect by DI GIACINTO *et al.* (2010).

Microbial ecology of oil extraction process

Cell concentrations of dominant microbial populations at different steps of oil extraction process from *Frantoio* and *Moraiolo* cultivar olives are shown in Tables 4 and 5, respectively.

Yeasts and/or moulds were always the dominant populations, independently of the sampling point. Cell density of bacteria only accounted for 1% of the total microbial counts on PCA plates.

The cell concentrations in olive paste after crushing (P) and in extracted olive oil (D) ranged between values below 10 and above 10^4 CFU/g or mL. These values were higher than that obtained from filtered olive oil (O), which, in most cases, was < 10^2 CFU/100 mL.

Microbial counts of each olive batch were often affected by high standard deviation values, as it typically occurs in manufacturing processes of raw materials (such as olives) at industrial scale. A rough general pattern for microbial evolution during olive processing could nonetheless be drawn.

Mould counts in olive paste after crushing (PM) were always significantly higher than those in extracted olive oil (DM), while yeast counts showed a different behaviour.

In most olive batches (from both *Frantoio* and *Moraiolo* cultivars) of the first harvesting date, yeast counts decreased by about one order of magnitude from olive paste after crushing (PY) to extracted olive oil (DY), as expected on the basis of olive oil yield. At the second harvesting date, yeast counts remained almost unchanged from olive paste (PY) to olive oil (DY), or even increased in extracted olive oil (DY), suggesting a progressive yeast colonization of the malaxation equipment and/or "decanter". Indeed, at the second harvesting date, olive paste (PY) harboured almost the same yeast concentration as that at the first harvesting date, with values ranging be-

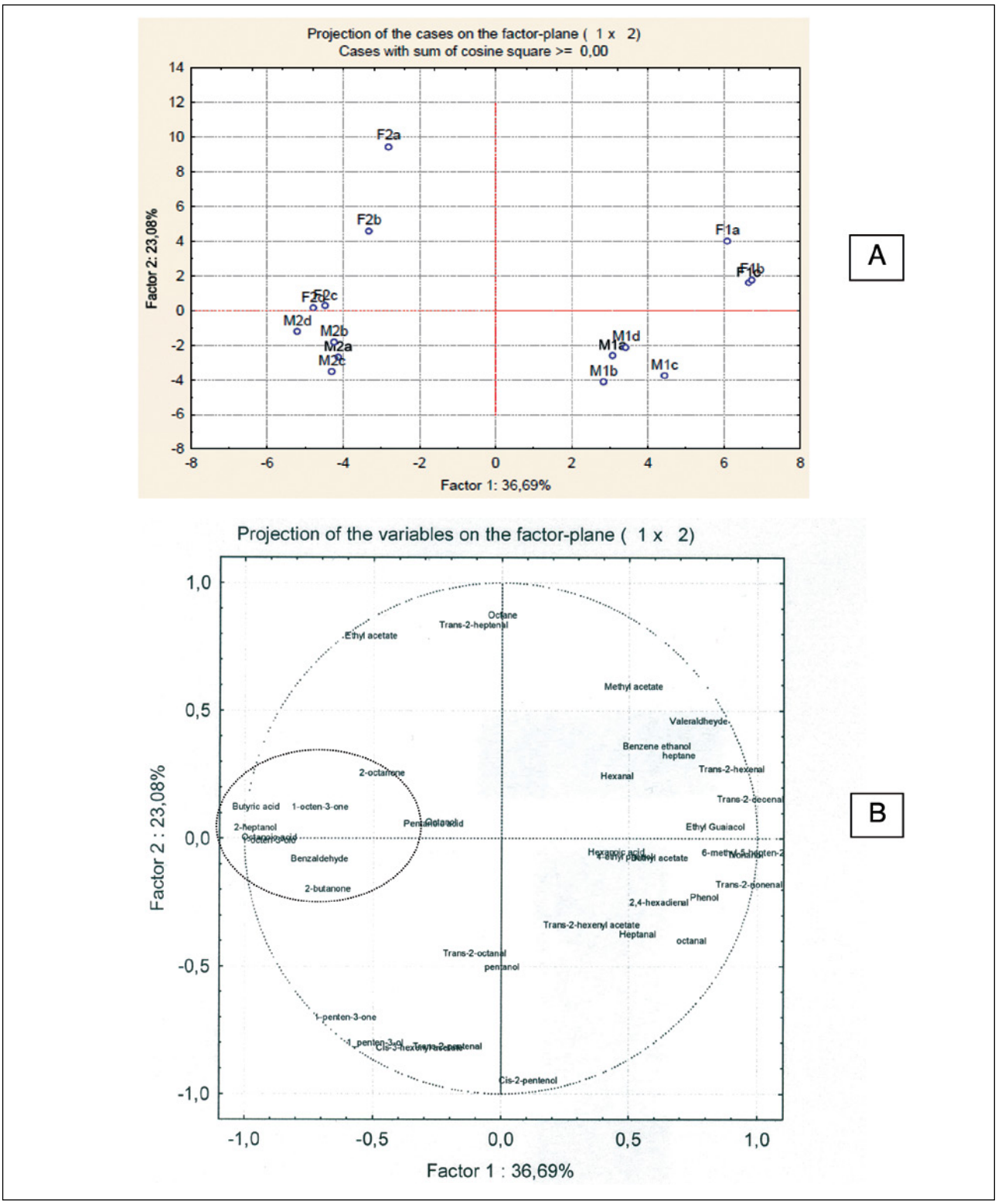


Fig. 2 - Principal Component Analysis carried out on volatile compounds content of olive oil samples. A: similarity map determined by Principal Component (Factor) 1 and 2; B: projection of the variables on the factor plane. Samples are coded as reported in Table 2.

tween 10^2 and above 10^3 CFU/g. On the contrary, the extracted olive oil of the second harvesting date (DY) harboured yeast concentrations, in most cases, of about one or two orders of magnitude higher than the extracted olive oil of the first harvesting date.

Correlation studies demonstrated that mould counts in olive paste after crushing (PM) and in extracted olive oil (DM) were positively related to each other, suggesting that mould contamination of unfiltered oil could be affected by the hygienic level of olives (Table 6). On the contrary, yeast cell densities in olive paste (PY) and in olive oil (DY) were statistically unrelated, sug-

Table 4 - Microbial cell counts at different steps of oil extraction process on two harvesting dates (HD) for *Frantoio* cultivar. P = olive paste after crushing; D = olive oil after extraction by "decanter"; O = olive oil after filtration; TMC = total microbial count; different letters indicate significant differences between different extractive steps of the same olive batch ($p < 0.05$); when no letter is reported, no significant difference was found.

HD	Batch code	Sampling point	Yeasts		Moulds		TMC	
			Mean	SD	Mean	SD	Mean	SD
1	F1a	P (CFU/g)	1.60×10^{3a}	1.40×10^{2}	1.00×10^{2}	0	1.40×10^{3a}	1.40×10^{2}
		D (CFU/mL)	4.50×10^{1b}	7.07	<10	-	4.00×10^{1b}	2.82
		O (CFU/100mL)	<1	-	<1	-	<1	-
	F1b	P (CFU/g)	8.50×10^{2a}	2.12×10^{2}	<10	-	1.60×10^{3a}	5.66×10^{2}
		D (CFU/mL)	1.00×10^{2b}	2.80×10^{1}	<10	-	4.00×10^{1b}	0
		O (CFU/100mL)	<1	-	<1	-	<1	-
	F1c	P (CFU/g)	1.10×10^{3a}	1.41×10^{2}	8.00×10^{2}	0	2.00×10^{3}	1.41×10^{3}
		D (CFU/ml)	3.25×10^{2b}	3.54×10^{1}	<10	-	5.00×10^{2}	2.83×10^{2}
		O (CFU/100mL)	<1	-	<1	-	<1	-
2	F2a	P (CFU/g)	1.00×10^{2a}	1.40×10^{1}	4.20×10^{4a}	2.82×10^{3}	4.80×10^{4a}	2.83×10^{3}
		D (CFU/mL)	3.00×10^{2b}	2.80×10^{1}	4.00×10^{1b}	2.82	3.00×10^{2b}	2.88×10^{1}
		O (CFU/100mL)	5.00×10^{1c}	1.41	<1	-	5.00×10^{1b}	2.82
	F2b	P (CFU/g)	2.70×10^{3}	1.84×10^{3}	2.85×10^{4}	2.32×10^{4}	8.75×10^{3a}	3.18×10^{3}
		D (CFU/ml)	2.92×10^{3}	1.99×10^{3}	3.33×10^{1}	3.27×10^{1}	1.00×10^{2b}	0
		O (CFU/100mL)	5.50×10^{1}	1.41	<1	-	6.50×10^{1b}	1.41
	F2c	P (CFU/g)	2.30×10^{3}	9.90×10^{2}	2.50×10^{4}	2.25×10^{4}	1.10×10^{3a}	1.41×10^{2}
		D (CFU/ml)	3.26×10^{3}	1.60×10^{3}	9.67×10^{1}	8.96×10^{1}	1.81×10^{3a}	7.66×10^{2}
		O (CFU/100mL)	5.50×10^{1}	2.82	<1	-	1.00×10^{1b}	2.82
	F2d	P (CFU/g)	4.00×10^{2a}	2.83×10^{1}	3.45×10^{4}	3.32×10^{4}	7.00×10^{3a}	1.41×10^{2}
		D (CFU/ml)	1.38×10^{4b}	6.36×10^{2}	1.20×10^{2}	2.83×10^{1}	1.35×10^{4b}	9.90×10^{2}
		O (CFU/100mL)	1.50×10^{1a}	1.40	5.00	0	4.00×10^{1c}	2.82

Table 5 - Microbial cell counts at different steps of olive oil extraction process on two harvesting dates (HD) for cultivar *Moraiolo*. P = olive paste after crushing; D = olive oil after extraction by "decanter"; O = olive oil after filtration; TMC = total microbial count; different letters indicate significant differences between different extraction steps of the same olive batch ($p < 0.05$); when no letter is reported, no significant difference was found.

HD	Batch code	Sampling point	Yeasts		Moulds		TMC	
			Mean	SD	Mean	SD	Mean	SD
1	M1a	P (CFU/g)	1.10×10^{3a}	1.41×10^{2}	4.00×10^{2a}	0	1.45×10^{3}	6.36×10^{2}
		D (CFU/mL)	4.50×10^{1b}	7.07	<10	-	4.00×10^{1}	1
		O (CFU/100mL)	<1	-	4.00×10^{1b}	1.41	1.00×10^{1}	1
	M1b	P (CFU/g)	3.75×10^{3a}	3.54×10^{2}	5.50×10^{2}	5.36×10^{2}	5.35×10^{3}	2.33×10^{3}
		D (CFU/mL)	5.00×10^{1b}	1.40	<10	-	<10	-
		O (CFU/100mL)	<1	-	2.00×10^{1}	1.00	2.00×10^{1}	1.40
	M1c	P (CFU/g)	1.10×10^{3a}	1.41×10^{2}	4.00×10^{2a}	0	2.35×10^{3a}	9.19×10^{2}
		D (CFU/mL)	6.90×10^{3b}	2.82×10^{2}	<10	-	1.50×10^{4b}	3.54×10^{3}
		O (CFU/100mL)	<1	-	1.00×10^{1b}	2.82	1.00×10^{1a}	1.40
	M1d	P (CFU/g)	1.10×10^{3}	1.41×10^{2}	4.00×10^{2a}	1.41×10^{1}	1.45×10^{3}	7.78×10^{2}
		D (CFU/ml)	3.20×10^{2}	3.11×10^{2}	<10	-	3.50×10^{1}	2.12×10^{1}
		O (CFU/100mL)	<1	-	2.00×10^{1b}	0	2.00×10^{1}	1.40
2	M2a	P (CFU/g)	1.70×10^{3}	2.83×10^{2}	2.60×10^{3a}	1.98×10^{3}	2.70×10^{3a}	4.24×10^{2}
		D (CFU/mL)	1.04×10^{3}	7.45×10^{2}	6.00×10^{1a}	5.66×10^{1}	9.73×10^{2ab}	8.60×10^{2}
		O (CFU/100mL)	<1	-	5.50×10^{1b}	2.82	5.50×10^{1a}	1.41
	M2b	P (CFU/g)	2.45×10^{3a}	7.78×10^{2}	6.00×10^{2}	5.66×10^{2}	2.35×10^{3a}	9.19×10^{2}
		D (CFU/ml)	3.27×10^{2b}	1.42×10^{2}	3.50×10^{1}	2.12×10^{1}	3.80×10^{2b}	2.31×10^{2}
		O (CFU/100mL)	1.00×10^{1c}	0	1.60×10^{2}	2.82×10^{1}	7.50×10^{1b}	3.53
	M2c	P (CFU/g)	7.45×10^{3}	2.19×10^{3}	1.00×10^{3}	2.82×10^{2}	4.15×10^{3a}	2.12×10^{2}
		D (CFU/mL)	9.72×10^{3}	5.04×10^{3}	4.00×10^{1}	3.66×10^{1}	1.16×10^{3b}	1.07×10^{3}
		O (CFU/100mL)	8.00×10^{1}	14.00	<1	-	1.65×10^{2b}	3.00
	M2d	P (CFU/g)	1.65×10^{3}	1.61×10^{3}	6.75×10^{3}	1.77×10^{3}	5.80×10^{3}	3.11×10^{3}
		D (CFU/mL)	3.08×10^{3}	3.02×10^{3}	6.00×10^{1}	5.66×10^{1}	2.53×10^{3}	2.04×10^{3}
		O (CFU/100mL)	1.10×10^{3}	2.00	<1	-	5.50×10^{1}	1.41

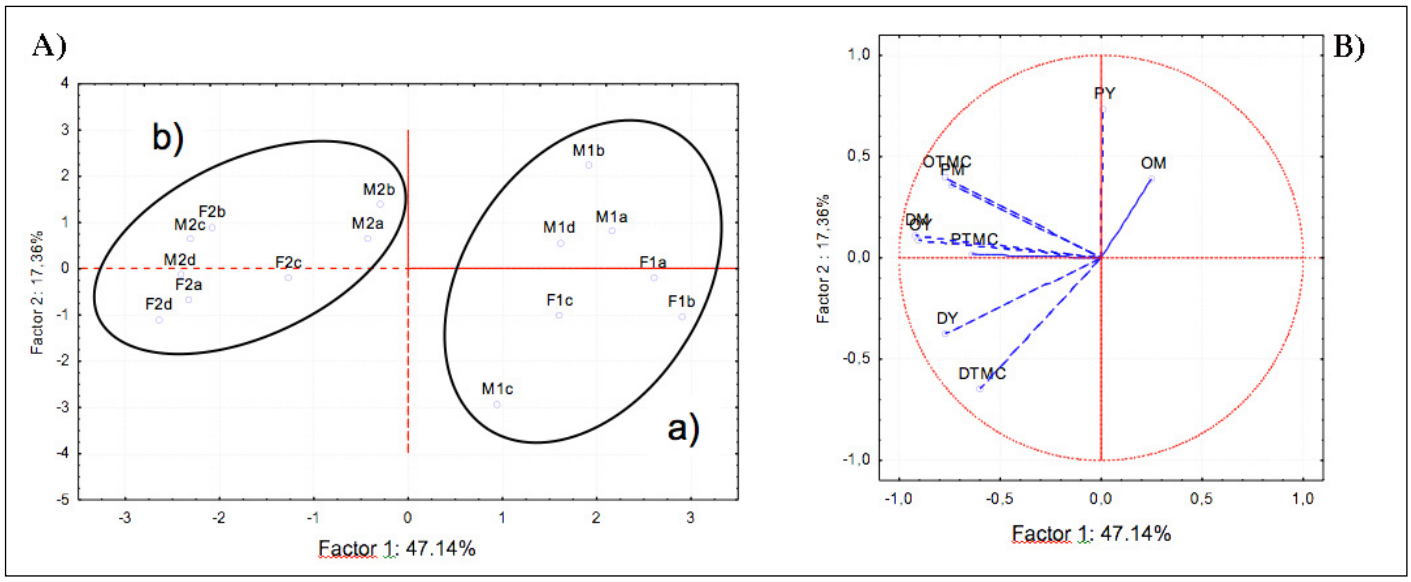


Fig. 3 - Principal Component Analysis of the various olive batches tested by considering as variables the microbial cell concentrations during various extraction process steps. Samples are coded by combination of letters which identify both samples at processing steps (P = olive paste after crushing; D = olive oil after centrifugation by "decanter"; O = olive oil after filtration) and microorganisms (TMC = total microbial count; Y = yeasts; M = moulds). A: similarity map determined by Principal Component (Factor) 1 and 2; B: projection of the variables on the factor plane.

gesting that yeast growth could be encouraged by malaxation and/or "decanting" steps. Finally, no correlation was found between yeast and mould concentrations in both olive paste (PY and PM, respectively) and filtered oil (OY and OM, respectively).

According to PCA of all microbiological data (Fig. 3), processed olive batches clustered into two different groups, independently of the olive cultivar: The samples of the first harvesting date, harboring the lowest microbial cell densities, clustered in group a), while all batches of the second harvesting date resulted to be included in group b). It is worth noting that both the PCA resulting from all microbiological data (Fig. 3) and the PCA resulting from volatile compounds (Fig. 2) are in full agreement, as olive batches from both statistical analyses are clustered in the same way.

Finally, some statistically significant correlations were found between microbial cell densities at the different steps of oil processing and some volatile compounds of olive oil. The significant correlations between yeast (Y) and mould (M) counts, in both extracted (D) and filtered olive oil (O), and volatile compounds content of the final olive oil samples are reported in Table 7. In particular, correlation coefficients (i.e. Pearson and Spearman) agreed on indicating significant positive correlations between yeast and mould counts in olive oil, both before and after filtration, and some volatile compounds; among the latter, the highest significance was related to ethyl acetate, 2-butanone, butyric acid, pentanol, 2-heptanol, octanoic acid and 1-octen-3-ol contents.

Since most of these compounds were identical to those correlated to olive oil batches with sensory defects, as described in the previous paragraph, yeast and mould contamination may have been responsible for those sensory defects. Which specific sensory defects were associated with the above-mentioned compounds could not be explained, as in the literature "rancid", "fusty", "winey–vinegary" and "musty" defects have been associated with both yeasts and moulds. As an example, a recent study demonstrated the capability of some oil born strains of *Candida*

Table 6 - Correlation coefficients calculated between microbial contaminations (Y = yeasts; M = moulds) of olive paste after crushing (P) and microbial contaminations of extracted (D) and filtered olive oil (O). Statistically significant correlations ($p<0.05$) are underlined.

	DY		DM		OY		OM	
	Spearman r	Pearson r	Spearman r	Pearson r	Spearman r	Pearson r	Spearman r	Pearson r
PM			<u>0.8304</u>	<u>0.7347</u>			-0.1575	-0.2485
PY	0.08641	0.05563			0.2841	0.1241		

Table 7 - Correlation coefficients calculated between yeast (Y) and mould (M) counts of extracted and filtered olive oil (D) and volatile compounds of the final olive oil samples (O). Statistically significant correlations ($p<0.05$) are underlined.

	DY		DM		OY		OM	
	Spearman r	Pearson r	Spearman r	Pearson r	Spearman r	Pearson r	Spearman r	Pearson r
Esters, acids and hydrocarbons								
Methyl acetate	-0.006737	-0.3253	-0.08564	-0.4042	0.01485	-0.2926	-0.8317	-0.5387
Ethyl acetate	0.4464	0.2619	0.6978	0.5603	0.7348	0.6665	-0.4953	-0.4413
Butyl acetate	-0.3046	-0.27	-0.5824	-0.5091	-0.3481	-0.3366	-0.1901	-0.1524
Cis-3-hexenil acetate	0.3194	0.325	0.1589	0.2075	-0.0114	0.01915	0.652	0.6335
Trans-2-hexenil acetate	-0.5621	-0.4382	-0.862	-0.4494	-0.7775	-0.3811	0.243	0.2902
Butyric acid	0.5532	0.5918	0.8694	0.9727	0.7434	0.8125	0.03821	-0.0028
Pentanoic acid	-0.0685	-0.1613	-0.1662	-0.1045	-0.3671	-0.331	0.5344	0.5813
Hexanoic acid	-0.1944	-0.3239	-0.337	-0.3943	-0.4902	-0.5172	-0.01623	-
Octanoic acid	0.4624	0.4945	0.8818	0.9251	0.6282	0.6242	0.1469	0.1633
Heptan	-0.6824	-0.5102	-0.5535	-0.4601	-0.5756	-0.4022	-0.0887	-0.218
Octan	-0.1092	-0.2505	0.0174	-0.04	0.205	0.0699	-0.6035	-0.5001
Aldehydes								
Valeraldheyde	-0.5583	-0.4607	-0.708	-0.7013	-0.5681	-0.5144	-0.291	-0.388
Hexanal	-0.3341	-0.3271	-0.3477	-0.3659	-0.4185	-0.4153	-0.3585	-0.315
Trans-2-Pentenal	0.3599	0.3423	0.2134	0.1599	0.06842	0.02669	0.5254	0.464
Cis-3-Hexenal	-0.2487	0.1017	-0.59	-0.1577	-0.4412	0.03461	0.3088	0.07831
Heptanal	-0.3023	-0.3369	-0.4689	-0.5529	-0.7091	-0.7582	0.2273	0.2932
Trans-2-Hexenal	-0.6784	-0.6681	-0.8182	-0.8242	-0.6991	-0.7192	-0.2628	-0.1959
Octanal	-0.405	-0.4349	-0.6645	-0.7347	-0.7142	-0.7745	0.1745	0.2041
Trans-2-Heptenal	-0.0552	-0.01303	0.1559	0.1205	0.2061	0.1596	-0.6411	-0.5767
2.4 Hexadienal	-0.4503	-0.3867	-0.7002	-0.6627	-0.4882	-0.42	-0.0957	-0.0823
Trans-2-Octanal	-0.0244	0.1057	-0.0918	-0.1199	0.08817	0.08237	-0.0718	-0.1306
Benzaldehyde	-0.5041	0.3715	0.5785	0.4958	0.4283	0.3689	-0.1509	0.1584
Trans-2-Nonenal	-0.6304	-0.6281	-0.8605	-0.9855	-0.7762	-0.8358	0.07103	0.04037
Trans-2-Decenal	-0.5946	-0.615	-0.8119	-0.895	-0.6682	-0.7092	-0.2806	-0.2243
Alcohols, ketones and phenols								
1-Penten-3-ol	0.6681	0.4847	0.5822	0.4303	0.3147	0.1095	0.5055	0.5417
2-Heptanol	0.6178	0.6498	0.8857	0.9774	0.7328	0.7846	0.06237	0.0213
Pentanol	0.4111	0.4182	0.8213	0.8118	0.7221	0.7422	-0.1372	-0.2154
Cis-3-Hexenol	0.3252	0.2959	0.2486	0.216	0.07623	0.05016	0.6277	0.5872
Trans-3-Hexenol	0.3032	0.2647	0.2875	0.1636	0.0247	-	0.5309	0.4904
1-Octen-3-ol	0.6212	0.6381	0.9304	0.9199	0.7286	0.7176	-0.0491	-0.0339
Cis-2-Pentenol	0.0486	0.08362	-0.1142	-0.16	-0.2467	-0.2997	0.685	0.618
2-Butanone	0.5204	0.5477	0.782	0.7111	0.5529	0.5283	0.00517	-0.1297
1-Penten-3-one	0.6461	0.5539	0.6247	0.5717	0.412	0.3304	0.3666	0.4029
2-Octanone	0.264	0.4949	0.5565	0.5658	0.4097	0.3878	-0.06146	-0.0623
1-Octen-3-one	0.2545	0.3958	0.5142	0.5573	0.5459	0.5804	0.1314	0.0085
6-methyl-5-Hepten-2-one	-0.5882	-0.5785	-0.8678	-0.9186	-0.7828	-0.779	-0.1013	-0.1062
Guaiacol	-0.0316	-0.1852	0.08201	-0.1711	-0.2404	-0.3518	-0.1757	-0.1935
Phenol	-0.4724	-0.258	-0.8245	-0.8028	-0.8142	-0.7034	0.1395	0.0637
Ethyl-guaiacol	-0.3957	-0.4097	-0.6943	-0.7573	-0.6262	-0.6423	-0.3215	-0.2771
4-Ethyl-phenol	-0.0820	-0.1033	-0.4472	-0.4672	-0.3997	-0.3962	-0.1999	-0.2132

spp. to induce defects such as "musty" and/or "rancid" in oil (ZULLO *et al.*, 2013).

CONCLUSIONS

This study was carried out on several olive oil samples extracted by olive batches from *Frantoio* and *Moraiolo* cultivars, harvested on two different dates. All extracted olive oil samples from the second olive harvesting date were classified as "non extra virgin", as they were affected by sensory defects.

By combining chemical, sensory, and microbiological data, it can be assumed that the olive oil samples with sensory defects were significantly correlated with specific volatile compounds (i.e., 2-butanone, butyric acid, 2-heptanol, octanoic acid, 1-octen-3-ol). The same volatile compounds were correlated to both yeast and mould counts. It could not be evidenced whether a specific sensory defect might result from specific volatile compounds, which in turn can be produced by specific yeasts and moulds.

Different processing steps were also identified, which resulted to be the most critical steps to cause the measured sensory defects: (i) the

mould contamination of olives; (ii) the two central steps of olive oil processing (i.e. malaxation and extraction by "decanter"), which were likely to have enabled some yeast species to grow. A study on identification of yeast isolates and determination of their enzymatic properties is being carried out to further investigate the incidence of yeast populations during olive oil extraction process.

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PAPER

PHYTOCHEMICAL COMPOSITION AND ANTIOXIDANT ACTIVITY OF TUSCAN BEE POLLEN OF DIFFERENT BOTANIC ORIGINS

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ABSTRACT

Within the apicultural products, the honey bee-pollen is growing in commercial interest due to its high nutritional properties. For the first time, bee-pollen samples from Tuscany (Italy) were studied to evaluate botanical origin, phytochemical composition and antioxidant activity. The investigated pollen loads were composed of three botanical families: *Castanea*, *Rubus* and *Cistus*. The highest levels of proteins and lipids were detected in *Rubus* pollen. *Castanea* pollen contained greater polyphenols, flavonoids and anthocyanins content, while the highest flavonols level was detected in *Cistus* pollen. These results were also confirmed by front-face fluorescence spectroscopy, used here, for the first time, as a fast tool to characterize bee-pollens.

- Keywords: antioxidant activity, pollen, bioactive compounds, chemical composition, fluorescence, Tuscany -

1. INTRODUCTION

For centuries the apicultural products have been used in phytotherapy as well as in diet for their health positive implications (KROYER and HEGEDUS, 2001; FERRERES *et al.*, 2010; ABOUDA *et al.*, 2011).

Bee-gathered pollen (bee-pollen) is an apicultural product of great commercial interest owing to its high nutritional value and physiological properties representing an important source of energy and proteins for human nutrition (ABOUDA *et al.*, 2011).

Worker-bees gather pollen from flowers and, after processing it with some proteic gland-secretion, they package it on their curbicula forming pollen loads before returning to the hive (PINZAUTI *et al.*, 2002; SCARSELLI *et al.*, 2005). Generally, a single pollen load is a one colored little pellet reflecting a homogeneous and monospecific pollen content. Bee-pollen loads are stilled from the worker bees at their entrance to the hive by special pollen loads traps. In accordance to the season, to the timing of collection by the beekeepers and to the post collection management is possible to obtain mono- or poly-pollen species loads. Amounts of pollen loads ready to be commercialized and consumed by human and animals is called bee-pollen. So it is possible to find bee-pollen of one specific flower (monoflora) or belonging to several flower species (polyflora), as well is possible to blend different monofloral in order to create mixtures of bee-pollen with characteristic organoleptic properties and quality attributes.

Nowadays, bee-pollen represents the richest and most complete natural food supplying high levels of carbohydrates (13-55%), proteins (10-40%), particularly free aminoacids, enzymes, cofactors, lipids (1-13%), including fatty acids and sterols, minerals, trace elements and vitamins, especially B group, A, C and E. Fresh and dry bee-pollen loads hold a different water content, ranging from 20-30% in the original form and 4-10% if dried, affecting organoleptic and "shelf-life time" properties (CAMPOS *et al.*, 2008; PASCOAL *et al.*, 2014).

Moreover, it is also an excellent source of bioactive compounds, such as phytosterols, carotenoids and polyphenols (especially flavonoids), that exert antioxidant, anti-inflammatory, antimicrobial, anti-allergic and antitumoral effects (MORAIS *et al.*, 2011; PÉREZ- PÉREZ *et al.*, 2012; FRATINI *et al.*, 2014). Very recently, several research works were published showing that bee products, such as propolis and pollens, possess a sedative effect and may be effective in protecting humans against depression and similar diseases (YILDIZ *et al.* 2014); additionally, preliminary studies show that pollens have a hepatoprotective potential (YILDIZ *et al.* 2013). Pollens effects on improving immune, cardiovascular and digestive systems as well as their therapeutic effects have been mainly related to the polyphenol content and chemical composition (PASCOAL *et al.*, 2014). In particular, the phenolic profile of bee-pollen consists of flavonol, glycosides and aglycones, and hydroxycinnamic acids, that can be present in free forms or combined with other pollen components (CHANTARUDEE *et al.*, 2012; FANALI *et al.*, 2013). However, as well as chemical composition, the phytochemical profile is affected by soil type, beekeeping management, climatic and preservation conditions, and especially by botanical origin (ALMEIDA-MURADIAN *et al.*, 2005; ARRUDA *et al.*, 2013; CAMPOS *et al.*, 2008). Aromatic aminoacids, many polyphenols, some enzyme cofactors, but also some water- or lipid-soluble vitamins and pigment's derivatives contained in bee-pollen are fluorescent intrinsic compounds (JØRGENSEN *et al.*, 1992; LAKOWICZ, 2006).

Front-face (FF) fluorescence spectroscopy is a non-destructive, rapid and sensitive technique suitable for complex and opaque samples, otherwise traditional right-angle florescence (KAROUI *et al.*, 2007; ZANDOMENEGHI *et al.*, 2005). In particular, this technique has been proved to be very effective in studying powders, crystalline or amorphous samples, and complex matrix, such as food (ZANDOMENEGHI, 1999; ZANDOMENEGHI and ZANDOMENEGHI, 2009; AIRADO-RODRÍGUEZ *et al.*, 2011; KULMYRZAEV *et al.* 2005). In this context, FF fluorescence could be a useful instrument to obtain a fingerprint of different bee-pollen types, advantageous to study and compare them.

In this study, we examined the botanical origin, the chemical composition (moisture, proteins, carbohydrates, lipids, ash) and the antioxidant profile (the total polyphenols and the flavonoids, flavonols and anthocyanins subclasses) of different color fractions of an organic Tuscan (Italian) bee-pollen sample. Moreover, we proposed for the first time a rapid qualitative evaluation of emission, excitation and synchronous spectra, obtained by front-face spectroscopy, of bulk state and ethanolic extracts of the different pollen types showing the main classes of identified fluorescent molecules. Lastly, to evaluate the synergic effect of the bee-pollen bioactive components we analyzed by DPPH and ORAC assay the free radical scavenging activity and the antioxidant capacity of both separate and mixed color fractions.

2. MATERIALS AND METHODS

2.1 Chemicals and reagents

All standards and reagents were of analytical grade. Absolute ethanol, methanol, hydrochloric acid, trichloroacetic acid, diethyl ether, sodium carbonate, sodium idrosside, potassium chloride, sodium acetate, Folin-Ciocalteu rea-

gent, catechin, gallic acid, 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), 1,1-diphenyl-2-picrylhydrazyl (DPPH) and fluorescein sodium salt were purchased from Fluka-Sigma-Aldrich, Inc. (St. Louis, MO), as well as the solid standards used in spectroscopic analysis tryptophan, b-carotene, gallic acid, 3,4-dihydroxybenzoic acid, 4-hydroxybenzoic acid, vanillic acid, caffeic acid, ferulic acid, p-coumaric acid, quercetin dihydrate and the vitamins C, B3, B2, B6 and B9. Hydroxide peroxide, sulfuric acid, boric acid and Kjeldahl tablets were purchased from Merck (Readington, NJ). Sodium nitrite and aluminum chloride were purchased from Carlo Erba (Milan, IT), while 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH) was purchased from Polysciences, Inc. (Warrington, PA).

2.2 Plant materials

Bee-pollen resulted from the blend of pollen loads collected during sunny days by the beekeeper using 10 beehives equipped with bottom-fitted pollen traps located in Massa Macinaia (Latitude 43.80638- Longitude 10.54213) in Lucca Province (Tuscany, Italy) between April and July 2013. In total 1 kg of bee-pollen was collected. The blended fresh bee-pollen was stored at -20°C in the dark until further analysis.

2.3 Palynological analysis

Within the bee-pollen sample the pollen loads were divided by colour into three groups (Fig. 1). Twenty single pollen loads of each colour were analyzed by microscope. Each single pollen load prepared by washing the pollen with distilled water and using glycerin jelly for permanent preparations. Pollen grains identification was performed by optical microscope with total magnification (400X and 1000X).

A reference collection of Pisa University and different pollen morphology guides were used for the recognition of the pollen types.

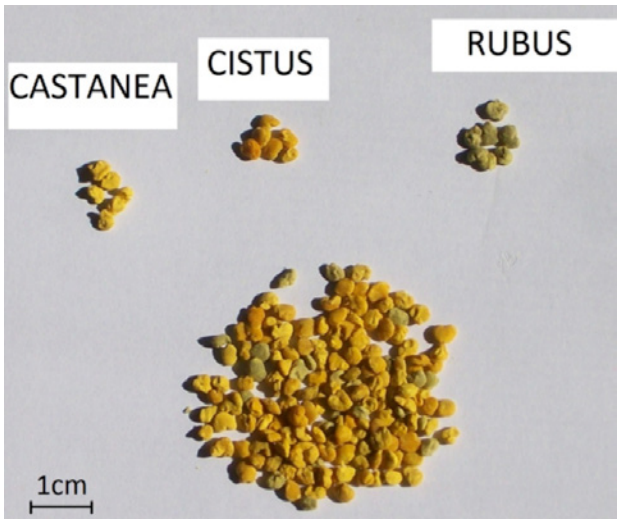


Fig. 1 - Picture of the original blended bee-pollen sample and *Castanea* sp. (yellow), *Rubus* sp. (green) and *Cistus* sp. (ochre) pollen.

2.4 Chemical composition

Dry matter, proteins, lipids and ash quantification were performed for the pollen-load samples according to AOAC guidelines (AOAC, 2000) and values were expressed as percentage on fresh matter basis. Dry matter content was made through gravimetry until constant weight, using oven at 105°C. Protein content was obtained using Kjeldahl method, while lipid content was determined by Soxhlet extractor using diethyl ether as solvent. Ash content was determined by gravimetry until constant weight, using oven at 550°C for one day. Moisture content was obtained by subtracting the dry matter from 100, while total carbohydrates content was determined according to the following formula: Carbohydrates (g) = 100 - [protein (g) + fat (g) + moisture (g) + ash (g)] (Ketkar, Rathore, Lohidasan, Rao, Paradkar, and Mahadik, 2014).

2.5 Bee-pollen extraction and phytochemical characterization

Pollen-loads were separated in three color groups and the mixed pollen sample was made blending the three pollen type in equal part. Bee-pollen extracts were obtained after 1 hour incubation at room temperature in 95% ethanol while being shaken gently. Then samples were centrifuged 10 minutes at 3500 rpm at 4°C and the supernatants were collected and kept in the dark at 4°C.

Total polyphenols were determined by the Folin-Ciocalteu colorimetric method (SINGLETON *et al.*,1999). Briefly, 100 µL of each natural extract were mixed with 500 µL of 0.2 N Folin-Ciocalteu reagent and incubated in the dark for 5 minutes. Then, 400 µL of 0.7 M sodium carbonate (Na2CO3) were added. The absorbance was measured at 760 nm, after 2 hours incubation at room temperature in the dark. Five serial dilutions of gallic acid (0.009, 0,017, 0,043, 0,085, 0.17 mg/mL) were used to obtain the standard calibration curve with the following equation: conc=0.079Abs-0.004 (R^2=0.996). Total polyphenols were expressed as mg of gallic acid equivalents (GAE)/g dry weight (dw).

The aluminum chloride colorimetric method was used for the total flavonoids determination (Kim D.O., Chun, Kim Y.J., Moon, and Lee, 2003). Briefly, 200 µL of extracts were mixed with 800 µL of dH_2O and 60 µL of 5% $NaNO_2$, followed by incubation of 5 minutes at room temperature. Then 60 µL of 10% $AlCl_3$ were added, incubated for 6 min and finally reactions were neutralized with 400 µL of 1M NaOH. Absorb-

ance was measured at 430 nm after 30 minutes of incubation and catechin was used to make the calibration standard curve (0.016, 0.032, 0.063, 0.125, 0.25, 0.5 mg/mL). Flavonoids concentration was obtained from the following calibration curve: conc=0.464Abs-0.005 (R^2=0.998) and expressed as mg catechin equivalent (CE)/g dw.

Flavonols content was measured according to the method described by Romani, Mancini, Tatti and Vincieri (1996). Briefly, 25 μL of extracts were mixed with 225 μL of 10% EtOH, 250 μL of 0.1% HCl in 95% EtOH and 1 mL of 2% HCl. After incubation at room temperature for 30 minutes, the absorbance of the reaction mixture was measured at 360 nm and quercetin was used to make the calibration standard curve (0.0006, 0.00125, 0.0025, 0,0050, 0.0075, 0.01, 0.0125, 0.015 mg/mL). Flavonols content, derived from the calibration curve equation: conc=0.536Abs-0.0001 (R^2=0.997), was expressed as mg quercetin equivalent (QE)/g dw,

Total monomeric anthocyanins were determined according to the pH differential method described by Lee, Durst, and Wrolstad (2005), a spectrophotometric method based on the change in pigmentation pH-dependent of anthocyanins. Absorbance was measured at 520 and 700 nm and anthocyanin concentration was expressed as mg cyanidin-3-glucoside equivalents (C3GE)/g dw (cyd-glu, molar extinction coefficient of 26,900 L cm^{-1} mol^{-1} and molecular weight of 449.2 g mol^{-1}).

2.6 Front-Face Fluorescence spectroscopy analysis

Each pollen sample was studied previous in its bulk state by FF fluorescence spectroscopy, then their ethanolic extracts were studied by UV-Vis absorption and FF fluorescence spectroscopies. The precipitate obtained after the extraction was also studied by FF fluorescence spectroscopy. To recognize the main classes of fluorescence compounds in pollen loads, standard solutions of the reagents reported in paragraph 2.1 were studied by Uv-Vis Absorbance and FF fluorescence spectroscopy.

For bulk analysis pollen loads were finely powdered with a pestle in a mortar. A little amount of powder was put between two quartz windows of 1 mm optical path with the help of few water drops to homogenized the sample (about 1:1 mg/ml sample: water). These quartz windows are held against a support in the spectrofluorometer by a laminar spring. For extraction procedure 2 mL of ethanol were added to 100 mg of pollen loads finely powdered. They were stirred for 30 minutes with a magnetic stir bar and then filtered through filter paper 0.45 mm pore size (Sartorious), to obtain a clear solution. To do it, Spectrum S-25-10 stirred cell device for ultra-filtration was used. A quartz cell with 2 mm optical path was used to record absorbance spectra and low capacity quartz cells with 5 mm optical path was used to record FF fluorescence spectra. The residual material was kindly removed from the filter paper and it was put between two quartz windows of 1 mm optical path. Fluorescence spectra were recorded using a ISA Fluoromax II photon counting spectrofluorometer, with Xenon arc lamp and a device for front-face measurements with a cell holder designed to set the incident angle of the excitation beam at 31°, eliminating or reducing self-absorption effects, light-reflected and scattering (ZANDOMENEGHI *et al.*, 2005). The excitation and the emission slits were 2 and 5 nm, respectively. The integration constant time was 0.5 s and the wavelength increment was 1 nm. The intensity of the spectra was determined as the ratio between the emisson signal (counts per second, cps) and the intensity of light from the excitation monochromator (mA), measured by means of a photomultiplier and a photodiode, respectively. For each sample emission spectra (280 nm < lambda_ex < 550 nm, with a step of 10 nm), excitation spectra (at the wavelength of the maximum position of fluorescence emission spectra, l_{em}) and synchronous spectra (20 nm < Deltalambda < 120 nm, with a step of 10 nm) were recorded. For bulk analysis emission spectra with l_{ex}=650 nm were recorded. Absorbance spectra were recorded with a Jasco V-550 spectrophotomer, between 200 nm and 750 nm with scanning speed of 400 nm/s, band width and data pitch of 1 nm and 0.5 nm, respectively.

2.7 Antioxidant activity

2.7.1 DPPH radical scavenging assay

The free-radical scavenging activity of ethanolic bee-pollen extracts was evaluated using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay (FRASSINETTI *et al.* 2011). The reduction of DPPH radicals was recorded at 517 nm and the radical scavenging activity (RSA) was calculated as percentage of DPPH inhibition according to the following equation: % RSA = $[(A_{DPPH}-A_S)/A_{DPPH}]$ x 100, where A_{DPPH} is the absorbance of DPPH solution and A_S the absorbance of sample. The extract concentration corresponding 50% of DPPH inhibition (EC_{50}) was measured by interpolation from the graph of RSA percentage versus bee-pollen concentration (Morais *et al.*, 2011). Lower EC_{50} values indicate higher antioxidant activities.

2.7.2 Oxygen Radical Absorbance Capacity (ORAC) Assay

The antioxidant capacity of ethanolic bee-pollen extracts was quantified using the oxygen radical absorbance capacity (ORAC) assay, modifying some reagent concentration adapted to our requirements (NINFALI *et al.*, 2005).

The final reaction mixture of our assay contained 0·04 mM fluorescein sodium salt in 0.075

M phosphate buffer, pH 7.4, at diluted sample or 5 mM Trolox. The control was 0.075 M phosphate buffer, pH 7.4. AAPH was used as peroxyl radicals generator and fluorescein as probe. Fluorescein fluorescence decay was read at 485 nm excitation and 514 nm emission using a VictorTM X3 Multilabel Plate Reader (Waltham, MA) and Trolox was used as antioxidant standard. ORAC values were expressed as micromoles of Trolox equivalents (TE)/g dw.

2.8 Statistical analysis

The statistical analysis was performed using GraphPad Prism, version 5.00 for Windows (GraphPad software, San Diego, CA). Assays were carried out in triplicate and results were expressed as mean values ± standard deviation (SD). Differences between bee-pollen samples were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's post test. A *p*-value lower than 0.05 is considered as statistically significant. Interdependence between the antioxidant capacity and the phytochemical profile was evaluated by Pearson's correlation coefficient (r).

3. RESULTS

3.1 Palynological analysis

The blended bee-pollen sample resulted to be a mixture of *Castanea* sp. (yellow), *Rubus* sp. (green) and *Cistus* sp. (ochre). Each pollen load owned a homogeneous and monospecific pollen content. The *Castanea* sp. was the most representative (70%), followed by *Rubus* sp. (23%) and *Cistus* sp. (7%).

3.2 Chemical composition

The chemical composition of *Castanea*, *Cistus* and *Rubus* pollen samples is listed in Table 1 and values are expressed as percentage on fresh matter basis. The nutritional content measured is in agreement with literature values (Balkanska and Ignatova, 2012; Carpes, Mourão, Alencar, and Masson, 2009; Nogueira, Iglesias, Feás, and Estevinho, 2012). Significant variations among samples were showed, with the highest protein ($p<0.01$) and lipid ($p<0.001$) content in *Rubus* compared to all other pollens. No difference among groups was found for moisture, dry matter, ash and carbohydrates content (p=ns).

3.3 Phytochemicals profile of ethanolic pollen-load extracts

Pollen-load extracts were screened for total polyphenols, flavonoids, flavonols and monomeric anthocyanins content. Phytochemical profile of *Castanea*, *Cistus* and *Rubus* pollen-load samples is listed in Table 2 and significant differences were found ($p<0.001$). In particular, *Castanea* pollen extracts contained the highest levels of polyphenols (24.75±0.78 mg GAE/g fw), flavonoids (15.86±0.62 mg CE/g fw) and anthocyanins (77.37±2.55 mg C3GE/L), while the highest levels of flavonols (4.93±0.05 mg QE/g fw) were detected in *Cistus* pollen samples. Otherwise, *Rubus* pollen extracts showed the lowest

Table 1 - Moisture, dry matter, proteins, lipids, ash and carbohydrates of pollen-load samples.
[a],[b],[c] Different superscript letters indicate statistical differences among the bee-pollen extracts ($p<0.001$ ANOVA). Assays were carried out in triplicate and results were expressed as mean values ± SD.

	***Castanea* pollen**	***Cistus* pollen**	***Rubus* pollen**	***p***
Moisture (%)	10.75 ± 1.25	12.03 ± 1.01	11.06 ± 1.50	n.s.
Dry matter (%)	89.24 ± 2.22	87.97 ± 2.43	88.31 ± 2.07	n.s.
Proteins (%)	26.57^{a} ± 0.34	25.87^{a} ± 0.29	28.42^{b} ± 0.31	<0.001
Lipids (%)	2.07^{a} ± 0.14	1.92^{ac} ± 0.13	2.83^{b} ± 0.13	<0.001
Ash (%)	2.63 ± 0.18	2.55 ± 0.16	2.85 ± 0.15	n.s.
Carbohydrates (%)	57.98 ± 1.91	57.63 ± 1.59	54.84 ± 2.09	n.s.

Table 2 - Total polyphenols, flavonoids, flavonols and anthocyanins concentration of ethanolic pollen-loads extracts. [a,b,c] Different superscript letters indicate statistical differences among the bee-pollen extracts ($p<0.001$ ANOVA). Assays were carried out in triplicate and results were expressed as mean values ± SD.

	Castanea pollen	*Cistus* pollen	*Rubus* pollen	*p*
Total polyphenols (mg GAE/g fw)	24.75±0.78[a]	21.19±0.24[b]	13.53±0.4[c]	<0.001
Flavonoids (mg CE/g fw)	15.86±0.62[a]	14.21±0.56[b]	5.91±0.27[c]	<0.001
Flavonols (mg QE/g fw)	4.77±0.09[ac]	4.93±0.05[a]	2.52±0.14[b]	<0.001
Anthocyanins (mg C3GE/L)	77.37 ±2.55[a]	57.19±5.84[b]	53.44±2.36[b]	<0.001

content of polyphenols, flavonoids and flavonols. The discrepancies in phytochemical composition observed among the different pollen samples might depend on their botanical origin (ARRUDA *et al.*, 2013; CAMPOS *et al.*, 2008; MORAIS *et al.*, 2011).

3.4 Fluorescence spectroscopy

3.4.1 Bulk analysis

Pollen is a complex matrix and, as a consequence, the fluorescence spectra are characterized by broad and overlapped bands, caused by the presence of many fluorophores, which limit the quantification and identification of all of them. However, it is possible to get several information as well as qualitative features, which can be used as fingerprint of the investigated pollen.

The fluorescence spectra are indeed characterized by three main intervals of excitation wavelength: 280-290 nm, 320-370 nm and 420-480 nm. In Fig. 2a the emission spectra obtained with excitation wavelength (l_{ex}) of 280 nm are reported. Two bands can be recognized: the first one, less intense, is centered at 340 nm for *Rubus* and at 360 nm for *Castanea* and *Cistus* pollens. This band is probably due to the aromatic aminoacids, which can be residues of proteins or free. The second one is the dominant band and its shape is different between *Rubus* and *Castanea*/*Cistus* pollens: it is more intense in the range 420-620 nm for *Rubus* and red shifted of about 40 nm for *Castanea* and *Cistus* pollens. This emission band is probably due to hydroxycinnamic acids, compounds belonging to polyphenols family, fluorescent water soluble vitamins such as B6, B9 and B2, and flavoinoid compounds. All these classes of compounds have two bands of absorption (three in the case of B2 vitamin), the first one centered in the 280-290 nm interval. This hypothesis is supported by the emission spectra obtained at highest l_{ex} 320-370 nm, where the above mentioned substances are excited in their second absorption band; these pollens' bands are indeed less intense but retain the same shape of previous broad band. It is interesting to observe that *Castanea* and *Cistus*'s spectra are again much different from *Rubus* (Fig. 2b), probably due to the presence of characteristic fluorophores in the latter pollen type.

For all pollens, fluorescence intensity decreases until λ_{ex}=400 nm; thereafter, it slightly increases again, to define a new band centered at 530 nm for *Cistus* and *Castanea* and at 510 nm for *Rubus* (λ_{ex}=450 nm). At this wavelength, B2 vitamin has its third absorption band, moreover, other fluorophores are excited, such as xanthophylls and carotenoids derivatives, although they are very weak emitter (JØRGENSEN *et al.*, 1992). Changing the λ_{ex} to 550 nm, only *Cistus* pollen presents a weak fluorescence at about 600 nm, probably caused by the presence of other polar carotenoids derivatives. At higher excitation wavelength, collected to investigate the eventual presence of chlorophyll derivatives (λ_{ex}=650 nm), no relevant fluorescence is recorded.

The synchronous spectra obtained with 60 nm offset (Fig. 2c) underlines the presence of three different bands of absorption and they are useful to compare the pollen types. The fluorescence profile of *Rubus* pollen is much different than that of *Castanea*/*Cistus*' one, especially in the second absorption bands (IIR and IIC respectively). In this spectrum, also the third band differs between *Rubus* and *Castanea*/*Cistus*, centered

at 440 nm (IIIR) and 480 nm (IIIC), respectively. Moreover, the *Cistus* pollen is the only one presenting an absorption centered at 550 nm (IV). All above data are consistent with the emission spectra. Vitamins C and B_3 did not show relevant fluorescence so they resulted undetectable.

3.4.2 Extracts analysis

In order to facilitate the spectral interpretation, an extraction procedure to separate the water soluble fraction from the lipid soluble one was adopted. The solvent used are not strong and the treatment of samples is such to limit the alteration of the matrix, and to use the potentiality of a rapid and direct spectroscopic technique. In the present work ethanolic extracts were studied.

The most interesting region of the UV-Vis absorption spectra is in the visible range (400-500 nm). Here, only *Cistus* extract presents an absorption spectrum with the typical shape of carotenoid or xanthophyll pigments, as shown in Fig. 3a.

Considering fluorescence, the emission spectra at λ_{ex}=280 nm (Fig. 3b) are characterized by a broad band, extended to the interval 340-520 nm. *Castanea* and *Rubus* pollen show maximum emission at about 420-430 nm, while the *Cistus* one shows a different profile with an emission band centered at about 400-410 nm and a shoulder at 520 nm. In this region, the fluorescence is probably due to the hydroxycinnamic acid, that at this wavelength begin to absorb. The polyphenols belonging to the hydroxybenzoic family (e.g. gallic acid, 3,4-dihydroxybenzoic acid, 4-hydroxybenzoic acid, vanillic acid) are not the mainly fluorophores. In fact, they have their absorption and emission maxima included in the interval 280-297 nm and 325-370 nm, respectively.

In Fig. 3b emission spectra of two representative compounds of the hydroxybenzoic's family (gallic and 4-hydroxybenzoic acids) compared with pollen's spectra are reported. The hypothesis of the presence of hydroxycinnamic acid is supported by the emission spectra recorded with highest λ_{ex} (310-340 nm), characterized by the same shape, but higher intensity. In fact, polyphenols belonging to hydroxycinnamic family (e.g. caffeic acid, ferulic acid, p-cumaric acid) have their absorption and emission maxima included in the interval 310-350 nm and 410-440 nm, respectively. Emission spectra of two representative compounds of this group (caffeic and p–coumaric acid) recorded with λ_{ex}=310 nm are reported in Fig. 3c and compared with pollen's spectra. *Castanea* pollen spectrum differs from the two others for the higher intensity.

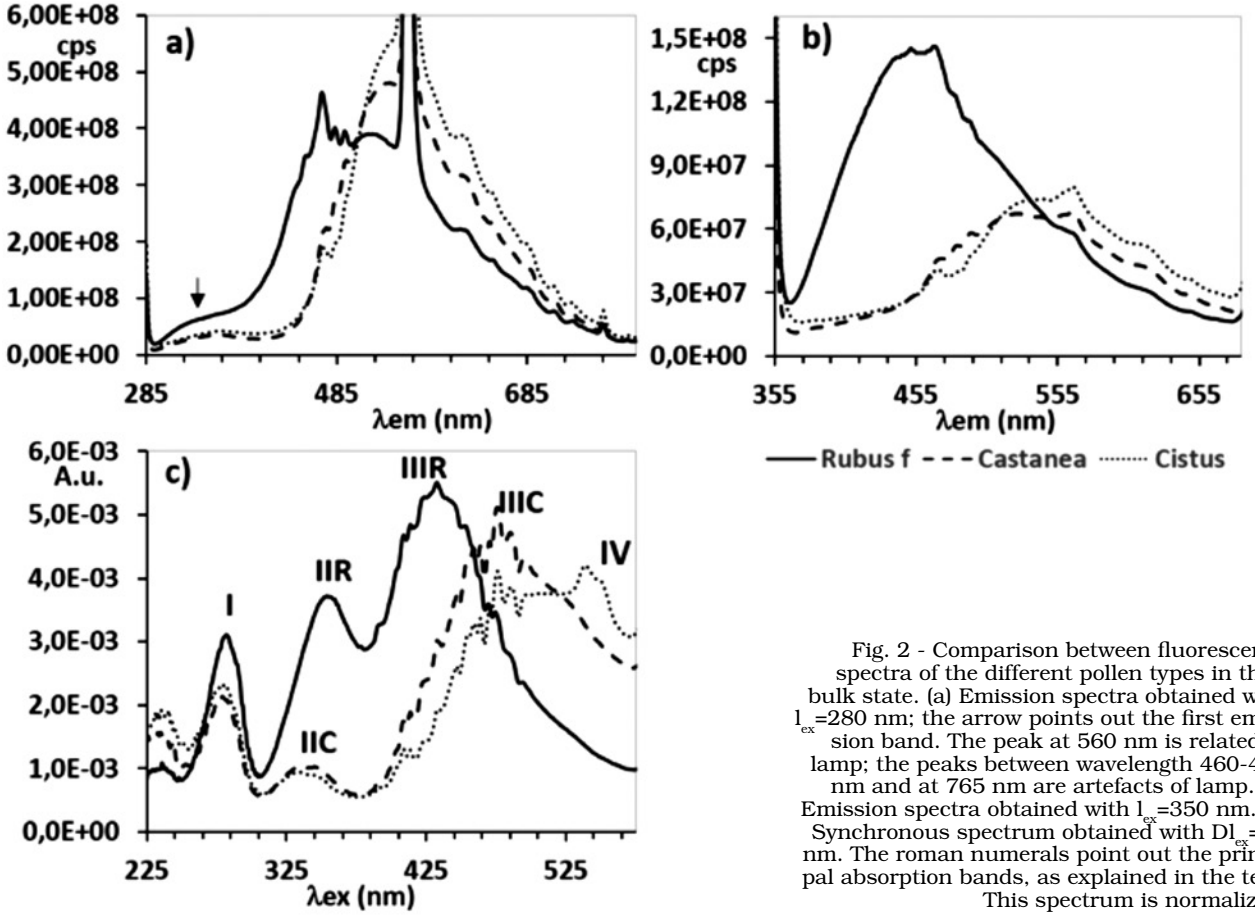


Fig. 2 - Comparison between fluorescence spectra of the different pollen types in their bulk state. (a) Emission spectra obtained with l_{ex}=280 nm; the arrow points out the first emission band. The peak at 560 nm is related to lamp; the peaks between wavelength 460-490 nm and at 765 nm are artefacts of lamp. (b) Emission spectra obtained with l_{ex}=350 nm. (c) Synchronous spectrum obtained with Dl_{ex}=60 nm. The roman numerals point out the principal absorption bands, as explained in the text. This spectrum is normalized.

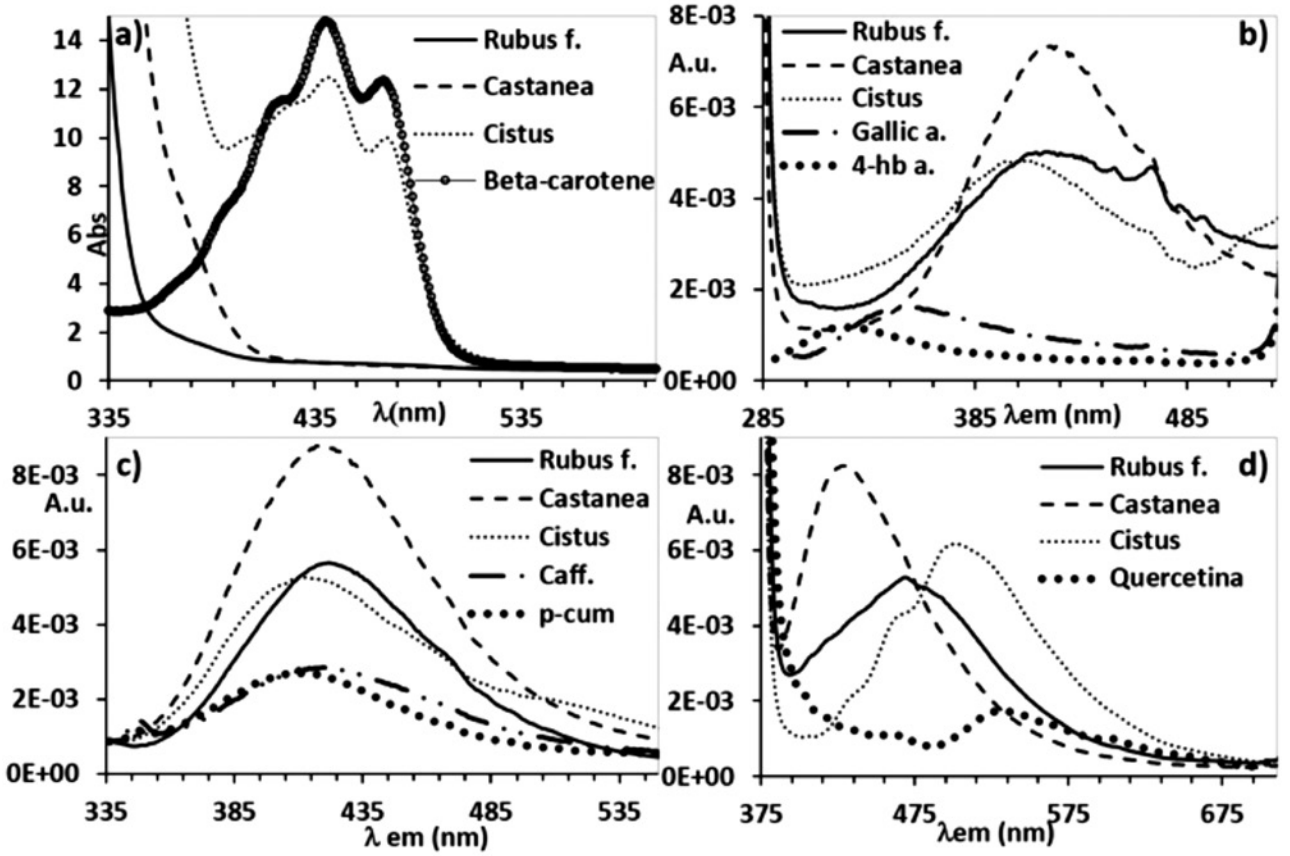


Fig. 3 - Spectra of ethanolic extracts of the different pollen types. (a) Uv-Vis absorpotion spectra (optical path=1 cm) of pollens compare to a b-carotene solution (3.2 mg/ml). Series of emission spectra of pollens compare to: (b) solutions of gallic acid and 4-hydroxybenzoic acid (0.01 mg/ml 90% H_2O-10% CH_3OH) (l_{ex}=280 nm); (c) solutions of caffeic acid and p-coumaric acid (0.01 mg/ml 90% H_2O-10% CH_3OH) (l_{ex}=310 nm); (d) solution of quercetin (20 mg/ml in CH_3OH) (l_{ex}=370 nm). All the fluorescence spectra are normalized. Caffeic and p-cumaric' spectra intensity are multiplied by 2.4.

Considering the excitation spectra recorded with the emission wavelength fixed in the maximum of emission (430 nm for *Castanea*/*Rubus* and 410 nm for *Cistus*) it is clear that their fluorescence is due to different fluorophores. In particular, they are a single fluorophore, with an absorption band centered at 330 nm for *Rubus*, a single fluorophore, with two absorption bands centered at 285 and 330 nm for *Castanea*, and two different fluorophores, centered at 290 and 325 nm excitation wavelength for *Cistus*. This latter hypothesis is confirmed by synchronous spectra obtained with offset 100 nm, in which it is possible to recognize two different contributions with absorption at 295 and 320 nm, respectively.

Changing the λ_{ex} to 350 nm (Fig. 3d), the fluorophores involved are others, thus confirming the complexity of the pollen matrix.

Castanea and *Rubus* samples present the same profile, while *Cistus*' spectrum shows a particular shape characterized by three shoulders, most likely due to three different fluorophores, confirmed by excitation spectra recorded with λ_{em}=460nm. These fluorophores are probably other hydroxycinnamic compounds (in the cases of emission at 430 nm and 460 nm) and flavonoids compounds (in the case of emission at 500-520 nm). For example, the flavonol quercetin has a weak emission, but the shape of its fluorescence spectrum is compatible with that of *Cistus* pollen, as reported in Fig. 3d.

The richness in fluorescent compounds of *Cistus* pollen is confirmed by the synchronous spectrum obtained with offset 120 nm, where it is possible to recognize four bands of absorption, centered at 295-320 nm, 350 nm, 370 nm and 400 nm. In the case of the first broad band it is possible to discriminate the contribution of two fluorophores with offset 100 nm, like previously discussed.

With higher excitation wavelength, collected to check the presence of carotenoids or xanthophyll with front-face fluorescent of liquid extract, no relevant fluorescence was recorded. It is important to underline that there are several xanthophylls and carotenoids derivatives, and only some of them have weak emission, as previous discussed in section "Bulk analysis".

Finally is interesting to analyze the fluorescence spectra obtained for solid samples. For example in Fig. 4 the comparison between emission spectra (λ_{ex}= 280 nm) of *Rubus* in its bulk

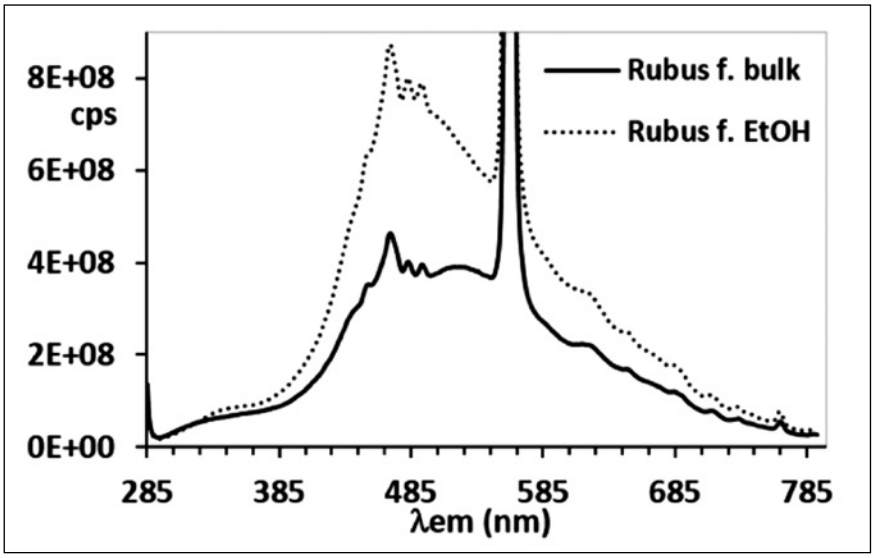


Fig. 4 - Emission spectra obtained with λ_{ex}=280 nm for solid *Rubus* samples differently processed. The dotted line shows the precipitate after ethanol extraction (spectrum intensity is divided by 1.7). The peak at 560 nm is related to lamp; the peaks between wavelength 460-490 nm and at 765 nm are artefacts of lamp.

state with the precipitate after ethanol extraction (dotted line) is reported. The first band due to water soluble aminoacids and proteins, not extracted in ethanol, is present into both spectra. Instead the shape of main broad emission band centered at 500 nm, differs from each other. This can be due to the lack of the classes of compounds extracted with the solvent: hydroxycinnamic acid and flavonol compounds. It is important to remember that fluorescence sensitive changes in relation of the physical state of compounds and their environment (LAKOWICZ, 2006). Although the broad emission band, these results are coherent with extracts' spectra.

3.5 DPPH radical-scavenging activity

The DPPH assay has been widely used to test the free radical scavenging activity of apicultural products, either honey, propolis or bee-pollen. As reported in literature the radical scavenging activity of bee-pollen is very dissimilar among different flower species with a value range of 0-97% that also depends on their chemical composition and solvent extraction (BASUNY *et al.*,2013; LEBLANK *et al.*, 2009; SILVA *et al.*, 2006). We evaluated both percentage of DPPH radical scavenging activity (% RSA) and EC50, the extract concentration providing 50% DPPH inhibition. As shown in Table 3, ethanolic bee-pollen extracts exhibited high free radical scavenging activity with DPPH inhibition values ranged from 37.95±0.19% (EC_{50} = 641.3±11.4 μg/mL) of *Rubus* extracts and 94.45±0.01% (EC_{50} = 215.2±2.7 μg/mL) of *Castanea* extracts.

ANOVA with Tukey post-test showed significant differences among samples with the highest and lowest activity detected in *Castanea* and *Rubus* extracts. As well as radical quenching capacity, *Castanea*, *Cistus* and mixed bee-pollen

Table 3 - ORAC and DPPH assay results of ethanolic pollen-load extracts expressed as μmol TE/g fw and % RSA and EC_{50} (μg/mL), respectively.
[a,b,c] Different superscript letters indicate statistical differences among the bee-pollen extracts (p<0.001 ANOVA). Assays were carried out in triplicate and results were expressed as mean values ± SD.

	Castanea pollen	*Cistus* pollen	*Rubus* pollen	Mixed pollen	*P*
ORAC (μmol TE/g fw)	544.01±4.75[a]	540.01±17.2[a]	519.45±15.07[a]	677.70±12.92[b]	<0.001
% RSA	94.45±0.01[a]	94.19±0.03[b]	37.95±0.19[c]	94.07±0.03[b]	<0.001
EC_{50} (μg/ml)	215.2±2.7[a]	224±12.3[ac]	641.3±11.4[b]	249.3±6.1[c]	<0.001

possessed similar EC_{50} values, three times lower than *Rubus* extracts, suggesting a lower radical scavenging activity of *Rubus* bee-pollen as antioxidant.

Our results also revealed a strong relation between the antiradical activity and the total phenolics, flavonoids and flavonols content, resulting in a significant positive correlation (r=0.9645, r=0.9888, and r=0.9847, respectively). Lastly, a moderate correlation was obtained between the anthocyanins and the DPPH scavenging activity (r=0.5541).

3.6 Oxygen Radical Absorbance Capacity (ORAC)

The antioxidant capacity of ethanolic bee-pollen extracts was also screened using ORAC assay and expressed as ORAC units (μmol Trolox equivalents/g fw). The ORAC values were listed in Table 3 and ranged from 519.45±15.07 μmol TE/g of *Rubus* fraction and 677.70±12.92 μmol TE/g of mixed bee-pollen. One-way analysis of variance with Tukey's post test showed a significant increase of ORAC values in mixed bee-pollen extracts respect to each one separate fractions ($p<0.001$), suggesting a synergic or additive effect among *Castanea*, *Cistus* and *Rubus* antioxidant compounds. In fact, besides to their specific effects, many antioxidants can interact in synergistic ways, maybe protecting another against oxidative degradation, exhibiting greater antioxidant effects (MĂRGHITAŞ *et al.*, 2009). The results showed no strong correlation between the ORAC values and the anthocyanin (r=0.1524) and flavonoid (r=0.4586) compounds; however, a moderate interdependence was obtained between the antioxidant capacity and the polyphenols (r=0.6638) and flavonols (r=0.5572) content.

4. CONCLUSIONS

In this study, an organic bee-pollen sample from Tuscany was analysed for the first time investigating the botanical origin, the chemical composition, the phytochemicals profile and the antioxidant activity. Different techniques were used. In particular, we propose an original application of FF fluorescence spectroscopy, a promising approach to put in evidence differences and analogies among pollens with different floral origin. This unconventional technique presents the advantage to require no particular sample pre-treatment; moreover, it is economic, fast and easy to use and it could be useful for both further scientific researches and commercial applications. The FF fluorescence results are coherent and uphold the spectrophotometric data obtained in this investigation. Specifically, the differences among *Castanea*/*Cistus* and *Rubus* fluorescence profiles, arisen from bulk study, are comparable and in agreement with the significant differences found in lipids and proteins composition. Furthermore, the ethanolic extracts fluorescence analysis, as well spectrophotometric results, displays a higher content of flavonols and polyphenols in *Cistus* and *Castanea*, respectively. Moreover, the FF fluorescence analysis shows the greater presence of hydroxycinnamic acids than hydroxybenzoic acids, in agreement with literature data (FANALI *et al.*, 2013; KETKAR *et al.*, 2014). Lastly, UV-Vis ethanolic extracts' spectra reveal the presence of carotenoid or xanthophyll pigments only in *Cistus*, confirmed also by bulk state's emission and synchronous spectra λ_{ex}=550 nm and offset 60 nm, respectively).

Besides nutritional and phytochemical composition, the antioxidant and free radical scavenging activity of Tuscan bee-pollen and its monofloral groups was measured. The redox properties of phenolic compounds, especially flavonoid components, play a key role in decomposing peroxides and quenching oxygen, as well as in absorbing and neutralizing free radicals (MĂRGHITAŞ *et al.*, 2009). Moreover, specific bioactive compounds or a combination of them can exert a different antioxidant activity, strongly dependent on structure and polyphenols composition, rather that the phytochemical concentration (MĂRGHITAŞ *et al.*, 2009).

In particular, we showed that mixed bee-pollen exhibit a much better antioxidant activity than the separate fractions with an ORAC value significantly greater than other samples; whereas, *Castanea, Cistus* and mixed bee-pollen showed a comparable DPPH radical scavenging activity greater than *Rubus* antiradical capacity. These results are in agreement with literature data that strongly associate the high ability to neutralize reactive oxygen species to the phenolic compounds structure, mainly flavonoids and cinnamic acid derivatives (LEJA *et al.*, 2007), maybe more representative in *Cistus* and *Castanea* pollen. Furthermore, according to MĂRGHITAŞ *et al.* (2009) the antioxidant effects of bioactive compounds change differently depending on the antioxidant method used. Therefore, we suppose a synergic or additive effect among *Castanea, Cistus* and *Rubus* antioxidant compounds or resulting from new antioxidant substances with greater antioxidant activity.

In conclusion, the use of spectroscopic techniques applied to bee-pollen samples is a suitable tool to underline differences and analogies in their micronutrient composition. The data obtained with different or complementary techniques are coherent and in agreement with literature concerning the variability in chemical composition and antioxidant activity of pollens from different floral sources (LEBLANC *et al.*, 2009; PASCOAL *et al.*, 2014).

Further investigations should be performed to identify and quantify the main fluorescence

compounds present, as well as to investigate on the nature of *Cistus'* pigments. Moreover, future analysis is required to separate and identify the specific profile of antioxidant compounds in mixed pollen sample in order to elucidate, strengthen and confirm the hypothesis of the antioxidant compound's synergic or additive effect or the presence of new antioxidant compounds.

Finally, the results obtained show for the first time the high nutritional value and the good antioxidant activity of Tuscan bee-pollen, which make it an excellent food supplement and a good candidate for a potential nutraceutical product that could be useful in the prevention of free radical associated diseases. As suggested by findings in a previous paper (LUCCHESI *et al.*, 2014), cellular studies could be useful to investigate the intracellular pathways involved in the bee-pollen antioxidant response.

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PAPER

COMPARATIVE STUDY OF OXIDATION IN CANNED FOODS WITH A COMBINATION OF VEGETABLES AND COVERING OILS

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ABSTRACT

The effects of sunflower (SFO), extra-virgin olive (EVO), and soybean oils (SBO), in combination with canned aubergins and dried tomatoes were studied during an accelerated shelf-life trial. Hydrolytic and oxidative quality parameters was determined and a sensorial test was run. For both canned vegetables, the SBO showed greater resistance to the oxidation at the end of the shelf-life trial. The SBO in both vegetables yielded similar results for peroxide formation, whereas a reduced formation of secondary oxidation products was observed in aubergins. The results highlighted a higher oxidation stability of canned vegetables in SBO and EVO than those in SFO. The sensorial test underlined differences between the oils, in aubergins and dried tomatoes, after 30 days of accelerated storage (corresponding to the sell-by date). Flavour and texture were judged better for vegetables in SBO.

- Keywords: canned vegetables, extra-virgin olive oil, oxidation, preservation, soybean oil, sunflower oil -

1. INTRODUCTION

Canned foods are products packed and hermetically sealed in metal (tin, aluminum), glass, or polymer containers that are thermally treated to destroy spoiling microorganisms and their enzymes with the aim to prolong the shelf-life. (LEISTNER, 1992). Moreover, the process of preparation and preservation could increase the quality of vegetable products because of the presence of added oils as cover liquids (LEISTNER, 1992). In canned vegetables: i) the covering oil promotes anaerobic conditions (i.e., less than 2% oxygen); ii) an adequate blanching treatment reduces numbers of contaminating microorganisms, inactivates enzymes, modifies texture, preserves color, flavor, and nutritional value, and removes trapped air; and iii) pasteurization guarantees commercial sterility (REYES DE CORCUERA *et al.*, 2004; BAIANO *et al.*, 2005a). In the food industry, virgin and extra-virgin olive oil, olive oil, seed oils, or various oil blends, are used as covering oils to prepare preserved vegetables. The quality of canned vegetables in-oil depends on the complex interactions between traits of the vegetables and those of the covering oils. During storage, many bioactive molecules migrate from the vegetable to the oil and vice versa, in a dynamic equilibrium that depends on the characteristics (e.g., chemical composition, structure, size, and shape) of vegetables and oils as well as on the technology used for preparation and storage (LUCCHETTI *et al.*, 2011). Spices and aromatic herbs, generally used as flavor enhancers in preparation of canned vegetables, also contribute to the quality of the canned product. They contain substances with documented antimicrobial, antioxidant and anti-inflammatory activities (GAMBACORTA *et al.*, 2007).

The shelf-life of in-oil canned vegetables depends on the quality of the vegetable and the covering oil and its composition (BAIANO *et al.*, 2005a). During processing and over the time, vegetables and oils undergo modifications because of mechanical and thermal degradation and hydrolytic and oxidative degradation (affecting the quality of oil and preserved food) (DE GIORGI *et al.*, 2000; BAIANO *et al.*, 2005b). Some studies have examined the oxidative and hydrolytic reactions that occur in the covering oil, and on the effects of the use of different oils as a covering medium on the canned vegetables preservation (LEISTNER, 1992; BAIANO *et al.*, 2005a; BAIANO *et al.*, 2005b; BAIANO *et al.*, 2005c).

The aim of the present paper was to compare the effects of the combination of different vegetables and covering oils in canned foods. Sunflower oil (SFO), extra-virgin olive (EVO) and soybean oil (SBO) in combination with canned aubergins and dried tomatoes were studied. The quality of covering oils was studied to verify the effect of these combinations during storage. Hydrolytic and oxidative quality parameters were measured together with sensory analysis. SFO was examined because of its wide use in the production of canned foods, EVO was examined because of its sensorial and nutritional properties, and SBO was examined for its nutritional value, widespread use, and low cost.

Moreover, the effects of aromatic herbs and spices (ingredients included in the recipes for canned vegetables) on oxidative degradation of the three oils were examined as a separate test on pure oils. The herbs and spices in the recipe were: i) garlic, rich in flavonoids and sulfur-containing compounds (LEELARUNGRAYUB *et al.*, 2006); ii) chili pepper, containing allicin, carotenoids, ascorbic acid, and phenolic compounds (SUHAJ, 2006); iii) oregano, containing various flavonoids (KYOJI *et al.* 2006); and iv) mint, rich in polyphenols and flavonoids (KANATT *et al.*, 2007; PADMINI *et al.*, 2008).

2. MATERIALS AND METHODS

Both commercial in-oil canned aubergins and dried tomatoes were prepared according to traditional recipes with three different vegetable oils (SFO, EVO, and SBO). The oil samples were provided by Vizzino "Orto Buono," Minervino di Lecce, Italy. All canned products were produced on the same day and analysed, at time zero (T0) and 15 days after processing (T1).

2.1. Preparation of in-oil canned vegetables

The canned vegetables used were aubergins in SFO (AUSFO), EVO (AUEVO), or SBO (AUSBO), and dried tomatoes in SFO (DTSFO), EVO (DTEVO), or SBO (DTSBO). Traditional recipes included the addition of chili pepper (0.001 g kg^{-1} of total product), oregano (0.001 g kg^{-1}), mint (0.001 g kg^{-1}), and garlic (0.004 g kg^{-1}) for canned aubergins and mint (0.001 g kg^{-1}) and garlic (0.004 g kg^{-1}) for canned sun-dried tomatoes. A batch of 150 kg of 1 cm slices of peeled aubergins were previously treated with coarse salt (20% w/w), then drained and washed with water and centrifuged. Then the slices of aubergins dehydrated were mixed with powdered herbs and spice (chili pepper, garlic, oregano, and mint) and a dose of 280g was put in transparent glass vessels, wrapped with metal caps. Vessels were then filled in the three considered oils (vegetables 65%, oil 33%, w/w) and hermetically sealed. A batch of 150 kg of dried tomatoes were blanched in boiling white vinegar for 30 seconds, then drained and dried, mixed with aromatic herbs (garlic and mint). Transparent glass vessels were filled with 280g of tomatoes and in the three considered oils (vegetables 65%, oil 33%, w/w), and hermetically sealed. The canned samples were pasteurized at 90°C for 40 minutes, and then quickly cooled to room temperature.

2.2. Aromatic herbs and spices added to oils

A case study (separate test on pure oils with herbs and spices) was conducted to investigate the efficacy as antioxidant, and to verify the protective effects of aromatic herbs and spices present as ingredients in the traditional recipes for considered canned foods. For this purpose SFO, EVO, and SBO, used in canned food preparation, were prepared with the different aromatic herbs and spice used in the recipe: chili pepper, garlic, oregano and mint (to investigate the effect of each single herb or spice); a mixture of mint and garlic (to simulate the dried tomatoes recipe); and a mixture of all the aromatic herbs and spice (to simulate the aubergins recipe). The spice and aromatic herb contents were chosen to simulate the same concentrations used in the recipes for canned vegetables in oils. The samples to analyze were: i) SFO, EVO, or SBO, with added garlic (0.1 g/100mL of oil); ii) SFO, EVO, or SBO with added chili pepper (0.03 g/100mL); iii) SFO, EVO, or SBO with added oregano (0.03 g/100mL); iv) SFO, EVO, or SBO with added mint (0.03 g/100mL); v) SFO, EVO, or SBO with added mint and garlic (0.03 g/100mL and 0.1 g/100mL, respectively); and SFO, EVO, or SBO with all aromatic herbs and spice under consideration added.

The samples were subjected to the same Accelerated Shelf-Life Test (ASLT) for canned vegetables and the extent of the hydrolytic and oxidative degradation of oils was determined by assessing peroxide numbers and *p*-anisidine values.

2.3. Accelerated Shelf-Life Test (ASLT)

To evaluate the shelf-life of the canned vegetables and of the pure oils with added herbs and spices, an accelerated shelf life test (ASLT), in which the storing of canned foods for 1 day at 55 °C corresponds to 18 days at room temperature (20°C), was performed (ROBERTSON, 1993; KILCAST, 2000; MAN, 2015). Accelerated aging was defined according to a common industrial method based on the Arrhenius equation, which defines the relationship between product shelf-life and the temperature (ROBERTSON, 1999; GIMÉNEZ *et al.*, 2012; MARCONI *et al.*, 2014;). The glass vessels with the vegetables canned in different covering oils were kept in a laboratory oven (Thermo Fisher Scientific, Milan, Italy) at 55°C for 10, 20, 30, and 40 days, which corresponds to 6 (T1), 12 (T2), 18 (T3) and 24 (T4) months at room temperature, respectively. The vegetable oils were then subjected to chemical and sensorial analysis.

2.4. Chemical analyses

The quality of the crude oils and the ongoing hydrolysis and oxidation of the covering oils were monitored by measurements of the acidity, expressed as g of oleic acid per 100 g of oil; peroxide values, expressed as milliequivalents (mEq) of active oxygen per kg of oil (E. U, 1348/2013), *p*-anisidine values (AOCS, 1998). The hydrolytic and oxidative parameters were determined by conventional methods of analysis. After separation from the vegetable matrices, the covering oil samples were filtered on anhydrous sodium sulfate and analysed after different storage times.

2.5. Sensory evaluation

Sensorial test was conducted to evaluate the palatability of the canned vegetables preserved in different oils at T0 and T3 (at the sell by date of canned food). Nine trained panelists (six women and three men), 30–50 years, evaluated the canned vegetables using the quantitative descriptive analysis technique.

The panelists were trained in 10 sessions, using standards and similar food products, to identify and determine descriptors relating to appearance, taste, and texture. The terms and the corresponding definitions (Table 1) were available to the panelists during all sessions. The evaluation of the canned vegetables was performed over two days in two sessions. Canned vegetables were served at room temperature. Crackers were used as a carrier for tasting. Different canned vegetables, in the different covering oils, were evaluated for sensory attributes, which included appearance (color), flavor (rancid, salty, and bitter), and texture (hardness and chewiness). A 10-point

Table 1 - Definition of physical and flavor descriptors used in the quantitative descriptive analysis.

Descriptor	Definition
Physical	
Color	Intensity of vegetable color.
Hardness	By steadily compressing the vegetable between the molars, the force required for compression.
Chewiness	The lenght of the time required to masticate the vegetable to a state of swallowing.
Flavour	
Rancidness	Unpleasant, stale smell or taste proper of oils and fats.
Saltiness	Taste of salt perceptible on the tip of the tongue and on the sides around it.
Bitterness	Harsh, disagreeably acrid taste.

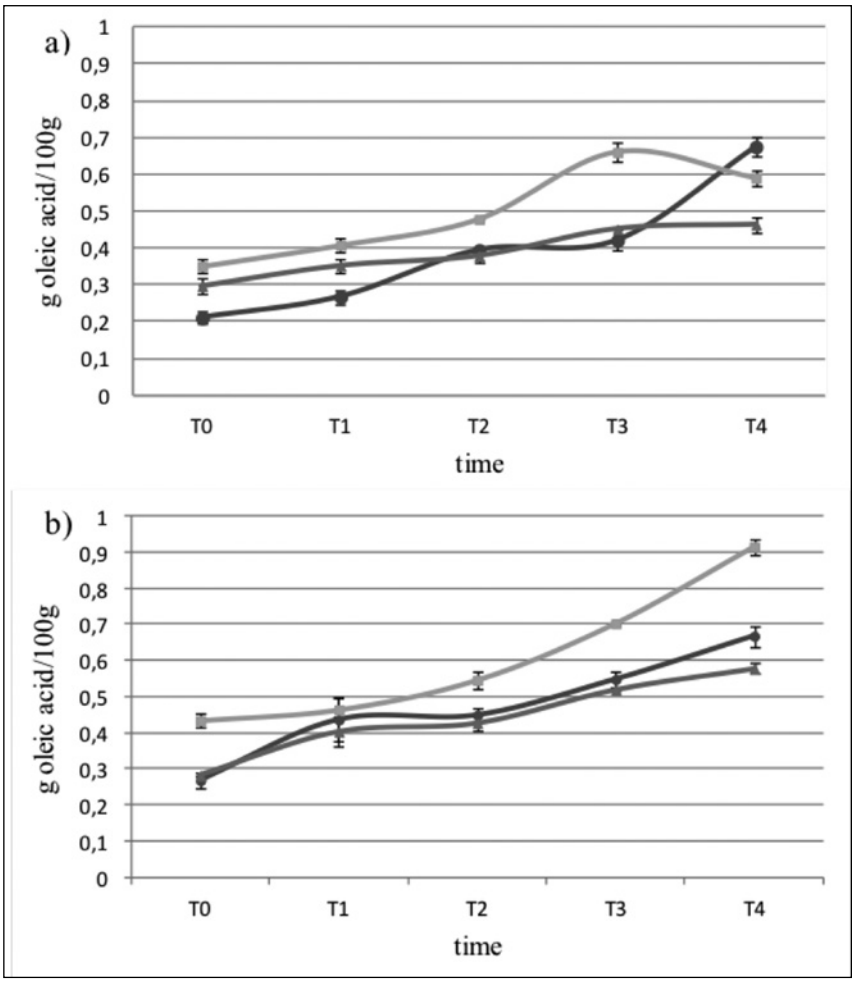


Fig. 1 - Free acidity of covering oils in canned aubergins (a) and in canned dried tomatoes (b) as a function of storage.
n = 6; bars in the figures represent standard deviation values; EVO = extra-virgin olive oil.
T0: 0 days; T1: 10 days; T2: 20 days; T3: 30 days; T4: 40 days

scale (from 0 to 9) was used where color (0 = extremely light to 9 = extremely dark); hardness (0 = extremely soft to 9 = extremely hard); chewiness (0 = none to 9 = extremely gummy); and rancidity, saltiness, and bitterness (0 = none to 9 = extremely strong) were assessed.

2.6. Statistical analysis

All data were analysed using SigmaStat (version 11, Jandel Scientific, San Rafael, CA) software to perform the appropriate statistical tests. Comparisons of the different vegetable and oil samples were made using one-way repeated measures analysis of variance, and the results obtained were further analysed using the Holm-Sidak test.

Each canned vegetable sample was produced in duplicate and all chemical analyses were performed in triplicate. Values were considered significantly different at $p < 0.05$.

3. RESULTS AND DISCUSSION

3.1. Oils

The quality parameters (free acidity, peroxide values, *p*-anisidine values, and fatty acid composition) of the three oils, employed as covering oils in the preparation of canned vegetables, are discussed. The acidity values were lower than 0.3% in all samples (0.11, 0.28, and 0.15 g oleic acid/100g for SFO, EVO, and SBO, respectively), which confirms low levels of hydrolytic activity in the oils comparable with cold pressed and virgin oils (CODEX-STAN 210-1999; EC, 1513/2001). The peroxide values were low for all oils (4.22, 13.55, and 2.93 meqO_2/kg, for SFO, EVO, and SBO, respectively), even if comparatively high in EVO (a value that was below the legal limits (EEC, 2568/91). The *p*-anisidine values in the three oils were low according to GUPTA (2005) and LIST *et al.* (1974), and the lowest value was observed for SBO (6.39, 6.42, and 1.95 for SFO, EVO, and SBO, respectively).

3.2. Preserved vegetables

The free acidity values of the covering oils for canned aubergins and dried tomatoes during the ASLT are reported in Fig. 1. Free Fatty Acids (FFA) are formed by chemical or enzymatic hydrolysis of triglycerides and may get promoted by the reaction of oil with moisture (naturally present in vegetables), FFAs content is an important measure of alteration for oils. In all considered sample the free acidity increased during the accelerated aging.

For canned aubergins (Fig. 1a), the FFAs percentage increased from 0.21 to 0.68% for SFO, from 0.35 to 0.60% for EVO, and from 0.30 to 0.46% for SBO.

For canned dried tomatoes (Fig. 1b) and the three different covering oils used, the free acidity increased during the ASLT. The FFAs contents increased from 0.27 to 0.67% for SFO, from 0.43 to 0.92% for EVO, and from 0.28 to 0.58% for SBO. Despite contact with the moist vegetable matrix, the acidity values were satisfactory. After 30 days of ASLT (18 months of conventional storage at room temperature), the changes in acidity for both aubergins and tomatoes were less than 1%. For aubergins, the acidity value for EVO was under the maximum limit for extra-virgin olive oil (EU, 61/2011), and for tomatoes, the value exceeded the maximum legal limit after 30 days of accelerated aging, anyway after the sell-by date of the canned product.

Fig. 2 shows the peroxide values and the *p*-anisidine numbers of the different covering oils for the canned aubergins (Fig. 2a) and dried tomatoes (Fig. 2b), respectively. The peroxide value indicates the level of rancidity that normally occurs in oils because of progressive unsaturated fatty acid oxidation. For canned aubergins (Fig. 2a), at T0, which corresponds to the fresh product, the peroxide values were unaltered or, for SBO, slightly increased with respect to the pure oils used as covering medium. The peroxide values for the product at the beginning of the shelf-life trial was similar for the canned dried tomato covering oils, nearly the same for SFO and EVO, and slightly increased for SBO as compared to the peroxide values of the pure oils (Fig. 2b). In all preserved vegetables, the peroxide values decreased after the beginning of the shelf-life trial while peroxide compounds were progressively decomposed into secondary oxidation products (aldehydes and ketones and polymers) which corresponds in an increase of *p*-anisidine value. The *p*-anisidine value reveals the presence of secondary oxidation products. In canned aubergins, the peroxides were reduced to one-half the initial value at the end of ASLT (Fig. 2a); in canned dried tomatoes, the decrease in peroxides was more rapid and was about 1 mEq O_2/kg after 40 days of accelerated aging (Fig. 2b).

As shown in Fig. 2, the *p*-anisidine values of the oils used for covering the preserved vegeta-

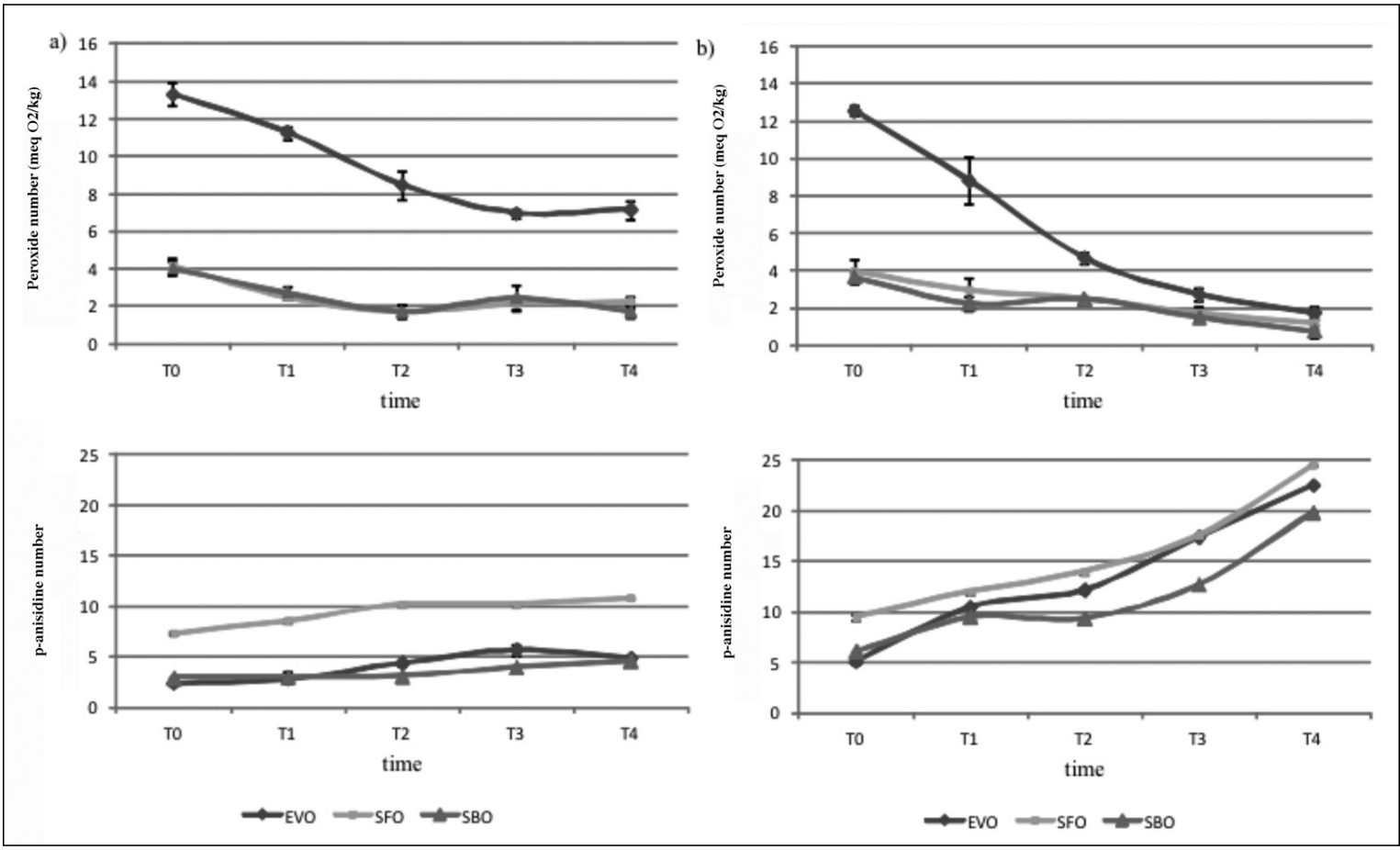


Fig. 2 - Peroxide number and *p*-anisidine value in canned aubergins (a) and in canned dried tomatoes (b), covered with sunflower oil, extra-virgin oil, and soybean oil, as a function of storage.
n = 6; bars in the figures represent standard deviation values.
T0: 0 days; T1: 10 days; T2: 20 days; T3: 30 days; T4: 40 days

bles showed a progressive increase during the ASLT.

For canned aubergins (Fig. 2a), *p*-anisidine values for EVO and SBO covering oils were significantly lower ($p < 0.05$) than that for SFO for the period of storage, and the decomposition of the hydroperoxides proceeded at a greater rate in SFO than in the EVO or SBO. As compared with the *p*-anisidine values found for EVO, the *p*-anisidine values of soybean oil were lower from two to four weeks of accelerated storage.

SBO had the lowest oxidative rancidity during the ASLT. Comparing the results of the two types of canned vegetables, the *p*-anisidine values of the covering oils for dried tomatoes were higher than those for aubergins during ASLT. However, as in canned aubergins, the *p*-anisidine numbers of tomatoes in SBO were significantly lower than those of the other oils considered at the sell-by date (Fig. 2b).

Since the data underlined the lowest content of hydroperoxides and the simultaneous lowest value of *p*-anisidine for aubergins and dried tomatoes in SBO and since the SBO showed the lowest value of free acidity, we may conclude that SBO has appreciable characteristics of stability to oxidation as covering oil in canned vegetables. A high stability of SBO under the similar conditions of storage was underlined by WARNER *et al.* (1989) in a previous study.

Dried tomatoes exhibited a higher index of secondary oxidation (*p*-anisidine value) than aubergins. At the sell-by date (18 months, 30 days of accelerated aging), the *p*-anisidine value of canned dried tomatoes was 12.66 ± 0.02, 17.38 ± 0.10, and 17.53 ± 0.10 for SBO, EVO, and SFO respectively. Conversely, in aubergins, the highest *p*-anisidine value at the sell-by date was 10.80 ± 0.13 in SFO. Differences in oxidation of covering oils for aubergins and dried tomatoes could justify consideration of the additive protective effects of antioxidant compounds present in aubergins (such as phenolic compounds, flavonoids, ascorbic acid, and vitamin A) and in dried tomatoes (lycopene, ascorbic acid, phenolic, flavonoids, and vitamin E) (HUNG and DUY, 2012), and the antioxidant effects of aromatic herbs and spices added (chili pepper, garlic, oregano, and mint).

Aubergin is one of the most active vegetables in its free radical scavenging capacity because of its phenolic constituents (JUNG *et al.*, 2011; HUNG and DUY, 2012). Considering aromatic herbs and spices, the antioxidant effects of the pungent component of chili pepper, capsaicin, has been documented in previous studies (REYES-ESCOGIDO *et al.*, 2011). HENDERSON and HENDERSON (1992) observed that the oxidation of oleic acid at cooking temperatures was inhibited by the presence of capsaicin. In addition, capsaicin is reported to inhibit lipid peroxidation (SALIMATH *et al.*, 1986; PULLA REDDY and LOKESH, 1992; ASAI *et al.*, 1999; HENDERSON *et al.*, 1999; OKADA and OKAJIMA, 2001; KOGURE *et al.*, 2002). Garlic has been reported to reduce free radical-induced oxidative damage in animal and human models. Extensive studies performed on garlic extracts (*Allium sativum* L.) highlighted the presence of two main classes of antioxidants: flavonoids and sulfur-containing compounds (LEELARUNGRAYUB *et al.*, 2006). The four main garlic antioxidant compounds are alliin, allyl cysteine, allyl disulfide, and allicin (BENKEBLIA, 2005; EL SHENAWY *et al.*, 2008). Oregano (*Origanum majorana*) is one of the aromatic herbs known to possess antioxidant compounds such as rosmarinic acid, caffeic acid, and various flavonoids. Oregano extracts have shown a pronounced effect on stabilizing lipids against autoxidation (KYOJI *et al.* 2006). The effectiveness of mint, a common aromatic herb, as a natural antioxidant is widely documented (KANATT *et al.*, 2007; PADMINI *et al.*, 2008; SAZHINA *et al.*, 2011). For canned aubergins, the antioxidant capacity arises from the typical antioxidant compounds of the vegetable and of garlic, mint, oregano and chili pepper; for preserved dried tomatoes, the antioxidant capacity arises from the vegetable and garlic and mint. The antioxidant capacity of aubergin is higher than that of dried tomato (WU *et al.*, 2004). The aromatic herbs added to canned dried tomatoes were mint and garlic, and the additive effect of these two herbs may be lower than that of the canned aubergins and the herbs and spices used for it. Different results for the two canned foods may have been caused by their different antioxidant properties and the different added aromatic herbs and spices.

To confirm the above hypothesis, aromatic herbs and spices were added to the pure oils used as packing oils for the vegetables considered. Each oil used for each vegetable had different aromatic herbs and spices in the recipe (chili pepper, garlic, oregano, and mint), a mixture of mint and garlic, or a mixture of all aromatic herbs and spices together, and was analysed for peroxide numbers and *p*-anisidine values. The results are reported in Figs. 3, 4 and 5.

The peroxide numbers and *p*-anisidine values of the three oils considered were influenced by the added aromatic herbs and spices. In particular, during the ASLT, the oil samples with added garlic, with added mint and garlic, and with all herbs and spices added presented lower values of the oxidation parameters. In SFO, a higher peroxide number was observed in the sample without spices and herbs, whereas chili pepper, oregano, and mint, showed an antagonistic effect on peroxide formation. For *p*-anisidine, lower values were observed in oil with added garlic and in oil with all herbs and spices added, whereas the oil with added mint and garlic had higher values; however, these values were lower than those found with other added herbs and spices.

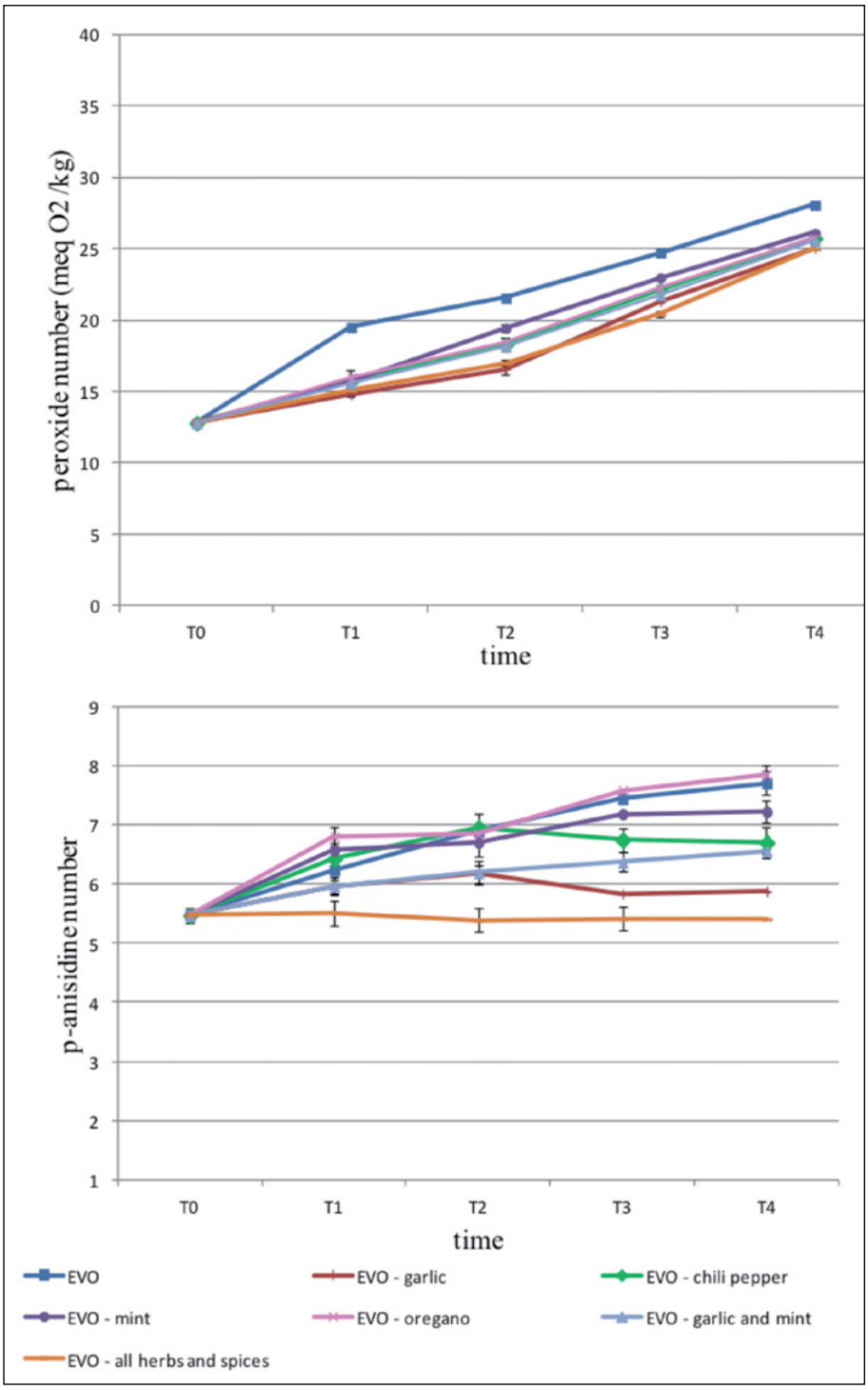


Fig. 3 - Peroxide number and p-anisidine value of extra-virgin olive oil (a), sunflower oil (b), and soybean oil (c), without herbs and spices and with different herbs and spices, as a function of storage.
n = 6; bars in the figures represent standard deviation values.
EVO = extra-virgin olive oil.
T0: 0 days; T1: 10 days; T2: 20 days; T3: 30 days; T4: 40 days.

A similar result was found for EVO where a low peroxide number was observed in oil samples with added garlic, and with all herbs and spices added. Samples with added mint and garlic had the same results as EVO with added oregano. The lower *p*-anisidine value for EVO was observed with added garlic, added mint and garlic, and when all herbs and spices were added. For SBO, each of the aromatic herbs and spices influenced the peroxide number and *p*-anisidine value. A higher protective effect was found for added garlic and for samples with all herbs and spices added. The oil with mint added had a *p*-anisidine value similar to the sample with add-

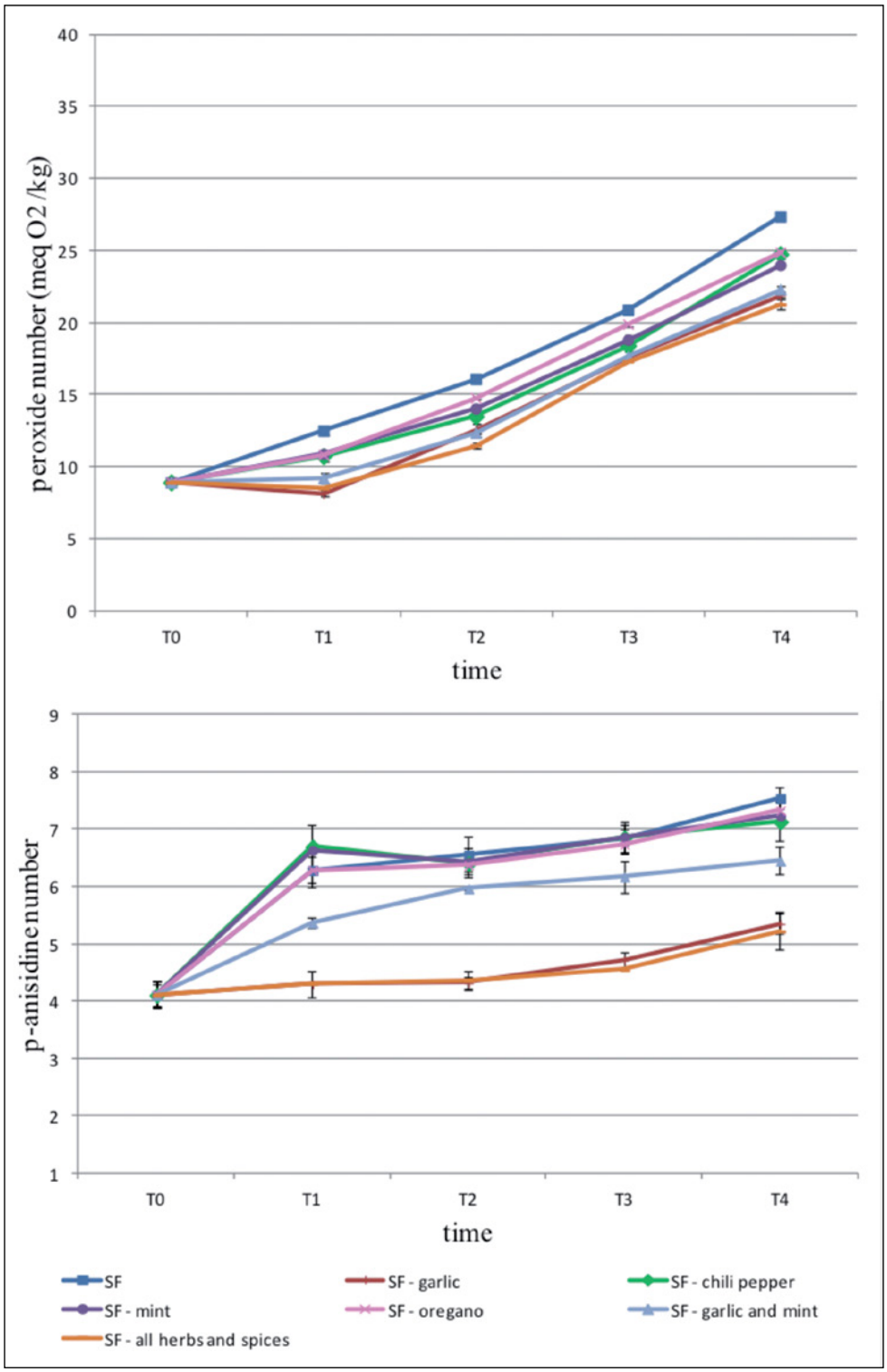


Fig. 4 - Peroxide number and p-anisidine value of sunflower oil, without herbs and spices and with different herbs and spices, as a function of storage.

ed mint and garlic, which was lower than that for other herbs and spices. For SBO, the worst *p*-anisidine values were found in SBO with added oregano (the oregano showed, in the case of SBO, an unusual pro-oxidant effect).

The sensorial test underlined differences among the covering oils for each vegetable at T0 and at T3 (fresh product and product at its sell-by date). Table 2 shows the results for quantitative descriptive analysis for AUSFO compared to AUEVO and with AUSBO at T0 and at T3. At T0, AUEVO was significantly different ($p < 0.05$) from AUSFO and AUSBO with respect to its color and bitterness, which were judged to be higher than

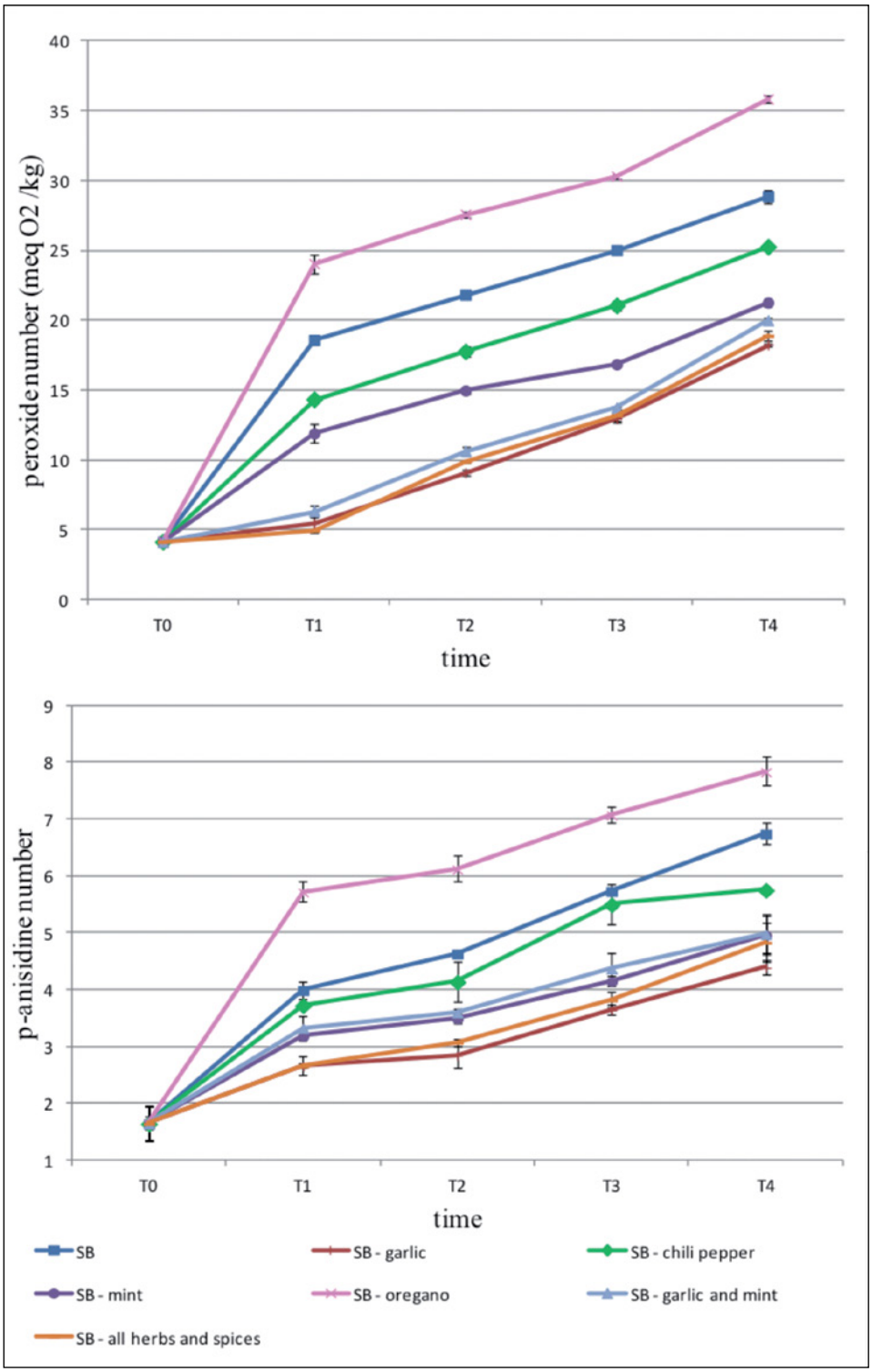


Fig. 5 - Peroxide number and *p*-anisidine value of soybean oil, without herbs and spices and with different herbs and spices, as a function of storage.

those found for the other oils. These differences could be explained by the characteristics of EVO. AUSBO was found to have lower rancidity when compared with the other samples. At the sell-by date, the quality of AUSBO differed from the other oils in its hardness, chewiness, and bitterness. AUSBO was judged slightly harder, less gummy, and less bitter than the other products. Table 3 shows the results for DTSFO as compared to DTEVO and DTSBO at T0 and at T3. At T0, DTEVO was judged to differ from DTSFO and DTSBO with respect to its bitterness, which was slightly higher than that of the other products. DTSBO was found to have lower

Table 2 - Sensory evaluation scores of AUSFO, AUEVO and AUSBO at T0 and T3.

Sample	Color[A]	Hardness[B]	Chewiness[C]	Rancidness[D]	Saltiness[D]	Bitterness[D]
AUSFO T0	6.70[a]	6.93[a]	5.96[a]	1.33[a]	4.46[a]	2.67[a]
AUEVO T0	7.27[b]	7.10[a]	5.6[a]	1.13[b]	4.16[a]	3.13[b]
AUSBO T0	6.63[a]	7.03[a]	5.30[a]	0.91[c]	4.13[a]	2.47[a]
AUSFO T3	6.30[a]	5.39[a]	6.65[a]	1.22[a]	4.78[a]	3.96[a]
AUEVO T3	6.57[a]	5.48[a]	6.21[b]	1.30[a]	4.48[a]	3.60[a]
AUSBO T3	6.29[a]	6.43[a]	5.43[a]	1.45[a]	4.71[a]	2.58[b]

n = 18; T0 = 0 months; T3 = 18 months.
Pairs (AUSFO T0 compared with AUEVO T0 and with AUSBO T0; AUSFO T3 compared with AUEVO T3 and with AUSBO T3) with the different letters within the same column are significantly different ($p < 0.05$).
AUSFO: aubergins in sunflower oil.
AUEVO: aubergins in extra-virgin olive oil.
AUSBO: aubergins in soybean oil.
[A] = Color: 0 = extremely light to 9 = extremely dark.
[B] = Hardness: 0 = extremely soft to 9 = extremely hard.
[C] = Chewiness: 0 = none to 9 = extremely gummy
[D] = Rancidness, saltiness, and bitterness: 0 = none to 9 = extremely strong

Table 3 - Sensory evaluation scores of DTSFO, DTEVO and DTSBO at T0 and T3.

Sample	Color[A]	Hardness[B]	Chewiness[C]	Rancidness[D]	Saltiness[D]	Bitterness[E]
DTSFO T0	6.88[a]	6.73[a]	4.73[a]	1.01[a]	5.98[a]	2.66[a]
DTEVO T0	6.67[a]	6.81[a]	4.83[a]	1.23[a]	6.32[a]	3.12[b]
DTSBO T0	6.65[a]	6.26[a]	4.69[a]	0.86[b]	7.39[b]	2.53[a]
DTSFO T3	6.81[a]	6.63[a]	4.93[a]	1.67[a]	5.57[a]	3.12[a]
DTEVO T3	7.43[b]	6.43[a]	5.13[a]	2.13[b]	6.37[b]	3.16[a]
DTSBO T3	7.06[a]	6.72[a]	4.13[b]	1.72[c]	5.45[a]	3.06[a]

n = 9; T0 = 0 months; T3 = 18 months.
Pairs (DTSFO T0 compared with DTEVO T0 and with DTSBO T0; DTSFO T3 compared with DTEVO T3 and with DTSBO T3) with the different letters within the same column are significantly different ($p < 0.05$).
DTSFO: dried tomatoes in sunflower oil.
DTEVO: dried tomatoes in extra-virgin olive oil.
DTSBO: dried tomatoes in soybean oil.
[A] = Color: 0 = extremely light to 9 = extremely dark.
[B] = Hardness: 0 = extremely soft to 9 = extremely hard.
[C] = Chewiness: 0 = none to 9 = extremely gummy.
[D] = Rancidness, saltiness, and bitterness: 0 = none to 9 = extremely strong.

rancidity and saltiness than the other samples. At T3 the DTEVO was judged to differ from the other samples in its color and rancidness, and DTSBO was reported to be less gummy and less rancid than the other oil samples.

4. CONCLUSIONS

The results obtained from this study underline the higher resistance to oxidation of vegetables canned in SBO and in EVO. A higher concentration of secondary oxidation catabolites was observed in oils covering dried tomatoes than in oils covering aubergins. This might be related to the additive protective effects of antioxidant compounds found in the vegetables and the antioxidant effects of added aromatic herbs and spices (HUNG and DUY, 2012). Data obtained from the case study, conduct to investigate the efficacy as antioxidants of aromatic herbs and spice present as ingredients in the traditional recipes for considered canned vegetables, confirm the higher antioxidant power found upon addition of all herbs and spices considered in the recipe, of an added mixture of mint and garlic, or of added garlic alone, as compared to the pure oils or the oils with other aromatic herbs and spices added, and the minor effects on secondary oxidation of the oils with added mint and garlic. The sensorial investigation partially confirmed the analytical data at T0 with the finding of lower rancidity and, at the sell-by date, a better consistency and lower bitterness of aubergins in SBO. A lower rancidity at T0 and at T3 a better consistency and a lower rancidity were found for dried tomatoes in SBO. The lowest free acidity value, peroxides number, and *p*-anisidine value for aubergins and dried tomatoes in SBO, and the results of sensorial investigation underline the stability to oxidation and the validity of SBO as covering oil in canned vegetables production.

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A ONE-YEAR SURVEY ON AFLATOXIN M1 IN RAW MILK

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ABSTRACT

In the year 2012, 288 raw milk samples were collected from six different dairy cow farms and analyzed for the presence of aflatoxin M_1 (AFM_1) using the ELISA technique. The AFM_1 levels ranged from 5 to 25 ng/kg and the highest concentrations were found in autumn, with a significant difference ($p<0.05$) between February and November. The EU legal limit of 50 ng/kg has never been exceeded. Even if the results of the present study show a low risk for AFM_1, its occurrence in dairy products has to be regularly monitored due to their importance as foodstuffs for people and children above all.

- Keywords: *Aspergillus*, carry-over, ELISA -

INTRODUCTION

Nowadays food industry has the responsibility to develop and implement a Hazard Analysis and Critical Control Point (HACCP) system aiming at identifying and preventing important hazards to food safety. The presence of aflatoxins (AFs) in dairy products is one of the most important critical control points to be checked in raw milk supplies. Aflatoxins are secondary metabolites mainly produced by three species of *Aspergillus* including *Aspergillus flavus*, *Aspergillus parasiticus* and *Aspergillus nomius* (CRAPPY, 2002). Even if eighteen AFs have been identified, only four out of them have been found in food and feed, i.e. AFB_1, AFB_2, AFG_1 and AFG_2 (HESHMATI and MILANI, 2010). The toxic effects of these compounds can be both acute, thus causing hepatitis, oedema or hemorrhagic necrosis, and chronic, thus resulting in liver, lung and kidney carcinomas as well as immunosuppression (WILLIAMS *et al.*, 2004). In particular, AFB_1 shows different toxic activities, including teratogenicity, mutagenicity and carcinogenicity (MCLEAN and DUTTON, 1995). Therefore, the International Agency for Research on Cancer (IARC) has included AFB_1 in group 1 as a human carcinogen (IARC, 1993). Animals eating contaminated feed rapidly adsorb and transfer AFB_1 to the liver, where it is metabolized into the 4-hydroxylated derivate AFM_1 and excreted through faeces and urine (POLONELLI *et al.*, 2011). Consequently, AFM_1 may be secreted in mammalian milk by means of a carry-over process, within 12-24 h after the ingestion of AFB_1 (KAV *et al.*, 2011). AFM_1 exhibits a high level of genotoxic activity due to its possible accumulation and linkage to DNA (SHUNDO and SABINO, 2006). It can cause DNA damage, gene mutation, chromosomal anomalies and cell transformation in *in-vitro* mammalian cells, insects, lower eukaryotes and bacteria (PRANDINI *et al.*, 2009). For that reason, AFM_1 has been included in group 1 (IARC, 2002) and removed from group 2B (i.e. as a possible human carcinogen).

Sources of AFs contamination in animal foodstuffs may vary geographically, with prevalence in areas with favorable environmental and climatic conditions. *Aspergillus flavus* and *A. parasiticus* colonize plants when still in the field, mainly when damaged; the highest AFs production occurs at temperatures between 20° and 30°C. In particular, *A. parasiticus* prefers a soil environment and can be found more commonly on peanuts, while *A. flavus* is better adapted to an aerial environment and colonizes cotton and corn (PRANDINI *et al.*, 2009). These molds can also colonize products in post-harvest if not adequately stored. However, the relationship between the amount of AFB_1 ingested by animals and the quantity of AFM_1 in milk is quite variable, as many factors – such as the individual variability among animals, the presence of udder infections, and the lactation period (at the beginning the carry-over is 3.3-3.5 times greater compared to the advanced lactation) – can affect carry-over (VAN EGMOND, 1989). The maximum limit for AFM_1 concentration in food varies in the legislation of different countries (GODIČ TORKAR and VENGUŠT, 2008). The European Community has prescribed a limit of 50 ng/kg in raw milk, heat-treated milk and milk for the manufacture of milk-based products (EC, 2006) while, according to US regulations, the action level of AFM_1 in milk should not be higher than 500 ng/kg (GHANEM and ORFI, 2009). AFM_1 is relatively stable in raw and processed milk products and is not affected by pasteurization or cheesemaking processes performed in dairy industry (KAV *et al.*, 2011). It has been reported that AFM_1 concentration in dairy products can be 3 to 4 times higher than in milk, as it is associated with milk proteins (BATTACONE *et al.*, 2003).

The present study aims at detecting the AFM_1 levels in bovine raw milk designed to a dairy factory located in the Marche region, central Italy. The preventive action used by such factory to control this hazard will also be discussed. The samples were analyzed by means of an enzyme-linked immunosorbent assay (ELISA) that is the most representative method for the fast screening analysis of AFs.

MATERIALS AND METHODS

A total of 288 samples of raw milk collected from six different suppliers (named 1 to 6) located in the Marche region, central Italy, were examined over the year 2012. Raw milk samples from each farm were provided to that dairy factory four times in a month and were transported in tanks at 0-4°C. All samples were analyzed in duplicate. Such raw milk samples (10 mL) were at first centrifuged at 3,500 *g* for 10 min at 4°C, then the upper cream layer was completely removed. A sample unit of 100 μL was used for the quantitative analysis of AFM_1 using the commercial kit RIDASCREEN (R-Biopharm, Germany). Such kit includes microtiter plates coated with capture antibodies, AFM_1 standard solutions used for the construction of the calibration curve, peroxidase-conjugated AFM_1, substrate (urea peroxidase), chromogen (tetramethylbenzidine) and stop reagents 1 N sulfuric acid. The test procedure was performed according to HESHMATI and MILANI (2010). The evaluation of AFM_1 was obtained dividing the absorbance values of the standards and the samples by the absorbance value of the first standard (zero standard), then multiplying the result by 100 (percentage of maximum absorbance). The adsorption was inversely proportional to the AFM_1 concentration in samples. The limit of quantitation according to the kit was 5 ng/kg.

A statistical analysis was carried out by GraphPad InStat Version 3.0, GraphPad Software (San Diego, California, USA). All the obtained data were assessed for normality by means of Kolmogorov-Smirnov test. Since the values were not normally distributed, non-parametric tests were applied. The differences among the values obtained from the six different suppliers and among the milk samples collected over 12 months were evaluated by Kruskal-Wallis Test (non-parametric ANOVA). When the *p* value was lower than 0.05, the Dunn's Multiple Comparisons Test was used.

RESULTS AND CONCLUSIONS

The mean AFM_1 concentrations in four collections (analyzed in duplicate) of raw milk over a month (for a total of 12 months) from each dairy farm are reported in Table 1. The levels ranged from the limit of quantitation (5 ng/kg) to a maximum of 25 ng/kg, with the highest values observed in the months of September, October and November. However, no sample exceeded the maximum levels (50 ng/kg) set for AFM_1 in milk by EU legislation (EC, 2006). No significant difference ($p>0.05$) was also observed among the AFM_1 concentrations in samples from the different suppliers, while a significant difference ($p<0.05$) was noticed only between the values obtained in February and November.

According to the HACCP plan implemented in the dairy factory of the present study, AFM_1 content is regularly monitored four times in a month but, when it results to be higher than 10 ng/kg, the supplier is contacted (as a preventive action) and analyses of raw milk from the matching dairy farm are repeated at the next supply. In this study (Table 2), 68.4% of the samples contained AFM_1 in the range of 5-10 ng/kg, while 27.1% was in the range of 11-19 ng/kg, exceeding the above mentioned preventive limit (10 ng/kg). Moreover, the dairy factory has set an internal system of corrective actions when AFM_1 content exceeds 20 ng/kg, defined as action limit. In the present study, the action limit was exceeded only in 4.5% of the samples collected from some dairy farms in different months (i.e. January, September, October, November and December), with values ranging from 20 ng/kg to a maximum of 25 ng/kg. In that case the supply of milk from the dairy farm is suspended until concentrations return to regular values. Whereas, if such value exceeds 50 ng/kg (the maximum level by law), the positive sample is analyzed by means of the HPLC as confirmatory assay, and milk has then to be intended as "Category 2 material" according to the EU regulations on animal by-products (EC, 2009). However, as a preventive measure, the HPLC procedure is routinely performed every four months.

Table 1 - Concentrations of AFM_1 (ng/kg) and range (in parentheses) for each six suppliers in the year 2012.

Supplier	January	February[a]	March	April	May	June	July	August	September	October	November[b]	December
1	ND	ND	12.5±2.9* (5.0-15.0)	10.0±3.3 (5.0-15.0)	ND	7.5±2.9 (5.0-15.0)	ND	9.10±3.2 (5.0-15.0)	7.5±2.9 (5.0-15.0)	9.0±3.2 (5.0-15.0)	11.8±4.8 (5.0-22.0)	10.0±3.6 (5.0-18.0)
2	8.8±4.3 (5.0-20.0)	ND	6.8±2.0 (5.0-12.0)	9.3±2.9 (5.0-15.0)	9.0±3.2 (5.0-15.0)	ND	10.0±3.3 (5.0-15.0)	13.4±2.8 (5.0-17.0)	15.8±0.9 (15.0-18.0)	15.2±4.1 (5.0-25.0)	9.3±2.9 (5.0-15.0)	10.0±3.3 (5.0-15.0)
3	13.0±5.5 (5.0-24.0)	ND	ND	7.5±2.9 (5.0-15.0)	9.0±3.2 (5.0-15.0)	ND	ND	ND	17.3±0.9 (15.0-18.0)	10.4±4.3 (5.0-19.0)	11.0±4.1 (5.0-19.0)	7.5±2.9 (5.0-15.0)
4	6.8±2.0 (5.0-12.0)	7.0±2.6 (5.0-15.0)	ND	ND	9.0±3.2 (5.0-15.0)	6.8±2.0 (5.0-12.0)	ND	8.0±2.6 (5.0-15.0)	10.8±3.9 (5.0-18.0)	10.6±4.7 (5.0-23.0)	14.0±6.1 (5.0-25.0)	12.8±3.0 (5.0-16.0)
5	ND	ND	9.3±2.9 (5.0-15.0)	ND	7.6±3.4 (5.0-18.0)	ND	7.5±2.9 (5.0-15.0)	9.2±3.3 (5.0-16.0)	17.8±1.2 (15.0-20.0)	17.6±1.7 (15.0-22.0)	15.8±4.5 (5.0-23.0)	10.0±3.3 (5.0-15.0)
6	ND	ND	7.5±2.9 (5.0-15.0)	ND	8.8±4.9 (5.0-24.0)	ND	ND	10.0±4.1 (5.0-15.0)	ND	ND	7.5±2.9 (5.0-15.0)	9.3±4.9 (5.0-22.0)

*These data are expressed as mean ± standard error; ND= $AFM_1 \leq 5$ ng/kg; a,b ($p<0.05$)

Table 2 - Distribution of AFM_1 in raw milk samples.

Months	Range of AFM_1 concentrations (ng/kg)		
	5-10 Number of samples (%)	11-19 Number of samples (%)	20-25 Number of samples (%)
January	20 (83.4)	2 (8.3)	2 (8.3)
February	23 (95.8)	1 (4.2)	-
March	17 (70.8)	7 (29.2)	-
April	19 (79.2)	5 (20.8)	-
May	16 (66.7)	8 (33.3)	-
June	22 (91.7)	2 (8.3)	-
July	21 (87.5)	3 (12.5)	-
August	15 (62.5)	9 (37.5)	-
September	9 (37.5)	14 (58.3)	1 (4.2)
October	9 (37.5)	12 (50.0)	3 (12.5)
November	12 (50.0)	8 (33.3)	4 (16.7)
December	13 (54.2)	10 (41.6)	1 (4.2)

- = no sample.

In Figure 1 the mean content of AFM_1 in milk per month was reported, without considering the different suppliers.

In the present study, the overall contamination levels of AFM_1 in milk samples were lower than those reported by other authors (HAN *et al.*, 2013; HUSSAIN and ANWAR, 2008; RAHIMI and AMERI, 2012; TAJIK *et al.*, 2007). These differences could be due to several factors, including different analytical techniques, samples size, season of the year, livestock management, and dairy processing systems. Moreover, the AFM_1 levels in milk seemed to be significantly influenced by the geographical region. The outcomes of some studies carried out in Italy showed an AFM_1 concentration range of 2-90 ng/kg (NACHTMANN *et al.*, 2007) and < 23 ng/kg (GALVANO *et al.*, 2001). In 2003, the risk of mycotoxins was brought to public attention following the indication of the presence of unusual amounts of AFM_1 in milk, in northern Italy in particular. At the beginning controls aimed at checking that the levels in milk did not exceed the limit established by law, but special monitoring plans were coordinated for milk and feed towards the end of 2003 due to an alarming amount of positivity in the self-check plan carried out on milk. Maybe the positive levels found in feed at the end of 2003 were the consequence of particularly unusual climatic conditions (high temperatures and drought lasting more than four months) that characterized the summer in the year 2003 (DECASTELLI *et al.*, 2007). Such approach – i.e. paying particular attention to the correlation in milk-feed monitoring procedures – could be considered particularly valid in order to find contaminated batches starting from controls on milk. In fact, many countries in Europe have shown relatively low levels of AFM_1 contamination in milk samples as a result of stringent rules on AFB_1 in dairy cattle feed (TRUCKSESS, 2006). In the present study AFM_1 concentrations were not very high and that result could be due to the feeding practices in dairy cow farms. The lower limits adopted by this dairy factory could be particularly effective, above all when the Italian Ministry of Health established an increase in milk analyses in order to detect AFM_1 following a series of notifications on AFB_1 in maize of European origin by the Rapid Alert System for Food and Feed (RASFF) since the last maize harvest in autumn 2012 (ANONYMOUS, 2012). In order to control AFM_1 levels in milk it is necessary to reduce AFB_1 contamination of feed for dairy cattle by preventing fungal growth and AFB_1 formation in agricultural commodities. That purpose can be achieved through some agricultural practices, such as the choice of hybrids, seeding time and density, suitable ploughing and fertirrigation, and stricter chemical or biological controls. Cereals harvested with the lowest possible moisture and conservation moisture close to or less than 14% are necessary to reduce contamination risks. Furthermore, kernel mechanical

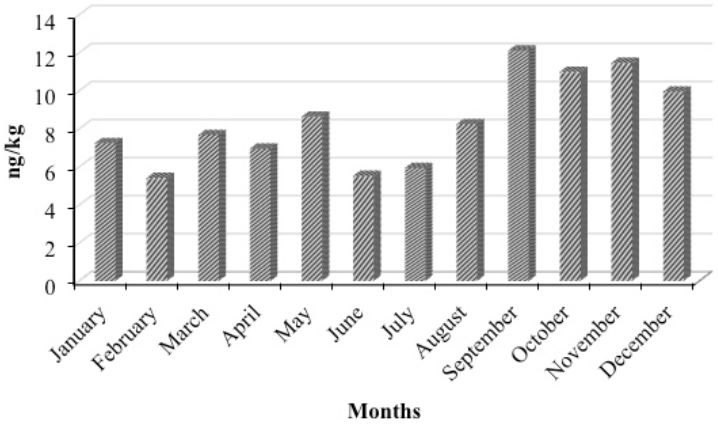


Fig. 1 - Mean content of AFM_1 in milk per month.

damage, grain cleaning practices and conservation temperature are also factors which need to be carefully controlled (PRANDINI *et al.*, 2009).

A marked seasonal variation in AFM_1 levels in milk has been previously reported (KAMKAR, 2005; RAHIMI and AMERI, 2012; RUANGWISES and RUANGWISES, 2010). It has been reported that AFs levels in feed are higher in rainy than in dry seasons. Moreover, the use of high amounts of contaminated concentrates is more frequent in cold months (KAMKAR *et al.*, 2011). Although no significant differences were observed in AFM_1 levels among the different months, except between February and November, the results of the present study shows that the mean concentrations in raw milk samples collected in autumn were higher than in other seasons. Such variation may be a result of toxin accumulation when storage occurs in hot and humid conditions. Many authors (BLANCO *et al.*, 1988; LOPEZ *et al.*, 2003; KAMKAR, 2005) reported on a higher number of yeasts, moulds and consequently on a higher concentration of mycotoxins in ensiled feed, mostly used in autumn or winter. Also DASHTI *et al.* (2003) observed that the contamination levels in the samples from local companies were higher in winter than in summer. That could be explained by the prolonged storage required for feed, which would provide favorable conditions for fungi to grow; or by the use of contaminated feed for the animals in winter, in addition to other factors such as temperature and relative humidity, agricultural products used as animal feed as well as seasonal effects from the country of origin of feed. Two other studies showed similar results – i.e., AFM_1 contamination is higher in winter than in summer. The first study was conducted in five regions of Iran on ninety-eight samples of raw milk analyzed in order to observe the possible presence of AFM_1. All samples resulted positive for AFM_1 with an overall mean level of 53 ng/L. The levels of AFM_1 were also higher in winter and spring than in summer and autumn (TAJKARIMI *et al.*, 2007). The second study was carried out in Sarab City, Iran, and showed that 76.6% of 111 raw milk samples was positive, with AFM_1 levels ranging between 15 and 280 ng/L. The lowest AFM_1 levels (24 ng/L) were found in August and the highest (118 ng/L) in December (KAMKAR, 2005).

In conclusion, the occurrence of AFM_1 in milk intended for human consumption is a critical control point to be steadily monitored in dairy products. Controls of the supply chain from feedstuffs for lactating cows to milk production represents the key to guarantee the safety of the end product, due to the large variation in the content of AFB_1 in animal feed and consequently of AFM_1 in milk. Even if the risk of a high AFM_1 content appears limited, it is certainly of great interest to implement a valid system of regular monitoring in order to have always safe raw materials. Establishing more restrictive limits, as those chosen by the dairy factory, taken as a case-study in the present paper, could be a good approach to achieve a better food quality for consumers.

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