ITALIAN JOURNAL OF FOOD SCIENCE

Rivista italiana di scienza degli alimenti

IJFS

Volume XXVIII
Number 4
2016

CHIRIOTTI EDITORI

ITALIAN JOURNAL OF FOOD SCIENCE

(RIVISTA ITALIANA DI SCIENZA DEGLI ALIMENTI) 2nd series

Founded By Paolo Fantozzi under the aeges of the University of Perugia
Official Journal of the Italian Society of Food Science and Technology
Società Italiana di Scienze e Tecnologie Alimentari (S.I.S.T.Al)
Initially supported in part by the Italian Research Council (CNR) - Rome - Italy
Recognised as a "Journal of High Cultural Level"
by the Ministry of Cultural Heritage - Rome - Italy

Publisher:
Alberto Chiriotti - Chiriotti Editori srl, Viale Rimembranza 60, I-10064 Pinerolo, Italy - Tel. +39 0121 393127 - Fax +39 0121 794480 e-mail: alberto@chiriottieditori.it - URL: www.chiriottieditori.it

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Frequency:

Quarterly - One volume in four issues. Guide for Authors is published in each number and annual indices are published in number 4 of each volume.

Impact Factor: 5-Year Impact Factor: 0.73 published in 2015 Journal of Citation Reports, Institute for Scientific Information; Index Copernicus Journal Master List 2009 (ICV): 13.19
IJFS is abstracted/indexed in: Chemical Abstracts Service (USA); Foods Adlibra Publ. (USA); Gialine - Ensia (F); Institut Information Sci. Acad. Sciences (Russia); Institute for Scientific Information; CurrentContents®/AB&ES; SciSearch® (USA-GB); Int. Food Information Service - IFIS (D); Int. Food Information Service - IFIS (UK); EBSCO Publishing; Index Copernicus Journal Master List (PL).

IJFS has a publication charge of € 350.00 each article.
Subscription Rate: IJFS is now an Open Access Journal and can be read and downloaded free of charge at http://www.chiriottieditori.it/ojs/index.php/ijfs/index
Journal sponsorship is € 1,2010.00

RECOVERY OF WINEMAKING BY-PRODUCTS FOR INNOVATIVE FOOD APPLICATIONS

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ABSTRACT

Winemaking by-products are potential resources for second-generation biorefineries, i.e., biorefineries fed with biowaste to produce added-value products, particularly for the food sector. In fact, winemaking by-products are outstanding sources of oil, phenolic compounds and dietary fibre and possess numerous health benefits and multifunctional characteristics, such as antioxidant, colouring, antimicrobial and texturizing properties. The present review highlights promising developments for the conversion of winemaking by-products into novel food ingredients, as well as their use in innovative foods, focusing on the type of recovered ingredients, dosage, formulation and processing. In addition, the primary benefits of winemaking by-products to new foods are described.

Keywords: by-products, dietary fibre, grape phenolics, grape seed oil, winemaking

1. INTRODUCTION

Food supply chains have significant environmental impacts due to their use of resources and production of emissions, effluents and wastes. According to the European Union (EU) Commission Council Directive 2008/98/EC, "waste" is defined as "any substance or object, which the holder discards or intends or is required to discard". The importance of food waste stretches from environmental pressures to economic and social impacts, including negative effects on food and nutrition security (OTLES *et al.*, 2015).
To meet the overall objective of increasing the sustainability of production chains, waste prevention/minimization is the main priority and best option, followed by reuse, recycling and energy recovery. Alternatively, disposal (the use of landfills or incineration with low energy recovery) must be considered the worst environmental option. Reuse and recycling strategies have drawn attention on the valorisation of by-products. A "by-product" is defined as a product that must originate from a production process without being the main goal of production, be usable in the same production process or in a subsequent production or utilization process and be directly re-usable without further treatment outside normal industrial practices. Moreover, a by-product must have a market value, and the final use should be integral without negatively impacting human health or the environment (GALANAKIS, 2015).
Like all agro-food productions, the winemaking process generates a series of by-products that are important from both a quantitative and qualitative point of view and have been considered potential resources for second-generation biorefineries, i.e., biorefineries fed with biowaste to produce added-value products (SCOMA *et al*., 2016). In fact, grapes are one of the most cultivated fruits worldwide. According to the FAO (www.faostat3.fao.org), 77 Mt of grapes were produced throughout the world in 2013 (most of which were used in winemaking), along with 3.4 Mt of grape pomace (otherwise referred to as grape marc). The latter represents, by weight, the primary winemaking solid by-product (60% on average), followed by lees (approximately 25%) and stalks (approximately 14%). Minor solid by-products mainly include wine filtration residues. On average, depending on the grape variety and winemaking process, 100 kg of processed grapes generates 20-25 kg of pomace, a mixture of skins and seeds, 3-5 kg of stalks and 8-10 kg of lees (SPIGNO, 2015).
All of the aforementioned by-products pose serious environmental concerns because their production is typically concentrated in a limited time frame, and their high organic matter content prevents direct disposal into the soil, except for limited and regulated amounts. Although these materials are re-used for other applications, being correctly considered as by-products, landfill additions and incineration are also conducted, depending on the country. Conventional applications for winemaking by-products include: agronomic use, animal feed production and compost production for all residues; distillation for pomace and lees; tartaric acid manufacturing and the production of colouring additives and nutritional supplements from pomace, lees and filtration residues; oil recovery from seeds. Agronomic use, animal feed production, composting and distillation, a relatively new approach, are not considered remunerative strategies (SPIGNO, 2015).
If properly recovered, winemaking by-products show a wide range of potential and remunerative applications in many industrial sectors, including cosmetics, pharmaceuticals, biomaterials and food (BORDIGA, 2015; YU and AHMEDNA, 2013). In fact, grape pomace is a rich source of both dietary fibre and various phenolic compounds (TEXEIRA *et al.*, 2014). The amount of phenolic compounds that remain in the pomace depends on the initial, genetically dependent content of grapes, as well as the processing conditions and skin thickness, which is another genetically dependent parameter that is crucial for the maceration phase (BATTISTA *et al*., 2015). A study of various cultivars of

Vitis vinifera L. has revealed that the content of soluble proanthocyanidins in the skin ranges between 1.16 and 44.6 g/kg d. w., while the content of soluble proanthocyanidins in seeds ranges between 23.1 and 68.5g/kg d. w. (TRAVAGLIA *et al.*, 2011). The total anthocyanin content of red grape skins is in the range of 2.5-132 g/kg d. w. (KAMMERER *et al.*, 2004; SRI HARSHA *et al.*, 2013). The presence of anthocyanins in the red grape seed fraction, due to mash constituents adhering to the seeds, is generally neglected or reported to be low (KAMMERER *et al.*, 2004; LAVELLI *et al.*, 2015a). However, a recent patent was focused on the extraction of anthocyanins from grape seeds, suggesting that their content deserves attention in a full recovery strategy (BI and RUI, 2014). The total flavonol content of grape skins is in the range of 0.3-2.6 g/kg d. w. (SRI HARSHA *et al.*, 2013; SRI HARSHA *et al.*, 2014), whereas these compounds are generally less than 0.1 g/kg d. w. in grape seeds (MAIER *et al.*, 2009b). Compared to the above-mentioned phenolic compounds, phenolic acids and stilbenes are present in considerably lower amounts in winemaking by-products (KAMMERER *et al.*, 2004). Grape seeds contain oil with a high nutritional value. Among various vegetable oils, grape seed oil shows the largest percentage of linoleic acid (C18:2 ≈70%). Other major fatty acids present in grape seed oil include oleic acid (C18:1 ≈15%), palmitic acid (C16:0 ≈7%) and stearic acid (C18:0 ≈3%) (HANGANU *et al.*, 2012; FERNANDES *et al.*, 2013; FIORI *et al.*, 2014). In addition to the interesting fatty acid profile, grape seed oil contains significant amounts of bioactive compounds such as tocopherols and tocotrienols, presenting a total tocol content up to 1208 mg/kg (BEVERIGE *et al.*, 2005; CREWS *et al.*, 2006; FIORI *et al.*, 2014).

Winemaking by-products are of particular interest for food uses when they are obtained via organic production because consumer preference is positively influenced by information on sustainable production practices (LAUREATI *et al.*, 2013). In particular, a great deal of interest in sustainability issues has been expressed for winemaking (LAUREATI *et al.*, 2014). The purpose of using winemaking by-products in foods may be fortification or enrichment. The distinction between these terms is not always recognized in scientific studies but has been clarified as follows: a fortified product is defined as a food containing additional nutrients, while an enriched product is defined as a food with additional novel nutrients or components not normally found in a particular food (SIRO' *et al.*, 2008).

Tartaric acid, enocyanine (E163) and grape seed oil are classical examples of successful commercial products obtained from winemaking by-products. Additionally, in the last several years, grape seed and grape skin powders have been commercialized by different companies and promoted as highly nutritional ingredients to enrich conventional cereal flours and baked products with fibre, minerals, antioxidants, colour and aroma. The concept of antioxidant dietary fibre was first proposed by SAURA-CALIXTO (1998), who set the criteria that 1 g of antioxidant dietary fibre should possess a free radical scavenging capacity equivalent to at least 50 mg of vitamin E and should contain more than 50% dry matter of dietary fibre from the natural constituents of the material. Whole grape pomace, grape seeds and grape skin generally meet these criteria and are often referred to as antioxidant dietary fibre. Enocyanine and grape antioxidant dietary fibre represent the two basic solutions for the reintroduction of grape pomace into the food chain, including indirect and partial use as concentrated extracts, or direct use as ground, dehydrated and micronized antioxidant dietary fibre. In both of the above-mentioned cases, a new production process must be implemented.

Fig. 1 shows a schematic depiction of the basic conventional process for antioxidant dietary fraction production.

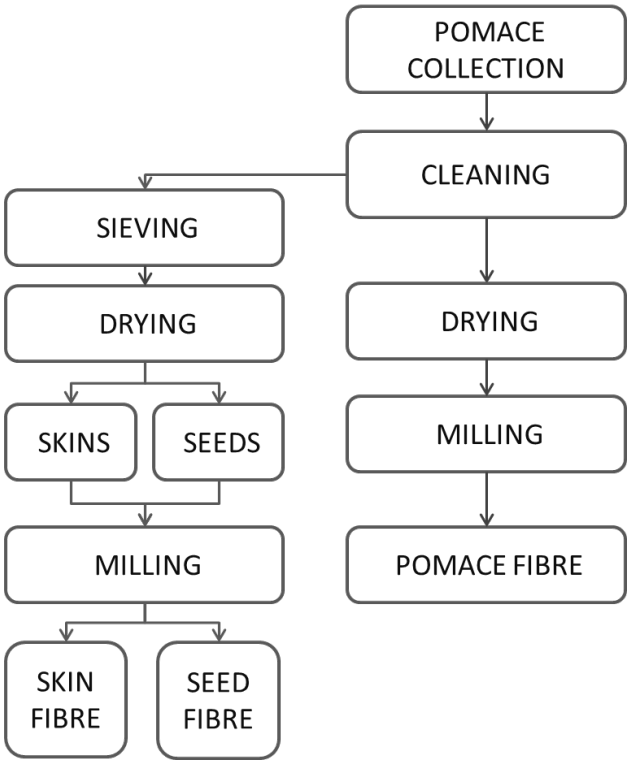

Figure 1: Scheme of the basic process for the production of grape pomace, skin or seed antioxidant dietary fibre.

Pomace collection should follow preliminary care selection to identify the best pomace for the production of food grade ingredients, based on the content of functional constituents (fibre, polyphenols and minerals), as well as possible contaminants (heavy metals, pesticides residuals and mycotoxins) (CORRALES *et al.*, 2010; SOLFRIZZO *et al.*, 2012). Washing and cleaning operations should be required, while the recovery of phenolic compounds would be reduced. If needed, skins and seeds can be separated by sieving, before or after drying. Of course, drying is necessary to obtain a final powder but is also the most common stabilisation treatment. Grape pomace has a high moisture content (greater than 60%) and undergoes rapid fermentation if not properly treated. Low temperature preservation may precede the drying step for logistic and timing reasons. Drying, which is an energy consuming process, should be reduced to a minimum because the thermal degradation of antioxidant compounds is detrimental to the nutritional profile. However, drying allows for the inhibition of enzymatic activity and can be considered a mild sanitization process. The operating temperature should not exceed 60°C to limit the degradation of phenolic compounds (AMENDOLA *et al.*, 2010). In the production of antioxidant dietary fibre from skins and seeds, the seeds can be defatted in a previous step to recover the oil and produce a fibre-rich ingredient with limited rancidity issues. The final milling step must set the ideal particle size, depending on the expected application. If dried skins and seeds are destined for the production of an extract, a particle size range of 0.5 to 2 mm is acceptable. As outlined in the following paragraphs, the particle size should be less than 0.5 mm for use in bakery products or pasta. For applications into fruit-based and dairy products, an even lower particle size is required, which leads to additional energy consumption.
Fig. 2 shows the basic process for the production of grape pomace, skin or seed extracts (SPIGNO, 2015).

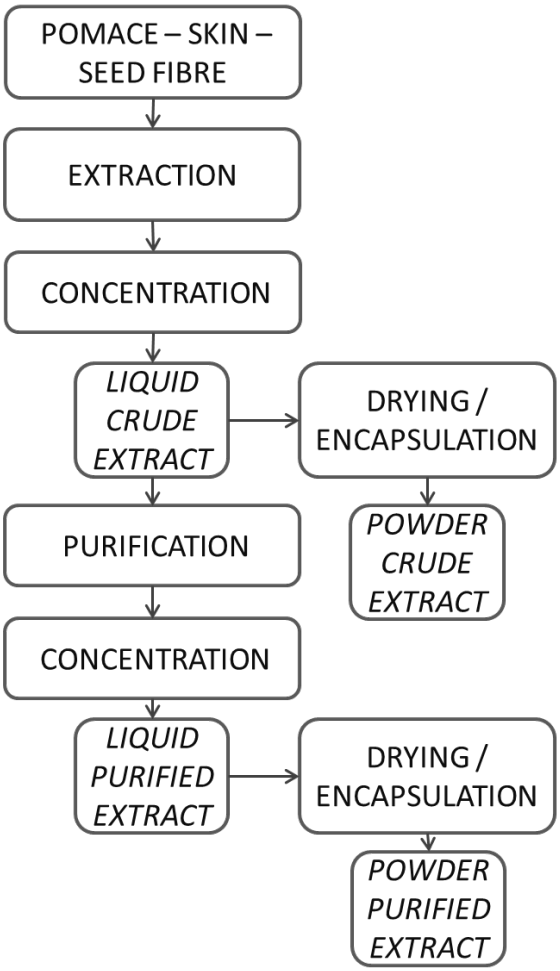

Figure 2: Scheme of the basic process for the production of extracts from grape pomace, skins or seeds.

As indicated in the production of antioxidant dietary fibre, the operating temperature should be less than 60°C. Different extraction techniques can be applied, such as conventional solvent extraction using food-grade solvents (AMENDOLA *et al.*, 2010), or non-conventional solvents and systems for the development of sustainable and environmentally friendly processes. The ultrasound-assisted extraction has been successfully applied for the extraction of grape pomace phenolics, using water as a solvent and achieving high extraction yield with a short extraction time (MARINELLI *et al.*, 2015). Microwave-assisted solvent extraction using ethanol/water (PEDROZA *et al.*, 2015) and high-pressure extraction using ethanol/water (PAINI *et al.*, 2016), as well as the use of aqueous solutions of organic acids (TZIMA *et al.*, 2015) have also been proposed. In general, all of these systems are characterized by low selectivity, and other compounds (such as sugars, minerals and organic acids) are co-extracted with the phenolic compounds, producing a crude extract. For food applications, the purification of extracts may be omitted without further increasing the production costs. The crude extract can then be simply concentrated to give a liquid extract or dried to give a powder extract. In this case, using a tailored approach (that takes into account the food category of the final target), the addition of suitable carrier materials (e.g., maltodextrins) can be exploited to increase and modify the stability and solubility of phenolic compounds (SPIGNO *et al.*,

2013; LAVELLI *et al*., 2016b). To obtain an extract with a higher purity in total phenolic compounds or within a selected class of phenolic compounds, a purification step is required. Adsorption resins (SOTO *et al.*, 2011) and membranes (ZAGKLIS and PARASKEVA, 2015) are the most commonly investigated purification systems, along with other non-conventional approaches, such as the use of colloidal gas aphrons (SPIGNO *et al.*, 2015). In any case, microfiltration is also suggested as a non-thermal technology to produce crude extracts while promoting microbiological stability.
Independent of the production process used to obtain grape pomace antioxidant dietary fibre, phenolic extracts or seed oil, this review describes literature examples of innovative food applications into meat, fish, cereal, fruit-based and dairy products, with a focus on the type of recovered ingredient, dosage level and primary results achieved by the application.

2. PHENOLIC EXTRACTS AND ANTIOXIDANT DIETARY FIBRE FROM GRAPE SKINS AND SEEDS AS INGREDIENTS IN INNOVATIVE FOODS

2.1. Functional effects

New ingredients recovered from winemaking by-products have the potential to provide a wide range of food products with numerous health benefits (SAURA-CALIXTO, 1998; TEXEIRA *et al*., 2014). Moreover, as outlined in the following sections, these by-products possess multifunctional properties and could be used as natural antioxidants, colorants, antimicrobial agents and texturizers.

2.1.1 Meat products

Due to the growing interest in convenience foods, ready-to-eat products such as dehydrated meat, frozen and precooked hamburgers, patties and meatballs have become a major category in the meat industry. The quality and shelf-life of these products is primarily dependent on the inhibition of lipid oxidation, which affects the colour, flavour, odour, texture and nutritional value of foods (FERNANDEZ *et al*., 1997). Consequently, research efforts have been devoted to the application of winemaking by-products in various meat products to prevent lipid oxidation during precooking and storage under refrigerated or frozen conditions, representing natural alternatives to the use of synthetic antioxidants (Table 1). In addition to auto-oxidation, microbial contamination is another serious factor that affects the quality and shelf-life of ready-to-eat meat products. However, only a few studies have investigated the antimicrobial properties of winemaking by-products in meat products (AHN *et al*., 2007). Regarding chicken meat, when red grape skin extract powder was added to the dehydrated product at a level of 1 g/kg_{meat}, the content of hexanal and thiobarbituric acid reactive substances (TBARS) formed during processing and storage at 22°C under air decreased. However, the efficacy was lower than those of rosemary extract and synthetic antioxidants, such as butylated hydroxyanisole and butylated hydroxytoluene (NISSEN *et al*., 2000). The antioxidant dietary fibre obtained from red grape pomace with particle sizes < 0.5 mm (total dietary fibre: 782 g/kg; soluble phenolics: 49.3 g/kg) has been applied to chicken hamburger, delivering fibre and imparting antioxidant effects during processing and refrigerated storage under air (SAYAGO-AYERDI *et al*., 2009). Alternatively, the extract obtained from the entire pomace (skins and seeds) has been proven to act as an antioxidant in uncooked and cooked chicken meat at a concentration corresponding to 60 mg phenolics/kg_{meat} during processing and frozen storage under vacuum (SELANI *et al*., 2011).

Table 1: Applications of winemaking by-products as new food ingredients in meat products.

Food product	Recovered ingredient	Main results and references
Chicken meat (dehydrated)	Red grape skin extract powder (soluble TP: 1.60 mmol phenol Eq./g) Integration: 1 g/kg_{meat}	Decrease in hexanal and TBARS content during processing and storage at 22°C in aluminized sachets sealed in air. Lower efficacy than rosemary extract. NISSEN *et al.*, 2000
Chicken hamburger (uncooked and pre-cooked)	Red grape pomace antioxidant dietary fibre (particle size < 0.5 mm; TDF: 782 g/kg; soluble TP: 49.3 g GAE/kg) Integration: 5-20 g/kg_{meat}	Decrease in TBARS content during processing and storage in polyvinyl chloride bags (OTR: 13.500 cm^3/m^2d) at 4°C. High antioxidant activity and fibre content. SÁYAGO-AYERDI *et al.*, 2009
Chicken meatballs (uncooked and pre-cooked)	Red and white grape pomace extract (soluble TP: 7.8-9.4 g GAE/kg) Integration: 60 mg TP/kg_{meat}	Decrease in TBARS content during processing and storage at -18°C under vacuum. SELANI *et al.*, 2011
Pork patties (pre-cooked)	Red grape skin extract powder (soluble TP: 1.60 mmol phenol Eq./g) Integration: 0.2 g/kg_{meat}	Decrease in hexanal and TBARS content during processing and storage at 4°C in polyethylene bags (OTR > 2000 cm^3/m^2d). Lower efficacy than rosemary extract. NISSEN *et al.*, 2004
Beef and pork patties (pre-cooked)	Grape seed extract powder (TP: 980 g/kg) Integration: 0.1-0.2 g/kg_{meat}	Decrease in TBARS content during processing and storage in polyvinyl chloride bags (OTR: 880 cm^3/m^2d) at 4°C. ROJAS and BREWER, 2007
Pork patties (pre-cooked)	Grape seed extract powder (TP: 865 g/kg) Integration: 0.05-1 g/kg_{meat}	Decrease in TBARS content during processing and storage in barrier film packs (OTR: 3 cm^3/m^2d) under 75% O_2 and 25% CO_2, at 4°C. CARPENTER *et al.*, 2007
Beef ground meat (pre-cooked)	Grape seed extract powder (TP: not specified) Integration: 10 g/kg_{meat}	Reduced numbers of *Escherichia coli* O157:H7, *Salmonella typhimurium*, *Listeria monocytogenes* and *Aeromonas hydrophila* during storage in bags (OTR: not specified) at 4°C. AHN *et al.*, 2007
Beef sausage (pre-cooked)	Grape seed extract powder (TP: 800 - 990 g/kg) Integration: 0.1-0.5 g/kg_{meat}	Decrease in TBARS content during processing and storage in polyvinyl chloride bags (OTR: 880 cm^3/m^2d) at -20°C. Higher efficacy than ascorbate. KULKARNI *et al.*, 2011

OTR: oxygen transmission rate; TBARS: thiobarbituric acid reactive substances; TDF: total dietary fibre; TP: total phenolics (expressed as GAE: gallic acid equivalents or phenol Eq: phenol equivalents or PAs: proanthocyanidins).

Considering pork meat, red grape skin extract powder added at a level of 0.2 g/kg_{meat} enhanced the oxidative stability of cooked patties during processing and refrigerated storage under air but showed lower efficacy than rosemary extract, as observed for chicken meat (NISSEN *et al.*, 2004). Grape seed extract appears to be more effective than grape skin extract. In fact, grape seed extract powder added at a level of 0.1-0.2 g/kg_{meat} had better antioxidant effects compared to rosemary oleoresin and oregano water extract during the processing and refrigerated storage under air of cooked beef and pork patties (ROJAS and BREWER, 2007). Grape seed extract powder is also an effective antioxidant in cooked pork patties during processing and frozen storage under a high-oxygen atmosphere, when added at a concentration as low as 0.05 g/kg_{meat} (CARPENTER *et al.*, 2007).

In ground beef, grape seed extract powder has been demonstrated to inhibit the growth of *Escherichia coli* O157:H7, *Salmonella typhimurium*, *Listeria monocytogenes* and *Aeromonas hydrophila* (AHN *et al*., 2007) at a level of 10 g/kg_{meat} during refrigerated storage. However, the required addition of grape seed extract is higher than the effective amount for the inhibition of meat oxidation. In fact, at an extract concentration of 0.5-1 g/kg_{meat}, grape seed extract powder prevented the oxidation of beef sausage during processing and frozen storage under air, more effectively than ascorbic acid (KULKARNI *et al*., 2011).

2.1.2 Fish products

Fish tissues have a high content of polyunsaturated fatty acids (PUFA), which undergo degradation via auto-oxidation. The use of natural antioxidants has become as an effective strategy for controlling the stability of these products, either during the frozen storage of minced tissue or during the processing and refrigerated storage of pre-cooked fish-based products. For this purpose, winemaking by-products have also been considered (Table 2).

Table 2: Applications of winemaking by-products as new food ingredients in fish products.

Food product	Recovered ingredient	Main results and references
Atlantic mackerel minced muscle (uncooked)	Phenolic fractions of white grape pomace. Integration: 0.1 g monomeric flavonoids or PAs/kg_{fish}	Longer induction period for the formation of peroxides and aldehydes during storage in Erlenmeyer flasks under air at -10°C. Maximum protection by PAs with high degree of polymerization and percentage of galloylation. PAZOS *et al*., 2005
Horse mackerel minced muscle (uncooked)	White grape pomace antioxidant dietary fibre (particle size < 0.25 mm; TDF: 760 g/kg; soluble TP: 78 g GAE/kg) Integration: 20 - 40 g/kg_{fish}	Inhibition of formation of conjugated dienes and trienes and TBARS during storage in Cryovac BB4L bags (OTR: 30 cm^3/m^2d) at -20°C. Significant antioxidant activity and high fibre content. SANCHEZ-ALONSO *et al*., 2008
Chub mackerel minced muscle (uncooked)	Red grape seed extract (soluble TP: 66 g GAE/kg) Integration: 20 g/kg_{fish}	Inhibition of lipid hydroperoxides and TBARS formation during storage in cartoon trays under air at -20°C. OZEN *et al*., 2011
Meagre sausage (pre-cooked)	White grape skin antioxidant dietary fibre (particle size < 1 mm; TDF: 820 g/kg; soluble TP: 42 g GAE/kg) Integration: 30 g/kg_{fish}	Inhibition of TBARS formation and oxidation during storage in barrier bags (OTR: < 2.1 cm^3/m^2d) at 2°C. Significant antioxidant activity and high fibre content. Antimicrobial effect on H_2S producers and a reduction in total viable counts. RIBEIRO *et al*., 2013

OTR: oxygen transmission rate; TBARS: thiobarbituric acid reactive substances; TDF: total dietary fibre; TP: total phenolics (expressed as GAE: gallic acid equivalents or sum of monomeric flavonoids or PAs: proanthocyanidins).

Fractionated grape pomace phenolic compounds at a concentration of 0.1 g/kg_{fish} have been proposed as inhibitors for fatty fish species, such as the muscle of Atlantic mackerel (*Scomber scombrus*) during frozen storage under air. The induction period for the formation

of peroxides and aldehydes was significantly increased in samples treated with grape phenolic fractions, and the maximum protection was achieved using procyanidins with a high degree of polymerization and percentage of galloylation (PAZOS *et al*., 2005). Non-fractionated grape seed phenolics also increased the oxidative stability of minced fish during frozen storage under air (OZEN *et al*., 2011).
The antioxidant dietary fibre obtained from white grape pomace with a particle size < 0.25 mm (total dietary fibre: 760 g/kg; soluble phenolics: 78 g/kg) at a concentration of 20-40 g/kg_{fish} can also increase the oxidative stability of the minced muscle of horse mackerel (*Trachurus trachurus*) during frozen storage under a low oxygen atmosphere (SANCHEZ-ALONSO *et al*., 2008). Similarly, white grape skin antioxidant dietary fibre with a particle size < 1 mm (total dietary fibre: 820 g/kg; soluble phenolics: 42 g/kg), which was used at a concentration of 30 g/kg_{fish} in precooked meagre (*Argyrosomus regius*) sausage, showed antioxidant effects as well as antimicrobial effects on H_2S producer counts and total viable counts, during refrigerated storage under a low oxygen atmosphere (RIBEIRO *et al*., 2013).

2.1.3 Bakery products and pasta

Bread is a staple food, and fortification with polyphenols and dietary fibre derived from winemaking by-products has been investigated to improve the diet of consumers (Table 3).
Grape seed extract powder added at a level of 0.6-2 g/kg_{bread} greatly increased the antioxidant activity of the final product, despite the loss of phenolic compounds during processing due to either thermal treatment or interaction with the food matrix. Interestingly, a decreased amount of N-(carboxymethyl) lysine, an advanced glycation end-product associated with health risks, was observed in bread containing grape seed extract. Moreover, the addition of grape seed extract powder did not significantly affect the hardness of bread but did increase the darkness (PENG *et al*., 2010). To obtain another vehicle for grape seed phenolics, the entire grape seed can be milled to fine particle sizes (< 0.150 mm). However, upon the addition of this ingredient (total dietary fibre and total phenolic contents not specified) to dough at a level of 25-100 $g/kg_{flour,}$ a decrease in loaf brightness and volume, along with an increase in hardness and porosity, was observed (HOYE and ROSS, 2011). These effects were likely due to the inhibition of yeast activity, which reduced the gassing power. Moreover, phenolics can inhibit the activity of endogenous amylases in dough, leading to inadequate maltose release for yeast activity during proofing (MILDNER-SZKUDLARZ *et al*., 2011). Because grape phenolics also inhibit mammalian α-glucosidase and α-amylase, white grape skin antioxidant dietary fibre with particle sizes < 0.250 mm (soluble phenolics: 20.0 g/kg) at a level of 100 g/kg_{flour} has been used in functional flat bread for diabetic people (LAVELLI *et al*., 2016a). Sourdough fermentation improves the textural properties of wheat and rye breads. Hence, the fortification of mixed wheat-rye bread with red grape pomace dietary fibre (total dietary fibre: 593 g/kg; soluble phenolics: 58.9 g/kg) has been investigated (MILDNER-SZKUDLARZ *et al*., 2011). An increase in hardness, gumminess and springiness was once again observed in wheat-rye mixed sourdough bread, but the cohesiveness and resilience did not change (MILDNER-SZKUDLARZ *et al*., 2011).
To increase the fibre and/or phenolic content, the fortification of brownies and biscuits with winemaking by-products has been achieved. In brownies, upon addition of 150-250 g/kg_{flour} of red grape pomace antioxidant dietary fibre with particle size < 0.589 mm (total dietary fibre and total phenolic contents not specified), hardness and chewiness decreased, while springiness increased. Hence, the fortification of brownies showed an opposite trend compared to bread fortification, likely due to the presence of fat (WALKER *et al*., 2014).

Table 3. Application of winemaking by-products as new food ingredients in bakery products and pasta.

Food product	Recovered ingredient	Main results and references
Wheat bread	Grape seed extract powder TP: not specified Integration: 0.6-2 g/kg$_{bread}$	Decreased level of N-(carboxymethyl)lysine, an advanced glycated end-product related to health risk. No significant difference in hardness. Increase in darkness. PENG *et al.*, 2010
Wheat bread	Grape seed antioxidant dietary fibre (particle size < 0.150 mm; TDF and TP: not specified) Integration: 25-100 g/kg$_{flour}$	Decrease in loaf volume, and increase in hardness and porosity. HOYE and ROSS, 2011
Wheat bread	White grape skin antioxidant dietary fibre (particle size < 0.25 mm; soluble TP: 1.5 g monomeric flavonoids/kg and 18.5 g PAs/kg) Integration: 100 g/kg$_{flour}$	Inhibition of mammalian α-glucosidase and α-amylase. LAVELLI *et al.*, 2016a
Wheat-rye bread (sourdough)	Red grape pomace antioxidant dietary fibre (particle size not specified; TDF: 593 g/kg; soluble TP: 58.9 g GAE/kg). Integration: 40-100 /kg$_{flour}$	Significant increase in TDF and TP. Increase in hardness, gumminess and springiness. No change in cohesiveness and resilience. MILDNER-SZKUDLARZ *et al.*, 2011
Brownies	Red grape pomace antioxidant dietary fibre (particle size < 0.589 mm; TDF and TP: not specified). Integration: 150-250 g/kg$_{flour}$	Decrease in firmness and chewiness and increase in springiness. WALKER *et al.*, 2014
Biscuits	Grape seed extract encapsulated in mesquite gum, zein and maltodextrin (soluble TP: 21 g GAE/kg) Integration: 0.6 g TP/kg$_{dough}$	Increase in TP content and thermal stability Partial masking of darkness DAVIDOV-PARDO *et al.*, 2012
Biscuits	White grape pomace antioxidant dietary fibre (particle size < 0.150 mm; TDF: 509 g/kg; soluble TP: 31 g GAE/kg) Integration: 100 - 300 g/kg$_{flour}$	Significant increase in TDF and TP. Decrease in hardness, brightness and yellowness MILDNER-SZKUDLARZ *et al.*, 2013
Biscuits	Red grape marc extract (soluble TP: 2.1 g GAE/L) Integration: 450 mL/kg$_{semolina}$	Increase in TP and antioxidant activity Increase in compounds derived from the Maillard reaction, except pyrazines, and lipid oxidation PASQUALONE *et al.*, 2014
Pasta	Red grape pomace antioxidant dietary fibre (particle size < 0.811 mm; TDF: 689.5 g/kg; TP: not specified). Integration: 25-75 g/kg$_{flour}$	Increase in TP and antioxidant activity Increase in cooking loss SANT'ANNA *et al.*, 2014
Pasta	Red grape marc (soluble TP: 4.43 g GAE/kg) Integration: 300 g/kg$_{semolina}$	Increase in TP and antioxidant activity Decrease in cooking loss MARINELLI *et al.*, 2015

TDF: total dietary fibre content; TP: total phenolic content (expressed as GAE: gallic acid equivalents or sum of monomeric flavonoids or PAs: proanthocyanidins).

Regarding biscuits, the addition of white grape pomace antioxidant dietary fibre with particle size < 0.150 mm (total dietary fibre: 509 g/kg; soluble phenolics: 31 g/kg) up to 300 g/kg$_{flour}$ led to a decrease in hardness, brightness and yellowness (MILDNER-

SZKUDLARZ *et al*., 2013). In contrast, the incorporation of grape seed phenolics encapsulated with mesquite gum, zein and maltodextrin (soluble phenolics: 21 g/kg) partially masked the dark colour (DAVIDOV-PARDO *et al*., 2012). Alternatively, biscuits containing red grape extract (soluble phenolics: 2.1 g/L) at a level of 450 $mL/kg_{semolina}$ displayed a particular red colour and aromatic profile and possessed greater contents of Maillard-reaction compounds, except pyrazines, as well as higher amounts of compounds derived from lipid peroxidation than the control biscuits (PASQUALONE *et al*., 2014).
The fortification of pasta with winemaking by-products is also promising. Grape pomace antioxidant dietary fibre with particle size < 0.811 mm (total dietary fibre: 689.5 g/kg; total phenolic content not specified) formulated into pasta (25-75 g/kg_{flour}) increased the total phenolic content and antioxidant activity but also caused a slight increase in the cooking loss at high levels of addition. This effect could be attributed to changes in the gluten protein network due to the interference of grape pomace fibre, which reduces the gluten strength and interrupts the overall structure of pasta (SANT'ANNA *et al*., 2014). However, the incorporation of red grape marc extract (soluble phenolics: 4.43 g/kg) into pasta at a ratio of 1:10, w/v, led to an increase in the phenolic content and antioxidant activity, as well as improved cooking performance. In fact, the cooking loss decreased due to the presence of grape pomace phenolics, which were complexed with proteins around starch granules, encapsulating phenolic compounds during cooking and restricting excessive swelling and amylose diffusion. The fortified sample was also characterized by a low adhesiveness value due to the formation of a stronger gluten network in the presence of phenolics, which entrapped the starch granules, slowing down amylose release during cooking. However, the hardness of fortified pasta was similar to that of the control pasta (MARINELLI *et al*., 2015).

2.1.4 Fruit-based products

The replacement of synthetic additives with natural compounds such as winemaking by-products and the development of functional foods are emerging trends in the fruit processing industry (Table 4). When the phenolic extract of red grape pomace (soluble phenolics: 30 g/kg) was added to a model fruit gel at a concentration of 8.2 $g/kg_{mixture}$ (prior to concentration), a stable red colour and a marked increase in the antioxidant capacity were observed. These effects were maintained, even after storage for 24 weeks at room temperature, likely due to intermolecular associations between pectins and anthocyanins (MAIER *et al*., 2009a). The formulation of 0.01 g/kg_{juice} of white grape skin extract (phenolic content not specified) in model fruit juice containing *Lactobacillus rhamnosus*, *Bifidobacterium lactis* and *Lactobacillus paracasei* improved the stability of probiotic bacteria during storage due to the presence of a more stable anaerobic environment (SHAH *et al*., 2010). Red and white pomace extracts (soluble phenolics: 75-280 g/kg) were added to apple and orange juice to achieve a concentration of 20-100 g/kg_{juice} of phenolics, and antimicrobial effects toward microbial contaminants were observed, including *Zygosaccharomyces rouxii* and *Z. bailii* (SAGDIC *et al*., 2011). Alternatively, the use of antioxidant dietary fibre with particle size in the range of 0.125-0.5 mm obtained from white grape pomace (total dietary fibre: 505 g/kg; soluble phenolics: 30 g/kg; insoluble phenolics: 139 g/kg) imparted multiple functional effects. When this antioxidant- and fibre-rich ingredient was added to tomato puree at a level of 30 g/kg_{puree}, the reducing capacity and inhibitory effect of hyperglycaemia-induced damage increased due to the ability of grape phenolics to act as both oxygen-radical and carbonyl-radical scavengers (LAVELLI *et al*., 2014; TORRI *et al*., 2015).

Table 4: Application of winemaking by-products as new food ingredients in fruit-based products.

Food product	Recovered ingredient	Main results and references
Model fruit gel	Red grape pomace extract soluble TP: 30 g GAE/kg Integration: 8.2 g /$kg_{mixture}$ (before concentration)	Brilliant red colour and strong antioxidant capacity during storage at room temperature. MAIER *et al.*, 2009a
Model fruit juice containing probiotic bacteria	White grape skin extract TP: not specified Integration: 0.01 g/$kg_{mixture}$	Improved stability of the probiotic bacteria *Lactobacillus rhamnosus*, *Bifidobacterium lactis*, and *Lactobacillus paracasei* during storage. SHAH *et al.*, 2010
Orange and apple juices	Red and white pomace extracts (soluble TP: 75-280 g GAE/kg) Integration: 20-100 g/kg_{juice}	Antifungal activity towards *Zygosaccharomyces rouxii* and *Z. bailii* with variety-dependent efficacy. SAGDIC *et al.*, 2011
Tomato puree	White grape skin antioxidant dietary fibre (particle size in the range of: 0.125-0.5 mm; TDF: 505 g/kg; soluble TP: 30 g flavonoids/kg; insoluble TP: 139 g PAs/kg). Integration: 30 g/kg_{puree}	Increase in reducing capacity and potential ability to inhibit hyperglycaemia-induced damage. LAVELLI *et al.*,2014; TORRI *et al.*, 2015
Tomato puree	White grape skin antioxidant dietary fibre (particle size in the range of 0.125-0.5 mm; TDF: 505 g/kg; soluble TP: 22 g GAE/kg). Integration: 30 g/kg_{puree}	Increase in Bostwick consistency, storage (G) and loss (G) moduli, and complex viscosity (η*). LAVELLI *et al.*, 2015b
Apple-based fruit jelly	Red grape skin antioxidant dietary fibre (particle size in the range: 0.125 - 0.5 mm; TDF: 600 g/kg; soluble TP: 26 g flavonoids/kg). Integration: 63 g/$kg_{mixture}$ (before concentration)	Increase in puncture energy, i.e., stronger texture. Requires less dehydration. High antioxidant and fibre content. CAPPA *et al.*, 2015

TDF: total dietary fibre content; TP: total phenolic content (expressed as GAE: gallic acid equivalents or sum of flavonoids or PAs: proanthocyanidins).

In addition, the fibre content increased, which improved the Bostwick consistency, storage and loss moduli and complex viscosity, providing a means to modulate the textural properties of puree (LAVELLI *et al.*, 2015b). A similar antioxidant- and fibre-rich ingredient obtained from red pomace with particle size in the range of 0.125-0.5 mm (total dietary fibre: 600 g/kg; soluble phenolics: 26 g/kg) added at a level of 63 g/$kg_{mixture}$ to apple-based candy modified the textural properties, resulting in a stronger structure that required greater puncture and penetration energy. As a result, during candy processing, the dehydration step could be reduced (CAPPA *et al.*, 2015).

2.1.5 Dairy products

The addition of fruit phenolics into food products has increased due to the emerging popularity of functional foods, including phenolic addition in the dairy sector (O'CONNELL and FOX, 2001). The fortification of phenolics into dairy products may increase the heat and foam stability of milk and enhance nutritional benefits (O'CONNELL and FOX, 2001). However, many phenolics are bitter and astringent (VIDAL *et al.,* 2004), and humans instinctively reject bitter substances (DREWNOWSKI

and GOMEZ-CARNEROS, 2000). Although phenolic compounds interact with proteins during the cheesemaking process, these interactions are dependent on the pH and molar ratio and molecular properties of the polyphenols and involve both hydrophobic and hydrophilic bonds (FELIX DA SILVA *et al.,* 2015). The application of winemaking by-products to the dairy sector is shown in Table 5.

Table 5: Application of winemaking by-products as new food ingredients in dairy products.

Food product	Recovered ingredient	Main results and references
Cheese	Single phenolic compounds and whole grape extract Integration: 0.5 mg single phenolic or GAE/mL_{milk}	Slightly reduced hydration capacity of cheese curd network. No variation in gel strength. Less smooth and less dense internal structure of cheese curds. More granular outer surfaces than control cheese by SEM microstructure analysis. HAN *et al.*, 2011a
Cheese	Single phenolic compounds and whole grape extract Integration: 0.5 mg single phenolic or GAE/mL_{milk}	High retention of grape phenolics in the curd. High radical scavenging activity. Increase in gel-formation rate due to a slight decrease in pH. HAN *et al.*, 2011b
Cheese	Red and white grape pomace antioxidant dietary fibre before and after distillation (particle size < 0.25 mm; TP: 3.64-16.0 g GAE/kg). Integration: 8 and 16 g/kg_{curd}	Highest radical scavenging activity and TP content, yielding 16 g/kg of grape powder after distillation. No effect on lactic bacteria or proteolysis. MARCHIANI *et al.*, 2015
Yogurt and salad dressing	Red grape pomace antioxidant dietary fibre (particle size < 0.18 mm; TDF: 613.2 g/kg, TP: 67 g GAE/kg). Integration: 10-30 g/kg_{yogurt}; 5-10 g/$kg_{salad\ dressing}$	Increase in TDF, TP and radical scavenging activity (but slight decrease in TP during storage at 4 °C). Decrease in peroxide values for both yogurt and salad dressing. Stable value for lactic acid percentage and syneresis during 3 weeks of storage at 4 °C for yogurt. TSENG and ZHAO, 2013
Yogurt	Grape seed extract TP: 76 - 150 g GAE/kg Integration: 50-100 mg TP/kg_{yogurt}	Increase in TP (slight decrease in TP during storage at 4 °C). No change in pH or Lactobacilli counts. CHOUCHOULI *et al.*, 2013
Yogurt	Red and white grape skin antioxidant dietary fibre (particle size < 0.25 mm; TDF: 345-481 g/kg). Integration: 60 g/kg_{yogurt}	Increase in acidity, total phenolic content and antioxidant activity with respect to control, but lower pH, syneresis and fat. Lactic acid bacteria, phenolic content and antioxidant activity were stable during 3 weeks of storage. MARCHIANI *et al.*, 2016
Milk	Grape seed extract powder TP: 842-927 g/kg Integration: 2 g/L_{milk}	Phenolic content equal to that of one serving of fresh apple. Increase in potential health benefit. AXTEN *et al.,* 2008

TDF: total dietary fibre content; TP: total phenolic content (expressed as GAE: gallic acid equivalents).

HAN *et al.* (2011a,b) studied the effect of single phenolic compounds (catechin, epigallocatechingallate, tannic acid, homovanillic acid, hesperetine and flavone) and natural extracts, such as whole grape extract, green tea extract and dehydrated cranberry powder as functional ingredients in cheese at a level of 0.5 mg of total phenolics/mL_{milk}. Three gel-forming parameters were evaluated, including T_{lag} (the lag-time before the beginning of milk coagulation), V_{max} (the maximum rate of gel formation) and TV_{max} (the time required to reach the maximum rate of gel formation). The results demonstrated that the addition of phenolic compounds to milk affected all of the gel-forming parameters. In fact, the change in pH due to the addition of phenolics resulted in faster gel formation in cheese samples fortified with single phenolic compounds than those fortified with natural extracts. In addition, several phenolic sources improved the antioxidant properties of cheese products. Among the tested single phenolic compounds and natural extracts, the greatest radical scavenging activity was achieved using whole grape extracts, likely due to the improved retention of grape phenolics in the curd, representing a promising utilization of winemaking by-products.

MARCHIANI *et al.* (2015) used antioxidant dietary fibre obtained from three grape pomaces (Barbera, Chardonnay prior to distillation and Chardonnay after distillation) with particle size < 0.25 mm (soluble phenolics: 3.64-16.0 g/kg) in semi-hard and hard cheeses (Italian Toma-like and Cheddar) to increase the content of phenolic compounds. Powders were added at two different concentrations (8 and 16 g/kg_{cheese}), and the results showed that the amount and type of powder did not significantly affect the physicochemical parameters of cheese, except the pH. Cheeses containing Chardonnay powder after distillation showed the highest phenolic content and radical scavenging activity at the end of ripening. Proteolysis and microbial counts did not show significant differences between fortified and control cheeses.

TSENG and ZHAO (2013) applied grape pomace antioxidant dietary fibre with particle size < 0.18 mm (total dietary fibre: 613.2 g/kg; soluble phenolics: 67 g/kg) at a level of 10-30 g/kg to yogurt and 5-10 g/kg to salad dressing to enhance the nutritional value and improve the storability. The authors demonstrated that this ingredient can be used as an alternative source of antioxidants and dietary fibre to delay the oxidation of lipids during refrigerated storage. However, the total phenolic content and radical scavenging activity of fortified yogurts decreased slightly during storage, likely due to interactions between proteins and phenolic compounds. Consequently, further research is necessary to determine the mechanism of the long-term retention of grape phenolics and radical scavenging activity of the aforementioned products.

CHOUCHOULI *et al.* (2013) used grape seed extracts from two grape varieties (50-100 mg of phenolics/kg_{yogurt}) to fortify full-fat and non-fat yogurt. The addition of the extract did not affect the pH or *Lactobacilli* count, while fortified yogurts showed higher antioxidant and antiradical activity than control samples, even after 3-4 weeks of cold storage. However, the phenolic content and radical scavenging activity decreased during storage.

The fortification of yogurt with up to 60 g/kg_{yogurt} of red and white grape skin antioxidant dietary fibre with particle size < 0.25 mm (total dietary fibre: 345-481 g/kg) was performed by MARCHIANI *et al.* (2016). In this case, a reduction in the total phenolic content did not occur during refrigerated storage, but the radical scavenging activity decreased. Grape addition did not affect the growth of lactic acid bacteria.

Moreover, UHT low fat milk was fortified with different grape seed extract powders to obtain a concentration of 2 g/L_{milk} of phenolics in the final product, representing the amount of phenolics ingested when consuming one serving of fresh apple (AXTEN *et al.*, 2008).

2.2. Sensory effects

Understanding the impact of new ingredients on consumers' perception is considered a key step in new product development (VERBEKE, 2006; TUORILA, 2007). Therefore, including a sensory- and consumer-based approach in product innovation is strategic for determining the properties that have the greatest effect on consumer preference, as well as relevant attributes that drive product optimization (TORRI *et al.*, 2016).

2.2.1 Meat products

The sensory effects of grape by-products on meat products have been thoroughly analysed in the literature. For chicken meat, grape skin extract added to dehydrated products (1 g/kg_{meat}) prevented changes in sensory attributes due to oxidation (hot wash, boiled chicken, subcutaneous fat and rancidity), with comparable efficacy to synthetic antioxidants and other natural antioxidants, such as rosemary, coffee and tea (NISSEN *et al.*, 2000). In chicken hamburger, the use of grape antioxidant fibre (5-20 g/kg_{meat}) increased the lipid stability without affecting the desirability of the odour, flavour, and tenderness during 5 days of storage. Only the colour was modified by the addition of grape skin extract (15-20 g/kg_{meat}), which did not affect the acceptability of samples. The sensory test comparing the effect of four different addition levels revealed that hamburgers preferred by consumers had the highest extract content (15-20 g/kg_{meat}) (SÀYAGO-AYERDI *et al.*, 2009). The incorporation of grape pomace extracts in raw and cooked chicken meatballs (60 mg of phenolics/kg_{meat}) also provided satisfactory results in terms of odour and flavour properties during frozen storage, which were not different from those observed using synthetic antioxidants (SELANI *et al.*, 2011).
Regarding pork meat, the addition of grape skin extract (0.2 g/kg_{meat}) to cooked patties was not sufficient to reduce the intensity of rancid and linseed odours and flavours correlated to lipid oxidation indexes (TBARS and hexanal content); thus, acceptable sensory properties for consumption could not be guaranteed (NISSEN *et al.*, 2004). Nevertheless, grape seed extract (0.1-0.2 g/kg_{meat}) has been proven to have a positive effect on both cooked pork and beef patties and was even more effective than rosemary oleoresin and oregano water-extracts. In fact, sensory evaluation performed over eight days of storage at 4°C demonstrated the efficiency of grape seed extract at controlling several negative sensory characteristics associated with a warmed-over flavour, such as rancidity, wet cardboard (for beef patties) and grassy (for beef and pork patties) odour descriptors (ROJAS and BREWER, 2007). Accordingly, the addition of grape seed extract (0.05-1 g/kg_{meat}) did not significantly affect the average scores obtained for cooked pork patties for any of the quality parameters evaluated (colour, flavour, texture, juiciness and off-flavour) over four days of storage at 4°C under a modified atmosphere (CARPENTER *et al.*, 2007).
In beef patties, grape seed extract (0.1-0.2 g/kg_{meat}) led to visual green discoloration (ROJAS and BREWER, 2007). This effect was also observed in precooked beef sausage containing grape seed extract (0.3-0.5 g/kg_{meat}). Moreover, grape seed extract improved the persistence of fresh cooked beef odour and flavour during the storage of fortified products with respect to the control group and prevented the formation of a rancid odour (KULKARNI *et al.*, 2011).

2.2.2 Fish products

Very little information is available on the sensory effects of grape by-products in fortified fish products. In fact, only one paper out of those cited in the present review included a sensory evaluation of fortified fish sausage. The sensory description of this product

showed that samples containing grape skin antioxidant dietary fibre (30 g/kg_{fish}) were significantly darker, less elastic, cohesive, succulent and oily and possessed a more unpleasant texture and flavour than the control product. The unpleasant odour and flavour has been described as a sour note, but a rancid aroma or flavour did not develop after 98 days of storage at refrigerated temperatures (RIBEIRO *et al.*, 2013).

2.2.3 Bakery products and pasta

Among the food categories considered in this review, cereal products have been the most commonly investigated from a sensory perspective. By adding different amounts of grape seed extract to white bread (0.6-2 g/kg_{bread}), significant alterations in quality attributes (sweetness, porosity, astringency and stickiness) were not detected (PENG *et al.*, 2010). The recommended replacement of hard red spring flour with grape seed antioxidant dietary fibre in bread was 50 g/kg_{flour}. Above this threshold, low sensory acceptance of astringency, sweetness, bitterness and overall liking was observed (HOYE and ROSS, 2011). Similarly, the overall acceptance for sourdough mixed rye bread decreased as the content of red grape pomace antioxidant dietary fibre increased from 40 to 100 g/kg_{flour}, indicating that a maximum of 60 g/kg_{flour} could be used to prepare acceptable products. Higher levels were associated with a decrease in the volume, porosity and typical aroma of freshly baked breads and an increase in hardness, acidity and alcoholic, sharp and fruity notes (MILDNER-SZKUDLARZ *et al.*, 2011).
Regarding brownies, fortification with red and white grape pomace antioxidant dietary fibre at levels higher than those acceptable for bread, up to 150 g/kg_{flour}, did not impact the sensory properties and acceptability of the products (WALKER *et al.*, 2014). The acceptance of biscuits enriched with white grape pomace antioxidant dietary fibre was also dependent on the level of addition: 100 g/kg_{flour} incorporation in wheat flour was adequate, while concentrations of 200 or 300 g/kg_{flour} induced a fruity-acidic note and an intense brown colour, which was undesirable to consumers (MILDNER-SZKUDLARZ *et al.*, 2013). However, microencapsulation of grape seed extracts prevented a decrease in the likeability ratings by consumers (DAVIDOV-PARDO *et al.*, 2012). Biscuits enriched with a red grape pomace extract (450 $mL/kg_{semolina}$) have been described by a trained panel of assessors as having a more intense colour, fruity odour and sour taste and lower friability than control samples. Moreover, consumers were able to discriminate among biscuits samples based on both colour and taste. However, neither the modifications in sensory profiles nor the differences perceived during the affective test influenced the acceptability or willingness of the subjects to buy anthocyanin-enriched biscuits. Regarding both the colour and taste, in a global evaluation, the number of consumers who preferred enriched biscuits was not significantly different than those who preferred control biscuits (PASQUALONE *et al.*, 2013).
In fettuccini pasta formulated with red grape pomace antioxidant dietary fibre (25-75 g/kg_{flour}), the overall liking and acceptance of aroma, aftertaste, flavour and appearance decreased, regardless of the concentration of the ingredient (SANT'ANNA *et al.*, 2014). However, upon addition of red grape pomace extract (300 $g/kg_{semolina}$), the sensory properties of fortified, fresh-extruded, fresh-pasteurized and dry spaghetti pasta were as acceptable as control products (MARINELLI *et al.*, 2015).

2.2.4 Fruit-based products

Information regarding the effect of grape by-products on the sensory properties of fruit-based products is scarce. The incorporation of different granulometric fractions of white grape skins into either smooth or rough tomato puree (30 g/kg_{puree}) induced a clear increase

in the textural attributes (crispiness and granularity), a decrease in the perceived homogeneity and a change in the vegetable odour notes (spicy hay). The intensity of these effects depended on the fraction particle size, which also influenced consumers' preferences. A cluster of subjects was found to significantly prefer the smallest particle size fraction (< 0.125 mm), especially when combined with smooth tomato puree, while another group of consumers showed opposite preferences, preferring the largest particle size (0.250-0.500 mm) and rough tomato puree cells (LAVELLI *et al*., 2014; TORRI *et al*., 2015).

2.2.5 Dairy products

A limited number of applications of grape pomace have been investigated in dairy products, especially in terms of sensory effects; however, yogurt fortified with winemaking by-products has been investigated. Fortification with grape seed extract amounts corresponding to 50-100 mg of total phenolics/kg_{yogurt} did not result in any major defects in sensory properties (consistency, colour and flavour) compared to control samples (CHOUCHOULI *et al.*, 2013). On the contrary, higher levels of addition strongly modified the sensory properties of yogurt, which was perceived as too sour by consumers, possessing an unpleasant flavour and grainy/sandy texture. However, significantly different hedonic scores were observed using by-products from different grape varieties, suggesting that Chardonnay was more suitable than Pinot noir and Muscat (MARCHIANI *et al.*, 2016). The fortification of yogurt with red grape pomace in the range of 10 to 20 g/kg_{yogurt} provided satisfying overall likeability values. Nevertheless, lower likeability scores for flavour and texture were observed for the sample with the highest concentration of grape pomace (TSENG and ZHAO, 2013). In salad dressing, the addition of red grape pomace antioxidant dietary fibre at a level of 10 g/$kg_{mixture}$ was best received by consumers (TSENG and ZHAO, 2013). Fortification with grape skin antioxidant dietary fibre clearly influenced the sensory properties of soft cow milk cheeses, especially the appearance and texture. In particular, the marbling aspect, granularity, sandiness, sourness and astringency (due to the presence of fibre and polyphenols from grape skin) negatively impacted the overall likeability of the cheese when the amount of Barbera and Chardonnay grape was greater than 8 and 16 g/kg_{curd}, respectively (TORRI *et al.*, 2016). The addition of grape seed extracts to low fat UHT milk had a significant effect on the product sensory attributes, tending to suppress sweetness and UHT odour and flavour and to increase the perception of bitterness, sourness, astringency, odours and flavours (fresh raisin, honey, inka, ashy and tobacco), as well as chalkiness (AXTEN *et al.*, 2008).

3. GRAPE SEED OIL EXTRACTION BY GREEN TECNOLOGIES

Grape seed oil extraction can be applied in parallel with the recovery of both antioxidant dietary fibre and phenolic extracts from grape skins and defatted grape seeds, thus making the overall recovery strategy more sustainable. With the recent technological advancements, "green technologies" have been proposed to replace the traditional oil recovery process by solvents.

The recovery of grape seed oil requires the preliminary separation of seeds from other grape pomace constituents, including skins and stalks. Separation occurs by mechanical devices, usually after grape pomace drying.

The conventional "non-green" solvent extraction allows for nearly complete oil recovery. Non-polar solvents are used for oil extraction, including *n*-hexane and petroleum ether, which are the most common extracting solvents and have the lowest cost. After extraction,

the oil must be separated from the solvent in which it is dissolved, and the solvent may be recycled. Separation is achieved by evaporating off the solvent. For *n*-hexane, evaporation occurs at 69 °C (the boiling point of *n*-hexane at ambient pressure).
High quality oil is obtained by mechanical extraction performed at ambient temperature. Unfortunately, mechanical extraction does not afford a high yield of oil, particularly for grape seeds, whose woody texture makes them mechanically resistant and, more importantly, whose oil content is reduced to the range of 4 to 17% (FERNANDES *et al.*, 2013; FIORI *et al.*, 2014). To increase the oil recovery, mechanical extraction can be performed at higher temperatures: however, at increased extraction temperatures, some of the noble oil constituents, which are thermally unstable, tend to degrade.
An emerging solvent for use in the food industry is high pressure CO_2, more precisely termed supercritical CO_2 (SC-CO_2) (DUBA and FIORI, 2015a). A fluid is in the supercritical state when the actual temperature and pressure are higher, respectively, than the critical temperature (T_c) and pressure (P_c) of the fluid. For CO_2, T_c and P_c are 31°C and 73 bar, respectively. Therefore, SC-CO_2 can be used while operating at temperatures only slightly higher than ambient temperature, making SC-CO_2 particularly interesting for thermally unstable compounds, which is often the case in the food sector.
An important advantage in the use of supercritical fluids as solvents is the extreme ease in separating the solvent and solute after extraction: separation occurs by simple depressurization. When using SC-CO_2 for extracting grape seed oil, after flowing SC-CO_2 is contacted with a static bed of milled grape seeds, the mono-phase stream consisting of SC-CO_2 and extracted and dissolved grape seed oil is expanded through a back-pressure valve. After expansion, low pressure gaseous CO_2 separates from grape seed oil, which is recovered solvent-free. Solvent-free defatted grape seeds can also be recovered and used for further food applications (LAVELLI *et al.*, 2015a). The process and corresponding equipment for an industrial-scale plant for SC-CO_2 extraction of grape seed oil have been fully explained in the literature (FIORI, 2010; FREITAS *et al.*, 2013). The effect of process parameters on the extraction kinetics and yield of the SC-CO_2 extraction of grape seed oil has been recently outlined (DUBA and FIORI, 2015b). Other obvious advantages of using SC-CO_2 in the food sector are represented by the peculiarities of CO_2, which is non-toxic, non-flammable and inexpensive.
Unfortunately, the proposed process includes drawbacks of an economic nature. The solubility of grape seed oil in SC-CO_2 is lower than 10 g_{oil}/kg_{CO2} for pressures lower than 350 bar (DUBA and FIORI, 2016). To achieve relatively high solubility values for oil in SC-CO_2, the equipment must be operated at not less than 400 bar, preferably at 500-600 bar, which translates into high investment costs for the facility, where the extractors must be employed at high pressure (FIORI, 2010).

4. CONCLUSIONS

The studies summarized in the present review demonstrate an increase in interest in potential food applications of winemaking by-products and provide a new production scenario for winemakers. Winemaking by-products can be processed into various food ingredients, including antioxidant dietary fibres, crude phenolic extracts or encapsulated extracts, and applied to produce new foods. Grape seed oil can be recovered using SC-CO_2 extraction. However, economic and regulatory factors prevent these applications from achieving large-scale application. First, the proposed applications imply new production cycles across winemaking and other food sectors. The establishment of these connections demand improved logistic organization, including appropriate technologies for the collection, storage, transportation and processing of grape pomace. Investment costs for

new processes are sometimes high. Second, although food legislation strongly promotes the recycling of by-products, a recovery strategy based on value addition and by-product use in functional food production results in additional regulatory issues. Actually, the application of by-products in foods generally leads to novel foods, which opens the debate regarding safety. Fortification/enrichment with the appropriate amount of grape antioxidant dietary fibre only allows for the labelling of foods as fibre-rich, while other possible health claims must be substantiated by specific studies. Thus, further scientific research is necessary to surpass economic and regulatory barriers and achieve significant advance towards the establishment of a biorefinery fed with winemaking by-products.

ACKNOWLEDGEMENTS

This research was supported by AGER (project number 2010-2222).

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Paper Received April 26, 2016 Accepted May 20, 2016

REVIEW

ORGANIC AND CONVENTIONAL FOODS: DIFFERENCES IN NUTRIENTS

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ABSTRACT

Organic agriculture represents a sustainable crop system focused on producing food without environmental degradation. Consumer confidence in organic food is based both in lowering environmental impacts and health diseases. The aim of the present review is to compare nutritional properties of agricultural products cultivated following organic and conventional procedures. The heterogeneity of published results does not permit to conclude definitely the higher presence of nutrients in organic foods; even there are clear evidences for some antioxidants. Comparative studies are challenging for the several factors influencing plant quality such as clime, soil type, cultivars and time of storage that should be considered. Holistic approaches whit nutritional, technological parameters and sensorial quality of food seem to be the best way for future comparative studies.

Keywords: food quality, nutrients, organic food, sustainable agriculture

1. INTRODUCTION

Organic agriculture consists of many practices that emphasize farming based on ecosystem management, integrated cropping and livestock systems, diversity of products, reliance on natural pest and disease control without the use of chemical inputs. The principal objectives of organic agriculture are to produce healthy and sustainable food only using biological and ecological processes (AZADI et al., 2011).
The increase in production and consumption of organic foods is one of the major market trends of last years. In fact U.S. sales of organic products were an estimated $28.4 billion in 2012 - over 4%of total food sales - and will reach an estimated $35 billion at the end of 2014 (USDA, 2014). In Europe, while sales in some countries were rather stagnating in 2012, others displayed a growth of more than 10% (Finland, Norway and the Netherlands) (FIBL, 2014).
Fresh fruits and vegetables have been the top selling category since the organic food industry started retailing products over 3 decades ago. Produce accounted for 43% of U.S. organic food sales in 2012, followed by dairy, packaged/prepared foods, beverages, bread/grains, snack foods, meat/fish/poultry and condiments.
The growth of organic agriculture, its production and trade needed of an increase in national and international legislation, in order to set the specific requirements and create the institutional framework for certification.
Organic farming and production has been regulated in the context of EU farm policy reform since 1991, when the European Council of Agricultural Ministers adopted Regulation (EEC) No 2092/91 on organic farming and labelling of organic farm produce and foods. Evolutions in the regulations are set by Council Regulation (EC) No 834/2007 defining the official EU aims, objectives and principles of organic farming and production, and by two implementing regulations (No 889/2008 and No 1235/2008) detailing the organic production, labelling and import rules. The principal topics regard sustainable management system for the respect of nature's cycle based on risk assessment and precautionary measures (exclusion of chemical fertilizers and mechanical treatments, respect of biodiversity, use of natural sources and respect of animal welfare standards).
In recent years, several studies confirm people belief in healthier properties of food from organic agriculture as consequence of the environmental-friendly management (HARPER and MAKATOUNI, 2002; YIRIDOEA et al., 2005). But until now, there is a lack of strong scientific evidences that organic food is significantly different from the conventional regarding nutritional properties and health impact. This is due to several important aspects that need to be considered in the experimental design of comparative studies and, consequently, in their interpretation. In fact, many factors impact the nutrient density of crops, whether they are grown organically or conventionally. Some factors impact both production systems equally, while a few factors tend to have a larger impact on one production system over the other. In order to carry out a valid comparison between organic and conventional agriculture food products at first, plants have to be of the same cultivar, and have to be cultivated in near farms, with similar soils, under similar climatic conditions (GASTOL and DOMAGALA-SWIATKIEWICZ, 2013). Furthermore, products must be sampled at the same time and pre-treated similarly, analyzed by accredited laboratories employing validated methods and results statistically treated. Generally, studies are divided into three classes: market-oriented studies, surveys and cultivation tests. In the first type of works, products are taken from organic and conventional shops; no information about origin, ripeness, variety, clime and condition of production is considered during comparisons. In surveys, instead, products derived from selected organic and conventional farms (SIDERER *et al*., 2005). Environmental factors and condition of production can be used in studies comparison selecting neighbouring farms,

but the precision of these parameters cannot be verified. The last category of works is represented by cultivation tests that permit to assess the difference in quality between organic and conventional products; unfortunately results can be applied only in the specific farm situation considered (RIGBY and CÀCERES, 2001).
Organic foods differ from the conventional ones predominantly because the absence of pesticides, fertilizers and heavy metals residues as application of regulated production rules; the majority of literature studies dealing with organic food quantify these compounds to verify the limits. The absence of pesticides use and nitrogen fertilization influences the production of bioactive compounds and plant metabolites; an example is given by those involved in the defensive mechanisms of plants. As consequence, a natural functional food is expected to have higher contents of health promoting substances if cultivated under organic agricultural system.
The present review focuses attention on such bioactive compounds, noted to improve human health, to evaluate if their content in foods can be use to discriminate between the two type of agriculture systems. For this scope, in this review we have considered the most relevant scientific papers published in the period 2000-2015, searched in three databases (Scopus, PubMed, Web of Science), written in English and comparing chemical composition of organic and conventional foods. The exposure terms searched were: "organic" and "conventional" combined with "food", "agricultural crops", "livestock", "agriculture" and terms for nutritionally relevant substances.
Results are organized on the base of polyunsaturated fatty acids, essential amino acids, vitamins, minerals and polyphenols contents.

2. ORGANIC *VS* CONVENTIONAL COMPARATIVE STUDIES

The debate about the differences in nutritional properties between organic and conventional food interested largely researchers, as shown by the consistent number of papers and reviews published in few years.
All the reviews existing about this topic reported different results. Some of these, concluded that organic foods have higher content of such constituent instead others underlined the absence of differences in nutritional values between the two alternatives (WOESE *et al*., 1997; BOURN and PRESCOTT, 2002; HUNTER *et al*., 2011; GASTOL and DOMAGALA-SWIATKIEWICZ 2013; JENSEN *et al*., 2013). The opposite outcomes were principally ascribed to the lack of coherence in study design and implementation. In fact, frequently, inaccurate comparisons led to assert the superior quality of organic respect to conventional foods.
At first, nutrient content is strictly affected by varieties; furthermore, other factors such as geographic locations of crops, characteristics of soil and clime, maturity from harvest to storage and testing must not be neglected in the way of comparison between organic and conventional agricultural methods. In the last years, systematic reviews were realized to compare the content of chemical compounds in different foods, checking at the same time for differences in study methodologies and implementation. In this way, the available scientific literature on the subject of interest is screened and the outcomes of all articles meeting predefined quality criteria, analysed by a systematic approach. (BENBROOK *et al*., 2008; DANGOUR *et al*., 2009; BRANDT *et al*., 2011; SMITH-SPANGLER *et al*., 2012; BARAŃSKI *et al*., 2014).
In 2008, the meta-analysis by Benbrook et al., analyzed differences in nutrient content between organic and conventional food samples within 236 matched pairs. Nutrients considered were Vitamin C, beta-carotene, Vitamin E, potassium and phosphorous, nitrates, total proteins, total phenolics, total antioxidant capacity, and the polyphenols

quercetin and kaempferol. This review found that total phenolics, vitamin E, vitamin C, quercetin, and total antioxidant capacity of organics exceeded that of conventionally grown produce in the case of total antioxidant capacity, by 80%. Conventional products had higher levels of potassium, phosphorous, and total protein, all basic constituents of conventional fertilizers.

DANGOUR *et al*. (2009) systematic review, based only on studies of satisfactory quality including field trials, farm surveys and basket studies, underlined the absence of differences in nutrient parameters between organically and conventionally produced foodstuffs. The small differences in nutrient content detected were ascribed as biologically plausible and generally related to differences in cultivation methods.

HUNTER *et al*. (2011) evaluated the micronutrient composition of organic and conventional plant foods with a systematic analysis. Organic plant foods (vegetables, legumes and fruit) were found to have a 5.7% higher content of vitamins and minerals than their conventionally grown counterparts. Irrespective of cultivar, soil type, harvest conditions, and chemical analysis, organic plant foods contained significantly higher amounts of minerals, including phosphorus, compared to conventional foods. These results were explained by the hypothesis of accelerated growth, as a result of conventional agricultural methods, that down-regulates the synthesis of carbon-containing metabolites, such as ascorbic acid. Furthermore, it has been proposed that organically produced plants synthesize higher levels of ascorbic acid than conventionally-grown plants, in response to biological and ecological stresses, and the absence of protection conferred by synthetic pesticides.

BRANDT *et al*. (2011) meta-analysis, focused on secondary metabolites and vitamins content in fruits and vegetables, collecting data from different studies had the scope to detect effects of systematic factors, separately from factors that occur randomly.

The results obtained show that organic plant material had higher levels of all analyzed secondary metabolites and of vitamins C than conventional vegetables and fruits, together with higher content of phenolic acids and total phenolics.

Instead, for the content of flavones and flavonols (non-defence-related compounds), differences between two cultivations systems were heterogeneous, while for the content of carotenes no significant differences were found.

The systematic analysis by SMITH-SPANGLER *et al*. (2012) based on 223 literature study on nutrient and contaminant levels underlined that heterogeneity in results were too high to estimate significantly higher nutritional properties of organic food. About pesticides residues, lower levels were found in organic than conventional product, but differences in risk for exceeding maximum allowed limits were small. Of the entire nutrient evaluated, only phosphorus content was homogeneous and significantly higher in organic food, but this difference was not statistically significant.

GASTOL and DOMAGALA-SWIATKIEWICZ (2013) made an evaluation on Polish organic and conventional fruit and vegetables juices. The results of this comparative study that covered 33 neighbouring pairs of organic/conventional fields with six evaluated species, confirmed the absence of differences in nutritional properties between the organic and conventional food analyzed. Only in few species, parameters such as polyphenolic content, antioxidant activity and dry matter significantly discriminate the two types of cultivation products.

The systematic review by JENSEN *et al*. (2013) dealt with the comparison between organic and conventional agriculture in terms of nutrients content, bioavailability of nutrients and potential effect on human health. About nutritional differences between the two type of agricultural products, results highlighted the difficulty of make direct comparison because of the variability of influencing factors and study designs. The approach of using a systematic review permitted to conclude that organic food contained higher levels of

vitamin E, vitamin C, phosphorus and lower content of pesticides than organic produce; no clear effects were established on health-related biomarkers.
The most recent systematic review by BARAŃSKI *et al*. (2014), based on an extensive data set of 343 peer-reviewed publications, indicated that organic crops and foods have a higher antioxidant activity and contain higher concentrations of a wide range of nutritionally desirable antioxidants/polyphenolics, but lower concentrations of Cd metal. This study, for plant secondary metabolites is in accordance with results carried out by BRANDT *et al*. (2011), but it contradicts the results of the most systematic reviews/meta-analyses published previously, which indicated that there are no significant composition differences between organic and conventional crops. The main reason for the inability of previous studies to detect composition differences was addicted, by the authors, probably to the highly limited number of studies or data sets available or included in the analyses, which could decrease the statistical power of the meta-analyses. In addition, authors used a weighted meta-analysis based on SMD (standardised mean difference), not used in most of the previous studies that is recommended when combining data from studies that measure the same parameter (e.g. the major phenolic compounds found in different crops), but use different scales.
Results of relevant scientific works considered in this review, from cultivation tests and surveys, are described below. Differences are underlined considering the content of functional compounds (polyunsaturated fatty acids, essential amino acids, vitamins and minerals, polyphenols) in organic and conventional foods.

2.1. Polyunsaturated fatty acids

The majority of studies dealing with PUFAs in conventional and organic products are focused on olive oil, for high contents in this matrix (Table1). GARCÍA-GONZÁLEZ *et al*. (2014) compared certified organic and conventional olive oil samples from different Spanish cultivars (*Arbequina, Cornicabra, Hojiblanca* and *Picual*). By application of multivariate algorithms (Principal Component Analysis and Multidimensional Scaling), authors concluded that fatty acids and sterols profile and content were only able to discriminate olive oil samples according to fruit cultivar. Differences in total PUFAs content were not statistically significant between organic (0.28±0.06 $mgKg^{-1}$) and conventional (0.28±0.07 $mgKg^{-1}$) oils. Also SAMMAN *et al*. (2008), comparing organic and conventional edible oils (coconut oil, olive oil, canola oil, mustard oil seed and sesame oil) found insignificant differences in the content of PUFAs. The influence of the cultivation method on the quality indices of virgin olive oils was also investigated by ANASTASOPOULOS *et al*. (2013). Olive oils (*Koroneiki cv*.) produced in different geographical origins and seasons, years 2000 and 2004, showed differences in PUFAs content. Those from organic production system and season 2004 had a PUFAs level (expressed as Mean±SD% on total content) of 79.25±0.18 that resulted significantly higher of 79.05±0.18, content of conventional products.
ROUPHAEL et al. (2015) investigated the presence of PUFAs in the seeds of Perilla, an annual plant of the mint family *Lamiaceae*.
Irrespective of the farming systems, the linolenic acid was the predominant fatty acid in seeds, representing 62% of the total fatty acids in the lipid fraction. No differences were recorded among treatments for the content of palmitic C16:0 (avg. 6.0%), stearic C18:0 (avg. 1.0%), and oleic C18:1 (avg. 14.0%), and linoleic acid (avg. 15.0%).

Table 1: Comparison of polyunsaturated fatty acids contents in organic and conventional foods.

References	Study design	Compounds analyzed	Compounds contents		Units	Statistical comparison
			organic	conventional		
García-González *et al*., 2014	16 samples of extra virgin olive oil of 4 Spanish cultivars taken from cooperative societies.	palmitic acid, palmitoleic acid, oleic acid, linoleic acid, linolenic acid, arachidic acid.	0.28±0.06	0.28±0.07	Means ± SD of PUFAs in mg kg^{-1}	No differences in PUFA content
Samman *et al*., 2008	59 certified organic and 53 conventional oils purchased from markets in Sydney. Edible oils considered: Coconut oil (1), Olive oil (2), Canola oil (3), Mustard seel oil (4), sesame oil (5).	palmitic acid, palmitoleic acid, oleic acid, linoleic acid, linolenic acid, arachidic acid.	(1) 2.63±0.86 (2)10.59±3.26 (3)25.73±9.08 (4)46.07±0.45 (5)48.2±1.09	3.86±0.46 12.09±9.6 29.51±0.53 28.21±13.79 44.18±3.8	Means ±SD % of PUFA on total fatty acids content	No differences in PUFA content
Anastasopoulos *et al*., 2013	Virgin olive oil (Koroneiki variety) produced in Messinia, Peloponnesus, Greece from different harvesting periods: season 2000(1) and 2004(2).	palmitic acid, palmitoleic acid,oleic acid, linoleic acid,linolenic acid,arachidic acid.	(1)77.43±1.95 (2)79.25±0.18	77.90±1.40 79.05±0.18	Means±SD% of PUFAs on total content	Higher PUFA content in organic olive oil
Rouphael *et al*., 2015	Perilla plants grown under conventional and organic farming in a typical Mediterranean area such as Southern Italy,season 2005.	palmitic acid oleic acid linoleic acid linolenic acid stearic acid	6.3 13.5 14.7 61.9 1.9	6.3 13.9 14.6 61.6 1.9	Means% of PUFAs on total content	No differences in PUFA content

2.2. Essential amino acids

Few literature studies investigated for differences in amino acids content in organic and conventional foods (Table 2). Furthermore, essential and non-essential amino acids were generally analyzed together in the same food. RÖHLING and ENGEL (2010) analyzed the influence of the input system on several metabolites of three maize cultivars (*Amadeo, Lukas and Flavi*). In the text numeric data results were not shown. Authors only reported results of statistical tools used: Principal component analysis (PCA) and analysis of variance (ANOVA). Amino acids levels, as well other compounds investigated (polar compounds, organic acids, sugar and sugar alcohols) showed the absence of a direct relationship between metabolites content and agricultural practice. Authors suggested that only genotype and environment contributed to differentiations in metabolite profiles of maize.

An absence of differences among organic and conventional food was also found by MADER *et al*. (2007) analyzing protein and amino acids content in wheat (*Triticum aestivum L.*). MAGGIO *et al*. (2008), investigating in tubers (*Agria* and *Merit cv*), found that the organically grown ones contained only significant higher levels of threonine (24.5 mg $100g^{-1}$ of Fresh Weight) respect to conventional tubers (24.0 mg $100g^{-1}$ of Fresh Weight).

Table 2: Comparison of essential aminoacids contents in organic and conventional foods.

Reference	Study design	Compounds analyzed	Compounds contents		Units	Statistical Comparison
			organic	conventional		
Rohling and Engel, 2010	Three maize cultivars grown in the season 2004 at two locations. The same procedure was repeated in the season 2005.	histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine	numeric data not reported.	numeric data not reported.	-	No differences in essential amino acids content
Mader *et al.*, 2007	Wheat (*Triticum aestivum* L.) grown in a 21 year agrosystem comparison in central Europe	histidine isoleucine leucine lysine methionine phenylalanine threonine tryptophan valine	47.2 23.1 34.6 67.1 26.3 13.5 45.9 31.0 11.4 42.8	46.6 22.8 35.4 67.0 25.4 13.3 46.4 30.5 10.8 42.8	means g kg^{-1} total protein	No differences in essential amino acids content
Maggio *et al.*, 2008	Tubers harvested on July, 2003. Six potatoes, collected from each plot, treated and analyzed	histidine isoleucine leucine lysine methionine phenylalanine threonine tryptophan valine	104.9 14.8 11.8 36.6 16.7 16.0 24.5 10.6 34.1	98.5 17.1 15.1 38.4 16.7 19.2 24.0 11.2 37.3	mg $100g^{-1}$ fresh Weight	Only threonine is in significant higher levels in organic tubers
Pieper and Barret, 2009	Processing tomatoes of the same cultivar (*Lycopersicon esculentum* var. AB2) grown and harvested in season 2006 (1)and 2007(2) from three commercial growers in California.	Phenylalanine Histidine Methionine Lysine Threonine	(2) 1.41±0.27 0.56±0.09 0.10±0.02 0.68±0.10 0.76±0.15	(1) 1.81±0.28 0.78±0.14 0.15±0.03 0.89±0.17 1.15±0.28	g kg^{-1} fresh weight	No differences in essential amino acids content

Conventional potatoes were grown with varying levels of nitrogen fertilization and as result, a significant decrease in some amino acids content (including alanine, glutamate and histidine) was correlated to a highest nitrogen levels used. Other amino acids levels varied principally with cultivar variety. PIEPER and BARRET (2009) analyzed the effect of production system on quality and nutrient content of tomatoes (*Lycopersicon esculentum var. AB2*) from two consequential years of cultivation, 2006 and 2007. Results indicated that differences in nutrient content were not statistically significant between production systems. Furthermore, non-essential amino acids such as glutamate, glutamine and tyrosine, were significantly higher in conventional tomatoes. Authors justified results obtained depending by the amount of available nitrogen, generally greater in conventional crops.

2.3. Vitamins and minerals

Many studies investigated micronutrient levels in organic and conventional products, due to their vital importance in human diet (Table 3).

Table 3: Comparison in minerals and vitamins content in organic and conventional foods.

References	Study design	Compounds analyzed	Compounds content		Units	Statistical Comparison
			organic	conventional		
Gastol *et al.*, 2013	66 fruit and vegetable fields (36 farms) producing organic and conventional crops	Cu, B, Fe, Mn, Zn, Ni, Pb, Cd, Ca, P, Mg, S, Na,	Numeric data not shown	Numeric data not shown	-	Higher amounts in organic product
Colla *et al.*, 2002	10 years of organic and conventional management practices on soil chemical properties, processing tomato yields and fruit mineral composition	N,P,K,Ca,Mg, Na	Numeric data not shown	Numeric data not shown	-	Organic fruit contain higher amounts of Ca and P
Laursen *et al.*, 2011	Samples of winter wheat,spring barley, faba bean, and potato(1), obtained from field trials undertaken in 2007 and 2008 at three different Danish geographical locations.	(1) K Mg P S Ca Fe Mn B Zn Cu Mb Sr Na	 2.28±0.24 0.11±0.01 0.24±0.03 0.16±0.01 180±67.4 21.0±7.66 6.16±1.01 4.46±0.46 11.6±0.77 5.30±0.99 0.35±0.07 0.75±0.20 69.3±16.0	 2.08±0.16 0.11±0.01 0.21±0.04 0.15±0.01 206±78.4 20.0±7.57 6.19±0.90 4.57±0.51 10.1±0.51 4.31±0.78 0.21±0.02 0.91±0.26 48.7±15.4	mg kg^{-1}	No differences in mineral levels
Gorenjak *et al.*,2012	52 samples of lettuce, conventional and organic, from 15 different areas of northeast Slovenia.	Nitrate/nitrite concentration	1258±1018.3	1359±960.6	mg kg^{-1} fresh weight basis	Nitrate levels higher in conventional foods
Ismail and Fun, 2003	5 types of green vegetables grown organically and conventionally selected based on popular consumption among Malaysian market.	vitamin C	124.80	114.70	mg 100 g^{-1}	No differences in vitamins content
Weibel *et al.*, 2000	Apples (Golden delicious cv) harvested of 5 pair of organic/conventional fruit farms with similar micro climate, soil condition and planting system.	P Vitamin C	Numeric data not shown Numeric data not shown	Numeric data not shown Numeric data not shown	-	No differences in vitamin C content

The most investigated essential elements were phosphorus (P), potassium (K), magnesium (Mg), iron (Fe), copper (Cu), sulphur (S), calcium (Ca), zinc (Zn) and sodium (Na). Differently by vitamins, minerals are present in the soil and are bio-available for plant acquisition. In conventional agricultural management many minerals (P, K and N), are commonly used in the form of soluble chemical fertilizers, so it could be expected a highest quantity of such minerals in conventional products than in the organic alternatives. But different results were obtained considering levels of minerals in organic and conventional fruit and vegetables (BRANDT *et al*., 2011). Many scientific studies showed how different agricultural systems have a strong influence on mineral content in products; in particular organic food were higher in such compounds content (Table 3). COLLA *et al*. (2002), investigating tomatoes elemental composition, reported only

statistical results from which, organically grown fruits had highest amounts of P and Ca instead of conventionally grown tomatoes richest in N and Na. GASTOL and DOMAGALA-SWIATKIEWICZ (2012), found none relationships between agricultural processes and minerals content in some fruits because of heterogeneity in results. Only in blackcurrant juice, results shown with the use of histograms, authors found significant higher amounts of minerals in the organic fruit. Further, a multi-element fingerprinting of potatoes and cereals, stated the absence of systematic differences between levels of minerals in products and different factors (crop management, location and year of cultivation) (LAURSEN *et al*., 2011). In Table 3 levels of minerals are indicated only for potatoes.

Also nitrate content was evaluated in organic food for the risks on human health. GORENJAK *et al*. (2012), estimated nitrate content in lettuce (*Lactuca sativa*) of different geographical origins. The mean of nitrate content, expressed as mg kg^{-1} of fresh weight basis, was significantly lower in organically cultivated lettuce (1258±1018.3) than in conventional products (1359±960.6).

On the contrary of minerals, plant itself is responsible for the production of vitamins depending manly by variety, ripeness of fruits and crop size. For such these factors, heterogeneous results came from research studies about the content of vitamins in organic and conventional plant foods. Some studies underlined a significant relation between vitamin C content in fruit and vegetables and organic procedure; others didn't found a consistent trend (WEIBEL *et al*., 2000; REMBIALKOWSKA, 2007). Few studies focused on the presence of other vitamins and their precursor.

ISMAIL and FUN (2003) determined vitamin C, β-carotene and riboflavin contents in five green vegetables from organic and conventional systems. Only organic swamp cabbage was highest in all vitamins content; for this vegetable only statistical results are shown. Organic Chinese mustard resulted in significant higher levels of β-carotene and in lower content of riboflavin. No significant differences were found in β-carotene content for Chinese kale, lettuce and spinach grown using the two different agricultural techniques while riboflavin content in conventionally grown Chinese kale and spinach was not detected compared to the organically grown vegetables.

To understand evidences of higher content of some vitamins in organic fruit and vegetables, influence on nitrogen fertilization was studied in deep. Nitrogen fertilization, belonged to conventional agriculture, was found to decrease vitamin C content in different fruits and vegetables (potatoes, tomatoes and citrus fruit) as well as increase beta-carotene content (MOZAFAR, 1993; LEE and KADER, 2000).

2.4. Polyphenols

Higher contents of polyphenolic compounds in organic fruits and vegetables were demonstrated in different reports (Table 4).

Organic apples (cv. Golden Delicious) originated from ten neighbouring organic and conventional fruit farms in Switzerland were examined by WEIBEL *et al*. (2000). The content of phenolic compounds (in particular flavanols) was 19% higher in organic apple.

The effect of cultivation methods on the antioxidant capacity of bluesberry (cv. *Vaccinum corymbosum* L.) was evaluated in random samples of commercial late harvest fileds in New Jersey by WANG *et al*. (2008). Results showed that blueberry fruit grown from organic culture contained significantly higher total phenolics (67.8%), total anthocyanins (59.2%), and antioxidant activity (49.8%) than fruit from the conventional culture.

Higher levels of some polyphenolic compounds were also found in organic peach (cv. Regina Bianca) and pear(cv. Williams) respect to the corresponding conventional samples (CARBONARO *et al*., 2002).

Table 4: Comparison of polyphenols contents in organic and conventional foods.

Reference	Study design	Compounds analyzed	Compounds content		Units	Statistical Comparison
			organic	conventional		
Weibel *et al.*, 2000	Apples harvested of 5 pair of organic-conventional fruit farms with similar micro climate, soil condition and planting system.	flavanols,cinnamon acids, phloretin-glycosides, quercetin-glycosides	only statistical result reported	only statistical result reported	-	Higher levels of phenolics in organic food
Wang *et al.*, 2008	Organic and conventional samples of blueberry collected from 5 certified organic farms in New Jersey.	anthocyanins: delphinidin 3gal. delphinidin 3-glu. cyanidin 3-gal. delphinidin 3-ara. petunidin 3-gal. petunidin 3-glu. petunidin 3-ara. malvidin 3-gal. malvidin 3-gluc. malvidin 3-ara.	 171.59 69.77 29.22 93.53 184.86 127.13 95.70 303.03 303.35 227.12	 41.74 24.64 14.24 37.00 75.26 79.00 66.70 289.38 184.92 199.62	μg g^{-1}	Higher levels of anthocyanins in organic food
Carbonaro *et al.*, 2002	Peaches (1) and pears (2), either grown on tilled soil (of the same age, 5 years), obtained from the Istituto Sperimentale per la Frutticoltura (Ciampino, Rome).	(1) Caffeic acid chlorogenic acid catechol α-tocopherol ϒ-tocopherol tocopherolquinone (2) caffeic acid chlorogenic acid catechol α-tocopherol ϒ-tocopherol tocopherolquinon	 2174.50±198.20 2655.30±171.20 - 0.57±0.01 0.37±0.01 1.34±0.03 865.1±43.8 3020.7±235.4 401.4±110.3 0.71±0.05* 0.68±0.15 2.0±0.10	 2451.9±126.4 2053.2±145.0 - 0.65±0.02 0.46±0.01 1.80±0.27 674.2±50.5 959.1±100.9 557.1±143.2 0.58±0.03 0.68±0.09 1.43±0.30	PPO activity (unit min-1/100 g f.w.) μg 100 g^{-1} fresh weight PPO activity (unit min-1/100 g f.w.) μg 100 g^{-1} fresh weight	Higher levels of polyphenolics in organic peach and pear
Lombardi-Boccia *et al.*, 2004	Yellow plums,conventionally or organically grown in the same farm (Fruit FarmingInstitute, Rome, Italy).	caffeic acid trans-*p*-cumarico acid ferulic acid chlorogenic acid neo-chlorogenic acid myricetin quercetin kaempferol	22.6±1.05 8.9±0.32 9.3±0.42 37.5±2.94 46.0±6.9 1.1±0.1 30.2±0.8 0.6±0.2	20.6±1.23 8.5±0.34 8.0±0.63 25.2±1.25 52.0±2.76 0.9±0.2 19.6±1.2 1.7±0.3	mg kg^{-1} fresh weight	Higher polyphenols content in conventional plums
Gastol *et al.*, 2013	66 fruit and vegetable fields (36 farms) producing organic and conventional crops.	Total polyphenols	Data not shown	Data not shown	-	No differences in polyphenols content
Valverde *et al.*, 2015	2 varieties of broccoli grown over 2 years (1),(2) in a split-plot factorial system comparison trial.	(1) Total phenolics total flavonoids	 345.70±51.30 16.60±6.90	 290.80±3.90 10.20±1.80	mg 100 g^{-1} fresh weight	No differences in polyphenols content
Granato *et al.*, 2015	Purple grape juices (n = 31) produced in Europe	Total phenolics	826.60±382.99	714.42±244.63	mg of chlorogenic acid equivalents per liter of juice	No differences in polyphenols content

In particular organic peach had statistically higher levels of chlorogenic acid (29.3%) and of total polyphenols (36.1%); organic pear had higher levels of total polyphenols (10.4%). and of caffeic acid (28.3%).
LOMBARDI-BOCCIA *et al*. (2004) found conventional plums (cv. *Shiro*) richest in total polyphenols content. Quercetin was higher in conventional plums (54.1%), but myrecitin (22.2%) and kaempferol (183.3%) were higher in organic plums; both in organic and conventional fruits caffeic acid, chlorogenic acid and quercetin were the predominant compounds. In opposite to these results, in vegetable juices from celery, carrot and red beet, GASTOL *et al*. (2013), did not found differences in polyphenolic content between organic and conventional products. In broccoli (*Brassica oleracea* var. *italica*), grown over two years of a split-plot factorial system trial, VALVERDE *et al*. (2015) didn't find differences in total phenols and flavonoids levels between the two agricultural systems. No differences in total phenolics contents was also found by GRANATO *et al*. (2015) analyzing organic and conventional purple grape juices.
Polyphenols, as phytonutrient compounds, are involved in the defensive mechanism of plants after attack by pests or diseases (FALLER and FIALHO, 2010). Many hypotheses were formulated to justify higher concentration of such compounds in organic crops and foods.
At first, plant subjected to stress, tend to accumulate higher content of secondary compounds. When pesticides are avoided, these compounds are also accumulating by natural protection system of plants. Furthermore, polyphenols are mainly synthesized during ripening of plant products. Conventional crop management consisting in higher amounts of nitrogen fertilizers than organic farming, generally accelerates plant growth with the consequent decreasing of plant metabolites production.

4. CONCLUSIONS

Food quality aspects, human health and environmental concerns influence organic food consumer preferences. The rapid growth of organic market, in recent years, is due principally to people belief of more nutritive properties of foods from organic agriculture. In this regard, literature studies considered in this review, dealing with the comparison of nutritive properties between organic and conventional fruits and vegetables, showed a high variability in results. Small differences in nutritive contents exist in foodstuffs belonged to the different cultivation methods. But in almost every study claiming large nutritional and sensory quality differences between organically and conventionally grown produce, the experimenters failed to control or to "pair-up" similar environmental and cultivar inputs that affect plant and fruit development, yield and quality. When this lack of methodological rigor was overcome by the application of a systematic review and meta-analysis approach, significant higher levels of antioxidant compounds and lower cadmium levels in organic food products were demonstrated. A way to improve comparative researches on nutritional value of organic food is to unify the methodologies applied and to better consider the various factors influencing nutrients content of agricultural food. There is also the need to include analysis of food during every step of the production chain and to consider further the effect of processing on nutritional parameters content. In accordance to this aspect, some authors suggest for future studies, the holistic approach in which nutritional and technological parameters together with sensor quality are needed to evaluate in total quality of foods (KÄHL *et al*., 2010).

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Paper Received July 27, 2015 Accepted April 14, 2016

PAPER

CHEMICAL, NUTRITIONAL, PHYSICAL AND ANTIOXIDANT PROPERTIES OF *PECORINO D'ABRUZZO CHEESE*

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ABSTRACT

Proximate composition, minerals, cholesterol, fat soluble vitamins, fatty acids (FA), colour and antioxidant properties of *Pecorino d'Abruzzo* cheese, manufactured at three different months, were investigated. Protein, calcium ($P \leq 0.05$) and polyunsaturated FA content was higher in winter samples than in summer, whereas cholesterol and total fat were lower. Summer cheese samples showed a lower content in saturated FA (55.4%, $P \leq 0.05$) and higher content of monounsaturated FA (38.3%, $P \leq 0.05$) than winter samples. Colour and FT-IR spectra varied ($P \leq 0.05$) seasonally. The dairy products supplied a good level of coverage (%) of some nutrients that are daily required according to Italian recommendations.

Keywords: fatty acids, antiradical activity, cholesterol, minerals, colour parameters, fat soluble vitamins

1. INTRODUCTION

Traditional food products, closely linked to a system of historic and cultural traditions and connected to a specific geographical area, have an important role in maintaining and supporting agro-biodiversity as well as sustainability. Traditional food products are a good source of nutrients, and the levels thereof can be influenced by a range of parameters related to the environment, as well as to food preparation and processing.
Generally speaking, *Pecorino* is a term indicating cheeses made of raw or thermised ewes' milk, among which there are cheeses with a defined geographical origin or labelled as Protected Designations of Origin (PDO) reporting also the specification of the Region, e.g., *romano, sardo, siciliano*.
In this study, the name of "*Pecorino d'Abruzzo"* cheese indicates an Italian traditional food product, recognised by the Italian Ministry of Agriculture, Food and Forest (MiPAAF). This hard or semi-hard cheese, made from ewes' milk, is a local produce linked to the sheep breeding tradition of the homonymous Italian region (*Abruzzo*) and it is processed according to a traditional cheese-making technique in farms located in mountain areas.
The quality of a product is expression of its own nutrient composition. The information on the physicochemical and nutritional characteristics of "*Pecorino d'Abruzzo"* is scarce or currently not available. In addition, there are many literature references on Fatty Acid (FA) profile and Conjugated Linoleic Acid **(**CLA) contents applying to *Pecorino* cheese in general, however the information on seasonal variation of other nutrients is very limited.
According to some authors, variations in milk and cheese ewe's composition may occur due to different factors. RAYNAL-LJUTOVAC *et al.* (2008) reported that protein content for ewes' milk can be different depending on the stage of lactation, season, age and animal feeding. JAEGGI *et al.* (2005) studied the influence of seasonal changes in ovine milk, in details, on the composition and yield of hard-pressed cheese, and found significant differences in the physico-chemical characteristics of the ovine milk collected at different times of the year (February, May and August), as well as in the cheese produced with it. TODARO *et al.* (2014) also reported that the season greatly affects the quality of milk and cheese, as it is the case of the milk and cheese produced by sheeps in *Valle del Belice*, mainly fed at pasture for most of the year. ADDIS *et al*. (2015) analyzed Pecorino cheeses manufactured from March to June and they also found that the month of production affected cheese composition. PRANDINI *et al.* (2011) reported that ewes' milk and products thereof are richer in CLA than other cheeses (e.g. cows, goats). NUDDA *et al*. (2005) reported substantial differences in the fatty acids pattern in ewes' cheeses from March to June, and explained that the progressive variation of fatty acid profile was due to the temporal variation of the animal diet, in terms of grass matured. The most significant seasonal variations were found by DE LA FUENTE *et al.* (2009) in polyunsaturated FA (in particular CLA and linolenic acid) contents, with the highest values occurring in spring and summer and the lowest in winter.
Based on the above, this research, that falls within the framework of the Italian project "TERRAVITA" (MiPAAF), investigated the difference between chemical, physical and nutritional characteristics of "*Pecorino d'Abruzzo"* cheeses manufactured at different months (February, July, December). In particular, macronutrients, minerals (sodium, calcium, potassium, magnesium and phosphorous), vitamin A and E, cholesterol, antioxidant properties and colour (CIE L*a*b*) parameters were studied, in order to update available data on composition and nutritional properties of this dairy product and investigate how and/or if these results are influenced by the manufacturing season.
Finally, a qualitative analysis of the major functional groups was also performed by Fourier Transformed Infrared Spectroscopy (FTIR) on Attenuated Total Reflectance (ATR) to show the fingerprint of these cheeses.

2. MATERIALS AND METHODS

2.1. Samples

Traditional *Pecorino* cheeses were supplied by a farm located at *Anversa degli Abruzzi*, in the province of *L'Aquila* (Italy), at 40 km from the two most important National Parks of the region: the Majella Park and the National Park of *Abruzzo*. This organic farm was founded in 1977 and generation by generation followed traditional farming practices, typical of that geographical area, in the production of their products.
The *Pecorino d'Abruzzo* cheeses samples are made of raw organic ewes' milk, rennet and salt, and are sold with the label "made with organic". They contain, apart from water and salt, at least 95% percent organically produced ingredients. Besides the manufacturer's name - or transformation/distribution company - the product shows in the label the code of the national control expressly authorized by the Italian Ministry of Agriculture, Food and Forestry (MiPAAF) (EU REGULATION, No. 271, 24 March 2010).
In this research, *Pecorino d'Abruzzo* cheeses, made by ewes' milk collected in different seasons, were studied: the PEC-A and PEC-C batches belonged to cheeses manufactured in winter time (February and December, respectively), whereas the PEC-B batch was produced in summer (July). For each batch (PEC-A, PEC-B and PEC-C), three different samples were analyzed.
Regarding the manufacture of *Pecorino d'Abruzzo* cheeses, after milking the ewes' milk is warmed up to 38°C, and natural microflora and lamb rennet paste are added. The milk is left curdling for less than one hour, and then the curd is broken, pressed with hands and fitted into special baskets (*'fiscelle' in Italian*) to facilitate whey drainage. Afterwards, it is salted and stored into ripening rooms. If the maturing process lasts over one month, the rounds periodically grease with olive oil.
After purchase, the cheeses under investigation were sampled in appropriate aliquots and stored at -20°C until analysis.

2.2. Chemicals

All used reagents were of HPLC grade or at least of the highest available purity. Standards were obtained from Sigma Aldrich (St Louis, MO, USA) and Merck KGaA (Darmstadt, Germany). Ultrapure water, of the grade required for critical laboratory applications, was prepared by an ion exchange system to >18 mΩ resistivity (ElgaPurelab Ultra, Veolia, UK).

2.3. Analytical Methods

2.3.1 Proximate composition

Water, protein, fat and ash contents were determined according to the Italian Official methods of Cheeses analysis (1986), and total carbohydrates were determined by difference to 100 of macronutrient contents. Total energy was calculated using the conversion factors according to Regulation (EU) No. 1169/2011.
The designation according to firmness characteristics was also calculated by the Moisture on Fat Free Basis (MFFB %), i.e. percent ratio between weight of moisture in the cheese and difference between total weight of cheese and weight of fat in cheese, in accordance to the Codex Standard for cheese (CODEX STAN 283-1978, Amendments 2006, 2008, 2010, 2013).

2.3.2 Mineral

Calcium, magnesium and phosphorous were determined according to the AOAC method (AOAC, 2002), which was also applied for potassium and sodium. The samples were analyzed after ashing: 1.0 g of sample was weighed into platinum crucibles and ashed in the furnace at 525C° for 16 hours. Calcium, sodium, potassium and magnesium were determined using an Atomic Absorption Spectrometer (Perkin Elmer Model: A. Analyst 300, Norwalk, CT), while phosphorus was measured at 400 nm by a spectrophotometer (Shimadzu Model: 1800 Tokyo, Japan).

2.3.3 Fatty Acid

The extraction and determination of fatty acids were performed according to a modified version of the method by PRANDINI *et al.* (2007). 2 g of sample were dissolved in ethanol/water (2:1, v/v) and extracted twice with diethyl ether/petroleum ether mixture (1:1 v/v). Fatty acid methyl esters (FAME) were prepared according to the methods of PRANDINI *et al.* (2007). The samples were analysed by GC (Clarus 500-Perkin Elmer) equipped with flame ionization detector (FID), using a fused silica capillary column SP 2380 (Supelco), 60 m x 0.32 mm x 0.2 μm film thickness and helium carrier gas. The oven temperature was programmed as follows: initial temperature 60°C for 5 min, 5°C/min to 180°C and then 3°C/min to 240°C. The injection and detector temperatures were held at 240°C and 260 °C, respectively.

2.3.4 Unsaponifiable fraction

α-tocopherol, β-carotene, *trans* retinol and cholesterol were determined according to PANFILI *et al.* (1994). Briefly, samples were subjected to alkaline digestion and extracted twice with hexane/ethyl acetate (9:1, v/v). The organic phase was collected and evaporated to dryness. The residue was dissolved in 2 ml mobile phase (2-propanol 1% in n-hexane), injected and analysed by an HPLC analytical system Alliance (Waters Model: 2695, Milford, MA). In the chromatographic procedure, a Phenomenex, Kromasil 5μm Si 250x4.6 mm, a fluorescence detector (Waters Model: 2475, Milford, MA, USA) and UV/VIS detector (Waters Model: 2487. Milford, MA, USA) connected in series were utilized to determine cholesterol (208 nm), β-carotene (450 nm), α-tocopherol (excitation 280 nm, emission 325 nm) and retinol (excitation 325 nm, emission 475 nm).

2.3.5 Degree of Antioxidant Protection (D.A.P.)

The *DAP index* was calculated as a molar ratio between Antioxidant Compounds (AC) and Oxidation Target (OT), as follows (PIZZOFERRATO *et al.*, 2007):

$$D.A.P. = \frac{\sum_{i=1}^{n} A.C._{i}(n°moles)}{O.T.(n°moles)}$$

Generally, α-tocopherol and β-carotene, when present, are used as AC, while cholesterol content was used as OT molecule.

2.3.6 Radical scavenging activity

The radical scavenging activity was evaluated on fat-soluble vitamins-rich extracts dissolved in 2-propanol 1% in n-hexane by the DPPH assay according to the methods of BRAND-WILLIAMS *et al.* (1995), with appropriate modifications. The radical scavenging activities of these extracts were evaluated towards the stable radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) as follows: 1.5 ml of the extract was reacted with 1.5 ml of DPPH radical (60μM), the decrease in absorbance at 515 nm for 400min was monitored and ethyl acetate was used as blank. The control was prepared using 1.5 ml of 2-propanol 1% in n-hexane. The percentage of inhibition of DPPH was calculated using the formula: % Radical scavenging activity (Inhibition of DPPH activity) = $[(Abs_{control}-Abs_{sample}) / Abs_{control}] *100$; where $Abs_{control}$ is the absorbance of the reaction control and Abs_{sample} is the absorbance in the presence of the sample measured at 400 min, and then the radical scavenging activity was expressed as μmol α-tocopherol/100g.

2.3.7 Colour measurements

The CIE L, a* and b* measurements were made on grated and sliced samples using an handheld tristimulus colorimeter (Konica Minolta CR-400, Minolta Limited, Milton Keynes, UK) controlled with the software CM-S100w (SpectraMagic NX) with D65 illuminant and 10° viewing angle, calibrated with a white tile. The following parameters were determined: L* value, that is an indicator of luminosity (the degree of lightness) and varies from black to white; the a* value, indicator of green (negative values) and red (positive values); the b* value, indicator of blue (negative values) and yellow (positive values); Hue angle (h) and Chroma (C*) colour intensity, which is the distance of a colour from the origin (a*=b*=0) in the a*, b* space.

2.3.8 Fourier Transform Infrared (FTIR) analysis

The FTIR spectra were acquired according to LERMA-GARCÍA *et al.* (2010). Different slices of each sample were examined by FT-IR (Nicolet iS10 Thermo Fisher Scientific, USA) with an ATR (Attenuated Total Reflectance) accessory (with diamond crystal). Each sample was placed in ATR surface and pressed with the clamp to obtain a thin layer. All infrared spectra were recorded (32 scans/sample) in the range between 4000 and 700 cm^{-1} at a resolution of 4 cm^{-1}, with automatic atmospheric suppression. Data were elaborated with Omnic 8.2.0.387 (Thermo Fisher Scientific Inc., USA) software.

2.3.9 Statistical analysis

Analysis of variance, one-way ANOVA test with the Tukey's Post hoc comparison, was used for multiple comparison of mean values of nutrients and parameters regarding cheeses produced at different months (February, July, December). P-values ≤ 0.05 were considered significant. All statistical analyses were performed using the KaleidaGraph 3.6 software (Synergy Software, Reading, PA. USA).

3. RESULTS AND DISCUSSIONS

3.1. Proximate composition

In Table 1 proximate composition, percentage ratio of fat/protein and Moisture/Fat Free Basis (MFFB), energy values, pH, mineral contents, calcium (Ca), phosphorous (P), sodium

(Na), potassium (K), magnesium (Mg) and the molar ratio of calcium/phosphorous of traditional *Pecorino d'Abruzzo* cheeses are reported. In all samples, the pH values were moderately acid, and a slightly but not significant increase from February to December was observed according to cheese manufacturing season. These pH values were higher when compared with those reported by ADDIS *et al.* (2015) for PDO *Pecorino Romano* cheeses and by JAEGGI *et al.* (2005) for ovine hard-pressed cheese. TODARO *et al.* (2014) found that there were slight changes in milk pH during the year, although it was lower in summer and spring. As shown in Table 1, the samples in February (PEC-A) exhibited a higher value of water 47.5 g/100g and a lower value of protein, fat and ash content than those produced in July (PEC-B) and December (PEC-C).

Table 1: Proximate composition (g/100g), percentage ratio of fat/protein and Moisture/Fat Free Basis (MFFB)°, energy values (kcal/100g and kJ/100g)°°, pH, mineral contents (mg/100g) and molar ratio of calcium/phosphorous on a fresh matter (F.M.) and dry matter (D.M.) basis of traditional *Pecorino d'Abruzzo* cheeses.

Samples	**PEC-A**	**PEC-B**	**PEC-C**	**PEC-A**	**PEC-B**	**PEC-C**
Month of production	**February**	**July**	**December**	**February**	**July**	**December**
		F.M. (%)			**D.M. (%)**	
water (g/100)	47.5±0.0^{a}	39.1±0.0^{b}	36.9±0.0^{c}			
° MFFB (%)	64.2	57.4	54.9			
*** protein (g/100g)**	21.6±0.1^{c}	23.2±0.1^{b}	25.1±0.1^{a}	41.2±0.1^{a}	38.0±0.1^{c}	39.8±0.1^{b}
fat (g/100g)	26.1±0.1^{c}	32.0±0.2^{b}	32.8±0.1^{a}	49.6±0.2^{b}	52.5±0.3^{a}	51.9±0.2^{a}
ash (g/100g)	3.5±0.0^{c}	4.1±0.0^{b}	4.8±0.0^{a}	6.7±0.1^{b}	6.7±0.0^{b}	7.6±0.1^{a}
**** carbohydrates**	1.3	1.7	0.4			
fat/protein (%)	1.2	1.4	1.3			
°° energy kcal (kJ)	327 (1355)	387 (1606)	397 (1645)			
pH	5.4±0.0	5.6±0.1	5.8±0.0			
Ca (mg/100g)	669.2±5.9^{b}	661.0±4.3^{b}	720.0±5.3^{a}	1273.8±11.2^{a}	1085.0±7.1^{c}	1141.9±8.5^{b}
P (mg/100g)	458.6±1.9^{c}	484.9±6.4^{b}	504.0±0.7^{a}	872.9±3.7^{a}	795.9±10.5^{b}	799.3±1.1^{b}
Na (mg/100g)	580.5±5.1^{c}	840.5±0.7^{b}	1074.5±2.0^{a}	1104.9±9.8^{c}	1379.5±1.2^{b}	1704.0±3.1^{a}
K (mg/100g)	84.0±0.2^{a}	80.3±0.1^{b}	76.7±1.1^{c}	159.9±0.4^{a}	131.8±0.1^{b}	121.6±1.7^{c}
Mg (mg/100g)	41.0±0.4	39.5±0.9	39.2±0.6	78.0±0.7^{a}	64.8±1.6^{b}	62.2±0.9^{b}
molar ratio Ca/P	1.1±0.0	1.1±0.0	1.1±0.0			

Data are means ± Standard Deviation (SD) of triplicate analyses; values in the same row with different superscript letters are significantly different (P≤0.05).
°Moisture/Fat Free Basis (%): in accordance to the Codex Standard for cheese (CODEX STAN 283-1978);
*proteins: total nitrogen 6.38;
**carbohydrates calculated by difference to 100 of macronutrient contents;
°°energy calculated using the conversion factors according to Regulation (UE) No. 1169/2011.

However MFFB ranged between 54% and 69% for all samples, and in terms of firmness it designated samples as firm or semi-hard cheeses. On the other hand, when taking into consideration the ripening times, cheeses can be designated as unripened/fresh (CODEX STAN 283-1978).
Generally, macronutrient content of commercial *Pecorino* cheeses, as reported by RAYNAL-LJUTOVAC *et al*. (2008), is very wide. Fat and protein contents are reported in literature to range 29.0 to 36/37 g/100g and 25.0 to 28.0 g/ 100g, respectively, in different ewe milk cheeses (*Pecorino cheeses*). In a study of MANZI *et al.* (2007) on different PDO Italian cheeses (*Pecorino romano, Pecorino toscano, Pecorino sardo*), the following ranges were found: 23.8 - 40.8 g/100g for moisture content, 21.8 - 30.3 g/100g for protein content 30.8 - 40.2 g/100g for fat content and 3.4 - 10.1 g/100g for ash. So, the macronutrients of *Pecorino d'Abruzzo* cheeses within this study had lower values than those obtained by MANZI *et al.* (2007) for the analysed PDO Italian *Pecorino* cheeses.
RAYNAL-LJUTOVAC *et al.* (2008) reported that the protein content for ewes' milk can range between 4.75 g/100g and 7.20 g/100g and that the main non-individual factors of protein content variation were the stage of lactation, season, age and feeding.
JAEGGI *et al.* (2005) found that the ewe's milk produced in February had a higher fat and a lower protein content (37.19% and 24.84%, respectively) with a lower casein:fat ratio than milk used in May (35.46%; 26.59%) and August (35.70%; 26.13%); and the same applied to cheeses. Moreover, TODARO *et al.* (2014) reported that the monthly levels of fat in milk showed a decreasing trend from January to April, and then an increase until reaching a maximum value in July/August. This result could explain the lowest ($P \leq 0.05$) fat content among *Pecorino d'Abruzzo* samples in February. However, as reported by TODARO *et al.* (2014), the higher fat content in summer cheese depends on the higher percentage of fat observed in summer ewes' milk, and this fact is the consequence of milk concentration in late lactation and a high level of dry and fibrous forage in ewes' diet.
ADDIS *et al.* (2015) analyzed several samples of *Pecorino* cheeses manufactured at different times of the year (from March to June) and found that the cheeses produced in late winter and spring were characterized by a less fat and salt content and a higher protein level with respect to those produced in early summer.
The outcomes by ADDIS *et al.* (2015) and TODARO *et al.* (2014) are in accordance with this study, and probably they could explain the differences observed and also expressed on a dry matter (D.M.) basis, for protein, fat and some mineral (Ca, P, K, Mg) concentrations (Table 1). In particular, as regards mineral content, the samples produced during winter had a major protein and Ca ($P \leq 0.05$) content than those manufactured in summer.

3.2. Minerals

The content of Ca, Na and K was significantly ($P \leq 0.05$) different among all samples (Table 1), whereas a significant difference ($P \leq 0.05$) for P and Mg contents was found only among samples of cheese produced in February and in those produced in July and December. On dry matter basis, the samples produced in February exhibited the highest value for minerals, such as Ca, P, K and Mg (1273.8: 872.9: 159.9: 78.0 mg/100g, respectively) and the lowest value for Na (1104.9 mg/100g). RAYNAL-LJUTOVAC *et al.* (2008) also reported for sheep milk cheeses that Mg content is less variable, by showing a mean value of 80 mg/100g D.M. that is higher than what was obtained in this study for *Pecorino d'Abruzzo* samples (on average 68.3mg/100 D.M.).
However, depending on the pH obtained during the cheese-making process, Ca and P content ranged between 661.0 and 720.0 mg/100g and 458.6 to 504.0 mg/100g, respectively. Regarding the mineral components, few data are available in literature for

sheep milk cheeses. As for the other investigated nutrients, the data obtained for the mineral composition of *Pecorino d'Abruzzo* samples were lower than those reported by literature. For instance, in PDO Italian cheeses, such as *Pecorino romano, Pecorino sardo* and *Pecorino toscano,* the average Ca content was, respectively, 938.5, 940.4 and 860.0 mg/100g, and P content was 634.5, 714.8 and 658.9 mg/100g (MANZI *et al.,* 2007).
In all samples the percentage ratio of fat/protein and the molar ratio Ca/P were always higher than 1.0 (Table 1). These results may be easily explained by the high content in fat, protein and minerals of the ewes' cheeses under investigation that thus resulted a food with high nutritional value.
The experimental results of this study matched ADDIS *et al.* (2015) that reported that ewes' milk produced in June and July is characterized by a higher fat content, generally not offset by an equal increase in protein content. The fat to protein ratio thus ranges from 1.03 to 1.13 in March/April, whereas in June/July it exceeds 1.20 (ranging from 1.22 to 1.35).

3.3. Unsaponifiable fraction

In Table 2, cholesterol, α-tocopherol, *trans*-retinol and β-carotene contents, and the Degree of Antioxidant Protection (DAP exp-3) in samples of traditional *Pecorino d'Abruzzo* cheese are shown.

Table 2: Cholesterol (mg/100g), α-tocopherol, *trans*-retinol, β-carotene contents (μg/100g) and Degree of Antioxidant Protection (DAP exp-3) on a fresh matter (F.M.) and dry matter (D.M.) basis of traditional *Pecorino d'Abruzzo* cheeses.

Samples	**PEC-A**	**PEC-B**	**PEC-C**	**PEC-A**	**PEC-B**	**PEC-C**
Month of production	**February**	**July**	**December**	**February**	**July**	**December**
		F.M. (%)			**D.M. (%)**	
cholesterol	72.9±0.4^{c}	96.1±1.1^{a}	81.1±0.0^{b}	138.7±0.8^{b}	157.7±1.9^{a}	128.6±0.0^{c}
***α*-tocopherol**	769.2±4.8^{c}	898.8±2.0^{b}	945.9±5.5^{a}	1464.0±9.2^{b}	1475.3±3.3^{ab}	1500,1±8.8^{a}
***trans* -retinol**	308.3±0.7	299.2±18.5	309.8±3.4	586.9±1.4^{a}	491.1±30.4^{b}	491.3±5.4^{b}
***β*-carotene**	nd*	nd*	nd*			
DAP (exp-3)	9.5±0.1	8.4±0.1	10.5±0.1			

Data are means ± Standard Deviation (SD) of triplicate analyses; values in the same row with different superscript letters are significantly different (P≤0.05);
*nd= not detectable; β-carotene detection limit 0.16ng/20 μl injected.

As regards cholesterol content, cheese samples exhibited on average a value of 83.4 mg/100g, and PEC-B cheeses (summer produces) had on average the highest cholesterol content (96.1 mg/100g). However, the amount of cholesterol is quite variable between winter and summer samples, and the values ranged in accordance with the data obtained by MANZI *et al*. (2007) on different PDO Italian Pecorino cheeses.
The winter samples had significantly a lower cholesterol value than those manufactured with milk produced in summer (P≤0.001), even when expressed on dry matter basis (Table 2). The cholesterol content seems therefore to be influenced by the manufacture month.

Regarding fat-soluble vitamins, there was a great variability in this study: α-tocopherol ranged between 945.9 and 769.2 μg/100g and *trans* retinol between 299.2 and 309.8 μg/100g. As expected, the β-carotene was not detectable in all studied cheese samples (NOZIERE *et al*., 2006).
Upon comparison of cheeses, PEC-C samples (December) showed the highest values for α-tocopherol, whereas no significant differences (P≤0.05) were observed for *trans* retinol. On the contrary, significant (≤0.05) differences for both α-tocopherol and *trans* retinol were found (Table 2) by comparing cheeses produced in February with the other samples on dry matter basis. This result could be explained by literature data. According to MANZI *et al.* (2007), in some *PDO* Italian cheeses, the average α-tocopherol content was 850 μg/100g and the range of variability for *trans* retinol was very wide: 322.1 to 545.9 μg/100g in *Pecorino romano*, 256.2 to 484.6 μg/100g in *Pecorino sardo* and 337.4 to 602.4 μg/100g in *Pecorino toscano*.
CALDERÒN *et al.* (2007) reported that the concentration of Vitamin E in cow milk was linearly related to the experimental amount of feed in the diet, and milk characteristics differed according to season. The vitamin E content in milk was higher during the grazing period (May-September) than during the winter feeding period (February-March), and this fact was linked to the proportion of grazed grass or grass silage in the forage, unlike vitamin A (AGABRIEL *et al*., 2007). Indeed, REVILLA *et al*. (2014) investigated the effects of milk origin (i.e., sheep, goat and cow) and season on the vitamin composition of different types of cheese, and found that seasonality had a significant effect on the concentration of vitamin A (i.e. higher during summer and autumn), but not on vitamin E. They also reported that the seasonal changes in animal feeding practices were the main reason for the differences observed.
So, in this study, the main chemical characteristics of *Pecorino d'Abruzzo* cheeses also seem to be affected by the month of manufacture; there is, in fact, a significant (P≤0.05) effect of seasonal change on cheese composition.
The Degree of Antioxidant Protection (DAP), that is a parameter to estimate the potential oxidative stability of fat in foods, has been also evaluated (Table 2). This parameter is related to farm systems and/or cheese manufacture. As reported by PIZZOFERRATO *et al*. (2007), it can discriminate products from grazing and zero-grazing feeding systems, and when this value is ≥7.0 the pasture is predominant (grazed herbage exceeds 15% in animals' total diet). In this study, the DAP reached the highest values in winter samples (PEC-A and PEC-C), although it can be stated that in all samples the values were always ≥7.0.
Despite several studies on quantification of single antioxidants in cheeses are published in literature, there are only few investigations on antioxidant properties of cheeses (GUPTA *et al*., 2009). In this research, the radical scavenging activity of fat soluble vitamin-rich extracts, derived from *Pecorino d'Abruzzo* samples, was studied. The DPPH assay is a simple, quick and cheap method to evaluate the free radical scavenging activity of compounds and/or samples. This evaluation is related to the structure of the active substances present in lipophilic extracts and their possible interactions. This measurement might hence be considered an index of the antioxidant properties of cheeses. LUCAS *et al.* (2006) studied the antioxidant properties in French cheeses, such as *Abondance, Tomme de Savoie, Cantalet, Salers* and *Rocamadour* and concluded that the variation of the Total Antioxidant Activity in cheeses was probably due to variations in antioxidants, such as α-tocopherol, retinol, β-carotene. DPPH values of fat soluble vitamin-rich extracts, obtained from *Pecorino d'Abruzzo* cheese, were higher in winter than in summer samples (Fig. 1) and that thus reflected the behaviour of DAP.

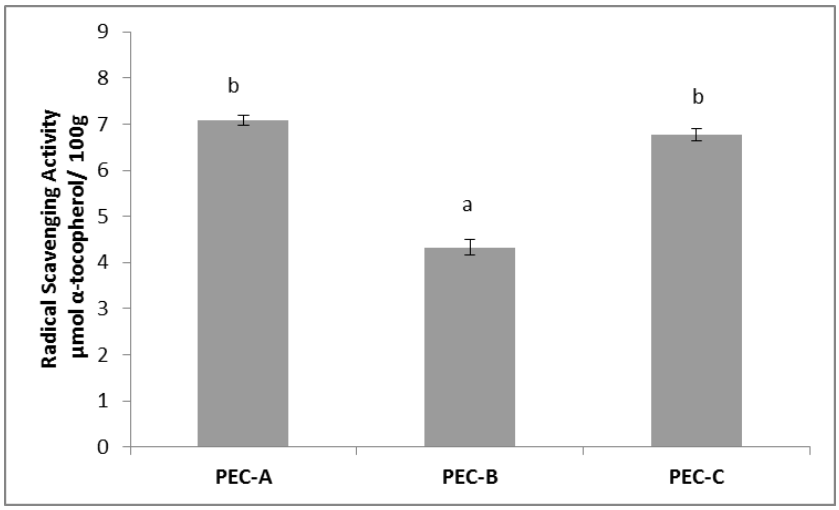

Figure 1: Radical Scavenging Activity (μmol α-tocopherol/100g) of fat-soluble vitamins-rich extracts derived from traditional *Pecorino d'Abruzzo* cheeses. Means followed by different letters are significantly different ($p \leq 0.05$).

3.4. Fatty acid composition

Fatty acid (FA) composition of traditional *Pecorino d'Abruzzo* cheeses is reported in Table 3. The samples produced during winter (December) had the highest percentage of Short and Medium Chain Fatty Acids (SCFAs, 5.25% and MCFAs 21.59%, respectively, $P \leq 0.05$), and consequently of Saturated Fatty Acids (SFAs 66.25%). On the contrary, the samples produced during summer (July) had the highest percentage of Long Chain Fatty Acids (LCFAs 81.99%, $P \leq 0.05$) and were the richest in MUFA (38.30%), primarily *all cis* and *trans* C18:1 (PEC-B samples 36.58 g/100g of total FA; $P \leq 0.05$). The cheeses produced during winter showed on average a large amount of PUFA (8.33% and 7.90% in PEC-C and PEC-A, respectively). TODARO *et al.* (2014) explained any increase in MUFA, especially oleic acid, with a major content in ewes' milk produced during summer and, hence in cheeses, as a result of the mobilization of long-chain FA from the body fat deposits of the sheep to balance the energy deficits that they undergo in summer, when the feeding regime may not be sufficient to satisfy their energy needs. DE LA FUENTE *et al.* (2009) reported that in ewes' milk there were significant seasonal variations in PUFA content with higher values in spring and summer than in winter. It is possible that factors, such as season and feeding system, could explain also in this study the differences observed. The period of cheese manufacture seems to affect the type and proportion of FA present in milk and in cheeses as well. During summer, the proportion of SCFA decreased, while LCFA increased (Table 3). In particular, SFA such as lauric acid (C12:0), myristic acid (C14:0) and palmitic acid (C16:0), that are defined atherogenic, resulted more concentrated ($P \leq 0.05$) in the *Pecorino d'Abruzzo* cheeses produced in winter. These results matched the data reported by several authors (NUDDA *et al.*, 2005; TODARO *et al.*, 2014; DE LA FUENTE *et al.*, 2009). On the contrary, the content of stearic acid (C18:0) was higher in *Pecorino d'Abruzzo* produced in summer (15.55 g/100g of FA in PEC-B sample; $P \leq 0.05$) than in winter cheese samples (PEC-C). This result is important because it can be used for the biosynthesis of oleic acid in human metabolism, and differed from the other SFAs for the beneficial effect on blood cholesterol level: it lowers LDL-Cholesterol without affecting blood concentrations of HDL-Cholesterol (HUNTER *et al.*, 2010).

Table 3: Fatty acid composition given as mean ± S.D. (g/100g of total Fatty Acids) of traditional *Pecorino d'Abruzzo* cheeses.

Samples		**PEC-A**		**PEC-B**		**PEC-C**	
Month of production		**February**		**July**		**December**	
Free Fatty Acids							
lipid numbers	*Usual names*	Means	SD	Means	SD	Means	SD
C4:0	Butyric	2.25	0.10	1.96	0.04	1.81	0.21
C5:0		0.05	0.00	0.12	0.00	0.15	0.06
C6:0	Caproic	1.37^{ab}	0.09	1.23^{b}	0.06	1.61^{a}	0.08
C8:0	Caprylic	1.16^{b}	0.09	1.09^{b}	0.05	1.68^{a}	0.05
C10:0	Capric	3.35^{b}	0.24	3.12^{b}	0.13	5.61^{a}	0.13
C12:0	Lauric	2.18^{b}	0.15	2.04^{b}	0.08	3.34^{a}	0.06
C13:0		0.06	0.01	0.04	0.00	0.06	0.00
C14:0	Myristic	8.01^{b}	0.51	6.89^{b}	0.17	10.44^{a}	0.11
C14 :1 c9	Myristoleic	0.81^{a}	0.04	0.52^{b}	0.01	0.72^{a}	0.03
C15:0		1.04^{a}	0.05	0.75^{b}	0.01	1.16^{a}	0.02
C15:1		0.37^{a}	0.02	0.24^{b}	0.00	0.25^{b}	0.01
C16:0	Palmitic	24.88^{a}	1.02	21.28^{b}	0.04	26.93^{a}	0.25
C16 :1 c9	Palmitoleic	1.11^{a}	0.05	0.69^{b}	0.00	0.97^{a}	0.06
C17:0	Margaric	0.83	0.03	0.68	0.00	0.85	0.12
C17:1		0.26^{a}	0.01	0.18^{b}	0.00	0.26^{a}	0.02
C18:0	Stearic	14.23^{a}	0.67	15.55^{a}	0.19	11.98^{b}	0.33
C18:1 (t+c)		29.28^{ab}	3.25	36.58^{a}	0.32	23.15^{b}	1.62
C18:2, t9 t12	Linolelaidic	1.08	0.12	1.57	0.15	1.60	0.27
C18:2, c9 c12 (n-6)	Linoleic acid (LA)	3.28^{a}	0.13	2.15^{c}	0.02	2.69^{b}	0.17
C20:0	Arachidic	0.53	0.02	0.45	0.00	0.44	0.02
C18:3 (n-6)	g-Linolenic acid	0.12^{bc}	0.01	0.08^{c}	0.02	0.19^{a}	0.00
C:18:3 (n-3)	a-Linolenic acid (ALA)	1.41^{a}	0.06	0.82^{b}	0.01	1.61^{a}	0.11
C20:1	Gondoic	0.08^{ab}	0.00	0.10^{a}	0.00	0.06^{b}	0.00
C18:2, c9 t11 - CLA	Rumenic	1.38^{b}	0.04	1.23^{b}	0.00	1.63^{a}	0.09
C20:2		0.29^{a}	0.01	0.21^{b}	0.00	0.29^{a}	0.02
C22:0	Behenic	0.26^{a}	0.01	0.17^{b}	0.00	0.18^{b}	0.01
C20:4 (n-6)	Arachidonic	0.12^{ab}	0.00	0.08^{b}	0.00	0.14^{a}	0.02
C20:5 (n-3)	EPA	0.12^{a}	0.00	0.08^{b}	0.00	0.12^{a}	0.01
C22:6 (n-3)	DHA	0.11^{a}	0.00	0.10^{a}	0.00	0.05^{b}	0.00
ω3/ω6 ratio°		0.47		0.43		0.59	
*Σ SHORT CHAIN FATTY ACIDS (SCFA)**		*4.84*	*0.90*	*4.40*	*0.76*	*5.25*	*0.78*
*Σ MEDIUM CHAIN FATTY ACIDS (MCFA)***		*15.81*	*2.78*	*13.61*	*2.44*	*21.59*	*3.81*
*Σ LONG CHAIN FATTY ACIDS (LCFA)****		*79.35*	*8.90*	*81.99*	*9.91*	*73.16*	*8.13*
Σ SATURATED FATTY ACIDS(SFA)		*60.21*		*55.37*		*66.25*	
Σ MONOUNSATURATED FATTY ACIDS (MUFA)		*31.89*		*38.30*		*25.42*	
Σ POLYUNSATURATED FATTY ACIDS (PUFA)		*7.90*		*6.33*		*8.33*	

Fatty acids are expressed in g/100g of total fatty acid (FA). Data are means ± Standard Deviation (SD) of triplicate analyses; different letters in the same row correspond to statistical differences ($P \leq 0.05$); °ω3/ω6 ratio of total omega 3 fatty acid (ALA; EPA; DHA) and total omega 6 fatty acid (LA; γ-Linolenic acid; Arachidonic acid) *SCFA: C4:0 - to C9:0; **MCFA: C10:0 to C15:1; ***LCFA: C16:0 to C22:6.

TODARO *et al.* (2014) reported that the ewes' cheeses produced in summer were lower in saturated FA and higher in linoleic acid (LA), MUFA and ω3 PUFA than those produced in autumn and spring; these results, except for LA and ω3 PUFA content, are in accordance with the results obtained within this research study.
Among Essential Fatty Acids (EFAs), LA (C18:2 cis9, cis12 -ω6) differed significantly ($P \leq 0.05$) among samples, and reached higher values in winter cheeses (3.28:2.69 g/100g of total FA in PEC-A: PEC-C, respectively); a similar behaviour was reported for α-linolenic acid (ALA, C18:3, ω3). ALA is present in food at lower concentrations than LA, and it is an important component in cheeses for healthy nutrition (XU, 2015). Indeed, ALA and γ-linolenic acid significantly increased in cheeses produced during winter: 0.12 and 0.19 g/100g total FA for γ-linolenic acid and 1.41 and 1.61 g/100g total FA for *a*-linolenic acid in PEC-A and PEC-C, respectively.
Several studies (ASTRUP *et al.,* 2011) underline that replacing SFAs by PUFAs in the diet decreases the risk of cardiovascular diseases (CVDs) and that dietary short-term intake of a cheese naturally rich in *cis*-9, *trans*-11 CLA appears to cause favourable biochemical changes of atherosclerotic markers in comparison with commercial cheese (SOFI *et al*., 2010). As regards that, it was important to highlight in the studied cheeses the presence of long chain fatty acids (LC-PUFAs), such as Eicosapentaenoic (C20:5 -EPA) acid and Docosahexaenoic (C22:6 -DHA) acid, especially in the samples produced during late winter (February). However, the values obtained in this study for C20:5 and C22:6 were on average higher than those reported by NUDDA *et al.* (2005).
Conjugated Linoleic Acid (CLA), detected as C18:2, c9 t11 (Rumenic Acid -RA), got higher value in the samples produced in December (1.63 g/100g total FA PEC-C) than in samples produced in July. The importance of CLA in food, as shown by *in vivo* studies on animal models, is linked to positive physiological effects, in terms of reduction of the growth rate of cancer cells, improvement of the immune system, and more in general beneficial effects on health (JAHREIS *et al.,* 1999; WHIGHAM *et al.,* 2000; XU, 2015).
Recently, more information on the effects of CLA on body composition and energetic metabolism are reported by LEHNEN *et al.* (2015). The consumption of foods naturally enriched with CLA (and not supplemented) during lifetime should contribute to reducing increased adiposity and hence the risk of other diseases related to obesity. Ewe milk is the richest in CLA, among the milk of other ruminant species (except for goat); it also has the highest content on total *trans* FA, as well as *trans* Vaccenic Acid (VA), and this was season-dependent (JAHREIS *et al.,* 1999). Thus, the manufacture time, i.e. season, of *Pecorino* cheese is of paramount importance.
Apropos of that, NUDDA *et al.* (2005) reported substantial differences in the fatty acid pattern in ewes' cheeses from March to June: a decrease in the CLA e VA concentration in milk fat as lactation progressed. This was probably due to the availability of pasture feeding and a different FA composition of grass lipids. Similarly, as reported by ADDIS *et al.* (2005), the nutritional value of cheese fat, associated in particular with LA, RA and VA contents, decreased exactly with the progress of season. However, it is known that other factors, such as the period of lactation (KIM *et al*., 2009), the processing temperature (SHANTHA *et al*., 1992) or ripening time (PRANDINI *et al*., 2011; KIM *et al*., 2009) may also affect the total amount of CLA in cheeses.
As regards the ω3/ω6 ratio was higher in winter cheeses than in summer samples (0.59, 0.47, 0.43 for PEC-C, PEC-A, PEC-B, respectively), while a meta-analysis study on dairy products (PALUPI *et al.,* 2012) shows that the ω3/ω6 ratio is higher in summer than in winter cheeses.
The profile of fatty acid composition confirms the important role of ewes' dairy products, in particular of *Pecorino d'Abruzzo* cheese, as a natural source of PUFA and CLA in human nutrition, especially when manufactured in winter.

3.5. Nutritional information

The knowledge of the nutritional supply of some nutrients may help to drive dietary choices among consumers and understand the tight relationship between food and health. It is therefore interesting for a nutrient dense food like *Pecorino* cheese to add nutritional information (Table 4) on the level of coverage (%) for both energy and some nutrients for human adults (aged 18-59) according to Italian recommendations (SINU, 2014) for daily requirements.

Table 4: Daily coverage requirements (%) for i) calcium, phosphorous and Vitamin A, out of *Population Reference Intake* (**PRI**); ii) Vitamin E, out of *Adequate Intake* (**AI**); and iii) sodium, out of *Suggested Dietary Target* (**SDT**) for the Italian population^, and average content of minerals (Ca, P, Na), Vitamins A and E supplied by one serving (50 g) of traditional *Pecorino d'Abruzzo* cheeses (SINU, 2014).

^ Adult	*Calcium: coverage (%) out of PRI	*Phosphorous: coverage (%) out of PRI	°Sodium: coverage (%) out of SDT	*Vitamin A: coverage (%) out of PRI	°°Vitamin E: coverage (%) out of AI
Man				21.8	3.4
	34.2	34.5	20.8		
Woman				25.5	3.6
			Average content for serving (50g)		
Mineral (mg)	341.7	241.3	415.9		
Vitamin A (µg RE)				152.9	
Vitamin E (mg α-TE)					0.4

^aged 18-59
*the Italian *Population Reference Intake* (PRI) is 1000 mg/day for calcium, 700 mg/day for phosphorous; 700µg RE/day for men and 600µg RE/day for women for vitamin A (µg Retinol Equivalents);
°the *Suggested Dietary Target* (SDT) for Sodium is 2.0 g/day (corresponded to NaCl ≤ 5 g/day);
°°the *Adequate Intake* (AI) for vitamin E (mg α-Tocopherol Equivalents) is 13mg α-TE /day for men and 12mg α-TE /day for women.

From a nutritional point of view, one serving (50 g) of *Pecorino d'Abruzzo* cheese added, on average, only 185 kcal (768 kJ) to total daily calories intake (Table 1), but it is worth pointing out that 50 g of this cheese (Table 4) supply 0.4 g of sodium (1.1 g of sodium chloride), that is about 20.8% of the *Suggested Dietary Target* (SDT; ≤ 2000 mg/day), and 341.7 mg of calcium, that is, about 34.2% out of the Italian *Population Reference Intake* (PRI) for adults (1000 mg/day) daily requirements. In addition, this serving (50 g) supplies 0.44 mg of vitamin E, that cover about 3.4% and 3.6% of daily *Adequate Intake* (AI) for respectively men and women, and 152.9 μg of vitamin A, that cover in adults about 21.8% and 25.5% of *Population Reference Intake* (PRI) for men and women, respectively, in accordance with Italian recommendations (SINU, 2014).

3.6. Colour measurements

The colour is the first quality parameter evaluated by consumers and an indicator for severe heat treatments, storage, ripening times, etc.; it could also be related to bioactive

compounds profile. However, few studies have been published on the evaluation of colour parameters in *Pecorino* cheeses. For example, JUAN *et al.* (2008) when investigating the effect of High-Pressure (HP) treatment at 300MPa on ewes' milk cheese in real industry conditions found that this treatment induced a colour variation, that is, a decrease in lightness and an increase in yellowness related to the structural changes of cheeses. Other authors (ROHM and JAROS, 1996; BUFFA *et al.*, 2001) studied the changes in colour parameters of cheeses during ripening time, and several changes were observed: a decrease of L and an increase of a* and b* values. The colour parameters measured in both grated (a) and sliced (b) *Pecorino d'Abruzzo* cheeses are shown in Table 5.
In this study (Table 5), all colour parameters presented significant differences ($P \leq 0.05$) among samples depending on the production period, that is, either in winter or summer, but they were not actually influenced by the shape (grated or sliced).

Table 5: Colour measuraments of a) grated or b) sliced traditional *Pecorino d'Abruzzo* cheeses.

Samples	**PEC-A**	**PEC-B**	**PEC-C**	**PEC-A**	**PEC-B**	**PEC-C**
Month of production	**February**	**July**	**December**	**February**	**July**	**December**
		a) grated			**b) sliced**	
L*	73.9±0.2^{a}	69.0±0.5^{c}	71.0±0.6^{b}	85.7±1.1^{a}	78.5±0.4^{c}	80.5±0.6^{b}
a*	-3.3±0.1^{b}	-3.3±0.1^{b}	-2.9±0.1^{a}	-5.9±0.2^{b}	-6.5±0.1^{a}	-6.1±0.1^{b}
b*	14.0±0.2^{b}	17.0±0.7^{a}	13.9±0.2^{b}	18.3±0.5^{b}	23.0±0.8^{a}	17.9±1.1^{b}
C*	14.4±0.3^{b}	17.3±0.7^{a}	14.2±0.2^{c}	19.3±0.5^{b}	23.9±0.8^{a}	18.9±1.0^{b}
h	103.3±0.1^{a}	101.0±0.5^{b}	101.6±0.2^{a}	107.9±0.1^{a}	105.7±0.5^{b}	108.9±1.0^{a}

*D65;
Data are means ± Standard Deviation (SD); values in the same row with different superscript letters are significantly different ($P \leq 0.05$).

In particular, all samples showed significant ($P \leq 0.05$) differences for L* (brightness). For cheeses manufactured in summer time (July), the brightness (L*) was lower, whereas the b* (yellowness) and C* (Croma) values reached higher values than those reported for the samples manufactured during winter (February and December). These results applied to both grated and sliced samples.
In the *Pecorino d'Abruzzo* samples produced during winter (December or February), the C* value tended to decrease, thus indicating that the samples became grey or looked less brilliant during this period, and suggesting that cheese might run a major risk of loosing colour in winter. Moreover, the hue angle (h), that is a qualitative attribute of colour related to the differences in absorbance at different wavelengths, resulted higher in winter samples than in the cheeses produced in July. Indeed, higher h values corresponded to lower b* values, and matched a minor yellowness in the cheeses where the β-carotene was not detectable. These are, nevertheless, preliminary results and need further investigation and more specifically targeted studies.

3.7. Qualitative application of FTIR-ATR technique to *Pecorino d'Abruzzo* cheeses

Cheeses are heterogeneous food products, and hence show qualitative differences related to a different chemical composition, maturation process, seasonality and/or various ripening times. The FTIR-ATR technique has been widely applied in food science for

different purposes, e.g., determination of the geographical origin of cheeses, of sensory and textural properties, shelf life, as well as classification of cheeses according to the manufacture month and manufacturing technique (LERMA-GARCÍA *et al.*, 2010; KAROUI *et al.*, 2005; KOCA *et al.*, 2007; VLACHOS *et al.*, 2006). Moreover, it is worth highlighting that mid-infrared spectroscopy allows to monitor specific functional groups and smaller molecules (KOCA *et al.*, 2007).
The FTIR-ATR spectra (range 4000-700 cm^{-1}) of the studied traditional *Pecorino d'Abruzzo* cheese provided a qualitative information by characterization of typical absorption bands (Fig. 2). Indeed, several significant differences appeared on the typical absorption bands arising from amide (1718 to 1581 cm^{-1} [NH-I] and 1581 to 1483 cm^{-1} [NH-II]), from lipids (1765 to 1718 cm^{-1} [C=O ester] and 2984 to 2831 [CH_3 and CH_2]), from -NH (in the region 3300 to 2984 cm^{-1}) and -OH (3300 to 3700 cm^{-1}).

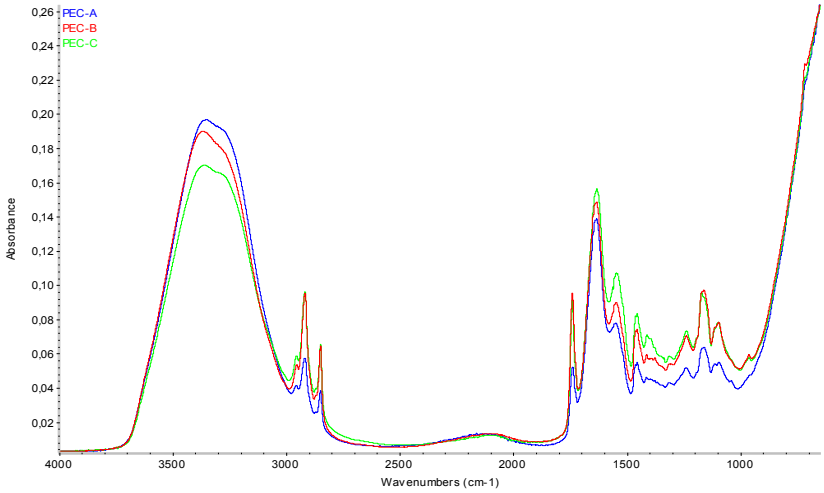

Figure 2: ATR-IR spectra average of traditional *Pecorino d'Abruzzo* cheeses.

In particular, the FT-IR qualitative study of the *Pecorino* d'Abruzzo cheeses under investigation allowed the identification of several significant ($P \leq 0.05$) changes in peak intensities in absorption bands of specific functional groups vibrations, that identify aliphatic and carbonyl stretching of acyl chain of fatty acids among samples (Table 6). However, the ratios CH_3 asym/CH_2 asym (2957 cm^{-1}/2920 cm^{-1}) and CH_3 sym/CH_2 sym (2873cm^{-1}/2851 cm^{-1}) were not significant.
Moreover, as reported in Table 6, the region of the carbonyl groups presents a maximum at 1741 cm^{-1} (VLACHOS *et al.*, 2006; ROHMAN *et al.*, 2011). The total amount of formed carbonyls or other secondary oxidation products causes a shoulder at 1728 cm^{-1} (VLACHOS *et al.*, 2006) that overlaps the stretching vibration at 1741 cm^{-1}, and thus a broadening of this peak to lower wavenumbers results. No significant differences ($P \geq 0.05$) were found among winter and summer samples when peak intensity changes are considered in this spectral area.

4. CONCLUSIONS

Results show that the main chemical characteristics of traditional *Pecorino d'Abruzzo* cheese samples, manufactured at different times of the year, are affected by the month of

production (season). As expected, in this traditional dairy product there was a great compositional variability: winter samples have a major protein content (P≤0.05) and a lower cholesterol and fat content than those manufactured in summer.

Table 6: Wavelengths and peak intensities of main functional groups that identify aliphatic and carbonyl stretching of acyl chain of fatty acids of traditional *Pecorino d'Abruzzo* cheeses.

Samples		**PEC-A**	**PEC-B**	**PEC-C**
Month of production		**February**	**July**	**December**
Functional Group	Wavelength cm^{-1}		Peak Intensities	
Asym $-CH_3$	2957	0.047±0.01^{b}	0.050±0.00^{b}	0.058±0.00^{a}
Asym $-CH_2$	2920	0.073±0.01^{b}	0.092±0.01^{ab}	0.098±0.02^{a}
Sym $-CH_3$	2873	0.028±0.00^{b}	0.032±0.01^{b}	0.037±0.01^{a}
Sym $-CH_2$	2851	0.046±0.01^{a}	0.061±0.00^{ab}	0.065±0.01^{b}
C=O	1741	0.060±0.02	0.087±0.02	0.090±0.02
Oxidation shoulder peak	1728	0.040±0.01	0.046±0.00	0.047±0.01
CH_3 and CH_2 Ratio	Wavelength cm^{-1} Ratio		Peak Intensities Ratio	
Asym/Asym	2957/2920	0.663	0.548	0.597
Sym/Sym	2873/2851	0.614	0.524	0.579

Data are result of means ± Standard Deviation (SD) of at least 15 spectra; values in the same row with different superscript letters are significantly different (P≤0.05).

Cheeses produced during summer showed a lower content in SFAs and a higher content of MUFAs than those produced in winter, whereas PUFAs and CLA were higher in winter samples. However, the ratio ω3/ω6 of FA was higher in winter cheese samples than in those manufactured in summer. The different FA profiles between winter and summer samples are also confirmed by the differences observed in the peak intensities in specific absorption bands of fatty acids.

In addition, the DAP parameter, as well as the DPPH values found in fat-soluble vitamins-rich extracts derived from *Pecorino* cheeses, reached the highest values in winter samples, whereas the information about the differences of colour, e.g. lower brightness (L*) and major yellowness (b*) in summer samples, can help to improve the physical knowledge of this traditional cheese.

As regard the nutritional value, *Pecorino d'Abruzzo* cheese exhibited appreciable nutritional properties due to a good level of coverage (%) of some nutrients for human adults (aged 18-59) according to Italian recommendations for daily requirements.

This work thus contributes to improving the information about the nutritional characteristics of *Pecorino d'Abruzzo*, that is currently scarce or not available, as well as to updating the Italian Food Composition Data Base.

ACKNOWLEDGEMENTS

This research was financially supported by MiPAAF "TERRAVITA" Project ((DM 25870/7303 - 02.12.2011).
The authors thank Dr. Francesca Melini for the editing and the linguistic revision of this paper.

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Paper Received November 26, 2015 Accepted May 24, 2016

POTENTIAL TECHNOLOGICAL INTEREST OF INDIGENOUS LACTIC ACID BACTERIA FROM ALGERIAN CAMEL MILK

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ABSTRACT

Nine isolates of lactic acid bacteria (LAB) obtained from the predominant microbiota of different camel milk samples collected in South-West Algeria, were selected in accordance with their growth ability in (cow) milk. The isolates were phenotypically and genotypically assigned to the following species: 4 *Leuconostoc mesenteroides* subsp. *dextranicum*; 2 *Lactobacillus brevis*; 2 *Lb. plantarum*; and 1 *Lactococcus lactis* subsp. *lactis*. One isolate from each of the *Leuconostoc* and *Lactobacillus* species were selected on the basis of their highest proteolytic and aminopeptidase activities. The selected isolates were used in combination with a commercial mesophilic O-type culture to make fermented milks. Sulfury flavor was detected as predominant in the sensory analysis of the milks made with only the *Ln. mesenteroides* and *Lb. brevis* adjuncts, which were characterized by the highest abundances of sulfur volatile compounds. Butter flavor was perceived in the milks made with the *Lb. plantarum*$^{(cit+)}$ adjunct, and was related to the presence of acetoin. Finally, cheese flavor prevailed in the milks made with both the *Lb. brevis* and the *Lb. plantarum* adjuncts, characterized by their high contents of short-chain free fatty acids. The results suggest the potential interest of these microorganisms in the manufacture of dairy products, particularly the combination of the *Lb. brevis* and *Lb. plantarum* isolates for cheese making.

Keywords: camel milk, lactic acid bacteria, *Lactobacillus brevis, Lactobacillus plantarum*, sensory analysis, volatile compounds

1. INTRODUCTION

One of the main challenges of modern dairy industry is the development of a range of varied products to meet the needs and tastes of the various sectors of an increasingly numerous and demanding population. The request for novel cultured products development having different and improved sensorial attributes requires the use of microbial strains with interesting properties for application in dairy fermentations (WOUTERS *et al.*, 2002). The strict hygiene measures and the high standardization of the production systems make the milk and dairy cultured products manufactured in the developed countries a poor source of novel and distinctive LAB strains. So, novel strains should be searched in little standardized raw materials and products, and in ecological niches with particular and unusual environmental conditions.
Camel (*Camelus dromedarius*) milk, besides being produced in traditional low-tech systems, has compositional characteristics that make it very special milk with clearly different environmental conditions (FARAH, 1993; KONUSPAYEVA et al., 2009) that may influence the metabolic abilities of the microbiota that settles it. The majority of the studies carried out on camel milk in the world, and particularly in Algeria focuses on the problem of its low clotting ability, which has been widely investigated (FARAH and BACHMAN, 1987; BOUDJENAH-HAROUN *et al.*, 2012). However, few works have been conducted regarding the microbiota of *Camelus dromedarius* milk or the biochemical and technological properties of LAB isolated from this source (ASHMAIG *et al.*, 2009; DRICI *et al.*, 2010; BENDIMERAD *et al.*, 2012; AKHMETSADYKOVA *et al.*, 2015).
In this context, the aims of the present study were: (i) to identify LAB isolates with potential technological interest belonging to the predominant microbiota of Algerian camel milk, and (ii) to characterize and to assay the isolates with a view to their use as adjunct cultures in the manufacture of fermented milks and cheeses with differentiated sensory characteristics.

2. MATERIALS AND METHODS

2.1. Bacterial cultures, media, and growth conditions

Nine LAB isolates obtained on pH 5.5 MRS medium (Oxoid Ltd., Basingstoke, UK) from six different camel milk samples collected in Bechar and Tindouf cities (southwest Algeria) were selected for this study. The selected isolates were able to clot cow milk after 12-24 h incubation at 30ºC. The bacterial cultures were maintained at –30ºC in MRS broth containing 20% glycerol (v/v). Working cultures were prepared by two consecutive transfers in MRS broth at 30 ºC. Lyophilized commercial mesophilic O-type culture FD-DVS R-704 (Chr. Hansen, Denmark), containing *Lactococcus lactis* subsp. *lactis* and *Lc. lactis* subsp. *cremoris* (phage-resistant) strains was used alone or in combination with selected indigenous LAB cultures for the preparation of fermented milks. This culture was stored at –20ºC and was directly inoculated in milk following the manufacturer's instructions.

2.2. Phenotypic identification of LAB isolates

Isolates were phenotypically assigned to the genus level as described by GARABAL *et al.* (2008). Utilization of citrate was also determined on citrate calcium agar (KCA) (NICKELS and LEESMENT, 1964), after 72 h incubation at 30ºC. Criteria followed for phenotypic identification were those compiled by WOOD and HOLZAPFEL (1995). Assignation to species level was made by means of the API 50 CHL carbohydrate fermentation strips

(bioMérieux, Marcy l'Etoile, France), following the manufacturer's instructions. The results at 48 h were analyzed using the API CH Lab software package (bioMérieux).

2.3. Genotypic identification of LAB isolates

For molecular identification of (presumptive) leuconostocs and lactobacilli isolates, chromosomal DNA from bacterial isolates was extracted from single-colony by using the SPEEDTOOLS DNA Extraction kit (Biotools B&M Labs, S.A., Madrid, Spain) following the manufacturer's instructions. From the obtained DNA, 20 μL aliquots were made and stored at – 20ºC until needed.
All DNA samples were tested using universal primers amplifying a 1000-bp region of the 16S rRNA gene 616V (forward): 5'-AGAGTTTGATYMTGGCTC AG-3' and 699R (reverse): 5'-RGGGTTGCGCTCGTT-3' (ARAHAL *et al.*, 2008). The primers were synthesized by Invitrogen Co. (Invitrogen, Carlsbad, CA, USA), diluted at a final concentration of 1 μg/μL with sterilized deionized water upon reception and stored at –20ºC. PCRs were performed in a reaction volume of 50 μL, containing 20 pM each of forward and reverse primers and 1 mL DNA template and prepared by using the DreamTaq DNA polymerase kit (Thermo Scientific, Waltham, MA, USA).
PCR amplifications were monitored in a Gene Amp thermal PCR System 2700 thermal cycler (Applied Biosystems, Foster City, CA, USA) under the following conditions: an initial cycle of 95ºC for 15 min; then 35 cycles of 95ºC for 30 s, 55ºC for 30 s, 72ºC for 1 min; and a final elongation step of 72ºC for 10 min. The PCR products were purified using the NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany). DNA sequencing was carried out with the v3.1 BigDye Terminator Cycle Sequencing kit (Applied Biosystems) on the ABI 3130xl capillary automate sequencer (Applied Biosystems) following the manufacturer's instructions. The obtained sequences were aligned to 16S rRNA gene sequences in the Gen Bank NCBI data base (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov) using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST) database.

2.4. Technological characterization of the selected LAB isolates

Leuconostocs and lactobacilli isolates were characterized for the following abilities: (a) carbohydrate fermentation, using the miniaturized API 50CHL system (bioMérieux), after 48 h incubation at 30ºC; (b) acidifying activity in sterile (110ºC, 15 min) reconstituted (10%, w/v) skim milk (pH 6.70) (Oxoid) after 6 h incubation at 30ºC (IDF, 1995); (c) proteolytic activity in sterile reconstituted skim milk (Oxoid) after 24 h incubation at 30ºC, as evaluated by the OPA method (Church et al. 1983); (d) aminopeptidase (AP) activity in sterile reconstituted skim milk (Oxoid) using leucine- and lysine-*p*-nitroanilide (both reagents from Sigma-Aldrich Corp., St. Louis, MO, USA) as substrates (EL SODA and DESMAZEAUD, 1982); (e) diacetyl-acetoin production in sterile reconstituted skim milk (Oxoid) after 48 h incubation at 30ºC (IDF 1997); (f) extracellular proteolytic activity on calcium caseinate agar (Merck GmbH, Darmstadt, Germany) (RODRÍGUEZ-ALONSO *et al.*, 2008); (g) extracellular lipolytic activity on 1% (w/v) tributyrin agar (Merck), containing 1% arabic gum (Panreac) (RODRÍGUEZ-ALONSO *et al.*, 2008); and (h) antimicrobial activity of the nine isolates against each other and against the commercial culture R-704 (Chr. Hansen, Hørsholm, Denmark), determined by the agar well diffusion assay as described by CENTENO *et al.* (2002).
All assays for testing technological abilities were performed in triplicate, and numerical results were expressed as the mean values obtained for each isolate.

2.5. Preparation of fermented milks

Eight fermented or acidified milks were prepared per assay. One of the milks was acidified with the mesophilic commercial culture R-704 alone, and the other seven milks were fermented with the commercial culture plus one among three selected isolates (*Leuconostoc mesenteroides* subsp. *mesenteroides* C8M, *Lactobacillus brevis* C21B, *Lactobacillus plantarum* C22P), or their combinations (C8M+C21B, C8M+C22P, C21B+C22P, and C8M+C21B+C22P). Fermented milks were prepared in 1 L of retail pasteurized (80ºC, 20 s) homogenized whole (3.6 g fat 100 mL^{-1}) milk (Leyma, A Coruña, Spain) contained in polyethylene bottles. The milks used for each assay corresponded to the same industrial batch and had been pasteurized the day before purchasing. The bottles were inoculated with 0.1 units of the DVS R-704 commercial culture. The DVS culture was previously rehydrated and strongly stirred in sterile (reconstituted) skim milk (Oxoid) at a ratio of 10 units per liter of milk, and then inoculated at 1% (v/v) in the pasteurized milk. The LAB isolates were cultured in sterile skim milk (Oxoid) at 30ºC for 16 h, and then inoculated at 1% (v/v) in the pasteurized milk. The inoculated milks were incubated at 30ºC for 24 h. Assays were made in triplicate.

2.6. Sensory analysis and volatile compounds produced in the fermented milks

For sensory analysis, the (clotted) fermented milks were intensely agitated and then distributed in 50 mL amounts in sterile polyethylene containers. Flavors of the fermented milks were perceived by smelling and tasting, as evaluated by a panel of ten regular consumers of fermented milks who had been trained as previously described (RODRÍGUEZ-ALONSO *et al.*, 2008). The judges were asked to agitate the milks before smelling and tasting. Odor and taste preference as well as overall acceptance were scored on a scale from 1 to 7. The acceptability indexes (AI) of the fermented milks were calculated by the formula: overall acceptance × 100/7 (DUTCOSKY, 1996).
Volatile compounds were determined in 20 mL samples taken after agitation of the clotted milks, and stored at –80 ºC until analysis. Volatile compounds were extracted by using the solid phase microextraction (SPME) technique and detected by gas chromatography coupled to mass spectrometry (GC–MS). For samples equilibration, 10 mL headspace vials containing 1 g of fermented milk were sealed with a PTFE-faced silicone septum (Supelco, Bellefonte, PA, USA) and maintained at 35ºC in a thermo block (Memmert model 100-800, Schwabach, Germany) during 15 min. Then, a 75 μm film thickness carboxen/polydimethylsiloxane (CAR/PDMS) fibre (Supelco) was exposed to the headspace while maintaining the sample at 35°C during 30 min. The SPME adsorbed compounds were injected to the chromatograph with a splitless mode injection at 260ºC for 8 min. The separation of volatiles was performed on a Hewlett-Packard 6890N (Agilent Technologies, Santa Clara, CA, USA) gas chromatograph equipped with a DB-624 capillary column (30m × 0.25 mm id, 1.4 μm film thickness; J&W Scientific, Folsom, CA, USA), following the method described by LORENZO and FONSECA (2014). Compounds were identified by comparing their mass spectra with those contained in the NIST05 (National Institute of Standards and Technology, Gaithersburg, MD, USA) library, and by comparing their mass spectra and retention time with authentic standards (Supelco). Abundances of volatile compounds are provided as peak area units/10^6 values.

2.7 Statistical analysis

Numerical data corresponding to technological characteristics, sensory analysis and volatile compounds were subjected to analysis of variance (ANOVA) and where statistical

differences were noted, differences among the distinct groups (isolates or fermented milks) were determined by the Duncan's test at a significance level of $P < 0.05$. All statistical procedures were performed with the SPSS software, version 20.0 (SPSS Inc., Chicago, IL, USA).

3. RESULTS AND DISCUSSIONS

3.1. Isolation and identification of LAB isolates

Low quantities (mean value of 2.50 log cfu mL^{-1}) of LAB were found in the six samples of camel milk used for LAB isolation (data not shown). This fact may be attributed to the high content of lysozyme and ascorbic acid in camel milk (FARAH, 1993). Among the 45 LAB isolates initially obtained, 27 (60%) were phenotypically assigned to *Leuconostoc/Weissella* genus (data not shown). Similarly to the results of this study leuconostocs have often been found as the major LAB in camel milk (ASHMAIG *et al.*, 2009; AKHMETSADYKOVA *et al.*, 2015), this may be attributed to the higher resistance to lysozime of this microbial group as compared to other LAB (LIMONET *et al.*, 2004). As regards the remaining 18 isolates, eight were identified as mesophilic lactobacilli, one was assigned to *Lactococcus* genus, and nine LAB isolates (unable to clot cow milk) could not be assigned to genus level (data not shown).
The selected nine isolates able to clot (cow) milk after 12-24 h incubation at 30°C were identified by means of the API 50 CHL system as follows: four *Leuconostoc mesenteroides* subsp. *dextranicum* (similarity level of 95.2-99.8%); two *Lactobacillus brevis* (99.7% similarity); two *Lactobacillus plantarum* (77.8-79.3% similarity); and one *Lactococcus lactis* subsp. *lactis* (72.3% similarity) (data not shown). Both the *Lb. plantarum* and the *Lc. lactis* subsp. *lactis* isolates were able to metabolize citrate on KCA medium (data not shown). The low proportion of leuconostoc isolates showing the ability to coagulate milk may be explained by the fact that these microorganisms are adapted to growth on vegetables and roots and therefore lack sufficient proteolytic ability to grow in milk (VEDAMUTHU, 1994). The lactococcal isolate was not further considered in this study because of the known susceptibility of lactococcal cells to bacteriophage infection, as lytic phage infection is at present a major cause of fermentation failure (GARNEAU and MOINEAU, 2011).
Phenotypic identification of the selected (presumptive) leuconostocs and lactobacilli isolates was confirmed by sequencing of the fragments of the 16S rDNA gen amplified by PCR (data not shown). Sequences obtained from the four presumptive *Ln. mesenteroides* isolates produced significant (97-99%) alignments with the complete genome of *Ln. mesenteroides* subsp. *dextranicum* DSM 20484. Sequences from the two presumptive *Lb. brevis* isolates aligned (97%) with that of *Lb. brevis* ATCC 367. Lower percentages of similarities (91-94%) were found between the presumptive *Lb. plantarum* isolates and the *Lb. plantarum* JDM1 strain (genomes of all the type strains included in the NCBI data base). Similarly to the results of this study, the species *Lb. plantarum* and *Lb. brevis* have been isolated from Sudanese fermented camel milk (ASHMAIG *et al.*, 2009), and *Lc. lactis* subsp. *lactis*$^{cit+}$ has been found in Algerian fermented camel milk (DRICI *et al.*, 2010; BENDIMERAD *et al.*, 2012).

3.2. Technological properties of the selected leuconostocs and lactobacilli isolates

The most relevant carbohydrate fermentation abilities exhibited by the selected LAB strains are shown in Table 1. Despite of their ability to hydrolyze lactose in the API 50 CHL test tube, all the selected isolates (*Ln. mesenteroides* subsp. *dextranicum* C2M, C5M,

C8M and C14M; *Lb. brevis* C21B and C27B; and *Lb. plantarum* C14P and C22P) showed low acidifying activities (mean pH values of 6.42-6.65, and mean titratable acidities of 0.06-0.21 g lactic acid 100 mL^{-1}) after 6 h incubation at 30°C (Table 1).

The pH values of the milk cultures of the lactobacilli isolates were significantly ($P < 0.05$) lower than those of the leuconostocs isolates. The lowest mean pH values corresponded to the two isolates of *Lb. brevis* (Table 1). On the basis of their low acidifying activities, *Lb. brevis*, *Lb. plantarum* and *Ln. mesenteroides* have been generally classified as adjunct LAB to be used together with a lactococcal starter in order to enhance the acidification of milk (SETTANNI and MOSCHETTI, 2010).

The proteolytic activities evaluated by means of the OPA test ranged between 0.124 lysine mM for one *Lb. brevis* isolate and 0.230 lysine mM for one *Ln. mesenteroides* isolate (Table 1). These values are much lower than those reported by HERREROS *et al.* (2003) for 17 isolates of leuconostoc and mesophilic lactobacilli obtained from raw goat's milk cheeses (mean value equivalent to 0.296 lysine mM) and by GARABAL *et al.* (2008) for 42 isolates of mesophilic lactobacilli from raw cow's milk cheeses (mean value equivalent to 0.298 lysine mM). Nevertheless, this low proteolytic activity could be a suitable trait if these bacteria are used as adjunct cultures, in order to prevent development of bitterness in the final product (NANDAN *et al.*, 2010).

All the leuconostocs and lactobacilli isolates exhibited both Leu- and Lys-aminopeptidase activities, with higher Lys-AP than Leu-AP values (Table 1). Differences among species were pronounced, with the two *Lb. brevis* isolates showing significant ($P < 0.05$) higher values for both activities (highest mean values of 200 U for Leu-AP and 530 U for Lys-AP) than the *Lb. plantarum* and *Ln. mesenteroides* isolates. Values obtained for the *Lb. plantarum* isolates were also significantly ($P < 0.05$) higher than those of the *Ln. mesenteroides* isolates (Table 1). High Leu- and Lys-aminopeptidase activities have been detected for cell-free extracts of some strains of *Lb. brevis*, *Lb. plantarum* and *Ln. mesenteroides* isolated from raw ewe's and goat's milk cheeses (MACEDO *et al.*, 2000; HERREROS *et al.*, 2003). Similarly to the results of the present study, NIETO-ARRIBAS *et al.* (2009) found low Leu- and Lys-AP activities for intact cells of 19 *Ln. mesenteroides* subsp. *dextranicum* isolates obtained from artisanal Manchego ewe's cheese, with Lys-AP activity being higher than Leu-AP activity for all the isolates. The same authors (NIETO-ARRIBAS *et al.*, 2010) reported higher values of both Leu-AP and Lys-AP activities for most of 10 *Lb. plantarum* isolates than those of *Ln. mesenteroides* isolates from the same cheese variety. Aminopeptidases play a key role in the degradation of bitter peptides and flavor formation during cheese ripening (URBACH *et al.*, 1995; NANDAN *et al.*, 2010).

Only the two *Lb. plantarum* isolates able to metabolize citrate on KCA produced diacetyl-acetoin in skim milk (Table 1). The production of diacetyl-acetoin by *Lb. plantarum* C22P (120 mg diacetyl L^{-1}) was significantly ($P < 0.05$) higher than those observed for *Lb. plantarum* C14P (45 mg L^{-1}) and for the initially selected *Lc. lactis* subsp. *lactis*$^{(cit+)}$ isolate (35 mg L^{-1}, data not shown). GARABAL *et al.* (2008) reported a mean production equivalent to 84 mg diacetyl L^{-1} for 33 isolates of mesophilic facultatively heterofermentative lactobacilli obtained from Galician (northwest Spain) raw cow's milk cheeses. Production of diacetyl by LAB could be considered an interesting technological property since this compound is related to positive (butter) flavor and antimicrobial effect in dairy products (DRICI *et al.*, 2010).

None of the isolates exhibited extracellular proteolytic or lipolytic activities in spite of their growth on calcium caseinate agar and on tributyrin agar media (data not shown).

Table 1: Technological characterization of the selected LAB isolates obtained from camel milk (quantitative results are means of three replicates).

	Leuconostoc mesenteroides				*Lactobacillus brevis*		*Lb. plantarum*		S.E.M.[1]	*P* value
Fermentation of:	**C2M**	**C5M**	**C8M**	**C14M**	**C21B**	**C27B**	**C22P**	**C14P**		
L- arabinose	–	–	–	–	+	+	+	+		
D-cellobiose	–	–	–	–	–	–	+	+		
D- fructose	+	+	+	+	+	+	+	+		
D-galactose	+	+	–	–	+	+	+	+		
D-glucose	+	+	+	+	+	+	+	+		
D-lactose	+	+	+	w[2]	+	+	+	+		
D-maltose	+	+	+	+	+	+	+	+		
D-mannose	+	+	+	+	–	–	+	+		
D-melibiose	–	–	–	–	+	+	+	+		
D-raffinose	–	–	–	–	–	–	–	–		
L-rhamnose	–	–	–	–	–	–	–	–		
D-ribose	w	–	w	w	+	+	+	+		
D-saccharose	+	+	+	+	–	w	+	+		
L-sorbose	–	–	–	–	–	–	–	–		
D-trehalose	+	+	+	+	–	–	+	+		
D-xylose	w	w	w	w	+	+	–	–		
Acidifying activity (skim milk; 30 ºC, 6 h)										
pH	6.60^{b}	6.65^{a}	6.63^{a}	6.65^{a}	6.42^{d}	6.45^{cd}	6.48^{c}	6.50^{c}	0.003	0.000
Titratable acidity[3]	0.10^{bc}	0.06^{d}	0.08^{cd}	0.09^{c}	0.21^{a}	0.21^{a}	0.14^{b}	0.12^{b}	0.0002	0.000
Proteolytic activity (OPA)[4]	0.210^{ab}	0.212^{ab}	0.230^{a}	0.189^{b}	0.126^{d}	0.124^{d}	0.180^{bc}	0.178^{c}	0.009	0.012
Aminopeptidase activity[5]										
Leu-AP	17.2^{d}	14.6^{d}	24.0^{d}	19.4^{d}	200^{a}	197^{a}	109^{b}	51^{c}	66	0.000
Lys-AP	73^{c}	68^{c}	73^{c}	71^{c}	530^{a}	528^{a}	129^{b}	134^{b}	161	0.000
Producton of diacetyl-acetoin[6]	0.0^{c}	0.0^{c}	0.0^{c}	0.0^{c}	0.0^{c}	0.0^{c}	120^{a}	45^{b}	6.9	0.000

[a-d]Mean values within a row with different superscripts are significantly different ($P < 0.05$; Duncan's test)
[1]S.E.M.: Standard error of the mean
[2]w: weak reaction
[3]Expressed as g lactic acid 100 mL^{-1}
[4]Expressed as mM lysine
[5]Expressed as enzymatic units (1 enzymatic unit = amount of enzyme giving an absorbance of 0.001 units at 410 nm min^{-1})
[6]Expressed as mg of diacetyl per liter of milk

The absence of extracellular enzymatic activities in LAB other than enterococci has been generally reported (HERRERO *et al.*, 1996; BUFFA *et al.*, 2004; NIETO-ARRIBAS *et al.*, 2009, 2010). According to HERRERO *et al.* (1996), LAB used as starter cultures should ideally present low lipolytic activity, as the degradation of milk fat must be slight in order to induce aroma production without giving rancid flavors.
One isolate from each one of the identified species (*Ln. mesenteroides* subsp. *dextranicum* C8M; *Lb. brevis* C21B; and *Lb. plantarum* C22P) were selected in accordance with their expected better technological performance (highest proteolytic and AP activities, and production of diacetyl for *Lb. plantarum* isolates). It should be pointed out that the similarity in the results of biochemical and genotypic (data not shown), and technological assays for *Lb. brevis* C21B and C27B could indicate that these two isolates probably belong to the same strain, although they were obtained from different camel milk samples (data not shown). The selected isolates were used to make fermented milks in combination with the commercial culture R-704 (Chr. Hansen) after confirming the absence of antibacterial activity against each other and against the commercial culture (data not shown).

3.3. Sensory analysis and volatile compounds produced in the fermented milks

The milks fermented with the commercial culture alone (control) and those cultured with the commercial starter in combination with both *Ln. mesenteroides* C8M and *Lb. plantarum* C22P, and in combination with the three selected isolates received the highest scores for sensory attributes (Table 2). The scores reached for the odor attribute were significantly ($P < 0.05$) higher for these last two fermented milks than for the other milks, and the scores for the taste attribute were significantly ($P < 0.05$) higher for the milk fermented with both the C8M and C22P adjuncts than for the other cultured milks. The highest acceptability index (86.1%) corresponded to the milk cultured with both the C8M and C22P isolates, followed by the milk fermented with the three selected isolates (76.7%) and by the control milk (74.5%) (Table 2).

Table 2: Mean sensory scores and main flavor descriptors of the fermented milks made with the commercial FD-DVS R-704 O-type starter alone (control) and in combination with cultures of the selected isolates obtained from camel milk.

	Control	+C8M	+C21B	+C22P	+C8M +C21B	+C8M +C22P	+C21B +C22P	+C8M +C21B +C22P	S.E.M.[1]	*P* value
Odor	5.0^{b}	4.8^{bc}	4.7^{bc}	4.9^{bc}	4.4^{c}	5.8^{a}	4.8^{bc}	5.7^{a}	0.060	0.039
Taste	5.3^{b}	3.9^{d}	3.8^{d}	4.8^{bc}	5.3^{b}	6.2^{a}	5.0^{bc}	5.0^{bc}	0.087	0.000
Main flavor descriptors	acid yoghurt	sulfury metallic	sulfury garlic	butter vanilla	sulfury sour	butter yoghurt	cheese butter	cheese butter		
AI (%)[2]	74.5^{bc}	62.8^{e}	61.5^{e}	70.1^{d}	70.2^{d}	86.1^{a}	71.7^{cd}	76.7^{b}	1.9	0.000

[a-e]Mean values within a row with different superscripts are significantly different ($P < 0.05$; Duncan's test)
[1]S.E.M.: Standard error of the mean
[2]AI (%): acceptability index of the fermented milk calculated by the formula: overall acceptance × 100/7 (in accordance with the used 7-point scale)

The flavor descriptors used by the majority of the judges include: acid and yoghurt for the control milk; butter and yoghurt for the milk fermented with both the C8M and C22P adjuncts; and butter and cheese for the milk cultured with the three selected isolates (Table

2). The butter and vanilla notes described for the milks made with the *Lb. plantarum* C22P adjunct may be related to the production of diacetyl and acetoin by this culture. These compounds are responsible for butter and nuts flavors in cheese (KONDYLI *et al.*, 2003; KAMINARIDES *et al.*, 2007). Sulfury and garlic nuances were detected by most of the judges in the milk fermented with the sole *Lb. brevis* C21B adjunct, which showed the lowest AI (61.5%) (Table 2). The sulfur flavors are related to the production of sulfur compounds principally from L-methionine (Met). It has been reported that sulfur compounds provide particular flavor notes in cheese such as garlic taste (KAMINARIDES *et al.*, 2007). The milks fermented with the C21B adjunct in combination with either the C8M or the C22P isolates received significant ($P < 0.05$) higher scores for the taste attribute than the milk made with the C21B isolate as the only adjunct culture (Table 2). This suggests that the "sulfury defect" was moderated due to the presence of other flavor compounds.

Only ten volatile compounds were definitely identified in the fermented milks with the methodology used in the present study. These volatiles include: the alcohol 2-heptanol; the ketones 3-hydroxy 2-butanone (acetoin), 2-heptanone and 2-nonanone; the fatty acids acetic acid, butanoic acid, hexanoic acid and octanoic acid; and the sulfur compounds dimethyl disulfide (DMDS) and dimethyl trisulfide (DMTS) (Table 3).

Table 3: Mean abundances (expressed as peak area units / 10^6) of each of the volatile compounds determined in the fermented milks made with the commercial FD-DVS R-704 O-type starter alone (control) and in combination with cultures of the selected isolates obtained from camel milk.

	Control	+C8M	+C21B	+C22P	+C8M +C21B	+C8M +C22P	+C21B +C22P	+C8M +C21B +C22P	S.E.M.[1]	*P* value
ALCOHOLS										
2-Heptanol	nd[d2]	4.1[c]	5.2[c]	14.0[a]	8.6[b]	14.2[a]	10.8[ab]	9.8[ab]	0.67	0.003
KETONES										
3-Hydroxy 2-butanone	nd[d]	nd[d]	nd[d]	93[a]	nd[d]	20.1[b]	nd[d]	5.4[c]	21.8	0.031
2-Heptanone	1.1[b]	2.8[b]	1.5[b]	29.3[a]	0.8[b]	28.2[a]	4.8[b]	4.7[b]	3.06	0.001
2-Nonanone	nd[d]	1.3[b]	0.4[c]	3.1[a]	nd[d]	2.8[a]	nd[d]	nd[d]	0.39	0.043
FATTY ACIDS										
Acetic acid	128[e]	239[c]	149[de]	329[a]	160[d]	265[bc]	131[e]	281[b]	18.9	0.005
Butanoic acid	3.8[e]	5.8[d]	9.7[bc]	9.2[bc]	8.1[c]	7.4[c]	15.4[a]	10.7[b]	0.66	0.006
Hexanoic acid	nd[f]	2.7[e]	7.3[bc]	6.5[c]	5.6[d]	6.7[c]	12.7[a]	7.8[b]	0.56	0.004
Octanoic acid	nd[c]	nd[c]	0.8[ab]	0.5[b]	0.5[b]	0.5[b]	1.2[a]	1.1[a]	0.073	0.019
SULFUR COMPOUNDS										
Dimethyl disulfide	0.7[cd]	2.0[a]	2.3[a]	1.3[b]	2.1[a]	1.0[bc]	0.5[d]	0.6[d]	0.21	0.011
Dimethyl trisulfide	nd[b]	nd[b]	0.3[a]	nd[b]	0.2[a]	nd[b]	nd[b]	nd[b]	0.01	0.001

[a-e]Mean values within a row with different superscripts are significantly different ($P < 0.05$; Duncan's test)
[1]S.E.M.: Standard error of the mean
[2]nd: Compounds not detected (considered as 0.0 values for statistical analysis)

The poor volatile profiles of the fermented milks, with the absence of aldehydes and the practical absence of alcohols, may be partially explained on the basis of the SPME fiber performance. It has been reported that the porous CAR/PDMS fiber coating shows a higher affinitiy for low-molecular-weight compounds, including a high proportion of ketones (LORENZO, 2014). In addition, absorption for the most compounds may reach equilibrium with a longer time of exposure than that assayed in this work (MARCO *et al.*, 2004), although longer exposure times could result inappropriate due to oxidation phenomena that can produce competitive effects between compounds (LORENZO, 2014).
The secondary alcohol 2-heptanol, and its probable precursor 2-heptanone were present in all the fermented milks made with the adjunct cultures, but not in the control milk. The highest abundances of (2-heptanone and) 2-heptanol were found in the milk fermented with the *Lb. plantarum* C22P adjunct. The abundances of 2-heptanol in the milks made with the sole C22P adjunct and with the combination of the C8M and C22P adjuncts were significantly ($P < 0.05$) higher than those of the milks made without the C22P adjunct culture. This compound, associated to herbaceous and fruity aromas has been considered as a key odorant in many cheese varieties (DELGADO *et al.*, 2011).
Regarding the ketones group, 3-hydroxy 2-butanone (acetoin) was only detected in the milks cultured with the *Lb. plantarum* C22P adjunct (Table 3), as it could be expected from the results of the technological assays. Acetoin is produced by the reduction of diacetyl originated from citrate metabolism, or by decarboxylation of α-acetolactate (McSWEENEY *et al.*, 2000). Diacetyl, and to a lesser extent acetoin are generally appreciated for their buttery and nut-like notes (CURIONI *et al.*, 2002), and may be responsible to some extent for the butter flavors described in the sensory analysis. The abundances of the methyl ketones 2-heptanone and 2-nonanone were significantly ($P < 0.05$) higher in the milks made with the sole *Lb. plantarum* C22P adjunct culture and with the combination of the C8M and C22P adjuncts than in the other fermented milks (Table 3). The methyl ketones 2-heptanone and 2-nonanone are associated with cheesy odors, particularly with blue cheese notes (CURIONI *et al.*, 2002). Methyl ketones containing odd numbers of C atoms are produced from β-oxidation of fatty acids (McSWEENEY *et al.*, 2000), which may have been released through the esterase and/or lipase activities of the lactobacilli.
The four fatty acids identified were detected in all the fermented milks made with the adjunct cultures, with the exception of octanoic acid in the milk fermented with the sole C8M leuconostoc adjunct. The abundances of acetic acid were significantly ($P < 0.05$) higher in the milks made with the sole *Lb. plantarum* C22P adjunct culture than in the other fermented milks (Table 3). Citrate-fermenting microorganisms convert this compound to pyruvate, carbon dioxide, and acetic acid, together with various carbonyl compounds such as diacetyl and acetoin. Facultatively heterofermentative lactobacilli such as *Lb. plantarum* may also produce acetate from lactose or from amino acids (BUFFA *et al.*, 2004). Acetate is primarily formed from citrate and this may be the main reason why this compound is more abundant in the milks fermented with the *Lb. plantarum* C22P adjunct. Acetate also comes from pyruvate originated in the glycolysis from carbohydrates, and this would explain its presence in milks fermented with adjuncts unable to metabolize citrate. Acetic acid is responsible for sour flavor in dairy products (KAMINARIDES et al., 2007). The abundances of butanoic and hexanoic acids were significantly ($P < 0.05$) higher in the milks made with the combination of *Lb. brevis* C21B and *Lb. plantarum* C22P adjunct cultures than in the other fermented milks (Table 3). MENÉNDEZ *et al.* (2000) found higher volatile free fatty acids contents (3.05 vs. 1.33 meq/100 g) in 1-day samples of cow's cheeses made with a *Lb. plantarum* adjunct culture than in control samples. Butanoic acid may be derived from lipolysis of milk fat or produced through the fermentation of lactose and lactic acid (KAMINARIDES *et al.*, 2007). However, and despite the fact that the strains tested in the present study did not exhibit (exocellular) lipolytic activity when growing on

tributyrin agar, hexanoic and octanoic acids are probably released from triglycerides through the action of non-specific bacterial esterases and lipases as reported by GONZÁLEZ DE LLANO *et al.* (1996) and BUFFA *et al.* (2004). Short-chain fatty acids are related to rancid and pungent flavors (McSWEENEY *et al.*, 2000; DELGADO *et al.*, 2011), and butanoic acid has been found to be a potent odorant in cheese (KAMINARIDES *et al.*, 2007). The high contents of butanoic and hexanoic acids may relate to the cheese flavors detected in the milks made with both the C21B and C22P adjuncts.
As regards sulfur compounds, the abundances of DMDS were significantly ($P < 0.05$) higher in the milks made with the *Ln. mesenteroides* C8M and the *Lb. brevis* C21B adjuncts, both alone or in combination, than in the other fermented milks (Table 3). DMTS was only detected in the milks made with the sole C21B adjunct and with the combination C8M+C21B. The thioesters DMDS and DMTS are expected to be formed mainly from the amino acid Met by the metabolism of LAB and secondary microbiota (URBACH, 1995; ENGELS *et al.*, 1997). It has been reported that a number of lactobacilli strains, including *Lb. brevis* are able to degrade Met (ENGELS *et al.*, 1997; SREEKUMAR *et al.*, 2009) which agrees with our findings regarding the *Lb. brevis* C21B adjunct assayed in the present study. The sulfury flavors detected in the sensory analysis of milks made with the *Ln. mesenteroides* C8M and the *Lb. brevis* C21B adjuncts, and the garlic nuance described in the milk made with the sole C21B adjunct culture may be attributed to the high contents of sulfur compounds.

4. CONCLUSIONS

The selected leuconostocs and lactobacilli isolates from camel milk used in the present study as adjunct cultures to manufacture fermented milks in combination with an acidifying starter, conferred different flavor nuances and were responsible for different volatile profiles of the products. Sulfury flavor was detected as a main flavor in the milks made with only the *Ln. mesenteroides* and *Lb. brevis* adjuncts, characterized by the highest abundances of volatile sulfur compounds. Butter flavor, related to the presence of the volatile 3-hydroxy 2-butanone (acetoin) was perceived in the milks made with the *Lb. plantarum*$^{(cit+)}$ adjunct, and cheese flavor, associated to high contents of volatile free fatty acids prevailed in the milks made with both the *Lb. brevis* and the *Lb. plantarum* adjuncts. The results suggest the potential interest of these LAB isolates in the manufacture of dairy products, in particular the combination of the *Lb. brevis* and *Lb. plantarum* isolates for cheese making.

ACKNOWLEDGEMENTS

Authors are grateful to Dr. José Manuel Lorenzo (Food Technology Center of Galicia, Ourense, Spain) for assistance with SPME–GC–MS methodology. This study was financially supported by the Government of Algeria, within The Program National Exceptional (PNE). K. Belkheir acknowledge receive a research fellowship from the Algerian Ministry of High Studies and Scientific Research, and the University of Relizane during these studies. This work was also partially supported by the Xunta de Galicia (The Spanish Regional Government) under the Consolidation and Restructuring Program of Competitive Research Units: Strategic Research Partnerships (2009/060).

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Paper Received December 30, 2015 Accepted May 19, 2016

EVALUATION OF THE *IN VITRO* ANTIMICROBIAL ACTIVITY OF MIXTURES OF *LACTOBACILLUS SAKEI* AND *LACTOBACILLUS CURVATUS* ISOLATED FROM ARGENTINE MEAT AND THEIR EFFECT ON VACUUM-PACKAGED BEEF

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ABSTRACT

A *L. sakei* and a *L. curvatus*-based mixtures exerted an antagonistic activity *in vitro* against 12 different spoilage and pathogenic bacteria. No activity was exerted by the cell-free supernatants. The addition of the two mixtures to sliced vacuum-packaged beef showed a better capability of *L. sakei* to adapt to meat substrate. After 60 days, Total Viable Count (5.8 *vs* 7.4 Log CFU/g), Gram-negative bacteria (2.5 *vs* 6.4 Log CFU/g) and *Enterobacteriaceae* (2.3 *vs* 4.4 Log CFU/g) were significantly lower in *L. sakei*-inoculated samples if compared with control ones; TVC and *Enterobacteriaceae* were also significantly lower in *L. curvatus*-inoculated samples than control ones. The addition of mixtures gave no significant effects on meat pH and colour. The use of high dosage of viable cells could be suggestible in order to exert an early conditioning of meat environment.

Keywords: *Lactobacillus sakei, Lactobacillus curvatus*, biopreservation, vacuum-packed beef, antimicrobial activity

1. INTRODUCTION

Meat preservation is a hard race against spoilage and potential pathogenic microorganisms and restriction methods need to be applied in order to reduce their growth and prolong the shelf-life. Recently, alternative technologies for the decontamination of meat products have been developed and implemented such as bioprotective cultures, natural antimicrobials, gamma, electron and x-ray irradiation, ozone, active packaging, high hydrostatic pressure, ohmic heating and steam pasteurization among the others (DEVLIEGHERE *et al.*, 2004; AYMERICH *et al.*, 2008; ZHOU *et al.*, 2010; LORETZ *et al.*, 2011). All the alternative technologies effort to be mild: their combination, as in the hurdle theory proposed by LEISTNER (2000), may improve their efficacy against pathogens and spoilage microorganisms, without modifying the sensorial qualities of the products.

In chilled vacuum-packaged raw meat, the oxygen source is restricted determining a selective effect on the microbial population; the main spoilage microorganisms associated with these type of food result as psychrotrophic, both Gram-positive bacteria, mainly Lactic Acid Bacteria (LAB) (*Lactobacillus* spp., *Leuconostoc* spp., *Carnobacterium* spp.) and *Brochothrix thermosphacta,* and Gram-negative, mainly represented by *Enterobacteriaceae* (SHAW and HARDING, 1984; HOLZAPFEL, 1998; LABADIE, 1999; NYCHAS and DROSINOS, 2000; FONTANA *et al.*, 2006; ERCOLINI *et al.*, 2009; PENNACCHIA *et al.*, 2011). In vacuum packaged meat, the natural LAB population increases during storage, becoming the predominant microflora: in particular, at chilling temperatures, LAB are able to exert antagonistic actions towards the growth of spoilage and pathogenic microorganisms in beef, pork, poultry and fish (KATLA *et al.*, 2002; YAMAZAKI *et al.*, 2003; CASTELLANO *et al.*, 2008).

In the last years, LAB have received great consideration as bioprotective cultures, leading to the discovery and characterization of several antimicrobial peptides (mainly bacteriocins, organic acids, carbon dioxide, ethanol, hydrogen peroxide and diacetyl), whose activity is well known (VIGNOLO *et al.*, 2000; CLEVELAND *et al.*, 2001; CASTELLANO and VIGNOLO, 2006; AYMERICH *et al.*, 2008; DORTU *et al.*, 2008; RAVYTS *et al.*, 2008). Their action is also due to the lowering of food pH and to the competition for nutrients (VANDENBERGH, 1993).

Different studies indicated that, during the storage, a gradual selection of LAB population occurs in the meat ecosystems, leading to the predominance of few *Lactobacillus* species (VIGNOLO *et al.*, 2010; 2012). *L. sakei* and *L. curvatus* have been observed as the most widespread species in vacuum-packaged beef (YOST and NATTRESS, 2002; FONTANA *et al.*, 2006; STELLA *et al.*, 2013).

Previous studies underlined the abilities of these two species as bioprotective cultures for meat, and their application to vacuum-packaged Argentine beef has already been described (CASTELLANO and VIGNOLO, 2006; CASTELLANO *et al.*, 2008). Their mechanism of action is expressed through the ability to produce not only bacteriocins but even organic acids. Moreover the good adaption to meat environment of *L. curvatus* and *L. sakei* was already proved, showing an important competitiveness in this substrate and an efficient use as an extra hurdle to minimize the risk of listeriosis in different muscle foods (SCHILLINGER *et al.*, 1991; HUGAS, 1998; CASTELLANO and VIGNOLO, 2006; FADDA *et al.*, 2008).

In a previous work 73 Lactobacilli were isolated from 8 lots of vacuum-packaged bovine rump hearts imported in Italy from Argentina, submitted to random amplified DNA-polymerase chain reaction and identified, showing a prevalence of *Lactobacillus sakei* (56 strains grouped in 18 different clusters) and *Lactobacillus curvatus* (8 strains grouped in 6 different clusters) (STELLA *et al.*, 2013).

One strain from each of the most representative clusters obtained of *L. sakei* (≥5 strains) and *L. curvatus* (≥2 strains), for a total 6 *L. sakei* and 2 *L. curvatus* strains, were chosen. Two specific mixtures were prepared (one *L. sakei*-based mixture and one *L. curvatus*-based mixture) and evaluated *in vitro* for their antimicrobial activity against spoilage and potential pathogenic microorganisms. Moreover, the effect of the addition of the two mixtures to sliced vacuum-packaged beef was investigated, considering microbiological and physical-chemical parameters

2. MATERIALS AND METHODS

2.1. Preparation of *Lactobacillus* strains and spoilage and potential pathogenic bacteria

All *L. sakei* and *L. curvatus* strains were stored in cryovials (Microbank™, Pro-Lab Diagnostics, Richmond Hill, Canada) at -70°C until the use. For each strain, a loop of the frozen culture was transferred to a test tube containing 10 mL of MRS broth (Oxoid, Basingstoke, UK) and incubated overnight at 30°C in jars (Anaerojar, Oxoid) with anaerobiosis generators (AnaeroGen, Oxoid). All the strains were re-inoculated into cooled MRS broth tubes and the initial absorbance (540 nm) (Shimadzu, UV1601, McCormick Place, Chicago, IL, USA) was measured. All the tubes were incubated at 15°C and the absorbance was measured after 24 and 48 h. Precultures were collected in exponential growth phase, defined as a change of absorbance of 0.05-0.2 at 540 nm. If necessary, the cultures were diluted before preparing the mixture in order to obtain the similar OD (optical density). Two specific mixtures were prepared (*L. sakei*-based mixture of strains n. 3, 42, 55, 77, 106 and 111 and *L. curvatus*-based mixture of strains n. 25 and 65) adding the same aliquot of broth of each strain.
A selection of 12 spoilage or pathogenic microorganisms was used as target strains for the test: *Escherichia coli* ATCC 25922, *Escherichia coli* 0157:H7 DSM 13526, *Proteus vulgaris* ATCC 8427, *Salmonella* Typhimurium ATCC 14028, *Serratia marcescens* ATCC 14756, *Yersinia enterocolitica* ATCC 23715, *Pseudomonas aeruginosa* ATCC 27853, *Pseudomonas fluorescens* ATCC 13525, *Pseudomonas putida* ATCC 49128, *Listeria monocytogenes* ATCC 7644, *Listeria innocua* ATCC 33090 and *Staphylococcus aureus* ATCC 6538. Each strain, stored in cryovials at -70°C until the use, was subcultured aerobically overnight at 37°C (30°C for *P. fluorescens* and *P. putida*) in 10 mL TSB tubes (Triptyc Soy Broth, Oxoid). All the strains were re-inoculated into cooled TSB tubes and the initial absorbance was detected. All the tubes were incubated at 15°C and the absorbance was measured after 24 and 48 h. Precultures were collected as reported above.

2.2. Antimicrobial activity test

For the evaluation of the antimicrobial activity, each mixture, prepared as reported above, was inoculated into MRS broth tubes and incubated at 30°C for 48 h in anaerobiosis. After incubation, each of the two broths was spotted by a sterile swab (Carlo Erba, Rodano, I) onto the surface of MRS agar plates, subsequently incubated for 48 h at 30°C in an anaerobic jar. For each spoilage or pathogenic microorganism, 0.2 mL of bacterial suspension were added to a 5 mL share of semisolid agar (BHI, Brain Heart Infusion Broth, Oxoid + agar 0.7%), maintained in a water bath (45°C) and then poured over the MRS plates previously spotted with each mixture. To avoid the dispersion of Lactobacilli from the spot into BHI, a little amount (3-4 drops) of the inoculated semisolid medium was firstly distributed by a sterile Pasteur pipette (Carlo Erba) on the surface of the spot; after solidification (about 3 minutes at room temperature), the remaining BHI was poured

on the plates. After aerobic incubation at 37°C (30°C for *P. fluorescens* and *P. putida*) for 24 h, the plates were checked. A clear zone around the *Lactobacillus* spot indicated the inhibition of the target microorganisms. The tests were conducted in triplicate.

2.3. Antimicrobial activity of cell-free supernatants against spoilage and potential pathogenic microorganisms

In order to determine if the inhibition was due to the production of antagonistic compounds, the cell-free supernatants of the cultured mixtures were tested against the same bacteria. The mixtures were subcultured in MRS broth as described above. After 48 h of incubation, an aliquot of each culture was centrifuged at 7700 rpm for 10 min. For each broth, pH was measured by a pH meter (Amel Instrument, 334-B, Milan, I): three independent measurements were performed on each sample. The supernatants obtained were subsequently filtered by 0.2 μm filters (Sacco, Cadorago, I) and maintained at 4°C. Each of the 12 target strains was inoculated into 10 mL TSB tubes and prepared as described in section 2.2; 1 mL of inoculated TSB was then transferred into 20 mL flasks of Tryptic Soy Agar (Oxoid), maintained in a water bath at 45°C, carefully mixed and poured in sterile Petri plates. Once the medium was solidified, blank discs (Oxoid) were dipped with the supernatant of each mixture and placed onto the plates, subsequently incubated at 37°C (30°C for *P. fluorescens* and *P. putida*) for 24 h. Clear zones around the discs were recorded. Finally, in order to evaluate if the eventual inhibition was due to the production of organic acids, the pH of cell-free supernatants were adjusted to 6.5 with NaOH (1 N) (Sigma Aldrich, St. Luis, USA) and the same test was repeated. All the tests were performed in triplicate.

2.4. Preparation and inoculation of vacuum-packaged meat slices

Two bovine rump hearts were sliced in a commercial cutting plant. From each meat cut, a total of 42 slices (1-cm thick, 50 g of weight) were obtained and inserted into individual sterile plastic bags, with a diffusion coefficient of 6/14 cm^3 m^{-2} atm^{-1} 24 h^{-1} to oxygen at 25°C and 75% relative humidity (Cryovac, Elmwood Park, NJ). The 42 slices obtained from each rump heart were divided into two series (each series including 21 discs) inoculated as follows:

- CLS (Control samples *L. sakei*), inoculated with 0.5 mL of sterile saline solution;
- LS (*L. sakei*), inoculated with 0.5 mL of a mixture of the six strains of *L. sakei* (final concentration of 5 Log CFU g^{-1});

and

- CLC (Control samples *L. curvatus*), inoculated with 0.5 mL of sterile saline solution;
- LC (*L. curvatus*), inoculated with 0.5 mL of a mixture of the two strains of *L. curvatus* (final concentration of 5 Log CFU g^{-1}).

A loop of the frozen culture of each strain was transferred to a test tube containing 10 mL of MRS broth (Oxoid) and incubated overnight at 30°C in jars (Anaerojar, Oxoid) with anaerobiosis generators. All the strains were re-inoculated into cooled MRS broth tubes and the initial absorbance (540 nm) was detected. All the tubes were incubated at 15°C and the absorbance was measured after 24 and 48 h. Precultures were collected in exponential growth phase. The bacterial cells were pelleted by centrifugation at 7700 rpm for 10 min at 4°C and washed twice in 10 mL of 0.1 M phosphate buffered saline (PBS) with pH 7.0. Cell density of each strain was determined by microscopy (1000x) (Meiji Techno America,

USA). An average value from 10 randomly picked fields of view was considered. As needed, precultures were diluted in 0.85% NaCl solution to obtain 5 Log CFU mL^{-1} suspensions prior to inoculate the products.
L. sakei-based mixture of strains n. 3, 42, 55, 77, 106 and 111 and *L. curvatus*-based mixture of strains n. 25 and 65 were finally prepared adding the same aliquot of each strain at a final concentration nearly of 5 Log CFU mL^{-1} (each *L. sakei* strains has a final concentration of 4.22 Log CFU mL^{-1}, each *L. curvatus* strains has a final concentration of 4.70 Log CFU mL^{-1}).
After inoculation, the plastic bags were submitted to a vacuum pump (final vacuum of 99%), sealed using a packaging machine (Orved VM 16, Musile di Piave, I) and immediately stored at 4°C. Samples were submitted in triplicate to analyses after inoculation (T0) and after 10 (T10), 20 (T20), 30 (T30), 40 (T40), 50 (T50) and 60 (T60) days of storage.

2.5. Microbiological analyses

10 g of each sample were diluted in physiological saline (0.85% NaCl) with 0.1% peptone and homogenized in a Stomacher for 60 s (Seward Stomacher 400 Blender Mixer Homogenizer, International PBI, Milano, IT). Serial 10-fold dilutions were prepared and the following parameters were evaluated: Total Viable Count (TVC) was performed on Plate Count Agar (PCA, Biogenetics, Ponte San Nicolò, I) (ISO 4833:2003) and incubated at 30°C for 48h; Lactobacilli were enumerated on MRS agar (Oxoid) (ISO 15214:1998) incubated at 30°C for 48h in anaerobiosis, Gram-negative bacteria were enumerated on Tryptone Soy Agar (Oxoid) supplemented with 10 UI mL^{-1} of penicillin G (Oxoid) (TSAP) and incubated at 30°C for 48h; the number of *Enterobacteriaceae* was determined on Violet Red Bile Glucose Agar (VRBGA, Biogenetics) according to the ISO 21528-2:2004 method.

2.6. Determination of pH and colour parameters evaluation

At each sampling time, pH was measured by a pH meter (mod. XS pH6, Ghiaroni &C., Buccinasco, Italy): three independent measurements were performed on each trimmed sample diluted 1:5 with distilled water; means were then calculated. The surface colour of the meat was assessed 45 min after opening the packages, in order to allow blooming (deoxymyoglobin oxygenation) on six randomly chosen spots of each sample surface using a Minolta CR-200 Chromameter (Minolta, Osaka, J). *L** (lightness), *a** ("red" index) and *b** ("yellow" index) parameters were determined. Chroma was calculated as a^2+b^2, the hue angle (h) was calculated as h = arctan (b*⁄a*), where h = 0 for red hue and h = 90 for yellowish hue. Total colour differences (ΔE*) between treated and control samples were calculated as: $\sqrt{(L1^*-L2^*)^2 + (a1^*-a2^*)^2 + (b1^*-b2^*)^2}$.
A ΔE* more than 2.3 means a variation hardly perceptible to the human eye, while ΔE* more than 3.0 a variation well perceptible to the human eye.

2.7. Statistical analysis

The experimental data from inhibition halos were analyzed by a two-way univariate analysis of variance, performed with MIXED procedure of SAS software (SAS Inst. Inc., Cary, NC, 2006) in order to in order to test mean inhibition halos size differences for the comparisons of interest at *Lactobacillus* mixtures by target strains levels.
Data from meat inoculation tests were also analyzed by a two-way univariate analysis of variance using the same SAS procedure to test response variable mean differences at the levels of interest of *Lactobacillus* mixtures by time. For all statistical evaluations, threshold

levels of $P \leq 0.05$ and $P \leq 0.01$ were considered for significance. A two-way multivariate analysis of variance was also performed on color parameters, considering the vector of values L*, a*, b* as the response variable; GLM Procedure of SAS software was used.

3. RESULTS AND DISCUSSIONS

3.1. Antimicrobial activity against spoilage and potential pathogenic microorganisms

The mean rays of the inhibition halos obtained from antimicrobial evaluation of *L. sakei* mixture and *L. curvatus* mixture are reported in Table 1. The two mixtures exerted an antagonistic activity, producing evident halos against all the 12 target strains tested (66.7% of the halos induced by *L. curvatus* mixture and 52.8% of halos produced by *L. sakei* mixture were >10 mm). Generally, *L. curvatus* mixture resulted significantly more effective if compared to *L. sakei* mixture ($P = 0.0383$), showing also a higher prevalence of halos > 20 mm (19.4% of the plates inoculated with the *L. curvatus* mixture *vs* 5.5% of those inoculated with *L. sakei* mixture). Considering the different target strains, *L. curvatus* mixture produced significantly wider halos against *Y. enterocolitica* ($P = 0.0383$) and *P. aeruginosa* ($P = 0.0325$).
If we consider the results of the target strains clustered in homogenous categories, it is evident that the most sensitive belonged to the genus *Pseudomonas*, whose components produced significantly higher halos if compared with *Enterobacteriaceae* ($P < 0.0001$), *Listeria* spp. ($P = 0.0004$) and *Staphylococcus aureus* ($P = 0.0117$), according to the results obtained by Moore et al. (2006) and Tirloni et al. (2014) who underlined that most of the species of *Pseudomonas* fail to grow under acid conditions.
In particular, *P. fluorescens* resulted to be the most susceptible among the 12 target strains tested as significantly wider halos were observed if compared with all the other strains ($P < 0.01$). Secondly, *P. putida* resulted to be significantly more susceptible if compared to *E. coli* O157:H7 ($P = 0.0357$), *E. coli* ($P = 0.0215$), *L. innocua* ($P = 0.0200$), *L. monocytogenes* ($P = 0.0411$), *P. vulgaris* ($P = 0.0084$), *S. marcescens* ($P = 0.0003$) and *S.* Typhimurium ($P = 0.0215$). Finally *P. aeruginosa* produced significantly wider halos if compared to *S. marcescens* ($P = 0.0185$).
Moreover, *Enterobacteriaceae* showed a high variability in susceptibility with differences among the various species due to the many interspecific and intraspecific differences among the bacteria tested (LIU *et al.*, 2013). *Serratia marcescens* was by far the most resistant target strain, and it showed significantly smaller halos if compared to *Y. enterocolitica* ($P = 0.0116$), the most sensitive of *Enterobacteriaceae*.
Many authors highlighted the presence of an evident antagonistic activity of LAB against *Listeria monocytogenes,* microorganism typically related to vacuum-packaged meat products (Jones *et al.*, 2008; Awisheh and Ibrahim, 2009). Even in our study, both *L. monocytogenes* and *L. innocua*, showed the production of modest halos (between 9.7 and 14 mm).
Considering the cell-free supernatants and the pH-adjusted supernatants, no activity was recorded for all the target strains tested, highlighting that the antagonistic effect originates probably from the nutrient competitive exclusion while the involvement of extracellular compounds was not detected in the species considered in this test. The mechanism of the antibacterial activity of *Lactobacillus* strains usually appears to be multifactorial: the well-known production of bacteriocins by *L. sakei* and *L. curvatus* strains, reported in many previous studies (CASTELLANO and VIGNOLO, 2006; CASTELLANO *et al.*, 2008; 2010) was not confirmed in our research.

Table 1: Halos (in mm) expressed as a mean of three replication induced by *Lactobacillus curvatus* mixture and *Lactobacillus sakei* mixture against spoilage or pathogenic target microorganisms.

Target strains	*L. curvatus* mixture	*L. sakei* mixture
Escherichia coli ATCC 25922	8.3±1.5	13.3±3.8
Escherichia coli O157:H7 DSM 13526	14.0±2.6	10.0±2.0
Proteus vulgaris ATCC 8427	10.3±1.2	7.3±1.5
Salmonella Typhimurium ATCC 14028	13.3±4.9	8.3±2.9
Serratia marcescens ATCC 14756	2.3±1.2	2.7±1.2
Yersinia enterocolitica ATCC 23715	19.7±3.1a	13.7±1.5b
Pseudomonas aeruginosa ATCC 27853	21.3±3.5a	10.0±5.0b
Pseudomonas fluorescens ATCC 13525	56.7±23.1	21.7±13.5
Pseudomonas putida ATCC 49128	30.0±34.7	17.3±2.5
Listeria monocytogenes ATCC 7644	14.0±3.6	10.7±1.5
Listeria innocua ATCC 33090	11.7±5.7	9.7±2.3
Staphylococcus aureus ATCC 6538	13.7±1.5	12.7±1.5

Values are expressed as mean ± standard deviation. Different lower-case letters are pointing out significant difference ($P<0.05$) at the levels of interest of experimental mixtures by target strains.

The potential antagonistic activity of LAB towards spoilage microorganisms is strongly favored by the packaging technique: actually, vacuum or modified atmosphere packaging are the main methods for distribution and commercialization of fresh meat. These systems help the extension of shelf-life limiting the replication of *Enterobacteriaceae* and *Pseudomonas* spp., bacterial communities that often dominate aerobic spoilage of fresh meat at temperatures between -1 and 25°C. Dominating microflora composed by LAB contribute to the quality of meat thanks to their carbohydrate and protein catabolism. As underlined also from our results, the inhibitory properties of LAB, are not only related to the production of organic acids (mainly lactic and acetic) or other compounds (organic acids, bacteriocins, hydrogen peroxide e.g.) but bioprotective actions are also due to the competition for nutrients. In fact, apart from the metabolic activity, starter cultures occupy vital niches, thereby discouraging the colonization of undesired microorganisms. In this context, LAB interact with other microorganisms and with the environment, increasing their biomass at a rate that depends on the physical and chemical characteristics of the substrates: in chilled fresh meat, competition for a growth-limiting substrate such as glucose and oxygen interaction among species occurs (GILL, 1976). In this context, the prevalence of certain species will be determined by their relative initial level, affinity for the substrates, substrate availability as well as the relative growth rate of the competing species at different temperatures (CASTELLANO et al., 2008).
As a matter of fact, in order to obtain an important growth inhibition of the target strains, the presence of high loads of live and metabolically active cells, is fundamental.

3.2. Inoculation of vacuum-packaged meat slices

LAB cultures, and in particular *L. sakei* and *L. curvatus*, have been often studied for the application to food with good results thanks to the inhibition of pathogens and spoilage microorganisms and with the aim to extend the shelf-life of raw meat without important changes in its sensorial properties (CASTELLANO and VIGNOLO, 2006).
In this study, the antagonistic activity observed *in vitro* was also revealed on meat (Fig. 1).
Considering the global effect of the application of *L. sakei* mixture to meat during the whole trial, TVC resulted significantly lower in treated samples (LS) if compared with the control ones (CLS) (P=0.0089), reaching at the end of the trial the loads of 5.8±0.4 and 7.4±0.5 Log CFU g^{-1}, respectively.
The addition of *L. sakei* mixture resulted, since the beginning of the trial, in a constantly higher level of LAB in LS samples if compared with CLS ($P < 0.0001$). In particular, in LS samples, LAB reached the plateau level between 8 and 8.5 Log CFU g^{-1} after only 20 days from the beginning of the experiment. In CLS samples, the LAB naturally present on the slices showed a rapid increase from the beginning until T20; afterwards, they reached a plateau level between 6.6 and 7.4 Log CFU g^{-1}. Considering Gram-negative bacteria for the whole period, LS samples values resulted to be significantly lower than CLS ones ($P = 0.0029$). Moreover, *Enterobacteriaceae* resulted to be significantly lower in LS samples considering the whole trial ($P < 0.0001$). In particular LS samples showed a very stable trend (LS T0=2.3±0.5 *vs* T60=2.3±0.5 Log CFU g^{-1}), while in CLS samples, *Enterobacteriaceae* reached a value of 4.4±1.9 Log CFU g^{-1} at T60; anyway such level of contamination is not generally associated to evident sensorial spoilage of raw meats.
Considering the effect obtained from the application of *L. curvatus* mixture to meat in the whole experimental period, TVC resulted significantly lower in treated samples (LC) than in control (CLC) ones ($P = 0.0013$). The addition of *L. curvatus* mixture resulted, since the beginning of the trial, in a constantly higher level of LAB in LC samples if compared with CLC ($P < 0.0001$). In particular, in LC samples, LAB reached the plateau level between 7.9 and 8.4 Log CFU g^{-1} after only 20 days from the beginning of the experiment, according with LS results. In CLC samples, the LAB naturally present in the product, characterized in this case by a higher load if compared with CLS (3.5±0.6 Log CFU g^{-1} at T0), showed a rapid increase from T20; afterwards, they reached a plateau level between 6.1 and 7.5 Log CFU g^{-1}.
Considering Gram-negative bacteria, the loads resulted to be quite comparable between LC and CLC samples until T20 and then very highly fluctuant data were obtained; considering the whole period no significant differences were recorded ($P = 0.3325$).
Enterobacteriaceae resulted to be constantly lower in LC samples until T60, showing a general significant difference ($P = 0.0225$): in particular they showed a very stable trend for the whole study (LC T0=2.3±0.6 *vs* T60=3.0±1.5 Log CFU g^{-1}). In CLC samples, *Enterobacteriaceae* showed an increasing trend since the beginning of the trial, even if not reaching the threshold level of 5 Log CFU g^{-1} (CLS T0=2.5±0.8 *vs* T60=4.0±1.8 Log CFU g^{-1}).
The effect of the inoculation with *L. sakei* mixture resulted generally more evident than the treatment with *L. curvatus* mixture, suggesting a better capability to adapt to vacuum packaged meat substrate. The better adaptation of *L. sakei* mixture (LS) confirmed the preponderance of *L. sakei* in long shelf-life vacuum packaged meat LAB population, as highlighted in the previous study (STELLA *et al.*, 2013).

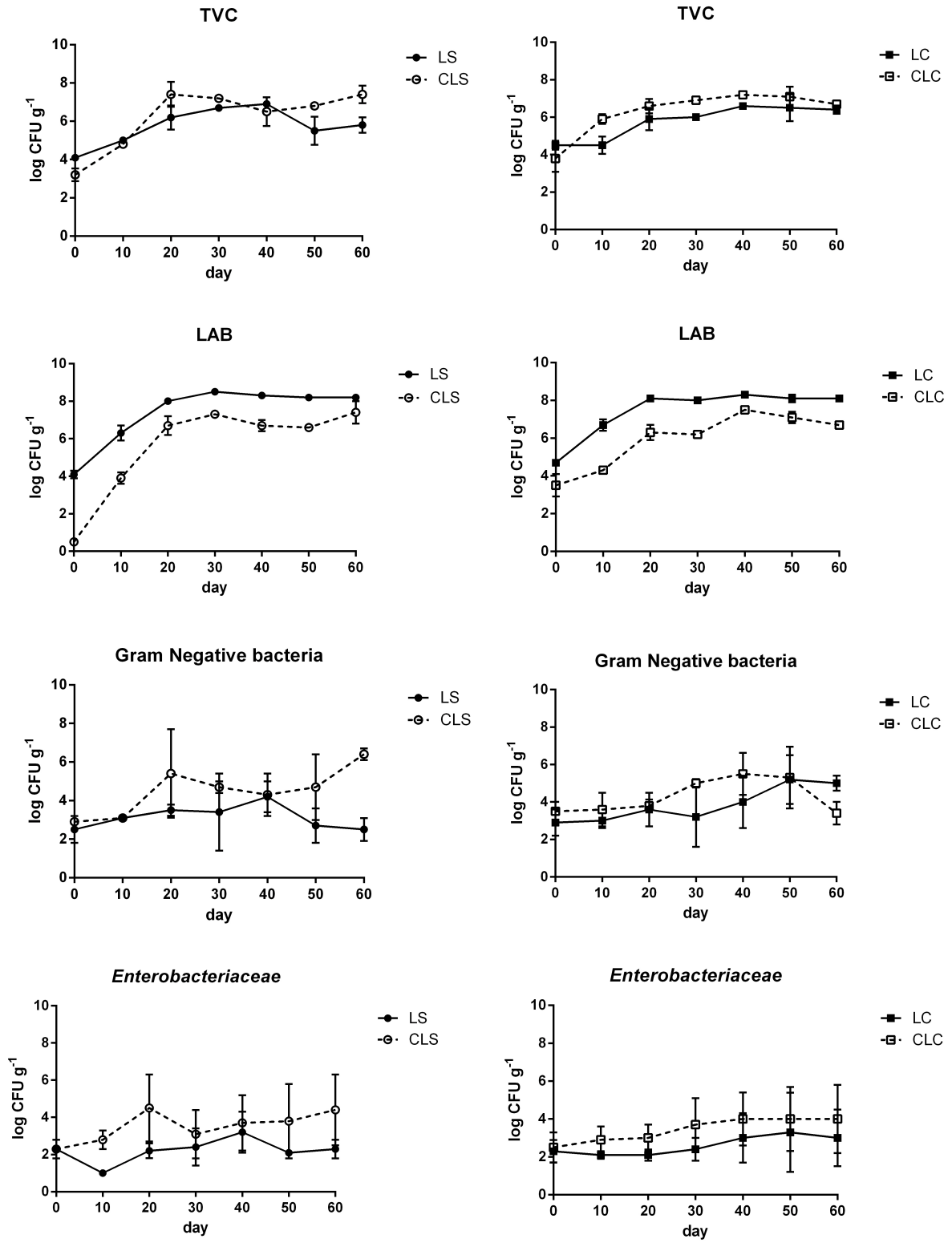

Figure 1: Results of total viable count (TVC), Lactic Acid Bacteria (LAB), Gram-negative bacteria and *Enterobacteriaceae*.
CLS= Control samples for *Lactobacillus sakei*; LS= samples inoculated a mixture of the six strains of *Lactobacillus sakei*; CLC= Control samples for *Lactobacillus curvatus*; LC= samples inoculated with a mixture of the two strains of *Lactobacillus curvatus*.

In any case, the capability of both of the two LAB mixtures to inhibit the growth of spoilage bacteria, clearly demonstrated *in vitro*, was also confirmed on meat substrate, also if it resulted more limited. This could be explained by the different growth rates and competitiveness of the cultures if applied to a complex matrix like meat: the adaptation to a substrate depends especially on the metabolic activity of cultures, which occupy vital niches, thus discouraging colonisation of undesired microorganisms. Generally, an antagonistic effect was detected both for *L. sakei* and *L. curvatus* treatments: despite the promising current knowledge and laboratory studies, LAB strains often suffer from a limited effectiveness in foods; among the others, the main factors involved are the poor adaptation to food environment, the inactivation of antimicrobial compounds through proteolytic enzymes or the binding to food ingredients and the pH buffering action (HOLZAPFEL *et al.*, 1995). In our case, the production of organic acids by the cultures inoculated on meat is supposable, even if their activity could be limited by the buffering capacity of meat. The metabolic activity of LAB population of vacuum packaged meat could be deduced by the slight reduction of pH of meat during the trial, with a 0.25 and 0.39 decrease in meat treated with the two mixtures (LS: T0 = 5.48 *vs* T60 = 5.23; LC: T0 = 5.80 *vs* T60 = 5.51), very close to the decrease observed (0.30-0.44) in control samples (CLS: T0 = 5.71 *vs* T60 = 5.27; CLC: T0 = 5.75 *vs* T60 = 5.45).

3.3. Determination of colour parameters

The addition of LAB mixtures did not result in an evident modification of meat colour. The multivariate analysis of the data evidenced some significant differences in whole meat colour between inoculated and control samples both for *L. curvatus* (T10 and T40) and *L. sakei* (T10, T30, T40 and T60). In any case, the analysis of the single colour parameters (Table 2) did not give univocal results, with few significant differences but without any clear trend.

However, the application of LAB cultures did not negatively affect meat colour for the whole period considered.

During the storage the hue value, a form of data reduction involving both a* and b*, and plottable in cylindrical coordinates when chroma and L* are known, is the main parameter used to attest the colour display life: in this study an increase of hue values between T0 and T10 was detected, afterwards they reached a stability level, without pointing out any significant difference among the series.

Chroma, also termed saturation index, used as an indicator of the loss of colour saturation, was characterized by a slight reduction during the whole period and did not show important differences between treated and control samples.

In three sampling times (T10, T20 and T50) a perceptible total colour difference (expressed as ΔE) between LS samples and CLS samples was not detected ($\Delta E<2.3$), in two sampling times (T0 and T30) this difference was acceptable ($2.3<\Delta E<3$), while in 2 sampling times (T40 and T60) the samples got $\Delta E >3$, highlighting very strong, perceivable differences in meat colour (Table 2). Considering LC and CLC, in four sampling times (T0, T20, T30 and T50) a perceptible total colour differences between LS samples and CLS samples (expressed as ΔE) was not detected, in four sampling times (T0, T20, T30 and T50) this difference was acceptable ($2.3<\Delta E<3$), while just only in one sampling time (T40) the samples got $\Delta E >3$.

Table 2: Values of L*, a*, b* and Hue angle values measured on LS, CLS, LC and CLC samples.

Samples	T0	T10	T20	T30	T40	T50	T60
LS							
L*	44.5±1.7	44.2±1.5	43.1±2.1	42.5±5.5	43.7±2.8B	44.8±2.0	44.8±3.0a
a*	23.1±2.2	20.8±3.6	20.1±1.2	19.7±1.6B	20.8±1.1A	16.9±2.1	16.9±1.1B
b*	15.8±1.9	12.8±4.1b	13.3±1.0	13.0±1.0b	14.5±0.3	13.2±0.9	13.4±1.2
Hue-Angle	34.41	40.82	42.06	41.40	44.56	41.69	42.25
Chroma	27.95	19.50	19.88	19.67	20.71	19.76	19.94
CLS							
L *	44.6±3.5	43.5±1.9	41.7±4.3	44.4±1.9	51.0±1.3A	45.7±1.9	40.9±5.4b
a*	20.9±1.6	21.7±0.8	19.8±1.1	23.1±3.8A	17.6±3.2B	17.4±1.5	20.0±1.2A
b*	14.0±1.5	14.9±0.8a	12.4±2.1	15.0±2.2a	14.9±1.2	13.7±1.0	13.7±1.3
Hue-Angle	33.88	45.17	39.96	45.51	45.25	42.84	42.95
Chroma	25.14	20.93	19.25	21.06	20.96	20.13	20.16
ΔE (LS-CLS)	2.83	2.23	1.68	2.73	7.38	1.03	3.89
LC							
L*	39.7±3.5	42.0±1.6	44.8±3.8	47.2±3.1	46.3±1.8	42.5±1.4	45.4±6.0
a*	22.3±2.4	22.8±2.4	20.6±1.4	19.6±1.7	19.0±2.0B	19.3±1.9	21.1±1.8
b*	13.5±1.1	15.2±2.3A	14.8±1.1	14.5±1.3	13.1±2.2b	14.3±0.6	15.4±1.1
Hue-Angle	31.12	45.78	45.02	44.55	41.55	44.12	46.15
Chroma	26.09	21.16	20.88	20.71	19.72	20.56	21.30
CLC							
L *	40.7±3.2	42.0±4.9	44.5±1.8	48.2±3.5	43.8±3.1	42.8±3.6	48.2±4.4
a*	23.0±2.7	20.9±1.3	19.8±1.5	20.2±0.9	22.5±1.6A	21.0±4.0	20.1±1.2
b*	14.8±2.7	12.4±3.2B	13.4±1.4	14.5±1.2	15.1±1.6a	15.0±1.7	15.2±1.1
Hue-Angle	32.66	39.96	42.20	44.45	45.61	45.38	45.77
Chroma	27.34	19.25	19.92	20.67	21.10	21.01	21.16
ΔE (LC-CLC)	1.73	2.80	1.44	1.06	3.27	0.70	2.77

Values are expressed as mean ± standard deviation. CLS= Control samples for *Lactobacillus sakei*; LS= samples inoculated a mixture of the six strains of *Lactobacillus sakei*; CLC= Control samples for *Lactobacillus curvatus*; LC= samples inoculated with a mixture of the two strains of *Lactobacillus curvatus*.
Different lower-case or upper-case letters are pointing out significant difference, respectively at $P<0.05$ or $P<0.01$, at the levels of interest of *Lactobacillus* mixtures by time.

4. CONCLUSIONS

Historically *L. sakei* and *L. curvatus* have been recognized for their useful role in food biopreservation by contrasting the growth of spoilage and pathogenic microorganisms without the production of sensorial changes. *L. sakei* mixture and especially *L. curvatus* mixture tested in this work showed promising antimicrobial activity *in vitro* against a wide number of spoilage and pathogenic bacteria. No activity was recorded in the supernatants and in the pH adjusted supernatant, for all the target strains tested, highlighting that the antagonistic effect originates probably from the nutrient competitive exclusion. Moreover, the effect of the addition of the two mixtures to sliced vacuum-packaged beef was investigated: the high loads detected on meat, that is a lesser inhibiting effect of the two bacteria on meat than that demonstrated *in vitro*, could be related to the slighter competitiveness of the cultures if applied to a complex substrate and to the buffering

capacity of meat, which decreased the potential action of organic acids. The use of higher dosage of LAB cultures could be suggested as an effective mean to determine an early conditioning of meat environment, in order to prevent the growth of spoilage bacteria and prolong vacuum packaged raw meat shelf-life.

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Paper Received July 10, 2015 Accepted October 18, 2015

PAPER

THE CHARACTERIZATION OF BLOSSOM HONEYS FROM TWO PROVINCES OF PAKISTAN

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ABSTRACT

This study characterized fifteen blossom honeys collected from eleven locations in the Punjab and Khyber Pakhtunkhwa (KPK) provinces of Pakistan. Mean values for physicochemical parameters (i.e., moisture, water activity, free acidity, pH, electric conductivity, diastase activity, total acidity, ash, total protein, hydroxymethylfurfural, fructose, glucose, sucrose, maltose, raffinose, reducing sugars, total sugars, and fructose/glucose ratio) were 18.09%, 0.57, 13.52 mEq kg^{-1}, 4.27, 414.41μS cm^{-1}, 10.56 DN, 26.80 mEq kg^{-1}, 0.15%, 313.30 mg 100 g^{-1}, 15.39 mg kg^{-1}, 35.49%, 30.77%, 4.41%, 2.12%,0.11%, 66.25%, 72.88%, and 1.16, respectively. The sucrose content was slightly high in three honeys. In general, all of the remaining honey samples met the criteria for international honey standards.

Keywords: chemical analyses, comparison, honeys, qualitative properties

1. INTRODUCTION

Honey is a natural sweet material made by honeybees from floral nectar, plant secretion or excretion of insects (which suck the sap from living plant parts), that honeybees collect, modify and intermix with their own particular substances, deposit and leave it in the cells of the comb to ripen and mature (MENDES *et al.*, 1998).
Humans have used honey as a reliable sweetener for centuries. Honey holds a particular place in the food and medical industries, and it has been regarded as a highly nutritive food in many civilizations (FEÁS *et al.*, 2010 a).
Pakistan has diverse landscape, climate and environmental conditions, including sandy beaches, deserts, high mountains and pictorial valleys, each featuring specific vegetation. The country has a great potential for honey production because of its congenial climate conditions and variety of bee flora. Punjab, the most populous province of Pakistan, has the majority of its bee flora in the northern and central regions, where an ample amount of honey is harvested (IZHAR-UL-HAQ *et al.*, 2010). Khyber Pakhtunkhwa (KPK) also has environmental conditions that are conducive for the growth of high floral biodiversity that contributes to honey production, and its export to the Middle East and western countries (MAIRAJ *et al.*, 2008).
Variation in the composition of honey largely arises from the climate and environmental conditions of an area, its nectar and pollen, and the abilities of beekeepers (WHITE, 1978). The physicochemical properties of honey affect its honey storage, quality, granulation, texture, flavor, and nutritional characteristics as well as its medicinal qualities (IFTIKHAR *et al.*, 2011). The characterization of honey promotes the understanding of its medicinal properties as well as its antibacterial and antioxidant characteristics (ADEBIYI *et al.*, 2004). Most of its physicochemical properties can be used to reveal adulteration; therefore, studies of certain quality parameters are needed to ensure the purity of honey (KHAN *et al.*, 2006).
This study sought to characterize the quality parameters and discriminative properties of 15 different blossom honeys collected from diverse areas of two provinces of Pakistan. Previous work has mainly focused on a few samples from specific locality.

2. MATERIALS AND METHODS

All of the chemicals and reagents employed in this study were of analytical grade. Fructose, glucose, sucrose, maltose, raffinose, bovine serum albumin, Folin & Ciocalteu's phenol reagent, phosphoric acid and 5-(hydroxymethyl) furfural were purchased from Sigma-Aldrich (St. Louis, MO, USA). Hiper Solv for HPLC™, water for high-performance liquid chromatography HPLC, was obtained from BDH Laboratory Supplies (Poole, UK). Acetonitrile (HPLC grade) was acquired from Fisher Scientific UK Ltd. (Leicestershire, UK).

2.1. Honey samples

In this study, fifteen samples of blossom honey were collected from different apiaries of Punjab and KPK provinces between March and April 2014. Nine samples, i.e., eucalyptus (*Eucalyptus* spp.), sunflower (*Helianthus annuus*), acacia (*Acacia* spp.), mustard (*Brassica campestris*), ziziphus (*Ziziphus mauritiana*), clover (*Melilotus officinalis*), citrus (*Citrus* spp.), currant bush (*Carissa opaca*), and multifloral honeys, were collected from seven locations of Punjab, whereas six honey samples, i.e., loquat (*Eriobotrya japonica*), eucalyptus (*Eucalyptus* spp.), ziziphus (*Ziziphus mauritiana*), acacia (*Acacia* spp.), citrus (*Citrus* spp.), and clover

(*Melilotus officinalis*), were collected from four locations of KPK (Fig. 1). These samples were brought to the Chair of Engineer Abdullah Ahmad Buqshan for Bee Research, Plant Protection Department, College of Food and Agriculture Sciences, King Saud University, Riyadh, Saudi Arabia and placed in a refrigerator at 4°C until analysis in May 2014.

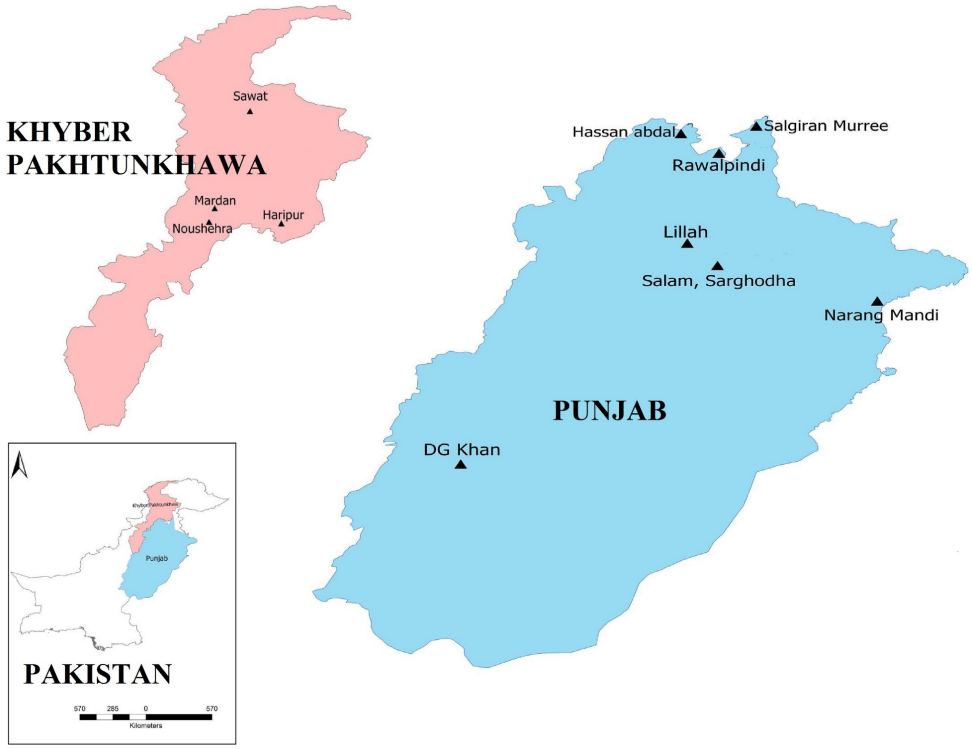

Figure 1: Map of Pakistan showing the honey collection sites in Punjab and Khyber Pakhtunkhwa.

2.2. Moisture content

The moisture content (expressed as a percentage) of all honey samples was determined via refractometery using a refractometer (Abbe Mark II model 10480, Cambridge Instruments Inc., Buffalo, NY, USA) at 20°C according to the guidelines of the International Honey Commission (IHC) (BOGDANOV, 2002).

2.3. Water Activity (a_w)

Water activity in honey samples was measured using a bench-top Aqualab CX-2 water activity meter (Decagon Device, Inc., WA, USA) at 20°C (HABIB *et al.*, 2014).

2.4. Electrical conductivity (EC)

EC was measured by making a 20% (w/v) honey solution in distilled water according to IHC guidelines (BOGDANOV, 2002) via an HI-9835 EC/TDS/NaCl meter (HANNA Instruments, Woonsocket, RI, USA). The measurements were recorded in μS cm^{-1}.

2.5. Ash content

Ash content was measured using a predetermined EC value for the honey samples and by substituting those values into the following formula: X1 = (X2 - 0.143)/1.743, where X1 denotes the ash content and X2 represents the EC (in mS cm^{-1}) at 20°C (PIAZZA *et al.*, 1991). The ash content is expressed as a percentage.

2.6. pH determination

To measure pH, 5 g of honey was mixed in 25 mL of distilled water, and a pH reading was taken using a professional benchtop BP3001 pH meter (Trans Instruments, Singapore).

2.7. Acidity (free and total)

Ten grams of honey was dissolved in 75 mL of CO_2-free water (Micropure UV Tank-Thermo Scientific, Hungary) in a 250-mL beaker. This solution was titrated with 0.1 M sodium hydroxide solution until the pH reached 8.3. Then, the free and total acidity were measured according to IHC guidelines (BOGDANOV, 2002) and are expressed in mEq kg^{-1}.

2.8. Diastase Activity (DA)

DA was determined according to FEÁS *et al.* (2010a). Five grams of honey was placed in a beaker and dissolved completely in 20 mL of distilled water and 2.5 mL of an acetate buffer solution. This mixture was transferred to a flask containing 1.5 mL of NaCl solution. Then, 10 mL of this solution was placed in a 50-mL volumetric flask and placed in a thermostatic bath (Thermolab Industries) at 40°C with a second flask containing 10 mL of starch solution. After 15 min, 5 mL of starch solution was pipetted into the honey solution and mixed. After the first 5 min, 1 mL aliquots from this solution were removed, and 5 mL of iodine solution was added at periodic intervals. The sample absorption was monitored at 660 nm against a water blank in a 1 cm cell using a PerkinElmer Lambda 25, UV/VIS/Spectrometer (Shelton, CT, USA). DA is expressed in Gothe units per gram of honey, i.e., the "diastase number" (DN). One Gothe unit is the amount of enzyme that converts 0.01 g of starch into a given point in 1 h at 40°C.

2.9. Sugar content

In this experiment, 5 g of honey were mixed in distilled water; to create a total volume of 100 ml, it was transferred to a 100 mL volumetric flask and adjusted with water. This honey solution was filtered using a 0.55 mm Whatman filter paper (Whatman International Limited, Maidstone, UK) and stored in vials at 4°C. The sugar content was determined using an HPLC system equipped with a refractive index (RI) detector (PerkinElmer Series-200a, USA). Sugar separation was performed at 85°C in a Sugar-Pak™ 1 column (6.5×300 mm) manufactured by Waters (USA). The HPLC pump, column oven, auto sampler, and RI detector were observed using a TotalChrom Workstation, version 6.3.1 (2006). The mobile phase consisted of 100% HPLC-grade water (HiprSolv for HPLC, BDH Laboratory Supplies, Poole, UK). The injection volume of the honey samples was adjusted to 1 μl accompanying a flow rate of 0.6 mL min^{-1}. The peaks were recognized by matching respective retention times with those standards. Furthermore, honey samples were spiked with standards, to confirm the identity of the chromatographic peaks. The average peak areas of the triplicate injections were used for peak quantification. A calibration curve was generated for each sugar using standard solutions (10–30 mg ml^{-1}).

The honey samples in the crystallized form were liquefied using a water bath (Thermolab Industries) at 40°C. The sugar results are expressed in g 100 g^{-1} honey.

2.10. Total protein content

Lowry's method of protein estimation was used to determine the total protein content of the honey samples. The basic principal in this method was the formation of a copper-protein complex and the reduction of phosphomolybdate and phosphotungstate present in Folin & Ciocalteu's reagent to hetero polymolybdenum blue and tungsten blue, respectively. Bovine serum albumin (0–100 μg ml^{-1}) was used as a standard to prepare the calibration curve (HABIB *et al.,* 2014). The results of the protein content were measured in mg 100 g^{-1}.

2.11. Hydroxymethylfurfural (HMF)

One gram of honey was mixed in 10 mL of acetonitrile:water (1:1) solution. This mixture was homogenized via constant shaking for 10 min and then filtered using a 0.45-μm syringe filter into vials and set for injection into HPLC system equipped with Series 200 UV/VIS detector (PerkinElmer Series-200a, USA). The injection volume was 10 μl, and the separation was performed using a Symmetry® C18 5 μm (3.9 × 150 mm) column manufactured by Waters (Ireland), maintained at 22°C with a run time of 5 min. The mobile phase was 0.01 N phosphoric acid (86%) and acetonitrile (14%), with a flow rate of 0.2 mL min^{-1} (CHINNICI *et al.,* 2003). The HMF found in the honey samples is expressed in mg kg^{-1}.

2.12. Statistical analysis

All of the tests were conducted in triplicate. SPSS for Windows (version 17, IBM, Armonk, NY, USA) was used to analyze the data. The mean ± standard deviation differences among samples were ascertained using a one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. The cluster analysis of 12 parameters (moisture content, free acidity, pH ,EC, diastase activity, total acidity, ash, HMF, fructose, glucose,sucrose, and maltose) was applied to Pakistani (Punjab and KPK) and Mediterranean honey samples in order to determine the similarity among the honeys of different countries. For this analysis, each parameter data were represented by mean value and the fractions of zero were used for the parameters (free acidity, EC, diastase activity, and ash), which were not available (N.A.) for compared honeys. The dendrogram was constructed by Ward's Linkage method with Euclidean distances. Discriminat analysis was performed using Past v.3.12 software.

3. RESULTS AND DISCUSSION

The physicochemical analyses of honey samples from Punjab and KPK provinces are summarized in Tables 1 and 2 and their sugar profiles are mentioned in Tables 3 and 4. All the data were reported as mean values ± standard deviations. Table 5 gives a comparison of relevant published data of honey parameters from some Mediterranean countries to the present study.

Moisture content is one of the factors that determine the shelf life of honey during storage (PEŔEZ–ARQUILLUE *et al*., 1994). It can be as low as 13% or as high as 23%, depending on the source of the honey and the climate conditions (BRADBEAR, 2009). The moisture level

of honey can increase under high humidity permeability and high storage humidity because of its hygroscopic property (ÖZCAN and ÖLMEZ, 2014). Higher moisture content can promote honey fermentation during storage (IDRIS *et al*., 2011). The moisture content values of all tested honey samples from both provinces varied from 16.32 to 19.91% (Tables 1 and 2) and were in acceptable range (≤ 20%) of international quality regulations according to the Codex Alimentarius Commission (2001). During the comparison of different Mediterranean honeys, the moisture values of Pakistani honeys were not statistically different from each other and from Turkish honeys reported previously (ÖZCAN and ÖLMEZ, 2014). Among the six types of compared honeys, the lowest mean moisture content of 17.01% was in Italian honeys (TRUZZI *et al*., 2014) while the Moroccan honeys (AAZZA *et al*., 2014) had the highest (18.97%). The variant moisture content might be the result of the diverse prevailing weather conditions across the disparate geographical locations of different countries or the different beekeeping practices that affect the degree of honey maturity.

The water activity of analysed honey samples varied from 0.51 to 0.66 (Tables 1 and 2). These results were in accordance with arid regions and Mexican honeys, with *aw* ranges of 0.52 to 0.64 (HABIB *et al*., 2014) and 0.569 to 0.613 (MONDRAGO´ N-CORTEZ *et al*., 2013), respectively. The water activity is a key factor that controls food stability by limiting or preventing microbial growth. Osmotolerant yeasts can grow under minimal *aw* conditions of 0.6 (CHIRIFE *et al*., 2006). The mustard honey from Punjab, which had the highest moisture content, had the highest *aw* and might be candidate for fermentation.

The presence of organic acids and inorganic ions in honey results in its acidity (TERRAB *et al*., 2004).

The total acidity of honey samples from both provinces ranged from 15.90 to 39.06 mEq kg^{-1} (Tables 1 and 2), both of which were acceptable (i.e., below 50 mEq kg^{-1}; Codex Alimentarius Commission, 2001). The lowest total acidity was associated with citrus, whereas the highest value was found in multifloral and eucalyptus honeys. The difference in total acidity of honey is due to the harvesting season (HABIB *et al*., 2014). The free acidity of the honey samples ranged from 3.97 to 24.77 mEq kg^{-1}. The lowest free acidity was observed in clover and loquat honeys of Punjab and KPK, respectively, whereas the highest values were found in eucalyptus honey samples of both provinces. The total and free acidity of the analyzed honey samples approximated the values recorded at other geographical locations (HABIB *et al*., 2014). A comparison between Pakistani and some Mediterranean honeys presented a substantial variation (Table 5). Nectar source, climatic conditions and soil properties of different countries might explain the acidity variation among the honey samples.

The pH values of tested honey samples of both provinces ranged from 3.22 to 5.18 (Tables 1 and 2). Lower pH was in acacia honey, whereas higher values were associated with the multifloral and ziziphus honeys of Punjab and KPK, respectively. The pH of honey is related to its storage and microbial growth and is responsible for its changes in texture and stability. The pH value of honey provides clues about its origin (i.e., floral vs. forest, where forest honey typically has a higher pH value; FEÁS *et al.,* 2010 a). The IHC has not described the limit of pH in honey; however, a low pH inhibits microbial contamination (HABIB *et al.,* 2014). The pH of the honey samples obtained from both provinces matched the values recorded by GULFRAZ *et al.* (2011) and RODRÍGUEZ *et al.* (2014). Honey samples from Pakistan showed a significant variation in pH from other Mediterranean honeys.

Table 1: Physicochemical properties of blossom honeys taken from the Punjab province of Pakistan.

S. No.	Honey Type	Location	Moisture Content (%)	Water Activity (a_w)	Free Acidity (mEq kg^{-1})	pH	EC μS cm^{-1}	Diastase Activity (DN)	Total Acidity (mEq kg^{-1})	Ash (%)	Total Protein (mg 100 g^{-1})	HMF (mg kg^{-1})
1	Eucalyptus	Hassanabdal	18.64±0.04^{b}	0.59±0.00^{c}	19.62±0.08^{a}	4.31±0.02^{b}	448.59±0.41^{d}	13.56±0.24^{b}	36.19±0.72^{a}	0.17±0.00^{c}	407.41±2.84^{a}	18.40±0.52^{c}
2	Sunflower	DG Khan	18.78±0.03^{b}	0.55±0.01^{f}	17.09±0.09^{bcd}	3.92±0.01^{e}	319.17±0.41^{f}	9.12±0.53^{cd}	29.59±1.42^{bc}	0.10±0.00^{e}	241.72±1.69^{f}	29.16±0.73^{b}
3	Acacia	Rawalpindi	16.32±0.35^{e}	0.53±0.00^{g}	14.80±0.21^{d}	3.85±0.01^{f}	467.87±0.41^{c}	10.15±0.24^{c}	26.91±0.49^{cd}	0.18±0.00^{c}	346.84±3.61^{c}	9.31±0.24^{f}
4	Mustard	Lillah	19.91±0.42^{a}	0.66±0.00^{a}	15.57±0.14^{cd}	4.34±0.01^{b}	302.28±0.41^{h}	9.26±0.53^{cd}	26.02±0.54^{d}	0.09±0.00^{e}	242.37±2.46^{f}	37.25±0.86^{a}
5	Ziziphus	Lillah	17.88±0.06^{d}	0.56±0.01^{e}	17.05±1.81^{bcd}	5.16±0.02^{a}	569.60±0.41^{a}	16.64±0.24^{a}	28.00±0.76^{cd}	0.24±0.00^{a}	289.40±1.21^{d}	9.10±0.17^{f}
6	Clover	Narang Mandi	18.58±0.03^{b}	0.61±0.00^{b}	3.97±0.21^{e}	4.12±0.01^{d}	367.81±1.60^{e}	8.98±0.53^{cd}	19.02±0.51^{e}	0.13±0.00^{d}	207.07±0.80^{g}	13.19±0.35^{e}
7	Citrus	Salam, Sargodha	18.42±0.03^{bc}	0.58±0.00^{d}	5.07±0.65^{e}	4.29±0.01^{b}	308.03±1.55^{g}	5.73±0.75^{e}	18.92±0.50^{e}	0.09±0.00^{e}	235.77±3.43^{f}	15.48±0.22^{d}
8	Currant bush	Salgiran, Murree	17.98±0.08^{cd}	0.56±0.00^{ef}	17.29±1.49^{abc}	4.21±0.01^{c}	446.56±0.41^{d}	8.72±0.53^{d}	31.50±2.19^{b}	0.17±0.00^{c}	272.18±1.17^{e}	7.43±0.53^{g}
9	Multifloral	Rawalpindi	17.67±0.06^{d}	0.51±0.00^{h}	18.77±0.46^{ab}	5.18±0.02^{a}	503.53±0.41^{b}	12.90±0.24^{b}	36.59±0.61^{a}	0.21±0.00^{b}	357.83±1.88^{b}	19.38±0.36^{c}

All analyses were performed in triplicate, and the mean value ± standard deviation (SD) are reported. Mean values in the same column but with different superscript letters differ significantly ($P > 0.05$).

Table 2: Physicochemical properties of blossom honeys taken from the Khyber Pakhtunkhwa province of Pakistan.

S. No.	Honey Type	Location	Moisture Content (%)	Water Activity (a_w)	Free Acidity (mEq kg^{-1})	pH	EC (μS cm^{-1})	Diastase Activity (DN)	Total Acidity (mEq kg^{-1})	Ash (%)	Total Protein (mg 100 g^{-1})	HMF (mg kg^{-1})
1	Loquat	Harripur	18.04±0.09^{c}	0.59±0.00^{a}	4.37±1.19^{d}	4.19±0.02^{c}	322.11±2.15^{e}	9.51±0.53^{cd}	20.42±2.13^{bc}	0.10±0.00^{e}	391.40±2.12^{b}	11.48±0.31^{d}
2	Eucalyptus	Mardan	17.37±0.05^{de}	0.54±0.01^{e}	24.77±1.58^{a}	4.11±0.01^{d}	439.14±0.41^{c}	11.51±0.24^{b}	39.06±4.47^{a}	0.16±0.00^{c}	377.60±1.08^{c}	23.33±0.43^{a}
3	Ziziphus	Noushehra	17.49±0.07^{d}	0.55±0.00^{d}	12.70±0.25^{c}	5.01±0.01^{a}	591.64±2.36^{a}	14.68±0.24^{a}	25.89±1.54^{b}	0.26±0.00^{a}	270.77±2.65^{e}	6.22±0.16^{e}
4	Acacia	Noushehra	17.21±0.06^{e}	0.57±0.00^{c}	11.35±0.88^{c}	3.22±0.01^{e}	487.53±0.41^{b}	9.43±0.53^{cd}	18.65±0.87^{c}	0.20±0.00^{b}	412.67±3.03^{a}	4.71±0.39^{f}
5	Citrus	Swat	18.29±0.08^{b}	0.58±0.01^{b}	5.25±0.95^{d}	4.27±0.02^{b}	286.45±1.28^{f}	9.75±0.53^{c}	15.90±1.12^{c}	0.08±0.00^{f}	311.36±5.65^{d}	13.98±0.26^{c}
6	Clover	Mardan	19.24±0.08^{a}	0.58±0.01^{b}	17.60±0.55^{b}	4.12±0.03^{d}	357.03±0.41^{d}	8.44±0.53^{d}	33.16±3.30^{a}	0.12±0.00^{d}	262.02±1.15^{f}	19.17±0.44^{b}

All analyses were performed in triplicate, and the mean value ± standard deviation (SD) are reported. Mean values in the same column but with different superscript letters vary significantly ($P > 0.05$)

Tunisian and Italian honeys were statistically similar to average pH values, while the Turkish honeys had comparatively high values (Table 5). The difference observed in the pH values among the different honey samples might be because of their acidity and mineral concentrations (KAMAL *et al.*, 2002).

The EC results showed variations based on the floral origin of the honey samples from both provinces, and they ranged from 286.45 to 591.64 μS cm^{-1}. Citrus honey was associated with less EC, whereas ziziphus honey with more EC (Tables 1 and 2). The EC of analysed honey samples matched the values reported by IDRIS *et al.*, (2011). TRUZZI *et al*., (2014) reported a low average EC of Italian honey samples among the compared Mediterranean honeys while the Tunisian honeys (BOUSSAID *et al*., 2014) showed high values (Table 5). The EC of honey is correlated with the intensity of its organic acids, mineral salts, and proteins; furthermore, it varies with changes in floral origin and is essential for differentiating the floral origins (HABIB *et al*., 2014). The EC values of blossom honeys should be below 800 μS cm^{-1}, whereas honeydew honeys have EC values above 800 μS cm^{-1} (FEÁS *et al.*, 2010 b). In this study, the EC measurements were below 800 μS cm^{-1}, which suggests that the origin of all the tested honey samples was floral.

The ash content is an important parameter to determine floral origin and differentiates nectar honey and honeydew honey (WHITE, 1978). Floral honey samples have a lower (≤ 0.6%) ash content than honeydew honeys (≤ 1.2%) (FEÁS *et al.*, 2010 b). The ash content of a honey is primarily due to certain nitrogen compounds, vitamins, minerals, aromatic substances and pigments (MAIRAJ *et al.*, 2008). The ash content of the tested honey samples ranged from 0.08 to 0.26% (Tables 1 and 2). The ash content in this study was below 0.6%, which indicates that the tested samples were nectar honeys (Codex Alimentarius Commission, 2001). The average ash content of analysed honeys was compared to some Mediterranean honeys. Turkish honeys (ÖZCAN and ÖLMEZ, 2014) had the minimum average ash content while the Moroccan (AAZZA *et al*., 2014) and Tunisian honeys (BOUSSAID *et al*. 2014) reflected greater values than Pakistani honeys (Table 5).

The differences in the ash content of the tested honey samples might be because of soil type where the nectar plant was located (GÓMEZ-DÍAZ *et al.*, 2012), the atmospheric conditions, and plant physiology (KAMAL *et al.*, 2002).

Diastase is an enzyme found in honey, and its level changes based on the geography, plant source, and the freshness of honey. DA might indicate aging and point out the treated temperature during the processing of honey (FALLICO *et al.*, 2006). The lowest acceptable DA value is 8 on Gothe's scale according to international regulations (Codex Alimentarius Commission, 2001). The range of DA in the current study was 5.73 to 16.64 DN. In Punjab, the lowest DA was found in citrus honey, whereas the highest was in the ziziphus honey (Table 1). Similarly, the lowest and highest DA values were observed in the clover and ziziphus honeys of the KPK, respectively (Table 2). The DA of the honey samples corroborated previously reported values (FEÁS *et al.*, 2010 a; IFTIKHAR *et al.*, 2011).

The DA of Pakistani honeys was lower than Turkish and Moroccan honey samples (Table 5). The results indicate that the honey samples were natural because their DA was within the acceptable range.

The total protein content of the honey samples of both provinces ranged from 207.07 to 412.67 mg 100 g^{-1}. These results are similar to the honey samples taken from arid regions, with the protein content ranging from 204.84 to 578.87 mg 100 g^{-1} (HABIB *et al*., 2014). The total protein content of the tested honey samples of both provinces was higher than that of samples from India, where it varied from 48 to 229.3 mg 100 g^{-1} (SAXENA *et al*., 2010). The protein content of honey depends on the presence of the enzymes introduced by honeybees as well as that putatively derived from floral nectar (SAXENA *et al*., 2010); therefore, this value varies among the honey samples.

Table 3: Sugar content (expressed as a percentage) of blossom honeys taken from the Punjab province of Pakistan.

S. No.	Honey Type	Location	Fructose (g 100 g^{-1})	Glucose (g 100 g^{-1})	Sucrose (g 100 g^{-1})	Maltose (g 100 g^{-1})	Raffinose (g 100 g^{-1})	Reducing Sugars (g 100 g^{-1})	Total Sugars (g 100 g^{-1})	F/G ratio
1	Eucalyptus	Hassanabdal	35.71±0.02^{e}	30.79±0.01^{d}	7.23±0.02^{a}	3.15±0.03^{b}	0.111±0.006^{c}	66.50±0.02^{g}	76.99±0.08^{b}	1.16±0.00^{d}
2	Sunflower	DG Khan	37.13±0.05^{b}	32.93±0.02^{a}	2.52±0.02^{g}	1.47±0.03^{f}	0.043±0.002^{f}	70.06±0.07^{b}	74.09±0.04^{d}	1.13±0.00^{e}
3	Acacia	Rawalpindi	35.62±0.03^{e}	32.82±0.08^{a}	1.12±0.04^{i}	2.93±0.05^{c}	0.160±0.010^{b}	68.44±0.05^{d}	72.65±0.13^{f}	1.08±0.01^{f}
4	Mustard	Lillah	34.90±0.02^{f}	32.31±0.03^{b}	4.36±0.01^{e}	0.62±0.08^{h}	N.D.*	67.21±0.03^{f}	72.19±0.05^{g}	1.08±0.00^{f}
5	Ziziphus	Lillah	33.65±0.08^{g}	24.75±0.06^{f}	2.76±0.03^{f}	2.08±0.04^{d}	0.064±0.002^{de}	58.41±0.04^{i}	63.30±0.09^{h}	1.36±0.01^{a}
6	Clover	Narang Mandi	38.03±0.03^{a}	32.24±0.02^{b}	6.15±0.02^{c}	1.04±0.06^{g}	0.021±0.002^{g}	70.27±0.05^{a}	77.48±0.03^{a}	1.18±0.00^{c}
7	Citrus	Salam Sargodha	36.63±0.03^{d}	31.04±0.02^{c}	1.41±0.03^{h}	3.39±0.11^{a}	0.406±0.015^{a}	67.67±0.01^{e}	72.88±0.06^{f}	1.18±0.00^{c}
8	Currant bush	Salgirran Murree	36.55±0.01^{d}	29.45±0.03^{e}	6.41±0.01^{b}	1.17±0.04^{g}	0.084±0.002^{d}	66.00±0.02^{h}	73.66±0.14^{e}	1.24±0.00^{b}
9	Multifloral	Rawalpindi	36.93±0.03^{c}	32.35±0.04^{b}	5.18±0.01^{d}	1.69±0.05^{e}	0.052±0.007^{ef}	69.28±0.04^{c}	76.20±0.07^{c}	1.14±0.00^{e}

* Not Detected ; Limit of detection (LOD) = 1.2 mg 100 g^{-1} All analyses were performed in triplicate, and the mean value ± standard deviation (SD) are reported. Mean values in the same column but with different superscript letters vary significantly ($P > 0.05$).

Table 4: Sugar content (expressed as a percentage) of blossom honeys taken from the Khyber Pakhtunkhwa province of Pakistan.

S. No.	Honey Type	Location	Fructose (g 100 g^{-1})	Glucose (g 100 g^{-1})	Sucrose (g 100 g^{-1})	Maltose (g 100 g^{-1})	Raffinose (g 100 g^{-1})	Reducing Sugars (g 100 g^{-1})	Total Sugars (g 100 g^{-1})	F/G ratio
1	Loquat	Harripur	36.40±0.02^{b}	31.30±0.02^{d}	4.10±0.02^{c}	3.67±0.06^{a}	0.097±0.003^{b}	67.70±0.03^{b}	75.57±0.07^{b}	1.16±0.00^{b}
2	Eucalyptus	Mardan	37.03±0.06^{a}	32.86±0.01^{a}	9.71±0.01^{a}	2.06±0.05^{d}	0.082±0.002^{cd}	69.89±0.07^{a}	81.74±0.05^{a}	1.13±0.00^{c}
3	Ziziphus	Noushehra	32.45±0.04^{f}	25.03±0.06^{f}	4.36±0.02^{b}	1.74±0.07^{e}	0.093±0.002^{bc}	57.48±0.06^{f}	63.67±0.08^{f}	1.30±0.01^{a}
4	Acacia	Noushehra	34.74±0.01^{d}	32.72±0.04^{b}	1.97±0.06^{e}	2.43±0.05^{c}	0.072±0.004^{d}	67.46±0.05^{c}	71.93±0.04^{d}	1.06±0.00^{e}
5	Citrus	Swat	35.51±0.03^{c}	31.58±0.01^{c}	4.01±002^{d}	2.82±0.07^{b}	0.236±0.012^{a}	67.09±0.02^{d}	74.16±0.11^{c}	1.12±0.01^{c}
6	Clover	Mardan	32.99±0.03^{e}	29.92±0.02^{e}	3.97±0.01^{d}	0.96±0.08^{f}	0.050±0.002^{e}	62.81±0.11^{e}	67.89±0.08^{e}	1.10±0.00^{d}

All analyses were performed in triplicate, and the mean ± standard deviations (SD) are reported. Mean values in the same column but with different superscript letters vary significantly ($P > 0.05$).

Honey freshness is widely judged based on its HMF content (BACANDRITSOS *et al*., 2006; CORBELLA and COZZOLINO, 2006) as fresh honeys are mainly deprived of this compound therefore, HMF content might increase during honey processing and/or aging (HABIB *et al.,* 2014). The HMF values of the honey samples ranged from 4.71 to 37.25 mg kg^{-1}. The lowest HMF values were in currant bush and acacia honeys, and the highest HMF values were observed in mustard and eucalyptus honeys (Tables 1 and 2). The HMF values of the honey samples in this study corroborated those of MAIRAJ *et al.* (2008), AKHTER *et al.* (2010), FEÁS *et al.* (2010 a), and FEÁS *et al.* (2010 b). Pakistan honey samples presented a substantial variation in average HMF from other Mediterranean honeys. Italian honeys (TRUZZI *et al*., 2014) had the minimum average HMF while the Tunisian honeys (BOUSSAID *et al*., 2014) had maximum values (Table 5). HMF values of Punjab honeys were statistically similar to Turkish honeys (ÖZCAN and ÖLMEZ, 2014). Many factors, including temperature and heat time as well as pH, storage environment, and floral origin, influence the levels of HMF in honey samples; therefore, HMF indicates overheating and poor storage conditions (FALLICO *et al.,* 2006). Long-term heat treatments of honey samples inactivate its natural enzymes, and increased HMF occurs due to fructose degradation (MAIRAJ *et al.,* 2008). All of the analyzed samples from both provinces of Pakistan showed HMF values within the acceptable range (< 80 mg kg^{-1} for tropical or arid regions) according to the international quality regulations of the Codex Alimentarius Commission (2001).

Honey is primarily composed of sugars (RODRÍGUEZ *et al.,* 2014). The monosaccharides glucose and fructose are important components of honey while fructose is always the primary sugar, followed by glucose (HABIB *et al.,* 2014).Fructose and glucose were the major sugars in all of the tested honey samples. The amounts of fructose and glucose in the honey samples of both provinces ranged from 32.45 to 38.03 g 100 g^{-1} and from 24.75 to 32.93 g 100 g^{-1}, respectively, whereas other minor sugars containing sucrose, maltose and raffinose ranged from 1.12 to 9.71 g 100 g^{-1}, 0.62 to 3.67 g 100 g^{-1} and 0.021 to 0.406 g 100 g^{-1}, respectively (Tables 3 and 4). Raffinose was not detected in the mustard honey sample from Punjab. The total sugar content of the honey samples ranged from 63.30 to 81.74 g 100 g^{-1}. The fructose/glucose (F/G) ratio and the reducing sugars (fructose + glucose) of the honey samples ranged from 1.06 to 1.36 and from 57.48 to 70.27 g 100 g^{-1}, respectively (Tables 3 and 4). Because glucose is comparatively less soluble in water than fructose, the F/G ratio can most likely be used to evaluate the granulation of honey (ANKLAM, 1998). The results of the current study were in line with the international quality regulations of the Codex Alimentarius Commission (2001). The reducing sugars of all of the tested honey samples were higher than 60%, except for the ziziphus honeys of both provinces. The comparison of examined Pakistani honeys with the Moroccan and Tunisian honeys (based on available data) revealed some variations. The fructose, glucose, sucrose and maltose contents were statistically different in compared honeys (Table 5). The average sucrose content of the examined Pakistani honeys slightly higher than Moroccan and Tunisian honeys but overall within the permissible limits (Codex Alimentarius Commission, 2001); however, the clover and currant bush honeys from Punjab were slightly high (Table 3). Early honey harvesting might explain the high sucrose content (AZEREDO *et al*., 2003).

Cluster analysis (Ward's Method- Euclidean distances) (Fig. 2) data categorized honeys into two main groups. The first group consisted of Turkish and Italian honeys and the second was comprised of Pakistani (Punjab and KPK), Moroccan and Tunisian honeys. It is interesting to note that in the second group, Pakistani honeys were quite similar even though these belonged to different provinces (Punjab and KPK). In second group, Moroccan honeys were close to Pakistani honeys while the Tunisian honeys were away from the remaining group members. Turkish and Italian honeys fell in first group and were close to each other.

Table 5: Comparison of physico-chemical properties of honeys from Pakistan (I & II) and some Mediterranean countries (III-VI).

	Honey origin	Moisture Content (%)	Free Acidity (mEq kg^{-1})	pH	EC (μS cm^{-1})	Diastase Activity (DN)	Total Acidity (mEq kg^{-1})	Ash (%)	HMF (mg kg^{-1})	Fructose (g 100 g^{-1})	Glucose (g 100 g^{-1})	Sucrose (g 100 g^{-1})	Maltose (g 100 g^{-1})
I	**Punjab, n=9** (Mean values of present study)	18.24±0.12^{ab}	14.36±0.54^{c}	4.38±0.02^{b}	414.83±0.67^{c}	10.56±0.43^{b}	28.08±0.86^{bc}	0.15±0.01^{c}	17.63±0.44^{c}	36.13±0.03^{c}	30.96±0.03^{b}	4.13±0.02^{b}	1.95±0.05^{d}
II	**KPK, n=6** (Mean values of present study)	17.94±0.07^{ab}	12.67±0.88^{c}	4.15±0.02^{c}	413.98±1.17^{c}	10.55±0.44^{b}	25.51±2.24^{cd}	0.15±0.00^{c}	13.15±0.32^{d}	34.85±0.03^{d}	30.57±0.02^{c}	4.69±0.03^{a}	2.28±0.06^{c}
III	**Morocco, n=17** (AAZZA *et al.*, 2014)	18.97±0.76^{a}	22.93±2.73^{a}	3.94±0.06^{d}	500.71±1.66^{b}	12.42±1.01^{ab}	29.66±1.16^{b}	0.36±0.01^{a}	44.80±0.78^{a}	38.47±0.04^{a}	30.76±0.05^{bc}	0.13±0.01^{d}	3.47±0.05^{a}
IV	**Tunisia , n=6** (BOUSSAID *et al.*, 2014)	18.71±0.47^{a}	N.A.	3.86±0.07^{d}	548.33±1.80^{a}	N.A.	22.59±0.92^{d}	0.26±0.02^{b}	20.01±0.37^{b}	36.93±0.10^{b}	33.49±0.19^{a}	1.89±0.07^{c}	2.64±0.03^{b}
V	**Italy, n=43** (TRUZZI *et al.*, 2014)	17.01±0.80^{b}	18.16±1.01^{b}	4.55±0.05^{a}	87.00±0.17^{d}	N.A.	21.56±0.82^{d}	N.A.	1.67±0.21^{e}	N.A.	N.A.	N.A.	N.A.
VI	**Turkey, n=8** (ÖZCAN and ÖLMEZ, 2014)	18.30±0.26^{ab}	N.A.	4.24±0.07^{bc}	N.A.	13.78±1.43^{a}	34.93±1.65^{a}	0.05±0.01^{d}	18.36±0.50^{c}	N.A.	N.A.	N.A	N.A.

The data are mean values ± standard deviation (SD). Mean values in the same column, but with different superscript letters differ significantly ($P > 0.05$). NA: not available.

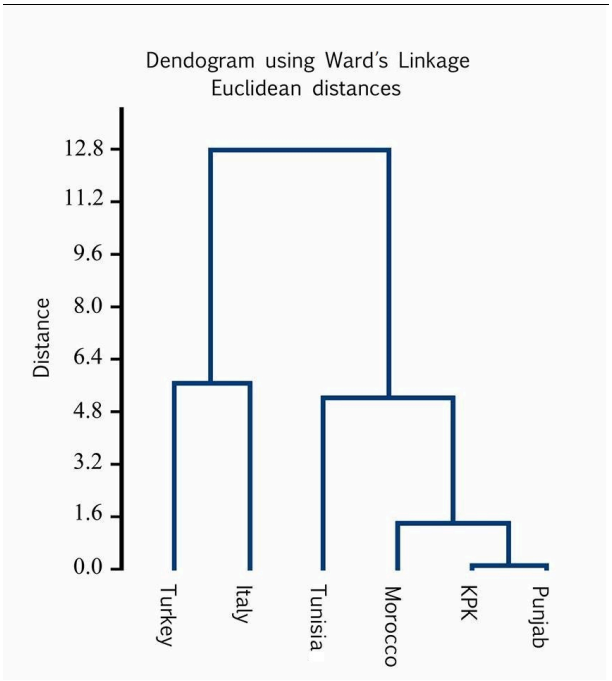

Figure 2: Dendogram (Ward's Method- Euclidean distances) showing the comparison of honeys from different countries.

These observations indicated the key role of climate, floral origin and soil properties of a particular region that determine the physicochemical characteristics of the honey.

4. CONCLUSIONS

The present study characterized nine Pakistani blossom honey samples from Punjab and six from KPK. A comparison was also made to some Mediterranean honeys. The results of this study allow us to assess the quality of Pakistani honeys and help to establish certain standards. Based on the studied quality parameters (i.e., moisture, a_w, acidity, pH, electric conductivity, diastase activity, ash, HMF, and sugar content) of the different honey samples from both provinces, Pakistani honeys meet international standards. However, the slightly higher sucrose content of certain honey samples and the lower values of reducing sugars in ziziphus honey indicate early harvesting by beekeepers.

ACKNOWLEDGEMENTS

The project was financially supported by King Saud University, Vice Deanship of Research Chairs.

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Paper Received September 29, 2015 Accepted June 1, 2016

PAPER

DRYING KINETICS OF SAFFRON FLORAL BIO-RESIDUES

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ABSTRACT

The kinetics of hot-air drying of saffron floral bio-residues was studied at two air-drying temperatures (70 and 90ºC) and four air-flow rates (2, 4, 6 and 8 $m \cdot s^{-1}$). No constant-rate drying period was observed during drying. Ten thin-layer drying models and the theoretical Fick's diffusion model were fit by non-linear regression to drying data. Three statistical parameters, Chi-squared, correlation coefficient and relative percentage deviation were used to compare the models. Effective moisture diffusivity, calculated using Fick's diffusion model, was in the range of 0.78-1.86 x 10^{-10} $m^2 \cdot s^{-1}$. According to the statistical parameters, three drying models (the logarithmic, two-term and Midilli-Kucuk models) were equally good to describe the drying curve and fit the data better than the other models. The model constants were independent of air-flow rate. The use of air at 90 ºC decreased drying time in half compared with drying at 70ºC.

Keywords: saffron, floral waste, drying, thin-layer models

1. INTRODUCTION

Saffron (*Crocus sativus* L.) spice is the dehydrated stigma of the flowers of this plant of the *Iridiaceae* family. Saffron spice production worldwide is about 250 tons. According to the Ministry of Agriculture of Iran (GHORBANI, 2008), the main producer exporter of saffron spice is Iran (93.7% of world production in 2005), with an export value of $100 million. Other countries, such as India, Greece, Spain, Morocco and Italy, are also producers and marketers of saffron spice. Spain is noted for producing saffron spice with the highest quality recognized worldwide. Moreover, Spain is also the leader in its trade, because it processes and re-exports saffron spice produced in other countries (i).

Stigma is only 7.4% of the fresh weight of a flower (SERRANO-DÍAZ *et al*., 2013a). Tepals, stamens and styles are also part of the flowers of saffron, but they have traditionally been thrown away. About 173,250 flowers weighing over 68 kg were used in Castilla-La Mancha (Spain) in 2009 to obtain 1 kg of saffron spice. As a result, 63 kg of these floral bio-residues (53 kg of tepals, 9 kg of stamens and 0.5 kg of styles) were generated (SERRANO-DÍAZ *et al*. 2013b). The introduction of the forced cultivation and mechanization of saffron production (GARVI PALAZÓN, 1987; GRACIA *et al*., 2008) will cause an increase in the production capacity and the concentration of these bio-residues in companies producing saffron spice, as stated in the white book of saffron (ii). This new situation is raising interest in the exploitation of saffron floral bio-residues. Many studies have demonstrated the biomedical properties of saffron tepal extracts. MOSHIRI *et al*. (2006) demonstrated the efficacy of the extracts in the treatment of mild-to-moderate depression. FATEHI *et al*. (2003) showed that they lower blood pressure and reduce the contractions induced by electrical field stimulation. HOSSEINZADEH and YOUNESI (2002) concluded that they have antinociceptive and anti-inflammatory effects. ZHENG *et al*. (2011) found that the saffron stamen and perianth possess significant antifungal, cytotoxic and antioxidant activity. BERGOIN (2005) extracted and characterized the volatile fraction and colorant compounds from fresh flowers and explored their use for the cosmetic, perfume and fragrance industries. The high phenolic content of the saffron floral bio-residues (SERRANO-DÍAZ *et al*., 2014b; NØRBÆK *et al*., 2002), their antioxidant properties (SERRANO-DÍAZ *et al*., 2012) their adequate nutritional composition (SERRANO-DÍAZ *et al*., 2013b) and the absence of cytotoxicity (SERRANO-DÍAZ *et al*., 2014a) show that these products could be used as food ingredients with high added value.

Traditionally, the remains of flowers that are generated in the production of saffron spice have been thrown near the saffron field; deterioration within hours has been observed, even though they were exposed to the sun. This spoilage could be due to their high moisture (SERRANO-DÍAZ *et al*., 2013b), which favors microbial attack. As in saffron stigmas, there is a need to dewater the saffron floral bio-residues the same day as the flowers are harvested. The technique used for dehydration of the stigma to produce saffron spice differs by country: sun drying, drying at room temperature in air-ventilated conditions (India, Iran and Morocco), drying at moderate temperatures (Greece and Italy) and drying at high temperatures (Spain). CARMONA *et al*. (2005) characterized the time-temperature profile during the traditional dehydration process in Castilla-La Mancha (Spain) compared with other dehydration processes. DEL CAMPO *et al*. (2010) studied the effect of mild temperature during dehydration in the main components responsible for the quality of saffron spice.

Drying is the most common way to preserve the quality of aromatic and medicinal plants (ROCHA *et al*., 2011). Hot air dehydration, by itself or combined with infrared radiation, has been successfully used to dry a number of flower commodities, such as marigold flower (SIRIAMORNPUN *et al*. 2012), torch ginger (JUHARI *et al*., 2012), chrysanthemums, roses (CASTRO *et al*., 2003), chamomile (BORSATO *et al*., 2009), daylily (MAO *et al*., 2006)

and oregano (CESARE *et al*., 2004) among others, while preserving their color, antioxidant properties and/or bioactive compounds. SERRANO-DÍAZ *et al*. (2013a) studied the conditions of hot-air drying of saffron floral residues to achieve minimal deterioration of the physicochemical quality of these products and concluded that the best quality was achieved with air at 90 ºC combined with a flow rate of 2, 4 and 6 m s^{-1}, but the kinetics of the drying process of these products have never been studied.
The aim of this study was to select and test the best drying model for hot-air dehydration of saffron floral bio-residues and to determine the influence of temperature and air-flow rate on dehydration kinetics.

2. MATERIALS AND METHODS

2.1. Plant material

The floral bio-residues generated by saffron spice production were from the Agrícola Técnica de Manipulación y Comercialización S.L. company (Minaya, Spain) during the 2010-2011 harvest season. The floral bio-residues were collected after separating the stigma from flowers using traditional procedures for the Protected Designation of Origin Azafrán de La Mancha. The thickness of the different floral tissues was measured with calipers. Fresh floral bio-residues were stored at -20ºC.

2.2. Hot-air drying

Hot-air drying was performed in a laboratory-scale hot-air dryer (Fig. 1). The dryer was equipped with four 500-W electric resistors, coupled to an automatic temperature (± 0.1ºC) controller. The air was impelled trough the drying bed by a 0.5-CV fan equipped with an automatic air velocity controller (± 0.1 m·s^{-1}). The evolution of the product was monitored by weighing the sample periodically with a Mettler (Switzerland) PM2000 balance (± 0.01 g) linked to a computer.

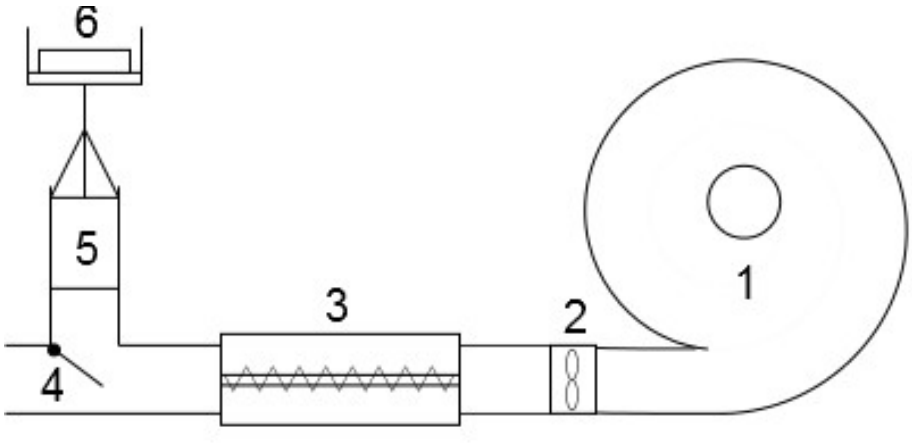

Figure 1: Hot air dryer set-up. 1: Fan, 2: Air velocity meter, 3: Electric heater, 4: Diversion valve, 5: Sample holder, 6: Scales.

Fresh floral tissues were placed on a cylindrical sample holder (9.2-cm diameter) with a perforated bottom. Sample size was kept constant (50 ± 2 g) for each experiment. Weight loss was recorded at 5-min intervals, and drying was continued until the moisture difference was lower than 5% (w/w). Dry runs were performed at temperatures of 70°C and 90 °C, with air-flow of 2 m·s^{-1}, 4 m·s^{-1}, 6 m·s^{-1} and 8 m·s^{-1}. Reynolds numbers, calculated using the slab half-thickness as the characteristic dimension (about 5·10^{-4} m), were on the order of 50, 100, 150 and 200, respectively. The sample temperature during the drying process was determined with an infrared laser thermometer (range: -33 to +250°C, accuracy: ± 2°C).
Sample moisture level was determined with a halogen lamp moisture balance, model XM-120T (Cobos, Barcelona, Spain) at 105 °C, in triplicate. When moisture loss was less than 0.1% in 180 s, samples were considered to have reached constant mass. Nine measurements were obtained for each combination of temperature-air flow.

2.3. Mathematical models

The moisture ratio (*MR*) was defined as:

$$MR = \frac{M - M_e}{M_0 - M_e} \qquad \text{Eq. 1}$$

where M is sample moisture at time t, Me is equilibrium moisture content, and M_o is initial moisture content. All moisture content was determined on a dry basis. Because drying experiments were carried out using hot air, with very low relative humidity, the moisture ratio was simplified to M/M_0.
The experimental data were fit to ten thin-layer drying models and to the theoretical Fick's diffusion model for a slab (Table 1). References for the model equations can be found elsewhere (AKPINAR, 2006).

Table 1: Mathematical drying models.

Model name	Model equation
Exponential	$MR = \exp(-kt)$
Page	$MR = \exp(-kt^n)$
Modified Page	$MR = \exp(-kt)^n$
Henderson and Pabis	$MR = a \exp(-kt)$
Logarithmic	$MR = a\exp(-kt) + c$
Two term	$MR = a\exp(-k_0 t) + b\, exp(-k_1 t)$
Two term exponential	$MR = a\exp(-kt) + (1-a)\, exp(-kat)$
Wang and Singh	$MR = 1 + at + bt^2$
Verma	$MR = a\exp(-kt) + (1-a)\, exp(-gt)$
Midilli-Kucuk	$MR = \exp(-kt^n) + bt$
Fick's diffusion	Eq (2)

The thin-layer drying models are simple empirical models that give good results when the assumptions needed for developing the analytical solutions to Fick's second law, namely the surface resistance or the geometry, are not truly met. Their main drawback is that their parameters lack physical meaning. Conversely, rigorous or phenomenological models can give a hint to the mechanism of the underlying process. An innovative approach to mathematical modelling of the drying of eggplant slabs considering the shrinkage effect can be found elsewhere (BRASIELLO *et al*., 2013; RUSSO *et al*., 2013).
The analytical solution to Fick's second law for a slab, in the case of negligible surface resistance, is (CRANK, 1975):

$$MR = \frac{8}{\pi^2}\sum_{n=0}^{\infty}\frac{1}{(2n+1)^2}\exp\left(-\frac{(2n+1)^2\pi^2 Dt}{4L^2}\right) \quad \text{Eq. 2}$$

where D is the effective water diffusivity ($m^2 \cdot s^{-1}$), and L is the half-thickness of the slab.
For long drying times, Eq. 2 can be simplified by taking only the first term in the summation, leading to the Henderson and Pabis equation with a theoretical value for the constant a of $8/\pi^2$.
The data were also fit to Eq. 2, and the effective diffusivity was calculated. The number of summation terms was adjusted to ensure that the error in MR was less than 0.1%.

2.4. Statistical analysis

All non-linear regressions were performed using the SOLVER optimization tool (GRG nonlinear method) included in the Microsoft Excel 2010™ spreadsheet, by minimizing the sum of the square differences between the experimental and calculated moisture ratios.
Comparison of the goodness of fit for each equation was determined by means of the following parameters: correlation coefficient (R), reduced chi-square (χ^2) and mean relative percentage deviation (P):

$$\chi^2 = \frac{1}{N-n}\sum_{i=1}^{n}(MR_{ei} - MR_{ci}) \quad \text{Eq. 3}$$

$$P(\%) = \frac{100}{N}\sum_{i=1}^{n}\frac{|MR_{ei}-MR_{ci}|}{MR_{ei}} \quad \text{Eq. 4}$$

where MR_{ei} and MR_{ci} are experimental and predicted moisture ratios, respectively; N is the number of experimental data-points; n is the number of model parameters.
Multiple linear regressions were performed to determine the influence of temperature and air-flow rate using SPSS 19.0 for Windows (SPSS Inc., Chicago, IL, USA).

3. RESULTS AND DISCUSSIONS

Figure 2 shows that the drying rate was decreasing from the beginning of the drying process, and there was no constant rate period. This pattern suggests that the drying resistance would be inside the product rather than in the outside air layer and is in agreement with the results reported for thin-layer drying of similar products, such as

saffron (AKHONDI *et al*., 2011), betel leaves (PIN *et al*., 2009), mint leaves (DOYMAZ, 2006) and spinach leaves (DOYMAZ, 2009).

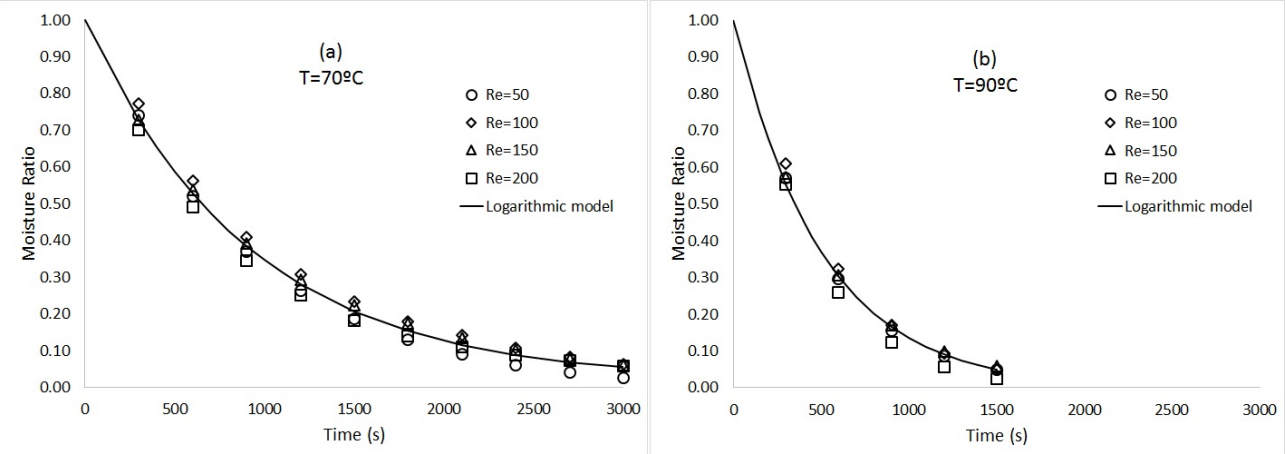

Figure 2: Drying rate of saffron flowers versus moisture ratio.

Table 2 shows the results of the statistical parameters obtained for each model. The best model was the one with the highest R-values, the lowest χ^2 values and the lowest P values. The two-term model was the best with regard to the R and χ^2 values, with all R values higher than 0.99987 and χ^2 values lower than $1.15 \cdot 10^{-5}$, but the logarithmic model also gave very good agreement, with R values higher than 0.99986 and χ^2 values lower than $1.56 \cdot 10^{-5}$. These two models also had very low P values (<4.8%). The Midilli-Kucuk model had the lowest P values, all lower than 4.2%, very high R values (> 0.99981) and low χ^2 values (< $1.89 \cdot 10^{-5}$). Therefore, the three best models were the two-term, logarithmic and Midilli-Kucuk models. The Verma model also gave a good fit, with R > 0.99924, $\chi^2 < 9.40 \cdot 10^{-5}$ and P < 5.4%. In contrast, the theoretical Fick's diffusion model (Eq. 2) gave one of the worst fits, with R > 0.98751, $\chi^2 < 3.30 \cdot 10^{-3}$ and P in the range of 9.7-41.3%, while the Henderson and Pabis model gave better results than the theoretical model (R > 0.99810, $\chi^2 < 2.09 \cdot 10^{-4}$ and P < 10.8%).

The logarithmic model has been reported by several authors as the one that gave the best fit for thin-layer drying of a number of products, such as finger millet (RADHIKA *et al*., 2011), olive cake (AKGUN and DOYMAZ, 2005), mint leaves (DOYMAZ, 2006), spinach leaves (DOYMAZ, 2009) and apricots (TOGRUL and PEHLIVAN, 2002). PIN *et al*. (2009) found that the logarithmic model gave the best results for drying betel leaves at 40-60ºC, while the Midilli and Kucuk model was better for drying at 70ºC. In some instances, the two-term drying model proved to be better than the logarithmic model for drying sultana grapes (YALDIZ *et al*., 2001). Other authors have claimed that the Midilli and Kucuk model was the best thin-layer drying model for drying potato, apple and pumpkin slices (AKPINAR, 2006) or saffron (AKHONDI *et al*., 2011). Our results agree with those of previous works and confirm that the two-term, the logarithmic and the Midilli-Kucuk model are the three best models for drying saffron floral bioresidues. This suggests that the Verma model could also be acceptable.

Table 2: Statistical parameters of the drying models.

Model	Temperature	Re	χ^2	*R*	*P* (%)
Exponential	70	50	1.15E-04	0.99995	10.0
		100	1.10E-04	0.99903	4.4
		150	1.30E-04	0.99974	7.2
		200	3.14E-04	0.99905	13.9
	90	50	1.38E-04	0.99935	3.7
		100	5.32E-04	0.99920	11.2
		150	7.52E-05	0.99935	3.1
		200	4.83E-04	0.99917	19.0
Page	70	50	1.24E-05	0.99989	3.0
		100	1.10E-04	0.99903	4.6
		150	2.44E-05	0.99976	2.3
		200	1.17E-04	0.99876	7.4
	90	50	3.86E-05	0.99960	5.0
		100	4.00E-05	0.99964	4.7
		150	4.90E-05	0.99946	4.7
		200	1.81E-05	0.99982	4.5
Modified Page	70	50	1.24E-05	0.99989	3.0
		100	1.10E-04	0.99903	4.6
		150	2.44E-05	0.99976	2.3
		200	1.17E-04	0.99876	7.4
	90	50	3.86E-05	0.99960	5.0
		100	4.00E-05	0.99964	4.7
		150	4.90E-05	0.99946	4.7
		200	1.81E-05	0.99982	4.5
Henderson and Pabis	70	50	1.15E-05	0.99991	4.8
		100	9.40E-05	0.99924	5.4
		150	6.04E-05	0.99945	4.5
		200	2.09E-04	0.99810	10.8
	90	50	1.62E-05	0.99985	3.7
		100	2.00E-06	0.99998	1.2
		150	3.28E-05	0.99968	4.2
		200	4.03E-07	1.00000	1.1
Logarithmic	70	50	1.15E-05	0.99991	4.8
		100	1.56E-05	0.99986	2.2
		150	1.21E-05	0.99987	2.1
		200	3.93E-06	0.99996	1.4
	90	50	4.07E-07	1.00000	0.3
		100	8.22E-07	0.99999	0.4
		150	8.63E-07	0.99999	0.7
		200	4.03E-07	1.00000	1.1
Two term	70	50	1.15E-05	0.99991	4.8
		100	2.84E-06	0.99997	0.7
		150	5.95E-06	0.99994	1.0
		200	2.31E-06	0.99997	0.9
	90	50	1.15E-05	0.99987	2.1
		100	1.37E-06	0.99999	0.5
		150	8.63E-07	0.99999	0.7
		200	4.96E-07	1.00000	1.2

Two term exponential	70	50	1.15E-04	0.99995	10.0
		100	1.10E-04	0.99903	4.4
		150	1.30E-04	0.99974	7.2
		200	3.42E-04	0.99876	12.9
	90	50	1.38E-04	0.99935	3.7
		100	5.32E-04	0.99920	11.2
		150	7.52E-05	0.99935	3.1
		200	4.83E-04	0.99917	19.0
Wang and Singh	70	50	1.60E-03	0.99235	38.7
		100	1.23E-03	0.99270	18.4
		150	1.99E-03	0.99091	22.7
		200	2.94E-03	0.98678	31.0
	90	50	1.05E-03	0.99390	23.7
		100	4.54E-04	0.99680	16.1
		150	1.12E-03	0.99341	21.5
		200	1.19E-03	0.99288	42.1
Verma	70	50	1.13E-05	0.99991	4.6
		100	9.40E-05	0.99924	5.4
		150	6.64E-06	0.99993	1.2
		200	5.64E-06	0.99994	1.4
	90	50	1.62E-05	0.99985	3.6
		100	2.53E-06	0.99998	1.4
		150	3.28E-05	0.99968	4.2
		200	1.42E-06	0.99999	0.8
Midilli-Kucuk	70	50	1.09E-05	0.99991	4.2
		100	1.12E-05	0.99990	1.4
		150	1.89E-05	0.99981	2.0
		200	8.33E-06	0.99991	2.0
	90	50	5.10E-06	0.99995	1.2
		100	3.43E-06	0.99997	1.3
		150	1.03E-05	0.99988	1.7
		200	6.33E-06	0.99993	2.3
Fick's diffusion	70	50	2.55E-03	0.99768	33.2
		100	2.43E-03	0.99488	13.0
		150	1.26E-03	0.99682	9.7
		200	1.41E-03	0.98751	16.6
	90	50	1.87E-03	0.99625	20.0
		100	3.30E-03	0.99609	27.4
		150	1.59E-03	0.99628	15.8
		200	2.55E-03	0.99586	41.3

Table 3 shows the constants for the four best drying models, together with the values of the effective diffusivity obtained with the solution to Fick's equation (Eq. 2). AKGUN and DOYMAZ (2005), DOYMAZ (2006) and DOYMAZ (2009) calculated the effective diffusivities for the simplified Fick's equation for drying olive cake. Note that they calculated D_{eff} using the traditional method of computing the slope of *ln* (*MR*) versus time by linear regression, while we obtained D_{eff} by non-linear regression. Our results for D_{eff} were in the range of 0.78-0.93 x 10^{-10} $m^2 \cdot s^{-1}$ at 70ºC and 1.55-1.86 x 10^{-10} $m^2 \cdot s^{-1}$ at 90 ºC, which is lower than the results for olive cake at the same temperatures (6.252 x 10^{-9} $m^2 \cdot s^{-1}$ and 7.887 x 10^{-9} $m^2 \cdot s^{-1}$, respectively) or spinach leaves at 70ºC (1.5 x 10^{-9} $m^2 \cdot s^{-1}$).

Table 3: Constants of selected drying models.

Model	Temperature	Re				
Logarithmic			*a*	*k*	*c*	
	70	50	1.0527	0.0012	0.0000	
		100	1.0238	0.0011	0.0311	
		150	0.9638	0.0011	0.0252	
		200	0.9676	0.0013	0.0417	
	90	50	1.0948	0.0023	0.0130	
		100	1.1547	0.0021	0.0037	
		150	1.0677	0.0022	0.0198	
		200	1.1786	0.0025	0.0000	
Two term			*a*	k_0	*b*	k_1
	70	50	0.0059	0.0012	1.0468	0.0012
		100	0.4423	0.0018	0.6488	0.0008
		150	0.4598	0.0016	0.5523	0.0007
		200	0.9275	0.0014	0.0915	0.0002
	90	50	0.0072	0.0003	1.0666	0.0021
		100	0.0068	0.0005	1.1454	0.0021
		150	0.0198	0.0000	1.0678	0.0022
		200	0.0051	0.0016	1.1737	0.0025
Verma			*a*	*k*	*g*	
	70	50	1.0587	0.0012	0.0089	
		100	1.0186	0.0010	0.0380	
		150	0.3303	0.0006	0.0013	
		200	0.0399	0.0000	0.0013	
	90	50	1.0747	0.0021	0.0276	
		100	1.1576	0.0021	0.0135	
		150	1.0426	0.0020	0.0283	
		200	1.2324	0.0026	0.0090	
Midilli-Kucuk			*a*	*k*	*n*	*b*
	70	50	1.0229	0.00087	1.0390	5.62×10^{-7}
		100	1.0871	0.00167	0.9352	3.73×10^{-6}
		150	0.9729	0.00093	1.0107	6.06×10^{-6}
		200	1.0076	0.00135	0.9838	1.12×10^{-5}
	90	50	0.9820	0.00087	1.1307	1.22×10^{-5}
		100	1.0437	0.00087	1.1274	9.03×10^{-6}
		150	0.9701	0.00089	1.1232	1.55×10^{-5}
		200	1.0173	0.00089	1.1472	4.28×10^{-6}
Fick's diffusion			D (m^2/s)			
	70	50	0.92×10^{-10}			
		100	0.78×10^{-10}			
		150	0.82×10^{-10}			
		200	0.93×10^{-10}			
	90	50	1.66×10^{-10}			
		100	1.55×10^{-10}			
		150	1.61×10^{-10}			
		200	1.86×10^{-10}			

The model constants were regressed against the drying air temperature and flow rate to determine the influence of these variables (Table 4). The model constants that gave non-

significant regressions ($p > 0.05$) were omitted from the table. Note that the flow rate variable was non-significant for all model constants and does not appear in the equations.

Table 4: Influence of drying temperature on model constants. T: absolute temperature (K).

Model	Constant	Regression equation	R^2
Logarithmic	*a*	-1.044+0.006T	0.699
	k	$-1.240+5.64\cdot10^{-5}T$	0.969
Two term	*a*	7.688-0.022T	0.722
	b	-8.023+0.026T	0.722
	k_1	$-0.025+7.585\cdot10^{-5}T$	0.907
Verma	*k*	$-0.025+7.565\cdot10^{-5}T$	0.853
Midilli-Kucuk	*n*	-1.403+0.007T	0.865
Fick's diffusion	*D*	$-8.985\cdot10^{-10}+4.044\cdot10^{-12}T$	0.959

RADHIKA *et al*. (2011) developed the relation equations between the constants of the logarithmic model and the drying temperature for the drying of finger millet. Their results were in agreement with ours for *a* and *k* constants. However, we did not find a significant relationship between *c* and drying temperature. AKPINAR (2006) obtained regression equations for the four Midilli-Kucuk model constants and found a significant influence of both air temperature and air-flow rate. In our results, only the *n* constant depended significantly on temperature.
Figure 3 shows the variation with time of the experimental moisture ratio for 70 and 90°C, together with the prediction lines obtained using the logarithmic, two-term and Midilli-Kucuk models.

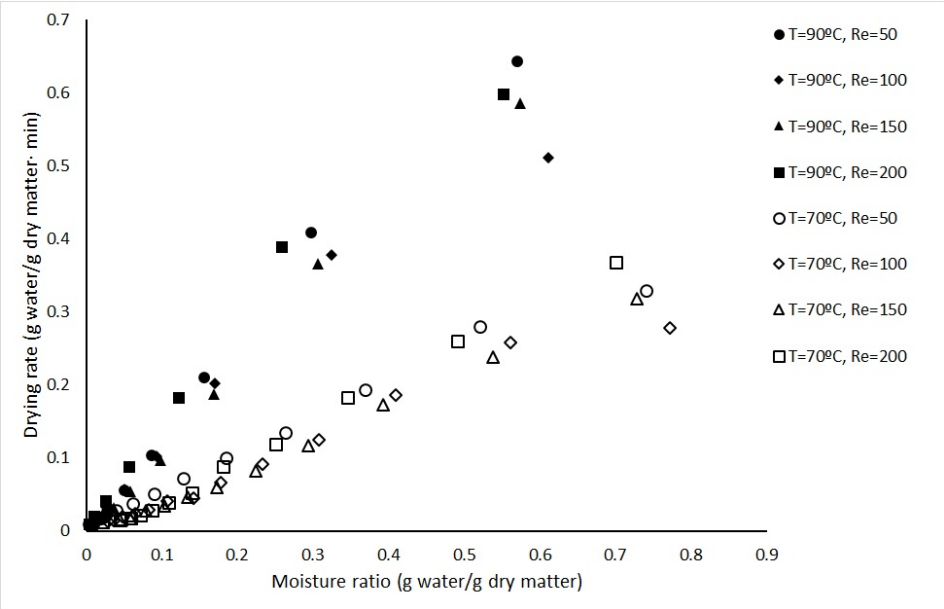

Figure 3: Experimental moisture ratio during drying time and prediction lines with the logarithmic model at 70°C (a) and 90°C (b).

It can be seen that the three model lines overlap almost completely; therefore, the three models describe the data equally well. Although a small influence of the variable flow rate on *MR* variation may be apparent from Fig. 3, the effect was not statistically significant. The drying time needed to arrive at *MR* values below 0.05 at 90ºC was about one half the time needed at 70ºC.

4. CONCLUSIONS

Drying rate curves for saffron floral bio-residues did not show a constant-rate drying period, and all the drying occurred during the falling-rate drying period. Moisture diffusivity obtained using the theoretical Fick's diffusion model was in the range of 0.78-1.86 x 10^{-10} m^2 s^{-1} at 70-90 ºC. The logarithmic, two-term and Midilli-Kucuk models were the best thin-layer model to fit the drying data, but other models, like the Verma model, gave also good agreement to the experimental data. The model constants were independent of air-flow rate. Regression equations were obtained to describe the influence of temperature on model constants. Increasing the temperature from 70 to 90 ºC halved the drying time.

ACKNOWLEDGEMENTS

The authors thank the Agrícola Técnica de Manipulación y Comercialización S.L company (Minaya, Spain) for providing the samples. They are also grateful to the Consejería de Educación y Ciencia of the JCCM and FEDER for funding this project (ref.: POIC10-0195-984). Thanks to Marco A. García and Eulogio López for technical assistance.

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Paper Received December 5, 2015 Accepted June 5, 2016

PAPER

NEW SUSTAINABLE PROTEIN SOURCES: CONSUMERS' WILLINGNESS TO ADOPT INSECTS AS FEED AND FOOD

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ABSTRACT

The aim of the study was to investigate the willingness of Italian consumers to adopt insects, suitable candidates for providing sustainable animal proteins, as part of animal and human diets. Furthermore, we evaluated the effect of information about the benefit of introducing insects into the diet on consumers' acceptance. The results showed that respondents were clearly not ready to accept insects as food, whereas a major positive trend was observed regarding their use as feed. The principal factors affecting the Italian consumers' readiness to adopt insects as food and feed were age, gender, cultural background and food neophobia. Contrary to our expectation, subjects' involvement in sustainability issues did not play a role in the acceptance of insects. Information about the environmental and nutritional benefits of introducing insects as food had a marginal but positive effect on their visual acceptability.

Keywords: consumer acceptance, entomophagy, familiarity, novel protein sources, sustainability

1. INTRODUCTION

The consumption of insects is traditionally practiced in many parts of the world, particularly in the Southern Hemisphere, with more than 2000 species of insects consumed not only because of their nutritive value but also because of their taste (DEFOLIART, 1997; NONAKA, 2009).
The world population, which will reach 9.6 billion people in 2050, will create a growing demand for animal protein that will require increased food and feed outputs (United Nations, 2015). Many insect species can be regarded as suitable candidates for providing sustainable animal proteins (RUMPOLD and SCHLÜTER, 2013a; SÁNCHEZ-MUROS *et al*., 2014). Consuming insects (or insect protein) instead of protein derived from livestock has numerous advantages. Insects are poikilothermic, have a higher feed-conversion efficiency than that of conventional livestock (i.e., insects need less feed for the production of 1 kg of biomass than livestock do) (NAKAGAKI and DEFOLIART, 1991), have a much higher fecundity level (i.e., produce more than one generation during a single season) and are mostly omnivorous, therefore could be raised on various organic waste; they might contribute a smaller amount of greenhouse gases than conventional livestock (OONINCX *et al*., 2010), and they have low requirements for space and water during the rearing process. In addition to these numerous advantages, their nutritional value has long been recognized (RUMPOLD and SCHLÜTER, 2013b). In fact, several articles have been published addressing the nutritive value and nutrient composition of various insects (i.e high level of polyunsatured fatty acids, high concentration of macronutrient and vitamins) (MAKKAR *et al*., 2014; HENRY *et al*., 2015). However, the presence of potentially harmful ingredients in insects, such as allergens, should be investigated to ensure that they are safe food and feed products (BELLUCO *et al*., 2013; RUMPOLD and SCHLÜTER, 2013b).
Currently, in Europe, there is a restrictive legislation about the use of insects as food and feed (Regulation (EC) No. 178/2002; Regulation (EC) No. 1069/2009; Regulation (EC) No. 999/2001; Regulation (EC) No. 258/1997; Commission Regulation (EU) No. 142/2011). Nevertheless, due to the growing interest in insects as alternative sources of proteins, initiatives have begun to emerge to create an enabling environment for the development of regulations and standards for the use of insects in feed and food. In particular, New Regulation on Novel Food had been approved by the EC and whole insects had been inserted among novel food (Regulation (EC) No 2015/2283).
Consumers' acceptance, in addition to the legislative obstacle, remains one of the largest barriers to adopting insects as sources of protein in many Western countries (VAN HUIS *et al*., 2013). There are few examples of traditional insect dishes that are consumed, such as "maggot" cheese in Italy (i.e. casu marzu) and palm larvae in Reunion Island. In fact, particularly in urban and Western societies, insects are rarely eaten or their consumption is perceived to be culturally inappropriate (VAN HUIS, 2013b) and disgusting (NONAKA, 2009).
Although it is an innate reaction (HERZ, 2012), disgust plays a major role in people's food rejection. Like other emotions, the origins of disgust are rooted in one's culture. In fact, food culture defines the rules of what is edible and what is not. In Western societies, insects have been rarely considered an edible food source. Consequently, they are rejected because they are considered to be non-food, unclean and a health risk (HARTMANN *et al*., 2015; LOOY *et al*., 2014).
From an evolutionary point of view, when a new food product is introduced into a culture, it generally induces feelings of fear and refusal called neophobia (PLINER and SALVY, 2006). Food neophobia, defined as the fear and aversion to new foods, is expected to reduce the likelihood of readiness to incorporate insects into the diet (MEGIDO *et al*., 2014; VERBEKE, 2015). The relation between entomophagy and food neophobia can be

explained in at least two ways (ROZIN and FALLON, 1987): first, the rejection of insects as food may depend on the knowledge of their origin and habitat; and second, this rejection may be based on negative post-ingestional consequences.
One of the factors shown to be most effective in establishing behavioural changes regarding food is exposure to it. Food exposure increases the familiarity to a stimulus through a mechanism of learned safe behaviour, thus reducing neophobic reactions (LAUREATI *et al*., 2015a). Information is also a factor that has been reported to play a role in consumers' acceptance (LAUREATI *et al*., 2013; 2016). Information about the ecological and nutritional benefits of employing insects as feed and food may be used to promote entomophagy (VERBEKE, 2015). Despite this, to our knowledge, there are no studies investigating how information provided to consumers might modulate their willingness to adopt insects as food and no studies are available on Italian consumers, who are known to have a deeply rooted culinary culture.
In addition, research studies to evaluate the readiness of Western consumers to incorporate insects into animal and human diets have been conducted mainly on Dutch and Flemish subjects (MEGIDO *et al*., 2014; TAN *et al*., 2015; DE BOER *et al*., 2013; VERBEKE, 2015) but never considering the willingness of consumers' from other European countries. This issue is of particular relevance in view of the role played by cross-cultural differences in accepting insects as food (TAN *et al*., 2015; SCHÖSLER *et al*. 2012; MEGIDO *et al*. 2014).
Based on these assumptions, the first aim of the present study was to evaluate the willingness of Italian consumers to adopt insects as part of animal and human diets and to investigate which are the main factors (e.g., socio-demographic factors, food neophobia and involvement in sustainability issues) that affect readiness to use this alternative food source. These factors have been reported to play a role in the willingness to adopt new and sustainable food (LAUREATI *et al*., 2013; VERBEKE, 2015). The second objective of the study was to investigate whether and how information can influence willingness to taste insects and their acceptability. This was achieved by comparing willingness to adopt insects in consumers with different awareness of the topic (i.e. students and staff from the Faculty of Agricultural and Food Science of the University of Milan and consumers from outside the university context) and by investigating how the information about the benefits of including insects in animal and human diets influenced the consumer acceptance of this type of food.

2. MATERIALS AND METHODS

2.1. Participants

Three hundred and forty one adults (223 females and 118 males) aged 18 to 80 years (M = 31.9; sd = 15.6) were recruited from the students and employees of the Faculty of Agriculture and Food Sciences of the University of Milan and from consumers from outside the university. This choice was made in order to compare two groups of consumers with a different awareness of the topic: university students and staff are expected to be more involved in the topics of insects and sustainability since these topics are studied, investigated, and debated in University courses. The inclusion criteria were being ≥ 18 years of age and being Italian. These subjects were involved in the first part of the study, which involved completing a questionnaire aimed at investigating their propensity towards incorporating insects into animal and human diets. A subset of these subjects consisting of university students (n=68, 42 females and 26 males, age 21.4 ± 3.9) was asked to participate in a second experiment, which consisted in evaluating the visual

acceptability of a series of food products made using insects. Unfortunately, at this stage consumers from outside the university were not available or not willing to come to the laboratory. Written informed consent was obtained from each subject after the aim of the experiment was described.

2.2. Questionnaire: assessment of consumers' willingness to incorporate insects into diets

The questionnaire for the evaluation of the willingness of the subjects to use insects as a food source for humans and animals was administered via the web and social networks. The questionnaire, which consisted of 52 questions, was divided into four sections.

2.2.1. Section 1 – Socio-demographic information

The first section concerned information about gender, age, awareness of the topic (i.e., university student/staff or consumers from outside the university), place of residence and monthly family income. The characteristics of the participants are reported in Table 1.

Table 1: Characteristics of the participants (n=341) reported as mean ± standard deviation (sd) or percentage of answer.

Variable		Percentage/mean ± sd
Age (years)		31.9±15.6
Gender	Male	34.6%
	Female	65.4%
Income	Low (< 1500€/month)	30.3%
	Medium (1500€ < income < 3000€)	45.3%
	High (≥ 3000 €/month)	24.4%
Place of residence	City	40.5%
	Small town	59.5%
Awareness of the topic	University students and staff	47.2%
	Consumers from outside the university	52.8%

2.2.2 Section 2 – Willingness to incorporate insects into diets

The second section consisted of questions related to the propensity towards the consumption of insects by humans and animals. The questions about the willingness to consume insects were structured to investigate consumers' readiness to use insects as feed ("If insects were used as a supplement in feed formulae for aquaculture and livestock, would you be willing to eat the meat and fish from animals that had been fed in that way?"), food ("Would you be willing to eat food obtained from insects, e.g., biscuits produced using insect flour?") and in a specific eating context ("If in an ethnic restaurant you were offered a dish based on insects, would you be willing to taste it?"). For each question, each subject had to indicate the degree of agreement using a 5-point scale (1= "I strongly disagree", 2= "I disagree", 3= "I neither agree nor disagree", 4= "I agree" and 5= "I strongly agree").

2.2.3. Section 3 – Consumers' food neophobia evaluation

Food-neophobia was assessed because this personal trait has been indicated as one of the major predictors of rejection of insects as food (VERBEKE, 2015). Food neophobia was measured using the food-neophobia scale (FNS) developed by PLINER and HOBDEN (1992). The FNS consisted of five neophilic ("I am constantly sampling new and different foods"; "I like foods from different countries"; "At dinner parties, I will try a new food", "I will eat almost anything", "I like to try new ethnic restaurants ") and five neophobic ("I do not trust new foods"; "If I do not know what is in a food, I won't try it"; "Ethnic food looks too weird to eat"; "I am afraid to eat things that I have never had before"; "I am very particular about the foods I will eat") statements about food or situations related to food consumption. The participants were asked to indicate the level to which they agreed or disagreed with the 10 statements. Responses were given on a 7-point agreement scale, ranging from ''strongly disagree'' to ''strongly agree.'' After reverse coding the responses for the neophilic statements, a total FNS score ranging from 10 to 70 was then calculated by summing the ratings for each item; the higher the FNS score, the higher the food-neophobia level. Cronbach's alpha was satisfactory (alpha=0.90).

2.2.4. Section 4 – Consumers' sustainable behaviour

Two batteries of questions were used. Both questions were based on the Theory of Planned Behavior (TPB) (AJIZEN, 1991), which has been proven to be a proper theoretical framework for understanding sustainable and ethical consumer behaviours concerning food (LAUREATI *et al*., 2013). The first question investigated the consumer's actual sustainable behaviour ("Recently, how often have you performed the following actions?"), by asking them to indicate how often (never = 0 times, rarely = 2-3 times a month; sometimes = 1-2 times a week, often = 3-4 times a week; and always = every day) they performed a series of sustainable and non-sustainable actions (e.g., to separate their waste, save energy or consume foods of exotic origin). Cronbach's alpha was satisfactory (alpha=0.81). The second question was related to the subject's level of involvement and interest in some of the major sustainability issues ("For each of the following items, please indicate the statement that best fits your experience"), for instance, the exploitation of Third World people, respect for the environment and the promotion of organic farming. Subjects had to answer the question by choosing one of the following options: "I am not aware/I never heard or paid attention to this matter"; "I know what it is but I'm not interested"; "I know what it is; I'm interested but I have never done anything about it"; "I am interested and I did some small action, for example, I spoke with someone about it" and "I am interested and I've done something meaningful, for example, I changed brand". Cronbach's alpha was satisfactory (alpha=0.77).
These two questions were used to categorize the consumers according to their sustainability level as indicated by LAUREATI *et al*. (2013) and to verify whether this behaviour might influence their willingness to accept insects as food.

2.3. Expected liking of insect-based food

A group of consumers (n= 68, 42 females, 26 males, mean age = 21.4y ± 3.8) who had previously completed the questionnaire participated in a visual hedonic assessment of a series of insect-based foods. The evaluation was conducted in two distinct phases. The first phase consisted of viewing eight images of foods containing insects or insect derived proteins, accompanied by a brief description of the food to inform them about what the product was when this was not interpretable from the image alone. After viewing each

image, the consumers had to express their expected liking using an unstructured linear scale anchored at the extremes by "Extremely disliked" (corresponding to 0) to "Extremely liked" (corresponding to 100). Then, they were presented with information about the nutritional and environmental benefits of using insects as food and feed. The information provided was as follows: *"The global increase in population resulting in a higher demand for food has led to the need to find new and more sustainable sources of protein. The consumption of insects, already practiced in some Eastern cultures, could spread to our culture. Insects are increasingly recognized as an excellent alternative protein source for use in animal feed and human diets. Many species are highly nutritious, and the production of insects has less environmental impact compared to that of traditional sources of protein. Insects can also be raised inexpensively and rapidly on a wide range of organic materials, such as the vegetable waste of households and industries, reducing the overall quantity of waste by up to 60%"*.
After viewing this information, consumers were asked again to express their expected liking for the same eight insect-based foods previously viewed and described. The purpose of providing the information was to determine whether and how being informed of the potential benefits of using insects as food could improve their acceptance of the food shown. The images were displayed on a screen in a randomized order in individual booths at the sensory laboratory of DeFENS (University of Milan). Fizz software version 2.43 was used for hedonic data acquisition (Biosystemes, Couternon, France).
The appearance and visual components of foods are critical for their acceptance; hence, the images were chosen according to the level of visibility of the insects. Thus, insects were not visible in some of the chosen foods (e.g., biscuits made using insect flour), insects were present but in a disguised form or were partially hidden in other foods (e.g., chocolate-coated grasshoppers), or insects were clearly visible in a third category of foods (e.g., cheese with larvae). Furthermore, the foods selected for viewing included sweet products (i.e., biscuits, chocolate, snack-bar and lollipop) as well as non-sweet formulations (i.e., tequila, rice, salad and cheese). The images were selected from the internet and referred in some cases to existing foods, e.g., tequila with worms that is traditionally consumed in South America and casu marzu (literally "rotten cheese"), a typical Sardinian gastronomic product notable for containing live insect larvae (maggots). In other cases, the images referred to dishes that were completely new and unusual for the Italian consumer, such as an apple salad containing insects or a risotto containing larvae. However, all of the products should be considered extremely unusual in the Italian gastronomic culture. The images selected for the hedonic assessment are shown in Fig. 1.

2.4. Data analysis

To evaluate the effect of the questionnaire variables on consumers' readiness to accept insects as feed, as food and in a specific eating context (i.e., in an ethnic restaurant), the willingness data were assigned a score to each answer, as follows: "I strongly disagree" = -2; "I disagree" = -1; "I neither agree nor disagree" = 0; "I agree" = 1; "I strongly agree" = 2. After having verified that the data were normally distributed, a mixed analysis of variance (ANOVA) using a generalized linear model (GLM) was applied considering the main factors subjects, gender, income (low, medium, high), place of residence (city vs small town), awareness of the topic (from the university vs outside the university), the consumer's food neophobia (low, medium, high) and the consumer's sustainable behaviour (low, medium, high) and the willingness data as the dependent variables. The subjects were considered as a random factor in the model.
The consumers were categorized as having a low, medium or high level of food neophobia and sustainable behaviour according to LAUREATI, BERTOLI *et al*. (2015a) and LAUREATI *et al*. (2013), respectively. The frequency distribution of the FNS scores was

calculated and the subjects were divided into the 3 following groups: "low neophobia" (subjects in the lowest quartile, FNS scores ≤ 23, n=86), "medium neophobia" (subjects in the second and third quartile, FNS scores ≥ 24 and ≤ 41, n=166) and "high neophobia" (subjects in the highest quartile, FNS scores ≥ 42, n=89).

Figure 1: Images of the insect-based foods shown to the consumers in the expected liking assessment.

In the same way, for each subject, an index for the actual sustainable behaviour and an index for awareness and interest in sustainable issues were calculated as the mean value of the scores for the different items in each of the two questions. Then, the distribution frequency of the scores for each index was calculated. The subjects with a score within the lowest quartile of both distributions (actual sustainable behaviour: ≤ 3.33, awareness: ≤ 3.25) were considered as having a "low sustainability level" (37 subjects), whereas the subjects with a score over the highest quartile of both distributions (actual sustainable behaviour: ≥ 4.00, awareness: ≥ 4.12) were considered as having a "high sustainability level" (52 subjects). The rest of the subjects were considered as having a "medium sustainability level" (251 subjects). The size of these groups was consistent with the results of a previously mentioned study (LAUREATI *et al*., 2013).
The effect of age on the propensity towards insect consumption in the three situations (i.e., as feed or food, in an ethnic restaurant) was investigated using Pearson's correlation test.
The liking data were subjected to a mixed ANOVA model using a GLM, considering the subjects, gender, food neophobia level (low, medium, high), the foods (the 8 pictures), the condition (non-informed *vs* informed) and the interaction foods by condition as the factors and the hedonic scores as the dependent variable. The subjects were considered a random factor in the model. The other background variables (e.g., age and income) were not included in this model because only students were involved in the hedonic test or because of the small number of participants was inappropriate (e.g., sustainability level).
When the ANOVAs indicated significant differences ($p < 0.05$), t-tests (SAS option lsmeans pdiff) were used as the multiple comparison tests. All of the statistical analyses were performed using SAS 9.3 software (SAS Institute Inc., Cary, NC, USA).

3. RESULTS

3.1. Willingness to adopt insects as feed and food

In a preliminary phase of the analysis, the data obtained using the questionnaire concerning consumer's willingness to incorporate insects into human and animal diets were reported as frequency responses to allow an overview of the results. In this first stage, the consumers were categorized into the following three groups: willing (the sum of people who answered "I agree very much" and "I agree"), uncertain (the people answered "I neither agree nor disagree") and unwilling consumers (the sum of people who answered "I disagree very much" and "I disagree"). The proportions of answer to the questions related to using insects as feed ("If insects were used as a supplement in feed formulae for aquaculture and livestock, would you be willing to eat the meat and fish from animals that had been fed in that way?"), food ("Would you be willing to eat food obtained from insects, e.g., biscuits produced using insect flour?") and in a specific eating context ("If you were offered a dish based on insects in an ethnic restaurant, would you be willing to taste it?") are reported in Table 2.
Approximately 53% of the consumers (of which 19.1% strongly agreed and 33.7% agreed) declared themselves to be ready to incorporate insects into animal diets and to eat fish and livestock reared upon insect-containing feed. Concerning incorporating insects into the human diet, a considerable decrease in the percentage of willing people was observed, with only 21.1% (16.7% agreed; 4.4 strongly agreed) and 31.1% (21.4% agreed, 9.7% strongly agreed) of people ready to eat food derived from insects and to consume insects in a specific eating context, respectively. The percentage of uncertain people was approximately the same for the three situations, with 25.5% uncertain regarding insects as

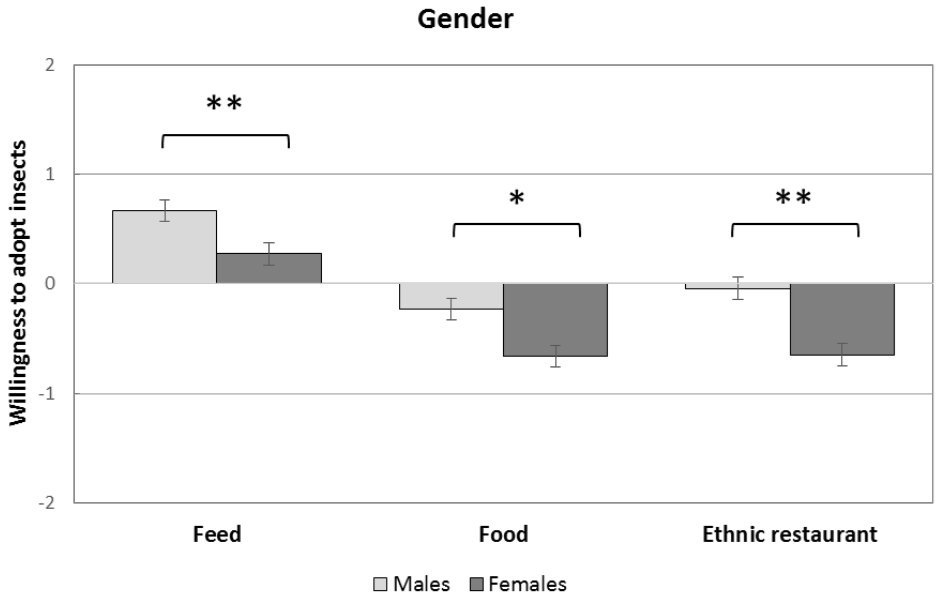
feed, 28.5% uncertain regarding insects as food and 20.5% uncertain regarding eating insects in a restaurant.

Table 2: Percentage of answer to the questions related to the consumer's willingness to accept insects as feed, as food and in a specific eating context (i.e., in an ethnic restaurant).

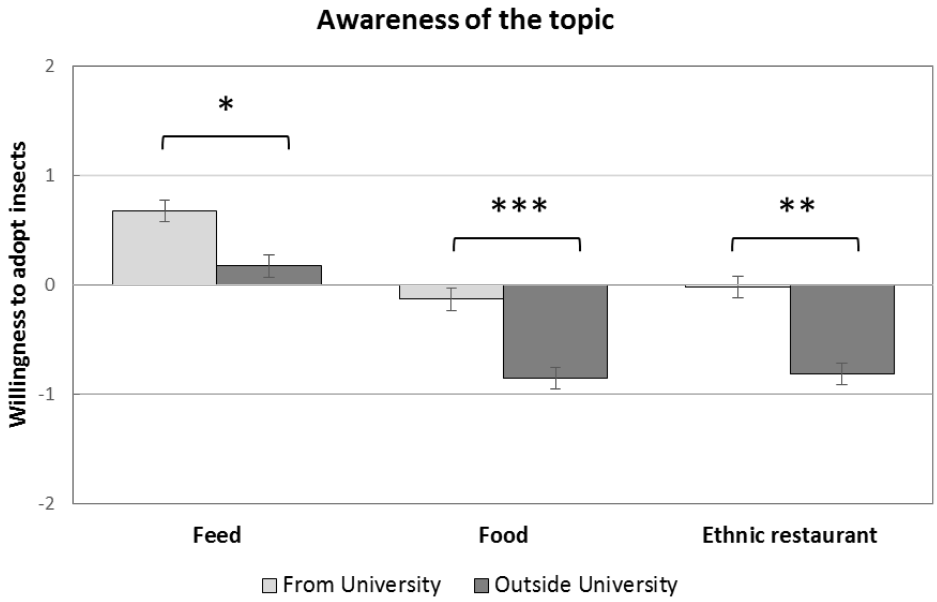
Willingness	Answer	Insect use		
		Feed	Food	Specific context (ethnic restaurant)
Unwilling	I strongly disagree	9.1	26.1	36.1
	I disagree	12.6	24.3	12.3
Uncertain	I neither agree nor disagree	25.5	28.5	20.5
Willing	I agree	33.7	16.7	21.4
	I strongly agree	19.1	4.4	9.7

To quantitatively compare the effect of each variable considered in the questionnaire (e.g., socio-demographic, eating behaviour and sustainability), the data on the willingness to accept insects were analysed using an ANOVA. Only the factors gender (Feed: $F=6.71$, $p<0.01$; Food: $F=4.40$, $p<0.05$; Restaurant: $F=9.71$, $p<0.01$), awareness of the topic (Feed: $F=6.65$, $p<0.05$; Food: $F=16.39$, $p<0.0001$; Restaurant: $F=10.94$, $p<0.01$) and food-neophobia level (Feed: $F=10.54$, $p<0.0001$; Food: $F=32.82$, $p<0.0001$; Restaurant: $F=37.12$, $p<0.0001$) had a significant effect on the consumers' willingness to incorporate insects into both animal and human diets, whereas income, place of residence, and involvement in sustainability issues did not play a role. Income had a marginal effect ($F=2.64$, $p<0.10$) only on the willingness to consume insects within a specific eating context (i.e. restaurant).
The mean values of the consumers' willingness to accept insects according to gender, awareness of the topic and food-neophobia level are shown in Figs. 2 a-c.

a



b



c

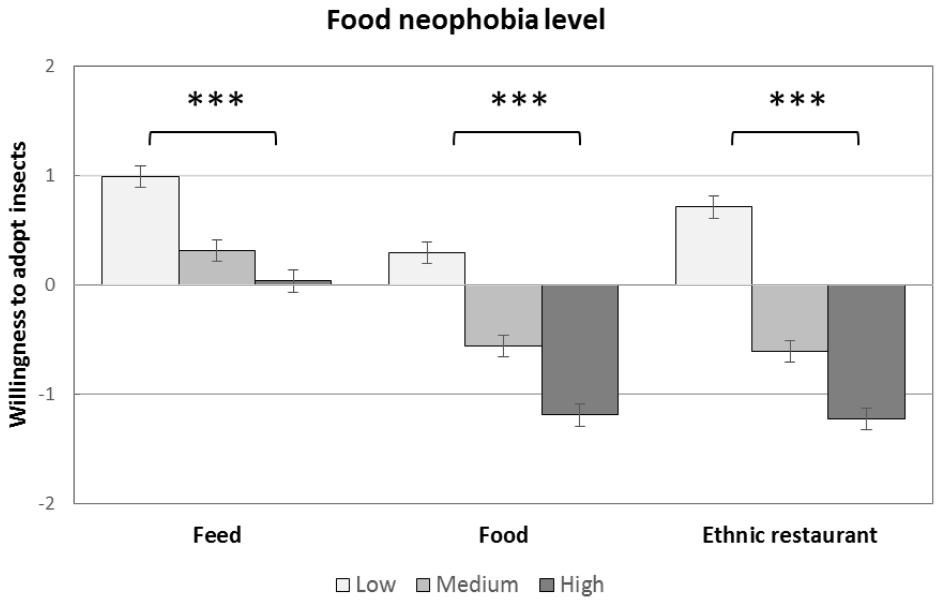


Figure 2a-c: Mean values (±SEM) of consumers' willingness to accept insects in the three situations, according to gender (a), awareness of the topic (b) and food-neophobia level (c).
* significant difference at $p<0.05$; ** significant difference at $p<0.01$; *** significant difference at $p<0.001$.

Males were significantly more willing than females to consume insects in all situations (Fig. 2a). People with a higher level of education about the topic (i.e., university students and staff) were significantly more ready to accept insects as feed, as food and in an ethnic restaurant than less-aware people (Fig. 2b). Furthermore, people with a low level of food

neophobia (i.e., neophilic people) were significantly more willing to accept insects as feed, as food and in an ethnic restaurant than people with a medium level of food neophobia who, in turn, showed a significantly higher readiness than neophobic consumers (Fig. 2c). Although the correlation coefficients were somewhat low (feed: r= -0.41, food: r= -0.43, restaurant: r= -0.45), a significant ($p<0.01$) negative relationship was seen between age and the willingness to accept insects in all of the situations, indicating that younger people were more ready to accept insects.

3.2. Expected liking of insect-based foods

A total of 68 students who had completed the questionnaire were involved in a hedonic test that evaluated their degree of liking for a series of insect-based foods shown in images. Liking was evaluated before and after the consumer received information regarding the environmental and nutritional benefits of consuming insects as part of animal and human diets. The mean hedonic values before (non-informed condition) and after the information was provided (informed condition) are reported in Table 3. As expected, the mean acceptability ratings were in general very low (ranging from 21.0 to 50.7 on a scale of 0 to 100). ANOVA results showed a significant effect of the main factor Food ($F=10.40$; $p<0.0001$). According to the results of post-hoc test, before receiving the information about the benefits of consuming insects, biscuits made using insect flour (M=44.8) and chocolate-coated grasshoppers (M=38.9) were significantly more liked than were the other products. A cereal bar containing insects (M=31.8), an apple salad containing insects (M=29.2) and tequila containing a larva (M=24.8) were very much disliked; however, they received significantly higher ratings compared with risotto containing larvae (M=22.1), cheese with larvae (M=21.9) and lollipops containing larvae (M=21.0), which were extremely disliked. A similar ranking of products was seen after the consumers had received the information (Informed condition, Table 3).

Table 3: Mean acceptability ratings (range 0-100) before (non-informed condition) and after (informed condition) the consumer had received information about the environmental and nutritional benefits of using insects in animal and human diets. The different superscripted letters within the columns indicate significant differences ($p<0.05$).

Food	Condition		
	Non-informed (N)	Informed (I)	I-N [1]
Biscuits made using insect flour	44.8^{a}	50.7^{a}	*
Chocolate-coated grasshopper	38.9^{a}	44.5^{a}	*
Cereal bar containing insects	31.8^{b}	44.8^{a}	**
Apple salad containing insects	29.2^{bc}	31.7^{b}	n.s.
Tequila containing a larva	24.8^{cd}	27.3^{bc}	n.s.
Risotto containing maggots	22.1^{d}	22.4^{c}	n.s.
Maggot cheese	21.9^{d}	23.3^{c}	n.s.
Lollipops containing larvae	21.0^{d}	25.5^{bc}	n.s.

[1] Significance of the difference between the hedonic mean values before (N) and after the information (I) was provided, according to the t-test.
* indicates a significant difference at $p<0.05$;
** indicates a significant difference at $p<0.01$; n.s. indicates a non-significant difference

The main factor condition had a significant effect on the acceptability of insects as food ($F=5.51$; $p<0.02$) effect. Overall, the mean acceptability ratings for all of the products increased under the informed condition (Non-informed condition: M=29.3; Informed condition: M=33.8), even though the increase was significant only for the biscuits made using insect flour, chocolate-coated grasshopper and the cereal bar containing insects. The interaction Food by Condition was not significant, meaning that under the informed condition, the acceptability ratings were systematically higher than they were under the non-informed condition regardless of what food was concerned. However, this result also indicated that the information provided generally had little impact on the acceptability ratings. Finally, regarding the willingness to accept insects as food, gender ($F=47.11$; $p<0.0001$) and food-neophobia level ($F=15.30$; $p<0.0001$) strongly affected the level of expected liking. According to the results of a post-hoc test, males (M=37.2) liked the insect-based foods more than females (M=24.1), and neophilic people (M=37.8) provided significantly higher ratings than people with a medium (M=29.2) or a high degree of food neophobia (M=25.0).

4. DISCUSSION

The results of the present study indicated that the interviewed Italian consumers are clearly not ready to incorporate insects into their diets. A more positive attitude was observed for utilizing insects in farming. More than half of the consumers declared themselves to be favourable towards the use of insects in animal feed and to eating livestock that had been reared with insects as a supplement in the diets. This outcome was understandable because fishes and many other farmed animals, such as poultry and pigs, eat insects when they are reared in natural environments. Thus, this phenomenon could make the consumer more willing to accept the systematic use of insects or relevant derivatives thereof (e.g., flour) in farming.

It should be noted that, although the percentages of people who were willing to introduce insects into their diets (21%) or to consume them in an ethnic restaurant (31%) were apparently low, these values are similar to or even higher than those observed in other studies conducted with other European consumers (VANHONACKER *et al*., 2013; VERBEKE, 2015; DE BOER *et al*., 2013). MEGIDO *et al*. (2014) reported a higher percentage of willing people than that found in our study, corresponding to 78% of their Belgian participants. This very high rate, however, is explained by the sample of people who were surveyed in their study, who had purposely visited an insectarium and thus were very interested in insects (and in eating them). This consideration can also be applied to our sample of consumers because approximately half of them were students and staff from a university in which the topic of insects is studied. However, when only the percentage of willing consumers from outside the university was considered, 13% and 21% of them declared themselves to be willing to introduce insects into their diets or consume them in an ethnic restaurant, respectively. These data demonstrate that one of ten or five people claimed to be ready to incorporate insects in their diets, thus indicating some degree of readiness to try to eat insects, which is consistent with the idea that a market niche for insects or insect protein may develop in Western countries (VERBEKE, 2015). Analysing the recent literature concerning the readiness to accept insects as meat substitutes or more generally as part of the human diet, it is evident that most of the studies were conducted using Belgian or Dutch people. In this context, the results of the present study provide an insight into the willingness to adopt insects as food of a consumer target living in a Mediterranean region with different eating habits compared with those in Belgium and The Netherlands. In this sense, Italian subjects' attitude towards insects appeared to be

comparable to or even a little more positive than those of consumers from other European countries.

A series of variables were investigated to determine their impact on consumers' willingness to accept insects as food or feed. Age, gender, awareness of the topic and food neophobia were found to be the most influential factors in this regard for the Italian consumers. The readiness to accept insects was stronger among males than females and was stronger among younger consumers than among older consumers. These findings are consistent with those of SCHÖSLER *et al*. (2012) and VERBEKE (2015). Age-related differences might be explained by the higher level of openness and curiosity of the young consumer regarding novel foods compared with those of older people, for whom a higher degree of food neophobia has been reported (DOVEY *et al*., 2008). Consistent with this assumption, the results of the present investigation indicated that food neophobia had the greatest effect on Italian consumers' willingness to accept insects as food or feed, as was demonstrated in other studies (MEGIDO *et al*., 2014; VERBEKE, 2015). What was particularly interesting about our results was the attitude of neophilic people towards insects; indeed, we observed positive and somewhat high willingness scores for these people in all of the situations tested (M_{feed}=1.0, M_{food}=0.3, $M_{restaurant}$= 0.7 on a scale ranging from -2 to +2), suggesting that this consumer group is a potential target for marketing insects.

As expected, consumers attending university courses or working in an environment in which the topic of insects as well as sustainability are studied, investigated, and debated, positively affected consumers' willingness to incorporate insects into animal and human diets. Students and staff from the university showed a more conscious attitude and were more open to the theme of insect as food or feed than were people from outside the university context, indicating that information plays a fundamental role in accepting new food.

Contrary to our hypothesis, the consumers' sustainability level did not affect their readiness to incorporate insects into animal and human diets. Most of the consumers who declared themselves to behave sustainably and to be aware of sustainability issues indicated their uncertainty and disagreement regarding the possible use of insects in both animal and human diets. This outcome was not consistent with data previously reported, which showed that people who are interested in the environmental impact of their food choice are the most likely adopters of insects as a novel and more sustainable protein source to be used as a meat substitute (VERBEKE, 2015). Furthermore, in a context different from adopting insects as food, LAUREATI *et al*. (2013) found that sustainability awareness may influence individuals' expectations about ''sustainable'' products (i.e., organic yogurt). A possible explanation for the discrepancy between our findings and the data in the literature is that asking people to accept insects as meat substitute is conceptually different - and most likely less troubling - than asking them to include insects in their diet or to eat them in a restaurant. Thus, a positive attitude towards sustainability might prevail when a person must choose insects instead of a single component of the diet (i.e., meat), whereas when one must consider the possibility of consuming different foods that contain insects in various forms (e.g., in flour or as an ingredient), the disgust might be a too strong determinant to overcome.

Information about the environmental and nutritional benefits of introducing insects into animal and human diets only marginally affected the visual acceptability of a series of insect-based foods. In comparing the acceptability ratings before and after the consumer had received the information, an overall significant increase was observed. As familiarity might reflect the receipt of the information, this finding suggested that people were sensitive to the information they were exposed to concerning the use of insects (VERBEKE, 2015). The acceptability results also indicated that although the ratings were generally very low, sweet products were more appealing than were non-sweet ones. The only

exception was the lollipops, which the consumers considered extremely unpleasant, most likely because the insects were much more visible in the lollipops than they were in the cereal bar containing insects, biscuits made using insect flour and chocolate-covered grasshoppers. Similar findings were reported by SCHÖSLER *et al*. (2012), who found that chocolate-coated locusts were preferred to other dishes containing more visible insects. Accordingly, MEGIDO *et al*. (2014) prepared a series of sweet and savoury insect-based foods, which were actually tasted by consumers, and found that sweet preparations (i.e., crispy mealworms covered with chocolate) were more liked than were the others. These findings corroborated the hypothesis that pairing something that is traditionally well liked and known (e.g., sweets) with a food that is initially unfamiliar and unpleasant might be an effective strategy to enhance liking (LAUREATI *et al*., 2014) even with insect-based foods. This hypothesis was recently confirmed by TAN *et al*. (2015), who reported that reducing the visibility of the insect and incorporating it into a familiar and well-liked product generally improved the consumer's willingness to consume insect-based food formulations. Interestingly, "casu marzu", a typical Sardinian cheese containing live insect larvae, was not accepted by the consumers involved in the present study. This is might be explained by the fact that this is a niche, traditional product from Sardinia, which is not indeed well known in the rest of Italy. Considering that the experiment was performed in Milan, it is likely that subjects involved in the hedonic test did not associate the cheese to a food from their culture, thus rejecting it. This outcome further stresses the importance of culture and tradition and how this may lead to consumer's acceptance or rejection even within the same country (LAUREATI *et al*., 2006).

One of the strengths of our study was that it combined a large survey to assess consumers' willingness to adopt insects as food or feed with an evaluation of the expected liking for a series of insect-based foods in a sub-group of these consumers. This strategy allowed evaluating the drivers of both the willingness to consume insects and the expected liking for insects of the interviewed Italian consumers. Furthermore, the readiness to accept insects was investigated using three separate items concerning introducing insects into animal feed, introducing insects into the human diet and consuming insects in a specific eating context. We did not refer to the use of insects as a substitute for a specific type of food, such as meat or animal protein, because in our opinion, such an approach would have been reductive by ignoring the possibility that insects can be eaten for reasons other than as a substitute for meat.

Of course, there were also weaknesses in our study that must be noted. First, the hedonic assessment was conducted using a small number of consumers. Most importantly, these consumers were selected from university students (of the Faculty of Agronomy and Food Science) who had a scientific cultural background, and therefore, on one hand our findings may represent a best-case scenario in terms of the level of acceptance of young Western consumers of insect-based foods. Second, while the survey enabled us to acquire a relatively large amount of data, in some cases, the participants' characteristics (mainly the place of residence and the gender) were not well balanced. Unfortunately, this bias is common in studies in which electronic recruitment and web-based surveys are used (VERBEKE, 2015). Another limitation of this study is that we did not provide the consumers with actual products to taste during the hedonic assessment. However, there is evidence that preference expressed in an image-based analysis is a good predictor of the actual preference (OLSEN *et al*., 2012). Furthermore, the choice to have a specific subgroup of the consumers perform the hedonic test did not allow investigating the effect of the information they were given on a series of background variables regarding their expected liking (e.g., age). Most importantly, the observed marginal effect of the information on liking might have been stronger if consumers with a lower level of topic awareness had been involved.

In conclusion, the results of this study revealed that there is more potential for the use of insects in livestock farming than in the human diet. Although it has been reported that simply stressing the sustainability and nutritional value of insects as a source of food was unlikely to provide sufficient motivation to drive a change in diet (DEROY *et al*., 2015), we found that the consumer's acceptability ratings for a variety of insect-based foods increased systematically after they had received that information. Of course, changing people's existing food choice is not an easy task and requires much effort to increase the degree of the perceived familiarity of insects as food. Because a neophobic reaction was the main driver of rejecting insects as food, it is important that authorities launch campaigns to raise the awareness of the benefits of eating insects and thus facilitate increasing the willingness to accept insects as human nutrition.

ACKNOWLEDGEMENTS

This study was funded by the University of Milan project "Sviluppo Unimi" - Linea B: Istituzione di fondi di ricerca da assegnare a giovani ricercatori. Project title: Insects to feed the future: a new sustainable protein source (INSPIRE).

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Paper Received March 17, 2016 Accepted June 1, 2016

PAPER

CONVECTIVE DEHYDRATION KINETICS AND QUALITY EVALUATION OF OSMO-CONVECTIVE DRIED BEETROOT CANDY

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ABSTRACT

The Beetroot cubes were osmotically pretreated in 60°Bx of sucrose solution at 55°C osmotic solution temperature for 180 min with fruit to solution ratio 1:4 (w/w). The osmotically dehydrated Beetroot cubes were further dehydrated convectively at different drying air temperatures of 55, 65 and 75°C up to final moisture content of 9±1% (w.b). Among the models investigated, the Page model fitted the experimental data for convective drying of natural and osmosed beetrooot cubes. During convective dehydration, the effective moisture diffusivity of natural samples and osmosed samples at drying air temperatures ranged from 55 to 75°C varied between 8.09917×10^{-9} to 1.45785×10^{-8} m^2s^{-1} and 1.13388×10^{-8} to 1.61983×10^{-8} m^2s^{-1} respectively. The activation energy of convective dried natural beetroot cubes was 27.92 kJ mol^{-1} as compared to 16.98 kJ mol^{-1} for osmosed samples. Finally osmo-convectively dried beetroot cubes were coated with sucrose for candy preparation.

Keywords: activation energy, beetroot, convective dehydration, effective diffusivity

1. INTRODUCTION

Beetroot (*Beta vulgaris*) is considered a very important vegetable from the nutritional point of view, which is rich in valuable, active compounds such as carotenoids (DIAS *et al*., 2009), glycine betaine (DE ZWART *et al*., 2003), saponins (ATA-MANOVA *et al*., 1975), betacyanines (PATKAI *et al*., 1997), betanin, polyphenols and flavonoids (VALI *et al*., 2007). Therefore, beetroot ingestion can be considered a factor in cancer prevention (KAPADIA *et al*., 1996). The moisture content of fresh fruits and vegetables is more than 80%, they are classified as highly perishable commodities (ORSAT *et al*., 2006). The moisture content of fruits and vegetables is removed by drying with an aim to preserve and store them for a long period of time by ensuring microbiological safety. Drying is a complex process where heat and mass transfer occurs simultaneously in transient conditions. India is one of the leading producers of fruits and over 20% of the world perishable crops are dried to increase shelf-life and food security (ERTEKIN and YALDIZ, 2004).

The preservation of fruits and vegetables by dehydration should be accomplished in such a manner that the quality effect on the structural configuration of these products is minimized. Among the different methods of food preservation, convective dehydration is the most popular and efficient way to reduce the moisture content and preserve foods. The modes involved in convective drying of foods are heat and mass transfer. Transport of the heat energy flow throughout the medium designated as heat transfer that causes the movement of internal moisture within the foods followed by the movement of water vapor from the food surface is called mass transfer that depends upon external conditions of temperature, air humidity, air flow and area of exposed surface in convective drying. A lot of studies have reported that convective drying is the most popular method applied to reduce the moisture content of fruits and vegetables including beetroot (HENDERSON *et al*., 1961; LEWICIKI, 2006; SHYNKARYK *et al*., 2008). However, this method takes long time and high temperature for drying that affects the nutritive value of products (MARFIL *et al*., 2008).

Product quality notably depends on texture, colour and flavour and they deteriorate with convective dehydration (LENART, 1996). A well-known process to achieve good quality product is freeze drying, but this is an expensive method of food preservation. Therefore, there is a need for simple economic and technically feasible alternative drying processes, which have low capital cost and offer a method that save highly perishable products, making them available to the regions away from production zones. Furthermore, osmotic dehydration is one of such methods that involves the partial removal of water from food, such as fruit or vegetables, by immersion in a hypertonic solution leaving a material that will need shorter drying time than the original food material, thereby, making this process more economical (LENART, 1996; SHI and LEMAGUER, 2002; FASOGBON *et al*., 2013). Osmotic pretreatment can also minimise drying colour losses (RAOULT-WACK *et al*., 1991), as well as reducing nutrient losses (SHI *et al*., 1999). This paper assesses the improvement of the quality of the final product to prevent oxidative browning and the loss of volatile flavoring constituents, reducing the fruit acidity and structural collapse during subsequent air-drying (DIAS *et al*., 2009). It has been reported that when convective drying is conducted between temperatures ranging from 40 to 60°C the moisture curves follow sigmoid shape characteristics of the drying process and shows reduction in drying time with increase in temperature (BARROCA, 2012). Hence, osmo-convective dehydration of beetroot is an interesting alternative for the development of confectionery based functional food with extended shelf life.

One of the most important aspects of drying technology is the modeling of the drying process. The basic principle in modeling is based on a set of mathematical equations that adequately explore the system. The solution of these equations must allow calculation of

the process parameters as a function of time at any point in the dryer based only on the primary condition (PREMI *et al*., 2010). Hence, the use of a simulation model is an important tool for prediction of performance of drying systems. The best model describing the drying characteristics of samples should be chosen as the one with the highest coefficient of determination (R^2), reduced chi-square (χ^2) and RMSE (MADAMBA *et al*., 1996).
(SHI and LEMAGUER, 2002) calculated effective diffusivity using the slope method but this method gives only a single value of diffusivity for the entire process and therefore does not predict the kinetics of the entire osmotic dehydration process, because the value of diffusivity changes with time and with moisture content of the commodity. Some researchers calculated effective diffusivity by using only first term of the analytical solution of the Fickian model assuming that the effect of terms other than first on the value of diffusivity was non-significant (RASTOGI *et al*., 1999; SHARMA *et al*., 2003). Thermodynamically, the activation energy is the ease with which the water molecules pass the energy barrier when migrating within the product (LOPEZ *et al*., 2009).
In this context, the objective of this work aim to: (i) study the experimental investigation to know the effect of osmotic pre-treatment and drying air temperature on the convective dehydration kinetics and determination of effective moisture diffusivity and activation energy. (ii) develop the preparation of beetroot candy from osmo-convective dried beetroot cubes.

2. MATERIALS AND METHODS

2.1. Sample preparation

Fresh, well graded, beetroot was procured from the local market of Sirsa, Haryana (India). Beetroot was washed properly and cut into 1cm×1cm×1cm size with the help of cutter equipped with a knife moving perpendicularly to a horizontal base. Two types of samples like natural beetroot and osmotic dehydrated beetroot were used for the study of the osmo-convective dehydration kinetics .The initial moisture content of natural beetroot cubes was found to be between 85.71 to 86.29% (w.b.).

2.2. Osmotic dehydration

A known amount (20-25 g approximately) of beetroot cubes were transformed in stainless steel containers containing calculated volume of an osmotic solution of different concentrations at pre-set desired temperature in hot water bath. Temperature of the osmotic solution was maintained by hot water bath agitating at the rate of 75 oscillations per min to reduce the mass transfer resistance at the surface of beetroot and for good mixing (GUPTA *et al*., 2012).
Optimization of osmotic dehydration process was carried out with the purpose of maximizing water loss, solute gain and quality of the product. The optimum conditions were 60°Bx osmotic solution concentration, 55°C osmotic solution temperature and 180 min process duration at fruit to solution ratio 1:4 (w/w). Following osmotic pre-treatment at optimum conditions, the moisture content of the beetroot cubes reduced to 74.86% (w.b.) and the solid content increased up to 8% designated as osmotic dehydrated beetroot.

2.3. Convective dehydration

The natural and osmosed samples were taken separately in plastic containers before drying and then divided into 175 g portions each. To prepare a shelf stable product, the beetroot cubes was dehydrated up to final moisture content 9±1% (w.b.) at an air temperature of 55, 65 and 75°C and air velocity of 1.6 m s^{-1} (REPPA *et al*., 1999). The dried samples were packed in high-density polyethylene bags after cooling in desiccators and placed at ambient temperature for further analysis. Initial moisture content was determined by the oven-drying method, for the natural and osmosed beetroot samples at a temperature of 135°C for 2 h until constant weight was reached with repetition in order to assure moisture content average values (AOAC, 2000).

3. ENGINEERING ANALYSIS OF DRYING DATA

3.1. Determination of moisture content

The moisture content of the samples during the drying process was calculated according to the following formula.

$$M_t = \frac{W_t - W_d}{W_d} \quad (1)$$

Where,
M_t = Moisture content at time t (g water/ g dm)
W_t = Dry matter at any time t(g).
W_d = dry matter (g).

3.2. Determination of moisture ratio

However, moisture data are used in non-dimensional form so moisture ratio is defined by the following equation:

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

Where,
MR= Moisture ratio, M_o and M_e Initial moisture content and Equilibrium moisture , g water/ g dry matter content, respectively .M_t, Moisture content at any time t on dry basis (g water/ g dry matter).

3.3. Determination of drying rate

To study the drying behavior at different drying air temperature, moisture content (d.b.) and drying rates were calculated. The drying curves were plotted to observe the effect of process variables. Corresponding to drying curves, the drying rate curves were also plotted (KAR and GUPTA, 2003). The instantaneous drying rate (DR) was calculated from the drying data by estimating the change in moisture content, which occur in each

consecutive time interval (dt) and was expressed as a gram of water/gram of dry matter per minutes.

$$DR = -(M_t + d_t - M_t) / d_t \quad (3)$$

Where,
M_t = Moisture content at time t (g water/g dm)
M_t+d_t = Moisture content at time t+ d_t (g water/g dm min).

3.4. Selection of models

The experimental data were fitted using the four models listed in Table 1. In order to find the best suitable model for describing the drying behavior of natural and osmosed beetroot, non-linear regression analysis was used for determination of the constant of each model. The effectiveness of each model used was evaluated critically analyzing coefficient of determination (R^2), reduced chi square (χ^2) and root mean square error (RMSE). The best model describing the convective drying characteristics was chosen based on the higher R^2 value and lower χ^2 and RMSE.

Table 1: Selected convective dehydration models.

Model name with reference	Model
Newton (SINGH *et al.*, 2007)	MR= exp(-Kt)
Henderson and Pebis (GRABOWSKI *et al.*, 2003)	MR= A exp(-Kt)
Page (PAGE, 1949)	$MR = \exp(-Kt^n)$
Wang and Singh (WANG and SINGH, 1978)	$MR= 1+At+Bt^2$

3.5. Effective moisture diffusivity

An analytical solution of Fick's model of mass diffusion equation for drying biological products in falling period was explained by (CRANK, 1975). When the plot of logarithm of moisture ratio (ln MR) versus drying time is linear, the moisture diffusivity assumes an independent function of moisture content. In this case, the change of moisture content can be described by the following equation (LOPEZ *et al.*, 2009)

$$MR = \frac{M_t - M_e}{M_o - M_e} = \frac{M_t}{M_o} = \frac{8}{\pi^2} \sum_{n=0}^{\infty} \frac{1}{(2n+1)} \exp\left[- \frac{(2n+1)^2 \pi^2 D_{eff} t}{4L^2} \right] \quad (4)$$

Where,
D_{eff} is the effective moisture diffusivity (m^2/sec) and MR is the moisture ratio. Since the top surface of slices was only exposed to hot air, the length, L, in Eq (4) was the thickness of the slabs. For long drying times; n = 0, then Eq. (4) can be written as;

$$\ln(MR) = \ln\left(\frac{8}{\pi^2}\right) - \left(\frac{D_{eff}.\pi^2}{4L^2}.t\right) \quad (5)$$

The effective moisture diffusivity was calculated using the method of slopes. When logarithm of MR values v/s drying time were plotted in accordance with Eq. (5), straight lines were obtained at all temperatures and sample thickness was investigated. With the help of linear regression analysis, numerical values of diffusion coefficients were obtained for different drying conditions from the slope of the straight lines.

3.6. Determination of activation energy

Effective diffusivity dependence on drying air temperature was obtained from Arrhenius relationship to calculate the activation energy (LOPEZ *et al*., 2009)

$$D_{eff} = D_o \exp\left(\frac{-E_a}{R_g(T+273)}\right) \quad (6)$$

Where,
T is temperature (°C), R_g is gas constant having a constant value of 8.314 KJ/mol K, D_o is the pre-exponential factor of Arrhenius Equation, m^2S^{-1}. E_a is the activation energy kJ mol^{-1}.
The above exponential form of Arrhenius equation can be expressed as;

$$\ln D_{eff} = \ln D_o - \frac{E_a}{R_g(T+273)} \quad (7)$$

A plot of ln D_{eff} versus 1/(T+273) gives a straight line of slope E_a/R_g slope and consequently activation energy.

3.7. Preparation of beetroot candy

Osmo-convectively dried beetroot cubes at different temperatures were immersed in sucrose syrup (70°Bx) for candy preparation. Sugar in powder form was crystallized on the surface of beetroot cubes during the cooling process (GUPTA *et al*., 2012). The resulting beetroot candy was packed in low-density polyethylene bags and kept at room temperature for sensory analysis.

3.8. Sensory evaluation

Organoleptic attributes like color, flavour, taste, texture, and overall acceptability of beetroot candy were determined using a 9-point Hedonic scale with the help of a 10-member consumer panel (WANG *et al*., 2009). The average scores of all 10 panelists were computed for different characteristics.

3.9. Statistical analysis

All the data obtained from convective drying of osmosed and natural beetroot were analysed to find out statistical parameters like R^2, χ^2 , RMSE and drying constants for different models using statistical package for social sciences (SPSS). Intra-pair significant differences especially for sensory quality attributes were determined using Duncan's tests at 5% level of significance.

4. RESULTS AND DISCUSSION

4.1. Effect of osmotic pre-treatment on convective drying kinetics

Table 2 indicates that, the total convective dehydration time at 55°C of natural beetroot was 690 min, but was 570 min for samples given osmotic pre-treatment with the sucrose solution. Therefore, beetroot samples given osmotic pre-treatment in sucrose solution consequently reduced convective drying time approximately by 120 min when compared with the convective drying of natural beetroot samples (not treated with the sucrose solution) at 55°C air temperature. Similar behaviors have also been observed at 65°C and 75°C drying air temperatures where the convective drying time reduced for the beetroot samples given osmotic pretreatment in sucrose solution by approximately 90 min and 140 min respectively. This might have happened due to leaching of some volatile components of the cellular structure during soaking in osmotic solution. This reduces cell wall resistance and increases drying of beetroot, the results obtained were consistent with data reported by other authors for apricot cubes and melons (RIVA *et al*., 2004; RODRIGUES and FERNANDES, 2007).

Table 2: Total convective drying time for natural and osmosed dried Beetroot.

Temperature (°C)	Average drying time (min)	
	Natural	Osmosed
55	690	570
65	570	480
75	460	320

4.2. Effect of drying air temperature on drying kinetics

The drying curves for all the drying experiments performed are reported in Figs. 1 and 2. Fig 1 shows the variation of moisture content of osmosed and natural beetroot samples with time for different temperatures. To dry natural and osmosed beetroot sample to final moisture content of 9±1% (w.b.), the drying time at 75 °C was lower as compared to drying time at 65°C and 55°C. Temperature increase cause diffusion coefficient to get higher values, and then drying rate increases. Similar results have been obtained in case of carrot (DIAS I., 2009), garlic (MADAMBA *et al*., 1996) and eggplant (DOYMAZ and ISMAIL, 2011). It can be seen (Fig. 1) that, the osmosed and natural beetroot did not have any constant rate drying period and complete drying took place during the falling rate period. The absence of a constant rate period was because the product could not provide a constant supply of water for an appreciable period of time for rapid thin-layer drying of

the product for initial stages of drying (LAHSANSI *et al*., 2004; PRAKASH *et al*., 2004). Drying in the falling rate period showed that internal mass transfer occurred by diffusion. Similar results have been obtained by different authors for drying of vegetables and fruits (DIAS *et al*., 2009; MADAMBA *et al*., 1996).

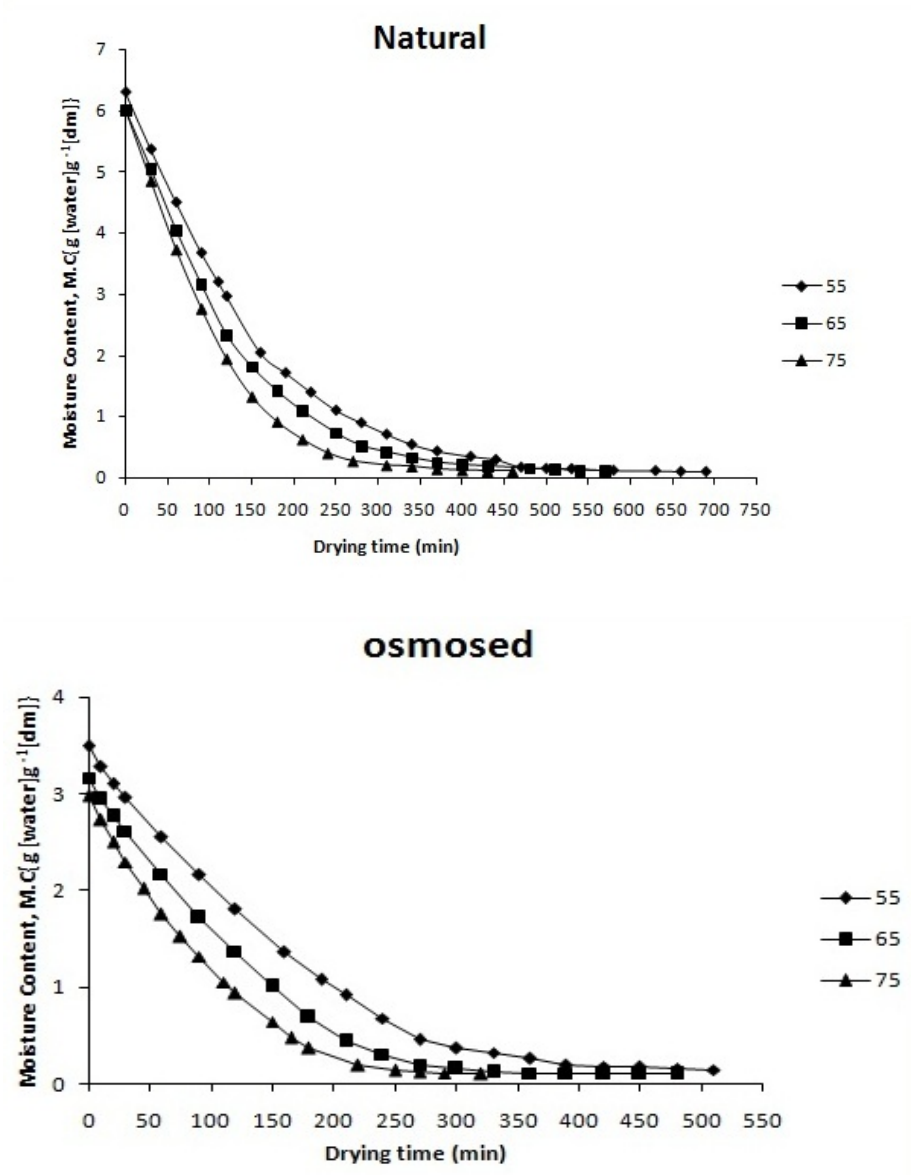

Figure 1: Effect of air temperature on drying behavior of osmosed and natural beetroot cubes at different drying air temperature.

Effect of drying temperatures on a variation of the drying rate with moisture content for osmosed and natural beetroot samples is shown in Fig. 2. Increasing the drying temperature results in an increase of the drying rate and a decrease of the total time of drying. Drying rate later decreased with decreasing moisture for natural and osmotically pre-treated samples under all the conditions of convective dehydration.

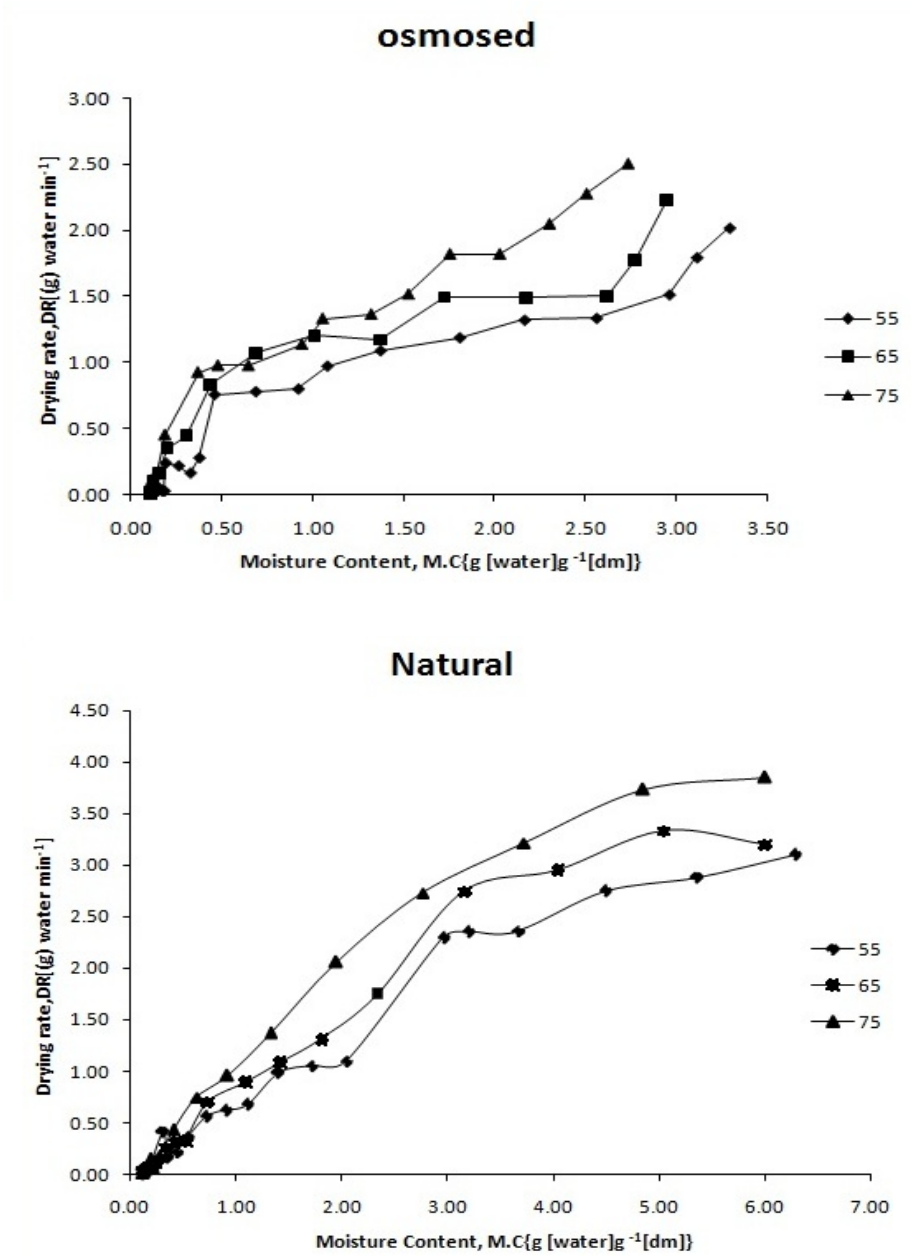

Figure 2: Effect of air temperature on drying behavior of Natural and osmosed beetroot cubes at different drying temperature.

The reason for the reduction of drying rate might be due to the reduction in porosity of the material and also due to shrinkage as the drying process advances. There was a decline in drying rate for both osmosed and natural beetroot. At the end, when moisture content of beetroot cubes neared 1 g [water] g^{-1} [dm], drying rate curves showed very low drying rate. Therefore, a considerably long drying period would be necessary to achieve final moisture content lower than 1 g [water] g^{-1} [dm]. As indicated by drying rate curves in Fig. 2, the migration of moisture to the surface and evaporation (drying) rate from the surface decreased with decreasing moisture in the product. However, closer examination of drying rate for experimental data for values below moisture content of 1 g [water] g^{-1} [dm]

for natural beetroot having higher drying rate compared to osmosed beetroot. This might be due to the resistance offered by solute gain during osmotic pre-treatment.

4.3. Validity of empirical models for convective dehydration

The statistical results with respect to terms of R^2, χ^2, RMSE and drying constants k for Newton, a and k for Henderson-Pabis and k, n for Page and a, b for Wang & Singh models are summarized in Tables 3 and 4, where T is the drying temperature.

Table 3: Statistical results obtained for different convective drying conditions for natural beetroot cubes.

T (°C)	Model	K	Drying coefficients	R^2	RMSE	χ2
55	Newton	0.002	------	0.969	0.06848	0.00498
	Henderson-Pabis	0.002	a=1.106	0.972	0.08645	0.00781
	Page	0.001	n=1.471	0.997	0.05524	0.00293
	Wang and singh	-----	a=-0.002 b=3.97E-07	0.987	0.16862	0.02972
65	Newton	0.003	-------	0.954	0.06888	0.00496
	Henderson-Pabis	0.003	a=1.113	0.973	0.05375	0.00303
	Page	0.001	n=1.467	0.997	0.04860	0.00243
	Wang and Singh	------	a=0.002 b=0.00001	0.986	0.04919	0.00253
75	Newton	0.004	--------	0.983	0.07998	0.00682
	Henderson-Pabis	0.004	a=1.111	0.958	0.06528	0.00453
	Page	0.002	n=1.587	0.997	0.04913	0.00298
	Wang and Singh	------	a=-0.003 b=0.000000115	0.985	0.11529	0.01418

R^2 values greater than 0.95 indicate a good fit, the appropriateness of the selected model is also confirmed by lower value of RMSE and χ^2 (DIAS *et al*., 2009). Among the models selected for convective drying, the Page model implies an excellent consistency in all the ranges of drying temperature (bold numbers in Tables 3 and 4) and this model may be assumed to represent the drying behavior of Beetroot cubes in a convective dryer within examined range.

4.4. Effective diffusivity for convective dehydration

During convective dehydration, the effective moisture diffusivity of natural samples and osmosed samples at drying air temperatures ranging from 55 to 75°C varied between 8.09917×10^{-9} to 1.45785×10^{-8} m^2s^{-1}, between 1.13388×10^{-8} to 1.61983×10^{-8} m^2s^{-1}, respectively. The results obtained are in good agreement with that reported in the literature (ZOGZAS *et al*., 1996).

Table 4: Statistical results obtained for different convective drying conditions for Osmosed beetroot cubes.

T (°C)	Model	K	Drying coefficients	R^2	RMSE	χ2
55	Newton	0.003	--------	0.957	0.07316	0.00563
	Henderson-Pabis	0.004	a=1.086	0.972	0.05969	0.00375
	Page	0.002	n=1.415	0.987	0.05543	0.00324
	Wang & Singh	-------	a=-0.002 b=0.0000013	0.979	0.13709	0.01978
65	Newton	0.004		0.961	0.06868	0.00498
	Henderson-Pabis	0.005	a=1.097	0.977	0.05568	0.00327
	Page	0.001	n=1.373	0.986	0.04717	0.00228
	Wang & Singh	-------	a=-0.003 b=0.0000029	0.976	0.09645	0.00982
75	Newton	0.006		0.966	0.06306	0.00428
	Henderson-Pabis	0.007	a=1.062	0.974	0.05904	0.00375
	Page	0.001	n=1.451	0.996	0.05869	0.00347
	Wang & Singh	-----	a=-0.004 b=0.0000047	0.992	0.09136	0.00899

4.5. Activation energy for convective dehydration

The activation energy of convective dried natural Beetroot cubes were 27.92 kJ mol^{-1} compared to 16.98 kJ mol^{-1} for osmosed samples. Activation energy was higher for natural beetroot cubes compared to osmosed beetroot cubes, it may be due to the presence of high initial moisture content of 85% (w.b.) for natural Beetroot samples compared to 74% (w.b.) for pre-osmosed samples. Therefore, more thermal energy would be required to remove greater amounts of water from natural beetroot cubes. Effect of convective drying temperature and time of activation energy were also reported for osmosed and natural samples (REPPA *et al.*, 1999). Similar reports were also found for pears where activation energy was 26.46-31.21 kJ mol^{-1} for pears without osmotic dehydration and 24.34-28.20 kJ mol^{-1} for pears with osmotic dehydration (PARK *et al.*, 2002).

4.6. Sensory analysis for osmo-convective dried beetroot candy

The average score of colour of osmo-convective dried beetroot candy at temperature 65°C was 8.73 compared to temperature at 55°C and 75°C which obtained the score of 8.50 and 8.26 (Table 5) respectively.

Table 5: Effect of convective drying Temperature on sensory quality of Beetroot candy.

Temperature (°C)	Colour	Flavour	Taste	Texture	OA
55	8.50 ± 0.10^{b}	8.30 ± 0.17^{a}	8.26 ± 0.20^{b}	8.3 ± 0.17^{a}	8.45 ± 0.15^{b}
65	8.73 ± 0.25^{a}	8.53 ± 0.40^{a}	8.4 ± 0.15^{a}	8.56 ± 0.42^{a}	8.59 ± 0.16^{a}
75	8.26 ± 0.15^{a}	8.23 ± 0.05^{a}	8.0 ± 0.10^{a}	8.0 ± 0.12^{a}	8.20 ± 0.05^{a}

Means in the same column with different letters as superscripts are significantly different ($p<0.05$).

In case of flavor of osmo-convective dried beetroot candy, the average value at temperature 65°C was higher compared to temperature at 55 and 75°C. Highest score values of 8.4 and 8.56 were awarded to osmo-convective dried beetroot candy at temperature 65°C for its taste and texture respectively. So the overall acceptability score of beetroot candy dried at temperature 65°C was highest over other two temperature treatments. The above results indicated that osmo-convective dried beetroot candy at temp 65°C was the most-preferred candy.

5. CONCLUSIONS

In the present study for preparing a shelf-stable product having final moisture content of 9 ±1% (w.b.), osmotic pre-treatment before convective dehydration of beetroot cubes results in a decrease of the total convective dehydration time. Osmotic pre-treatment also results in increases in drying rate and effective moisture diffusivity and decreasing activation energy during convective dehydration. Among the empirical models applied to the data, the Page model best describes the convective drying characteristics of natural and osmosed cubes. Among osmo-conevctively dried beetroot candies at different temperatures, most preferred candy was obtained at 65°C.

NOMENCLATURE

D_{eff}	effective moisture diffusivity (m^2s^{-1})	M_t	Moisture content at any time t on dry basis (g water/ g dry matter)
E_a	Activation energy	MR	Moisture ratio (Dimensionless)
R^2	Coefficient of determination	M_o	Initial moisture content
t	Time	M_e	Equilibrium moisture content
T	Temperature (^oC)	$\chi 2$	Reduced chi-square
R_G	Gas constant (8.3143KJ $mol^{-1}K^{-1}$)	RMSE	Root mean square error
D_o	pre-exponential factor of Arrhenius equation, m^2s^{-1}		

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Paper Received February 13, 2015 Accepted June 5, 2015

PAPER

COMPOSITION OF INTRAMUSCULAR PHOSPHOLIPID FATTY ACIDS OF INRA RABBIT AT DIFFERENT AGES

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ABSTRACT

The composition of intramuscular phospholipids fatty acids in *Longissimus dorsimuscle* (LD), left-hind leg muscle (LL) and abdominal muscle (AM) of Inra rabbit slaughtered between 35 to 90 days old were investigated. Significant decreasing of intramuscular phospholipids (% total intramuscular lipids) was observed in three muscles as age increased ($p < 0.05$). The highest phospholipids content was found in LL in both male and female rabbits during the growth period, and the phospholipids content in three muscles of the males were higher than that of the females. Abundant amount of unsaturated fatty acids (UFA), especially polyunsaturated fatty acids (PUFA) characterised the fatty acid composition of the intramuscular phospholipids (32.94-55.79%), and the percentage of PUFA in the muscles were all significantly decreased during the growth of male and female Inra rabbit ($p < 0.05$). In addition, a significant reduction of PUFA/SFA ratio and a significant increase of SFA + MUFA were observed ($p < 0.05$). Major fatty acids, such as Palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1n-9), linoleic acid (C18:2n-6) and arachidonic acid (C20:4) changed more obviously than other fatty acids. The analysis of partial least square regression (PLSR) showed that the composition of phospholipid fatty acids varied in age, muscle and gender, and the nutritional value of the phospholipid fatty acids decreased with age distinctly, the AM had better nutritional value of phospholipids.

Keywords: rabbit, fatty acids, intramuscular phospholipids, composition

1. INTRODUCTION

Functional foods are a tool that can be easily used in reducing public health costs. Compared to meats of other animal species, rabbit meat is characterized by high levels of polyunsaturated fatty acids (PUFA) and n-3 fatty acids, high levels of protein with essential amino acids, high digestibility value, lower cholesterol contents and significant source of vitamin B family (vitamins B_2, B_5, B_6, B_3, B_{12}) etc. (DALLE ZOTTE and SZENDRÖ, 2011). Moreover, rabbit meat consumption could become a good way of providing bioactive compounds to human consumers, since the rabbit meat fatty acids profile may be favorably modified by the inclusion of raw materials rich in unsaturated fatty acids (UFA) in the diet (DAL BOSCO *et al*., 2004; HERNÁNDEZ, 2008; KOUBA *et al*., 2008). Rabbit meat is considered as dietetically healthy, relatively rich in n-3 PUFAs and with a lower n-6 to n-3 ratio (7～12) than pork, veal or chicken meats (DALLE ZOTTE, 2002; HERNÁNDEZ and GONDRET, 2006). Unlike pork or beef meat, it contains two important metabolites from α-linolenic acid (ALA), docosahexaenoic acid (DHA, C22:6n-3) and eicosapentaenoic acid (EPA, C20:5n-3) in detectable levels (COMBES and DALLE ZOTTE, 2005; EIBEN *et al*., 2010). Inra rabbit is imported from France and have high breeding efficiency. In recent years, interests have focused not only on the amount of intramuscular phospholipids but also on the composition of fatty acids. The intramuscular fat (IMF), which characterises the amount of fat, is one of the major factors affecting the palatability of meat (HOCQUETTE *et al*., 2010). Muscle lipids are composed of polar lipids, mainly phospholipids (rich in PUFA) located in the cell membranes, and triacylglycerols (high levels of saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA)) along the muscle fibres (DE SMET *et al*., 2004). In the IMF, PUFA are restricted almost exclusively to the phospholipids fraction (WOOD *et al*., 2003). Thus, the amount of intramuscular phospholipids in the meat is an important factor (GRAY *et al*., 1996). Phospholipids consist of long-chain fatty acids attached to a phosphoryl group. Since the fatty acids chains can vary in length and degree of saturation, each phospholipid class possesses numerous molecular species with different chemical and biological properties (MARCO *et al*., 2004; WANG *et al*., 2009).

CAMBERO *et al*. (1991) first analysed the phospholipid content and classes in rabbits and they provide important information on phospholipid prevalence according to breed and feeding, specifying that phospholipid differs also according to age and gender. Generally, the analysis of phospholipids is based the determination of the phospholipid classes (phosphatidyleth-anolamine, phosphatidylinositol, phosphatidylserine, phosphatidylcholine, sphingomyelin, lysophosphatidylcholine etc.) with high performance liquid chromatography (ALASNIER and GANDEMER, 1998; PETERSON and CUMMINGS, 2005; BOSELLI *et al*., 2008). However, practically limited literature data are available on the determination of the fatty acids in intramuscular phospholipids by gas chromatography after purification of the polar lipid fraction, especially the intramuscular phospholipids from Inra rabbit. Hence, in order to provide a database for the characterization of nutritional quality of Inra rabbit meat, the composition of intramuscular phospholipids fatty acids and the effect of ages, genders and muscles on the composition and nutritional value of fatty acids were investigated by gas chromatography.

2. MATERIALS AND METHODS

2.1. Sampling of Inra rabbit meat

A total of 200 35 days old weaned Inra rabbits (20 males+20 females per age) were provided by College of Animal Science and Technology, Southwest University**.** The ingredients and proximate chemical composition of the diet were shown in the Table 1.

Table 1: The ingredients and proximate chemical composition of the diet.

Item	**Diets**
Ingredients	***Proportion(%)***
Corn	24.2
Wheat bran	19
Soybean meal	10.82
Alfalfa meal	36
Corn germ cake	4
Rapeseed	3
Powder	0.5
Dicalcium	0.8
Lysine	0.07
Methionine	0.11
Salt	0.5
Premix[a]	1
Nutrition	***Proportion (%)***
Dry matter	89.8%
Crude protein	16.0%
Fat	3.3%
Lysine	0.7
Methionine	0.6
Calcium	0.95
Phosphorus	0.59
Acid detergent fiber	33.2%
Neutral detergent fiber	21.4%
Digestible energy[b]	10.5 MJ/kg

[a] The premix contains (per kg of diet): Vitamin A, 10000 IU; Vitamin D_3, 1000 IU; Vitamin E, 30 mg; Vitamin K, 1 mg; Vitamin B_1, 1 mg; Vitamin B_2, 3.5 mg; Vitamin B_6, 2 mg; Vitamin B_{12}, 0.01 mg; niacin, 50 mg; folic acid, 0.3 mg; choline, 1000 mg; Zn, 30 mg; Cu, 5 mg; Mn, 15 mg; Fe, 30 mg; I, 1 mg.
[b] Digestible energy (kcal/kg DM) = TDN×4400 (NRC, 1985).

They were maintained in a closed building under natural environmental conditions in individual wire mesh cages, equipped with metal troughs and automatic nipple drinkers. The rabbits had free access to feed and water.
The rabbits were bred under similar production system and slaughtered at the age of 35, 45, 60, 75, and 90 d in a local commercial slaughterhouse. The facilities of the slaughterhouse met the requirements of the Institute of Animal Care and Use Committee (IACUC), which is funded by the United States National Institutes of Health. After 24 h post-mortem, the longissimus dorsi muscle (LD), left-hind leg muscle (LL), and abdominal muscle (AM) (ventral musculus) of the carcass were removed and immediately vacuum-packed and frozen at -20°C until analyzed.

2.2. Intramuscular lipid content and fatty acid composition analysis

Intramuscular lipids were extracted according to FOLCH *et al*. (1957). Total lipid content was measured by weighing after solvent evaporation. The content of IMF was expressed as percent of the muscle weight. Fractions of intramuscular phospholipids were prepared with silica cartridges (Sep-Pack, Waters, Milford, MA, USA) by the method of JUANEDA and ROCQUELIN (1985). Phospholipids were quantified by phosphorous determination (BARTLETT, 1959). The relative content of phospholpids was expressed as percent of the IMF weight, while the absolute content was expressed as percent of the muscle weight. The phospholipids were methylated with boron fluoride-methanol (Sigma Aldrich) according to MORRISON and SMITH (1964). The fatty acids methyl esters were analyzed by a QP-2010 gas chromatograph (Shimadzu, Kyoto, Japan) equipped with a flame ionization detector and a split injector. One microliter of FA methyl esters was injected in split mode (5:1) onto a Rtx-Wax capillary column (Restek, Bellefonte, PA, USA; 30 m × 0.25 mm id × 0.25 μm film thickness). The temperature of the column was programmed as follows: 1 min at 140°C, increments of 8°C/min to 180°C and held at 180°C for 2 min, increments of 3°C/min to 210°C then increments of 5°C /min to 230°C and held at 230°C for 10 min. The temperature of the injector and the detector both were 250°C. The flow rate of the carrier gas (N_2) was 1.5 mL/min. Identification of fatty acids was performed by comparison of the retention times with those of standards (Sigma). The results were expressed as percent of the total fatty acids methyl esters present.

2.3. Statistical analysis

The Statistical Analysis System (1996) was used to determine means, standard errors and analysis of variance. Duncan's multiple range test was used to compare differences among means. An alpha level of $p < 0.05$ was considered significant.
The effect of ages, muscles and genders on the composition of intramuscular phospholipid fatty acids were performed by ANOVA-partial least squares regression (A-PLSR). Ten 0/1 indicators variables (35, 45, 60, 75, 90 d, female, male, LD, LL, AM), SFA+MUFA, and PUFA/SFA in the X-matrix and 21 kinds of fatty acids (C12:0 - C22: 6n-3 were represented by the number 1-21) in the Y-matrix. Ellipses represent R^2=0.5 (50%) and 1.0 (100%). A PLSR was performed using the Unscrambler Software, version 9.7 (CAMO ASA, Trondheim, Norway). All data was centered and standardized before analysis.

3. RESULTS AND DISCUSSIONS

3.1. Variation of content of total intramuscular lipids and phospholipids of Inra rabbits

3.1.1. Variation of intramuscular lipid content

The intramuscular lipid content (% muscle weight) of LD, LL and AM from male and female Inra rabbits were significantly increased (p <0.05) with age (Table 2). The intramuscular lipid content of the three muscles of male and female rabbits was all increased. During the growth of Inra rabbits, the AM showed the highest content of intramuscular lipid, followed by LL and LD. HERNÀNDEZ and DALLE ZOTTE (2010) reported that the leanest cut of meat in the rabbit carcass was the loin and the hindleg was the quantitatively important cut because of its low lipid content compared to the other meats, which was consistent with our investigation (male: 0.77-1.21%, female: 0.79-1.33%).

Hence, the lipid content depended greatly on the age, gender and muscle. During the growth period from 35 d to 90 d, the deposition degree of total intramuscular lipid of the females in AM (2.88% to 5.42%) was significantly higher than that in the LL (1.19% to 1.78%) and LD (0.79% to 1.33%).

Table 2: Comparison of intramuscular lipid content of Inra rabbit at different ages[a].

	LD[a]		LL		AM	
	Male	**Female**	**Male**	**Female**	**Male**	**Female**
35 d[bc]	0.77±0.02d	0.79±0.08C	1.16±0.12d	1.19±0.15C	2.41±0.16d	2.88±0.12E
45 d	0.80±0.08cd	0.82±0.06C	1.20±0.06d	1.24±0.12C	2.67±0.09c	3.18±0.08D
60 d	0.96±0.11bc	1.01±0.10BC	1.30±0.03c	1.45±0.13B	2.74±0.01c	3.58±0.20C
75 d	1.15±0.13ab	1.25±0.24AB	1.49±0.12b	1.64±0.07AB	4.60±0.03b	4.83±0.05B
90 d	1.21±0.13a	1.33±0.21A	1.66±0.02a	1.78±0.06A	5.16±0.20a	5.42±0.17A

[a] LD, Longissimus dorsimuscle; LL, left-hind leg muscle; AM, abdominal muscle.
[b] Results were expressed as means ± SE, data were means of three replicates.
[c] Values in the same column with different letters were significantly different ($p<0.05$), male: a-d, female: A-E.

3.1.2. Variation of intramuscular phospholipids content

The percentage of phospholipids in the total intramuscular lipids of both male and female Inra rabbits was significantly decreased at the three muscles with age ($p<0.05$) (Table 3). A higher percentage of phospholipids characterized the total lipids (22.35-53.81%), however, the phospholipid contents in the meat of both New Zealand white and the commercial hybrid ranged from 9% to 19% total lipid (CAMBERO *et al*., 1991).

Table 3: Comparison of intramuscular phospholipids content (intramuscular lipid weight %) of Inra rabbit at different ages[a].

	LD[a]		LL		AM	
	Male	**Female**	**Male**	**Female**	**Male**	**Female**
35 d[bc]	43.47±1.33a	42.11±1.96A	53.81±2.82a	51.03±2.22A	42.21±2.36a	37.01±1.98A
45 d	35.02±1.88b	33.64±1.53B	38.98±1.48b	36.78±1.65B	34.94±2.88b	31.99±1.68B
60 d	32.28±1.02b	31.14±1.92B	36.35±1.44b	33.87±1.60B	28.93±1.22c	25.31±1.16C
75 d	27.93±1.58c	24.13±0.17C	30.21±2.14c	27.84±2.39C	27.87±1.91c	23.36±2.96C
90 d	26.84±1.72c	23.52±0.86C	28.89±2.18c	26.78±1.16C	25.40±1.32c	22.35±1.29C

[a] LD, Longissimus dorsimuscle; LL, left-hind leg muscle; AM, abdominal muscle.
[b] Results were expressed as means ± SE, data were means of three replicates.
[c] Values in the same column with different letters were significantly different ($p<0.05$), male: a-c, female: A-C.

The highest relative phospholipids content was found in the LL in both male and female Inra rabbits at different ages. In addition, the relative content of phospholipids in Inra rabbit abdomen showed significant gender differences, whereas that in legs and back phospholipids content appeared more pronounced gender differences after the age of 60 d. However, the absolute percentage of intramuscular phospholipids in the three muscles

did not vary obviously ($p > 0.05$) (Fig. 1). Among the three muscles, the AM showed the maximum absolute percentage of intramuscular phospholipids, followed by LL and LD, and the absolute percentage of intramuscular phospholipids in males were higher than that in females. According to wood *et al* (2008), phospholipids remained constant or increase little, as muscle fatness increases, whilst triacylglycerols increased to a higher extent, which may explain this phenomenon.

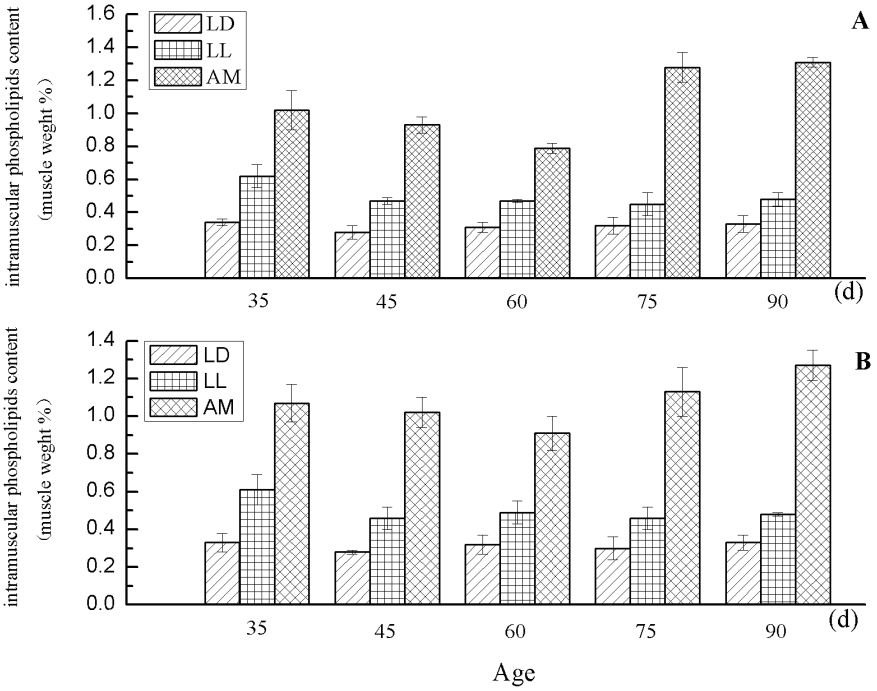

Figure 1: Comparison of intramuscular phospholipids content (muscle weight %) of Inra rabbit at different ages (A: male rabbits, B: female rabbits).

3.2. Effect of age, muscle and gender on composition of intramuscular phospholipids

The comparative intramuscular phospholipids fatty acids in LD, LL and AM of both male and female Inra rabbits at 35, 45, 60, 75, 90 d were shown in Table 4 (LD), Table 5 (LL), and Table 6 (AM), respectively. High levels of UFA (the sum of PUFA and MUFA), especially the abundance of PUFA, including the long chain (C20-22) PUFA in muscle, were observed in all samples. In muscle, significant percentage is phospholipids, which has a much higher PUFA content in order to perform its function as a constituent of cellular membranes (Wood *et al*., 2008). The ratio of SFA and MUFA in LD, LL and AM increased significantly with age in both gender rabbits ($p < 0.05$), whereas the ratio of PUFA among the muscles were all significantly decreased ($p < 0.05$). In addition, a significant reduction of PUFA/SFA ratio and a significant increase of SFA + MUFA were observed ($p < 0.05$).
During the growth, the phospholipids PUFA percentage (% intramuscular fatty acids) was significantly higher in the LL than that in the LD and AM of male rabbits, corresponding to the lowest MUFA percentage in male LL. The phospholipids PUFA percentage in female LD was the lowest, while the MUFA in female legs was the minimum. Compared to the LD and LL, the AM existed more obvious gender differences in SFA and UFA percentage of phospholipids.

Table 4: Composition of the fatty acids of intramuscular phospholipids (%) in Longissimus dorsi of Inra rabbit at different ages[a].

	35d		45d		60d		75d		90d	
	male	female	male	female	male	female	male	female	male	female
C12:0[b]	0.10±0.02a	0.03±0.01C	0.05±0.01b	0.08±0.02B	0.12±0.03a	0.17±0.02A	-[d]	-	-	-
C14:0	0.26±0.05e	0.28±0.05B	0.47±0.01d	0.52±0.15A	0.60±0.17c	0.57±0.07A	0.79±0.05b	0.58±0.05A	0.83±0.05a	0.53±0.04A
C14:1	0.36±0.02b	0.41±0.02A	0.26±0.02c	0.46±0.04A	0.11±0.02d	0.41±0.06A	0.34±0.03b	0.46±0.11A	0.50±0.02a	0.46±0.02A
C15:0	1.19±0.11d	1.46±0.22E	1.65±0.16c	1.57±0.10D	1.81±0.22b	2.47±0.31C	2.02±0.18a	3.20±0.32A	1.10±0.03d	2.79±0.13B
C16:0	20.03±0.03e	17.16±0.19E	21.64±0.38d	20.30±0.05D	22.37±0.11c	21.25±0.09C	23.71±0.14b	23.34±0.12B	25.11±0.30a	28.22±0.02A
C16:1n-7	0.24±0.03c	0.29±0.03D	0.31±0.02c	0.48±0.06A	0.34±0.03c	0.39±0.02C	0.52±0.02b	0.44±0.04B	1.75±0.24a	0.44±0.01B
C17:0	0.50±0.02a	0.48±0.02A	0.42±0.03b	0.47±0.04A	0.38±0.02b	0.39±0.02B	0.48±0.02a	0.40±0.01B	0.41±0.01b	0.50±0.02A
C17:1	0.56±0.04d	0.72±0.09E	0.82±0.05a	1.34±0.07A	0.92±0.07b	1.15±0.07B	0.66±0.05c	0.93±0.05D	0.41±0.01e	1.01±0.03C
C18:0	11.04±0.03e	12.58±0.40D	11.72±0.07d	13.75±0.10C	12.56±0.13c	13.82±0.03C	13.11±0.02b	14.31±0.24B	13.50±0.03a	14.45±0.04A
C18:1n-9	13.34±0.02e	13.54±0.15E	13.99±0.53d	13.69±0.15D	15.01±0.03c	14.43±0.03C	15.80±0.04b	14.61±0.06B	17.89±0.02a	17.75±0.07A
C18:1n-7	1.63±0.03a	1.64±0.21A	1.52±0.31a	0.88±0.12D	1.00±0.03b	1.06±0.01C	0.86±0.02b	1.15±0.02B	0.65±0.02c	0.65±0.02E
C18:2n-6	25.91±0.07a	25.00±0.23A	24.29±0.28b	20.24±0.13B	22.67±0.02c	19.14±0.10C	20.77±0.02d	17.65±0.11D	20.21±0.07e	15.09±0.08E
C18:3n-3	0.27±0.03d	0.35±0.03B	0.41±0.02b	0.58±0.02A	0.48±0.02c	0.58±0.03A	0.78±0.06a	0.56±0.02A	0.37±0.02b	0.38±0.12B
C20:0	0.05±0.01a	0.06±0.01B	0.08±0.03a	0.17±0.01A	0.11±0.03a	0.17±0.02A	0.09±0.02a	0.17±0.01A	0.13±0.02a	0.16±0.06A
C20:1n-9	0.18±0.01ab	0.17±0.02B	0.11±0.03b	0.11±0.03C	0.18±0.06ab	0.22±0.02A	0.37±0.03a	0.14±0.01BC	0.16±0.02ab	0.10±0.02C
C20:2n-6	0.68±0.03d	0.73±0.03E	0.71±0.03d	0.83±0.06D	1.19±0.02c	0.90±0.03C	1.65±0.03a	1.15±0.02B	1.74±0.03b	1.23±0.01A
C20:3n-6	0.63±0.02e	0.68±0.04C	0.91±0.02d	1.21±0.07A	1.26±0.03c	1.20±0.06A	1.54±0.02b	1.21±0.04A	1.88±0.03a	0.82±0.05B
C20:4n-6	13.80±0.08a	14.58±0.05A	11.78±0.08b	14.01±0.13B	9.72±0.14c	13.64±0.15C	9.47±0.04d	12.99±0.11D	8.81±0.05e	10.36±0.10E
C20:5n-3	5.01±0.04a	5.53±0.03A	4.84±0.02b	5.03±0.12B	4.60±0.04c	4.60±0.03C	3.91±0.03d	4.04±0.06D	2.77±0.10e	3.40±0.02E
C22:5n-3	2.58±0.02a	2.49±0.03A	2.47±0.01b	2.33±0.08B	2.34±0.02c	2.21±0.04C	1.84±0.01d	1.62±0.04D	1.12±0.03e	1.11±0.03E
C22:6n-3	1.65±0.02a	1.83±0.02A	1.56±0.02b	1.41±0.04B	1.40±0.03c	1.23±0.01C	1.29±0.02d	1.03±0.01D	0.69±0.02e	0.56±0.03E
SFA[c]	33.16±0.12e	32.06±0.06E	36.03±0.69d	36.86±0.78D	37.96±0.11c	38.83±0.34C	40.19±0.13b	41.99±0.26B	41.07±0.31a	46.65±0.07A
PUFA	50.54±0.17a	51.18±0.24A	46.96±0.34b	45.64±0.07B	43.68±0.17c	43.51±0.35C	41.26±0.07d	40.27±0.30D	37.56±0.09e	32.94±0.09E
MUFA	16.30±0.07e	16.76±0.28D	17.02±0.77d	17.50±0.85C	18.36±0.18c	17.66±0.06BC	18.55±0.19b	17.74±0.04B	21.37±0.22a	20.41±0.02A

[a] Results were expressed as means ± SE, data were means of three replicates.
[b] Values in the same column with different letters were significantly different ($p<0.05$), male: a-e, female: A-E.
[c] SFA, total saturated fatty acids; MUFA, total monounsaturated fatty acids; PUFA, total polyunsaturated fatty acids.
[d] "-" : undetected.

Table 5: Composition of the fatty acids of intramuscular phospholipids (%) in left-hind leg muscle of Inra rabbit at different ages[a].

	35d		45d		60d		75d		90d	
	male	female	male	female	male	female	male	female	male	female
C12:0[b]	0.05±0.01b	0.04±0.01B	0.04±0.01b	0.09±0.01A	0.27±0.02a	0.05±0.02B	-[d]	-	-	-
C14:0	0.16±0.02b	0.23±0.06D	0.22±0.07b	0.41±0.11C	0.34±0.09b	0.54±0.13BC	0.77±0.16a	0.95±0.11A	0.26±0.01b	0.68±0.02B
C14:1	0.35±0.03b	0.37±0.02B	0.22±0.02c	0.14±0.02C	0.14±0.01d	0.51±0.03A	0.23±0.04c	0.32±0.01B	0.52±0.01a	0.52±0.03A
C15:0	1.12±0.25d	1.55±0.09B	1.46±0.35c	1.60±0.09B	1.51±0.36bc	2.39±0.47A	1.53±0.03b	1.77±0.13B	1.66±0.01a	1.84±0.07B
C16:0	19.57±0.05e	16.98±0.13D	20.65±0.08d	20.48±0.07C	21.55±0.11c	21.95±0.06B	22.63±0.13b	22.01±0.22B	24.08±0.05a	24.66±0.20A
C16:1n-7	0.34±0.02c	0.37±0.02D	0.32±0.02c	0.39±0.02CD	0.36±0.02c	0.41±0.02C	0.51±0.18b	0.75±0.02A	1.94±0.03a	0.48±0.01B
C17:0	0.40±0.03c	0.43±0.01B	0.44±0.01b	0.36±0.01C	0.38±0.02c	0.50±0.02A	0.55±0.03a	0.40±0.03B	0.40±0.02c	0.49±0.01A
C17:1	0.51±0.09bc	0.67±0.02B	0.94±0.12a	2.08±0.29A	0.86±0.13a	0.89±0.11B	0.57±0.01b	0.54±0.05B	0.35±0.02c	0.78±0.02B
C18:0	11.79±0.06e	13.12±0.06E	13.15±0.04d	14.78±0.10D	14.61±0.03c	15.29±0.10C	15.97±0.04b	15.91±0.03B	16.12±0.02a	16.32±0.03A
C18:1n-9	11.78±0.05e	12.09±0.12E	12.48±0.02d	12.34±0.06D	13.24±0.16c	13.60±0.12C	14.83±0.02b	13.94±0.15B	15.10±0.02a	15.75±0.07A
C18:1n-7	1.48±0.06ab	1.39±0.03A	1.48±0.02ab	0.85±0.01C	1.41±0.10a	0.99±0.05B	1.53±0.02b	1.00±0.08B	0.61±0.01c	0.70±0.03D
C18:2n-6	29.86±0.11a	26.34±0.02A	26.58±0.07b	20.62±0.25B	24.59±0.01c	18.77±0.72C	21.37±0.05d	18.21±0.02D	20.84±0.02e	15.45±0.05E
C18:3n-3	0.37±0.02c	0.37±0.02CD	0.37±0.02c	0.72±0.06A	0.54±0.01ab	0.40±0.02C	0.83±0.37a	0.56±0.01B	0.50±0.03c	0.35±0.04D
C20:0	0.05±0.01b	0.07±0.01A	0.06±0.01b	0.17±0.03A	0.13±0.03a	0.17±0.18A	0.09±0.02b	0.12±0.03A	0.08±0.01b	0.13±0.01A
C20:1n-9	0.18±0.02b	0.18±0.02BC	0.17±0.02b	0.11±0.02D	0.27±0.02a	0.20±0.02B	0.28±0.03a	0.28±0.01A	0.27±0.01a	0.15±0.02C
C20:2n-6	0.83±0.02d	0.83±0.02E	0.87±0.02c	0.93±0.01D	1.11±0.02b	1.03±0.02C	1.15±0.01b	1.41±0.02B	1.29±0.02a	1.53±0.02A
C20:3n-6	0.83±0.02d	0.78±0.02E	0.93±0.02c	1.08±0.02D	1.08±0.04b	1.04±0.01C	1.18±0.02a	1.10±0.02B	0.95±0.02c	1.16±0.01A
C20:4n-6	12.33±0.10a	15.29±0.07A	11.95±0.18b	14.42±0.07B	10.29±0.02c	13.94±0.05C	9.95±0.05d	13.56±0.09C	9.84±0.02d	12.59±0.07D
C20:5n-3	3.77±0.06a	4.93±0.02A	3.58±0.03b	4.73±0.05B	3.34±0.03c	4.45±0.11C	3.12±0.01d	4.21±0.03D	3.09±0.02e	4.17±0.05D
C22:5n-3	2.40±0.04a	2.33±0.02A	2.34±0.02b	2.31±0.03B	2.33±0.02b	2.06±0.04C	1.87±0.02c	1.79±0.03D	1.34±0.04d	1.59±0.08E
C22:6n-3	1.82±0.15a	1.63±0.02A	1.75±0.03a	1.37±0.02B	1.66±0.03b	1.27±0.28C	1.05±0.01c	1.17±0.02D	0.83±0.02d	0.67±0.04E
SFA[c]	33.14±0.29e	32.44±0.06D	36.02±0.56d	37.90±0.01C	38.77±0.11c	40.89±0.33B	41.53±0.23b	41.16±0.04B	42.60±0.07a	44.13±0.11A
PUFA	52.22±0.45a	52.50±0.10A	48.37±0.26b	46.19±0.22B	41.95±0.07c	42.51±0.39C	40.51±0.35d	42.01±0.19D	38.67±0.03e	37.50±0.12E
MUFA	14.64±0.16e	15.07±0.14D	15.61±0.09d	15.91±0.21C	16.28±0.14c	16.61±0.07B	17.96±0.13b	16.83±0.19B	18.72±0.08a	18.37±0.08A

[a] Results were expressed as means ± SE, data were means of three replicates.
[b] Values in the same column with different letters were significantly different ($p<0.05$), male: a-e, female: A-E.
[c] SFA, total saturated fatty acids; MUFA, total monounsaturated fatty acids; PUFA, total polyunsaturated fatty acids.
[d] "-" : undetected.

Table 6: Composition of the fatty acids of intramuscular phospholipids (%) in abdominal muscle of Inra rabbit at different ages[a].

	35d		**45d**		**60d**		**75d**		**90d**	
	male	female	male	female	male	female	male	female	male	female
C12:0[b]	0.17±0.01a	0.07±0.02A	0.07±0.02b	0.04±0.01B	0.07±0.02b	0.06±0.01AB	-[d]	-	-	-
C14:0	0.53±0.16a	0.39±0.05D	0.38±0.07ab	0.65±0.19A	0.30±0.02b	0.41±0.06C	0.44±0.14ab	0.59±0.04B	0.27±0.02b	0.69±0.03A
C14:1	0.25±0.02b	0.15±0.02D	0.21±0.02b	0.18±0.01C	0.60±0.35a	0.27±0.01B	0.24±0.02b	0.28±0.02B	0.47±0.05ab	0.56±0.19A
C15:0	1.82±0.56a	1.36±0.02D	2.53±0.76a	1.68±0.39C	2.34±0.79a	1.80±0.57B	2.88±0.52a	1.83±0.51B	1.98±0.13a	1.94±0.04A
C16:0	21.22±0.07c	12.65±0.01D	22.17±0.08bc	12.90±0.26D	22.67±0.31b	14.06±0.13C	22.97±0.06b	17.23±0.06B	23.87±1.09a	21.37±0.11A
C16:1n-7	0.34±0.03d	0.34±0.02E	0.30±0.02d	0.48±0.02D	1.02±0.32b	0.65±0.02C	2.02±0.24a	1.05±0.02B	2.04±0.06a	1.56±0.12A
C17:0	0.43±0.02bc	0.44±0.02A	0.44±0.01b	0.39±0.02B	0.41±0.03c	0.33±0.01C	0.52±0.01a	0.38±0.03B	0.37±0.02d	0.47±0.01A
C17:1	0.82±0.23ab	0.61±0.11BC	0.93±0.28ab	1.27±0.14A	1.16±0.25a	0.74±0.19B	0.92±0.18ab	0.46±0.11C	0.65±0.03b	0.47±0.01C
C18:0	10.00±0.08e	13.24±0.03E	10.58±0.02d	15.50±0.06D	10.95±0.28c	17.04±0.11C	11.42±0.34b	17.39±0.05B	13.48±1.35a	17.67±0.40A
C18:1n-9	13.93±0.03b	13.22±0.02D	14.21±0.78b	13.65±0.50CD	14.66±0.24b	14.10±0.07C	16.42±0.28a	14.65±0.06B	16.89±0.85a	15.40±0.38A
C18:1n-7	1.20±0.04a	1.42±0.03A	1.12±0.27a	0.90±0.19C	0.65±0.03b	0.71±0.07D	0.50±0.04b	1.13±0.04B	0.99±0.38ab	1.34±0.07A
C18:2n-6	24.23±0.07a	25.26±0.10A	23.91±0.03b	22.09±0.09B	23.48±0.58b	20.80±0.19C	22.78±0.29bc	19.73±0.13D	22.56±0.99c	17.21±0.21E
C18:3n-3	0.35±0.01c	0.35±0.02E	0.31±0.02d	0.60±0.02B	0.56±0.03b	0.51±0.02C	0.85±0.02a	0.67±0.02A	0.54±0.01b	0.45±0.02D
C20:0	0.13±0.03a	0.10±0.02AB	0.07±0.01b	0.08±0.01B	0.11±0.01ab	0.11±0.02A	0.09±0.01b	0.12±0.01A	0.07±0.01b	0.13±0.01A
C20:1n-9	0.26±0.01b	0.22±0.02B	0.17±0.02b	0.23±0.02B	0.30±0.02ab	0.27±0.02A	0.22±0.02b	0.27±0.02A	0.37±0.12a	0.20±0.03C
C20:2n-6	0.96±0.02c	0.76±0.04E	1.11±0.03d	0.98±0.02D	1.14±0.03b	1.14±0.02B	1.23±0.07a	1.07±0.02C	1.08±0.05b	1.24±0.02A
C20:3n-6	0.95±0.01d	0.83±0.02E	1.00±0.03c	1.13±0.02D	1.28±0.03b	1.43±0.01C	1.38±0.07a	1.78±0.05B	0.99±0.05cb	1.96±0.02A
C20:4n-6	12.93±0.14a	17.88±0.08A	11.71±0.03b	17.40±0.15B	10.72±0.41c	16.08±0.12C	8.92±0.40d	13.64±0.10D	8.67±0.41d	11.22±0.17E
C20:5n-3	5.65±0.06a	5.84±0.07A	5.23±0.02b	5.48±0.07B	4.44±0.15c	5.16±0.02B	3.48±0.10d	4.59±0.04C	2.88±0.14e	3.59±0.09D
C22:5n-3	2.24±0.03a	2.96±0.03A	2.12±0.02b	2.73±0.02B	1.76±0.07d	2.72±0.02B	1.87±0.08c	1.91±0.07C	1.08±0.01e	1.56±0.03D
C22:6n-3	1.60±0.14a	1.92±0.03A	1.45±0.01b	1.61±0.06B	1.37±0.08c	1.59±0.03B	1.08±0.04d	1.24±0.01C	0.76±0.01e	1.08±0.02D
SFA[c]	34.30±0.30d	28.25±0.27E	36.22±0.98c	31.25±0.95D	36.84±0.73c	33.82±0.28C	38.31±0.35b	37.54±0.43B	40.03±0.50a	42.26±0.36A
PUFA	48.91±0.45a	55.79±0.37A	46.84±0.14b	52.03±0.22B	44.75±0.21c	49.43±0.34C	41.59±0.82d	44.63±0.12D	38.56±1.65e	38.31±0.53E
MUFA	16.79±0.16a	15.96±0.09B	16.94±0.77a	16.72±0.56C	18.41±0.48b	16.75±0.07D	20.10±0.49c	17.83±0.06B	21.41±1.28d	19.43±0.21A

[a] Results were expressed as means ± SE, data were means of three replicates.
[b] Values in the same column with different letters were significantly different ($p<0.05$), male: a-e, female: A-E.
[c] SFA, total saturated fatty acids; MUFA, total monounsaturated fatty acids; PUFA, total polyunsaturated fatty acids.
[d] "-" : undetected.

In terms of fatty acids composition of intramuscular phospholipids of Inra rabbit, SFA among the muscles were mainly composed of palmitic (C16:0) and stearic (C18:0), MUFA were mainly represented by oleic (C18:1), whereas PUFA consisted of linoleic (C18:2) and arachidonic acid (C20:4). According to CAMBERO *et al*. (1991), the C16:0, C18:0, C18:1 and C18:2 were together representing more than 70% of the total fatty acids. A higher percentage of PUFA characterized the fatty acids composition of phospholipids (KANATT *et al*., 2006). In our study, the percentage of PUFA in males and females were accounted for 37.56-52.22% and 32.94-55.79% respectively in the intramuscular phospholipids during growth period from 35 d to 90 d. Long chain n-3 and n-6 PUFA were mainly found in phospholipids (ENSER *et al*., 2000; COOPER *et al*., 2004), which was also in good agreement with our investigation. However, the fatty acids composition was rarely detected in different rabbit muscles during different feeding days, especially on a particular rabbit species. Comparing the variety of intramuscular phospholipids from LD, LL and AM during the growth stages from 35 d to 90 d, C16:0, C18:0, palmitoleic acid methyl ester (C16:1n-7), C18:1n-9, cis-11,14-eicosadienoic acid methyl ester (C20:2n-6) and cis-8,11,14-eicosatrienoic acid methyl ester (C20:3n-6) increased significantly ($p < 0.05$) in both genders, whereas C18:2n-6, C20:4n-6, C20:5n-3, C22:5n-3 and C22:6n-3 decreased significantly ($p < 0.05$). The percentage of C16:0 and C18:0 in female-LD significantly increased ($p < 0.05$), and both C18:2n-6 and C20:4n-6 in female-LL significantly decreased ($p < 0.05$). Moreover, the percentage of C20:4n-6 in female-AM decreased faster than other samples during the test days. However, other fatty acids did not showed apparent changes. According to ALASNIER and GANDEMER (1998), the fatty acid composition of individual phospholipid classes was related to metabolic type of fibre in the rabbit, and the differences in fatty acid composition of phosphatidyl ethanolamine, phosphatidyl choline and cardiolipin explained a large part of the differences in fatty acid compositions of the total phospholipids of glycolytic and oxidative muscles.

As the major ingredient of feeds for all species, the incorporation of C18:2n-6 into the muscles, in relation to the amount in the diet, was greatest among other fatty acids. C18:2n-6 was deposited in muscle phospholipids at a high level where it and its long chain products C20:4n-6 competed well for insertion into phospholipids molecules (WOOD *et al*., 2008). Comparing the changes of fatty acids in the LD, LL and AM, the deposition rate of C16:0 was faster in the LD of Inra rabbits (both males and females) than that in the LL and AM. However, the C16:0 had lowest percentage and slowest deposition rate in AM. In terms of C18:0, LL was sequentially higher than LD and AM in male rabbits, while AM was higher than LL and LD in female rabbits. Meanwhile, the percentage and deposition rate of C18:0 in AM were also higher in females than that in males. For the C18:1n-9 percentage, LD showed the highest and fastest deposition rate in both genders. The percentage of C18:2n-6 in LL was the highest, and decreased in the maximum levels with age. According to WOOD *et al* (2008), the higher percentage of C18:2n-6 in phospholipids compared with neutral lipids in all species mean that muscle from lean animals has relatively higher percentages of this major PUFA. In addition, during the growth period from 35 d to 90 d, the initial content of C20:4n-6 in LD was higher compared with other muscles, and showed the fastest reducing rate. No significantly variation was found among other fatty acids components.

During the growth period of Inra rabbit, the n-6/n-3 values for LD, LL, and AM ranged from 4.02 to 6.61, 4.71 to 5.72 and 3.97 to 6.33 in males, and 3.88 to 5.05, 4.05 to 4.67 and 3.95 to 4.73 in females, respectively. There is an increasing recognition of the health benefits of PUFA in general, and of n-3 PUFAs in particular, because these fatty acids are essential for humans (ALESSANDRI *et al*., 1998; CONQUER *et al*., 2011). Nutritional value is determined primarily by the ratio between SFA and PUFA in meat and the balance between fatty acids of the n-6 and n-3 series. Unfortunately, Western diet is very high in n-

6 fatty acids relative to n-3 fatty acids (ENSER *et al*., 2000; HARGIS and VAN ELSWYK, 1993). Nutritionist recommendations are for a ratio of n-6/n-3 PUFA of less than 5 (WOOD *et al*., 2003; KOUBA *et al*., 2003), and a ratio of n-6/n-3 below about 4.0 is required in the diet to combat various ''lifestyle diseases'' such as coronary heart disease and cancers (SIMOPOULOS, 2004; WILLIAMS, 2000). According to FAO/WHO, the recommended dose of essential PUFA in a healthy daily diet is 5/1 to 10/1 (n-6/n-3) (DALLE ZOTTE and SZENDRÖ, 2011), and a lower ratio is more desirable in reducing the risk of many of chronic diseases, even if the optimal ratio may vary depending on the disease under consideration (SIMOPOULOS, 2002). Therefore, it can be suggested that the intramuscular phospholipids of Inra rabbits is recommended. ALASNIER and GANDEMER (1998) reported that the phosphatidyl ethanolamine of oxidative muscles contains less 18:2n-6 and more 18:0 and long chain PUFA of the n-6 and n-3 series than that of glycolytic ones; phosphatidyl choline of oxidative muscles contains more 18:0 and less 16:0 and 18:2n-6 than that of glycolytic ones; cardiolipin of the oxidative muscles contains less 18:2 n-6 than those of the glycolytic ones. In addition, they suggested that a part of the composition difference could be related to high mitochondria content of the oxidative muscles compared to the glycolytic ones. To check this hypothesis, further investigations are required to determinate the fatty acid composition of individual phospholipid classes of both mitochondria and microsomes in rabbit muscles.

3.3. Analysis of PLSR on composition of intramuscular phospholipids

The analysis of PLSR showed that the first and second main ingredients explained 43% and 33% Y variables, respectively. From the nutritional point of view, the PUFA/SFA value is often used to evaluate the nutritional value of the meat, and higher value represents better nutritional value. However, the higher SFA + MUFA value of the meat are, the tenderness juiciness and the better flavor are (CAMERON and ENSER, 1991). On the contrary, if the content of PUFA is too high, the tenderness, flavor and juiciness of meat are poor. Hence, the SFA + MUFA value can be used to measure the quality indicators of samples after processing.

The SFA + MUFA value of intramuscular phospholipid fatty acids of Inra rabbit was located in the bottom right renderings, indicating the higher the nutritional value of the sample in the bottom right, and the PUFA/SFA values were located in the top left of the renderings (Fig. 2). Thus, the better flavor of the sample after processing is closer to the top left. On the first principal component, the composition of phospholipid fatty acids showed obviously different in ages, genders and muscles. The LD was closer to the SFA + MUFA, indicating the better phospholipid processed-flavor after processing of LD. However, the AM was closer to PUFA/SFA, indicating the better phospholipid nutritional value of AM. On the second principal component, the composition of phospholipid fatty acid of the raw material showed obviously different in ages and genders. The nutritional value of the total lipid decreased with age.

In addition, the 35 d-feedstock located in the top left oval, closely to 12 (C18: 2n-6), 18 (C20: 4n-6), 19 (C20: 5n-3), 20 (C22: 5n-3), 21 (C22: 6n-3) and other PUFAs, while the 90 d-feedstock located in the bottom right of the ellipse, closely to 6 (C16:1n-7,), 2 (C14:0), 3(C14:1n-6), 5 (C16:0), 10 (C18:1n-9) and some other saturated and monounsaturated fatty acids. Moreover, the intramuscular phospholipids of male rabbits was closely related to the 5 (C16:0) and 12 (C18:2n-6), that is the C16:0 and C18:2n-6 percentage in muscle was higher in male rabbits than that in the females. The intramuscular phospholipid of female rabbits was closely related to the percentage of 9 (C18:0), 14 (C20:0) and 18 (C20:4n-6), that is the C18:0, C20:0 and C20:4n-6 levels in muscle were higher in female rabbits than that in the males. Among the three sections, the AM had better phospholipid nutritional

value, which may be due to the higher percentage of PUFA, and the LD and LL showed better phospholipid flavor after processing, which may be due to the higher percentage of 13 (C18:3n-3) and 7 (C17:0), 15 (C20:1n-9), respectively.

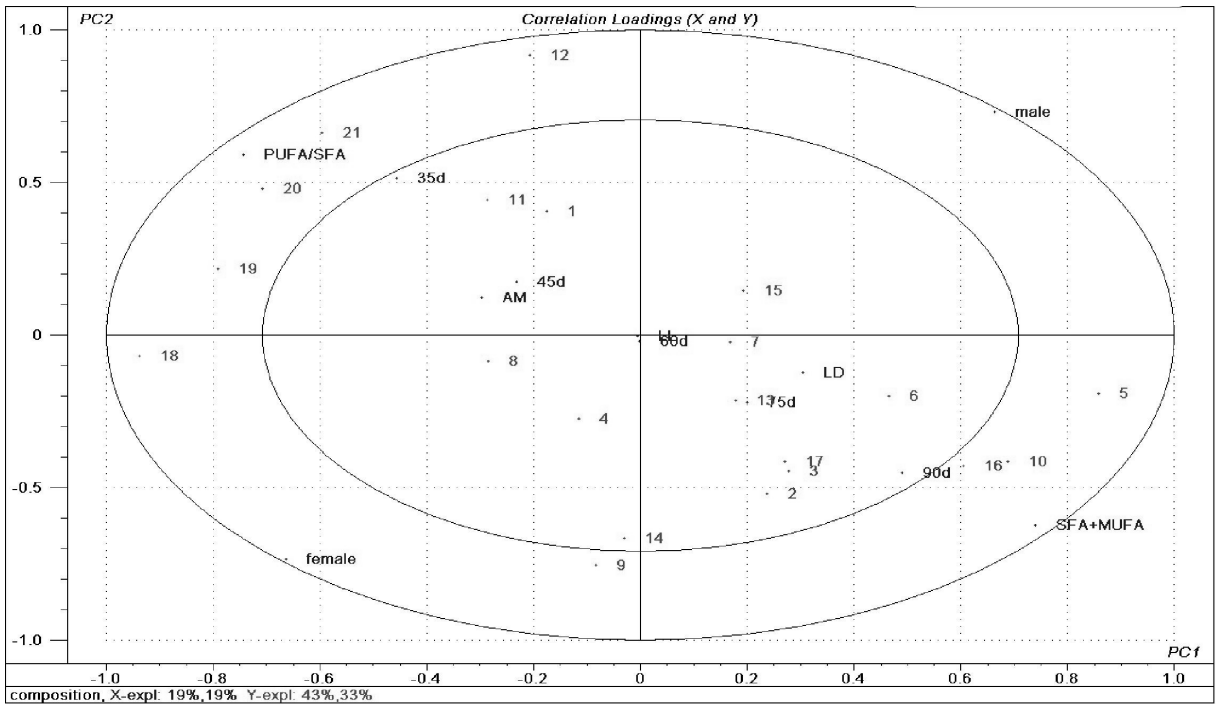

Figure 2: A PLSR correlation loadings plot for first 2 principal components (PCs). Ten 0/1 indicators variables (35 d, 45 d, 60 d, 75 d, 90 d, female, male, LD, LL, AM), SFA+MUFA, and PUFA/SFA in the X-matrix and 21 kinds of fatty acids (C12:0 - C22:6n-3 were represented by the number 1-21) in the Y-matrix. Ellipses represent R^2=0.5 (50%) and 1.0 (100%).

Overall, the composition of phospholipid fatty acids at different ages, genders and muscles showed significant difference. The effects of age, gender and muscle on the composition of phospholipid fatty acids mainly reflected on the first principal component. However, on the second principal component, only ages and genders showed the obvious difference of phospholipid fatty acids composition.

4. CONCLUSIONS

Inra rabbits are a meat source of nutritious quality, containing low content of intramuscular lipids, low ratio of n-6/n-3, whilst high content intramuscular phospholipids (% lipid). A higher content of PUFA characterised the fatty acids composition of the phospholipids, and the significantly decrease of PUFA in intramuscular phospholipids during growth were observed. Among the fatty acids from intramuscular phospholipids, SFA mainly consists of C16:0 and C18:0, MUFA consist of C18:1, and PUFA consist of C18:2 and C20:4. There is a wide variation of total lipids content and fatty acid composition at different ages, genders and muscles in Inra rabbit. By the analysis of PLSR, the nutritional value of the phospholipid fatty acids decreased with age, and the AM showed better nutritional value than LD and LL. The absolute data analysis is important for recommendations and suggestion of the consumption of dietary

phospholipids from animal sources. Further investigation is necessary to explore the properties of processing and nutritional characteristics of different sections from Inra rabbit meat.

ACKNOWLEDGEMENTS

The authors are grateful to College of Animal Science and Technology, Southwest University for the help in the meat sample supply. This study was funded by The Special Public Welfare Industry (Agriculture) Research Program of China (Grant No. 201303144), the National Rabbit Industry Technology System Program (Grant No. CARS-44D-1), the Doctoral Scientific Research Foundation of Minnan normal university (Grant No. 2006L21513), and the Program of education and scientific research for young and middle-aged teachers in Fujian Province (Grant No. JAT160296).

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Paper Received December 11, 2014 Accepted September 10, 2015

PAPER

THE EFFECT OF INFRARED RADIATION IN MODIFYING NUTRITIONAL AND MECHANICAL PROPERTIES OF GRASS PEA SEEDS

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ABSTRACT

The aim of this work was to evaluate the effect of exposure of grass pea seeds to IR (infrared) radiation applied at varying time intervals on their mechanical properties, trypsin inhibitor activity, and reactive lysine content. Grass pea seeds cv. Derek were exposed to IR radiation at 180°C for 30, 60, 90, 120, and 180 s, respectively.

Compared with that of raw seeds, IR heating of grass pea seeds reduced trypsin inhibitor activity. Reactive lysine proved to be relatively stable in the applied heating conditions. In addition, the process led to the reduction in the value of breaking load required for the destruction of a single seed, which may facilitate further processing, for example, flaking. Therefore, IR heating can be used in processing grass pea seeds.

Keywords: grass pea, infrared radiation, reactive lysine, breaking load

1. INTRODUCTION

Grass pea (*Lathyrus sativus* L.) is one of the oldest legumes cultivated by man. Currently, it is still an important nutritional component in Arabic countries, the Caucasus, and the Indian subcontinent. The advent of cultivation of this species in Poland is related to Tartar settlers who relocated to the Podlasie region in the 17th century. However, nowadays, it can be found only in limited regional cultures. The recent increasing interest in unconventional food products of plant origin also involves grass pea.

The nutritional value of grass pea seeds is primarily associated with their relatively high protein content (24-36%), accompanied by a very rich amino acid composition (MILCZAK *et al*., 2001). The significant amount of oleic, linoleic, and linolenic acids in fat, which is similar to the composition and utility of soybean oil, contributes to the high dietary value of the seeds (GRELA et. al., 2010). It should also be noted that they contain non-starchy polysaccharides (ca. 5%), which can stimulate Bifidobacteria in the digestive tract, enriching the organism with B-group vitamins, with a beneficial effect in absorbing calcium and reducing both the content of putrefactive bacteria and cholesterol absorption (TOMOMATSU, 1994). Grass pea seeds are suitable for human consumption and can be used to manufacture the feed and the production of the so-called "plant casein" (DZIAMBA, 1997).

However, the usability of grass pea seeds may be limited by bioactive components with anti-nutritional activity, such as protease inhibitors, tannins, or neurotoxins. The negative effect of anti-nutrients found in the seeds of leguminous plants, including grass pea, can be minimized through thermal processing (GRELA *et al*., 2001; SZMIGIELSKI and MATYKA, 2004). It seems highly advisable to assess the effect of infrared (IR) radiation on the seeds, including both modifications of the chemical composition and accompanying changes in the mechanical properties. Special attention should be paid to lysine because thermal processing may reduce its reactivity, thus limiting the availability of this essential amino acid (SAGAN and JAŚKIEWICZ, 2011). Lysine is involved in building proteins and producing hormones, enzymes, and antibodies. Lysine is needed for proper bone formation in children, as it helps in absorbing calcium (SARWAR *et al*., 2012).

The aim of the present work was to evaluate the effect of exposure of grass pea seeds to IR radiation, at varying time intervals, on the mechanical properties and selected nutritional components of grass pea seeds.

2. MATERIALS AND METHODS

The research material was seeds of grass pea (*Lathyrus sativus* L.) cv. Derek. The material harvested in 2011 was provided by "Spójnia" Hodowla i Nasiennictwo Ogrodnicze (Breeding and Horticultural Company) in Nochowo, Poland. The seed moisture content was 11±0.2%. Table 1 presents the geometric data and the basic physical properties of grass pea seeds.

Table 1: Basic physical properties of grass pea seeds (cv. Derek) (± standard deviation).

Mass of 1000 seeds (g)	Mean size of seeds (mm)	Angle of tipping over plate (°)	Bulk density ($kg \cdot m^{-3}$)	Bulk density tapped ($kg \cdot m^{-3}$)
116.3±2.49	5.90±0.03	18.3±1.15	810.66±23.09	896.13±10.99

Six 500-g samples of grass pea seeds were prepared. Five of them were exposed to IR radiation at 180°C (the temperature adopted for the process was the temperature of the seed surface exposed to heating) for 30, 60, 90, 120, and 180 s, respectively. Processing was carried out using a laboratory apparatus, which was designed and constructed especially for this purpose, with a wavelength emitted $\lambda = 2.5 - 3.0$ μm (ANDREJKO *et al*., 2011).

By applying uniaxial compression of individual seeds between two parallel plates, mechanical properties were evaluated using a dynamometer (Instron 4302, Instron Ltd., High Wycombe, UK). Individual seeds of grass pea were placed with their seed leaves parallel to the surface of the fixed plate and then they were compressed with the help of the upper plate. The speed of the compression plate shift applied during the test had a constant value of 10 mm/min. Force required for destructing the structure of a single seed was determined. The values of maximal forces were read out using specialist computer software (Instron 12) provided by the Instron Company. The result was reported as the arithmetic mean of 50 replications.

Protein, fat, ash, and raw fiber of the grass pea seeds were determined following AOAC - 984.13, 920.39, 942.05, 962.09, respectively (AOAC, 1990). Amino acids were determined by ion-exchange chromatography, following PN-EN ISO 13903 (2006). Prior to the analysis, the sample was hydrolyzed with 6 M HCl at 105°C for 24 h. Next, automatic amino acid analyzer AAA 400 was used to determine amino acids (Ingos, Praha, Czech Republic). Sulfur-containing amino acids were determined after oxidation to methionine sulfone and cysteic acid with performic acid (prepared by mixing 30% hydrogen peroxide and 99.9% formic acid in a ratio of 1:9) at 0°C for 16 h. Tryptophan was determined after alkaline hydrolysis with a saturated barium hydroxide solution at 105°C for 20 h. According to the procedure of OSER (1951), essential amino acid index (EAA index) was calculated taking into account the ratio of EAA in the test protein relative to their respective amounts in the whole egg protein. The fatty acid composition was determined in conformity with PN-EN ISO 5508 (1996) and PN-EN ISO 5509 (2001). The extracted fat was saponified with a 0.5 M methanolic sodium hydroxide solution at 85-95°C for 10 min. Fatty acid salts were esterified with anhydrous methanol using BF_3 as a catalyst at 100°C for 10 min. The methyl esters of the fatty acids were analyzed using a gas chromatograph with a flame ionization detector (Varian 450-GC, Middelburg, The Netherlands) on 30 m long column Select Biodiesel for FAME (Agilent Technologies, Santa Clara, USA). Based on the study by KAKADE *et al*. (1974), trypsin inhibitor activity (TIA) was determined. The analytical evaluation of TIA is based on the estimation of the part of trypsin activity that is blocked by a buffered extract containing trypsin inhibitors obtained from a seed sample and takes place under conditions ensuring the maximum activity of this enzyme. N-*α*-Benzoyl-DL-arginine-*p*-nitroanilide (BAPA), a synthetic substrate, was used. The reaction between trypsin and BAPA yields yellow *p*-nitroaniline whose concentration is registered at 410 nm. The calculation of the result is based on a diagnostic reaction between the standard BAPA solution and the standard trypsin solution. The absorbance of this solution corresponds to 40 conventional trypsin units (TU). Extracts obtained from the tested samples containing a trypsin inhibitor characterized by TIA reduce the activity of this enzyme, which is reflected in a proportional decrease in the quantity of produced *p*-nitroaniline and reduced absorbance of the analyzed solution converted into trypsin units inhibited (TUI) per 1 mg dry seed mass ($TUI \cdot mg_{DM}^{-1}$). Reactive lysine was determined by using the HPLC technique according to RAMÍREZ-JIMÉNEZ *et al*. (2004). The method consists in creating a colored complex of ε-DNP-lysine in the reaction with dinitrofluorobenzene (DNFB), and thereafter, hydrolyzing the sample and determining the content of ε-DNP-lysine using a liquid chromatograph with UV-VIS Spectroflow 773 detector (Kratos Analytical, Manchester, UK). Reducing sugars were extracted from the sample with 40% ethanol for 1 h and determined with the Luff-Schoorl method (PN-R-

64784, 1994). The method is based on the reduction of copper salt from the Luff solution (copper citrate with sodium carbonate) by reducing sugars contained in the extract. The reaction was carried out at a boiling point for 10 min. After cooling, the excess of copper ions was reduced with hydrogen iodide generated after the addition of 30% KI solution and 6 M H_2SO_4. Liberated iodine was titrated with a 0.1 M sodium thiosulfate solution using starch as an indicator near the end point. All chemical analyses were performed in three replications.

The results of the determination of the TIA of reactive lysine and reducing sugars in the grass pea seeds were subjected to the analysis of variance (ANOVA). The significance of differences between the mean values was verified using Tukey's test, with $P<0.05$. Calculations were done using Statistica 8.0 software.

3. RESULTS AND DISCUSSIONS

The value of the physical parameters determined for the grass pea seeds (Table 1) were similar to those presented by other authors (ALTUNAS and KARADAG, 2006). The mass of 1000 seeds (116.3±2.49 g) was similar to the average quoted by DZIAMBA (1997).

Seed resistance to compression plays an important role in many technological processes. Thus, an attempt has been made to determine the changes caused by the thermal activity of IR radiation in the mechanical resistance of the grass pea seeds. The data presented in Fig. 1 show that the thermal effect of IR radiation on the grass pea seeds led to a decrease in breaking load, in accordance with the exposure time. The highest values of breaking load were registered for seeds that had not been thermally processed (1.019 kN), while heating the seeds for even a short period of 30 s with IR radiation reduced the value of breaking the load to 0.885 kN. Furthermore, increasing the time of the seed exposure to IR radiation led to a reduction in the measured values. The lowest value, i.e., 0.463 kN, was obtained from the seeds subjected to heating for 180 s. The dependence presented in Fig. 1 has a nearly linear character; hence, it was described by means of the first-degree regression equation. The good correspondence of the experimental data and the equation is confirmed by the high value (close to 1) of the determination coefficient R^2.

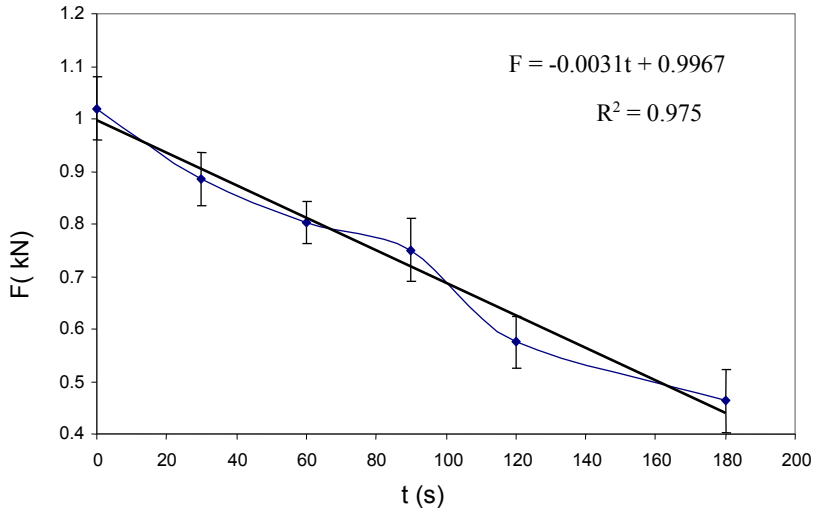

Figure 1: Effect of heating time (t) with infrared radiation on the value of breaking load (F) of the grass pea seeds.

The dependencies obtained are not characteristic of one type of material only, namely, grass pea seeds. On the basis of the results recorded previously (ANDREJKO *et al*., 2011), it was concluded that the values of the breaking load causing destruction of wheat cv. Zawisza were lower following IR radiation. FASINA *et al*. (2001) recorded the similar observations as follows: after the thermal processing of beans, peas, and lentils, reduced forces destroying the seeds were noted and were found to be different for each individual material, despite the same conditions of processing.
The content of the chemical components in the grass pea seeds (Table 2) was similar to the values quoted in the available sources (GRELA *et al*., 2010; SMULIKOWSKA *et al*., 2008; TAMBURINO *et al*., 2012). The analyzed seeds contained 24.35% of protein with a high nutritional value, resulting from their amino acid composition (Table 3).

Table 2: Chemical composition of grass pea seeds (± standard deviation).

Basic components (%)	
Protein	24.35±0.30
Fat	0.88±0.18
Ash	2.62±0.08
Raw fiber	5.16±0.42
Fatty acids (% of FA total)	
C 16:0	9.09±0.31
C 18:0	4.41±0.22
C 18:1	12.87±0.42
C 18:2	57.30±1.02
C 18:3	14.53±0.21
SFA	14.87
MUFA	13.09
PUFA	72.09
n-3	14.42
n-6	57.41
PUFA *n*-6/n-3	3.98

Table 3: Content of amino acids in grass pea seeds (± standard deviation).

Amino acids ($g \cdot kg^{-1}$)	
Phenylalanine	10.7±0.35
Tryptophan	4.2±0.05
Methionine	2.0±0.03
Threonine	9.3±0.10
Leucine	16.3±0.11
Isoleucine	10.1±0.10
Valine	11.2±0.18
Lysine	15.7±0.20
EAAI[1]	72.0

[1]Essential Amino AcidIndex.

Among the essential amino acids, lysine and leucine were the most abundant, which is consistent with the results obtained for an Italian variety (TAMBURINO *et al*., 2012). The content of the essential amino acids in the grass pea seeds was similar to soybean, except for methionine, which was definitely less abundant in grass pea than in soybean. The index of essential amino acids, EAAI, amounting to 72.0, remained within the range of the values obtained for Polish varieties of bean seeds (SAGAN and JAŚKIEWICZ, 2011).
The fatty acid profile in the analyzed seeds was similar to that quoted by other authors (GRELA *et al*., 2010). The high share of PUFA in the sum of fatty acids is notable, as well as the high ratio of *n*-6:*n*-3. The high level of polyunsaturated fatty acids is extremely important in preventing cardiovascular disease.
The content of trypsin inhibitors was at a level of 37.09±1.41 TUI/mg of dry mass of raw seeds. GRELA *et al*. (2001) and SZMIGIELSKI and MATYKA (2004) noted a lower activity of trypsin inhibitors in raw grass pea seeds (19.64 and 23.13 TUI/mg of dry mass). Heating grass pea seeds with IR radiation led to a reduced TIA, which becomes more significant with the longer time of processing. Statistically significant changes were already observed after 60 s exposure of the seeds to IR radiation (Table 4). After 60 s of radiation, the TIA was 90% of its value in the raw samples and was only 41% after 180 s. This confirms some previous analyzes, which revealed that thermal processing reduces the content of anti-nutrients in the seeds of grass pea. The reduction in the activity of trypsin inhibitors is extremely important because of their anti-nutritional effects. These inhibitors block trypsin, which leads to the inhibition of some processes, thereby restricting the use of the protein and reducing proteolysis in the gastrointestinal tract; in turn, this may result in the inhibition of the growth of the organism (SARWAR *et al*., 2012).

Table 4: Trypsin inhibitor activity, content of reactive lysine, and reducing sugars in grass pea seeds (± standard deviation).

Time of processing (s)	Trypsin inhibitors activity ($TUI \cdot mg_{DM}^{-1}$)	Reactive lysine ($g \cdot kg_{DM}^{-1}$)	Reducing sugars ($g \cdot kg_{DM}^{-1}$)
0	37.09^{a}±1.41	15.21^{a}±0.53	7.00^{a}±0.54
30	35.93^{a}±0.89	15.18^{a}±0.25	6.89^{a}±0.42
60	33.49^{b}±1.39	15.20^{a}±0.04	6.72^{ab}±0.39
90	27.57^{c}±0.96	14.84^{ab}±0.76	5.95^{ab}±0.25
120	22.80^{d}±0.68	14.86^{ab}±0.42	5.71^{b}±0.14
180	15.34^{e}±0.74	13.95^{b}±0.14	5.65^{b}±0.32

[a,b,c]Statistically significant differences in columns ($P<0.05$).

In order to determine the influence of the processing procedures on the nutritional value of the food, the content of reactive lysine may be used as an indicator. Exposing grass pea seeds to high temperature may pose a risk of lowering the nutritional value of protein, for example, by reducing the content of reactive lysine, which at higher temperatures reacts with other native compounds. This amino acid becomes inaccessible for the digestive processes after blocking the free ε-amino group. In this process, the susceptibility of other leguminous seeds occurring during the thermal processing was also observed by SAGAN and JAŚKIEWICZ (2011), and ŽILIĆ *et al*. (2006), among others. Reactive lysine in the analyzed seeds was relatively resistant to the IR radiation. Statistically significant losses of this compound were noted only as a result of the 180 s exposure to the IR radiation (Table 4). The retention of reactive lysine in the sample heated in this manner amounted to 92%,

and it was higher in comparison with soybean seeds of Serbian varieties subjected to heating with IR radiation at a temperature of 150°C and flaking for 2-3 min (retention after the process: 79% and 55%) (ŽILIĆ *et al*., 2006). The results may confirm earlier studies on the influence of thermal processes on the content of reactive lysine in bean seeds (SAGAN and JAŚKIEWICZ, 2011), which suggest that prolonged heating may lead to a reduction in the content of this amino acid.
The content of reducing sugars exceeded the sum of glucose and fructose determined by PIOTROWICZ-CIEŚLAK *et al*. (2008) in the seeds of Derek and Krab lines and was lower than that in Ethiopian varieties (URGA *et al*., 1995). A significant reduction was noted in the content of reducing sugars after 120 and 180 s of heating (Table 4). After this period, the content of reducing sugars in the grass pea seeds was 81% of the initial amount. This may result from the reaction of these compounds with other components occurring at higher temperature, for example, with amino acids (Maillard's reactions), including reactive lysine (NARANJO *et al*., 1998).

4. CONCLUSIONS

IR heating of grass pea seeds resulted in a decreased TIA, compared with that of raw seeds. Reactive lysine proved to be relatively stable in the applied heating conditions. In addition, the process reduced the value of breaking load required for destructing a single seed. This may facilitate further processing, for example, flaking. Therefore, IR heating can be applied in processing of grass pea seeds.

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Paper Received February 10, 2016 Accepted May 10, 2016

PAPER

TALC EFFECT ON THE VOLATILES OF VIRGIN OLIVE OIL DURING STORAGE

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ABSTRACT

The aim of the study was to assess the influence of talc on the extra virgin olive oil volatile profile during production and storage. The obtained results showed that talc used at 1% level did not cause significant differences, whereas at 2% level showed only slight differences were revealed compared to control. During storage a significant increase of the volatiles deriving from the oxidative process was observed and a concomitant significant decrease of the C6-LOX aldehydes. The evolution of the volatile compounds of extra virgin olive oils during storage was not significantly influenced by talc addition, with the exception of the sum of C6-LOX aldehydes, which showed a significant higher decrease.

Keywords: extra virgin olive oil, storage, talc, volatile compounds

1. INTRODUCTION

Volatile compounds, together with phenolics, are responsible for the sensory attributes of extra virgin olive oil (MORALES *et al.*, 1995; APARICIO *et al.*, 1996) and play a key role in the marketing process, by influencing the choice of the consumer. Volatiles arise during olive (*Olea europaea* L.) ripening (ANGEROSA and BASTI, 2001; APARICIO and MORALES, 1998) and are influenced by oil extraction processes (ANGEROSA *et al.*, 2000; KIRITSAKIS, 1998). Moreover, they may be altered during storage, especially if the main factors promoting product oxidation - such as heat, light, and air - are not well-managed (FRANKEL, 1985; MORALES and TSIMIDOU, 2000; BRKIĆ BUBOLA *et al.*, 2014).

It is well known that in fresh extra virgin olive oils the volatile compounds derive from the degradation of polyunsaturated fatty acids, through the lipoxygenase (LOX) pathway (ANGEROSA *et al.*, 2000; KALUA *et al.*, 2007), which produce C5 and C6 volatiles, mainly responsible for the "green" odor notes (ANGEROSA *et al.*, 2004). Among these volatiles, C6 unsaturated and saturated aldehydes, whose amount depends on the activity of the enzymes involved in LOX pathway (ANGEROSA *et al.*, 2001), are the most abundant compounds in high quality virgin olive oils (ANGEROSA *et al.*, 2004).

In recent years the use of coadjuvants in the oil mills has increased considerably, bringing increments of extraction efficiency during oil production and decreasing pomace moisture. It is common knowledge that the extraction systems can only recover about 80-90% of the oil contained in the starting olives, with the remainder being trapped in microgels or emulsified with the water phase (AGUILERA *et al.*, 2010). The extraction yield is further lowered in case of the so-called "difficult pastes" (CAPONIO *et al.*, 2014a), derived by batches of olives having a heterogeneous ripening index.

To increase extraction effectiveness, malaxation is the key phase of virgin olive oil production process and, generally, increasing the temperature determines an increase in oil extraction yield, due to a reduction of viscosity in the oily phase; however, the collateral effects of increasing temperature and time at this step are well known (ANGEROSA *et al.*, 2001). More recently, new technologies were also proposed for oil industries, among which ultrasounds, microwaves, and mechanical vibrations (BEJAOUI *et al.*, 2016; TAMBORRINO *et al.*, 2014; TOSCHI *et al.*, 2013).

On the other hand, innovation in the field of olive oil extraction lead to the spread of a new generation of two-way decanters, which do not require the addition of water to the olive paste and result in obtaining high quality oils, without losing the minor compounds responsible for sensory, nutritional, and healthy features of olive oil. However, these decanters generate pomaces with a moisture content of higher than 55% (CAPONIO *et al.*, 2014a; TAMBORRINO *et al.*, 2015), which is an excessively high value for pomace refineries, necessitating to expensive preliminary drying.

Talc is a commonly used coadjuvant, not excluded by the EC Regulation No. 1513/2001 in the production of extra virgin olive oil, due to its exclusively physical action (CERT *et al.*, 1996). It is added during malaxation and has a positive effect on oil yield, especially in the case of difficult olive pastes (CAPONIO *et al.*, 2016). All investigations highlighted an effect of talc on the chemical parameters of extra virgin olive oil. Nevertheless, few studies are available regarding the influence of talc on the volatile compounds of extra virgin olive oil. In particular, CAPONIO *et al.* (2015) highlighted that talc addition did not significantly influence the sums of aldehydes, alcohols, and ketones; on the contrary, the sums of acids and esters significantly increased with talc addition. KOPRIVNJAK *et al.* (2016) did not find significant changes of C6 aldehydes and C5 compounds, whereas with 3% of talc a significantly higher value of C6 alcohols was observed. The authors concluded that correlations between the degree of talc addition and levels of volatile compounds are not clearly evident. Moreover, no information is available about the evolution of the volatile

compounds of extra virgin olive oil obtained with talc addition to olive paste malaxation during storage.
In this framework, the aims of the present work were: i) to further investigate the influence of talc on the volatile compounds of extra virgin olive oils; ii) to evaluate whether the addition of talc to olive paste during malaxation influenced the evolution of the volatile fraction of extra virgin olive oils during storage. Olive fruits of Coratina, a cultivar that often leads to difficult olive pastes, were processed with and without talc addition at two different levels, and the corresponding oils were analyzed over a period of 6 months.

2. MATERIALS AND METHODS

2.1. Material

Olive fruits (*Olea europaea* L.) from Coratina cultivar, mechanically harvested in December 2013 were transported, immediately after harvesting, to a local plant (Andria, Bari, Italy) where, after leaf-removal, were milled within 24 h. Talc (hydrated magnesium silicate) was kindly furnished by Imerys Talc (Luzenac, France).

2.2. Oil extraction process

Three lots of about 2,000 kg of olives were considered in the trials. Each lot was divided into three homogeneous batches: one batch was processed without micronized natural talc addition (Co, control) and the remaining two batches were processed by adding 1% and 2% of talc (T1 and T2), respectively. Talc was added at the beginning of the malaxation phase.
For each batch, the olive paste after crushing was transferred into the malaxer, where it was mixed with talc only for the trials that provided its addition. After malaxation (50 min at 22±1°C) the paste was pumped into a two-phase decanter, operating at 2,800 rpm, with a processing capacity of 3,000 kg h^{-1}. Finally, the oily phase was separated from any aqueous residue by centrifugation at 6,400 rpm.
Then, the obtained oil was poured into 1-L clear glass bottles and hermetically sealed with headspace of about 3 mL. Three bottles for each batch were sampled. One bottle for each batch was immediately analyzed (Time 0), whereas the others were placed in a carton box and stored in the dark to be analyzed after three (Time 3) and six (Time 6) months of storage. The samples were stored at room temperature.

2.3. Volatile compounds determination

For the determination of the volatile compounds, the oil samples (0.5 ± 0.005 g) were weighed into 20 mL vials, sealed with a screw aluminium cap and silicone/PTFE septa, and submitted to the SPME/GC-MS in the conditions reported in a previous paper (CAPONIO *et al.*, 2014b). In particular, the extraction was performed by exposing a 75 μm Carboxen/polydimethylsiloxane (CAR/PDMS) fiber (Supelco, Bellefonte, PA, USA) in the headspace of the sample at 40°C for 20 min. When the extraction process was completed, the fiber was inserted into the injector port (set at 230°C) of the gas chromatograph for thermal desorption of volatiles. The GC/MS instrumentation included an Agilent model 6850 gas chromatograph coupled to a mass spectrometer Agilent 5975. The volatile compounds were separated on a HP-Innowax (60 m × 0.25 mm, 0.25 μm film thickness) polar capillary column (Agilent) under the following conditions: flow 1.5 mL min^{-1}; injector

temperature, 250°C; pressure of the carrier (helium), 30 kPa. The oven temperature was held for 5 min at 35°C, then increased by 5°C min^{-1} to 50°C and held constant for 5 min, then raised to 230°C at 5.5°C min^{-1} and finally held at 230°C for 5 min. The mass spectrometer was operated in the electron impact mode (electron energy = 70 eV), and the ion source temperature was 250°C. A continuous scan mode was employed with a scan time of 7.7 scans/s over a mass range of 33-200 amu. The volatile compounds were identified both by comparison of their mass spectra and retention times with those of authentic reference compounds and by their LRI and by comparison with the mass spectra present in the NIST and Wiley libraries. The volatile compounds were expressed as integrated area and its relative percentage.

2.4. Statistical analysis

Analysis of variance (one-way and two-way ANOVA) was carried out on the experimental data using the Minitab software (Minitab Inc., State College, USA). Two-way ANOVA was performed considering the amount of talc added (*talc*) and oil storage time (*time*), as well as the first order interaction (*talc*time*), as independent variables; Tukey's HSD test was applied for multiple comparisons.

3. RESULTS AND DISCUSSION

All the samples showed values of free fatty acids, peroxides, K_{232}, K_{270}, and DK which fulfilled the extra virgin olive oil marketing requirements according to current rules (OFFICIAL JOURNAL OF THE EUROPEAN COMMUNITIES, 2011) (data not shown).
Table 1 reports the volatile compounds detected in the oil samples, obtained with and without the addition of talc during processing, immediately after their production (fresh oils). The volatiles were grouped on the basis of their most probable origin.

The overall volatile profile agreed with the findings of other authors for Italian extra virgin olive oils (ANGEROSA *et al.*, 2004; KALUA *et al.*, 2007), which were found to be richer in C6 volatile compounds and poorer in esters than Spanish and Moroccan extra virgin olive oils (REINERS and GROSCH, 1998). The low amounts of volatiles generated by auto-oxidation and/or sugar fermentation evidenced the high quality level of the starting olives. The most represented volatile compound was *trans*-2-hexenal, accounting for more than 80% headspace. It derives from the LOX activity involving polyunsaturated fatty acids and is responsible for bitter, almond, and green notes (MORALES *et al.*, 1997; BENDINI *et al.*, 2009). CAVALLI *at el.* (2004) analyzing seven French Cailletier olive oils, found a content of *trans*-2-hexenal ranging from 37-64%.
The volatile profile of the oils obtained with talc addition generally corresponded to data reported in literature (BRKIĆ BUBOLA *et al.*, 2014; CAPONIO *et al.*, 2015; CAVALLI *et al.*, 2004; KOPRIVNJAK *et al.*, 2016). In particular, talc used at 1% level did not cause significant differences, whereas at 2% level a significant increase of ethanol (responsible for alcoholic, ripe apple, and floral notes) (REINERS and GROSCH, 1998; SERVILI *et al.*, 2001) and a decrease of 1-penten-3-one (associated with the green and pungent notes), *trans*-2-penten-1-ol (responsible for green notes), and ethyl acetate (responsible for sweet and aromatic notes) (ANGEROSA *et al.*, 2004; BENDINI *et al.*, 2009) were observed.

Table 1: Integrated area mean value and results of one-way ANOVA ($p < 0.05$) of the volatile compounds detected in oil samples obtained without (Co) and with addition of 1% and 2% of talc during processing (T1 and T2, respectively), analyzed immediately after production (fresh oils).

Volatile compounds	Co			T1			T2		
	Area		%	Area		%	Area		%
C_6 LOX pathway									
Hexanal	6.84E+07	a	3.26	6.96E+07	a	3.38	6.59E+07	a	3.24
trans-2-hexenal	1.72E+09	a	81.90	1.67E+09	a	81.31	1.66E+09	a	81.59
Hexan-1-ol	2.61E+07	a	1.25	2.45E+07	a	1.19	2.59E+07	a	1.28
cis-3-hexenal	1.12E+07	a	0.53	9.06E+06	a	0.44	9.02E+06	a	0.44
trans-3-hexen-1-ol	1.01E+06	a	0.05	1.07E+06	a	0.05	1.03E+06	a	0.05
cis-3-hexen-1-ol	4.15E+07	a	1.98	4.42E+07	a	2.15	4.42E+07	a	2.18
cis-2-hexen-1-ol	4.40E+07	a	2.10	4.52E+07	a	2.20	4.63E+07	a	2.28
C_5 LOX pathway									
1-penten-3-one	4.54E+07	a	2.17	4.48E+07	a	2.18	3.92E+07	b	1.93
trans-2-pentenal	6.26E+06	a	0.30	6.67E+06	a	0.32	5.99E+06	a	0.30
1-penten-3-ol	9.99E+06	a	0.48	8.72E+06	a	0.42	9.08E+06	a	0.45
trans-2-penten-1-ol	2.50E+06	a	0.12	2.30E+06	a	0.11	1.85E+06	b	0.09
cis-2-penten-1-ol	2.47E+07	a	1.18	2.69E+07	a	1.31	2.47E+07	a	1.22
Carbohydrate fermentation									
Ethyl acetate	1.46E+07	a	0.70	1.26E+07	a	0.61	7.63E+06	b	0.38
Ethanol	3.22E+07	a	1.54	4.06E+07	ab	1.97	4.49E+07	b	2.21
Acetic acid	1.74E+07	a	0.83	1.44E+07	a	0.70	1.55E+07	a	0.76
Other origins									
Methyl acetate	3.34E+06	a	0.16	3.06E+06	a	0.15	3.11E+06	a	0.15
Pentan-3-one	1.02E+07	a	0.49	8.53E+06	a	0.41	8.58E+06	a	0.42
cis,*cis*,*cis*-1,3,6-Octatriene, 3,7-dimethyl	3.14E+06	a	0.15	3.17E+06	a	0.15	3.00E+06	a	0.15
Nonanal	3.75E+06	a	0.18	4.20E+06	a	0.20	3.95E+06	a	0.19
trans,*trans*-2,4-hexadienal	5.36E+06	a	0.26	5.79E+06	a	0.28	5.78E+06	a	0.28
trans,*trans*-2,4-heptadienal	8.28E+05	a	0.04	1.01E+06	a	0.05	7.34E+05	a	0.04
Benzaldehyde	1.85E+06	a	0.09	2.29E+06	a	0.11	1.90E+06	a	0.09
Propanoic acid	6.20E+05	a	0.03	5.87E+05	a	0.03	7.29E+05	a	0.04
Hexanoic acid	1.44E+06	a	0.07	1.32E+06	a	0.06	1.53E+06	a	0.08
Phenylethyl alcohol	3.56E+06	a	0.17	4.02E+06	a	0.20	3.45E+06	a	0.17

With the aim of evaluating the effect of talc on the volatiles, the variations of groups of compounds having the same origin, and belonging to the same chemical class, have been monitored during storage in the oils with or without talc addition. Moreover, some specific compounds considered to be effective indices of oil oxidations have been focused.

Figures 1 and 2 show the evolution of C6 and C5 volatile compounds, respectively, both derived from the LOX pathway.

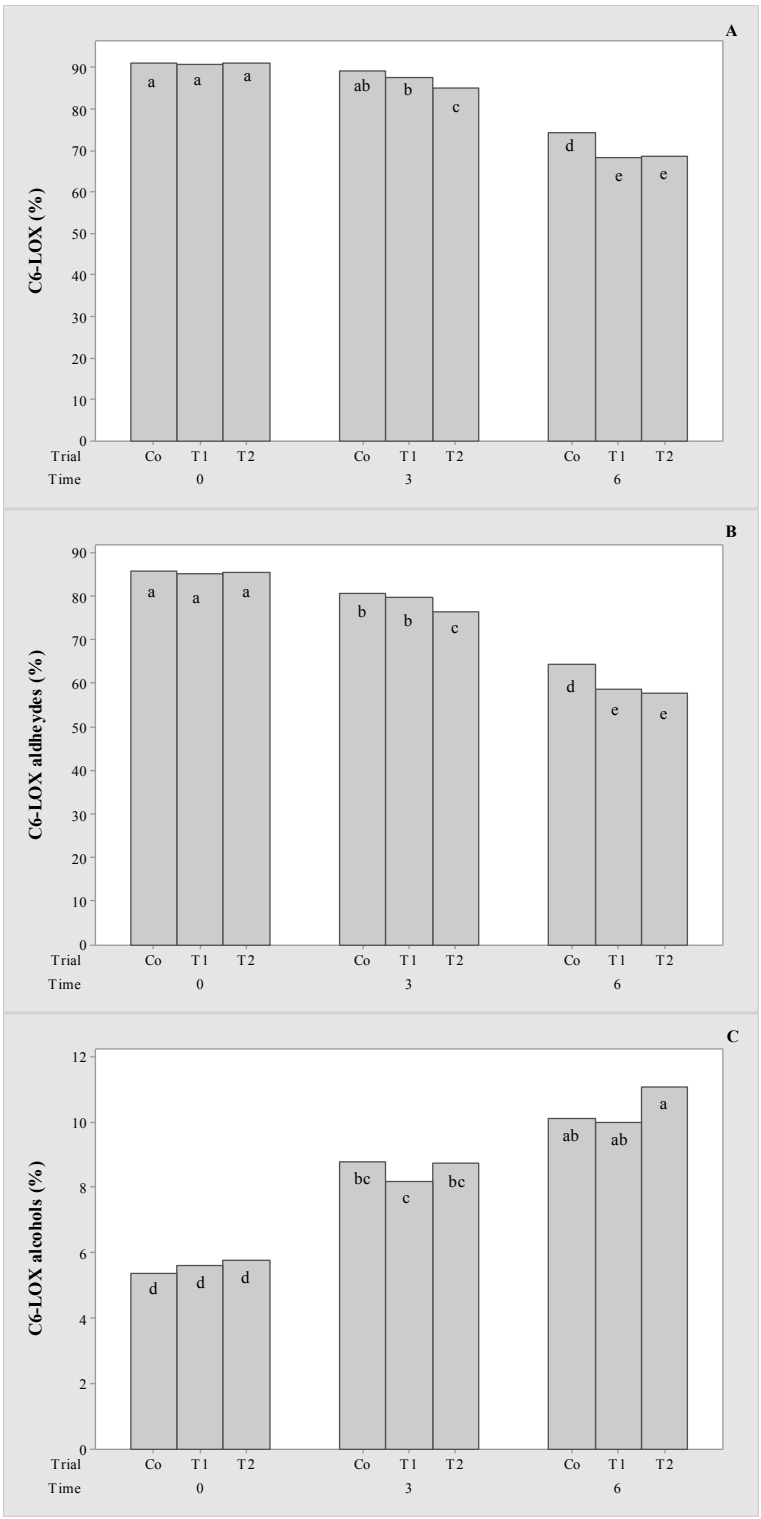


Figure 1: Percent mean value and results of two-way ANOVA ($p < 0.05$) of C6 volatile compounds deriving from the lipoxygenase (LOX) pathway of the oils obtained without (Co) and with addition of 1% and 2% of talc (T1 and T2, respectively) during storage: total (A), aldehydes (B), and alcohol (C). 0, fresh oils; 3, oil stored for three months; 6, oil stored for six months.

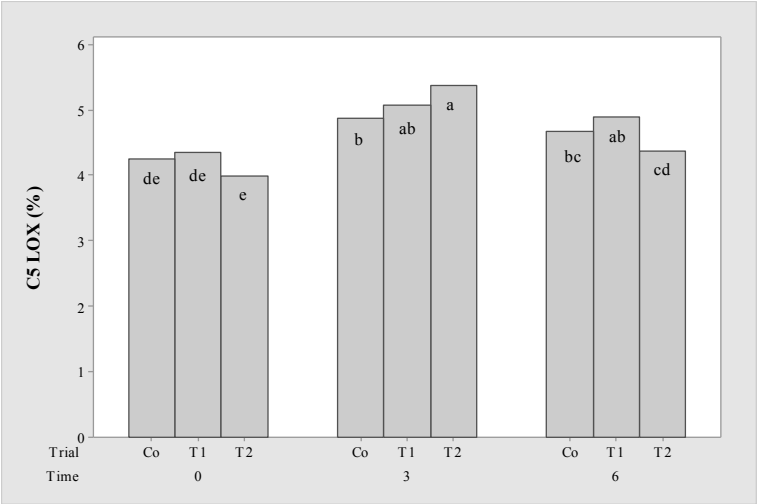


Figure 2: Percent mean value and results of two-way ANOVA ($p < 0.05$) of C5 volatile compounds deriving from the lipoxygenase (LOX) pathway of the oils obtained without (Co) and with addition of 1% and 2% of talc (T1 and T2, respectively) during storage. 0, fresh oils; 3, oil stored for three months; 6, oil stored for six months.

The C6-LOX compounds (Fig. 1A), which represent about 90% of the volatile compounds, showed a significant decrease during storage, as observed also by other authors (BENDINI *et al.*, 2009), more evident in the trials involving the use of talc. The observed trend was mainly attributable to the evolution of the C6-LOX aldehydes (Fig. 1B), which significantly decrease during storage, while C6-LOX alcohols (Fig. 1C), that representing about 5% of the volatile fraction, did not show a significant variation among trials. The significant increase of the latter during time, as reported by CAVALLI *et al.* (2004), could be attributed to the formation of alcohols by decomposition of methyl linolenate hydroperoxides (FRANKEL, 1980).

The C5-LOX compounds (Fig. 2) showed a significant increase during storage, more evident after 3 months. The increase of C5-LOX compounds was mainly due to the increase of *trans*-2-pentenal (Fig. 4B) and of 2-penten-1-ol (data not shown), that could arise from the decomposition of methyl linolenate hydroperoxides (FRANKEL, 1980). In fact, BENDINI *et al.* (2009) reported that *trans*-2-pentenal increases significantly during the oxidation of virgin olive oils in presence of metals. Ketones (Fig. 3) did not show significant variation up to 3 months of storage whereas a significant increase after six months of storage was observed, in agreement with the findings of CAVALLI *et al.* (2004). Talc addition did not show to significantly influence the variations of ketones (Fig. 3) and of C5-LOX compounds (Fig. 2).

Several authors report that the hexanal/nonanal ratio is an appropriate index to detect the beginning of olive oil oxidation and to follow its evolution (MORALES *et al.*, 1997; KIRITSAKIS, 1998; ANGEROSA *et al.*, 2004; KANAVOURAS *et al.*, 2004; BENDINI *et al.*, 2009). Moreover, also *trans*-2-pentenal, an unsaturated aldehyde originated by secondary reactions of the primary auto-oxidation products (13-LnOOH, 9-LOOH and 9-OOOH, respectively), could effectively monitor the oxidative process evolution of extra virgin olive oil (LUNA *et al.*, 2006). Finally, propanoic and hexanoic acids, the latter due to the oxidation of hexanal, and 2,4-decadienal, derived from fatty acid oxidation (REINERS and GROSCH, 1998), have also been detected during the oxidation (GUTIERREZ *et al.*, 2002; VICHI *et al.*, 2003).

Fig. 4 shows the variations of the above reported indices during storage.

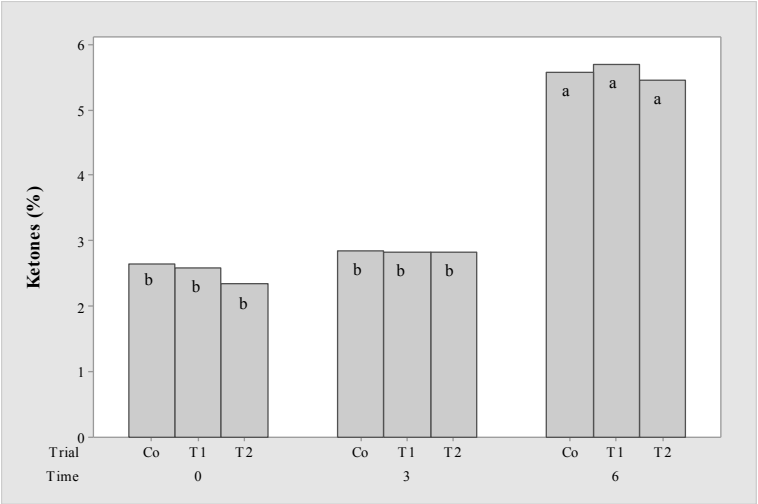
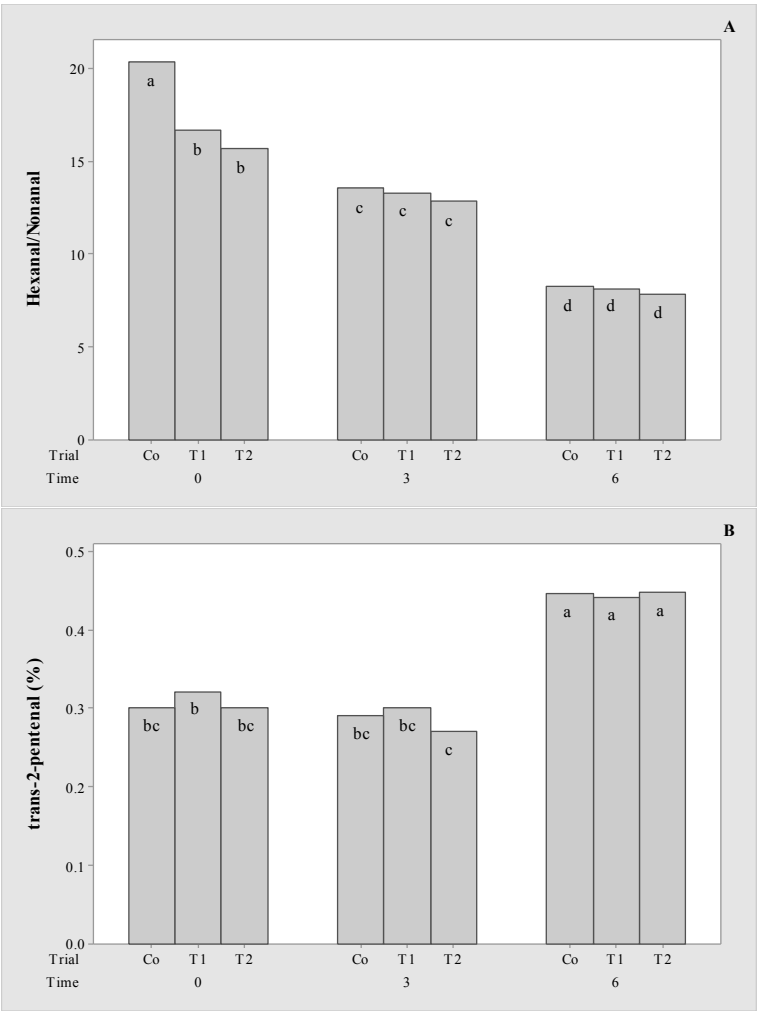


Figure 3: Percent mean value and results of two-way ANOVA ($p < 0.05$) of sum of ketone volatile compounds of the oils obtained without (Co) and with addition of 1% and 2% of talc (T1 and T2, respectively) during storage. 0, fresh oils; 3, oil stored for three months; 6, oil stored for six months.



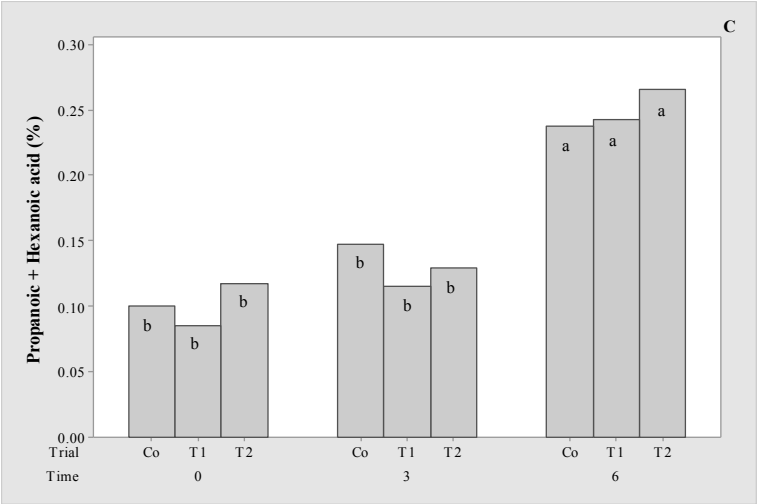


Figure 4: Percent mean value and results of two-way ANOVA ($p < 0.05$) of hexanal/nonanal ratio (A), *trans*-2-pentenal (B), and sum of propanoic and hexanoic acids (C) of the oils obtained without (Co) and with addition of 1% and 2% of talc (T1 and T2, respectively) during storage. 0, fresh oils; 3, oil stored for three months; 6, oil stored for six months.

An increase of auto-oxidation phenomena occurred during 6 months, irrespective of the addition of talc, with the only exception of the hexanal/nonanal ratio of fresh oil that indicated a stronger oxidation of talc-added oils than in the control.

4. CONCLUSIONS

The obtained results confirm that talc addition during olive processing determines only slight differences in the volatile compounds profile of the corresponding extra virgin olive oil compared to control obtained without talc addition. In the majority of cases, these differences were devoid of statistical significance. During storage a significant increase of the volatiles deriving from the oxidative process was observed, as shown by the hexanal/nonanal ratio and the sum of propanoic and hexanoic acids, and a concomitant significant decrease of the C6-LOX aldehydes responsible for bitter, almond, and green notes of extra virgin olive oil. The evolution of the volatile compounds of extra virgin olive oils during storage was not significantly influenced by talc addition, with the exception of the sum of C6-LOX aldehydes which showed a significant higher decrease.

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Paper Received February 5, 2016 Accepted June 14, 2016

PAPER

THE STABILITY OF SPRAY-DRIED MICROENCAPSULATED β-CAROTENE IN THE MIXTURE OF GUM ARABIC, OSA-TYPE MODIFIED STARCH AND MALTODEXTRIN

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ABSTRACT

The study analyzed effects of mixtures of three carriers: gum Arabic, OSA-type modified starch and maltodextrin, applied in different ratios on the properties of β-carotene microencapsulated by spray drying. β-Carotene emulsions were prepared using high-pressure two-stage homogenization. Emulsion properties and microcapsule features were characterized. Correlations between the studied characteristics were determined with the use of Principal Component Analysis (PCA).
The study showed that the use of media mixed in equal proportions increased pigment retention during the storage of microcapsules to a lesser extent than the mixtures with different ratios of the carriers. The matrix which was the most effective in protecting the core was made of a mixture of modified starch, gum Arabic and maltodextrin in the ratio of 1:3:2. It was also found that the higher content of gum Arabic in the mixture of carriers was more effective in preventing β-carotene degradation than an increasing content of OSA-type modified starch.

Keywords: carotenoids, food colorant, storage stability, microstructure, principal component analysis (PCA)

1. INTRODUCTION

β-Carotene arouses interest among carotenoid colorants because it has the highest provitamin activity and is commonly used as a food colorant (FERREIRA and RODRIGUEZ-AMAYA, 2008). Furthermore, it is an antioxidant, which prevents aging (DESOBRY *et al*., 1997) and certain diseases such as heart disease and molecular degeneration (MELÉNDEZ-MARTÍNEZ *et al*., 2007). However, carotenoids are sensitive to thermal and chemical oxidation, and also isomerization influenced by oxygen, light and heating during technological processing and storage (FERREIRA and RODRIGUEZ-AMAYA, 2008). Degradation of β-carotene may also be induced by active prooxidative compounds (e.g. radicals) (CAO-HOANG *et al*., 2011). Moreover, high hydrophobicity of β-carotene makes it insoluble in water and slightly soluble in oil at room temperature (LIANG *et al*., 2013), which also limits its use as a food ingredient and in various medicines (YIN *et al*., 2009). The bioavailability of β-carotene depends on the type of food, factors influencing gastrointestinal absorption and the factors that control its metabolism and distribution in the body. The composition, freshness and content of fat in consumed food are of particular importance. Excessive intake of essential fatty acids causes an increase of β-carotene demand in the body. A low content and poor quality of fat reduce intestinal absorption of β-carotene and carotenes conversion into vitamin A. The intake of vegetable oils and natural antioxidants increases β-carotene bioavailability. Therefore, dietary β-carotene should be incorporated into lipid micelles to ensure effective absorption by intestinal cells (HORNERO-MÉNDEZ and MÍNGUEZ-MOSQUERA, 2007). The bioavailability of β-carotene is often limited and depends on food constituents (RIBEIRO *et al*., 2008).

In order not to prevent the loss of bioactive properties of β-carotene, it should be protected from adverse effects of the environment (BONNIE and CHOO, 1999). The process of spray-dried microencapsulation, based on the inclusion of β-carotene into oil-in-water emulsion, can be considered as an effective, inexpensive and convenient method for increasing stability of hydrophobic carotenoids (RIBEIRO *et al*., 2008). In this encapsulation method it is important to select a proper coating material. Usually gum Arabic, maltodextrin and modified starch are used as coatings. Their usability in the microencapsulation process has been described in literature (PRZYBYSZ *et al*., 2012), however none of them meets all requirements of coating materials, thus only their combination may provide a good protection of an active substance.

Literature data shows that the use of a mixture of modified starch, gum Arabic and maltodextrin is more effective than the application of only one of these carriers (BUFFO and REINECCIUS, 2000, KRISHNAN *et al*., 2005, KANAKDANDE *et al*., 2007, DONHOWE *et al*., 2013). Studies of BUFFO and REINECCIUS (2000) showed that a high content gum Arabic in spray-dried emulsions increased retention of microencapsulated substances. KANADANDE *et al*. (2007) found that a mixture of three carriers (gum Arabic, modified starch and maltodextrin) with the highest contribution of gum Arabic was more efficient in the microencapsulation of cumin oleoresin than other mixtures and better than gum Arabic applied as the carrier alone. A similar conclusion was made by KRISHNAN *et al*. (2005) who encapsulated cardamom oleoresin.

The aim of this study was to evaluate the effect of different ratios of gum Arabic, OSA-type modified starch and maltodextrin applied in mixtures used as microcapsules coating on storage stability of β-carotene microencapsulated by spray drying.

2. MATERIALS AND METHODS

2.1. Emulsion preparation

A commercial oil preparation of β-carotene E160a (ii) 1 % OS (Food Colours – Komponenty do żywności) containing 1 % of pigment was used in the study. According to the manufacturer's declaration it was a nature-identical colorant Oil-in-water type pigment emulsions were spray dried. They were made of mixtures of three media: instant gum Arabic E414 (GA), maltodextrin 150 LOW-DE 15.6 (M) and commercial OSA-type modified starch from tapioca "Capsule TA" (sodium starch octenylsuccinate) E1450 (MS), obtained by starch esterification with n-octenyl succinic anhydride acid (<3%). Eight types of emulsions were prepared with the following composition: oily pigment preparation: 10 %, carrier mixture: 25 % and distilled water: 65 %. The media were mixed in the following ratios: sample 1: MS:GA:M – 1:1:1, sample 2: MS:GA:M – 1:2:1, sample 3: MS:GA:M – 1:3:1, sample 4: MS:GA:M – 2:1:1, sample 5: MS:GA:M – 3:1:1, sample 6: MS:GA:M – 1:1:2, sample 7: MS:GA:M – 3:1:2, and sample 8: MS:GA:M – 1:3:2. Sample 1 was taken as a control.
Carrier solutions were prepared by dispersion of the matrix made of gum Arabic, maltodextrin and OSA-type modified starch in distilled water. The carriers were added to distilled water at 40 °C and stirred with a laboratory stirrer (IKA LABORTECHNIK, RW 20 DZM, Germany) at 380 rpm for 30 min. The continuous phase was left at room temperature (20 ± 2 °C) for about 24 h in order to fully hydrate the carrier. On the following day, the β-carotene oil solution of core was added to wall material solutions and the mixtures were stirred together using a laboratory stirrer at 380 rpm for 10 min. To prepare pre-emulsions, all mixtures were homogenized in a high-shear homogenizer (IKA LABORTECHNIK, Ultra Turrax Model T25, Germany) at 13,500 rpm for 10 min. The pre-emulsions were then subjected to two-stage homogenization using a high-pressure homogenizer (Model APV-1000, Albertslund, Denmark). In the first stage of homogenization, the pressure was 55 MPa and in the second stage 18 MPa, according to DŁUŻEWSKA and LESZCZYŃSKI (2005).

2.2. Emulsion analysis

Stability and color parameters of the obtained emulsions were determined. All experiments were done in triplicate.

2.2.1. Emulsion stability

Emulsion stability (SE) was determined by forced dissolution by centrifugation, which involved 24 h of storage at 37°C (thermostable conditions). Subsequently, the samples were centrifuged (centrifuge type MPW-340, Poland) at 3,500 rpm for 10 min. The volume of separated phases was measured and SE was calculated from the formula:

$$SE = \frac{(V_0 - V)}{V_0} \times 100 \qquad (1)$$

where: SE: emulsion stability, V_0: total volume after centrifugation [cm^3], V: volume of separated non-emulsified phase [cm^3].

2.2.2. Color measurements

Emulsions and solutions (10 % microencapsulated pigment solutions) were subjected to color analysis in CIE L*a*b* system using a colorimeter (Konica-Minolta, CM-3600d, Japan) at the wavelength of 450 nm. The assay was performed in reflected light using an observer 10° and illuminant D_{65}, in cuvettes with a thickness of 10 mm. The thickness of aperture was 25.4 mm. The color values represented brightness (+L/-L), redness/greenness (+a/-a) and yellowness/blueness (+b/-b). Based on the a* and b* parameters, color chromaticity (C*) and hue angle (h^0) were calculated using the following equations:

$$C^* = (a^{*2} + b^{*2})^{1/2} \quad (2)$$

$$h^0 = \arctan (b^*/a^*) \quad (3)$$

h^0 equal to 0 indicates red color, 90: yellow, 180: green and 270: blue.

2.3. Drying

The obtained emulsion was dried in a laboratory spray drier (type A/S Niro Atomizer, Copenhagen, Denmark; diameter of a drying chamber: 0.85 m, height: 1.3 m, spraying mechanism: disk). It was fed to spray disk using a peristaltic pump (Elpan, type 372.1, Poland) with a speed of 4 rpm. The emulsion was heated to a temperature 40±2°C before inserting it into a dryer in order to reduce its viscosity. It facilitates atomization and ensures more efficient microencapsulation. Countercurrent drying was used, the temperature of the inlet air was 190±5°C, that of the outlet air was 80±5°C and the pressure of air propelling the spray disk was at 3.0±0.2 kg cm-2 (PRZYBYSZ *et al*., 2012). Microcapsules were obtained in six parallel series.

2.4. Microcapsules analysis

200 g of microencapsulated pigment were stored in colorless glass jars at 20±2°C with the access of daylight for 64 days. The properties of microcapsules were studied immediately after spray drying and during storage by determining: the total content of pigment in microcapsules and on their surface, the color of microcapsules, the color of β-carotene solutions obtained after dissolution of microcapsules, apparent density and morphology of powders. All measurements were done in triplicate.

2.4.1. Determination of β-carotene content in microcapsules and on their surface

The spectrophotometric measurement of β-carotene content in microcapsules and their surface was performed according to POLISH STANDARD PN-A-75101-12:1990. Extraction of pigment from microcapsules and their surface was carried out according to the method of PRZYBYSZ *et al*. (2012) on the day of manufacturing the microcapsules, as well as after 28, 49 and 64 days of storage (the content of pigment in oily preparation was measured at the same time). The extraction was carried out 3 times. The extraction solution was a mixture of hexane and acetone (1:1).
Microencapsulation efficiency (ME) is the ratio of pigment content inside the microcapsules to the total pigment content (inside and on the surface) expressed as a percentage. ME was calculated according to the equation proposed by McNAMEE *et al*. (2001) based on the determined β-carotene content in microcapsules and on their surface:

$$ME = \frac{(c_b - p_b)}{c_b} \times 100 \quad (4)$$

where: ME: microencapsulation efficiency [%], c_b: total pigment content in microcapsules [mg (100 g)$^{-1}$], p_b: pigment content on microcapsule surface [mg (100 g)$^{-1}$].

2.4.2. Color of microcapsules

The color of microcapsules was determined with a colorimeter (Minolta, type CR-200, Japan), which defines L*, a*, b* color parameters. Light D_{65} was used for the study. Samples of microcapsules were placed in Petri dishes (the dishes were filled up to the full volume). A measuring head was applied to the surface of microcapsules. Measurements were taken at three different points and results were expressed as an average. Before the test, the camera was calibrated with a white pattern.

2.4.3. Apparent particle density

The apparent density ϱ_p was determined in a helium pycnometer (Stereopycnometer/Quantachrome Instruments, BoyntonBeach, USA) with specification as described by DOMIAN and BIALIK (2006). The apparent density is the ratio of powder mass and volume of powder particles. This volume does not include the volume of the air between particles.

2.4.4. Scanning electron microscopy (SEM)

The morphology of microencapsulated β-carotene was analyzed based on images taken with a scanning electron microscope (Hitachi Tabletop Microscope TM 3000, Tokyo, Japan) using a Multi Scan Base v. 18.03 software (computer scanning system, Warsaw, Poland) operating at 15 kV. Microcapsules were applied to a SEM platform with a double-sided adhesive tape. Samples were not sprayed with a coating material before observation. The paper presents selected images of microcapsules (magnification at 200x and 500x).

2.5. Statistical analysis

Statistical analyses were performed using Statistica 10.0 software (StatSoft). The significance of differences between mean values was evaluated with one-way ANOVA at a significance level of $p = 0.05$. The least significant difference was determined with the Tukey's test. The linear model was used for the random in the variance analysis.
Correlations between the evaluated properties and division of samples into groups were interpreted with a multivariate statistical method: principal component analysis (PCA). Results are presented in a two-dimensional plot.
Half-life and decay constant of β-carotene were calculated according to SZTERK and LEWICKI (2007). The half-life was calculated from the regression formula:

$$y = at + b \quad (5)$$

where: y: concentration of pigment [mg/100 g]; t: half-life [days].

The half-life of β-carotene ($T_{1/2}$) was determined by substituting "y" for half of the initial content of β-carotene. Decay reaction rate constant of β-carotene (K) was calculated from the equation:

$$K = (CA_0 - CA)/T \quad (6)$$

where: K: Decay reaction rate constant of β-carotene [(mg/100 g)/day]; CA_0 and CA: initial and final content of β-carotene [mg/100 g]; T: time [day].

3. RESULTS AND DISCUSSIONS

3.1. Emulsion stability

Oil-in-water emulsions may become instable which reduces the efficiency of the microencapsulation process. Stability of emulsions containing OSA-type modified starch, gum Arabic and maltodextrin ranged from 71.75 to 90.27 % and depended significantly on carrier ratios in the mixture. The emulsion containing the above-mentioned before carriers in the ratio of 1:1:1 was taken as a control sample. Its stability was 85.54 %. A higher content of gum Arabic and OSA-type modified starch in the mixture increased emulsion stability. However, emulsions with a higher content of modified starch than gum Arabic were more stable. The increase in emulsion stability results from a higher ratio of carriers with good emulsifying properties: gum Arabic and modified starch. Gum Arabic is a good emulsifier due to the presence of the arabinogalactan and protein complex. However based on the conducted study, the OSA-type modified starch is a better emulsifier than gum Arabic. In the emulsion systems with modified starch, crosslinking occurs in the aqueous phase. The starch forms a thick adsorptive layer on the surface of the oil droplet. Due to the polymeric stevic integration it prevents the release and migration of the oil phase (CHANAMAI and McCLEMENTS, 2000). The least stable emulsion obtained in this study was a mixture with the highest content of maltodextrin (sample 6), which is related to its very poor emulsifying properties (REINECCIUS, 1991). The low emulsifying ability of maltodextrin makes the dispersion of lipid components harder and reduces stability of the emulsion (DRURI and PAWLIK, 2001). Also, studies of LEWANDOWICZ *et al*. (2005) on the functional properties of maltodextrin in emulsion systems demonstrated that emulsions obtained from solutions of maltodextrin and oil were delaminated immediately. Based on the conducted study, it was found that the most stable emulsion system was obtained with the combination of MS, GA and M in the ratio of 3:1:2.

3.2. Apparent particle density

The apparent density of powders (i.e. the mass ratio of the particle to its reduced volume by open pore volume) depended significantly on matrix composition. In a preliminary study, the carriers were analyzed individually and process parameters were the same for all of them. The highest apparent density (1438 kg m^{-3}) was shown for the powders made of maltodextrin and the lowest one for the powders with modified starch (1107 kg m^{-3}), while that of the powders with gum Arabic was estimated at 1340 kg m^{-3}. It may, therefore, be hypothesized that the apparent density of obtained microcapsules, made of three carriers mixture, will have additive properties. Powders obtained from a stabilized emulsion of a mixture of carriers with the highest content of gum Arabic (sample 3 and 8) were characterized by the highest apparent density (1241 kg m^{-3}), which is indicative of the smallest internal porosity (Fig. 1).

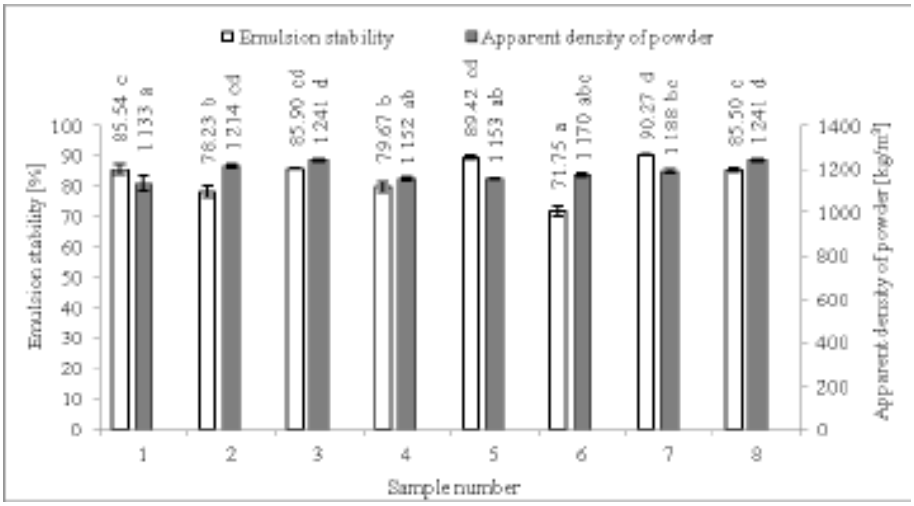

Figure 1: Selected physical properties of emulsions and powders.
Explanations:
Numbers represent variants of the microcapsules, in which the matrix is a mixture of: MS – modified starch, GA: gum Arabic, M: maltodextrin used in the following ratios: sample 1: MS:GA:M – 1:1:1, sample 2: MS:GA:M – 1:2:1, sample 3: MS:GA:M – 1:3:1, sample 4: MS:GA:M – 2:1:1, sample 5: MS:GA:M - 3:1:1, sample 6: MS:GA:M - 1:1:2, sample 7: MS:GA:M - 3:1:2, sample 8: MS:GA:M - 1:3:2.
Occurrence of at least one the same letter means no significant difference between the compared mean values in the group ($p<0,05$). Comparisons were made separately for each of the storage times.

It was found that the higher ratio of this carrier in the matrix (samples 1-3) tended to increase apparent density of the obtained powders. Increased apparent density of powders may result from a higher degree of structure condensation of these powders (DOMIAN, 2005). The obtained results shows that the powders with gum Arabic formed a more dense structure than modified starch. It may be explained by the fact that it forms emulsions with a higher viscosity compared to other carriers. The spraying of emulsion with a higher viscosity by a disk in the spray-drying chamber results in the formation of larger droplets, hence powder particles are larger as well. Many gas particles are not retained, which makes them more compact. The lower apparent density of powders produced from stabilized emulsions by a mixture of carriers with a higher content of modified starch than gum Arabic, may indicate greater amount of air spaces inside powder particles (i.e. internal pores, which are not connected with the surrounding atmosphere). Particularly, greater aeration of the sample occurred and a larger amount of foam was created during the mixing and homogenization of emulsion with an increased content of modified starch. The lower apparent density of powders may also be associated with their dustiness. Increasing the ratio of modified starch to two parts boosted the apparent density (sample 4). Further increase of modified starch addition in the mixture to three parts did not affect the value of this parameter. A higher content of maltodextrin in the carrier mixture (sample 6) resulted in an increase of apparent density due to the fact that among the applied carriers, it forms powders with the highest apparent density. Only in the case of sample 8 with the highest content of gum Arabic and the highest content of maltodextrin in the matrix, no influence of carrier on apparent density was observed and its value was the same as for sample 3, with the lower content of maltodextrin. According to CUPIAŁ *et al*. (2010), the addition of maltodextrin during spray drying to hydrolyzed protein resulted in a decrease of apparent density of the resultant powders compared to the hydrolyzed protein applied alone. The authors explain that maltodextrin has a lower

density and is a "skin-forming" material, which is characterized by the ability to form an impermeable film on the surface of droplets during evaporation. As a result, gas bubbles remain trapped inside particles, and powders have a lower apparent density because of the increased internal porosity. An important parameter that should be examined is the wettability of the powders.

3.3. Stability of microencapsulated β-carotene

The microencapsulated pigment as well as the control (i.e. oil β-carotene solution) underwent a 64-day storage test. The microencapsulation process increased pigment retention during storage. Pigment retention reached 44.8 % in the control (oil β-carotene solution) and ranged from 67.9 to 86.7 % in the microencapsulated β-carotene (Table 1).

Table 1: Comparison of the stability of β-carotene in the oil solution and stability of the microencapsulated pigment.

Analyzed factor	Oil β-carotene solution	Type of microcapsules							
		MS:GA:M 1:1:1	MS:GA:M 1:2:1	MS:GA:M 1:3:1	MS:GA:M 2:1:1	MS:GA:M 3:1:1	MS:GA:M 1:1:2	MS:GA:M 3:1:2	MS:GA:M 1:3:2
Microencapsulation efficiency [%]	-	88.6±0.4	81.2±0.2	81.0±5.3	79.3±9.1	66.9±2.7	81.5±4.4	75.6±5.5	81.5±9.5
Total β-carotene retention after 64 days of storage [%]	44.8±0.8	75.7±3.6	85.9±12.4	83.1±0.9	70.8±7.4	67.9±5.3	84.4±7.3	68.4±1.6	86.7±2.8
Retention of β-carotene on microcapsules surface after 64 days of storage [%]	-	23.7±7.1	14.0±0.3	21.8±5.6	16.4±6.1	14.6±0.1	15.8±2.5	14.2±2.5	14.0±6.6
Decay constant of total β-carotene [mg/100g/24 hours	8.82±0.11	1.37±0.23	0.71±0.60	0.88±0.05	1.41±0.37	1.57±0.35	0.77±0.42	1.55±0.12	0.70±0.14
Decay constant of β-carotene on microcapsules surface [mg/100g/24 hours]	-	0.49±0.02	0.84±0.04	0.79±0.28	0.85±0.44	1.37±0.04	0.74±0.13	1.03±0.24	0.85±0.48
The half-life [days]	53±0	123±2	230±14	189±1	105±1	95±5	187±17	97±3	288±5

β-Carotene content inside microcapsules depended on time and varied from 33.8 to 94.1 %, whereas on the microcapsule's surface it varied from 75.5 to 90.6 % for different variants (the percentage is based on the determination coefficient R^2). The loss of the pigment resulted from degradation of this labile compound caused by daylight, oxygen and temperature (RODRÍGUEZ-HUEZO *et al*., 2004). A decrease in the amount of microencapsulated pigment during storage test was also observed by ELIZALDE *et al*. (2002), who encapsulated β-carotene inside trehalose and gelatin shells. DESOBRY *et al*. (1999) reported on the loss of β-carotene enclosed within maltodextrin microcapsules during storage. The above findings were consistent with results of the study by DESOBRY *et al*. (1997).

The stability of β-carotene depended significantly on the content of different types of carriers in the emulsion. It was found that it is necessary to apply carriers mixed in

different ratios, because when evenly mixed the protection of microencapsulated pigment was weaker (the highest efficiency of microencapsulation was neither related to a high retention coefficient and a high decay constant of total β-carotene at a relatively short half-life, which additionally confirmed its short stability (Table 1).

The microcapsule's core was best protected when the matrix was made of a mixture of carriers, containing mainly gum Arabic reinforced with maltodextrin (sample 8). The pigment inside such a matrix had the highest retention and the longest half-life (it had the lowest decay constant of total β-carotene: [(0.70 mg / 100 g) / 24 hours]. This was also associated with the highest apparent matrix density due to the lowest pore number inside powder particles, which as a consequence led to the most effective protection of the core against oxygen. Gum Arabic is flexible and resistant both to microcapsules deformation and to cracking, which makes the loss of β-carotene much lower. Moreover, the high maltodextrin addition has reinforced the protective properties of the gum Arabic carrier. According to literature, maltodextrin is a strong oxygen barrier due to its ability to form a glassy structure (REINECCIUS, 1991; GOUIN, 2004). Maltodextrin combined with gum Arabic has a stronger protective effect on the microencapsulated substance. This relation was true for the sample 6, which contained an increased amount of maltodextrin (retention reached 84.4% and the decay constant of total β-carotene: [(0.77 mg / 100 g) / 24 hours].

In turn, the weakest protection of the core against oxidation was observed for the matrix containing the highest percentage of modified starch in the mixture of carriers (sample 5). In this case, the total microencapsulation efficiency as well as pigment retention were the lowest and the decay constant for β-carotene was the highest – both in total and on the microcapsule's surface.

Carotene degradation results from its oxidation initiated by isomerization. This process occurs easily at high temperature, in daylight, in acidic environment and in the presence of catalysts. In those conditions, a typical *trans* isomer of carotene is converted into *cis* isomer (RODRIGUEZ-AMAYA, 2001). *Cis* isomer is in fact much more susceptible to oxidation. As a result of this process, carotenyl peroxide is formed, which upon the activity of free radicals gives epoxy carotenoids or dioxetanes. The latter can decompose, thus producing aldehydes and ketones (MORDI *et al*., 1993). It is noteworthy that the emulsion used to prepare microcapsule 5 turned out to be the most stable. However, although the modified starch is capable of forming emulsion as a main component of the shell it does not provide sufficient protection against pigment oxidation. REINECCIUS (1991) came to a similar conclusion. Thus, the relatively high stability of the emulsion is not necessary to achieve high microencapsulation efficiency if dried emulsion formula has been properly chosen. When only starch content was increased in the wall material (samples 1, 4, 5), we have observed a decrease in microencapsulation efficiency as well as shortening of both β-carotene total retention and its retention on the microcapsule's surface. In the same time, an increase was observed in β-carotene decomposition rate leading to its degradation. Therefore it was concluded that the following dependency is true: the higher the amount of modified starch in the carrier mixture, the faster degradation of β-carotene is. Changes in the total content of microencapsulated pigment and pigment absorbed on the microcapsule's surface are shown in Fig. 2 and 3. For different ratios of the carriers, the total pigment content and pigment content on the surface of the microcapsules ranged from 308.3 to 361.6 mg $(100\ g)^{-1}$ and from 41.1 to 102.4 mg $(100\ g)^{-1}$ at the beginning of the storage period.

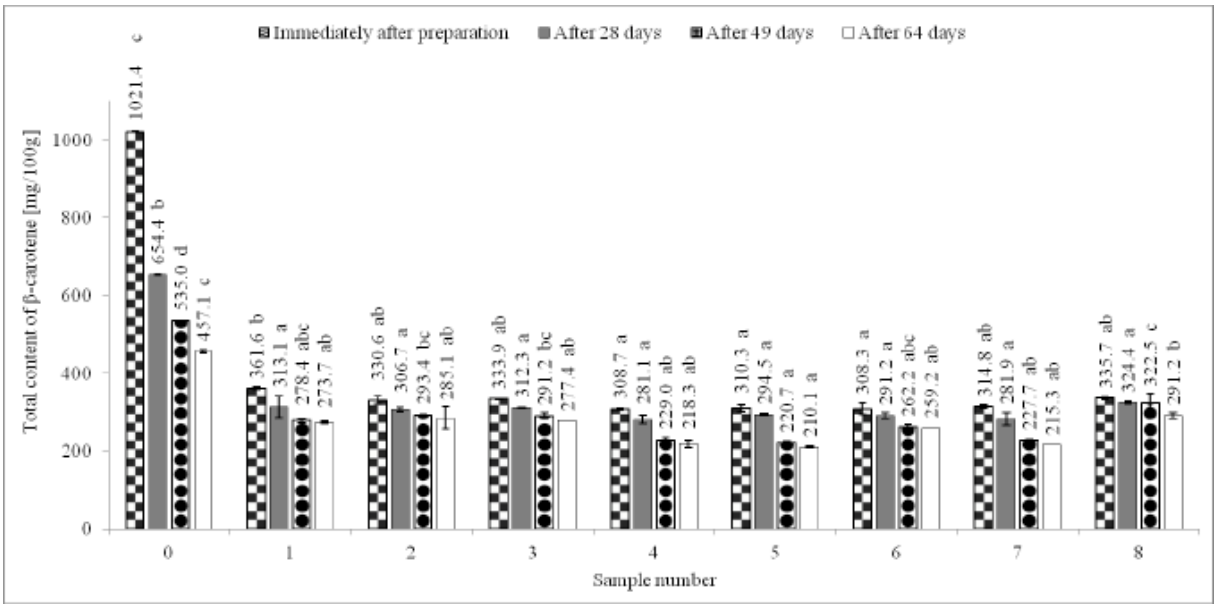

Figure 2: Contents of total β-carotene in microcapsules and in the oil formulation.
Explanations:
Numbers represent variants of the microcapsules, in which the matrix is a mixture of: MS: modified starch, GA: gum Arabic, M: maltodextrin used in the following ratios: sample 1: MS:GA:M – 1:1:1, sample 2: MS:GA:M – 1:2:1, sample 3: MS:GA:M – 1:3:1, sample 4: MS:GA:M – 2:1:1, sample 5: MS:GA:M - 3:1:1, sample 6: MS:GA:M - 1:1:2, sample 7: MS:GA:M - 3:1:2, sample 8: MS:GA:M - 1:3:2. Sample 0: oil β-carotene solution. Total amount of β-carotene for sample 0 [mg/100 g oil], for samples 1 - 8 [mg/100 g powder].
Occurrence of at least one the same letter means no significant difference between the compared mean values in the group ($p<0,05$). Comparisons were made separately for each of the storage times

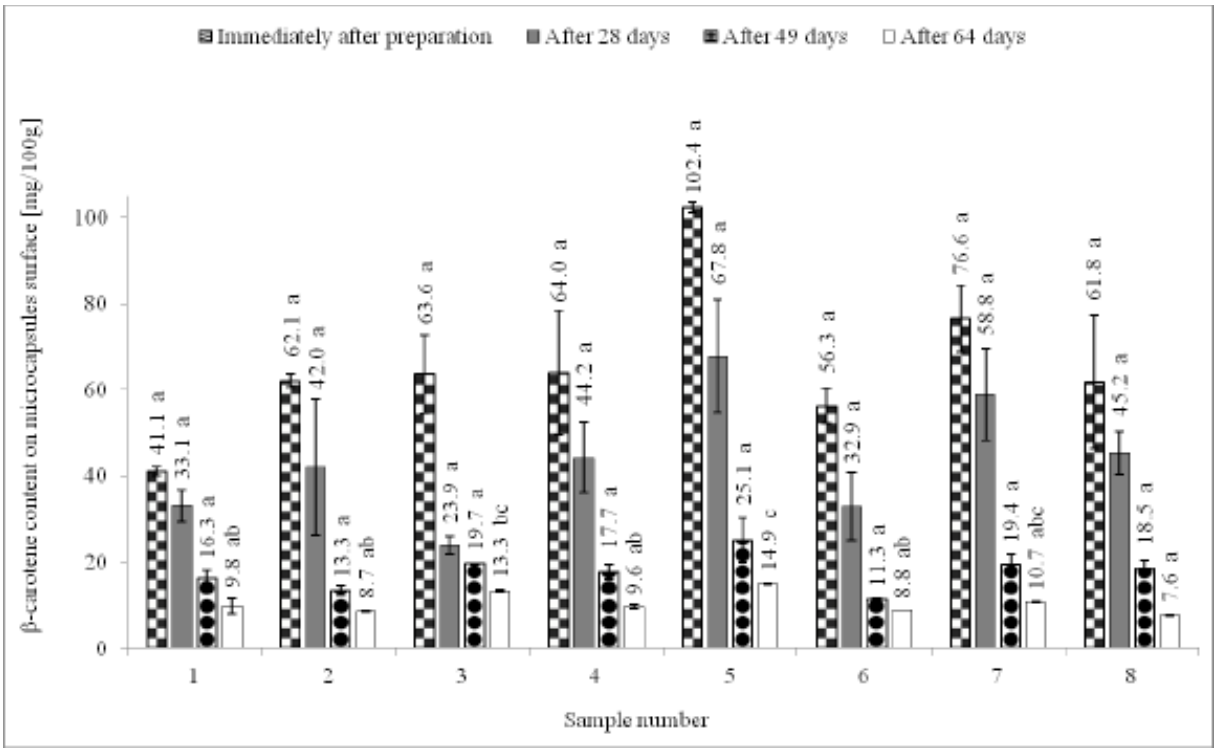

Figure 3: Content of β-carotene on microcapsules surface.
Explanations as under Fig. 1.

However, an in-depth analysis shows that the surface of microcapsules containing an increased amount of modified OSA starch had the largest content of the pigment. This has also been confirmed in studies by PRZYBYSZ *et al*. (2012) and by DŁUŻEWSKA *et al*. (2011). Thus, the poor β-carotene stability in those matrices might result from its high concentration on the microcapsule's surface, which leads to its more rapid degradation (DESOBRY *et al*., 1997). Both in the case of total β-carotene and surface β-carotene content changes, it was found that in all the samples the highest average loss of the pigment occurred between the 28th and the 49th day of storage and averaged 25.8 mg $(100\ g)^{-1}$. The minimal pigment level at the end of the storage period was on average 7.2 mg $(100\ g)^{-1}$. The above results are typical according to literature, which proves that the loss of β-carotene during the initial period of sample storage as microcapsules is higher than during the final period (RODRÍGUEZ-HUEZO *et al*., 2004).

3.4. Color changes during storage

β-Carotene has 11 conjugated double bonds and is an orange pigment (RODRIGUEZ-AMAYA, 2001). The color of emulsion and solutions containing the microencapsulated pigment may help to determine its stability (PESEK and WARTHESEN, 1990, SPADA *et al*., 2012). The color analysis should be preceded with a note that the color parameters of the emulsion as well as the colors of the solution of microcapsules are a sum of particular color parameters: both of β-carotene oil solution and of carriers dissolved in water. In this case, the color was largely determined by the ratio of all types of carriers, whereas in the case of the color parameters of the obtained microcapsules, color will be largely determined only by the color of β-carotene absorbed on their surface. The color of a carrier mixture will be developing in time. It cannot be determined whether color measurement of powders indicates the oxidation of pigment on the surface. During β-carotene degradation, the color of the carrier on the surface of microcapsules is revealed. Therefore color measurements were not performed during storage. Average values of brightness (L*) for microcapsules, emulsions and solutions of the dissolved microcapsules were: 74.54; 57.27 and 65.69, respectively (Fig. 4).
Regardless of the type of the tested material, the same tendencies were observed: the higher the ratio of modified starch, the higher brightness value (for samples 1, 4, 5). Similar results were obtained at a higher ratio of maltodextrin. Microcapsules with the highest luminescence (L* = 75.13) are those with an increased addition of maltodextrin only (sample 6), while the most bright emulsions (L* = 60.39) are those with an increased addition of both modified starch and maltodextrin (sample 7). The high brightness of microcapsules is caused by white color of the carriers, for both those made of modified starch and of maltodextrin, and by the fact that in the case of samples with a higher content of maltodextrin the quantity of pigment on microcapsule's surface was lower. It may also result from high brightness of maltodextrin solution itself. Chromatic parameters of microcapsule color were positive for both a* (indicating the dominant red hue) and for b* (indicating the dominant yellow hue).

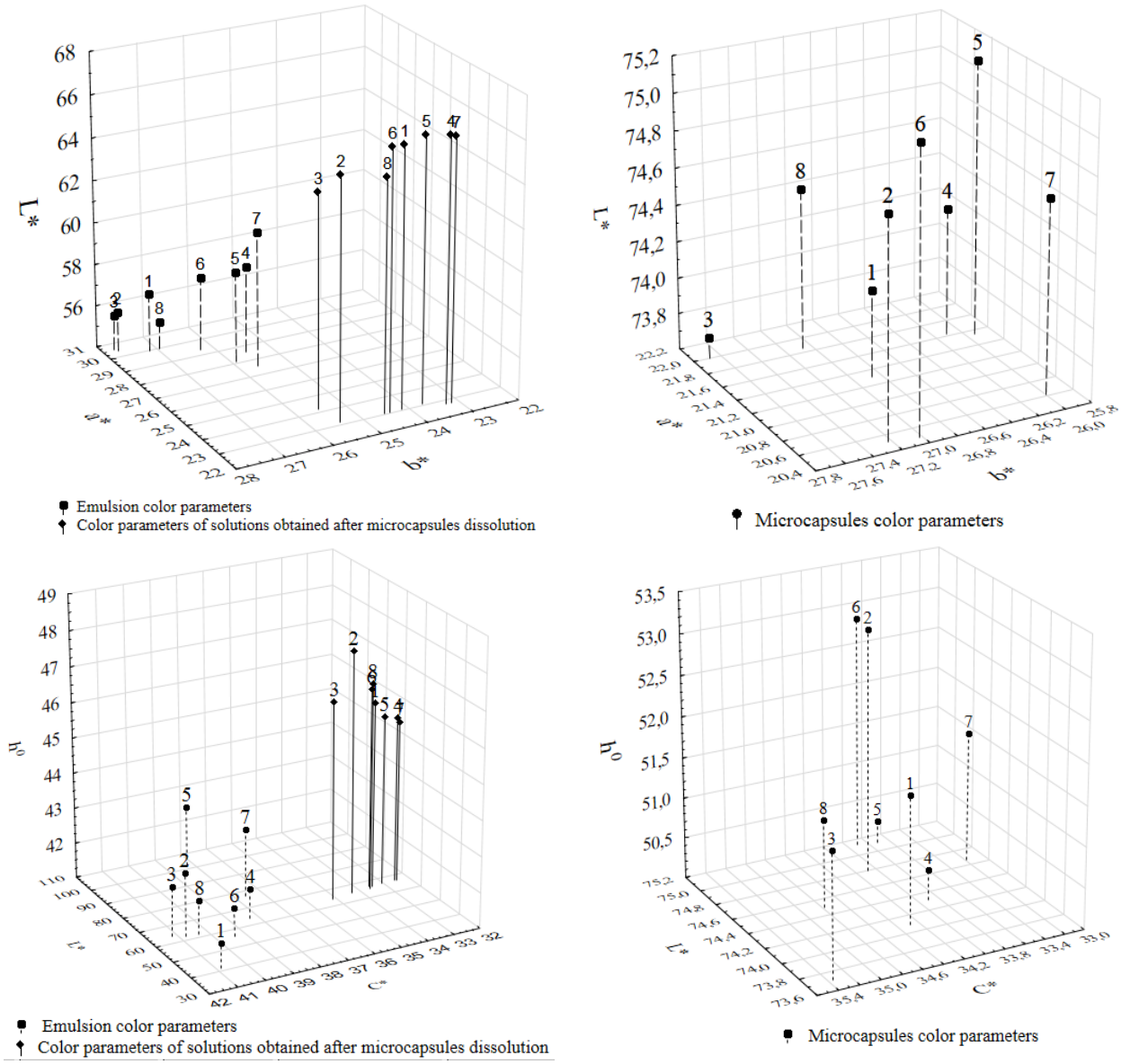

Figure 4: Color values of emulsions, microcapsules and solutions obtained from the dissolved microcapsules.
Explanations as under Fig. 1.

Average values of red parameter (a*) for microcapsules, emulsions and solutions prepared from dissolved microcapsules were 21.24; 29.30 and 23.12 respectively. The carrier mixture redness parameter (a*) of the analyzed samples increased as well with the increasing ratio of the gum Arabic. The same dependency was observed for the chromatic parameter b*. Sample 3 with an increased addition of gum Arabic had the highest values of both red parameter (a*) and yellow parameter (b*), regardless of the type of the material analyzed. However, gum Arabic could have caused differences in chromatic parameters due to its yellow color. Sample 3 (with the highest content of GA) showed the highest chroma, i.e. 41.19, 32.47, and 33.91 respectively for emulsions, powder solutions and powders. Sample 7 (with the highest content of MS and M) had the smallest chroma value. The hue angle for all samples ranged from 41.71° to 53°, which indicates that they were red to yellow in color. Sample 2 (high GA amount) was characterized by the largest tone hue angle, i.e.

47.80°and 53.00° respectively for powder solutions and powders, which shows that these samples were mostly yellow.

3.5. SEM analysis

Fig. 5 (a, b) shows some photomicrographs of β-carotene microencapsulated in a mixture of carriers GA: MS: M used in a ratio of 1: 1: 1.

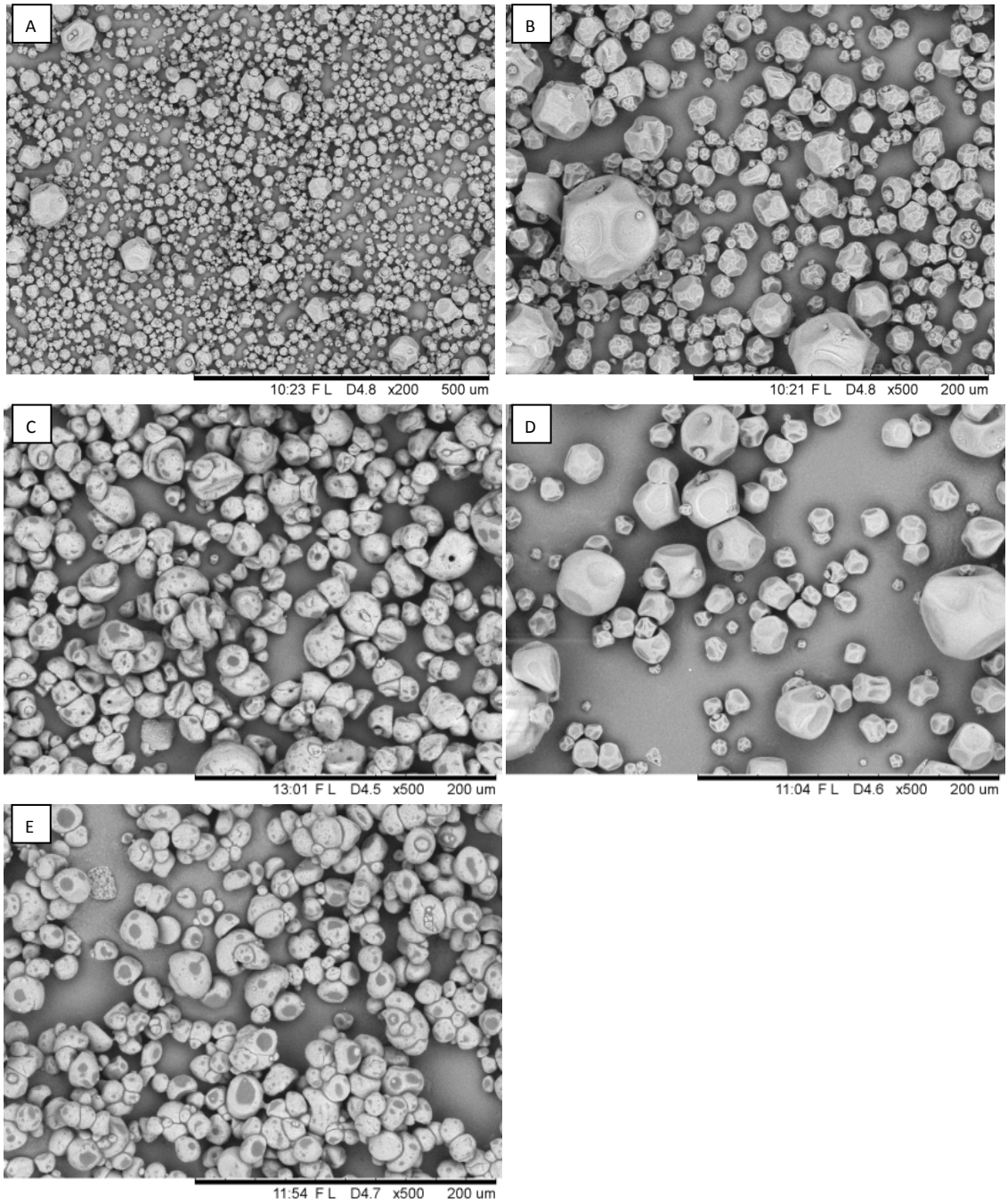

Figure 5: Morphology of microcapsules with GA:MS:M ratio of 1:1:1 (magnification at A: 200x, B: 500x), microcapsules morphology made of A) 25% gum Arabic, B) 25% modified starch, C) 25% maltodextrin (magnification at 500x), accelerating voltage 15 kV.

Observations of powder, performed with a scanning electron microscope (SEM), showed microparticles of powder of mean diameters reaching 15-20 μm and some larger ones as well, but never exceeding 60 μm. Microcapsule shape resembles a distorted sphere with characteristic holes and indentations on its entire surface. Noteworthy is the high degree of morphological similarity of microcapsules produced from a combination of gum Arabic, modified starch and maltodextrin applied in both identical and different proportions (hence this study presents only one variant). Preliminary drying experiments performed with β-carotene emulsion and single carriers (the SEM analysis is attached in Fig. 5 (c, d, e)) have confirmed that the nature and origin of the microencapsulating material strongly affect morphological properties of microcapsules produced with the use of spray-drying method, and in the case of different proportions of the three types of carriers used in the study the OSA modified starch had the key impact. Similar pictures of β-carotene microcapsules consisting solely of the OSA CAPSULTA modified starch were obtained by LIANG *et al*. (2013).

3.6. Principal component analysis (PCA)

Results obtained for carrier mixture properties were analyzed with the PCA method. Two main components explained 72.75 % of total variation: the first one (factor 1) accounted for 55.40 % and the second (factor 2) for 17.35 % (Fig. 6).
The PCA analysis proved that several expected correlations between the analyzed parameters were true. The apparent density of the particles was positively correlated with the total β-carotene content in microcapsules (measured immediately after microcapsules preparation and after 28 days of storage) and pigment retention on the surface of the microcapsules. They were negatively correlated with the brightness of solutions obtained from dissolved microcapsules (Fig. 6). Moreover, the microencapsulation efficiency was negatively correlated with pigment content measured on the surface of microcapsules immediately after their preparation and with the decay constant of β-carotene on their surface. A negative correlation was also found between total β-carotene retention in microcapsules and the decay constant of total β-carotene and pigment on the surface of microcapsules after 28 days of storage. Not all expected correlations were, however, confirmed, e.g. it had been expected the β-carotene content on the surface of the microcapsules would be correlated with chromatic parameters of their color, but it has not been confirmed in this study.
PCA classification of microcapsules allowed distinguishing two groups: the first for samples 1, 2 and 8, and the second for samples 4 and 7, which are similar to one another in terms of all examined traits. Moreover, there are some microcapsules that are distinctly different from others concerning both parameters analyzed (samples 3, 5 and 6). It was also observed that the increased content of gum Arabic (samples 2, 3, 8) caused a shift of the results to the left side of the X axis (factor 1). The first parameter was strongly negatively correlated with Rβ, T½, β28, β49, β64, aE, bE, bM, bR and positively correlated with Kβ, βp28, LE and LR. It indicates the positive effect of gum Arabic on the parameters negatively correlated with factor 2 and its negative effect on the variables correlated positively. On the contrary, microcapsules with matrices containing a higher amount of modified starch (samples 4, 5, 7) are shifted to the right part of the projection. This is related to the opposite effect of MS on the analyzed parameters compared to GA.

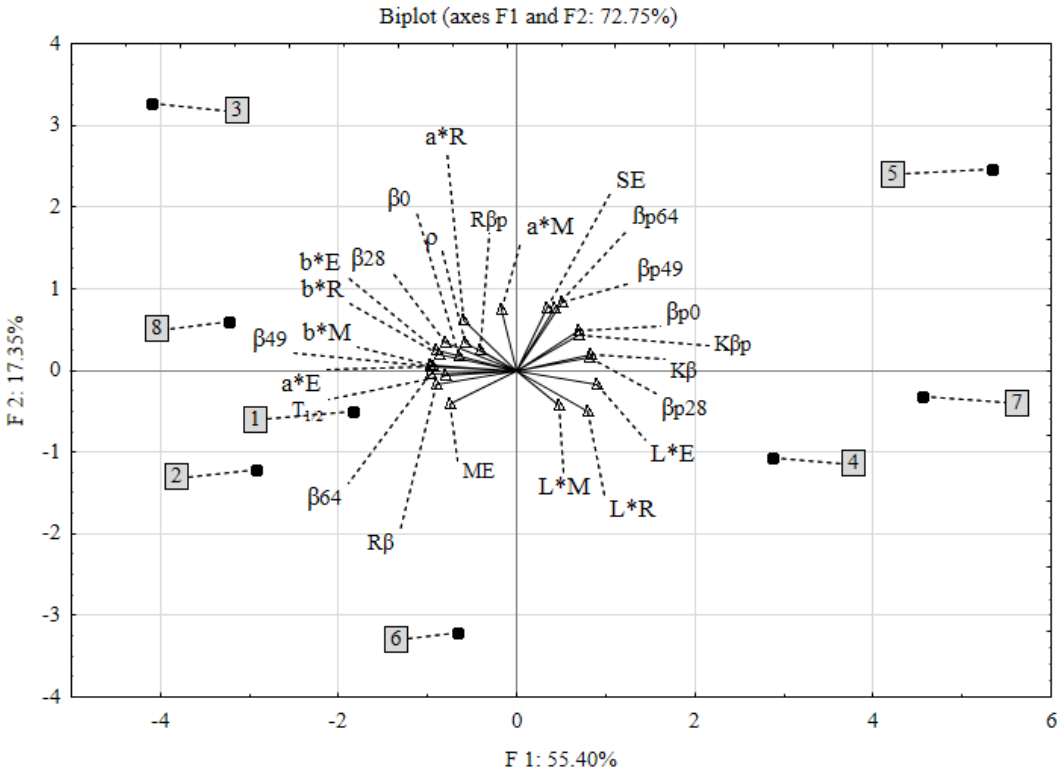

Figure 6: Examined characteristics and samples in the plot of the first two principal components.

Explanations:
SE: emulsion stability, ϱ: apparent density of powders, ME: microencapsulation efficiency, Rβ: overall β-carotene retention after 64 days of storage, Rβp: retention of β–carotene on microcapsules surface after 64 days of storage, Kβ: decay constant of total β-carotene, Kβp: decay constant of β-carotene on microcapsules surface, $T_{1/2}$: half-life, β0, β28, β49, β64: total content of β-carotene in microcapsules determined after 0, 28, 49 and 64 days of storage, βp0, βp28, βp49, βp64: content of β-carotene on the surface of microcapsules determined after 0, 28, 49 and 64 days of storage, L*E, a*E, b*E: emulsion color parameters, L*M, a*M, b*M: microcapsules color parameters, L*R, a*R, b*R: color parameters of solutions obtained after microcapsules dissolution.
Other explanations as under Fig. 1.

4. CONCLUSIONS

Stability of β-carotene emulsion depends on the type of carrier applied. Emulsion in the mixture of modified starch, gum Arabic and maltodextrins is stabilized by an increasing content of modified starch and is destabilized by an increasing content of maltodextrins.
The apparent density of β-carotene microcapsules obtained with the use of spray-drying method depended on the ratio of the carriers (modified starch, gum Arabic and maltodextrin) in the mixture. The higher content of gum Arabic in the emulsion allows obtaining a higher powder density, which results in an increase of β-carotene retention in microcapsules.
When the mixture consisted of modified starch, gum Arabic and maltodextrin in the ratio of 1: 3: 2, we obtained microcapsules that had the highest β-carotene retention, the lowest pigment degradation rate constant and the longest half-life in comparison to the other analyzed samples. Increased content of modified starch in the wall material of the microcapsules resulted in a decrease of their stability (retention reduction, increase of

microcapsule degradation rate constant - both total and on the surface) and reduced microencapsulation efficiency.
Regardless of carrier type microencapsulated, β-carotene was much more stable than in the form of oil solution.
The type of wall material has a significant effect on the value of brightness (L*) and redness parameters (a*) of microcapsules. With an increasing amount of modified starch in the carrier mixture, brightness (L*) of the emulsion, microcapsule, and solution obtained with the addition of microencapsulated β-carotene increases, while the higher content of gum Arabic causes an increase of the redness parameter (a*).

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Paper Received March 4, 2015 Accepted August 5, 2015

LISTERIA MONOCYTOGENES ADHESION TO FOOD PROCESSING SURFACES (BONING KNIVES) AND THE REMOVAL EFFICACY OF DIFFERENT SANITIZERS

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ABSTRACT

The adhesion and biofilm formation of *Listeria monocytogenes* on new and used boning knives (handle and blade) were observed. The number of pathogens on both surfaces increased with the contact time, forming a biofilm after 2 h. Peracetic acid or hot water effectively removed adhered *L. monocytogenes* at each of the contact times and concentrations evaluated. Biguanide showed lower removal efficacy on used surfaces, but had increased effectiveness at concentrations of 2.0%. The new knife blades had a lower roughness and flattened morphology in comparison with used surface

Keywords: bacterial adhesion, sanitizer, *Listeria monocytogenes*, nactivation, boning knives

1. INTRODUCTION

Food contact surfaces constantly must pass a microbiological evaluation for efficiency control and sanitizing procedures, in order to avoid contamination of the food produced (PINHEIRO *et al*., 2010; ALMEIDA *et al*., 2013). The exposure of surfaces to pathogens may take place either by direct contact with contaminated objects or indirectly through airborne particles. According to KUSUMANINGRUM *et al*. (2003), many bacteria, including *L. monocytogenes* may survive on utensils for hours or days after the initial contamination and can persist on industrial equipment and installations with high potential for adhesion and biofilm formation at low temperatures (GIAOURIS *et al*., 2014; CARPENTIER and CERF, 2011).
Cell surface hydrophobicity and production of extracellular polymeric substances influence the rate and extent of attachment of microbial cells. Another factor that can also influence bacterial adhesion is the surface roughness (RODRIGUEZ *et al*., 2008; SKOVAGER *et al*., 2013).
Bacterial adhesion to stainless steel, glass, rubber and polypropylene surfaces is a potential source of contamination that may lead to disease transmission (CHAVANT *et al*., 2007). The adhered cells are highly resistant to acids present in sanitizers, to desiccation and to heat. Tolerance to increased sub-lethal concentrations of disinfectants or resistance to lethal concentrations are documented, because sessile cells produce exopolysaccharides that protect against chemical agents (JOSEPH *et al*., 2001; CARPENTIER and CERF 2011; SILVA *et al*., 2010). Several sanitizers are available with different uses, but they do not always eliminate bacteria to the expected level (BELTRAME *et al*., 2012). In this way, it is very important to know the factors involved in the adherence of biofilms to surfaces, which could be useful to improve methods for disinfection of food processing surfaces (boning knives). Therefore, the purpose of this study was to investigate adhesion and biofilm formation of *L. monocytogenes* on the handle and blade of new and used boning knives. This study also evaluated the removal of bacteria on the surface of the knife using different contact times and concentrations of chemical sanitizers (peracetic acid and biguanide) or hot water.

2. MATERIALS AND METHODS

In this study, bacterial adhesion and inactivation were evaluated on new and used boning knives using Gram-positive *L. monocytogenes* (ATCC 7644). The bacterial culture was purchased from the Instituto Oswaldo Cruz, mantained at -80°C and revitalized in Luria Bertani broth (LB Merck, Darmstadt, Germany) at 30°C, 24 hours before the experiments.

2.1. Bacterial adhesion and quantification of adhered cells

In order to assess bacterial adhesion, the polypropylene (PP) handle and stainless steel (SS) blade of new boning knives (Professional line Mundial® model 5315-6) and the same model of used knives (use in a cattle slaughterhouse for 45 to 60 days) were studied.
The entire knife surface (handle and blade) was prepared according to the follows steps: cleaning by manual rubbing with water and neutral liquid detergent, rinsing with water and then by sterile distilled water and air drying. For sanitization, the surfaces were exposed to ultraviolet light (254 nm) for one hour. After cleaning, the entire knife surface was swabbed in order to confirm the absence of initial contamination (negative control), before carrying out the experiments.

The bacteria were incubated in LB broth for 24 h at 35-37°C. Next, 10 mL of inoculum (3 log CFU/mL) was inoculated into 1.5 L LB broth that had been previously poured into a polyvinylchloride (PVC) tube with a 10 cm diameter to accommodate the entire knife, and the samples were incubated for 24 h at 35-37°C. Afterward, the knives (new or used) were removed from the PVC tube with sterile forceps and rinsed with water to remove the planktonic cells. The entire surface of the knife handle and knife blade were swabbed separately to assess contamination. Dilutions were performed in peptone water, plated onto LB agar, and incubated at 35-37°C for 24 h. The number of adherent cells was assessed in intervals of 0.1, 0.5, 1, 2, 6, 24 and 48 h at 35°C. These times were selected in order to simulate the factory production time, which is the period that knives remain in contact with products without sanitization.

2.2. Characterization of the boning knife surface by contact angle

The hydrophobicity and hydrophilicity of the new and used blades with and without *L. monocytogenes* adhesion for 6 h, were determined by contact angle with a drop of water using a contact angle metre (KSV Instruments, Helsinki, Finland). The measurements were performed at 25°C and 45% humidity, by depositing 4.0 μL of water with a Hamilton syringe. The handles were not evaluated in this analysis because the texture of the handle produces differences in surface that did not permit the stable formation of a drop on the surface.

2.3. Characterization of the boning knife surface by Atomic Force Microscopy (AFM)

The morphology and average roughness (Ra) of the new and used blades with and without *L. monocytogenes* adhesion for 6 h were analyzed with a Dimension V (Veeco Instruments Inc.) AFM, using a silicon nitride tip, with a spring constant of 42 N/m and resonance frequency of 285 kHz. All images were obtained in tapping™ mode at a scan rate of 1 Hz. The images were processed with the aid of Gwydion© 2.1 data analysis software. The handles were not evaluated due to the characteristics of the non-slip coating that had very large differences in height, which did not permit a surface scan.
The Ra value (arithmetic mean deviation of the profile) is the most common measure used to define the surface roughness (VERRAN *et al*., 1991). AFM scans were performed in 500 x 500 nm^2 areas on each surface. The roughness was calculated from three scans in different areas.

2.4. *L. monocytogenes* inactivation

In this study, peracetic acid (Pluron 461 AP®) and biguanide (Pluron 463 AP®) sanitizers were studied; they were prepared in sterilized water immediately prior to testing, according to the supplier's instructions. In inactivation experiments, the entire knife was analyzed at intervals of 1, 2 and 6 h, simulating industrial conditions. After rinsing with deionized water, the knives were immersed in beakers containing 500 mL of the respective sanitizer solution at concentrations of 0, 0.2, 0.5, 1.0 or 2.0% (v/v) for 10 min at 25°C, to evaluate their efficacy against cell attachment.
The hot water treatment was performed by immersing the knife surface in a hot water bath (82.2°C) for 15 s, according to 175/2005/MAPA method 2 (BRASIL 2005).
Bacterial presence was quantified by the enumeration method as previously described (KIM *et al*., 2008) using swabs of the knives.

2.5. Statistical analysis

The results of *L. monocytogenes* counts were converted to decimal logarithmic values (log CFU/cm^2) and subjected to Tukey's test at a 5% significance level using Statistica 8.0 software (StatSoftInc®, USA). All experiments were run in triplicate and repeated with three separate Knives.

3. RESULTS AND DISCUSSIONS

3.1. *Listeria monocytogenes* adhesion

The adhesion of *L. monocytogenes* on new and used boning knives (handles and blades) is shown in Fig. 1. A rapid bacterial growth was observed for the first 6 h, with a tendency to stabilize at 48 h. Adherence occurred on handles and blades and the adhesion velocities were similar for used and new materials. However, statistical analysis showed a significant difference in adherence to new and used polyethylene handles between 6 and 48 h (Fig. 1a). The knives a non-slip coating on the surface of the handle, which conveys firmness and grip during handling, however, this feature promoted easy adhesion of bacteria on used surfaces.

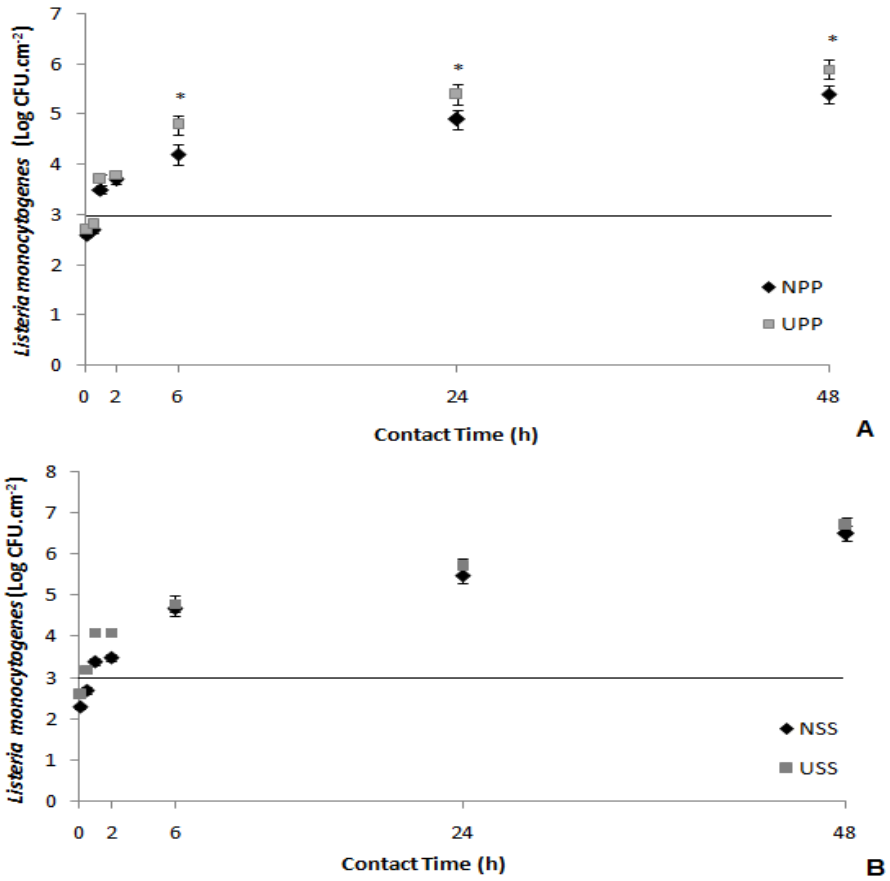

Figure 1: Adhesion kinetics of *L. monocytogenes*on(A) new polyethylene handles (NPP) and usedpolyethylene handles (UPP) and (B) newstainless steel blades (NSS) and usedstainless steel blades(USS). Biofilm formation with 3.0 Log CFU/cm^2. Means (± standard deviations), for each time tested, who having the * symbol are significantly different ($p<0.05$).

L. monocytogenes showed a greater capacity for adhesion onto SS than to PP (Fig. 1) from 6 h in new, and 24 h in used knives. These results confirm those by Teixeira *et al*. (2008), who observed greater adhesion on SS as compared with the extent of adhesion on PP (cutting board).
RONNER and WONG (1993) defined biofilm formation as a recovery of greater than 3.0 log CFU/cm^2 adhered cells. Thus, according to this criterion, *L. monocytogenes* biofilm growth occurred after 1 h of contact on the handles (new and used), and from 30 min (new) to 1 h (used) on the SS surface.

3.2. Characterization of the surface of the boning knife by contact angle

Water contact angle is a quantitative measurement of surface wettability, and also can be used to evaluate the cleanliness of the material surface. Throughout this study, a contact angle of 70±1.0 and 82±1.0 degrees was obtained for new stainless steel (NSS) and used stainless steel (USS) surfaces without microbial adhesion, respectively. These results agree with those found by BERNARDES *et al*. (2010), who found a value of 70±7.9 on SS surfaces. After 6 h of bacteria exposure, contact angles of 19±1.4 and 30±2.1 were obtained on NSS and USS respectively, showing that the surfaces became more wettable after microbial adhesion. These results corroborate with those of CHAVANT *et al*. (2007), who observed better adhesion and biofilm formation of *L. monocytogenes* on hydrophilic (SS) rather than on hydrophobic (PP) surfaces. These decreases in the value of contact angle from 70 down to 19 (NSS) and 80 down to 30 (USS) may be attributed to the *L. monocytogenes* surface composition (molecules such as proteins, teichoic and lipoteichoic acids) (Bereksi *et al*., 2002).

3.3. Morphological and roughness characterization

The morphology of new and used blades was analyzed through use of AFM before and after *L. monocytogenes* adhesion (Fig. 2).
Roughness values (Ra) of 5.1±2.3, 14.3±1.7, 17.4±2.4 and 28.8±2.1 nm were obtained from new and used blades without and with *L. monocytogenes* adhesion, respectively. New blades had a low roughness and flattening morphology compared with used surfaces, which is very important to note because studies have shown that an increase in Ra value will cause a corresponding increase in microbial adhesion on surfaces (Whitehead *et al*., 2004). This increase may be due to protective cells present in microscopic niches.
Roughness values of 800 nm or less are generally used to describe a hygienic surface (FLINT *et al*., 1997). These values were found for all surfaces evaluated in this work (Fig. 2). Teixeira *et al*. (2008), observed that SS is a material with high surface roughness, similar to what was measured in this study for the surface of the knife blade (Mundial®).
TAYLOR *et al*. (1998) observed a significant increase in the attachment of *Pseudomonas aeruginosa* and *Staphylococcus epidermidis* on polymethyl methacrylate when the surfaces had a small increase in roughness (40-1240 nm). Bacterial adhesion has been found to increase numerically with surface roughness, by comparing bacterial adhesion on used versus abraded SS and for Ra value increases from 602 to 706 nm or from 484 to 698 nm (Holah and Thorpe, 1990). Thus, it was found that the roughness data obtained after the adherence of *L. monocytogenes* are in agreement with those from the literature, but no correlation was demonstrated between Ra and the number of adhering cells. From this evaluation of the surface characteristics of boning knives, it can be observed that with an increase of the surface roughness, there was a decrease in the contact angle.

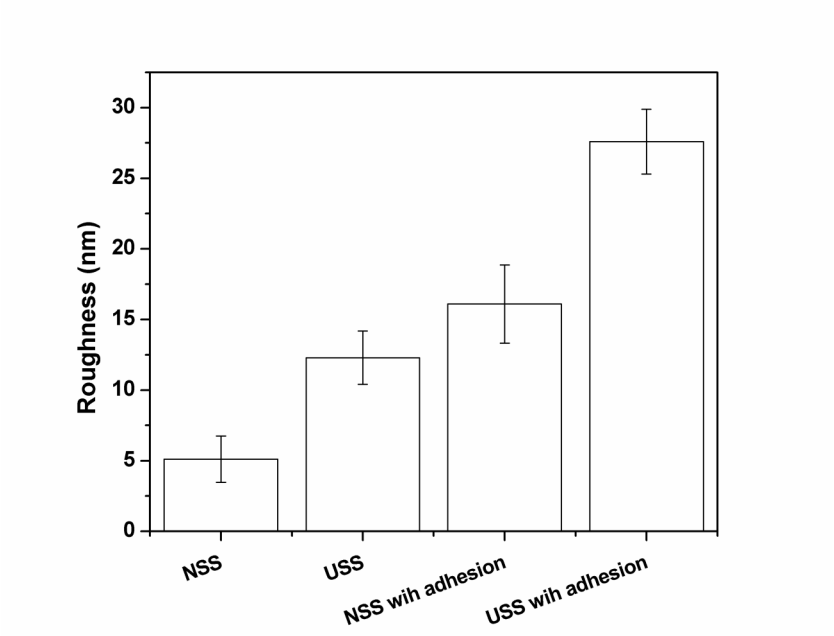

Figure 2: Roughness values on NSS and USS with and without*L. monocytogenes* adhesion. Mean values and standard deviations (error bars) are indicated. Observations correspond to on 500 x 500 nm scan area.

3.4. Efficacy of different sanitizers on food processing surfaces

To evaluate their ability to sanitize new and used knives, biguanide and peracetic acid sanitizers were used at different concentrations (0.2, 0.5, 1.0 and 2.0% v/v) for 10 min, or hot water (82.2°C) was used for 15 s. The boning knives remained in contact with *L. monocytogenes* for different contact times (1, 2 and 6 h) to simulate industrial conditions for adhesion. *L. monocytogenes* was resistant to biguanide sanitizer under the different contact times studied (Table 1).

Similar efficacy levels have been obtained by other researchers. MARTÍN-ESPADA *et al*. (2014) observed that 1.61% peracetic acid was effective against *P. aeruginosa* biofilms formed on polystyrene surfaces, inhibiting almost 100% of the microbial population. Similarly, BELTRAME *et al*. (2015) evaluated the efficacy of 0.5% peracetic acid at inactivating *L. monocytogenes* cells adhered to cutting boards and observed that it was able to reduce the amount of adhered cells by 100%, after 3 h of contact time with bacteria. Cabeça *et al*. (2012) observed adhesion of *L. monocytogenes* on SS surfaces and studied the inactivation after treatment with 0.5% biguanide and 0.5% peracetic acid. The authors verified an initial count of 6.2 log CFU/cm^2 with reduction to 2.9 log CFU/cm^2 and 1.1 log CFU/cm^2 using biguanide and peracetic acid, respectively, showing that peracetic acid was more effective against *L. monocytogenes* cells.

For the sanitizer evaluated, the suppliers recommend a maximum disinfection concentration of 0.5% for both biguanide and peracetic acid. Thus, there was a variation in the effectiveness of the procedures, based on decimal reductions, in microbial effect conveyed by the different sanitization process investigated, as demonstrated in Table 2.

In the maximum contact time (6h) between the knife and the microorganism, the low concentration of peracetic acid (0.2%), lower than that recommended by the supplier, showed reliable results from the point of microbiology safety. Similar results were observed for hot water, where the presence of surviving *L. monocytogenes* was not observed. On the other hand, the maximum concentration of biguanide recommended by

the supplier was not able to remove adherent cells, with maximum efficacy of only 50% on NPP (Table 2).

Table 1: Reduction of the *L. monocytogenes* count (Log CFU/cm²) as a function of contact time (1, 2 and 6 h) and the Biguanide sanitizer concentration (0.2, 0.5, 1.0 and 2.0%).Results in Log CFU/cm².

Contact time	Boning knives	Initial count	Biguanide Concentration (%) R* 0.2%	%	R* 0.5%	%	R* 1.0%	%	R* 2.0%	%
1h	NPP	3.5	2.0±0.2^{c}	57,1	2.7±0.1^{b}	77.1	3.3±0.1^{a}	94,3	3.5±0.3^{a}	100
	UPP	3.7	0.5±0.1^{c}	14.3	1.7±0.1^{b}	45,9	1.8±0.2^{b}	48.6	3.0±0.1^{a}	81.1
	NSS	3.4	0.6±0.1^{c}	17.6	1.3±0.1^{b}	38.2	3.2±0.1^{a}	94.1	3.4±0.1^{a}	100
	USS	4.1	0.2±0.1^{d}	4.9	1.7±0.1^{c}	41.5	2.8±0.1^{b}	68.3	4.0±0.1^{a}	97.6
2h	NPP	3.7	1.7±0.1^{b}	4.6	2.4±0.2^{ab}	64.9	2.8±0.2^{a}	75.7	2.7±0.1^{a}	73.0
	UPP	3.8	0.8±0.1^{c}	21.0	1.8±0.1^{b}	47.4	1.8±0.1^{b}	47.4	2.7±0.1^{a}	71.0
	NSS	3.5	0.2±0.1^{d}	5.7	0.9±0.1^{c}	25.7	1.9±0.1^{b}	54.3	3.3±0.1^{a}	94.3
	USS	4.1	0.6±0.1^{d}	14.6	1.6±0.1^{c}	39.0	2.1±0.1^{b}	51.2	3.3±0.1^{a}	80.5
6h	NPP	4.2	1.1±0.1^{c}	26.2	2.1±0.1^{b}	50.0	2.5±0.2^{a}	59.5	2.8±0.1^{a}	66.7
	UPP	4.8	0.1±0.1^{c}	2.1	2.3±0.2^{b}	47.9	2.7±0.2^{ab}	56.2	3.1±0.2^{a}	64.6
	NSS	4.7	1.0±0.1^{d}	21.3	2.0±0.1^{c}	42.6	2.7±0.1^{b}	57.4	3.9±0.1^{a}	83.0
	USS	4.8	0.7±0.1^{c}	14.6	0.5±0.1^{c}	10.4	2.4±0.1^{b}	50.0	3.7±0.1^{a}	77.1

NPP: new handles; UPP: used handles, NSS: new blades; USS: used blades.
*R Decimal reduction (Log CFU initial population-Log CFU end population submitted to the sanitizers application). Values followed for the same letters in the lines do not differ statistically according to theTukey Test, with 95% confidence range.

Table 2: Reduction of the *Listeria monocytogenes* count (Log CFU/cm²) in boning knives after hot water and sanitizer treatments (6 h of contact time, sanitizer concentrations - 0.5% biguanide and 0.2% peracetic acid).

Boning Knives	Initial Count	Biguanide R*	%	Peracetic acid R*	%	Hot water R*	%
NPP	4.2	2.1±0.1	50.0	4.2±0	100	4.1±0.1	100
UPP	4.8	2.3±0.2	47.9	4.8±0	100	4.8±0	100
NSS	4.7	2.0±0.1	42.6	4.7±0	100	4.7±0	100
USS	4.8	0.5±0.1	10.4	4.8±0	100	4.8±0	100

NPP: new handles; UPP: used handles; NSS: new blades; USS: used blades.
* Decimal reduction (initial population Log CFU - end population Log CFU).

Thus, this work confirms that the most effective treatments were the hot water and peracetic acid sanitizer, whereas biguanide showed lower performance. Additional studies should be performed to improve the action of biguanide on removal of *L. monocytogenes* from food preparation surfaces.
The treatments with hot water and peracetic acid were shown to be effective for the inactivation of bacteria that had initially adhered. This result can be associated to the disinfectant activity of peracetic acid based on the release of active oxygen, which may

disrupt the chemiosmotic function of the lipoprotein cytoplasmic membrane and transport through the dislocation or rupture of cell walls. This can also be effective against outer membrane lipoproteins, facilitating its action against Gram-negative cells. The intracellular peracetic acid may also oxidize essential enzymes, which may damage the bases of DNA molecules (KITIS, 2004). Moreover this sanitizing agent has low environmental hazards and does not produce toxic compounds upon reaction with organic materials.

Likewise, the application of hot water was also an effective means of bacterial inactivation. The mechanism of action of hot water treatment is multifactorial. The exposure to this high temperature is likely to affect most components of the bacterial cell including the cell wall, cell membrane, enzymes and proteins, DNA and RNA (PHUA *et al*., 2014).

Differences between the bactericidal effect of biguanide solutions and peracetic acid were observed, corroborating data obtained by SILVA *et al*. (2010), who showed that peracetic acid was a more effective bactericidal agent than the quaternary ammonium compound (whose mechanism of action is similar to biguanide).

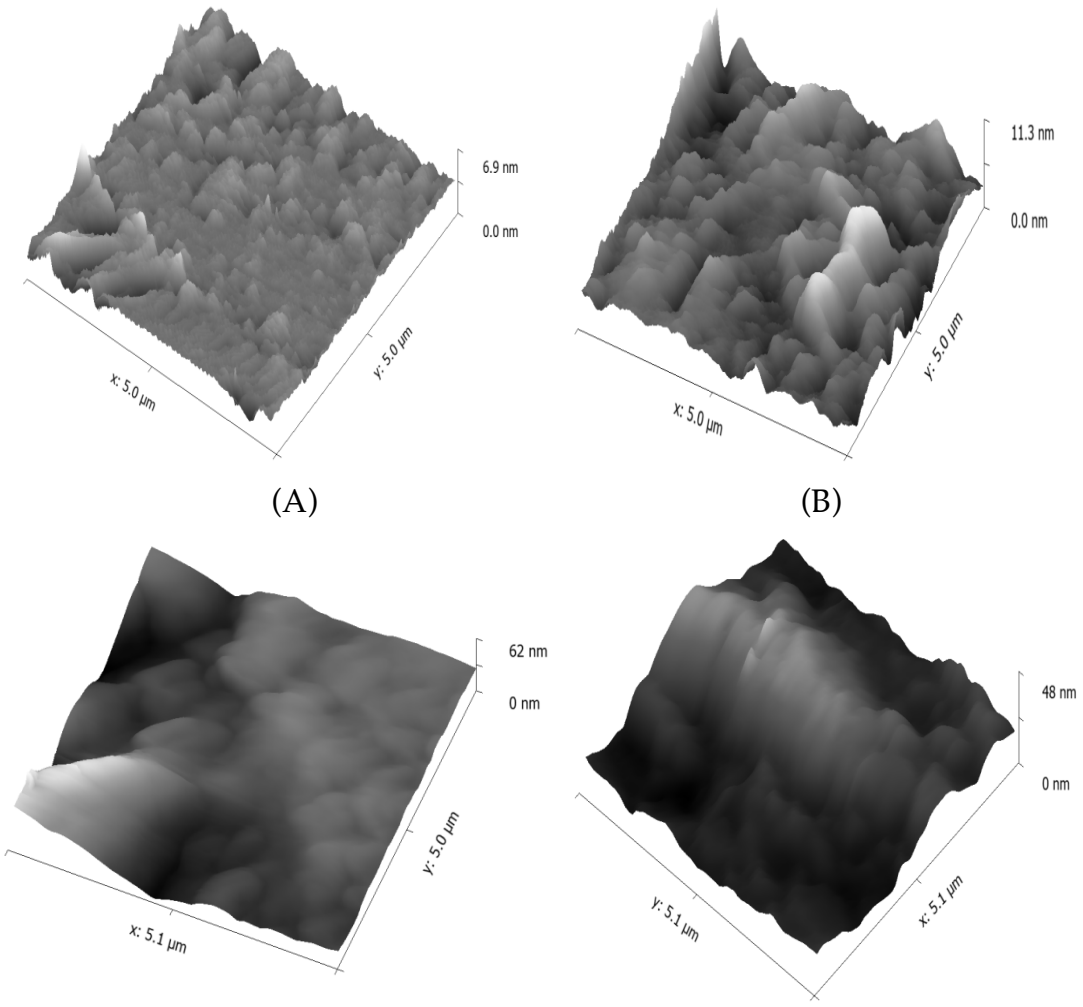

Figure 3: AFM characterization of the knives surface in 3D (A) new without bacterial adhesion, (B) used without bacterial adhesion, (C) new with bacterial adhesion and (D) used with bacterial adhesion.

Sodium dichloroisocyanurate, hydrogen peroxide and peracetic acid have been evaluated for their abilities to inactivate the biofilm formed by *S. aureus* on SS and glass surfaces; peracetic acid showed a significant difference ($p < 0.05$) compared with the three disinfectants used (MARQUES *et al*., 2007). The higher efficacy of peracetic acid was explained by its high capacity to oxidize cellular molecules.
The survival of bacteria after cleaning and sanitizing is a potential danger to the food industry and the consumer (RODGERS *et al*., 2001). This study demonstrates the need for specific tests in order to select products to be used to clean surfaces that come into contact with food. Furthermore, bacteria can obtain high resistance through adaptation, genetic elements, stress response and biofilm formation (BRIDIER *et al*., 2011).
It should be noted that an appropriate and effective cleaning process is extremely important, since the American Public Health Association (APHA) recommends a maximum tolerated limit of 2 CFU/cm^2 in order to consider a food contact surface appropriate (VANDERZANT and SPLITTSSTOESSER, 1992), whereas the World Health Organization (WHO) suggests limits of 30 CFU/cm^2. Based on the obtained results, this study confirms that hot water and peracetic acid was fully effective in removing *L. monocytogenes* cells, which had adhered onto new and used boning knives, whereas biguanide was not efficient in removing the bacterial cells.

4. CONCLUSIONS

Adherence of *L. monocytogenes* occurred on handles and blades, and the adhesion velocities were similar between the used and new materials. Regarding the morphology and contact angle of the surfaces, an increase of the wettability and roughness on the used stainless steel surface in relation to the new stainless steelsurface was observed.
Disinfection with peracetic acid was effective at all contact times and concentrations evaluated; no surviving bacteria was found after sanitizer application in all conditions investigated. The hot water treatment (82.2°C for 15 s) also was effective in reducing *L. monocytogenes* adhesion on the surfaces tested. Biguanide showed lower efficacy on new and used handles and blades, but had increased effectiveness at concentrations of 2.0% with 1 h of contact time.

ACKNOWLEDGEMENTS

The authors thank CNPq, CAPES, FAPERGS, Science and Technology Secretary RS and URI-Erechim for the financial support for this research.

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Paper Received March 21, 2015 Accepted June 12, 2015

SHORT COMMUNICATION

CLARIFYING AGENTS AND 3-SULFANYLHEXANOL PRECURSORS IN GRAPE JUICE

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ABSTRACT

We evaluated the impact of a number of clarifying agents on the concentration of S-3-(hexan-1-ol)-L-cysteine (Cys-3SH) and S-3-(hexan-1-ol)-L-glutathione (GSH-3SH). 19 clear grape juices were spiked with a grape skin tannin rich in Cys-3SH and GSH-3SH. Juices were then treated with Na-bentonite, PVPP or charcoal (1 g/L) and cold settled. The concentration of precursors was measured and compared to the corresponding untreated control juices in the devatted samples. Cys-3SH and GSH-3SH were analysed using UHPLC-MS/MS and accuracy was guaranteed with deuterated internal standards. Only charcoal caused a statistically significant depletion of both precursors, quantitatively limited even at the highest dose adopted. Technologically, the clarifiers used in juice affected the thiol precursors in a marginal manner.

Keywords: bentonite, charcoal, grape juice, polyvinylpolypyrrolidone, varietal thiols

1. INTRODUCTION

S-3-(hexan-1-ol)-l-cysteine (Cys-3SH) and S-3-(hexan-1-ol)-l-glutathione (GSH-3SH) are precursors, present in grapes and/or formed in juice (TOMINAGA and DUBOURDIEU, 2000; PEYROT DES GACHONS *et al*., 2002; SCHNEIDER *et al*., 2006; FEDRIZZI *et al*., 2009; ROLAND *et al*., 2011a), of 3-sulfanylhexanol (3SH), responsible - together with its acetate - for the tropical and grapefruit-like fruity notes produced during fermentation by some yeast strains having lyase activity (RONCORONI *et al*., 2011; WINTER *et al*., 2011). The grape variety, as well as the processing conditions of grape, pomace and juice, are very important in saving/producing a high level of precursors (ROLAND *et al*., 2011a; CERRETI *et al*., 2015; ROMÁN VILLEGAS *et al*. 2016). Technologically speaking, for example, 3SH precursors increase with longer skin-contact and stronger pressing conditions (MATTIVI *et al*., 2012) and GSH-3SH in particular increases when oxidative pre-fermentative maceration is adopted (LARCHER *et al*., 2013a). Nevertheless, the effects of the main clarifying agents on the content of the precursors cited are little known to date. For this reason, the aim of the experiment reported in this paper was to investigate whether certain common clarifiers used in juice can modify the concentration of Cys-3SH and GSH-3SH.

2. MATERIALS AND METHODS

2.1. Juice preparation

Nineteen lots of must (20 L) were produced from sound white and red grapes coming from different varieties and plots in Trentino (Northern Italy), selected in order to include a wide compositional variability. Grape lots (300 Kg) were destemmed, crushed and pressed (3.5 bar; press mod. UP600, Willmes, Lorsch, Germany) on a semi-industrial scale at the Edmund Mach Foundation experimental winery (San Michele all'Adige, Italy). To ensure a high concentration of thiol precursors, only the juice fraction over 65% w/v yield was used in the experiment (MAGGU *et al*., 2007; ALLEN *et al*., 2011; ROLLAND *et al*., 2011b). Moreover, the juices were indirectly enriched randomly with 500-1000 mg/L of grape skin tannin containing 224.2 mg/kg GSH-3SH and 25.5 mg/kg Cys-3SH, quantified according to LARCHER *et al*., (2013b), and supplemented with a volume of 15-25% of Sauvignon Blanc juice, a variety well-known for its richness in thiol precursors (CAPONE *et al*., 2010; LARCHER *et al*., 2013a). After sulfiting (20 mg/L SO_2), all juices were cold settled (< 20 nephelometric turbidity units), well beyond normal winemaking practice, in order to minimise the effect of solids suspended in the turbid juice. Clear juices were then devatted, divided into 4 fractions of 5 L each and supplemented with activated Na bentonite (Pentagel, 1 g/L; Perdomini-IOC S.p.A., S. Martino Buon Albergo, Italy), charcoal (Eno Anticromos, 1 g/L; Dal Cin S.p.A., Concorezzo, Italy) or polyvinylpolypyrrolidone (PVPP V, 1 g/L; Perdomini-IOC) in comparison with the unspiked fraction respectively. After treatment, all samples were cold settled again for 48h at 4°C.

2.2. Sampling

The settled juice was sampled (25 mL), supplemented with methanol (25 mL, -20°C) and stored at -20°C until analysis. The methanol solution was spiked with d_3-GSH-3SH and d_3-Cys-3SH as labelled internal standards and filtered through a 0.22 μm filter (Millex-GV, Millipore, Ireland) before analysis.

2.3. Chemical analysis

The juice composition was analysed using a WineScan FT 120 Type 77310 (Foss, Hillerød, Denmark), accurately aligned according to the official methods (OIV 2012).
An UPLC Acquity system coupled with a Xevo TQ MS mass spectrometer (Waters Corporation, Milford, USA) was used for LC-MS/MS quantification of thiol precursors. A 5 μL sample was injected into an Acquity UPLC HSS T3 C18 column (1.8 μm film thickness, 2.1 mm × 100 mm; Waters) set with a flow rate of 0.45 mL/min and a temperature of 40°C. MS isotopic dilution analysis was performed in positive ion mode (capillary voltage, 2.5 kV), using argon (0.20 mL/min) and nitrogen (1,000 L/h) as collision and desolvation gas respectively. Other characteristics of the method are specified in LARCHER *et al*. (2013a).

2.4. Statistical analysis

Anova (main effects: juice, clarifier) and Tukey's HSD test were carried out using STATISTICA v. 8.0 (StatSoft Inc., Tulsa, OK).

3. RESULTS AND CONCLUSIONS

The clarifiers were chosen because they are extensively used in winemaking during prefermentation manipulation of white grape must, due to their depletion features in relation to specific classes of compounds (bentonite vs. proteins; PVPP vs. polyphenols) or to their high but non-selective adsorption capacity (charcoal). To our knowledge, there are no reports that specifically link fining agents and thiol precursor content, while their depletion capacity has been previously reported in relation to free and bound primary aromas (MOIO *et al*., 2004) and other odour active compounds in juice (LAMBRI *et al.*, 2010).
The juices were chemically characterised by their base composition (mean ± st. dev.; min - max) for total soluble solids (21.2±2.0 °Brix; 18.6-26.0), pH (3.23±0.11; 3.01-3.45), titratable acidity (6.63±1.23 g/L; 4.70-10.00), tartaric acid (5.76±0.69 g/L; 4.93-7.62), malic acid (3.25±0.76 g/L; 1.93-4.68), potassium (1348±158 mg/L; 1086-1618). These data highlight the considerable compositional variability used to ensure the robustness of the results, since the grape cultivar and ripeness not only affect the precursor content (KOBAYASHI *et al.*, 2010; CERRETI *et al.*, 2015) but also influence either the composition (Pirie and Mullins, 1977; POCOCK *et al.*, 2000) or the haze (MESQUITA *et al.*, 2001) of the most usual target molecules for these clarifiers and hence the clarifying activity.
The ranges obtained for GSH-3SH (min-max: 240 - 564 μg/L) and Cys-3SH (36,5 - 244 μg/L) in the control juices match the literature (PEÑA GALLEGO *et al.*, 2012; LARCHER *et al.*, 2013a;). Comparison of the results of the corresponding control juices and treated samples showed that bentonite and PVPP had a limited and not statistically significant effect (Table 1) on the concentration of GSH-3SH and Cys-3SH. On the contrary, charcoal treatment significantly reduced (p<0.05) the two thiol precursors, however this reduction was limited, being roughly 20% for GSH-3SH and 10% for Cys-3SH.
The significance of these results is not limited to winemaking, but could also be of interest for the grape juice industry, where there is the possibility of using hybrid varieties, resistant to mold diseases and consequently with lower operating costs. Precursors are also present in their juices (LARCHER *et al*., 2014), and the release of 3SH through specific commercial enzymes could contribute to overall aroma.

Table 1: Thiol precursors in juice in relation to the clarifying agent used. (Values with the same letter are not statistically different in Tukey's HSD test, $p<0.05$; n.s. = non significant).

	GSH-3SH (μg/L)			Cys-3SH (μg/L)		
Treatment	**Mean (n=19)**	**S.D.**	**sign.**	**Mean (n=19)**	**S.D.**	**sign.**
Control	344	73	a	80	53	a
Bentonite (1 g/L)	336	75	a	79	53	a
Charcoal (1 g/L)	276	66	b	73	46	b
PVPP (1 g/L)	344	74	a	80	53	a

In conclusion, of the clarifying agents used in this experiment, only charcoal proved able to significantly reduce 3-sulfanylhexanol precursors in juice. Nevertheless, in the light of the usually lower doses of these products adopted for juice in modern white winemaking, the low conversion ratios of the precursors to the corresponding free thiols (ROLAND *et al*., 2011a), and the limited percentage changes observed in this experiment, it can be deduced that the clarifying agents used affect the content of thiol precursors in a technologically and sensorially negligible manner, despite the low sensory threshold of the relative derivatives in free and acetate form.

ACKNOWLEDGEMENTS

The authors wish to thank Cavit s.c. and D. Zatelli for their cooperation.

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Paper Received March 17, 2016 Accepted May 20, 2016

ITALIAN JOURNAL OF FOOD SCIENCE

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(4) Standard Usage, Abbreviations and Units. The Concise Oxford and Webster's English Dictionaries are the references for spelling and hyphenation. Statistics and measurements should always be given in figures, e.g. 10 min, except when the number begins a sentence. When the number does not refer to a unit of measurement it should be spelled out unless it is 100 or greater. Abbreviations should be used sparingly, only when long or unwieldy names occur frequently, and never in the title; they should be given at the first mention of the name. International Standard abbreviations should generally be used except where they conflict with current practice or are confusing. For example, 3 mm rather than $3x10^{-3}$ m. Abbreviations should be defined the first time they are used in the text and they should be used consistently thereafter. Temperatures should be expressed in the Celsius (centigrade) scale. Chemical formulae and solutions must specify the form used, e.g. anhydrous or hydrated, and the concentration must be in c1early defined units.
Common species names should be followed by the Latin binomial (italics) at the first mention. For subsequent use, the generic name should be contracted to a single letter if it is unambiguous.

3. Editorial and Review Policy

Scientific contributions in one of the following forms may be submitted:
Reviews –They can be submitted directly to the Editor-in-Chief or articles can be requested directly by the Editor-in-Chief.
Short Communications, Surveys and Opinions – They do not need to have the formal organization of a research paper; they will receive priority in publication; maximum of five pages allowed.
Papers – The paper must follow the guidelines as specified under the section Manuscript Preparation.

Reviews, Papers, Short Communications and Surveys will be **subjected to critical review by referees.**
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Gratitude is expressed to the following entities for contributing to the realization of the Journal by being supporting subscribers for 2016.

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Milano
Fax +39-02-50316427

ITALIAN JOURNAL OF FOOD SCIENCE
Rivista Italiana di Scienza degli Alimenti
DIRETTORE RESPONSABILE: Alberto Chiriotti
AUTORIZZAZIONE: n. 3/89 in data 31/1/1989
del Tribunale di Perugia
TIPOGRAFIA Giuseppini - Pinerolo

ISSN 1120-1770 © 2016

CHIRIOTTI EDITORI srl - 10064 Pinerolo - Italy

publishes the technical magazines:

VOLUME XXVIII IJFS No. 4, 2016

CONTENTS