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REVIEW

BIODEGRADABLE PACKAGING AND EDIBLE COATING FOR FRESH-CUT FRUITS AND VEGETABLES

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ABSTRACT

This work focuses on biodegradable packaging and edible coatings applied to fresh-cut fruits and vegetables and their effects on the product quality. Practical applications are mainly limited to the use of biodegradable materials that, however, do not allow full control of the product moisture loss. Better results can be achieved by the combined use of biodegradable packagings with edible coatings and recent research has shown that enrichment with silver-montmorillonite nanoparticles may be a promising technique. However, the actual utilization of these materials is still limited, due to the high costs of the raw materials and the limited production.

- Keywords: biodegradable packaging, biopolymers, edible coating, fresh-cut fruits and vegetables, minimally processed foods, nanocomposites -

INTRODUCTION

In recent years, the establishment of new lifestyles, characterized by a lack of time in domestic preparations, has been accompanied by an evolution of the structure of food consumption and a destructuring of meals. Therefore, the consumption of minimally processed fruits and vegetables has grown rapidly as a result of the consumer trend- "rich in cash, poor in time" (MARTÍN-DIANA *et al.,* 2007; CONTE *et al.,* 2009).

Minimally processed fruits and vegetables were developed in the 1980s to respond to the emerging consumer demand for both convenience and high quality aspects (DEL NOBILE *et al.,* 2008). These foodstuffs combine fresh-like and healthy characteristics with a minimal time of preparation before consumption (RAGAERT *et al.,* 2004). In fact, their success is also due to the beneficial health effects for the presence of antioxidants that act as receptors of free radicals; in particular, ascorbic acid and β-carotene are the antioxidants present in the greatest quantities in fruits and vegetables (RICO *et al.,* 2007).

The minimal processing operations ("mild technology") necessary to produce fresh-cut foods, such as peeling, cutting, washing, treatments with sanitizing agents, drying, alter the physical integrity of these products, making them more perishable than the original raw materials (CORBO *et al.,* 2006). This is due to respiration, transpiration, enzymatic activity of the living tissue after harvest and processing and, at the same time, to proliferation of spoilage and pathogenic microorganisms (GALGANO *et al.*, 2014; NGUYEN-THE and CARLIN, 1994). The industrial process accelerates the degradation of the minimally processed foods and leads to biochemical changes such as:

- increasing of respiration that accelerates the oxidation processes;
- degradation of cell membranes and enzymatic browning;
- loss of tissue texture.

The knowledge of factors influencing quality degradation after processing of fruits and vegetables is essential to develop technologies for shelf-life extending and maintaining quality during processing and distribution (CORBO *et al.,* 2006).

In order to reduce the microbiological, chemical and physical spoilage, it is possible to act on processing or, more usually, on packaging that represents a barrier to qualitative decay of the product (GALGANO *et al.*, 2014). The packaging operation should establish inside the packaging an optimal atmosphere for the best preservation of the product. Generally, low O_2 and elevated CO_2 atmospheres, associated with low storage temperature, reduce product respiration rate, limiting, in this way, losses in fresh weight (WATADA *et al.,* 1996). Therefore, a proper combination of product characteristics and film permeability results in the evolution of an appropriate atmosphere within packages (SMITH *et al.,* 1987).

Traditionally, food companies use polymeric films (polyethylene PE, polypropylene PP, polystyrene, PS) to package fresh fruits and vegetables because of their large availability at relatively low cost and their good mechanical performance, good barrier to oxygen, carbon dioxide (SIRACUSA *et al.,* 2008). Nowadays, there is a growing trend in fresh fruits and vegetables packaging sector to replace the petrochemical based packaging films with more environmentally-friendly biodegradable materials (THARANATHAN, 2003). The extensive use of synthetic packaging films has led to serious ecological problems due to their total non- biodegradability. Therefore, biodegradability is not only a functional requirement but also an important environmental attribute. Biologically-based packaging contains raw materials originated from agricultural sources, produced from renewable raw materials such as starch and bio-derived monomers (LUCERA *et al.,* 2010). These materials represent a viable alternative because:

- they are obtained from renewable sources;
- they are recyclable and degradable;
- they are an opportunity to reduce costs.

The applications range from the design of multilayer barrier *coating* consisting of biopolymers, to enrichment of the matrix of traditional plastics PP and PE with nanocomposite materials of natural origin. However, bio-packaging still represents a niche market because of the cost and poor overall performance of biodegradable films when compared to those of traditional plastic materials (DEL NOBILE *et al.,* 2009 a).

The present work focuses on the different packaging strategies for fresh-cut fruits and vegetables. In particular, the potential applications of biodegradable materials and edible coatings have been described, with their effects on the quality of these products. The application of nanotechnology, as a tool to improve the performance and thermal, barrier and mechanical properties of bio-polymers has also been assessed (AZEREDO, 2009).

BIO-BASED MATERIALS FOR FOOD PACKAGING

The term "*Bio-Based Materials" (BBM)* is assigned to packaging materials and to packaging produced from biological renewable raw materials (PIERGIOVANNI and MASCHERONI, 2007).

Polymers derived from renewable resources (biopolymers) are broadly classified according to method of production, as follows:

1. Polymers directly extracted/removed from natural materials (mainly plants). Examples are polysaccharides, such as starch and cellulose and proteins, like casein and wheat gluten, and lipids.

2. Polymers produced by "classical" chemical synthesis from renewable bio-derived monomers. A good example is polylactate, a biopolyester polymerised from lactic acid monomers. The monomer itself is produced via fermentation of carbohydrate feedstock.

3. Polymers produced by microorganisms or genetically transformed bacteria. The best known bio-polymer types are the polyhydroxyalkanoates, mainly polyhydroxy butyrates and copolymers of hydroxy-butyrate (HB) and hydroxyvalerate (HV) (PETERSEN *et al.,* 1999).

The compostability attribute is very important for biopolymer materials, because composting allows disposal of the packages in the soil, which is more energy efficient than recycling. During biological degradation water, carbon dioxide and inorganic compounds without toxic residues are produced.

According to the European Bioplastics, biopolymers made with renewable resources have to be biodegradable and especially compostable, so they can act as fertilizers and soil conditioners. Whereas plastics based on renewable resources must not necessarily be biodegradable or compostable, the bioplastic materials do not necessarily have to be based on renewable materials, because the biodegradability is directly correlated to the chemical structure of the materials rather than the origin. In particular, the type of chemical bond defines whether and in what time the microbes can biodegrade the material. Several synthetic polymers are biodegradable and compostable, such as starch, cellulose, which are naturally carbon-based polymers. Viceversa, the bioplastics based on natural monomer, can lose their biodegradability through chemical modification like polymerization, such as for example Nylon 9 type polymers obtained from polymerization of oleic acid monomer or Polyamid 11 obtained from the polymerization of castor oil monomer (SIRACUSA *et al.,* 2008).

The problems associated with renewable biopolymers are threefold: performance, processing and cost. Although these factors are somewhat interrelated, problems due to "performance and processing" are more pronounced with polymers extracted directly from biomass (cellulose, starch, proteins). Conversely, polymers belonging to categories 2 and 3 above, generally perform very well and are easily processed into films using standard plastic techniques, but tend to be expensive compared with synthetic analogues (PETERSEN *et al.,* 1999).

Polymers directly extracted/removed from natural materials

Polysaccharides

The polysaccharides show effective gas barrier properties although they are highly hydrophilic and show high water vapor permeability in comparison with commercial plastic films. The main polysaccharides that can be included in edible coating formulations are starch and starch derivates, cellulose derivates, alginate, carrageenan, chitosan, pectin, and several gums. Based on the molecular level, polysaccharides vary according to their molecular weight, degree of branching, conformation, electrical charge and hydrophobicity. Variations in these molecular characteristics will lead to variations in the ability of different polysaccharides to form coatings, as well as to variations in the physicochemical properties and performance of the coatings formed. For example, the linear structure of some of these polysaccharides such as cellulose, amylose (a component of starch), and chitosan makes their films tough, flexible, transparent, and resistant to fats and oils (VARGAS *et al.,* 2008).

Starch, a storage polysaccharide of cereals and legumes, is most commonly used in the formulation of edible coatings and films because it is inexpensive, abundant, biodegradable, and easy to use. Films based on starch have moderate gas barrier properties. Their mechanical properties are generally inferior to synthetic polymer films. When a plasticizer, such as water, is added starches exhibit thermoplastic behavior (KROCHTA and MULDER-JOHNSTON, 1997). Starch-based thermoplastic materials have been commercialized over the last few years and today dominate the market of bio-based, compostable materials (CLAUS, 2000). The properties of the starch-based materials can be improved by destructuring the native conformation of the starch and adding synthetic substances. It is common practice to add copious amounts of synthetic polymer to the starch, such as polyvinyl alcohol (PVA) or polycaprolactone.

Another important polysaccharide is chitosan, which is mainly obtained from crab and shrimp shells (HIRANO, 1999). Films and coatings based on chitosan have selective permeability to gases (CO_2 and O_2) and good mechanical properties. However, their uses are limited mainly because of their high water vapor permeability (BUTLER *et al.*, 1996; CANER *et al.*, 1998). Moreover, chitosan shows antifungal and antibacterial properties, which are believed to have originated from its polycationic nature. An edible film with innovative characteristics has been obtained by combining the antimicrobial properties of chitosan with the property of self-sealing banana starch, variety Kluai Namwa. The presence of starch in the composite film makes water soluble and sealable bags or wraps possible, while the presence of chitosan gives them the antimicrobial property. The composite bags were found to protect asparagus, baby corn and Chinese cabbage against *Staphylococcus aureus* activity by serving as a good barrier and as an antimicrobial agent. The properties of composite

films were more similar to starch films than to films made solely from chitosan as the amount of banana flour was greater than chitosan in the films. These composite films were cheaper and less flexible than chitosan films. Film extensibility was improved by the addition of banana flour and glycerol. Banana flour addition enhanced WVP and solubility, although, the films were not completely smooth. The biodegradable and hot water soluble films can be used in food packaging and to reduce microbial counts thus extending shelf-life of fruit and vegetables in convenient bags (PITAK and RAKSHIT, 2011).

Cellulose is the most abundant natural polymer on earth and it is an essentially linear polymer of anhydroglucose. As a consequence of its chemical structure, it is highly crystalline, fibrous, and insoluble. Many derivatives of cellulose have excellent properties of film-forming, but are simply too expensive for use on a large scale. Finally, the similarity of cellulose and chitosan in primary structures has facilitated the formation of homogeneous composite films. MÖLLER *et al.* (2004) reported that 1% of chitosan in chitosan-HPMC (hydroxypropylmethylcellulose) composite films is effective against *L. monocytogenes*.

Alginates can also be used to prepare edible coatings and films. Alginates are the salts of alginic acid, which is a linear copolymer of D-mannuronic and L-guluronic acid monomers. Alginate coating formation is based on the ability of alginates to react with di-valent and tri-valent cations such as calcium, ferrum or magnesium, which are added as gelling agents (CHA and CHINNAN, 2004). An original study (NORAJIT *et al.,* 2010) investigated the physical and antioxidant properties of alginate biodegradable film incorporating white, red and extruded white ginseng extracts. The major pharmacologically active constituents of ginseng are triterpene saponins called ginsenosides. The studies demonstrate that the ginseng extract can be successfully incorporated into biodegradable alginate films and retain excellent antioxidant activities. The incorporation of ginseng extract into the alginate films did not cause major changes in the moisture content values. On the other hand, significant reductions in tensile strength and elastic modulus values of ginseng-alginate film have been found compared to those of the film without ginseng extract.

Galactomannans, natural polysaccharides commonly used in the food industry, mostly as a stabilizer, thickener and emulsifier, are one of the alternative materials that can be used for the production of edible films/coatings based on their edibility and biodegradability. These gums are mostly obtained from the endosperm of dicotyledonous seeds of numerous plants, particularly the *Leguminosae*. The great advantage of galactomannans is their ability to form very viscous solutions at relatively low concentrations that are only slightly affected by pH, ionic strength and heat processing. The mechanical, barrier and rheological properties of galactomannan films/coatings may be used to improve the stability, safety, and quality of food products (CERQUEIRA *et al.*, 2011). LIMA *et al.* (2010) successfully blended collagen with two galactomannans from different species (*A. pavonina and C. pulcherrima*). The composition of films/coatings with different proportions of galactomannan, collagen and glycerol have been optimized based on the wettability of films, transport and mechanical properties; these films have been subsequently used to coat mangoes and apples. The results showed that the application of the coatings leads to a decrease of gas transfer rates of the fruits. For mangoes, a coating of *A. pavonina* galactomannan (0.5%), collagen (1.5%) and glycerol (1.5%) decreased oxygen consumption and carbon dioxide production in 28% and 11%, respectively. For apple, the oxygen consumption and carbon dioxide production decreased for both gases by approximately 50%, with the utilization of a coating of *C. pulcherrima* galactomannan (0.5%) and collagen (1.5%).

Proteins

Proteins that have received great attention for their capability of forming edible films and coatings include corn zein, wheat gluten (WG), soy protein, whey protein, casein, collagen/gelatin, pea protein, rice bran protein, cottonseed protein, peanut protein, and keratin (HAN and GENNADIOS, 2005). Casein based edible coatings are attractive for food applications due to their high nutritional quality, excellent sensory properties, and strong potential for providing food products with adequate protection against their surrounding environment. Whey proteins have been subjected to intense investigation over the past decade or so. With the addition of plasticizer, heat-denatured whey proteins produce transparent and flexible water-based edible coatings with excellent oxygen, aroma, and oil barrier properties at low relative humidity. However, the hydrophilic nature of whey protein coatings causes them to be less effective as moisture barriers (VARGAS *et al.,* 2008). The water vapor permeability limits their potential uses for food packaging and justifies the interest in natural hydrophobic substances, such as lipids.

Lipids

Edible lipids used to develop edible coatings are: beeswax, candelilla wax, carnauba wax, triglycerides, acetylated monoglycerides, fatty acids, fatty alcohols, and sucrose fatty acid esters. Lipid coatings and films are mainly used for their hydrophobic properties, representing a good barrier to moisture loss. This factor is extremely important, as a great number of studies deal with the use of coatings on fresh fruits

and vegetables to control their desiccation. In addition to preventing water loss, lipid-based coatings have been used to reduce respiration, thereby extending shelf life and to improve appearance by generating a shine on fruits and vegetables. In contrast, the hydrophobic characteristic of lipids forms thicker and more brittle films. Consequently, they must be associated with film forming agents such as proteins or cellulose derivatives (DEBEAUFORT *et al.,* 1993).

Polymers produced by "classical" chemical synthesis from renewable bio-derived monomers

Aliphatic polyesters belong to this category and are obtained from bio-derived monomers by means of classical polymerization procedures. One of the most promising biopolymer is the poly(lactic acid) (PLA), which is derived from the controlled depolymerization of the lactic acid monomer, obtained from the fermentation of sugar feedstock, corn, etc., which are renewable resources readily biodegradable (CABEDO *et al.*, 2006). Discovered in 1932 by CAROTHERS (JAMSHIDIAN *et al.,* 2010), it is a versatile polymer, recyclable and compostable, with high transparency, high molecular weight, good processability and water solubility resistance. In comparison to other biopolymers, the production of PLA has numerous advantages including:

a) production of the lactide monomer from lactic acid, which is produced by fermentation of a renewable agricultural source corn (usually based on the strain of *Lactobacillus*);

b) fixation of significant quantities of carbon dioxide via corn (maize) production by the corn plant;

c) significant energy savings;

d) the ability to recycle back to lactic acid by hydrolysis or alcoholysis;

e) the ability to tailor physical properties through material modification (DORGAN *et al.,* 2000).

PLA is generally recognized as safe (GRAS) by the United State Food and Drug Administration (FDA). Moreover, PLA can be easily processed by conventional processing techniques used for thermoplastics like injection moulding, blow moulding, thermoforming and extrusion. PLA can be used in a wide range of applications; the major PLA application today regards packaging (nearly 70%). PLA food packaging applications are ideal for fresh products, such as fresh meat (GALGANO *et al.*, 2009) or fruits and vegetables.

Polymers produced by microorganisms: polyhydroxyalkanoates (PHA)

In recent years poly-β-hydroxyalkanoates (PHA) have attracted a lot of attention as biocompatible and biodegradable thermoplastic materials with potential applications.

Poly(3-hydroxybutyrate) (PHB) is one of the well-known biodegradable poly(hydroxy alkanoates) (PHA). PHB is a natural thermoplastic polyester and has many mechanical properties comparable to synthetically produced degradable polyesters such as the poly-L-lactides (FREIER *et al.,* 2002). Since 1925 PHB has been produced by bacterial fermentation (ROSA *et al.,* 2004), which takes place in the presence of a wide variety of bacteria, as intracellular reserve material. At least 75 different genera of bacteria have been known to accumulate PHB as intracellular granules. This polymer is synthesized under limited culture conditions and its production has most commonly been studied with microorganisms belonging to the genera *Alcaligenes, Azobacter, Bacillus and, Pseudomonas* (UGUR *et al.,* 2002). PHB films are degraded by numerous microorganisms (bacteria, fungi and algae) in various ecosystems. When in contact with the polymer, the microorganisms secrete enzymes that break it into successively smaller segments, thereby reducing the average molecular weight (BUCCI *et al.,* 2005). These polymers, alone or in combination with synthetic plastic or starch produce excellent packaging films. The polyalkanoates are more hydrophobic than the polysaccharide-based materials, resulting in a material with good moisture barrier properties, whereas the gas barriers are inferior (PETERSEN *et al.,* 1999). PHB has been used in small disposable products and in packing materials. However, little is known about the application of PHB to packaging for food products. This polymer is not used in cut-fruits and vegetables, so there is no sense to have it here, unless you write some possible uses in cut-fruits and vegetables.

Another biopolymer produced from microorganisms is the pullulan, extracted from cells of *Aureobasidium pullulans, Tremella mesenterica* and *Cyttaria harioti*. It is characterized by high resistance to fats, good barrier to gases; moreover, it is completely biodegradable and can be processed with conventional techniques. Chemically pullulan is a polymer of maltotriose used in combination with sorbitol and fatty acids esterified with sucrose to achieve edible coatings for fruits (PIERGIOVANNI and MASCHERONI, 2007). In a recent study the possibility of using new biodegradable material such as kefiran, a microbial polysaccharide obtained from the flora of kefir grains has been investigated (GHASEMLOU *et al.,* 2011). It is a water-soluble polysaccharide containing approximately equal amounts of glucose and galactose (MICHELI *et al.,* 1999), used in the food industry as a texturing agent and gelling agent. It could be a viable alternative to synthetic materials for food packaging, also because it has antibacterial and anticancer properties (MAEDA *et al.,* 2004).

The biopolymers fulfill environmental requirements, but show some limitations in terms of

performance, such as thermal resistance, mechanical properties and barrier properties, associated with high costs. So, more research needs to be carried out on the packaging material with a view to introducing, for instance, intelligent molecules or inorganic material, preferably in the form of nano-particles, in order to expand the range of properties of the materials. The protagonist of the twenty-first century is another scientific innovation that is affecting the packaging industry: *nanotechnology.*

NANOTECHNOLOGY AND NANOCOMPOSITES

The concept of nanotechnology was introduced by RICHARD FEYMAN in 1959 at a meeting of the American Physical Society (KHADEMHOSSEINI and LANGER, 2006). Nanotechnology is defined as "The design, characterization, production and application of structures, devices and systems by controlling the shape and size at the nanometer scale" (Royal Society and Royal Academy of Engineering, Nanoscience and Nanotechnologies, 2004). Nanotechnology and its application in food science have recently been studied by several researchers. The use of nanoparticles, such as micelles, liposomes, nanoemulsions, biopolymeric nanoparticles aimed at ensuring food safety, are some novel nano-food applications. Nanotechnology is also applicable in the context of food packaging: however, the use of edible and biodegradable polymers has been limited because of problems related to performance (such as brittleness, poor gas and moisture barrier), processing (such as low heat distortion temperature), and cost. The application of nanotechnology to these polymers may open new possibilities for improving not only the properties but also the cost-price-efficiency (SORRENTINO *et al.,* 2007). The use of fillers with at least one nanoscale dimension (nanoparticles) produces nanocomposites. Most reinforced materials present poor matrix–filler interactions, which tend to improve with decreasing filler dimensions. Nanoparticles have proportionally a larger surface area than their microscale counterparts, which favors the filler–matrix interactions and the performance of the resulting material.

Polymer composites are mixtures of polymers with inorganic or organic fillers with certain geometries (fibers, flakes, spheres, particulates). A uniform dispersion of nanoparticles leads to a very large matrix/filler interfacial area, which changes the molecular mobility, the relaxation behavior and the consequent thermal and mechanical properties of the material. Although several nanoparticles have been recognized as possible additives to enhance polymer performance, the packaging industry has focused its attention mainly on layered inorganic solid like clay and silicates, due to their availability, low cost and relative simple processability (AZEREDO, 2009). The most widely studied type of clay fillers is montmorillonite (MMT), a hydrated alumina-silicate layered clay consisting of an edge-shared octahedral sheet of aluminum hydroxide between two silica tetrahedral layers (WEISS *et al.,* 2006). MMT is an effective reinforcement filler, due to its high surface area and large aspect ratio (50–1000) (UYAMA *et al.,* 2003). Furthermore, the presence of this nanoclay in polymer matrix increases its degradation rate, due to hydroxyl groups of MMT (SOUZA *et al.*, 2013).

MANGIACAPRA *et al.* (2005) reported that it is possible to reduce the oxygen permeability by adding clay montmorillonite to pectins. Also the combination of PLA film with MMT-layered silicates may be useful to create a nanocomposite material with good barrier properties (SOUZA *et al.*, 2013).

Moreover, a starch/clay nanocomposite film have been obtained by dispersing MMT nanoparticles via polymer melt processing techniques (AVELLA *et al.,* 2005). The results related to mechanical characterization showed an increase of modulus and tensile strength.

Besides nano-reinforcements, nanoparticles can have other functions when added to a polymer, such as antimicrobial activity, enzyme immobilization, biosensing (AZEREDO, 2009). The incorporation of antimicrobial compounds into food packaging materials has received considerable attention. Films with antimicrobial activity could help control the growth of pathogenic and spoilage microorganisms. An antimicrobial nanocomposite film is particularly desirable due to its acceptable structural integrity and barrier properties imparted by the nanocomposite matrix, and the antimicrobial properties contributed by the natural antimicrobial agents impregnated within (RHIM and NG, 2007). The most common nanocomposites used as antimicrobial films for food packaging are based on silver, which is well known for its strong toxicity towards a wide range of microorganisms (LIAU *et al.*, 1997), with high temperature stability and low volatility (KUMAR and MÜNSTEDT, 2005). Some mechanisms have been proposed for the antimicrobial property of silver nanoparticles (Ag-NPs): adhesion to the cell surface, degrading lipopolysaccharides and forming "pits" in the membranes, largely increasing permeability (SONDI and SALOPEK-SONDI, 2004); penetration inside bacterial cell, damaging DNA (LI *et al.,* 2008); and releasing antimicrobial Ag^+ ions by Ag-NPs dissolution (MORONES *et al.,* 2005). Moreover, Ag-NPs absorbs and decomposes ethylene (HU and FU, 2003), which may contribute to its effects on extending shelf life of fruits and vegetables. Indeed, LI *et al.* (2009) reported that a nanocomposite PE film with Ag-NPs can retard the senescence of jujube, a Chinese fruit. AN *et al.* (2008) reported that a coating containing Ag-NPs is ef-

fective in decreasing microbial growth and increasing shelf life of asparagus.

The three new requirements of food packaging are the following: "Bioactive, Biodegradable and Bionanocomposite". However, there are many safety concerns about nanomaterials, as their size may allow them to penetrate into cells and eventually remain in the system. One can assume that the application of nanoparticles with an antimicrobial function (the nanoparticles of silver or copper) poses the risk the consumer's direct exposure to metals. Although the migration of metals from biodegradable nanocomposite film of starch / clay applied to vegetable products is considered to be minimal (AVELLA *et al.,* 2005), it is necessary to have more accurate information about the likely impact on human health. In fact, many studies and experiments relating to the use of nanoparticles are still in progress in order to avoid premature generalizations that could undermine the potential benefits obtainable from this technology. In the next section, the potential applications of this new category of materials (biodegradable, edible coating and nanocomposites) and their possible effects on the quality characteristics of fresh-cut fruit and vegetables is discussed.

EDIBLE FILMS AND COATINGS FOR FRESH-CUT FRUITS AND VEGETABLES

Consumers usually judge the quality of fresh-cut fruit on the basis of appearance and freshness at the time of purchase (KADER, 2002). However, minimal processing operations alter the integrity of fruits, with negative effects on product quality, such as browning, off-flavour development and texture breakdown. Also, the presence of microorganisms on the fruit surface may compromise the safety of fresh-cut fruit.

Traditionally, edible coatings have been used in the fresh-cut industry as a strategy to reduce the deleterious effects that minimal processing imposes on intact vegetable tissues.

An edible coating may be defined as a thin layer of material that covers the surface of the food and can be eaten as part of the whole product. Therefore, the composition of edible coatings must be conform to the regulation that apply to the food product concerned (GUILBERT *et al.,* 1995; VARGAS *et al.,* 2008).

Edible coatings may contribute to extend the shelf-life of fresh-cut fruits by reducing moisture and solute migration, gas exchange, respiration and oxidative reaction rates, as well as by reducing or even suppressing physiological disorders (BALDWIN *et al.,* 1996; PARK, 1999). Edible coatings are capable of producing a modified atmosphere on coated fruits by isolating the coated product from the environment. Coatings with selective permeability to gases are capable of decreasing the interchange of O_2 and CO_2 between coated fruits and the environment (OLIVAS and BARBOSA-CÁNOVAS, 2005). High CO_2 concentration within fruit tissues also delays ripening by decreasing the synthesis of ethylene, a hormone essential for ripening (SALTVEIT, 2003). LEE *et al.* (2003) demonstrated that the respiration rate of apple slices decreases 20% when coated with a film based on whey protein. Edible coating based on aloe vera reduced respiration rate and microbial spoilage in sliced kiwifruit (BENÍTEZ *et al.*, 2013). Fresh-cut products are characterized by high water transpiration rates and the creation of a moisture and gas barrier may lead to weight loss and respiration rate reduction, with a consequent general delay of produce senescence (VALENCIA-CHAMORRO *et al.,* 2011). Coating apple slices with a carbohydrate/lipid bilayer film reduces water loss during storage between 12 to 14 times when compared to the water loss suffered by uncoated apple slices in similar storage conditions (WONG *et al.*, 1994). Alginate coatings prevented water loss on fresh-cut apple (LEE *et al.*, 2003; FONTES *et al.*, 2007), papaya (TAPIA *et al.,* 2007), pear (OMS-OLIU *et al.*, 2008a), and melon (OMS-OLIU *et al.*, 2008b). Chitosan coatings reduced water loss in sliced mango (CHIEN *et al.*, 2007) and carrageenan coatings prevented water loss on sliced banana (BICO *et al.*, 2009). The cassava starch edible coating with or without the calcium lactate was able to reduce weight loss in fresh-cut pineapple (BIERHALS *et al.*, 2011), while the cassava starch edible coating with or without potassium sorbate decreased the respiration rate in fresh-cut strawberries (GARCIA *et al.*, 2010). Minimally processed pummelo (*Citrus Maxima* Merr.), coated with starch-based coatings (derived from cassava and rice) had a lower weight loss of 4.8–7.7% compared to the control (non-coated minimally processed pummelo) (KERDCHOECHUEN *et al.*, 2011). Moreover, the combination of citric acid dipping and cassava starch or sodium alginate edible coatings was able to delay the quality deterioration of fresh-cut mangoes, decreasing the respiration rate of fruits with value up to 41% lower than the control fruit. Although the citric acid increases the weight loss of the product, causing a partial dehydration of the vegetable tissue, the use of cassava starch and alginate coatings was able to hinder this undesirable effect of citric acid, presenting a lower weight loss than the coated samples (CHIUMARELLI *et al.*, 2011). In a previous work, CHIUMMARELLI *et al.* (2010) reported that this treatment provided a better maintenance of colour characteristics, due to the combined effect of citric acid dipping and cassava starch coating. Citric acid delayed browning along the storage and the edible coatings acted as a gas barrier, decreasing the respiration rate of mango pieces and, consequently the formation of carotenoids.

Moreover, another important advantage of edible coating is the reduction of synthetic packag-

ing waste, because these coatings are composed of biodegradable raw material (DHALL, 2013).

The properties of edible coating depend primarily on molecular structure rather than molecular size and chemical constitution. Specific requirements for edible films and coatings are as follows (ARVANITOYANNIS and GORRIS, 1999):

- the coating should be water-resistant so that it remains intact and covers a product adequately, when applied;
- it should not deplete oxygen or build up excessive carbon dioxide. A minimum of 1–3% oxygen is required around a commodity to avoid a shift from aerobic to anaerobic respiration;
- it should reduce water vapor permeability;
- it should melt above 40°C without decomposition;
- it should be easily emulsifiable, non-sticky or should not be tacky, and have efficient drying performance;
- it should never interfere with the quality of fresh fruit or vegetable and not impart undesirable order;
- it should have low viscosity and be economical;
- it should be translucent to opaque but not like glass and capable of tolerating slight pressure.

The ability of edible coatings to preserve the quality of fresh-cut products may vary from product to product, then it is necessary to consider the variety and maturity of the product, food surface coverage, storage conditions and composition and thickness of the coating (GONZÁLEZ-AGUILAR *et al.*, 2010).

According to their components, edible films and coatings can be divided into three categories: hydrocolloids, lipids, and composites. Hydrocolloids include proteins and polysaccharides, while lipids include waxes, acylglycerols, and fatty acids. Composites contain both hydrocolloid components and lipids. Their presence and abundance determine the barrier properties of material with regard to water vapor, oxygen, carbon dioxide, and lipid transfer in food systems. However, none of the three constituents can provide the needed protection by themselves and so are usually used in a combination for best results (MCHUGH *et al.*,1994; GUILBERT *et al.,* 1996). The main objective of producing composite films is to improve the permeability or mechanical properties according to specific applications. These heterogeneous films are applied either in the form of an emulsion, suspension, or dispersion of the non miscible constituents, or in successive layers (multilayer coating or films), or in the form of a solution in a common solvent. Several other compounds such as plasticizers and emulsifiers may be added to edible films and coatings to improve their mechanical properties and form stable emulsions when lipids and hydrocolloids are combined (VALENCIA-CHAMORRO *et al.,* 2011). The application of edible coatings to fresh-cut fruits must address the problem regarding the difficult adhesion of materials to the hydrophilic surface of the sliced fruit. The layer-by-layer (LbL) electrodeposition can solve this problem (WEISS et al., 2006). LbL assembly, which is performed by alternating the immersion of substrates in solutions of oppositely charged polyelectrolytes with rinsing steps, produces ultrathin polyelectrolyte multilayers on charged surfaces. A requirement for multilayer formation is that the addition of an opposite charged polyelectrolyte to a charged surface results in a charge reversal, which permits the successive deposition of oppositely charge polyelectrolytes. Chitosan, poly-L-lysine, pectin, and alginate are the most common biopolymers that can be used in the formation of these multilayered structures (MARUDOVA *et al.*, 2005; KRZEMISKI *et al.*, 2006; BERNABÉ *et al.*, 2005). In a study of MANTILLA *et al.* (2013), an alginate-based multilayered coating (developed using the layer-by-layer technique) enhanced the quality and shelf-life of fresh-cut pineapple extending its shelf-life to 15 days at 4°C.

The main advantage of using edible films and coatings is that several active ingredients (such as antimicrobials, antibrownings, texture enhancer and nutraceuticals) can be incorporated into the polymer matrix and consumed with the food, thus enhancing safety or even nutritional and sensory attributes (ROJAS-GRAÜ *et al.,* 2009).

Antimicrobial agents

Fresh-cut fruits are more perishable than their corresponding whole uncut commodities due to wounding during preparation (BRECHT, 1995). The physical and chemical barrier provided by the epidermis, which prevents the development of microbes on the fruit surface, is removed during processing.

There are several categories of antimicrobials that can be potentially incorporated into edible films and coatings, including organic acids (acetic, benzoic, lactic, propionic, sorbic), fatty acid esters (glyceryl monolaurate), polypeptides (lysozyme, peroxidase, lactoferrin, nisin). ESWARANANDAM *et al.* (2006) incorporated malic and lactic acid into soy protein coatings aiming to evaluate its effect on the sensory quality of fresh-cut cantaloupe melon, without studying the antimicrobial effect of the coating. In general, organic acids incorporated in films did not adversely affect the sensory properties of coated fruit.

With reference to the use of natural antimicrobials, the development of coatings which use inherently antimicrobial polymers as a support matrix is very promising. For example, chitosan, produced from the deacetylation of crustacean chitin (poly-β-(1→4)-N-acetyl-D-glucosamine), is one of the most effective antimicrobial film

forming biopolymers. Chitosan is a cationic polysaccharide, which, among other antimicrobial mechanisms, promotes cell adhesion by the interaction of the positive-charged amines with the negative charges in the cell membranes, causing leakage of intracellular constituents (HELANDER *et al.*, 2001). Chitosan based coatings were shown to protect highly perishable fruits like strawberries, raspberries, grapes and fresh-cut-green pepper from fungal decay. (VARGAS *et al.*, 2006; EL GAOUTH *et al.,* 1991; ZHANG and QUANTICK, 1998; ROMANAZZI *et al.,* 2002; DEVLIEGHERE *et al.,* 2004; PARK *et al.,* 2005, RAYMOND *et al.*, 2012).

The application of chitosan edible coating on fresh-cut broccoli (MOREIRA *et al.*, 2011a) significantly reduced mesophilic and psychrotrophic counts and inhibited the growth of total coliform with respect to the control samples, throughout the whole storage period. Similar results were reported by GERALDINE *et al.* (2008) working with minimally processed garlic. At the end of the storage, yeasts and molds were the most dominant flora and represented the largest part of the total aerobic count in broccoli. On the contrary, DURANGO *et al.* (2006) founded an important fungicidal action of chitosan applied on minimally processed carrot. Moreover, the application of mild heat shock enhanced the chitosan inhibition action (MOREIRA *et al.*, 2011b). From a sensory point of view, the chitosan inhibited the yellowing and opening florets of fresh-cut broccoli. Moreover, chitosan-based edible coatings can also be used to carry other antimicrobial compounds such as organic acids (OUTTARA *et al.,* 2000), essential oils (ZIVANOVIC *et al.,* 2005), spice extracts (PRANOTO *et al.*, 2005), lysozyme (PARK *et al.,* 2004) and nisin (PRANOTO *et al.,* 2005; CHA *et al.,* 2003). Essential oils (EOs) (cinnamon, oregano, lemongrass) stand out as an alternative to chemical preservatives and their use in foods meets the demands of consumers for natural products. BURT (2004) reported that hydrophobicity is an important characteristic of EOs, which makes them able to pass through cell membranes and enter mitochondria, disturbing the internal structures and rendering the membranes more permeable.

Yet the application of EOs in foods is limited due to their impact on organoleptic food properties, variability of their composition, and their variable activity in foods due to interactions with food components. Nevertheless, the use of EOs to control microbial growth in foods has been proposed for several products including fresh-cut fruits and vegetables.

RAYBAUDI-MASSILIA *et al.* (2008b) studied the effect of malic acid and essential oils of cinnamon, palmarosa and lemongrass as natural antimicrobial substances incorporated into an alginate-based edible coating on the shelf-life of fresh-cut melon. The coating containing malic acid was effective in improving the shelf-life of fresh-cut melon from both the microbiological and physicochemical points of view in comparison with non-coated fresh-cut melon samples. The incorporation of the essential oils or their active compounds into the coating prolonged the microbiological shelf-life by more than 21 days in some cases, probably due to an enhanced antimicrobial effect of malic acid and the essential oils. However, some physicochemical characteristics, such as firmness and color, and also some sensory quality attributes were adversely affected, causing a significant reduction of fresh-cut melon shelf-life. In contrast, when malic or lactic organic acids incorporated in soy protein coatings were applied to fresh-cut cantaloupe, they did not adversely affect the sensory properties of the fruit after cold storage (ESWARANANDAM *et al.*, 2006). AYALA-ZAVALA *et al.* (2013), developed an edible pectin film enriched with the essential oil from cinnamon leaves and proved that this application can increase the antioxidant status and reduce bacterial growth of fresh-cut peach. Chitosan coatings enriched with bioactive compounds (bee pollen, ethanolic extract of propolis, pomegranate dried extract, resveratrol)/essential oils (tea tree, rosemary, clove, oreganum, lemon aloe vera calendula) could be a good alternative for controlling not only the microorganisms present in broccoli, but also the survival of *E. coli* and *L. monocytogenes* inoculated in the product, without introducing deleterious effects on the sensory attributes of minimally processed broccoli (ALVAREZ *et al.*, 2013).

ROJAS-GRAU *et al.* (2006, 2007a) have studied the effects of oregano, cinnamon, and lemongrass oils and their active compounds (carvacrol, cinnamaldehyde and citral) incorporated into apple puree and alginate apple puree edible films against *Escherichia coli* O157:H7. In these works, oregano oil or its active compound, carvacrol, was most effective against *E. coli* O157:H7. In line with these preliminary studies, ROJAS-GRAU *et al.* (2007b) combined the efficacy of alginate and gellan edible coatings with the antimicrobial effect of EOs (lemongrass, oregano oil and vanillin) to prolong the shelf-life of fresh-cut apples. A 4 log reduction of the inoculated population of *Listeria innocua* in fresh-cut apple was observed when lemongrass or oregano oils were incorporated into an apple puree alginate edible coating. In addition, the coating reduced respiration rate and ethylene production of coated fresh-cut apples. In a later work (RAYBAUDI-MASSILIA *et al.*, 2008a), the addition of cinnamon, clove and lemongrass essential oils or their active compounds, cinnamaldehyde, eugenol and citral into an alginate-based coating increased their antimicrobial effect, reduced the population of *E. coli* 0157:H7 by more than 4 log CFU/g and extended the microbiological shelf-life of "Fuji" apples for at least 30 days. According to these studies, AZARAKHSH *et al.* (2014), observed that the lemongrass incorporated into

an alginate-based coating for fresh-cut pineapple, reduces the microbial growth of the product during storage time. According to the Institute of Food Science and Technology (IFST), 10^6 CFU/g is considered the limit of acceptance for shelf-life of fruit-based products (BIERHALS *et al.*, 2011). In fact, the coated samples with 0.3% and 0.5% lemongrass reached 10^6 CFU/g after 12 and 16 days, respectively. On the contrary, the application of tapioca starch/decolorized hsian-tsao leaf gum coatings on minimally processed carrots with antimicrobial agents (cinnamon oil and grape seed extract) had no beneficial effect on controlling the mesophile aerobics and psychrophiles. As a result, the respiration rate of the product increased (LAI *et al.*, 2013). Instead, in the case of fresh-cut "Fuji" apples, cinnamon oil incorporated in tapioca starch/decolorized hsian-tsao leaf gum (dHG) based edible coatings, significantly reduced the growth of microorganisms, respiration rate, CO_2 and ethylene production (PAN *et al.,* 2013). MANTILLA *et al.* (2013) studied the effects of a multilayered edible coating with a microencapsulated antimicrobial complex (beta-cyclodextrin and trans-cinnamaldehyde) on the quality and shelf-life of fresh-cut pineapple. They reported that trans-cinnamaldehyde affects the pineapple flavour. However, the application of this antimicrobial coating extended the shelf-life of samples to 15 days at 4°C by inhibiting microbial growth. The same edible coating was applied on fresh-cut watermelon (SIPAHI *et al.*, 2013) and similar results were obtained: this type of alginate coatings extended the shelf life of fresh-cut watermelon from 7 (control) to 12-15 days. A microencapsulated beta-cyclodextrin and trans-cinnamaldehyde complex was also incorporated into a multilayered edible coating made of chitosan and pectin. This coating extended the shelf life of fresh-cut papaya up to 15 days at 4°C while uncoated fruits did not last this long (< 7days). Moreover, the coating reduced the losses of vitamin C and the total carotenoid content (BRASIL *et al.,* 2012).

Antibrowning agents

Fresh-cut fruit processing operations can induce undesirable changes in colour and appearance of these products during storage and marketing. The phenomena is usually caused by the enzyme polyphenol oxidase (PPO), which in the presence of oxygen, converts phenolic compounds into dark colored pigments (ZAWISTOWSKI *et al.,* 1991). Application of antioxidant treatments such as dipping after peeling and/or cutting is the most common way to control browning of fresh-cut fruits. Ascorbic acid is the most extensively used to avoid enzymatic browning of fruit due to the reduction of the *o*-quinones, generated by the action of the PPO enzymes, back to the phenolic substrates (MCEVILY *et al.,* 1992). For example, the antioxidants citric and ascorbic acid were incorporated into methylcellulose-based edible coatings in order to control oxygen permeability and reduce vitamin C losses in apricots during storage (AYRANCI and TUNC, 2004). The combination of chitosan coating and sodium chlorite dip treatment on pear slices adversely affected the quality of the fruit, accelerating the discoloration of cut surfaces and increasing the PPO activity. On the contrary, coating sodium chlorite-treated samples with carboxymethyl chitosan significantly prevented the browning reaction and inhibited PPO activity. (XIAO *et al.*, 2011). The effect of coatings in combination with anti-browning agents (1% chitosan; 2% ascorbic acid + 0.5% $CaCl_2$) on minimally processed apple slices has been studied during storage by HAIPING *et al.* (2011). Chitosan-coating treatments effectively retarded enzymatic browning on minimally processed apples during storage and effectively retarded tissue softening.

As an alternative to ascorbic acid, several thiol-containing compounds, such as cysteine, N-acetylcysteine, reduced glutathione and 4-hexylresorcinol have been investigated as inhibitors of enzymatic browning (GORNY *et al.*, 2002; SON *et al.*, 2001). These compounds react with quinones formed during the initial phase of enzymatic browning reactions to yield colorless additional products or to reduce o-quinones to o-diphenols (RICHARD *et al.,* 1992). 4-hexylresorcinol, in combination with sodium erythorbate, was effective in maintaining the color of pear slices "Anjou" (COLELLI and ELIA, 2009). Furthermore, carboxylic acids (citric acid and oxalic acid) have also been suggested as effective antioxidant agents in fresh-cut fruits (JIANG *et al.*, 2004; PIZZOCARO *et al.*, 1993; SON *et al.*, 2001). The incorporation of antibrowning agents into edible coatings applied on fresh-cut fruits has been studied by various authors. The application of alginate edible coating in conjunction with antibrowning agents (ascorbic and citric acid) to mango cubes increased vitamin C content compared to mango cubes treated only with alginate coating or control cubes, preserving the color of fresh-cut mangoes and increasing the antioxidant potential of cubes (ROBLES-SÁNCHEZ *et al.*, 2013). Moreover, PEREZ-GAGO *et al.*, (2006) reported a substantial reduction in browning of fresh-cut apples when using a whey protein concentrate-beeswax coating containing ascorbic acid, cysteine or 4-hexylresorcinol. They observed a significant improvement of the efficiency of antioxidant agents when incorporated into the coating formulation, being the most effective treatment with 0.5% cysteine. BRANCOLI and BARBOSA-CÁNOVAS (2000) decreased surface discoloration of apple slices with maltodextrin and methylcellulose coatings including ascorbic acid. BALDWIN *et al.* (1996) found that a carboxymethyl

cellulose-based coating with addition of several antioxidants, including ascorbic acid, reduced browning and retarded water loss of cut apple more effectively than an aqueous solution of antioxidants. Furthermore, ROJAS-GRAÜ *et al.* (2008) observed that both alginate and gellan edible coatings containing N-acetylcysteine prevented apple wedges from browning during 21 days of storage. More recently, the application of konjac glucomann (polysaccharide derived from the tuber of konjac, *Amorphophallus konjac*) with pineapple fruit extract represents an effective alternative to prevent browning of fresh-cut Taaptimjaan rose apple fruit during storage. This coating enhanced total phenols and inhibited both PPO and peroxidase (POD) activities (SUPAPVANICH *et al.*, 2012). Or even, pullulan-based coating treatments in combination with antibrowning and antibacterial agents (1% pullulan; 0.8% glutathione + 1% chitooligosaccharides) effectively inhibited enzymatic browning, retarded tissue softening, inhibited microbial growth, decreased weight loss and respiration rate of the minimally processed apple slices (WU and CHEN, 2013).

Nutraceuticals

Nutraceuticals can also be incorporated into the formulation of edible coatings. Despite the growing interest in incorporating nutraceutical compounds into food products, few studies have suggested their integration into edible films or coatings. In this sense, the concentration of nutrients added to the films/coatings must be carefully studied since it is important to know the effects on their basic functionality, namely on their barrier and mechanical properties. Some studies have reported the effect of the addition of active compounds in the functionality of edible films. For instance, MEI and ZHAO (2003) evaluated the feasibility of milk protein-based edible films to carry high concentrations of calcium (5 or 10% w/v) and vitamin E (0.1% or 0.2% w/v). They concluded that protein-based edible films can carry active compounds, although the film functionality can be compromised. In contrast, PARK and ZHAO (2004) reported that the water barrier property of the chitosan-based films was improved by increasing the concentration of mineral (5and 20% w/v zinc lactate) or vitamin E in the film matrix. Nevertheless, the tensile strength of the films was affected by the incorporation of high concentrations of calcium or vitamin E, although other mechanical properties, such as film elongation, puncture strength, and puncture deformation, were not affected. Several researchers have endeavoured to incorporate minerals, vitamins and fatty acids into edible film and coating formulations to enhance the nutritional value of some fruits and vegetables, where these micronutrients are present in low quantities. TAPIA *et al.* (2008) reported that the addition of ascorbic acid (1% w/v) to the alginate and gellan based edible coatings helped to preserve the natural ascorbic acid content in fresh-cut papaya, thus helping to maintain its nutritional quality throughout storage. In the last few years, attention has been paid to the addition of probiotics to obtain functional edible films and coatings. TAPIA *et al.* (2007) developed the first edible films for probiotic coatings on fresh-cut apple and papaya, observing that both fruits were successfully coated with alginate or gellan film-forming solutions containing viable *bifidobacteria*. In fact, values higher than 10^6 cfu/g *Bifidobacterium lactis* Bb-12 were maintained for 10 days during refrigerated storage of both papaya and apple pieces, demonstrating the feasibility of these polysaccharide coatings to carry and support viable probiotics on fresh-cut fruits.

Alginate offers the possibility of formulating a broad range of functional and innovative food products, increasing the nutritional properties of foods. Recently, an edible alginate coating containing prebiotics such as oligofructose and inulin has been applied on fresh-cut apples wedges. Fructan analysis showed that all prebiotics remained stable over the 14 day storage period and sensory and visual assessment indicated acceptable quality of apple wedges coated with prebiotics (RÖßLE *et al.*, 2011). The addition of prebiotics could be especially appealing to consumers as they are essential to human nutrition in the context of dietary guidelines.

However, more studies are necessary to understand the interactions among active ingredients and coating materials when developing new edible film and coating, before they are applied to the surface of a real food system (ROJAS-GRAÜ *et al.*, 2009).

Further progress comes from nanotechnology, a science that could provide new techniques for extending the shelf-life of foods. Among the inorganic agents, silver nanoparticles have received great attention from the scientific world, due to the high biocidal effects towards many species of microorganisms (KIM *et al.*, 2007). In a study by COSTA *et al.* (2012), the effects of both active calcium-alginate coating loaded with silver-montmorillonite (Ag-MMT) nanoparticles and film barrier properties on the shelf-life of fresh-cut carrots were assessed. The results of this study suggest that the active coating is the best treatment and could be used to control both dehydration and microbial spoilage of minimally processed carrots. Moreover, the combination of coating with Ag-MMT controlled the microbial growth better than the sole coating treatment. The combined use of proper packaging and active coating allowed the carrots to be kept in a good state of preservation thus prolonging the shelf-life to about 70 days, with respect to the uncoated samples (about 4 days). However, the

toxicological aspects of silver nanoparticles are well known (KIRUBA *et al.*, 2010; LU *et al.*, 2010); EU safety regulation limits the silver amount to 0.05 mg Ag/kg of food (FERNANDEZ *et al.*, 2009).

The application of edible coating either on fresh-cut apples or on fruits and vegetables are respectively summarized in Table 1 and in Table 2 (a and b).

Table 1 - Application of edible coating on fresh-cut apple.

Food product	Coating	Composition	Main results	References
"*Gala*" apple	Alginate	Alginate	Low weight loss; low enxymatic browning; greater firmness	OLIVAS *et al*. (2007)
Fresh-cut "*Fuji*" apple		Cinnamon, clove, lemongrass essential oil or their their active compound cinnamaldehyde, eugenol and citral	4 log reduction of the *E.coli* O157:H7population; shelf-life extension at least 30 days	RAYBAUDI-MASSILIA *et al*. (2008)
Fresh-cut apple wedges		Prebiotics, such as oligofructose and inulin	All prebiotics remained stable over the 14 days; good sensory characteristics	RÖßLE *et al*. (2011)
Fresh-cut "*Fuji*" apple	Alginate and gellan	Essential oils: lemongrass, oregano oil and vanillin	4 log reduction of the inoculated population of *Listeria innocua;* lower respiration rate and ethylene production	ROJAS-GRAÜ *et al*. (2007b)
Fresh-cut apple		Alginate or gellan film-forming solution containing viable *Bifidobacteria*	Higher values higher than 10^6 cfu/g of *Bifidobacterium lactis* Bb-12 were maintained for 10 days during refrigerate storage	TAPIA *et al*. (2007)
Fresh-cut apple		N-acetylcysteine	Enzymatic browning prevention during 21 days of storage	ROJAS-GRAÜ *et al*. (2008)
Apple slices	Methylcellulose coatings	Maltodextrin and ascorbic acids.	Decreased surface discoloration of apples slices	BRANCOLI *et al*. (2000)
Fresh-cut apple	Whey protein Concentrate beeswax	Ascorbic acid, cysteine or 4-hexyl-hexylresorcinol	Substantial reduction of browning	PEREZ-GAGO *et al*. (2006)
Apple "*Fuji*" slices		Ascorbic acid and citric acid	20% decrease of the respiration rate; shelf-life extension to 2 weeks.	LEE *et al.* (2003)
Apple slices	Carbohydrate/lipid bilayer film	Carbohydrate/lipid bilayer film	Water loss reduction	WONG *et al.* (1994)
Fresh-cut "*Fuji*" apple	Tapioca starch/decolorized hsian-tsao leaf gum	Cinnamon oil	Reduction of microbial growth, respiration rate, CO_2 and ethylene production	PAN *et al*. (2013)
Fresh-cut Taaptijaan rose apple	Konjac glucomannan	Pineapple fruit extract	Increase of total phenols; inhibi-tion of PPO and POD activities.	SUPAPVANICH *et al*. (2012)
Fresh-cut apple slices	Pullulan	Pullulan; glutathione and chitooligosaccharides	Delay of tissue softening, inhibition of microbial growth, decrease of weight loss and respiration rate.	WU and CHEN (2013)
Fresh-cut apple slices	Chitosan	Ascorbic acid and $CaCl_2$	Delay of enzymatic browning and tissue softening	HAIPING *et al.* (2011)

BIODEGRADABLE PACKAGING AND MINIMALLY PROCESSED FRUITS AND VEGETABLES

Fruits and vegetables are living organisms which continue to respire and transpire after harvesting, being characterized by a respiration rate; respiration and transpiration rates of fresh fruits and vegetables are often good indicators of their storage life; the higher the rate, the shorter the shelf-life (FLOROS, 1993). Moreover, the shelf-life of fresh-cut products is limited by microbiological deterioration and in order to reduce the effects of microbiological, chemical and physical events, it is possible to act on processing or, more usually, on packaging. The packaging operation should allow to establish inside the packaging an optimal atmosphere for the best preservation of the product. Generally, low and elevated atmospheres, together with low storage temperature, reduce the product respiration rate limiting, in this way, losses in fresh weight (WATADA *et al.*, 1996). The atmosphere that is established is the resultant of oxygen and carbon dioxide permeation through the walls of the packaging and of respiration

Table 2a - Application of edible coating on fresh-cut fruits and vegetables.

Food product	Coating	Composition	Main results	References
Sliced kiwifruit "Haiward"	Aloe vera	Aloe vera	Reduced O_2 consumption and CO_2 production; reduction of microbial spoilage	BENÍTEZ *et al*. (2013)
Sliced mango	Chitosan	Chitosan	Reduction of water loss	BICO *et al*. (2009)
Fresh-cut mango	Alginate and gellan	Ascorbic acid	Increase of Vitamin C	ROBLES-SÁNCHEZ *et al*. (2013)
Fresh-cut mango (pre-treated with citric acid dipping)	Cassava starch-edible coating	Cassava starch-edible coating	Reduction of weight loss and respiration rate; better maintenance of colour characteristics	CHIUMARELLI *et al*. (2010, 2011)
Sliced banana	Carrageenan	Carrageenan	Reduction of water loss	BICO *et al*. (2009)
	Soy protein	Soy protein, glycerol, malic acid, lactic acid	Good sensory characteristics	ESWARANANDAM *et al*. (2006)
Cantaloupe melon cube	Alginate	Malic acid and essential oils of cinnamon, pal- marosa and lemongrass	Microbiological shelf-life extension.	RAYBAUDI-MASSILIA *et al*. (2008)
Fresh-cut papaya	Alginate and gellan	Ascorbic acid	Preservation of the natural ascorbic acid content in papaya	MANTILLA *et al*. (2013); SIPAHI *et al*. (2013)
	Chitosan and pectin	Microencapsulated anti-microbial complex (beta-cyclodextrin and trans-cinnamaldehyde)	Shelf-life extension to 15 days; reduction of vitamin C and total carotenoid content loss	
Fresh-cut apple, papaya, pear, melon	Alginate	Alginate	Shelf-life extension to 15 days	FONTES *et al*. (2007); TAPIA *et al*. (2007); OMS-OLIU *et al*. (2008)
Fresh-cut pineapple, fresh-cut melon	Multilayered Sodium alginate coating	Microencapsulated antimicrobial complex (beta-cyclodextrin and trans-cinnamaldehyde)	Prevention of water loss	FONTES *et al*. (2007); TAPIA *et al*. (2007); OMS-OLIU *et al*. (2008)
Fresh-cut pineapple; Fresh-cut strawberries	Cassava starch-edible coating	Calcium lactate/ potassium sorbate	Reduction of weight loss	BIERHALS *et al*. (2011)
			Reduction of respiration rate	GARCIA *et al*. (2010)

Table 2b - Application of edible coating on fresh-cut fruits and vegetables.

Food product	Coating	Composition	Main results	References
Minimally processed pummel	Cassava or rice starch	Cassava or rice starch	Lower weight loss of 4.8–7.7% than the control	KERD-CHOECHUEN *et al*. (2011)
Fresh-cut peach	Pectin	Essential oil from cinnamon leaves	Reduction of bacterial growth; better antioxi- dant status	AYALA-ZAVALA *et al*. (2013)
Pear slices	Chitosan Carboxymethyl chitosan	 Sodium chlorite	Increase of PPO activity Inhibition of PPO activity	XIAO *et al*. (2011)
Fresh-cut pineapple	Alginate	Lemongrass oil	Reduction of microbial growth	AZARAKHSH *et al*. (2014)
Fresh-cut broccoli, garlic	Chitosan	Chitosan	Reduction of meso- philic and psychrotrophic counts; inhibition of the yellowing and opening florets of fresh-cut broccoli	MOREIRA *et al*. (2011) GERALDINE *et al*. (2008)
Fresh-cut broccoli	Chitosan	Bioactive compounds (bee pollen, ethanolic extract of propolis, pomegranatedried extract,resveratrol)/essential oils (tea tree, rosemary, clove, oreganum, lemon aloe vera calendula)	Better control of microorganisms (*E. coli and L. monocytogenes);* good sensory attributes	ALVAREZ *et al*. (2013)
Fresh-cut carrots	Tapioca starch/decolorized hsian-tsao leaf gum	Cinnamon oil	No beneficial effect on controlling the meso- phyles aerobics and psychrophiles	LAI *et al*. (2013)
	Active calcium-alginate coating loaded with silver montmori-llonite (Ag MMTT) nanoparticles	High biocidal effects	Good control of dehy- dration and microbial spoilage; of shelf-life extension to about 70 days, with respect to the uncoated samples	COSTA *et al*. (2012)

by the cells, until reaching an equilibrium condition aimed to slow the senescence and avoid sensorial defects to the packaged product. Either high CO_2 or low O_2 concentrations could indirectly induce *off-flavours* by stimulating the growth of homo- and hetero-fermentative bacteria and yeast, which produce organic acids, ethanol and volatile esters (CARLIN *et al.,* 1989; KAKIOMENOU *et al.,* 1996). Therefore, a proper combination of product characteristics and film permeability, which is a fundamental characteristic of the packaging materials, results in the evolution of an appropriate atmosphere within packages (SMITH *et al.,* 1987). Moreover, the packaged food actually interacts with the packaging material, changing the initial mechanical and barrier properties of the packaging materials (PETERSEN *et al.,* 1999).

In alternative to the traditional polymeric materials, biodegradable films and coatings could be used. In fact, studies and experiments relating to possible applications of biodegradable materials, possibly combined with coating technique, to minimally processed fruits and vegetables are not lacking. Results obtained by using biodegradable films for samples of *Iceberg* lettuce are interesting: the film that is traditionally used to package fresh-cut salads is the bi-oriented polypropylene, which is associated with a level of senescence, linked to the metabolic activity of the vegetable, which is always higher than that achieved by salads packaged in biodegradable film. So, the potential application of biopolymers for the packaging of fresh-cut lettuce is justified (DEL NOBILE *et al.,* 2008). Also the rate of quality loss of minimally processed grapes in relationship with packaging film barrier properties has been assessed (DEL NOBILE *et al.*, 2009a). In earlier work, DEL NOBILE *et al.* (2008b) investigated the influence of postharvest treatments (ethanol, chlorinated water and hot water) on the quality loss kinetics of freshly processed grapes packaged in biodegradable films. The results suggested that ethanol is the

best solution to preserve the microbial stability of the fresh produce without affecting its respiration rate to any great extent. Based on these results, clusters of table grapes (*Vitis vinifera* cv. Italia) were treated in ethanol solution prior to packaging in different bags made of two types of commercially available films (a multilayer film obtained by laminating nylon and a polyolefin layer (NP); an oriented polypropylene film (OPP)) and in three biodegradable polyester-based films (NVT-100, NVT-50, NVT-35). Results suggest that the respiratory activity of packed minimally processed produce is the main reason for its quality loss during storage. In fact, the best results in terms of grape quality were obtained using grapes with lower respiratory activity and high barrier films, such as NP and NVT-100. From a sensory point of view, no undesirable after-taste developed within the fruit, when the grapes were packaged in the thicker film (NP and NVT-100).

Also PLA has been tested for fresh-cut fruits and vegetables packaging; the coating of PLA silicon oxide (SiOx) improved the barrier oxygen and water vapor properties (PEELMAN *et al.*, 2013). In particular, the use of PLA containers with lids and pouches composed of VC999 Biopack PLA films coated with a barrier of pure SiOx to preserve quality of fresh-cut pears after 9 days of storage in comparison with conventional polyethylene PE material, has been investigated. The results showed that color and firmness color of fresh-cut pears is better maintained with biodegradable packaging materials (KRASNOVA *et al.*, 2013)

Biodegradable materials do not always fulfill the physiological requirements of the specific fruit and/or vegetable packaged in them: in fact, poor moisture barrier properties, linked to the hydrophilic character that marks most of the bio-based-materials, is sometimes responsible for higher percentages of weight loss in products, such as zucchini (LUCERA *et al.,* 2010), artichokes (DEL NOBILE *et al.,* 2009b) lampascioni (*Muscari comosum*) (CONTE *et al.,* 2009c) and melon (CONTE *et al.,* 2009b).

Often in several experiments, the technique of coating is used as a pretreatment to improve the performance of biodegradable materials and its effects on product quality. The coating treatment, containing sodium alginates, has been more effective than the *dipping* in delaying the metabolic activity associated to respiration rate in samples of lampascioni, subsequently packaged in NTV2, a film based biodegradable polyester (CONTE *et al.*, 2009c). Moreover, the browning process decreased, as well as, the microbial growth, since the availability of oxygen on the surface of the product was reduced. The same type of coating was also applied to fresh-cut artichokes (DEL NOBILE *et al.*, 2009 b): also in this case, the coating together with packaging in NTV2 film seems to represent the better strategy to preserve the quality of artichokes, also from a microbiological point of view. Interesting results also came from a study of fresh-cut cactus-pear: the shelf life of the minimally processed fruit, coated with an alginate and then packed with monolayer film (based on a blend of biodegradable polyesters), was prolonged to about 13 days, corresponding to an shelf-life increase of about 40%, compared to the control sample (untreated sample); on the contrary, produce immersion into either agar or fish protein strongly reduced the shelf life, most probably due to water migration from the surrounding hydro-gel to the fresh-cut produce (DEL NOBILE *et al.,* 2009 c).

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PLA is also been used in combination with silver; silver ions have been incorporated into polylactic film, in order to evaluate the silver-infused PLA films for inactivation of Salmonella and feline calicivirus in vitro and on fresh-cut vegetables (MARTÍNEZ-ABAD *et al.*, 2013). The results showed that in vitro the antibacterial and antiviral activity is more efficient than on food samples. Anyway, in lettuce samples incubated a 4°C at 6 days of storage, 4 log CFU of Salmonella was inactivated for film with 0.1% wt of silver ions and no viral infection has been found in the same conditions (MARTÍNEZ-ABAD *et al.*, 2013).

The results of the case studies on biodegradable packaging applied to fresh-cut fruits and vegetables and in some cases combined with edible coating are summarized in Table 3.

CONCLUSIONS

Foodstuffs are dynamic systems with a limited shelf-life and specific requirements in terms of packaging. In order to ensure the highest standards of product quality, the right combination of product characteristics and packaging film is the basis for the development of appropriate storage conditions.

This work focuses on the potential applications of biodegradable packaging and edible coating for minimally processed foods. The results highlight how the biodegradable films may have a more or less advantageous performance in relation to the physiological characteristics of

Table 3 - Application and combination of biopolymer with coating for fresh-cut fruits and vegetables packaging.

Food product	Biopackaging	Property of materials	Main results	Reference
Iceberg lettuce	Polyester-based biodegradable film	Good gas barrier properties	Shelf-life extension	DEL NOBILE *et al.* (2008)
Table grape (*Vitis vinifera*)	Polyester-based biodegradable film /nylon and polyolefin layer (ethanol pre-treatment before packaging)	High gas barrier	Good microbiological stability; good sensory characteristics	DEL NOBILE *et al.* (2009)
Zucchini (*Cucurbita pepo*)	Biodegradable co-extruded polyesters	High permeability to water vapor	High percentage of weight loss	LUCERA *et al.* (2010)
Artichoke	Biodegradable monolayer film based on a blend of biodegradable polyesters	Permeability to water vapor	High percentage of weight loss; good microbiological stability	DEL NOBILE *et al.*(2009)
	Coating with sodium alginate (pre-treatment before packaging)	Barrier to water vapour	Shelf-life extension (200%) low water loss	
Lampascioni (*Muscari comosum*)	Polyester-based biodegradable film	High permeability to water vapor	High percentage of weight loss	CONTE *et al.* (2009)
	Coating with sodium alginate (pre-treatment before packaging)	Barrier to water vapour and oxygen	Lower water loss; low microbial growth. reduced browning process	
Cactus pear	Monolayer film, based on a blend of biodegradable polyesters	High permeability to water vapour	High percentage of weight loss.	DEL NOBILE *et al.* (2009)
	Coating with alginates (pre-treatment before packaging)	Barrier to water vapour and other agents	Shelf-life extension to about 13 days	
Fresh-cut pears (*Pyrus communis*)	PLA films coated with a barrier of pure silicon oxide (SiOx)	Improved barrier properties	Better maintenance of color an firmness with respect to conventional PE packaging	KRASNOVA *et al.* (2013)
Melon	Blend of biodegradable polyester	High permeability to water vapor	High percentage of weight loss	CONTE *et al.* (2009)

complex matrices, such as fruit and vegetables, characterized by an active metabolism which significantly affects their conservation. Certainly, biopolymers fulfill the environmental requirements but they have some limitations in terms of performance, like thermal resistance, barrier and mechanical properties. Better results can be achieved by the combined use of biodegradable packagings with edible coatings.

The use of edible films and coatings represents an environmentally-friendly technology that offers substantial advantages for shelf-life prolongation of many food products, including fruits and vegetables. The development of new natural edible films and coatings with either inherent microbicidal activity or the addition of antifungal ingredients (food preservatives, essential oils, antagonistic microorganisms, etc.) to improve the quality of fresh-cut fruits and vegetables is a technological challenge for the industry and a very active field of research worldwide. Edible coatings can keep product characteristics, such as texture and hydration intact, and they can provide significant fruit senescence retardation. Moreover, they serve as a carrier for a wide range of food additives, including anti-browning or antimicrobial agents.

New challenges should focus on the development of tailor-made coatings, containing the most appropriate film forming constituents and active ingredients for fresh and minimally processed fruits and vegetables, according to specific industrial needs. New trends have shown that enrichment of biodegradable materials with silver-montmorillonite nanoparticles may be a promising technique.

Undoubtedly, biodegradation offers an attractive route to environmental waste management. However, the actual application of these packaging solutions to food is still limited, due to the high cost of raw materials and the small-scale production. In the future, when the quan-

tities processed have increase to allow a change of scale, there will be a considerable reduction in the cost of biopolymers along with new and interesting applications in fresh-cut fruits and vegetables packaging.

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PAPER

COMPARISON OF KNOWLEDGE IN THE FIELD OF NUTRITIONAL FATS AMONG STUDENTS

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ABSTRACT

The aim of this work was to analyze the knowledge in the field of *trans* fatty acids (TFAs) and nutritional recommendations related to fats among students at the University of Life Sciences in Warsaw (SGGW) and Wageningen (WUR). The research was done using a questionnaire composed of 16 questions among 194 students from SGGW and WUR in 2012.

In Poland 96% and in Holland 89% of students had heard the name "*trans* fat". More than half of the questionnaire respondents knew industrial sources of TFAs. After comparing the results of the research, in which students succeeded, it was concluded that differences in the level of knowledge were statistically not significant ($p<0.05$). Eating habits for fatty pastry products representing a potential source of TFAs were similar. WUR students' purchase of pastry products was dependent on price, whereas that of Polish students depended on information and ingredients listed on the package.

- Keywords: *trans* fatty acids, consumer awareness, Polish students, Dutch students, eating habits -

INTRODUCTION

Fats are one of the fundamental food ingredients, which play a significant role in the human body. Their nutritional value depends on the composition of fatty acids (FAs) and content of diluted vitamins. The most important for the organism are FAs, from the group of EFAs (essential fatty acids) (GURR, 2000; BAYIR *et al.,* 2011), and they are easily oxidized.

Fats used for technological purposes should be resistant to high temperatures and storage conditions. For pastry products and frying fats, mainly fats rich in saturated fatty acids (SFAs) and sometimes *trans* fatty acids (TFAs) are used. In the manufacture of many food products it is necessary to use fats with solid consistency. It could be a natural solid fat or a modified fat (McDONALD and MOSSOBA, 1996; MATTHÄUS, 2007). It is possible to alter the characteristics of fats by using hydrogenation, transesterification, mixing and fractionation. Hydrogenation is one of the oldest methods, where double bonds in triacylglycerols (TAG) are saturated. As a result of this treatment unsaturated fatty acids (UFAs) become SFAs. Moreover, *trans* isomerization of FAs occurs within the process of hydrogenation, which results in TFAs. The modified fat has a higher melting point and increased resistance to oxidation (FLACZYK and KORCZAK, 2002; MATTHÄUS, 2007; DOWNES *et al.,* 2013).

TFAs may occur in natural products originating from ruminants. As a result of enzyme activity in ruminants' bodies there occurs *trans* isomerization from *cis* bonds to *trans* (GOODMAN *et al.,* 2001; EFSA, 2004; GEBAUER *et al.,* 2007). The content of TFAs originating from natural sources (from meat and milk) may range from 2 to 8 g/100 g fat depending on kind, breed, way of feeding and season. In Europeans' diet on average 30% of consumed TFA comes from milk and 10% from meat. Fats developed with industrial hydrogenation way may include even 70% of TFA in the whole FAs (MATTHÄUS, 2007; JASTI and KOVACS, 2010; KRASNOWSKA and SALEJDA, 2011). *Cis-trans* isomerization may also happen during high temperature treatment, for example during deodorization of oils or frying (KRASNOWSKA and SALEJDA, 2011).

According to most researchers, *trans* fatty acid isomers have a negative impact on human health. Because of the hazards related to excessive consumption of TFAs, international organizations such as EFSA and WHO have stated that daily intake for adults and children should be reduced as much as possible (EFSA, 2004; DOWNES *et al.*, 2013).

Fats rich in TFAs and SFAs have a negative impact on the cardiovascular system. They contribute to the cholesterol rise in the blood and the higher risk of cardiovascular diseases. There are also reports indicating negative influences of TFA on other human body organs. Consumption of 5 g of SFAs a day may increase the risk of heart diseases by about 2% and of TFAs by even 25% (MARTIN, 2007).

Due to the unfavorable effect of TFAs on the human body, legal restrictions of their content in food products have been implemented (Denmark and Canada). A different solution to reduce the consumption of TFAs is to require labeling of food products with information about the content of TFAs (Canada, USA) (ŻBIKOWSKA, 2010; DOWNES *et al.,* 2013). Denmark is the first country in the world to introduce a tax on food products containing SFAs. The aim of this tax is to reduce the consumption of products rich in saturated FAs and increase the consumption of fruits and vegetables (SIMOPOULOS, 1996).

Due to the negative impact of TFA on the body and any actions concerning with reduction of their consumption, it is important to follow the level of consumer's education in this area. It can be assumed that people with higher education related to food and nutrition sciences, interested in the subject of nutrition, should avoid consumption of products that may adversely affect their health, eg: products with *trans* fatty acids.

The aim of this study was to verify how the education of students at both universities influenced the knowledge in the field of TFAs, purchases of food products and eating habits. Therefore students' scores from the test were compared among students from studies related to food and health sciences with different studies (economic, marketing, agriculture, etc.). In addition, it was hypothesized that students of a master's degree course, especially from majors related to food and nutrition, are characterized by a high level of knowledge in the field of TFAs and fats.

MATERIALS AND METHODS

The analysis was based on the results of a survey carried out in 2012 among students from the University of Life Sciences in Warsaw (SGGW) (in Poland) and the University of Life Sciences in Wageningen (WUR) (in the Netherlands).

Before the questionnaire was distributed to respondents, a pilot test had been done. 215 people participated in the survey, but 194 of these questionnaires were qualified. 90 questionnaires were completed by students with different nationalities from Wageningen University (Table 1), while the rest of the respondents were Polish students from SGGW. The survey was addressed to students finishing their education at universities, from fields of studies related to food and nutrition, and also from other majors, but declaring an interest in the issues of food and nutrition. At first the study was conducted in The Netherlands. Participants of courses related to food sciences, which are in the master's degree program, were invited to take part in the sur-

Table 1 - Nationality of WUR students.

Country	Contribution (%)
Netherlands	38
Spain	18
Czech Republic	10
Finland	4
Germany	4
Hungary	4
USA	4
Canada	4
China	4
France	2
Greece	2
Turkey	2
Denmark	2
Sweden	2

vey. Students taking the bachelor's degree were allowed to participate in these classes (Table 1) only if they had finished specialized courses. Participants of these elective classes were students representing different majors. Therefore people who took part in the study were divided into two groups: respondents studying subjects related (fs) and not related to food and nutrition sciences (nfs). Students from WUR came from different countries, mainly of the European Union (Table 1). Master's degree studies at this university are open, which means that students are recruited from all over the world and classes are taught in English. Then, the study was conducted on respondents from SGGW in Poland. They were mainly students taking a master's degree, from majors related and not related to food and nutrition science. Students not attending any food faculties declared that they were interested in food related topics. In this connection, although there was a small population size it is assumed that the obtained results will answer the question whether it is necessary to intensify the education of students (including those that theoretically should have a high nutritional knowledge) in the subject of food fats especially those that contain *trans* isomers.

Table 2 - Characteristics of the studied population.
Legends':*fs – students from studies related to food science or human nutrition;
**nfs – students from studies not related to food science.

Specification		Structure of population (%)	
		WUR	SGGW
sex	women	78	81
	men	22	19
age	avarage	24.6	24
	SD	5.60	0.99
field of studies	Fs*	38	81
	Nfs**	62	19
degree of studies	bechelor's	16	8
	master's	84	92

The survey was held in the classrooms and the questionnaire was distributed in a paper version to respondents. The interviewer was present with the students. Surveys that were distributed to students in The Netherlands were written in English, while in Poland they were in Polish.

Questions in terms of content were divided into those which were a test of the respondents' knowledge (10 questions) and observations of the students' eating habits. In the questionnaire there were also basic questions – legal information (Tables 1 and 2). Most of the respondents were women (Table 2). Most of the respondents were aged 24. In the case of WUR they constituted 24.4% of the whole population, while in SGGW 55.8%. The average respondents' age in Holland was around 25 and in Poland 23. In the Netherlands the age range was much wider than in Poland. The eldest respondent of the survey was 58 years old.

In the discussion results of students from studies related to food and human nutrition and students from studies not related to these fields were analyzed separately. With regard to questions concerning the knowledge test a scoring system was used in which each respondent received one point per correct answer. The maximum score was 13 points. Such presentation of responses helped to interpret the results and assess the level of knowledge of the students.

Survey results were analyzed in Excel 2011 and the statistical program Statistica v10.0. One-way analysis of variance and χ^2 test were applied, with the significance level $p<0.05$. In order to conduct statistical analysis the responses were expressed as a percentage of answers.

RESULTS AND DISCUSSION

Analysis of the answers given by respondents concerning knowledge of trans fatty acids

Over 90% of the examined populations were students from studies related to food or health and respondents interested in this topic.

All students studying in the field related to food and nutrition had heard earlier the name "*trans* fat". However, 65% of WUR and 80% of SGGW students from other studies had heard this term (Fig. 1). Similar studies were conducted in Canada, where since 2005 it has been obligatory to provide information on the content of TFAs. On this basis, it was found that food selection based on TFAs increased significantly (SMED, 2012). A study conducted in 2007 among students of different fields of study (related to food and not related) in the U.S. (where since 2006 it has been obligatory to label food products containing TFAs) showed that 92% of respondents were aware of the term "*trans* fat"

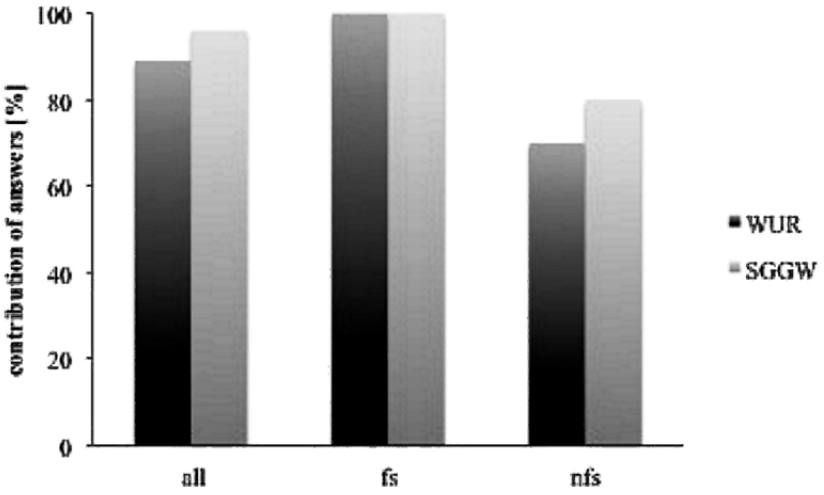


Fig. 1 - Students who had heard the term "*trans* fat".
SGGW - University of Life Sciences in Warsaw; WUR - Wageningen University; all – all of the respondents; fs – students from studies related to food science or human nutrition; nfs – students from studies not related to food science.

(STAMPFER *et al.*, 1991). Based on this, it can be assumed that the subject of TFAs is more widespread in the U.S. (also among students of subjects not related to food or nutrition).

Most of the respondents correctly indicate as the main source of TFAs 3 groups of products: shortening, hard margarines and pastry products (Fig. 2). The other correct answers related to natural sources of *trans* fatty acids (milk fat and dairy products) were selected much more often (30% more) by the students from WUR (Fig. 2a) than from SGGW (Fig. 2b). The aim of the following question was to verify whether respondents knew that food products might be a natural source of TFA developing naturally in bodies of ruminants. In research done by Stampfer (1991) 59% of students considered confectionery products as a source of TFAs in their diet.

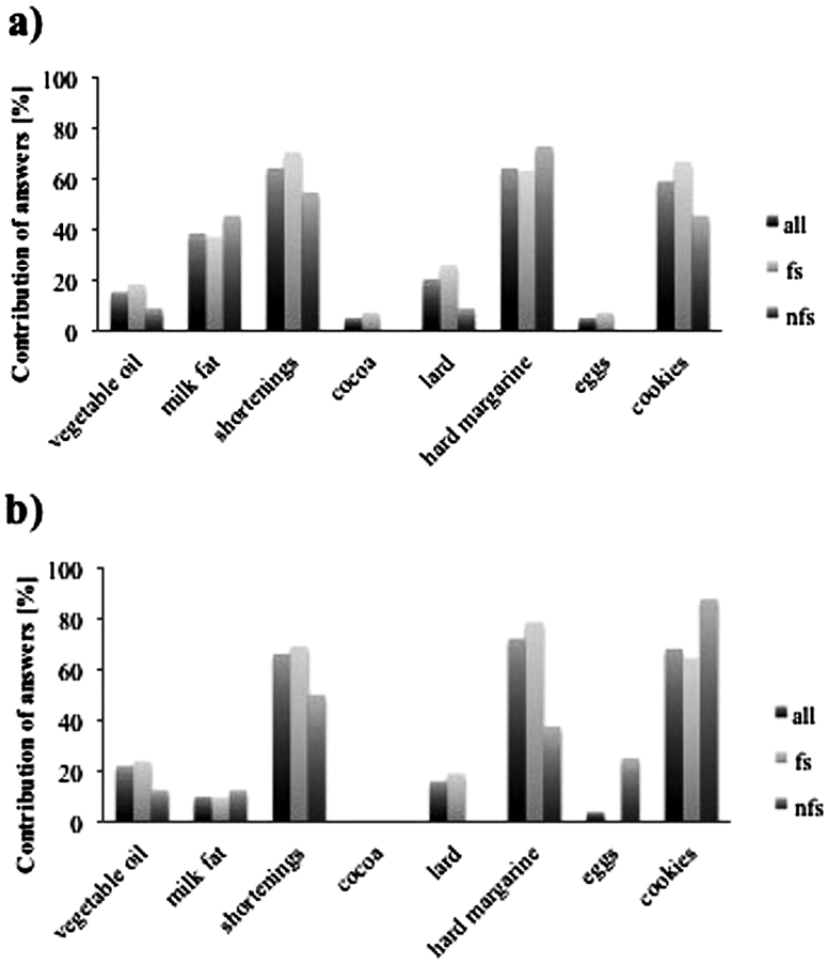


Fig. 2 - Sources of TFAs indicated by students of (a) WUR and (b) SGGW.
*all - all of the respondents; * fs, nfs - explanation under the Table 2.

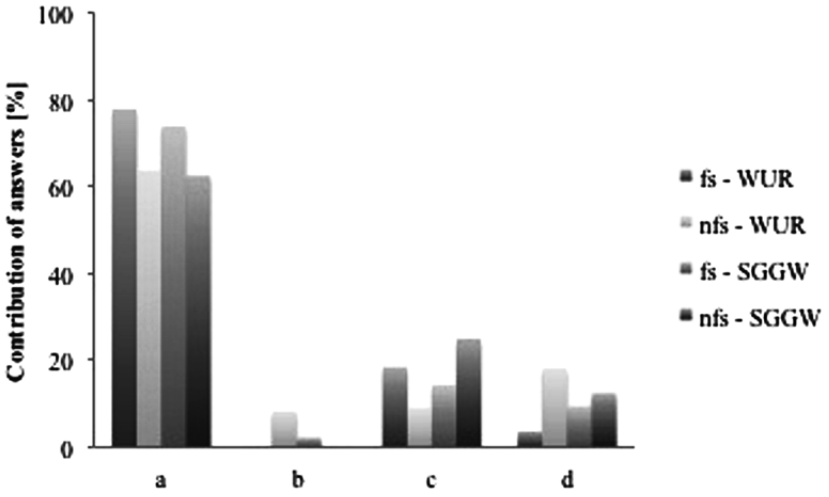


Fig. 3 - Structure of responses to the question "Are TFAs good for one's health?".
a - no, b - yes, c - it depends on their origin, d - I do not know;
explanation under the Fig.1.

Analysis of answers given by respondents concerning knowledge of the nutritional role of TFAs and fat

Most TFAs, mainly coming from industrial fats, have a negative influence on the human body. They contribute to increase of cardiovascular diseases. Only in the case of conjugated linoleic acid (CLA), which is present in the fat of ruminants, is it believed that the effect is positive (GEBAUER *et al.,* 2007). Therefore, the next question was to check the students' knowledge about the impact of TFAs on human health. At both universities more than two thirds of the students answered that TFAs have an adverse effect on human health (Fig. 3). Similarly, in another survey (STAMPFER *et al.,* 1991) 73% of American students considered TFAs as negative food components for health.

Another question, testing knowledge in the field of fats with particular emphasis on TFAs, considered nutritionally valuable elements which are supplied to the body from fat. As nutritionally beneficial elements, respondents chose the following answers: essential fatty acids (EFAs), vitamins and, unfortunately incorrectly, saturated fatty acids (SFAs). All of the students who study food and nutrition and related fields at SGGW selected EFAs and half of them vitamins (Fig. 4a). Among those students who study food science and nutrition at WUR, 89% of them selected the answer for EFAs and 32% for vitamins. Several people (12%) from the Dutch university thought that TFAs are nutritionally important (Fig. 4b).

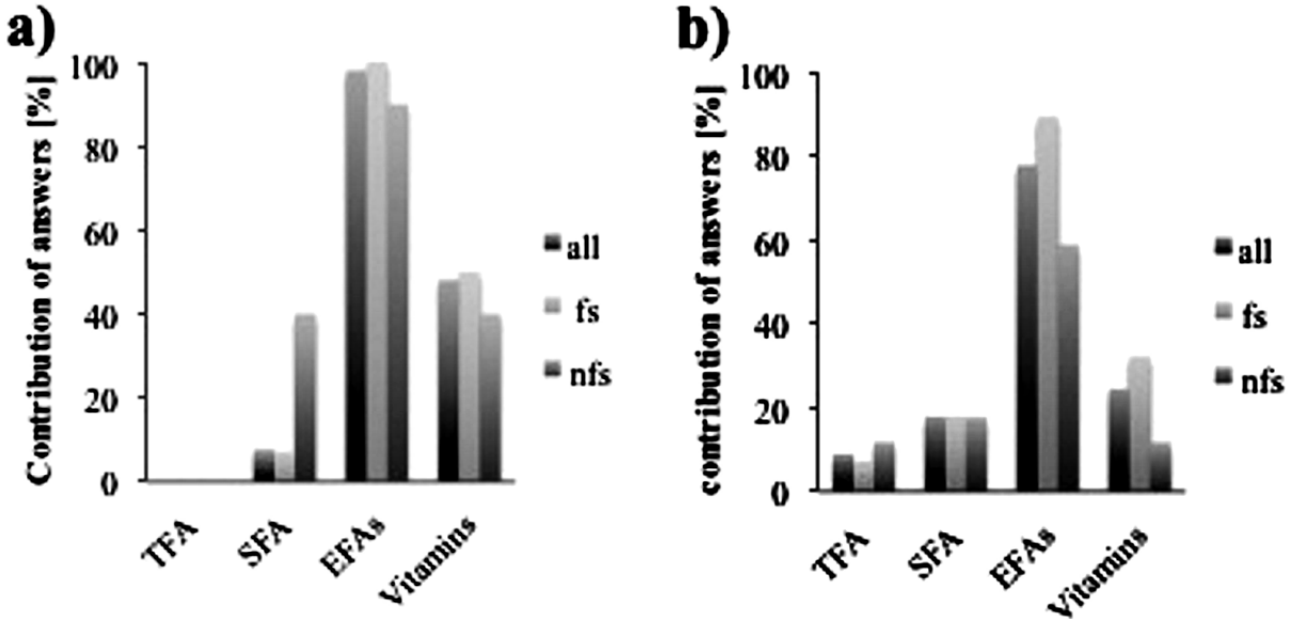


Fig 4 - Nutritionally valuable elements indicated by students of (a) SGGW and (b) WUR.
Explanation under the Fig. 1.

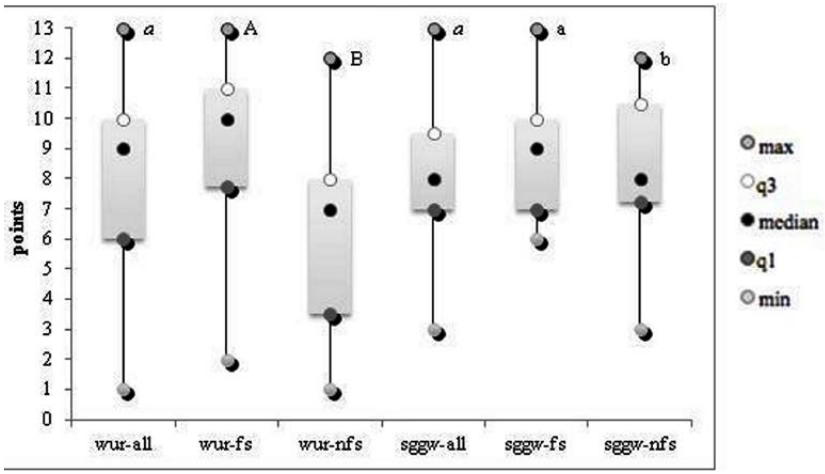


Fig. 5 - Averages of achieved points from students' test results.
a, a – statistically not significant; a, a – statistically not significant; A, B - statistically significant (p = 0,00291).
*explanation under the Fig. 1.

Results of the knowledge test

From the test of knowledge in the field of fats, students from SGGW in Warsaw scored around 9 points and students from WUR 8 points, from the 13 possible (Fig. 5). Polish students from studies related to food and nutrition sciences did not show any statistically significant differences ($p < 0.05$) in higher knowledge in the field of TFAs and fats than the respondents from unrelated studies (Fig. 5). However, differences were significant in the case of students from the Dutch university.

Eating habits of respondents

Students of both universities were eating pastry and confectionary products at a similar frequency (Table 3). It could be due to similar availability of these types of products in shops and vending machines at universities. Women were consuming much more of these types of products than men. In The Netherlands 80% and in Poland 62% of female students declared that they consume sweet snacks at least several times a week. Men had better eating habits, because two thirds of SGGW male students and nearly all male students from Wageningen University stated that they eat such products very rarely. Men from the Polish university were eating confectionary and pastry products more often than men studying in Holland. Researchers (URBAŃSKA and CZARNIECKA-SKUBINA, 2007) previously reported that 21% of high-school students were eating sweet snacks every day, while 31% consumed them several times a week. Thus, it can be concluded that university students eat fewer sweets than high-school students.

TFAs develop during frying. The most sensitive to *trans* isomerization in heat treatment are oils rich in unsaturated FAs. MORENO *et al.* (1999) reported that the TFA content substantially increases at 150°C and at 200°C reaches 357% compared to the initial content of *trans* isomers, while at 300°C it reaches the level of 3026%. That is why, for frying, it is advisable to use fats with a high thermal resistance. Almost all of the students declared that they use vegetable oil for this reason (94% of WUR respondents and 90% SGGW). FLACZYK and KORCZAK (2002) obtained a slightly different structure of answers of the question related to the fat used for frying. They found that, for frying meat, the most popular fat was lard (32% of answers) and vegetable oil (25%), for fish vegetable oil (91%),

Table 3 - Frequency of pastry product consumption among SGGW and WUR students.

Frequency of consumption	Contribution of answers (%)					
	WUR			SGGW		
	women	men	all	women	men	all
Once a month / not at all	11	10	11	19	10	17
2-4 times a month	9	90	27	19	60	27
2-4 times a week	43	0	33	31	0	25
almost everyday	37	0	29	31	30	31

and for eggs table margarine (63%). To fry flour-based products 40% of the respondents favored the use of vegetable oils.

Students' decisions related to purchase of pastry and confectionery were influenced by different factors (Table 4). In The Netherlands the most popular factors of purchase taken into consideration were price (29% of answers) and information on the package (24%). However, Polish students indicated first of all the ingredients and the information on the packages (38%) and then the brands (27%). Therefore the Polish students appreciated above all quality of these products and were guided as well by the trust of previously eaten products of the brands. Regardless of universities, one fifth of students claimed that they always chose the same products. In the following question the obtained answers among the students from both universities were different and statistically significant ($p = 0.04654$).

In turn, the studies of Krasnowska and Salejda [2011] showed that 37% of respondents were influenced by the price of products and then by the expiration date and brand (each 26%). The results of this research are very different from the answers obtained by the students from the Polish university.

Table 4 - Factors influencing students' decisions of the purchase of confectionery and pastry products ($p = 0.04654$).

Product features influencing the choice	Contribution of answers (%)	
	WUR	SGGW
ingredients and information on the package	24	38
price	29	10
brand	5	27
attractive package	20	4
always the same products	22	21

CONCLUSIONS

Differences in the level of students' knowledge, independently of university, were statistically not significant ($p<0.05$). Taking into account the fact, the all of the examined population's interest in nutrition aspects, it can be concluded that knowledge in the topic of *trans* FAs was not satisfactory. Most of the examined populations had heard the term '*trans* fats' before and were aware of their negative influence on human health. Some of the students, independently of the university, could not indicate all of the products constituting a potential source of TFAs (around 30%). Polish students were not aware of natural sources of TFAs (less than 10%) compared to students from the Dutch university (around 40%). Polish students from studies not related to food and nutrition sciences had less knowledge in the topic of TFA than respondents in the USA and Canada. Based on this, it can be concluded that Polish society should be further educated and food producers should be encouraged to label packages about nutritional characteristics of fat.

Eating habits related to consumption of pastry and confectionery products, which constitute a potential source of TFAs, of both populations were similar. Worrisome is the fact that women (independently of population) were consuming these kinds of products definitely too often.

Taking into account the level of knowledge of Polish students (SGGW) and from different regions of the EU (studying in the Netherlands – WUR) finishing master's degree studies (fs and nfs) it should be considered that there is still a need for education about the negative effect of TFAs on health and to inform consumers which products can be a source of them in the diet. Based on the obtained results it can be assumed that people coming from different EU regions who were not educated may have even less knowledge in this subject. Moreover, eating habits of women should change as they consume sweet snacks far more often than men, which could be a potential source of undesired fatty acids in the diet.

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PAPER

TURKISH CONSUMER DECISIONS AFFECTING ICE CREAM CONSUMPTION

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ABSTRACT

The aim of the study is to determine the main factors affecting the national-branded ice cream preferences of Turkish consumers, and to analyze the relationships between their preferences and consumption amounts. The data obtained from 400 households in Kahramanmaras, Turkey were used for *Principal Component*, *K-Means Cluster* and *Multiple Regression Analyses.* The results of the study highlighted clearly that the consumers of *the high (C1)*, *middle (C2)* and *low-income users (C3)* satisfied with the manufacturer brand, the individual private label, and the retailer brands on the ice cream purchase decision and consumption amounts, respectively. The manufacturers, retailers and marketers of the ice cream, therefore, should implement effectively the manufacturer brand, the private label and the retailer brand for *C1, C2* and *C3*, respectively, and then they could also increase the demand trends of the target consumers segments satisfied.

- Keywords: Ice cream attributes, manufacturer/national brand, principal component, cluster and multiple regression analyses, Turkish consumers' purchase decisions -

INTRODUCTION

Consumers purchase decisions towards the food products are a complex phenomenon influenced by a numerous factors classified as product-related (intrinsic and extrinsic food attributes), consumer-related (demographic, personal, psychological and physiological characteristics) and marketing environmental-related (economic, cultural, natural, technological, political and social environments) (REALINI *et al.*, 2013; TOPCU and UZUNDUMLU, 2012; TROY and KERRY, 2010; TOPCU *et al.*, 2009). Traditional sensory analyses focused on the intrinsic product attributes are not sufficient to meet not only the requirements of today's fast developing food markets but also the food choices of the consumers at the sale points. In order to be able to reply the consumers' need and willingness to buy, therefore, the suppliers must evaluate the food products with integrated approaches covering their intrinsic and extrinsic attributes by thinking of the consumers' socioeconomics characteristics.

In order to design the actual food product image optimized, the suppliers have focused on not only the intrinsic ice cream[1] attributes such as the sensory, structural, visual, nutritional, chemical and confidential properties including in aroma, taste, flavor, viscosity, color, texture and its content (REALINI *et al.*, 2013; MENICHELLI *et al.*, 2012; TOPCU and UZUNDUMLU, 2012; SOUKOULIS *et al.*, 2010; SIMEONE and MAROTTA, 2010; TOPCU *et al.*, 2009) but also the extrinsic ones consisting of hedonic quality attributes such as the price, country of origin, actual product image and quality, brand, labelling, packaging, promotion, advertising, etc. (TOPCU and UZUNDUMLU, 2012; TROY and KERRY, 2010; TOPCU *et al.*, 2009; SIRO *et al.*, 2008; McCARTY *et al.*, 2003; ORTH and FIRBASOVA, 2003) along with the consumers' socioeconomic characteristics such as income, food expenditure, education, age and lifecycle, occupation, etc. (TOPCU, 2012; TOPCU and UZUNDUMLU, 2012; TOPCU *et al.*, 2009).

While the consumers have considered the hedonic and visual quality attributes of the ice creams at point of sale before purchase and the sensory ones after consumption; the manufacturer, retailer and marketers have also interested with their various attributes to develop, improve and design the innovative ice creams based on their need and willingness to buy.

Providing an important communication among the manufacturer, retailer and the consumers, and then establishing a strong bridge between each group; the brands, one of the utmost important pieces of the information read a foodstuff, play an important role on the consumers' purchase decision making processes (TOPCU *et al.*, 2008; WULF *et al.*, 2005; GUERRERO *et al.*, 2000). Their effects on the purchase decisions, therefore, could be explained by several functions including in their identification and attributes, the reference function formalizing their purchase models, the guarantee function providing the quality image by reducing the feeling of risk, the personalization function allowing the consumers to locate themselves in their social environment, the entertainment function getting the consumers to motivate into the different brand choices, and the practical function allowing the consumers to learn and evaluate the results of different shopping experiences (TOPCU and UZUNDUMLU, 2012; AILAWADI *et al.*, 2011; ALDAZABAL *et al.*, 2006; GUERRERO *et al.*, 2000).

Not only has been consumed increasingly the ice cream by many consumer segments such as children, adolescents, adults and elder people during all the lifecycle due to their refreshing, sensational, nutritional and sanitarian attributes, on the other hand, but also the ice cream industry becoming a much profitable sub-sector owing to rapidly developing technological progresses has introduced 240 different types of ice cream resulting from the diverse ingredients and methods of freezing to the domestic markets under a strict competition between manufacturer and retailer in recent years (TOPCU and UZUNDUMLU, 2012; TURGUT and CAKMAKCI, 2009).

Therefore, the upmost motivation drives of the fundamental selection criteria in the ice cream purchase decision making process of all the consumers under various product depths and varieties are the brands. Therefore, it makes possible to distinguish among the consumers in terms of their sensitivity and loyalty to the brand highlighting the persistent purchase of a specific brand within a well-defined context and having a positive attitude towards it. In other word, there is a strong relationship between their varieties and brand types impacting on their purchase decisions (TOPCU, 2012a; ENNEKING *et al.*, 2007; GUERRERO *et al.*, 2000).

The brand type or names of the food products could be generally explained in two categories as the manufacturer brands created by manufacturers and bearing their chosen brand name (the local, national, international and global brands) and private label products derived and owned by retailers functioning at a distribution channel (the store/retailer, store sub-brand, generic and individual product brands) (TOPCU *et al.*, 2008). The effects of the brand types associated with the food choice and acceptability, and the consumers purchase attitude and behaviors were widely studied in marketing researches (FORNERINO and HAUTEVILLE, 2010; ZHOU *et al.*, 2010; GEHLHAR *et al.*, 2009; KUMAR *et al.*, 2009; TOPCU *et al.*, 2009; DIMOFTE *et al.*, 2008; ENNEKING *et al.*, 2007).

[1] Ice cream has a complex food colloidal system including in air bubbles, ice crystals and partially destabilized fat globules dispersed in a continuous aqueous phase. Therefore, its manufacturing as the most popular frozen dairy dessert has fairly complex processes and follows the subsequent steps such as preparation of the mixture, pasteurization, homogenization, cooling, aging of the mixture, addition of fermented milk, freezing, packaging and storage of the final mixture (SOUKOULIS *et al.* 2010).

Table 1 - Annual production, export, import, consumption (million liters) and per capita consumption amounts (liter) of ice cream, and macroeconomic indicators in Turkey.

Items and macroeconomic indicators	2000	2005	2010	2012	Annual change(%) 2005-2012
Production	60.0	113.0	324.0	260.0	7.1
Export	-	4.0	8.0	12.0	8.3
Import	-	0.8	0.2	1.5	5.8
Consumption	-	99.0	229.0	247.0	7.5
Per capita consumption	1.0	1.5	3.0	4.0	7.8
Population (million)	67.8	72.1	73.7	75.6	0.6
Grow rate of GDP (%)	6.8	8.4	9.2	2.1	2.9
Grow rate of GDP per capita (%)	5.3	7.1	7.5	0.8	1.9

Sources: (TUIK, 2013 and 2013a; ASUD, 2013).

The brands of the leader manufacturer manufacturing the ice cream with the national and international brands in Turkey consist of Unilever's Algida, Ulker's Natura, Has Food's Panda, Dinamik Food's Alaska, Izmir Dairy Products' MEMO, Nestlé's Nestle, Yasar Food's MADO and Ferah Food's EDO (FOOD, 2009). Their annual production, export, import, consumption and per capita consumption amounts of the ice cream along with some macroeconomic indicators in Turkey are indicated in Table 1.

According to Table 1, as considered the annual increasing production (7.1%), consumption (7.5%) and export (8.3%) trends between 2005 and 2012; it should be increased dramatically the domestic production amount responding to the needs and willingness to buy of Turkish consumers. Additionally, the annual increase rate in the consumer population (0.6%) and Gross Domestic Product (GDP) per capita (1.9%) between 2005 and 2012 in Turkey could increase considerably its consumption trends. Furthermore, it has been estimated that the ice cream consumption at selected European markets reported by MGMN for Turkey will also increase about 64% between 2012 and 2016.

Annual ice cream consumption amounts per capita of the leader countries such as New-Zealand, US, Finland and Sweden in the ice cream consumption in 2012, on the other hand; were calculated as 25, 21, 14 and 12 liters, respectively (FIIND, 2013). Furthermore, those per capita in Turkey, EU and the world in 2012 were calculated as 4, 12 and 6.0 liters, respectively (EICA, 2013; FAOSTAT, 2013; TUIK, 2013).

As a result of all this, compared with the consumers of the leader countries in the ice cream consumption, Turkish consumers have consumed about 3-6 times less ice cream than them, but this difference has continued to increase steadily. According to all the indicators, the ice cream production should be increased to be able to meet the increasing demands of Turkish consumers at domestic food markets.

In order to increase considerably the annual ice cream consumption amounts of Turkish consumers, traditional sensory analysis should be combined with integrated marketing approaches focused on the intrinsic and extrinsic ice cream attributes, and then they should develop/design the marketing tactic and strategies under homogenous consumer segments based on their socioeconomic characteristics. There is not any integrated scientific research taking into consideration all the conceptual frameworks with regard to the ice cream purchase decisions for the target consumers segments in the scientific literature. With the present study focused on integrated marketing approaches combined with all the factors influencing on the ice cream purchase decision making process of Turkish consumers, it could be filled an important gap in the literature by contributing considerably to the scientific literature.

This study, therefore, was designed to reach all the objectives mentioned above. In this scope, the main aims of the study are to explore the core factors related to the intrinsic and extrinsic product attributes impacting on the national-branded ice cream purchase decisions of Turkish consumers; and then to determine the target homogenous consumer segments based on their socioeconomic characteristics, and finally to analyze the effectiveness of the factors effecting on their consumption amounts.

MATERIAL AND METHODS

Material

The primary data used in this study which include in the variables based on the national-branded ice cream attributes and Turkish consumers' socioeconomic characteristics influencing on their consumption decisions and amounts were obtained from a face-to face questionnaire in Kahramanmaras[2] from autumn of 2012 until winter of 2013. The questionnaire was conducted with the heads of the households in their houses. The participants consuming the national-branded ice

[2] Kahramanmaras is located in the southeastern part of Turkey. The province lies on plain at the foot of the Taurus Mountains and has a total population of 1.063.174 in 2013, and about 42% of those live in the city centre.

cream, and accepting the voluntary contribution were selected randomly. They have demographic and socioeconomic characteristics such as average 46 years old, $1282 income, 3.7 liters ice cream consumption, 4.3 family sizes 51% male population and 61% high school education.

Methods

Method used in determination of the sample size

In order to determine the sample size, while minimizing sample bias and representing correctly the population; the city center was divided into four parts covering the west, east, south and north-sides of Kahramanmaras with 73.929 households consisting of about six family members (APDK 2013; TUIK 2013).

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

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

Where

n = sample size
Z = z value, (1.96 for 95% confidence level)
p = percentage making a choice, (0.5 used for sample size needed)
c = confidence interval, (used 0.05 = ±5)

The minimum sample size having the capability of representing the main population was calculated as 385 households (but the study was conducted with 400 households). By considering the information obtained from the food science and marketing literature under the expert consultancy and the prior experiences of the researcher, a draft questionnaire was prepared (TOPCU, 2012). In order to check out non-sampling error which occurs due to ambiguous definitions, unclear instructions, questionnaire wording, format and length, a pre-test was carried out 15 consumers selected randomly in the target regions. The flow and nature of the questionnaire were tested, and the order and timing of the questions were re-arranged. The questionnaire was then modified and refined before starting the fieldwork.

Methods used in the preparation of the questionnaires

It was asked the participants in the survey to respond to each statement indicating the significance levels of the ice cream attributes by using a *Likert-format* with 1-5 scale (where 1 refers to the least important and 5 refer to the most important attribute). Of eighteen ice cream attributes, five are related to the intrinsic ice cream attributes (ingredient quality, texture and aroma, taste and flavor, hardness and viscosity and organic content); six are covered by the extrinsic food attribute referring the marketing mix (the region of origin, advertisement and promotion, package material and appearance, product assortment, quality-price relation and price); two are determined by manufacturing process (the reliability of manufacturing process and food safety and hygiene); five are stated by the relationships between the consumer and marketing environments (brand recognition, store satisfaction and confidence, manufacturer brand satisfaction and image). The sources in the literature related to the intrinsic and extrinsic ice cream attributes and their items were showed in Table 2.

On the other hand, of the two string and three numeric variables referring to the consumer demographic and socioeconomic characteristics, three referred to the consumer demographic characteristics (age as a numeric variable; consumer education (0: primary and 1: high school and college graduate) and the occupation (0: others and 1: officer) as string variables); two included in the consumer socioeconomic characteristics (monthly consumer income ($) and the share of the ice cream expenditure within total food one as numeric variable).

Methods used in the statistics analyses

After editing and coding, the primary data were first used in *Principal Component Analysis (PCA)*[3] to determine the main factors related to the product attitudes influencing on the national ice cream purchase patterns of Turkish consumers. *PCA* is a data reduction technique that reduces the number of variables used in an analysis by creating new variables (called factors) that combine redundancy in the data (SPSS 15.0 2006). The first step in *PCA* is to determine the number of relevant factors. This was conducted by *PCA* using *Varimax Rotation Method (VRM)*[4]. *PCA* was used initially to identify underlying aspects explaining a correlation among a set of the food product attributes. The purpose of *PCA* was to identify those attributes accounting for a relatively large proportion of the variance in the sample.

In the second and final steps of the statistics analyses, the main factors obtained from *PCA* were used for *k-means cluster and multiple regression/correlation MRC Analyses,* respectively. In the second step, according to

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

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

Table 2 - The sources in literature related to the macro variables and their items.

Macro variables	Items	Sources
Intrinsic product attributes	Texture and aroma	SOUKOULIS *et al*., 2010; CRUZ *et al*., 2009 OZDEMIR *et al*., 2005; AIME *et al*., 2001
	Taste and flavor	TOPCU and UZUNDUMLU 2012; SOUKOULIS *et al*., 2010; TURGUT and CAKMAKCI, 2009; ENNEKING *et al*., 2007
	Ice cream content	SOUKOULIS *et al*., 2010; TURGUT and CAKMAKCI, 2009; ALVAREZ *et al*., 2005
	Hardness and viscosity	ATSAN and CAGLAR, 2008; KAYACIER and DOGAN, 2006; GRANGER *et al*., 2005
	Ingredient quality	KARAMAN *et al*., 2011; FARHOOSH and RIAZI, 2007; GRANGER *et al*., 2005
	Food safety and hygiene	TOPCU and UZUNDUMLU, 2012; GOFF, 2008; ROININEN *et al*., 1999
Extrinsic product attributes	Actual image quality	TOPCU and UZUNDUMLU, 2012; SIRO *et al*., 2008; WILDMOSER *et al*., 2004
	Ice cream assortment	SOUKOULIS *et al*., 2010; LANGE *et al*., 1999; GOFF, 2008
	Brand recognition	AILAWADI *et al*., 2011; GEHLHAR *et al*., 2009; WULF *et al*., 2005
	Store satisfaction	TOPCU and UZUNDUMLU, 2009; TOPCU *et al*., 2008; WULF *et al*., 2005; GUERRERO *et al*., 2000
	Store confidence	TOPCU and UZUNDUMLU, 2009; SOBERMAN and PARKER, 2006; GUERRERO *et al*., 2000
	Brand satisfaction	AILAWADI *et al*., 2011; DIMOFTE *et al*., 2008; STRIZHAKOVA *et al*., 2008
	Brand image	TOPCU, 2012; KUMAR *et al*., 2009; BRAKUS *et al*., 2009; LI and HOUSTEN, 2001
	Package appearance	TOPCU and UZUNDUMLU, 2012; TOPCU and ISIK, 2008; ENNEKING *et al*., 2007
	Promotion mix	KARRAY and MARTIN-HARRAN, 2009; TOPCU and ISIK, 2008; LI and HOUSTEN, 2001
	Price and quality relation	TOPCU and UZUNDUMLU, 2012; AMROUCHE and ZACCOUR, 2009; KUMAR *et al*., 2009; WOLK and SPANN, 2008; ENNEKING *et al*., 2007
	Region/country of origin	REALINI *et al*., 2013; BATRA *et al*., 2010; TOPCU *et al*., 2010; ORTH and FIRBASOVA, 2003

monthly income levels of Turkish consumers, therefore, the target consumers were separated to three homogeneous clusters including in low-income users (less than $500 per month), middle-income users ($500-1250 per month) and high-income users (more than $1250 per month) (TOPCU 2012), and then the main factors were allocated to the homogeneous consumer clusters based on monthly income levels of the target consumers by *k-means cluster analysis.*

In the final step, the main factors obtained from *PCA* were used *Multiple Regression/Correlation (MRC) Analysis. MRC analysis* was used to measure the effects of variable factors delivered from the ice cream attributes and Turkish consumers' socioeconomic characteristics effecting on their ice cream amounts.

In order to test whether the normal distribution of the main factors delivered from *PCA* and the socioeconomic variables collected from the consumers exhibited or not was applied the various transformations techniques, and it was tested that the closest distribution to the normal of all the factors provided. On the other hand, the coefficient estimations were estimated by using *ordinary least squares (OLS).* Individual and group significance of these coefficients were tested using *t* and *F* tests, respectively.

In order to evaluate whether to be any econometrical problem among the variables, it was tested the overall *multicollinarity* and *auto-correlation* problems by considering *Variance-Inflating Factor (VIF)* and *Durbin-Watson d statistics*, respectively. *Multicollinearity* among variables was detected by calculating *(VIF)* (GUJARATI 2005; SPSS 15.0 2006). The analysis techniques have been widely used in many marketing researches with regard to the dairy food product attributes (DAVIES and CLINE, 2005; ISHII *et al.*, 2007; BATRA *et al.*, 2010; CADENA and BOLINI, 2011).

SPSS 15.0 statistical software program was used to run the *PCA* and *MRC* analyses. *MRC* model could be written as follows:

ICC = f (RPRS, TMNF, MBST, HQLT, SQLT, ETH, INC, EXP,OCU, EDU, AGE,)

Dependent Variable

ICC: Monthly national ice cream consumption amount per household (l/month)

Independent Variables

RPRS: Retailer prestige
TMNF: Trust to manufacturer
MBST: Manufacturer brand satisfaction
HQLT: Hedonic quality
SQLT: Sensory quality
ETH: Ethnocentrism
INC: Monthly consumer income ($)
EXP: The shares of the ice cream expenditures within total food ones
OCU: Consumer occupation
EDU: Consumer education
AGE: Consumer age

RESULTS AND DISCUSSION

Demographic and socioeconomic profiles of the participants

The results of descriptive statistics related to the gender, marital status, tasks in the family, education levels, occupation and age of the household heads, and monthly income, national-branded ice cream consumption and expenditure of the households attaining in questionnaire were indicated in Table 3. The results of the statistics showed that 51.0, 90.2, 47.1, 61.3 and 28.0% and 46.2 years

of the participant household heads consisted of the male, married, husband, high school graduate and retailer, respectively. On the other hand, monthly average income, ice cream expenditure, ice cream consumption amount and family size of the households were calculated as $1281.9, $48.6, 3.9 *l* and 4.3 individuals, respectively.

The results of PCA related to the ice cream consumption satisfaction

Kaiser Normalization (KMO) which compares partial correlation coefficients with observed ones was calculated as 0.87 for the ice cream attributes, and this means that the data set was at a perfect level for the factor analysis since the test score was greater than 0.50 (Table 4).

The principal component analysis using *VRM* grouped the eighteen variables related to the ice cream attributes into the six factors with Eigenvalues greater than 1.0, which these factors explained the 71.96% of the total variance. *F1* being the first of these factors was explained by 19.00% of the total variance, and consisted of the retailer/store positioning. *F1*, therefore, could be called by *retailer prestige (RPRS)* (TOPCU *et al*., 2009). Explaining the 12.15% of the total variance, *F2* gave us information about the manufacturing process and the used material quality, and thus this factor could be determined by *trust to manufacturer (TMNF)* (TOPCU, 2012).

Reporting the 10.99% of the total variance, *F3* gathered together the variables related to the brand satisfaction, and it could be named as *manufacturer brand satisfaction (MBST)* (TOPCU and UZUNDUMLU, 2012). Referring to the 10.28% of the total variance, *F4* could be represented *hedonic quality (HQLT)* covering the relationship between the product quality and its price (TOPCU, 2012, 2012a). Considering the 10.08% of the total variance, *F5* was constituted by the sensory quality attributes of the ice cream, and could be entitled by *sensory quality (SQLT)* (TOPCU, 2012). Finally, referring the 7.20% of total variance, *F6* stated the relationship between the country of origin and the ice cream consumption with manufacturer brand, and thus it could be denominated by *ethnocentrism (ETH)* (ORTH and FIRBASOWA, 2003).

The results of cluster analysis related to the ice cream consumption satisfaction

The main factors derived from the *PCA* and effecting on the ice cream purchase decisions of Turkish consumers were separated into three homogeneous consumers segments through *k-means cluster* according to their income levels including in the low, middle and high-income users (Table 5).

Table 3 - Demographic and socioeconomic characteristics of the participants.

Demographic characteristics	Frequency	Percent	Cumulative	
Gender	400	100.0		
Male	204	51.0	51.0	
Female	196	49.0	100.0	
Marital status	400	100.0		
Married	360	90.2	90.2	
Not married	40	9.8	100.0	
Tasks in the family	400	100.0		
Husband	188	47.1	47.1	
Wife	212	52.9	100.0	
Education level	400	100.0		
First school	40	10.0	10.0	
High school	245	61.3	71.3	
College	115	28.7	100.0	
Occupation	400	100.0		
White-collar state employ	107	26.8	26.8	
Blue-collar state workers	50	12.5	39.3	
Businessman	40	10.0	49.3	
Retailer	112	28.0	77.3	
Pensioner	67	16.8	94.1	
Others	24	5.9	100.0	
Socioeconomic characteristics	**Minimum**	**Maximum**	**Mean**	**Std. Dev.**
Age	23.0	77.0	46.2	12.4
Monthly average income ($)*	190.0	3810.0	1281.9	1448.4
Ice cream consumption amount (l)	1.0	15.0	3.7	2.1
Monthly ice cream expenditure ($)*	2.0	200.0	48.6	36.5
Average family size	1.0	9.0	4.3	2.1

*The prices of the products were converted from Turkish Lira (TL) to US Dollar ($) using the exchange rate on February 25, 2014. The conversion rate used was 2.15 TL/$.

Table 4 - Factors and correlated variable loadings related to the ice cream attributes.

Variables	Factor loadings*					
	F1	F2	F3	F4	*F5*	*F6*
Retailer prestige (F1:RPRS)						
The ice cream assortment	**0.845**	0.048	0.032	-0.205	-0.061	0.249
Brand recognition	**0.727**	-0.343	-0.304	0.198	-0.005	-0.151
Store satisfaction	**0.640**	0.273	-0.086	-0.362	-0.222	-0.075
Store confidence	**0.558**	0.121	-0.214	-0.370	-0.051	-0.435
Advertisement and promotion	**0.532**	0.258	0.340	-0.210	0.070	-0.031
Trust to manufacturer (F2: TMNF)						
Ingredient quality of the ice cream	-0.056	**0.879**	0.126	0.054	-0.035	0.134
Food safety and hygiene	-0.009	**0.741**	-0.189	0.091	0.080	-0.210
Reliability of manufacturing process	-0.284	**0.729**	0.169	-0.052	-0.092	0.017
Manufacturer brand satisfaction (F3: MBST)						
Manufacturer brand satisfaction	-0.021	0.090	**0.828**	-0.053	0.058	-0.021
Package material and appearance	-0.090	-0.051	**0.790**	0.021	-0.200	0.091
Manufacturer brand image	0.368	0.220	**0.581**	0.230	0.450	0.098
Hedonic quality (F4:HQLT)						
Quality-price relation	-0.096	0.177	-0.186	**0.765**	0.031	-0.081
Product price	0.058	-0.088	0.196	**0.745**	-0.145	0.139
Sensory quality (F5: SQLT)						
Texture and aroma	0.112	0.035	0.094	-0.157	**0.866**	-0.097
Taste and flavor	-0.166	0.028	0.038	-0.097	**0.735**	-0.187
Organic content of the ice cream mix	-0.160	-0.172	-0.358	0.024	**0.731**	0.311
Hardness and viscosity	0.220	-0.202	0.220	-0.409	**0.516**	0.209
Ethnocentrism (F6: ETH)						
The region of the origin	0.025	0.070	-0.027	0.098	-0.002	**0.932**
Eigen-value	***3.230***	***2.449***	***1.868***	***1.748***	***1.713***	***1.223***
Share of explained variance (%)	19.00	14.41	10.99	10.28	10.08	***7.20***
Cumulative share of explained variance (%)	***19.00***	***33.41***	***44.40***	***54.68***	***64.76***	***71.96***
KMO (Kaiser-Meyer-Olkin) Statistic						***0.873***
Bartlett's test of Sphericity	***(Chi-square, df: 136): 2552.21 (p: 0.000)***					

*Bold numbers indicate the largest loading for each variable.

Table 5 - Final cluster centres and the number of cases in each cluster.

Main Factors	Clusters*		
	High-income users (C1)**	Middle-income users (C2)**	Low-income users (C3)**
RPRS (Retailer prestige)	-0.266	-0.586	0.991
TMNF (Trust to manufacturer)	0.276	0.256	-0.109
MBST (Manufacturer brand satisfaction)	0.813	-0.375	-0.599
HQLT (Hedonic quality)	0.301	-0.375	0.381
SQLT (Sensory quality)	-0.408	0.514	0.105
ETH (Ethnocentrism)	-0.332	0.380	-0.414
Number of total cases in each cluster ***	***140***	***187***	***73***
% of total cases in each cluster	***35%***	***47%***	***18%***

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

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

*** The total number of the cases (n): 400

The results of the study showed that *high-income users of the national-branded ice cream (C1)* formed their consumption satisfaction according to *trust to manufacturer (TMNF)* and *manufacturer brand satisfaction (MBST)* by taking into consideration *the manufacturer-branded ice cream willingness to buy* as an indicator of the main component of the hedonic quality attributes. The target homogeneous consumers in the *C1*, therefore, gave a much more attention to the manufacturer brand satisfaction on their purchase decision and satisfactions, and thus it could be designed/developed the manufacturer-branded ice creams for the target consumers at this segment.

The results of the study also indicated that *the middle-income users of the national-branded ice cream (C2)* focused on the purchase patterns constructed by a combination of the factors such as *the sensory quality (SQLT), trust to manufacturer (TMNF)* and *Ethnocentrism (ETH)*. The consumers in *C2* provided much stronger purchase motivations for the private labels under trust to local manufacturers by emphasizing the core benefits of the ice cream based on the sensory quality attributes. It could be introduced the private labeled local ice creams for the target segments to the region retailers.

The results of the study explained that *the low-income users of the national-branded ice cream (C3)* tended to buy the ice cream with *retailer prestige (RPRS)* under *hedonic quality attributes (HQLT)*. The target consumers in *C3* focused on the actual ice cream-imaged purchase decision and satisfactions based on the retailer/store brands. It could be presented, therefore, the retailer branded ice creams for this segment.

The results of MRC analysis with regard to the ice cream consumption amounts

VIF values calculated as 1.028 and 1.582 indicating the scores between 1.00 and 2.50 determining the acceptable reference range for *multicollinearity* problem showed that there was not it. *Durbin-Watson d statistics*, on the other hand, computed as 2.09 was not located between d_u *(1.89) and 4-*d_u *(1.65)*. There, therefore, was no problem related to *auto-correlation* in the *MRC* model (KALAYCI, 2005). According to these statistical test results diagnosing the econometrics problems, we could directly use this data set for the *MRC* model.

The determination coefficient *(Adj.*R^2*)* was calculated as 0.80 in the *MRC* model, this means that all the independent variables explained the 80% of the dependent variable. The *OLS* estimates of the model coefficients and other statistical measurements were presented in Table 6. The results of statistical measurements highlighted that the *F-statistic* rejecting the null hypothesis that makes all the coefficients equal to zero was calculated as 153.76 *(p<0.01)*. On the other hand, the partial regression coefficients of all the independent variables, except for those of *RPRS*, were statistically found to be meaningful *(p<0.00 and p<0.05)*. Their signs, moreover, were also found in conformity with economic theory. However, *RPRS* and *ETH* were not important statistically *(p=0.333 and p=0.384)*, and thus they were not evaluated for compliance with the economic theory. Additionally, they had no impact on the manufacturer/national-branded ice cream consumption amounts, and thus there was an inverse (negative) relationship between *ETH* and the consumption

Table 6 - The results of multiple linear regression (MRC) analysis.

n: 400 R2: 0.82 Adj R2: 0.80 F (11, 388): 153.76* dL=1.65 du=1.89 DW dh=2.09

Variables	Multiple linear regression model				Collinearity statistics		Correlations		
	Coefficients [a]	Sd.error	t_h-value	p-value	Tolerance	VIF	Zero-order	Partial	Part
Constant	0.672	1.729	4.437	0.000*	-	-	-	-	-
RPRS	0.023	0.099	0.970	0.333	0.866	1.154	0.196	0.049	0.021
TMNF	0.072	0.102	3.218	0.001*	0.973	1.028	0.011	0.161	0.075
MBST	0.045	0.095	1.996	0.047**	0.893	1.120	0.103	0.098	0.042
HQLT	0.066	0.077	2.503	0.010*	0.958	1.043	0.080	0.126	0.066
SQLT	0.090	0.091	4.045	0.000*	0.975	1.026	0.126	0.201	0.089
ETH	-0.019	0.089	-0.872	-0.384	0.968	1.033	-0.004	-0.044	-0.019
INC	0.080	0.511	2.944	0.003*	0.648	1.544	0.331	0.148	0.065
EXP	0.736	0.347	26.689	0.000*	0.632	1.582	0.358	0.305	0.385
OCU	0.062	0.379	2.281	0.018**	0.703	1.422	0.023	0.120	0.052
EDU	0.180	0.395	4.738	0.000*	0.648	1.543	0.081	0.106	0.048
AGE	0.424	0.018	18.958	0.000*	0.690	1.448	0.294	0.101	0.046

[a] Coefficients consist of the standardized coefficients.

*p<0.01 **p<0.05

amounts of the ice cream. The findings were supported by the similar results of some studies (BRAKUS *et al*., 2009; TOPCU *et al*., 2009; DIMOFTE *et al*., 2008; STRIZHAKOVA *et al*., 2008; ENNEKING *et al*., 2007).

The results of the study indicated that of the six main factors, the four ones such as *SQLT*, *TMNF*, *HQLT* and *MBST* had an important effect on the national-branded ice cream consumption amount per household in *C2; C1* and *C2; C1*and *C3;* and *C1*, respectively. *SQLT* was regarded as their main determinant on their satisfaction after consumed, and thus it played an important role in this process for *C2* by providing much important information about the consumer satisfaction associating with the intrinsic ice cream attributes. The results were similar with respect to those of the studies carried out by TOPCU (2012); TOPCU (2012a); TOPCU and UZUNDUMLU (2009); ENNEKING *et al*. (2007); ISHII *et al* (2007); GUERRERO *et al*. (2000).

The results of the study also showed that there was a positive relationship between *TMNF* giving much important information about the manufacturing process to Turkish consumers, reducing their health concern related to the ice cream based on the manufacturer confidence constructed with its ingredient quality at the manufacturing process under hygienic conditions and their consumption decision and amounts. This caused the consumers to increase dramatically the ice cream consumption trends in *C1* and *C2* due to the safe and hygienic ice cream manufactured by the manufacturers. This findings were quite similar to the results reported by several researchers (TOPCU, 2012; TOPCU and UZUNDUMLU, 2012; TOPCU *et al*., 2010; SOBERMAN and PARKER, 2006; SCHUILING and KAPFERER, 2004).

The results of the present study also reported that there was a string relationship between *HQLT* having a bigger impact effect on the consumer demands making possible the product differentiation through the quality and price rating in *C1* and *C3,* and their purchase decisions. The marketers could, therefore, separate target Turkish consumer masses into two homogenous segments, and then they could also stimulate much more effective marketing tactic and strategies for each segments (WOLK and SPANN, 2008; KUMAR *et al*., 2009; TOPCU *et al*., 2010; ZHOU *et al*., 2010).

The results of the study also revealed that *MBST* provided attitudinally a positive motivation on the national-branded ice cream consumption amounts in *C1*, and it had a string relationship between *TMNF* and *HQLT* of the manufacturer-branded ice cream. As a result, *MBST* was of a strong linear relationship with these factors influencing on not only the ice cream purchase decision at the sale points but also its consumption satisfaction and amounts after consumed. There were a lot of the studies referring to the relationship between *MBST* and the food consumption amounts (TOPCU, 2012, TOPCU, 2012a; TOPCU and UZUNDUMLU, 2012; ZHOU *et al*., 2010; AMROUCHE and ZACCOUR, 2009; STRIZHAKOVA *et al*., 2008).

The results of this study also provided the important information about how the demographic and socioeconomic characteristics of Turkish consumers affected their purchase decision and attitudes towards the national-branded ice cream. In this research considering their distinctive characteristics, the results of the study also referred that the share of the ice cream expenditure within total food ones *(EXP),* age *(AGE)*, education *(EDU)*, income *(INC)* and occupation *(OCU)* of Turkish consumers had a much bigger effect on the ice cream consumption amounts than the other preference factors, especially, *EXP, AGE*, *EDU.* These findings were supported by results of previous researches based on the consumers purchase attitude and behaviors towards the food products (TOPCU, 2012; TOPCU and UZUNDUMLU, 2012; TOPCU *et al*., 2010; ZHOU *et al*., 2010; TOPCU *et al*., 2009; DIMOFTE *et al*., 2008; ENNEKING *et al*., 2007; LI and HOUSTON, 2001).

CONCLUSIONS

In this study, the integrated approaches patterns based on not only the intrinsic and extrinsic attributes of the national-branded ice cream but also the socioeconomic characteristics of Turkish consumers impacting on their ice cream purchase decisions and consumption amounts were evaluated. The measurement results of the study highlighted clearly that the consumers in *C1, C2* and *C3* satisfied with the actual manufacturer brands linking between trust to manufacturer and the hedonic quality attributes, the individual private brands under trust to local manufacturers and the sensory ones, and the retailer brands emphasizing the hedonic ones on the ice cream purchase decision and consumption amounts, respectively. The manufacturers, retailers and marketers of the ice cream, therefore, should widely implement the manufacturer brand, the individual private label and the retailer brand for *C1, C2* and *C3*, respectively in order to be able to create the demand trend increases under the integrated marketing tactic and strategies affecting positively the ice cream purchase decisions and consumption amounts of the target consumer segments.

Although this study has some scientific merit for the academic and food manufacturing communities, there are some limitations. The results of this study have a limited generalizability since the data were obtained from only one city. If the survey is conducted nationally, more data will give more objective results about the purchase decisions of all the population. In future studies, furthermore, this model could be expanded to incorporate more factors and factor levels into the model.

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PAPER

TECHNOLOGICAL AND SENSORY PROPERTIES OF HAMBURGERS ENRICHED WITH CALCIUM STUDY OF THE *IN VITRO* BIOAVAILABILITY

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ABSTRACT

Hamburgers were supplemented with three calcium salts (calcium gluconate CG, calcium lactate CL and calcium citrate-malate CCM). They were added in sufficient amount to that 100 g of hamburger gives 20 or 30% of the Ca RDA (1000 mg). Their technological and sensory properties were studied. CG 30% gave the worst sensory properties and it was discarded. Bioavailability of calcium depends on the type of salt used and the highest value was obtained with CCM (14.5%). For that, this salt is proposed as the most adequate for the enrichment of fresh meat products.

- Keywords: bioavailability, calcium salts, hamburgers -

INTRODUCTION

Meat and meat products are important to the human diet; they contain proteins with all nine essential amino acids of high biological value, accounting for 40% of total amino acids. They are an excellent source of bioactive compounds, including vitamins (B-complex), iron, zinc, phosphorus (FERNÁNDEZ *et al.*, 2005; WEISS *et al.*, 2010).

The interest on the human health and the actual consumer tendencies, who prefer more nutritious food, have stimulated interest in developing meat products with bioactive compounds with attractive physiological activities (GRIGUELMO *et al.*, 1999; CENGIZ and GOKOGLU, 2005; DECKER and PARK, 2010). Greater emphasis has been placed on strategies involving the addition of bioactive compounds with recognized health benefits, such as proteins, fibre, polyphenols, unsaturated fatty acids, probiotics or minerals (ROBERFROID, 2002; SAIGA *et al.*, 2003; CACERES *et al.*, 2006; ARHIARA, 2006; JIMÉNEZ-COLMENERO *et al.*, 2006; DECKER and PARK 2010; ALONSO *et al.*, 2010; ZHANG *et al.*, 2010; KHAN *et al.*, 2011). Dietary minerals are essential for various physiological functions and they have been associated with the prevention of several diseases (MENÉNDEZ-CARREÑO *et al.*, 2008; DECKER and PARK, 2010; ZHANG *et al.*, 2010). Calcium (Ca) is one of the most important. It gives structural integrity to mineralized tissue preventing osteoporosis and contributing to the "bone health". Although interest in Ca primarily derives from this role, it also plays other essential physiological roles in arterial hypertension, cellular function, skeletal muscle contraction, blood coagulation and enzymatic reactions as a co-factor (PRINCE *et al.*, 2006; STRAUB, 2007, ADLURI *et al.*, 2010). Health authorities have recommended a Ca daily allowance (RDA) of 1000 mg for adults aged 19-50 (Institute of Medicine, IOM, 2004) or 800 mg (Directive 2008/100/EC), without concern for age.

Milk and dairy products account for much of the Ca in the human diet, (75% of Ca intake, aprox); only 16% comes from fish and vegetables and a 6-7% from mineral water (GUÉGUEN and POINTILLART, 2000; CHAROENKIATKIU *et al.*, 2008). Since meat and meat products are poor source of Ca, supplementing them with Ca salts could be a good option to increase its intake particularly those in which the consumption of milk can be a health problem. Previous studies have examined the addition of Ca to meat products, but mainly for reducing sodium levels (GIMENO *et al.*, 1998, 1999) and the final Ca content was not sufficient to consider them as *a source* of this mineral. Studies previously performed in our laboratory (CÁCERES *et al.*, 2006), it was reported that calcium could be successfully added to both cooked and dry-fermented sausages, but until now, fresh meat products have been not assayed.

In this way, the present work deals with the manufacture of fresh meat products (hamburgers) enriched with calcium. For that, three different calcium salts were assayed: Ca gluconate (CG), Ca lactate (CL) and Ca citrate-malate (CCM). They are permitted in food (Regulation EC No 1907/2006) and characterized because of their high bioavailability (KORSTANJE and HOEK, 2001). The technological and sensory properties of the hamburgers manufactured were studied and finally, a study of the Ca bioavailability have been performed, using an *in vitro* static method to simulate the passage through the intestinal cell-membrane (GLAHN *et al.*, 2002; PERALES *et al.*, 2005; SHIOWATANA *et al.*, 2006).

MATERIAL AND METHODS

Hamburger manufacture

Beef meat was obtained from a local abattoir and chopped in a grinder using a 3 mm plate (Grinder C10, Falsf Co., Spain).The Ca salts (Panreac, Castellar del Vallés, Spain) were added separately to the ground meat in sufficient amount to give a final Ca content of 20 or 30% of the RDA (1000 mg/day) (IOM, 2004). These calculations took into account the calcium content of the molecules and the purity of the Ca salts, which was >98% based on the anhydrous formula (Table 1). The Ca salts were homogeneously distributed into the ground meat in a mixer (Mainca, Pamplona, España). Then, hamburgers were moulded into plates (10 cm diameter, 1 cm height) and kept under refrigeration (2 °C) until analysis, less than 24 h.

Seven batches were manufactured: a control batch without Ca and 6 batches added with CG, CL or CCM at two concentrations (20 and 30% RDA). According to the type and Ca salt amount, the batches were named as CG20, CG30, CL20,

Table 1

Calcium salt amount added to raw hamburgers to provide 20 or 30% of the RDA

Batch	g/100 g
Control	0
CG20	2.24
CG30	3.36
CL20	1.54
CL30	2.31
CCM20	0.93
CCM30	1.40

CL30, CCM20 and CCM30. The hamburgers manufacture was made in triplicate.

Hamburgers were cooked on an electric grill preheated to 180 °C. They were placed for 2 min on each side, sufficient time to achieve a temperature of 60 °C in the inner of the hamburger and a good final degree of doneness (THORNBERG, 2005). Temperature was controlled using a digital thermometer (Testo Mod 735, Barcelona, Spain).

Physico-chemical analyses

Water activity was determined with a Decagon CX1 dew point hygrometer (Decagon Devices, Pullman, WA, USA). The pH was measured using a Crison 2001 pH meter using a glass electrode and according to the AOAC (2011). Water-holding capacity (WHC) was tested according to ZAMORANO and GAMBARUTO (1997). For that, a sample of 0.1 g was placed on a filter paper (Whatman No. 2), sandwiched between translucent plastic plates, and pressed for 1 min. The meat area and liquid area on the filter paper were measured with a planimeter. The following formulae were applied for WHC:

$$WHC = [\text{area of liquid } (cm^2) - \text{area of meat } (cm^2)] \times [9.47/\text{moisture in sample (mg)}] * 100$$

The measurements were made in quintuplicate and the final result was the average value.

Colour analysis

Colour was measured at room temperature on the surface of raw hamburgers, using a Chroma Meter CR-200 colorimeter (Minolta Co., Osaka, Japan) according to the Space colour CIE L*a*b* system and calibrated with a rose tile (L* 44.88, a* 25.99, b* 6.67). A D-65 illumination source was used. L*, a*, b*, Hue angle (tonality) and Saturation index (vivacity) were estimated according to ARTÉS and MÍNGUEZ, (2002). For each batch, twenty five measurements were taken.

Texture analysis

Textural properties were determined using a texturometer Stable Micro System Mod. TA.XT 2i/25 (Surrey, UK). Texture Profile Analysis (TPA) was performed on central cores of cooked hamburgers which were compressed twice to 50% of their original height. A cylindrical probe (2.5 cm diameter) of aluminium was used for the assay. The following parameters were determined: Hardness (N), Springiness (cm), Cohesiveness (ratio), Adhesiveness (N s), Gumminess (N) and Chewiness (N cm) (BOURNE, 1978). Shear force (N) and Work of shearing (N s) were estimated using a Warner-Bratzler blade. In both tests, the samples were 1 cm high and 2.5 cm in diameter; the crosshead speed was 2 mm/s. All determinations were carried out in quintuplicate for each batch.

Sensory analyses

The taste panel consisted of forty untrained assessors selected according to their eating habits, acquaintance with the product to be analyzed and sensitivity, as well as the reproducibility of their evaluations.

First, an Anchored Descriptive Analysis was performed in which the assessors evaluated the similarity between the external appearance of the enriched raw hamburgers and the control batch. This test was performed under a D-65 illumination source using a 5-point descriptive scale, in which the value of 3 points corresponded to the control batch. The value of 1 point meant *much worse than the reference*; 5 points, *much better than the reference;* 2 and 4 points were intermediate values. Three series were prepared: Control-CG20; Control-CL20-CL30 and Control-CCM20-CCM30. The series were presented to the panellists with 30 min of difference to avoid subjectivities.

After this test, a Hedonic Test was performed with cooked hamburgers. In this case the taste panel consisted by 15 trained panellists. They were in individual booths constructed according to ISO DP 6658 (ISO, 1985), under white fluorescent light. The assessors evaluated different attributes (odour, colour, texture, taste and overall acceptability) using a 10 cm non-structured scale (0 = extremely dislike and 10 = extremely like). Two sessions per day were carried out with an interval of at least 1 h between them to avoid panellist fatigue. Unsalted crackers and room-temperature water were provided to clean the palate between samples. The hamburgers were cooked and served in transparent Petri dishes. In each session three randomly selected hamburgers were served.

Bioavailability

Calcium bioavailability was studied using a static *in vitro* test that simulates the gastric and intestinal phases of the digestion process according to the methodology of SHIOWATANA *et al.*, (2006). All enzymes were from Sigma-Aldrich (Steinheim, Germany) and the reactives from Panreac (Barcelona, Spain). The samples were 10 g of cooked hamburgers which were homogenised (Polytron®, Littau-Luzerne, Switzerland) with 50 ml of 0.2 M phosphate buffer. The pH was adjusted to 2.0 with 5 N HCl. To simulate the gastric phase, 0.33 ml of suspension of pepsin (0.16 g pepsin (EC 232-629-3) per ml 0.1 N HCl) was added. Then, the samples were incubated in a shaker (130 rpm) at 37 °C for 2 h (Thermo Scientific MaxQ4000, Iowa, USA).

To simulate the intestinal phase, 20 g of the gastric digest was mixed with 5 ml of pancreatin-bile conjugate, [0.2 g pancreatin from porcine pancreas (EC 232-468-9) and 1.25 g porcine bile extract (EC 232-369-0) in 50 ml 1 M

$NaHCO_3$]. The pH was adjusted to 7-7.5 with 2 M $NaHCO_3$ and the total volume of the digest was measured.

Distilled water (25 ml) and the same volume of 2 M $NaHCO_3$ determined before were added inside to a cellulose dialysis tube with a molecular weight cut-off of 12000-14000 Da and a diameter of 25 mm (Sigma Aldrich, Steinheim, Germany). The tube was introduced into a flask containing other 20 g aliquots of the gastric digest and it was incubated at 37 °C in a shaker (130 rpm) until the pH reached a value of 5. Then, 5 ml of pancreatin-bile conjugate was added to the gastric phase and it was incubated again during 2 h at 37 °C.

After the dialysis process, the dialyzed calcium (inside the dialysis tube) and the non-dialyzed calcium (outside the dialysis tube) were determined. This last one, beside with the calcium in the solid delivery, corresponds to the calcium that would be eliminated. The difference between the amount of calcium detected in these two phases and that determined in the gastric phase was considered as the calcium remaining in the solid residue (solid delivery) and would be eliminated with the feces. It was calculated by difference.

The percentage of bioavailability was expressed as follows:

Bioavailability (%) = [Dialyzed Ca (mg)/ Ca sample (mg)] *100

Calcium determination

Calcium levels were determined according to IKEM *et al.*, (2002). For microwave digestion, 1.0 g of each sample was subjected to an acid digestion with 6 ml of HNO_3 and 2 ml of H_2O_2 (Suprapure, Merck) in a microwave digestion system and diluted to 10 ml with deionised water (Milli-Q, Millipore). Blank digestion was carried out in the same way. Digestion conditions for the microwave system were the following: 2 min - 250 W, 2 min - 0 W, 6 min - 250 W, 5 min - 400 W and 8 min - 550 W. A Perkin Elmer DV 3300 inductively coupled plasma-optical emission spectrometry (ICP-OES) was used to analyze the calcium level in the digested samples. Bovine muscle BCR No 184 was used as reference (CÁCERES *et al.*, 2006). Experimental values obtained were in the range of the certificate value with its uncertainty. All determinations were carried out in duplicate.

Statistical analyses

Results were statistically analysed by two-way ANOVA, which factors were represented by type and concentration of calcium salt. Values of $p<0.05$ were considered to be significant. The effect of the panellists in the sensorial responses was removed by rescaling all the scores given by each assessor from 0, which represents the minimum score used by a given assessor, to 10, which is the maximum score used by that assessor. After rescaling, the effect of the assessors in the sensorial responses was analysed by a two factor analysis of variance according to a randomised balanced block experimental design. The factor represented the type and percentage of calcium salt, while the block variable had 15 levels representing each of the panellists that collaborated in the trials. The F test showed that the effect of the panellists was not significant ($P > 0.05$). In consequence, the final model considered of only 1 factor (one-way ANOVA) representing the calcium salt content.

Statistical analyses were performed using the Statgraphics Centurion XVI.I (Statistical Graphics Corporation, Herndon, VA, USA).

RESULTS AND DISCUSSION

Physico-chemical parameters

Table 2 showed the results of the a_w and pH of the batches manufactured. The a_w of the reminded batches ranged between 0.945 and 0.989 and no significant differences were observed between control and calcium added batches. CL batches showed the lower values. The lower pH values corresponded to the CG20, CL20 and CL30 batches, which showed a pH of 5.70, 5.55 and 5.50, respectively. They differ significantly to the reminder batches. According to the FDA (FDA, 2014) these values assure the safety of these meat products.

GC30 values are not showed due to its technological attitude like it was explained in the following section (Colour parameters).

WHC showed similar values in all the batches and ranged between 36.0-39.0%. The addition of whichever calcium salts supposed no significant changes in the WHC ($p>0.05$).

The values of all these parameters are according to those reported for several authors in raw

Table 2

Effect of added calcium salts on water activity (a_w) and pH on of raw hamburgers

Batch	a_w	pH
Control	0.966 ± 0.016^{AB}	5.80 ± 0.02^{B}
CG20	0.958 ± 0.027^{AB}	5.70 ± 0.05^{C}
CL20	0.954 ± 0.030^{B}	5.55 ± 0.02^{D}
CL30	0.945 ± 0.031^{B}	5.50 ± 0.10^{D}
CCM20	0.989 ± 0.037^{A}	5.86 ± 0.04^{AB}
CCM30	0.955 ± 0.013^{AB}	5.92 ± 0.06^{A}

or ground meat (LAWRIE, 1998; LEE *et al.*, 1998; POULANNE and HALONEN, 2010).

Colour analysis

The worse results were obtained with the CG batches. The CG crystallized in contact with the water of the meat and formed visible small white crystals that appeared homogeneously distributed in the hamburger. These crystals gave an external appearance different to that usually expected by the consumers in a commercial hamburger. It was especially striking at the maximum concentrations (CG30) because the crystals were larger and numerous. For that, it was considered that this batch didn't have enough quality and it was discarded for this and the following tests. Batch with CG20 also had crystals, but they were smaller, as white dots. Therefore, it was considered adequate for continuing the experiment, although its visual appearance would be compromised.

Colour parameters (L*, a* and b*) were determined in raw hamburgers (Table 3). The differences observed were related to the type of salt added. Lightness was very similar in all the batches without significant differences ($p<0.05$). However, it can be observed as the L* parameter was slightly higher in batches manufactured with CL, while, the lower values corresponded to those manufactured with CCM. It is important to remember that CL batches were those that showed the lower pH values. The relationship between color and pH is widely accepted (MANCINI and HUNT, 2005) and several authors (KIM *et al.*, 2006; MANCINI and RAMANATHAN, 2008; NAIR *et al.*, 2014) have reported specifically the effects of lactate on meat color. These authors proposed that lactate plays an indirect role in color stability by generating NADH, which is subsequently used to maintain reduced forms of myoglobin, increasing in this way, the stability of meat colour.

The values of redness (a*) and yellowness (b*) were similar between the control and calcium added batches. In relation to the redness, the lower values were observed in the CCM batches and the same occurs in the yellowness (b*). The higher value was recorded in the batch CL20 but it seems to be an inconsistent data. It was described that the haem pigment contents are mainly related to a* and, consequently, it is considered as the most important parameter in meat, while redox state influenced b* (MANCINI and HUNT, 2005). This parameter reach less importance because colours represented (blue and yellow) are not typical or intuitively related to meat. According to these authors, and giving greater relevance to the a* parameter, it can be concluded that the colour is very similar in all the batches, although CL20 and CCM30 batches were the only ones that showed any difference. Hue Angle and Saturation Index behaved in a similar way and these batches showed again, the only differences in relation to the remainder ones. The colour lecture of hamburgers did not reflect the presence of the small white crystals due to their size. It is important to take into account, firstly, that the colorimeter project the light from the probe, which has 1 cm of diameter and secondly, that the crystals had a much smaller size. For that, the colorimeter read the colour of the meat and thus the data of CG20 and control batches (without calcium) were similar, without significant differences.

The colour of cooked samples (data not shown) was very similar in all batches without exceptions. It has been described that during heat treatment, the meat colour changes from red to grey-brown (Maillard reaction) and increase

Table 3

Effect of added calcium on colour properties of raw hamburgers

Batch	L*	a*	b*	Hue Angle	Saturation Index
Control	44,45±1,96^{AB}	21,72±1,15^{AB}	8,37±0,88^{BC}	20,62±1,85^{BC}	23,36±1,23^{AB}
CG20	44,63±6,64^{AB}	22,60±4,96^{AB}	9,20±3,21^{AB}	21,79±4,88^{BC}	24,49±4,78^{AB}
CL20	45,68±4,35^{A}	23,36±3,46^{A}	10,76±2,53^{A}	24,74±3,81^{A}	25,72±3,95^{A}
CL30	46,60±7,54^{A}	21,39±3,44^{AB}	9,30±3,42^{AB}	23,96±6,22^{AB}	22,36±4,18^{BC}
CCM20	43,64±5,72^{AB}	20,34±3,36^{BC}	7,34±1,91^{BC}	19,12±3,58^{C}	21,73±3,65^{BC}
CCM30	41,32±5,43^{B}	19,60±3,77^{C}	7,04±2,81^{C}	19,10±6,67^{C}	20,88±4.14^{C}

Data are mean ± S.D.

Values sharing the same letters are not significantly different ($p > 0.05$)

the opacity when the internal meat temperature is between 45 °C and 67 °C (PAKULA and STAMMINGER, 2012). In this way, TORNBERG (2005) reported that the increase in meat opacity is related to the myosin desnaturation, which starts at about 35 °C. Above 50 °C, the myosin molecules are completely coagulated and the meat appears opaque.

Textural analyses

Textural parameters were determined in cooked hamburgers (Table 4). It was observed that in both CL and CCM batches, the higher the calcium amount, the greater the hardness was. This is probably because calcium, which is a divalent cation, establishes bonds between meat proteins, mainly with myosin. This favors the formation of a stronger network which leads to the highest firmness (DAMODARAN, 2008). According to TORNBERG (2005) it could be also due to the relationships stabilized between calcium and meat proteins, partially denatured by the cooking. This last one favors the formation of a more compact network that increases the hardness.

Springiness was quite similar in all the calcium enriched batches and shown higher values to batch control. According to BELITZ *et al.*, (2009), the increase of the ionic strength leads the extraction to the surface of the particles of minced meat yielding sticky exudates. During heating, the proteins interact between them, yielding a structure consist on a protein gel that can modify the springiness of the cooked products. (TORNBERG, 2005).

Adhesiveness and cohesiveness parameters did not change significantly ($p>0.05$). Gumminess and chewiness behaved as the hardness because they are secondary parameters dependent on it. So, the lower values corresponded to the CG which was the batch which showed the lower harness

The results obtained in the shear test showed the same tendency than in the TPA and so,

Table 4

Effect of added calcium salts on textural properties of experimental cooked hamburgers[ß]

Batch	Hardness (N)	Adhesiveness (N s)	Springiness (cm)	Cohesiveness (ratio)	Gumminess (N)	Chewiness (N cm)
Control	27,58±3,58 ^{B}	-0,03±0,004 ^{A}	0,51±0,01 ^{C}	0,60±0,01 ^{A}	16,52±1,95 ^{B}	8,25±0,97 ^{C}
CG20	18,16±5,86 ^{C}	-0,03±0,019 ^{A}	0,54±0,04 ^{BC}	0,52±0,06 ^{B}	10,19±3,67 ^{C}	4,99±2,07 ^{B}
CL20	35,07±6,34 ^{A}	-0,03±0,015 ^{A}	0,59±0,04 ^{A}	0,62±0,04 ^{A}	21,64±4,28 ^{A}	12,30±2,60 ^{A}
CL30	38,44±5,59 ^{A}	-0,04±0,021 ^{A}	0,57±0,02 ^{AB}	0,60±0,06 ^{A}	22,41±3,34 ^{A}	13,81±1,89 ^{A}
CCM20	27,37±6,08 ^{B}	-0,03±0,011 ^{A}	0,55±0,05 ^{BC}	0,61±0,03 ^{A}	16,75±3,73 ^{B}	9,12±1,45 ^{B}
CCM30	38,74±8,15 ^{A}	-0,04±0,021 ^{A}	0,56±0,04 ^{ABC}	0,60±0,04 ^{A}	23,30±6,08 ^{A}	12,04±1,57 ^{A}

[ß] Data are mean ± S.D.

Values sharing the same lower case letters are not significantly different ($p > 0.05$)

Table 5

Effect of added calcium salts on the sensory properties of raw (Anchored Descriptive Analisys) and cooked hamburgers (Hedonic Test)ß

Batch	Anchored Descriptive Analysis	Hedonic Test		
		Texture	Taste	Overall Acceptability
Control	3.00*	6.06±1.40 ^{AB}	6.13±1.33 ^{A}	6.22±1,15 ^{AB}
CG20	1.87±0.21 ^{D}	6.24±1.80 ^{AB}	4.94±2,44 ^{AB}	5.18±2,05 ^{B}
CL20	2.40±0.35 ^{B}	7.42±3,23 ^{A}	5.13±2,30 ^{AB}	5.87±2,10 ^{A}
CL30	3.53±0.29 ^{A}	6.06±2,22 ^{AB}	4.56±2,30 ^{B}	5.33±1.97 ^{B}
CCM20	2.67±0.40 ^{B}	5.79±1.78 ^{B}	5.62±1.91 ^{AB}	5,38±1.99 ^{B}
CCM30	2.20±0.26 ^{B}	5.90±1.30 ^{AB}	5.60±1.33 ^{AB}	5.80±1.35 ^{AB}

[ß] Data are mean ± S.D.

Values sharing the same uppercase letters are not significantly different ($p > 0.05$).

* Reference

the principal differences were an increase of the Work of Shearing related with the calcium amount added, independently to the salt added (data not shown).

Sensory analyses

Table 5 shows the results of the Anchored Descriptive Analysis, performed with raw hamburgers. Control batch was the reference and it was awarded a score of 3 in the 5 points scale used. The panellists evaluated the Visual Appearance of enriched hamburgers in comparison to the control.

The worse results were obtained with the CG20 batch, mainly due to the presence of small white crystals (see Colour section). In this case, the number and the size of the crystal were lower than in the CG30, but they were sufficient to modify negatively the appearance of the hamburgers.

CL30 batch was the best evaluated, even more than the reference ($p<0.05$). This result is according to the instrumental colour measurement (Table 3) in which this batch was the one that had the greatest the higher L* and Hue Angle.

Table 5 also shows the results of the Hedonic Test performed with cooked hamburgers. Odour and colour were well-evaluated in all the cases obtaining similar values in all batches ($p>0.05$) (data not shown). The texture achieves punctuations higher than 6 except the batches enriched with CCM20 and CCM30 which achieved scores of 5.79±1.78 and 5.90±1.30, respectively. Significant differences were observed only between CL20 and CCM20, but, in general terms it can be observed a great similarity between the enriched batched and the control batch.

However, the presence of calcium salts influenced negatively on the taste and so, independently of the type of salt or the amount added, all the enriched batches achieved punctuation lowers than the control batch.

Significant differences were observed between control and CL30 batches; the panellists described this last batch as *slightly more acidic*.

The overall acceptability values behaved similar than the taste ones and, consequently, the taste seemed to be the most influential parameter. So, the punctuations obtained by the acceptability were very similar in all the enriched batches, although it was observed that the lowest values were those for batches CG20 and CL30. The control batch reached the best score although the difference was not significant with CL20 and CCM30 batches. In any case none of the batches were discarded because all of them exceeded the value of 5 points.

Bioavailability

In order to facilitate the calcium analysis, it was decided to perform the bioavailability study using the batches manufactured with the higher concentration: CL30 and CCM30. The CG batches were discarded for this study because of the low sensory quality. Table 6 shows the results obtained. The bioavailability was determined in the cooked hamburgers.

The calcium amount determined was slightly higher than the expected, a 20% approximately. This increase could be due to the loose of water during the cooking as it has been reported by PAN and SHING (2001). These authors established that the water losses during ground beef cooking (60 °C, 2 min) is closed to 15-20%. The final calcium amount determined was sufficient to give the calcium levels proposed in our objective (30% RDA).

In the gastric phase was detected all the calcium presented in the cooked hamburgers.

The dialyzed calcium was close two fold higher in the hamburgers enriched with CCM30 than those with CL30. The solid deliveries calculated were 171.29 mg in the CL batch and 129.12 mg in the CCM one. This difference represents a calcium loss close to 47% and 34%, respectively. It could be due to that the CCM interferes with proteins during the cooking and contributes, as it has been described above, to the formation of a more compact protein network that could difficult the activity of pancreatin-bile complex, and to favor the retention of a higher calcium amount.

Applying the formula (see Material and Methods section), the bioavailability percentage was 7.73% for CL and 14.55% for CCM.

The variability in calcium bioavailability is

Table 6

Calcium content in the different phases of the in vitro digestion and calcium bioavailability (%) from cooked hamburgers [ß]

Calcium Salt	Sample*	Gastric Phase Ca*	Non Dialyzed Ca*	Dialyzed Ca*	Bioavailability (%)
CL30	373.65±37.36	363.95±36.53	163.82±26.14	28.84±2.20	7.73
CCM30	361.85±25.33	382.6±23.00	200.82±12.01	52.66±3.08	14.55

[ß] Data are mean ± S.D.

*mg Ca/100 g hamburger

Bioavailability (%) = [Dialyzed Ca (mg) / Ca sample (mg)]*100

consistent with numerous studies. One of the first bioavailability assays performed (MORRISEY and FLYNN, 1972) reported that the calcium absorption from cow's milk ranged from 21 to 45% in healthy human adults. RECKER *et al.*, (1988) described that the absorption of calcium carbonate from enriched whole milk, chocolate milk, yogurt, imitation milk and cheese ranged between 21-26% in postmenopausal women. KRUGER *et al.*, (2003) concluded that calcium bioavailability was similar in milk fortified with calcium carbonate or milk calcium. In a study performed by SITTIKULWITIT *et al.*, (2004) with different calcium salts and milk powder, bioavailable calcium ranged between 28.5 and 58.7%. Other authors determined calcium absorption from infant milk formula of 39% (NELSON *et al.*, 1996) and 29% from enriched orange juices (GONELLI *et al.*, 2007). CILLA *et al.*, (2011) reported calcium efficiency uptake values from 10.47-19.82% for different milk-based fruit beverages and PERALES *et al.*, (2005) found calcium bioavailability to be 5.0-31% for infant and adapted milks.

The differences reported between the values of calcium dialyzable have be also attributed to the methodology applied, particularly with regard to the amount and activity degree of the enzymes used, the pH values and incubation times during the gastric and intestinal phases. In this way, VAN der HEE *et al.*, (2009) reported that calcium absorption from food depends on numerous factors, causing a range broadly from 15 to 44%. So, bioavailability described in this paper is according to the data collected by other authors. Furthermore, our results showed that CCM would be the most bioavailable salt from hamburgers, which suggests that is the most appropriate for the enrichment of fresh meat products.

SITTIKULWITIT *et al.*, (2004) reported that the dialysis rate of calcium (calcium bioavailable) in milk powder is 28%. Taking into account that 200 ml of milk would give 240 mg of calcium, the bioavailable amount would correspond to 67.2 mg. According to our data, if 100 g of hamburger enriched with CCM at the concentration of 30% IDR leaves bioavailable 52.66 mg (Table 6), one commercial hamburger of 120 g, would leave 63.2 mg calcium, a similar quantity to that reported for milk.

CONCLUSIONS

CCM and CL can be adequate for enriching fresh meat products (hamburgers). They can be added in sufficient amount to give a theoretical calcium level of 30% of RDA. GC crystallizes and it is not recommended for its use. The calcium bioavailability from CCM enriched hamburgers was the highest, close to 15%. This allows that one hamburger of 120 g enriched with CCM 30% RDA, would leave bioavailable 63.2 mg calcium, a similar calcium amount to 200 ml of milk. Consequently, CCM is proposed as the most adequate salt and the hamburger manufactured with it could be considered as a *source of* calcium, according to the Regulation Nº 1907/2006 of the European Parliament.

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PAPER

OPTIMIZATION OF MICROWAVE AND AIR DRYING CONDITIONS OF QUINCE (*CYDONIA OBLONGA*, MILLER) USING RESPONSE SURFACE METHODOLOGY

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ABSTRACT

Effects of slice thickness of quince **(***Cydonia oblonga* Miller**)** , microwave incident power and air drying temperature on antioxidant activity and total phenolic content of quince were investigated during drying in microwave and air drying. Optimum conditions were found to be: i) for microwave drying, 285 W and 4.14 mm thick (maximum antioxidant activity) and 285 W and 6.85 mm thick (maximum total phenolic content), and ii) for air drying, 75 °C and 1.2 mm thick (both maximum antioxidant activity and total phenolic content). Drying conditions were optimized by using the response surface methodology. 13 experiments were carried out considering incident microwave powers from 285 to 795 W, air temperature from 46 to 74 °C and slice thickness from 1.2 to 6.8 mm.

- Keywords: Microwave, air drying, antioxidant activity, total phenolic content, response surface methodology -

1. INTRODUCTION

Edible fruits are sources of nutrients such as carbohydrates, vitamins, and minerals as well as non-nutrient compounds such as polyphenols. Nowadays, it is commonly admitted that there is a positive relationship between a diet rich in vegetables and fruits and a reduced incidence of degenerative diseases such as cancer and cardiovascular events (GIBNEY *et al.*, 2009). Health beneficial properties of quince fruit (*Cydonia oblonga* Miller) have known from ancient times. Quince is the only species in the genus Cydonia, which falls into Pomoideae subfamily of the Rosaceae along with apple and pear (PACIFICO *et al.*, 2012). Quince is used extensively in Europe as a dwarfing rootstock for pear. Total world production of fresh quince was 540.337 tons in 2010 and about 25% of this was produced in Turkey (TSI, 2012).

Drying process is one of the most important preserving operations that causes time and energy consumption in the food industry. That is why new methods are aimed to decrease drying time and energy consumption. New methods combined different systems such as using microwave drying together with traditional drying methods to reduce drying time (SECMELER, 2003). Over the past two decades, there has been an increasing attraction in microwave drying to reduce drying time and increase the removal of water from agricultural products. Microwave drying has several advantages such as short drying time, higher drying rate, better quality of the dried products and decrease energy consumption (SANGA *et al.*, 2000).

Response surface methodology (RSM) is one of the most commonly used optimization technique in food science. This method is preferred because of the simplicity and high efficiency. RSM covers a group of techniques used to study the relationship between one or more measured responses and input variables. (ARTEGE *et al.*, 1994). It has been successfully applied to optimize food processing operations by many researchers (FRANK, 2001; LEE *et al.*, 2006; LUCIANE *et al.*, 2001; MIRHOSSEINI *et al.*, 2008; PIETRASIK and LI-CHAN, 2002).

2. MATERIAL AND METHODS

2.1. Material

Fresh quinces were obtained from a local market in Konya. These samples were transferred to laboratory in cool bags and they stored in refrigeration temperature (4 °C) until the assay, Initial moisture content of fruits was detected as 80% in average. Prior to drying, round shaped samples (2 cm in diameter) were obtained from fruit slices. Thickness varied from 1.2 to 6.8 mm according to experimental design.

2.2. Drying

Quinces were dried until the moisture content decreased to 40% of the initial moisture content, since burning was observed on the quince slices in microwave drying below this moisture content level. Microwave drying experiments were performed using a domestic microwave oven (ARCELIK ARMD 580, Turkey). The dimensions of the microwave cavity were 345 mm x 340 mm x 225 mm. Three power levels were selected as high (720 W), medium (540 W) and low (360 W) for drying experiments. One dish containing 1 slice of sample to make effective drying was placed on the centre of a turntable fitted inside the microwave oven. Quinces were placed uniformly as a thin layer onto the stainless steel trays (0.3 m x 0.2 m) and dried using air oven (Nüve FN055 Ankara, Turkey, 55 L volume) at three different temperatures (50, 60 and 70 °C). Quince slices were placed uniformly as a thin layer onto the stainless steel trays (0.3 m x 0.2 m) and dried under direct sunlight in April in Konya, Turkey (BALLADIN & HEADLEY, 1999).

2.3 Extraction

The phenolic compounds were isolated from samples using a modified version of the method described by SHAHIDI *et al.* (2001). One gram of sample was extracted 3 times using 10 ml of 70% (v/v) aqueous methanol (Merck, Germany) at room temperature by a homogenizer (Ika Ultra Turrax Tube Disperser) for 1 min. The slurry was centrifuged at 4000 rpm for 15 min. Supernatants were collected and combined in a rotary flask and then evaporated at 45 °C under vacuum by a Rota vapor R-3000 rotary evaporator (Laborato 4001, Heidolph). The extracted phenolics were dissolved in 25 ml methanol and then filtered using filter paper. Methanolic solutions of phenolic were stored -25 °C until analysis.

2.4 Determination of total phenolic content and antioxidant activity

Total phenolic compounds were prepared by using Folin-Ciocalteu Reagent (YOO *et al.*, 2004). The free radical scavenging activity of the extract was determined using 1, 1-diphenyl-2-picrylhydrazyl (DPPH) in order to determine antioxidant activity (LEE *et al.*, 1998). Total phenolic contents were calculated by a standard calibration curve prepared using gallic acid. Total phenolic content results were given as gallic acid equivalents in milligrams per 1000 g fruit. Antioxidant activity results were expressed as percentage activity (%).

2.5 Experimental Design

Response Surface Methodology (RSM) was used to optimize drying conditions, based on better preserve the antioxidant activity and total phenolic content of quince fruit. Box–Behnken design was selected for RSM analysis. Box–Behnken designs require only three levels, coded as −1, 0 and +1. The effects of the two independent processing parameters: slice thickness (X_1, mm), incident microwave power (X_2, Watt) in microwave operation and additionally slice thickness (X_1, mm), processing temperature level (X_2, °C) in air drying on two dependent variables (antioxidant activity and total phenolic content) were investigated using RSM. The total number of experiments in this study was 13 based on two levels and a two factor experimental design, with five replicates at the centre of the design for estimation of a pure error sum of squares. Minitab 16 (Minitab Inc. State College, PA) was used for the experimental design, data analysis and regression modeling. The independent variables were; X_1 (2–4 mm), X_2 (360-720 W) in microwave process and X_1 (2 – 4 mm), (X_2 50 - 70 °C) in air drying. Experimental data from the Box–Behnken design was fitted into a second-order polynomial model.

$$Y = b_o + b_1X_1 + b_2X_2 + b_1^2X_1^2 + b_2^2X_2^2 + b_1b_2X_1X_2 \quad (1)$$

Where Y is the predicted response, X_1 and X_2 are independent variables, b_0 is a constant; b_1, b_2, b_1^2, b_2^2, b_1b_2 are linear, quadratic and interaction coefficients, respectively.

3. RESULTS AND DISCUSSION

3.1 Microwave drying

Independent variables [thickness of slice (X_1) and microwave power level (X_2)] observed and predicted values of antioxidant activity and total phenolic content for microwave drying were given in Table 1. During drying in microwave oven antioxidant activity decreased in the studied variables. The influences of interaction between thickness-thickness were determined as statistically important ($p \leq 0.05$) whereas the other parameters were found insignificant ($p > 0.05$) (Table 2a). Statistical analysis indicated that the fitted model (Eq.1) to experimental results displayed high performance to predict the antioxidant activity of quince samples within the studied ranges of variables. Regression coefficient (R^2) and adjusted regression coefficient (R^2_{adj}) were calculated as 0.994 and 0.990, respectively (Table 2a). 3D surface plots for the significant terms were shown in Figs. 1 and 2 (MINITAB 16). Surface plot given in Fig. 1a indicated that antioxidant activity was not affected by thickness and power level. Thickness-thickness square parameter was the only parameter which affected on antioxidant activity ($p \leq 0.05$) (Table 2a). Antioxidant activity of quince increased up to 4 mm of slice thickness and then decreased (Fig. 1a).

The interaction between thickness-thickness, thickness-power, power-power, power and thickness alone showed significant effects ($p \leq 0.05$) (Table 2b) on total phenolic content of quince dried in microwave oven. Total phenolic content of quince dried in microwave oven was fitted in Eq.2. Re-

Table 1.Experimental Design of Microwave Drying and Corresponded Responses (Antioxidant Activity and Total Phenolic Content)

	X_1	X_2	Antioxidant Activity (%)		Total Phenolic Content (mg/1000g)	
Run	Thickness (mm)	Power (W)	Observed	Predicted	Observed	Predicted
1	4	285	83	82	1661	1635
2	2	720	66	65	796	791
3	1.2	540	68	68	810	791
4	6	360	77	77	1499	1503
5	4	540	70	73	882	898
6	2	360	75	76	1125	1157
7	4	540	75	73	879	898
9	4	795	63	66	691	718
10	4	540	73	73	895	898
11	6.8	540	67	68	859	880
12	4	540	77	73	916	898
13	6	720	68	65	605	572

gression coefficient (R^2) and adjusted regression coefficient (R^2_{adj}) were calculated as 0.880 and 0.795, respectively (Table 2b). The linear coefficient for thickness was over seventy times greater than that of power level. There was interaction of significance between thickness and power level.

$$Y_1 = 80.13 + 5.58\,X_1 - 0.66\,X_1^2 \quad (1)$$

$$Y_2 = 2088.05 + 290.44\,X_1 - 4.87\,X_2 + -7.86\,X_1^2 - 0.39\,X_1X_2 \quad (2)$$

Antioxidant activity and total phenolic content values were found higher in microwave drying than that of air drying. Drying time decreased 60-120 times in microwave drying. BERTELI & MARSAIOLI (2005) have studied the influence of drying methods on moisture content of product. Very short heating–cooling cycles take place in microwave drying. The use of microwave for drying has become common because it enhances the product quality and processing speed (DIAZ *et al.*, 2003). Fresh fruits are well known for their antioxidant activity which is usually attributed to the polyphenol content (WANG *et al.*, 1996). Total phenolic content of 2020 mg GAE/kg sample and 78% inhibition for antioxidant activity were reported in fresh quince samples before the drying experiment (HAMAZU *et al.*, 2005). KARADENIZ *et al.* (2005) reported that antioxidant activity of some quince varieties in Turkey found between 51-68%.

Table 2 A. Analysis of variance of antioxidant activity of quince in microwave drying
B. Analysis of variance of total phenolic content of quince during microwave drying

A

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	5	318.926	318.926	63.7851	10.34	0.004
Linear	2	268.848	48.119	24.0594	3.9	0.073
Thickness	1	0.239	26.655	26.6549	4.32	0.076
Power	1	268.608	6.093	6.0932	4.05	0.353
Square	2	49.997	49.997	24.9984	4.05	0.068
Thickness*Thickness	1	49.906	48.507	48.5071	7.86	0.026
Power*Power	1	0.091	0.091	0.091	0.01	0.907
Interaction	1	0.081	0.081	0.0812	0.01	0.907
Thickness*Power	1	0.081	0.081	0.0812	0.01	0.912
Residual Error	7	43.174	43.174	6.1677		
Lack-of-Fit	3	17.322	17.322	5.774	0.89	0.518
Pure Error	4	1328	1328	332		
Total	12	362.099				

B

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	5	1081629	1081629	216326	263.08	0
Linear	2	849631	300620	150310	182.79	0
Thickness	1	7932	72176	72716	87.77	0
Power	1	841699	122155	122155	148.55	0
Square	2	452240	152240	76120	92.57	0
Thickness*Thickness	1	17411	6877	6877	8.36	0.023
Power*Power	1	134829	134829	134829	163.97	0
Interaction	1	79758	79758	79758	97	0
Thickness*Power	1	79758	79758	79758	97	0
Residual Error	7	5756	5756	822		
Lack-of-Fit	3	4428	4428	1476	4.45	0.092
Pure Error	4	1328	1328	332		
Total	12	1087385				

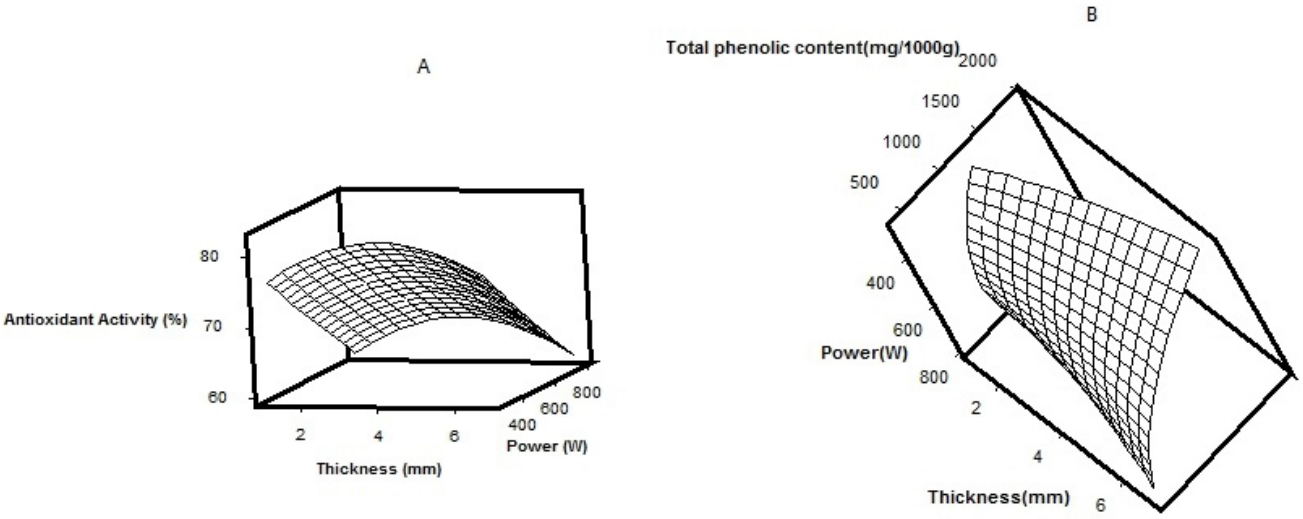


Fig. 1 - Effect of thickness and power on antioxidant activity during microwave drying (A) and on total phenolic content during microwave drying (B).

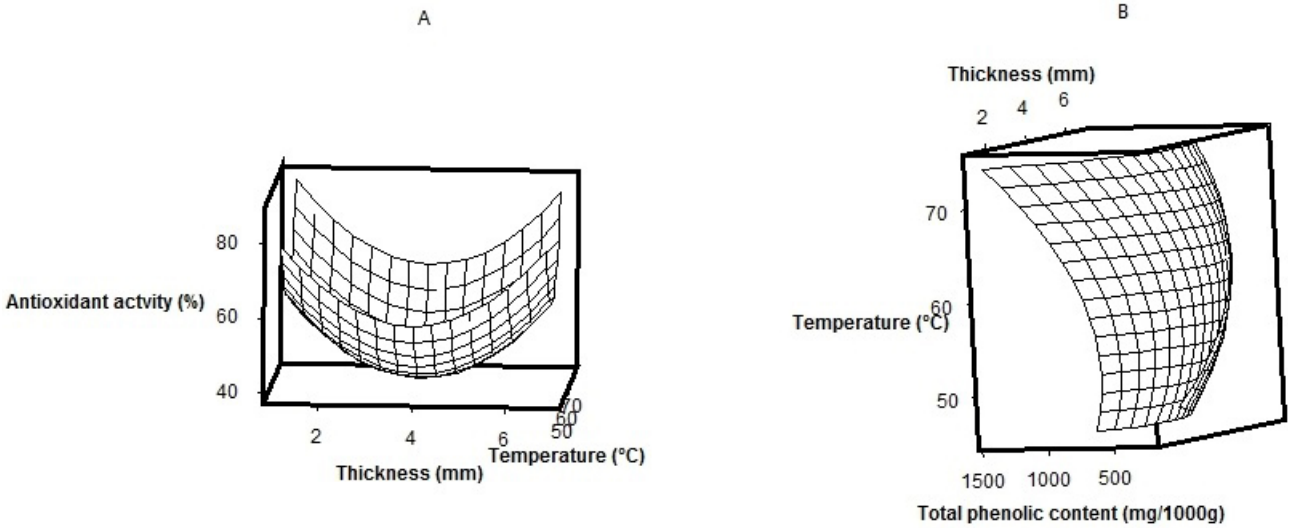


Fig. 2 - Effects of thickness and temperature on antioxidant activity oven drying (A) and on total phenolic content oven drying (B).

Table 3. Experimental Design of Oven Drying and Corresponded Responses (Antioxidant Activity and Total Phenolic Content)

Run	X_1 Thickness (mm)	X_2 Temperature (°C)	Antioxidant Activity (%) Observed	Antioxidant Activity (%) Predicted	Total Phenolic Content (mg/1000g) Observed	Total Phenolic Content (mg/1000g) Predicted
1	2	50	63	59	484	469
2	6	70	59	63	457	472
3	4	74	67	65	696	720
4	4	60	38	40	229	264
5	4	60	39	40	280	264
6	2	70	65	64	952	903
7	1.2	60	58	61	685	730
8	6	50	58	58	491	540
9	4	60	40	40	258	264
10	6.8	60	63	60	520	475
11	4	60	42	40	289	264
12	4	46	55	58	485	460
13	4	60	40	40	266	264

3.2 Air drying

Independent variables [thickness of slice (X_1) and temperature of air (X_2)], observed and predicted values of antioxidant activity and total phenolic content for air drying were given in Table 3. Antioxidant activity of quince samples decreased after air drying. Thickness of samples and process temperature had considerable effect on antioxidant activity. Additionally, the influences of interaction between thickness-thickness and temperature-temperature were statistically significant ($p \leq 0.05$) whereas the other parameters were insignificant ($p > 0.05$) (Table 4a). Effect of thickness and temperature on the antioxidant activity in air drying was given in Fig. 2a. Increase in thickness from 2 to 4 mm resulted in lower antioxidant activity at different drying temperatures. The fitted model to antioxidant activity results of quince samples after air drying was given in Eq. 3. Model parameters for Eq. 3 (R^2 and R^2_{adj}) were calculated as 0.953 and 0.920, respectively (Table 4a).

The interaction between thickness-thickness, temperature-temperature, thickness-temperature and temperature of process showed significant effects ($p \leq 0.05$) on total phenolic content after air drying (Table 4b). Changes in total phenolic content was given in Fig. 2b and increase in thickness and power level caused firstly decrease and then increase in total phe-

Table 4.A Analysis of variance of antioxidant activity of quince in oven drying
B Analysis of variance of total phenolic content of quince in oven drying

A

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	5	1420.7	1420.7	284.13	28.7	0
Linear	2	50.57	750.9	375.45	37.9	0
Thickness	1	1.7	141.46	141.46	14.3	0
Temperature	1	48.87	721.43	721.43	72.9	0
Square	2	1369.7	1369.7	684.87	69.2	0
Thickness*Thickness	1	571.44	749.98	749.99	75.8	0
Temperature*Temperature	1	798.3	798.3	798.3	80.6	0
Interaction	1	0.36	0.36	0.357	0.04	0.86
Thickness*Temperature	1	0.36	0.36	0.357	0.04	0.86
Residual Error	7	69.3	69.3	9.9		
Lack-of-Fit	3	58.38	58.38	19.46	7.13	0.04
Pure Error	4	10.92	10.92	2.729		
Total	12	1490				

B

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	5	533725	533725	106745	59.7	0
Linear	2	132129	125964	62982	35.3	0
Thickness	1	649.25	14	14	0.01	0.93
Temperature	1	67203	118914	118914	66.6	0
Square	2	338638	338638	169319	94.8	0
Thickness*Thickness	1	154271	198402	198402	111	0
Temperature*Temperature	1	184367	184367	184367	103	0
Interaction	1	62959	62959	62959	35.2	0
Thickness*Temperature	1	62959	62959	62959	35.2	0
Residual Error	7	12508	12508	1787		
Lack-of-Fit	3	10381	10381	3460	6.51	0.05
Pure Error	4	2128	2128	532		
Total	12	546233				

nolic content similar to the antioxidant activity. Total phenolic content of quince by air drying was fitted in Eq. 4. Model parameters for Eq 4 (R^2 and R^2_{adj}) were calculated as 0.977 and 0.960, respectively (Table 4b). The high values of regression coefficient indicate a high degree of correlation between the experimental and fitted values.

$$Y_3 = 449.60 - 20.10\,X_1 - 12.54\,X_2 + 2.59\,X_1^2 + 0.10\,X_2^2 \quad (3)$$

$$Y_4 = 4925.38 - 6.43\,X_1 - 161.10\,X_2 + 42.22\,X_1^2 + 1.63\,X_2^2 - 6.27\,X_1X_2 \quad (4)$$

3.3 Sun drying

Sun drying was chosen as traditional drying method and this process maintained about 24 h. The use of microwave oven seems to be more advantageous considering the time factor. In sun drying, total phenolic content and antioxidant activity were determined as 1544 mg GAE/ 1000 g and 74%, respectively. These values were close to the results observed after microwave drying and higher than that of air drying.

In order to determine the optimal residual activity, response optimizer tool in MINITAB 16 (Minitab Inc. State College, PA) was used. The optimum conditions were found as 4.14 mm thickness and 285 W power level for maximum antioxidant activity, 6.85 mm thickness and 285 W power level for maximum total phenolic content in microwave drying. When air drying was analyzed, 1.2 mm thickness and 75 °C temperature were selected for maximum antioxidant activity and total phenolic content.

4. CONCLUSIONS

The effect of power level and slice thickness on the antioxidant activity and total phenolic content were investigated for quince samples after drying. RSM was used to optimize the factors in order to obtain maximum level of antioxidant activity and total phenolic content of quince samples. All independent variables including thickness of slice (mm), power level (W) and processing temperature (°C) had significant effects on the response values. Furthermore square and interaction parameters showed significant effects ($p \leq 0.05$). A desirable quadratic mathematical model was built by using Box–Behnken design. The antioxidant activity and total phenolic content decreased after drying. Nevertheless optimum drying conditions were obtained for both microwave and air drying. Additionally, the use of microwave provides time saving compared to the other drying methods.

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PAPER

SE EFFECT ON BIOLOGICAL ACTIVITY OF *FLAMMULINA VELUTIPES*

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ABSTRACT

The goals of the study were evaluation of antioxidant, antifungal and anticancer potential of *Flammulina velutipes* mycelium ethanol extract and examination of Se effect on those activities. Both Se-amended and non-amended mycelium extracts exhibited significant antioxidant and antifungal potential. Se-enriched extract was more effective against *Candida krusei* and *C. albicans* and better DPPH• scavengers than non enriched one. Carriers of the antioxidant activity were phenol compounds. Contrary to antioxidant and antifungal potential, tested extracts were many times weaker cytotoxic agents against HeLa and LS174 cell lines than *cis*-DDP. Thus, Se-enriched mycelium could be supplement with antioxidant and antifungal capacity.

- Keywords: anticancer activity, antifungal activity, antioxidant activity, *Flammulina velutipes*, Se-enriched mycelium -

INTRODUCTION

A need for new antimicrobial agents exist for a long time due to the emergence of microorganism resistance as a result of the uncontrolled usage of commercial antibiotics and antimycotics. Preference is given to natural compounds due to their health-beneficiary and environmentally-friendly effect. Besides diseases caused by microorganisms, nowadays, cancer, diabetes, atherosclerosis, as well as neurodegenerative disorders occur frequently. One of the significant triggers for the mentioned diseases and disorders is oxidative stress (LIMÓN-PACHECO and GONSEBATT, 2009). Since the capacity of cellular antioxidant defence is insufficient in some cases and synthetic antioxidants could have toxic and mutagenic effects, and as awareness about healthy lifestyle is permanently raised, search for natural antioxidants presents current trend. Nowadays, natural remedies are also favoured for cancer treatment, because of the many side effects of the chemotherapy, such as the same effect on cancer and healthy cells, occurrence of mutations which could pass into future generations, as well as more aggressive metastasis of returned disease.

Based on traditional experience and scientific data, numerous mushroom species represent important resources of antimicrobial, antioxidant, and anticancer agents. Although special attention is given to the species of the genera *Ganoderma*, *Lentinus*, *Trametes*, and *Pleurotus*, other mushrooms also produce active compounds and their biological activities should be tested (ZHANG *et al.*, 2007; CHEN *et al.*, 2008; SHI *et al.*, 2012). One of them is *Flammulina velutipes* (Curt. Fr.) Karst, known as Golden needle mushroom, Enoki, Enokidake and Enokitake. This species is a famous edible and medicinal mushroom due to synthesis of numerous bioactive molecules, such as polysaccharides, proteins, sterols etc. Significant immunomodulating, antitumor, antioxidant, and antiviral potential of its extracts and compounds were reported (BAO *et al.*, 2010; CHANG *et al.*, 2010; YANG *et al.*, 2012). CHANG *et al.* (2010) demonstrated cytotoxic effect of immunomodulatory protein FVE against murine hepatocellular carcinoma, and YANG *et al.* (2012) showed anti-proliferation activity of polysaccharide FVP against human gastric and lung cancer cells. However, antibacterial and especially antifungal activities of the extracts were poorly studied (RODRIGUES de MELO *et al.*, 2009; WANG *et al.*, 2012).

Among essential microelements, Se takes significant place in animal and human diet, due to its participation in biosynthesis of selenoproteins and selenoenzymes involved in cell protection against free radicals and indirectly in cell prevention from malignant transformation (RAYMAN, 2005; BRIGELIUS-FLOHÉ, 2006). Organic Se compounds, depending on form and concentration, also have antimicrobial activity based on catalysis of O_2^- production which causes oxidative damage of bacterial cell membrane and cell disfunction (XU *et al.*, 2008). Considering that Se bioavailability from organic forms is better than from inorganic ones, and that mushrooms have ability to absorb inorganic Se and convert it to organic, their usage as food and dietary supplements could contribute the prevention of disease appearance (KALAĆ, 2010). However, there are just a few reports about biological activity of Se-enriched mushroom mycelia (MALINOWSKA *et al.*, 2009; TURLO *et al.*, 2010; SHI *et al.*, 2010), and there are no available data for *F. velutipes*. Considering everything mentioned, the aims of the study were to evaluate antioxidant, antifungal and anticancer potential of *F. velutipes* mycelial extract and examine in which way mycelium enrichment with Se affects those activities.

MATERIALS AND METHODS

Organism and cultivation conditions

The culture of *Flammulina velutipes* HAI 966 was obtained from Institute of Evolution, University of Haifa, Israel (HAI), and maintained on malt agar medium in the culture collection of the Institute of Botany, Faculty of Biology, University of Belgrade.

The inoculum preparation involved the following steps: *(i)* inoculation of 100.0 mL of synthetic medium (glucose, 10.0 g/L; NH_4NO_3, 2.0 g/L; K_2HPO_4, 1.0 g/L; $NaH_2PO_4 \times H_2O$, 0.4 g/L; $MgSO_4 \times 7H_2O$, 0.5 g/L; yeast extract, 2.0 g/L; pH 6.5) with 25 mycelial discs (Ø 0.5 cm, from 7-day-old culture from malt agar); *(ii)* incubation at room temperature (22 ± 2°C), on a rotary shaker (100 rpm), for 7 days; *(iii)* washing of obtained biomass (3 times) by sterile distilled water (dH_2O); *(iv)* biomass homogenization with 100.0 mL of sterile dH_2O in a laboratory blender.

Homogenized inoculum (30.0 mL) was used for inoculation of 400.0 mL modified synthetic medium (with glucose in the amount of 65.0 g/L and peptone in the concentration of 2.0 g/L, previously determined as the optimal carbon and nitrogen sources and concentrations for biomass production) enriched with sodium selenite (Na_2SeO_3) in the initial Se concentration of 1.3 mg/L. The medium without Se was used as the control.

Submerged cultivation was carried out at room temperature on rotary shaker for 21 days. The obtained biomass was filtered, washed 3 times with dH_2O at magnetic stirrer and temperature of 30°C with the aim of removing the remaining Se from cell wall, and dried at 50°C to constant weight.

Preparation of the fungal extracts

Dry Se-amended and non-amended mycelia (3.0 g) were extracted by stirring with 90.0 mL of 96% ethanol at the 30°C for 72 h. The

obtained extracts were filtered through Whatman No. 4 filter paper, concentrated under reduced pressure in a rotary evaporator (BÜCHI R-114, Switzerland) at 40°C to dryness, and redissolved in 96% ethanol (for the testing of antioxidant activity) or in 5% dimethylsulphoxide (DMSO) (for the analysis of antifungal and anticancer activity).

Antioxidant activity

DPPH assay

Antioxidant activity was defined by measuring bleaching of the purple-coloured methanol solution of stable 1,1-diphenyl-2-picryl-hydrazil radical (DPPH•) (BLOIS, 1958). 1800.0 μL of 4% methanol solution of DPPH• and 200.0 μL of extract of defined concentration (series of double dilutions from 32.0 mg/mL to 0.5 mg/mL) were mixed and shaken. After 30 min of incubation in the darkness, the absorbances of reactive mixtures were measured at 517 nm against methanol as blank by spectrophotometer (CECIL CE2501, U.K.). The negative control contained all the reaction reagents except the extract. Scavenging effects was calculated by equation:

$$\text{DPPH scavenging effect (\%)} = \\ = [(A_0 - A_{sample})/A_0] \times 100,$$

A_0 - the absorbance of the negative control; A_{sample} - the absorbance of reaction mixture.

The EC_{50} value (mg extract/mL) is the effective concentration at which the DPPH• were scavenged by 50% and was obtained by interpolation from linear regression analysis. Commercial antioxidant, butylated hydroxyanisole (BHA) was used as a positive control.

Determination of total phenolic content

Total soluble phenolic compounds in the ethanolic extracts of Se-amended and non-amended mycelium were estimated with Folin-Ciocalteu reagent according to the method of SINGLETON and ROSSI (1965), using galic acid as a standard. 1000.0 μL of 10% Folin-Ciocalteu reagent and 200.0 μL of the extract were reacted in the dark for 6 min before addition of 800.0 μL of 7,5% Na_2CO_3. The reaction mixture was vortexed vigorously and incubated on a rotary shaker (100 rpm) in the dark and at the room temperature for 2 h. The absorbance was measured at 760 nm by spectrophotometer against blank (mixture without extract). The total concentration of phenolic compounds in tested extracts determined as μg of galic acid equivalents (GAE) per mg of dry extract, using an equation that was obtained from standard galic acid graph as:

$$\text{Absorbance} = 0.012 \times \text{total phenols} + \\ + 0.041 \ (R^2 = 0.999)$$

Determination of total flavonoid content

Total flavonoid content was determined using the methods of PARK *et al*. (1997). 1000.0 μL of the mycelium extract was diluted with 4300.0 μL mixture containing 4100.0 μL of 80% ethanol, 100.0 μL of 10% aluminium nitrate and 100.0 μL of 1 M aqueous potassium acetate. The mixture was incubated at room temperature for 40 min, and absorbance was measured spectrophotometrically at 415 nm. The amount of total flavonoids was expressed as μg of quercetine equivalents (QE) per mg of dry extract, using an equation that was obtained from standard quercetin hydrate graph as:

$$\text{Absorbance} = 0.014 \times \text{total flavonoid} - 0.072 \\ (R^2 = 0.989)$$

Antifungal activity

The tested micromycetes (Table 1) are maintained on Malt agar at 4°C in the culture collection of the Institute of Botany, Faculty of Biology, University of Belgrade.

Tested micromycetes were cultivated on Sabouraud dextrose agar (SDA) at temperature of 25 ± 2°C for 21 days. Spore suspensions were prepared by washing of agar surface with sterile 0.9% saline containing 0.1% Tween 80 (v/v). Turbidity was determined spectrophotometrically at 530 nm and spore number was adjusted to 10^6 CFU/mL (NCCLS, 1998).

DMSO extracts of Se-amended and non-amended mycelia were sterilized by filtration through Whatman No. 4 filter paper and 0.2 μm membrane filter. Antifungal potential of the tested extracts was studied by microdilution method using 96-well microtiter plate (SARKER *et al*., 2007). Series of double extract dilutions (from 32.0 mg/mL to 0.5 mg/mL) was analyzed. Each well contained SDA, spore suspension, resazurine, and crude ethanol extract of defined concentration. The mixture without extract was used as the negative control, while positive control contained commercial antimycotic, ketoconazole, instead extract. Tested ketoconazole concentrations ranged from 0.0313 mg/mL to 0.0019 mg/mL (series of double dilutions). Effect of 5% DMSO on the spore germination was also analysed by its addition in the mixture instead SDA. Microtiter plates were incubated at 25 ± 2°C for 72 h. The lowest extract concentration without visible mycelium growth was defined as minimal inhibitory concentration (MIC). Minimal fungicidal concentration (MFC) was determined as the lowest extract concentration with no mycelial growth after reinoculation of 2 μL of the mixture on SDA. The experiments were repeated three times.

Cytotoxic activity

Cell lines

Human cervix adenocarcinoma HeLa and human colon carcinoma LS174 cell lines were ob-

Table 1 - Antifungal activity (MIC and MFC) of ethanol extracts of Se-non amended and Se-amended mycelium of *Flammulina velutipes* and commercial antimycotic.

Tested organisms	*Flammulina velutipes*				Ketoconazole	
	MIC (mg/mL)		MFC (mg/mL)		MIC (mg/mL)	MFC (mg/mL)
	Se non-amended mycelium	Se-amended mycelium	Se non-amended mycelium	Se-amended mycelium		
Acremonium strictum W. Gams	4.0	4.0	-	-	0.0078	0.0156
Aspergillus flavus Link	8.0	4.0	-	-	0.0078	0.0078
Aspergillus fumigatus Fresen.	32.0	16.0	-	-	0.0078	0.0156
Aspergillus niger Teigh.	32.0	16.0	-	-	0.0156	0.0313
Aspergillus terreus Thom	16.0	8.0	-	-	0.0156	0.0313
Candida albicans (C.P. Robin) Berkhout	8.0	16.0	-	-	0.0078	0.0156
Candida krusei (Castell.) Berkhout	8.0	2.0	-	-	0.0078	0.0156
Candida parapsilosis (Ashford) Langeron & Talice	16.0	2.0	-	-	0.0078	0.0156
Cladosporium sp.	-	-	-	-	0.0078	0.0039
Fusarium verticillioides (Sacc.) Nirenberg	8.0	4.0	-	-	0.0156	0.0156
Microsporum gypseum (E.Bodin) Guiart & Grigoraki	-	-	-	-	0.0019	0.0039
Penicillium funiculosum Thom	8.0	4.0	-	-	0.0039	0.0078
Trichoderma viride Pers.	8.0	8.0	-	-	0.0039	0.0078
Trichophyton mentagrophytes (C.P. Robin) Sabour.	-	-	-	-	0.0019	0.0039

tained from the American Type Culture Collection (ATCC) (Manassas, VA, USA). Both cancer cell lines were maintained in the recommended Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 100.0 g/L heat-inactivated (56°C) fetal bovine serum (FBS), 3 mM L-glutamine, 100.0 mg/mL streptomycin, 100.0 IU/mL penicillin, and 25 mM 4-(2 hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and adjusted to pH 7.2 with bicarbonate solution. Cells were grown in a humidified atmosphere of 95% air/5% CO_2 (v/v) at 37°C.

Treatment of cell lines

Stock solutions (100.0 mg/mL) of extracts, made in 50.0 g/L DMSO, were dissolved in enriched RPMI 1640 medium to the required working concentrations. Neoplastic HeLa cells (2000 cells per well) and neoplastic LS174 cells (7000 cells per well) were seeded into 96-well microtiter plates. 24 h later, after cell adherence, five different doubly diluted concentrations of the extracts were added to the wells. The final concentrations applied to target cells were 200.0, 100.0, 50.0, 25.0 and 12.5 μg/mL, except in the control wells where only the nutrient medium was added to the cells. The cultures were incubated for 72 h.

Determination of cell survival (MTT test)

The effect of extracts on cancer cell survival was determined by microculture tetrazolium test (MTT test), according to MOSMANN (1983) with modification by OHNO and ABE (1991). 20.0 μL of MTT solution [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide in phosphate-buffering saline] of concentration of 5.0 mg/mL was added to each well. Samples were incubated for 4 h at 37°C in a humidified atmosphere of 95% air/5% CO_2 (v/v). Then, 100.0 μL of 10% sodium dodecyl sulfate was added to extract to dissolve the insoluble product formazan resulting from the conversion of the MTT dye by viable cells. The number of viable cells in each well was proportional to the intensity of the light absorbance (A), which was read in an enzyme-linked immunosorbent assay (ELISA) plate reader at 570 nm 24 h later. The inhibition rate was calculated according to the formula:

$$\text{Cell growth inhibition rate (\%)} = \\ = (A_{control} - A_{sample})/A_{control} \times 100$$

It was implied that the A of the blank was always subtracted from the A of the corresponding sample with target cells. IC_{50} was defined as the concentration of the extracts inhibiting cell survival by 50%, compared with a vehicle-treated control. *Cis*-diamminedichloroplatinum (*cis*-DDP) was used as a positive control. All experiments were done in triplicate.

Statistical analysis

The results were expressed as the mean ± standard error of data obtained from three parallel measurements. One-way analysis of variance (ANOVA) followed by LSD post-hoc determinations were performed using STATISTIKA software, version 5.0 (StatSoft, Inc) to test any significant differences. P-values less then 0.01 were considered statistically significant.

RESULTS

Antioxidant activity

Ethanol extracts of both Se-amended and non-amended *F. velutipes* mycelium showed good antioxidant potential that was dependent on the concentration, at higher concentrations extracts were more effective in DPPH• scavenging (Fig. 1). However, significant difference between the extracts, especially at higher concentrations, was noted ($P<0.01$). Se-enriched mycelium extract had higher scavenging effect than the control, and it exhibited a progressive increase of the activity at concentrations up to 8.0 mg/mL (from 4% to even 15% at concentration of 32.0 mg/mL). These results were confirmed by EC_{50} values, which were 30.5 ± 0.3 mg/mL in Se-enriched mycelium extract and 43.8 ± 0.6 mg/mL in non-amended one. Commercial antioxidant BHA was more efficient comparing with *F. velutipes* mycelium extracts, with EC_{50} value of 13.4 μg/mL.

Total phenol compounds were detected in both tested extracts. Their content in the non-amended extract was 9.5 ± 1.8 μg/mg, while the higher concentration of 14.5 ± 1.1 μg/mg was noted in Se-amended extract. However, flavonoids were not detected in any of the extracts. Direct correlation between phenol content and DPPH• scavenging effect existed, and linear relationship with $R^2 = 0.989$ in non-amended mycelium extract and $R^2 = 0.957$ in Se-amended one were noted.

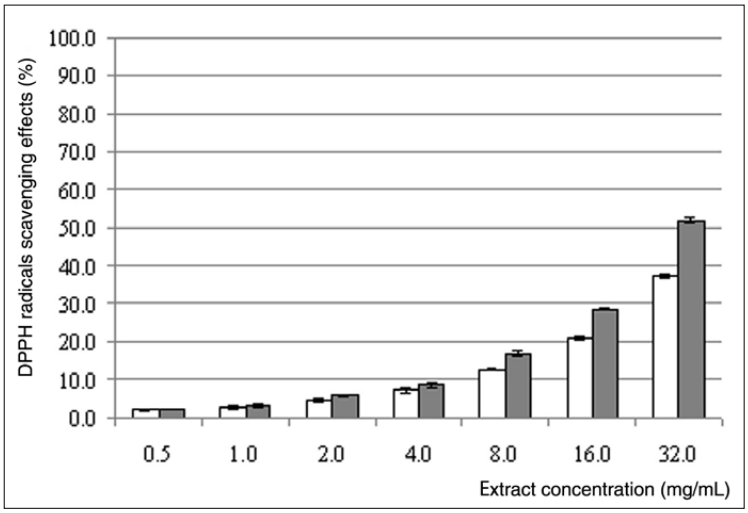


Fig. 1 - DPPH radical scavenging capacity of ethanol extracts of *Flammulina velutipes* mycelium. Se non-amended (□); Se-amended (■). (Data represent mean value of activities of three different samples. Variations are given as standard errors).

Antifungal activity

The antifungal potential of ethanol extracts of Se-amended and non-amended mycelium was tested against 14 micromycetes including saprobes as well as plant, animal and human pathogens. In the most cases, MICs of Se-enriched mycelium extract were lower, except for *Candida albicans* where control extract had twice more effect, and for *Acremonium strictum* and *Trichoderma viride* where MICs of Se-amended and non-amended extracts were the same (Table 1). The most sensitive species, with MIC of Se-enriched mycelium extract of 2.0 mg/mL, were *C. krusei* and *C. parapsilosis*. The most resistant species were *Aspergillus fumigatus* and *A. niger*, which growth was inhibited only with the maximum extract concentration (32.0 mg/mL for non-amended and 16.0 mg/mL for Se-amended one). Tested extract concentrations (from 0.5 mg/mL to 32.0 mg/mL) have no inhibitory effect on *Cladosporium* sp. and both causal agents of dermatomycosis, *Microsporium gypseum* and *Trichophyton mentagrophytes*. The maximum tested concentration of extract (32.0 mg/mL) did not show fungicidal effect for any tested fungal species (Table 1).

Sensitivity of the tested species to commercial antimycotic, ketoconazole, was more higher. Thus, the lowest tested concentration of 0.0019 mg/mL was MIC for *Cladosporium* sp., *M. gypseum*, and *T. mentagrophytes*. The concentration of 0.0039 mg/mL was MIC for *Penicillium funiculosum* and *T. viride* and MFC for *Cladosporium* sp., *M. gypseum*, and *T. mentagrophytes*. Mycelium growth of *A. strictum*, *A. flavus*, *A. fumigatus*, *C, albicans*, *C. krusei*, and *C. parapsilosis* was inhibited at ketoconazole concentration of 0.0078 mg/mL, which was also MFC for *A. flavus*, *P. funiculosum* and *T. viride*. Concentration of 0.0156 mg/mL was MIC for *A. niger*, *A. terreus*, and *Fusarium verticillioides* and MFC for most of the tested species, while the highest tested concentra-

Table 2 - Cytotoxic activity (IC_{50}) of Se-non amended and Se-amended *Flammulina velutipes* mycelium extracts and commercial cytostatic against HeLa and LS 174 cell lines.

Mycelial extract/cytostatic	IC_{50} (μg/mL)			
	HeLa		LS 174	
	Se-non amended extracts	Se-amended extracts	Se-non amended extracts	Se-amended extracts
F. velutipes	259.69 ± 0.70	331.91 ± 0.49	338.47 ± 0.97	348.46 ± 0.34
cis-DDP	0.72 ± 0.14		2.61 ± 0.11	

tion (0.0313 mg/mL) was MFC for *A. niger* and *A. terreus* (Table 1). 5% DMSO, used as a negative control, had no inhibitory effect on the tested micromycetes.

Cytotoxic activity

Comparing with *cis*-DDP, which was used as the positive control, the tested extracts showed low cytotoxic activity against both HeLa and LS174 cell line (Table 2). IC_{50} of non-amended mycelium extract against HeLa cells was 360- and 130-fold, respectively, higher than values obtained for *cis*-DDP. Se-amended mycelium extract was weaker cytotoxic agent than control one, especially for HeLa cell line.

DISCUSSION

Enrichment of cultivation medium with Se in the initial concentration of 1.3 mg/L and ability of *F. velutipes* HAI 966 mycelium to absorb and incorporate it in significant amount (10.0 μg/g) (MILOVANOVIĆ *et al.*, 2013) led to enhancement of antioxidant and antifungal capacity, while cytotoxic activity was reduced and was not in correlation with antioxidant potential.

DPPH• scavenging activity of *F. velutipes* ethanol extracts was reported by BAO *et al.* (2010), who demonstrated higher efficiency of mycelium extract than fruiting body one in almost 4-fold. According to SALTARELLI *et al.* (2009), the activity bases on redox properties of phenols, which enable them to act as reducing agents and hydrogen donators. Direct dependence of antioxidant capacity on phenol content was confirmed by CHEN *et al.* (2008) and BAO *et al.* (2010). Lower phenol content in *F. velutipes* HAI 966 mycelium extracts, compared with that in reported data, was responsible for negligible antioxidant potential that was significant only at the highest concentration. Lack of flavonoid in the ethanol extracts was in accordance with results of KARAMAN *et al.* (2009) who also have not found those compounds in mycelium extracts of *F. velutipes*. However, other compounds from those extracts could also be carriers of antioxidant activity (SHI *et al.*, 2012; WANG *et al.*, 2012). Thus, SHI *et al.* (2012) demonstrated significant scavenging effects of polysaccharides, even 90% at concentration of 2.5 mg/mL, while WANG *et al.* (2012) have noted high efficiency of sesquiterpenoids.

Enhanced antioxidant potential of Se-amended *F. velutipes* HAI 966 mycelium extract is in accordance with results of TURLO *et al.* (2010) who noted higher level of antioxidant activity in *Lentinus edodes* after cultivation in Se-enriched medium. The better effect could be explained by the fact that selenomolecules react with free radicals (•OH, •H) and successfully neutralize them (SHEN *et al.*, 2010).

WANG *et al.* (2012) tested antifungal potential of sesquiterpenoids isolated from *F. velutipes* mycelium against numerous species and reported weak activity against *A. fumigatus* and absence of any inhibitory effect on *C. albicans*. Similar, negligible activity of ethanol extract of *F. velutipes* HAI 966 mycelium against *A. fumigatus* was also noted but it was enhanced with Se addition. However, tested *Candida* spp. were very sensitive to the extracts, especially in the Se presence, which was contrary to the results of WANG *et al.* (2012). Results of this study showed that Se acts as an antifungal agent which is in accordance with results of SHAHVERDI *et al.* (2010) who clearly demonstrated antifungal activity of biogenic Se against selected clinical micromycetes. Mycelium enrichment with this element presents a way for getting biogenic Se nanoparticles that could be a potent ingredient for the preparation of antifungal formulations (SHAHVERDI *et al.* 2010).

Contrary to Se stimulatory effect on antioxidant activity, this trace element caused regression of cytotoxic activity. Numerous biologically active compounds, such as diverse types of sesquiterpenes, polysaccharides, glycoproteins, ribosome inactivating proteins, and sterols, isolated from mycelium of *F. velutipes*, take important place as cytotoxic agents against various cancer cell lines (LEUNG *et al.*, 1997; NG and WANG, 2004; WANG *et al.*, 2012; YANG *et al.*, 2012; YI *et al.*, 2013). LEUNG *et al.* (1997) showed that soluble homopolysaccharide, composed of glucose and isolated from *F. velutipes* fruiting bodies, were very efficient in Sarcoma-180 cells regression *in vivo*. The same effect was noted by YANG *et al.* (2012) for alkaline-soluble heteropolysaccharides with a glucan as backbone chain and triple helix structure, which also have strong anti-proliferation activity against lung cancer cells (A549) and human gastric cancer cells (BGC-823) (inhibitory rate was 32.3% and 95%, respectively). The cytotoxic effect of polysaccharides was based on proliferation of B-cell, T-cell or both cells and not on tumor cell kill. Activation of immune system and in such a way production of interferon-gamma with antiproliferate effect on tumor cells was also lied in the base of antitumor activity of *F. velutipes* glycoprotein FVE (CHANG *et al.*, 2010). However, ribosome inactivating proteins (flammulin, velutin, flammin, and velin) stopped cancer cell proliferation by ribosome inactivation and translation inhibition (NG and WANG, 2004). Efficiency of sesquiterpenoids against human liver carcinoma cell line (HepG2), breast cancer cell line (MCF-7), human gastric cancer cell line (SGC7901) and A549 was modest (IC_{50} was in range between 20.0 and 100.0 μM) (WANG *et al.*, 2012), while inhibition rate of human glioma cell line (U251) by sterols, at concentration of 20 μg/mL, was even 57% (YI *et al.*, 2013). However, the sterol was not so efficient against HeLa cell line (IC_{50} > 40.0 μg/mL), but more efficient comparing with tested Se-amended and non-amended mycelium extract.

Although tested extracts showed lower biolog-

ically activities than commercial antimycotic, antioxidant and cytostatic, preference should be given to natural products. Therefore, *F. velutipes* is not only food but also could be considered as a possible base for natural safe remedies for human therapy.

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PAPER

THE PHYSICOCHEMICAL PROPERTIES OF EDIBLE PROTEIN FILMS

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ABSTRACT

In the present study, edible films from isolated or concentrated protein sources and from proteins of two different fish species were produced. The texture properties, light transmission (LT) and oxygen permeability (OP) of producing films were determined. The CL film settled in the second range according to both tension test parameters, thus outclassing the other tested films. The WG film possessed the lowest LT, so making it more effective in protecting of food products from light than the other tested films. The SPI film with the lowest OP value can be used for the purpose of protecting of food products from harmful effects of oxidation.

- Keywords: Coating, edible protein films, permeability, smoked fish, texture -

1. INTRODUCTION

Edible films and coatings are thin layers that can be eaten with food. They are derived from natural sources and are formed on the surface of a food for the purpose of protecting food and to prolong its shelf-life. For this agricultural function, protective coatings were improved as an alternative to commercial packaging materials such as glass, tin and polymer. Edible films and coatings do not carry carcinogen risk and do not cause a waste problem, which are important problems with plastic-based food packaging.

If edible films are prepared in appropriate conditions, they can perform all the functions of a useful package. Edible films are prepared utilizing hydrocolloids (protein and polysaccharide), lipids and composites (hydrocolloid+lipid). Edible protein films are separated, however, into two groups: plant origin proteins (corn zein, wheat gluten, soy protein, pea protein, sunflower protein, peanut protein and cotton protein, etc.) and animal origin proteins (keratin, collagen, gelatin, fish myofibrillar protein, egg white protein, casein and whey protein, etc.). Edible protein films can be manufactured from isolates or concentrated protein products that are purified from various protein sources, and by the evaluation of processing waste products (RHIM and NG, 2007).

The use of edible films for food is very old. So far, the most important application of edible films and coatings is an emulsion, made from oil and waxes (in 1930) that protect the important features of fruits such as brightness and color, prevents complications such as softness, initialing paleness and for improving fungicides, to better control the maturation and to delay water loss (DEBEAUFORT *et al.*, 1998; BALDWIN, 1999).

The advantages of natural biopolymer films can be summarized as follows: they are edible and biodegradable; to supplement the nutritional value of foods; enhance organoleptic characteristics of food, such as appearance, odor, and flavor; reduce packaging volume, weight and waste; incorporate antimicrobial agents and antioxidants; extended shelf-life and improved quality of usually non-packaged items; control over inter-component migration of moisture, gases, lipids, and solutes; individual packaging of small particulate foods, such as nuts and raisins; function as carriers for antimicrobial and antioxidant agents; for microencapsulation and controlled release of active ingredients; and have a possible use in multilayer food packaging materials together with non-edible film. They are low-cost and abundant; annually renewable resources (KROCHTA, 2002).

Despite the positive effects in the literature (GENNADIOS *et al.*, 1997), the industrial application of edible films is not very common yet. The theoretical, experimental studies related to different polymers will be used to compose edible films or coatings (using fresh, frozen, processed products and determining the permeability properties of films) are currently underway.

In the present study were used soy protein isolates (SPI), whey powder protein (WP), egg white powder protein (EWP), wheat gluten (WG), corn zein (Z), cattle gelatin (G) and collagen (CL), as well as rainbow trout protein (RTP) and Atlantic mackerel protein (MP) as the protein source. The texture properties, the light transmission and the oxygen permeability of edible films produced from these sources were established.

2. MATERIAL AND METHODS

2.1. Edible protein film manufacturing

The materials for the production of edible protein film were obtained from the Smart Chemicals Company (Izmir, Turkey). The edible protein films were manufactured as explained below, by pouring a determined amount film of solution into a Teflon pan (18 cm×18 cm), drying for a determined time and at a determined temperature.

2.1.1. Soy protein isolates (SPI) film

5 g SPI (produced in Germany and containing 90% protein), 100 mL distilled water and 2.5 mL glycerol (GLY) was stirred for 15-20 minutes at 55-60°C. This solution was then stirred for another 10-15 minutes at 75-80 °C and filtered through a cloth, after the solution's pH was adjusted to 10.5±0.1 with 2 M sodium hydroxide (NaOH) solution (DENAVI *et al.*, 2009). Prepared film solution (70 mL) was poured into a Teflon pan and dried for 24 hours at 35°C.

2.1.2. Whey protein (WP) film

5 g WP (produced by Lactoprot Company in Germany and containing 80% protein), 100 mL distilled water and 5 mL GLY was stirred for 15-20 minutes at 55-60°C. This solution then was stirred for another 15-20 minutes at 75-80 °C and filtered through a cloth, after the solution's pH was adjusted to 8.0±0.1 with 2 N NaOH (SARIKUS, 2006). The WP film solution (70 mL) was poured into a Teflon pan and dried for 48 hours at 35°C.

2.1.3. Egg white powder protein (EWP) film

9 g EWP (produced in Turkey and containing 80% protein), 100 mL distilled water, 4.5 mL GLY was stirred for 5 minutes at room temperature. Then the solution's pH was adjusted to 11.25±0.1 with 1 N NaOH. The solution was kept for 20 minutes in a water bath at 45 °C and filtered through a cloth (GENNADIOS *et al.*, 1997). The prepared film solution (70 mL) was poured into a Teflon pan and dried for 24 hours at 35°C.

2.1.4. Wheat protein (gluten-WG) film

7.5 g WG (produced in Belgium and containing 75-82% protein), 55 mL 95% ethyl alcohol, 45 mL distilled water and 3.75 mL GLY was stirred for 15 minutes at 55 °C. The solution's pH was adjusted to 4.0±0.1 with 50% acetic acid. The solution was filtered through a cloth, then stirred for another 15 minutes at 70 °C (TANADA-PALMU, 2000). All the prepared film solution was poured into a Teflon pan and dried for 48 hours at 45°C.

2.1.5. Corn protein (zein-Z) film

The film solution was prepared from zein protein (produced by Sigma Aldrich in USA and containing 90% protein), by modifiying the method developed by BAYSAL *et al.* (2009). 2.5 g Z, 60 mL 95% ethyl alcohol, 1 mL GLY was stirred for 30 minutes at 75-80 °C (BAYSAL *et al.*, 2009). The pH of the prepared Z film solution was measured as 5.8. The Z film solution (40 mL) was poured into a Teflon pan and dried for 24 hours at 45°C.

2.1.6. Gelatin (G) film

2 g G (produced by Rousselot Company in Argentina and containing 83% protein), 100 mL distilled water, 1.1 mL GLY was stirred for 30 minutes at 55-60 °C. The solution was then filtered through a cloth (THOMAZINE *et al.*, 2005). The pH of the prepared G film solution was determined as 5.1. The G film solution (130 mL) was poured into a Teflon pan and dried for 48 hours at 45°C.

2.1.7. Collagen (CL) film

3 g CL (produced in Turkey and containing 90% protein), 200 mL 3% acetic acid, 1.5 mL GLY was stirred for 30 minutes at 75-80 °C (HO *et al.*, 2001). When prepared according to this method, the pH of the CL film solution was 3.3. All the prepared film solution was cast into Teflon pan and dried for 24 hours at 45°C.

2.1.8. Rainbow trout protein (RTP) film

Rainbow trout (*Oncorhynchus mykiss*) were obtained from the Istanbul Fish market. The fish were transported in ice to the laboratory. The gutted and beheaded fish were minced, after skinning. The fish mince was washed 2-3 times to remove the water-soluble proteins, blood and dirty components, by keeping it for 5-10 minutes in cold water (water:fish ratio of 4:1, w/w). Following the washing process, fish mince was pressed through a cloth in order to remove water. Prepared in this way, washed fish mince constituted the raw material for the edible fish film. Previously produced edible film were tested for protein and moisture in this mince. The RTP ratio was found to be 18.48%, and the moisture ratio was found to be 73.35%. Because the protein ratio of the fish mince should be 2%, it was adjusted by adding the necessary amount of distilled water and then GLY at a ratio of 50% of the protein ratio was added. The mixture was blended and filtered. The solution's pH was adjusted to 3 with 50% acetic acid, after the solution was stirred for 10-15 minutes at 75-80 °C. The solution was stirred for another 10-15 minutes at 75-80 °C (CUQ *et al.*, 1997). The prepared film solution (100 mL) was poured into a Teflon pan and dried for 24 hours at 35°C.

2.1.9. Atlantic mackerel protein (MP) film

Used in manufacturing of edible film, Atlantic mackerel minces (*Scomber scombrus*) were prepared by cleaning and washing, as in the case of rainbow trout flesh. The MP ratio was found to be 23.38%, and the moisture ratio was found to be 72.62%. The MP film manufacturing method was similar to the RTP film manufacturing method. The only difference was that less distilled water was added, as when adjusting the protein ratio was to 2%, because of the MP ratio was higher. The film solution's pH was adjusted to 3 again (CUQ *et al.*, 1997). The MP film solution (100 mL) was poured into a Teflon pan and dried for 24 hours at 35°C.

2.2. The texture Profile of the films

Tension tests were made for the purpose of determining the resistance of the film against breakage and tensile forces. Test measurement values were as follows: target type: distance, test type: tension, target value: 40-90 mm, trigger load: 0.04 n, test speed: 0.5 mm/second, return speed: 4.5 mm/second, probe type: TA3/100, fixture: TA-DGA, load cell: 1500 g. The measured parameters were the tensile force (N) and the maximum elongation (mm). Three measurements were taken from three protein films belong to each group.

2.3. The Light Transmission (LT) of the films

The LT of edible protein films was measured according to the method in ASTM (2009) by using working visible position dual beam spectrophotometer at 560 nm, at 23±2 °C. This test method covers the measurement of the transparency of plastic sheeting in terms of regular transmittance (T_r). Although generally applicable to any translucent or transparent material, it is principally intended for use with nominally clear and colorless thin sheeting. Three measurements were taken from three protein films belong to each group. The LT of the films was calculated with the formula below accord-

Table 1 - The texture parameters, the light transmission and the oxygen permeability values of edible protein films.

Edible protein film	Tensile force (N)	Maximum elongation (mm)	Light transmission (%)	Oxygen permeability (mL/mm/day)
SPI	2.883±0.155AF	31.85±1.11AG	20.08±0.01A	17.10±0.01A
WP	0.668±0.001B	5.69±1.03B	23.35±0.01B	322.00±0.01B
EWP	1.972±0.117AC	82.00±2.94C	23.22±0.01C	45.40±0.01C
WG	1.275±0.147BC	57.40±2.07D	13.29±0.01D	218.00±0.01D
Z	0.948±0.427BC	0.50±0.02B	19.92±0.01E	181.37±0.01E
G	5.267±0.559D	35.12±1.88AF	63.30±0.01F	45.50±0.01F
CL	4.145±0.198DE	68.03±1.57E	18.70±0.01G	105.10±0.01G
RTP	3.559±0.720EF	39.94±0.03F	14.53±0.01H	97.10±0.01H
MP	1.273±0.082BC	28.95±0.96G	39.35±0.01I	281.00±0.01I

*Different letters (A, B, C) in the same column indicate the significant difference (P<0.05).

ing to light intensity value, measured by equipment (ASTM, 2009).

$$T_r = (I_r/I_0) \times 100$$

T_r: Light transmission, %
I_r: Light intensity value of spectrophotometer bath with sample
I_0: Light intensity value of spectrophotometer bath without sample

2.4. The Oxygen Permeability (OP) of the films

The OP of the films was measured during 4 hours at 23±2 °C by using Systech mark gas permeability test equipment, at 170 bar O_2 of pressure (ASTM, 2010). Three measurements were taken from three protein films belong to each group.

2.5. Statistical analysis

The resulting analysis data were evaluated by using an IBM SPSS Statistics 20® program. The results were given as an average ± standard deviation. The one-way analysis of variance (One-Way ANOVA) was applied. In the data parametric assumptions for multiple comparisons occurred. The Tukey test was used to locate the sources of the differences found within different groups in this test. P<0.05 variation was accepted as the significant discrepancy between the groups and the parameters.

3. RESULTS

The texture parameters, the light transmission and the oxygen permeability values of edible protein films are presented in Table 1. The lowest and the highest tensile force of edible protein films were 0.668±0.001 N (in the WP film), and 5.267±0.559 N (in the G film), respectively. The Z film had the lowest maximum elongation (0.50±0.02 mm), and the EWP film had the highest maximum elongation (82.00±2.94 mm) (Table 1).

It was reported by SABATO *et al.* (2007) that mechanical and barrier properties of protein-based films were generally better than polysaccharide (PLS)-based films. The properties of protein-based films were dependent on the various factors, such as the protein source, the pH of protein solution, the species and the amount of plasticizer, film thickness, the manufacturing conditions (temperature and relative humidity), and the structures, included film forming solution (enzymes, antimicrobials, etc.) (BENJAKUL *et al.*, 2008).

TEMIZ and YESILSU (2006) indicated that when made from WG strong films have mechanical properties like rubber. WG films, containing a low amount of GLY, had a higher tensile resistance (TR) and lower elongation at break (EAB) (TANADA-PALMU *et al.*, 2000). When the WG film was prepared from three different flours (commercial bread flour, hard red winter flour and soft white flour), it had a smooth tissue and film resistance, measured as TR, and the ratio increased by 50% by adding cysteine (cross-linking agent) (RAYAS *et al.*, 1997). When the mechanical and physical properties of the WG films, plasticized with GLY, were examined at different temperature (20°C, 50°C and 80°C) and the relative humidity (35% and 70%), the tensile strength (TS) increased too, by increasing the drying temperature at 35% relative humidity. TS decreased, when the temperature increased, however, at 70% relative humidity. The film thickness decreased with increasing temperature (KAYSER-ILIOGLU *et al.*, 2003). LIEBERMAN and GILBERT (1973) reported that the mechanical properties of edible films were considerably affected by the ratio of the plasticizer, used.

The composite films can be manufactured taking into account the different barriers and mechanical properties needed by different products and thus the effectiveness of the material was increased. The use of the plasticizers such as GLY, polyethylene glycol (PEG), sorbitol (SOR), etc. in the film formulations or the composites is advan-

tageous to impart pliability and flexibility, which improves handling. Utilization of the plasticizers reduces the brittleness of film by interfering with the hydrogen bonding between the lipid and hydrocolloid molecules (THARANATHAN, 2003).

In the study of MARINIELLO *et al.* (2003), whole soy flour and apple pectin were used as raw materials in order to obtain films, which due to their consistency could be perfectly handled. The films were also prepared in the presence of transglutaminase (T-Gase). The latter films showed a smoother surface and higher homogeneity, as demonstrated by microstructural analyses, whereas studies on the mechanical properties indicated that T-Gase increased their TS and reduced their flexibility. It was reasoned that by there was a possible use of the T-Gase polymerized pectin-soy protein films as edible food or drug coatings.

The proper pH value to prepare the SPI film with good mechanical and barrier properties was 10. Addition of PEG as a plasticizer at 60% of SPI weight gave better film properties comparing with other used plasticizers. Cross-linking of the SPI film by adding formaldehyde or glutaraldehyde at a different level into the film forming solution and the combination of SPI with the starch, also caused a noticeable improvement in the mechanical and barrier properties of the films (SOLIMAN *et al.*, 2007). According to DENAVI *et al.* (2009), the applied drying conditions when preparing the SPI film affected the mechanical properties, with the optimal drying conditions of 70 °C and 30% relative humidity and 60 °C and 60% relative humidity. Dried under these conditions, the SPI films presented a higher TS, lower EAB values. SHIH (1998) indicated that the TS and the EAB belonging to SPI was affected by pH. The mechanical properties of edible films improved with some of the applied methods. The mechanical properties of the CL film improved with glyceraldehyde and alcoholdiols; the TS of the CL film increased by exposing to UV beams; the TS of the Z film improved with aldehydes; the TS of the WG film developed with keratin; the TS of the WP film improved by heat treatment; the TS of the SPI film developed by heat treatment, UV and gamma radiation application, and by adding calcium chloride and calcium sulphate (MCHUGH and KROCHTA, 1994).

It was expressed by GENNADIOS *et al.* (1997) that the EWP films are highly hydrophilic; they could be used for water-soluble packets (pouches) for ingredients in the food, chemical, and pharmaceutical industries similar to cellulose ether-based water soluble packets in commercial use. The mechanical and barrier properties of such films can be modified by varying the types and amounts of added plasticizers.

When microbial T-Gase was employed as the catalyst for preparing the chitosan (CH)-ovalbumin films, the mechanical resistance of the CH-ovalbumin films increased from 24 MPa to 35 MPa. The improved mechanical and solubility properties of these new materials confirmed that this enzymatic approach could be a useful tool for preparing edible films for food coating and pharmaceutical applications (DI PIERRO *et al.*, 2007). In the study conducted by KOLODZIEJSKA and PIOTROWSKA (2007), the effect of GLY on the mechanical and water barrier properties, as well as on the water solubility, of fish gelatin-CH films (4:1, w/w) cross-linked with T-Gase or 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) was determined. The TS of the films decreased after modification of the components with T-Gase or EDC, by about 25% and 40% respectively. The elongations of the enzymatically modified films containing 20% of GLY and of chemically modified films containing 15% of GLY were, respectively, about 8 and 13 times higher than those of unplasticized films. However, the TS of plasticized films were, respectively, 2.5 and 5 times lower. These properties widen the practical applications of modified films as packaging material. However, the cross-linking of components with commercial preparations of T-Gase, -different to modification with EDC- increased the fragility of films. Therefore, such films have should be plasticized.

The increase in the proportion of GLY caused a reduction of the puncture force, the TS, the modulus of elasticity, and an increase of the puncture deformation and the EAB of the G film (THOMAZINE *et al.*, 2005). When the G film and the Z film were prepared by incorporating nisin (NS) into the film-forming solutions, the Z film with the NS of 12.000 IU/mL had an increase of 11.6 MPa in the TS compared with the control, but the G film had a slight increase resulting from the increase of the NS concentration. These results suggested that the incorporation of NS into the Z film and the G film improved the physical properties of the films (KU and SONG, 2007).

In the study of CARVALHO *et al.*, (2008), the G films from the skins of Nile perch, a warm-water fish species, were reported to exhibit stress and EAB similar to that of bovine bone gelatin. The gelatin, extracted from halibut skins, showed a suitable filmogenic capacity, leading to transparent, weakly colored, water-soluble and highly extensible films. The intermediate evaporation step at 60 °C in the industrial procedure for drying the G film induced thermal protein degradation, caused the resulting films to be significantly less resistant and more extensible. It was seen that the G film with predominance of lower-molecular-weight fractions was apparently more plasticized by SOR molecules, favoring a higher extensibility and a lower resistance in the final film.

The edible films were successfully prepared from fish skin gelatin of brownstripe red snapper (*Lutjanus vitta*) and bigeye snapper (*Priacanthus macracanthus*). The films with the greater protein content had a higher thickness and me-

chanical properties (TS and EAB), but lower water vapor permeability than those with the lower protein content. The films without GLY were mostly brittle, and became more flexible in the presence of GLY. The TS generally decreased with increasing GLY concentration from 25% to 75% (JONGJAREONRAK *et al.*, 2006). IWATA *et al.* (2000) expressed that edible films prepared from water-soluble fish proteins, had better flexibility compared to most of the other protein films. The pH change, the various physical and chemical treatments and the properties of plasticizer affected the quality of fish protein film. Lower mechanical properties were found in the films prepared from the lower quality washed fish mince (BENJAKUL *et al.*, 2008). The TS of the films was higher, when prepared at acidic (pH 2, 3) and alkaline (pH 11, 12) conditions. The film with the lowest TS was made at pH 7. The mechanical resistance of the myofibrillar protein (MYFP) based films were substantially lower than the synthetic films -such as polypropylene (PP), polyethylene and polyvinyl dichloride- but was relatively close to low density polyethylene (LDPE) (SHIKU *et al.*, 2003).

GLY and PEG plasticizers gave flexible structure to the films prepared from water soluble fish protein. As the concentration of GLY increased, TS decreased with the concomitant increase of EAB. In contrast, PEG showed more marked influence on TS than on EAB. GLY:PEG ratio of 2:1 exhibited the maximum EAB value. It was confirmed that MYFP based films plasticized with the mixture of GLY-PEG showed lower TS (2.5-3.0 MPa) than with only GLY (5.0 MPa) or with only PEG plasticized films (7.0 MPa) (TANAKA *et al.*, 2001).

PARRIS and COFFIN (1997) observed that Z-based films plasticized with a mixture of GLY-polypropylene glycol (PPG) showed lower TS and lower elastic modulus than with only GLY plasticized films or PPG plasticized films. Moreover, the films plasticized with the mixture of GLY-PEG caused higher EAB (117.8%) than those with only GLY (2.6%) or PEG plasticized films (2.8%).

The functional properties of the fish protein films produced by the thermo-melting techniques were similar to known protein films and the TS of the films were similar to LDPE (CUQ *et al.*, 1998). The films obtained from Tilapia fish treated at 65°C/30 minutes were more resistant and more rigid than the films treated at 40°C/30 minutes (GARCIA and SOBRAL, 2005). When the protein concentration was treated at 90°C/30 minutes, the filmogenic solution was 2 g of sarcoplasmic protein (SRCP)-MYFP/100 g filmogenic solution, the prepared films were more resistant than the respective films (SOBRAL *et al.*, 2005). CUQ *et al.* (1995) expressed that MYFP films produced from sardine had the higher TR than that obtained from other protein films, such as Z, WG, SPI and WP.

The quality of Alaska pollack surimi (SRM) films was decreased in order to determine the influence of the SRM quality on the TS, and the EAB values. Thawed Alaska pollack SRM was incubated at 30°C for 20 minutes (slight protein denaturation) and at 30 °C for 5 hours (complete protein denaturation) in order to decrease the SRM quality. Slight protein denaturation caused a decrease in the EAB of the films and complete denaturation gave rise to a reduction of the TS, and the EAB values (SHIKU *et al.*, 2004). MYFP-based films were developed from a film-forming solution based on fish mince and the influence of plasticizers on the protein film quality was investigated. When no plasticizer was introduced in formulation the films were relatively brittle and needed to be handled very carefully, and GLY, SOR and sucrose were added in various concentrations. Plasticization of the MYFP-based films induced a large decrease in film strength and elasticity and an increase in deformation properties (CUQ *et al.*, 1997). SOR plasticized films were the most brittle, with the highest TS (3.14 MPa). In contrast, GLY and PEG plasticized films exhibited flexible structure, despite the low TS (2.13 MPa and 1.80 MPa, respectively). As plasticizer concentration increased, the TS decreased concomitant with an increase in EAB (BOURTOOM *et al.*, 2006).

Propylene glycol alginate (PGAL) was incorporated into Alaska pollack SRM film in order to improve their mechanical properties. The TS of the PGAL-SRM films prepared at pH 11 were twice as high compared to the SRM films prepared at pH 7. The EAB was not affected by the incorporation of PGAL. Results revealed that the formation of cross-linkages between epsilon-amino groups of lysine residues in SRM proteins and mannuronic acid esters of PGAL above pH 10 was responsible for the increased TS of the films (WENG *et al.*, 2006). The effect on the ratios of MYFP to SRCP from round scad (*Decapterus maruadsi*) muscle on the properties of the resulting films was investigated. The TS of the films decreased with an increased SRCP content. The films prepared from MYFP:SRCP ratio of 10:0 (w/w) exhibited the highest TS. The EAB of the films, prepared with SRCP content greater than 30%, had the decreased EAB (ARTHAN *et al.*, 2008).

A comparison could not be made, due to no mention in the literature to studies involving similar textural values of edible protein films obtained from the texture analyzer equipment used in our study. Moreover, when both tensile test parameters were evaluated, as seen clearly in Fig. 1 and Fig. 2, placing the CL film in the second range, this film group outclassed other film groups considerably. SARIKUS (2006) expressed that the CL film is used in sausage coating more than natural coatings. The most important advantages of these films are that they remain strong in producting process conditions, having a flexible structure with a high-tensility.

The G protein film obtained the highest LT value (63.30%±0.01); the WG protein film obtained, however, the lowest LT value (13.29%±0.01) (Table 1).

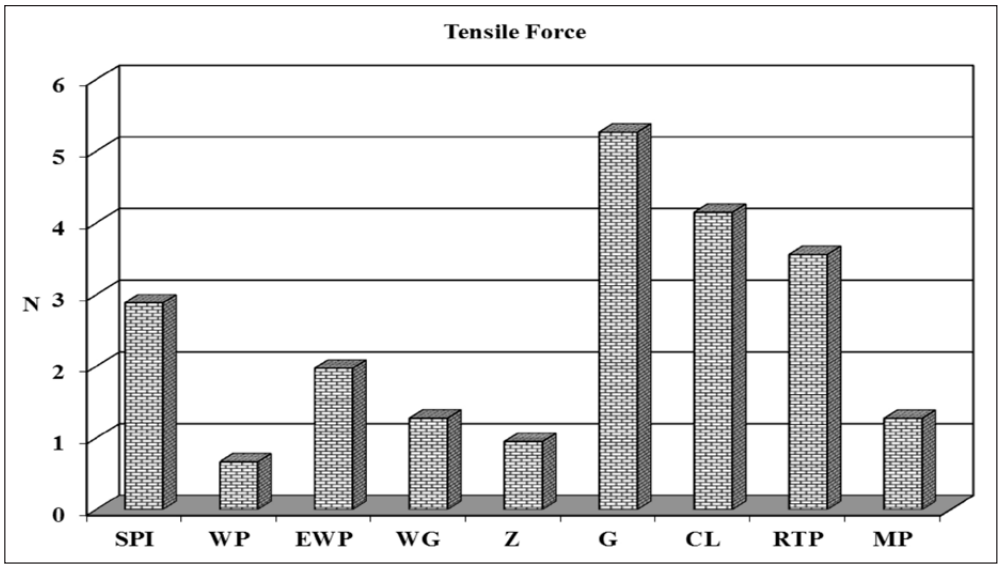


Fig. 1 -The changes of the tensile force values of edible protein films.

Because food products are exposed to light trigger oxidative reactions, the products were packed with non-light transmitting packaging material in order to protect them from the light. There are a limited number of studies mentioned in the literature about the LT value of edible films.

SHIKU *et al.* (2003) expressed that MYFP films, prepared from blue marlin flesh, have excellent barrier properties to UV light in the range of 200-280 nm regardless of pH. However, the UV barrier property of the films gradually became poor above the wavelength of 300 nm. In contrast, the MYFP film prepared at pH 7 blocked the most light in the UV-visible range from 350 to 800 nm because of its semi-transparency.

In our study, the LT of the WG film was measured as the lowest value (13.29%±0.01) at 560 nm/23±2 °C. The RTP film possessed the second lowest LT value after the WG film (Fig. 3). Comparisons could not be made, as there are no similar studies in the research literature of LT measurements conducted at the same wavelength as in our study. However, it is possible to say that the WG film can be more effective protecting products from light than other protein films.

The highest OP value was observed at 322.00±0.01 mL/mm/day in the WP film, and the lowest OP value was observed at 17.10±0.01 mL/mm/day in the SPI film (Fig. 4). The OP value of the EWP film and the OP value of the G film; the OP value of the CL film and the OP value of the RTP film was determined to be close to each other in.

The diffusion of gas and water vapor into film is called transition. These gases and the water vapor were absorbed by a surface of the polymer and oscillated by the other surface of the polymer during transition. The films have different barrier properties according to component properties and manufacturing techniques. The polar polymers, such as protein and carbonhydrates,

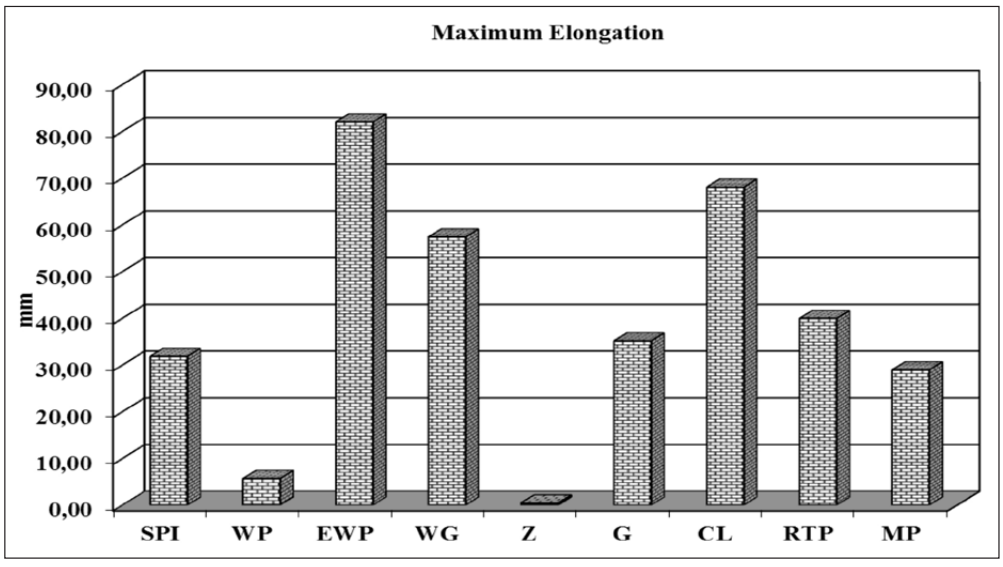


Fig. 2 -The changes of the maximum elongation values of edible protein films.

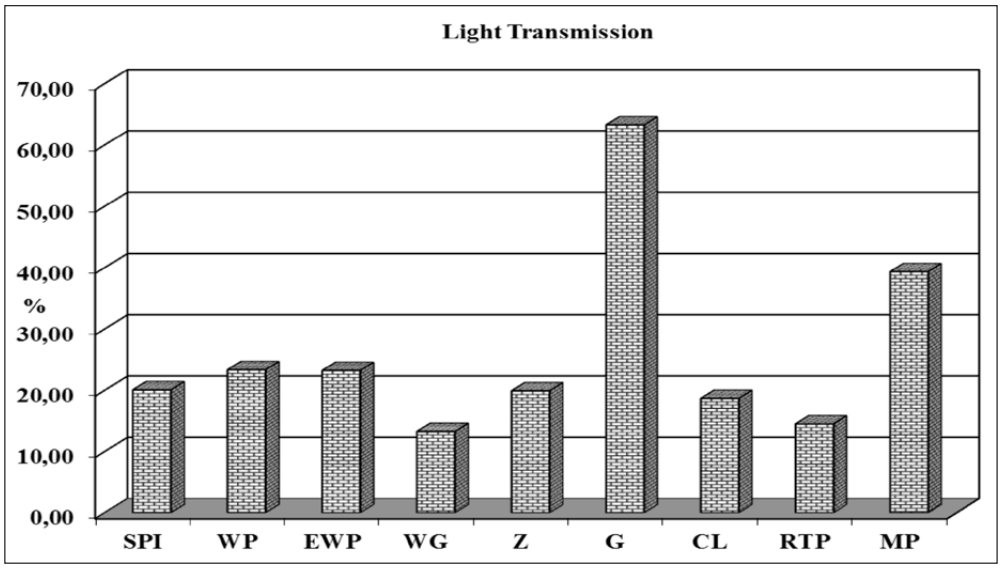


Fig. 3 -The changes of the light transmission values of edible protein films.

show low gas permeability and high water vapor permeability. In contrast, whilst materials (containing apolar hydrocarbon such as lipid) constitute an excellent barrier against the water vapor transition, they are not an effective barrier to gas transition. The chemical structure and the shape of the transition or absorbed film matrix substance is effective at the speed of diffusion and transfer. For example, the small molecules diffuse faster than the big molecules or the polar molecules diffuse faster than the apolar molecules in the polar films (CAGRI-MEHMETOGLU, 2010). The rate of rancidity, causing lipid oxidation and brown coloration, as well as causing myoglobin oxidation in meats, could be reduced by using edible coatings with the low OP (GENNADIOS *et al.*, 1997). It was implied by YILMAZ *et al.* (2007) that WP-based edible film and coating slowed gas (O_2, CO_2) transfer. The OP of the CL film was <0.04 $cm^3.\mu m/m^2.dk.Pa$; the OP of the Z film was 11.8 $cm^3.\mu m/m^2.dk.Pa$; the OP of the WG film was 3.9-6.1 $cm^3.\mu m/m^2.dk.Pa$; the OP of the SPI film was 1.6-4.5 $cm^3.\mu m/m^2.dk.Pa$; and the OP of the WP film was 18.5-76.1 $cm^3.\mu m/m^2.dk.Pa$ (LIEBERMAN and GILBERT, 1973). LIN and ZHAO (2007) expressed that the O_2 permeability ($7.84x10^{-19}$ $m^3.m/m^2.s.Pa$) of the Z-based coating was lower than the O_2 permeability of plastic films (such as LDPE, PP, polystyrene and polyvinylchloride) and of PLS, PLS/lipid composite coatings, but higher than the O_2 permeability ($2.89x10^{-17}$ $m^3.m/m^2.s.Pa$) of WG coatings. It was observed that the O_2 permeability of the WG coating was close to the O_2 permeability ($2.25x10^{-17}$ $m^3.m/m^2.s.Pa$) of LDPE. It was recognized in literature that the OP of edible films was affected considerably by factors such as the preparation conditions (temperature and relative

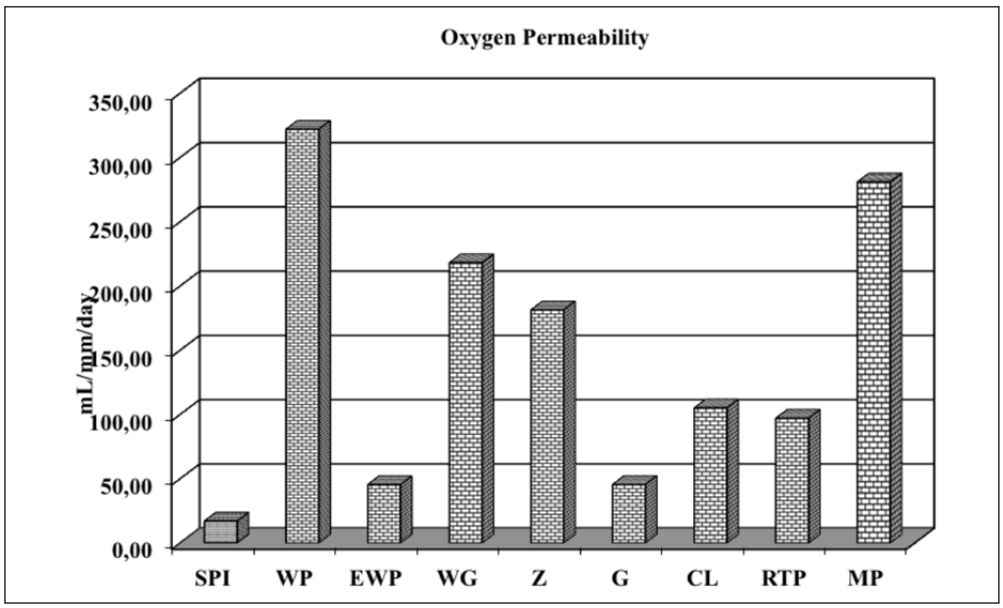


Fig. 4 -The changes of the oxygen permeability values of edible protein films.

humidity); the type and the amount of plasticizers, the incorporation of various chemicals; heat-treatments and pH levels. For example, MCHUGH and KROCHTA (1994) specified that the gas permeability of the CL film decreased with formaldehyde and chrome tannic acid, whilst the OP of the WP film and the SPI film improved with heat treatment. The OP (3.16 $cm^3.\mu m/m^2.dk.Pa$-at 23 °C and 50% relative humidity) of the WG film, made with 40% GLY, was found to be similar to the OP (3.82 $cm^3.\mu m/m^2.dk.Pa$-at 23 °C and %0 relative humidity) of the film obtained by GENNADIOS *et al.* (2006) (MUJICA-PAZ and GONTARD, 1997). Despite the fact that the OP of all WG films was low, the OP level could be raised by increasing the GLY concentration. It was seen that temperature has less effect on OP than the relative humidity. The low OP of the WG films may be due to their polar nature and linear structure, leading to the high cohesive-energy density and the low free volume (TANADA-PALMU *et al.*, 2000). SHIH (1998) expressed that the OP of the WG film decreased by adding of mineral oil and dipping in Ca^{+2}. It was specified that the SPI coatings were effective oxygen barriers (OP; $3.14x10^{-19}$ $m^3.m/m^2.s.Pa$) at low relative humidity conditions, especially as their high oxygen barrier properties made them suited to gave the facility their applications such as flavour and pharmaceutical microencapsulated agents or as coatings for fruit, vegetable and cheese. It was reported that the WP-based films also had excellent oxygen barrier properties (OP; $1.13x10^{-18}$ $m^3.m/m^2.s.Pa$) at low and medium relative humidity when they compared with synthetic polymers (LIEBERMAN and GILBERT, 1973). The proper pH value for the preparation of SPI film with good barrier properties was 10; the O_2 permeability of the SPI film was 1.06 $cm^3.\mu m/m^2.dk.Pa$ at this pH value. The addition of PEG as a plasticizer at 60% of SPI weight made the film properties better, compared with other plasticizers, and the O_2 permeability of the SPI film was 0.76 $cm^3.\mu m/m^2.dk.Pa$. The cross-linking of the SPI film by adding formaldehyde or glutaraldehyde at different levels into the film forming solution improved the barrier properties of the resulting films (SOLIMAN *et al.*, 2007). DENAVI *et al.* (2009) found that the applied drying conditions used in SPI film manufacturing affected the barrier properties of the film: the O_2 permeability of the film was 18.2 $cm^3.\mu m/m^2.dk.Pa$ dried at 70 °C and 30% relative humidity and the O_2 permeability of the film was 47.6 $cm^3.\mu m/m^2.dk.Pa$ dried at 60 °C and 60% relative humidity. In our study, the SPI film had the lowest O_2 permeability (17.10±0.01 ml/mm/day) (Table 1). The properties of SPI film can be improved with application of additional methods in light of the literature (LIEBERMAN and GILBERT, 1973; SOLIMAN *et al.*, 2007; DENAVI *et al.*, 2009) and it can be used for the purpose of protecting food products from the harmful effect of oxygen.

4. CONCLUSIONS

In our study of the mechanical properties of edible protein films, both tensile test parameters (tensile force and maximum elongation) were evaluated, placing of the CL film in the second range: this film group outclassed other film groups considerably. It was observed that the WG film possessed the lowest LT value. It is possible to say that the WG film is more effective in protecting products from light than other protein films. The lowest OP in our study belonged to the SPI film. The properties of the SPI film -which can be improved with additional methods in light of the literature- can be used for the purpose of protecting food products from the harmful effect of oxygen.

ABBREVIATIONS

CH:	Chitosan	PGAL:	Propylene glycol alginate
CL:	Collagen	PLS:	Polysaccharide
EAB:	Elongation at break	PP:	Polypropylene
EDK:	1-ethyl-3-(3-dimethylaminopropyl) carbodiimid	PPG:	Polypropylene glycol
EWP:	Egg white powder protein	RTP:	Rainbow trout protein
G:	Gelatin	SOR:	Sorbitol
GLY:	Glycerol	SPI:	Soy protein isolates
LDPE:	Low density polyethylene	SRCP:	Sarcoplasmic protein
LT:	Light transmission	SRM:	Surimi
MP:	Mackerel protein	T-Gase:	Transglutaminase
MYFP:	Myofibrillar protein	TR:	Tensile resistance
NaOH:	Sodium hydroxide	TS:	Tensile strength
NS:	Nisin	WG:	Wheat Gluten
OP:	Oxygen permeability	WP:	Whey protein
PEG:	Polyethylene glycol	Z:	Zein

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PAPER

SPECTROPHOTOMETRIC DETERMINATION OF TRACE QUANTITIES OF CADMIUM IN SEAFOOD SAMPLES BY SIMULTANEOUS CLOUD POINT EXTRACTION AND TERNARY ION ASSOCIATION SYSTEM

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ABSTRACT

A procedure for simultaneous separation/preconcentration of cadmium in various seafood samples, based on cloud point extraction (CPE) as a prior step to it's determination by spectrophotometric method in a ternary ion association system has been developed. In this work, the zwitterionic surfactant 3-[N,N-Dimethyl(3-palmitoylaminopropyl)ammonio]-propanesulfonate, ABS-16, functions not only as a chelating agent but also co-extraction agent. In fact, there are interactions between Cd (II), ABS-16, Ponceau 4R, Triton X-114, and N-cetyl-N-N-N-trimethyl ammonium bromide (CTAB). The parameters affecting the extraction efficiency such as pH, temperature and incubation time, concentration of surfactants and salt, anionic dye, and chelating agent were evaluated and optimized. Under the optimal conditions, the limit of detection (LOD) was 1.07 μg L^{-1}. The relative standard deviation (RSD) for ten replicate measurements of 100 and 500 μg L^{-1} of cadmium was 3.52% and 2.10, respectively. A linear calibration curve in the range of 10-500 μg L^{-1} of cadmium in the initial solution with r = 0.998 (n = 12) was acquired. The results showed the quantities of Cd^{2+} in the seafood samples from the rivers located in industrial area are much more than the nonindustrial one.

- Keywords: cadmium, cloud point extraction, seafood -

1. INTRODUCTION

Cadmium is known to be highly toxic for animals, plants and humans even at low concentrations and can be accumulated in several organs. The most important anthropogenic sources of this element include emissions from industrial plants, such as zinc smelters, steel works, incinerators and power stations.[1] Cadmium is among the six forbidden substances by the Directive Restriction of the use of Hazardous Substances in Electrical and Electronic Equipment (RoHS) issued by Europe Union in 2003.[2] Because of the toxicity of cadmium to plants and humans, its determination in the environment is increasingly important. Cadmium is known to damage organs such as kidneys, liver and lungs. It also causes high blood pressure and destruction of red blood cells. [3] Daily intake of food and water is an important pathway through which cadmium enters human body, thus there is an increasing need to monitor cadmium levels in food samples. [4] Consumption of seafood is also one of the dominant routes for human exposed to Cd. Seafood can accumulate Cd via waterborne and dietborne exposure pathways, posing a potential human health risk.[5] During the last few years, seafood consumption has undergone major changes. The rapid growth in aquaculture production has made the sector important to the economy of many developing countries and, in the case of some traded aquatic products. Due to the availability and variety of different analytical methods for the determination of many residues in food samples, it is necessary to ensure the quality and comparability of the analytical results generated by laboratories approved for official control. [6] As the rapid development of industry, more and more waste water containing hazardous heavy metals was discharged into river, which is harmful to living beings and the environment around. Hence, preconcentration and separation technologies have to be employed to extract analytes from the complex matrix so as to obtain suitable concentrations of the analytes for accurate determination.[7] The use of preconcentration steps based on phase separation by cloud point extraction offers a convenient alternative to more conventional extraction systems.[8] Cloud point extraction (CPE) has been applied to determination of trace metal ions from different matrices recently years.[9] Cloud-point extraction is based on the property that a solute present in aqueous solution of non-ionic surfactant is distributed between two phases. [10] Aqueous solutions of non-ionic surfactants become turbid when they are heated above the temperature known as the cloud point. The solution is then separated into two isotropic phases, i.e. a surfactant-rich phase and a bulk aqueous phase. The hydrophobic solutes and metal ions, after the formation of sparingly water soluble complex, can be enriched into the surfactant-rich phase.[11] Because of the importance of this issue, a lot of researchers have done studies regarding measuring of cadmium by various methods.[12-18] In order to study Cd (II) ion, zwitterionic (or amphoteric, containing both positive and negative charges) surfactant ABS-16 was chose as the chelating agent. The interaction between cadmium and ABS-16 led to the formation of $[Cd\ (ABS\text{-}16)_2]^{2+}$ cationic complex. Then small amount of Ponceau 4R was added to the $[Cd\ (ABS\text{-}16)_2]^{2+}$, cationic complex, led to the formation of $[Cd\ (ABS\text{-}16)_2\text{-Ponceau 4R}]^{-1}$ anionic complex. Since, hydrophobic ion-associated complexes could be more efficiently extracted into surfactant-rich phase than ionic ion-associated complexes, small amount of cationic surfactant (CTAB) was added for neutralizing the negative charge of complex. The result was the formation of $[Cd\ (ABS\text{-}16)_2\text{-Ponceau 4R-CTA}]$ hydrophobic complex.

In the present work we have applied ternary ion association system for cloud point extraction by the spectrophotometric method for determination of the cadmium quantities in the seafood samples from the rivers located in industrial and nonindustrial areas.

2. EXPERIMENTAL

2.1. Chemicals and Reagents

All chemicals and solvents were of analytical reagent grade and were used without further purification. The McIlvaine's buffer solution was provided by dissolving 7.71 ml of 0.2 M Na_2HPO_4 in 12.29 ml of 0.1 M citric acid.[19] Bidistillated water was used through the work. Cadmium (II) nitrate (Aldrich, WC, USA) was used for preparation of stock solution (1000 mg mL^{-1}) of cadmium (II). More diluted solutions (10-500 $\mu g\ L^{-1}$) of cadmium (II) were prepared by suitable dilution of the stock with bidistillated waters in presence of few drops of dilute H_2SO_4 (1.0 mol L^{-1}). A stock solution of $1.0 \leftrightarrow 10^{-4}$ mol L^{-1} of ABS-16 was prepared by diluting appropriate amount of ABS-16 (Merck Millipore, Germany) in bidistilated water. The non-ionic surfactant Triton X-114 (Sigma-Aldrich, Steinheim, Germany) was used without further purification. A 3% (v/v) nonionic surfactant solution was prepared by dissolving 3 mL of Triton X-114 in 100 mL distilled water. A solution of NaCl (0.1 mol L^{-1}) was prepared by dissolving 0.584 g of NaCl (Merck, Germany) in water and diluting to 50 ml in a volumetric flask. A stock solution of 1000 $\mu g\ ml^{-1}$ of Ponceau 4R (Sigma-Aldrich, Germany) was prepared by dissolving appropriate amount of the Ponceau 4R (Merck, Germany) in water and diluting to 100 ml in a volumetric flask. Stock solution CTAB ($1.0 \leftrightarrow 10^{-3}$ mol L^{-1}) was prepared by dissolving

0.0364 g of this reagent (Merck, Germany) in bi-distilled water and diluting to the mark in a 100 mL volumetric flask.

2.2. Instrumentation

A double beam spectrophotometer (UV-1800, Shimadzu, Japan) was used for absorption spectra and absorbance measurements using 10 mm(500 μ L) quartz cuvette cells. A digital pH meter (Metrohm, model 691) with a combined glass electrode was applied to measure pH values. A thermostat bath (Model Colora) maintained at the desired temperature was used for the cloud point temperature (CPT) experiments. A centrifuge was used to accelerate the separation process (Universal-320, Hettich centrifuges, England).

2.3. General procedure

An aliquot of the solution containing of cadmium (so that its final concentration would be in the range of 10-500 μg L^{-1}), 2.25 mL of $1.0\leftrightarrow10^{-4}$ mol L^{-1} of ABS-16, 1.5 mL of $1.0\leftrightarrow10^{-4}$ mol L^{-1} of Ponceau 4R, 1.75 mL of 3% (v/v) of Triton X-114, 0.75 mL of $1.0\leftrightarrow10^{-3}$ mol L^{-1} of CTAB, 1.25 mL of 0.1mol L^{-1} of NaCl and 3.5 mL of McIlvaine's buffer, pH 4, was transferred into a 15 mL tube, diluted to the mark with distilled water and equilibrated at 45°C in a thermostat bath for 10 min. Separation of the aqueous and surfactant-rich phase was performed by centrifugation for 5 min at 3500 rpm. After cooling in an ice bath, the surfactant-rich phase became viscous and the supernatant aqueous phase was then removed thoroughly by a syringe centered in the tube. The surfactant-rich phase was diluted to 0.5mL with methanol to decrease its viscosity prior to transferring into a 10 mm (500 μ L) quartz cuvette cell. The absorbance was measured at 644 nm against a reagent blank. The reagent blank was containing all the reagents except analyte.

2.4. Sample preparation

1 g of each of the samples were added to a PTFE bomb containing 5 ml HNO_3 and 2 ml H_2O_2. After 15 min the closed bombs were placed in the microwave oven, using the following program: 5min at 63 W, 5 min at 155 W, 10 min at 315 W and 5 min at 470 W. After each step the vessels were cooled down and gas was released. The clear solution was diluted to 10 ml using deionized water.

3. RESULTS AND DISCUSSION

3.1. Effect of pH

The pH is crucial factor influencing both the reaction between metal ions and chelating agent molecules (binary-complex, Cd-ABS-16), and CPE procedure (ternary-complex, [Cd(ABS-16)$_2$ -Ponceau 4R-CTA]. The impact of pH on the extraction of ternary ion association system (10 μg L^{-1} of Cd(II)) in aqueous solution was investigated in the range of 1.0-8.0. Fig. 1 shows the maximum absorbance and efficiency of CPE was obtained in pH 4 by the addition of McIlvaine's buffer. 1.5 mL of McIlvaine's buffer pH 4 was added to the sample solutions to maintain the pH at 4. At higher and lower pH values, ion association complex does not form completely in that ion pairing systems are very sensitive to changes in pH values.

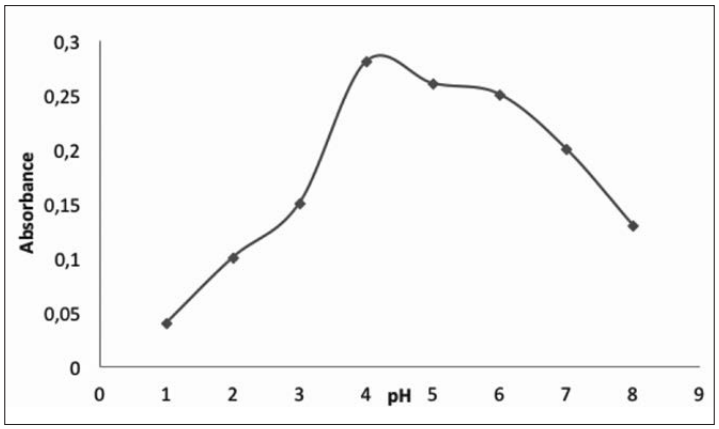


Fig. 1 - The effect of pH on the absorbance of Cd (II).

3.2. Effect of ABS-16 concentration

The effect of ABS-16 concentration was studied in range of $0.33\leftrightarrow10^{-5}$- $2.33\leftrightarrow10^{-5}$ mol L^{-1}. The results are given in Fig. 2. The extraction yield increased by increasing ABS-16 concentration up to $1.50\leftrightarrow10^{-5}$ mol L^{-1} and remained constant at higher ligand concentrations. So, $1.50\leftrightarrow10^{-5}$ mol L^{-1} of ABS-16 ligand was selected as an optimum concentration of ABS-16 for Cd^{2+} extraction process.

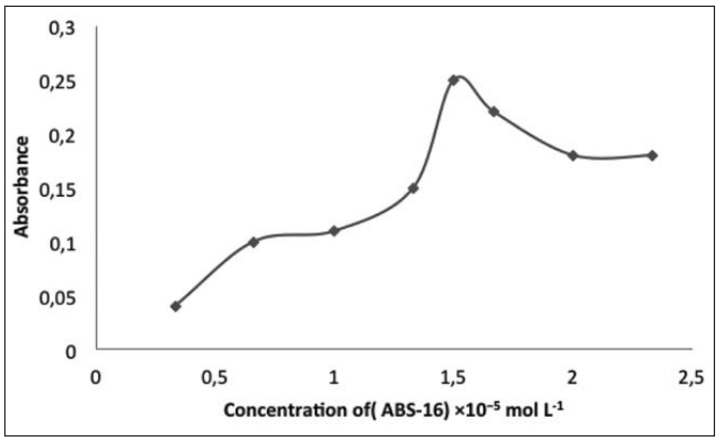


Fig. 2 - The effect of ABS-16 concentration on the absorbance of Cd (II).

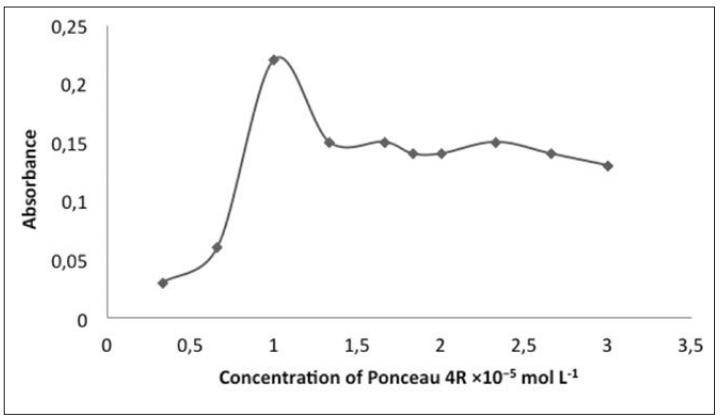


Fig. 3 - The effect of Ponceau 4R concentration on the absorbance of Cd (II).

3.3. Effect of Ponceau 4R dye concentration

The effect of concentration of Ponceau 4R on extraction yield is shown in Fig. 3. It is apparent in Fig. 3 the absorbance increased gradually by increasing Ponceau 4R dye concentration up to $1\leftrightarrow10^{-5}$ mol L^{-1} and decreased slowly at higher concentrations of Ponceau 4R. The excessive dye could be co-extracted into the surfactant rich phase, thus decreased the extraction efficiency of target complex. Additionally, it caused reduction of ion-pairing reagent, CTA^+. Therefore, 1.50 mL of $1.0\leftrightarrow10^{-4}$ mol L^{-1} Ponceau 4R (equivalent to a concentration of $1\leftrightarrow10^{-5}$ mol L^{-1} in final solution) was chosen as the optimal ligand concentration.

3.4. Effect of ionic surfactant concentration

Initially, three surfactants such cationic CPC, CTAB and anionic SDS were considered and investigated in range of 0-1.33×10^{-4} mol L^{-1}. The best extraction yield was obtained in presence of CTAB. Therefore, to the use of CTAB was decided for further studies. The effect of CTAB concentration, was studied in range of 0-1.33×10^{-4} mol L^{-1} (Fig. 4). The extraction yield gradually increased by increasing CTAB concentration up to 5×10^{-5} mol L^{-1}, so it was selected as the optimal concentration.

3.5. Effect of nonionic concentration

Initially, three nonionic surfactants such as Triton X-114, Ponpe 7.5 and Triton X- 45 (0.1-0.60% (v/v)) were used to CPE. However, the results showed the best extraction yield was obtained in the presence of Triton X-114. So, to the use of Triton X-114 was chosen for further studies. The results indicated that the absorbance of the surfactant-rich phase increased by increasing Triton X-114 concentration up to 0.35 % (v/v) and decreased at higher concentrations. Therefore, 0.35% (v/v) Triton X-114 was used as optimum concentration. The results are shown in Fig. 5.

3.6. Effects of equilibration temperature, time and centrifuge time

The effect of temperature was examined from room temperature to 75°C. It was found that 45 °C is adequate for the quantitative analysis. It is often necessitated to preconcentrate trace amounts of analytes with high efficiency at a minimum time. In order to attain desirable extraction the incubation time was studied in the range of 5–30 min. Consequently, an incubation time of 10 min was chosen for use in next experiments. Separation of the aqueous and surfactant-rich phase was performed by centrifugation for 5 min at 3500 rpm.

3.7. Effect of ionic salt concentration

It has been reported that the addition of electrolytes may accelerate the separation of the two phases of the CPE procedure [20]. In this work,

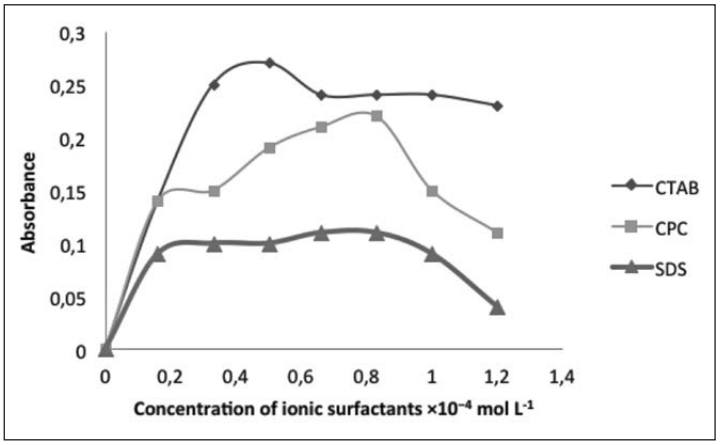


Fig. 4 - The effect of CTAB concentration on the absorbance of Cd (II).

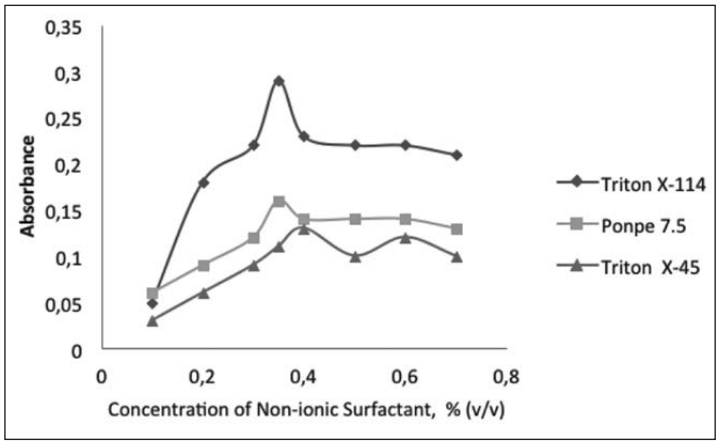


Fig. 5 - The effect of nonionic surfactant concentration on the absorbance of Cd (II).

salts which were used in the CPE included NaCl, KCl, and KI. However, the results showed the best extraction yield was obtained in the presence of NaCl. So, it was chosen for further studies. The effect of electrolyte concentration on the extraction of Cd (II) was examined by various amount of 0.1mol $L^{-1}$of NaCl solution. The result showed that the absorbance increased with increasing NaCl concentration up to $8.33\leftrightarrow10^{-3}$ mol L^{-1} in final solution and approximately remained constant above that.

3.8. Figure of merits

The calibration graph was linear in the range of 10-500 μg L^{-1} of Cd (II) solutions. The calibration graph for the preconcentration procedure was $A= 2.92\times10^{-3} C+ 1.49\times10^{-2}$ for 10-500 μg L^{-1} of Cd (II) with a correlation coefficient of 0.998 (n = 12), where A is the absorbance and C shows the concentration of cadmium in μg L^{-1}. The limit of detection of this method was 1.07 μg L^{-1}. The detection limit was calculated according to 3 S_{blank} /s, where S blank was obtained from the standard deviation for 10 replicate measurements of a blank solution, and s is the slope of the calibration graph. The relative standard deviation for 100 and 500 μg L^{-1} of Cd (II) was 3.52 and 2.1% (n = 10), respectively. The preconcentration factor was 33.3.

3.9. Inferences of co-existing ions

The impact of potential inference of some cationic and anionic species on the preconcentration of Cd (II) was investigated. 10 μg L^{-1} of Cd (II) solutions were prepared and different amounts of foreign ions were spiked and examined according to the proposed method under optimal conditions, and the tolerance of the foreign ions, defined as a relative error of ±5% with respect to the absorbance difference for the Cd (II) solution, is shown in Table 1.

Table 1 - The effect of other species on the determination of 10 μg L^{-1} of Cd(II).

Foreign ions	Tolerance limit (μg mL^{-1})
Na^{+}, K^{+}, $NH^{4+,}$ $CO3^{2-}$, F^{-}, Cl-	1000
Li^{+}, NO^{3-}	800
$Pd2^{+}$, Al^{3+} , Fe^{3+}, Mg^{2+}	500
SO_4^{2-}, HPO_4^{2-}, Pb^{2+}, Co^{2+}	250
Ca^{2+}, Fe^{2+}, Zn^{2+}	100
Cu^{2+}	50

3.10. Analytical applications

The presented procedure was successfully applied for the determination of Cd (II) in various seafood samples. Five replicates determinations were carried out and the obtained results (Table 2) were satisfactory. The high percentage recoveries were confirmed the accuracy, precision and the independence of the procedure from the matrix interference.

4. CONCLUSIONS

A new approach based on simultaneous mixed-micelle mediated extraction (MMME) and ion association system was developed for determination of trace cadmium ion in environmental seafood samples by using the UV-Vis spectrophotometric method. The proposed method has some advantages that are summarized as follows: first, mixed micelle extraction has much higher sensitivity; second, cloud point extraction based on ion association system has much higher sensitivity compared to CPE based on simple metal-chelate complex; third, in contrast to some familiar preconcentration techniques like solvent extraction methods, it is much safer, because only a small amount of the surfactant, which has a low toxicity, is used.

5. ACKNOWLEDGEMENTS

The authors would like to thank Young Researchers and Elite Club, Gachsaran Branch, Islamic Azad University, Gachsaran, Iran, for financial support of this work (Grant 1392).

Table 2 - Cadmium determination in samples.

	Cd^{2+}		Recovery
Sample	Added, μg L^{-1}	Found a, μg L^{-1}	(%)
fish[b]	-	NDe	
	50	51.23±0.16	102.46
	100	101.29±0.21	101.29
shrimp[b]	-	NDe	
	50	49.16±0.12	98.32
	100	99.29±0.18	99.29
fish[c]	-	16.78±0.17	
	50	65.7±0.03	98.38
	100	117.75±0.03	100.83
shrimp[c]	-	9.06±0.28	
	50	59.31±0.23	100.42
	100	110.43±0.18	101.26

[a] $\bar{x}\pm$ ts $\sqrt{n}$ at 95% confidence (n = 5)
[b] pole zohre river, located in a non industrial area
[c] zayande rood river, located in a industrial area
[e] Not Detected(under LOQ)

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PAPER

A COMPARATIVE STUDY ON FATTY ACID CONTENT OF MAIN ORGANS AND LIPID CLASSES OF LAND SNAILS *ASSYRIELLA ESCHERIANA* AND *ASSYRIELLA GUTTATA* DISTRIBUTED IN SOUTHEASTERN ANATOLIA

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ABSTRACT

In the present work, main organs (digestive gland, cephalopedal, gonad and mantle) and lipid classes (total, neutral and phospholipid) of land snails *Assyriella escheriana* and *Assyriella guttata* from southeastern Anatolia were examined for their fatty acids. The major components detected in both of the species were C16:0, C18:0, C18:1ω9, C18:2ω6, C18:3ω3, C20:2ω6 and C20:4ω6. C18:2ω6 was identified as the primary fatty acid ranging from17.07% to 28.12% in *A. guttata* and 18.02% to 27.43% in *A. escheriana*. The proportions of C20:4ω6 modified to form prostaglandins that are directly involved in regulation of reproduction, ranged from10.01% to 20.30% in *A. escheriana* and 11.05 % to 16.58% in *A. guttata*. Taking into consideration that ΣPUFA levels were always higher than ΣSFA and ΣMUFA levels in all treatments of both species. This was an expected finding for the snails collected during the breeding season because PUFA plays an important role as precursors for signal-transduction involved in the regulation of mating and reproduction. A significant amount of C20:2ω6 was concentrated in the cephalopedal of *A. guttata* (13.42%) and *A. escheriana* (14.93%). Probably, cephalopedal serves as a storage organ of this component. Consequently, the findings revealed that the snail's fatty acid profiles were qualitatively similar, but quantitatively there were some differences. Most important of all, tissues of the snails were good source of essential fatty acids (C18:2ω6 and C18:3ω3) and PUFA, particularly omega 6 fatty acids.

- Keywords: Fatty acids, Assyriella escheriana, Assyriella guttata, organs, lipid classes -

INTRODUCTION

Assyriella escheriana and *Assyriella guttata* (endemic to southeastern Turkey and North Iraq) species are common in moist and calciferous habitats of southeastern Turkey. Although they are not eaten and commercially not exported, they are big enough and fleshy as much as edible relatives *Helix lucorum, Eobania vermiculata* dwelling in the same region. The fatty acid distribution of a large number of commercially important marine and freshwater molluscs have been reported and reviewed in varying degrees of details (ACKMAN, 2000; KARAKOLTSIDIS *et al.*, 1995). Lipids from marine, freshwater and edible land molluscs are more extensively studied (ÖZOĞUL *et al.*, 2005; MILINSK *et al.*, 2006; MILETIC *et al.*, 1991; RAKSHIT *et al.*, 1997; EKİN and BAŞHAN, 2010; EKİN *et al.*, 2012, 2014) than those from nonedible terrestrial members. Nevertheless, nonedible land snails deserve special attention from the point of their evolutional relationship, roles in food chain, nutritional value, taxonomic and possible benefits in cosmetic, medicine and biochemistry. Nutritional, food chain and taxonomic studies are important in understanding interrelationship in marine, freshwater and terrestrial environment, however for the southeastern Anatolia; quite little data were available in the literature on edible and nonedible land snails.

A. escheriana and *A. guttata* species whose edible relatives including *Helix aspersa, H. asemnis, H. cincta* and *H. lucorum, Theba pisana, Eobania vermiculata* and *Cantareus apertus,* living in Turkey (YILDIRIM, 2004), can deserve more detailed studies. Many snail farms are being established in some countries in order to produce good quality snails for consumption and export. So, it seemed useful to make a comparative study of similarity and differences in biochemical and nutritional composition of edible and nonedible snail species.

A comparative biochemical study on fatty acid composition of snails belonging to same classes but living in different habitats is hoped to provide an insight into the adaptive capabilities and influence of the environment on their fatty acid distribution. This paper discusses the fatty acid distribution of main organs and lipid classes in two same genus gastropods from southeastern Anatolia, as data which is hoped to be basic to further comparative biochemical, nutritional, taxonomic and evolutionary studies.

MATERIALS AND METHODS

Sample collection and preparation

Fifteen adult *A. escheriana* species were collected from a woodland near Tizyan (Elmabahçe) village, 20 km north of Mardin (N 37° 49' / E 40° 68') at an altitude of 985 m and fifteen adult endemic *Assyriella guttata* species were collected from stony and rocky region of Diyarbakır city walls (N 37° 55.2' / E 40° 13.8') at an altitude 675 m. Both species were collected in April 2012. Similar size (length: 4 ± 0.60 cm, wet flesh weight: 13 ± 0.50 g) snail species were sampled for lipid analyses. The snails' shells were removed and divided into seven groups (digestive gland, cephalopedal, gonad, mantle, total lipid, neutral lipid and phospholipid) and their organs were dissected out. Then, tissues of each experimental set were conditioned in polyethylene bags and kept at -80°C until chemical analysis.

Extraction of fatty acids and GC analysis

Digestive gland, gonad, mantle, cephalopedal and whole body samples were homogenized in chloroform/ methanol (2:1, v/v) solution in order to extract total body lipids (BLIGH and DYER, 1959). Organ's lipid, total lipid, phospholipid and neutral lipid fractions were obtained according to the method of STANLEY-SAMUELSON and DADD, 1983.

Fatty acids methyl esters (FAMEs) were provided by capillary gas chromatography (GC) using Hewlett Packard (Wilmington, DE) gas chromatograph (model 6890), a DB-23 capillary column (60 m × 0.25 mm i.d. × 0.250 μm film thickness and Bonded 50% cyanopropyl) (J & W Scientific, Folsom, CA), a flame ionization detector, and Hewlett-Packard ChemStation software. The injection port and the detector temperatures were 270°C and 280°C, respectively. The split ratio was 1:20. The flow rates of compressed air and hydrogen were 300 ml/min, 30 ml/min, respectively. Carrier gas was helium (2.8 ml/min). The oven temperature was programmed at a rate of 6.5°C/min from 130°C (1 min hold) to 170°C, then increased at a rate of 2.75°C/min to a 215°C, then again increased at a rate of 40°C/min to 230°C, was held for 12 minutes. Each tissue fatty acids percentages and spectra of FAMEs are obtained by HP 3365 ChemStation computer program. FAMEs existence and retention times were determined by comparing the spectra of authentic standards (Sigma-Aldrich Chemicals). Individual FAME was identified by comparisons with the chromatographic behaviors of authentic standards.

Statistical analyses

The results were expressed as mean values ± SD (Standard Deviation). All analytical determinations were performed in triplicate and the mean values were reported. The analyses were performed using a commercial statistical program (SPSS 20). The percentages of fatty acid were compared by ANOVA variance analysis with 5% significance level. TUKEY's test was used for cooperation of average values.

RESULTS

In both snails, C16:0, C18:0, C18:1ω9, C18:2ω6, C18:3ω3, C20:2ω6 and C20:4ω6 were presented as predominant fatty acids. Of the detected fatty acids, amount of C18:2ω6 was the highest in all analyses from both *A. escheriana* and *A. guttata* (Tables 1 and 2).

Compared to the fatty acids of the species' organs, in *A. escheriana,* highest level of C16:0 (11.81%) and C18:1ω9 (20.56%) were presented in the gonad; C18:0 (15.18%) and C20:2ω6 (14.93%) were in the cephalopedal; C18:2ω6 (26.67%) and C18:3ω3 (7.36%) were in the digestive gland and C20:4ω6 (17.86%) was in the mantle (Table 1). In *A. guttata* highest level of C16:0 (9.72%), C18:1ω9 (19.65%) and C18:3ω3 (4.76%) were found in the gonad; C18:0 (15.21%) and C20:4ω6 (15.68%) were in the mantle; C18:2ω6 (28.12%) was in the digestive gland and C20:2ω6 (13.42%) was in the cephalopedal (Table 2). On the other hand, the fatty acids from lipid classes of the species showed some differences, in *A. escheriana,* highest level of C16:0 (10.16%) and C18:3ω3 (8.42%) were identified in the total lipid; C18:0 (11.21%), C18:1ω9 (13.33%) and C20:2ω6 (11.79%) were in the neutral lipid; C18:2ω6 (27.43%) and C20:4ω6 (20.30%) were in the phospholipid (Table 1). In *A. guttata,* highest level of C16:0 (9.61%) and C20:4ω6 (16.58%) were presented in the total lipid; C18:0 (13.01%), C20:2ω6 (10.99%) and C18:1ω9 (16.03%) were in the neutral lipid; C18:2ω6 (22.34%) and C18:3ω3 (10.70%) were in the phospholipid (Table 2).

In all treatments, results showed that concentration of ω6 (omega 6) always higher than concentration of ω3 (omega 3) family fatty acids. In *A. escheriana*, the ratio of Σω6 / Σω3 was 4.86, 6.71, 5.44 and 7.66 in the digestive gland, cephalopedal, gonad and mantle, respectively (Table 1). In *A. guttata* it was 5.13, 8.49, 5.29 and 4.84 in the digestive gland, cephalopedal, gonad and mantle, respectively (Table 2). Additionally, Σω6 / Σω3 ratio were 5.19 in the total lipid, 4.86 in the neutral lipid and 6.59 in the phospholipid of *A. escheriana* (Table 1). This ratio was observed 4.00 in the total lipid, 3.91 in the neutral lipid

Table 1 - Fatty acid profile of main organs total lipid and lipid classes from *Assyriella escheriana.*

	Fatty acid compositions of total lipid from *A. escheriana* organs				**Fatty acid compositions of lipid classes from *A. escheriana***		
Fatty Acids	**Digestive gland (Mean*±S.D.)****	**Cephalopedal (Mean*±S.D.)****	**Gonad (Mean*±S.D.)****	**Mantle (Mean*±S.D.)****	**Total lipid (Mean*±S.D.)****	**Neutral lipid (Mean*±S.D.)****	**Phospholipid (Mean*±S.D.)****
C10:0	0.12±0.02a	0.05±0.01b	0.08±0.02ab	-	0.02±0.01c	0.04±0.01b	0.10±0.02a
C12:0	0.03±0.01a	0.07±0.02b	0.04±0.01a	0.05±0.01ab	0.06±0.01ab	0.07±0.01b	0.09±0.02b
C13:0	0.08±0.01a	-	0.09±0.02a	0.03±0.01b	0.10±0.02a	-	0.05±0.01b
C14:0	1.31±0.11a	0.29±0.04b	0.49±0.06c	0.81±0.09d	0.58±0.07cd	0.73±0.08d	0.44±0.06c
C15:0	0.39±0.04a	0.19±0.03b	0.23±0.04b	0.30±0.04a	0.23±0.04b	0.68±0.06c	0.37±0.04a
C16:0	**8.41±0.67a**	**9.33±0.83a**	**11.81±1.03b**	**9.72±0.80a**	**10.16±0.91ab**	**9.99±0.82a**	**7.05±0.61c**
C17:0	1.63±0.14a	2.03±0.15b	0.87±0.09c	2.48±0.25b	1.60±0.14a	1.11±0.10d	1.08±0.09d
C18:0	**10.66±0.84a**	**15.18±1.05b**	**10.11±0.79a**	**11.06±0.87a**	**10.30±0.73a**	**11.21±0.91a**	**9.67±0.69a**
C14:1ω9	0.45±0.05a	0.90±0.10b	0.32±0.04a	-	0.15±0.03c	-	0.07±0.02c
C16:1ω7	0.88±0.09a	0.63±0.07b	1.07±0.09c	0.99±0.08c	1.80±0.16d	1.02±0.12c	0.98±0.09c
C18:1ω9	**19.50±1.41a**	**15.20±1.30b**	**20.56±1.52a**	**14.53±1.21b**	**12.68±1.10c**	**13.33±1.11c**	**13.04±1.10c**
C20:1ω9	0.60±0.05a	0.75±0.07a	0.76±0.06a	0.46±0.04b	0.34±0.04b	0.64±0.05a	0.96±0.08c
C22:1ω9	0.03±0.01a	0.05±0.01a	0.12±0.03b	0.14±0.03b	-	0.06±0.01a	0.18±0.03b
C18:2ω6	**26.67±1.72a**	**18.02±1.41b**	**24.42±1.55c**	**22.32±1.49d**	**20.45±1.39e**	**19.77±1.43e**	**27.43±1.66a**
C18:3ω3	**7.36±0.69a**	**1.74±0.15b**	**5.66±0.44c**	**3.96±0.29d**	**8.42±0.71a**	**8.00±0.70a**	**6.60±0.51e**
C20:2ω6	**7.35±0.68a**	**14.93±1.26b**	**8.51±0.75a**	**10.91±0.96c**	**10.72±0.94c**	**11.79±1.01d**	**7.25±0.64a**
C20:3ω6	1.41±0.12a	0.66±0.05b	1.42±0.13a	0.88±0.07b	0.73±0.06b	0.88±0.09b	1.30±0.10a
C20:4ω6	**11.14±1.04a**	**14.37±1.12b**	**10.01±0.94a**	**17.86±1.27c**	**19.51±1.39d**	**17.12±1.25c**	**20.30±1.44d**
C20:5ω3	1.07±0.09a	4.11±0.31b	1.90±0.13a	1.91±0.15a	1.03±0.14a	1.75±0.15a	1.00±0.09a
C22:2ω6	0.09±0.02a	0.22±0.03b	-	0.06±0.01a	-	-	0.05±0.01a
C22:5ω6	0.21±0.03a	0.45±0.04b	1.05±0.08c	0.73±0.05d	0.89±0.08d	0.78±0.06d	1.02±0.10c
C22:6ω3	1.22±0.12a	1.40±0.13a	0.78±0.06b	1.02±0.09a	0.63±0.05b	0.60±0.05b	1.10±0.10a
Σω6 / Σω3	4.86	6.71	5.44	7.66	5.19	4.86	6.59
ΣSFA	22.63±1.50a	27.14±1.63b	23.72±1.56a	24.45±1.58c	23.05±1.49a	23.83±1.51a	18.85±1.41d
ΣMUFA	21.46±1.47a	17.53±1.25b	22.83±1.44a	16.12±1.23b	14.97±1.17c	15.05±1.19c	15.23±1.18c
ΣPUFA	**56.52±2.28a**	**55.90±2.27a**	**53.75±2.20b**	**59.65±2.48c**	**62.38±2.55d**	**60.69±2.52c**	**66.05±2.61e**

Results expressed as percentage of total fatty acids methyl esters.

*Values are means ± S.D (Standard Deviation) for three samples of triplicate analysis.

Means followed by different letters in the same line are significantly different (P** < 0.05) by Tukey's test.

SFA: Saturated Fatty Acids, **MUFA:** Monounsaturated Fatty Acids, **PUFA:** Polyunsaturated Fatty Acids, **Σω6:** Total of omega 6 fatty acids, **Σω3:** Total of omega 3 fatty acid.

Table 2 - Fatty acid profile of main organs total lipid and lipid classes from *Assyriella guttata*.

Fatty Acids	Fatty acid compositions of total lipid from *A. guttata* organs				Fatty acid compositions of lipid classes from *A. guttata*		
	Digestive gland (Mean*±S.D.)**	Cephalopedal (Mean*±S.D.)**	Gonad (Mean*±S.D.)**	Mantle (Mean*±S.D.)**	Total lipid (Mean*±S.D.)**	Neutral lipid (Mean*±S.D.)**	Phospholipid (Mean*±S.D.)**
C10:0	-	0.09±0.02a	0.12±0.03b	0.08±0.02a	0.07±0.02a	0.24±0.04c	-
C12:0	0.70±0.08a	-	0.87±0.10b	0.95±0.10b	0.60±0.07a	0.77±0.08ab	-
C13:0	1.35±0.13a	1.16±0.12a	1.09±0.10a	1.30±0.12a	1.11±0.12a	1.05±0.11a	0.09±0.02b
C14:0	2.22±0.21a	1.47±0.16b	1.10±0.12a	1.78±0.19ab	1.85±0.20ab	2.03±0.21a	0.64±0.08c
C15:0	0.99±0.10a	1.02±0.11a	1.32±0.14a	1.44±0.15a	1.32±0.14a	1.82±0.20b	0.47±0.07c
C16:0	**7.01±0.64a**	**9.01±0.72b**	**9.72±0.78b**	**7.82±0.66ab**	**9.61±0.73b**	**7.78±0.58ab**	**5.92±0.41c**
C17:0	0.13±0.03a	0.33±0.06b	0.16±0.03a	0.18±0.04a	0.06±0.02c	0.09±0.02ac	0.08±0.02ac
C18:0	**8.62±0.64a**	**13.58±0.91b**	**10.11±0.74c**	**15.21±1.04d**	**12.33±0.86bc**	**13.01±0.94b**	**8.76±0.59a**
C14:1ω9	0.35 ±0.05a	0.21±0.04a	0.23 ±0.04a	-	0.25±0.04a	-	0.67±0.06b
C16:1ω7	1.34±0.12a	0.77±0.09b	0.87±0.10b	0.89±0.16b	0.40±0.03c	0.44±0.03c	0.68±0.07bc
C18:1ω9	17.42±1.32a	16.32±1.25a	19.65±1.49b	15.35±1.21c	15.06±1.21c	16.03±1.30a	16.02±1.29a
C20:1ω9	0.60±0.07a	0.75±0.08a	0.67±0.06a	0.16±0.03b	0.43±0.05c	0.31±0.05c	0.69±0.08a
C22:1ω9	-	-	0.21±0.04a	0.04±0.01b	-	0.09±0.02b	0.08±0.02b
C18:2ω6	**28.12±1.75a**	**21.60±1.48b**	**24.24±1.55c**	**20.23±1.45b**	**18.54±1.38d**	**17.07±1.35d**	**22.34±1.65a**
C18:3ω3	**4.06±0.31a**	**2.43±0.19b**	**4.76±0.44a**	**2.90±0.32a**	**6.24±0.51c**	**7.01±0.57c**	**10.70±0.41ac**
C20:2ω6	**6.14±0.53a**	**13.42±1.16b**	**9.15±1.02c**	**8.81±0.96c**	**9.22±1.04c**	**10.99±1.16bc**	**8.52±0.84c**
C20:3ω6	0.71±0.62a	0.61±0.56a	0.24±0.03b	0.19±0.03b	0.13±0.03c	0.08±0.02c	0.80±0.09a
C20:4ω6	**15.09±1.29a**	**13.73±1.12b**	**11.05±1.10c**	**15.68±1.25a**	**16.58±1.30d**	**15.71±1.28a**	**16.13±1.33ad**
C20:5ω3	4.53±0.39a	2.16±0.21b	2.45±0.23b	4.85±0.35c	3.75±0.38c	3.54±0.34c	4.76±0.41a
C22:2ω6	0.14±0.03a	0.02±0.01b	0.04±0.01b	-	-	0.59 ±0.05c	0.08±0.02ab
C22:5ω6	0.15±0.03a	0.13±0.03a	0.55±0.06b	0.37±0.04c	0.98±0.10d	0.67±0.08b	0.82±0.09d
C22:6ω3	1.23±0.13a	1.24±0.12a	1.38±0.14a	1.60±0.16a	1.36±0.15a	1.00±0.10a	2.08±0.24b
Σω6 / Σω3	**5.13**	**8.49**	**5.29**	**4.84**	**4.00**	**3.91**	**2.28**
ΣSFA	21.02±1.51a	26.66±1.62b	24.49±1.56ab	28.76±1.74c	26.95±1.67b	26.79±1.59b	15.96±1.32c
ΣMUFA	19.71±1.37a	18.05±1.33a	21.63±1.42b	16.44±1.28c	16.14±1.22c	16.87±1.30c	18.14±1.33a
ΣPUFA	60.17±2.49a	55.34±2.25b	53.86±2.15c	54.63±2.18b	56.80±2.39d	56.66±2.36d	66.23±2.51e

Results expressed as percentage of total fatty acids methyl esters.
*Values are means ± S.D (Standard Deviation) for three samples of triplicate analysis.
Means followed by different letters in the same line are significantly different (P** < 0.05) by Tukey's test.
SFA: Saturated Fatty Acids, **MUFA:** Monounsaturated Fatty Acids, **PUFA:** Polyunsaturated Fatty Acids, **Σω6:** Total of omega 6 fatty acids, **Σω3:** Total of omega 3 fatty acids.

and 2.28 in the phospholipid of *A. guttata* (Table 2). These high levels of Σω6/Σω3 were mostly on account of higher concentration of C18:2ω6 and C20:4ω6.

The most notable result was significantly high level of ΣPUFA (total polyunsaturated fatty acids) and low level of ΣSFA (total saturated fatty acids) and ΣMUFA (total monounsaturated fatty acids) in all organs and lipid classes. Among the organs, the maximum level of ΣPUFA was obtained in the mantle (59.65%) of *A. escheriana* (Table 1) and in the digestive gland (60.17%) of *A. guttata* (Table 2). On the other hand, maximum level of ΣSFA was detected in the cephalopedal (27.14%) of *A. escheriana* (Table 1) and in the mantle (28.76%) of *A. guttata* (Table 2). The level of ΣMUFA in all treatments was found significantly lower than ΣPUFA and ΣSFA. It ranged from 16.12% to 21.46% in *A. escheriana* and 16.44% to 21.63% in *A. guttata.* It was noteworthy that, the amount of ΣPUFA was significantly high; 66.05% in the phospholipid, 62.38% in the total lipid and 60.69% in the neutral lipid of *A. escheriana* (Table 1) and 66.23% in the phospholipid, 56.80% in the total lipid and 56.66% in the neutral lipid of *A. guttata* (Table 2).

DISCUSSION

The significance of fatty acids drives from their role as fuel to provide metabolic energy, their usage for storage products, eicosanoids, physiological activities, structural components such as membrane lipids particularly phospholipids and sterol esters. Most importantly, they fulfill a structural role and are also very important intermediates in cell physiology, formation of prostaglandins and other eicosanoids from ω3 and ω6 fatty acids (STANLEY-SAMUELSON, 1994). The importance of specific fatty acids as dilatory compounds for animals is partly because of almost all animals to introduce second or third double bond into fatty acids to synthesize polyunsaturated fatty acids (BEENAKKERS *et al.*, 1985).

The seriousness of fatty acids was mostly emphasized for freshwater molluscs. However, lipid data correlated with nutritional, physiologi-

cal, structural and environmental factors in terrestrial snails is notably limited in literatures and relatively little is known about terrestrial snails' fatty acid composition, particularly on their organs. There are only a few studies regarding of fatty acids of edible, nonedible snails and land slugs such as *H. aspersa* (ÇAĞILTAY *et al.*, 2011), *H. aspersa maxima* (MILINSK *et al.*, 2006), *H. pomatia* (ÖZOĞUL *et al.*, 2005), *E. vermiculata* (STAVRAKAKIS *et al.*, 1989), *Helix sp., Haplotrema sportella, Vespericola columbiana, Arion ater, Limax maximus, Prophysaon andersoni* (ZHU *et al.*, 1994). Furthermore, only few studies are present on fatty acid distribution of mollusc organs and tissues. *Macoma balthica* (WENNE and POLAK, 1989), *Telescopium telescopium* (RAKSHIT *et al.*, 1997), *Argopecten purpuratus* (CAERS *et al.*, 1999), *Bellamya bengalensis*, *Pila globosa* (MISRA *et al.*, 2002), *Unio elongatulus* (EKİN and BAŞHAN, 2010), *Corbicula fluminalis* (EKİN, 2012), *H. lucorum* (EKİN 2014) are some of known mollusc species, tissue and organs studied.

Qualitatively, fatty acid profiles of two species were similar. Similarity of the fatty acid content of both species is not surprising. Because they are close relatives and derived from the same origin. However, quantitative differences in the fatty acid profile were likely due to environmental, nutritional and physiological effects.

Generally, molluscs are well known to contain C16:0, C18:0, C18:1ω9, C18:2ω6, C18:3ω3 and C20:4ω6 as major fatty acids. These fatty acids have previously reported in most of the mollusc species and explored for their potential use in food chain studies. They were identified as predominant components in *Theodoxus jordani, Melanoides tuberculata, Pyrigula barroisi, Melanopsis praemorsum* freshwater snails (GO *et al.*, 2002); in *Helix sp. H. sportella, V. columbiana* (ZHU *et al.*, 1994), *H. aspersa* (ÇAĞILTAY *et al.*, 2011), *H. pomatia* (ÖZOĞUL *et al.*, 2004), *H. lucorum* (EKİN, 2014) land snails; in *T. telescopium* marine snail (RAKSHIT *et al.*, 1997); in *U. elongatulus* (EKİN and BAŞHAN, 2010), *C. fluminalis* (EKİN, 2012), *B. bengalensis*, *P. globosa* (MISRA *et al.*, 2002), *A. purpuratus* mussels (CAERS *et al.*, 1999); in *P. andersoni, A. ater, L. maximus* (ZHU *et al.*, 1994) slugs. As highlighted above, *A. escheriana* and *A. guttata* also contained high amount of C16:0, C18:0, C18:1ω9, C18:2ω6, C18:3ω3 and C20:4ω6, concentrated in the organs and fractions. In previous studies, it was stated that C20:4ω6 is more characteristic of sea urchins and starfish, C20:5ω3 is characteristic of invertebrates that feed on single-celled algae and occurs in almost all classes, additionaly C22:6ω3 is more characteristic of fish and crustacea (SINANOGLOU and MINIADIS-MEIMAROGLOU, 1998). Notably, C16:0, C18:0 and C18:1ω9 can be found in most of the animal tissues and very common among fatty acids.

PUFAs may be further modified to form prostaglandins that are directly involved in regulation of reproduction, renal function, ion regulation as known from mollusc species, (STANLEY-SAMUELSON, 1987). It is stated that egg production in freshwater snail *Helisoma durgi* was stimulated by prostaglandins (KUNIGELIS and SALEUDDIN, 1986). C20:4ω6 precursors of prostaglandins was found to be 15.09% in the digestive gland, 13.73% in the cephalopedal, 11.05% in the gonad, 15.68% in the mantle of *A. guttata* and 11.14% in the digestive gland, 14.37% in the cephalopedal, 10.01% in the gonad, 17.86% in the mantle of *A. escheriana*. Probably, this high value is related to reproduction and other physiological activities of the snails. In animal cells prostaglandins precursor C20:4ω6 is mostly obtained from phospholipid main source of PUFA. To remember, the phospholipids of the snails contained high level of C20:4ω6, 20.30% in *A. escheriana* and 16.13% in *A. guttata*. As a matter of fact, ΣPUFA levels in the phospholipid were detected much higher than other lipid fractions and organs both in *A. escheriana* and *A. guttata*. In the phospholipid of *A. escheriana* and *A. guttata*, ΣPUFA levels were presented to be 66.05% and 66.23%, respectively. Recognizing that snail species in this study are herbivores, therefore containing high proportion of PUFA was expected result. Because plant based diet is containing much more PUFA than flesh based diet.

In the present study, ΣPUFA levels were always found to be higher than ΣSFA and ΣMUFA. This finding was in agreement with garden snail *H. aspersa* stating that PUFA was most abundant fatty acids (ÇAĞILTAY *et al.*, 2011). It was also declared that C18:2ω6, C20:4ω6, C18:3ω3 and C20:5ω3 were the dominant fatty acids. Snails frequently feed on decaying plant materials to avoid high concentration of deterrent or toxic plant metabolites (SPEISER *et al.*, 1992). Aging of plant material results in a decrease of its PUFA content (KIS *et al.*, 1998), suggesting that snails eating old plant material may suffer from a shortage of PUFA. Therefore, it can be said that natural food sources vary seasonally in the composition of ingredients (WACKER, 2005). In the present study, the snails were collected in spring season and they mostly fed on fresh plant materials. Maximization of PUFA levels in all organs and fractions were probably because of fresh plant diets. The snails' mating activities are significantly reduced when snails were fed the PUFA-deficient diet. It is stated that PUFA played important role in reproductive allocation (WACKER, 2005).

A. escheriana species were collected from woodland, whereas *A. guttata* species were collected from stony and rocky region of city walls which is containing decaying organic matter, garbage, sediments, grass, shrubs and etc. Decaying organic matter containing places mostly shelters bacteria, protozoa, mold, invertebrates

and other microorganisms. C13:0, C14:0, C15:0, C17:0 and other short-chain saturated fatty acids are common in bacteria (WACKER, 2005). In the analyses, it was observed that *A. guttata* contained slightly higher amount of short-chain fatty acids than *A. escheriana*. C14:0 ranged from 1.10% to 2.22% in *A. guttata* organs and 0.49% to 1.31% in *A. escheriana* organs. C15:0 varied from 0.99% to 1.44% in *A. guttata* and from 0.19% to 0.39% in *A. escheriana*. Probably, it was stem from habitats of *A. guttata* which is suitable for living for microorganisms.

In all fractions and organs, C18:2ω6 essential fatty acid was the main components followed by C18:1ω9 and C20:4ω6. The highest concentration of the fatty acid was found to be 28.12% and 26.67% in the digestive gland of *A. guttata* and *A. escheriana*, respectively. Among the snails' lipid fractions, the phospholipid contained 27.43% in *A. escheriana* and 22.34% in *A. guttata* of C18:2ω6 (Table 1, 2). On the contrary, this fatty acid was found in low level in *T. telescopium* freshwater snail's organs; 2.5% in the digestive gland, 4.3% in mantle and 4.95% in the cephalopedal (RAKSHIT *et al.*, 1997). On the other hand, in edible snail *H. aspersa maxima*, C18:2ω6 was found rather a lot, between 44.79%-51.19% (MILINSK *et al.*, 2006) and this fatty acid was also found good amount in edible snail *H. lucorum* (EKİN, 2014). Most likely, these different data stem from the requirement of the fatty acid for snail species. This essential fatty acid plays central role in production of other PUFA and most animals cannot synthesize it, for this reason they are dependent on taking it from their diets.

An interesting fact was that snails had C20:2ω6 with high concentrations varying from 6.14% to 13.42% in *A. guttata* and from 7.25% to 14.93% in *A. escheriana*. In particular, the highest level of the fatty acid was detected in the cephalopedal of both species. In some studies, it is stated that snail cephalopedal served as a storage organ (JOHNS *et al.*, 1979), probably, the cephalopedal stored this fatty acid for further metabolic activities.

In comparison with *A. escheriana* and *A. guttata*, it was observed some strange results in *T. telescopium* snail, for instance C18:3ω3 was not detected in the digestive gland and mantle, but it was found 10.7% in the cephalopedal tissue as well as C16:1ω7 was found to be 11.3% in the digestive gland, 6.1% in the mantle, 4.9% in the cephalopedal (RAKSHIT *et al.*, 1997). In *A. guttata* and *A. escheriana*, C16:1ω7 was found at low concentrations, did not exceed 1.80%. However, C18:3ω3 was presented 7.36% in the digestive gland, 3.96% in the mantle of *A. escheriana* and 4.06% in the digestive gland, 2.90% in the mantle of *A. guttata*. For *A. escheriana,* the highest proportion of C18:3ω3 was found to be 8.42% in the total lipid in comparison with 10.70% in the phospholipid of *A. guttata*. C18:3ω3 was another essential fatty acid and its content was expected to be high in the phospholipid fractions, because phospholipid contains much more PUFA than MUFA and SFA. It is also noteworthy that, the content of fatty acids may differ from year to year, season to season, and depend on the nutrition of organism. Above all, the distribution of an organism is mostly influenced by many factors including temperature, reproduction season, growth, nutrient availability, genetic, physiology and etc.

In the treatments, neutral lipid and total lipid fatty acid distribution are more similar to each other than phospholipids. In particular, ΣSFA, ΣMUFA and ΣPUFA contents in neutral lipid and total lipid were detected so close to each other in both species. This kind of determination is very normal, because neutral lipids and total lipids are structurally and contently similar.

Both ω3 and ω6 fatty acids are important components of biomembranes and are precursors to many other substances in organisms. Researches indicate that omega fatty acids especially ω3 fatty acids reduce inflammation and may help lower risk of chronic diseases such as heart disease, cancer, and arthritis. The ratio of Σω6/Σω3 is usually received to be useful indicator for comparing nutritional values of the samples. In *A. escheriana*, the highest value of Σω6/Σω3 was in the phospholipid (6.59), whereas the lowest value was in the neutral lipid and digestive gland (4.86). On the other hand, in *A. guttata* the highest value of Σω6/Σω3 was in the cephalopedal (8.49), the lowest value was in the phospholipid (2.28). This wide difference between snails' phospholipids fractions was due to the high proportion of C18:2ω6 in *A. escheriana.* In agreement with our findings, Σω6/Σω3 ratio was also found to be high in *A. ater, L. maximus, P. andersoni,* slugs and *V. columbiana, H. sp. H. sportella* (ZHU *et al.*, 1994) and in *H. lucorum* land snails (EKİN, 2014). But, in marine molluscs, percentage of Σω6 was found to be lower than Σω3 (ABAD *et al.*, 1995; PAZOS *et al.*, 2003).

Eventually, the results showed that species were rich in PUFA, totally always over 50% in all analyses and maximization of C16:0, C18:0, C18:1ω9, C18:2ω6, C18:3ω3, C20:2ω6 and C20:4ω6 were observed. Particularly, the organs and lipid fractions of both snails contained good amount of essential fatty acid, C18:2ω6 taking role in the synthesis of other fatty acids. Moreover, Σω6/Σω3 and ΣPUFA/ΣSFA+ ΣMUFA ratios were found in good range. Herewith, the results can be important guide for further investigation on nutritional, physiological, biochemical and taxonomic studies of molluscs. Commercially some important edible snails *Cryptomphalus aspersus (H. aspersa), H. asemnis, H. cincta, H. lucorum, T. pisana, E. vermiculata* and *C. apertus* dwell in Turkish territories (YILDIRIM, 2004). Although *A. escheriana* and *A. guttata* are not edible snails; however they are very common in

the southeastern Anatolia region. It should not be forgotten, snails collected from the wild environment may accommodate poisonous chemicals, heavy metals, drugs, alkaloids and agricultural chemicals. Perhaps, *A. escheriana* and *A. guttata* land snails will be used as edible after the pathological and biochemical detailed studies in the future; however we can offer no adequate explanation about edibility at present.

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PAPER

COFFEE BEAN MYCO-CONTAMINANTS AND OXALIC ACID PRODUCING *ASPERGILLUS NIGER*

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ABSTRACT

Coffee bean-contaminating fungi were determined in random samples collected in Riyadh, Kingdom of Saudi Arabia, using the direct plating technique. Forty-five samples were examined and 12 fungal species belonging to 5 genera were isolated. *Aspergillus niger* was the most widely distributed and most frequently isolated fungus (86.67%). The ability of the predominant fungus, *A. niger*, to produce oxalic acid was evaluated using high-performance liquid chromatography. About 50% of the tested *A. niger* isolates produced oxalic acid; the amount produced was in the range of 90–550 ppm of oxalic acid. Because *A. niger* was the predominant and most widely distributed toxigenic fungus in the examined samples, more efforts should be directed to minimize the risk of oxalic acid contamination of commoditized coffee beans in the Kingdom of Saudi Arabia.

- Keywords: mycotoxins, seed-borne, *Coffea arabica* L., high-performance liquid chromatography, metabolites -

INTRODUCTION

Coffea arabica L. is considered to have the best flavour and quality, and coffee is one of the most popular beverages consumed in countries of the Arabian Peninsula. However, coffee beans may become poisonous because of contamination with mycotoxigenic fungi that may occur throughout all pre and/or post-harvest stages (BATISTA *et al.*, 2003; NOONIM *et al.*, 2008). Several fungal genera were found to contaminate coffee beans at each stage, from the farmer to the consumer (VEGA *et al.*, 2008; BATISTA *et al.*, 2009; VILELA *et al.*, 2010).

The main mycotoxigenic fungal genera found to be associated with commoditized coffee beans belong to the genera *Aspergillus, Penicillium,* and *Fusarium* (PARDO *et al.*, 2004; BOKHARI, 2007; LEONG *et al.*, 2007). These fungi not only affect the quality of coffee beans but also produce toxic secondary metabolites that are harmful to the consumers (BERNNETT and KLICH, 2003, VILELA *et al.*, 2010).

One of the most important mycotoxigenic and organotoxic metabolite-producing contaminant is *Aspergillus niger*, which is considered a class 1 containment agent (U.S. NPA, 1977; SCHUSTER *et al.*, 2002; ILIC *et al.,* 2007). This fungus is responsible for the *in vitro* and/or *in vivo* secretion of the nephrotoxic compound oxalic acid (MANDAL *et al.*, 2005; MAGNOLI *et al.,* 2008). The toxicity of oxalic acid is due to the deposition of calcium-oxalate complexes in renal tubules causing renal failure; in humans, the minimum lethal dose of orally ingested oxalic acid is 600 mg/kg (SAFETY OFFICER IN PHYSICAL CHEMISTRY, 2005; BOTHA *et al.*, 2009).

The purpose of this study was to examine coffee beans commoditized in Saudi Arabian markets for the presence of toxigenic fungi. Furthermore, we evaluated the production of oxalic acid by the isolated *A. niger* strains.

MATERIALS AND METHODS

Mycological analysis

Mycotoxin-producing fungi were determined in 45 random samples of coffee beans collected in Riyadh City, Kingdom of Saudi Arabia. For the isolation of fungi associated with coffee beans, the direct plating technique was applied using potato dextrose agar (PDA) medium. Beans were plated directly onto PDA medium after surface disinfection by 5% sodium hypochlorite solution. The plates were incubated for 5–7 days at 25°C and the growing fungal colonies were purified. The obtained fungal isolates were then identified to the species levels at the Mycological Centre, Assiut University, Egypt.

Table 1 - Distribution of isolated fungi in coffee bean samples.

Fungi	Distribution %
1. *A. alternata*	8.89
2. *A. f.columnaris*	17.78
3. *A. niger*	86.67
4. *A. ochraceus*	4.44
5. *A. terreus*	2.22
6. *Nigrospora* sp.	2.22
7. *P. brevicompactum*	8.89
8. *P. corylophilum*	6.67
9. *P. variabile*	2.22
10. *P. suchlasporia*	2.22
11. *R. stolonifer*	44.44
12.*T. flavus*	2.22

Mycotoxigenicity

The oxalic acid production of the tested *A. niger* isolates was determined using high-performance liquid chromatography. Briefly, 50 mL of Czapek-Dox broth medium was placed in 250-mL Erlenmeyer flasks and inoculated with *A. niger*. Inoculated media were incubated in triplicates at 30°C on an orbital shaker maintained at 215 rpm for 7 days. The culture supernatants were then analysed for their oxalic acid content. Separation of oxalic acid was carried out using a CLC-C825 CM cation exchange column. The mobile phase was 90% H_2O and 10% CH_3OH. The flow rate was 1 mL/min and the temperature was 35°C (GHORBANI *et al.,* 2007).

Statistical analysis

The SPSS-16 statistical package was used for the analysis of variance and correlation and cluster analyses. Cluster analysis was performed by the unweighted pair-group method with arithmetic averages. Data were processed by root square transformation of % frequencies + 0.5 to normalize and stabilize the variance before subsequent analyses were carried out. Means were statistically compared using the least significant difference test.

Table 2 - ANOVA of the isolation frequencies of mycotoxigenic fungi in coffee beans.

Source of variance	Df	M S	F	Sig.	R.C.*
Samples	44	2.070	1.558	0.012	0.35
Fungi	11	575.637	433.265	0.000	98.60
Samples x Fungi	484	6.086	4.580	0.000	1.04
Error	1620	1.329			

*Relative contribution.

Table 3 - Comparison of the isolation frequencies of fungi isolated from coffee beans.

S.	F1		F2		F3		F4		F5		F6		F7		F8		F9		F10		F11		F12	
No	Freq.	Trans.	Freq.	Trans.	Freq.	Trans.	Freq.	Trans.	Freq.	Trans.	Freq.	Trans.	Freq.	Trans.	Freq.	Trans.	Freq.	Trans.	Freq.	Trans.	Freq.	Trans.	Freq.	Trans.
1	0.00	0.71	25.00	3.04	50.00	5.37	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71
2	0.00	0.71	25.00	3.04	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71
3	7.30	2.29	0.00	0.71	36.46	4.63	0.00	0.71	0.00	0.71	0.00	0.71	0.00	1.79	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71
4	0.00	0.71	0.00	0.71	62.50	7.03	0.00	0.71	0.00	0.71	0.00	0.71	6.25	1.79	0.00	0.71	0.00	0.71	0.00	0.71	6.25	1.79	0.00	0.71
5	23.75	4.35	23.75	4.35	46.25	6.76	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	6.25	1.79	0.00	0.71
6	0.00	0.71	0.00	0.71	100.00	10.03	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71
7	50.00	5.37	25.00	3.04	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71
8	0.00	0.71	5.00	1.66	95.00	9.76	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71
9	0.00	0.71	3.57	1.49	42.86	4.98	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	3.57	1.49	0.00	0.71
10	0.00	0.71	0.00	0.71	20.09	3.54	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	6.70	2.22	23.21	3.78
11	0.00	0.71	0.00	0.71	70.83	7.48	0.00	0.71	0.00	0.71	0.00	0.71	4.18	1.57	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71
12	0.00	0.71	0.00	0.71	64.28	8.01	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	25.00	4.38	10.72	2.66	0.00	0.71	3.57	1.49	0.00	0.71
13	0.00	0.71	0.00	0.71	100.00	10.03	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71
14	0.00	0.71	0.00	0.71	10.00	2.12	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	15.00	2.48	0.00	0.71
15	0.00	0.71	0.00	0.71	83.33	9.16	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	16.70	4.15	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71
16	0.00	0.71	13.44	2.97	48.21	6.19	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	13.39	2.96	0.00	0.71
17	0.00	0.71	0.00	0.71	37.50	4.64	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	37.50	4.64	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71
18	0.00	0.71	3.57	1.49	88.69	9.42	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	7.75	2.35	0.00	0.71
19	0.00	0.71	0.00	0.71	100.00	10.03	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71
20	0.00	0.71	0.00	0.71	91.67	9.59	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	8.35	2.43	0.00	0.71
21	0.00	0.71	0.00	0.71	95.83	9.81	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	4.18	1.57	0.00	0.71
22	0.00	0.71	0.00	0.71	100.00	10.03	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71
23	0.00	0.71	0.00	0.71	60.00	7.65	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	27.50	4.07	12.50	2.31	0.00	0.71
24	0.00	0.71	0.00	0.71	62.50	7.82	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	37.50	5.49	0.00	0.71
25	0.00	0.71	0.00	0.71	100.00	10.03	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71
26	0.00	0.71	0.00	0.71	100.00	10.03	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71
27	0.00	0.71	0.00	0.71	48.21	6.19	0.00	0.71	0.00	0.71	0.00	0.71	23.21	4.26	0.00	0.71	0.00	0.71	0.00	0.71	3.57	1.49	0.00	0.71
28	0.00	0.71	0.00	0.71	70.83	7.48	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	4.18	1.57	0.00	0.71
29	0.00	0.71	0.00	0.71	100.00	10.03	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71
30	0.00	0.71	0.00	0.71	42.86	5.85	16.07	3.09	16.07	3.09	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71
31	0.00	0.71	0.00	0.71	76.19	8.69	0.00	0.71	0.00	0.71	23.82	4.34	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71
32	0.00	0.71	0.00	0.71	100.00	10.03	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71
33	0.00	0.71	0.00	0.71	100.00	10.03	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71
34	0.00	0.71	0.00	0.71	100.00	10.03	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71
35	0.00	0.71	0.00	0.71	95.83	9.81	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	11.32	2.74	0.00	0.71
36	0.00	0.71	0.00	0.71	0.00	0.71	50.00	5.37	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71
37	0.00	0.71	0.00	0.71	38.69	4.76	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	11.32	2.74	0.00	0.71
38	75.00	7.70	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71
39	0.00	0.71	0.00	0.71	100.00	10.03	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71
40	0.00	0.71	0.00	0.71	95.83	9.81	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	4.18	1.57	0.00	0.71
41	0.00	0.71	0.00	0.71	45.00	5.10	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	5.00	1.66	0.00	0.71
42	0.00	0.71	0.00	0.71	41.67	4.93	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	8.35	2.43	0.00	0.71
43	0.00	0.71	0.00	0.71	100.00	10.03	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71
44	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	75.00	7.70	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71
45	0.00	0.71	0.00	0.71	75.00	8.57	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	25.00	3.91	0.00	0.71

F1-F12= *A. alternate*, *A. f. columnaris*, *A. niger*, *A. ochraceus*, *A. terreus*, *Nigrospora* sp., *P. brevicompactum*, *P. corylophilum*, *P. variabile*, *P. suchlasporia*, *R. stolonifer* and *T. flavus* respectively.
Freq. = Frequency of fungal isolation.
Trans. = transformed data into root square of % values + 0.5.

Table 4 - Correlation among frequencies of fungi isolated from coffee beans.

Fungi	1	2	3	4	5	6	7	8	9	10	11	12
1. *A. alternata*	1	0.365*	-0.427**	-0.058	-0.042	-0.042	-0.048	-0.075	-0.042	-0.042	-0.133	-0.042
2. *A. f.columnaris*		1	-0.293	-0.084	-0.062	-0.062	-0.107	-0.109	-0.062	-0.062	-0.003	-0.062
3. *A. niger*			1	-0.306*	-0.060	0.078	-0.310*	0.007	0.045	0.027	-0.005	-0.172
4. *A. ochraceus*				1	0.439**	-0.031	-0.053	-0.055	-0.031	-0.031	-0.144	-0.031
5. *A. terreus*					1	-0.023	-0.039	-0.040	-0.023	-0.023	-0.106	-0.023
6. *Nigrospora* sp.						1	-0.039	-0.040	-0.023	-0.023	-0.106	-0.023
7. *P. brevicompactum*							1	-0.069	-0.039	-0.039	-0.108	-0.039
8. *P. corylophilum*								1	0.562**	-0.040	-0.119	-0.040
9. *P. variabile*									1	-0.023	0.010	-0.023
10. *P. suchlasporia*										1	0.130	-0.023
11. *R. stolonifer*											1	0.116
12. *T.flavus*												1

RESULTS AND DISCUSSION

The mycological examination of the coffee bean samples (Table 1) revealed the presence of 12 fungal species that belonged to 5 genera (SILVA *et al.*, 2008; BATISTA *et al.*, 2009). The predominance of *A. niger* in the tested coffee bean samples was in agreement with previously reported data (URBANO *et al.*, 2001; NEHAD et al., 2007; NOONIM *et al.* 2008). The main mycotoxigenic fungal genera, i.e. *Aspergillus* and *Penicillium*, have frequently been associated with coffee beans (JOOSTEN *et al.*, 2001; PARDO *et al.*, 2004; BOKHARI, 2007; LEONG *et al.*, 2007).

ANOVA indicated that the effects of fungus, sample, and fungus × sample interaction were highly significant sources of variation in the fungal isolation frequencies. Compared to the other sources, fungus was the most important source of variation (Table 2).

The isolation frequencies varied according to the tested samples. For example, *Alternaria alternata* and *A. flavus* var. *columnaris* showed equal isolation frequencies in sample No. 5, but the former was significantly more frequently isolated from sample No. 7 than the latter. Although *A. flavus* var. *columnaris* was isolated from samples No. 9 and 18 at equal frequencies, the frequency at which *A. niger* was isolated from these 2 samples varied significantly. Whereas *Penicillium brevicompactum* and *Rhizopus stolonifer* were isolated from sample No. 4 at equal frequencies, *P. brevicompactum* was significantly more frequently isolated than *R. stolonifer* (Table 3). However, the contamination of coffee beans with mycotoxigenic fungal genera (NOONIM *et al.*, 2008; VILELA *et al.*, 2010) may start in the field and subsequently extend to storages and markets, in particular under conducive conditions (JESTOI *et al.*, 2004; JUAN *et al.*, 2008; PATERSON and LIMA, 2010).

Correlation analysis showed that the fungal isolation frequencies were positively and/or negatively correlated with each other (Table 4). Some correlations between isolated fungal species and their isolation frequencies were significant or highly significant. Highly significant positive correlations were noted among *Asper-*

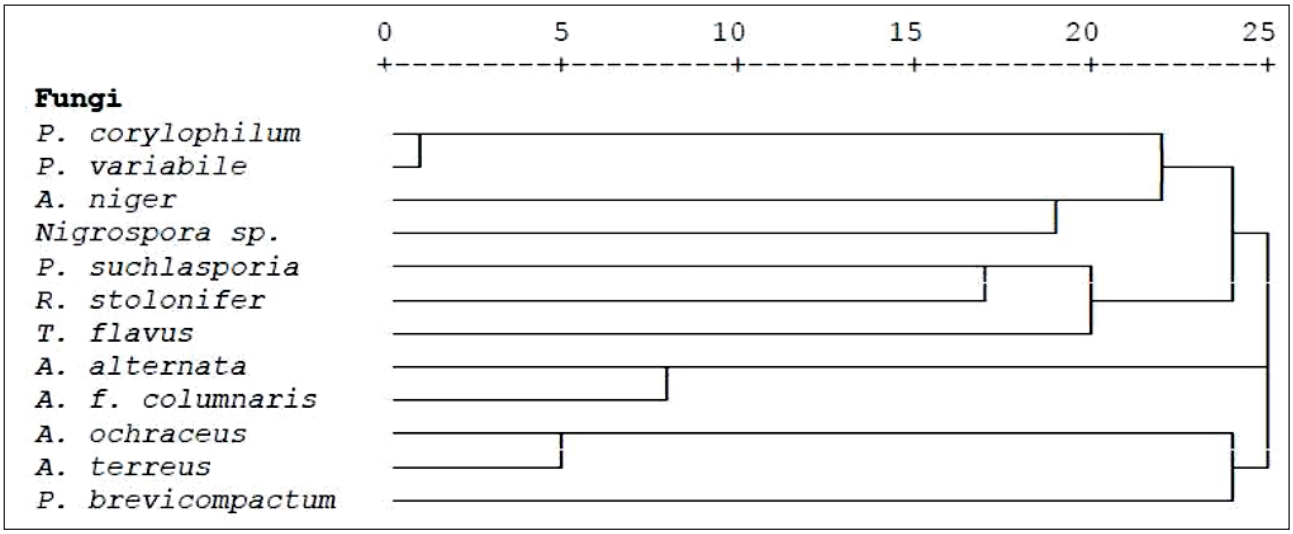


Fig. 1 - Phenogram based on average linkage cluster analysis of frequencies of fungi recovered from coffee beans.

Table 5 - Production of oxalic acid by *A. niger* isolates.

A. niger isolates	Oxalic acid (ppm)
***A.n.*1**	00.00
***A.n.*2**	200.00
***A.n.*3**	150.00
***A.n.*4**	00.00
***A.n.*5**	00.00
***A.n.*6**	00.00
***A.n.*7**	00.00
***A.n.*8**	550.00
***A.n.*9**	100.00
***A.n.*10**	90.00

gillus ochraceus and *Aspergillus terreus* as well as among *Penicillium corylophilum* and *Penicillium variabile*. Furthermore, a highly significant negative correlation was found between *A. alternata* and *A. niger*. The highly significant positive correlations between some isolated fungi found in this study imply that similar colonizing conditions are provided by the coffee beans for those fungi; the opposite conclusion can be drawn for negatively correlated fungi (YASSIN *et al.,* 2013).

The phenogram (Fig. 1) illustrates the cluster analysis of fungal isolation frequencies (%) on the basis of their distribution patterns using all samples. Three distinct groups of isolated fungi are shown; each of them is divided into subgroups. Strongly and positively associated fungi were grouped in the same cluster. The grouping pattern of the isolated fungi in the cluster analysis suggests the potential existence of sample- (environment-) related fungal groups (YASSIN *et al.,* 2011).

With regard to the mycotoxigenicity, 50% of the tested *A. niger* isolates were able to produce oxalic acid. Other tested isolates failed to produce any detectable amounts of oxalic acid (Table 5).

Aspergillus species are well known to be responsible for the secretion of different toxic metabolites (AL-ABDALALL, 2009; YASSIN *et al.,* 2010; EL-SAMAWATY *et al.,* 2011). Moreover, the organotoxic metabolite oxalic acid has also been shown to be produced by *A. niger* isolated from commoditized agricultural products (BAHKALI *et al.*, 2013; YASSIN *et al.,* 2013). Accumulation of such compounds produced by mycotoxigenic fungi could affect the quality of coffee (ARRUSA *et al.*, 2005; KUMAR *et al.*, 2008) and harm human consumers (PALANEE *et al.*, 2001; DAVID *et al.*, 2005).

CONCLUSIONS

The coffee beans that were examined in this study were found to be contaminated with many fungal genera that might affect the quality of coffee. *A. niger* was the most widely distributed and most frequently isolated fungus (86.67%). Furthermore, 50% of the tested *A. niger* isolates were mycotoxigenic. They were able to produce oxalic acid in the range of 90–550 ppm. Because *A. niger* was the predominant and most widely distributed fungus in the examined coffee bean samples, more efforts should be directed to minimize the risk of oxalic acid contamination of commoditized coffee beans in KSA.

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SHORT COMMUNICATION

EFFECTS OF NUTRITIONAL ELEMENTS LEVEL ON NUTRITIONAL CHARACTERS AND PHYTOCHEMISTRY OF STRAWBERRY IN HYDROCULTURE

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ABSTRACT

This study was conducted to evaluate the impacts of nutrient elements on phytochemistry characters and qualities of strawberry in soilless culture system. The experiment was carried out in a factorial experiment based on randomized complete design with three replications. Treatments consisted of 6 groups of strawberry growing on soilless medium made of perlite and coco peat that were treated with different ratio of nutrient solutions. According to the results modified nutrient improved fruit nutritional characters but it was not unique. TSS_S, TA, vitamin C and pH often were increased by increasing nutritional elements but anthocyanin was decreased by increasing some nutritional elements.

- Keywords: hydroculture, nutritional elements, nutrient solution, strawberry -

INTRODUCTION

Strawberry (*Fragaria* × *ananassa* Duch.) is one of the most commonly consumed berries, both in fresh and processed forms such as jams, yoghurts, desserts or juices. The relevant nutritional value of strawberry fruits has been remarkably correlated (HANNUM 2004) with the high level of micronutrients such as minerals, vitamin C and folate which are essential for health, and, more recently, to the high levels and different phytochemical constituents (TULIPANI *et al.*, 2009).

Since strawberry is an adaptable plant, and its fruit can be obtained almost in all seasons, its growing areas are being widely expanded in the world. On the other hand since the fruits can be obtained early in the season, when there are no fresh fruits in the markets, its marketability is high. Another important aspect is that it can bring back the investment in a short period; therefore it is suitable for family hobby (ILGM, 2006).

Because of increased demand for more products with high quality and offseason, greenhouse production is increasing. Soilless media are popularly used in greenhouse crop production because they are relatively lightweight, free from diseases, readily available, more uniform and more suitable for growing in containers than soil (YUAN *et al.*, 1996). The choice of the medium should be based on physical characteristics as well as availability and cost (LIETEN *et al.*, 2004; TABATABAEI and MOHAMMADREZAEI, 2006).

Hydroponics is a method of growing plants using mineral nutrient solutions without soil. In this method, growing substrate may be an organic material (peat moss, shredded bark, foam or other organic materials) or an inorganic material such as sand, perlite, vermiculite and rock wool. To support and anchor the root system; plant nutrition is provided through a nutrient solution circulating in the substrate.

One of the advantages of plant nutrition in soilless culture derives from the possibility of precise control of nutrient elements (JOHNSTON *et al.*, 2010) which is not possible with soil substrate (ARZANI, 2007). Coco peat is the best medium for growing summer crops, flowers and strawberry because it has high porosity, and it has a good capacity of holding water and nutrients. Porosity in perlite provides good air exchanges and soil watering and it improves soil aeration promoting the growth of the root system (NOGUERA *et al.*, 2003). Perlite has rich inorganic materials such as iron, sodium, calcium and rare organic materials, since it is based an organic feature (DJEDIDI, 1999; EBRAHIMI *et al.*, 2012).

Strawberry requires high amounts of potassium because this element is a major component of the fruit and has a positive correlation with fruits size, color and acidity (BEHNAMIYAN and MASIHA, 2002).

The research was designed and performed to evaluate the effect of different concentrations of nutrients elements via nutrient solution in soilless system composed of 50% perlite and 50% coco peat on phytochemical and quality characters of greenhouse grown strawberries cultivar Gaviata (AMERI *et al.*, 2012).

MATERIAL AND METHOD

Plant material and growth conditions

The study was conducted from March to August, 2013, in an experimental greenhouse of the plant production department, Imam Khomeini higher educational center Karaj, Iran. Strawberry plants cv Gavieta were grown in 2 liters pots on a soilless medium made of 50% perlite and 50% coco peat (v:v) with three plants per pot. Day/ night temperatures were kept at 22/17°C. and were treated with different ratios of a nutrient solution. The full nutrient solution formula was made up with the following stock solutions of the different nutrients: 2.6 KH_2PO_4, 1.9 KNO_3, 2.4 $Ca(NO_3)_2$ $4H_2O$, 0.65 $MgSO_4$ $7H_2O$ and 0.46 K_2SO_4. Microelements for the full nutrition solution were provided in the following amounts: 0.16 H3BO3, 0.09 MnSO4, 0.07 ZnSO4, 0.01 $CuSO_4$ and 0.002 H_2MoO_4. To provide iron, a stock solution containing 0.1 Fe-EDTA was prepared (ARZANI, 2007).

The following treatments were applied:

Full nutrient solution (G1), a modified nutrient solution with either 10% less (G2) or more (G3) amount of Fe, $ZnSO_4$, B_2SO_4, $MgSO_4$, KNO_3, a modified nutrient solution with either 10% more (G4) or less (G5) amount of Fe, $Ca(NO_3)2$, KHPO4, $MnSO_4$, $CuSO_4$, $MoSO_4$ and a modified nutrient solution consisting of +10% Ca $(NO_3)2$, KHPO4, MnSO4, $CuSO_4$, $MoSO_4$ (G6).

The hydroponic system was open. Nutrient solution formula for the group containing chemical treatment was prepared according to KEREJ *et al.* (1999) instruction (Table 1). The pH and EC of nutrient solution were adjusted to 5.7 and from 0.9 to 1.4 dS m^{-1}, respectively.

The pots were arranged in the glasshouse according to a randomized complete design with three replications per treatment.

Determination of Total Anthocyanin Content (ACY)

The ACY of the hydroalcoholic extract of fruits was determined using the pH differential method previously described by GIUSTI (2001). ACY concentration was calculated from the calibra-

Table 1 - Analysis of variance for strawberry phytochemical characters under different nutrient solutions.

Treatment	df	Phythochemical Characters				
		TSS (Brix)	TA (mg/100 g F.W.)	Vitamin C(mg/g F.W.)	Total Anthocyanin (mg/100 g F.w.)	pH
Nutrient Solution	5	2.957*	0.0024*	0.0306*	7527570.5**	0.045**
Error	15	0.944	0.0008	0.0084	435379.3	0.006
CV		10.1	10.9	8.3	26.6	1.9

**,*, ns and CV, significant at 1, 5% level of probability, non-significant and Coefficient of Variation, respectively.

tion curve using pelargonidin- 3-glucoside (Pg-3-gluc) as a standard. Results are expressed as mg of Pg-3-gluc equivalents per 100 g of fresh weight (FW) of strawberry. Data are reported as a mean value (SD for six measurements) (TULIPANI *et al.*, 2008).

Total soluble solids (TSSs), total titratable acidity (TA), and pH determinations

Twenty fruits from each replicate were wrapped in cheesecloth and squeezed with a hand press, and the juice was analyzed. TSSs, was determined at 20°C by an Atago DBX-55 refractometer (Atago Co. Ltd, Tokyo, Japan). pH was measured with a pH meter. TA was determined by titrating to pH 8.2 using 0.1 mol/l NaOH after appropriate dilution (AOAC, 2000).

Determination of vitamin C

Ascorbic acid was measured by HPLC as described by HELSPER *et al.* (2003). Briefly, vitamin C was extracted by sonication of 0.5 g of wet frozen powder in 2 ml of ice cold water with 5% metaphosphoric acid and 1 mM DTPA, followed by centrifugation at 2500 rpm for 10 min, filtering, and immediate analysis on an HPLC system. Quantification was made through a standard calibration curve prepared by running standard concentrations of vitamin C prepared similarly and measured in duplicate at the beginning and end of the analysis. Results are expressed as mg of vitamin C per g of FW (TULIPANI *et al.*, 2008).

Statistical analyses

Data were analyzed by the general linear model ANOVA by Minitab® Release 13.2 (Minitab Inc.). Following ANOVA, treatment means were compared using the LSD test at $P = 0.05$. Statistical procedures were performed using the PC SAS software package.

RESULTS

The results showed under G_1 nutrient solution (first group) the fruit regarded indexes that affect its aroma and taste are in acceptable level, however when we used modified nutritional solution for the other groups (G_2-G_6), increasing levels of some of the characters was detectable. The highest level of TSS and vitamin C observed in group four that had higher percentage of Fe, N, P, S, Mn, Cu and K against the group that treated with G_1 nutrient solution (first group). Fruits produced under treatment G_3 (group3) that received higher amount of Fe, S, Mg, Zn, N and K than the other groups exhibited the highest level of TA, whereas levels of TSS and vitamin C were almost similar to group G_4. Fruits treated with modified nutrient solution number 5 that contained lower level of Fe, N, P, S, Mn, Cu and K against normal nutrient solution, showed the highest level of anthocyanin and the highest amount of pH was detectable in fruits treated with modified nutrient solutions G_6 and G_2 that received higher level of N,Ca,P,K,S,Mn,Cu and Mo and Fe, Zn, B,S,N,K,Mg respectively (Table 2).

Table 2 Effect of different concentration of nutrient solution on strawberry phytochemical characters.

Treatment	Phythochemical Characters				
	TSS (Brix)	TA (mg/100 gF.W.)	Vitamin C (mg/g F.W.)	Total Anthocyanin (mg/100 g F.W.)	pH
G1	10.33ab	0.28b	1.03ab	3997.3ab	3.77b
G2	9.00b	0.26b	1.10ab	3744.3b	4.02a
G3	9.67ab	0.32a	1.10ab	2343.0c	3.85b
G4	11.00a	0.24b	1.26a	2067.3c	3.81b
G5	9.17b	0.25b	1.13ab	4996.8a	3.85b
G6	9.00b	0.26b	1.20ab	1378.1c	4.06a

Analysis of variance showed that interaction between nutritional elements of nutrient solution supplied with nutritional characters of the fruits was significant;_TSS, TA and vitamin C in 5% level and anthocyanin and PH in 1% level of probability (Table 1). Nutrient solution treatment with different ratio chemical nutritional elements showed significantly influence on total soluble solids (TSS), total acidity (TA), vitamin C ($P>0.05$), anthocyanin and pH ($P<0.001$).

DISCUSSION

Considering the results of the present study using a modified nutrient improved fruit nutritional characters but it was not unique; different nutritional formula showed different effect on each index. However often characters increased following enhanced nutritional elements (micro and macro), but anthocyanin decreased with increasing some nutritional elements (micro and macro) (Table 2). SEYYEDI (2005) studied the effect of four kinds of nutrient solution in hydroculture system on the quantitative and qualitative traits of Silva strawberry. He showed that by increasing potassium up to 3meq/l in nutrient solution, the soluble solid material increases. FARZANEH *et al.* (2009) studied the effect of different nitrogen and potassium levels on yield and density of nitrogen and potassium of tomato leaf in perlite environment and reported that the most yield of fruit was gained with 200 mg/L nitrogen consumption, and higher levels of nitrogen reduced the yield and different levels of potassium did not have any significant effect on the yield. HARTZ *et al.* (1999) studied different levels of potassium on the quality of muskmelon. They found that 240 mg/L potassium level caused a significant increase in total sugar, TSS, glutamic acid, aspartic acid and acetate volatile components in fruit flesh, which have an effect on its taste and flavor. MASHHADI-JAFARLOO *et al.* (2009) showed that the most strawberry yield was obtained in 100% coconut medium and cocopeat + perlite (50% + 50%) placed in the next stages (EBRAHIMI *et al.*, 2012; ANIEL *et al.*, 2007). According previous studies the result showed that increasing nutritional element until a definite level has positive effect on the fruit nutritional characters.

Whereas fruits treated with nutrient solution formula exhibited significant effect on nutritional characters in fruit quality, were not the highest level but almost all of them were in limited amount. However with using of modified nutrient solution, some of the indexes decreased though some of them increased, so it seems that under a usual condition and_using G_1 nutrient_solution we have strawberries with satisfying aroma and taste. When we used a growing medium containing 50% perlit and 50% coco peat treated with G_1 nutrient solution formula, produced strawberries with more acceptable nutritional characters comparing the fruits that produced under other nutrient solution. However in these conditions some nutritional characters exhibited to increase but the others decreased. Therefore based on the experiment results fruits produced under G_1 conditions are the best.

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SOCIO-ECONOMIC ASSESSMENT OF DIRECT SALES IN SICILIAN FARMS

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ABSTRACT

Many farmers today adopt direct sales as an entrepreneurial strategy in order to achieve a competitive advantage. The aim of this study has been to analyze the role that direct sales play in Sicilian farms and how the short food supply chain is able to valorize the endogenous resources of rural areas and increase the net income of farmer. Our results showed that direct sales, in conjunction with conventional sales, can represent a growing opportunity for farmers and lead to an improvement in the economic performances of agricultural businesses, an increase in farm investments and the creation of new job opportunities.

- Keywords: direct sales, entrepreneurial strategies, short supply chain, small and medium enterprises (SME) -

1. INTRODUCTION

Competitive advantage represents the result of a strategy that leads an enterprise to occupy and maintain a favorable position in the market in which it operates, thereby obtaining a higher profitability than its competitors (TUDISCA *et al.*, 2013a). In order to compete in the global market, farmers have to be able to change their entrepreneurial strategies and improve their economic performance, thus incorporating 'added value' (CHINNICI *et al.*, 2013a; STURIALE and SCUDERI, 2013; VEIDAL and KORNELIUSSEN, 2013). The higher profit margin obtained allows a higher level of self-financing and a greater return on the invested risk capital (SANTERAMO *et al.*, 2012). Considering the difficulties that farms have to be competitive (high production costs, low sale prices of agricultural products, shortage of labour), direct sales could represent a way to achieve a competitive advantage and improve profit margins.

Direct sales, through the reduction of intermediaries along the supply chain, can affect the annual budget of a farm by allowing the farmer's family to obtain a dignified remuneration for the use of their productive factors (RIZZO and MAZZAMUTO, 2009; POLIDORI *et al.*, 2008). With direct sales, the farmers are not subject to the price offered to them; they may decide to become price-makers (SACCOMANDI, 1999) and apply a different price, one that is higher than the one that is determined in the case of sales to fruit and vegetable wholesale markets or contracts with the Large Organized Distribution (LOD). This type of sale fully utilizes the work of the farmer's family and produces positive effects on the farm's economic performance because it increases the available liquid assets in the business and lowers the capital required for the coverage of the short-term debts that are present during the management activity of the farm (DI TRAPANI *et al.*, 2013). This is a worthy outcome, especially in rural territories where agriculture is the main economic activity and where the pricing of farm production represents the strategic variable for the success needed to relaunch farm competitiveness and boost the local economy, thereby avoiding the phenomena of rural exodus (TUDISCA *et al.*, 2014a; RIZZO and GIUDICE, 2013; TUDISCA *et al.*, 2011; BULIN, 2011; BRUNORI *et al.*, 2002). All of this is fostered by a general increase in the public interest in issues such as ecology and the health and welfare of animals, as well as a growing distrust in the quality of food products derived from conventional agriculture (TUDISCA *et al.*, 2014b; CHINNICI *et al.*, 2013b; BRUNORI *et al.*, 2012; BRIAMONTE and GIUCA, 2010; HARVEY *et al.*, 2004). Direct sales allow consumers to obtain more reasonably priced fresh, healthy food and foster ecological sustainability, as represented by the reduced food miles and carbon emissions flowing from sustainable farming (LITTLE *et al.*, 2009; FEAGAN, 2008). The decreased amount of food miles can result in a lower level of environmental pressure due to a reduction in key factors such as air pollution, soil pollution, loss of biodiversity and noise pollution and can also reduce social pressures that can contribute to problems arising from road accidents and animal welfare issues (VAN PASSEL, 2013). Finally, direct sales can reconfigure relations between producers and consumers, assuming a social justice characteristic (FEAGAN, 2007), and can encourage more harmonious community relations (WINTER, 2003) and more democratic participation of participants in the food supply chain (HINRICHS, 2003).

The aim of this paper, as well as in other studies (PETER *et al.*, 2010; HOLLOWAY, 2008; SONNINO and MARSDEN, 2006), has been to analyze the role of direct sales in Sicilian farms and to determine how the short food supply chain is able to valorize the endogenous resources of rural areas and, consequently, the economic profitability of farmers, who seek to enhance their value along the food supply chain. In particular, it has been carried out an empirical analysis on a sample of farms that adopted the direct sales in order to analyze their structural characteristics, the motivations that led farmers to undertake this selling strategy and its benefits for farms.

2. MATERIALS AND METHODS

In order to analyse how direct sales can contribute to obtaining a competitive advantage for Sicilian farms, we carried out an empirical analysis on 30 small and medium enterprises (SMEs) that adopted this entrepreneurial strategy. The survey was conducted in 2013 by means of face-to-face interviews with farmers, using a specific questionnaire (TUDISCA *et al.*, 2014c; RAFFAELLI *et al.*, 2009; MARBACH, 2000) divided into three parts. In particular, in the first section we collected the information related to the structural characteristics of farms, their agri food products (product portfolios), the socio-demographic characteristics of farmers, the quota of farm production destined to direct sales and its sales modality (farm outlets and/or farmers' markets). In the second one we asked to farmers the reasons that led them to adopt a short supply chain strategy and the benefits reflected in the business performance by its adoption. In this case interviewees had to assign a score to a 1-5 scale to each possible predefined question (TUDISCA *et al.*, 2013c; TRABALZI and DE ROSA, 2012). This scale, better know as Likert scale (LIKERT, 1932), is a psychometric scale commonly involved in research that employs questionnaires. It is the most widely used approach to scaling responses in survey research. When responding to a Likert questionnaire item, respondents specify their level of agreement or disagreement (strongly agree, agree, uncertain, disagree, strongly disagree) on a symmetric agree-disagree scale for a series of statements by attrib-

uting a score for each one (5, 4, 3, 2, 1). Thus, the range captures the intensity of their feelings for a given item (NORMAN, 2010; BURNS and BURNS, 2008; ALLEN and SEAMAN, 2007).

In the third section, we collected information in order to determine the net income of farmers, by means of the following formula:

$$NI = GPV - \sum C_i \quad (1)$$

where:

NI = net income of farmer;

GPV = gross production value of agri food products;

C_i = costs of productive factors that have not been conferred by entrepreneur.

Finally, in order to better quantify how direct sales allowed farmers to remain competitive in the market, it has been compared this value to the net income that farmer would obtain by conferring all farm production exclusively on traditional sales channels.

The empirical survey responses showed that the surveyed farms had an average area of 8.39 ha and ranged from a minimum of 4.50 ha to a maximum of 13.56 ha (Table 1).

The majority of enterprises produced fruit and vegetables (8), followed by milk, cheese and dairy products (6), olive oil and fruit (5), vegetables (4), grapes, wine and fruit (4), grapes and wine (2) and olive oil (1). All of the surveyed farms were worked directly by the farmer's family. The majority of our sample (18 farmers) had used direct sales for more than five years, while the remaining farmers had applied this entrepreneurial strategy in the past three years. Males accounted for 63.3% cent of the entrepreneurs and 36.7% were females. The majority of the entrepreneurs (60.0%) were aged between 31 and 40 years; only 13.3% were over 60 years. These responses highlighted how direct sales strategies have been used mainly by young entrepreneurs, unlike the Sicilian primary sector, where farmers are generally of an advanced age (MASSOLI

Table 1 - Surveyed farms and socio-demographic characteristics of farmers.

Farms (No.)	Farm size (ha)	Agrifood products	Age	Sex		Educational qualification
				Male	Female	
1	7,98	Milk, cheese and dairy products	32	x		Degree
2	6,50	Fruit and vegetables	40	x		Degree
3	8,90	Fruit and vegetables	60	x		Secondary school
4	4,50	Fruit and vegetables	36	x		Higher school
5	6,55	Milk, cheese and dairy products	40		x	Degree
6	4,80	Fruit and vegetables	33		x	Higher school
7	7,98	Milk, cheese and dairy products	28		x	Higher school
8	6,40	Olive oil and fruit	38	x		Degree
9	10,30	Milk, cheese and dairy products	42	x		Higher school
10	9,85	Milk, cheese and dairy products	34		x	Higher school
11	9,58	Grape and wine	35	x		Degree
12	7,89	Olive oil	55	x		Secondary school
13	8,65	Vegetables	26		x	Higher school
14	10,80	Milk, cheese and dairy products	38	x		Degree
15	12,80	Fruit and vegetables	48	x		Higher school
16	9,70	Fruit and vegetables	34		x	Higher school
17	12,63	Olive oil and fruit	65	x		Secondary school
18	13,50	Vegetables	37		x	Higher school
19	8,65	Grape, wine and fruit	72	x		Secondary school
20	8,90	Grape and wine	40		x	Degree
21	6,56	Vegetables	66	x		Secondary school
22	9,85	Grape, wine and fruit	33		x	Higher school
23	8,63	Vegetables	39	x		Higher school
24	13,56	Grape, wine and fruit	46	x		Higher school
25	6,90	Fruit and vegetables	35		x	Degree
26	7,80	Olive oil and fruit	29	x		Higher school
27	5,63	Olive oil and fruit	40	x		Higher school
28	4,89	Fruit and vegetables	31		x	Degree
29	6,50	Grape, wine and fruit	64	x		Secondary school
30	4,50	Olive oil and fruit	36	x		Degree
Total	**251,68**					

Source: our processing of directly collected data.

and DE GAETANO, 2004). Young farmers are able to respond significantly better to new opportunities and market changes than older entrepreneurs (Parker, 2006) and are able to change their entrepreneurial strategies and skills in order to remain competitive in an increasingly competitive market (PRASHANTHAM and YOUNG, 2013). Our surveyed entrepreneurs had a medium-high level of school education. In particular, 46.6% of farmers had higher school qualifications and 33.4% had a degree, indicating that the adoption of a new entrepreneurial strategy is correlated positively with the level of education (NAJJAR *et al.*, 2013; MANCINI *et al.*, 2008).

3. RESULTS AND DISCUSSION

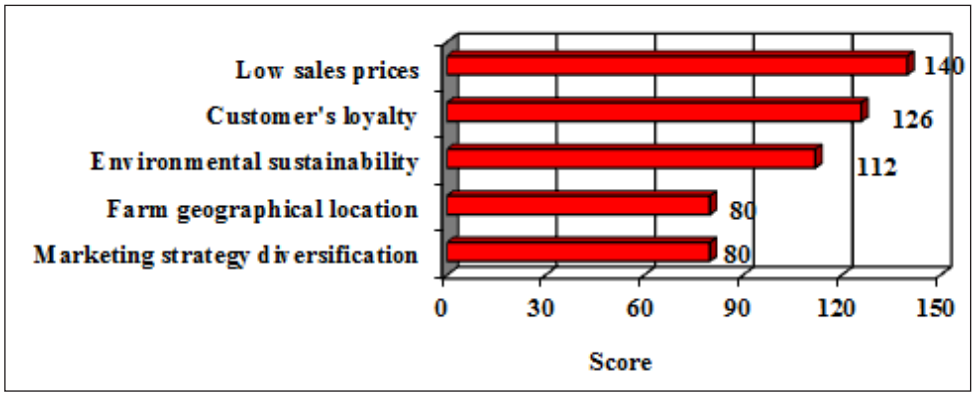
The results showed that direct sales of agrifood products were in all cases a portion of the agricultural production (ranging from 18 to 35%) of the farms we surveyed; the remaining production was marketed through traditional channels (fruit and vegetable wholesale markets, LDO, packing centres). The direct sale was conducted by the entrepreneur or his family (especially women and young people) and was crucial to achieving increases in the remuneration of the enterprise (HENKE and SALVIONI, 2010).

Our empirical survey showed that the majority of farmers (20) sold their products only in farm outlets. This was because it is relatively easy for a farmer to sell his products directly at the place of production; it requires only a simple organization and helps to improve the farm's image (UEMATSU and MISHRA, 2011). Eight of these farmers also conducted guided tours of their farms, with positive outcomes for the visitors such as nutrition education, the diffusion of rural culture and the valorization of territory and local products (METTEPENNINGEN *et al.*, 2012). The influx of customers was distributed throughout the year, thanks to the Sicilian favourable climatic conditions (GRILLONE *et al.*, 2014; D'ASARO and GRILLONE, 2012; AGNESE *et al.*, 2008).

Six of the farmers sold their products exclusively in farmers' markets and four adopted both sales channels. Participation in farmers' markets allowed the entrepreneurs to also sell their agrifood products in urban and periurban areas, thereby promoting their farm and increasing customers and annual revenues (PSARIKIDOU and SZERSZYNSKI, 2012; BRUNORI *et al.*, 2009; BROWN and MILLER, 2008).

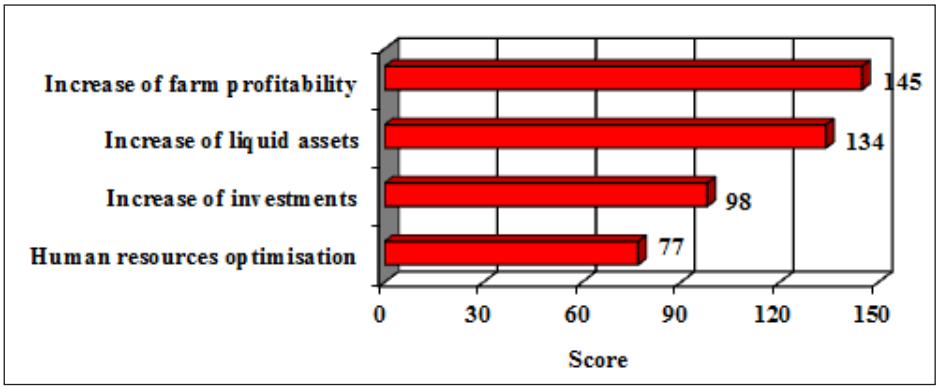
Entrepreneurs adopted direct sales mainly because of the low sale prices of agrifood products, by assigning them the highest score (140) (Fig. 1).

This was essentially attributable to the absence of intermediaries, so that despite the lower gross sales prices for agrifood products compared with conventional markets, the farmers were able to obtain a higher net level of remuneration for their productive factors, by obtaining a competitive advantage (HOLLOWAY *et al.*, 2006; RENTING *et al.*, 2003). They were essentially appropriating a portion of the value that is usually dispersed in the various stages of the long supply chain (BANDARRA, 2011). The second motivation, in order of importance, was customer loyalty with a score equal to 126. This is due to the fact that, compared to the traditional supply chain, direct sales can create a relationship between the consumer and producer that allows the farmer to valorise his production and to transmit his knowledge and links with the territory (GUARINO and DONEDDU, 2011; RENKO *et al.*, 2010). Consumers have the opportunity to purchase agrifood products at lower prices compared to traditional sales channels (SEYFANG, 2008; TAYLOR *et al.*, 2005; KNICKEL and RENTING, 2000) and the growth of face-to-face transactions has stimulated the development of markets in the region, which are considered for their status as oppositional sites to the mainstream food industry (SAGE, 2003). The third motivation, in order of importance, was environmental sustainability (112). This denotes a new multifunctional vision of farming that meets the EU guidelines and the new consumer's needs, respecting and recovering territorial, environmental and ecological values (TUDISCA *et al.*, 2013b; RENTING *et al.*, 2009; RENTING *et al.*, 2008; THILMANY *et al.*, 2008). Direct sales represent a sustainable alternative as the food miles (the distance between the place of production and consumption) are minimized in passing 'from farm to fork'



Marketing strategy diversification 2
Farm geographical location 2
Environmental sustainability 3
Customer's loyalty 4
Low sales prices 5

Fig. 1 - Reasons that led entrepreneurs to adopt direct sales.



Human resources optimisation 2
Increase of investments 3
Increase of liquid assets 4
Increase of farm profitability 5

Fig. 2 - Obtained benefits on the business performance by adopting direct sales.

and the area of distribution is limited. This results in a significant reduction of the negative externalities associated with transport over long distances, such as CO_2 emissions, traffic, road accidents and noise pollution (LANFRANCHI and GIANNETTO, 2013). In contrast to the results of other studies (HARRIS, 2010), farmers assigned to the geographical location of the farm and the diversification of the business marketing strategy the lowest score (80).

Our empirical analysis showed that the farmers felt that the increase in farm profitability was the main benefit to their business performance through the adoption of direct sales. In common with other authors (TRAVERSAC *et al.*, 2011), they assigned it the highest importance by attributing a score equal to 145 (Fig. 2).

Another benefit considered to be of fundamental importance was the increase in liquid assets (134), because timely availability of capital can lead to the adoption of modern technologies, which increase farm production and ultimately the growth rate (RIAZ *et al.*, 2012). The third highest-ranking benefit from adopting direct sales was the increase in investments (98). This was correlated to the increase in farm profitability that allowed for an increase in self-financing and thereby enabled farmers to realize investments (CRNČAN *et al.*, 2011). Finally, the farmers also identified direct sales as an opportunity for human resources optimization, by attributing it a score equal to 77. However, this presupposes that in the farm family there is a state of under-employment, because the possible economic advantage created would otherwise be absorbed by the need to employ external sales staff (TUDISCA *et al.*, 2014d).

Our results showed that farmers who adopted direct sales were able to update their skills and modify the market orientation of their enterprises in order to compete effectively in the current competitive system (FRANK *et al.*, 2012). This highlights the importance of human capital in an enterprise (GURĂU *et al.*, 2010).

Economic importance of direct sales adoption is showed by Table 2. Results denoted that in surveyed farms the net income deriving from the adoption of direct sales ranged from a minimum

Table 2 - Net incomes in surveyed farms

Farms (No)	NI deriving from traditional channels	NI deriving from direct sales adoption	% var.
1	6.200,00	7.563,00	22,0
2	3.500,00	5.650,00	61,4
3	4.750,00	5.860,00	23,4
4	2.500,00	3.052,00	22,1
5	3.650,00	3.850,00	5,5
6	5.500,00	6.500,00	18,2
7	4.750,00	6.523,00	37,3
8	6.500,00	7.980,00	22,8
9	7.800,00	9.500,00	21,8
10	6.520,00	9.562,00	46,7
11	8.562,00	10.523,00	22,9
12	7.650,00	9.632,00	25,9
13	9.560,00	12.065,00	26,2
14	7.500,00	9.652,00	28,7
15	9.850,00	13.000,00	32,0
16	8.950,00	11.250,00	25,7
17	9.560,00	12.000,00	25,5
18	10.520,00	12.360,00	17,5
19	6.500,00	8.500,00	30,8
20	6.850,00	9.020,00	31,7
21	5.932,00	7.500,00	26,4
22	8.952,00	9.650,00	7,8
23	7.560,00	10.325,00	36,6
24	12.350,00	14.630,00	18,5
25	5.900,00	7.500,00	27,1
26	6.500,00	8.000,00	23,1
27	4.600,00	6.253,00	35,9
28	3.678,00	4.321,00	17,5
29	5.690,00	6.980,00	22,7
30	3.690,00	5.936,00	60,9

Source: our processing of directly collected data.

of 3,052.00 to a maximum of 14,630.00 euro.

In all farms, as well as in other studies (HARDESTY and LEFF, 2010), it has been highlighted an increase of net income ranged from 5.5 to 60.9% respect to the one deriving from the selling of farm production exclusively through traditional channels. The higher increases were in farms that sold their products both in farm outlets and farmers' markets as they were able to reach a greater number of consumers.

4. CONCLUSIONS

Our results showed that SMEs employing a direct sales strategy are part of an entrepreneurial network characterized by entrepreneurs who have been able to reorient their business strategy in order to remain competitive in the market. The most important reason that has driven entrepreneurs to adopt direct sales was the need to overcome the consistently low sales prices of agrifood products and thereby obtain higher profit margins and create a competitive advantage. The increase in farm profitability resulted in higher liquid assets and an increase in farm investments and enabled the human resources in the farmer's family to be optimized. This highlights the fact that the use of direct sales in agriculture nowadays can have a positive impact on the many components of the territorial system in which it operates. Nevertheless, direct sales cannot be the only marketing strategy for a farm because the produced quantities cannot be absorbed exclusively by local demand. However, direct sales can represent a winning strategy for a farm if it is inserted within a wider business marketing strategy or if this strategy is used in conjunction with traditional sales methods, such as fruit and vegetable wholesale markets and contracts with LOD.

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ANALYSIS OF A DIRECT SELLING NETWORK FOR AGRIFOOD PRODUCTS

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ABSTRACT

Sicily has become a food and wine area of great interest. However, conflicts within the supply chains have caused the selling process to become long and complex to the disadvantage of farmers, thereby leading to an information asymmetry between producers and consumers.

In order to meet the new needs of the agrifood sector, we developed a theoretical model of organized direct selling that goes beyond regional boundaries, which is an alternative model to farmers' markets and that helps to promote the creation of a network among the operators of Sicilian agrifood supply chains. The aims of this study was to verify the potential of the proposed theoretical model based on a SWOT analysis, which was achieved by collecting data from interviews with the producers involved in the Sicilian agrifood supply chains, and with the main stakeholders involved.

- Keywords: agri-food, consumer, market, marketing -

INTRODUCTION

Farmers have always tried to shorten the supply chain between producers and consumers. They started by setting up consumer cooperatives and farmers' points of sale[1]. Recently, farmers have incorporated e-commerce within their selling activities, as well as farmers' markets, box schemes, pick-your-own initiatives, and community-supported purchasing groups (ABEL *et al.*, 1999; AGUGLIA, 2009; BRUNORI *et al.*, 2009; LA TROBE, 2001).

National and international studies make numerous references to these practices, which have focused on producers, consumers, those outside the established sources of supply, which have been compare with consolidated distribution systems (MURDOCH *et al.*, 2000) with respect to environmental or legislative issues. In particular, these studies focus on short supply chains, direct selling, alternative food networks (HOLLOWAY and KNEAFSEY, 2004), short food supply chains (RENTING *et al.*, 2003), community-supported agriculture (RAFFAELLI *et al.*, 2009), and food community networks (LOMBARDI *et al.*, 2012; PASCUCCI, 2010).

One of the main features that are debated frequently concerns the effective advantages of short supply chains for producers, consumers, and the community as a whole. Producers may have higher economic margins compared with those in the traditional agrifood supply chains, where different mediators take away part of the producers' margins (VAN DER PLOEG, 2006).

However, a feature that is often ignored is the fundamental difference between the roles, tasks, attitudes, and capacities of farmers and market experts, who have specific skills to address the market in an effective manner. It is inconceivable in terms of education, culture, or tradition for farmers to occupy specific commercial roles or to confront the "unfair" challenge of the large-scale retail trade, which has been present in cities for decades. Instead, it is possible to suggest the organization of an innovative market system, which can use its available efficiencies to move agriculture closer to urban consumers in different but stable ways, because consumers are increasingly keen to retain the precious nutritional value of food in general and of the "Mediterranean diet"[2] in particular, where UNESCO has recognized the latter as an intangible heritage of humanity (GROSSO *et al.*, 2013).

At present, the food industry and large-scale retailers are trying to exploit the information asymmetry that exists to direct consumption toward their needs and targets. This can only be overcome by farmers, particularly the producers of high quality food with good organoleptic characteristics (ABEL *et al.*, 1999; HUNT, 2007). The real advantages for consumers of short supply chains are lower prices but also better information about the nutritional value of raw materials, the characteristics of the production process, agri-industrial processing techniques, and food preservation techniques (HINRICHS, 2000; LA TROBE, 2001).

Accordingly, our goal was to design an experimental model of direct selling that may represent an innovative method for agrifood products and to promote the products from specific areas, thereby implementing an alternative networked commercial system that can communicate effectively and reliably with consumers about the value of the food produced.

ANALYSIS AND REFERENCE CONTEXT

Italian legislation has supported the direct commercialization of agrifood products since the 1960s (Law no. 59 of 1963) and legislative decree no. 228 of 2001[3] simplified the relative procedures for farmers who practice direct selling[4] (ALABRESE, 2008).

Subsequently, a decree has been applied to farmers' markets only (BELLETTI *et al.*, 2010). The growth of these markets was supported further by the Ministry of Agricultural, Food, and Forestry Policies on November 20, 2007[5] but the current economic recession is limiting food habits and life style, and reducing the purchas-

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[1] For example, citrus fruit producers' organizations in the province of Catania promote a network of points of sale in north-eastern Italy, which are run directly by the members (Rizzo and Mazzamuto, 2009).

[2] Hedonistic reasons accompany healthy ones in defining the value of food products. The increasing number of press columns and television formats dedicated to food confirm the widespread research into the pleasure of good food and conviviality, as well as best sellers and editorials about regional recipes and the increasing number of concept stores where furniture, atmosphere, and menus make consumers feel relaxed and comfortable while enjoying simple, traditional, but creative meals based on the Mediterranean diet.

[3] Article 4 of L.D. no. 228 dated 18 May 2001, "*Orientation and modernization of the agrifood sector, according to art. 7 of law, no. 57 dated 5 March 2001*".

[4] The new decree also allowed direct selling for products not produced directly by the farm itself. The previous law, no. 59/63, "Rules for Farmers to Directly Sell Agrifood Products," limited selling by farmers to the products obtained exclusively from their farms. They were identified as "owners of the land where they grow, their cooperatives or associations."

[5] This is an unprescribed decree since the legislative competence concerning commerce and agriculture is limited exclusively to regional governments according to article 117 of the Constitution. Only regional governments can issue rules and regulations about this matter. Thus, it is a decree aimed at guidance that is not mandatory (Alabrese, 2008).

ing power of families (FANTUZZI and BRUGNOLI, 2010).

Producers use direct selling as a way to sell their products in nearby markets. There are two reasons for this: first, the greater the distance from the origin of the food, the greater the problems of information asymmetry between producers and consumers become, while the information and communication costs related to products and production techniques are also higher (BRIAMONTE, 2010; GARDINI *et al.*, 2007; GUIDI, 2008); second, producers are hampered by the lack of organization, logistics, and the availability of finance when operating in distant markets. This makes it difficult for small-medium enterprises to place their products and operate at national and international levels (CHIFFOLEAU, 2009).

Several regional governments have issued specific regulations and financially supported market development[6], including numerous measures in the Rural Development Plan (RDP). In Sicily, measure 321/A1 of the 2007/2013 RDP, supports the development of equipped public areas for farmers' markets of typical products and handicrafts.

Fig. 1 shows the intervention areas, which include 85 projects and a total investment of 9 million Euros to develop farmers' markets, 79 of which will be activated in Local Action Groups (LAGs) areas and the remaining six in other areas.

However, direct selling within farmers' markets has limits and critical points (VERHAEGEN and VAN HUYLENBROECK, 2001; CHIFFOLAEU, 2009). Thus, previous studies have noted that farmers' markets provide a direct relationship between producers and consumers, which may guarantee fresh products, because of their excellent locations and temporal discontinuity, but they do not provide an effective organization for selling, they do not obtain appropriate sales volumes, and they do not meet demand in full (BRUNORI *et al.*, 2009). Farmers' markets are often combined with village fairs and with folkloristic and cultural characteristics, but they are rarely oriented towards a modern organization[7].

The exclusive selling of products grown and consumed in the same area cannot allow for temporal continuity or completeness in terms of product diversification and the quality level, which is the basis of the modern distribution

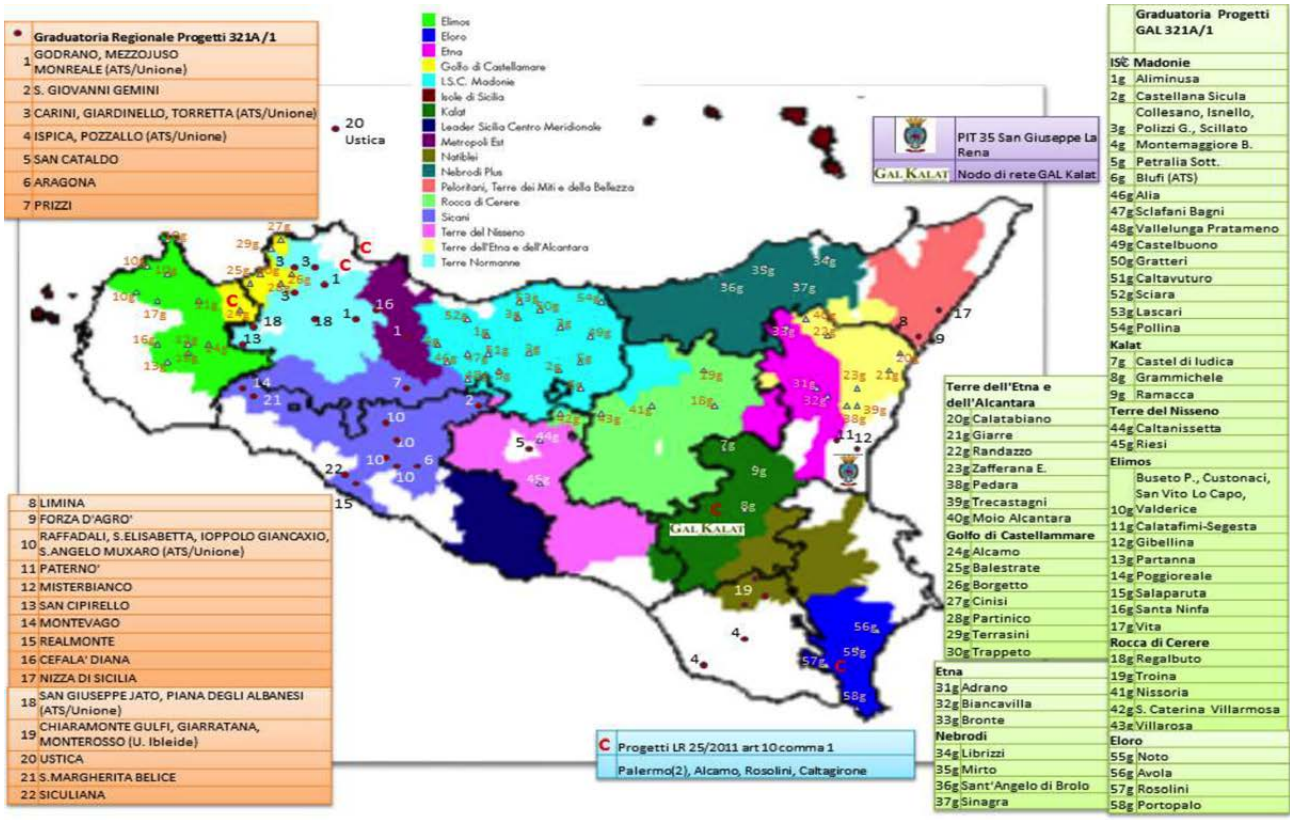


Fig. 1 - Farmers' market in Sicily (Source: Data elaboration 2007-2013 RDP Sicily).

[6] The Sicilian Regional Government intervened on this issue by article 83 of L.R. 11/2010, by allowing direct selling, particularly by certified farmers who carry out their activities within the Sicilian territory.

[7] The regulation gives municipalities central powers to organize, authorize, and finance such markets. Despite the marginal role assigned to regional governments by national legislation, the majority have regulated and financially sustained farmers' markets.

system thanks to the evolution of preservation techniques, transportation systems, and logistics (HINRICHS, 2000).

Several studies (RIZZO and VECCHIO, 2008) have shown that this approach is not a sustainable alternative because the volumes are too small. These low volumes are due to the lack of temporal continuity of production and the incapacity of the local agrifood system to offer an articulated range of products. If direct selling of agrifood products is to gain a higher economic weight, it cannot be confined to the farm location itself or to the neighboring area. Italian legislation has not limited direct selling to local products alone, which has allowed it to spread throughout the territory of the Republic after communication with the municipality to which the farm belongs[8] (TUDISCA *et al.*, 2014).

Recently, the Sicilian regional government issued a regulation (article 10 of Law 25/2011[9]) that goes beyond regional boundaries to support and promote "the direct and market selling activities" of Sicilian agrifood products via networked regional structures (section 1), that may interact in synergy with analogous networked structures at the national level (section 5) and at community level (section 6).

PREREQUISITES OF THE NETWORK MODEL

The European agrifood sector, particularly fruits and vegetables, has not experienced consumption increases for many years. By contrast, demand has diversified greatly where the dynamics have affected the structure of the offer (CSO 2012 data).

Consumers are increasingly keen to look for products that better meet their needs and their respect for a renewed linkage between purchasing processes. The points of sale within the framework of the complex income dynamics suggest new market segmentation models, which are function of a different "perception" of the quality-price relationship. Indeed, quality now includes aspects that go beyond its traditional concept, which was bound only to the organoleptic characteristics of products (BRUNORI *et al*, 2009; CHINNICI *et al.*, 2013).

An orientation toward "responsible" purchasing has been added to the reasons to buy, especially from the particular segment of consumers who have a mature awareness of high value products because of their organoleptic, nutritional, healthy, evocative, ethical, and solidarity-based characteristics compared with commodities (DI VITA *et al.*, 2013). This value is derived from, either jointly or singly, the fact that products come from specific territorial contexts (ABEL *et al.*, 1999), where they are grown with traditional and/or organic production techniques, thus the offer is organized directly by producers who bet their reputation on their products and they only receive a premium price.

Accordingly, Sicily's pedoclimatic characteristics may allow it to produce a wide range of agrifood and zootechnical products, which may satisfy all the nutritional needs of the regional market, but also the national market. Sicily is a food and wine "continent" because of the wide range of high quality agrifood products it offers and its millennial culinary tradition. Based on its range of high quality agrifood products, Sicily (Graphic 1) is the third highest ranked region in terms of the number of registered products (28), especially for fruits, vegetables, oils, and cheeses.

Despite the acknowledged excellence of several quality products, the tendency for territorial specialization and exploitation has not diminished, especially in those territories where the community politics for years have favored monocultures destined for "global" markets.

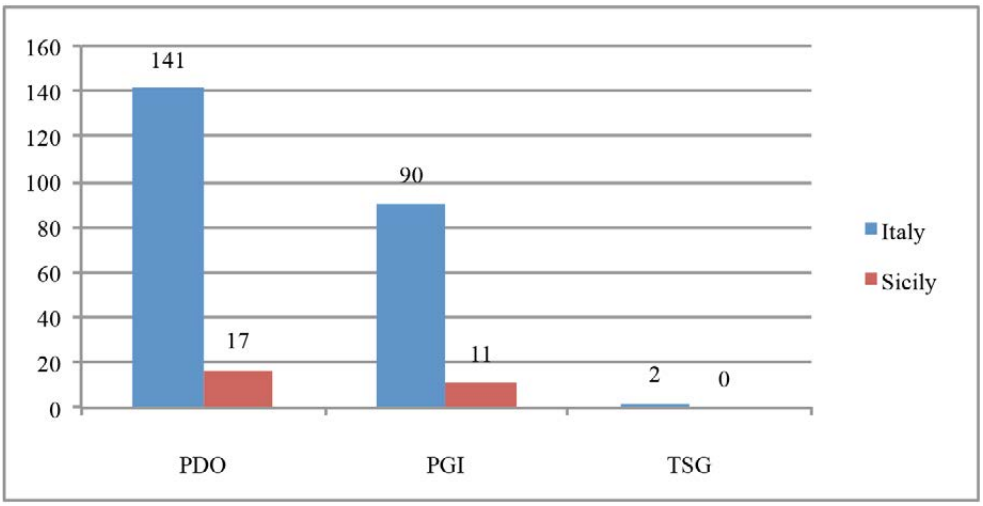
In Sicily, this tendency has caused (Fig. 2) the sellable gross production (SGP) to rely on a few typical products, i.e., citrus fruits, grapes, oil, wine, vegetables, and a wide range of zootechnical products, which together comprise 70% of the SGP of Sicilian agriculture, while 30% of the SGP includes other fruits, vegetables, and livestock, which have lower value despite their high quality.

Although Sicily may rely on a good range of agrifood products, its supply chains are inadequate due to a lack of organization and production volumes. Thus, it cannot account for a significant market share.

In many cases, single companies cannot face the problems related to the planning and management of the necessary promotion and communication activities. In addition, they cannot easily obtain the necessary information about the market situation and consumers' preferences in order to tailor their offer according to consumer needs. For example, it is difficult for them to standardize their quality, arrange for suitable packaging, ensure the constant presence of their product, or adopt an advanced traceability system (HAUSMANN and DE AMICIS, 2007; RAPISARDA and RIZZO, 2010). All of this would require

[8] The first national organization to undertake direct selling and overcome these critical points was sponsored by Coldiretti, an organization that represents Italian farmers. Coldiretti, via its initiative called "Campagna Amica," is promoting points of sale throughout the national territory. These points of sale are gathered in a single commercial network that offers consumers the products of their members regardless of the geographical location of the growing area within Italy.

[9] Regional Law dated November 24, 2011, no. 25, issued on GURS no. 50 of December 2, 2011, entitled: "Interventions to Support Agriculture and Fishing. Regulations for handicrafts, cooperation, and commerce."



Graphic 1 - PDO, PGI and TSG registered products in Italy *(Source: Qualivita data direct elaboration).*

resources, competencies, and a critical mass of products that SMEs often lack. The only way to overcome these problems is via group initiatives (GALISAI *et al.*, 2009; LOMBARDI *et al.*, 2012) that combine production as well as some steps of the distribution processes and logistic arrangements (BELLETTI and MARESCOTTI, 2012).

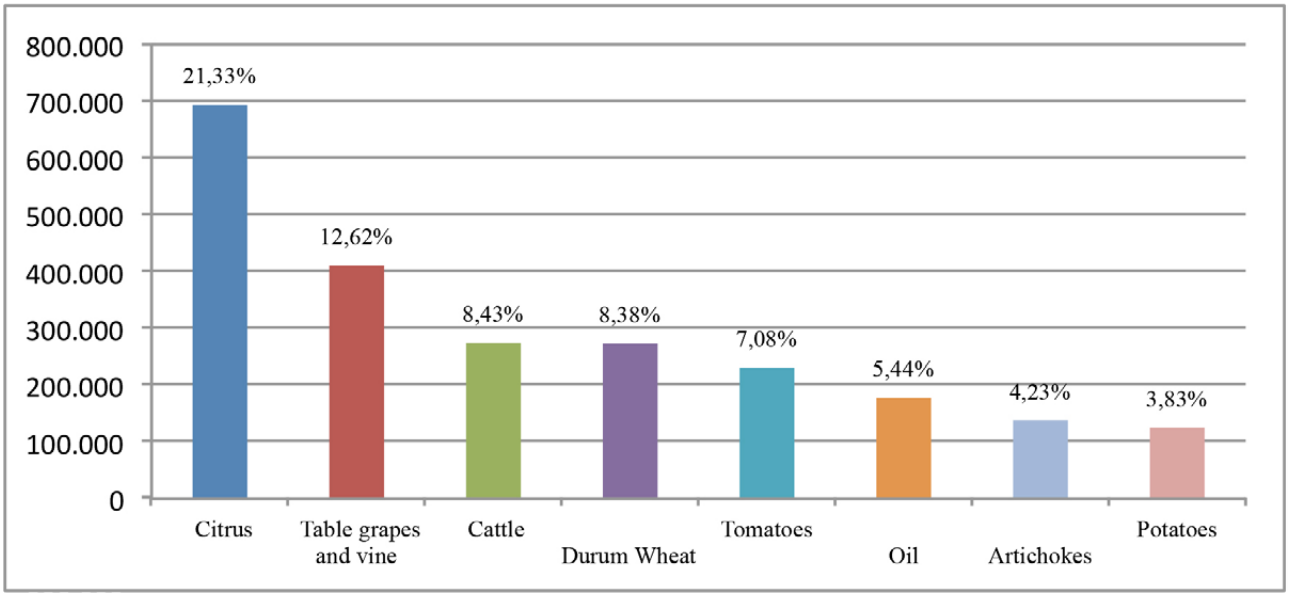
Based on these requirements and legislative interventions of the Sicilian regional government, we aimed to develop an organized direct selling (ODS) theoretical model, which goes beyond regional boundaries and provides an alternative to the farmers' markets, thereby promoting the creation of networks among Sicilian agrifood supply chain operators.

The model is structured and includes the following subject typologies.

- Subjects in the supply chains: farmers' associations: these subjects have to organize the offer and services for each farmer's direct shop[10] (from product preservation to shipment).

- Subjects for farmer's direct shop management: Specific or pre-existing companies will organize the farmer's direct shops at national and international levels, such as the organization of promotional events and the management of market activities. They will have the functional prerequisite of collaborating with the subjects of the supply chains to guarantee the direct selling of products and to form a network that agrees to perform all of the other related activities.

- Network junction: This is the organizational structure required to deal with the relationship between the subjects of the supply chains and



Graphic 2 - Sicilian gross sellable productions (2012) (Source: Inea data direct elaboration).

[10] Direct selling shop, a *"closed area,"* which is independent and included in an articulated structure with dedicated corner for the subjects of the supply chains to carry out their activities.

the subjects of the farmer's direct shop management to coordinate and organize common activities, and to ensure that regulations are observed (RIZZO and GIUDICE, 2013).

This ODS model allows the possibility of overcoming the limits of the most common short supply chains in the Italian territory, by joining producers as subjects in the supply chains, which are separated based on products, and by proposing the management of the direct selling commercial activities via a direct selling shop manager, who is a third subject.

This theoretical management model is based on the network junction, the function of which is to coordinate the bidirectional inputs from the subjects of the supply chains and the subjects of the farmer's direct shop management. The results of this information exchange will generate the product typology, the packaging typology, and the selling price, as well as linking producers directly to the subjects of the farmer's direct shop management, who have direct daily contact with consumers. All of the subjects of the supply chains will be represented inside farmer's direct shops with promotional and tasting initiatives for their products.

In order to make the model stronger and more significant, side activities are included within the agrifood product direct selling scope, such as the following:

- Tasting and distribution of quality regional agrifood products;
- Organization of "satellite market spots" within the commercial area of reference of each farmer's direct shop;
- Promotion of regional quality production within the hotel, restaurant, and café (Ho. Re.Ca.), and ethical purchasing groups (GAS) commercial scopes of reference;
- Acting as a structure that manages the organization of promotional activities at a regional level, including territorial marketing and customer retention;
- Acting as an intermediate logistics centre to carry out e-commerce activities.

This network includes a union point where producers and consumers meet to increase knowledge of the organoleptic and nutritional qualities of products based on tasting as well as on information that, thanks to modern IT tools, has become widely available and is articulated and updated in real time. In order to comply with the aim of this research and combine tradition, culture, gastronomy, and diet inside farmers' direct shops, traditional promotional activities will be developed, such as tasting, cultural, educational, and gastronomic activities involving the products and the territory.

The truly innovative element is the promotional function of farmer's direct shops, which will be integrated within the market and promotional activities, thereby producing a synergy between promotion and selling to help overcome the limits of the promotional activities carried out by public and/or territorial bodies, which often develop out of the commercial logic of private operators. Integration, in addition to the physical level, is a common operative project between the public promotional activity and the private commercial activity, which is a highly innovative element of the model suggested to the regional government.

METHODOLOGY

We decided to verify the potential of the proposed theoretical model by carrying out a SWOT analysis using data collected from interviews with producers and the main stakeholders involved with Sicilian agrifood supply chains.

SWOT analysis is a strategic planning tool used to evaluate the strengths, weaknesses, opportunities, and threats related to the ODS model in order to fulfill its goals.

The SWOT analysis includes:

- Strengths: the aspects of the model that help to fulfill its goals;
- Weaknesses: the aspects of the model that hamper the fulfillment of its goals;
- Opportunities: useful conditions outside the model that help to fulfill its goals;
- Threats: external conditions that may damage the performance of the model.

The analysis used aimed to meet the goals of our research. In fact, it links the knowledge of the context where producers operate to the politics of the economic development and promotion of agrifood products.

The analysis group collected information concerning the difficulties of Sicilian farmers, commercial solutions, market dynamics, the specific needs of producers who adopt short supply chains at organizational and management levels, and objective and official data related to the agrifood system, which was obtained from the main research institutes of Ismea, Inea, Istat, the Osservatorio sulla Vendita diretta, and Nomisma. This study helped to identify the strengths and weaknesses of the most widespread forms of short supply chains, specific data concerning Sicilian agrifood productions, the importance of this phenomenon, legislative aspects, and the fiscal and administrative supports of direct selling.

Direct interviews were conducted during 2013 in collaboration with the technical assistance operational sections (SOATs) of the Sicilian regional government, which allowed the nine provinces of reference to select farms that were interested in the proposed direct selling model, where 126 operators in the Sicilian agrifood supply chains were interviewed either jointly or singly.

The number of interviews was quite significant compared with the number of operators involved. The answers to the questionnaire and SWOT analysis entries were selected based on

the number of times they were iterated. Answers were given according to the personal experiences of the interviewees.

The originality of this research concerns the definition of a functional model of direct selling that includes producers, market managers, and consumers, as well as the creation of farmer's direct shops with the primary function of selling agrifood products, but also with a wider and complex economic meaning, which combines articulated functions, such as "farmer's direct shops", promotion, marketing, and tasting that are linked to the production territory.

RESULTS

The results of this study show that "Local Food" has emerged as an increasing interest due to the economic weight it is gaining in terms of "proximity," i.e., the physical distance between producers and consumers, but also because of the growing importance consumers allocate to the quality of products that come from a specific territory. Numerous typical Sicilian products possess the necessary characteristics to develop their own local market and to find places in the market that differ from their original roles, especially if they are characterized by clear traceability, sufficient critical mass, and the will to create a network of all supply chains and services for farms.

The SWOT analysis highlighted the main strengths, weaknesses, and threats, but also the opportunities that the proposed model offers to support Sicilian agrifood productions and to strengthen the role of producers in the supply chains (Table 1).

The SWOT analysis suggest that the ODS model has a strategic meaning and it may achieve the following.

Promote a base of knowledge and excellence beyond the regional scope, thereby spreading information and stimulating consumption.

Improve the competitiveness of producers who cannot easily find commercialization channels

Table 1 - SWOT analysis of the organized direct selling experimental model.
Source: elaborations of direct surveys and Nomisma dat

Strengths	**Weaknesses**
▪Wide product range ▪Creation of a network between producers and consumers ▪Selling far from the place of origin guaranteeing the origin of products, quality, freshness, product seasonality ▪Valorization and promotion of products within the selling stage ▪Higher added value for producers along the value chain ▪Daily selling activity	▪ Logistics organization ▪ Remote management of points of sale ▪ Logistics and transportation costs
Opportunities	**Threats**
▪ Creation of alternative selling channels ▪ Promotion of the territory of origin ▪ Possible interaction with local bodies and associations to develop community initiatives to the advantage of the territory ▪ Diversification towards non-agriculture-related activities (Ho.Re.Ca; catering) ▪ Advertising of the Mediterranean Diet and of the "born in Sicily" label	▪ Management cost of points of sale ▪ Purchase frequency ▪ Management of returns

beyond the regional scope, thereby motivating production differentiation in Sicilian agriculture and making traditional producers economically sustainable. Indeed, the latter remains an expression of the biodiversity of specific territorial contexts.

Exploit the well known advantages of short supply chains to allow the agrifood world to become closer, both significantly and stably, to the growing segment of consumers who look for and buy quality agrifood products.

Stimulating farmers' associations to concentrate, organize, and commercialize their offer, thereby improving the performance and competitiveness of members.

In addition, several weaknesses of direct selling in farmers' markets may be overcome by creating an organized network that strengthens the role of producers within the supply chains. However, the weaknesses and threats show that there is a need to strengthen the concept of direct selling within farmer's direct shops. By contrast, the model may become distorted given the difficulties of maintaining the producer-consumer relationship directly from a legislative-fiscal point of view.

Overall, this model is an example of organized supply chains with defined roles and a vertical distribution strategy directly from producers to consumers, thereby providing the opportunity to design intervention proposals and strategies to define the offer based on the specific characteristics of the demand.

CONCLUSIONS

The research results allowed us to evaluating our experimental model that aims to promote a network of direct selling operating promotional farmer's direct shops and points of sale throughout the national territory, thereby promoting Sicilian agrifood products. This is a step forward compared with today's "country markets" because it shortens the physical distance from the field to the table and optimizes the organizational and economic structure of this sector.

The proposed short supply chain management model provides tools that are more flexible for producers, by overcoming the current difficulty of being present in different places at the same time to meet consumers. A direct relationship with the farmer makes product commercialization easier but not all farmers are ready to assume this role. Indeed, many would prefer to continue playing their existing role, which is to dedicate themselves to their production activities because they lack sufficient time, resources, or the correct attitude to participate in selling activities.

Sicilian farmers may not face price competition, but the hypothetical shortening of the distribution chain by proposing direct contacts between the consumer (national, according to the whole range of the legislative intervention under study) and the producer (Sicilian) cannot be separated by the segmentation of a specific target group of consumers. Thus, the proposal of a "pact" as a sign of a philosophy that favors some qualitative aspects but does not aim to make it prevail over the price-quality relationship that is a prerequisite of large-scale retailers cannot be a characteristic element of the supposed distribution model's competitiveness.

This verification model could be a combined governance model for regional planning.

To evaluate the replicability of the model, it will be necessary to establish the trend in the relationships within the network and the evolution of the organizational system, as well as determining the extent to which this might help to develop the technical, organizational, and relational capacity of each farmer, as well as to preserve/reproduce local resources and biodiversity.

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END OF THE DEROGATIONS TO REGULATION (EC) 853/2004 FOR COW'S MILK IN ITALY

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ABSTRACT

Derogations for somatic cell and total bacterial count limits had allowed non-compliant milk to be used for cheesemaking in Italy. Commercial and health considerations prompted a decision to implement a program to gradually repeal the derogations. In this study, we report the statistical evaluation of the situation in 2007-2008, the outcomes of the program to close the derogation and observations of its effects during its implementation from 2010-2013 in the Lombardy region. The introduction of a progressive decrease of the limit allowed regulators to minimize the negative impact on production levels by focusing on the management of the most non-compliant farms first.

- Keywords: cow milk, derogations, somatic cell count, total bacterial count -

INTRODUCTION

After the adoption of European regulations for food safety (Reg. EC 178/2002, 852-853-854/2004), Italian farmers were still able to sell cow's milk that was non-compliant in somatic cell count (SCC) and total bacterial count (TBC), indicators of presence of udder pathogens and insufficient hygiene during production and storage, respectively, because of the derogation of article 10 of the Regulation (EC) 853/2004.

Specifically the derogation allowed for cow's milk with geometric means exceeding the legal limits (400,000 cells/ml for SCC and 100,000 CFU/ml for TBC) to be used for the production of cheese with ripening periods of at least 60 days. This derogation was based on the knowledge that potential risks linked to high SCC and TBC can be significantly reduced or even eliminated during the production processes and ripening periods (Annex IV of the Reg. EC 854/2004). In fact, many stages of processing have antimicrobial effects including: the cooking of curd, the acidification of curd, the salting of cheese and the reduction of free water. However, over the years unfavorable opinions about the use of non-compliant milk have increased in Italy. Aged dairy products, like Grana Padano and Parmigiano Reggiano cheese, represent the uniqueness and tradition of Italian raw milk cheeses. Given that they are some of the most popular cheeses on the international market, is the use of the "worst" milk appropriate for the "best" cheeses of Italy?

It became clear that the presence of the derogation did not promote improvement in the quality of Italian milk. Furthermore, according to the principles of the Community Regulations, a derogation has to be considered "temporary" and contingent on specific issues. A derogation, if it affects food safety, must always provide a time limit or an exit strategy that will lead to conformity with the other nations. For these reasons Italy began a gradual process to repeal the derogation. The project was developed in 2008, after which it was communicated to the European Commission (notification number 134/2010) and it was formalized by the "Agreement between the Government, Regions and autonomous Provinces of 09 September 2010". The goals of this study were to conduct a preliminary evaluation of the problem based on data from 2005 to 2008, to create a plan to phase out the derogation and to evaluate the results of its application over 2 years, from January 2011 to June 2013, in the Lombardy region (an area responsible for more than 40% of the national milk production).

MATERIALS AND METHODS

The data come from the analysis of the milk quality payment system instituted in the Lombardy region. The system requires at least 2 samples per month from each farm. The samples are taken from farms by trained and qualified dairy industry operators. For statistical evaluation and calculation of the geometric mean we selected the farms with continuous production and active participation in the milk quality payment system during the 2-year study period (roughly 4600 farms out of 6,000 total active farms in the region). The majority of farms can be characterized by rearing Holstein Friesian (85%) or Brown Swiss (15%) cows in loose housing with cubicles, with milking parlor, fed with mixed ration of corn silage, hay and concentrate. On these farms the average herd size is 70 cows and the average milk yield is 9,400 kg per cow per year.

TBC was determined with Bactoscan FC and the SCC was obtained using Fossomatic 5000 (Foss, DK). For descriptive statistics (frequency distribution of farms' geometric means of SCC and TBC) the free "R" software environment was used.

RESULTS AND DISCUSSION

Analysis of compliance

The distribution of the farms' yearly SCC and TBC geometric means during the year 2008 are represented in Figures 1 and 2, respectively (5,200 farms). The "yearly" geometric means were calculated for each farm from 24 or more samples per year. From the figures it is evident that the SCC situation was more critical than that of TBC with respect to Regulation 853/2004 limits (BERTOCCHI *et* al., 2012), (BOLZONI *et* al., 2007).

In Table 1 an evaluation of the farms' SCC rolling geometric means from 2005 to 2007 is presented. The "rolling" geometric means were calculated over periods of 2 or 3 months for TBC and SCC, respectively. The data show that 44% of the farms were consistently under the SCC limit, while 29% of farms exceeded it one or more times but returned under the limit within the 3-months observation period; the remaining 27% of the farms were still non-compliant after the observation period. Further analysis of the last group of non-compliant farms (Figure 3) revealed that only a small fraction of these farms returned to compliance shortly after the 3 months of observation. Most of the farms spent a long time in non-compliance and even some farms never became compliant. These were the farms that were able to avoid compliance by commercializing their non-compliant milk in a geographic area where most of the milk is used for aged cheese.

The end of the derogation: potential effects

According to the abovementioned observations, it could be hypothesized that closure of the derogation would cause economic problems

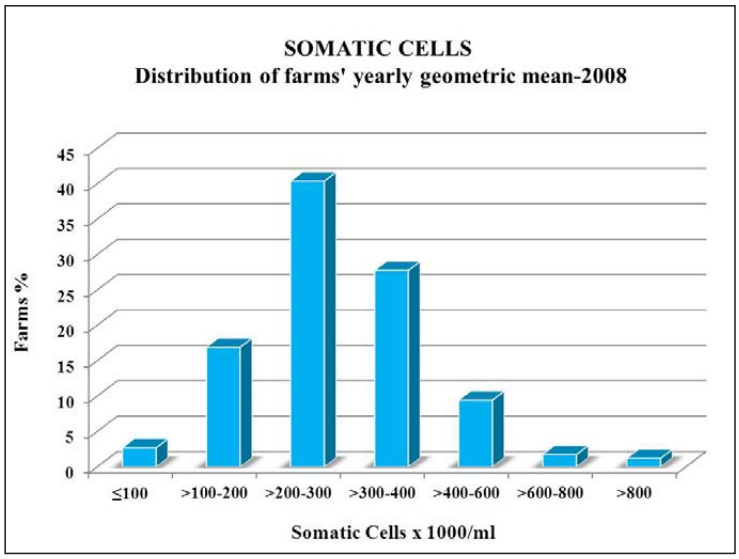


Fig. 1.

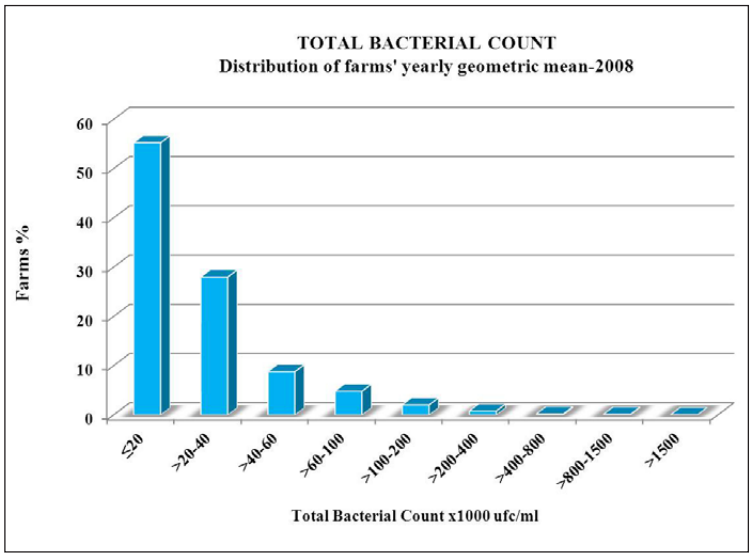


Fig. 2.

Table 1 - Somatic cell count compliance among 4,595 farms from 2005 to 2007.

Rolling geometric mean (cells/mL)	Farms (n)	Farms (%)
Always < 400,000	2,032	44%
> 400,000 with recuperation < 90 days	1,313	29%
> 400,000 without recuperation < 90 days	1,250	27%

for about 30% of the farms based on their SCC values and 4% of the farms based on their TBC values (data not shown). To explore these effects, we performed a statistical simulation on the 2008 data to quantify the effect of a gradual repeal of the derogation and its effects on farm production. The results of the simulation for SCC are presented in Table 2.

Program to repeal the derogation

A regional program was developed with a series of decreasing temporary limits. Given that controlling SCC levels is known to require both medium- and long- term actions, it was expected that some of the farmers would become proactive with control measures and improvements in order to reach compliance in time for the more restrictive future limits. The plan was approved by the Ministry of Health and then it was expanded into a national program with the "Agreement between the Government, Regions

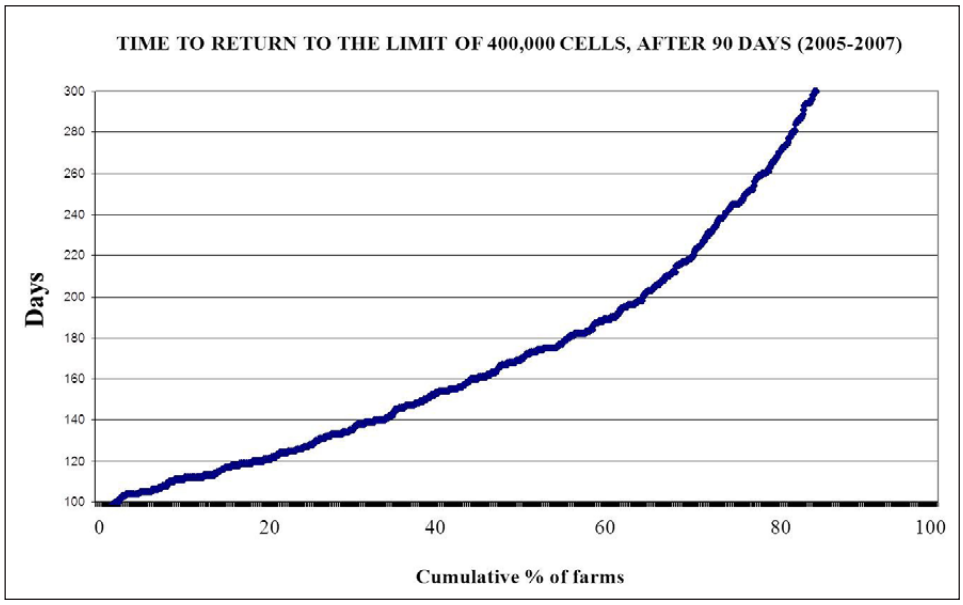


Fig. 3.

Table 2 - Geometric means of the compliance of 4,669 farms at different somatic cell count limits in 2008.

Somatic cell limit (cells/mL)	% of farms always under limit	% of farms over the limit with recuperation in 90 days	% of farms over the limit without recuperation in 90 days
700,000	89	8.4	2.7
600,000	83	12.1	4.6
500,000	73	19.6	7.7
400,000	50	32.1	18

Table 3 - Temporary limits for compliance during the program to close the derogation (geometric means calculated over periods of 2 or 3 months, respectively, for TBC and SCC).

PERIOD	Total Bacterial Count (Rolling GM calculated over 2 months)	Somatic Cell Count (Rolling GM calculated over 3 months)
January 2011 - June 2011	< 200,000	< 700,000
July 2011 - June 2012	< 100,000	< 600,000
July 2012 - June 2013	No derogation	< 500,000
From July 2013	No derogation	< 400,000

Abbreviation: GM, geometric mean.

and Autonomous Provinces". It banned the use of non-compliant milk for human consumption and set temporary limits for TBC and SCC as shown in Table 3. Currently milk with rolling geometric means > 400,000 for SCC or > 100,000 for TBC continues to be used in the production of cheeses with over 60 days of ripening.

Program application and results

Ten-year trends in SCC and TBC levels in the Lombardy region are shown in Figures 4 and 5, respectively. From these graphs it is possible to deduce a preliminary and general trend of decline in the two parameters. In particular, in fig-

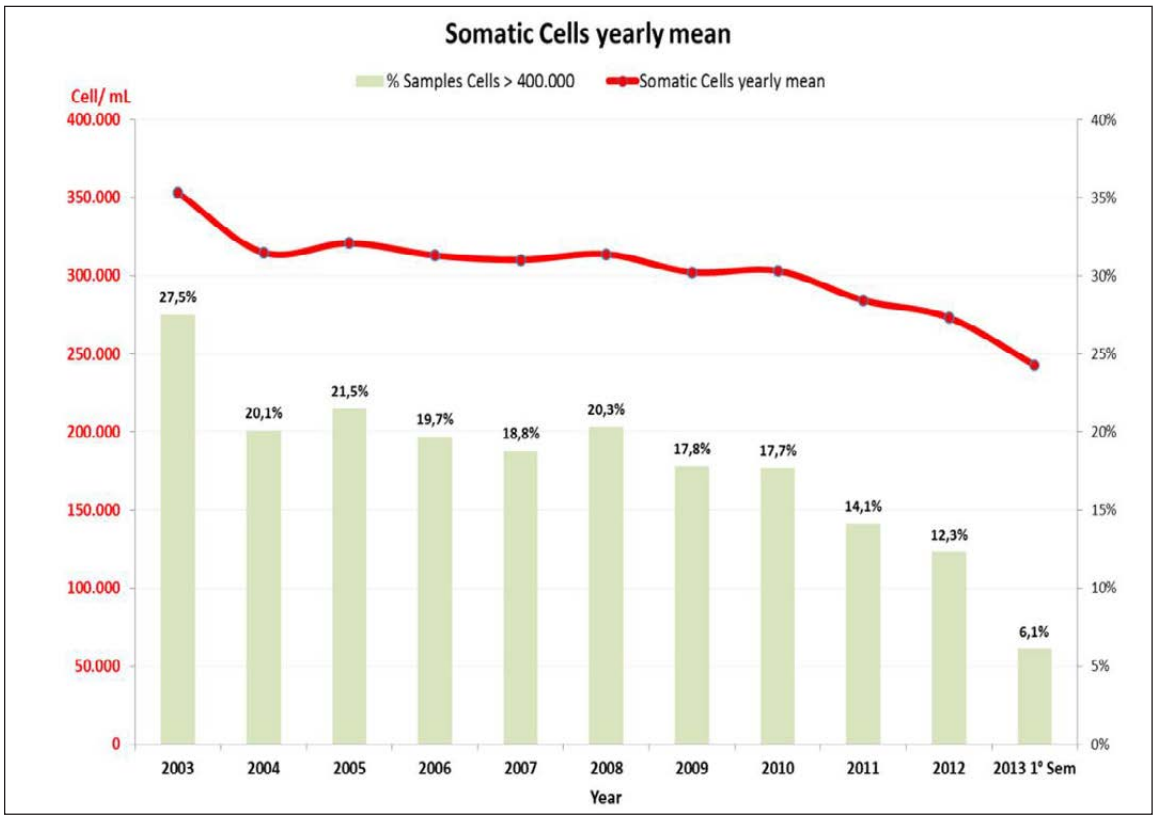


Fig. 4.

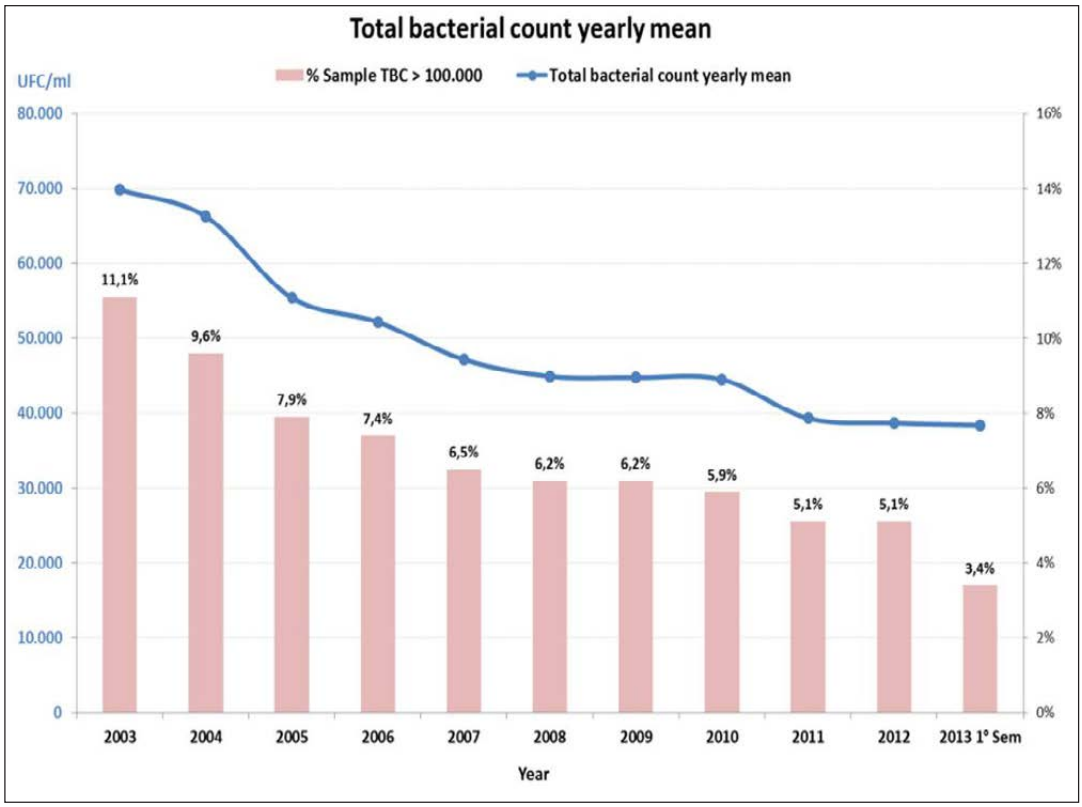


Fig. 5.

ure 5, the low percentage of samples over 100,000 UFC/ml (colored bars) and the low and decreasing value of the yearly regional mean (blue line), indirectly confirm the very low number of non-compliant farms in TBC during the last year. The number of samples used to generate the yearly means varies from a high of 165,000 in 2003 to a low of about 100,000 in 2013 which reflects the significant reduction in the number active farms in the region during this period.

Fig. 6 presents a specific assessment of the effect of the progressive application of the pro-

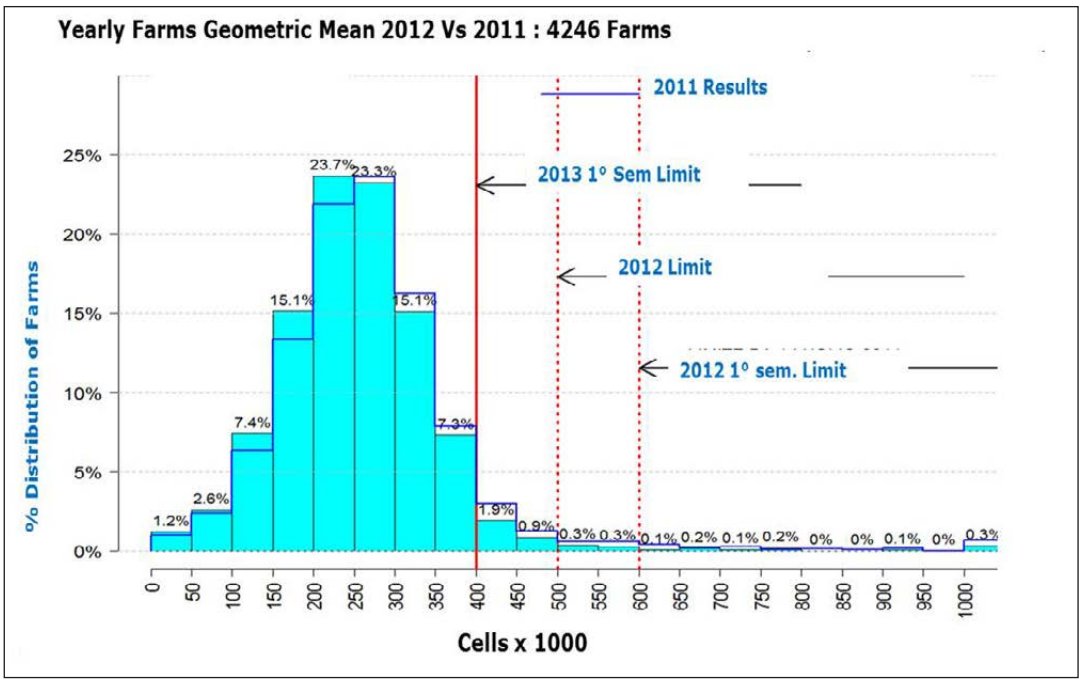


Fig. 6.

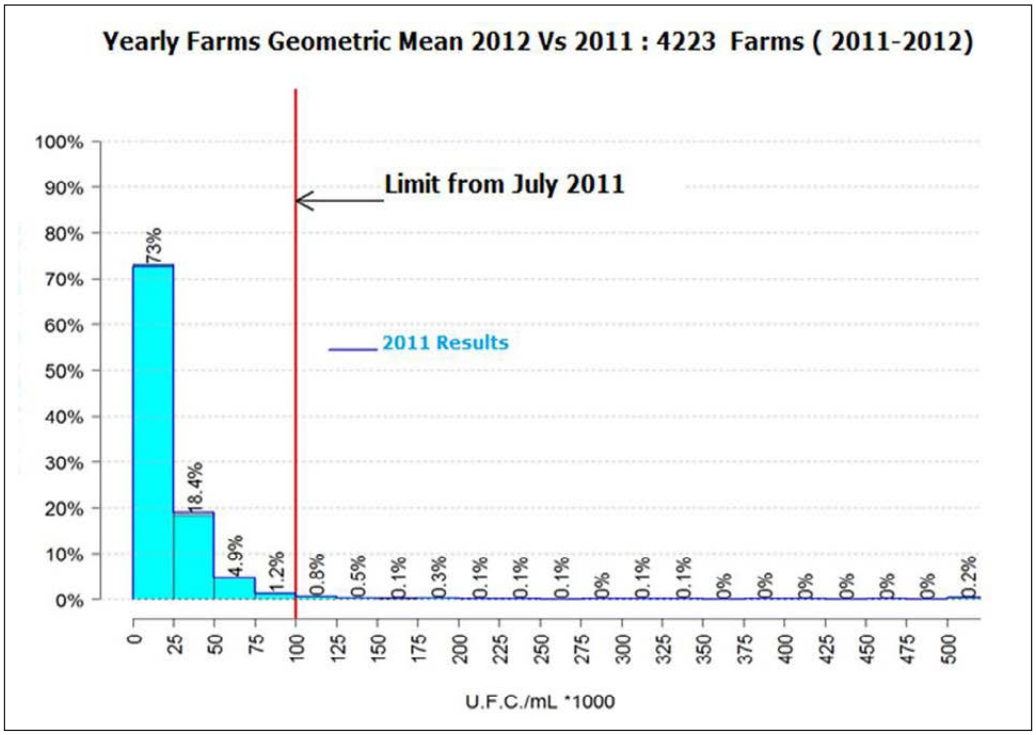


Fig. 7.

gram where the blue line indicates the distribution of the farms' geometric means in the first year (2011) while the blue bars indicate the situation in second year (2012). The difference between the two years is particularly evident in some areas (e.g. 200,000-250,000 SCC). It is interesting to note that there is a decrease in the percentage of farms presenting with 400,000-500,000 cells/ml in 2012 even though the limit was still set at 700,000-600,000 cells/ml. This again suggests that some farmers took preventive actions early with an eye to the more restrictive limits of 2013. Figure 7 presents the same comparison for the TBC. In this case, it is not possible to appreciate graphically the differences between the two years because of the minimal variations of the data across the board. One further analysis was performed on SCC to compare the first semester of 2010 (before the start of the program) and the first semester of 2013 and the data are presented in Figure 8; Figure 9 shows the same comparison between the first semesters of 2012 and 2013. It is evident that the percentage of geometric means with a lower SCC range increased over time while the higher SCC values decreased. For example, in Figure 8 the percentages of farms in the ranges of 300,000-350,000 and 350,000-400,000 cells/ml both decreased while the percentage of farms in the ranges of 100,000-150,000 and 150,000-200,000 cells/ml both increased almost equally. Even though the data come from thousands of different farms, the combination of these representations enabled us to notice a shift to the right in the distribution and through it the positive effects of the introduction of this progressive program. Its effects will be fully appreciated, of course, only in 2014 when the program will have been finished for over 6 months. However we still hypothesize that the impact on compliant productivity will be minimal due to the progressive shift of the majority of the farms to adopting long-term practices that should ensure their continued conformity within SCC limits (KELLY *et* al., 2009; NORMAN *et* al., 1995; SHUKKEN *et* al., 2003). The number of farms with TBC over the limit has already been negligible since 2011, likely because corrective actions against TBC can be effective in a very short time (KELLY *et* al., 2009).

CONCLUSIONS

The decision to repeal the derogations for SCC and TBC in raw milk was made for several reasons but particularly because of the need to stimulate improvements in the quality of milk and traditional dairy products. Statistical evaluation of the data from the previous years (2005-2007) suggested that a sudden closure of the derogations, especially for SCC, would create difficulties for farmers with downstream repercussions on the dairy industry as well as the Health Authority (regional veterinary service in Italy). The introduction of a progressive decrease of the limits allowed regulators to minimize these consequences and focus on the most non-compliant producers. At the same time, it allowed farmers to make improvements and preventive measures during the

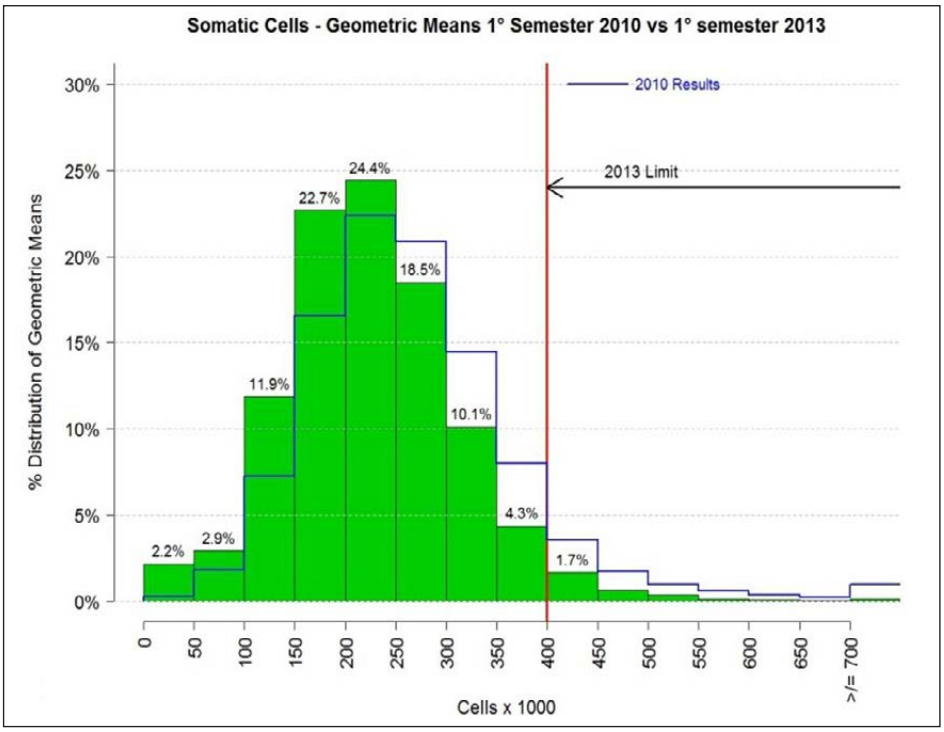


Fig. 8.

24-month program, without excessive conflict or serious effects on farm productivity.

The progressive reduction of the SCC limit also allowed the regional veterinary service to split the management of the non-compliant farms, giving priority to solving the most serious and significant problems first (starting from the first and highest limit of 700,000 cells/ml). The issue of non-compliance in TBC has been nearly resolved; since 2012 the cases of TBC non-compliance appear only occasionally and are often quickly resolved.

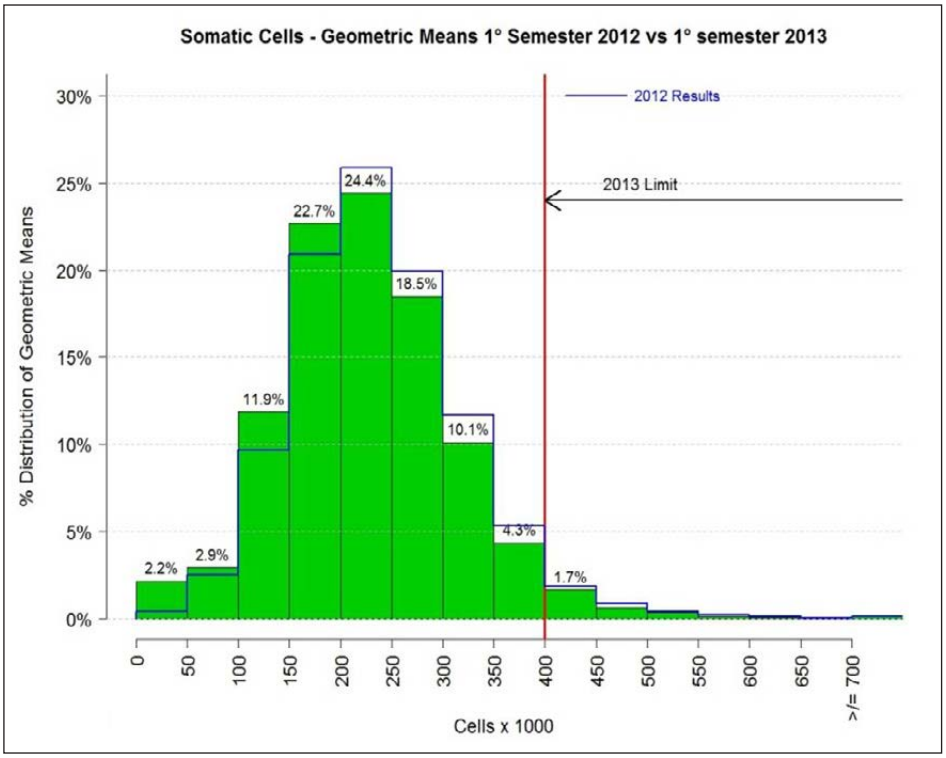


Fig. 9.

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GUIDE FOR AUTHORS
ITALIAN JOURNAL OF FOOD SCIENCE -IJFS

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Acknowledgments. Acknowledgments of assistance are appropriate provided they are not related to analyses or other services performed for a fee. Financial support, thanks for assistance, artic1e number or thesis fulfilment may be included.

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