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REVIEW

LISTERIA MONOCYTOGENES: BIOFILMS IN FOOD PROCESSING

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ABSTRACT

Contamination of food by *Listeria monocytogenes* (*L.m*) frequently occurs in food processing environments, where cells persist due to their ability to attach to surfaces. *L.m* is able to attach and colonize environmental surfaces by producing a three-dimensional matrix of extracellular polymeric substances (EPS) called biofilm; such structures are dynamic systems. Once established, biofilms can serve as a source of product contamination. Moreover, *L.m* in the biofilm state shows a reduced susceptibility to antimicrobial agents. The present review focuses on *L.m* biofilms in food processing environments. In addition, some aspects of biofilm control and eradication are highlighted.

- Keywords: biofilm, food industry, food safety, *Listeria monocytogenes* -

INTRODUCTION

Listeria monocytogenes is a ubiquitous facultative intracellular bacterium that is potentially pathogenic to humans (FARBER and PETERKIN, 1991). This pathogen is frequently isolated from a variety of food products and also from soil, vegetation, fecal matter, sewage, water, and animal feed (DONNELY, 2001; MOLTZ and MARTIN, 2005; TOMPKIN, 2002). Meat, poultry, dairy, vegetables, and ready to eat (RTE) products have all been implicated as vehicles of listeriosis (CONTER *et al.*, 2009a; EFSA, 2007; TEIXEIRA *et al.*, 2007). In fact, ingestion of food contaminated with *L.m* is the primary route of transmission to humans (DUSSURGET, 2008).

Although the incidence of listeriosis is low, it remains a public health concern because of its high mortality rate (20-30%) (SUTHERLAND *et al.*, 2004). *L.m* strains show heterogeneous levels of virulence (LIU *et al.*, 2007) that are regulated by the presence of pathogen-specific genetic factors, which affect the outcome of human and animal infection (CONTER *et al.*, 2009a; THEVENOT *et al.*, 2006). Additional factors, however, may contribute to the pathogenesis potential (CHATTERJEE *et al.*, 2006). *L.m* is able to colonize surfaces, and particular subtypes may persist in food production plants for several years (PIETTE and IDZIAK, 1991). One reason for this persistence may be the ability of *L.m* to form biofilms (SCHLECH *et al.*, 1983). Biofilms are assemblages of microorganisms that adhere to each other and/or to a surface and are embedded in a matrix of exopolymers (MORRIS and MONIER, 2003). Particularly, biofilms allow microorganisms to persist in the environment and resist desiccation, UV light, and treatment with antimicrobial and sanitizing agents (DI BONAVENTURA *et al.*, 2008).

BIOFILM FORMATION

The term "biofilm" was created to describe the sessile form of microbial life, characterized by adhesion of microorganisms to biotic or abiotic surfaces, with consequent extracellular production of polymeric substances (MUSK *et al.*, 2005). It is now generally accepted that bacteria grow preferentially as biofilms (HALL-STOODLEY and STOODLEY, 2005). In fact, in nature and food systems, microorganisms are attracted to solid surfaces. Initially, microorganisms are simply deposited; later, they get attached, grow, and actively multiply to form a colony of cells (ALLISON and SUTHERLAND, 1987). This mass of cells then becomes large enough to entrap organic and inorganic debris and other microorganisms, leading to the formation of a microbial biofilm. In food processing environments, these biofilms may be a few micrometers or several millimeters thick and contain 90-97% water (STOODLEY *et al.*, 2002). Biofilm development can occur on almost any surface in any environment in which viable microorganisms are present. Biofilm formation is a dynamic process and involves a series of steps. Initial attachment is via weak electrostatic and van der Waals' forces and occurs rapidly; but, within a short time, film growth begins with physical attachment of the cells to the surface by complex polysaccharides (OLIVEIRA *et al.*, 2010). In particular, the first step of biofilm formation is conditioning of the surfaces (HOOD and ZOTTOLA, 1997). This conditioning film favors biofilm formation and alters the physicochemical properties of the surface (e.g., changes in hydrophobicity and electrostatic charges) (DICKSON and KOOMARAIE, 1989). The second step of biofilm formation is the attachment of microorganisms to the conditioned surface. During this stage, bacteria can easily be removed. The irreversible attachment of cells happens in the third step, and the removal of cells requires much stronger forces. During this period, the attached cells also produce additional extracellular polymeric substances (EPS) that help anchor the cells to the surface (COSTERTON *et al.*, 1995). Multilayers of bacterial cells entrapped within the EPS-containing matrices develop within the biofilm. Besides EPS and microbial cells, biofilms are composed of proteins, nucleic acids, and lipids, which provide mechanical stability, surface adhesion, and scaffold formation for the three-dimensional architecture that interconnects and immobilizes biofilm cells (FLEMMING and WINGENDE, 2010). However, diverse polymers are used by different species or strains of the same species. The identification of these matrix components may provide clues for the identification and application of matrix-degrading enzymes that prevent formation and/or activate biofilm dispersion (KARATAN and WATNICK, 2009; LANDINI, 2009). Microorganisms within the biofilm grow in the matrix-enclosed microcolonies interspersed within highly permeable water channels (COSTERTON *et al.*, 1994). At some point after film formation, the attached bacteria or pieces of film are released in order to allow the cells to survive and colonize new niches. This release leads to contamination of the product stream. The vegetative cells may reattach in downstream parts of the plant and initiate biofilm formation, completing the cycle. When planktonic cells attach to a surface, there may be a lag before growth commences as they adapt to the sessile state. The cells released from the film may behave like biofilm cells when they reattach, showing no lag and growing more rapidly than primary cells (RASMUSSEN *et al.*, 2005). The drivers for biofilm formation are the following: defence against harmful conditions; colonization of a favorable niche; utilization of potential benefits of the community; protection from the bulk phase environment, where there may be toxins, antibiotics, detergents, or sanitizers; the possibility of organization through inter-cell

signalling; and transfer of genetic information, including resistance genes (JEFFERSON, 2004).

BIOFILMS IN THE FOOD ENVIRONMENT

Microbial adhesion and biofilms are of great importance for the food industry and occur on a variety of food contact surfaces (OLIVEIRA *et al.*, 2007). Biofilms continue to pose concerns to food manufacturers as they are one of the major reasons for limiting the shelf life and favoring pathogen contamination of food products. Moreover, biofilms form a reservoir of contamination that persists where manufacturing plant cleaning is ineffective (SENCZEK *et al.*, 2000). Depending on the specific systems investigated and the nature of the microorganism, biofilms can display a wide range of phenotypes. *L.m* biofilms, grown under static conditions, generally consist of a homogeneous layer of cells and/or microcolonies, with biofilm cells displaying morphology similar to that of planktonic cells. In contrast, *L.m* biofilms grown under continuous flow conditions consist of spherically shaped microcolonies that are surrounded by a network of knitted chains composed of elongated cells (RIEU *et al.*, 2008).

Studies have shown the capacity of *L.m* to persist in the environment for years. Biofilms form not only on processing environment surfaces but also on food itself, which offers the potential for cross-contamination and post-process contamination (KUMAR and ANAND, 1998). Moreover, environmental surfaces such as floors and walls may also be indirect sources of contamination, e.g., transference to food products by vectors such as air, people, and cleaning systems (GIBSON *et al.*, 1999). In food systems, improperly cleaned and sanitized equipment and airborne microbiota are usually considered important niches of contamination (Fig. 1) (SIMÕES and VIEIRA, 2009). Other common sources involved in biofilm accumulation include floors, waste water pipes, bends in pipes, rubber seals, conveyor belts, stainless steel surfaces, glasses, etc. Buna-N and Teflon seals have also been implicated as important sites for biofilm formation (FRANK and KOFFI, 1990; RODAS-SUAREZ *et al.*, 2006). HERALD and ZOTTOLA (1988) observed that *L.m* can attach to stainless steel via produced attachment fibrils. In meat and dairy processing industries, the presence of *L.m* has been found on equipment and utensil surfaces (LÓPEZ *et al.*, 2008).

Importantly, bacterial biofilms have been consistently described as being more resistant to biocides than planktonic cells (GILBERT *et al.*, 2002; SCENHIR, 2009). The reasons for this decrease in susceptibility or "tolerance" is a biofilm-associated phenotype (ASHBY *et al.*, 1994; BROWN and GILBERT, 1993; DAS *et al.*, 1998), which includes decreased metabolism, quiescence, reduced penetration due to the extracellular polymeric matrix (PAN *et al.*, 2006), and enzymatic biocide inactivation (SCENHIR, 2009).

In nature, biofilms may be composed of a single species or represent a consortium of numerous species. Several types of microorganisms (spoilage or pathogenic) are capable of participating in adhesion processes and biofilm formation. Mixed species biofilm formation of several *Staphylococcus aureus* strains with *L.m* has been demonstrated (RIEU *et al.*, 2008). In addition, some researchers have found that *L.m* grows preferably as a biofilm as part of a consortium of bacterial species (JEONG and FRANK, 1994a-b; KALMOKOFF *et al.*, 2001). Other reports examining longer-term biofilm formation have noticed that *L.m* is a poor organism for cell attachment and biofilm formation, and this has led to suggestions that *L.m* may use a primary colonizing bacterium of a different bacterial species to form a biofilm consortium on a surface (MOLTZ and MARTIN, 2005). CARPENTIER and CHASSAING (2004) and TOMPKIN (2002) showed that the "house flora" of food processing premises has a strong effect on the likelihood of finding *L.m* on inert surfaces. A study on mixed biofilm formation of *L.m* in combination with various secondary species showed that mixed species biofilms were established, and depending on the specific combination, they showed increased, reduced, or no effect on the number of *L.m* cells in the biofilm (CARPENTIER and CHASSAING, 2004). Furthermore, an elaborate study on the formation of mixed species biofilms of *L.m* and *Lactobacillus plantarum* showed that mixed species biofilms have the capacity to be more resistant against disinfectant treatments than a single species biofilm or planktonic cells (VAN DER VEEN and ABEE, 2010). Another interesting study on the adherence of *L.m* to preformed *Lactococcus lactis* biofilms with different architectures, matrices, and cell surface properties showed that *L.m* biofilm formation can be influenced by resident biofilms (HABIMANA *et al.*, 2009). The impact of secondary species on *L.m* settlement, biofilm for-

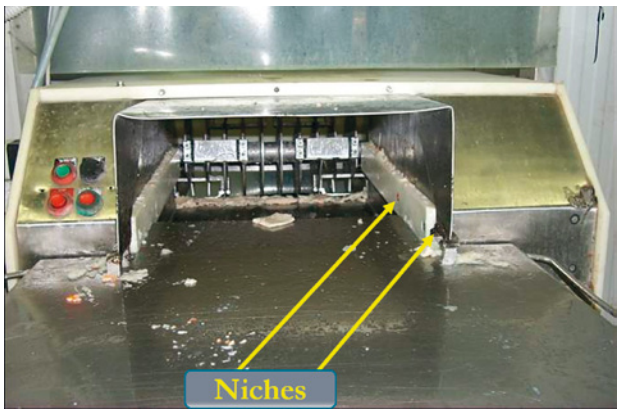


Fig. 1 - Example of possible niches of *L. monocytogenes* in a battering and breading machine.

mation, and persistence in food processing environments remains to be characterized.

Bacterial attachment to surfaces is influenced by the physicochemical properties of both the surface and microorganism, such as temperature and hydrophobicity (GORSKY *et al.*, 2003; HOOD and ZOTTOLA, 1997). It has been suggested that flagellation and motility play a role at the cell level in several stages of biofilm formation (MOENS and VANDERLEYDEN, 1996). Motility has been shown to facilitate attachment to both biotic and abiotic surfaces (BUTLER *et al.*, 1979), since flagella, in addition to being the locomotive organelles of bacterial motility, have also been reported to serve as adhesive structures (MOENS and VANDERLEYDEN, 1996). With regard to food surfaces, many studies have shown the attachment of *L.m* to meat and poultry surfaces (CHUNG *et al.*, 1989; DICKSON and KOOHMARAIE, 1989; THÉNEVOT *et al.*, 2006). However, these studies did not clearly reflect the formation of biofilms with regard to these surfaces.

LISTERIA MONOCYTOGENES AND BIOFILM

L.m can adhere rapidly and firmly to inert surfaces commonly found in the food processing industry. This bacterium has been found to form biofilms on surfaces such as plastic, polypropylene, rubber, stainless steel, and glass and on the interface between two different materials (e.g., plastic and stainless steel) (CHAE *et al.*, 2006; HOOD and ZOTTOLA, 1997; MOLTZ and MARTIN, 2005; SIMÕES *et al.*, 2010). *Listeria* sources in processing plants include conveyor belts, cutters, slicers, bringing and packaging machines, coolers, freezers, floors, and drains (D'ORIO *et al.*, 2007; EKLUND *et al.*, 1995; MIETTINEN *et al.*, 2001).

The mechanisms by which *L.m* survives under harsh physical and chemical stress conditions are, at least in part, due to its ability to form biofilms on surfaces within the food processing environment (CHAE *et al.*, 2006; HOLAH *et al.*, 2004). Several researchers have reported that *L.m* is not capable of forming thick biofilms made up of several layers (9 to 12 Log CFU/cm^2), but rather it adheres to surfaces at levels ranging from 4 to 6 Log CFU/cm^2 (GRAM *et al.*, 2007).

The ability of *L.m* to produce biofilm significantly differs according to the growth temperature and growth surface. For example, *L.m* has the capacity to adhere rapidly to stainless steel surfaces, and this risk is aggravated considering that this bacterium is able to reach an irreversible stage in a few hours (OH and MARSHALL, 1996). The ability to form biofilms at 4°, 12° and 22°C is significantly more pronounced on glass than on polystyrene and stainless steel. Furthermore, at 37°C, both stainless steel and glass allow comparable amounts of biofilm formation, significantly higher than on polystyrene (Figs. 2 and 3) (DI BONAVENTURA *et al.*, 2008). The ability of *L.m* to colonize a surface at low temperatures, used in the food industry to process and store a significant amount of foodstuffs, added to the recent finding that *L.m* cells in biofilms can survive storage at 4° or 10°C for at least 5 days (SMITH *et al.*, 2004), increases the propensity for cross-contamination to foodstuffs.

Recent studies have shown significant differences in biofilm formation on polyvinyl chloride (PVC) (BORUCKY *et al.*, 2003; DJORDJEVIC *et al.*, 2002) or stainless steel (FOLSOM and FRANK, 2006) between different lineages, although the findings were contradictory (CHAE *et al.*, 2006; DJORDJEVIC *et al.*, 2002). DI BONAVENTURA *et al.* (2008) did not find any relationship between phylogeny and the ability to form a biofilm on different surfaces at different temperatures. Furthermore, no differences among environmental and food strains were observed in the experiment. At the same time, differences in biofilm production have been shown (KALMOKOFF *et al.*, 2001; PALMER *et al.*, 2007): serotype 1/2c forms a higher average amount of biofilm than serotype 1/2a on stainless steel and serotypes 1/2a and 4b on glass, at 37°C.

Food contact surfaces have different physicochemical characteristics: hydrophilic (glass, stainless steel) and hydrophobic (polystyrene). Some studies have failed to find a correlation between hydrophobicity and surface attachment. Particularly, CHAE *et al.* (2006) recently found that attachment on glass is independent of hydrophobicity level. Other authors (DI BONAVENTURA *et al.*, 2008) suggested that the hydrophobicity level is correlated with biofilm formation on glass. These conflicting results are probably due to the fact that hydrophobicity can differ between serotypes or strains, and it can change with variation in growth conditions (GIOVANNACCI *et al.*, 2000).

The cell surface is generally considered a significant factor in bacterial attachment to surfaces. Many studies suggest that microbial cell surface charge and hydrophobicity play an important role in the initial steps of microbial adhesion. CHAE *et al.* (2006) demonstrated that high levels of extracellular carbohydrates produced by *L.m* increase their ability to form biofilms, indicating the importance of this characteristic for the biofilm forming ability of a given strain.

Bacterial attachment to surfaces is influenced not only by cell surface and hydrophobicity but also by the presence of particular surface appendages such as flagella and fimbriae. Very little is known about the relationship between motility and biofilm formation and, in any case, only swimming motility has been considered. Recent findings suggest that Gram-positive organisms, including *L.m*, have evolved multiple molecular strategies for the formation of pili on microbial surfaces (HUNG and SCHNEEWIND, 2004). Furthermore, DONS *et al.* (2004) showed

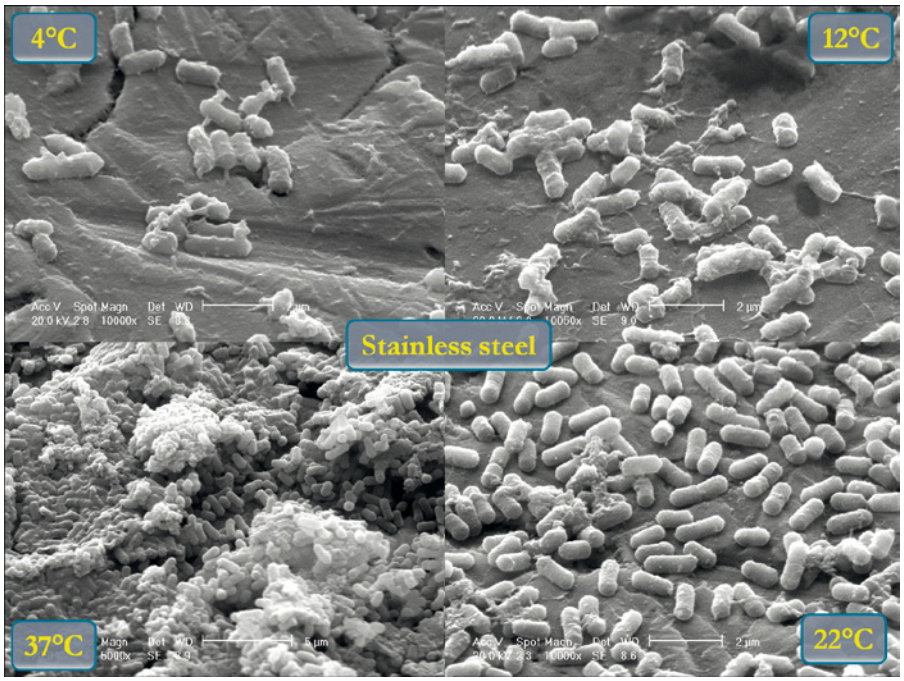


Fig. 2 - Scanning Electron Microscopy (SEM) images of biofilm formed by *L. monocytogenes* on stainless steel at different temperatures.

that motility by swarming is critical in host-cell invasion and virulence of *L.m*. In an attempt to individuate a relationship between *L.m* flagellum-mediated motility (swimming) and biofilm formation, it has been shown that temperature strongly influences flagellum production in *Listeria* spp. (DJORDJEVIC *et al.*, 2002). On the contrary, motility did not show a positive correlation with biofilm-forming ability, regardless of the substratum (DJORDJEVIC *et al.*, 2002). On the whole, functionally active flagella (motility) are probably not required for biofilm formation in *L.m*, and changes in surface structures other than flagella contribute to bacterial attachment to a solid surface.

At both 4° and 12°C, *L.m* produces a rudimentary biofilm consisting only of sparse clusters of cells and minimal amounts of EPS, re-

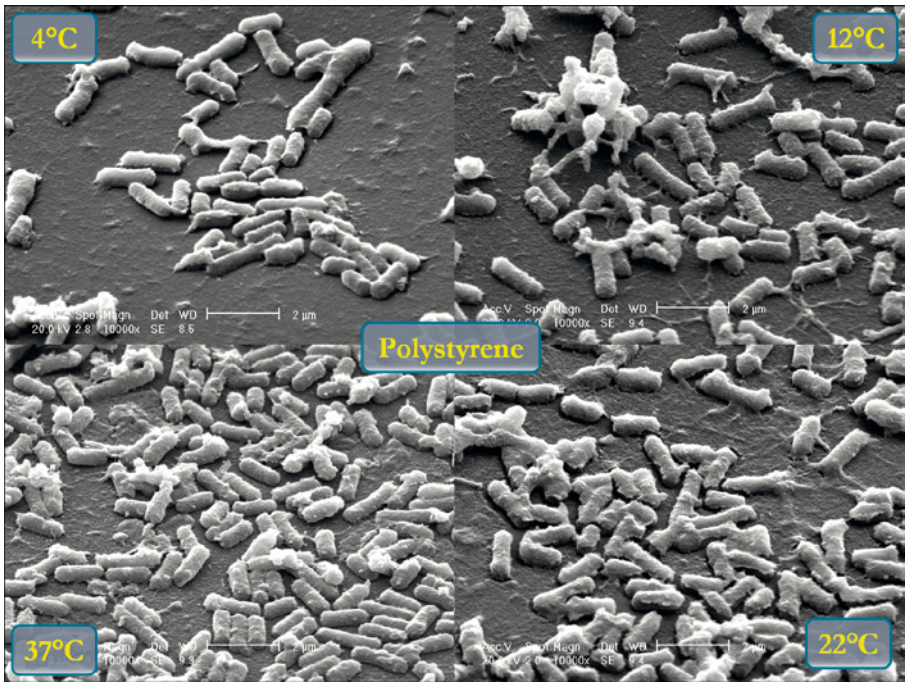


Fig. 3 - Scanning Electron Microscopy (SEM) images of biofilm formed by *L. monocytogenes* on polystyrene at different temperatures.

gardless of the substratum. On the contrary, at 22° and 37°C, the biofilm formed is more complex in terms of cell number and EPS produced. Although only on glass it appeared as a complex three-dimensional architecture, consisting of dense cell aggregates held together by extracellular matrix and surrounded by void areas, probably representing water channels for nutrient circulation and facilitation of waste removal in the biofilm (COSTERTON *et al.*, 1995). The relevant amount of EPS that can be observed in biofilms formed on glass raises a relevant hygienic/sanitary issue, since the chlorine resistance of *L.m* strains may be closely associated with the amount of EPS produced by the biofilm cells (FOLSOM *et al.*, 2006).

The time available for biofilm formation depends on the frequency of cleaning regimes. Product contact surfaces are typically cleaned several times per day, while environmental surfaces such as walls are cleaned once per week. Therefore, there is more time for biofilm formation on environmental surfaces. GIBSON *et al.* (1999) found that although attachment to a variety of surfaces in the food processing environment readily occurred, extensive surface colonization and biofilm formation only occurred on environmental surfaces. Finally, routine cleaning in place (CIP) cycles in food plants may not remove all cells, and the remaining biofilm cells may allow more rapid plant recolonization by a seeding process or by providing a surface to which new cells may readily adhere (MASHALL, 1994). The situation is exacerbated if fouling has occurred, and it is not removed during CIP (HINTON *et al.*, 2002).

BIOFILM AND SANITIZING AGENTS

The elimination of biofilms is a very difficult task because many factors affect detachment. Sanitation, i.e., cleaning and disinfection, is carried out in food processing plants in order to produce safe products with an acceptable shelf life and quality. An effective sanitation program is the major method to control surface contamination. When *L.m* is in the biofilm state, it exhibits an increased tolerance to disinfectants than their free living counterparts; and thick complex biofilms are more difficult to remove than adhered single bacterial cells (KLAEBOE *et al.*, 2006). The recalcitrance of biofilms towards biocide treatment is not in dispute, and the existence of resistance phenotypes induced by various nutrient limitations has been postulated to explain this observation (BROWN and GILBERT, 1993).

SINDE and CERBALLO (2000) found that sanitizers alter the surface properties of materials, and this alteration is directly related to the degree of attachment. Thus, sanitizers have different effects on bacterial adhesion depending on food contact materials. In particular, quaternary ammonium compounds were more effective against *L.m* attachment on stainless steel and rubber than on polytetrafluorethylene (PTFE), for which the effect was very similar. In some studies, it has been reported that *L.m* attached to food contact surfaces exhibited a reduced susceptibility to conventional sanitizers like acid anionic sanitizers and quarternary ammonium compounds (FRANK and KOFFI, 1990). A reasonable explanation for the reduced efficacy of such agents against biofilms is incomplete biofilm penetration by such reactive biocides (HUANG *et al.*, 1995) and the wide variation of environmental conditions that exist on food contact surfaces.

Antimicrobial agents are far more effective against actively growing cells, i.e., the best disinfectants for planktonic cells are not necessarily the most suitable ones for biofilm cells (HOLAH *et al.*, 1990). Caustic chlorine washes can be very effective in removing well-established biofilms by breaking down the polysaccharide matrix, rather than inactivating the microorganisms, probably because high pH favors ion-promoted detachment (COSTERTON *et al.*, 1995). PAN *et al.* (2010) demonstrated that biofilm cells show a reduced susceptibility to a sanitizer. This resistance to a sanitizer was greater on a Teflon substrate than on a stainless steel substrate. Similar results were observed by KRYSINSKI *et al.* (1992), who found that the resistance of *L.m* biofilms on stainless steel was lower than that on polyester or polyester/polyurethane. In addition, BREMER *et al.* (2002) reported that there was a significant difference in the effectiveness of sanitizers against cells attached to stainless steel surfaces than to conveyer belt surfaces (PVC/polyester).

The hydrodynamic conditions under which biofilms grow also have a significant effect on structure (STEWARD and COSTERTON, 2001). Thus, LIU and TAY (2001) found that at relatively high shear stress, as might be found in a food production plant, the biofilm was denser than at lower shear. Therefore, shear stress during biofilm development may profoundly affect the results of disinfection trials and the success of cleaning procedures.

Physical surface phenomena such as increased nutrient concentration at the surface are also often quoted as improving the tolerance to antimicrobial stress of biofilm bacteria. A recent work showed that micro- and nano-emulsions may be effective anti-biofilm agents, although *L.m* films appeared to be resistant (TAKEUCHI *et al.*, 2000). The widespread use of antimicrobial agents such as sanitizers or disinfectants in food processing or equipment cleaning and their effect on antimicrobial resistance is being investigated (CONTER *et al.*, 2009b; SPLENDIANI *et al.*, 2006).

Genetic factors such as the mobility of antibiotic resistance genes found on plasmids and transposons can increase the transfer of anti-

biotic resistance between bacteria (SOMERS and WONG, 2004). The ability of bacteria to adapt to adverse environmental conditions is an important factor in the development of resistance. This is because exposure of the organism to a sublethal level of an antimicrobial agent can lead to adaptation and development of resistance to higher levels of the antimicrobial agent or even cross-resistance to other agents. Although several authors report interactions between the bacterial biofilm physiological state and resistance to antibiotics or biocides, very little information is available on the cross resistance of sessile bacteria to antibiotics and biocides (SCENHIR, 2009). The emergence of resistant bacteria to conventional antimicrobial agents clearly shows that new biofilm control strategies are required (SIMÕES and VIEIRA, 2009).

SOLUTIONS: CONTROL AND REMOVAL OF BIOFILMS

The evaluation of biofilm sanitation procedures should be part of the Hazard Analysis and Critical Control Points (HACCP) development plan in order to control those biofilms prevalent in processing areas (SENCZECK *et al.*, 2000). There are several steps in normal cleaning/sanitation procedures, including cleaning, rinsing, and sanitizing, in that order (GIBSON *et al.*, 1999). The purpose of cleaning is to remove residual materials that may interfere with the sanitation procedure, because the presence of organic material on the biofilms significantly reduces the log kill. This reinforces the importance of adequate cleaning before the use of sanitizers (KRYSINSKY *et al.*, 1992).

Biofilms in the food industry can be eliminated by adopting different strategies such as physical, chemical, and biological methods. The use of enzyme-based detergents as bio-cleaners can serve as a viable option to overcome biofilm problems in the food industry. Due to EPS heterogeneity, a mixture of enzymes may be necessary for sufficient biofilm degradation. However, the use of enzymes in biofilm control is still limited due to the low prices of chemicals used today compared to the costs of enzymes. As a matter of fact, the technology and production of these enzymes and enzyme-based detergents are mostly patent-protected. Moreover, the low commercial accessibility of different enzyme activities limits their current usage. Enzymes and detergents have also been used as synergists to improve disinfectant efficacy (JOHANSEN *et al.*, 1997).

An interesting study on the control of *L.m* in a biofilm by competitive exclusive microorganisms indicated that *L.m* in biofilms can be strongly inhibited (> 5 Log CFU of *L.m*/cm^2) by metabolites of *Enterococcus durans* and *Lactococcus lactis* subsp. *lactis*. These two strains were isolated and screened from biofilms obtained from the floor drains of food processing plants that were free of *L.m* according to records. *Enterococcus durans* and *Lactococcus lactis*, subsp. *lactis* are beneficial organisms and can be used as starter cultures for food fermentation, and they have great potential for controlling *L.m* biofilms in food processing environments (ZHANG *et al.*, 2007).

Studies have shown that some detergents are bactericidal, and some disinfectants may even depolymerize EPS, thus enabling biofilm detachment from surfaces, e.g., oxidants such as chlorine and hydrogen peroxide (JUVEN and PIERSON, 1996). Monolaurin (glycerol monolaurate) was also found to be lethal to *L.m* at low concentrations. In addition, a synergistic interaction between monolaurin and organic acids like acetic acid also caused a pronounced inhibition of *L.m* (NIKOLAEV and PLAKUNOV, 2007). More recently, several authors (GUERRIERI *et al.*, 2009; SORUM and L'ABEE-LUND, 2002) demonstrated that bacteriocin producers showed the best antilisterial potential.

Microbial molecules, commonly used as biopreservatives, such as nisin, lauricidin, reuterin, and pediocin, have been well documented for their biofilm control potential against microorganisms commonly found in dairy processing facilities, including *L.m* (MAHDAVI *et al.*, 2007; ZHANG *et al.*, 2007). Bacteriocin application has also been tested on food packaging materials for the biocontrol of *L.m* on meats (MING *et al.*, 1997). The antagonistic effect was made more effective by decreasing the pH due to lactic acid production in the *Lb. plantarum* biofilm; the outcome was confirmed by the considerable activity in the *Lb. plantarum* biofilm and suspension. Comparing the antilisterial activity of lactic acid bacteria (LAB) biofilms against both planktonic and adherent cells, the *L.m* adherent cells showed a higher resistance. This finding can be explained by the greater resistances that microorganisms display to exogenous agents in biofilms (SAFDAR and ARMSTRONG, 2003).

The use of bacteriophages to control biofilms may provide a natural, highly specific, nontoxic, and feasible approach for controlling several microorganisms involved in biofilm formation (KUDVA *et al.*, 1999). A bacteriophage (*L.m* phage ATCC 23074-B1) was used successfully in *L.m* biofilm inactivation (HIBMA *et al.*, 1997). LU and COLLINS (2007) engineered a bacteriophage to express a biofilm-degrading enzyme. This enzymatic phage had the ability to attack bacterial cells in the biofilm and the biofilm matrix, substantially reducing the biofilm cell counts (more than 99.9% removal). The technology for this process has not yet been successfully developed, and relatively little information is available regarding the action of bacteriophages on biofilms (STOODLEY *et al.*, 2002).

More recently, the use of chlorine dioxide (CD) as a decontamination agent has demonstrated its high effectiveness against a wide variety of

microorganisms. It is a strong oxidizing agent with several advantages, such as the formation of low toxic disinfection by-products, effectiveness at low concentrations, low reaction time, ease of generation, and effectiveness over a broad range of pH values (CHANG *et al.*, 2000). The efficacy of CD gas to disinfect biofilms has not been studied in detail. A recent study demonstrated that low levels of gaseous CD (0.3 mg/L) and aqueous CD (7 mg/L) have equivalent inactivation of *L.m* cells in a biofilm matrix compared to conventional sodium hypochlorite treatment (50 mg/L). Additional research is needed to establish the CD levels and treatment times required for complete inactivation of biofilm cells. The potential use of CD to inactivate biofilms from food processing equipment surfaces should be further explored.

The discovery that many bacteria use quorum sensing to form biofilms makes it an attractive target for their control (POYART-SALMERON *et al.*, 1990). It is conceivable that quorum sensing inhibition may represent a natural, widespread, antimicrobial strategy with significant impact on biofilm formation. A good understanding of the cell-cell signaling phenomena of bacteria such as *L.m* can be used to control the biofilm formation process by the identification of products that can act as quorum sensing antagonists (SHARMA and ANAND, 2002; SINDE and CERBALLO, 2000). This property can lead to the development of new and efficient natural products for biofilm control.

The literature demonstrates that there is no unique strategy with absolute biofilm control efficiency. Nevertheless, the importance of adequate cleaning and disinfection procedures, in order to avoid *L.m* becoming established in processing environments and thus posing a product contamination threat, is widely accepted. Furthermore, continuous environmental monitoring schemes for *L.m* are of major importance to identify potential contamination sources and as an early warning system for food business operators, especially in food processing plants with low *L.m* prevalence in their food products.

CONCLUSIONS AND FUTURE DIRECTIONS

Pathogenic microorganisms in biofilms are the major source of food contaminations and clinical infections. Preferably, preventing biofilm formation would be a more logical option than treating it. Nutrient and water limitation, equipment design, and temperature control are important in biofilm control. In addition, the choice of material herein is crucial in terms of biofilm formation. The hygienic properties of the material can be altered by specific modifications to render it intrinsically antibacterial and/or less susceptible to attachment (VAN HOUDT and MICHIELS, 2010). The deposition of antifouling layers on stainless steel can influence their hygienic status, as demonstrated by the 81-96% decrease in *L.m* attachment and biofilm formation on polyethylene glycol-modified stainless steel. The modified surface properties were obtained by plasma-enhanced cross-linking of polyethylene glycol on stainless steel (DONG and ZHANG, 2005).

Biofilm detectors have been developed to monitor surface colonization by bacteria and allow the control of biofilms in the early stages of development (PEREIRA *et al.*, 2008). Many authors have suggested that *L.m* may be inhibited by some bacteriocin-producing LAB (SABIA *et al.*, 2002; TYOPPONEN *et al.*, 2003). Newer strategies devised for the bio-control of *Listeria* in planktonic form or in biofilms could include the adsorption of bioactive compounds, such as bacteriocins, onto food-contact surfaces (GUERRIERI *et al.*, 2009). Several attempts have been made to avoid biofilm formation by incorporating antimicrobial products into surface materials, coating surfaces with antimicrobials (GOTTENBOS *et al.*, 2001), and/or modifying the surface physicochemical properties (RODRIGUEZ *et al.*, 2007).

Biosurfactants are compounds with surface-active properties, which are produced by microorganisms. Their use has been suggested as an alternative to synthetic products. Their major advantages over synthetic detergents are their low toxicity and highly biodegradable nature. Biosurfactants may also show antimicrobial and anti-adhesive activities. Biosurfactants have been mentioned as promising multipurpose ingredients, which simultaneously exhibit emulsifier, anti-adhesive, and antimicrobial activities, and they are consequently suitable for many food applications (NITSCHKE and PASTORE *et al.*, 2002; NITSCHKE and COSTA, 2007). Therefore, these compounds of microbial origin could be used as detergent formulations to clean surfaces that come in contact with food and prevent food contamination (FREIRE *et al.*, 2009). Thus, biosurfactants could be utilized in the development of new strategies to retard *L.m* surface colonization and biofilm formation (NITSCHKE *et al.*, 2009; ARAUJO *et al.*, 2011).

Plant-derived compounds have gained widespread interest in the search to identify alternatives for microbial control (ESSAWI and SROUR, 2000). It has been postulated that surface pretreatment with plant extracts produces an unfavorable film that promotes microorganism detachment (SANDASI *et al.*, 2010).

To date, rational equipment design that minimizes laminar product flow, reduces static product, and facilitates cleaning and CIP processes can result in reduced bacterial attachment to processing equipment. The next step is the choice of materials modified by a set of coatings and surface modifications in order to reduce surface adhesion and biofilm formation (SZE, 1981; MANDRACCI and RICCIARDI, 2007). The increased biofilm resistance to convention-

al treatments enhances the need to develop new control strategies (SHARMA and ANAND, 2002). Research on microbial biofilms is proceeding on many fronts, with particular emphasis on elucidation of the genes specifically expressed by biofilm-associated organisms, evaluation of various control strategies (including medical devices treated with antimicrobial agents and antimicrobial locks) for either preventing or remediating biofilm colonization of medical devices, and development of new methods for assessing the efficacy of these treatments.

The majority of bacteria are able to form biofilms displaying a large diversity in architecture, phenotypes, and matrix components. Novel insights include factors contributing to phenotypic heterogeneity within biofilms, such as the identification and characterization of a range of matrix building blocks. To date, the prevention and control of *L.m* biofilms in food processing environments should be based on integrated efforts. The food industry should develop cleaning plans and disinfection programs and monitor their efficacy. In addition, the process equipment should be designed with high standards of hygiene in mind. Finally, a better understanding of how *L.m* attaches, grows, and detaches is urgently needed, and much effort should be invested in research on novel biofilm prevention and control strategies.

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PAPER

SOURCES OF *LISTERIA MONOCYTOGENES* CONTAMINATION IN TRADITIONAL FERMENTED SAUSAGE PROCESSING PLANTS IN ITALY

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ABSTRACT

Environmental samples, raw materials and fermented sausages produced in Sardinia (Italy) were analysed in order to investigate the prevalence and enumeration of *L. monocytogenes*. Isolates were identified by single PCR and characterised by multiplex PCR-based serogrouping. The contamination routes of *L. monocytogenes* in the plants were traced using PFGE. In addition, a quantitative assessment of the *in vitro* biofilm formation was carried out. Fermented sausages seem to be regularly contaminated with *L. monocytogenes*: results showed the ability of the pathogen to overcome the hurdles of the manufacturing process and adapt to the processing plant environments, forming biofilms.

- Keywords: biofilm, fermented sausages, *Listeria monocytogenes*, polymerase chain reaction, pulsed-field gel electrophoresis -

INTRODUCTION

Listeria monocytogenes is a ubiquitous organism, widely distributed in the environment. The principal reservoirs are soil, forage and water (EFSA, 2011). Other reservoirs include healthy humans and animals (ILSI, 2005) or infected domestic and wild animals (EFSA, 2011). *L. monocytogenes* has been isolated throughout the pork processing industry (NESBAKKEN *et al.,* 1996), with an increase of contamination along the production line (CHASSEIGNAUX *et al.,* 2002). One of the most recent listeriosis outbreaks (Canada 2008) was linked to the consumption of ready to eat (RTE) pork meat products, causing 22 deaths and 57 confirmed cases (PHAC, 2009). Fermented RTE pork meat products, such as dry and semi-dry sausages have rarely been implicated in food poisoning. Nevertheless, in the manufacturing of traditional fermented products, an empirical application of hurdle technologies often occurs and these products are regularly contaminated with *L. monocytogenes*. This fact may present a major public health concern if the pathogen is able to multiply and reach high levels of contamination (THEVENOT *et al.*, 2006[1]). Previous surveys carried out on traditional fermented products at the end of ripening showed a prevalence of 10% in France (THEVENOT *et al.,* 2005), 10.6% in Chile (CORDANO and ROCOURT, 2001) and 15.2% in Italy (DE CESARE *et al.,* 2007). In a former study, *L. monocytogenes* was found in 40% of "*Salsiccia Sarda*" at the end of ripening (MELONI *et al.,* 2009) with contamination levels always lower than 100 CFU/g. "*Salsiccia Sarda*" is the primary meat product (37% of consumption) of the meat supply chain in the Sardinia region (Italy) and is included on the national list of traditional food products. It is one of the typical Italian semi-dry sausages (a_w ranging from 0.90 to 0.95), naturally fermented (GRECO *et al.,* 2005) and marketed locally and regionally in Italy (COMI *et al.,* 2005). The main composition and characteristics of "*Salsiccia Sarda*" include: minced lean pork (87%); pork back fat (8%); salt (3%); a mixture of sugars and additives (0.8%); garlic (0.15%); ground pepper (0.25%); and a mixture of whole pepper, nutmeg, cloves and pimento (0.8%). The mixture, refrigerated overnight, is stuffed into natural pork casings. After they are first warmed up at 20°-22°C for 4-6 h, the products are dried for six days in a fermentation chamber. On the first day of drying, the products are stored at 20°-22°C and 60% relative humidity. Over the next five days of drying the temperature is gradually reduced to 15°C and the relative humidity is gradually increased to 70%. Ripening is then carried out for 15 days in store-rooms at 15°C and 70-75% relative humidity. The final products are cylindrical (about 30 mm Ø), about 40-45 cm long, shaped like horseshoes, and about half a kg each in weight. The normal pH of "*Salsiccia Sarda*" at the end of ripening is 5.28 (GRECO *et al.,* 2005). *L. monocytogenes* may survive during the processing of the fermented meat products due to its high tolerance to low pH conditions and high salt concentrations (FARBER and PETERKIN, 1991) and if the standard hurdle technologies are ineffective. The main hurdles used during the processing of dry fermented products are nitrite and salt content, the decrease in redox potential and water activity (a_w) that inhibit many aerobic bacteria in the early stages of production (*Pseudomonas spp.* and other Gram-negative bacteria), and selecting the lactic acid bacteria (LAB) which cause the pH to decrease (BARBUTI and PAROLARI, 2002). These hurdles, with the coagulase negative *Staphylococci* development and the length of the ripening period, are essential for the microbial safety and stability of quick-ripened fermented sausages, which are not greatly dried. Pork meat used for the manufacturing of sausages may be contaminated from a wide variety of sources and *L. monocytogenes* once introduced in the processing plants can persist over time in the environment, contaminating food processing machines (LOPEZ *et al.,* 2008). *L. monocytogenes* forms assemblages of surface-associated microbial cells that are enclosed in hydrated extracellular polymeric substances and grow in biofilms on surfaces in contact or not with the food (GANDHI and CHIKINDAS, 2007), such as floor drains, storage tanks, hand trucks, conveyor belts and other food contact materials (MAFU *et al.,* 1990). The presence of the pathogen on surfaces in contact and without contact with food increases the food safety risk (KIM and FRANK, 1995). Thus, *L. monocytogenes* may become an important source of secondary contamination of food products, and without suitable sanitisation procedures, cross-contamination of the meat products may occur (SAMELIS and METAXOPOULOS, 1999). The main objective of the present study was to evaluate the occurrence of *L. monocytogenes* in the fermented sausage production of the Sardinia region (Italy). Isolates were identified by single PCR and further characterised by multiplex PCR-based serogrouping. The contamination routes of *L. monocytogenes* in the plants were traced using PFGE. In addition, a quantitative assessment of the *in vitro* biofilm formation was carried out in order to investigate the potential for persistence.

MATERIALS AND METHODS

Sampling

A total of 170 samples from environments, raw materials and final products from two large (A and D > 300 tons of "*Salsiccia Sarda*" per year)

and two small (B and C from 50 to 100 tons of "*Salsiccia Sarda*" per year) processing plants, located in different provinces of Sardinia (Italy), were sampled in order to investigate the prevalence, ecology and genetic profile of *L. monocytogenes*. All of the processing plants were representative of the regional fermented sausage production and some characteristics are summarised in Table 1. Three of the processing plants (A, B, D) were only sampled once (S1). In order to evaluate the role of swine carcasses as a possible source of plant contamination and the persistence of *L. monocytogenes*-adapted strains, in plant C (randomly chosen) sampling was repeated within three months (S2). Details on samples collected from each plant are provided in Table 2. A total of 132 environmental samples were collected: 96 swabbed surfaces without contact with meat (SWCM) and 36 surfaces in contact with meat (SCM). SWCM and SCM were sampled during the production stages by swabbing with 10 cm by 10 cm sterile gauze pads rehydrated with 10 mL of neutralising buffer (Solarcult sampling kit, Biogenetics, Padova, Italy) and using a sterile template to delineate the swabbed area of 100 cm^2. Sampling locations were chosen in order to represent those most likely to present *L. monocytogenes* contamination. Sampling sites for SWCM included walls and floor drains of the ground meat store-rooms, drying and ripening rooms, processing and packaging/shipment rooms. Regarding SCM, the sampling sites included work tables, sausage trolleys, hooks, mincing, mixing and stuffing machines. A total of 16 samples from ground meat and 10 from fermented sausages at the end of ripening were collected. Only during the repeat sampling (S2) in plant C12 samples from swine carcasses were collected: 6 of raw pork meat with and without rind and 6 swab samples of vertebral canal between the first thoracic vertebra and the seventh lumbar vertebra, in correspondence with the bone saw surface of the swine carcasses. The carcasses were stored at +4°C and were sampled before their entry in the processing line. Samples were collected by swabbing as previously described. All of the items were aseptically sampled, placed in sterile bags (kept in ice boxes at +3°C) during transport and were immediately analysed upon arrival at the laboratory.

Detection and enumeration of *L. monocytogenes*

Detection and enumeration of *L. monocytogenes* was carried out using the ISO 11290-1:1996 and 11290-2:1998 protocols, respectively. Samples of raw pork meat, ground meat, fermented sausages and swabbed samples were homogenised 1/10 with Fraser broth base (Biolife, Milan, Italy) in a Stomacher Lab Blender 400 (Seward Medical, London, UK) for 2 min. The homogenates were incubated at 20°C for 1 h, in order to resuscitate stressed microorganisms. For the enumeration of *L. monocytogenes*, 1 ml of each inoculum was distributed over the surface of three Aloa (Biolife) 90 mm plates using a sterile spreader. The plates were incubated at 37°C for 48 h. For detection of *L. monocytogenes*, the homogenates were supplemented by Fraser half-selective supplement (Biolife) and incubated at 30°C for 24 h. Afterwards, 0.1 mL of the primary enrichment was inoculated in 10 mL of Fraser broth supplemented (Biolife) by Fraser selective supplement (Biolife) and incubated at 37°C for 48 h. Cultures were streaked onto Oxford (Oxoid, Milan, Italy) and Aloa (Biolife) plates and incubated at 30° and 37°C for 48 h, respectively. From each plate of the primary and secondary enrichment, five colonies presumed to be *Listeria spp.* were streaked on Tsyea plates (Biolife) and incubated at 37°C for 24 h. Colonies were selected for typical appearance on Tsyea and submitted to Gram staining, catalase and oxidase tests. Haemolytic activity and CAMP tests on sheep blood agar were performed for confirmation of *L. monocytogenes*. Biochemical characterisation of all the isolates was performed using the API Listeria identification system (bioMérieux, Marcy l'Etoile, France).

Molecular identification and characterisation

Single PCR-based identification

The phenotypic identification of *L. monocytogenes* isolates was confirmed by a single PCR-based method (sPCR) aimed at *prfA* gene fragment detection. *L. monocytogenes* isolates were grown on BHI (Oxoid) at 37°C for 16-18 h. The cells were pelleted by centrifugation of 1 mL at

Table 1 - Some characteristics of the four Sardinian processing plants.

Plants	Tons/year	Processing line complexity	Origin of the groundmeat	Characteristics of the ground meat	Ripening period (fermented sausages)
A	300-500	+++	Domestic	Fresh	15 days
B	50-100	++	Domestic	Fresh	15 days
C	50-100	++	Domestic	Fresh	15 days
D	>500	++++	Domestic /European	Fresh	7-15 days

Processing line complexity: ++ (simple equipment); +++ (more equipment, more complex); ++++ (a lot of equipment, with a complex design).

12,000 rpm for 5 min at 4°C and washed twice in phosphate-buffered saline (PBS). DNA was extracted by suspending the pellet in 1 mL of PBS, which was boiled for 5 min and centrifuged at 10,000 rpm. The supernatant was quantified with a UV-1700 PharmaSpec spectrophotometer (Shimadzu, Kyoto, Japan) at OD_{260} and then stored at -20°C until use. Heterologous DNA of *S. xylosus* ATCC 29971 was used as a negative control and DNA of *L. monocytogenes* reference strain ATCC 19115 was used as a positive control. The sPCR was carried out with the primer set Lip1 and Lip2 (D'AGOSTINO *et al.,* 2004; JOFRÉ *et al.,* 2005) which produces a fragment of 274_{bp} (SIMON *et al.,* 1996). All amplification reactions were performed in a final volume of 50 µL containing 5 µL of DNA, 5 µL of 10X PCR buffer (Invitrogen, Carlsbad, USA), 2.5 mM of $MgCl_2$, 0.3 mM each of dNTP, 0.3 mM each of primer and 1U of Platinum Taq DNA polymerase (Invitrogen). All amplification reactions were performed in a GeneAmp 2700 Thermal Cycler (Applied Biosystems, Foster City, CA, USA) programmed as follows: denaturation at 94°C for 2 min, annealing at 55°C for 30 sec and elongation at 74°C for 1 min, followed by a final extension period at 74°C for 5 min. The amplified fragments were separated by 1.5% agarose gel electrophoresis (Roche diagnostics, Milan, Italy) in 1X Tris-acetate EDTA (TAE, Invitrogen) and stained with ethidium bromide (0.1 mg/mL) for 20 min. The gels were observed and digitalised by the Gel-Doc UV trans-illuminator (Bio-Rad, Hercules, CA, USA).

Multiplex PCR-based serogrouping

Multiplex PCR-based serogrouping was carried out using the target genes *lmo0737*, *lmo1118*, *ORF2819*, *ORF2110* and *prs* (DOUMITH *et al.,* 2004). The multiplex PCR products were resolved by electrophoresis on 1.5% agarose gel in 1X TAE (Invitrogen) and stained with ethidium bromide (0.1 mg/mL) for 20 min. The gel images were visualised and captured using the Gel-Doc UV trans-illuminator (Bio-Rad).

DNA macrorestriction and pulsed-field gel electrophoresis (PFGE)

Isolates were submitted to DNA macrorestriction with *Apa*I and *Asc*I (New England Biolabs, Beverly, MA, USA). The separation of the restriction fragments was carried out by PFGE in a CHEF Mapper XA system (Bio-Rad) using the PFGE-PulseNet protocol (GRAVES and SWAMINATHAN, 2001). Gel images were visualised and captured using the Gel-Doc UV trans-illuminator (Bio-Rad). The banding patterns for each enzyme were assigned through visual analysis of the restriction profiles. Isolates were designated genetically indistinguishable (same pulsotype) when their restriction patterns had the same number of bands and the corresponding bands were the same apparent size (TENOVER *et al.,* 1995; GRAVES *et al.,* 2005). *Asc*I and *Apa*I macrorestriction patterns were analysed using BioNumerics software (Applied Maths, Sint-Martens-Platen, Belgium). The similarity between restriction patterns, based on bands position, was expressed as a Dice coefficient correlation. The position tolerance was optimal when set at 1.0 and 2.0% for *Asc*I and *Apa*I, respectively. Clustering and construction of dendrograms were performed by the Unweighted Pair Group Method using arithmetic averages (UPGMA) combining both *Asc*I and *Apa*I macrorestriction patterns into one unique PFGE profile.

Quantitative assessment of *in vitro* biofilm formation

Isolates were tested for their ability to attach to abiotic surfaces forming biofilm. The quantitative assessment of the *in vitro* biofilm formation was carried out on sterile 96-well polystyrene microtiter plates using the method described by STEPANOVIC *et al.* (2004), with some modifications. Isolates were grown for 24 h in 2 mL of BHI broth. All the wells of a polystyrene microtiter plate were filled with 230 µL of BHI broth. Afterwards, 21 wells per strain were filled with 20 µL of culture. Each plate included 12 wells of BHI broth without inoculum, as a negative control. Microtiter plates were incubated at 30° for 24 h. At the end of the incubation the content of the wells was removed and the microtiter plate washed three times with 300 mL of sterile, distilled water in order to remove loosely attached bacteria. The remaining attached bacteria were fixed with 250 µL of methanol per well, and after 15 min the wells were emptied and air-dried. Each well was stained with 250 µL of crystal violet for 5 min. After staining, the microtiter plates were washed under running tap water, then air-dried and the dye bound to the adherent cells was solubilised with 250 µL of 33% (v/v) glacial acetic acid per well. The microtiter plates were read spectrophotometrically (OD_{620}) using a Sunrise RC absorbance reader (Tecan, Maennedorf, Switzerland). The strains were divided into four categories: no biofilm producers (NP, OD=<0.5), weak producers (WP, OD= ≥0.5<1.0), moderate producers (MP, OD= ≥1.0<1.5) and strong producers (SP, OD= ≥1.5).

RESULTS

Detection and enumeration of *L. monocytogenes*

The results (Table 2) showed the presence of *L. monocytogenes* in all of the fermented sausage processing plants (overall prevalence in the environments: 15%). None of the samples from pork carcasses was positive for *L. mono-*

Table 2 - *Listeria monocytogenes* in environments, raw materials and final products of four fermented sausage processing plants.

Plants		SCM***		SWCM****		Pork carcasses		Ground meat		Fermented sausages	
		Positive/Total	%	Positive/Total	%	Positive/Total	%	Positive/Total	%	Positive/Total	%
A	S1	0/6	-	2/18	11.11	-	-	2/4	50	2/2	100
B	S1	2/6	33.33	4/18	22.22	-	-	2/4	50	0/2	-
C	S1*	0/10	-	6/18	33.33	-	-	2/4	50	2/2	100
	S2**	0/8	-	2/20	10	0/12	-	-	-	-	-
D	S1	2/6	33	2/22	9	-	-	0/4	-	4/4	100
Grand total		4/36	11.11	16/96	16.66	0/12	-	6/16	37.50	8/10	80

*S1: first sampling; **S2: repeated sampling; ***SCM: Surfaces in contact with meat; ****SWCM: Surfaces without contact with meat.

cytogenes. The occurrence was 37% in ground meat and 80% in the fermented sausages. These products did show detectable levels always below 10 CFU/g, complying with the food safety criteria provided for RTE foods able to support the growth of *L. monocytogenes* (EFSA, 2005). According to THEVENOT *et al.* (2006[2]), several factors, such as the manufacturing process, the use of spices with antioxidative or antimicrobial properties, and the growth of the natural competitive microflora should significantly reduce the count of *L. monocytogenes* in fermented sausages. Altogether, 170 strains of *Listeria spp.* were isolated: 39% were *L. welshimeri*, 23% *L. monocytogenes*, 21% *L. innocua*, 10% *L. grayi*, 4% *L. ivanovii*, and 3% *L. seeligeri*. A subset of 20 *L. monocytogenes* isolates was selected and primarily identified by single PCR. From each processing plant, *L. monocytogenes* strains were selected in order to include almost 50% of each category of positive samples. Isolates were further characterised by multiplex PCR-based serogrouping, PFGE and *in vitro* biofilm formation. A summary of the phenotypic and genotypic characteristics of the 20 *L. monocytogenes* isolates is reported in Table 3.

Table 3 - Distribution of 20 *Listeria monocytogenes* isolates in the four processing plants, in relation to serotype, pulsotype, PFGE profile and *in vitro* biofilm production.

Plant and source of contamination		Strain code	Serotype	Pulsotypes		PFGE profile	Biofilm production
				*Asc*I	*Apa*I		
A	Ground meat store room: floor drains	*A1*	*4b*	I	I	*9*	NP
	Ground meat	*A2*	*4b*	II	II	*11*	MP
	Fermented Sausage	*A3*	*1/2b*	III	III	*13*	NP
B	Ground meat store room: floor drains	*B1*	*1/2b*	IV	IV	*4*	MP
	Ground meat store room: floor drains	*B2*	*1/2b*	IV	IV	*4*	NP
	Ground meat	*B3*	*1/2b*	V	V	*2*	WP
	Drying room: floor drains	*B4*	*1/2b*	V	V	*2*	WP
	Drying room: floor drains	*B5*	*1/2b*	VI	VI	*7*	NP
	Ripening room: hooks	*B6*	*1/2b*	VII	VII	*1*	WP
	Ripening room: hooks	*B7*	*1/2b*	VII	VII	*1*	WP
C	Drying room: floor drains	*C1*	*1/2b*	VIII	VIII	*12*	NP
	Ground meat	*C2*	*1/2b*	IX	IX	*5*	WP
	Fermented sausage	*C3*	*1/2b*	IX	IX	*5*	NP
	Shipment room: floor drains	*C4*	*1/2b*	X	X	*8*	WP
	Ripening room: floor drains	*C5*	*1/2b*	X	X	*8*	NP
	Ripening room: floor drains	*C5bis*	*1/2b*	X	X	*8*	MP
D	Fermented sausage	*D1*	*1/2a*	XI	XI	*6*	WP
	Fermented sausage	*D2*	*1/2a*	XII	XII	*3*	WP
	Processing room: work tables	*D3*	*1/2a*	XIII	XIII	*10*	WP
	Processing room: floor drains	*D4*	*1/2a*	XIV	*n.t.**	*n.t.**	NP

*n.t.: not typeable; NP: no biofilm producers; WP: weak producers; MP: moderate producer.

Molecular identification and characterisation

Single PCR-based identification

The amplification product of 274_{bp} was found in all the strains phenotypically identified as *L. monocytogenes*. The amplification products always exhibited the same electrophoretic pattern. These results confirm that the *prfA* gene is a reliable target gene for identification of the pathogen (VAZQUEZ-BOLAND *et al.*, 2001).

Multiplex PCR-based serogrouping

The prevalent serotype was 1/2b (70%), followed by 1/2a (20%) and 4b (10%). No specific serotype was recovered during the processing and ripening of the sausages (THEVENOT *et al.*, 2005).

DNA macrorestriction and pulsed-field gel electrophoresis (PFGE)

A high heterogeneity of pulsotypes (14 with *Asc*I and 13 with *Apa*I) occurred within the plants (Table 3). Restriction patterns were combined in 13 PFGE profiles appearing to be plant-specific. One strain from plant D was typeable only with *Asc*I (pulsotype XIV) and consequently was not assigned to any PFGE profile. Within each processing plant, particularly in the two large ones (A and D), a high heterogeneity distribution of PFGE profiles was observed. The presence of PFGE profile five both in ground meat and in the fermented sausages, may evidence the ability of specific strains of *L. monocytogenes* to survive during "*Salsiccia Sarda*" fermentation. Furthermore, the recovery of PFGE profile eight in floor drains from different rooms in both samplings (S1 and S2) showed the ability of the pathogen to adapt and persist in the different processing environments of plant C. The PFGE profiles were allotted into three major PFGE clusters (similarity ≥ 70%) labelled A-B-C (Table 4 and Fig. 1). Cluster A included 9 isolates of serotype 1/2b (8) and 1/2a (1) from three processing plants (B-C-D). Cluster B included 6 isolates belonging to different serotypes: 1/2a (1), 1/2b

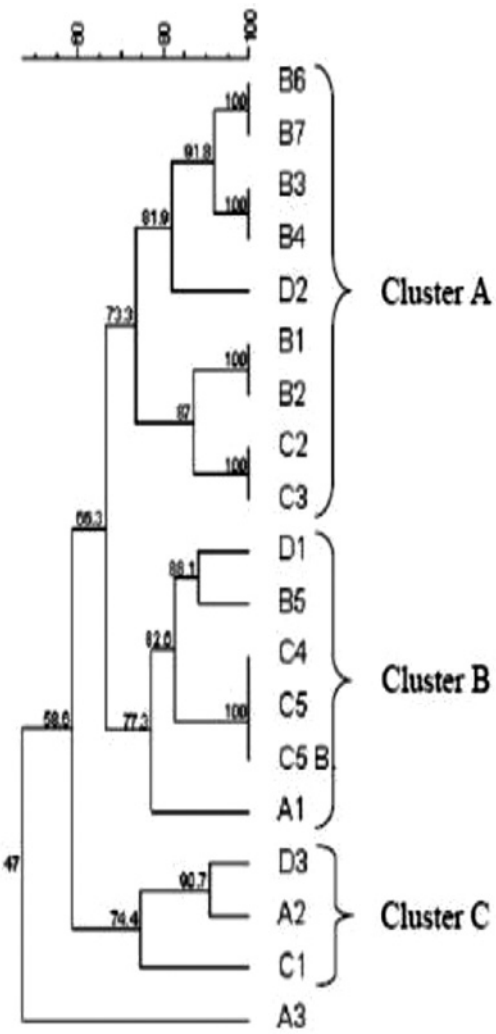


Fig. 1 - UPGMA clustering of the 19 *Listeria monocytogenes* PFGE profiles.

Table 4 - Clustering of 18 *Listeria monocytogenes* strains.

Cluster	Overall similarity	PFGE profile	N° of isolates	Serotype	Source of contamination in the plants
A	73.3%	1	2	*1/2b*	Hooks in the ripening room
		2	2	*1/2b*	Ground meat and floor drains in the drying room
		3	1	*1/2a*	Fermented sausage
		4	2	*1/2b*	Floor drains in the store room
		5	2	*1/2b*	Ground meat and Fermented sausage
B	77.3%	6	1	*1/2a*	Fermented sausage
		7	1	*1/2b*	Floor drains in the drying room
		8	3	*1/2b*	Floor drains in the shipping room (1) and ripening room (2)
		9	1	*4b*	Floor drains in the store room
C	74.4%	10	1	*1/2a*	Work tables in the processing room
		11	1	*4b*	Ground meat
		12	1	*1/2b*	Floor drains in the drying room

(4) and 4b (1) from all of the processing plants (A-B-C-D). Cluster C included three isolates of serotype 1/2a, 1/2b, and 4b from plants A-C-D. One strain (A3) of serotype 1/2b isolated from fermented sausage was an outlier, showing low similarity (47%) with the other clusters.

Quantitative assessment of *in vitro* biofilm formation

More than half (60%) of the strains previously identified and characterised by molecular methods were able to attach to abiotic surfaces forming biofilm. The results of this experiment are shown in Table 3 and Fig. 2. Serotypes 1/2a and 1/2b showed weak *OD* (*OD*=≥0.5<1.0) or moderate *OD* (*OD*=≥1.0<1.5) ability in biofilm formation. The microtiter plate assay is a useful method to assess the ability of *L. monocytogenes* strains to attach to abiotic surfaces (STEPANOVIC *et al.*, 2004).

DISCUSSION AND CONCLUSION

In this study, the contamination of four "*Salsiccia Sarda*" processing plants representative of Sardinian fermented sausage production was investigated on samples from surfaces (with and without contact with meat), ground meat and fermented sausages. Our results show the overall presence of *L. monocytogenes* in environmental niches, raw materials and final products. *L. monocytogenes* was not detected in the pork carcasses prior to processing: contamination of the final products appears to be due to strains already present in the processing environments (LOPEZ *et al.*, 2005), with all of the facilities serving as the source of product contamination (CHASSEIGNAUX *et al.*, 2002). In the Sardinian processing plants and their products, *L. monocytogenes* serotype 1/2b predominated, followed by serotypes 1/2a and 4b. Previous studies have also reported the presence of these serotypes in meat processing environments (CHASSEIGNAUX *et al.*, 2001; THEVENOT *et al.*, 2006[1]). The recovery of serotypes frequently associated with epidemic or sporadic cases of listeriosis (McLAUCHLIN *et al.*, 2004) is an interesting result in terms of public health protection. Previous surveys have shown that lineage I strains of serotype 4b belonging to a clonal group (DUP-ID 1038) linked to several listeriosis outbreaks (DE CESARE *et al.*, 2001) were recently recovered in the same meat products (MELONI *et al.*, 2009). It must be emphasised that the presence of such strains in the processing plants, and as a consequence in fermented sausages, may represent a hazard if the pathogen is able to multiply during the ripening of the product and reach levels higher than 1,000 CFU/g (ROSS *et al.*, 2002; THEVENOT *et al.*, 2006[1]). Thirteen different PFGE profiles were obtained indicating a great level of diversity among the strains collected from the Sardinian processing plants and their products. The high number of PFGE profiles and their heterogeneous distribution within the plants is in agreement with the results of previous surveys carried out in meat processing plants (THEVENOT

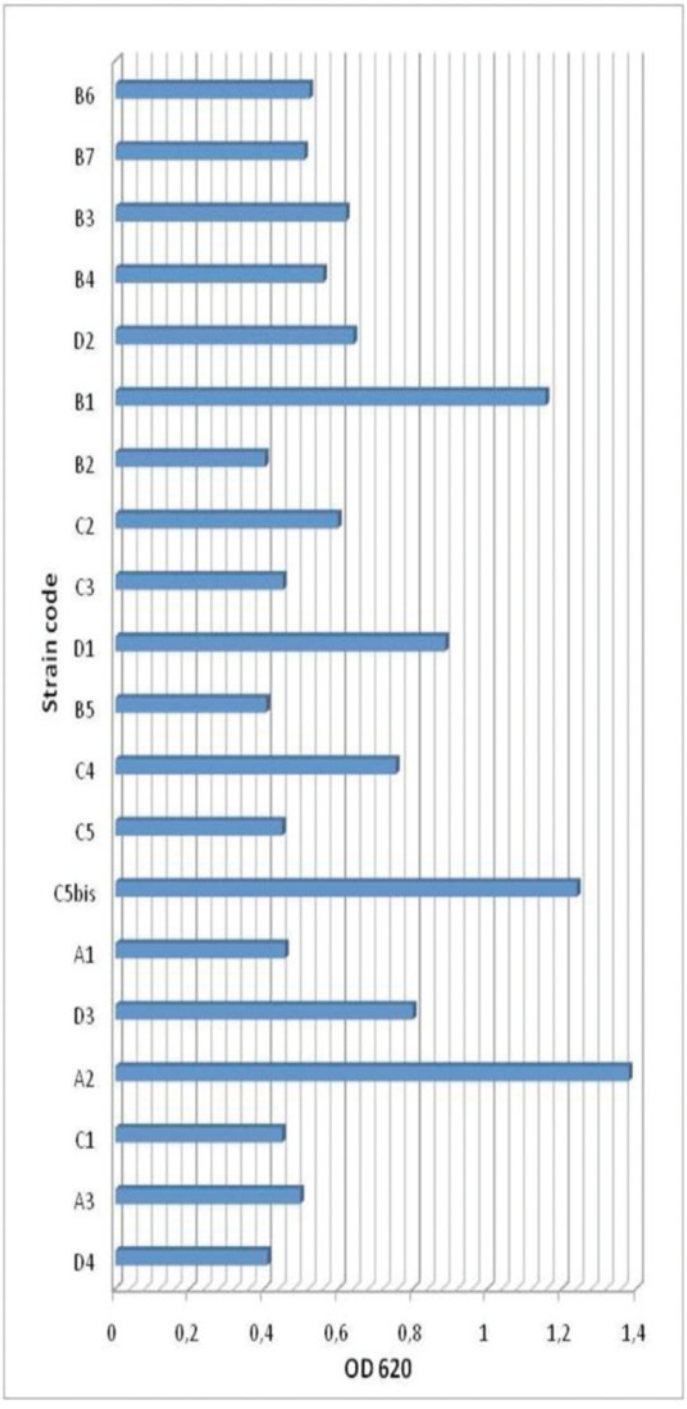


Fig. 2 - Biofilm production of the *Listeria monocytogenes* strains.
The quantitative assessment of the *in vitro* biofilm formation was evaluated spectrophotometrically (OD620). The strains were divided up into four categories: no biofilm producers (NP= OD <0.5), weak producers (WP= OD ≥0.5<1.0), moderate producers (MP= OD ≥1.0<1.5) and strong producers (SP= OD ≥1.5). Altogether, 60% of the strains were able to attach to abiotic surfaces forming biofilm, showing weak or moderate ability in biofilm formation.

et al., 2006[1]). Such subtypes appear to be unique to each processing plant (FUGETT *et al.,* 2007). These results may be due to the limited number of isolates included in this study or to the great diversity of the *L. monocytogenes* strains collected from ground meat, but also to the quantities used by each plant. The large plants exhibited the highest PFGE profile heterogeneity, since these plants also used the greatest amount of raw meat from several different sources (domestic and European). Numerical analysis of the PFGE profiles showed that the isolates included in the study could be allotted into three major PFGE clusters labelled A-B-C. Contrary to all expectations, these three genomic divisions were not linked with the flagellar antigen type, in spite of what was described by several authors (CHASSEIGNAUX *et al.,* 2001; AUTIO *et al.,* 2003; THEVENOT *et al.,* 2006[1]). In this study, *L. monocytogenes* strains were thought to be persistent when the same PFGE profile occurred in samples collected from the same plant equipment C after an interval of three months: such strains were found in several floor drains. The persistence of the same PFGE profile (eight) is probably due to two interacting factors. First of all, the nature of the strains themselves, which have a moderate ability to form biofilms and have adapted to environments where meat is processed. Several authors have shown that certain *L. monocytogenes* strains were more capable of causing persistent contamination of meat processing plants than others (AUTIO *et al.,* 2003; THEVENOT *et al.,* 2006[1]) probably due to their ability to form biofilms. The second factor affecting persistence may be the presence of ineffective cleaning and disinfection measures. Treatments need to reach the contamination site in sufficient quantities and duration in order to be effective (THEVENOT *et al.,* 2006[1]). This may not always occur with routine cleaning procedures because of the complexity of the processing line structure and because environmental niches, such as floor drains can be a site critical for controlling contamination of the processing plant environment and food products (TOMPKIN, 2002). Decontaminating floor drains is especially challenging because, when entrapped in a biofilm, *L. monocytogenes* is afforded unusual protection against available disinfectants and treatments (ZHAO *et al.*, 2004). *L. monocytogenes* strains can become well established in the floor drains and persist as resident microbial flora for up to several years. This study has shown the presence of adapted *L. monocytogenes* strains able to survive during sausage fermentation, overcoming the current hurdles of the "*Salsiccia Sarda*" manufacturing process. Our results highlighted that *L. monocytogenes* is able to survive in meat processing plants by forming biofilms on abiotic surfaces. The surface used for the *in vitro* experiment (polystyrene) approximately mimics the plastic material used in the plants (e.g., conveyor belts). Further testing with appropriate steel specimens of each plant is needed in order to understand better the mechanism of biofilm formation *in vivo*. To decrease the presence of *L. monocytogenes* in the traditional fermented sausages at the end of ripening, food business operators should adhere to accurate application of hurdle technologies. Products can also become contaminated through contact with work surfaces and equipment, even after routine cleaning and disinfecting operations (THEVENOT *et al.,* 2006[1]). More attention should be focused on respecting good manufacturing practices and the application of HACCP principles.

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PAPER

USE OF RUSTIC PIGS TO MANUFACTURE PORK SALAMI FREE OF COLOUR AND TASTE ENHANCERS

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ABSTRACT

Two different pork sources (early and heavy-rustic pig breed) were evaluated to manufacture semi-ripe Salami free of two additives with enhancing properties (Ponceau red and sodium glutamate). Two trials were performed using salamis from the above pork sources. The eating quality and several related ripening properties were determined. Ponceau red colouring improved lean colour in early Salami, but masked the natural pinkness as a result of nitrous-myoglobin formation in rustic Salami; however, better colour can be reached by using pork from heavy rustic pigs, without adding Ponceau red. The resulting flavour of Salami was similar with and without sodium glutamate ($P \leq 0.05$).

- Keywords: dry-cured sausage, carmine, glutamate, Ponceau red -

INTRODUCTION

The elimination or reduction of chemical additives in food is increasingly demanded by consumers. In the case of pork Salami, industrial sausage is often manufactured by the rapid drying-ripening of pork cuts from early pigs (fast-growing breeds that are slaughtered at 4-5 months). These are poor in muscle pigments and so additives with sensory enhancing properties are added to prevent defects in colour and flavour. However, the additives commonly used as enhancers in Salami, such as carmine and sodium glutamate, may be rejected by some consumers due to the potential health risks associated with their intake. For example, sodium glutamate intake may increase appetite and the risk of obesity, and has also been related with "Chinese restaurant syndrome", characterized by symptoms such as headache, nausea, facial pressure, burning sensation in the back of the neck, forearms, and chest, numbness in the back of the neck, tingling, warmth, weakness in face, temples, drowsiness, weakness and even asthma, nettle rash, angioedema and rhinitis (WILLIAMS and WOESSNER, 2009). Carmine colouring intake can cause allergic reactions due to the cochineal proteins or carminic acid it contains (TABAR *et al.*, 2003), and symptoms include hives, itching and even anaphylactic reactions (Beaudouin *et al.*, 1995; Di Cello *et al.*, 1999; CHUNG *et al.*, 2001). REGULATION (EC) No. 1333 (2008) obliges manufacturers to mention the health risk of ingesting azoic carmine colouring, such as E-124 Ponceau red "1-(4-sulpho-1-napthylazo)-2-napthol- 6,8-disulphonic acid, trisodium salt"; for its possible effect on the activity and attention of children.

The omission of Ponceau red and sodium glutamate would therefore imply a potential nutritional benefit to consumers; however, the lack of sensory enhancers may negatively affect sensory properties, especially in the salami processed by rapid drying, as it has little ripening time to develop pinkness and for fats to be oxidized. The processing of more pigmented and tastier pork may improve the eating quality of semi-ripe Salami, without the need for increasing the temperature or drying time. The sources of this type of pork are often two: heavy pigs slaughtered at an advanced age and rustic pig breeds. The term "rustic" refers to pigs, that often belong to slow-growing, traditionally reared local breeds, that are usually slaughtered at an older age, providing carcasses and meat with high muscle pigmentation and fattiness (SERRA *et al.*, 2003; ESTÉVEZ *et al.*, 2003; FRANCI *et al.*, 2005; GALIÁN *et al.*, 2008). Previous studies (MORETTI *et al.*, 2004; MADONIA *et al.*, 2007; BAÑÓN *et al.*, 2010) have suggested that rustic pork improves the colour and flavour of Salami, compared with commercial pork, although the possibility of removing sensory enhancers was not considered.

Rustic pig breeds have traditionally been used to manufacture local meat specialities; however, these breeds have gradually disappeared due to their low productivity, but there are now new opportunities to rear rustic pigs. The EU greatly values genetic diversity and has implemented programmes to recover endangered rustic pig breeds, which are raised extensively or semi-extensively in Europe. For example, a recent review (PUGLIESE, 2010) listed thirty-two local pig breeds of several European Mediterranean countries: Black Slavonian in Croatia; Blanc de l'Ouest, Limousin, Gascon, Bayeaux, Basque and Nustrale in France; Apulo-Calabrese, Casertana, Cinta Senese, Macchiaiola Maremmana, Mora Romagnola, Nero dei Liepini, Nero dei Monti, Nero Siciliano, Dauni Meridionali, Nero Reatino, Napoletana Fulva, Parmigiana nera, Pugliese and Sarda in Italy; Greek in Greece; Bisara, Malhado de Alcobaça and Alentejano in Portugal; Krskopolje in Slovenia; and Ibérica, Chato Murciano, Celta, Negra Canaria, Negra Mallorquina, Gochu Asturcelta and Euskal Txerria in Spain. The sustainability of most of these and other rustic breeds will depend on the suitable production of meat products with differentiated eating quality and/or nutritional benefits for consumers. The elimination of chemical additives that represent a potential health risk could be a first step. The aim of the present study was determine the effects of omitting Ponceau red and sodium glutamate on the quality of semi-ripe Salami made with pork from commercial or heavy-rustic carcasses. The addition of both sensory enhancers in Salami is discussed.

MATERIALS AND METHODS

Pigs and raw materials

All the pigs were reared in a two-phase system, where pigs were transferred to an on-growing installation after weaning on the birth farm. All farms were situated in Lorca, Murcia, S.E. Spain. Commercial early pigs (from Landrace x Large White crossbreed) were reared in an intensive system and were fed *ad libitum* with a standard feed composed (g kg^{-1}) of 462 barley, 250 wheat, 50 maize, 115 soya flour, 23 biscuit flour, 35 dry residues from maize distillation, 32 pig fat, and 10 cane molasses. The Fatty Acids "FA" profile of the feed was (g kg^{-1}): 330 saturated FA, 310 unsaturated FA, and 360 polyunsaturated FA. Rustic pigs (from Chato Murciano breed) were reared in a semi-extensive system and were fed *ad libitum* with a feed composed (g kg^{-1}) of 224 barley, 300 wheat, 200 maize, 140 soya flour, 100 peas, 80 wheat bran, 20 lucerne, 17 pig fat, and 10 cane molasses. The fatty acid (FA) profile of the feed was (g kg^{-1}): 300 saturat-

ed FA, 400 monounsaturated FA, and 300 polyunsaturated FA. Rustic pigs occasionally ate figs and carobs from the farm.

All the pigs were slaughtered in an industrial abattoir (La Comarca, Lorca, Murcia, Spain) according to EU sanitary Regulations. Early pigs were slaughtered at 5.5 months and 100 kg live weight, providing around 80 kg carcass weight. Rustic pigs were slaughtered at 18 months old and 180 kg live weight, providing around 140 kg carcase weight. The shoulders were removed from the carcasses, boned and skinned to obtain pork and backfat to manufacture Salami.

Preparation of dry-cured sausage

Three trials were performed in commercial and rustic Salami. Three batches of each experimental type of Salami were manufactured in the same process by a local factory (Elaborados Cárnicos de Lorca S.L, Murcia, Spain). Each batch was made using pork from six different carcases. Table 1 shows the main characteristics of the different pork and Salami types. For further details, refer to BAÑÓN *et al.* (2010). Optionally, 10 g kg^{-1} of sodium glutamate "E-621" and 0.25 g kg^{-1} of Ponceau 4R red (1-(4-sulpho-1-napthylazo)-2-napthol- 6,8-disulphonic acid, trisodium salt) "E-124" were added to the two enhanced Salami types. Both doses correspond to the maximum permitted by REGULATION (EC) No. 1333 (2008). The additives and spices were provided by Cargill Texturizing Solutions Cultures SAS, La Frete Sous Jouarre, France. A mix of pediococci and staphylococci cultures (Ref. CXP, Degussa Ferment´s Aromatization SAS, La Frete Sous Jouarre, France) was used to start the fermentation of Salami (see Table 1). *P. pentosaceus* culture was used as rapid acidifying lactic acid bacteria, while *S. xylosus* and *S. carnosus* cultures contribute to lean colour stabilization and flavouring due to their nitrate reductase, protease and lipase activities. The tolerance data of staphylococci cultures were 120 g NaCl l^{-1}, 10°-35°C of temperature and pH<4.5, while the tolerance data of pediococci cultures were 70 g NaCl l^{-1}, 15°-35°C and pH <3.8.

Table 1 - Pork sources (early and rustic pig) and Salami recipe.

	Early Salami	Rustic Salami
Pork source	**Landrace x Large white**	**Chato Murciano**
Pork protein/fat ratio	0.69	0.68
Fatty acid "FA" composition	**g kg^{-1}**	**g kg^{-1}**
Saturated SFA	347	389
Monounsaturated MUFA	482	438
Polyunsaturated PUFA	140	158
Recipe	**g kg^{-1}**	
Boned pork shoulder	880	
Water	44	
Sodium chloride	22	
Black and white pepper	10	
Dextrose, lactose and sucrose	20	
Dextrin	20	
Potassium nitrate	0.25	
Sodium nitrite	0.25	
Sodium isoascorbate	0.5	
Sodium citrate	0.3	
Sodium glutamate*	10	
Ponceau 4R red*	0.25	
Starter culture	**CFU kg^{-1}**	
Pediococcus pentosaceus	$3\ 10^7$	
Staphylococcus carnosus	$1.5\ 10^7$	
Staphylococcus xylosus	$1.5\ 10^7$	

*Optional.

The meat was minced in an atmospheric mincer using a 6 mm plate (Laska GMBH, WW1302, Nu-Meat Technology, Girona, Spain). Prior to mixing, the total fat and total protein contents (g 100 g^{-1}) of minced meat were estimated using an optical analyser (NIR FoodScan TM; Barcelona, Spain) with a precision of 1 g 100 g^{-1}. Calculations were made using prediction models (Artificial Neural Net; Foss). The lyophilised cultures were rehydrated (15 g in 200 mL chlorine-free water) for 4 hours and then sown in the mass at a rate of 6×10^7 cfu g^{-1}. The meat was then mixed with the starter cultures, additives and spices in an AMU102 vacuum mixer (Maquinaria Vall, Miralcamp, Lleida, Spain). The meat mass was allowed to marinate in a cold chamber at 2°-4°C for 24 hours to facilitate mixing of the ingredients, starter cultures and additives. The paste was stuffed on a WF-612 automatic line (Albert Handtmann Mahcinefabrik, Biberach an der Riss, Germany), using swine natural casing, slightly curved, 40-43 mm calibre and 300-320 mm in length. The casing was previously desalted and washed with dechlorinated water. The recently stuffed pieces were bathed in mould solution of *Penicilium chrysogenum* (0.8 g l^{-1} water) (PS5.1, Cargill) and hung from steel racks 1.2 m wide, 1.2 m deep and 2.2 m high. The loading density on the trolleys was 19 kg m^3. The trolleys were placed in an air-drying room (Sabroe S.A., Barcelona, Spain) set at 15°±1°C. The RH was gradually reduced: the first day at ambient humidity to eliminate water through dripping followed by 6 days at 80±5% RH and 5 days at 70±5% RH. The temperature and RH were verified using a P 650 thermohygrometer (Dostmann Electronic GmbH, Waldenbergweg, Germany) with a precision of 0.2°C and 0.5% RH. The samples were stored at 4°C for up to 24 hours prior to analysis.

Physical-chemical analyses

A total of twenty-four sausage units per Salami type were analysed in duplicate. The water activity "a_w" was measured at 25°C using

a TH200 water activity meter (Novasina Axair AG, Pfäffikon, Switzerland) (ISO 21807, 2004). The pH was measured with a Crison micropH 2001 pHmeter (Crison, Barcelona, Spain) using a combined Cat nº 52-22 electrode (Ingold, Inc. Wilmington, USA) (ISO 2917, 1999). Lipid oxidation was expressed as thiobarbituric acid reactive substances (TBARS) (mg malondialdehyde kg^{-1}), as determined by BOTSOGLOU *et al.* (1994), using a Unicam UV2 spectrophotometer. Colour was measured in sausage cuts using a CRF200/08 Chroma Meter II (Minolta Ltd., Milton Keynes, United Kingdom). Results were expressed as a*, b*, Lightness (L*) (CIELab units), Chroma (C*) and Hue angle (H*) (sexagesimal degrees); $C^* = (a^{*2} + b^{*2})^{1/2}$. $H^* = tg^{-1}$ (b*/a*). Non-protein nitrogen (NPN) and total nitrogen (TN) were determined by Kjeldahl's method (ISO 937: 1978), using a Nº 323 digestion-distillation unit (Buchi Labortechnik AG Flawil, Switzerland) an automatic Titrino 702 SM equipped with a No. 6.0233.100 combined pH electrode (Methrom Schweiz, Zofingen, Swizerland). The non-protein nitrogen was determined after precipitating the proteins with trichloroacetic acid. The proteolysis index was calculated as g NPN per 100 g TN by subtracting the added nitrogen from Ponceau Red and sodium glutamate (0.9 g kg^{-1}). The fat acidity index (expressed as g KOH kg^{-1} fat) was determined in fat extracted with chloroform-methanol (FOLCH *et al.*, 1957), by titrating with KOH, and using phenolphthalein as indicator.

Microbiological analyses

Total Viable Counts (TVC), Lactic Acid Bacteria (LAB), *Micrococcaceae,* moulds and yeasts were determined (log cfu g^{-1}). The samples were weighed with a sterile tweezers into masticator bags and blended with 0.1% (w:w) peptone water (Oxoid Ltd. CM0087, peptone water) in a masticator (IUL Instruments, GMBH, Königswinter, Germany). The bags used for microbiological analyses were aseptically manipulated inside a microbiology cabinet (Telstar, Bio-II-A, Terrasa, Barcelona, Spain). Total Viable Counts were counted on PCA (Plate Count Agar) (Oxoid Ltd. CM0325, Basingstoke, Hampshire, United Kingdom) after incubation at 30°C for 72 h (ISO 4833: 2003). Lactic acid bacteria were counted on MRS Agar plates (de Man, Rogosa, Sharpe) (Oxoid Ltd. CM0361) and incubated at 30°C for 72 h in a ST 6120 culture incubator (Heraeus, Madrid, Spain) (ISO 15214, 1998). *Micrococcaceae* were counted on Mannitol Salt Agar (Oxoid Ltd. CM0085) and incubated at 37°C for 72 h. Moulds and yeasts were counted on RB (Rose-Bengal) plates with chloramphenicol (Oxoid Ltd. CM0549) after incubating at 25°C for 120 h (ISO 21527-2, 2008). A model 207 cultures oven (Selecta, Abrera, Barcelona, Spain) was used.

Sensory analyses

The eating quality of Salami was determined by Quantitative Descriptive Analysis (QDA) (ISO 4121, 2003). Slices (8 mm thick) were cut with a MAS9101 slicer (Rober Bosch Hawgeräte GmbH, Ljubljana, Slovenia). Sensory analysis was made at a room temperature of 25°C. The sensory panel was composed of sixteen panellists (eight men and eight women) selected and trained according to ISO 8586-1 (1992). Most of the panellists had previous experience in QDA applied to dry-cured fermented sausage. The average age of the panellists was 28±9 years. There were five training sessions. In the first session, the main sensory descriptors of dry-cured fermented sausage were studied; in the second session, potential sensory descriptors of pork Salami and their relationship with the colour and taste enhancers were studied; the others three sessions were concerned with identifying, selecting and quantifying the main sensory attributes: colour intensity, dry-cured fermented meat odour and flavour, pepper odour and flavour and acid flavour. Attributes were quantified on a five points scale according to their sensory intensity, using reference sausages as the scale ends. The reference sausages used to define the minimum values were: similar pork Salami ripened for four days (for colour, odour and flavour; pepper odour and flavour); unfermented raw-cured pork sausage (for acid flavour). The sausages used as maximum values were: Iberian pork Salami (for colour); similar pork Salami manufactured with cured meat flavouring (for odour and flavour); spicy pork Salami (for pepper odour and flavour); and the similar pork Salami ripened for four days (for acid flavour). Each panellist evaluated six samples from each treatment.

Statistics

A randomized design using the addition of enhancers as treatment was performed. The effects of the treatment on the quality of Salami were determined by one-way ANOVA (Scheffe's means test). Data from each trial were analysed separately. Two-way ANOVA was not performed because different causes of variability (pig breed, feeding, farming and slaughter weight) were simultaneously acting. Statistical calculations were carried out with the Statistix program 8.0 for Windows (Analytical Software, USA).

RESULTS

Early Salami

Table 2 shows the effects of the addition of enhancers on the ripening properties of Salami enhanced or not with Ponceau red and sodium glutamate. Mean L* was similar (P>0.05) in Enhanced-Early-Salami "EES" (53.4) and Free-Ear-

Table 2 - Ripening properties of salami made with different pork sources (early and rustic pig) enhanced or not (free) with Ponceau red and sodium glutamate.

	Early Salami		Rustic Salami	
	Enhanced M±SD	Free M±SD	Enhanced M±SD	Free M±SD
L* Lightness (CIE units)	53.4±3.04	53.1±1.88	47.5±2.40^{b}	51.7±2.34^{a}
a* Redness (CIE units)	14.4±1.78	13.8±2.85	14.1±1.62^{b}	18.7±1.03^{a}
b* Yellowness CIE units	5.57±0.82^{a}	3.83±1.27^{b}	3.02±0.89^{a}	1.88±0.95^{b}
C* Chroma CIE units	15.4±1.91	14.5±2.60	14.5±1.69^{b}	18.9±1.04^{a}
H* Hue Angle CIE units	21.3±1.64^{a}	16.2±7.26^{b}	13.4±2.65^{a}	4.08±3.31^{b}
a_w	0.89±0.02	0.89±0.03	0.90±0.01	0.90±0.01
pH - log H^+	4.72±0.05	4.86±0.45	4.72±0.22	4.80±0.14
Proteolysis (g NPN 100 g^{-1} TN)	12.1±1.75	11.3±3.01	10.4±1.36	10.9±2.82
Fat acidity g (KOH 100 g^{-1} fat)	12.0±1.50^{a}	9.51±3.98^{b}	11.7±1.50^{a}	8.72±0.54^{b}
TBARS (mg MDA kg^{-1})	n.d.	n.d.	n.d.	n.d.
Total Viable Counts (log cfu g^{-1})	8.38±0.24	8.29±0.28	8.92±0.24	8.71±0.35
Lactic Acid Bacteria (log cfu g^{-1})	8.25±0.21	8.06±0.47	8.80±0.32	8.64±0.19
Micrococacceae (log cfu g^{-1})	7.35±0.32^{a}	6.10±0.37^{b}	6.88±0.50	6.51±0.47
Moulds and Yeasts (log cfu g^{-1})	6.37±0.30^{a}	5.34±0.60^{b}	5.46±0.45^{b}	6.06±0.81^{a}

M: Mean; SD: Standard Deviation; n.d.: not detected.
TBARS: Thiobarbituric Acid Reactive Substances.
Means with different superscripts and same pork source are significantly different for P≤0.05.

ly-Salami "FES" (53.1). As regard chromatic coordinates, no significant differences (P>0.05) was found between EES and FES for mean a* values, while mean b* was higher (P≤0.05) in EES (5.6) than in FES (3.8). The addition of Red Ponceau increased the Hue Angle although it did not affect the value of Chroma, changing the colour tonality from red-bluish to red-yellowish in the early-type Salami. Minor differences between EES and FES were found for other ripening properties. Water activity was not influenced (P>0.05) by the addition of enhancers. The mean pH was similar in EES (4.72) and FES (4.86) and there were no relevant (P>0.05) differences in acidification. The mean proteolysis indexes were also similar in EES (12.1) than in FES (11.3), while fat acidity was higher in EES (12.0) than in FES (9.5). No appreciable levels of TBARS were detected in any Early Salami. Some differences in the fermentative groups were found; *Micrococacceae,* mould and yeast mean counts were higher in EES (7.3 and 6.4) than in FES (6.1 and 5.3), while mean counts of TVC and LAB were similar for both treatments, reaching microbial charges above 8 log cfu g^{-1}. Of all the ripening properties studied, the reflectance changes may be explained by the addition or not of Ponceau red.

Table 3 shows the effects of the addition of enhancers on the eating quality of Salami enhanced or not with Ponceau red and sodium glutamate. In accordance with the reflectance values, the lean colour score was slightly higher

Table 3 - Eating quality of salami made with different pork sources (early and rustic pig) enhanced or not (free) with Ponceau red and sodium glutamate.

	Early Salami		Rustic Salami	
	Enhanced M±SD	Free M±SD	Enhanced M±SD	Free M±SD
Lean colour	3.13±0.61^{a}	2.71±0.77^{b}	3.63±0.53^{b}	4.46±0.63^{a}
Odour	3.09±0.54	3.06±0.68	3.21±0.64	3.11±0.55
Pepper odour	2.54±0.52	2.72±0.68	2.67±0.71^{a}	2.17±0.66^{b}
Flavour	3.00±0.54	2.86±0.58	3.17±0.59	3.11±0.61
Pepper flavour	2.58±0.54	2.62±0.68	2.83±0.67^{a}	2.39±0.69^{b}
Acid flavour	2.87±0.66	2.70±0.73	2.96±0.76^{a}	2.45±0.74^{b}

M: Mean; SD: Standard Deviation.
Scale: (1: minimum; 5: maximum).
Means with different superscripts and same pork source are significantly different for P≤0.05.

in EES (3.1) than in FES (2.7), confirming that Ponceau red intensified meat reddening, fulfilling its role as red colouring in Salami. However, no relevant differences between EES and FES were found in the mean scores for odour or flavour, pepper odour and pepper flavour. Likewise, acidity scores were similar in EES (2.9) and FES (2.7), reflecting their similar pH and BAL counts, while rancidity was not detected in early Salami. The addition of Ponceau red and sodium glutamate, at the maximum dose permitted by the EC Regulations intensified the reddening, improving the colour, but did not enhance the odour and flavour of the early Salami ripened for two weeks. Therefore, sodium glutamate could well be removed from the recipe with no significant flavour loss.

Rustic Salami

All CIE L*a*b* colour coordinates of Rustic Salami was clearly affected ($P<0.05$) by the addition of Ponceau red. Enhanced-Rustic-Salami "ERS" had lower mean L* (47.5) than Free-Rustic-Salami "FRS" (51.7). Mean a* value was also lower in ERS (14.1) than in FRS (18.7), while mean b* was higher in ERS (3.0) than in FRS (1.9). Difference in L* could be explained by the different lipid contents in E and F Rustic Salami; however, when no Ponceau red was added, the Hue Angle decreased strongly and the value of Chroma increased. These chromatic arose from the formation of red-bluish pigments (nitrous-myoglobin) in the dry-cured meat. The differences between treatments were less pronounced for other ripening properties of rustic-type Salami. The mean value of water activity was similar in ERS (0.90) and FRS (0.90), and there were no significant differences in acidification since the mean pH was similar in ERS (4.72) and FRS (4.80). The mean proteolysis index was also similar in FRS (10.4) and ERS (10.9), and mean fat acidity was strongly higher in ERS (11.7) compared with FRS (8.72). No appreciable levels of TBARS were detected in rustic Salami. There were similar mean counts of TVC, BAL and *Micrococacceae* for both treatments, while, in contrast, a lower growth of moulds and yeasts had was observed in ERS (5.5) compared with FRS (6.1). As in the case of the early Salami, the reflectance changes may be explained by the addition of Ponceau red, since differences found for other ripening properties could be due to a certain lack of standardization of rustic Salami.

In accordance with reflectance data, the lean colour score was much higher in FES (4.5) than in EES (3.6). This means that colouring by Ponceau red does not really enhance the red colour of rustic Salami because it masks the natural colour of the dry-cured pork. No differences between ERS and FRS were found for the mean odour or flavour scores, although acid flavour and pepper odour and flavour were slightly more intense in ERS (2.7 and 3.2) than in FRS (3.0 and 2.4). Therefore, the addition of Ponceau red and sodium glutamate, at the maximum dose permitted by the EC Regulations, did not improve reddening or flavouring of the rustic-type Salami ripened for two weeks. Both Ponceau red and sodium glutamate could be removed from the recipe with no significant colour or flavour loss. Indeed, the lean colour of Rustic Salami improved when Ponceau red was not added.

DISCUSSION

Chato Murciano farmers seek to produce a pig identified with the local culture, capable of providing pork with different sensory traits from those of "commercial" pork. Besides the use of local breed, additional strategies were simultaneously followed to achieve this objective: diet, semi-extensive rearing and slaughter at higher age / weight of pigs. Chato Murciano breed (and probably other similar rustic pig breeds) tends to fatness, and in particular, to the infiltration of fat in muscle (GALIÁN *et al.*, 2008). Pig age increases the levels of muscle myoglobin, intra and extra muscular fat and the MUFA/PUFA ratio in meat (MAYORAL *et al.*, 1999; VIRGILI *et al.*, 2003). In addition, the diet of Chato Murciano pigs is modified to increase the level of oleic acid in meat, while pigs exercise in the field aimed to increase myoglobin formation and meat redness. Different studies have reported that Chato Murciano meat may be more pigmented, juicier and tastier, while fat may be more stable against excessive oxidation, softer and creamier (PEINADO *et al.*, 2009).

The results show that the same dry-fermented sausage may or may not require sensory enhancers, depending of the quality on the pork used. The colour of Salami made with early pork benefitted from being enhanced by red coloring. Both reflectance measurements and the sensory panel detected deficiencies in the red colour when Red Ponceau red was not added. Ponceau red provided a red-yellow colour, increasing the hue angle with no relevant change in Chroma. In contrast, Red Ponceau did not enhance the colour of rustic Salami, but masked the natural red-blue colour (pink) of raw-cured meat, as seen from the reduced Chroma value and the strong increase in Hue angle. Sensory evaluation confirmed the higher intensity of the lean colour in the Ponceau Red-free rustic Salami. These colour differences were mainly due to differences in the meat pigmentation, as the sensory panel assessed the lean colour regardless of the quantity of the fat grains present in the slices. Thus, sensory evaluation seems a more accurate discriminator of Salami colour than L*a*b* coordinates, because fat grains and lean meat contribute to light reflection of slices, and it may be more difficult to determine colour differences due to nitrous-myoglobin formation.

A greater concentration of nitrous-myoglobin in Salami is associated with increased Chroma and lower Hue angle (CHASCO *et al.*, 1996; PÉREZ-ÁLVAREZ *et al.*, 1998). L*a*b* ranges of 45-50, 8-11 and 5-8 CIE units, respectively, have been reported in semi-ripe pork Salami without red enhancer (SAYAS *et al.*, 1998; PÉREZ-ÁLVAREZ *et al.*, 1999), while L*a*b* values of 56, 17 and 11, respectively, were reported in a semi-ripe pork Salami with 0.25 g kg^{-1} added Ponceau red (GIMENO *et al.*, 2001). These values averaged light reflection from lean and fat. MADONIA *et al.* (2007) also found a greater intensity of lean colour in rustic (Nero Siciliano) compared with commercial (Large white) pork Salami. Chato Murciano pork also improved the colour and flavour of dry-cured loin compared with commercial pork loin (SALAZAR *et al.*, 2009).

Muscle pigmentation seems to be a major factor in the lean colour of semi-ripe Salami. Early pigs are often slaughtered at 4-5 months and usually provide less pigmented and leaner meat than heavy and/or rustic pigs. A preliminary trial determined a reddish-pink to pale-pinkish colour (NATIONAL PORK PRODUCERS COUNCIL STANDARDS, 1999) and L*a*b* values of 48.8±2.2, 5.1±0.9 and 1.3±0.6 CIE units in the *Longissismus dorsi* muscle of Large White x Landrace pigs (N=40 carcasses). In contrast, a reddish-pink to purplish-red colour and L*a*b* values of 48.5±5.7, 14.4±3.4 and 9.1±3.2 CIE units were found in the same muscle of heavy Chato Murciano pig (N=52 carcasses). Other authors reported L*a*b* ranges of 45-46, 9-15 and 3-4 CIE units, respectively, in loin from Chato Murciano slaughtered at around 8 months and 120 kg live weight (POTO *et al.*, 2007; GALIÁN *et al.*, 2008). The myoglobin content of pork is correlated positively with redness (CIE a*) (Kim *et al.*, 2010).

Sodium glutamate did not enhance the odour and flavour in either Salami and this taste enhancer could be removed from the recipe without affecting quality. The odour and flavour in Salami strongly depends on the formation of oxidized lipids (aldehydes, ketones and others) and non-protein nitrogen compounds (peptides, nucleotides and amino acids), e.g. glutamic acid. The odour and flavour of semi-ripe Salami strongly depends on the fermentation process of the mixture of meat and spices, since it has little time to develop pinkness and an adequate degree of fat oxidation, and only weak odour and flavour are reached after two weeks of drying. Acidity and pepper flavours are often perceived in Salami and can mask the flavour of dry-cured meat (BERIAIN *et al.*, 2000; BENITO *et al.*, 2004; BAÑÓN *et al.*, 2010). No rancidity or appreciable levels of TBARS were detected in early or rustic Salami, which agrees with previous studies on semi-ripe Salami made with added isoascorbate and sodium citrate (BAÑÓN *et al.*, 2010). In contrast, quite high levels of carbonyl compounds have been described in early Salami (BERIAIN *et al.*, 2000); while SUMMO *et al.* (2010) reported TBARS higher than 1 mg MDA kg^{-1} in smoked pork Salami with added sodium ascorbate.

Other minor ripening or sensory differences point to a certain lack of standardization in Salami, since only a small amount of enhancers is added. Variations in the composition of minced sausage are often caused by the processing of heterogeneous meat cuts (e.g. boned shoulders, including backfat). Mixing can also produce differences in the lean, fat or starter cultures distribution. Differences in proteolysis or lipolysis may often be related with the staphylococci and lactobacilli used as starter cultures (CASABURI *et al.*, 2008; ARO *et al.*, 2010), although the endogenous enzyme activity in muscle may also play a role in this respect. ARMERO *et al.* (1999) observed differences in endogenous protease and lipase activity between the pork from Iberian (18 months old) and Landrace (6 months old) pigs. No data have been found concerning the influence of sodium glutamate and Ponceau red on LAB or staphylococci growth in dry-cured sausage. Fat acidity of 9.6 mg KOH per g fat and proteolysis index of 17 g NPN per 100 g TN were reported by CHASCO *et al.* (1996) in pork-beef Salami after two weeks of ripening, although NPN from additives was not subtracted.

CONCLUSIONS

A way is proposed to link the production of healthier dry-cured pork sausages, free of unnecessary additives, with the preservation of swine biodiversity. The addition of Ponceau Red modifies the natural pink colour of the dry-cured pork formed by the reaction of myoglobin with nitrifying agents. Similar flavour and better colour can be reached in semi-ripe Salami by using more pigmented pork from heavy and/or rustic pigs, without adding red colouring or sodium glutamate. Endangered pig breeds, such as Chato Murciano, may be preserved by increasing awareness of their potential for the production of more natural semi-ripe Salami.

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PAPER

RELATIONSHIP BETWEEN BASIC BEEF TEXTURE ATTRIBUTES AND THEIR PERCEPTION BY POLISH CONSUMERS

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ABSTRACT

There is a possibility of defining beef desirability, using formulas based on texture attributes (tenderness, juiciness, and fibrousness). The aim of the study was analysis of beef texture consumer survey and defining relationships between attributes. Object were results of evaluation of roast beef (24 batches, each evaluated by at least 30 randomly chosen consumers) conducted using 23-point intensity scale (method of the Texas A&M University). Statistical analysis was conducted using Statistica computer program. Using obtained predictive models, complex beef texture evaluation may be conducted – tenderness evaluation accompanied by fibrousness and juiciness prediction or fibrousness evaluation accompanied by tenderness and juiciness prediction.

- Keywords: beef, consumer survey, fibrousness, juiciness, tenderness, texture attributes -

INTRODUCTION

The constant trend of decline the per capita beef consumption is observed, especially in Poland. Although the situation may result from several combined factors, the lack of suitable advertisement of beef of culinary quality, inadequate guidance for preparation techniques and inadequate quality of meat from dairy-cattle used instead of meat-cattle are the factors, that have contributed to the intensifying of the problem (JEREMIAH and GIBSON, 2003). Such situation on the market does not promote the growth of beef consumption. To improve the situation, it is essential to characterize basic quality attributes desired by consumers and to elaborate possibilities of efficient prediction of beef attributes, on the basis of chosen factors.

In consumer opinion the most characteristic and desirable attributes of beef are tenderness (MOLINA *et al.*, 2005), chewingness, and juiciness (ZIMOCH and GULLET, 1997), accompanied by typical taste, flavor (ADHIKARI *et al.*, 2011) and color of meat (OLIVEROS *et al.*, 2009). The three main texture attributes in decisive way determine sensory value, contribute to palatability perception and, as a consequence, to its acceptance or rejection, as well as to satisfaction derived from beef consuming. Therefore, the researchers, seeking for proper predictive coefficients of beef quality, focused on possibility of defining the beef desirability, using formulas based on mentioned attributes (MCDONALD and CHEN, 1991; JEREMIAH and PHILLIPS, 2000).

The tenderness is one of texture attributes, which has been established as the most important factor in determining beef taste and consumer quality perception, which causes, that explorers dedicated the most of their attention to its analysis (STRYDOM *et al.*, 2000; LI *et al.*, 2001; DENOYELLE and LEBIHAN, 2004; JEREMIAH *et al.*, 2003; HOFFMAN *et al.*, 2007). The juiciness is another important texture attribute of beef. Some research suggest that the higher palatability of beef results not from increased tenderness, but rather from increased juiciness and flavor. It is one of the reasons why the marbling of beef is so important, as a factor associated with both juiciness and flavor (EGAN *et al.*, 2001). Some research suggest that quality grades of beef are more strongly related to juiciness than to tenderness or flavor (KIM, 2003). The third important feature of beef assessed by sensory methods is fibrousness, that cannot be evaluated instrumentally (OTREMBA *et al.*, 1999).

Tenderness, juiciness and fibrousness attributes depend mainly on the properties of muscle fibers as well as the amount and type of connective tissue and water. Tenderness, juiciness and fibrousness are variable between carcasses (CHAMBAZ *et al.*, 2003), muscles (BELEW *et al.*, 2003) and even within the same muscle (REUTER *et al.*, 2002) and are related to age, gender, breed and factors of processing conditions such as suspension, chilling and aging (DENOYELLE and LEBIHAN, 2004). Aiming to improve above mentioned attributes, some following procedures may be applied: suitable cattle cross-breeding, slaughter in proper age, but of a great importance are also control of maturation processes, appropriate feeding and reduction of pre-slaughter stress (DENOYELLE and LEBIHAN, 2004). Mentioned interventions are conducted in order to improve profitability of production, which is connected with the fact that consumer would be willing to pay the higher price, if beef was characterised by desired sensory value (BOLEMAN *et al.*, 1995; MILLER *et al.*, 2001).

Tenderness may be evaluated by using objective instrumental methods, while juiciness and fibrousness are rather evaluated using sensory methods with trained panel, or subjective methods with a consumer panel. Objective methods of tenderness evaluation enable the quick comparison of samples and analysis of influence of its preparation on characteristics, but do not provide information about consumer beef acceptability and preference (WHEELER *et al.*, 1997). Therefore, long-lasting consumer survey providing information about market needs and purchase decision is essential to improve intrinsic quality attributes.

Hitherto investigations relating beef intrinsic quality attributes concentrated inter alia on tenderness, juiciness and palatability attributes (NORTHEN, 2000). There were also some trials connected with color and marbling of beef, as factors which would be significantly correlated with general meat quality. However there are observations, that color and marbling scores have limited predictive potential in beef quality and tenderness estimation (MCDONALD and CHEN, 1991). It is one of the reasons why, in spite of numerous investigations connected with beef quality expectation, researchers and beef industry all the time postulate new coefficients, enabling effective and simple beef intrinsic quality attributes prediction (LI *et al.*, 1999). Explanation of beef attributes, which may be determined only in sensory evaluation by other factors (especially these, which may be measured instrumentally) would enable effective analysis conducting.

The aim of the study was analysis of consumer survey of beef texture attributes (tenderness, fibrousness, juiciness) and defining the relationships between them.

MATERIALS AND METHODS

Sample preparation and sensory evaluation

The object of the study was result of the consumer survey, connected with sensory evaluation of randomly chosen samples of beef (the

roast beef). Sensory evaluation was related to 860 samples of beef, from 24 different batches, differing with Country of origin (Poland, Argentina, Brazil, and USA), the way of breeding (extensive and intensive) and the way of feeding, which guaranteed diverse samples, typical to market variety.

Population that participated in the research constituted beef consumers aged over 21. Randomly chosen group of consumers was characterised by diverse age, education and level of monthly income per person. The research was conducted in 2 provincial cities of Poland (Olsztyn and Warsaw), where the quantity of questionnaire interviews was proportional to inhabitants' quantity. The research was conducted in second and third quarter of 2008.

The object of the sensory analysis was the beef steaks, prepared from roast beef as the principal element of beef carcass. Roast beef was cut into slices, characterised by 30 ± 5 mm of thickness and subjected to identical thermal treatment (grilling, using medium power, to temperature of 71°±1°C), which treatment guarantees the sensory value of meat, accompanied by microbiological safety (RHEE *et al.*, 2003; KILLINGER *et al.*, 2004; OLIVER *et al.*, 2006). Temperature of 71°±1°C obtained in geometrical center of culinary element corresponds to the "medium" degree of doneness (LÓPEZ OSORNIO *et al.*, 2008). Meat was grilled without addition of fat, salt and spices.

Sensory evaluation was conducted under controlled conditions. The steaks labeled with three-digit random numbers, were cut into 2cm-2cm-2cm cubes and presented warm to the panellists. From each of 24 batches, from 30 to 40 samples were prepared, which resulted in 860 samples of beef. Each beef batch was planned to be evaluated by at least 30 randomly chosen consumers, to guarantee obtaining the representative material.

In the sensory evaluation participated 235 consumers. Each of them received from 3 to 4 randomly chosen samples of beef, but not from the same batch. The consumers had assured the possibility to add salt on plate, according to their preferences. Simultaneously with the sample, consumers received the questionnaire of consumer survey. Completed questionnaires were the basis of conducted analysis.

Consumers conducted sensory evaluation using 23-point intensity scale, according to an American method of the Texas A&M University. Panellists were estimating the intensity of beef texture attributes (tenderness, fibrousness, and juiciness) on scale, with 23 points marked, anchored at the end by the words "not intensive"/"very intensive".

Gathered investigative material enabled elaborating predictive formulas, to estimate the conditional value of variables of beef textural attributes.

Analysis of data

Data are presented as mean ± standard deviation with minimum and maximum value. To define relationships between analysed factors, they were tested using Pearson's correlation coefficient. Calculation of the prediction formulas, as well as analysis of efficacy of prediction and degree of accuracy of regression function to empirical data, was conducted using the multiple regression model, according to widely accepted and used method (HILL and LEWICKI, 2007) using the Statistica version 8.0 by StatSoft Inc. To define significance of differences, the level of significance $\alpha \leq 0,05$ as well as two independent levels of precision: ±10 and ±25% were accepted. The figures (3-dimensional diagram and 2-dimensional diagrams) were created using the graph creator of the Statistica version 8.0 by StatSoft Inc. The statistical analysis was also conducted using the computer program Statistica (data analysis software system) version 8.0 (2007) by StatSoft Inc.

RESULTS AND DISCUSSION

The obtained results of consumer analysis of beef texture attributes (tenderness, fibrousness, and juiciness) are presented in Table 1. The results concern the average ratings of consumer opinions on 24 different beef samples. The average, minimum and maximum values of studied texture parameters of analysed beef are presented. The results of analysed attributes' ratings of beef are characterised by the parametric distribution. Because of the fact, that analysed culinary elements of beef were derived from cattle of meat performance, they were characterised by specific sensory profile, highly desired by consumers. The most desirable for consumer profile of meat is described by three main texture attributes: tenderness, fibrousness and juiciness. On the basis of mentioned above, these attributes were taken into account to create predictive models of analysed attributes.

The majority of analysed beef elements were characterized, in consumers' opinion, by high tenderness, low fibrousness and high juiciness, which attributes are of the high value in sensory evaluation of meat intrinsic quality attributes. The other researches also indicate, that the at-

Table 1 - Beef texture attributes analysed in the consumer survey – mean, minimum and maximum of mean values of analysed 24 beef sample batches.

	Mean ± SD	Minimum	Maximum
Tenderness	14.26±3.9	6.21	20.44
Fibrousness	8.87±3.2	3.05	14.63
Juiciness	15.80±3.0	6.75	20.12

Table 2 - Correlations between beef texture attributes analysed in the consumer survey.

		Tenderness	Fibrousness	Juiciness
Tenderness	r		-0,9246	0,5431
	p	-	0,000	0,006
	r^2		0,8548	0,2950
Fibrousness	r	-0,9246		-0,5785
	p	0,000	-	0,003
	r^2	0,8548		0,3347
Juiciness	r	0,5431	-0,5785	
	p	0,006	0,003	-
	r^2	0,2950	0,3347	

tributes, influencing consumer taste perception in the most significant way, are tenderness, meat texture connected with absence of perceptible fibers (MORGAN *et al.*, 1999; OTREMBA *et al.*, 2000; DESTEFANIS *et al.*, 2008) and juiciness (ROBBINS *et al.*, 2003).

While in case of tenderness and juiciness of beef, high values are associated with high quality, in case of fibrousness of beef, inverse situation is observed – low values are associated with high quality. In the conducted research, fibrousness was ranked lower than tenderness and juiciness – it achieved lower average, minimum and maximum values, which is positive. It suggests that assessed beef, in spite of different origin and essential differences in sensory profile, was generally favourably estimated by consumers, which is testified by high notes of attributes distinguished positively – tenderness and juiciness, accompanied by low notes of negatively perceived fibrousness. The identified characteristics of highly preferable sensory profile of beef correspond to results of other researches (CAMPO *et al.*, 1999).

The statistical analysis of obtained results indicated numerous dependences between assessed factors and enabled creating a correlation between studied attributes (tenderness, juiciness, and fibrousness). Consumer survey conducted on representative group, enabled to prove statistically significant dependences between meat attributes, perceived by consumers. The results of the analysis of correlation are presented in Table 2.

Almost full negative correlation between tenderness and fibrousness texture attributes was affirmed. The correlation indicates that the more fibers were perceptible, the lower notes tenderness received, which may be a result of remaining the original structure of meat. Consumers are prone to rate higher the tenderness of meat, which is characterised by lower fibrousness, which attributes, in other research are also highly correlated (OTREMBA *et al.*, 2000).

The beef fibrousness attribute correlated negatively also with juiciness attribute on the quite strong level. The physiology of mastication and swallowing food requires to crack the product (the less fibrous and softer samples exert less intense resistance) and to mix with saliva which facilitates swallowing (the high juiciness admits the most efficient course of the processes). It is one of the reasons why high notes of juiciness attribute accompanied by low notes of fibrousness attribute result in positive consumer perception of beef samples. It is important to emphasize that, according to BOLEMAN *et al.* (1995) and MILLER *et al.* (2001), the consumer would be willing to pay a higher price in the marketplace for beef as long as it's positive texture attributes are guaranteed.

The beef juiciness attribute correlated with its tenderness. The analysis of the results shows that softer meat was simultaneously perceived by consumers as more juicy, which corresponds to results of other research (OTREMBA *et al.*, 2000).

It is important to emphasize, that tenderness, juiciness and fibrousness attributes, correlated in strong or very strong way. It may be associated with the fact, that among analysed samples were observed some, which were positively distinguished from others, in regards to all the analysed texture quality attributes. The appearance of beef samples, differing significantly in the profile of texture attributes is observed. The groups of samples characterised by higher general quality (softer, juicier, with lower presence of fibers) and characterised by lower sensory value, were emerged. Observed dependences are presented in graphic form (Fig. 1). In the analysis of culinary beef studied samples, it is observed that the softer samples were also perceived by consumers as more juicy, which corresponds to earlier observations (RESURRECCION, 2003). Simultaneously, in case of fibrousness attribute, it may be certified that its value was diverse, and on the basis of presented graphic

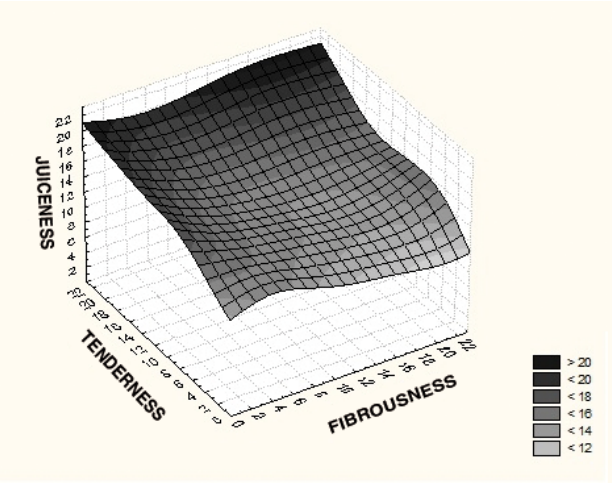


Fig. 1 - Three-dimensional model of correlations between analysed beef texture attributes.

Table 3 - Efficacy of prediction of beef texture attributes using predictive models obtained in the study.

	Precision	Efficacy of prediction on the basis of:			
		tenderness	fibrousness	juiciness	2 attributes
Tenderness	±10%	-	0.71	0.17	0.42
	±25%		0.92	0.62	0.87
Fibrousness	±10%	0.50	-	0.17	0.37
	±25%	0.87		0.54	0.71
Juiciness	±10%	0.71	0.62	-	0.75
	±25%	0.96	0.92		0.92

model, conclusions about its dependences with other beef texture attributes may not be formulated. Therefore different tools were necessary, to enable global conclusions.

Observed dependences, mentioned above, are so significant (Table 2) that the presumption about linear dependences between analysed texture attributes was possible. Moreover, the basis of further considerations was that perception of the attributes by consumers may be of the resultant nature. The gathered investigative material, confirming essential correlations between analysed attributes, as well as the reliable professional literature knowledge and experience were fundamental in elaborating predictive formulas, based on regression equation. They enabled, on the basis of one beef texture attribute, to predict, with high probability, the rest of them, by estimation of conditional value of expected variables, associated with beef texture attributes. The analysis of numerous samples enabled to elaborate the model, estimating beef texture attributes with very high efficacy. The modelling was based on three main texture attributes, analysed in presented research.

Mathematical analysis of all the hypothetic possibilities of estimating beef texture attributes (tenderness, fibrousness, juiciness) on the basis of other analysed attributes was conducted. The efficacy of attributes estimation on basis of every other attribute and both of them simultaneously was verified.

The analysis of formulas' efficacy was executed, with presumed two levels of precision (±10, ±25%). The degree of practical realization of planned prediction of the studied samples' attributes was verified, accepting two levels of precision – 10 and 25%. This analysis enabled selection of formulas, which are characterized by the highest level of efficacy (verifiability) (Table 3).

It was affirmed that the attribute, which may be predicted, with the highest certainty, on the basis of any other attribute, is the juiciness, as it is presented in Table 3. In obtained model, value of the beef tenderness, fibrousness or tenderness and fibrousness attributes enable to estimate the beef juiciness attribute, with efficacy over 0.9, when analysis is conducted on the level of precision ±25%. Level of precision ±10% enables to predict juiciness attribute with efficacy of 0.75, if it is estimated on the basis of the tenderness and fibrousness attributes. If estimation is made on the basis of tenderness attribute, efficacy is not much lower – about 0.71. It is observed that especially tenderness attribute alone is highly useful to predict juiciness with high efficacy (it is better than fibrousness alone and even better than tenderness accompanied by fibrousness) but fibrousness attribute may be also used. Conducted analysis suggest, that the juiciness attribute analysed in consumer survey, is not in practice indispensable parameter to evaluate, because it may be predicted on the basis of other beef texture attribute (tenderness or fibrousness). Additionally, tenderness may be estimated also, using simpler, quicker and cheaper instrumental objective method (AMSA, 1995), which is often applied in industry, because of its repeatable results (DESTEFANIS *et al.*, 2008).

From the conducted analysis of studied beef samples, it is concluded, that the most effective beef juiciness prediction may be obtained on the basis of the following formula:

$$\text{Juiciness} = 0.4133 * \text{tenderness} + 9.9035 \quad [1]$$

If fibrousness attribute is also evaluated, on the basis of both juiciness and fibrousness, using the following formula:

$$\text{Juiciness} = 0.2066 * \text{tenderness} - 0.2729 * \text{fibrousness} + 15.2703 \quad [2]$$

estimation may be made with better certainty, than estimation on the basis of fibrousness attribute alone:

$$\text{Juiciness} = -0.5459 * \text{fibrousness} + 20.6372 \quad [3]$$

Juiciness prediction may be based mainly on the tenderness attribute, nevertheless inclusion of the fibrousness, not as leading, but supplementary attribute, may be applicative. Results of the research of other authors also suggest tenderness as better predictor of juiciness, than fi-

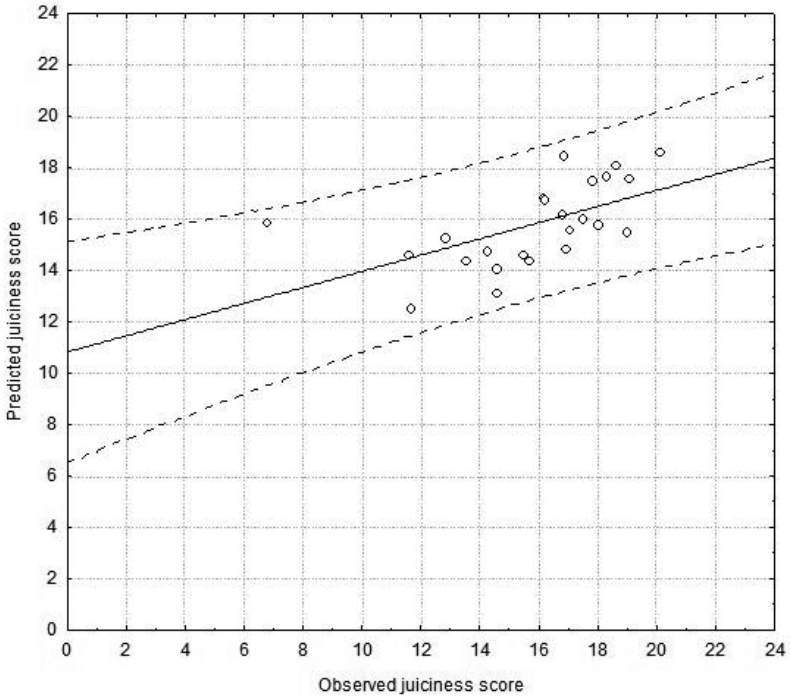


Fig. 2 - Comparison of predicted juiciness scores by the model based on tenderness and fibrousness attributes with actual scores (r=0.5722, p=0.0035, r^2= 0.3275)

brousness (RESCONI *et al.*, 2010). Comparison of three obtained predictive models, estimating beef juiciness attribute, on the basis of its tenderness, fibrousness or both tenderness and fibrousness, may demonstrate some advantages connected with formula estimating attribute on the basis of two factors. Such model will be in practice less susceptible to disturbances, which may be caused by incorrect evaluation of attributes, than formula estimating on the basis of only one factor, which will be more prone to such mistakes.

Comparison of predicted juiciness scores by the model based on tenderness and fibrousness attributes with actual scores is presented in Fig. 2. In spite of inclusion two factors to juiciness estimation and the high dependence between juiciness and analysed attributes, the degree of accuracy of regression function to empirical data is realized only in 32,7%. This observation marks that in spite of existing dependence, because of the complexity of factors influencing juiciness attribute, to explain the rest of variation range, the juiciness prediction based also on other factors should be conducted. It needs to be emphasized, that the model, based on too many factors, causes that in practice the formula is too complicated and its usefulness is reduced. In case of analysed attributes, the high interdependence was certified, and simultaneously high efficacy degree and simplicity of the formula guarantee its high practical usefulness.

It is important that the best model should be simultaneously highly accurate, simple, with high utility, so creating the formula, based on too many factors, leads to lowering its usefulness and limitation possibility of its practical use. Hence, obtained formulas may be of a great value to conduct the fast estimation of the beef juiciness attribute, on the basis of its tenderness, fibrousness, or both factors.

Estimating the fibrousness and tenderness attributes on the basis of juiciness attribute turned out to be entirely impossible to use in practice, with regard to low efficacy of formulas, particularly on the level of precision ±10%. The level of precision ±25% was associated with higher efficacy of obtained formulas, but it was also unsatisfactory. It must be concluded that the juiciness attribute does not enable effective prediction of any of analysed texture attributes – neither tenderness, nor juiciness.

The prediction of tenderness attribute may be based on the fibrousness and juiciness attributes simultaneously, and prediction of fibrousness attribute – on the basis of tenderness and juiciness simultaneously. The estimation, based on two factors is more effective, than prediction on the basis of juiciness attribute alone. Expectation on the basis of two factors was associated with effective estimation on the level of precision ±25%, but on the level of precision ±10% efficacy was unsatisfactory. It may be concluded that on the basis of juiciness attribute, prediction of neither tenderness, nor fibrousness, should be made, even if the juiciness is, in formula, not leading attribute, but only a supplementary one.

The beef tenderness attribute may be estimated effectively, using predictive model based on fibrousness attribute. In presented research, such function was established, which was associated with practical efficacy of 0.71 (on the level of precision ±10%) and 0.92 (on the level of precision ±25%). From conducted analysis of

beef, it is concluded that effective prediction of tenderness attribute may be conducted, using the following formula:

$$\text{Tenderness} = -1.341 * \text{fibrousness} + 26.1491 \quad [4]$$

Comparison of predicted tenderness scores by the model based on fibrousness attribute with actual scores is presented on Fig. 3. It should be emphasized, that in case of the model of beef tenderness prediction on the basis of its fibrousness, not only high efficacy is assured, but also very high degree of accuracy of regression function to empirical data, which is realized in 85.5%. Results of the research of other authors also suggest fibrousness as better predictor of tenderness, than juiciness (RESCONI *et al.*, 2010). Existing variation of beef tenderness attribute corresponds strongly to fibrousness and its variation. Taking it into account, there is no necessity of tenderness prediction on the basis of other factors, because in case of beef tenderness attribute, complexity of factors influencing it, is not as high, as in case of, analysed above, juiciness attribute.

It should be also emphasized that the model of beef tenderness attribute, created on the basis of its fibrousness, is characterized not only by high interdependence of factors – p=0.0000, but also by high degree of efficacy. Additionally, taking into account only one factor – fibrousness attribute, the formula is characterised by low complication, simplicity of use, and resulting from them – high practical usefulness.

The most efficacy estimation of the beef fibrousness attribute may be conducted on the basis of its tenderness. This model is characterised by average efficacy on the level of precision ±10%, but on the level of precision ±25% efficacy is considerable - 0.87. In the presented research, such function was elaborated, according to which, the most effective beef fibrousness attribute prediction may be conducted on the basis of the following formula:

$$\text{Fibrousness} = -0.7457 * \text{tenderness} + 19.4994 \quad [5]$$

Comparison of predicted fibrousness scores by the model based on tenderness attributes with actual scores is presented on Fig. 4. This predictive model, similarly to tenderness attribute estimation on the basis of fibrousness, is characterized by high interdependence of factors, high efficacy and very high degree of accuracy of regression function to empirical data. The observed dependence is highly expected – the beef tenderness and fibrousness attributes are, as mentioned above, factors characterized by almost full correlation, hence majority of one factor's variation is explained by variation of the other one. Results of the research of other authors also suggest tenderness as better predictor of fibrousness, than juiciness (RESCONI *et al.*, 2010). Concluding, it is not necessary to predict the beef fibrousness attribute on the basis of other factors, because the factors influencing fibrousness are not complex. Additionally, taking into account the fact that ob-

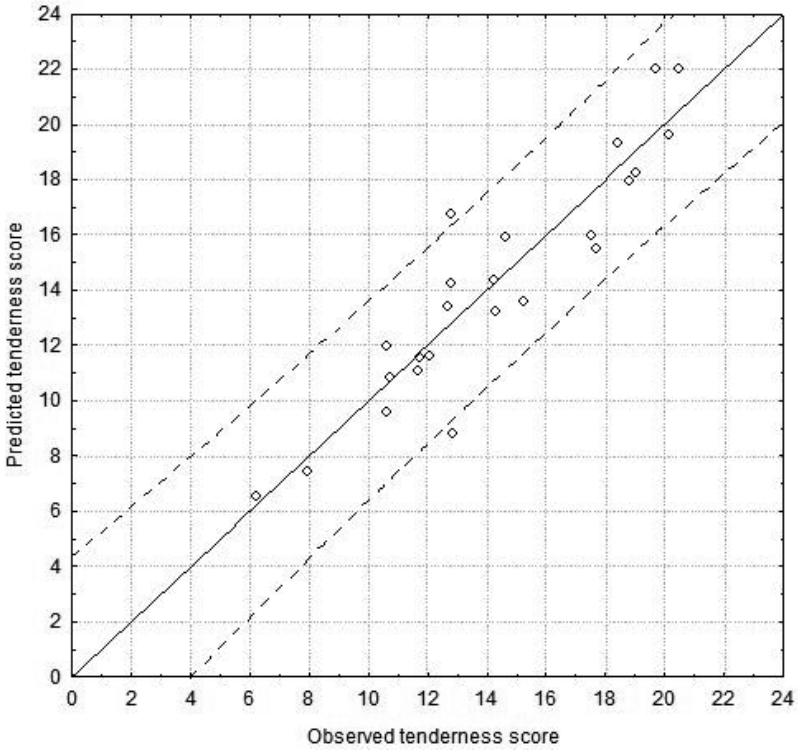


Fig. 3 - Comparison of predicted tenderness scores by the model based on fibrousness attribute with actual scores (r=0.9246, p=0.0000, r^2= 0.8548).

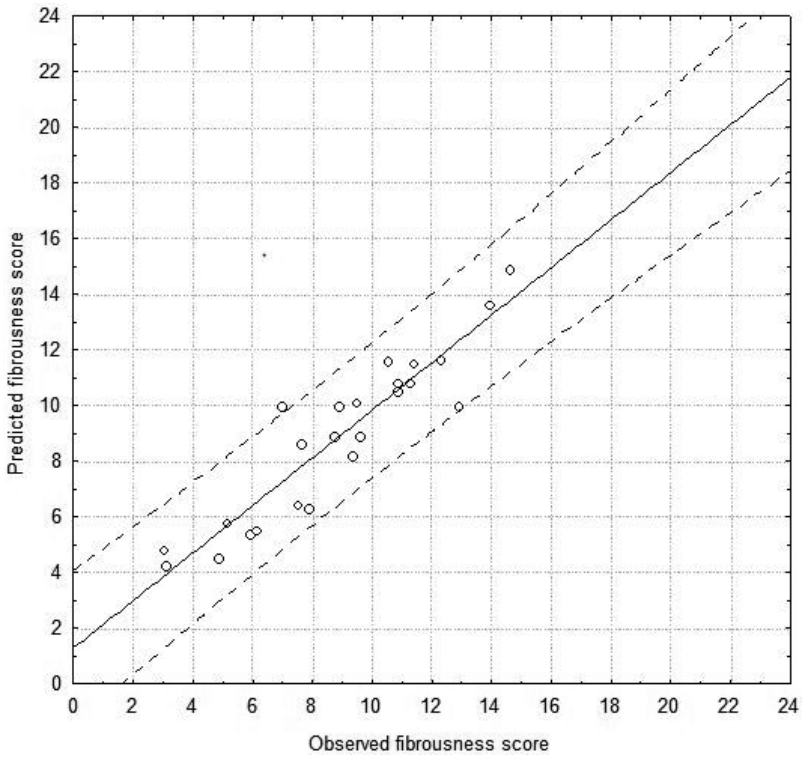


Fig. 4 - Comparison of predicted fibrousness scores by the model based on tenderness attribute with actual scores (r=0.9246, p=0.0000, r^2= 0.8548).

tained model is based only on one factor (tenderness), it is highly useful, because of simplicity of use in practice.

Is should be emphasized, that beef tenderness attribute may be also evaluated using quick, cheap and easy instrumental method. Taking into account the high correlation of beef instrumental analysis' and consumer survey's results (DENOYELLE and LEBIHAN, 2004; CIERACH and MAJEWSKA, 1997), probably obtained predictive formulas may enable estimation the fibrousness and juiciness attributes on the basis of instrumental beef tenderness evaluation. Such possibilities would be of a great value in industry, where instrumental methods are applied, as easier in practical use. Also other authors are analysing correlations between tenderness, juiciness and fibrousness of beef as well as the possibilities of predictive formulas applications (OTREMBA *et al.*, 2000). The possibilities of predictive formulas exploitation, using instrumental evaluation of beef texture, need further researches, inter alia the further testing of presented formulas is needed.

CONCLUSIONS

It must be concluded, that using obtained models, characterised by high efficacy, may be conducted prediction of analysed beef key texture attributes. The tenderness, fibrousness and juiciness attributes may be predicted mainly on the basis of tenderness and fibrousness. It is of the a great value in practice – consumer analysis of beef tenderness or fibrousness attribute will suffice to estimate with high precision the rest of texture attributes. Explanatory value of presented predictive models is associated also with the fact that best-fit model, based on indispensable number of variables, enables to identify the features determining analysed attribute (SEPÚLVEDA *et al.*, 2008).

Using obtained predictive models, complex evaluation of beef texture may be conducted – tenderness evaluation accompanied by fibrousness and juiciness prediction or fibrousness evaluation accompanied by tenderness and juiciness estimation. Prediction based on specific models is in meat industry highly recommended, because of numerous advantages, connected with its use (HOUTSMA *et al.*, 1996; MCDONALD and SUN, 1999). Such approach enables not only quicker, but also more economical and effective analysis of beef texture.

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PAPER

VALIDATION OF AN ELISA ACCORDING TO EC DECISION 2002/657/EC FOR DETECTION OF ZEARALENONE IN FEEDSTUFF

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ABSTRACT

This study presents data from a single laboratory for internal validation of an enzyme-linked immunoassorbent assay (ELISA) for determination of levels of the mycotoxin zearalenone in complementary and complete feedstuff for dairy cattle, calves, sheep and goats. The study was carried out at the request of European Commission (EC) Recommendation 576/2006/EC, in agreement with EC Decision 2002/657/EC on the implementation of EC Directive 96/23/EC and concerning the performance of analytical methods and interpretation of results. This screening detection method has useful advantages: it can analyse large numbers of samples in a relatively short time, with a small quantity of sample required; it is sufficiently robust, reliable and rugged to be statistically sound; and it generates few suspected false negatives (<5%).

- Keywords: animal feed, EC Decision 2002/657/EC, ELISA, mycotoxin, validation, zearalenone -

INTRODUCTION

Mycotoxins are secondary metabolites that are produced by microscopic fungi, and which are toxic for higher animals. They tend to have relatively low molecular weight, moderate or high volatility, high stability, and a specific structure that is related to the taxonomy of the microorganism in which it is produced (BENNET and KLICH, 2003).

Contamination from mycotoxins in man generally occurs through their transfer from contaminated vegetables or alimentary products (BHATNAGAR *et al.*, 2002 and 2004). As well as direct human health problems, contamination of alimentary products can also result in large economic losses that arise from reduced productivity of animals that are nourished with feedstuff that contain even only small levels of mycotoxins (D'MELLO *et al.*, 1999).

Zearalenone is a non-steroidal oestrogenic mycotoxin that can be produced by at least eight toxigenic species that belong to the genus *Fusarium*. These include, in particular, *Fusarium graminearium*, *Fusarium culmorum* and *Fusarium equiseti*, which can colonise a wide variety of cereal crops, and hence their derived food products, where they find excellent conditions for mycotoxin synthesis (HIDY *et al.*, 1977; TANAKA *et al.*, 1988). This mycotoxin is produced in these species as a consequence of prolonged cold or humidity, or through interactions of multiples factors, such as the moisture content of the grain, an infection, the temperature, various microbial interactions, and for other related reasons (MOSS, 1991).

The chemical structure of zearalenone shows it as a resorcyclic acid lactone, and it can bind to oestrogen receptors (ERa, ERb), resulting in oestrogen-like responses (KUIPER-GOODMANN *et al.*, 1998). For this reason, as well as having a specific and acute toxicity, zearalenone can have actions on the reproductive organs that parallel those of 17-β-oestradiol (FITZPATRICK *et al.*, 1989; DIEKMAN and GREEN, 1992; MIKSICEK, 1994). Furthermore, although it is acknowledged that zearalenone has a relatively low acute toxicity (oral LD_{50} >2,000-20,000 mg/kg body weight [bw]) after oral administration in mice, rats and guinea pigs (FLANNIGAN, 1991), it has also been shown to be hepatoxic, haematotoxic, immunotoxic and genotoxic in *in vitro* and *in vivo* studies (ZINEDINE *et al.*, 2007).

The particular concern here arises from the carry-over and entrance of zearalenone into to the food chain through products of animal origin, which can occur when animals are given mycotoxin-contaminated feedstuff. Based on a no-adverse-effects level of 40 μg/kg bw/day obtained in a 15-day study in pigs, the human exposure assessment for zearalenone has been indicated as a provisional maximum tolerable daily intake of 0.5 μg/kg bw (CCFAC, 2000). Thus the Joint Committee of the FAO/WHO experts on food additives recommended that the total intake of zearalenone and its metabolites (including α-zearalenone) should not exceed this value (CCFAC, 2000).

In this respect, the European Commission (EC) has defined some guidance values for of zearalenone and other mycotoxins (e.g., ochratoxin, fumonisin B1 and B2, and T-2 and HT-2 toxins) in products intended for animal feedstuff, with EC Recommendation 576/2006/EC of 17 August, 2006. This provided guidance to the EC Member States on the acceptability of the presence of these mycotoxins in cereal products and compound feedstuff for animals. This had the intention of avoiding the disparities that existed in the mycotoxin levels accepted by the different EC Member States. It was also designed to increase monitoring for the presence of these mycotoxins in animal feedstuff, and to ensure that the lower guidance levels for cereals and cereal products were applied by the feedstuff manufacturers. These recommendations also take into account the different sensitivities of the different animal species, to ensure compliance with the guideline values determined for compound feedstuff for each animal species.

To ensure a high level of protection for human and animal health, and for the protection of the environment, Italy instituted a National Plan on 3 May, 2007, for animal feedstuff that repealed and replaced the previous National Plan for health vigilance and monitoring of the feeding of animals. In agreement with what was largely already established through EC Regulations 178/2002/EC and 882/2004/EC, the aim of the new National Plan was to provide an official system for the control of animal feedstuff along the entire animal food supply system.

Using feedstuff samples collected in the territories of Puglia and Basilicata as part of the National Plan, we have here carried out a validation of an enzyme-linked immunoassorbent assay (ELISA) for the detection and quantification of zearalenone in the feedstuff of dairy cattle, calves, sheep and goats, according to the criteria of European Union Decision 2002/657/EC. The aim was to examine the quality and correct interpretation of the analytical data, and to evaluate some of its performance characteristics, such as the specificity, precision, detection capability, robustness and ruggedness, in agreement with the standards of UNI CEI EN ISO/IEC 17025 (2000).

MATERIALS AND METHODS

Sampling

The sampling was carried out from the animal-feed materials supplied to the feed mills, along with samples from complementary and complete

feedstuff for dairy cattle, calves, sheep (including lambs) and goats (including kids). This was carried out at the request of EC Recommendation 576/2006/EC, of 17 August, 2006.

Complementary feedstuff samples include a mixture of ingredients, and they have a high content of certain substances (e.g., additives such as vitamins and trace elements). These mixtures are not sufficient to provide the full daily ration of nutrients for the animals, and they have to be used in combination with other feedstuff. Complete feedstuffs are mixtures that can provide the total dietary needs of an animal.

The samples were obtained and analysed according to the methods indicated in the Ministerial Decree of 20 April, 1978, relating to samples for official monitoring of animal feedstuff, and in EC Regulation 401/2006/EC of 23 February, 2006. These thus provided the methods for this sampling and analysis for official monitoring of the levels of mycotoxins in feedstuff, according to the Programme for Surveillance and Monitoring of Animal Nutrition (PNAA) and the National Residue Monitoring Plan 2007-2008, as carried out in the territories of Puglia and Basilicata by local veterinary services.

Of note, as zearalenone is light sensitive, the samples were conserved in cotton mail bags to protect them from light, which were stored in a cool, dry place until the analysis.

Feedstuff for validation and sample preparation

The feedstuff samples analysed for this validation study were for dairy cattle (n = 6), calves (n = 12) and sheep/goat (n = 2), as representative samples, as indicated by the PNAA. All of these feedstuffs were ground in a grinder (Retsch) to a mesh size of 20.

For each of these representative samples, a 5 g portion was thoroughly mixed with 25 mL methanol/ water (7:2; v/v) for 3 min on a horizontal shaker (ISCO) at room temperature. The suspensions were then filtered through Whatman n° 1 filter paper, and the filtrates were collected. These sample extracts were diluted a further 1:5 with methanol/ water (7:2; v/v). The methanolic extracts were stored in glass vials at 2°-8°C for approximately 2 weeks, while protected against light.

Test kit assay method

The ELISA competitive enzyme immunoassay for quantitative analysis of zearalenone that was used was the Ridascreen zearalenone kit provided by R-Biopharm (Darmstadt, Germany); the manufacturer instructions for the development of the test were followed, with some small changes.

The results of the analysis were obtained photometrically at a wavelength of 450 nm, using a microplate reader (Anthos HT-2). The specificity of the test was established by analysing the cross-reactivity to the corresponding mycotoxin: zearalenone, 100.0%; α-zearalenol, 41.6%.

The limit of detection (LOD) was 50 ng/kg. The standard solutions provided in the kits were of 0, 50, 150, 450, 1350, 4050 ng/kg zearalenone in aqueous solution. Further dilutions were carried out (1:7) with the buffer solution provided in the kits, and 50 ml of the resulting samples were analysed in duplicate.

Specificity and detection capability

The detection capability according to EC Decision 2002/657/EC is the smallest content of the substance (in this case, zearalenone) that can be detected, identified and/or quantified in a sample with false compliance rate <5% (β-error). In the case of substances with an established permitted limit, this means that the detection capability is the concentration at which the method can detect this permitted limit concentration with a statistical certainty of 1-β.

Estimations of the specificity and the error β were carried out in parallel, and the same assay included a representative blank feedstuff sample with the 20 feedstuff samples for the different animals (dairy cattle, 6; calves, 12; sheeps/ goats, 2) and related samples fortified at the guidance value of 0.5 mg/kg zearalenone. The samples were analysed in two equivalent analytical steps carried out within a short time of each other (Table 1). The precision (repeatability and within-laboratory reproducibility) and statistical evaluation were assessed by variance analysis (ANOVA test), Fischer tests and Students' t-tests of the paired data between ten blank samples and ten fortified samples at the guidance value chosen, across these two analyses (Table 2).

Ruggedness of the tests

The ruggedness of the methodology (with the included minor changes) was also determined. This was carried out as a test for ruggedness at 0.50 mg/kg zearalenone by the introduction of seven small, but deliberate, changes in the operating parameters (factors or variables) and through the consequent assessment of the influences of these changes on the results. These deliberate changes were designed to reflect those that can occur when a method is transferred between different laboratories, with the modified factors of: sample preparation; kit condition; filter type; reagent temperature; animal age; time of absorbance readings; and methanol producer.

RESULTS AND DISCUSSION

We thus validated an immunoenzymatic method (ELISA) and determined its suitability for the

Table 1 - First (left) and second (right) analytical datasets for specificity.

N.	Sample	Relative absorbance (%[B/B$_0$])		N.	Sample	Relative absorbance (%[B/B$_0$])	
		Blank	Fortified			Blank	Fortified
1	*Calves*	102.06	22.01	*11*	*Calves*	101.62	21.75
2	*Dairy cattle*	105.09	19.05	*12*	*Calves*	92.24	20.09
3	*Calves*	91.55	20.08	*13*	*Calves*	91.07	18.72
4	*Calves*	92.27	20.19	*14*	*Dairy cattle*	103.34	18.82
5	*Calves*	90.49	18.94	*15*	*Calves*	91.71	23.69
6	*Sheep/Goat*	92.34	20.01	*16*	*Dairy cattle*	104.68	19.07
7	*Dairy cattle*	95.79	19.44	*17*	*Calves*	98.02	21.72
8	*Dairy cattle*	104.66	19.44	*18*	*Sheep/Goat*	91.85	19.71
9	*Calves*	96.93	21.83	*19*	*Dairy cattle*	95.76	19.35
10	*Calves*	88.49	20.51	*20*	*Calves*	88.32	20.31
Mean		***95.97***	***20.15***	***Mean***		***95.86***	***20.32***
S.D.		***6.04***	***1.05***	***S.D.***		***5.75***	***1.60***
Variance		***36.48***	***1.119***	***Variance***		***33.062***	***2.56***

routine monitoring and analysis of low levels of the mycotoxin zearalenone in complementary and complete feedstuff for dairy cattle, calves, sheep (including lambs) and goats (including kids) on the basis of EC Decision 2002/657/EC (DESIMONI and BRUNETTI, 2003). The reference concentration used for the final determination of the fitness of this analysis methodology according to the method performance characteristics was the guidance value of 0.5 mg/kg zearalenone, as determined in these complementary and complete feedstuffs for the animal species given, according to EC Recommendation 576/2006/EC.

Specificity

Each value used in the data processing for the data given in Table 1 derived from the mean of two readings of the sample relative absorbance (%[B/B$_0$]). For the blank samples, the mean %(B/B$_0$) was 95.97% for the first ten samples, and 95.86% for the second ten. For the samples fortified with 0.5 mg/kg zearalenone, the mean %(B/B$_0$) was 20.15% for the first ten samples, and 20.32% for the second ten (Table 1).

Table 2 shows that for both of these analytical sessions, the t critical value (Stat t) is higher than the experimental one (t critical one-tail), which thus verifies that the differences between blank samples and the corresponding fortified samples are significantly different from zero.

In addition, the semi-interval confidence of the blanks was calculated using the formula:

$$\bar{S}_o - \frac{t^* S_0}{\sqrt{n}}$$

where S_0 is the mean signal of the 20 blank samples, t is Students' t tabulated for nine degrees of freedom at a confidence level of 5% (1.83), n is the number of tests, and s_0 is the standard deviation of the signal obtained from the 20 trials of the blank samples.

For both the first and the second sessions, the fortified samples showed a %(B/B$_0$) below the semi-interval confidence (92.46 and 92.54 %), so all 20 of these fortified samples are recognised as being "non-compliant" and are subsequently processed by a confirmatory method. The β error is thus satisfied.

Table 2 - First (left) and second (right) sessions for the t-test results of the paired data.

Statistic	Variable 1	Variable 2	Variable 1	Variable 2
Mean	0.95972218	0.20153129	0.958674148	0.20327923
Variance	0.003656499	0	0.003309525	0.000256437
Observations	10	10	10	10
Pearson correlation	0.024941152		-0.166469138	
Hypothesised mean difference	0		0	
gdl	9		9	
Stat t	39.22288437		38.38559639	
P (T<=t) one-tail	1.13141E-11		1.37248E-11	
t critical one-tail	1.833112923		1.83	

Robustness

The different levels and variables that were monitored here were mixed following a Youden experimental plan, as described in Tables 3 and 4. The effects of each factor were calculated by subtracting the mean result obtained with the variable at a high level (the reference value, indicated by the capital letter) and the mean result achieved with the factor at a low level (the investigated factor, indicated by the corresponding small letter) (see Table 3). The differences were indicated as D_i, and the standard deviations of the differences, SD_i, were calculated according to the following equation:

$$SDi = \sqrt{2 \cdot \Sigma (Vi^2 / 7)}$$

In the ruggedness study performed at a concentration of 0.5 mg/kg zearalenone using the Youden approach, the SD_i obtained was 0.0308, and was compared to the within-laboratory reproducibility estimated under the nominal conditions S_r, of 0.066. This latter indicates the precision obtained in the same laboratory under these predetermined conditions (e.g., concerning the method, test materials, operators and environment) over justified long time intervals. As this difference is not significant ($SD_i < S_r$), this demonstrates that taking all of these investigated factors together, they do not have effects on the performance of this method, and thus the method is strong.

For all of the investigated factors, the t value obtained was compared with the 1-tailed t-critical value (t crit) at n-1 degrees of freedom and a = 0.05 (95% confidence level). Each t-value is lower than the corresponding t-crit, which thus confirms that the method is not affected by any slight variations in each of the critical factors.

Precision and recovery

As this screening procedure is used as a quantitative method and the results of the compliant samples are reported in accordance with a numeric value in the final test reports, precision tests were also carried out. This precision relates to the closeness of agreement between the independent test results obtained under predetermined conditions. The measure of precision is expressed in terms of imprecision and computed as a standard deviation. The precision is calculated either in terms of the repeatability, as the variability of the independent test results obtained with the same method on identical test samples in the same laboratory by the same operator using the same equipment, or in terms of the within-laboratory reproducibility, as the variability of the independent test results ob-

Table 3 - Variables and their levels in the Youden ruggedness test experimental design. Upper case letters represent high level; lower case letters represent low level values of variables.

N.	Selected variable	Units	Change in operating parameter (abbreviation)	
			High level (reference)	Low level (investigated)
1	Sample preparation	-	Prepared the day before the analysis (A)	Prepared fresh that day (a)
2	Kit condition	-	Already open (B)	Opened immediately prior to use (b)
3	Filter type	µm	Whatman 1(C)	Blotting paper (c)
4	Kit temperature	Min	Room temperature for 1 h before use (D)	Room temperature for 45 min before use (d)
5	Animal age	-	Calves (E)	Dairy cattle (e)
6	Absorbance reading	Min	Immediate (F)	After 30 min (f)
7	Methanol producer	-	Carlo Erba (G)	Baker (g)

Table 4 - Experimental design for the ruggedness (minor changes) using the fractional Youden approach.

Selected variable	Experiment number							
	1	2	3	4	5	6	7	8
Sample preparation	A	A	A	A	a	a	a	a
Kit conditions	B	B	b	b	B	B	b	b
Filter type	C	c	C	c	C	c	C	c
Kit temperature	D	D	d	d	d	d	D	D
Animal age	E	e	E	e	e	E	e	E
Absorbance reading	F	f	f	F	F	f	f	F
Methanol producer	G	g	g	G	g	G	G	g
Observed results (mg/kg)	0.484	0.479	0.545	0.483	0.428	0.484	0.481	0.481

tained with the same method used with identical test samples in the same laboratory by different operators at different times but using the same equipment.

For the simultaneous determination of precision and recovery, analyses were performed on two sets of blank feed samples (six replicates each) fortified at two different concentrations of standard solutions of zearalenone: 0.25 mg/kg and 0.5 mg/kg (Sigma Aldrich). As no certified reference materials were available, instead of the accuracy, the recovery parameter was determined, according to the performance criteria of EC Decision 657/2002. The mean recovery at each of the two zearalenone fortification levels was 96.8%, and the full results are summarised in Table 5.

Table 5 - Performance parameters of repeatability for the determination of two different zearalenone levels in spiked feedstuff samples (0.25 and 0.5 mg/kg).

Parameter	Fortification level	
	0.25 mg/kg	0.5 mg/kg
Mean (mg/kg)	0.225	0.518
Recovery (%)	90.00	103.60
Standard deviation (%)	0.03	0.03
Coefficient of variation (%)	11.11	6.37

Proficiency test

To calculate the inter-laboratory reproducibility, we took part in a proficiency test that was organised by the Milk Standard Laboratory of the Italian Association of Breeders (Associazione Italiana Allevatori; AIA), in March 2009. Two unknown samples were analysed in duplicate and the results were evaluated in terms of the Z-score, as calculated by the proficiency test organiser.

The Z-score obtained in this proficiency test that was performed on unknown samples analysed in duplicate (Z_1 0.548; Z_2 0.181) and the value of the laboratory Z-score (Z_{LAB} 0.083) were all below the reference value of 1.0, which thus demonstrated the technical competence of the laboratory according to the international harmonised protocol for proficiency testing of analytical laboratories (THOMPSON and WOOD, 1993).

CONCLUSIONS

In this study, the examination of the analytical method of this ELISA and the provision of the relevant evidence confirm its validation in our laboratory in a single laboratory study (i.e., in-house validation) according to EC Decision 2002/657/CE, with a high performance obtained. This is demonstrated by means of the experimental recovery, which effectively fulfils the requirements of the detecting of the mycotoxin zearalenone in samples of feedstuff for cattle, sheep and goats (and their young) at a level of interest that is in conformity with EC Recommendation 576/2006/EC. The applicability of this method of screening is also verified by carrying out tests on samples taken during an official inspection in the field of animal nutrition, as obtained from the competent authorities.

According to EC Regulation 882/2004, the methods of sampling and analysis used within the context of official monitoring must be fully validated in compliance with EC legislation or with the relevant accepted international protocols, in order to ensure the quality and comparability of analytical results generated by laboratories approved for official residue monitoring.

The method so validated here has also been recently accredited as compliant with the international standards of the series ISO 17000 and of the guidelines and the harmonised series of EC norms EN 45000, by ACCREDIA, the new Italian National Accreditation Body that was created recently by the merger of SINAL (Italian National System for Laboratory Accredition) and SINCERT (Italian National System for Accredition). ACCREDIA is a non-profit association that has been recognised by the Italian State since 22 December, 2009, as the sole National Accreditation Body for accreditation under the authority of the Italian State. ACCREDIA thus operates in full compliance with the national accreditation system according to Regulation N° 765 of the European Parliament and Commission of July 9, 2008, which has been applicable since January 1, 2010, for accreditation and market compliance in all EU countries. Moreover, the competence, behaviour and performance of ACCREDIA is continuously and carefully monitored, particularly by means of the construction of relevant monitoring charts with a biennial frequency, and by their participation in proficiency testing organised by the AIA and by the Food Analysis Performance Assessment Scheme (FAPAS)Further studies are in progress at present to analyse the effects of the moisture content on zearalenone determination in feedstuff samples, in accordance with Annex III of EC Regulation N° 152/2009/EC, which details the methods of sampling and analysis for the official monitoring of animal feedstuffs.

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PAPER

SURVIVAL OF HEPATITIS A VIRUS IN PASTEURIZED MANILA CLAMS

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ABSTRACT

Reports of infections from enteric viruses, particularly hepatitis A virus (HAV), and norovirus, have resulted in consumers boiling or steaming molluscs before eating them and, currently, ready to use, pasteurized and packed products are available from the food industry.

The present study investigated HAV survival in manila clams (*Ruditapes philippinarum*) after infection and subsequent pasteurization treatments of different duration and temperature according to established industrial procedures. The results showed that HAV was completely inactivated only after application of heat treatment at 90°C for 10 minutes and only when that temperature was reached in the core of the mollusc.

- Keywords: HAV, manila clams, pasteurization, PCR-seminested, virus isolation -

1. INTRODUCTION

Bivalve molluscs, being filtering organisms, are capable of concentrating particles, microorganisms, algal toxins and chemical substances internally. For this reason and due to consumer habits of eating partially cooked or raw molluscs, they are associated with the transmission of gastroenterological diseases of varying importance. Indeed, up until the Fifties, bivalve molluscs were involved in extended epidemics of typhoid fever and cholera (FAO, 2008).

Subsequently, with the introduction of discharge water treatment techniques and the development of criteria for the classification of shellfish farming and harvesting areas, the occurrence of such pathologies has begun decreasing. The etiological agents mostly involved in cases of infections from bivalve molluscs are Vibrios (*V. parahaemolyticus* and *V. vulnificus*) and enteric viruses such as hepatitis A virus (HAV) and norovirus (WHO, 2010).

Several notifications of clinical cases caused by HAV have been reported in the past, one of which was the occurrence of HAV infection due to mollusc consumption which involved over 300,000 people in Shanghai in 1988 (HALLIDAY *et al.*, 1991).

Hepatitis A infection represents the most common form of hepatitis in Italy where it still poses a serious problem for public health, even as epidemics have been progressively decreasing [data provided by the Integrated Epidemiology System for the control of Acute Viral Hepatitis (SEIEVA). In the past twenty years the average incidence rate is still equal to 5 cases per 100,000 inhabitants (SEIEVA, 2009)].

Over the last few years, in some regions of the country (i.e. Campania and Puglia) periodically repeated epidemics have been reported. Among the most recent cases, the hepatitis A epidemics in Puglia between 1996 and 1997 (11,000 recorded cases) (MALFAIT, 1996; LOPALCO, 1996) and in Campania in 2004 (421 recorded cases). The latter was associated with the consumption of bivalve molluscs, in particular mussels (RMI CNESPS OER Campania, 2004; BOCCIA, 2004).

Notwithstanding the evidence for the risk of contracting enteric viruses from bivalve molluscs, the current European regulation on microbiological criteria for foodstuffs does not include the detection of viruses in bivalve molluscs (EC, 2005).

Water decontamination systems currently in use are not effective against enteric viruses (SCHWAB *et al.*, 1998; UEKI *et al.*, 2007; LE GUYADER *et al.*, 2006) while the presence of the *Escherichia coli* reference faecal marker used as an indicator of product quality prior to harvesting is not an indicator of the presence of HAV (ROMALDE *et al.*, 2002), leaving the consumer exposed to the risk of infections from enteric viruses.

Proper cooking can remove these risks. HAV is known to be resistant to cooking and as such it is the most suitable of the enteric viruses for heat resistance tests (ICMSF, 1996). The aim of the present study was the assessment of the resistance of hepatitis A virus in infected clams (*Ruditapes philippinarum)*, of which Italy is the most important producer in Europe with a yearly output of 50,000 tons (PELLIZZATO *et al.*, 2010), to different pasteurization treatments.

In the present study clams were artificially infected and subsequently subjected to various heat treatments. Virus detection was performed using two different analysis methods i.e. virus isolation in cell cultures in order to detect viable infecting virus, and a seminested-PCR analysis to confirm the presence of isolated virus.

2. MATERIALS AND METHODS

2.1 Samples

The tests were performed on fresh bivalve molluscs known by their common name as manila clams (*R. philippinarum*) collected in a lagoon area of the southern Po river delta, located in the province of Rovigo (North-Eastern Italy).

2.2 Virus

Hepatitis A virus, (cytopathic strain HM175 kindly provided by IZSLER, Brescia), used for experimental contamination of samples, was grown on the FrP3 cell line, derived from the FRhK-4 (Fetal Rhesus Monkey Kidney cell line) and kept at 37°C in a 5% CO_2 atmosphere.

2.3 Experimental tests

2.3.1 Contamination and preparation of samples

Clams were infected by direct injection into the clam body of 50 µL of a viral suspension containing 1.6 x 10^7 $TCID_{50}$/mL. The viral titre, expressed in TCID50/mL (50% tissue culture infectious dose) according to the Reed and Muench formula (REED and MUENCH, 1938), was calculated after the preparation of tenfold serial dilutions of the virus and the inoculation of FrP3 cell monolayers grown on 24-well tissue culture plates.

For the infection, the shells of the clams were gently opened just enough to see the clam body and allow injection of the virus. The clams, still alive and with sealed shells, were then packed in double layered bags (10 clams per each bag) consisting of a polyamide layer of 15 microns thickness and a polypropylene layer of 100 microns thickness. These bags, suitable for steam sterilization, were sealed using a heat-sealer that also created a vacuum inside the bags.

In order to compare titre reduction of the virus

in the suspension used to inoculate the clams to that of the virus injected into the mollusc tissue, 5 mL aliquots of each of the viral suspensions at the same titre as that used to inoculate the clams were prepared in hermetically sealed glass test-tubes, and also put into vacuum bags.

2.3.2 Heat treatment of samples

Three groups of ten clams, infected and packed as described above, were exposed to four different temperatures (60°, 70°, 80°, and 90°C) for two different periods of time, e.g. 5 and 10 minutes. Treatments for 20 and 30 minutes were also carried out at 90°C, which was considered the most efficient temperature for virus inactivation. Another six groups of ten clams, three infected but not heat treated, and three not infected and not heat treated, were used as positive and negative control for the tests respectively.

2.3.3 Pasteurization and temperature detection system

The heat treatment of the clams was performed with an industrial gas-powered oven (Angelo Po Grandi Cucine S.p.A.). In the present study the oven was used in the steam mode: inside the oven a steam dispenser injects super hot steam directly into the cooking chamber and temperature homogeneity is ensured by the presence of fans that allow a uniform diffusion. The oven is equipped with a probe (Delta OHM S.r.L., mod. DO 9847, probe ppt 100, range T -40, + 100°C, tolerance +/- 0.06°C) for temperature measurement at the core of the product.

A receptacle (Ecklund-Harrison Technologies Inc., mod. C-5.2) guaranteed the maintenance of the vacuum in the bags with the probe.

2.4 Virological and molecular analyses

2.4.1 Cell culture infection

All ten clams were processed separately immediately after each bag, in which heat treatment had been carried out were opened. Each clam, without shell, was homogenized, and diluted 1:2 (weight/volume) with Phosphate-Buffered Saline (PBS pH 7.2-7.4) supplemented with antibiotics (penicillin G 10,000 IU/mL, streptomycin 10 mg/mL, nystatin 5,000 IU/mL). The homogenized samples were then centrifuged at 2,500/3,000*xg* for 30 minutes and the supernatants obtained were collected and maintained at 4°C overnight to allow for the action of the antibiotics. After this incubation, the supernatant of each sample was diluted 1:5 with D-MEM (Dulbecco's Modified Eagle Medium) supplemented with 1% L-glutamine (200 mM) and 1% antibiotics (penicillin G 10,000 IU/mL, streptomycin 10 mg/mL, nystatin 10,000 IU/mL and gentamicin 250 μg/mL) but without fetal bovine serum (FBS). FrP3 monolayers grown for 24 hours at 37°C in a 5% CO_2 atmosphere on 24-well plates were inoculated with 0.2 mL of the diluted sample supernatant, in duplicate. The remaining supernatant was immediately stored at -80°C.

Monolayer infection was performed with an adsorption phase of 90 minutes. After adsorption, cells monolayers were washed once with 1 mL of D-MEM and then overlaid with the same D-MEM (without FBS to avoid cellular aging), added to a final volume of 1 mL in each well. The infected monolayers were incubated for 10 days at 37°C in a 5% CO_2 atmosphere and periodically observed under a microscope to check for cytopathic effects. After 10 days, all the infected plates (positive and negative for cytopathic effects) were frozen at -80°C and then thawed. The cryolysate obtained was used for a second infection of new monolayers.

After the second passage on cell culture, two other subcultures were performed as described above, for a total of four passages. Cell culture cryolysate of the final subculture was analyzed by seminested-PCR for RNA-HAV to determine the presence of HAV.

2.5 Molecular analyses

2.5.1 Viral extraction and purification

Total RNA extraction from cell culture cryolysate was performed using the commercial kit "*High PureRNA Isolation Kit*" (*Roche*). The RNA was eluted with *Elution Buffer* and after the addition of *RNAse inhibito*r was stored at -80°C prior to use.

2.5.2 RT-PCR

The viral RNA was reverse transcribed using the commercial kit "GeneAmp ® RNA PCR and Core Kit" (Applied Biosystems).

The reverse transcription reaction was optimized for a volume of 20 μL containing 2.5 μL of nucleic acid extract and 17.5 μL reaction mixture [5 mM $MgCl_2$, 1X PCR Buffer II, 1 mM of each deoxynucleotide triphosphate, 2.5 μM random hexamers, 1U/μL of RNase inhibitor and 2.5 U/μL of reverse transcriptase (murine leukemia virus MuLV Moloney)]. The temperature profile of the reverse transcription reaction consisted of a reverse transcription cycle at 42°C for 60 minutes followed by a denaturation cycle at 94°C for 5 minutes.

2.5.3 Seminested PCR and electrophoresis

The resulting cDNA was used in a seminested PCR reaction for the determination of hepatitis A virus in accordance with LE GUYADER *et al.* (1994). The gene segment amplified in the PCR reaction corresponds to the junction between VP1 and VP3. The method consists of

two consecutive amplification steps: from the first, using primers HAV-1 (reverse) and HAV-2 (forward), a PCR product of 247 pb is obtained. This amplicon is the template for the second amplification, which involves the use of primers HAV-2 (forward) and HAV-3 (reverse), resulting in an amplicon of 210 bp. The first amplification product was obtained using the commercial kit "GeneAmp RNA PCR core kit" (Applied Biosystems) in a final volume of 25 μL of reaction mixture (1 mM $MgCl_2$, 1X PCR Buffer II, 0.5 mM deoxynucleotide triphosphate, 0.5 μM of primer HAV-1, 0.5 μM of primer HAV-2, 0.025 U/μL AmpliTaq® DNA Polymerase and water DNAse/RNAse free) containing 5 μL of cDNA. The mixture was incubated at 94°C for 5 min, and then subjected to 35 cycles (94°C for 30 sec, 55°C for 1 min, 72°C for 30 sec). A final extension was carried out at 72°C for 7 min.

The amplified products were analysed by agarose gel electrophoresis (1.5% agarose) in the presence of ethidium bromide. The "PCR 100 bp Low Ladder (Sigma-Aldrich)" molecular weight marker was used. The gel was visualized with the "UV Bio-Rad GelDoc XR" image acquisition system. The sample was considered positive if a band of 200 bp was visible on the gel.

2.6 Virus quantification by titration on cell cultures

Three clam homogenates, chosen at random among the clam samples and collected from different bags, that resulted positive for isolation on FrP3 cell cultures and subsequently analysed in seminested PCR for RNA - HAV, were subjected to virus titration. Similarly, the aliquots of virus suspension used to infect the clams were also titrated.

Titration was performed on FrP3 cell monolayers grown on 24-well tissue culture plates; the infection of monolayers was performed by virus adsorption (200 μL) in quintuplicate for 1 hour at 37°C in a 5% CO_2 atmosphere. Well plates were incubated for 10 days at 37°C in a 5% CO_2 atmosphere and observed daily to check for cytopathic effects. At the end of the incubation, titres were expressed in $TCID_{50}$/mL according to the Reed and Muench formula (REED and MUENCH, 1938) (Table 1).

Table 1 - Effect of different heat treatment on HAV.

Sample	Temperature	Time	Titre expressed as $TCID_{50}$/mL
Clam homogenate (C+)	No treatment		$2.7x10^5$
Viral suspension			$1.6x10^7$
Clam homogenate	60°C	5 min	$1.3x10^{5*}$
Viral suspension			$2x10^5$
Clam homogenate	60°C	10 min	$2.7x10^{3*}$
Viral suspension			$3.2x10^1$
Clam homogenate	70°C	5 min	$7.1x10^{3*}$
Viral suspension			$1.1x10^1$
Clam homogenate	70°C	10 min	$1.6x10^{1*}$
Viral suspension			<1^
Clam homogenate	80°C	5 min	$3.8x10^{2*}$
Viral suspension			<1^
Clam homogenate	80°C	10 min	$1.1x10^{1*}$
Viral suspension			<1^
Clam homogenate	90°C	5 min	<1^
Viral suspension			<1^
Clam homogenate	90°C	10 min	0§
Viral suspension			0§
Clam homogenate	90°C	20 min	0§
Viral suspension			0§
Clam homogenate	90°C	30 min	0§
Viral suspension			0§

C+ = positive control.
*Average value of three titres of clam homogenate tested.
^Presence of cytopathic effect but titre not determinable.
§No cytopathic effect.

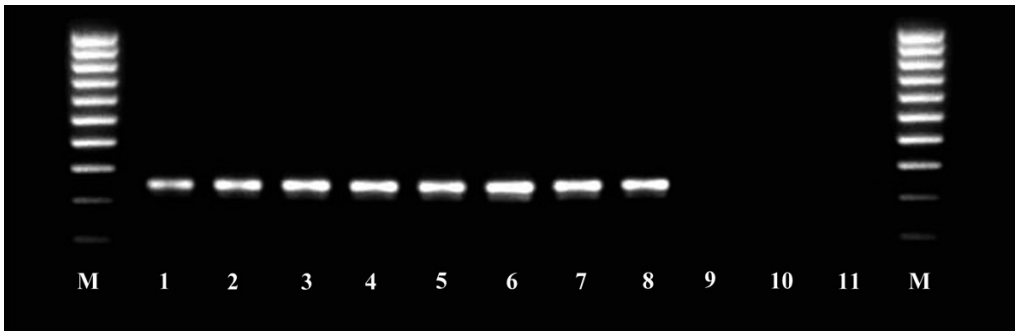


Fig. 1 - RNA-HAV detection by seminested RT-PCR in cell culture cryolisate Lane M: Marker Sigma-Aldrich (Low Ladder 100 bp). Lane 1: positive control (clam samples infected but not heat treated). Lane 2: clam samples after heat treatment of 5 min at 60°C. Lane 3: clam samples after heat treatment of 10 min at 60°C. Lane 4: clam samples after heat treatment of 5 min at 70°C. Lane 5: clam samples after heat treatment of 10 min at 70°C. Lane 6: clam samples after heat treatment of 5 min at 80°C. Lane 7: clam samples after heat treatment of 10 min at 80°C. Lane 8: clam samples after heat treatment of 5 min at 90°C. Lane 9: clam samples after heat treatment of 10 min at 90°C. Lane 10: clam samples after heat treatment of 20 min at 90°C. Lane 11: clam samples after heat treatment of 30 min at 90°C.

RESULTS

All of the homogenates from each infected clam, subjected to a heat treatment of 5 minutes at 60°, 70° and 80°C and 10 minutes at 60° and 70°C showed a cytopathic effect on cell cultures following the first passage. In contrast, the samples heat treated at 80°C for 10 minutes and 90°C for 5 minutes, showed a cytopathic effect (CPE) in cell cultures only after a second passage in cells. Clam samples heat treated for 10, 20 and 30 minutes at 90°C did not show any cytopathic effect and were negative after four consecutive passages in cell culture. All cell culture supernatants, both from cell cultures with or without CPE, including positive and negative controls, were tested at the end of the fourth passage, by seminested PCR for HAV. These molecular analyses confirmed the virus isolation results, i.e., no amplicon was observed for samples heat treated for 10, 20 and 30 minutes at 90°C (see Fig. 1).

The titres obtained from the heat treated clam homogenates were compared with the titres of the virus suspension subjected to the same heat treatment. The comparison of the titres gave similar results with regard to the progressive reduction of the viral titre with increasing time and temperature treatment, but clearly demonstrated that, within the tissues of the mollusc, the virus has a higher resistance to the effect of temperature. In fact, titres of virus present in the clam homogenates (although with a starting titre almost 60 times lower than those of viral suspensions i.e. $2.7x10^5$ *vs* $1.6x10^7$) are visibly higher after the same heat treatment and this difference is more evident with increasing time and temperature (see Table 1 and Figs. 2 and 3).

CONCLUSIONS

To our knowledge, this is the first work concerning the resistance of HAV in pasteurized and packed products although the HAV sensi-

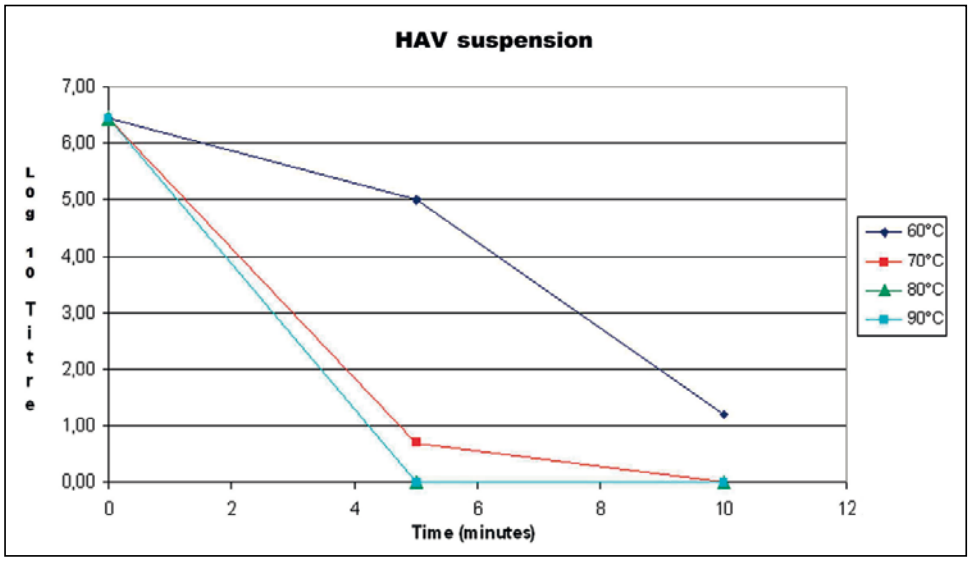


Fig. 2 - Hepatitis A virus determination on viral suspension treated at 60°, 70°, 80°, 90°C for 5 and 10 minutes. The titrations of the viral suspensions were performed in duplicate obtaining identical results.

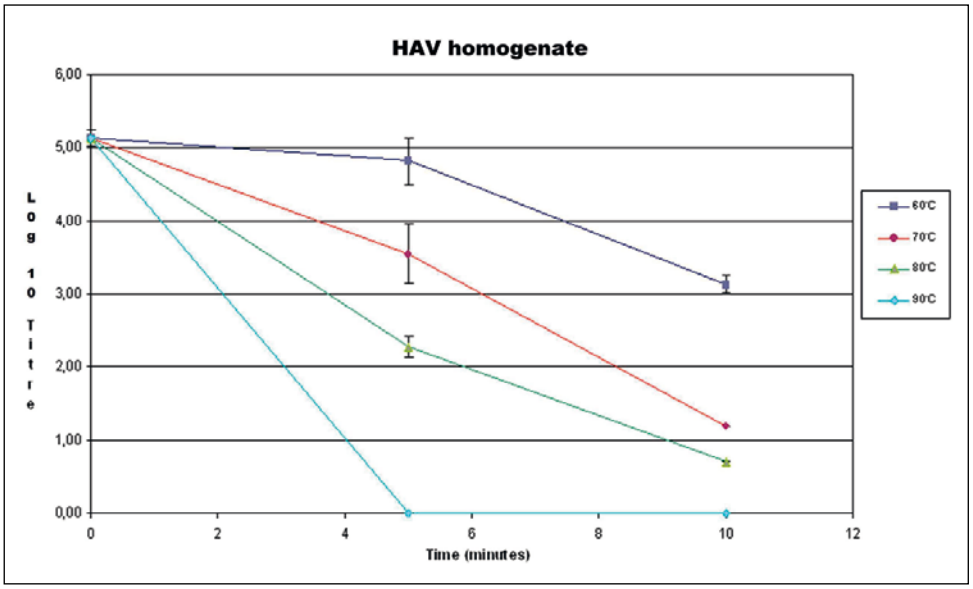


Fig. 3 - Hepatitis A virus determination on three homogenates of clams treated at 60°, 70°, 80°, 90°C for 5 and 10 minutes. The titrations of the homogenates were performed in triplicate. Points on the graph are the average of the three titres calculated for each time point and each temperature. The bars represent the standard deviation.

tivity to heat treatment has already been demonstrated in various scientific reports. With the aim of establishing a temperature to completely inactivate hepatitis A virus in molluscs, MILLARD *et al.* (1987) carried out tests on samples of *Cerastoderma edule* (common cockle) by artificially inoculating them with the virus and heat treating them both by immersion in boiling water and hot steam cooking. The authors concluded that after one-minute immersion in boiling water or steam heating for 1.5 minutes, the core of the molluscs reached a temperature of 85°-90°C and if such a temperature was maintained for another minute it was sufficient to inactivate the virus completely.

Other tests, carried out more recently, report the need for longer treatments and higher temperatures. CROCI *et al.* (2005) reported that it took 8 minutes in boiling tomato juice to inactivate HAV in mussels. HEWITT and GREENING (2006) used steam cooking for 3 minutes (average core temperature of 63°C) to reduce the viral titre by about 1.5 logarithms and immersion of mussels in boiling water for 3 minutes (average core temperature 92°C) was required to completely inactivate HAV in lip mussels (*Perna canaliculus*).

The results of the present work only partially confirm previous data, but comparison is not simple due to the different temperatures, exposure time, type of mollusc and method used to test the viability of the virus and infection dose applied. Our results highlight a higher survivability of hepatitis A virus to the temperatures tested compared to other studies as well as the importance of applying an appropriate experimental procedure that allows even small amounts of viable infectious virus to replicate and to be identified following heat treatment. The treatment of virus at a core temperature of 60°C for 5 minutes applied in this work to infected molluscs did not cause a significant reduction of viral titre nor was treatment at 90°C for 5 minutes able to inactivate the virus completely.

Some authors have investigated the effect of protection provided by animal tissues to heat treatment by comparing titre reductions between virus samples inoculated into molluscs and uninoculated samples (CROCI *et al.*, 1999). It is known that hepatitis A virus can be easily inactivated by heat but the viruses' sensitivity to heat depends on the environment in which it finds itself. Indeed, the present study shows that the virus is able to resist more if protected by the tissue of the mollusc pulp than if free in a protein solution.

CROCI and SUFFREDINI (2003) report that some components of food can protect the virus from the effect of temperature and this important aspect has already been reported in the past by other authors. For example, the fat percentage in some milk-based food products is important in determining HAV virus stability when exposed to heat (BIDAWID *et al.*, 2000). Other studies (DI GIROLAMO, 1970) have reported that molluscs provide good protection against heat treatment; in fact, in oysters infected with poliovirus and subsequently treated with different cooking methods (steamed, fried, oven-baked, etc.), the virus had a survival rate of between 7 and 13%. The present study also confirmed the protective role of tissues in clams for HAV: to inactivate the virus inoculated in the mollusc a 10-minute treatment at 90°C was necessary, while 5 minutes at 70°C were sufficient for inactivation of an equal amount of "unprotected" virus.

In conclusion, the results of this study indicate that the industrial steam pasteurization treatment of whole clams capable of reaching a temperature of 90°C in the core of the clam for a minimum of 10 minutes can result in the complete inactivation of HAV with an infective dose $\leq 2.7 \times 10^5$ $TCDI_{50}$/mL.

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PAPER

THE INFLUENCE OF STERILIZATION ON FREE-RADICAL GENERATION, DISCOLORATION AND THE ANTIOXIDANT PROPERTIES OF CERTAIN SPICE HERBS

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ABSTRACT

The aim of this work was to assess the influence of steam sterilization on free-radical generation in herbs and on changes in their chromatic parameters, as well as in their antioxidant activity and the content of polyphenols and flavonoids.

The research conducted showed that the Hight Temperature Short Time (HTST) sterilization method decreased the number of free radicals in the herbs tested. At the same time, the antioxidant activity as well as the polyphenol and flavonoid contents increased. Changes in chromaticity in sterilized herbs were determined; darkening and shift towards red and yellow hues were observed. The antioxidant properties of herbs before and after sterilization decreased in the following sequence: oregano>thyme>marjoram>basil.

- Keywords: EPR, CIELab, antioxidant activity, spices, steam sterilization -

INTRODUCTION

Herb spices have been used for many centuries to imbue foods with desired flavor and aroma characteristics. Among these herbs are basil, (*Ocimum basilicum* L.), oregano (*Origanum vulgare* L.), marjoram (*Origanum majorana* L.) and thyme (*Thymus vulgaris* L.). These plants belong to the mint family, or Labiatae. They contain essential oils of varied composition, organic acids and flavonoids. Thus, they are characterized by specific sensory properties and may positively influence, among others, digestion processes, as they have an antispasmolytic effect and stimulate the excretion of gastric acid. The components in these herbs give them anti-inflammatory properties (OCAÑA-FUENTES *et al.*, 2010), as well as bactericidal and fungicidal properties, which are used in the treatment of various infections, mainly of the upper and lower respiratory tract and the skin (TOHIDPOUR *et al.*, 2010; ROTA *et al.*, 2008; DE SOUZA *et al.*, 2010). They also stimulate the excretion of mucus from the bronchi and allow for easier expulsion (GROSSO *et al.*, 2010), in addition to having a tranquilizing effect and countering depression (NGUYEN *et al.*, 2010). The herbs are also used as an auxiliary in treating colic and diarrhea. Tests have also shown that they have cholesterol-lowering properties in the blood (BRAVO *et al.*, 2008) and inhibit blood-platelet aggregation (YAZDANPARAST and SHAHRIYARY, 2008).

Apart from their well-known medicinal properties, the herbs and spices are primarily an abundant source of antioxidant compounds, which play an important role in the prevention of lifestyle diseases (YU *et al.*, 2010; LEE *et al.*, 2005). These characteristics are due to the presence of polyphenol compounds in the herbs, such as phenolic diterpen, phenolic acids and flavonoids (HOSSAIN *et al.*, 2011; SELLAMI *et al.*, 2009), as well as vitamin C, tocopherols and carotenoids (SUHAJ, 2006; SGHERRI *et al.*, 2010).

Herbs and spices can become contaminated with microorganisms that pose a serious threat to human health. The contamination can be primary, as in the case of soil, dust, bird feces and fertilizer pollution, or secondary, as caused by incorrect gathering, drying, storage, packaging or transportation of plants (KNEIFEL *et al.*, 2002; SCHWEIGGERT *et al.*, 2007). Various methods of removing microorganisms from food products exist. The first method, currently forbidden in European countries, was sterilizing with ethylene oxide gas (TATEO and BONONI, 2006). Food is also sterilized by means of radiation (FARKAS, 2006; POLOVKA and SUHAJ, 2010). Because the influence of radiation on sterilized products is not fully known, the method of irradiation is not very popular in many countries. When using radiation in the sterilization process, special information must be included on the product packaging. The most popular method used in the herb- and spice-processing industry involves steaming (ALMELA *et al.*, 2002). Modern steam sterilization methods are ecologically benign and ensure microbiological purity. One such method is Natural Microbiological Clear (NMC®) steam sterilization, which employs the High-Temperature, Short-Time (HTST) principle, as well as a special Nauta® mixer. Sterilization is performed using saturated steam, brought in under a pressure of 10 bar at a temperature of 110°-140°C in a relatively short time (30-180 seconds). The product is constantly stirred during sterilization. Steam vapor together with essential oils pass through the condenser, where the oils liquefy and are introduced back into the cooled product. Thanks to this, the loss of essential oils is marginal. The humidity of the products obtained does not exceed 2-10%.

Oregano, thyme, basil and marjoram are usually added to dishes at the beginning of the culinary process and are subject to prolonged heat exposure. They also might be added at the end of cooking and thus heated for a short duration.

Considering the possibility of changes in the composition and the quality of herb material exposed to high temperature (BAGDATLIOGLU and ORMAN, 2010), the aim of this work was to assess the influence of steam sterilization on the appearance of free radicals and the changes both in chromaticity parameters in dried herbs and in the antioxidant activity and the content of polyphenols and flavonoids in their water extracts.

MATERIALS AND METHODS

Materials

The plant material for this study were unsterilized (NST) and sterilized (ST) dried herbs: basil, (*Ocimum basilicum* L.), oregano (*Origanum vulgare* L.), marjoram (*Origanum majorana* L.), and thyme (*Thymus vulgaris* L.).

The material for the study was provided by K.P.P.S. Interjarek company (Gołuchów, Poland) where the herbs and spices were sterilized by means of the NMC® steam sterilization method.

Sample preparation and conditions of EPR spectra measurements

Powdered samples in their initial state as well as samples of the sterilized leaves were placed in thin-walled glass test tubes with an outer diameter of 3 mm. Empty tubes gave no electron paramagnetic resonance (EPR) signal at the maximum line amplification and maximum microwave power (70 mW).

EPR spectra of the samples tested were registered at room temperature using an X-band frequency spectrometer (9.3 GHz) with a magnetic field modulation of 100 kHz (RADIOPAN compa-

ny, Poznań, Poland). The frequency of microwave radiation was registered by means of an MCM 101 meter (EPRAD company, Poznań, Poland).

The samples in the test tubes were placed between the electromagnetic poles, and EPR spectra were registered by computer in the form of the first derivative of absorption. Numerical acquisition of data (JAGMAR company, Kraków, Poland) was used, together with the LabView program and spectroscopic analysis software.

Measuring the concentration of free radicals in the samples

The integral intensity of the EPR line registered as the first derivative of absorption was measured by integration of the area under the curve of absorption resonance performed twice. The integral intensity of the EPR line was used in calculating the concentration of free radicals in the sample (WEIL *et al.*, 1994; EATON *et al.*, 1998).

Ultramarine (sodium aluminosilicate containing sulphur) was used as the pattern of concentration because the paramagnetic centers present in it are quite stable. To increase the accuracy of the measurement, a ruby crystal (Al_2O_2: Cr^{3+}) was used as the so-called 'internal pattern'. For each sample analyzed and the ultramarine, the ruby EPR signal was also registered. The amplitude of the EPR line of the ruby was measured.

The concentration of free radicals in the samples was measured according to the following equation:

$$N = n_u (I /WA) / (I_u / W_u A_u)$$

where:

n_u – the number of paramagnetic centers in ultramarine (the reference) ($n_u = 1.2x10^{19}$spin);

W, W_u – the receiver gains for sample and ultramarine, respectively;

A, A_u – the amplitudes of the ruby signal for the sample and ultramarine, respectively;

I, I_u – the integral intensities for the sample and ultramarine, respectively;

m – the mass of the sample.

Chromatic parameter measurement

Chromatic parameters of unsterilized and sterilized herbs were measured using a CM-5 spectrophotometer (Konica, Minolta). The samples were placed on a Petri dish and measured in reflected light with the mirror-surface reflection component attenuated. A ∅ 30 mm aperture was used as well as a standard colorimetric observer with a 10° field of vision and D65 illuminant. Before each measurement, the device underwent white tile calibration. Chromatic coordinates were measured according to the conventional CIELab colorimetry system. Differences in the parameters of color brightness were measured in terms of the L* coordinate and the tone of color as coordinates a* and b*. Additionally, the absolute difference of color DE*ab was measured (NSONZI and RAMASWAMY, 1998):

$$\Delta E^*ab = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2}$$

where ΔL*, Δa*, and Δb* are indicators of chromatic difference between sterilized and unsterilized herbs.

Preparation of water extracts

Water extracts of the herbs were used in the study, prepared in the form of infusions and decoctions.

For the infusions, 0.5 g of the material was measured, soaked in 5 mL of deionized boiling water and cooked for 15 min in a lid-covered vessel. The infusion was set aside for another 15 minutes and then filtered and filled with water to achieve a final volume of 10 mL.

For the decoctions, 0.5 g of the material was measured, soaked in 5 mL of deionized room-temperature water and cooked for 30 min in a lid-covered vessel, then filtered and filled with water to achieve capacity final volume of 10 mL.

Measurement of TAS, polyphenol and the flavonoid contents

In the extracts obtained and after having diluted the sample, the Total Antioxidant Status (TAS) was measured, as well as the polyphenol content, using colorimetric techniques. The measurement of TAS was performed using Randox Laboratories Ltd., United Kingdom. The method of measuring TAS consists of the inhibition of the appearance of $ABTS^{\bullet+}$ cation radicals proportional to the concentration of antioxidants contained in the sample. The total antioxidant activity was expressed as the Trolox antioxidant activity or TEAC (Trolox Equivalent Antioxidant Capacity).

The technique of measuring polyphenols (SINGLETON and ROSSI, 1965) is based on a chromatic reaction with the use of the Folin-Ciocalteau reagent. The total polyphenol concentration was calculated as mg gallic acid equivalents (GAE). The flavonoid content was measured using the Woisky and Salatino method (WOISKY and SALATINO, 1998) based on the production of chromatic complex compounds with $AlCl_3$. The flavonoid concentration was expressed in mg quercetin equivalents (QE).

Statistical analysis

All data were expressed as the means ± standard deviation of five measurements. ANOVA analyses and the STATISTICA PL v. 8.0 software were used to evaluate the results of the experiments. A value of $p < 0.05$ was regarded as significant.

Table 1 - Parameters of the EPR spectra recorded for herbs.

		Parameters of EPR spectra			
		A [a.u.] (±0.01)	ΔB_{pp} [mT] (±0.02)	I [a.u.] (±0.01)	N x 10^{18} [spin/g] (±0.1x10^{18})
Before sterilization	Oregano	0.15	0.59	0.47	1.3
	Basil	0.56	0.60	2.03	3.4
	Thyme	0.21	0.59	0.67	1.9
	Marjoram	0.38	0.58	1.42	1.3
After sterilization	Oregano	0.14	0.61	0.42	1.2
	Basil	0.47	0.62	1.67	3.0
	Thyme	0.19	0.58	0.53	1.5
	Marjoram	0.32	0.60	1.32	1.2

RESULTS AND DISCUSSION

The sterilization process, which aims at removing microorganisms from stored products, employs steam at a very high temperature and pressure. Herbs owe their medicinal properties mainly to the high content of antioxidants and essential oils. The latter in the form of fatty acid esters might undergo hydrolysis and oxidation resulting in the generation of superoxide radicals if exposed to conditions favorable to such changes. EPR results showed that HTST steam sterilization does not cause a significant increase in the amount of free radicals. These tests demonstrated that both the initial samples and the sterilized samples were paramagnetic. EPR spectra were registered for samples of all the herbs tested, both the sterilized herbs and those in their initial state. EPR spectra were obtained at low microwave power and at 15 dB of attenuation. The EPR spectral parameters used for testing the herbs are shown in Table 1. The amplitude (A) of the EPR line increases with the number of free radicals present in the sample, and the width (ΔB_{pp}) of the EPR line depends on magnetic interactions between unpaired electrons in the sample. The amplitude (A) of the EPR line decreases following sterilization in all herbs tested, and the width of the EPR line (ΔB_{pp}) of the sterilized herbs is greater than the width of the EPR line of the initial samples (Table 1). This means that in the sterilized samples more powerful dipole interactions take place between free radicals. The EPR lines of both the initial samples and the sterilized herbs are characterized by a large width, which indicates powerful dipole interactions between the unpaired electrons of free radicals, and thus the distances between them are short. The relationship between the amplitude and width of the EPR lines and the microwave power are characteristic of a homogenous distribution of free radicals in the sample. In the case of sterilized herbs, these results are especially important because they indicate that the sterilization process influences the samples in a homogenous manner, and spin islands do not appear.

Both in the initial samples and in the sterilized samples, free radicals are present. Their concentrations are similar and amount to 10^{18} spin/g (Table 1). It has been determined that in all the herbs tested the concentration of free radicals decreases following sterilization (Fig. 1).

The highest concentration of free radicals was observed in basil, both sterilized and unsterilized, while the lowest concentration was found in oregano and marjoram. The decrease in the number of free radicals might be connected with the increased antioxidant activity observed in the herbs following sterilization (Fig. 1). In the case of oregano, which has the highest antioxidant value, the smallest number of free radicals was observed. Conversely in the case of basil, the greatest number of free radicals was accompanied by the lowest antioxidant activity among the herbs tested.

During sterilization, a change in the color of the plant products took place, which was demonstrated by changes in the CIELab values in (Fig. 2). In sterilized herbs, a decrease in the L* chromaticity coordinate was observed in comparison with the unsterilized samples. This decrease means that less light was reflected by the surface of the material, which is most likely due to a loss of water during sterilization and greater porosity of the dried herb surface. This change results in dried herbs being darker and the L* coordinate shifting towards black. The value of the L* parameter, that is, the brightness of the material, also depends among other things on the number of compounds of brown hue. During the process of drying, enzymatic and non-enzymatic reactions of browning take place in the plant tissue, causing an increase in the content of brown pigments and thus a darkening of the dry material (PERERA, 2005). The greatest change in the parameter (ΔL^* = -3.5) was observed in basil and the least in thyme (ΔL^* + -1.05) and oregano (ΔL^* + -1.16).

The value of the a* chromaticity coordinate

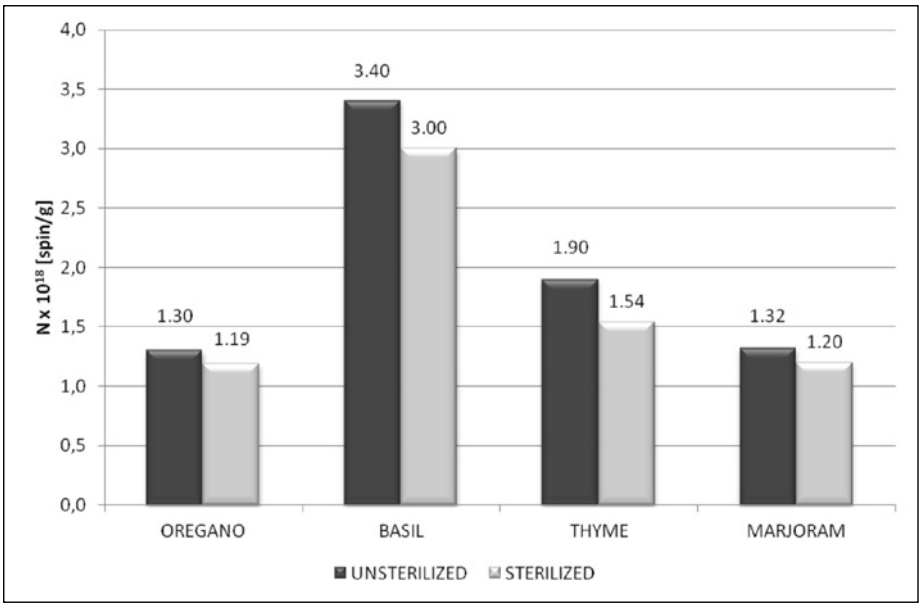


Fig. 1 - The influence of sterilization on the free radical concentration (N) in the tested herbs.

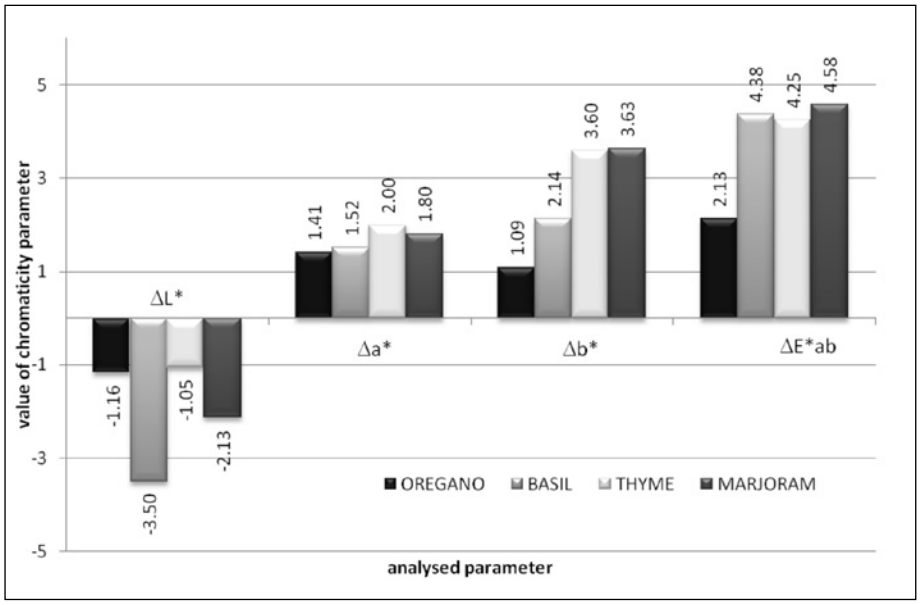


Fig. 2 - Change in the chromaticity parameters in sterilized herbs.

responsible for red (+) and green (+) hues increased in the sterilized herbs, that is, following the shift towards red, which is confirmed by other reports in the literature (ALIBAS, 2007; ALIBAS *et al.*, 2007). In the unsterilized herbs, the value of the a* coordinate is characterized by a greater amount of green hue. Evidently, sterilization causes a decrease in the share of green color, which might be caused by degradation of chlorophyll a and b during the high-temperature process. The changes in Da* are similar for all herbs tested and amount to between 1.41 and 2.0 (Fig. 2). The positive value of the b* parameter is responsible for a yellow hue. As a result of sterilization, the value of this parameter increased from b*=1.09 in oregano to Δb*=3.63 in thyme (Fig. 2). The increase in the share of yellow might be caused by the process of browning as well as by the depletion of chlorophyll, which often conceals the carotenoids of yellow, orange and red hues present in the leaves. Similar changes in the b* chromaticity parameter were observed in mint and parsley leaves (THERDTHAI and ZHOU, 2009; DOYMAZ *et al.*, 2006).

The value of the total chromaticity difference (ΔE*ab) was determined based on the brightness (L*), red color (a*) and yellow color (b*) of the final product. The more distant these values were from the model – the unsterilized herbs – the greater was the total chromaticity difference.

Assessing the total chromaticity difference (ΔE*ab) between the sterilized and the unsterilized herbs, it may be said that the oregano sample is characterized by a color that was altered the least by sterilization (ΔE*=2.13). The remaining herbs displayed higher values of ΔE* (from 4.25 in thyme to 4.58 in marjoram) (Fig. 2).

Higher values were found for all parameters analyzed, i.e., the antioxidant activity, as well as the polyphenol and flavonoid concentrations in the extracts of the sterilized herbs in comparison with the extracts of the unsterilized ones. The influence of temperature on the antioxidant activity of plant products is not single-valued and depends, among other things, on the temperature value and the time of exposure (KATSUBE *et al.*, 2009; TOMAINO *et al.*, 2005). The increase in the antioxidant activity observed as a result of exposure to high temperatures might be caused by many factors. As a result of heating, increased pressure or enzymatic hydrolysis, damage to cell walls occurs and an increase in the bioaccessibility of the antioxidant substances might result (MCINERNEY *et al.*, 2007). Furthermore, during thermal processing new antioxidant compounds might be created (JEONG *et al.*, 2004). What seems the most important consideration, however, is that thermal processing, which, on the one hand, is detrimental to many components, inactivates enzymes responsible for enzymatic oxidation of natural antioxidants on the other hand, contributing to the retention of antioxidant activity (GONÇALVES *et al.*, 2010). An example of this effect might be fruits and vegetables subjected to so-called blanching, which retain their antioxidant activity during storage to a greater extent than non-blanched fruits and vegetables (PATRAS *et al.*, 2010). An important factor influencing the antioxidant content is not only the increased temperature itself but also the environment in which it acts. Heating plant products in water causes relatively fast penetration of heat to the inside of the tissues, which causes longer exposure of the whole mass of the processed product to the agent and high antioxidant-compound loss.

However, when heated in the air, the inside of the product remains at a lower temperature than the surface, which causes lesser antioxidant loss (TURKMEN *et al.*, 2005; FRANCISCO *et al.*, 2010). As Munyaka has demonstrated (MUNYAKA *et al.*, 2010), blanching by the HTST method (high temperature short time) is the best way of retaining vitamin C while processing broccoli.

The antioxidant activity in herbs both before and after sterilization decreased in the following sequence: oregano>thyme>marjoram>basil (Fig. 3). Decoctions displayed slightly lower values of antioxidant activity than infusions, which might be caused by a longer period of heating and the decomposition of antioxidant substances vulnerable to high temperatures, such as for example ascorbic acid. Changes in the polyphenol concentrations were similar to changes in the antioxidant activity (Fig. 4). The highest polyphenol concentration in the sterilized herbs was measured in oregano (20.67-21.22 mg GAE/mL), while the lowest concentration was measured in basil (6.13-7.40 mg GAE/mL). Contrary to the antioxidant activity, higher concentra-

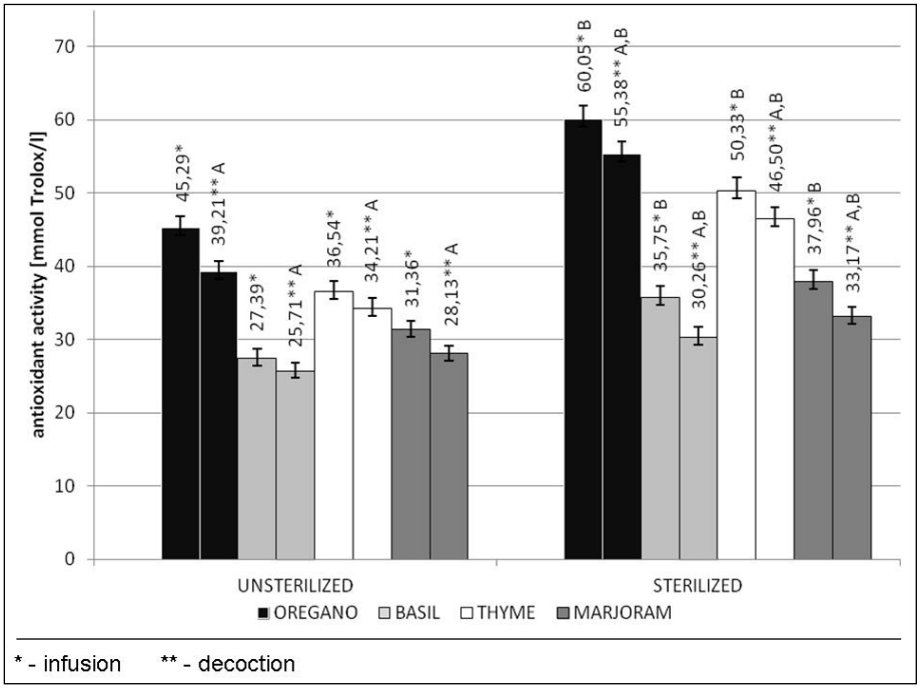


Fig. 3 - Antioxidant activity of water extracts of sterilized and unsterilized herbs. A – $p < 0.05$ *vs* infusion; B – $p < 0.05$ *vs* unsterilized.

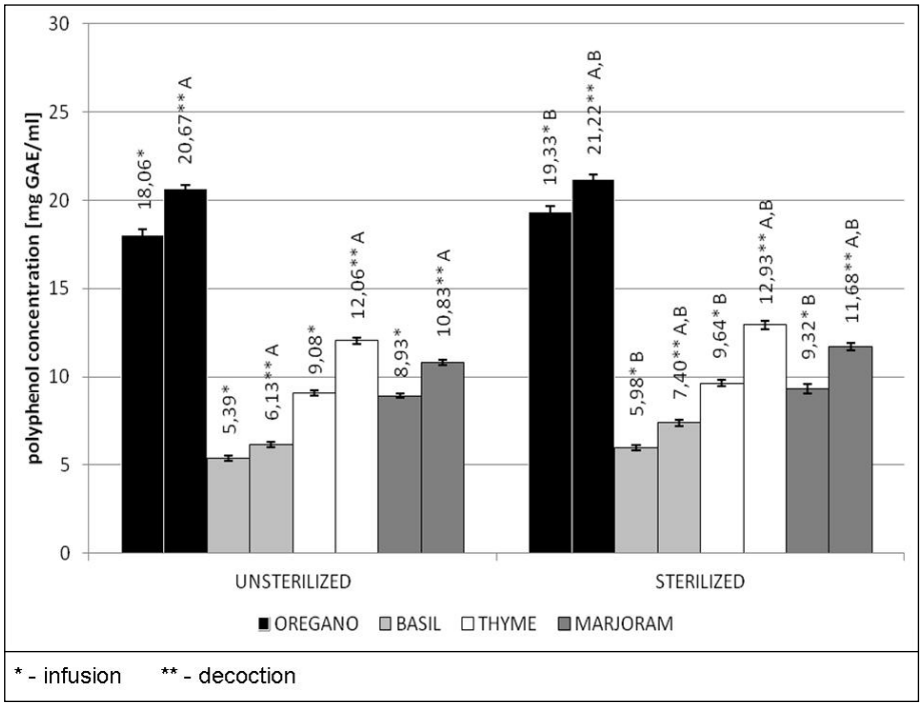


Fig. 4 - Polyphenol concentrations in water extracts of sterilized and unsterilized herbs. A – $p < 0.05$ *vs* infusion; B – $p < 0.05$ *vs* unsterilized.

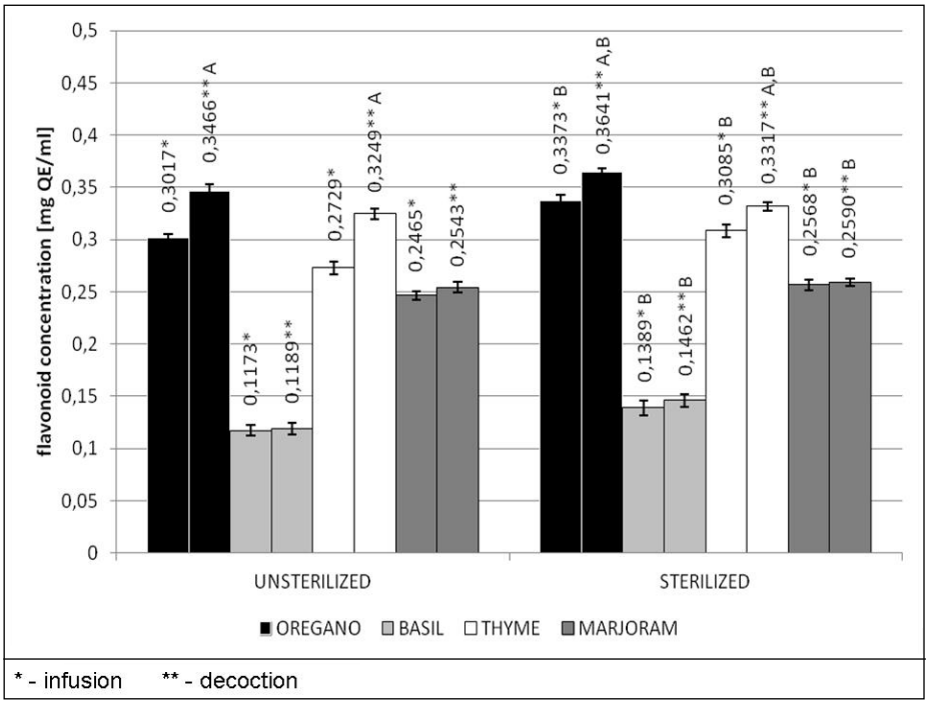


Fig. 5 - Flavonoid concentrations in water extracts of sterilized and unsterilized herbs. A – $p < 0.05$ *vs* infusion; B – $p < 0.05$ *vs* unsterilized.

tions of polyphenols were measured in the decoctions than in the infusions. This slight increase in polyphenol concentration in decoctions might be caused by longer heating and the aforementioned increase in bioaccessibility. The tendency of the changes in herb flavonoids before and after sterilization is similar to the trend in polyphenols. However, the changes are very minute (Fig. 5). Both sterilization and the way in which the water extracts were prepared had little influence on the flavonoid concentration. Higher flavonoid concentrations were found in the sterilized herbs and their decoctions. The concentrations of flavonoids meas-

ured in the herbs decreased in the following sequence: oregano>thyme>marjoram>basil.

An attempt at assessing the correlation between the antioxidant activity and the polyphenol concentration in the sterilized ($r=0.878$, $p<0.05$) and unsterilized ($r=0.875$, $p<0.05$) herb extracts resulted in determining a linear correlation. The correlation between the content of phenolic compounds and antioxidant activity in various plant extracts was proven by MILIAUSKAS *et al.* (2004). Lower correlations in the sterilized ($r=0.839$, $p<0.05$) and unsterilized ($r=0.762$, $p<0.05$) herb extracts were found between the antioxidant activity and the flavonoid content, as well as between the polyphenol and flavonoid concentration in the sterilized ($r=0.873$, $p<0.005$) and unsterilized ($r=0.768$. $p<0.05$) herb extracts.

CONCLUSION

The research conducted showed that the HTST sterilization method decreased the number of free radicals in the herbs tested. At the same time, the antioxidant activity as well as the polyphenol and flavonoid contents increased. Changes in chromaticity in sterilized herbs were observed in the form of darkening and a shift towards red and yellow hues. The antioxidant properties of herbs before and after sterilization decreased in the following sequence: oregano>thyme>marjoram>basil.

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PAPER

OPTIMIZATION OF PROCESS VARIABLES FOR EXTRACTION OF ZINC, COPPER AND IRON BY MICROWAVE-ASSISTED EXTRACTION FROM PISTACHIO NUT USING RESPONSE SURFACE METHODOLOGY

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ABSTRACT

In this study, a full factorial design and three-level, three-factor Box-Behnken experimental design based on response surface methodology (RSM) were used for optimizing zinc, copper and iron mineralization from pistachio nuts by microwave-assisted extraction (MAE) prior to its determination using flame atomic absorption spectrometry (FAAS). Four independent variables including power, temperature, time and volume of nitric acid were chosen in this study. The results of full factorial design showed that the power, temperature and volume of nitric acid were significant. Therefore, a Box-Behnken experimental design was performed in order to determine the optimum conditions. The working conditions for results obtained by Box-Behnken for three elements were established as a compromise between optimum values found for each analyte (zinc, copper and iron). These values were 100°C, 425 W and 4.0 mL, for temperature, irradiation power and volume of nitric acid, respectively. A high regression coefficient between the variables and the response ($R^2 \approx 0.97$ for all elements) indicated excellent evaluation of experimental data by a polynomial regression model.

- Keywords: copper, zinc, iron, response surface methodology, microwave-assisted extraction, pistachio nut -

INTRODUCTION

The pistachio is a small tree originally from Iran, which now can also be found in regions of Turkey, Syria, Greece, Turkmenistan, Kyrgyzstan, Egypt, India, Pakistan and possibly Afghanistan. The pistachio, in particular, significantly reduced levels of low-density lipoprotein (LDL cholesterol) while increasing levels in the serum of volunteers (KAY *et al.*, 2007; 2010). In rats, consumption of pistachio as 20% of daily caloric intake increased beneficial high-density lipoprotein without lowering LDL cholesterol, and while reducing LDL oxidation (AKSOY *et al.*, 2007).

Trace elements are very important both nutritionally and toxicologically. Copper, iron and zinc are very essential, but ingestion of excessive levels can be toxic to the human body. Humans acquire copper, iron and zinc from numerous sources, such as food, beverages and water. Dietary intake is considered to be the major source of these elements for the body (DEMIROZU *et al.*, 2002). Hence, the consumed levels of food products should be investigated. For this reason, the goal of the present study was to determine the level of copper, zinc and iron in commercial pistachio products. Several methods, such as mineralization with concentrated reagents (HNO_3, HCl and $HClO_4$; OLIVEIRA, 2003) have been used in previous research. These methods involve a large sample mass and require both the attention of the operator and a long completion time (HUIE, 2002). However, the analytical sequence time can be reduced using microwave-assisted extraction (MAE), hence leading to an increase in the analytical sampling rate (OLIVEIRA, 2003).

One of the main advantages of MAE is the reduction of the extraction time. It also allows for a reduction in organic solvent consumption. The principle of heating using microwave energy is based on the direct influence of microwaves on molecules by ionic conduction and dipole rotation. If MAE is carried out in closed vessels, extractions can be performed at temperatures above the boiling point of the solvent; therefore, increasing the extraction rate and efficiency. Parameters that affect extraction include extraction time, temperature, power and solvent volume (PIZARRO *et al.*, 2007).

Response surface methodology (RSM) has several advantages compared to the classical experimental or optimization methods in which one variable at a time is changed. Firstly, RSM provides a large amount of information from a small number of experiments. Indeed, classical methods are time consuming and a large number of experiments are needed to explain the behavior of a system. Secondly, with RSM, it is possible to observe the interaction effect of the independent parameters on the response. The model equation easily clarifies effects for binary combinations of the independent parameters. In addition, the empirical model that relates the response to the independent variable is used to obtain information about the process. Finally, RSM also represents a more economical approach, as the number of experiments can be significantly reduced (SHOKUHFAR *et al.*, 2008). Clearly, RSM is a useful tool for optimizing analytical methods.

The optimization of analytical methods by multivariate experimental techniques is faster, more effective and more economical than the traditional "one-at-a-time" technique. Moreover, circumstances which are not explained by traditional methods, such as interactions between the parameters that affect the analytical response, can be revealed (FERREIRA *et al.*, 2003).

In this research, the MAE system was used for the mineralization of zinc, copper and iron from samples of pistachio nuts and prior to its determination by FAAS. The optimization of the experimental variables (power, time, volume of nitric acid, and temperature) is described using response surface methodology.

EXPERIMENTAL

Apparatus

The measurements were performed with a Konik Won M300 (Barcelona, Spain) flame atomic absorption spectrometer, equipped with a conventional pneumatic nebulizer and nebulization chamber. A hollow cathode lamp was used for determination of copper, zinc and iron at wavelengths of 324.8, 213.9 and 248.3 nm, respectively. The microwave extractions were carried out in an Ethos SEL (Milestone, Sorisole, Italy) equipped with 10 closed vessels. The HPR-1000/10s high-pressure segmented rotor was used. The maximum operating temperature is 260°C; and pressure is 100 bar. At full power, the Ethos delivers approximately 2,500 W of microwave energy at a magnetron frequency of 2,450 MHz.

Materials

The nitric acid (HNO_3) used was of the highest purity (65%) available from Merck (Darmstadt, Germany). Reagent grades of $ZnCl_2$ (97%), $CuSO_4 \cdot 5H_2O$ (98.5%) and $FeCl_3 \cdot 6H_2O$ were used. Stock solutions of zinc, copper and iron (100 $mg \cdot L^{-1}$) were prepared by dissolving a proper amount of the above salts in doubly distilled water in a 10 mL flask. Dilute solutions were prepared by an appropriate dilution of the stock standard solution with doubly distilled water.

MAE procedure

The pistachio nuts were purchased from a local market in Iran. The microwave-assisted extraction was used for the mineralization of pistachio. A 0.5-g sample of pistachio nut was transferred to the extraction vessel. According to a

Table 1 - Full factorial design matrix with 2 center point and the value of responses.

No.	P (W)	T (°C)	time (min)	V (mL)	Y(μgg^{-1}:RSD%)
1	100	60	5	4	7.92±0.5
2	300	60	5	4	5.89±0.3
3	100	80	5	4	5.73±0.6
4	300	80	5	4	5.69±0.5
5	100	60	15	4	4.74±0.1
6	300	60	15	4	8.87±0.4
7	100	80	15	4	6.04±0.6
8	300	80	15	4	9.72±0.3
9	100	60	5	8	5.33±0.5
10	300	60	5	8	9.32±0.4
11	100	80	5	8	8.58±0.6
12	300	80	5	8	10.20±0.3
13	100	60	15	8	7.64±0.1
14	300	60	15	8	5.01±0.5
15	100	80	15	8	7.86±0.4
16	300	80	15	8	9.15±0.6
17	200	70	10	6	8.85±0.1
18	200	70	10	6	8.81±0.4

preliminary experimental design, extractions were performed at various conditions of volume of nitric acid (for factorial design ranging from 4 to 8 mL and for RSM design ranging from 6 to 10 mL), temperature (for factorial design ranging from 60° to 80°C and for RSM design from 80° to 110°C), irradiation time from 5 to 15 min and power (for factorial design ranging from 100 to 300 W and for RSM design from 300 to 500 W). After extraction, the irradiated sample was transferred to a 10 mL volumetric flask and the volume was completed to the mark. The concentration of the zinc, copper and iron in this solution was analyzed by FAAS.

Statistical analysis

The data were Pareto chart and analysed using analysis of variance (ANOVA) to examine the statistical significance of factors. A P value of 0.05 was considered statistically significant. Box-Behnken experimental design was used for final optimization. The Design-Expert 8.0.7 software was used for statistical analysis.

RESULTS AND DISCUSSION

Factorial design

A two-level full factorial design (2^4) was performed for preliminary evaluation of the significance of each factor and their interactions in the extraction system. In this study, the experiments were planned to obtain a full factorial design containing 16 trials plus 2-center points (runs 17 and 18). The factors chosen were power (A; 100-300 W), nitric acid volume (D; 4-8 mL), time (C; 5-15 min) and temperature (B; 60°-80°C). The experimental design and the results derived from each run are shown in Table 1. ANOVA was used to check the significance of the factor effects. The Pareto chart (Fig. 1) also shows the significance of these

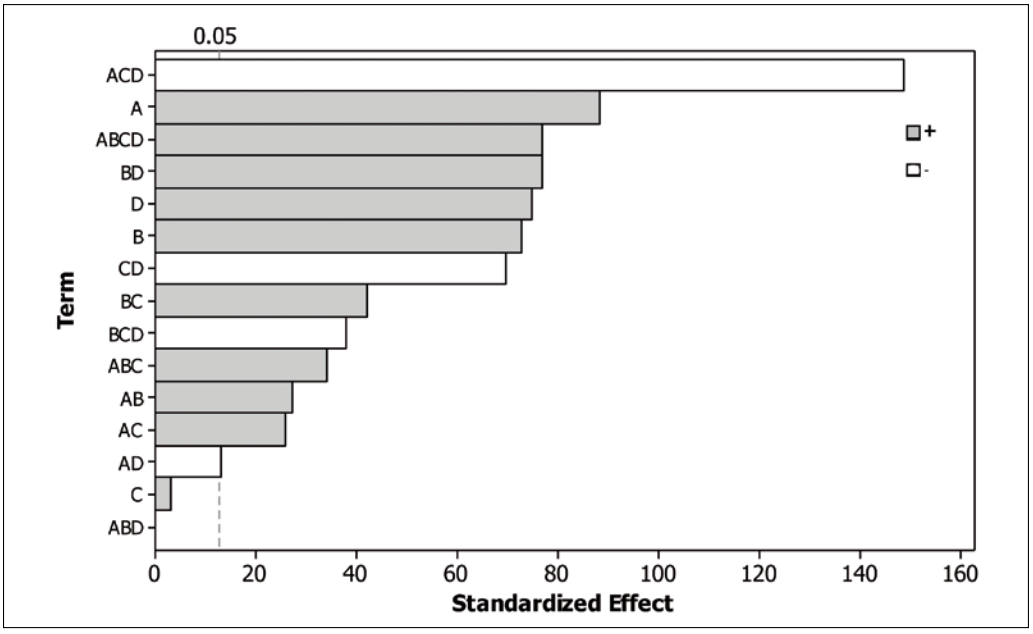


Fig. 1 - Pareto chart of main effects and interaction obtained from the 2^4 full factorial designs.
The vertical line defines the 95% confidence interval [A = power (P); B = temperature (°C); C= time (min); D = volume of nitric acid (mL); AB = interaction between power and temperature; AC = interaction between power and time; AD = interaction between power and volume of nitric acid; BC = interaction between temperature and time; BD = interaction between temperature and volume of nitric acid; CD = interaction between time and volume of nitric acid; ABC = interaction between power, temperature and time, ABD = interaction between power, temperature and volume of nitric acid; BCD = interaction between temperature, time and volume of nitric acid; ACD = interaction between power, time and volume of nitric acid; ABCD = interaction between power, temperature, time and volume of nitric acid].

factors. The lengths of the bars in the Pareto chart are proportional to the absolute value of the estimated effects. The results of the Pareto chart indicate that the power (A), temperature (B) and volume of nitric acid (D) were significant. However, the extraction time (C) was not significant. The interactions between ACD, ABCD, BD, CD, BC, BCD, ABC, AB, AC, and AD were significant. The interaction between power, temperature and volume of nitric acid (ACD) was very significant, for this purpose, a Box-Behnken experimental design was used for final optimization.

Box-Behnken design

Factorial design showed which factors, namely temperature, volume of nitric acid and power, in the studied levels needed final optimization. Thus, a Box-Behnken experimental design was used for this purpose. In this research, the three-level, three-factor Box-Behnken technique was used to investigate and validate parameters affecting the mineralization of zinc, copper and iron. The input variables were power (P; 300-500 W), volume of nitric acid (V; 6-10 mL) and temperature (T; 80°-110°C). The factor levels were coded as -1 (low), 0 (central point), and 1 (high). The design of real Box-Behnken experiments is shown in Table 2.

The behavior of the system is explained by the following quadratic equation (BOX *et al.*, 1997).

$$Y = \beta_0 + \sum \beta_i \chi_i + \sum \beta_{ii} \chi_{ii}^{\ 2} + \sum \sum \beta_{ij} \chi_i \chi_j + \varepsilon \quad (1)$$

where Y is the process response or output (dependent variable), β_0 is the constant, x_1, x_2, ..., x_k are the coded independent variables, β_i is the linear effect, β_{ii} is the quadratic effect, β_{ij} is the interaction effect, and ε is the random error or allows for description or uncertainties between predicted and measured value.

Modelling

The optimization step of the MAE was performed using a Box-Behnken design. Several variables that could potentially affect the extraction efficiency were chosen including irradiation power, volume of solvent, and temperature. Other parameters implicated in the extraction were kept constant, namely the amount of dried sample (0.5 g) and the extraction time (10 min).

In this study, experiments were planned to obtain a quadratic model containing 12 trials plus 3-center points. The range and levels of the three independent variables are shown in Table 2, as well as the experimental and predicted response values. Each run was carried out in triplicate and mean values are presented. The optimum values of the selected variables were obtained by solving the regression equation. An empirical relationship between the response and the independent variables has been expressed by the following quadratic models:

$$\begin{aligned} Y\,(Cu) = {} & 16.9276 + 0.018\,(P) - 0.5998\,(T) + \\ & + 3.0015\,(V) + 0.0001\,(P)^2 + 0.005\,(T)^2 - \\ & + 0.2189\,(V)^2 - 0.001\,(P)(T) - 0.0008\,(P)(V) + \\ & + 0.0069\,(T)(V) \end{aligned}$$

$$\begin{aligned} Y\,(Fe) = {} & 81.6928 - 0.2\,(P) - 1.5\,(T) + \\ & + 3.2356\,(V) + 0.0002\,(P)^2 + 0.0057\,(T)^2 - \\ & + 0.1744\,(V)^2 + 0.0004\,(P)(T) - 0.0002\,(P)(V) - \\ & + 0.0052\,(T)(V) \end{aligned}$$

$$\begin{aligned} Y\,(Zn) = {} & -1.13264 + 0.3\,(P) + 1.1\,(T) - \\ & + 1.77479\,(V) - 0.0003\,(P) + 0.00083\,(T) + \\ & + 0.14719\,(T) - 0.00044\,(P)(T) + \\ & + 0.00101\,(P)(V) - 0.01017\,(T)(V) \end{aligned} \quad (2)$$

Table 2 - Box-Behnken experimental design with the independent variables. Observed and predicted values.

No.	P (W)	T (°C)	V (mL)	Observed, Y_o[a] (μgg^{-1}:RSD%)			Predicted, Y_p (μgg^{-1})		
				Cu	Fe	Zn	Cu	Fe	Zn
1	300	80	8	10.23±0.4	11.43±0.1	9.88±0.5	10.70	11.33	9.90
2	500	80	8	16.41±0.5	11.27±0.3	12.95±0.1	16.40	10.98	12.76
3	300	110	8	13.65±0.6	10.34±0.2	11.13±0.3	13.66	10.63	11.32
4	500	110	8	13.87±0.3	12.39±0.5	11.56±0.5	13.40	12.49	11.54
5	300	95	6	10.45±0.5	9.17±0.1	11.23±0.6	10.33	9.25	11.17
6	500	95	6	12.99±0.1	9.84±0.3	12.17±0.2	13.35	10.11	12.32
7	300	95	10	10.41±0.6	9.01±0.4	10.99±0.4	10.05	8.74	10.85
8	500	95	10	12.35±0.4	9.48±0.3	12.74±0.6	12.47	9.40	12.80
9	400	80	6	12.65±0.2	9.02±0.2	11.85±0.1	12.31	9.04	11.89
10	400	110	6	11.76±0.3	10.12±0.4	12.73±0.3	11.87	9.75	12.60
11	400	80	10	11.43±0.6	8.37±0.5	12.45±0.2	11.32	8.74	12.58
12	400	110	10	11.37±0.4	8.84±0.3	12.11±0.4	11.72	8.82	12.07
13	400	95	8	11.36±0.2	8.43±0.6	11.28±0.3	11.56	8.50	11.51
14	400	95	8	11.84±0.3	8.75±0.1	11.85±0.4	11.56	8.50	11.51
15	400	95	8	11.49±0.4	8.32±0.2	11.40±0.1	11.56	8.50	11.51

[a]Average of triplicate extraction.

Table 3 - Analysis of variance for the fitted quadratic polynomial model of extraction of element.

Source	Sum of Squares			df	Mean of Square			F-Value			p-value		
	Cu	Fe	Zn		Cu	Fe	Zn	Cu	Fe	Zn	Cu	Fe	Zn
Model	35.279	20.746	8.969	9	3.919	2.305	0.997	17.645	16.118	14.842	0.0028	0.0035	0.0042
P	0.643	7.631	1.415	1	0.643	7.631	1.415	2.895	53.356	21.067	0.1496	0.0008	0.0059
T	1.569	6.0441	0.047	1	1.569	6.044	0.047	7.064	42.263	0.694	0.0450	0.0013	0.4426
V	1.144	1.331	0.400	1	1.144	1.330	0.400	5.15	9.299	5.959	0.0725	0.0284	0.0586
PT	8.881	1.2211	1.742	1	8.881	1.221	1.742	39.973	8.538	25.949	0.0015	0.0329	0.0038
PV	0.09	0.01	0.164	1	0.09	0.01	0.164	0.405	0.0699	2.443	0.5525	0.8020	0.1788
TV	0.172	0.0991	0.372	1	0.172	0.099	0.372	0.775	0.694	5.542	0.4189	0.4428	0.0652
$(P)^2$	2.744	9.130	0.369	1	2.744	9.130	0.369	12.352	63.841	5.499	0.0170	0.0005	0.0660
$(T)^2$	4.586	6.097	0.128	1	4.587	6.097	0.128	20.647	42.631	1.908	0.0061	0.0013	0.2258
$(V)^2$	2.829	1.796	1.279	1	2.829	1.796	1.279	12.734	12.5601	19.061	0.0161	0.0165	0.0072
Residual	1.111	0.715	0.3396	5	0.222	0.143	0.067						
Lack of Fit	0.988	0.615	0.155	3	0.329	0.205	0.052	5.341	4.110	0.573	0.1618	0.2019	0.6859
Pure Error	0.123	0.0998	0.181	2	0.062	0.0499	0.0903						

The critical points in the surface response are determined by solving these equation systems for the conditions of

$$\frac{\delta(Y)}{\delta(T)} = 0, \frac{\delta(Y)}{\delta(P)} = 0 \text{ and } \frac{\delta(Y)}{\delta(V)}$$

The method of calculating these critical points has been published previously (SANTOS *et al.*, 2004). The calculated values for the critical points are as follows: temperature (T) = 98.0°C, irradiation power (P) = 428.0 W and volume (V) of nitric acid = 8.0 mL for copper, temperature (T) = 99.0°C, irradiation power (P) = 406.0 W and volume (V) of nitric acid = 7.0 mL for iron and temperature (T) = 106.0°C, irradiation power (P) = 437.0 W and volume (V) of nitric acid = 8.2 mL for zinc. Working conditions of 100°C, 425 W and 8.5 mL, for temperature, irradiation power and volume of nitric acid, respectively, were established as a compromise between optimum values found for copper, zinc and iron.

The results of the response surface model fitting in the form of ANOVA are shown in Table 3. The ANOVA is needed to test the significance and adequacy of the model. The ANOVA results (Table 3) suggest that the model was significant, as evident from the Fisher's F-test (F_{model} = 17.645, 16.118 and 14.842 for copper, iron and zinc, respectively) with very low probability values (P_{model} = 0.0028, 0.0035 and 0.0042 for copper, iron and zinc, respectively). Therefore, the calculated F-values were compared with the critical F-value ($F_{\alpha,df,(n-df+1)} = F_{0.05,9,5} = F_{tab}$ = 4.77) for the considered probability (P = 0.05) and degrees of freedom. The results show that the critical F-value is less than the calculated F-values of 17.645, 16.118 and 14.842 for copper, iron and zinc, respectively. Thus, the computed Fisher's variance ratios at these levels were large enough to justify a very high degree of adequacy of the quadratic models and significance of the combinations of variables (YETILMEZSOY *et al.*, 2009). The interaction between power and volume of nitric acid and temperature and volume of nitric acid were non-significant (P>0.05) for copper, zinc and iron. Moreover, the second-order power for zinc was non-significant. The goodness of fit of the model was checked by the correlation coefficient (R^2). The values of adjusted R^2 (0.915, 0.907 and 0.90 for copper, iron and zinc, respectively) indicated that only about 10.0% of the total variations was not explained by the model. Furthermore, the value of the correlation coefficient (R^2 = 0.969, 0.967 and 0.964 for copper, iron and zinc, respectively) shows good relation between the experimental and predicted values of the response variables. The non-significant values (P>0.05) of lack-of-fit indicate that the quadratic model is statistically significant for the response variables of copper, iron and zinc. The significance of the quadratic model coefficients was evaluated by the student's t-test and p-values listed in Tables 3 and 4, for copper, iron and zinc. A larger magnitude of the t-value and a smaller p-value are associated with increasing significance of the corresponding factors in the regression model (YETILMEZSOY *et al.*, 2009). The percent contribution (PC%) of each of the individual terms in the final model was computed (Table 4) using the SS values of the corresponding term.

To obtain a better understanding of the influences of the independent variables and their interaction on the dependent variables, three-dimensional response surface plots for the meas-

Table 4 - Estimated regression of relationship between response variables and independent variables.

Factor (coded)	Parameter	SE[a]			*t*-ratio			(PC%)[b] PC = $\frac{SS}{\sum SS}\times 100$		
		Cu	Fe	Zn	Cu	Fe	Zn	Cu	Fe	Zn
Intercept	β_0									
P	β_1	0.0264	0.0212	0.0145	1.701	-7.305	4.59	2.84	22.88	23.92
T	B_2	0.2257	0.1811	0.1241	-2.658	-6.501	0.833	6.92	18.12	0.79
V	B_3	1.3224	1.061	0.7270	2.27	3.05	-2.441	5.05	3.99	6.76
PT	B_{12}	0.0002	0.0001	0.00009	-6.322	2.922	-5.094	39.20	3.66	29.45
PV	B_{13}	0.0012	0.0009	0.0007	-0.634	-0.264	1.563	0.40	0.03	2.77
TV	B_{14}	0.0079	0.0063	0.0043	0.88	-0.833	-2.354	0.76	0.30	6.29
(P)2	B_{11}	0.00001	0.00001	0.00001	3.515	7.99	-2.345	12.11	27.37	6.24
(T)2	B_{22}	0.0011	0.0009	0.0006	4.544	6.529	1.381	20.24	18.28	2.16
(V)2	B_{33}	0.0613	0.0492	0.0337	-3.569	-3.544	4.366	12.49	5.38	21.62

[a]Standard error.
[b]Percentage contribution (%).

ured responses were constructed based on the quadratic model. The three-dimensional response surface plots for the amount of copper, iron and zinc as a function of P, T and V are shown in Fig. 2.

Model validation

To verify the proposed model, several additional experiments were performed in the experimental area of the Box-Behnken design, and each experimental response was compared with the predicted response. As similarly conducted by YETILMEZSOY (2009), these extra experiments were selected randomly in the experimental domain. The validation results of the model with these experimental points are shown in Table 5. Predicted values were in very good agreement with observed values (R^2 = 0.977, R^2_{Adj} = 0.935 for copper, R^2 = 0.969,

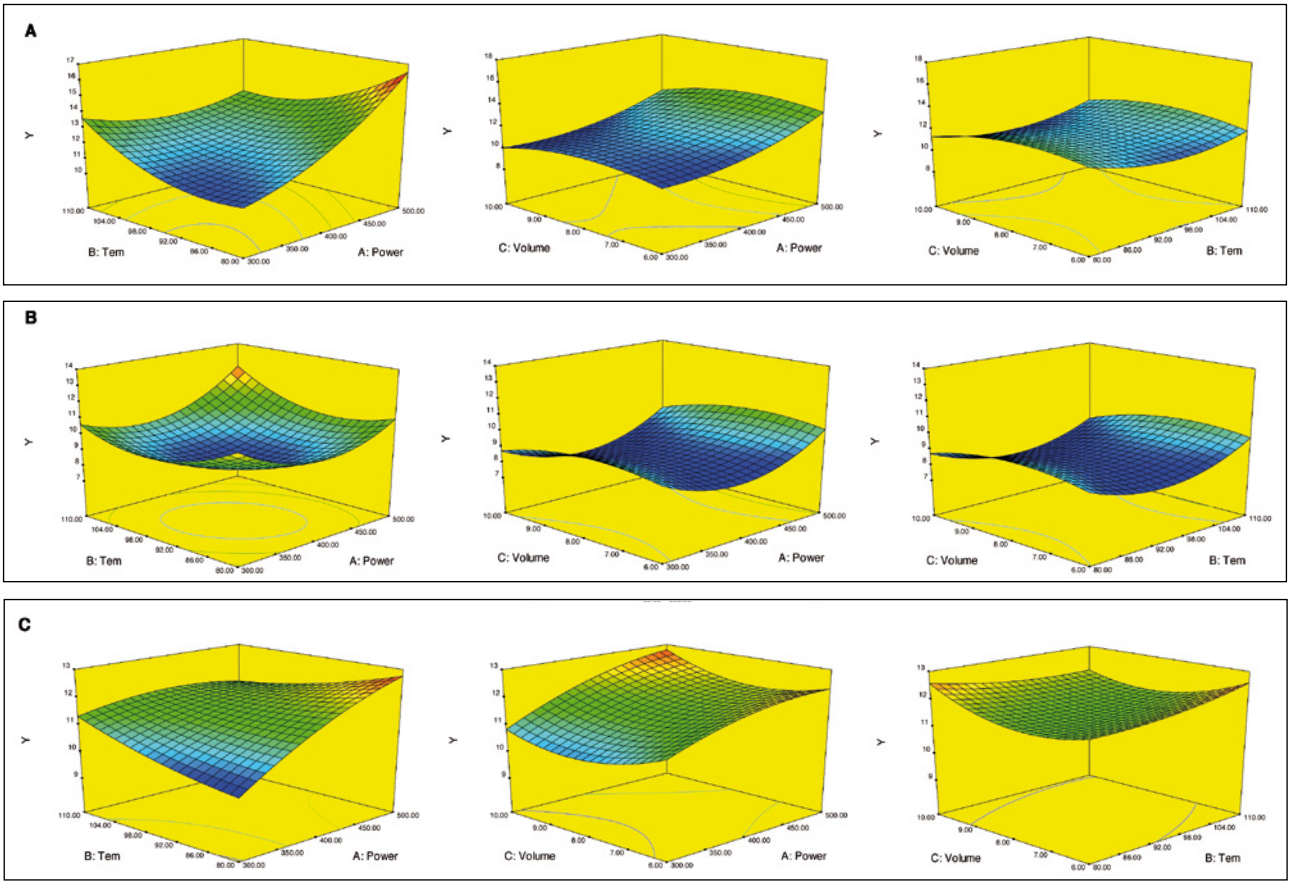


Fig. 2 - Response surface obtained from Box-Behnken design for extraction efficiency of copper (A), iron (B), and zinc (C).

Table 5 - The Box-Behnken design for model validation.

No.	P (W)	T (°C)	V (mL)	Observed, Y_o (μgg^{-1}:RSD%)			Predicted, Y_p (μgg^{-1})		
				Cu	Fe	Zn	Cu	Fe	Zn
1	448	110	10.0	11.81±0.1	9.40±0.4	12.01±0.4	11.78	9.61	12.15
2	300	80	10.0	9.35±0.3	10.58±0.2	10.75±0.3	9.47	10.54	10.63
3	370	97	6.0	10.56±0.5	8.26±0.3	11.79±0.4	10.65	8.17	11.94
4	500	110	10.0	12.42±0.4	11.14±0.2	12.21±0.5	12.3	11.28	12.07
5	326	97	6.0	10.39±0.1	8.52±0.4	11.68±0.3	10.47	8.67	11.58
6	448	97	6.0	11.85±0.3	8.65±0.5	12.25±0.6	11.93	8.78	12.27
7	370	97	7.7	11.26±0.2	8.71±0.3	11.46±0.5	11.32	8.59	11.31
8	448	97	7.7	12.49±0.5	8.83±0.1	11.75±0.3	12.37	9.17	11.78
9	448	97	10.0	10.74±0.1	8.25±0.3	12.44±0.1	11.13	8.08	12.46
10	448	80	10.0	12.76±0.4	8.82±0.3	13.19±0.1	12.81	8.99	13.29

Table 6 - Determination of zinc, copper and iron in the pistachio nut.

Sample No.	(μgg^{-1})(RSD%)		
	Zinc	Iron	Copper
Sample 1	12.47±0.14	9.3±0.08	11.52±0.12
Sample 2	12.93±0.12	9.1±0.10	12.1±0.15

R^2_{Adj} = 0.914 for iron and R^2 = 0.974, R^2_{Adj} = 0.928 for zinc). Verification experiments performed at the predicted conditions show the validity and adequacy of the predicted models. As shown in Table 5, the observed results accorded well with the predicted results and indicate that the developed model is accurate and reliable.

The optimized MAE method was applied to the determination of zinc, iron and copper in the pistachio nut. The results obtained are showed in Table 6.

CONCLUSION

Application of microwave-assisted extraction for mineralization of copper, iron and zinc from pistachio nuts is a practical approach. This research showed that response surface methodology was an appropriate method to optimize the best culture conditions for achieving maximum extraction of the zinc, copper, and iron. The validation results clearly confirmed that the proposed optimization modelling is an effective procedure to understand the relationships among the independent and response variables and to maximize the extraction efficiency.

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SHORT COMMUNICATION

INFLUENCE OF MARINADES ON THE LEVEL OF PAHS IN GRILLED MEAT PRODUCTS

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ABSTRACT

This manuscript addresses the effect of meat marinating and applying aluminum trays during grilling on the content of PAHs in pork steaks of neck.

Based on survey evidence it was found that the use of marinades with beer, wine and oil increased the content of PAHs in grilled meat products. The results obtained in this study clearly indicate that the use of the aluminum trays in grilling process, significantly reduces the level of PAHs in finished products (BaP - below quantification limit of the method). The application of aluminum trays resulted in the reduction of the total level of PAHs, i.e. by 66.9% - dry marinate and by 86.3% - wine marinate.

Sensory analysis confirmed the highest value in steaks from the grate after dry marinating; the least tasty appeared to be those steaks marinated in wine.

- Keywords: polycyclic aromatic hydrocarbons, benzo[*a*]pyrene, meat, grilling, marinades, sensory evaluation -

INTRODUCTION

The technique of thermal treatment of raw materials, namely grilling, is highly popular amongst consumers. This may be affected by several factors including, most of all, the desirable sensory values and reduced calorific value of the final products. An important aspect is the availability of a variety of appliances, recipes and raw materials as well as the fact that grilling usually takes place in the fresh air, in the company of family and friends. Consumers pay a lot of attention to the sensory and nutritive values obtained in grilled food products (TKACZ *et al.*, 2007). Apart from such desirable traits as the pleasant and specific aroma and taste, thermal treatment over an open fire induces the formation of cholesterol oxidation products one of the causative agents of atherosclerosis, as well as compounds with carcinogenic potential, including: polycyclic aromatic hydrocarbons, heterocyclic amines and free radicals (DYREMARK *et al.*, 1995; JÄGERSTAD and SKOG, 2005; KEATING and BOGEN, 2004; LARSSON *et al.*, 1983; MU LEE and AE SHIM, 2007; SINHA and ROTHMAN, 1999; STRATIL and KUBAŃ, 2005; WENZL *et al.*, 2006). The generation of these detrimental substances may be counteracted by the appropriate conduct of the grilling process – grilling on incandescent coal, not allowing the dripping fat to burn, grilling smaller portions of food which enables the shortening of the high-temperature treatment and not applying grilling to marinated or cured meats (FARHADIAN *et al.*, 2011; REINIK *et al.*, 2007, KUHN *et al.*, 2008).

Polycyclic aromatic hydrocarbons (PAHs) are toxic substances that constitute a numerous group of compounds (over 100) containing from two to several or even ten to twenty aromatic rings in a molecule (CIECIERSKA and OBIEDZIŃSKI, 2007; FARHADIAN *et al.*, 2010; FELTON and KNIZE, 1995; HYLLAND, 2006; JIRA *et al.*, 2006; MOTTIER *et al.*, 2000). PAHs do not occur alone, but always in a mixture. Investigations have shown that the presence of one compound from the group of PAHs in environmental samples indicates that other compounds belonging to that group occur in those samples as well. The Scientific Committee on Food (SCF) concluded that 15 PAHs, namely benz[*a*]anthracene (BaA), benzo[*b*]fluoranthene (BbF), benzo[*j*]fluoranthene (BjF), benzo[*k*]fluoranthene (BkF), benzo[*ghi*]perylene (BghiP), benzo[*a*]pyrene (BaP), chrysene (CHR), cyclopenta[*cd*]pyrene (CPP), dibenz[*a,h*]anthracene (DhA), dibenzo[*a,e*]pyrene (DeP), dibenzo[*a,h*]pyrene (DhP), dibenzo[*a,i*]pyrene (DiP), dibenzo[*a,l*]pyrene (DlP), indeno[1,2,3-*cd*]pyrene (IcP) and 5-methylchrysene (5MC), have shown substantial genotoxicity in standard assays *in vitro* and *in vivo*. The Joint FAO/WHO Expert Committee on Food Additives (JECFA) has added a 16th analyte to the list – *7H*-benzo[*c*]fluorene (EFSA 2008). The best recognized hydrocarbon belonging to the group of PAHs is benzo[*a*]pyrene (BaP), which owing to the strength of its carcinogenic action and common occurrence in the environment has been acknowledged as an indicator of the entire group of PAHs (BENFORD *et al.*, 2010; PHILLIPS, 1999; REINIK *et al.*, 2007; SEIDEL *et al.*, 2008; ŠIMKO, 2002; WENZL *et al.*, 2006).

In 2005, the European Union implemented the recommended values for BaP content in selected food products. The maximal admissible levels of BaP were provided for foodstuffs containing fats and oils - 2 μg kg^{-1}, for infants and small children formulas - 1 μg kg^{-1}, for smoked meat and smoked meat products - 5 μg kg^{-1}, as well as for oysters - 10 μg kg^{-1} (EC REGULATION No. 1882/2006; EC REGULATION No. 333/2007; WENZL *et al.*, 2006). However, that regulation does not stipulate the admissible levels of BaP contents in grilled products.

Marinating is widely used by consumers in the case of meat in order to influence its sensory parameters. Therefore, the effects of marinating on the level of PAHs in marinated grilled meat products, were analysed.

The research was aimed at showing consumers such technology of raw materials preparation for grilling as would enable obtaining as low possible a concentration of indicatory hydrocarbons in finished products to be obtained.

MATERIALS AND METHODS

Grilling

In Poland, the most popular raw meat used for grilling is a pork neck steak. Such raw material was used in the experiment. Pork neck was purchased in a local store. The proximate chemical composition of the raw material on a Food Check 22 FC 90 60 80 (Bruins Instruments Spectrophotometer) was as follows: water 67.7%, protein 19.4%, fat 12.4%, and collagen 2.45%. It was used to prepare steaks weighing ca. 180 g, with a similar shape and a thickness of ca. 2 cm. Next, the steaks were marinated in 4 marinates. Following the producer's recommendations, 25 g of classic marinade per kg of meat were used on each of them. The composition of a classic marinade available on the market was as follows: salt, garlic, sweet paprika, hot paprika, sugar, coriander, mustard, marjoram, black pepper, juniper, onion, rosemary, clove, bay leaves, and monosodium glutamate. Next, oil, beer and wine were added to 3 marinades: A - dry classic marinade 25 g - without any additives; B - classic marinade 25 g + half a glass of oil; C - classic marinade 25 g + half a glass of red dry wine; D - classic marinade 25 g + half a glass of pale beer (quantities of seasonings and additives used per kg of raw material). The steaks were marinated for 12 h at a

temperature of 4°C (6 steaks were marinated in each). Afterwards, they were weighed to determine mass gain during marinating.

Next, the steaks were grilled. They were weighed before and after the thermal treatment in order to determine their mass losses. Control samples were non-marinated steaks.

A coal grill with a grate diameter of 50 cm, and distance between fire and sample reaching 15 cm was employed. After 30 min since kindling (the charcoal briquette was glowing), the heated briquette was evenly distributed over the grill and left for 10 min for temperature stabilization – the temperature of the grate was 250°C. Afterwards, meat samples were grilled directly onto the grate and with the use of aluminum trays to the internal temperature of 82°C. The temperature was measured by thermometer Hanna Instrument HI 92804C.

Polycyclic aromatic hydrocarbons

Experimental

The following methods were used to determine BaP in meat products: extraction of lipid fraction, SEC (Size Exclusion Chromatography) technique to separate hydrocarbons from fat, and high-performance liquid chromatography with fluorescence detection (HPLC/FLD).

The BaP determination technique with the use of HPLC with fluorescence detection was conducted in reversed phase configuration (nonpolar stationary phase). The mobile phase was water and acetonitrile (WĘGRZYN *et al.*, 2005).

Reagents and standards

The following reagents and standards were used for determination of BaP:

acetonitrile - ACN, dichloromethane - DCM, chloroform and methanol – (HPLC Labscan, Dublin, Ireland), benzo[*b*]chrysene – BbCh (Dr Ehrenstorfor GmbH (10 ng/μL ACN)], BaP [Standard Reference Material 1647 d-NIST (4.91 μg/mL)). The remaining reagents were of "*pure for analysis*" grade. Water was sourced from Milli-Q System (Millipore, Bedford, USA).

For quantitative evaluation of BaP, use was made of the mixture of standards and an internal standard in ACN with Bap and BbCh concentrations of: 49.1 ng/mL and 50 ng/mL, respectively, and of a solution of internal standard BbCh in CAN with a concentration of 50 ng/mL.

Equipment

The study made use of a UM4EV2A - colloidal mill (Bosch, Germany) for disintegration, Vortex (JWE-electric, Warsaw, Poland) separator 317b high-speed, with 310b rotations controller (Precision Mechanics, Warsaw, Poland), and water bath (Falc, Luvarno, Italy).

To isolate the hydrocarbon fraction from the extracted fat of the analysed samples, the following instruments were employed: ASI-100 preparative chromatograph with automatic sample dispenser, gradient chromatographic pump with P-580 degassifier, photometric detector with UVD-340S diode matrix, UCI-100 universal chromatographic adapter (DIONEX-SOFTRON, Germering, Germany), fraction collector Foxy Jr. Fraction Collector (Isco, Lincon, USA), column of steric exclusion – Plgel on PS/DVB bed, 5 μm, 50 Å, 600 x 7.8 mm i.d., together with precolumn (Polymer Laboratories, Amherst, Usa). The run of analyses was controlled by the Chrom-Leon v. 620 program (DIONEX-SOFTRON, Germering, Germany).

For quantitative determination of BaP, the following instruments were employed: Agilent 1100 analytic chromatograph, equipped with a G-1322A vacuum degassifier of samples, G-1313A automatic sample dispenser, G-1311A four-channel pump, G-1316A column thermostat, G-1321A fluorimetric detector with the possibility to sweep the spectra (Agilent Technologies, Palo Alto, Usa), column with precolumn Hypersil Green PAHs, 5 μm, 250 x 3 mm i.d. (Thermo Electron Corporation, Runcorn, USA). The run of analyses was controlled by the Chem Station LC 3D program (Agilent Technologies, Palo Alto, Usa).

Analytical procedure

To glass test tubes, 1 g of previously homogenized sample was weighed, then 100 μL of internal standard of BbCh (50 ng/mL) was added and then, 1.5 mL of methanol was added. The samples were shaken, using a Vortex, for one minute and then, 3 mL of chloroform and 1.5 mL of water were added. The whole content was shaken, using a Vortex, for 3 minutes and then centrifuged for 10 minutes at 10,000 r.p.m. The chloroform solution containing the lipid fraction of PAHs was filtered to a test-tube (10 mL) on Whatman's paper. The sample was again extracted with 3 mL of chloroform, shaken, using a Vortex, for 3 minutes and then, centrifuged for 10 minutes at 10,000 r.p.m. The chloroform fraction was again filtered on a filter. The combined chloroform fractions were evaporated to dryness in a nitrogen stream in a water bath (40°C). The residues were dissolved in 4 mL of dichloromethane. The prepared extracts of the samples were subjected to purification, with the application of preparative chromatography of steric exclusion (WĘGRZYN *et al.*, 2005).

The purification stage utilizing preparative chromatography of steric exclusion was performed under isocratic conditions with the flow of the mobile phase, DCM at the rate of 1 mL/min. UV detector – 254 nm. Before performance of chromatographic measurements, the column was preliminarily stabilized for 1 hour. Analy-

sis covered 400 μL of the prepared extract of the sample in dichloromethane. PAHs-containing eluate with a retention time equal to 18-24 minutes was collected in a fraction collector and then, was evaporated to dryness in a nitrogen stream in a water bath (40°C). Dry residues were dissolved in 200 μL of ACN. The prepared samples were transferred onto a high performance liquid chromatograph.

Determination of BaP was performed, using a gradient of the mobile phase water /acetonitrile. The sample was transferred with a 50:50 composition of mobile phase. Within 20 minutes, the concentration of ACN increased by 100%; remained at this level for 15 minutes and then returned to the initial value (50:50). The flow of the mobile phase was 0.8 mL/min and the temperature was 25°C. The volume of the transferred sample was 20 μL. Before performing the measurements, the column was thermostatically controlled at a temperature of 25°C for 1 h. On the basis of retention times for BaP and BbCh, the optimal conditions of work of the fluorescence detector were established (WĘGRZYN *et al.*, 2005):

BaP – λ_{ex} 256 nm, λ_{em} 446 nm;
BbCh - λ_{ex} 295 nm, λ_{em} 410 nm.
BaP - content was calculated according to the following formula:

$$C_{BaP} = \frac{A_{BaP}}{A_{is}} * M * k$$

C_{BaP} – content of BaP in the sample (μg kg^{-1})
A_{BaP} – area of BaP peak
A_{is} – area of internal standard peak
M – concentration of the added standard (μg kg^{-1})
k – correction coefficient

Sensory evaluation

The sensory assessments of the samples were carried out by a panel consisting of 10 members selected and trained according to ISO guidelines (ISO 8586-1:1993). All assessors passed the basic taste test, the odour test and the colour vision test. Prior to their participation in the experiments, the subjects were trained on sensory descriptors for marinated (with oil, beer, and wine) and unmarinated grilled pork.

Having been grilled the samples were served hot to the sensory panels. The ready grilled steaks were sensory evaluated according to the ISO 8587 ranking method in order to determine which of the marinates is the most acceptable to consumers. Each of the 10 assessors was asked to rank 4 samples obtained by ascribing the 1st rank to the most desirable sample and the 4th rank to the least acceptable sample.

Statistical analysis

The average value, standard deviation and the test of the significance of differences test were estimated using the Statistica 8.0 software. Results of the sensory evaluation were analyzed using the Friedman test.

RESULTS

Figure 1 demonstrates mass increase in the samples after marinating and thermal drip that occurred during grilling directly on a grate and with the use of aluminum trays. Mass gain determined in the samples marinated in oil, beer and wine did not differ significantly and

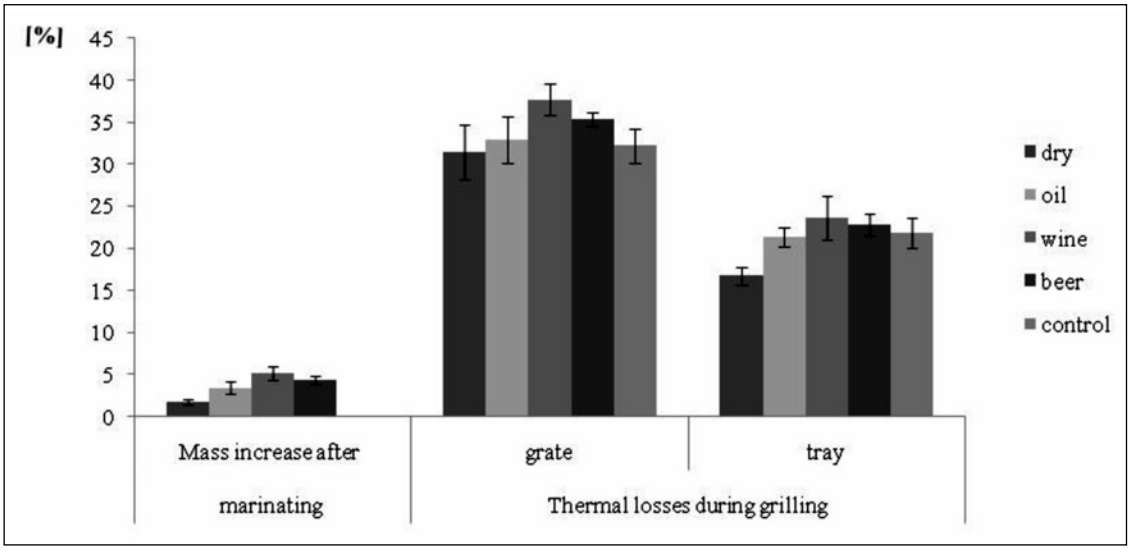


Fig. 1 - Mass increase after marinating and thermal losses generated during grilling on a grate and with the use of aluminum trays.
Control samples were unmarinated steaks grilled directly on the grate.

reached 4.3±0.7% on average in turn, a significantly lower ($p<0.05$) mass increase was observed during marinating without the addition of liquids, i.e. 1.7±0.4% when compared to the marinating in wine – 5.2±0.8% and in beer – 4.3±0.5% (Fig. 1).

The marinating had no significant effect on mass losses generated during the grilling (Fig. 1). Analyses demonstrated, in turn, that the application of aluminum trays may reduce thermal drips by ca. 12% (Fig. 1). The grilling of the steaks directly on the grate results in a thermal drip at the level of 33.8%, whereas with the use of aluminum trays the thermal drip accounts for 21.6%.

The influence both of the type of marinates used and marinating on the thermal weight loss was also observed. This dependency was also demonstrated by, among others, QUELHAS *et al.* (2010) and MELO *et al.* (2008). They determined the thermal weight loss of marinated and unmarinated pan-fried beef to reach, respectively: 45 - 49%, 39 - 40% (QUELHAS *et al.*, 2010), and 52 - 55%, 48 - 50% (MELO *et al.*, 2008). GIBIS and WEISS (2010) show that the extent of the thermal weight loss upon the heat treatment process is determined by the time. Different heat treatment times differentiate thermal weight loss value by 31 to 44% (GIBIS and WEISS, 2010).

Polycyclic aromatic hydrocarbons

The obtained detection values (LOD-μg kg^{-1}), quantification (LOQ-μg kg^{-1}) and recovery (%) methods were respectively for: fluoranthene 0,2/0,4/102; pyrene 0,2/0,4/96; BaA 0,2/0,4/101; CHR 0,1/0,3/78; BbF 0,1/0,3/98; BkF 0,1/0,2/104; DBahA 0,1/0,3/90; BghiP 0,1/0,3/76; BaP 0,1/0,2/102. The obtained values of LOD, LOQ and recovery showed enough sensitivity for the detection of the compound in the samples (EC DIRECTIVE 2005/10/EC).

Table 1 collates results of contents of the PAHs analyzed in marinated pork steaks grilled directly on the grate and with the use of aluminum trays.

In the steaks grilled directly on the grate, the concentration of the BaP in the control samples and those prepared with the applied marinates dry, based on oil and beer did not differ significantly and ranged from 1.05 to 1.22 μg kg^{-1}. A significantly higher ($p<0.001$) concentration of BaP, i.e. 2.78 μg kg^{-1}, was reported for the steaks marinated in wine. In all samples grilled on aluminum trays, the level of BaP was below the quantification limit of the method.

A similar tendency was noted while analyzing the total (Σ) content of the examined hydrocarbons. The steaks grilled on the grate, both the control ones and those marinated in the dry, oil, and beer marinades were characterized by similar Σ PAHs – 15.32, 17.81, 22.51, and 19.38 μg kg^{-1} respectively. In contrast, in the case of the samples marinated in wine that value was significantly higher and accounted for 42.12 μg kg^{-1} (Table 1).

The use of the aluminum trays was observed to significantly decrease the sum of all analyzed hydrocarbons to the same level in all the marinated samples examined – ca. 5.85 μg kg^{-1} (Table 1).

Sensory evaluation

The sensory assessment involved determinations of the acceptance and preference ranks of the consumers. Each assessor was provided with four samples arranged in a row (steaks marinated in different marinades: A, B, C, and D, as described in the Materials and Methods section) for evaluation and ascribed a respective rank in a row for each analyzed sample – from the best to the worst. Results of the ranking made by the assessors are presented in Fig. 2.

For 10 assessors and 4 samples, the critical value of Friedman test accounts for $F=11.34$ at a significance level of $p<0.01$.

Based on the evaluation performed, the critical F value was calculated (ISO 8587) taking into account the number of assessors, the number of samples and the sum of ranks. The calculated value reached $F_D=20.4$. The value obtained in the assessment is higher than the critical value $F_D>F$, which enables the conclusion that the samples assessed differed significantly between one another. In addition, differences were determined between particular samples in a row (ISO 8587).

Analyses demonstrated that samples B and D did not differ significantly between one another, and that the other samples were statistically significantly different at a significance level of $p<0.05$.

The sensory evaluation carried out in the study enables the conclusion that the steaks marinated in the dry marinade are the most acceptable by the consumers. The assessors most frequently ranked those samples the 1st place in a row. The steaks marinated in oil and beer were ranked second, whereas the least desirable turned out to be the steaks marinated in wine – ranked fourth by the consumers (Fig. 2).

DISCUSSION

Grilling in Poland is somehow specific due to the raw material preferred by consumers. The most popular is pork meat – neck, loin and bacon. In the present study, analyses were conducted for a pork neck steak, which – unfortunately – is difficult to compare with results reported in other countries. In the United States and Canada (KAZEROUNI *et al.*, 2001; KEATING and BOGEN, 2004; SALMON *et al.*, 1997;

Table 1 - Contents of the PAHs [μg kg^{-1}] analyzed in marinated pork steaks grilled directly on the grate and with the use of aluminum trays.

PAHs			Fluor anthene	Pyrene	BaA	CHR	BbF	BkF	DBahA	BghiP	BaP	∑ PAHs	Reduction of ∑ PAHs tray/control* [%]	Reduction of BaP tray/control*# [%]
Control sample	grate	x	5.22	5.06	0.59	0.99	1.2	0.39	blm	0.82	1.05	16.3 ^{aA}	73.6	81
		SD	0.03	0.12	0.08	0.03	0.07	0.028	-	0.006	0.19	0.6		
	tray	x	2.28	2.05	blm	blm	blm	blm	blm	blm	blm	4.30 ^{bB}		
		SD	0.013	0.008	-	-	-	-	-	-	-	0.021		
Dry marinate	grate	x	6.25	6.135	0.755	0.995	1.26	0.44	blm	0.88	1.08	17.81 ^{cA}	66.9	81.5
		SD	0,014	0,021	0,007	0,007	0,028	0,014	-	0,014	0,04	0,11		
	tray	x	2.675	2.43	blm	0.415	0.365	blm	blm	blm	blm	5.9 ^{dC}		
		SD	0.007	0.014	-	0.007	0.021	-	-	-	-	0.5		
Oil marinate	grate	x	8.37	7.67	0.99	1.29	1.67	0.455	blm	0.83	1.22	22.5 ^{eD}	74.2	83.6
		SD	0.12	0.15	0.03	0.08	0.04	0.007	-	0.014	0.028	0.5		
	tray	x	3.105	2.69	blm	blm	blm	blm	blm	blm	blm	5.80 ^{dC}		
		SD	0.021	0.014	-	-	-	-	-	-	-	0.04		
Wine marinate	grate	x	13.18	14.09	2.565	3.06	3.045	0.99	blm	2.4	2.78	42.12 ^{fE}	86.3	92.8
		SD	0.028	0.04	0.021	0.014	0.021	0.014	-	0.028	0.06	0.14		
	tray	x	3.295	2.45	blm	blm	blm	blm	blm	blm	blm	5.75 ^{dC}		
		SD	0.021	0.014	-	-	-	-	-	-	-	0.04		
Beer marinate	grate	x	6.74	6.76	0.77	1.365	1.345	0.41	blm	0.88	1.1	19.38 ^{gF}	70	81.8
		SD	0.07	0.014	0.014	0.021	0.007	0.014	-	0.014	0.04	0.19		
	tray	x	2.915	2.07	0.415	blm	blm	blm	blm	blm	blm	5.82 ^{dC}		
		SD	0.021	0.014	0.021	-	-	-	-	-	-	0.06		

BaA: benz[*a*]anthracene; CHR: chrysene; BbF: benzo[*b*]fluoranthene; BkF: benzo[*k*]fluoranthene; DBahA: dibenz[*a,h*]anthracene; BghiP: benzo[*ghi*]perylene; BaP: benzo[*a*]pyrene.

x, SD – mean and standard deviation (n=3).

blm: below quantification limit of the method.

Means with different superscript letters are significant differences at $p \leq 0.05$ for small letters and at $p \leq 0.001$ for big letters.

*control sample were unmarinated steaks grilled directly on the grate.

to determine the reduction of BaP the quantification limit of the method for BaP was used (0.2 μg kg^{-1}).

SINHA and ROTHMAN, 1999), in Europe (MOTTIER *et al.*, 2000; RODRIGUES-ESTRADA *et al.*, 1997; SUNDARAJAN *et al.*, 1999; TIKKANEN *et al.*, 1996) consumers usually grill beef, poultry and hamburgers. The results obtained in the study were, thus, discussed in respect of those products.

Results obtained in the experiment demonstrate that marinating does not exert any explicit effect on PAH content. Of significance in this case are the composition of the marinade and the method of treatment. The addition of wine considerably increases all the mentioned indicators – 2.78 μg kg^{-1}-BaP and 42.12 μg kg^{-1}-ΣPAHs. This may be due to the high compactness of the wine polyphenolic compounds such as anthocyanins and tannins, which play an important role in the formation of Maillard compounds and are an additional organic substrate undergoing pyrolysis.

It should be borne in mind, however, that none of the consumers grills meat with no seasonings added, but each additive may be the primary source of pollution from the environment or the process carried out.

The content of BaP in grilled food varies to a considerable extent, and is affected by the grilling temperature, the extent of frying and type of the raw material grilled.

Depending on the raw material to be grilled, the content of BaP is adopted at a level of: 6 μg kg^{-1} for a beef steak (RIVERA *et al.*, 1996); 0.1 – 4.85 μg kg^{-1} for a hamburger (ELHASSANEEN, 2004); 4,57 and 4.6 μg kg^{-1} for chicken with bone and skin (KAZEROUNI *et al.*, 2001; CHEN *et al.*, 1996); as well as 0.4 μg kg^{-1} and 0.12 μg kg^{-1} for chicken fillet (KAZEROUNI *et al*,. 2001; CIEMNIAK, 2007). MOTTIER *et al.* (2000) did not detect BaP in barbecued meat sausages from turkey, beef, and pork. In turn, the concentration of BaP in veal sausages ranged from 0.32 to 2.81 μg kg^{-1}.

The effect of the extent of roasting on the content of BaP was reported in a study by KAZEROUNI *et al.* (2001) and CIEMNIAK (2007). The content of BaP in grilled beef hamburgers accounted for: medium – 0.09 μg kg^{-1}, well done – 0.56 μg kg^{-1}, very well done – 1.52 μg kg^{-1}; in beef steaks: medium – 4.15 μg kg^{-1}, well done – 4.75 μg kg^{-1}, very well done – 4.86 μg kg^{-1} (KAZEROUNI, 2001), and in chicken leg: medium – 0.32 μg kg^{-1} and well done – 0.73 μg kg^{-1} (CIEMNIAK, 2007).

A very interesting result of our experiment is the fact that in all the samples examined the use of aluminum trays, constituting a barrier between the fire and the raw material reduced the content of benzo[*a*]pyrene to a level below the detection limit of the method, and the Σ PAHs by 66.9% in the samples prepared in dry marinade, by 74.2% in the samples marinated in oil, by 86.3% in those marinated in wine and by 70% in those prepared in beer (Table 1).

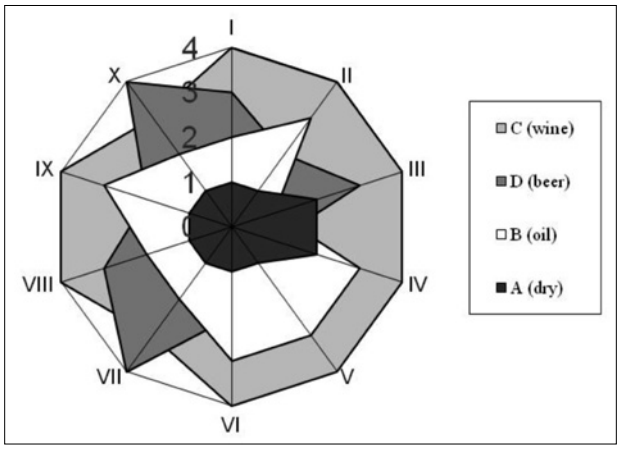


Fig. 2 - Results of the sensory evaluation of marinated pork steaks grilled on a grate.
I - X - Assessor.
1-4: place in a row.
1 place: the best sample; 4 place: the worst sample.

Similar results were obtained in research by CIEMNIAK (2007). As a result of using the aluminum trays, the reduction of BaP in medium-done grilled chicken meat reached: leg – 53%, leg skin – 99%, wing – 97%, wing skin – 99%, and fillet – 83%.

The grilling conditions applied in the study enabled a product to be obtained which was fully acceptable to consumers with the least possible exposure to the generation of detrimental substances. Despite the growing interest in the health aspects of meat, good sensory properties remain a key priority as a consumer choice criterion (TKACZ *et al.*, 2007; VERBEKE *et al.*, 1999). Results of the sensory assessment are correlated with those noted for concentrations of carcinogenic substances, namely PAHs. It turns out that the respondents were the most fond of steaks from the grate marinated in the dry marinade, in which the content of ΣPAHs (17.81 μg kg^{-1}) and the content of BaP (1.08 μg kg^{-1}) were the lowest. The least tasty appeared to be the steaks from the grate marinated in wine that were characterized by the highest content of both ΣPAHs (42.12 μg kg^{-1}) and benzo(a)pyrene (2.78 μg kg^{-1}).

In conclusion the following tips can be formulated for consumers:

- meat may be marinated in any marinate, however aluminum trays should be used at each grilling – this will enable the risk of human organisms being exposed to polycyclic aromatic hydrocarbons to be minimised;

- if, due to sensory reasons, aluminum trays are not used liquids such as oil, beer and especially red wine for marinades should be avoided in view of the need to limit exposure to PAHs.

ACKNOWLEDGEMENTS

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SHORT COMMUNICATION

AQUEOUS TWO-PHASE SYSTEMS COMPOSED OF DIFFERENT MOLECULAR WEIGHT OF POLYETHYLENE GLYCOL AND DIAMMONIUM PHOSPHATE FOR EXTRACTION OF BOVINE SERUM ALBUMIN

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ABSTRACT

The effect of polymer molecular weight and pH on the partitioning of Bovine Serum Albumin (BSA) has been studied. The protein partitioning decreased on increasing the molecular weight of PEG, whereas the partitioning increased with pH increasing of the system. The maximum recovery percentage of extraction was in pH=8.5 and PEG_{1000}.

- Keywords: aqueous two-phase systems, bovine serum albumin, polyethylene glycol, partition coefficient -

INTRODUCTION

Aqueous two-phase systems (ATPS) form an extensive separation technique in biochemistry and biotechnology for the separation and purification of biological materials such as proteins, nucleic acids, microorganisms, animals and plant cells (AZEVEDO *et al.*, 2009; KARAKATSANIS and LIAKOPOULOU-KYRIAKIDES, 2007). Usually downstream process accounts for 50-80% of the total production costs of proteins (ALBERTSSON, 1986). Conventional methods used for protein purification are usually expensive because it involves several steps of unit operation, which require high-energy input and are difficult to scale up. Hence, in recent years there has been an ongoing interest in biotechnology for the development of innovative separation and purification methods that are both economically viable and gentle enough to preserve biological activity of proteins. ATPS provides a secure separation and purification technique for bio-molecules that meet the abovementioned requirements (WALTER *et al.*, 1985).

The partitioning of proteins in the standard biphasic aqueous systems depends on the concentration and molecular weight of the polymers, the concentration and type of the salt, and pH. So the protein partition coefficient can be modified by changing the medium experimental conditions such as pH, salt concentration, and polymer molecular mass (WALTER *et al.*, 1985). Proteins are partitioned between two phases with a partition coefficient that can be modified by changing the medium experimental conditions such as pH, salts, ionic strength, molecular mass and polymer concentration, pH and temperature (SPELZINI *et al.*, 2005). The aim of this work was to study the behavior of the partition coefficients of Bovine Serum Albumin (BSA) in PEG/$(NH_4)_2HPO_4$ systems at 25°C. In order to enhance the recovery percentage of the protein in the top phase, the influences of process parameters like different polymer molecular weights and pH on BSA partitioning were studied.

MATERIALS AND METHODS

Materials

The Bovine serum albumin, salt and other chemicals used were of analytical grade. Double distilled and deionized water were used throughout. The salt was dried in an oven at about 120°C for 24 h before use. All chemicals were used without further purification.

Preparation of aqueous two-phase systems

The diammonium phosphate solutions were prepared by adding small amount of ammonium hydroxide or phosphoric acid to 40% (w/w) diammonium phosphate solutions, until the desired pH was reached. ATPSs were prepared in 10 mL centrifuge tubes by weighing the appropriated amounts of 40% (w/w) PEG (1,000, 4,000 and 8,000) stock solution, 40% (w/w) diammonium phosphate solutions (desired pH). A known concentration (20 μg/mL, 500 μL) of soluble protein was added to ATPS at desired pH of the system. All systems were mixed using a vortex mixer and centrifuged for 10 min at 1,500 g, then placed at 25°C for 30 min. The volume of each phase was measured when the system turned clear. Samples from top and bottom phases were then carefully removed and assayed for protein concentration.

Partition coefficient (*K*) was defined as the ratio of equilibrium concentration of BSA extracted in the top phase to that in the bottom phase. The recovery percentage (Y_e) was also calculated based on the partition coefficient to study the efficiency of the system. Yield percentage was calculated by using the following equation:

$$Y_e(\%) = \frac{100}{((RK)^{-1}+1)}$$

The ratio (*R*) was defined as the volume ratio of the top phase to the bottom phase.

Determination of BSA concentration

The protein concentration was determined by the Bradford (1972) method with Coomassie Blue G250. The optical density was measured at 595 nm.

All experiments and measurements were conducted at least in triplicates.

RESULTS AND DISCUSSION

Effect of PEG molecular weight on the protein partitioning

The strong influence of polymer molecular mass on protein partitioning has been shown experimentally by several researchers (DALLORA *et al.*, 2007; DONG-QIANG *et al.*, 2003). In the present work, the influence of polymer molecular weights and pH on protein partitioning have been studied for PEG - $(NH_4)_2HPO_4$ system, and the result is shown in Fig. 1. From the figure, it has been observed that the partitioning of proteins in PEG - $(NH_4)_2HPO_4$ system is dependent on the molecular weight of the PEG. In this figure, *K* is the partition coefficient. The partitioning behavior of proteins in aqueous two-phase systems results from concurrent effects that cannot be individually measured, but insight can be gained by relating the observed tendencies with features of the proteins and of the systems themselves.

From the figure it is seen that the partition-

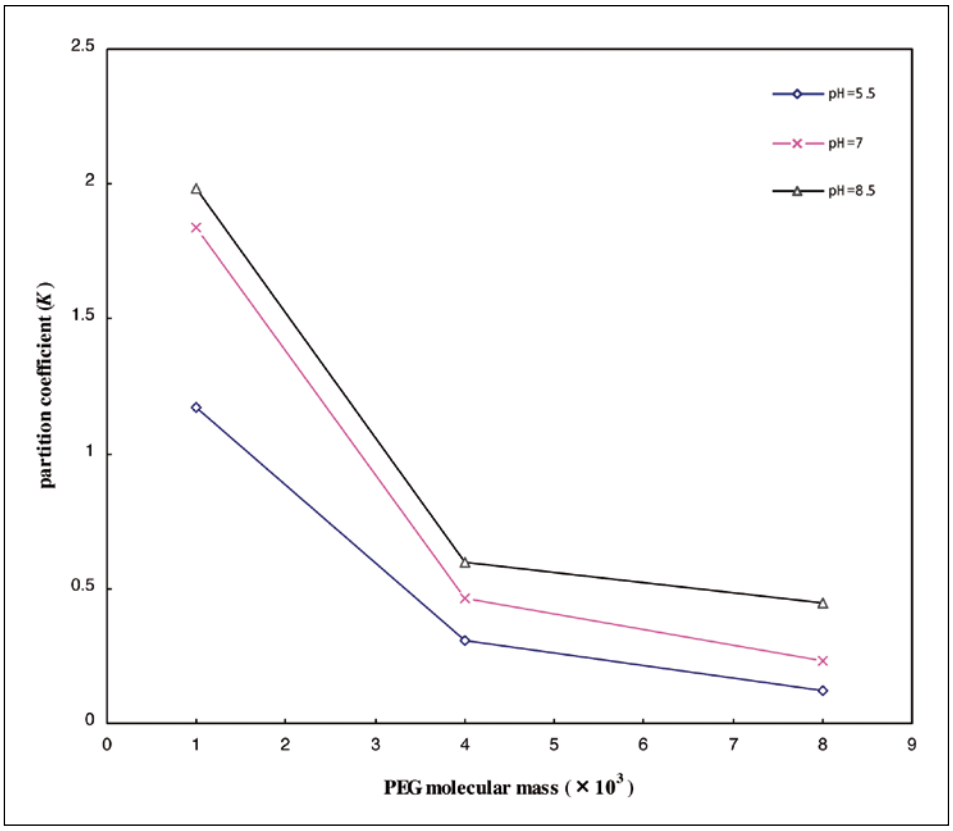


Fig. 1 - Effect of PEG molecular weight on protein partitioning in PEG - $(NH_4)_2HPO_4$ systems.

ing of soluble proteins can be altered by varying the molecular weight of PEG. With an increase in the molecular weight of PEG from 1,000 to 8,000, the partition coefficient of BSA decreases at PEG - $(NH_4)_2HPO_4$ system. For a lower molecular weight protein, the partition coefficient is high and thereby the extraction efficiency of the system is increasing due to an excluded volume effect (ZASLAVSKY, 1995).

Our data have showed that different parameters and conditions such as PEG molecular weight and pH could be effect on BSA extraction rates (Table 1).

BASKIR *et al.* (1989) stated that the protein partition is driven by not only an entropic mechanism but also enthalpic effects as well. According to PICO *et al.* (2007), from a molecular point of view, PEG of low molecular mass may interact strongly with proteins, while PEGs of higher molecular mass have ability to form intramolecular bonds. The protein transfer to one of the phases requires the breaking of the interaction between the phase components to create a cavity where the protein will be included. Therefore, the energy balance can be negative or positive according to whether protein/polymer interactions are attractive or repulsive, thus it depends on the PEG molecular mass (SARAVANAN *et al.*, 2008).

Better protein partitioning was achieved with low PEG molecular weight due to the low interfacial tension for low molecular weight of PEG. It was reported that interfacial tension between the phases is an important factor that determines the partitioning behavior of particles and cells (JOHANSSON, 1974).

Effect of pH on protein partitioning

The pH values chosen were near neutral, so as to make the system biocompatible. The partition coefficient increased with pH increasing in of the system as shown in Fig. 2. The pH

Table 1 - Effect of PEG molecular weight and pH on BSA extraction in PEG - $(NH_4)_2HPO_4$ systems.

	pH=5.5			pH=7.0			pH=8.5		
	PEG_{1000}	PEG_{4000}	PEG_{8000}	PEG_{1000}	PEG_{4000}	PEG_{8000}	PEG_{1000}	PEG_{4000}	PEG_{8000}
Y_e (%)	55.71	8.70	3.92	62.03	12.46	15.20	62.72	32.47	26.43

could affect the partitioning, either by changing the charge of the solute or by altering the ratio of the charged species present. At low pH, the proteins have a net positive charge because the amine gains an extra proton and at high pH, they have a net negative charge because the carboxyl loses its proton. At higher pH, the protein is more negatively charged than at low pH, and therefore, the partition coefficient of the protein increases with increasing the pH (Fig. 2), which may be caused by the electrostatic interactions between the protein and PEG units. Several authors have reported that negatively charged proteins partition to the top phase (PEG rich) and positively charged protein to the bottom phase (JOHANSSON *et al.*, 1994; SARAVANAN *et al.*, 2008).

BSA better partitioning obtained at pH 8.5. This pH is above the isoelectric point of BSA (pH_t=4.7). Increased in partition coefficient value could be due to hydrophobic interaction and net charge effect, which is a function of the polymer concentration and solution pH (SARAVANAN *et al.*, 2008).

CONCLUSIONS

Aqueous two-phase extraction have successfully used for BSA extraction. There are many factors affecting on extraction rates. Our examination results showed at pH=7-8 and molecular weight of PEG_{1000} extraction rate was increased to 62%. In summary, ATPS is a promising technique for the separation of BSA with the PEG - $(NH_4)_2HPO_4$ system.

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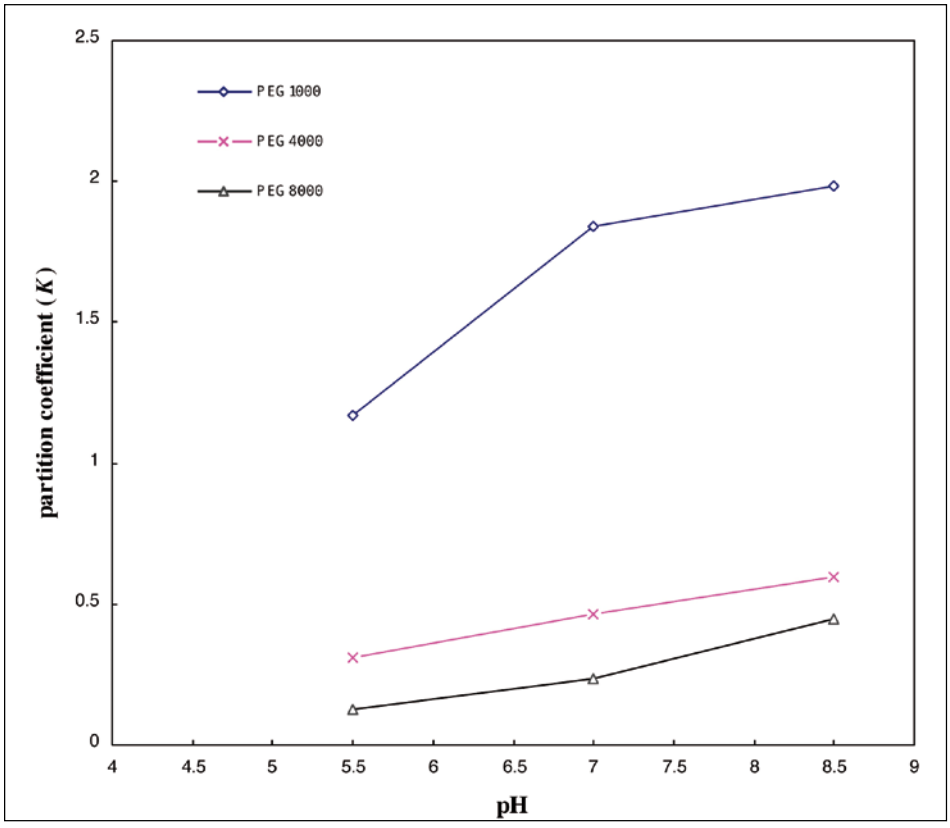


Fig. 2 - Effect of pH on protein partitioning in PEG - $(NH_4)_2HPO_4$ systems.

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SHORT COMMUNICATION

COMPOSITIONAL ANALYSIS AND ANTIOXIDANT PROFILE OF YELLOW, ORANGE AND PURPLE POLIGNANO CARROTS

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ABSTRACT

In the Puglia region, a multicolor landrace called the yellow-purple Polignano carrot has been cultivated since 1940 by local smallholder farmers and has been added to the Slow Food list of traditional products. Composition analyses of these carrots were carried out including carbohydrate assay, inorganic cations, nitrate content, and dry weight of each colour. Besides, antioxidant activity, β-carotene, total phenols and carotenoid content on the full carrots and on the cortex and inner core separated tissues were determined. The same analyses were performed on a commercial carrot cultivar.

On average, total glucose, fructose, and saccharose content was 22% lower in the yellow-purple Polignano carrot than in the commercial one. In addition, the highest contributor to the relative sweetness in the yellow-purple Polignano carrot was fructose (43.5%), which affects its well-known taste, as well as its glycemic index. As for the nutritional parameters, purple carrots showed the highest content in antioxidant activity, total phenols, carotenoids and β-carotene, mainly detected in the cortex.

- Keywords: coloured carrots, antioxidant activity, β-carotene, carotenoids, sweetness -

1. INTRODUCTION

The consumption of carrot (*Daucus carota* L.) is increasing steadily due to its recognition as an important source of natural antioxidants with anticancer activity (SHARMA *et al.*, 2011). Carrot is a significant source of phytochemicals, including phenolics (BABIC *et al.*, 1993) and carotenoids (BLOCK, 1994). Due to their appreciable level of different compounds, carrots are considered a functional food with significant health-promoting properties (ARSCOTT and TANUMIHARDJO, 2010).

An increasing interest in coloured carrots due to their high antioxidant activity and nutrient content has been reported (ALASALVAR *et al.*, 2001; ALASALVAR *et al.*, 2005; GRASSMANN *et al.*, 2007). In particular, purple carrots, such as purple-yellow or purple-orange, have higher antioxidant capacity and antioxidant content in comparison with other colours (SUN *et al.*, 2009).

Recently, new hybrids of coloured carrots have been commercialized. In particular, yellow and purple carrots (the latter also named *Daucus carota* ssp. *sativus* var. *atrorubens* Alef.) were first domesticated in oriental countries (such as Afghanistan and Turkey) about ten centuries ago and have since been cultivated in Asia and Europe. Finally, documentary evidence reported by BANGA (1963) showed that coloured carrots have been present in Italy since at least the 13-14th century (RUBATZKY *et al.*, 1999; BARANSKA *et al.*, 2006). In the Puglia region (Southern Italy), a multicolor landrace called the yellow-purple Polignano carrot has been grown since 1940 by local smallholder farmers (personal communication: V.V. Bianco 2011). The length of these carrots ranges from 15 to 25 cm, while the diameter can reach 5 cm. They are characterized by a special sweetness, crispiness and fragrance and by a great variety of colours in the external (outer core or cortex) and internal (inner core) root tissues. The cortex pigmentation or colour ranges from yellow or deep orange to dark purple, while the inner core colour ranges from pale yellow to light green. This landrace has been added to the Slow Food list of traditional products by the Slow Food Association (SLOW FOOD, 2011). In addition, it is included in the list of vegetables at risk of genetic erosion in the Rural Development Program of the Puglia region (2007-2013). The interesting aspect of this landrace is that the product can achieve excellent prices, generally twice or three times more than orange commercial carrots. Nevertheless, although information is available on the composition of other coloured carrots (ALASALVAR *et al.*, 2001), there is no data on the chemical characterization of the Polignano carrot.

In the present study, compositional analyses of traditional yellow, orange and purple Polignano carrot were performed. According to LUTALADIO *et al.* (2010), the aim was to evaluate the antioxidant activity, total phenol, carbohydrate, carotenoids, and mineral content of this landrace to preserve its genetic variability and to promote its diffusion in the market.

In addition to highlight the quality of this landrace, commercially-grown orange carrots were also included in these analyses in order to compare their chemical compositions.

2. MATERIALS AND METHODS

2.1 Plant material and growth conditions

Commercial (*cv* "Presto", Vilmorin http://www.vilmorin.com) and traditional carrots were collected from a farm located in Polignano a Mare (Ba), Southern Italy and transported in refrigerated conditions in the laboratory to be analyzed. Based on the epidermis colour, also known as skin pigmentation, the traditional Polignano carrots, hence indicated as "Polignano", were sorted into three groups, and designated yellow, orange and purple (Table 1). Thirty carrots for each group of "Polignano" and commercial carrots (10 samples x 3 replications) were analyzed for chemical traits as described.

2.2. Colour analysis

Ten samples for each group of "Polignano" and commercial carrots were used for the colour analysis. Colour parameters L* (lightness), a* (redness) and b* (yellowness) were measured on five points on each carrot surface for a total of 50 measures for each carrot type. After that,

Table 1 - Skin colour of commercial and yellow, orange, and purple Polignano carrots.

Skin colour	L*	a*	b*	h°
Commercial	50.98±0.54	22.25±1.03	37.18±1.09	59.47±0.44
Traditional				
Yellow	53.21±1.14	-1.34±1.29	28.95±7.81	92.36±1.67
Orange	51.24±0.32	12.72±1.74	25.61±1.98	63.66±1.30
Purple	34.99±1.95	6.91±1.30	4.56±0.42	33.91±7.59

Mean value of 50 measurements for each carrot type ± standard deviation.

the same carrots were cut longitudinally and the colour of the cortex and inner core with the same method was measured. A colorimeter (CR-400, Konica Minolta, Osaka, Japan) equipped with a D65 illuminant, in the reflectance mode and in the CIE L* a* b* colour scale was used. Hue angle (h°= arctg b*/a*) from primary a* and b* readings, was then calculated. The colorimeter was calibrated with a standard reference having values of L*, a* and b* corresponding to 97.55, 1.32, and 1.41 respectively.

2.3. Carbohydrate assay and sweetness

Glucose, fructose and saccharose were determined as reported by BUONO *et al.* (2009). Briefly, 25 g carrots were homogenized with boiling distilled water (1:4, w/v) in a blender. The filtered solution (Whatman 1) was diluted and analyzed by ion chromatography (Dionex model DX500; Dionex Corp., Sunnyvale, CA) with a pulsed amperometric detector. Peak separation was performed using a Dionex CarboPac PA1 and isocratic elution with 50 mmol L^{-1} NaOH.

The relative sweetness was calculated relative to all naturally occurring carbohydrates (fructose, 1.73; glucose 0.74; saccharose, 1.00) as reported by ALASALVAR *et al.* (2001).

2.4. Antioxidant activity and total phenol content

Antioxidant activity and total phenol content were analysed on the full carrots and on the separated cortex and inner core. The latter were obtained by cutting the carrots longitudinally, and sampling the external (cortex) and middle (inner core) root sections, which presented the same uniform colour. The extraction procedure reported by CEFOLA *et al.* (2011) for both antioxidant activity and total phenols was used.

Antioxidant assay was performed following the procedure described by BRAND-WILLIAMS *et al.* (1995) with minor modifications. The diluted sample (50 μL) was pipetted into 0.95 mL of diphenylpicrylhydrazyl (DPPH) solution to initiate the reaction. The absorbance was read after about 30 min at 515 nm. Trolox was used as a standard and the antioxidant activity was reported in milligrams of Trolox equivalents per 100 grams of fresh weight (fw).

Total phenols were determined according to the method of SINGLETON and ROSSI (1965). Each extract (100 μL) was mixed with 1.58 mL water, 100 μL of Folin-Ciocalteu reagent (Sigma-Aldrich, Milan, Italy) and 300 μL of sodium carbonate solution (200 g L^{-1}). The absorbance was read after 2 hours at 765 nm (UV-1800, Shimadzu, Kyoto, Japan). The content of total phenols was calculated on the basis of the calibration curve of gallic acid and was expressed as milligrams of gallic acid per 100 grams of fw.

2.5. Total carotenoids and β-carotene content

Total carotenoids and β-carotene content were analysed on the full carrots and on cortex and inner core separated tissues. For the analysis of total carotenoids, five grams of tissue were placed in capped 50 mL centrifuge tubes, frozen at -80°C until extracted in 80% acetone, containing a few mg of magnesium carbonate, with a homogenizer (Ultraturrax T-25, IKA Staufen Germany) (LICHTENTHALER, 1987). To remove all pigments, tissues were extracted five times by adding 10 mL of 80% acetone to the pellets and finally the extracts were combined. Samples were centrifuged, and the absorbances at 471 and 477 nm were measured against an acetone blank. Total carotenoids were calculated according to the equation reported by ALASALVAR *et al.* (2005).

AOAC method (AOAC, 2000) was used for determination of β-carotene by a spectrophotometric assay. β-carotene was directly quantified in the acetone phase by absorbance measurement at 436 nm.

To obtain the calibration curve at 436 nm, the standard of β-carotene supplied by Sigma-Aldrich (Milan, Italy) was used. Thereafter, the β-carotene content was calculated on the basis of the calibration curve and data were expressed as mg 100 g^{-1} of fw.

2.6. Dry weight, nitrate content, and inorganic cations

In order to measure dry weight, chopped carrots were maintained in a forced-draft oven at 65°C until constant weight was reached.

The dehydrated carrots were finely milled using an MF 10.1 cutting grinding head, 25 mm sieve (IKA Werke GmbH, Staufen, Germany) and used for ion-exchange chromatography analysis of nitrate as reported by SANTAMARIA *et al.* (1999). Nitrate (NO_3^-) content was expressed on a fw basis.

Sodium, potassium, magnesium, and calcium were determined on juice as reported by SERIO *et al.* (2004). Briefly, 25 g of carrots were homogenized with distilled water (1:4, w/v) in a blender. The filtered solution was diluted and analyzed by ion chromatography (Dionex model DX120; Dionex Corporation, Sunnyvale, CA) with a conductivity detector, using the pre-column IonPack CG12A and the column of separation IonPack CS12A.

2.7. Statistical analysis

The compositional analysis of "Polignano" and commercial carrots was tested performing a one-way ANOVA, with data means arranged in a completely randomized design. A two-way ANOVA for carrot types, carrot tissues (inner core and cortex) and their interaction was performed. Mean

values for carrot types were separated using the Student-Newman-Keuls (SNK) test and standard deviation was also calculated.

3. RESULTS AND DISCUSSION

3.1. Carbohydrate assay and sweetness

The Polignano carrot showed a higher glucose and fructose content (on average, 1.20 and 1.25 g 100 g^{-1} fw, respectively) than the commercial type (Table 2). On the other hand, the commercial carrots presented a total glucose, fructose and saccharose content that was 20% higher than the mean value detected in the "Polignano" (Table 2). The relative sweetness of commercial carrots was mainly affected by saccharose (69.1%), which in "Polignano" accounted for only 38.5%. The highest contribution to the relative sweetness in "Polignano" was ascribed to fructose (43.5%). These results confirmed that the "Polignano" are characterized by the well-known special taste (sweetness), which distinguishes them from the commercial type. In addition, due to its lower saccharose and higher fructose content, the latter sugar having a very low glycemic index of 19, this landrace could be recommended as a vegetable to use in the hypoglycemic diet (JENKIS *et al.*, 1981).

3.2. Antioxidant activity, total phenols, total carotenoids and β-carotene

"Polignano" showed various skin colours, which allowed us to separate them into three groups (yellow, orange and purple) as previously described. Generally, the cortex presented the same colour as the skin, whereas the inner core was characterized by yellow-green components in purple, orange and yellow carrots (h° mean value 86.17±5.2, 89.07±1.4 and 98.2±1.0, respectively). These colour variations, from the cortex to inner core, are typical of "Polignano" and they are not present in commercial carrots (Fig. 1). For this reason, antioxidant activity, total phenol, total carotenoids, and β-carotene were measured both in all carrot tissues and separately in the cortex and inner core tissues.



Fig. 1 - Yellow, orange, and purple Polignano carrots used in the trial.

"Polignano" and commercial carrots showed differing antioxidant activity, as reported in Table 3. In particular, purple carrots presented an antioxidant activity 4-fold higher than the commercial and 10-fold higher than yellow and orange "Polignano" (Table 3). These results are in agreement with data reported by different authors (ALASALVAR *et al.*, 2005; HABEGGER and SCHNITZLER, 2007). As shown in Fig. 2A, the elevated antioxidant activity recorded in the purple carrots is only ascribed to the cortex tissues. By contrast, in orange and common carrots, antioxidant activity in the cortex tissues was 50% higher than in the inner core (Fig. 2A). No significant differences were measured between the cortex and inner core tissues for the yellow "Polignano" (Fig. 2A).

As previously reported by ALASALVAR *et al.* (2001), sizeable differences in the content of total phenolics were found among the different coloured carrots (yellow, orange and purple). According to these authors, the total amount of phenolics in purple carrots was about 3.5-

Table 2 - Carbohydrate assay (g 100 g^{-1} fw) in commercial and yellow, orange, and purple Polignano carrots.

Carrots	Glucose	Fructose	Saccharose	Total	Relative sweetness
Commercial	0.83±0.14 b	0.69±0.16 b	4.08±0.69 a	5.60±0.76 a	5.89±0.78 a
Traditional					
Yellow	1.27±0.10 a	1.30±0.14 a	1.40±0.22 c	3.97±0.14 b	4.59±0.20 b
Orange	1.13±0.04 a	1.16±0.07 a	2.19±0.13 bc	4.48±0.23 b	5.03±0.27 ab
Purple	1.20±0.05 a	1.28±0.09 a	2.24±0.46 b	4.70±0.39 b	5.33±0.38 ab
ANOVA	**	**	***	*	*

Significance: *, ** and *** = significant at P ≤ 0.05, 0.01 and 0.001, respectively. Mean values of 30 samples ± standard deviation. For each attribute, means followed by a different letter are significantly different (P = 0.05) according to the SNK test.

Table 3 - Antioxidant activity, total phenols, total carotenoids and β-carotene contents in commercial and yellow, orange, and purple Polignano carrots.

Carrots	Antioxidant activity ($mg \cdot kg^{-1}$)	Total phenols ($mg \cdot kg^{-1}$)	Total carotenoids ($mg \cdot kg^{-1}$)	β-carotene ($mg \cdot kg^{-1}$)
Commercial	113.4±2.7 b	215.7±11.6 b	127.1±1.9 b	113.6±5.0 b
Traditional				
Yellow	44.6±0.4 c	163.9±11.8 c	91.9±1.8 c	26.2±1.9 c
Orange	49.9±0.1 c	170.6±1.9 c	76.8±3.7 d	44.5±9.1 c
Purple	426.6±54.4 a	676.0±9.1 a	433.5±5.2 a	155.4±24.2 a
ANOVA	***	***	***	***

Significance: *** = significant at P ≤ 0.001. Mean values of 30 samples ± standard deviation. For each attribute, means followed by a different letter are significantly different (P = 0.05) according to the SNK test.

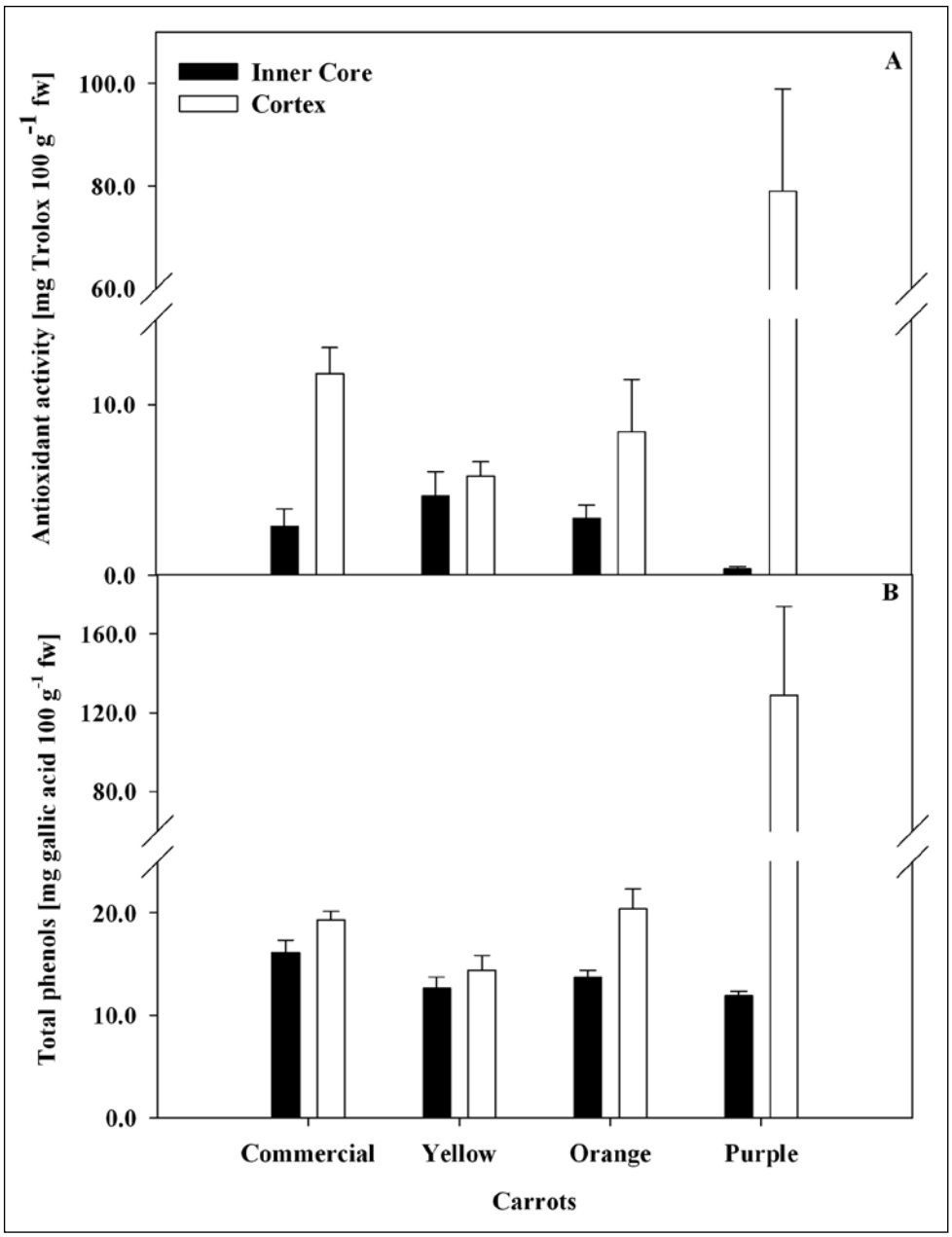


Fig. 2 - Antioxidant activity (A) and total phenol content (B) in the inner core and cortex of commercial and of yellow, orange, and purple Polignano carrots (P-value < 0.0001). Mean value of 3 replications ± standard deviation.

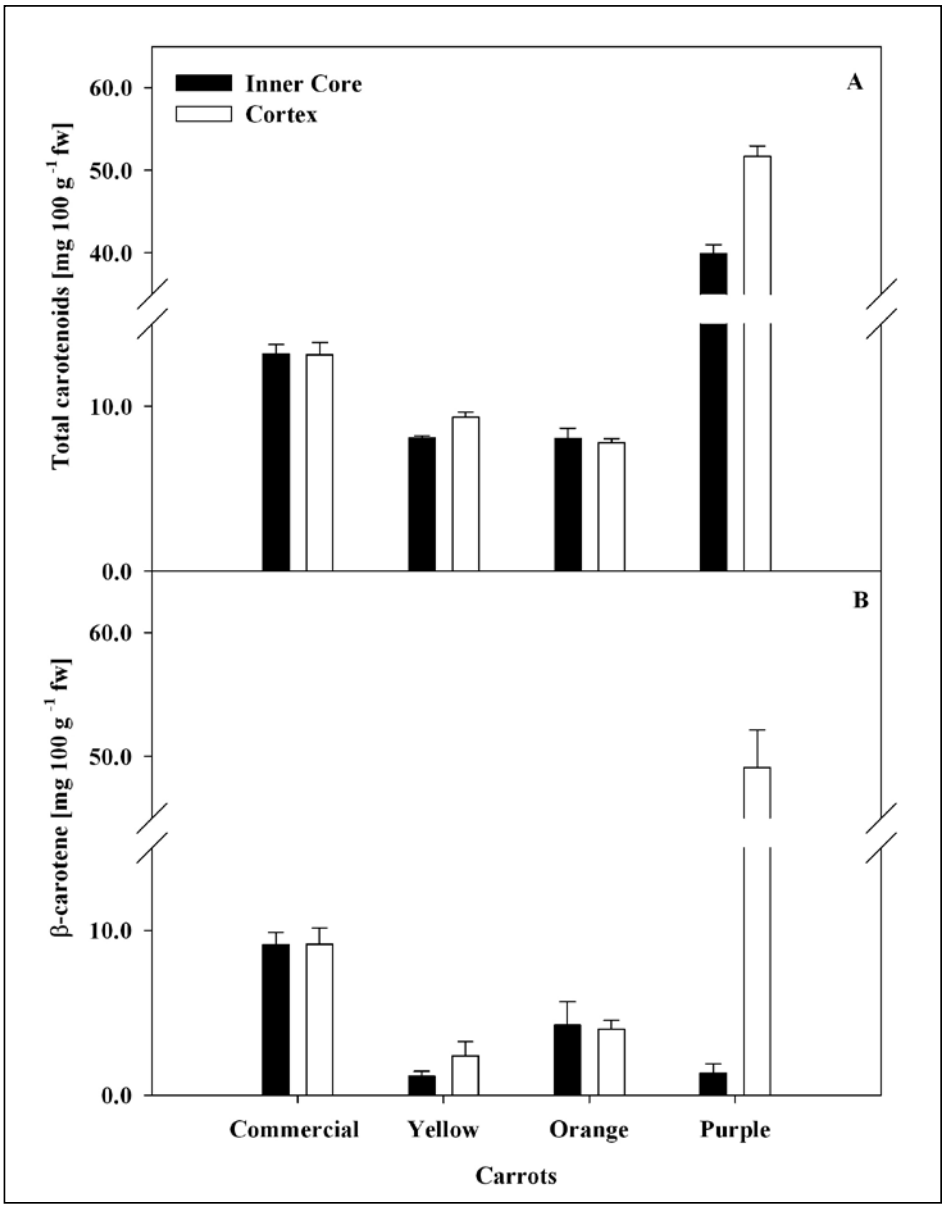


Fig. 3 - Total carotenoids (A) and β-carotene content (B) in the inner core and cortex of commercial and of yellow, orange, and purple Polignano carrots (P-value < 0.0001). Mean value of 3 replications ± standard deviation.

fold higher than in commercial carrots. In addition, yellow and orange "Polignano" showed a mean total phenol content about 77 and 24% lower than in purple and commercial carrots, respectively (Table 3). As previously reported for antioxidant activity, the main source of phenols was in the cortex of purple carrots. In the other carrots analysed, no relevant differences were detected between cortex and internal tissues (Fig. 2B).

Total carotenoid content of purple carrots was 3.4-fold higher than the commercial type, whereas yellow and orange showed a mean content 75 and 22% lower than purple and commercial ones, respectively (Table 3), in agreement with ALASALVAR *et al.* (2001). In addition, our data agreed with that previously reported on the range of total carotenoid content in the edible portion of carrot roots (6.0 to 54.8 mg 100 g^{-1}) (SIMON and WOLFF, 1987; BARANSKI *et al.*, 2011). Sampling the cortex separately by the inner core, the total carotenoid content was 20% higher in the outer tissues than in the inner core of purple carrots (Fig. 3A). By contrast, commercial and yellow or orange "Polignano" showed no differences in carotenoid content between cortex and inner core (Fig. 3A).

The range of β-carotene recorded was from 2.6 to 15.5 mg 100 g^{-1} according to data reported by different authors (ALASALVAR *et al.*, 2001; LEMMENS *et al.*, 2009). Purple carrots contained roughly four times more β-carotene than yellow and orange "Polignano", whereas data for commercial types (Table 3) were similar to those reported in the literature (CARNOVALE and MARLETTA, 2000; KARNJANAWIPAGUL *et al.*, 2010).

By analyzing the cortex separately from the inner core, the β-carotene detected in purple carrots was essentially located in the cortex. Finally, as for total carotenoids, no differences were measured between the inner and outer tissues for the commercial and yellow or orange "Polignano" (Fig. 3B).

The average daily intake of β-carotene is 5 mg, as estimated from a pooled analysis of 500,000 women living in the Usa, Canada and some European Countries (KOUSHIK *et al.*, 2006). Fifty g of commercial carrots are able to provide this amount, whereas, only 10 g of cortex tissues of the purple carrots are enough to provide the same intake. This give us an idea about the nutritional value of the purple "Polignano" in comparison with the commercial carrots usually consumed in the diet.

3.3. Dry weight, mineral composition, and nitrate content

The "Polignano" showed a dry weight content (on average, 8.2 g 100 g^{-1} fw) about 40% lower than that measured in commercial carrots.

As for mineral composition, no significant differences were detected among the different carrots when data were calculated on the basis of the fresh weight, with the exception of K^+ content (Table 4). The data are in agreement with those reported by CARNOVALE and MARLETTA (2000) in their food composition tables. Generally, the nitrate content was very low (under 100 mg kg^{-1} fw) in all carrot samples analyzed (Table 4). The highest nitrate content was detected in commercial carrots, whereas lower concentrations were measured in "Polignano". In particular, purple carrots showed a 50% lower nitrate content than the commercial ones.

The major dietary source of nitrates is from vegetables (SANTAMARIA, 2006), and their organs can be listed in order of decreasing nitrate content as follows: petiole > leaf > stem > root > inflorescence > tuber > bulb > fruit > seed (SANTAMARIA *et al.*, 1999). With regard to carrot, the problem of nitrate concentration level is particularly important because it constitutes one of the most frequently used raw materials for baby food. For these kinds of products, European regulations (1258/2011) impose a maximum nitrate concentration of 200 mg kg^{-1} fw. On the other hand, EU legislation has set no limits for nitrate content in carrot. It has instead been reported that, when nitrate is consumed in a normal diet containing vegetables, other concomitantly consumed bioactive substances, such as antioxidants, may inhibit the endogenous formation of nitrosamines from nitrite (EFSA, 2008). Starting from this finding, purple carrots could be considered a vegetable of high nutritional value due to their well-known low nitrate content and their very high antioxidant activity, phenolic, and carotenoid content. This represents added value for vegetable products that are already rich in nutrients.

4. CONCLUSIONS

The yellow-purple Polignano carrot has a qualitative profile that is characterized by high concentrations of antioxidants, phenols and carotenoids, especially β-carotene. This profile, combined with its low glycemic index, may account for the high nutritional quality of this landrace, especially the purple type. These traits might encourage future research aimed to study the postharvest behaviour and suitable storage conditions to preserve these nutritional qualities during the shelf-life.

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Table 4 - Dry weight, mineral composition and nitrate content in commercial and yellow, orange, and purple Polignano carrots.

Carrots	Dry weight	Mineral composition (mg kg^{-1} fw)				
	(g 100 g^{-1} fw)	Ca^{2+}	K^+	Mg^{2+}	Na^+	NO_3^-
Commercial	13.80±0.30 a	248.5±29.50	2011.5±111.50 b	81.50±8.50	633.0±86.00	81.67±17.37 a
Traditional						
Yellow	8.30±0.20 b	224.7±12.42	2032.3±112.52 b	76.67±8.08	623.3±89.19	69.33±8.15 a
Orange	7.90±0.44 b	250.0±25.24	2617.0±407.99 a	95.67±18.15	582.0±98.73	67.00±12.14ab
Purple	8.40±0.52 b	220.0±28.93	2343.7±155.13 ab	91.67±14.98	448.3±51.60	45.33±3.99 b
ANOVA	****	ns	*	ns	ns	*

Significance: ns, * and **** = not significant or significant at P ≤ 0.05 and 0.0001, respectively. Mean values of 30 samples ± standard deviation. For each attribute, means followed by a different letter are significantly different (P = 0.05) according to the SNK test.

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CLASSIFICATION OF COMMERCIAL OMANI HALWA BY PHYSICO-CHEMICAL PROPERTIES AND INSTRUMENTAL TEXTURE PROFILE ANALYSIS (TPA)

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ABSTRACT

Commercially available Omani halwa from the local market was classified based on its composition and instrumental texture profile. The products were grouped into four classes based on cluster analysis. None of the attributes of the instrumental Texture Profile Analysis (TPA) were correlated significantly with the ash, colour and pH values ($p<0.05$), while specific attributes did correlate with specific aspects of the chemical composition. All TPA attributes were significantly correlated with specific types of fatty acid. Hardness was correlated significantly with the moisture and sugar contents, while adhesiveness was correlated significantly with the moisture, sugar and non-sugar carbohydrate contents ($p<0.05$). Springiness was correlated with sugar and non-sugar carbohydrate. The firmness and chewiness were correlated with moisture and the total fat, saturated and unsaturated fat contents ($p<0.05$). Cohesiveness-1 was correlated only with the protein, total fat, saturated and un-saturated fat contents, while cohesiveness-2 was correlated with sugar, total fat, saturated and unsaturated fat ($p<0.05$). The resilience was correlated with the total fat, saturated and unsaturated fatty acids, while gumminess was only correlated with the moisture content. The link between TPA and physico-chemical characteristics was established using multivariate matrix correlations and revealed that the pattern of texture attributes was mostly linked to the moisture and fat contents. Principal component analysis was carried out to identify the main physico-chemical properties and TPA attributes of the four classes of halwa as determined by cluster analysis. The four classes of halwa could be characterised as soft-resilient, soft-springy-cohesive, soft-springy and hard-chewy.

- Keywords: food classification, fatty acids, sugar, multivariate analysis, cluster analysis, principal component analysis -

INTRODUCTION

Halwa is a dessert (i.e., sweet jelly) made up mainly of starch, sugar, water, ghee, and flavoured with saffron, nuts and/or rose water. Omani halwa is usually made with sugar (50%), water (25%), mill flour (10%) and ghee (clarified butter) (15%) (AL-SHAMSI *et al.*, 2013). Traditional halwa is prepared as follows. First, sugar is added to boiling water in an open pan. Starch is added to the sugar syrup after all of the sugar has dissolved. The heating process continues with manual mixing until the mixture turns into a thick, jelly-like substance. At this stage, fats, nuts, and spices are added, and then, the gels are poured into dishes of varying sizes for retail sale. There are mainly 3 types of Omani halwa, based on their final colour: white, yellow or black. They differ in the sweetener that is used, the flavour and texture. AL-SHAMSI *et al.* (2013) used a structured survey questionnaire to determine consumer preferences and behaviour with regard to 22 attributes of halwa. Five types of consumer groups were identified through hierarchical cluster analysis (AL-SHAMSI *et al.*, 2013). The type and amount of fat and sugar content in halwa can be considered part of an unhealthy diet. The successful development of healthier halwa (i.e., one that is lower in fat and sugar with the incorporation of health functionality) by the food industry requires an understanding of the different classes of halwa in the market and their generic attributes in order to explore the sensory preferences of consumers.

Product classification systems are considered a viable approach to support a specific surrogate. This system could be used as the basis for developing new products with varied specification or characteristics as well as for exploring the generic characteristics or attributes of low- or high-grade products to avoid adulteration. This classification could include general categories that can lead to specific relationships between product attributes (SOUSA and WALLACE, 2006). Qualitative information is difficult to use in high dimensions. PENNINGTON and FISHER (2009) pointed that the classification of fruits and vegetables is most helpful for dietary assessment if the classification is based on composition, as well as for teaching students about food composition and to assist dieticians in providing dietary guidance to patients and clients. They determined levels of food components in fruits and vegetables correlated with classification criteria based on botanical family, colour, the part of the plant, and the total antioxidant capacity. However, the level of total antioxidant capacity did not match well with the identified clusters. RELEAUX (1904) devised one of the first product classification systems after recognising the identicalness among various properties of different products. Products have been classified in several different ways. Different perspectives on what a product is and the purpose of the classification lead to distinct classification systems (SOUSA and WALLACE, 2006).

Food products can be classified by flavour compounds, chemical composition, and texture. MARTIN *et al.* (1999) classified vegetable oils by linear discriminant analysis of electronic nose data for flavour compounds. Classification studies have been performed on a variety of food products, such as food, beverages and perfumes (PENZA *et al.*, 2001); commercial cheeses based on their volatile profiles using multivariate analysis of complex data of GC/MS (DIRINCK and WINNE, 1999) and cheddar cheese based on flavour profiles (O'RIORDAN *et al.*, 2003); Greek white wines from 6 grape varieties based on their amino acid profile (SOUFLEROS *et al.*, 2003); apple fruit maturity according to polyphenol composition (ALONSO-SALCES *et al.*, 2005); medicinal plants of different species based on 10 triterpenoid acids (GUO *et al.*, 2009); vegetable oils based on fatty acid profile (BRODNJAK-VONCINA *et al.*, 2005); commercial potato chips based on colour and image texture (MENDOZA *et al.*, 2007); and tomato fruit using acoustic impact and colorimetry (BALTAZAR *et al.*, 2008). However, food products could be classified by their textural attributes. Instrumental TPA attributes were correlated with the composition of sandesh (a soft cheese made by direct acidification of heated milk with dilute acid followed by removal of whey and then mixed with sugar) available in various markets. The textural attributes were significantly influenced by the moisture, fat and ash contents (KHAMRUI and SOLANKI, 2010). There are few classifications of foods based on texture and composition.

The objective of this study was to classify Omani halwa available in the market based on chemical composition, physico-chemical properties and instrumental TPA attributes. This classification through a product survey could help the industry to understand existing products and to develop low-fat and low-sugar products (with new ingredients) by mimicking the TPA attributes of existing products.

MATERIALS AND METHODS

Halwa

Halwa samples (4 batches) were purchased from different local supermarkets in Muscat. The samples were selected from different types with various prices (very high, high, medium and low), colours (black and yellow) and flavours (royal, special, and saffron). After purchasing, the samples were stored at room temperature (20°C) for one day before analysis.

Proximate chemical analysis

Moisture, protein, fat, and ash were determined by following the AOAC Official Methods (2000). The moisture content was determined by drying 5 g samples in a mechanical convection oven for at least 18 hours at 105°C. Crude protein was determined by the Kjeldahl method using a conversion factor of 5.55 × N. Digestion and analysis were performed in a Digestion System 2000 and a Kjeldahl analyser unit 2300 (Foss Tecator, Höganäs, Sweden). The ash content of the samples was determined by burning the sample in a furnace in accordance with AOAC methods (2000). Approximately 5 g of each sample was weighed and burned at 530°C in a muffle furnace for 18 hours. The fat content was determined gravimetrically after extraction with light petroleum ether and evaporated at 103°C in an oven for at least 1 hour. The non-sucrose carbohydrate content was calculated by the difference of the total proximate composition and the water, protein, fat, ash and sucrose. All of the values were calculated in units of g/100 g sample. All of the analyses were performed in triplicate.

Sugars

Individual free sugars were determined by HPLC according to the technique of MYHARA *et al.* (1998). Sugars were extracted by refluxing halwa samples twice with aqueous ethanol (850 and 750 mL^{-1}), and the residues were washed with ethanol (750 mL L^{-1}). The extracts were then treated with lead acetate (10 g L^{-1}) over gentle heat to precipitate proteins. After centrifugation, the supernatant solutions were dried in a rotary evaporator under reduced pressure. The dry residues were dissolved in a known volume of water, decolourised by passing through a C18 Sep-Pak solid-phase extraction tube (Waters Associates, Milford, MA, USA) and filtered through a 0.45 μm cellulose acetate filter.

Sugars were separated on a Hewlett-Packard (Palo Alto, CA, USA) HP1090 system, equipped with a 10 μL sample loop, a refractive index detector (model HP1047A) and a silica Nova-Pak Radial-Pak cartridge column (100 mm x 8 mm id, particle size 4 μm; Waters). Both the column and detector were maintained at 40°C. Prior to analysis, the silica column was modified by passing 1 L of acetonitrile/water (65:35) containing 1 g L^{-1} of 3-aminopropyltriethoxysilane at a flow rate of 1 mL min^{-1}. The column was then stabilised with 1 L of acetonitrile/water (65:35) containing 0.1 g L^{-1} of 3-aminopropyltriethoxysilane. The latter solution was also used as the mobile phase. External standards of fructose, glucose and sucrose (filtered through C18Sep-Pak and 0.45 μm cellulose acetate filters) were used for quantification.

Colour

The colour of the halwa samples was determined using a colour meter at room temperature (Minolta CR-310, Minolta, Japan). The equipment was calibrated with a white standard calibration plate provided by the manufacturer. The halwa samples were placed on a flat plate, and the tip of the measuring head was pointed at the sample for measurement. Each value was the mean of 6 measurements. The results were expressed in Hunter L, a, b values, in which L is the lightness or darkness (black, L=0; white, L=100), +a is the redness, -a is the greenness, +b is the yellowness, and -b is the blueness. The colour (CL) was also calculated as CL= (L*b)/a (RAHMAN *et al.*, 2002).

pH

The pH of the halwa solution was measured by preparing a 10% (w/v) halwa solution in distilled water (RAHMAN *et al.*, 2008). The pH was then measured in triplicate with a glass electrode (Toledo MPC 227 pH meter, Mettler-Toledo GmbH, Schwerzenbach, Switzerland).

Fatty acids

Triplicate samples, each weighing approximately 2 g, were freeze-dried for 4 days. The fatty acids were extracted according to the method described by AOAC (2000) with some modifications. Halwa samples were mixed with 50 mL of 1 N KOH, and 4 mL of tricosanoic acid internal standard (C23) was added. The mixture was heated for 30 min at 150°C, followed by cooling at room temperature. The samples were transferred to a 500 mL separating funnel, and 150 mL of distilled water was added followed by 0.1% methyl orange drop-wise until the colour changed to yellow. A solution of 5 N HCl was added until the colour turned light pink (pH=2), and the solution was then partitioned vigorously for 5 min with 100 mL of diethyl ether. The diethyl ether layer was collected, and the coloured aqueous phase was re-extracted with a further 100 mL of diethyl ether. The diethyl ether layer extracts were pooled and washed four times with 80 mL of distilled water and subsequently dried over anhydrous Na_2SO_4. The diethyl ether extract was then concentrated to 2 mL in a rotary vacuum evaporator at less than 40°C and transferred to a screw-cap test tube following addition of 2 mL of BF_3-methanol reagent (14%). The test tube was heated at 100°C for 15 min in a water bath. After cooling to room temperature, 3 mL of hexane and 5 mL of saturated NaCl were added, and the mixture was vigorously shaken for 5 min. The hexane layer was then carefully transferred using a Pasteur pipette into an amber crimp cap and stored at -20°C until assayed by gas chromatography.

Fatty acid samples were analysed with an Agilent Plus gas chromatograph (GC) 6890N equipped with a 30 m × 0.25 mm SP-2380 (Supeclo Inc., USA) fused-capillary column attached to a flame ionisation detector. Helium was used as the carrier gas at a velocity of 20 cm/sec with electronic pressure control and split ratio of 50:1. The GC temperature was programmed from 50° to 250°C at a heating rate of 4°C/min. The temperatures of the injector and detector were 250° and 260°C, respectively. Fatty acids were identified by comparison of the retention times with a 37 FAME Standard mix solution (Supeclo). The concentration of individual fatty acids was calculated using tricosanoic acid (C23) as an internal standard.

Instrumental Texture Profile Analysis (TPA)

Instrumental TPA attributes were measured using a TA.XT2 Texture Profile Analyser (Stable Microsystems, Godalming, Surrey, UK) with two cycles of compression-decompression. The texture analyser was coupled with the computer software (texture expert). The load cell was calibrated with a 5 kg weight. The equipment was set to zero automatically before each experiment (i.e., the point when the top plate just touched the surface of the table). Compression mode was used for the textural analysis. Halwa samples were cut into 10, 20 and 30 mm cubes manually with a knife. The halwa specimens were placed on the stage of the texture analyser and compressed with a flat, 75 mm plunger (for 10, 20, and 30 mm cubes) or a flat, 20 mm, edge cylindrical plunger (for a 30 mm cube) or a plunger 5 mm in diameter (for a 10 mm cube). Halwa samples were compressed to 60% of their original height at a compression rate of 1.0 mm/s at room temperature (i.e., 20°C). The force-displacement curve was automatically recorded by a computer attached to the TPA. At least six replicates were analysed for each halwa sample.

The TPA attributes were determined from force-time curves based on the following attributes: hardness (HA), the maximum force during the first compression cycle (N); adhesiveness (AD), the negative area of the first compression (N m); springiness 1 (SP1), the distance between the end of the first compression and start of the second (mm); springiness 2 (SP2), the distance between the start of the first compression and the starting point; firmness 1 (FR1), the slope of the initial linear part of the first compression (N/s); firmness 2 (FR2), the slope of the second linear part of the first compression (N/s); cohesiveness 1 (CO1), the ratio of the positive area of the second compression and the positive area of the first compression; cohesiveness 2 (CO2), the ratio of difference of the positive and negative areas of the second compression and difference of the positive and negative areas of the first compression; resilience (RE), the ratio of the negative area to positive area of the first compression; gumminess 1 (GU1), the product of hardness and cohesiveness 1 (N); gumminess 2 (GU2), the product of hardness and cohesiveness 2 (N); chewiness 1 (CH1), the product of gumminess 1 and springiness 1 (N m); and chewiness 2 (CH2), the product of gumminess 2 and springiness 1 (N m) (RAHMAN and AL-MAHROUQI, 2009).

Multivariate analysis

Multivariate analysis (Spearman's correlation matrix, cluster analysis, and principal component analysis) was used to identify different groups of halwa and their characteristics based on instrumental texture and physico-chemical properties. These analyses were performed with PAST software (HAMMER *et al.*, 2001). Correlations were used to determine the relationships between each variable. The Spearman's correlation matrix of the TPA attributes and the physico-chemical variables were determined, and the results were summarised for the p values ≤ 0.05 and 0.01. A cluster analysis was performed to group similar halwa. A hierarchical clustering using Ward's method of variance minimisation within groups was applied to the TPA standardised attributes and the physico-chemical variables (WARD, 1963). The number and composition of the clusters retained were estimated by visual observation of the clustering tree. The multivariate linkage between the overall texture and the overall physico-chemical composition was investigated using the Bio-Env procedure of "Primer" (CLARKE and GORLEY, 2006). This procedure measures the correlations between a single matrix of similarities/distances calculated from the texture profile and all of the matrices of similarities calculated from all of the possible combinations of physico-chemical composition variables [$(2^n - 1)$ matrices, where n is the number of variable]. The best correlation identifies the best linkage between the texture profile matrix and one of the $2^n - 1$ physico-chemical composition matrices. The better correlation matrices are based on "ranks of values" in the two similarity matrices.

The Spearman's and Pearson's correlations were plotted to determine the degree of non-linearity of the variables. Principal component analysis was used to classify and to characterise the products. In the case of principal component and cluster analysis, the TPA attributes and physico-chemical properties were standardised to remove undesired effects related to the size of the measurement by the following equation:

$$y = \frac{(x - \bar{x})}{\sigma} \quad (1)$$

where y is the standardised data (or variable), x is a variable, is the average, and σ is the standard deviation of the data for this variable.

RESULTS AND DISCUSSION

In this study, batch 1 of the halwa was used to check the reproducibility of instrumental TPA data generated by compression and punching methods. Table 1 shows the TPA attributes measured by punching with a 20 mm probe provided more reproducible data compared with the 75 mm probe compression and punching with a 5 mm probe. For example, the standard deviations of hardness compared with the average values were 88.5, 21.4 and 6.3% for the 5 mm probe puncher, the 75 mm compression disk (sample size of 30 mm) and the 20 mm probe puncher methods, respectively. To determine the sensitivity of the 20 mm punch-probe method, batch 2 with varying moisture contents of halwa were used. The TPA attributes using the 20 mm probe did not show much sensitivity (Table 2) while moisture varied from 13.1 to 18.9 g/100 g halwa ($p>0.19$) (Table 3). However, the compression disk method was found to be more sensitive as a function of sample size and moisture content (Tables 1 and 4). Thus, the compression method was used to determine the instrumental TPA attributes for a 10 mm cube of halwa (the most reproducible sample size). The variability of the TPA data using different probes was reported. The sensitivity results of 3 types of punching probes (blade, sphere and cylinder) indicated that cylindrical probe produced the most sensitivity with respect to the TPA attributes of salmon fillet (CASAS *et al.*, 2006). Similarly, BENEDITO *et al.* (2006) compared the instrumental attributes of cheese as a function of its maturity using different types of probes (compression, punching and needle). They observed a similar correlation in the cases of compression and punching, which was much higher compared to the needle-type probe.

Batch 3 was purchased and included 15 samples (black and yellow halwa) from different shops. The TPA attributes were determined from the typical, instrumental TPA force-time graph using a 75 mm disk (Fig. 1), which shows compression-decompression cycles. The instrumental TPA attributes of 15 samples are shown in Table 4, and their chemical compositions are given in Table 5. The extraction of fat through Gas Chromatography was considered for classification. Hardness, adhesiveness, firmness, cohesiveness, resilience, gumminess, and chewiness de-

Table 1 - Instrumental TPA attributes with flat and punch probes of different diameters and sizes of sample (Batch 1).

Probe Type	Probe Size (mm)	Sample Size (mm)	HA (N)	AD (N m)	SP1 (mm)	FR (N/s)		CO1	CO2	SP2	RE	GU1 (N)	GU2 (N)	CH1 (N m)	CH2 (N m)
						FR1	FR2								
Flat	75	30	14 (3)	-1.0 (0.8)	5.2 (0.4)	1.3 (0.5)	5.5 (2)	0.41 (0.1)	0.41 (0.1)	0.87 (0.1)	-0.06 (0.1)	5.5 (0.7)	5.5 (0.6)	29 (5)	29 (4)
		20	26 (9)	-3 (1)	3 (1)	2 (1)	9 (4)	0.242 (0.05)	0.23 (0.1)	0.42 (0.2)	-0.09 (0.02)	6 (2)	6 (2)	14 (5)	13 (5)
		10	106 (30)	-2.9 (0.6)	1.1 (0.2)	13 (3)	69 (11)	0.383 (0.04)	0.38 (0.1)	0.20 (0.1)	-0.03 (0.01)	41 (15)	41 (15)	46 (20)	46 (20)
Punch	20	30	3.2 (0.2)	-2.0 (0.5)	5.5 (0.6)	0.38 (0.1)	0.63 (0.1)	0.41 (0.1)	0.38 (0.1)	0.92 (0.1)	-0.19 (0.1)	1.3 (0.1)	1.2 (0.1)	7.1 (0.8)	6.5 (0.6)
Punch	5	10	26 (23)	-0.72 (0.3)	4.0 (2)	10 (13)	57 (42)	0.50 (0.1)	0.46 (0.1)	0.66 (0.3)	-0.18 (0.2)	14 (13)	13 (13)	44 (33)	42 (34)

HA: Hardness, AD: Adhesiveness, SP1: Springiness1, FR1: Firmness1, FR2: Firmness2, CO1: Cohesiveness1, CO2: Cohesiveness2, SP2: Springiness2, RE: Resilience, GU1: Gumminess1, GU2: Gumminess2, CH1: Chewiness1, CH2: Chewiness2.

Table 2 - Instrumental TPA attributes of different types of Omani halwa measured with a 20 mm punch probe and a 30 mm sample size (Batch 2).

Type	HA (N)	AD (N m)	SP1 (mm)	FR (N/s)		CO1	CO2	SP2	RE	GU1 (N)	GU2 (N)	CH1 (N m)	CH2 (N m)
				FR1	FR2								
B1	3.0 (0.75)	-2.3 (1.27)	5.7 (0.03)	0.3 (0.08)	0.7 (0.20)	0.5 (0.03)	0.4 (0.02)	0.1 (0.00)	-0.3 (0.10)	1.4 (0.40)	1.3 (0.23)	8.2 (2.12)	7.3 (1.34)
B2	3.7 (0.29)	-2.6 (0.67)	5.8 (0.08)	0.4 (0.06)	0.8 (0.08)	0.4 (0.02)	0.4 (0.02)	0.1 (0.01)	-0.3 (0.06)	1.6 (0.15)	1.5 (0.15)	9.3 (0.97)	8.5 (0.93)
B3	4.3 (0.67)	-1.7 (0.90)	5.8 (0.11)	0.4 (0.06)	0.9 (0.12)	0.4 (0.05)	0.4 (0.04)	0.1 (0.02)	-0.2 (0.09)	1.7 (0.26)	1.7 (0.16)	9.5 (1.52)	1.0 (0.95)
Y1	3.0 (0.77)	-1.2 (0.31)	5.6 (0.08)	0.4 (0.13)	0.6 (0.13)	0.3 (0.04)	0.4 (0.08)	9.3 (0.01)	-0.1 (0.05)	1.0 (0.13)	1.4 (0.43)	7.0 (0.72)	7.7 (2.34)
Y2	3.0 (0.44)	-1.8 (0.58)	5.8 (0.08)	0.3 (0.03)	0.64 (0.09)	0.5 (0.02)	0.5 (0.02)	1.1 (0.24)	-0.2 (0.06)	1.4 (0.25)	1.4 (0.18)	8.2 (1.36)	8.1 (0.99)
Y3	3.0 (0.24)	-1.6 (0.84)	5.0 (1.8)	0.4 (0.05)	0.6 (0.04)	0.4 (0.02)	0.3 (0.02)	0.9 (0.31)	-0.1 (0.08)	1.1 (0.12)	1.0 (0.13)	5.7 (2.31)	5.3 (2.21)

HA: Hardness, AD: Adhesiveness, SP1: Springiness1, FR1: Firmness1, FR2: Firmness2, CO1: Cohesiveness1, CO2: Cohesiveness2, SP2: Springiness2, RE: Resilience, GU1: Gumminess1, GU2: Gumminess2, CH1: Chewiness1, CH2: Chewiness2, B: Black, Y: Yellow.

Table 3 - Chemical compositions of different types of halwa. Same halwa as in Table 2 (Batch 2).

	Composition (g/100 g sample)				
Type	Moisture	Protein	Fat	Ash	Carbohydrate
B1	13.4 (0.4)	0.51 (0.04)	3.9 (0.2)	0.30 (0.02)	81.8
B2	18.9 (0.3)	0.87 (0.29)	5.7 (0.4)	0.48 (0.02)	74.1
B3	15.8 (0.6)	1.05 (0.07)	0.8 (0.1)	0.27 (0.03)	82.2
Y1	15.5 (0.1)	2.27 (0.85)	14.4 (2.2)	0.36 (0.01)	67.5
Y2	13.0 (0.4)	1.60 (0.57)	5.7 (0.1)	0.15 (0.04)	79.5
Y3	13.1 (0.4)	1.02 (0.36)	14.0 (0.9)	0.14 (0.01)	72.1

Note: B: Black, Y: Yellow.

creased with an increase in the water content, while springiness increased with an increase in the moisture content (Table 4). Table 5 shows detailed chemical compositions of 15 commercial products. Moisture, protein, fat, ash, sucrose, and pH varied from 11.2-19.9, 0.15-1.10, 4.3-13.5, 0.10-0.84, 14.9-56.3 g/100 g halwa, and 4.26-5.46, respectively. It is interesting to see that most of the black halwa contained more ash compared with the yellow halwa. This greater amount of ash could have resulted from the use of brown or raw sugar in preparing black halwa whereas yellow halwa was prepared with white sugar. Table 6 shows the significant relationship between the variables at $p<0.05$ and $p<0.01$. For example, hardness was significantly correlated with AD, SP1 and SP2, GU1 and GU2, XW, SU, and C20:2 and C20:3n6 fatty acids ($p<0.05$). Moisture, the types of fat and sugar significantly affected most of the TPA attributes (Table 6). In the case of ready-to-eat meat products, TPA attributes were significantly correlated with the water and fat contents (PROBOLA and ZANDER, 2007). The textural attribute matrix found using the Bio-Env procedure showed the highest correlation with the moisture and unsaturated fat contents followed by total fat and non-sugar, and sucrose and saturated fat (Table 7).

Hardness was negatively correlated with moisture content ($p<0.05$). Similarly, cheese with resistance starch showed that hardness decreased with increasing water content using a 35 mm disk (NORONHA *et al.*, 2007). In food products, water acts as plasticiser of the solid constituents, thus making the food less elastic and more susceptible to fracture upon compression (FOX *et al.*, 2000). Water molecules are bound within a three-dimensional matrix and weaken the structure of the network. Thus, increasing the moisture content reduced the coherence of the matrix and resulted in softer products (DIMITRELI and THOMAREIS, 2007). In the case of compression of cheese using a 35 mm disk, the hardness increased with fat content when most of the composition remained the same, except for the pH and butanoic acid content (NORONHA *et*

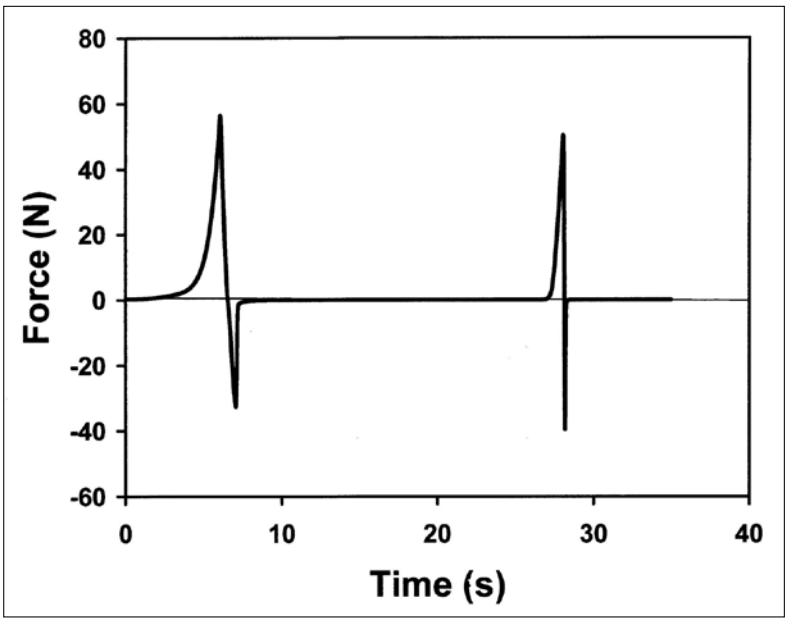


Fig. 1 - Typical force-time graph of halwa.

Table 4 - Instrumental TPA attributes of 15 types of Omani halwa measured with a 75 mm compression flat probe on a 10 mm sample size (Batch 3).

Type	HA (N)	AD (N m)	SP1 (mm)	FR (N/s)		CO1	CO2	SP2	RE	GU1 (N)	GU2 (N)	CH1 (N m)	CH2 (N m)
				FR1	FR2								
B1	40 (18)	-3.8 (1.3)	0.84 (0.10)	5 (2)	30 (14)	0.24 (0.04)	0.23 (0.04)	0.14 (0.02)	-0.10 (0.01)	10 (6)	9 (5)	9 (5)	8 (5)
B2	76 (44)	-3.8 (1.1)	1.0 (0.3)	8 (5)	49 (27)	0.33 (0.08)	0.33 (0.08)	0.19 (0.06)	-0.06 (0.04)	29 (21)	28 (20)	32 (27)	32 (27)
B3	66 (9)	-1.4 (0.3)	1.6 (0.3)	93 (13)	441 (49)	1.1 (0.1)	1.2 (0.1)	0.34 (0.06)	-0.13 (0.03)	72 (18)	82 (18)	113 (35)	129 (38)
B4	7 (3)	-0.14 (0.08)	3.7 (1.4)	7 (3)	25 (17)	0.44 (0.07)	0.61 (0.07)	0.62 (0.22)	-0.06 (0.02)	3.3 (0.1)	4.6 (1.8)	11 (2)	15 (3)
B5	73 (32)	-4.1 (1.0)	1.3 (1.6)	11 (5)	62 (18)	0.37 (0.04)	0.36 (0.04)	0.26 (0.03)	-0.07 (0.03)	28 (17)	27 (17)	36 (23)	35 (23)
B6	7 (1)	-0.17 (0.04)	4.1 (0.7)	6 (1)	20 (5)	0.49 (0.05)	0.71 (0.05)	0.68 (0.11)	-0.09 (0.01)	3.6 (0.3)	5.2 (0.9)	14 (2)	20 (3)
B7	26 (12)	-0.39 (0.24)	3.0 (0.9)	32 (16)	167 (99)	0.59 (0.16)	0.67 (0.15)	0.52 (0.22)	-0.08 (0.03)	17 (13)	19 (13)	58 (37)	64 (39)
B8	66 (24)	-1.0 (0.4)	2.0 (0.4)	74 (29)	414 (149)	1.2 (0.3)	1.2 (0.3)	0.42 (0.06)	-0.09 (0.02)	83 (40)	86 (49)	158 (81)	164 (79)
Y1	86 (31)	-2.5 (0.5)	0.87 (0.18)	12 (5)	65 (19)	0.36 (0.06)	0.35 (0.06)	0.17 (0.03)	-0.03 (0.01)	33 (17)	32 (17)	31 (23)	30 (23)
Y2	65 (19)	-1.3 (0.3)	1.4 (0.2)	77 (26)	451 (120)	1.1 (0.3)	1.3 (0.3)	0.31 (0.04)	-0.13 (0.02)	79 (44)	90 (41)	107 (61)	122 (57)
Y3	89 (42)	-3.9 (1.8)	1.2 (0.2)	12 (7)	65 (27)	0.38 (0.08)	0.37 (0.07)	0.23 (0.04)	-0.07 (0.07)	37 (22)	36 (22)	46 (32)	46 (32)
Y4	11 (6)	-0.29 (0.17)	3.4 (1.2)	9 (5)	49 (47)	0.47 (0.05)	0.73 (0.05)	0.56 (0.19)	-0.12 (0.03)	5.2 (2.5)	8.2 (4.5)	15 (2)	23 (3)
Y5	69 (35)	-1.1 (0.7)	3.6 (0.9)	86 (52)	417 (196)	1.3 (0.5)	1.3 (0.4)	0.81 (0.25)	-0.08 (0.01)	103 (78)	106 (82)	319 (192)	326 (203)
Y6	35 (18)	-0.66 (0.34)	2.2 (0.8)	37 (18)	219 (98)	0.73 (0.36)	0.82 (0.36)	0.39 (0.09)	-0.11 (0.01)	32 (11)	35 (14)	55 (28)	60 (37)
Y7	42 (21)	-0.80 (0.41)	1.5 (0.4)	49 (26)	317 (158)	0.83 (0.45)	0.91 (0.45)	0.28 (0.05)	-0.12 (0.01)	43 (33)	45.59 (34)	53 (42)	56 (38)

HA: Hardness, AD: Adhesiveness, SP1: Springiness1, FR1: Firmness1, FR2: Firmness2, CO1: Cohesiveness1, CO2: Cohesiveness2, SP2: Springiness2, RE: Resilience, GU1: Gumminess1, GU2: Gumminess2, CH1: Chewiness1, CH2: Chewiness2, B: Black, Y: Yellow.

Table 5 - Chemical proximate composition and physico-chemical properties of 15 types of halwa. Same halwa as in Table 4 (Batch 3).

Type	Composition (g/100 g sample)									
	Moisture	Protein	Fat	Ash	Sucrose	(Non-sucrose) Carbohydrate	L	a	b	pH
B1	16.3 (0.6)	1.10 (0.19)	5.2 (1.9)	0.42 (0.01)	33.6 (0.4)	43.06	26.4 (1.1)	1.6 (0.3)	2.9 (0.3)	5.39 (0.02)
B2	13.0 (0.2)	0.99 (0.26)	4.3 (2.1)	0.37 (0.01)	56.3 (1.1)	24.98	24.9 (1.5)	2.1 (0.2)	3.5 (0.6)	4.75 (0.01)
B3	12.7 (0.1)	0.33 (0.01)	13.5 (2.6)	0.24 (0.07)	29.3 (0.6)	43.80	24.7 (1.2)	1.9 (0.3)	3.8 (0.6)	5.45 (0.01)
B4	20.2 (0.4)	0.84 (0.05)	8.9 (1.8)	0.84 (0.03)	16.8 (1.2)	52.27	27.2 (1.2)	1.8 (0.2)	2.7 (0.5)	4.97 (0.03)
B5	12.6 (0.5)	0.87 (0.40)	4.7 (2.4)	0.37 (0.02)	40.1 (1.9)	41.24	23.9 (1.3)	1.5 (0.2)	2.5 (0.4)	5.37 (0.01)
B6	19.9 (0.5)	0.70 (0.25)	7.3 (2.7)	0.34 (0.07)	14.9 (1.3)	56.76	27.5 (1.8)	2.4 (0.3)	3.3 (0.2)	5.11 (0.02)
B7	19.1 (1.2)	0.47 (0.14)	7.9 (2.3)	0.36 (0.01)	32.6 (0.8)	39.40	24.6 (1.7)	1.7 (0.3)	3.2 (0.5)	5.46 (0.03)
B8	14.8 (1.0)	0.15 (0.03)	8.2 (2.2)	0.20 (0.03)	29.3 (1.7)	47.20	24.5 (1.3)	2.2 (0.2)	3.4 (0.7)	4.80 (0.01)
Y1	17.5 (2.8)	0.85 (0.24)	5.7 (1.9)	0.21 (0.01)	33.3 (0.4)	42.31	34.1 (1.6)	5.8 (0.2)	9.4 (0.2)	5.02 (0.01)
Y2	13.3 (0.3)	0.96 (0.26)	11.4 (3.9)	0.26 (0.05)	25.8 (0.5)	48.19	30.2 (1.2)	6.3 (0.2)	7.5 (0.3)	5.17 (0.02)
Y3	11.2 (0.5)	0.18 (0.02)	8.2 (3.1)	0.10 (0.03)	42.3 (1.0)	37.85	33.8 (1.2)	5.3 (0.3)	7.2 (0.2)	5.32 (0.02)
Y4	19.0 (1.0)	0.45 (0.07)	7.0 (3.1)	0.14 (0.05)	23.5 (0.9)	46.65	30.1 (1.1)	6.8 (0.3)	9.9 (0.9)	5.41 (0.01)
Y5	8.6 (0.5)	0.51 (0.13)	8.2 (3.2)	0.16 (0.01)	28.9 (1.1)	53.56	35.7 (1.3)	6.2 (0.2)	9.7 (0.3)	5.38 (0.02)
Y6	14.2 (1.5)	0.50 (0.20)	10.3 (2.3)	0.15 (0.03)	37.7 (0.5)	37.05	34.4 (1.4)	5.7 (0.3)	7.9 (0.4)	4.26 (0.02)
Y7	15.0 (1.8)	0.34 (0.05)	10.6 (2.0)	0.16 (0.04)	31.5 (1.4)	42.35	34.4 (1.1)	5.1 (0.2)	8.8 (0.2)	4.52 (0.01)
C.V[a]	22.72	49.37	31.60	63.77	32.11	17.88	15.56	56.27	51.26	7.26

[a]: Coefficient of variation, B: Black, Y: Yellow.

Table 6 - Correlation between TPA attributes and physico-chemical compositions measured on 15 types of Omani halwa with p values ≤ 0.05 and 0.01.

Attributes	List of significant variables	P value
HA	Ad, SP1, SP2, GU1, GU2, Xw, SU, C20:2, C20:3n6	P<0.05
	Ad, SP1, Xw, SU	P<0.01
AD	SP1, SP2, Xw, SU, NS, C17:0, C18:0, C15:1, C18:1n9t, C18:1n9c, C20:2, C20:3n6, C20:3n3	P<0.05
	SP1, SP2, Xw, SU, C20:2, C20:3n6, C20:3n3	P<0.01
SP1	CO1, CO2, SP2, SU, NS, C17:0, C18:0, C15:1, C18:1n9t, C18:1n9c, C18:2n6t, C20:2, C20:3n6, C20:3n3	P<0.05
	SP2, SU, C20:2, C20:3n6, C20:3n3	P<0.01
FR1	FR2, CO1, CO2, GU1, GU2, CH1, CH2, Xw, FT, SF, UF, C16:0, C15:1, C18:1n9t, C18:1n9C	P<0.05
	FR2, CO1, CO2, GU1, GU2, CH1, CH2, FT, UF, C16:0	P<0.01
FR2	CO1, CO2, GU1, GU2, CH1, CH2, Xw, FT, SF, UF, C16:0, C18:1n9t	P<0.05
	CO1, CO2, GU1, GU2, CH1, CH2, FT, UF, C16:0	P<0.01
CO1	CO2, SP2, RE, GU1, GU2, CH1, CH2, PR, FT, SF, UF, C16:0, C17:0, C18:0, C15:1, C18:1n9t, C18:1n9c, C20:2, C20:3n6, C20:3n3	P<0.05
	CO2, SP2, GU1, GU2, CH1, CH2, FT, SF, UF, C16:0, C15:1, C18:1n9t, C18:1n9c, C20:2, C20:3n6, C20:3n3	P<0.01
CO2	SP2, RE, GU1, GU2, CH1, CH2, FT, SU, SF, UF, C4:0, C16:0, C17:0, C18:0, C14:1, C15:1, C18:1n9t, C18:1n9c, C20:2, C20:3n6, C20:3n3	P<0.05
	RE, GU1, GU2, CH1, CH2, FT, SF, UF, C16:0, C17:0, C18:0, C15:1, C18:1n9t, C18:1n9c, C20:2, C20:3n6, C20:3n3	P<0.01
SP2	SU, NS, C17:0, C18:0, C14:1, C15:1, C18:1n9t, C18:1n9c, C18:2n6t, C20:1, C20:2, C20:3n6, C20:3n3	P<0.05
	SU, NS, C17:0, C18:0, C18:1n9t, C20:2, C20:3n6, C20:3n3	P<0.01
RE	FT, SF, UF, C4:0, C16:0, C17:0, C18:0, C14:1, C15:1, C18:1n9t, C18:1n9c, C20:2, C20:3n6	P<0.05
	FT, UF, C16:0	P<0.01
GU1	GU2, CH1, CH2, Xw, C16:0	P<0.05
	GU2, CH1, CH2, Xw	P<0.01
GU2	CH1, CH2, Xw, C16:0	P<0.05
	CH1, CH2, Xw	P<0.01
CH1	CH2, Xw, FT, UF, C16:0, C18:1n9t, C18:1n9c	P<0.05
	CH2, C16:0	P<0.01
CH2	Xw, FT, UF, C16:0, C18:1n9t, C18:1n9c	P<0.05
	C16:0	P<0.01

HA: Hardness, AD: Adhesiveness, SP1: Springiness1, FR1: Firmness1, FR2: Firmness2, CO1: Cohesiveness1, CO2: Cohesiveness2, SP2: Springiness2, RE: Resilience, GU1: Gumminess1, GU2: Gumminess2, CH1: Chewiness1, CH2: Chewiness2, XW: Moisture, PR: Protein, FT: Fat, SU: Sucrose, NS: Non-Sucrose Carbohydrate, AS: Ash, CL: Color, SF: Saturated Fat, UF: Unsaturated Fat.

Table 7 - Overall texture matrix correlated with different combination of physico-chemical attributes and composition.

Selections of variables	Correlation
XW, UF	0.573
XW, FT	0.556
XW, NS, UF	0.548
XW, FT, UF	0.545
XW, FT, NS, UF	0.537
XW, FT, NS	0.531
XW, NS, SF, UF	0.520
XW, SU, UF	0.519
XW, FT, SU, UF	0.512
XW, SF, UF	0.508

XW: Moisture, FT: Fat, SU: Sucrose, NS: Non-Sucrose Carbohydrate, SF: Saturated Fat, UF: Unsaturated Fat.

al., 2008). Similarly, VOLIKAKIS *et al.* (2004) observed that the hardness increased with increasing fat content. However, DIMITRELI and THOMAREIS (2007) observed a decrease in hardness with increasing fat content. Fat could behave as an active filler, and thus, hardness increased with increasing fat content. However, if fat acts as a lubricant, the hardness could decrease with fat content (VOLIKAKIS *et al.*, 2004). MUGUERZA *et al.* (2002) observed increasing hardness with decreasing fat content in the case of meat sausage. In the case of sausage, hardness was significantly correlated with water but not with fat content (HERRERO *et al.*, 2008). In the case of dairy products such as sandesh, hardness was positively ($p<0.01$) correlated with fat content but negatively ($p<0.01$) correlated with the moisture and ash contents and did not show any significant effect from the sugar and protein content or acidity (KHAMRUI and SOLANKI, 2010).

Adhesiveness was significantly correlated with moisture, sucrose, non-sucrose starch, and some fatty acids (C17:0, C18:0, C15:1, C18:1n9t, C18:1n9c, C20:2, C20:3n6, C20:3n3) ($p<0.05$). For the AD of milk-based sweets determined compression with a 75 mm disk, the moisture showed negative correlations while fat showed positive correlations ($p<0.01$) (KHAMRUI and SOLANKI, 2010). Springiness 1 and springiness 2 were significantly correlated with sucrose, non-sucrose and some fatty acids (C17:0, C18:0, C14:1, C15:1, C18:1n9t, C18:1n9c, C18:2n6t, C20:1, C20:2, C20:3n6, C20:3n3) ($p<0.05$). In the case of compression of cheese with a 75 mm disk, VOLIKAKIS *et al.* (2004) observed no significant effect of fat on springiness 1. The SP1 of milk-based sweets showed positive and negative correlations ($p<0.05$) with the fat and moisture contents (KHAMRUI and SOLANKI, 2010). Firmness 1 was significantly correlated with the water, total fat, saturated fat and unsaturated fat contents, and fatty acids (C16:0, C15:1, C18:1n9t, C18:1n9c) ($p<0.05$). Firmness 2 was as significantly correlated as firmness 1 with the exception of fatty acids C15:1 and C18:1n9c.

Cohesiveness 1 was significantly correlated with the protein, total fat, saturated and unsaturated fat contents and fatty acids (C16:0, C17:0, C18:0, C15:1, C18:1n9t, C18:1n9c, C20:2, C20:3n6 and C20:3n3) ($p<0.05$). Similarly, cohesiveness 2 was significantly correlated to the same variables as cohesiveness 1 except for protein and with the addition of sucrose. In the case of cheese, cohesiveness 1 did not show any specific trends with fat content when most of the composition remained the same except for pH and butanoic acid content (NORONHA *et al.*, 2008). Similarly, VOLIKAKIS *et al.* (2004) observed that cohesiveness 1 did not show any trend with increasing fat content. In the case of sausage, cohesiveness 1, adhesiveness and springiness 1 did not show any correlation with the water or fat contents ($p<0.05$) (HERRERO *et al.*, 2008). Cohesiveness 1 and springiness 1 of ready-to-eat meat products were correlated (i.e., *r* values) with water, fat and protein as follows: 0.25, -0.21, -0.38 and 0.50, -0.54, -0.38, respectively (PROBOLA and ZANDER, 2007). In the case of dry fermented sausage (i.e., considering water, fat, water activity and pH), HA did not correlate with any parameters ($p>0.05$), CO1 correlated with water content ($r^2 = 0.306$, $p<0.005$), AD correlated with water content and pH ($r^2 = 0.282$, $p<0.005$), and SP correlated with only water content ($r^2 = 0.216$, $p<0.05$) (HERRERO *et al.*, 2008). CO1 of milk-based sweets was not significantly correlated with the composition parameters (water, fat, protein, ash, sugar contents and acidity) ($p>0.05$).

Resilience was significantly correlated with the total fat, saturated and unsaturated fat contents and fatty acids (C4:0, C16:0, C17:0, C18:0, C14:1, C15:1, C18:1n9t, C18:1n9c, C20:2, C20:3n6) ($p<0.05$). In the case of dairy sweets, RE was correlated positively ($p<0.01$) with the ash and water contents, whereas the fat content negatively influenced RE (KHAMRUI and SOLANKI, 2010). Gumminess 1 and gumminess 2 were significantly correlated with the water content and C16:0 fatty acids ($p<0.05$). In the case of high and low fat cheese punctured with a 10 mm probe, firmness 1, cohesiveness 1, and gumminess 1 significantly increased with an increase in water (SAINT-EVE *et al.*, 2009). In the case of dairy products such as sandesh, gumminess 1 was positively ($p<0.01$) correlated with the fat content but negatively ($p<0.01$) correlated with the moisture and ash contents and did not show any significant effect from the sugar and protein content or acidity (KHAMRUI and SOLANKI, 2010).

Chewiness 1 and chewiness 2 were significantly correlated with water, total and unsaturated fat, and fatty acids (C16:0, C18:1n9t, C18:1n9c) ($p<0.05$). In the case of cheese, gumminess 1 and chewiness 2 did not show any trend with fat content (VOLIKAKIS *et al.*, 2004). CH1 of milk-based sweets was negatively correlated with water content and was positively influenced by the fat content ($p<0.01$). The salt content also affected cohesiveness 1 differently in low-fat and high-fat cheeses. The cohesiveness 1 was only significantly affected by the fat content at low salt levels but not at high salt levels. The TPA parameters (FA1, AD, CO1, SP1, and GU1) differentiated the products according to salt content rather than at low-fat content. However, fat played a major role in aroma release and olfactory perception, whereas salt played a predominant role in sensory texture and TPA parameters (SAINT-EVE *et al.*, 2009). Considering all of the physico-chemical properties and TPA attributes, a cluster analysis based on Ward's method revealed 4 distinct groups of formulation, named G1, G2, G3, and G4 at a level of similarity/distance of 9 (Fig. 2).

The plot of Pearson's versus Spearman's cor-

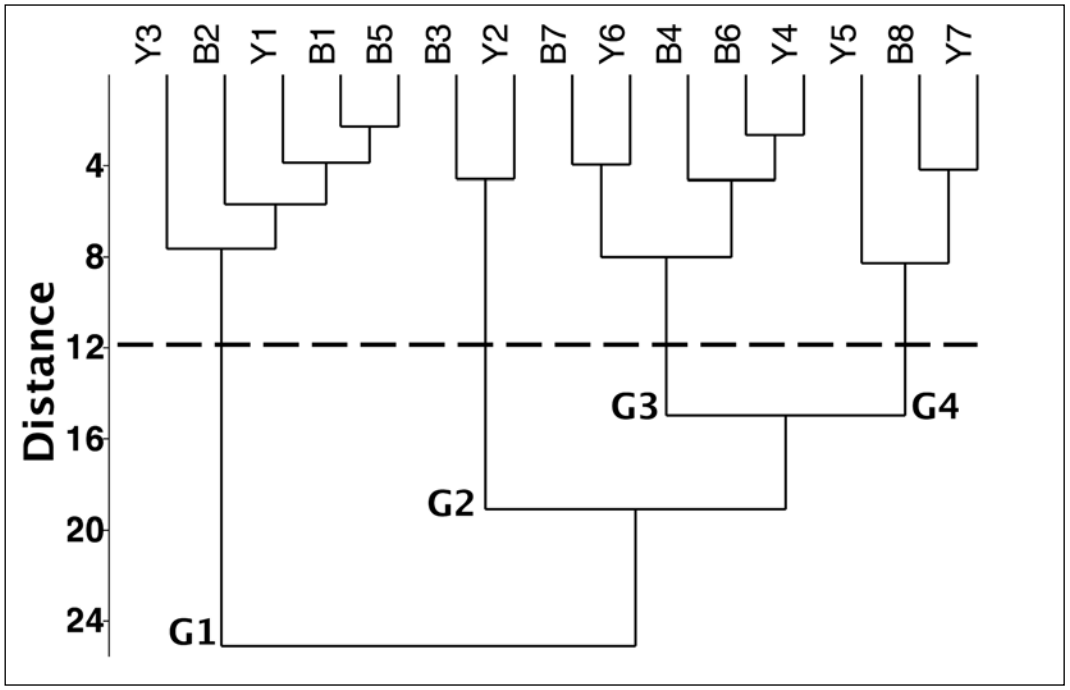


Fig. 2 - Dendogram of the cluster analysis for the 15 halwa samples.

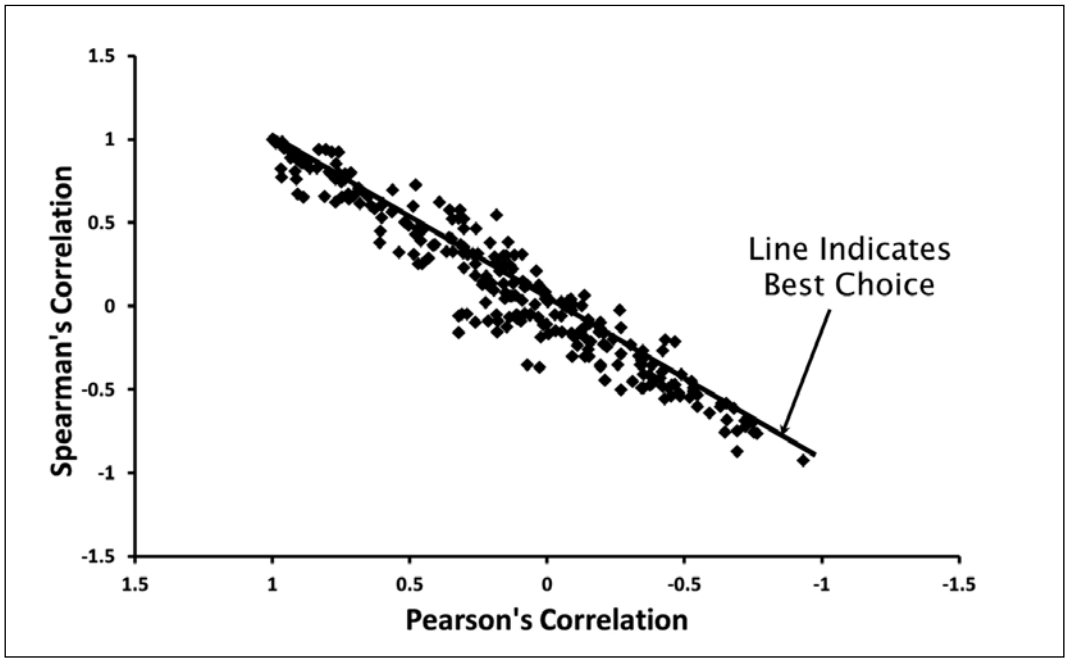


Fig. 3 - Relationship between Spearman's and Pearson's correlations.

relations suggests no real bias and supports the idea of linkages between variables being mostly linear (Fig. 3). The PCA analysis shows that seven principal components (93% of the total variance) had eigenvalues close to 1 (Kaiser criterion; RAHMAN and AL-FARSI, 2005). These components explained 41, 19, 15, 8, 4, 3 and 3% of the total variance (Table 8). The first axis was highly correlated with firmness 1, firmness 2, cohesiveness 1, cohesiveness 2, total fat, saturated and unsaturated fat and fatty acids (C4:0, C14:0, C15:0, C16:0, C17:0, C18:0, C14:1, C15:1, C18:1n9t, C18:2n6t) and negatively correlated with adhesiveness, resilience and fatty acids (C18:1n9c, C20:2, C20:3n6) (Table 8) and corresponds to a descriptor of plasticity (deformation and structural damage of first and second compression). The second axis was correlated to firmness 2, cohesiveness 1, chewiness 1, chewiness 2, protein and fatty acids (C8:0, C10:0, C12:0, C18:2n6c) and corresponds to the elasticity (force and deformation of first and second compression). The

Table 8 - Correlations of attributes with principal components, eigenvalues and per cent variance explained.

	HA	AD	SP1	FR1	FR2	CO1	CO2	SP2	RE	GU1	GU2	CH1	CH2	XW	PR	FT	SU	NS	AS	CL	pH	SF	UF	C4:0	C6:0	C8:0
PC1	-0.20	0.67	0.40	0.75	0.73	0.78	0.88	0.49	-0.66	0.52	0.58	0.48	0.53	-0.13	-0.32	0.88	-0.54	0.49	-0.13	-0.07	0.04	0.89	0.77	0.81	0.67	0.53
PC2	-0.17	-0.20	-0.01	-0.47	-0.50	-0.51	-0.39	-0.13	0.06	-0.48	-0.45	-0.51	-0.50	0.28	0.60	-0.13	-0.12	0.04	0.40	-0.45	0.39	0.11	-0.40	0.26	0.59	0.70
PC3	0.92	-0.67	-0.76	0.40	0.41	0.25	0.09	-0.59	0.04	0.62	0.61	0.36	0.35	-0.82	0.01	0.10	0.60	-0.44	-0.46	0.12	0.15	0.20	-0.04	0.21	0.37	0.36
PC4	0.09	0.00	0.45	0.11	0.03	0.20	0.16	0.58	0.37	0.29	0.26	0.60	0.58	-0.22	0.16	-0.39	-0.17	0.43	0.07	-0.11	0.48	-0.32	-0.43	-0.34	-0.06	-0.01
PC5	-0.02	0.04	0.18	-0.12	-0.19	-0.15	-0.16	0.18	0.10	-0.14	-0.16	0.05	0.04	-0.12	-0.46	0.09	0.13	-0.11	-0.41	0.54	0.16	0.18	-0.04	-0.02	-0.14	-0.03
PC6	-0.04	-0.04	-0.06	0.05	0.03	0.00	0.01	-0.05	-0.12	0.00	0.00	-0.03	-0.03	0.13	-0.12	0.12	-0.49	0.54	0.01	0.18	0.49	0.08	0.15	0.11	-0.01	-0.01
PC7	-0.10	0.01	-0.10	0.11	0.08	-0.01	-0.06	-0.07	-0.01	-0.03	-0.03	0.07	0.07	0.05	0.39	0.02	0.11	-0.19	0.39	0.42	0.13	-0.02	0.08	0.16	0.11	-0.10

	C10:0	C12:0	C14:0	C15:0	C16:0	C17:0	C18:0	C20:0	C14:1	C15:1	C16:1	C18: 1n9t	C18: 1n9c	C18: 2n6t	C18: 2n6c	C20:1	C20:2	C20: 3n6	C20: 3n3	EGV[a]	PVE[b]	CPVE[c]
PC1	0.53	0.42	0.75	0.69	0.74	0.88	0.88	0.51	0.83	0.94	0.63	0.74	-0.75	0.75	0.01	0.57	-0.76	-0.80	-0.66	18.60	41	41
PC2	0.65	0.70	0.57	0.59	-0.36	0.42	0.30	0.39	0.34	0.10	0.51	-0.51	0.30	0.55	-0.82	0.57	0.31	0.30	0.28	8.40	19	60
PC3	0.50	0.41	0.26	0.13	0.14	-0.07	-0.12	-0.17	-0.12	-0.02	0.03	-0.32	0.50	-0.06	-0.11	-0.01	0.46	0.46	0.31	6.94	15	75
PC4	0.00	-0.32	-0.04	0.12	-0.44	0.01	-0.12	0.01	0.16	-0.10	0.08	-0.26	-0.03	0.07	-0.47	0.22	0.03	0.02	0.09	3.43	8	83
PC5	-0.11	-0.11	0.17	0.25	0.22	-0.07	0.24	-0.10	0.00	-0.28	0.50	-0.02	0.15	-0.27	-0.06	0.34	0.07	0.03	-0.07	1.83	4	87
PC6	-0.01	0.04	-0.04	-0.10	0.17	-0.03	-0.10	0.01	-0.10	0.05	0.03	0.03	0.10	-0.03	0.17	-0.15	0.29	0.22	0.48	1.41	3	90
PC7	-0.10	-0.16	-0.03	-0.11	-0.01	0.09	0.07	-0.55	0.22	0.06	0.01	0.10	-0.10	0.02	-0.02	0.29	-0.01	0.02	0.17	1.21	3	93

[a]EGV: eigenvalue of principal component expressed in %.
[b]PVE: percentage of explanation of the component by each variable.
[c]CPVE: Cumulative Percentage Variance Explain.
HA: Hardness, AD: Adhesiveness, SP1: Springiness1, FR1: Firmness1, FR2: Firmness2, CO1: Cohesiveness1, CO2: Cohesiveness2, SP2: Springiness2, RE: Resilience, GU1: Gumminess1, GU2: Gumminess2, CH1: Chewiness1, CH2: Chewiness2, XW: Moisture, PR: Protein, FT: Fat, SU: Sucrose, NS: Non-Sucrose Carbohydrate, AS: Ash, CL: Color, SF: Saturated Fat, UF: Unsaturated Fat.

third axis was strongly correlated with hardness and moisture content as well as adhesiveness, springiness 1, springiness 2, gumminess 1, gumminess 2 with sugar content and corresponds to the hardness (force needed to deform the product). The fourth axis correlated with springiness 2, chewiness 1, chewiness 2 and corresponds mainly to chewiness (the work required to chew the product for swallowing), while the fifth axis correlated with protein, colour and C16:1 fatty acid and corresponds mainly to protein and colour. The sixth axis correlated with sucrose, non-sucrose and pH, which correspond to the sugar and carbohydrate content. The seventh axis is correlated with colour and C20:2 fatty acids, thus corresponding to the colour. In general, specific fatty acids contribute to most of the principal components.

The four identified groups by cluster analysis are shown on a PCA biplot with the projection of the original TPA variables (Fig. 4). Figure 4 shows that group G1 (on the left-hand side of the PCA plot) includes yellowish and black halwa leading to soft and resilient attributes (low-fat and high sugar). On the opposite direction to that of the resilient component, group G2 includes halwa that are soft and cohesive-springy (high-fat and low sugar). G4 includes soft and springy halwa (high-fat and medium-sugar). This result indicates that most of the products in the market are soft with different textural attributes. Group G3 opposite to the G4 corresponds to hard and chewy (fatty and medium sugar) halwa. The springiness of the softer products indicated that structural damage due to compression was rapidly repaired. SOUKOULIS *et al.* (2008) identified two classes of ice cream formulated with different hydrocolloids. LASSOUED *et al.* (2008) classified 15 baked products (i.e., bread) with various types of dough similar to commercial products, and they classified the products based on sensory texture and image analysis. They grouped the products into 6 clusters based on Hierarchical Cluster Analysis and identified stiffer products (i.e., stiffness, elasticity and flexibility) (group 1), products with larger-crumb cells (group 2), elastic products (i.e., elasticity, softness, crumbliness) (group 3), products with fine-crumb cells (group 4), products with cell regularity (group 5), and crumble-bread

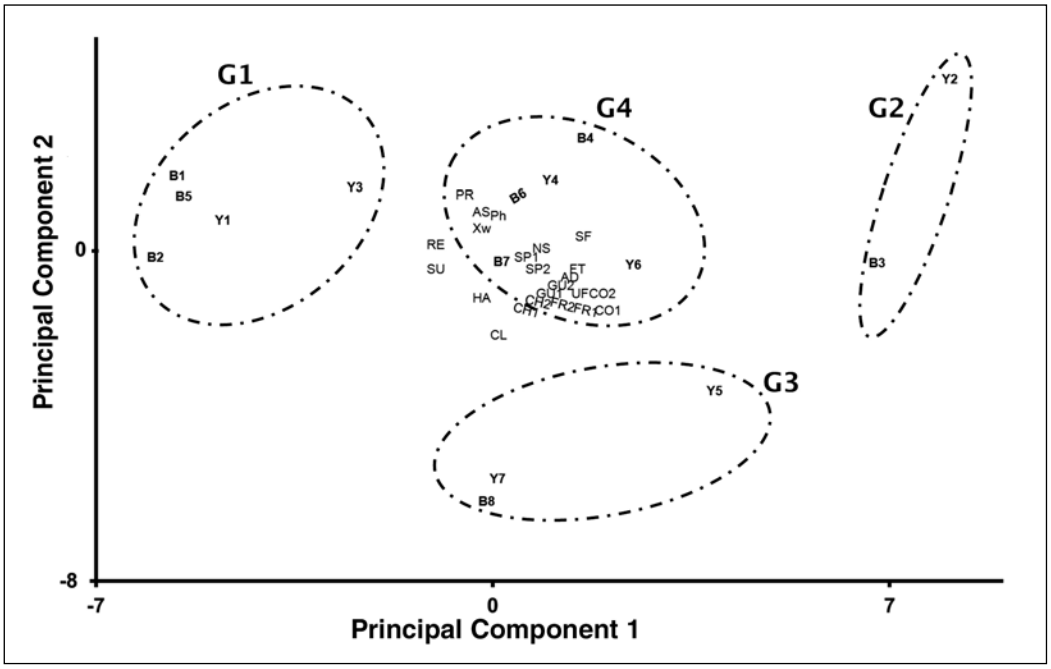


Fig. 4 - Biplot of different products on two principal components with variables loading on the principal components.

that was easy to tear (group 6). In the case of 12 commercial custard desserts, DE WIJK *et al.* (2003) identified 4 cluster groups based on sensory and instrumental mouth feel. They used PCA and cluster analysis and characterised the classes by roughness, thickness, melting, creaminess/softness. The halwa industry could produce their products based on these categories in order to imitate the types of products available in the market today and possibly to please the consumer (AL-SHAMSI *et al.*, 2013).

CONCLUSION

Commercial halwa products purchased in the market were surveyed and classified by measuring their physico-chemical properties and TPA attributes. The instrumental TPA attributes of halwa were correlated with the moisture, fat, fatty acid and sugar contents. Based on the physico-chemical properties and TPA attributes, halwa in the local market could be classified into 4 groups: hard-chewy (fatty and medium-sugar), soft-resilient (low-fat and high-sugar), soft-springy (fatty and medium-sugar) and soft-cohesive-springy (high-fatty and low sugar). PCA was used to identify 7 principal components, which correspond to the plasticity, elasticity, hardness, chewiness, protein-colour, sugar-carbohydrate, and colour. Halwa producers could develop new products based on the characteristics of these four classes in order to please the consumer base.

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PREVIEW ON VOLUME XXIV No. 4, 2012

PAPERS

Determination of Contamination Stages of Frozen Chicken Döner
N. Değirmencioğlu, R. Irkin and A. Değirmencioğlu

Volatile Organic Compounds of Parma Dry-Cured Ham as Markers of Ageing Time and Aged Ham Aroma
A. Pinna, N. Simoncini, T. Toscani and R. Virgili

Effects of Incorporation of Clear Flour on the Quality of Chinese Noodles
Su-Yi Lin, Hua-Han Chen and Yu-Tuan Chen

Moisture Adsorption Characteristics of Pistachio Nut Cream: a New Food Product
O.F. Gamli, I. Hayoglu and H. Turkoglu

Degradation Kinetics of Total Phenols, Anthocyanins and Antiradical Activity of Blackcurrant Nectars Stored at Room and Refrigerator Temperatures
J. Piljac-Žegarac, A. Piljac, S. Basha, M. Pinter, D. Šamec and V. PetraviéTominac

Evaluation of Riboflavin Content in Dairy Products and Non-Dairy Substitutes
S. Sunaric, M. Denic and G. Kocic

Accelerated Kashar Cheese Ripening with Encapsulated Lipase and Protease Enzymes
M.B. Güler-Akın, M.S. Akın, A.F. Atasoy, H. Avni Kırmacı and L. Eren-Karahan

Effect of Enzymatic Hydrolysis on the Resistant Starch Yield
Rui Xu, Dan-Hong Xu and Hong-Hua Xu

The Effect of Chips Processing on the Content of Toxic Compounds
E. Rytel, K. Kułakowska and A. Nemś

SHORT COMMUNICATION

Adaptability of the Sicilian Cultivar Nero d'Avola in Tuscany
M.C. Cravero, F. Bonello, C. Tsolakis, F. Piano and D. Borsa

SURVEYS

Durum and Common Wheat Imports into Puglia During 2010: Mycotoxins and Grain-Quality Monitoring
R. De Pace, V. Menga, V. Vita, C. Franchino, M.A. Dattoli, C. Fares

A Survey of Ergosterol Content of Tomato Products in Turkey During 2006-2010
Ç. Kadakal

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CONTENTS

REVIEW
Listeria Monocytogenes: Biofilms in Food Processing
P. Di Ciccio, M. Conter, E. Zanardi, S. Ghidini, A. Vergara, D. Paludi, A.R. Festino and A. Ianieri 203

PAPERS
Sources of *Listeria Monocytogenes* Contamination in Traditional Fermented Sausage Processing Plants in Italy
D. Meloni, F. Piras, A. Mureddu, R. Mazza, D. Nucera and R. Mazzette 214

Use of Rustic Pigs to Manufacture Pork Salami Free of Colour and Taste Enhancers
M. Bedia, R. Serraino and S. Bañón 223

Relationship Between Basic Beef Texture Attributes and Their Perception by Polish Consumers
D. Guzek, D. Głąbska, A. Wierzbicka, J. Wierzbicki and M. Cierach 231

Validation of an Elisa According to EC Decision 2002/657/EC for Detection of Zearalenone in Feedstuff
R. De Pace, C. Franchino, F. Floridi and V. Vita 240

Survival of Hepatitis A Virus in Pasteurized Manila Clams
E. Cappellozza, G. Arcangeli, M. Rosteghin, S. Kapllan, C. Magnabosco, E. Bertoli and C. Terregino 247

The Influence of Sterilization on Free-Radical Generation, Discoloration and the Antioxidant Properties of Certain Spice Herbs
E. Kurzeja, M. Stec, P. Ramos, B. Pilawa and K. Pawłowska-Góral 254

Optimization of Process Variables for Extraction of Zinc, Copper and Iron by Microwave-Assisted Extraction from Pistachio Nut Using Response Surface Methodology
M. Khajeh 263

SHORT COMMUNICATIONS
Influence of Marinades on the Level of PAHs in Grilled Meat Products
K. Tkacz, A. Więk and M.S. Kubiak 270

Aqueous Two-Phase Systems Composed of Different Molecular Weight of Polyethylene Glycol and Diammonium Phosphate for Extraction of Bovine Serum Albumin
G. Khayati and M. Anvari 279

Compositional Analysis and Antioxidant Profile of Yellow, Orange and Purple Polignano Carrots
M. Cefola, B. Pace, M. Renna, P. Santamaria, A. Signore and F. Serio 284

SURVEY
Classification of Commercial Omami Halwa by Physic-Chemical Properties and Instrumental Texture Profile Analysis (TPA)
M. Shafiur Rahman, Q. Al-Shamsi, A. Abdullah, M.R. Claereboudt, B. Al-Belushi, R. Al-Maqbaly and J. Al-Sabahi 292

NEWS 305
PREVIEW ON VOLUME XXIV No. 4, 2012 306
GUIDE FOR AUTHORS 307
CONTRIBUTORS 310