ISSN 1120-1770

ITALIAN JOURNAL OF FOOD SCIENCE

Rivista italiana di scienza degli alimenti

IJFS

Volume XXII
Number 2
2010

CHIRIOTTI EDITORI

ITALIAN JOURNAL OF FOOD SCIENCE

(RIVISTA ITALIANA DI SCIENZA DEGLI ALIMENTI) 2nd series

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

Editor-in-Chief:
Paolo Fantozzi
Dipartimento di Scienze Economico-Estimative e degli Alimenti, Università di Perugia, S. Costanzo, I-06126 Perugia, Italy - Tel. +39 075 5857910 - Telefax +39 075 5857939-5857943 - e-mail: paolofan@unipg.it

Co-Editors:
Lina Chianese - Università degli Studi di Napoli Federico II, e-mail: chianese@unina.it
Giovanni Lercker - Università Alma Mater di Bologna, e-mail: giovanni.lercker@unibo.it
Carlo Pompei - Università degli Studi di Milano, e-mail: carlo.pompei@unimi.it
Bruno Zanoni - Università degli Studi di Firenze, e-mail: bruno.zanoni@unifi.it
Marco Gobbetti - SIMTREA - Università degli Studi di Bari, e-mail: gobbetti@agr.uniba.it

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

Aim: The Italian Journal of Food Science is an international journal publishing original, basic and applied papers, reviews, short communications, surveys and opinions on food science and technology with specific reference to the Mediterranean Region. Its expanded scope includes food production, food engineering, food management, food quality, shelf-life, consumer acceptance of foodstuffs. food safety and nutrition, and environmental aspects of food processing.
Reviews and surveys on specific topics relevant to the advance of the Mediterranean food industry are particularly welcome.
Upon request and free of charge, announcements of congresses, presentations of research institutes, books and proceedings may also be published in a special "News" section.

Review Policy:
The Co-Editors with the Editor-in-Chief will select submitted manuscripts in relationship to their innovative and original content. Referees will be selected from the Advisory Board and/or qualified Italian or foreign scientists. Acceptance of a paper rests with the referees.

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

Impact Factor: 5-Year Impact Factor: 0.651 published in 2008 Journal of Citation Reports, Institute for Scientific Information; Index Copernicus Journal Master List 2009 (ICV): 13.19
IJFS is abstracted/indexed in: Chemical Abstracts Service (USA); Foods Adlibra Publ. (USA); Gialine - Ensia (F); Institut Information Sci. Acad. Sciences (Russia); Institute for Scientific Information; CurrentContents®/AB&ES; SciSearch® (USA-GB); Int. Food Information Service - IFIS (D); Int. Food Information Service - IFIS (UK); EBSCO Publishing; Index Copernicus Journal Master List (PL).
IJFS has a page charge of € 25.00 each page.

Subscription Rate: IJFS is available on-line in PDF format only.
2010: Volume XXII: Ordinary € 50.00 - Supporting € 1,000.00

ITALIAN JOURNAL OF FOOD SCIENCE



ADVISORY BOARD

R. Amarowicz
Editor-in-Chief
Polish J. Food and Nutrition Sci.
Olsztyn, Poland

A. Bertrand
Institut d'Oenologie
Université de Bordeaux
Talence Cedex, France

L.B. Bullerman
Dept. of Food Science and Technology
University of Nebraska-Lincoln
Lincoln, NE, USA

C. Cannella
Ist. Scienza dell'Alimentazione
Università di Roma (La Sapienza)
Roma, Italy

F. Devlieghere
Dept. Food Technology and Nutrition
Faculty of Agricultural and Applied Biological Sciences
Gent University
Gent, Belgium

S. Garattini
Ist. di Ricerche Farmacologiche "Mario Negri"
Milano, Italy

J.W. King
Dept. Chemical Engineering
University of Arkansas
Fayetteville, AR, USA

T.P. Labuza
Dept. of Food and Nutritional Sciences
University of Minnesota
St. Paul, MN, USA

A. Leclerc
Institut Pasteur
Paris, France

C. Lee
Dept. of Food Science and Technology
Cornell University,
Geneva, NY, USA

G. Mazza
Agriculture and Agri-Food Canada
Pacific Agri-Food Research Centre
Summerland, BC, Canada

J. O'Brien
School of Biological Sciences
University of Surrey
Guildford, Surrey, UK

J. Piggott
Dept. of Bioscience and Biotechnology
University of Strathclyde
Glasgow, Scotland

S. Porretta
Associazione Italiana di Tecnologie Alimentari (AITA)
Milano, Italy

J. Samelis
Dairy Research Institute
National Agricultural Research Foundation
Ioannina, Greece

M. Suman
Food Research Lab
Barilla C.R. F.lli spa
Parma, Italy

M. Tsimidou
School of Chemistry,
Artisotle University
Thessaloniki, Greece

Prof. Emeritus J.R. Whitaker
Dept. of Food Science and Technology
University of California
Davis, CA, USA

REVIEW

SUCCINIC ACID AND ITS DERIVATIVES: FERMENTATIVE PRODUCTION USING SUSTAINABLE INDUSTRIAL AGRO-FOOD BY-PRODUCTS AND ITS APPLICATIONS IN THE FOOD INDUSTRY

F. CHIMIRRI, F. BOSCO*, R. CECCARELLI, A. VENTURELLO and F. GEOBALDO
Dipartimento di Scienza dei Materiali e Ingegneria Chimica, Politecnico di Torino,
Corso Duca degli Abruzzi 24, 10129 Torino, Italy
*Corresponding author: francesca.bosco@polito.it

ABSTRACT

The fermentative production of succinic acid (SA) is becoming an attractive alternative to traditional chemical synthesis. SA can be used directly or transformed into some derived chemicals, with applications in the food industry. For example, SA and sodium succinate are flavour enhancers; adipic acid and its salts can be used as acidity regulators, acidifiers, flavour enhancers, gelling aids and slow raising agents. Moreover, SA-derived biodegradable plastics, such as aliphatic polyesters and aliphatic-aromatic co-polyesters have a wide range of applications in food packaging. The replacement of a chemical process with a biological one offers many environmental advantages. To make it cost-competitive, however, process optimization is required and the substrate cost has to be reduced. To reach this goal, renewable low-cost resources, such as agro-food by-products (molasses, cheese whey, wood hydrolysates, wheat flour) can be used in place of pure, expensive ones. In the present paper, the use of agro-food industry by-products as low cost carbon sources in the fermentative production of SA are reviewed. The use of SA and its derivatives in the food industry are also highlighted. Attention is focused on the innovative "cyclic" aspect of SA fermentative production and its applications from agro-food by-products to food products.

- Key words: agro-food by-products, biodegradable polymers, fermentation, food additives, succinic acid -

INTRODUCTION

The depletion of fossil fuel sources, increasing greenhouse gas emissions, climate changes and interest in using locally available, renewable raw materials have spurred the development of appealing bio-based processes to produce chemicals and fuels as an alternative to conventional petrochemicals. In many cases, biological processes are still more expensive than petrochemical ones (McKINLAY *et al.*, 2007). Consequently, the key to a successful production of bio-based compounds is the development of an efficient, economically feasible manufacturing process.

Succinic acid (SA) is one of the most interesting and promising chemicals that can be successfully produced from renewable resources through a fermentation process (DU *et al.*, 2007). At present, SA is produced through the hydrogenation of maleic anhydride, that is, using a petroleum source as the starting material (BECHTHOLD *et al.*, 2008). Considering its wide range of potential chemical derivatives, its commercial value (LANDUCCI *et al.*, 1994) and the potential market, the United States Department of Energy (DOE) has listed SA among the top twelve chemicals of greatest interest (WERPY and PETERSON, 2004). SA can be used directly or processed into many other value-added chemicals and commodities having several applications in various industrial fields (ZEIKUS *et al.*, 1999; POTERA, 2005; SAUER *et al.*, 2008). Therefore, SA is a so-called "building block chemical" or "platform chemical". Focusing attention on the food industry, SA and its derivatives are used as food additives and for manufacturing biodegradable polymers.

In 2007, the total SA market and its derivatives amounted to 30 kton, of which 10% was used directly in the agricultural and food fields, 7% in SA-polymer production, 31% for coatings, pigment, dye and ink production and the remaining 52% was used in other fields of application (RUPP DAHLEM, 2008). The total market value for all of these SA applications is more than 400,000,000 US$/year (ZEIKUS *et al.*, 1999). This value could potentially increase (US$ 1.3 billion/year) (http://www.wisbiorefine.org/prod/sacid.pdf) thanks to the production of SA through microbial fermentation.

In order to develop a bio-based industrial SA production, that would be cost-competitive with traditional chemical processes, three main issues must be addressed: 1) the need to select low-cost raw materials; 2) the need to select high-yield producing microorganisms, that can metabolize a wide range of sugar feedstocks (WERPY and PETERSON, 2004); and 3) the need to reduce recovery and purification costs.

The price of raw materials greatly influences the overall process cost. The possibility of using agro-food industry by-products as low-cost carbon sources (C-sources) in the fermentative production of SA is described in this review and the promising future of SA building blocks is discussed.

Production of succinic acid from microbial fermentation

SA is an intermediate in the TCA cycle (tricarboxylic acid cycle) in plants, animals and in many microorganisms. It is also one of the fermentation end-products of anaerobic microbial metabolism. Some microorganisms can also produce SA in aerobic fermentation through the glyoxylate pathway shunt (SANFACOR *et al.*, 1976; McKINLAY *et al.*, 2007).

Recent studies (LEE *et al.*, 2002; LIU *et al.*, 2008; MEYNIAL-SALLES *et al.*, 2008) have focused attention on anaerobic rumen bacteria (e.g. *Actinobacillus succinogenes, Anaerobiospirillum succiniciproducens, Mannheimia succiniciproducens*) because they are naturally able to produce and accumulate large amounts of SA (SONG and LEE, 2006). Furthermore, in the last few years, the use of recombinant strains has also been expanding (VEMURI *et al.*, 2002a; LEE *et al.*, 2006; KERN *et al.*, 2007).

Currently, the fermentative production of succinic acid is generally carried out using defined carbon sources (glucose, fructose, xylose, saccharose, lactose, maltose) (VEMURI *et al.*, 2002b; LEE *et al.*, 2002; ISAR *et al.*, 2006; LIU *et al.*, 2008). However, some studies have demonstrated that agro-food by-products can be used as cheaper substrates, which would be important in a future perspective of an industrial scale production of SA.

Food by-products as low-cost substrates

Italian food industries produce an annual capital turnover of 113,000 billion €; the main contributors are dairy products (14.2 billion €), wine and liquor (10.7 billion €), confectionary (10.1 billion €), and fresh and processed meat (7.4 billion €) (http://www.federalimentare.it/Documenti/Cibus2008/Sottoprodotti.pdf). In addition to these main products, food plants and industries produce huge quantities of by-products. According to Italian legislation (D. lgs. 152/2006), by-products are different from waste, in that they can be used to obtain value-added products. Their valorisation is a basic goal in a sustainable development strategy. Furthermore, exploitation of food by-products is an environmentally friendly approach which reduces their disposal as waste and could be an additional source of profit for industries themselves.

While SA-producing bacteria can use some by-products directly as a substrate (cheese whey and molasses), others (lignocellulosic materials and wheat flour) cannot be used directly because of their complex composition and structure. In the latter case, a two-step fermentative process

is needed. In the first step, an inexpensive carbon source is converted into a stream of nutrients that are readily accessible to a variety of microorganisms; in the second step, the nutrient stream is converted into the desired product. The first step is usually a fungal bioprocess and the second is a bacterial one (DU *et al.*, 2007).

To date, the fermentative production of SA has been reported for the following cost-competitive renewable feedstocks: cane molasses (0.073 US$/kg) (AGARWAL *et al.*, 2006; NGUYEN and GHEEWALA, 2008), cheese whey (0.58-0.62 US$/kg) (WAN *et al.*, 2008; http://usda.mannlib.cornell.edu/usda/current/DairProdPr/DairProdPr-07-10-2009.pdf), wood hydrolysates (0.025 US$/kg) (KAYLEN *et al.*, 2000; KIM *et al.*, 2004b; LEE *et al.*, 2003a) and wheat flour (0.09-0.134 US$/kg) (AKERBERG and ZACCHI, 2000; DU *et al.*, 2007). The cost of a pure substrate (e.g. glucose, fructose, lactose or sucrose) is much higher: 40- 120 US$/kg (http://www.sigmaaldrich.com/sigma-aldrich/home.html).

It is crucial to take into consideration that the cost of each by-product can fluctuate appreciably, depending on: 1) the location of the agro-food plants and, consequently, on the availability of the by-product in a specific geographical area and 2) the annual production of the main-product. For example, the current price of cane molasses is 0.196 US$/kg (http://www.agerborsamerci.it/listino/listino.html). All the above-mentioned feedstocks will now be described in detail.

Cane molasses is a by-product from the sugar industry, with the following composition: total sugars such as fructose, glucose and sucrose (about 50%), suspended colloids, heavy metals, vitamins and nitrogenous compounds (ROUKAS, 1998). This by-product has been employed in "green" SA fermentation with *A. succinogenes* (LIU *et al.*, 2008), *Escherichia coli* (AGARWAL *et al.*, 2006) and *Enterococcus flavescens* (AGARWAL *et al.*, 2007). *A. succinogenes* has proved to be the most efficient, giving the highest SA concentration (55.2 g/L) and productivity (1.15 g/L.h) in a fed-batch process (LIU *et al.*, 2008).

Cheese whey is a major by-product in the manufacturing of cheese or casein from milk, and makes up about 80% of the volume of processed milk; it contains lactose (4.5-5% w/v), soluble proteins (0.6-0.8% w/v), lipids (0.4-0.5% w/v) and mineral salts (8-10% of dried extract). Cheese whey also contains appreciable quantities of other components, such as lactic and citric acids, non-protein nitrogen compounds (urea and uric acid) and group B vitamins (GONZALES SISO, 1996). In the past, whey was considered to be a pollutant because of its high Biological Oxygen Demand (BOD) (approximatively 45,000 mg/L). Hence, the disposal of huge quantities of this "waste" was an environmental and economic problem for dairy industries. At present, whey is recycled into useful products such as food ingredients and animal feed or used for the recovery of value-added substances (such as lactose and proteins). The possibility of using cheese whey as a low-cost substrate is becoming a very appealing alternative.

Whey has been successfully fermented by *E. flavescens* (AGARWAL *et al.*, 2007), *A. succinogenes* (WAN *et al.*, 2008), *A. succiniciproducens* (SAMUELOV *et al.*, 1999) and *M. succiniciproducens* (LEE *et al.*, 2003b) to produce SA. A high concentration (34.7 g/L) and yield (90.6%) of SA were obtained using fed-batch fermentation by *A. succiniciproducens*; this strategy offers the advantages of both batch and continuous processes (SAMUELOV *et al.*, 1999). *M. succiniciproducens* had the highest SA productivity (3.90 g/L.h) in a continuous mode (LEE *et al.*, 2003b); *A. succinogenes* 130Z performed well in batch fermentation, producing 27.9 g/L of SA (WAN *et al.*, 2008).

Lignocellulosic biomass can be obtained as a by-product from the agro-food industry. This substrate is not easily fermentated by bacteria but, considering the huge amount available, it is one of the most preferable inexpensive substrates. A two-step fermentative process is applied to a lignocellulosic biomass: in the first step, the lignocelluloses are hydrolyzed using a physical-chemical process (thermal, mechanical or acidic hydrolysis) or a biological one (treatment with ligninolytic fungal enzymes). In the second step, the cellulosic and hemicellulosic fractions are depolymerised to fermentable sugars (glucose and xylose) which can be used as substrate in microbial SA-fermentation (LEE, 1997). Unfortunately, some toxic or inhibitory compounds derived from the hydrolysis process are also present: this causes a decrease in the fermentative process yield and productivity (KIM *et al.*, 2004b). These molecules (furfural, hydroxymethylfuran, p-hydroxybenzoic aldehyde and vaniline) (CONVERTI *et al.*, 1998) can be removed but with a consequent increase in operational costs. Alternatively, an alkaline hydrolysate treatment is an effective and rather inexpensive method that reduces the formation of inhibitory compounds (KIM *et al.*, 2004b).

Some researchers have reported fermentation by *M. succiniciproducens* and *A. succiniciproducens* in a wood-hydrolysed-based medium for SA production (LEE *et al.*, 2003a; KIM *et al.*, 2004b). *A. succiniciproducens* (LEE *et al.*, 2003a) and *M. succiniciproducens* (KIM *et al.*, 2004b) can metabolise wood hydrolysate for a "green" SA production with high SA yields (55-88% g SA/g substrate). The highest productivity (3.19 g/L.h) was obtained with continuous fermentation on a wood hydrolysate by *M. succiniciproducens* (KIM *et al.*, 2004b).

As in the case of wood hydrolysates, SA can be

produced in a two-stage bioprocess using wheat flour as starting material. In the first step, wheat flour is used as a substrate in fungal fermentation and converted into microbial feedstock. In the second step, the microbial feedstock (flour hydrolysate) is used in bacterial SA production (DU *et al.*, 2007).

A. succinogenes is able to use flour hydrolysate (DU *et al.*, 2007; 2008; LIN *et al.*, 2008); the highest SA concentration obtained was 64.2 g/L and the yield was 0.81 g/g substrate, in a batch fermentation (DU *et al.*, 2008).

The same microorganism can be efficiently used for SA fermentation in a wheat hydrolysate medium. A high SA concentration was produced (62.1 g/L) when a hydrolysate of a by-product of wheat flour milling was employed (DORADO *et al.*, 2009).

The production of SA using food by-products and different bacterial strains in batch or fed-batch lab-scale fermentation are summarized in Table 1 where the working volume and the succinic acid concentration are reported.

Apart from substituting an expensive, pure C-source with a cheaper one, other expensive constituents of the medium can also be replaced with more economical ones. Some studies have reported the use of corn steep liquor (CSL), a by-product of the corn wet milling industry (LEE *et al.*, 2000; 2003b), or rice bran, a by-product of the rice-milling process (MOON *et al.*, 2004), as low-cost nitrogen sources (N-sources) instead of complex, more expensive ones (peptone, yeast extract or trypton). These substrates also supply the medium with vitamins and minerals, in addition to proteins and amino acids. Quite comparable SA yields have been obtained in a fermentative process using both pure and cheaper N-sources (LEE *et al.*, 2003a, b). If wood hydrolysate is used as the C-source, the SA yield is 88-89% (g/g) in the presence of polypeptone plus yeast extract and 85-88% (g/g) using CSL (LEE *et al.*, 2003a). If whey is used as the C-source, the SA yield is 67-69% (g/g) in the presence of yeast extract and 65-66% in the presence of CSL (LEE *et al.*, 2003b).

Potential applications of succinic acid and its derivatives in the agro-food industry

Since SA is a linear saturated dicarboxylic acid, it can be used as an intermediate chemical. It can be converted into 1,4-butanediol (BDO), tetrahydrofuran (THF), γ-butyrolactone (GBL), and other four-carbon chemicals that have a world-wide market. For example, SA can be readily hydrogenated to 1,4-butanediol, which can then be further carbonylated to adipic acid (ZEIKUS *et al.*, 1999) that is used as a food additive (see below).

Food additives

In Directive 95/2/EC (http://ec.europa.eu/food/fs/sfp/addit_flavor/flav11_en.pdf) on food additives which include colours and sweeteners, it is also possible to find SA, adipic acid and their salts. In the European Community, food addi-

Table 1 - Succinic acid production from various microorganisms using food by-products as low-cost substrates.

C source	Bacterium strain	Fermentation strategy	Working volume (L)	[SA] (g/L)	Reference
Cane molasses	*E. coli*	Batch	7.5	17	Agarwal *et al*., 2006
Cane molasses	*A. succinogenes* CGMCC1593	Fed-batch	3.5	55.2	Liu *et al*., 2008
Cane molasses	*E. flavescens*	Batch	0.3	0.5	Agarwal *et al*., 2007
Cheese whey	*E. flavescens*	Batch	0.3	0.54	Agarwal *et al*., 2007
Cheese whey	*M. succiniciproducens* MBEL55E	Batch	1	13.4	Lee *et al*., 2003b
Cheese whey	*A. succiniciproducens* ATCC 29305	Fed-batch	1	34.7	Samuelov *et al*., 1999
Cheese whey	*A. succinogenes* 130Z (ATCC 55618)	Batch	1.2	27.9	Wan *et al*., 2008
Wood hydrolysate	*A. succiniciproducens* ATCC29305	Batch	1	17.4	Lee *et al*., 2003a
Wood hydrolysate	*M. succiniciproducens* MBEL55E	Batch	1	11.73	Kim *et al*., 2004b
Flour hydrolysate	*A. succinogenes* ATCC 55618	Batch	0.8	27.2	Du *et al*., 2007
Flour h ydrolysate	*A. succinogenes* ATCC 55618	Batch	0.8	64.2	Du *et al*., 2008
Flour hydrolysate	*A. succinogenes* ATCC 55618	Batch	0.5	59.3	Lin *et al*., 2008
Wheat hydrolysate	*A. succinogenes* ATCC 55618	Batch	0.45	62.1	Dorado *et al.*, 2009

Table 2 - Food additives and their applications in the food industry. The reported maximum level of use refers to foodstuffs ready for consumption prepared following the manufacturers' instructions.

Food additives	Uses	Maximum level of use
Succinic acid (E363)	in desserts	< 6 g/kg
	in soups and broths	< 5 g/kg
	in powders for the home preparation of drinks	< 3 g/L
Adipic acid (E355) and its salts	in fillings and toppings for fine bakery products	< 2 g/kg
	in dry powdered dessert mixes	< 1 g/kg
	in gel-like desserts	< 6 g/kg
	in fruit-flavoured desserts	< 1 g/kg
	in powders for the home preparation of drinks	< 10 g/L (expressed as adipic acid)
Starch sodium octenyl succinate (E1450) Acetylated distarch adipate (E1422)	in weaning food in infant formulas and follow-on formulas	< 50 g/kg < 20 g/kg

tives are identified with a code composed of the letter E followed by a number. SA (E363) is a flavour enhancer or a buffer and neutralizing agent for food and beverages. Its applications and maximum level of use are reported in Table 2.

Sodium succinate can replace monosodium glutamate and dilysine succinate as a salty flavour enhancer for low-sodium food (ZEIKUS *et al.,* 1999). Other potential applications of SA include: 1) the succinylation of lysine residues to improve the physical and functional attributes of soy proteins in food and 2) the use of succinylated starch as a thickening agent.

Adipic acid (E355) and its salts (sodium adipate E356 and potassium adipate E357) have different applications, which are reported in Table 2. Adipic acid is an acidity regulator, acidifier, flavour enhancer, gelling aid and a slow raising agent.

Starch sodium octenyl succinate (E1450) and acetylated distarch adipate (E1422) are used in weaning food or in infant formulas and follow-on formulas as emulsifiers, stabilizers, thickeners and bulking agents.

Succinic acid derived biodegradable plastics

Of all the SA-derived chemicals, 1,4-butanediol and adipic acid are considered very important for the production of biodegradable polyesters and polyamides.

The Showa Denko company, produces 2 different series of aliphatic polyesters, under the trademark BIONOLLE. Bionolle1000 is a polybutylene succinate (PBS) constituted by 1,4-butanediol (BDO) and SA; Bionolle3000 is a polybutylene succinate adipate (PBSA), copolymer that is made by using 1,4-butanediol and a mixture of SA and adipic acid. Bionolle has some physical properties that are similar to traditional plastics (PE, PET) (McCARTHY *et al.*, 1999). Furthermore, thanks to its excellent processability, it can be processed into various moulded injected, extruded and blown products in conventional equipment commonly used for polyolefins (FUJUMAKI, 1998). The Bionolle series have a wide range of applications in the food sector: films (food packaging and wrapping films, bags, labels), lamination (paper lamination, bags, cups, containers) (KOBAYASHI *et al.*, 2002), sheet extrusion (cards, can holders, food trays), blow moulding (drink bottles) and foam (cups and food trays). Bionolle can also be used coupled with starch to give blends that can be used in food packaging (GORMAL, 2002).

These SA polyesters are also manufactured under other trademarks: GS Pla (Mitsubishi Chemical), Skygreen (Sk chemicals), Lunare SE (Nippon Shokubai), EnPol (IRe Chemicals Ltd). Different series, characterized by different physical properties, exist according to the required applications.

Polybutylensuccinate terephthalate (PBST) is produced by DuPont under the trademark Biomax. This material is usually tailored to mimic polyethylene or polypropylene. Biomax® resins are used for different applications, such as injection moulding for disposable cutlery, paper coating, thermoformable cups and trays, and certain films, such as lidding stock. This material possesses good barrier properties, which are among the best of all the biodegradable polymers. Biomax is mainly intended for final disposal through composting and in-soil degradation.

Polybutylenadipate terephthalate (PBAT) is an aliphatic-aromatic copolyester which is sold under the trade names Ecoflex (Basf) and Eastar-Bio (Eastman). This copolyester combines the biodegradable properties of aliphatic polyester and the performance properties of aromatic polyesters. Two of the three monomers are

constituted by 1,4-butandiol and adipic acid and can be obtained by using SA as the starting material. PBAT can be processed in different ways and then used for bags, cast films (agricultural mulching sheetings) and extrusion coating (paper coating, wrapping papers), fast-food packaging (drinking cups and hamburger boxes), food trays, disposable packaging for meat, fish, poultry, fruit and vegetables. It is biodegradable and compostable within about a maximum of 180 days. Consequently, these items can be eliminated organically, thus reducing their processing costs. PBAT looks and performs like low density polyethylene (LDPE). It is waterproof, sealable, printable and tear- and temperature variation- resistant; it is also characterized by flexibility, anti-fogging performance and transparency.

The golden future of the bio-based succinic acid platform

The fermentative production of SA is a very promising way for expanding the use of SA and its derivatives on the market. Taking into account the potential use of agro-food by-products as low-cost substrates, the fermentation approach becomes more interesting from an economic and environmental point of view.

The economic interest is associated with the transformation of the by-products used as raw materials into value-added products and the reduction in the costs of by-product disposal.

The environmental advantage is gained by replacement of petrochemical resources with renewable ones, such as feedstocks, the fixation of carbon dioxide in microbial fermentation (lower emissions of greenhouse gases) and replacing traditional plastics (non-degradable) with SA-derived biodegradable ones.

From an economic point of view and that of process bottlenecks, consideration must be given not only to the cost of raw materials, but also the cost of the downstream process. The recovery and purification steps are difficult and crucial. In fact, an industrial agro-food by-product has SA in the medium but also large amounts of additional undesirable substances. These substances must be removed, resulting in an increase in the overall process costs.

At present, three methods are used for the downstream process: electrodialysis (GLASSNER and DATTA, 1992), acidification (DATTA *et al.*, 1992) and solvent extraction (KIM *et al.*, 2004a). These methods however cannot be applied on an industrial level to recover and purify SA because they are too costly. Furthermore, they must be improved in order to obtain purer SA. A new method has recently been set-up that consists of reactive extraction, vacuum distillation and crystallization (HUH *et al.*, 2006). It should noted that all of these methods were first developed in culture broth containing pure C-sources. A great deal of effort is necessary to set up and optimize the downstream process that uses a culture medium that contains complex C-sources, such as agro-food by-products.

The low cost of raw materials having a complex substrate is offset by the need to remove the toxic or inhibitory molecules (e.g. wood hydrolysate-derivatives) during the downstream steps (GLASSNER and DATTA, 1992; YEDUR *et al.*, 2001; HUH *et al.*, 2006). Moreover, all the fermentation products derived from bacterial metabolism, other than those from SA, must also be purified.

The main goals of the SA production process are: to optimize the culture conditions and fermentation parameters, to use engineered microorganisms, to increase SA yield and productivity, to reduce by-products formation and to set up a better performing SA recovery method.

"Green" SA and its derivatives make up a more "natural" food-additive category, that can be used instead of traditional chemical ones. Biodegradable plastics produced from "green" SA are also an attractive alternative in the food packaging sector. Both these aspects could have a successful impact on public opinion.

REFERENCES

Agarwal L., Isar J., Meghwanshi G.K. and Saxena R.K. 2006. A cost effective fermentative production of succinic acid from cane molasses and corn steep liquor by *Escherichia coli*. J. Appl. Microbiol. 100: 1348.

Agarwal L., Isar J., Meghwanshi G.K. and Saxena R.K. 2007. Influence of environmental and nutritional factors on succinic acid production and enzymes of reverse tricarboxylic acid cycle from *Enterococcus flavescens*. Enzyme Microb. Technol. 40: 629.

Akerberg C. and Zacchi G. 2000. An economic evaluation of the fermentative production of lactic acid from wheat flour. Biores. Technol. 75: 119.

Bechthold I., Bretz K., Kabasci S., Kopitzky R. and Springer A. 2008. Succinic acid: a new platform chemical for biobased polymers from renewable resources. Chem. Eng. Technol. 31: 647.

Converti A. and Borghi M.D. 1998. Inhibition of the fermentation of oak hemicellulose acid hydrolysate by minor sugars. J. Biotechnol. 64: 211.

Datta R., Glassner D. A., Jain M. K. and Roy J. R. V. 1992. Fermentation and purification process for succinic acid. U.S. Patent 5168055.

Decreto Legislativo (D.Lsg.) 3 Aprile 2006, n. 152 Norme in materia ambientale (G.U. n. 88 del 14 aprile 2006, supplemento ordinario n. 96).

Dorado M.P., Lin S.K.C., Koutinas A., Du C., Wang R. and Webb C. 2009. Cereal-based biorefinery development: utilisation of wheat milling by-products for the production of succinic acid. J. Biotechnol. (doi:10.1016/j.jbiotec.2009.06.009).

Du C., Lin S.K.C., Koutinas A., Wang R. and Webb C. 2007. Succinic acid production from wheat using a biorefining strategy. Appl. Microbiol. Biotechnol. 76: 1263.

Du C., Lin S.K.C., Koutinas A., Wang R., Dorado P. and Webb, C. 2008. A wheat biorefining strategy based on solid-state fermentation for fermentative production of succinic acid. Biores. Technol. 99: 8310.

Fujimaki T. 1998. Processability and properties of aliphat-

ic polyesters "Bionolle", synthesized by polycondensation reaction. Polym. Deg. Stab. 59: 209.

Glassner D.A. and Datta R. 1992. Process for the production and purification of succinic acid. US Patent 5143834.

Gonzales Siso M.I. 1996. The biotechnological utilisation of cheese whey: a review. Biores. Technol. 57: 1.

Gormal S. 2002. Compatibilisation of biodegradable starch/Bionolle™ blends. CHEE 4006 Individual inquiry.

Huh Y.S., Jun Y-S., Hong Y.K., Song H., Lee S.Y. and Hong W.H. 2006. Effective purification of succinic acid from fermentation broth produced by *Mannheimia succiniciproducens*. Process Biochem. 41: 1461.

Isar J., Agarwal L., Saran S. and Saxena R.K. 2006. Succinic acid production from *Bacteroides fragilis*: process optimization and scale up in a bioreactor. Anaerobe 12: 213.

Kaylen M., Van Dyne D.L., Choi Y-S. and Blase M. 2000. Economic feasibility of producing ethanol from lignocellulosic feedstocks. Biores. Technol. 72: 19.

Kern A., Tilley E., Hunter I.S., Legisa M. and Glieder A. 2007. Engineering primary metabolic pathways of industrial micro-organisms. J. Biotechnol. 129: 6.

Kim B.S., Hong Y.K. and Hong W.H. 2004a. Effect of salts on the extraction characteristics of succinic acid by predispersed solvent extraction. Biotechnol. Bioprocess Eng. 9: 207.

Kim D.Y., Yim S.C., Lee P.C. and Lee W.G. 2004b. Batch and continuous fermentation of succinic acid from wood hydrolysate by *Mannheimia succiniciproducens* MBFL55E. Enzyme Microb. Technol. 34: 648.

Kobayashi Y., Mori H.I. and Iwasaki Y. 2002. Food packaging bag, method of food packaging and use of laminated film as food-packaing bag. European Patent EP1182142A1.

Landucci R., Goodman B. And Wyman C. 1994. Methodology for evaluating the economics of biologically producing chemicals and materials from alternative feedstocks. Appl. Biochem. Biotechnol. 45-46: 678.

Lee J. 1997. Biological conversion of lignocellulosic biomass to ethanol. J. Biotechnol. 56: 1.

Lee P.C., Lee W.G., Lee S.Y., Chang H.N. and Chang Y.K. 2000. Fermentative production of succinic acid from glucose and corn steep liquor by *Anaerobiospirillum succiniciproducens*. Biotechnol. Bioprocess Eng. 5: 379.

Lee P.C., Lee S.Y., Hong S.H. and Chang H.N. 2002. Isolation and characterization of a new succinic acid-producing bacterium, *Mannheimia succiniciproducens* MBEL55E, from bovine rumen. Appl. Microbiol. Biotechnol. 58: 663.

Lee P.C., Lee S.Y., Hong S.H., Chang H.N. and Park S.C. 2003a. Biological conversion of wood hydrolysate to succinic acid by *Anaerobiospirillum succiniciproducens*. Biotechnol. Lett. 25: 111.

Lee P.C., Lee S.Y., Hong S.H. and Chang H.N. 2003b. Batch and continuous cultures of *Mannheimia succiniciproducens* MBEL55E for the production of succinic acid from whey and corn steep liquor. Bioprocess Biosyst. Eng. 26: 63.

Lee S.J., Song H. and Lee S.Y. 2006. Genoma-based metabolic engineering of *Mannheimia succiniciproducens* for succinic acid production. Appl. Environ. Microbiol. 72: 1939.

Lin S.K.C., Du C., Koutinas A., Wang R. and Webb C. 2008. Substrate and product inhibition kinetics in succinic acid production by *Actinobacillus succinogenes*. Biochem. Engin. J. 41: 128.

Liu Y-P., Zheng P., Sun Z-H., Ni Y., Dong J-J. and Zhu L-L. 2008. Economical succinic acid production from cane molasses by *Actinobacillus succinogenes*. Biores. Technol. 99: 1736.

McCarthy S.P., Gross R.A. and Ma W. 1999. Polylactic acid-based blends. US Patent 5883199.

McKinlay J.B., Vieille C. and Zeikus J.G. 2007. Prospects for a bio-based succinate industry. Appl. Microbiol. Biotechnol. 76: 727.

Meynial-Salles I., Dorotyn S. and Soucaille P. 2008. A new process for the continuous production of succinic acid from glucose at high yield, titer and productivity. Biotechnol. Bioeng. 99: 129.

Moon S-K., Wee Y-J., Yun J-S. and. Ryu H-W. 2004. Production of fumaric acid using rice brain and subsequent conversion to succinic acid through a two-step process. Appl. Biochem. Biotechnol. 113-116: 834.

Nguyen T.L.T. and Gheewala S.H. 2008. Fuel ethanol from cane molasses in Thailand: environmental and cost performance. Energy Pol. 36: 1589.

Potera C. 2005. Making succinate more successful. Environ. Health Perspect. 113: 832.

Roukas T. 1998. Pretreatment of beet molasses to increase pullulan production. Proc. Biochem. 33: 805.

Rupp Dahlem C. 2008. "Bio-succinic acid, an attractive product for biorefineries" International Conference on renewable resources & biorefineries – Rotterdam 1st-4th June 2008 (http://www.rrbconference.com/bestanden/downloads/142.pdf).

Samuelov N.S., Datta R., Jain M.K. and Zeikus J.G. 1999. Whey fermentation by *Anerobiospirillum succiniciproducens* for production of a succinate-based animal feed additive. Appl. Environ. Microbiol. 65: 2260.

Sanfacor R., Roulliard R. and Heick H.M. 1976. The accumulation of succinate by yeast *Brettanomyces bruxellensis*. Can. J. Microbiol. 22: 213.

Sauer M., Porro D., Mattanovich D. and Branduardi P. 2008. Microbial production of organic acids: expanding the markets. Trends Biotechnol. 26: 100.

Song H. and Lee S.Y. 2006. Production of succinic acid by bacterial fermentation. Enzyme Microb. Technol. 39: 352.

Vemuri G.N., Eiteman M.A. and Altman E. 2002a. Effects of growth and pyruvate carboxylase on succinic acid production by metabolically engineered strains of *Escherichia coli*. Appl. Environ. Microbiol. 68: 1715.

Vemuri G.N., Eiteman M.A. and Altman E. 2002b. Succinate production in dual-phase *Escherichia coli* fermentations depends on the time of transition from aerobic to anaerobic conditions. J. Ind. Microbiol. Biotechnol. 28: 325.

Wan C., Li Y., Shahbazi A. and Xiu S. 2008. Succinic acid production from cheese whey using *Actinobacillus succinogenes* 130 Z. Appl. Biochem. Biotechnol. 145: 111.

Werpy T. and Peterson G. 2004. Top value added chemicals from biomass. Volume I – Results of screening for potential candidates from sugars and synthesis gas. US Department of Energy (http://www1.eere.energy.gov/biomass/pdfs/35523.pdf).

Yedur S., Berglund K.A. and Dunuwila D.D. 2001 Succinic acid production and purification. U.S. Patent n. 6265190 B1.

Zeikus J.G., Jain M.K. and Elankovan P. 1999. Biotechnology of succinic acid production and markets for derived industrial products. Appl. Microbiol. Biotechnol. 51: 545.

Revised paper received July 30, 2009 Accepted October 30, 2009

PAPER

MICROSTRUCTURE OF NATURAL, EXTRUDED AND CO-EXTRUDED COLLAGEN CASINGS BEFORE AND AFTER HEATING

S. BARBUT
Food Science Department, University of Guelph
Guelph, Ontario, Canada N1G 2W1
Corresponding author: Tel. (519) 824-4120 ext. 53669
e-mail: sbarbut@uoguelph.ca

ABSTRACT

The microstructures of natural and manufactured collagen casings were evaluated by light and scanning electron microscopy. Sheep and hog casings showed a typical natural casings structure consisting mainly of the submucosa layer. Polarized light microscopy clearly showed the connective tissue fiber alignment. Manufactured, extruded casings showed a gelled matrix in which connective tissue fibers were embedded. A special chemically hardened casing showed a much denser alignment of the connective tissue fibers, and higher protein content per unit area. In co-extruded casings, a structure fairly similar to the manufactured, extruded casings was observed. Scanning electron microscopy revealed three dimensional microstructures showing the connective tissue fibers' orientation and distribution.

- Key words: casing, co-extrusion, collagen, hog, meat, microscopy, structure, sausage, sheep -

INTRODUCTION

For generations, natural collagen casings have been considered to be the standard for fresh and a variety of cooked sausages. The international trade in natural casings has been estimated at about $2.5 billion a year (INSCA, 2004). However, over the last three to four decades, manufactured collagen casings (i.e. prepared from re-generated collagen) and more recently co-extruded casings (i.e. directly deposited on the meat batter as it comes out of the stuffer) have been introduced on the market. Exact trade figures for these casings are difficult to obtain, and therefore are not provided here. When looking at market figures of natural casings it is important to note that ≈60% of the trade is in sheep casings. However, due to the relatively high cost of using natural casings (e.g. preparation cost, efforts at the meat processing plant) and shelf life considerations (high initial bacteria counts; BAKKER *et al.,* 1999; GABIS and SILLIKER, 1974), various processors have been moving towards using manufactured collagen casings and/or the new co-extrusion technology.

In any case, it should be emphasized that for various high end meat products on the market today, natural casings with their old-fashion appearance are still the norm. Natural casings have been routinely used to stuff different meats as well as other food products (e.g. dairy) for storage, preservation (e.g. by fermentation) and processing. An example of the latter can be a raw minced meat system, in a semi fluid state, that is stuffed into casings and heated until it gels and forms a coherent elastic structure which is sliceable and spreadable and can be handled even without the casings.

The casing plays both direct and indirect roles in the volumetric, structural and chemical changes that occur in the sausage through its processing steps (SAVIC and SAVIC, 2002). Therefore the unique requirements of each sausage product should be matched with the characteristics of the casings to be used. To accomplish this goal, a number of producers offer a variety of casings, with different functional properties. However, since there is very little or no information in the scientific literature on the microstructure of manufactured collagen casings, the goal of this study was to examine and compare different types with histological methods and scanning electron microscopy, including the new co-extruded casings, to the traditional natural casings.

MATERIAL AND METHODS

Meat sample preparation

A common sausage meat batter was prepared at the University of Guelph meat laboratory, and used to stuff five of the six different collagen casings. The sixth formulation, a co-extruded casing, was produced at a different location (ie. equipment not available at the University). The common sausage meat batter used at the University included 20% chilled beef trims (80/20), 25% pork trims (80/20), 20% pork jowls, 15% pork back fat, 20% ice. The following non meat ingredients were used: 1.7% salt, 1.0% starch, 0.6% seasonings, 0.02% phosphate, and in addition, 300 ppm sodium erythorbate, and 160 ppm sodium nitrite. The meat was ground through a 3 mm plate, chopped in a silent cutter (Schneidmeister SMK 40, Berlin, Germany) while adding all the non meat ingredients for 4 min.; temperature maintained below 12°C. The meat was then stuffed (Mainca model EM20R, Berkshire, UK) into the five different casings (Table 1); approximately 10 kg per casing type. The co-extruded casings (QX Co-extruder, Stork-Townsend, Des Moines, IA) were produced at a different location and shipped to the University meat laboratory. Samples of the raw casings and heated casings (products placed in boiling water for 3 min) were evaluated by either cutting thin sausage samples (5 mm) from different link, or taking pieces of casings and attaching them onto 2x2 cm cardboard squares (using stainless steel pins) prior to chemically fixing them for microscopical evaluation. Such casing sections were used when casings would not stay attached to the meat product after cutting the sausage sample.

Light microscopy

Ten thin sausage or casing sections, per sample, were taken from the ends and middle of different sausage links and fixed in 10% neutral buffered formalin for 1.5 h, followed by dehydrating in 70, 95 and 100% isopropanol; each for 2 h. The samples were soaked in xylene for 2 h and embedded in paraffin for 3 h, using an automated vacuum infiltration unit (Sakura Tissue-Tek VIP, Sakura Finetek, Torrance, CA). The embedded samples were sectioned (Microtome HM 200, Ergostar, Walldoft, Germany) into 5-7 micron sections, allowed to float on water, and transferred onto a glass slide. Slides were dried, and stained with either a general Hematoxylin/Eosin stain or a specific Masson stain for collagen. A computerized image analysis system attached to the microscope (Model BX60F5, Olympus Optical Co, Ltd., Japan) was used to view (x 200 magnification) the samples and capture images (Image-Pro Plus, Version 4.5-1.29, Media Cybernetics Inc., Silver Spring, MD).

Scanning electron microscopy

Six cubes (approx. 1 cm^3) from the edge of each raw sausage were fixed overnight in 2.5% glutaraldehyde washed with a Sorensen's buffer, post fixed in 1% OsO_4, dehydrated in ethanol, criti-

cal point dried, fractured to expose inside surfaces, placed on stubs and sputter-coated with 200-300A of gold/palladium (Emitech K550; Ashford, Kent, England). The scanning electron microscope (S-570, Hitachi, Japan) was used at an accelerating voltage of 5Kv.

Protein content

Protein level in the casings, as received, was determined in triplicate according to the AOAC (1996) method.

RESULTS AND DISCUSSION

The raw natural sheep casing (Fig. 1a) reveals a typical microstructure of cleaned sheep intestine showing the submucosa layer which is the basic structure of this kind of natural casing. The image is consistent with other examples of sheep casings published by KOOLMEES *et al.* (2004) who looked at the efficiency of mechanical versus manual cleaning and preparing of casings. During the process the serosa, mucosa and muscularis layers are removed, only the submucosa layer for sausage production remains (KOOLMEES and HOUBEN, 1997; SAVIC and SAVIC, 2002; WIJNKER *et al.*, 2008). During this process the thickness of the intestine is reduced by 91-92% and weight by about 87-89% (COMER and BRADLEY, 2002; KOOLMEES *et al.*, 2004) and a thin elastic layer rich in connective tissue fibers which includes both collagen and elastin fibers is left over. The connective tissue fibers can be clearly seen by polarized light (Fig. 1b). The collagen fibers are birefringent (PIMENTAL, 1981), meaning that by rotating the plane of polarized light, the fibers appear bright against an otherwise dark background (SWATLAND, 1984). This is because collagen has two to three principle refractive indices (such materials are also called anisotropic). A Masson stain was used to colorize the collagen (dark blue), but it was also easy to visualize the fibers by polarized light (i.e., micrographs of the Masson stain are not shown here because a black and white reproduction is not suitable to show the color effect). When meat is stuffed into the casings the connective tissue fibers can stretch and consequently put pressure on the filling inside. This pressure is important for keeping the product's integrity during the cooking process and provides a unique firm texture when the product is consumed (BARBUT, 2002). The thickness of the casing was about 100 μm (at the middle of the sausage link), which is in line with the 110 μm (0.11 mm) measurement published by BAKKER *et al.*, (1999). They also reported that the whole small intestine wall (prior to cleaning and processing) was 1.5 mm and cleaning resulted in about 90% reduction in thickness.

The hog casings (Fig. 1c) showed a thicker structure of about 225 μm (range 200 250; measured at the middle of the sausage link) with more connective tissue fibers (Fig. 1d) compared to the sheep casings. BAKKER *et al.* (1999) reported an average thickness of 300 μm for hog casings. However, the exact thickness would depend on the age and feed of the animal as well as the climatic conditions it lived in, the degree of cleaning, and stretching of the casing during stuffing. In any case, hog casings have a larger diameter, are stronger than sheep casings, and also have moderately higher protein content per unit (20.9% vs. 16.5% protein, respectively; Table 1). In the present study, the sausage batter stuffed into sheep casings had a diameter of 20 mm (Table 1), and the hog casing was 32 mm. These stuffing diameter values are in line with values reported by SAVIC and SAVIC (2002); i.e., 16-25 mm for sheep and 20-28 mm for hog runners (casings obtained from the small intestine).

The manufactured collagen casings for fresh sausage (Fig. 1e) show a much more dense and compact matrix compared to the natural casings. Overall, the manufactured casings are dried and shipped to the customer in a moisture proof packaging to prevent hydration during storage (67.2% protein in the dried form; Table 1). This is opposite to natural casings which are commonly shipped in a saturated salt solution which includes over 50% water. The manufactured casings are made from collagenous connective tissue extracted from different parts of the animal (e.g. hides). The collagen extraction method and the later extrusion are usually proprietary processes, but they basically consist of separating the corium layer from the hide, decalcification, regeneration, dry extrusion process (usually referred to as"German proces", where HCl is used) or wet extrusion process "American proces", where H_2SO_4 is used). SAVIC and SAVIC (2002) emphasized that during the first phase (corium layer removal and modification) care should be taken to avoid converting all the collagen into a soluble form (i.e., need to maintain actual fiber structure). An example of the fibers can be seen in Fig. 1e and more distinctly under polarized light in Fig. 1f. When the casings are hydrated (during or before the meat stuffing process) the fibers can regain some of their elasticity, stretch and consequently exert pressure on the meat batter placed inside. The thickness of this specific manufactured collagen casing was about 50-75 μm.

The second type of manufactured collagen casing examined here was for smoked sausages (Fig. 2a), which was obtained from another supplier. The colour was different from the first manufactured casings (Table 1), but the basic microstructure was fairly similar, with a difference in the number of intact fibers; i.e., higher number in the fresh sausage casings than in the one for smoked sausage (see polarized

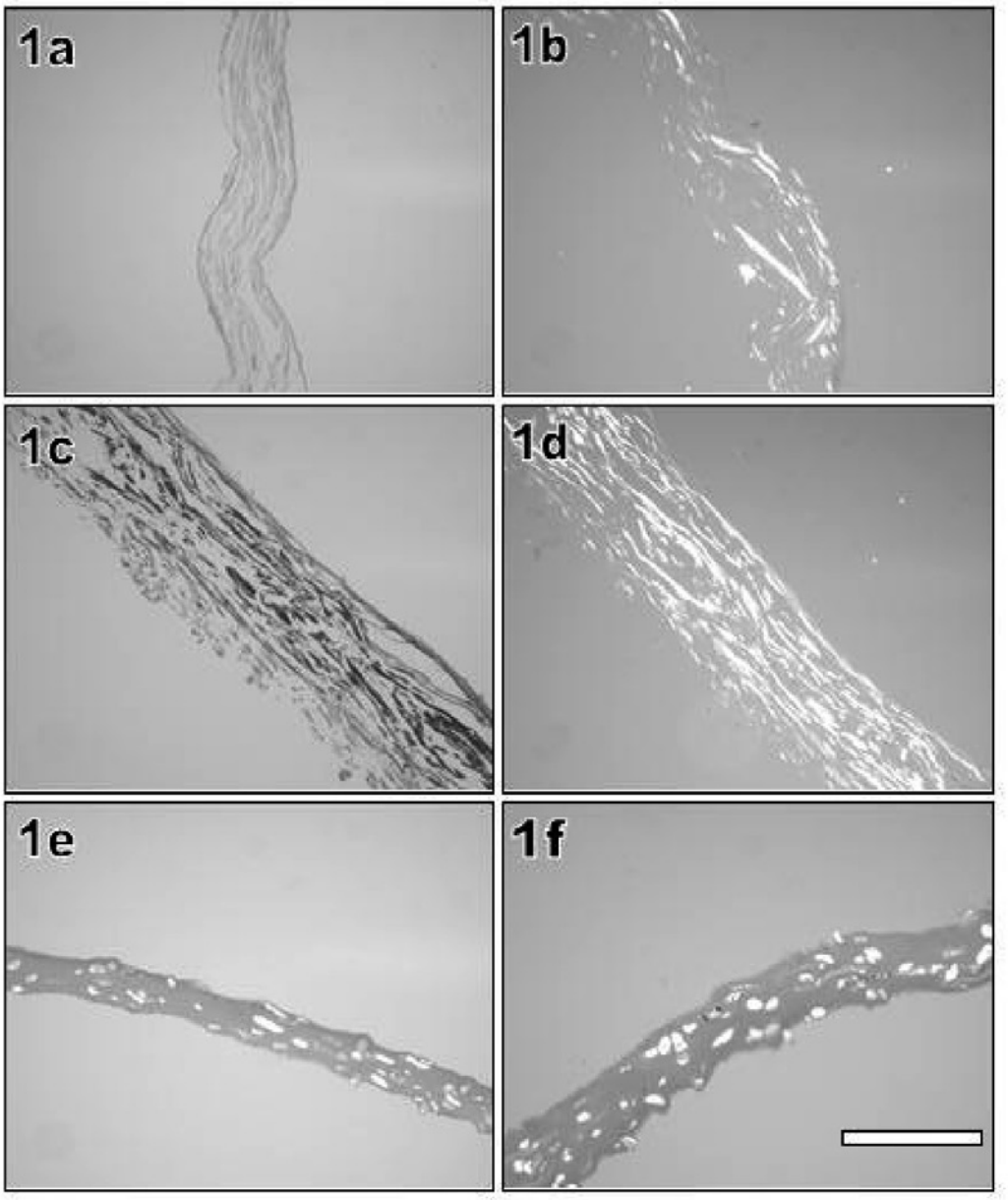


Fig. 1 - Light micrographs of raw collagen casings: (a) and (b) natural sheep; (c) and (d) natural hog; (e) and (f) manufactured collagen for fresh sausage. Polarized light was used in (b), (d) and (f) to reveal connective tissue fibers. Bar = 200 μm.

Table 1 - Casings evaluated and some of their characteristics.

Casings type	State of raw casings	Stuffed diameter (mm)	Color raw	Treatment prior to stuffing	Protein* (%)	Supplier (casings ID) Location
1. Natural sheep	Pliable. Shipped in a salt solution	20 can stretch	Transparent	Rinse salt	16.5	F. Marie (sheep casings) Markham, ON
2. Natural hog	Pliable. Shipped in a salt solution	32 can stretch	Transparent	Rinse salt	20.9	F. Marie (hog casings) Markham, ON
3. Manufact. collagen (for fresh sausage)	Flexible. Shipped dried	35	Cream white	None	67.2	Mar/Co (freshsausage) Bloomington, MN
4. Manufact. Collagen (for smoked sausage)	Flexible. Shipped dried	32	Yellowish	None	83.9	Naturin (NCC 32) Weinheim, Germany
5. Manufact. collagen (for ring sausage)	Flexible. Shipped dried	50	Brownish	None	76.8	Naturin (Kranzdarm) Weinheim, Germany
6. Co-extruded	Gel like	20 can make to size	Semi transparent	Keep cold. Pump to deliver	6.3	Devero (Co Ex Paste) Colombia, SC

*Protein content in casings as received.

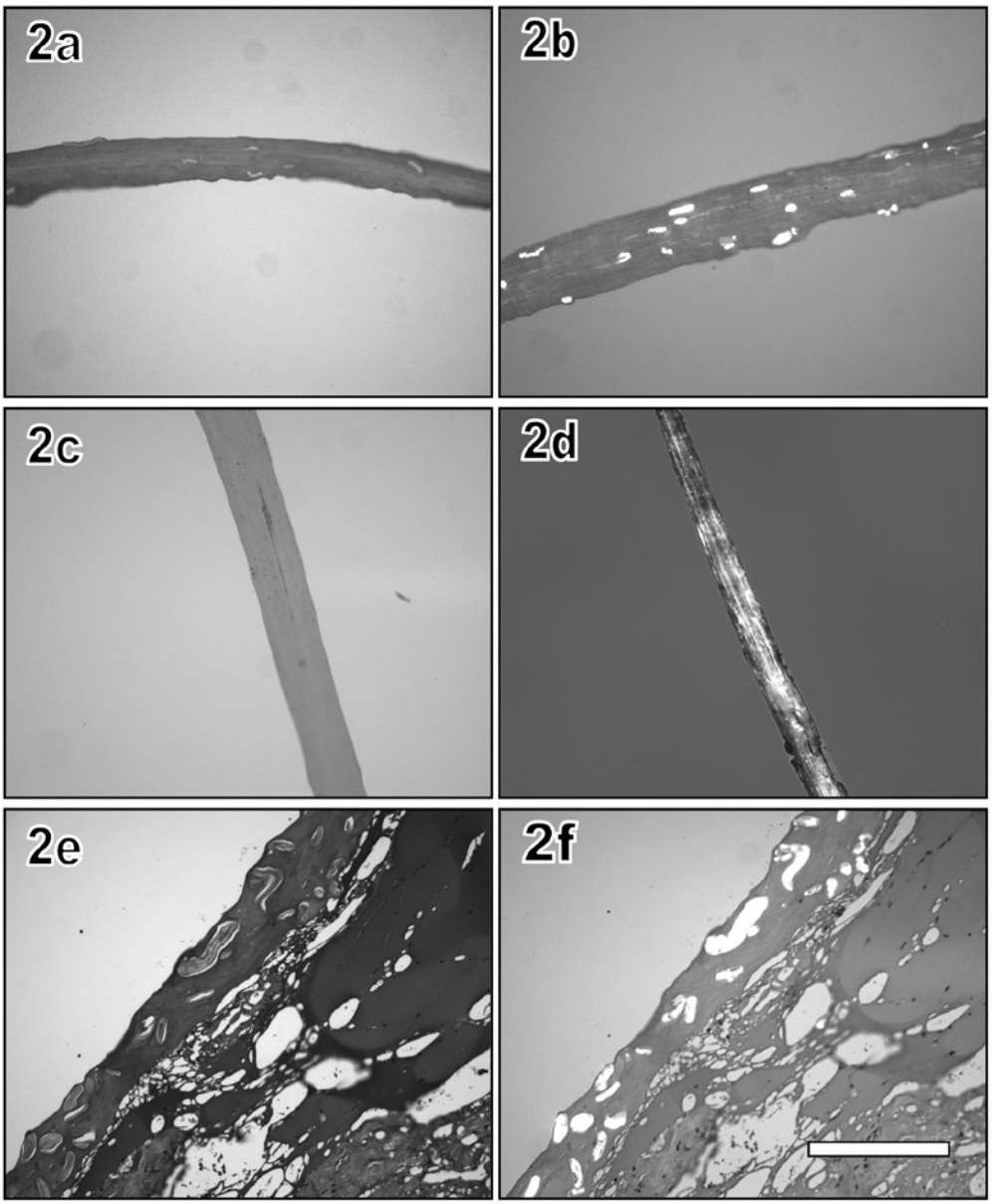


Fig. 2 - Light micrographs of raw collagen casings: (a) and (b) manufactured for smoked sausage; (c) and (d) manufactured for large diameter ring sausage; (e) and (f) co-extruded; the lower right part shows the meat batter. Polarized light was used in (b), (d) and (f) to reveal connective tissue fibers. Bar = 200 μm.

light micrographs Fig. 1f vs. Fig. 2b). The latter can be used by manufacturers to vary the strength, and degree of stretching of a particular casing they produce.

The third manufactured collagen casing was designed for a large diameter (50 mm) ring type sausage (Table 1). This casing is much stronger than the other two manufactured ones because it needs to hold a stuffed meat mass in excess of 1 kg while hanging on a smoke house tree. This casing has to be removed prior to eating the product because it has a tough texture that can not be easily broken down by chewing. The thickness of this casing was actually not different from the previous ones. However, a closer look under the microscope, and especially under polarized light, reveals a high concentration of connective tissue fibers (mostly aligned parallel to each other) compared to the other two manufactured casings. This gives the casing its strength and it is most likely that the manufacturer used organic/ inorganic cross linking agents such as aldehydes, smoke condensate, aluminum or ferric salts to treat the casings (SAVIC and SAVIC, 2002). Another common way is to use heat for cross linking but as indicated above, these are usually steps within a proprietary process not revealed to the customer.

The co-extruded casing, which is extruded around the sausage meat as it comes out of the stuffer, reveals a microstructure (Fis. 2e and 2f) pretty similar to the manufactured casings for the fresh and smoked sausages described above (Fis. 1f and 2b, respectively). This should not be a surprise because the raw materials for both types are pretty much the same and actually the so-called casing-dough (used for co-extrusion) is commonly supplied by the same companies that produce the dried manufactured casings. The main difference is in the way the casing is

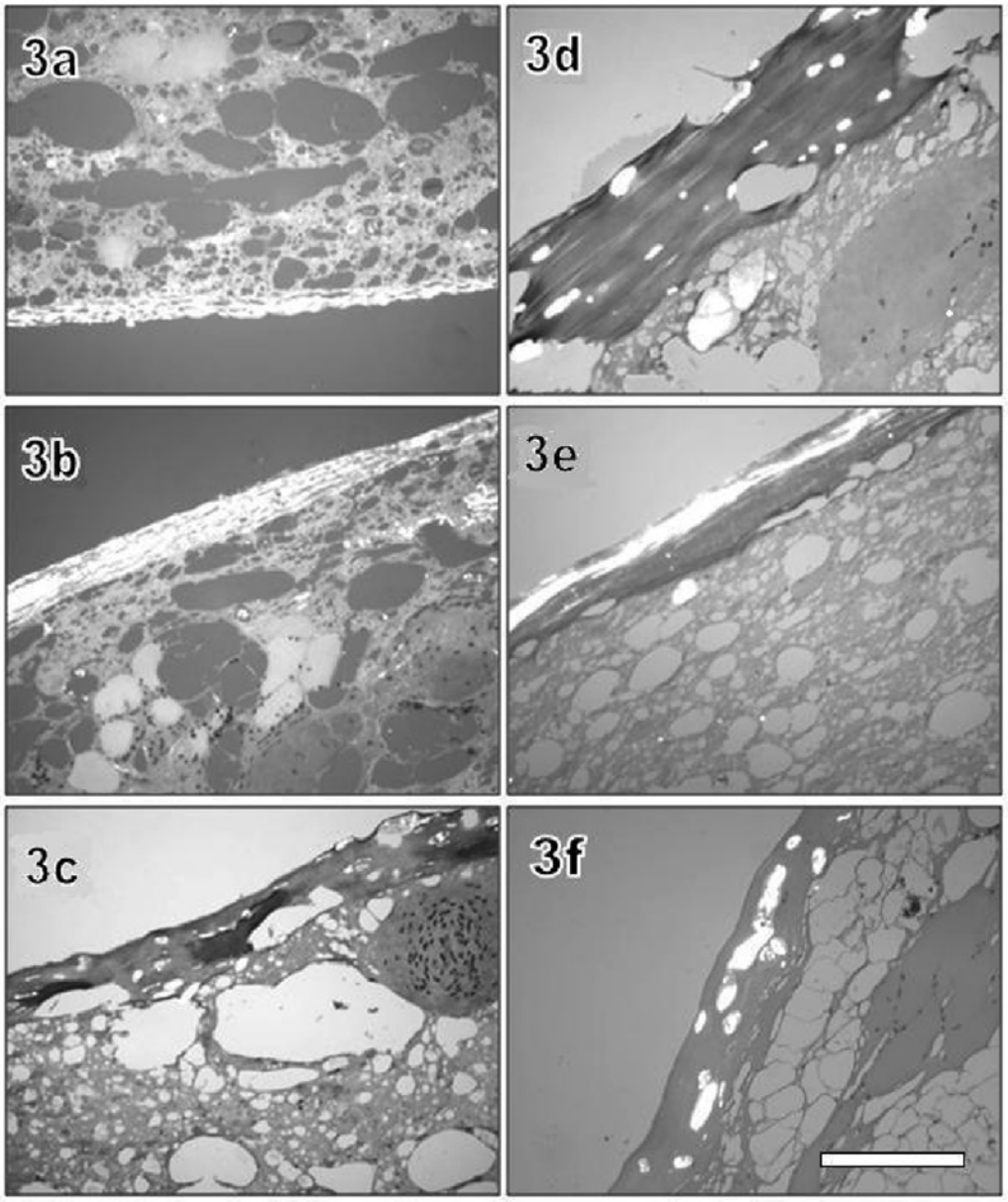


Fig. 3 - Light micrographs of cooked collagen casings using polarized light to reveal connective tissue fibers: (a) natural sheep; the upper part shows the meat batter; (b) natural hog; (c) manufactured collagen for fresh sausage; (d) manufactured for smoked sausage; (e) manufactured for large diameter ring sausage; (f) co-extruded. Bar = 200 μm.

applied to the meat product; i.e. applied in a gel form with an initial low protein level (6.3 %; Table 1). However very soon after application (within a few seconds) the processor starts drying it by exposure to a high salt concentration and/or heat. In the micrographs presented (Figs. 1e and 1f), the sausage meat is shown at the bottom because the casing stayed well attached to the meat; i.e. did not separate as was the case with the other treatments when a thin sausage sample was prepared for observation. In general, the co-extrusion technology has started to gain momentum over the past two years. Overall, the technology was introduced earlier, but the lack of proper equipment and gel formulations delayed its industrial application (LAZAR, 2006). Currently more meat processors are recognizing the advantages of using the technology (e.g., increased automation, labour savings, improved hygiene). Although becoming popular, this technology can benefit from some additional refining to make it more widely used.

The microstructure of the casings on the heated sausages (placed in boiling water for 3 minutes) shows basically identical structures as before cooking, but with some modifications. The two natural casings showed a thinner profile (Fis. 3a and 3b) but that is very probably due to stretching during the sausage filling process (i.e. as indicated in the Methods section, when casings did not adhere to the product, particularly when dealing with the raw product, a section of the casings was taken and pinned to a piece of cardboard; no attempt to control the stretching, only to ensure that no folds were present). The cooking might have induced the beginning of some collagen fiber gelatinization, but not to the extent that the casing would fall apart. Overall, a certain degree of collagen solubilization is an expected situation when sausages are scald-

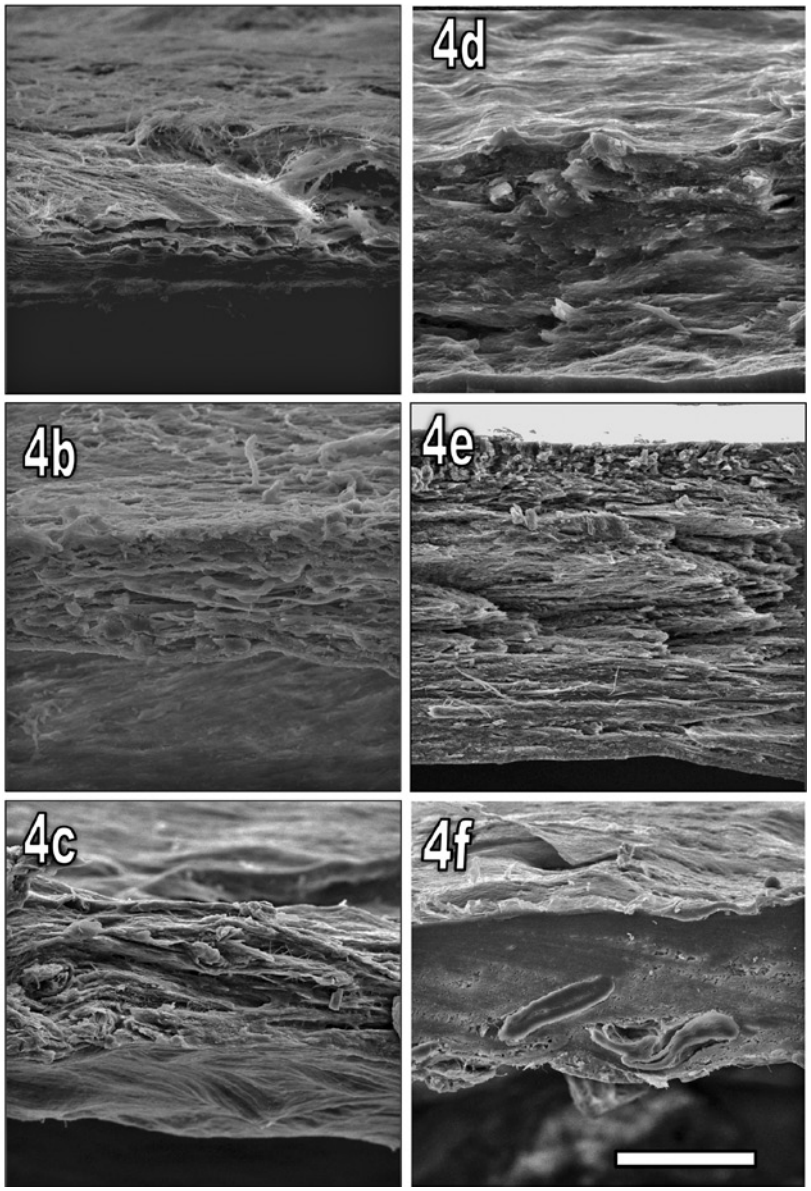


Fig. 4 - Scanning electron micrographs of raw collagen casings: (a) natural sheep; (b) natural hog; (c) manufactured collagen for fresh sausage; (d) manufactured for smoked sausage; (e) manufactured for large diameter ring sausage; (f) co-extruded. Bar = 30 μm.

ed for a longer period of time. In addition, casing stability is also due to the presence of elastin fibers which are not affected by heat. BAKKER *et al.* (1999) have also indicated that, "there were no microscopic differences between cooked and smoked sausage skins made in hog and sheep casings cured with additives and those made with salted casing."

The three manufactured casings showed the same basic microstructure after heating. The fresh sausage casing (Fig. 3c) stayed at the same thickness as prior to being heated. The smoked sausage casing (Fig. 3d) showed a thicker profile which might have been due to swelling during the boiling process. The heated casings of the ring sausage (Fig. 3e), stayed at the same thickness as prior to cooking, but if it was strengthened by cross linking (as postulated earlier), it would not be expected to swell. The cooked co-extruded casings (Fig. 3f) maintained the same microstructure as prior to heating.

Scanning electron microscopy showed a three dimensional structure of the casings. The sheep casings (Fig. 4a) show the arrangement of numerous, relatively thin, connective tissue fibers of the submucosa layer. The fibers resemble the SEM picture of collagen fibrils published by NOITUP *et al.* (2006). The hog casings (Fig. 4b) show a similar structure but with what appears to include some thicker connective tissue fibers. This was also evident in the light micrographs (Figs. 1a and 1b), but here the higher SEM magnification allows a closer examination. The manufactured smoked sausage casings (Fig. 4d) also show the connective tissue fiber arrangement within the surrounding proteinaceous matrix. The manufactured casings for ring sausage (Fig. 4e) show the unique arrangement of the parallel fibers packed together. This confirms the light

micrograph showing the tightly packed configuration (Fig. 2d). Within the co-extruded casings (Fig. 4f) two connective tissue fibers sticking out of the structure (i.e. towards the viewer) can be observed. The fibers are embedded in a dense coagulated proteinaceous matrix.

CONCLUSIONS

In summary, the study compared the microstructure of collagen casings produced in different ways (ranging from natural to manufactured and co-extruded). The light and electron microscopy micrographs reveal differences in the connective tissue fiber, number and orientation as well as casing thickness The information could be useful for the industry in understanding and developing new types of casings.

REFERENCES

Association of Official Analytical Chemist (AOAC). 1996. "Official Methods of Analysis", (3rd Ed). Washington, D.C.

Bakker W.A.M., Houben J.H., Koolmees P.A., Bindrich U. and Sprehe L. 1999. Effect of initial mild curing, with additives, of hog and sheep sausage casings on their microbial quality and mechanical properties after storage at difference temperatures. Meat Sci. 51:163.

Barbut S. 2002. Casings. In "Poultry Products Processing". p. 267, CRC Press. New York, USA.

Comer P. and Bradley R. 2002 Note on weights of intestine in small ruminants and expected risk reduction Report for European Natural Sausage Casing Association. August 26, 2002, Hamburg, Germany.

Gabis D.A. and Silliker J.H. 1974 Salmonella in natural animal casings Appl. Micro. 27:66

INSCA. 2004 International Natural Sausage Casing Association Scientific Working Group. Personal communication

Koolmees P.A. and Houben J.H. 1997. Inventory part: histology and microbiology of hog and sheep casings. In "Color Print Leaflet EU-CRAFT Project BRE 2. CT 94. 1495: Improved Treatment of Natural Sausage Casings for Quality Improvement in Automated Stuffing Processes". Deutches Institut für Lebensmitteltechnik eV, Quakkenbrück, Germany

Koolmees P.A., Tersteeg M.H.G., Keizer G., Van Den Broek J. and Bradley R. 2004. Comparative histological studies of mechanically versus manually processed sheep intestines used to make natural sausage casings J. Food Prot. 67:2742

Lazar V. 2006. Casing technology – co extrusion as well as natural. Meat Proc. Magazine. 5:20

Noitup P., Morrissey M.T. and Garnjanagoonchorn W. 2006 *In vitro* self-assembly of silver-line grunt type 1 collagen effects of collagen concentrations, pH and temperatures on collagen self-assembly J. Food Biochem. 30:547

Pimental E.R. 1981. Collagen. Acta Histochem. et Cytochem. 14:35.

Savic Z. and Savic I. 2002. Animal casings. In "Sausage Casings". p. 127. Victus Publishing. Vienna, Austria.

Swatland H.J. 1984. The connective tissues. In "Structure and Development of Meat Animals". p. 56. Prentice Hall Publication. Englewood Cliffs, NJ.

Wijnker J.J., Tersteeg M.H.G., Berends B.R., Vernooij J.C.M. and Koolmees P.A. 2008. Quantitative histological analysis of bovine small intestines before and after processing into natural sausage casings. J. Food Prot. 71:1199.

Revised paper received May 7, 2009 Accepted January 28, 2010

PAPER

EVALUATION OF SOME SENSORY PROPERTIES AND COOKING LOSS OF DIFFERENT BURGER FORMULATIONS

K.M. AL-MRAZEEQ, B.M. AL-ABDULLAH* and K.M. AL-ISMAIL
Department of Nutrition and Food Technology, Faculty of Agriculture, University of Jordan, Amman 11942, Jordan
*Corresponding author: Tel. +962 6 5355000, Fax +962 6 5355577
e-mail: basem@ju.edu.jo

ABSTRACT

Five burger formulations were prepared to study the effect of replacing beef and chicken fat with olive oil, of mixing chicken meat with beef on some of the sensory properties and cooking loss during storage at -18°C for 1 and 3 months and after grilling at 75°C for 20 min. The five burger formulations were: beef, chicken, mixed beef and chicken (50:50), beef with olive oil (90:10), and chicken with olive oil (90:10). Cooking loss, after formulation was between 43.28-51.30%; chicken with olive oil showed the lowest cooking loss, while the mixed treatment showed the highest cooking loss. At the end of storage (3 months), cooking loss significantly ($P \leq 0.05$) increased for all treatments. Mixing of chicken with beef significantly ($P \leq 0.05$) enhanced the sensory characteristics of the beef burger, whereas addition of olive oil had a variable influence on the sensory characteristics of the freshly prepared and stored burgers.

- Key words: cooking loss, meat burger, sensory property -

INTRODUCTION

Burger is a meat product prepared from minced lean meat, with or without the addition of other ingredients. The total fat content must not exceed 15% (JS: 1334/2002). In Jordan, burgers are prepared from two main meat sources: beef or chicken.

Many efforts have been made to improve the quality and stability of burgers because consumer demand for healthy fast food has increased rapidly in recent years (PAPADINA and BLOUKAS, 1999). Olive oil is the most mono-unsaturated vegetable oil. It contains 56.3-86.5% mono-unsaturated fatty acids (MUFA), 8-25% saturated and 3.6-21.5% polyunsaturated fatty acids (PUFA). It also has tocopherols and phenolic substances which act as antioxidants. Olive oil has a high biological value attributable to its high vitamin E/polyunsaturated fatty acid ratio (VIOLA, 1997). Olive oil has been used in the countries surrounding the Mediterranean Basin for more than 8,000 years. It has never been associated with undesirable consequences for human health (INTERNATIONAL OLIVE OIL COUNCIL, 1983).

There is convincing evidence that high MUFA diets may be of benefit in both the prevention and management of diabetes. The majority of these beneficial effects, particularly those on carbohydrates and lipid metabolism, are due to the substitution of MUFA for either saturated fats or carbohydrates in the diet. The pharmacological properties of olive oil phenolics provide new insights about the mechanisms by which good quality olive oil may contribute to lowering chronic heart disease (CHD) mortality (QUILES *et al.*, 2006). Nutritionists generally recommend a change in the composition of the fat associated with meat products by substituting MUFA with saturated fatty acids (ZANARDI *et al.*, 2000). Unsaturated fatty acids in the diet have been shown to decrease plasma low density lipoprotein (LDL) cholesterol in the blood. However, polyunsaturated fatty acids (PUFA) reduce high density lipoprotein (HDL) cholesterol levels, while lowering LDL cholesterol. It is known that HDL cholesterol is correlated with a reduced risk of CHD. On the other hand, increasing mono-unsaturated fatty acid contents (MUFA) in the diet does not decrease the plasma HDL-cholesterol level but lowers LDL cholesterol in the plasma (MATTSON and GRUNDY, 1985).

The type of meat and fat that are used in meat products affects the following quality parameters of these products: water-holding capacity, lipid binding and stability, oxidative stability, sensory properties, emulsion characteristics, textural characteristics, nutritional value and other characteristics. Little information has been published in the literature on the effect of partial replacement of beef meat with chicken meat and partial replacement of beef tallow or chicken fat with olive oil on the sensory properties of burgers. The aim of this study was to determine the effect of partial replacement of beef tallow and chicken fat with olive oil, the effect of partial replacement of beef tallow and meat with chicken meat and fat (50:50) and the effect of grilling the above-mentioned formulations on some sensory properties of freshly prepared and stored burgers.

MATERIALS AND METHODS

Burger preparation

The raw materials (meat and olive oil) used to prepare the burgers were purchased from the local markets. The combinations of beef and chicken burgers with beef tallow, chicken fat and olive oil (Table 1) were prepared as follows: samples were prepared in the meat technology laboratory at the University of Jordan. The fresh meat was coarsely ground in a Hobart mincer (Hobart, London, UK) through an 8 mm plate; water was added and then all was thoroughly mixed. The level of fat was adjusted to about 15% by adding beef or chicken fat or olive oil; the olive oil was added as such (olive oil) without any modification. Fat adjustments were carried out after determining the actual level of fat in the mixture by using the Soxhlet method. The prepared mixtures were then shaped into (100 g) patties using a household burger mould press (Meat Technology Laboratory, University of Jordan). Some of the freshly prepared burger samples were taken and analyzed for cooking loss and for some sensory properties after grilling. The remaining samples were kept in a freezer (at -18°C); the frozen samples were analyzed after one and three months of storage. Cooking loss and some sensory properties after grilling were determined.

Proximate composition

Samples were analyzed before and after grilling for moisture, fat and protein following AOAC (1995) procedures (analyses were carried out in duplicate).

Grilling

Burgers were cooked directly from the frozen state by contact grilling on a preheated electric grill (model LT15, Ankara, Turkey) for 20 min until they reached the brown cooked-color (grilling temperature 115°C, internal temperature 75°C for 15 sec). The upper part of the grill was closed and came into direct contact with the burgers. The internal temperature of the burgers was monitored with a thermocouple (Digitec, Modena, Italy) placed at the geometric center of the sample.

Table 1 - Burger formulations of beef, chicken, a combination of beef and chicken with beef tallow and chicken fat, beef with olive oil and chicken with olive oil.

Ingredients	Treatments				
	Beef burger	Chicken burger	Beef and chicken with burger (1:1)	Beef burger with olive oil (90:10)	Chicken burger with olive oil (90:10)
Meat (kg)	5	5	5 (2.5 kg beef and 2.5 kg chicken)	5	5
Fat content adjustment to 15%	Approx. 15% by addition of beef tallow	Approx. 15% by addition of chicken fat	Approx. 15% by addition of beef tallow and chicken fat (1:1)	Approx. 15% by addition of olive oil	Approx. 15% by addition of olive oil
Water (kg)	0.25	0.25	0.25	0.25	0.25

Cooking loss

Percent cooking loss was determined by using weight differences between raw and cooked burgers as follows:

$$\% \text{ cooking loss} = \frac{\text{= Raw frozen burger weight (g) – – Cooked burger weight (g)} \times 100}{\text{Raw frozen burger weight (g)}}$$

The frozen samples were weighed directly and then grilled. The samples were left to cool to room temperature, and were then weighed. The cooking loss was calculated using the sample weight data. Triplicate samples were used for each treatment.

Sensory evaluation

Cooked burgers from each treatment were evaluated by 18 panelists from the sensory evaluation team at the Department of Nutrition and Food Technology. The panelists were both male and female, and were of different ages; they were requested to taste each sample separately without comparing it with other samples. Panelists were familiarized with the questionnaire form used. The samples were evaluated for desirability in appearance, color, tenderness, flavor, juiciness and overall acceptability using a 9-hedonic scale test as described by LARMOND (1991), varying from 9 (like extremely) to 1 (dislike extremely). Pieces of bread and water were used to neutralize the taste between samples.

Statistical analysis

Statistical analysis of data was carried out using statistical analysis system package (SAS Inc., 2000). The data obtained were analyzed using a Split Plot Design to study the effect of treatments on the cooking loss and sensory evaluation results. The significant difference of the mean was determined using the Least Significant Difference (LSD) method. The coefficient of correlation test between the sensory scores for the burgers was estimated (SAS Inc., 2000).

RESULTS

Moisture, fat and protein contents

The moisture, fat and protein contents of all the raw and grilled burgers during the storage time are presented in Table 2. These data were collected in order to have a better understanding of the product.

Cooking loss

The cooking loss data obtained from the difference between the weight before and after grilling is illustrated in Table 3. The freshly prepared and stored chicken with olive oil sample showed the lowest cooking loss (43.28-47.39%), while the mixed sample showed the greatest loss (51.30-53.17%). It can be observed that at each storage period, the cooking loss of all the samples, except those of chicken treated with olive oil, showed comparable values; the difference in cooking loss of these samples did not exceed 1.6%. Cooking loss at the start and after one month of storage was not significantly ($P>0.05$) different, but there was a significant ($P\leq0.05$) difference after three months of storage, since cooking loss was higher in the third month compared with the loss at the start and after one month.

Table 4 shows the percentages of moisture and fat loss due to grilling. The moisture loss percentage of the freshly prepared samples was between 20.37 and 25.62%, whereas the fat loss percentage was between 18.85 and 21.79%.

Table 2 - Effect of formulation, storage time and grilling on moisture, fat and protein contents of burger samples.

Characteristic	Time of storage (month)	Treatment									
		Beef		Chicken		Mixed		Beef with olive oil		Chicken with olive oil	
		raw	grilled	raw	grilled	raw	grilled	raw	grilled	raw	grilled
Moisture%	0	65.55	49.32	66.73	51.61	66.61	49.55	65.42	48.88	66.51	52.96
	1	65.42	49.25	66.68	51.53	66.59	49.50	65.40	48.79	66.41	52.84
	3	65.48	48.31	66.54	50.42	66.41	48.93	65.35	48.01	66.30	52.12
Fat%	0	15.11	11.83	15.33	12.31	15.02	11.79	15.23	11.55	15.00	12.16
	1	15.06	11.85	15.34	12.34	15.10	11.82	15.18	11.57	15.14	12.11
	3	15.10	11.46	15.40	11.89	15.15	11.39	15.13	11.22	15.11	11.86
Protein%	0	18.24	37.53	17.23	35.62	17.31	37.40	18.13	37.71	17.52	34.14
	1	18.28	37.58	17.26	35.65	17.32	37.45	18.01	37.76	17.49	34.29
	3	18.37	38.09	17.30	36.11	17.43	37.92	18.21	38.85	17.51	34.76

Table 3 - Percentage cooking loss of burger samples in relation to storage period.

Characteristic	Time of storage (month)	,Treatment**				
		Beef	Chicken	Mixed	Beef with olive oil	Chicken with olive oil
Cooking loss%	0	$_{b}49.69^{c}$	$_{b}50.22^{b}$	$_{b}51.30^{a}$	$_{b}50.26^{b}$	$_{b}43.28^{d}$
	1	$_{b}49.86^{c}$	$_{b}50.48^{b}$	$_{b}51.53^{a}$	$_{b}50.21^{b}$	$_{b}43.02^{d}$
	3	$_{a}51.70^{c}$	$_{a}52.63^{b}$	$_{a}53.17^{a}$	$_{a}52.78^{b}$	$_{a}47.39^{d}$

Each value is the mean of three replicates.
*Values within the same column with different subscripts are significantly ($p \leq 0.05$) different according to LSD.
**Values within the same row with different superscripts denote significant differences ($p \leq 0.05$) according to LSD.

Table 4 - Moisture and fat loss of burger samples due to grilling in relation to formulation and storage.

Characteristic	Time of storage (month)	,Treatment**				
		Beef	Chicken	Mixed	Beef with olive oil	Chicken with olive oil
Moisture loss%	0	$_{b}24.76^{c}$	$_{b}22.66^{d}$	$_{b}25.62^{a}$	$_{b}25.28^{b}$	$_{b}20.37^{e}$
	1	$_{b}24.72^{c}$	$_{b}22.72^{d}$	$_{b}25.66^{a}$	$_{b}25.40^{b}$	$_{b}20.43^{e}$
	3	$_{a}25.70^{b}$	$_{a}23.72^{c}$	$_{a}26.10^{a}$	$_{a}26.25^{a}$	$_{a}21.03^{d}$
Fat loss%	0	$_{b}21.06^{b}$	$_{b}19.70^{c}$	$_{b}21.51^{a}$	$_{b}21.79^{a}$	$_{b}18.85^{d}$
	1	$_{b}21.00^{b}$	$_{b}19.56^{c}$	$_{b}21.72^{a}$	$_{b}21.88^{a}$	$_{b}18.94^{d}$
	3	$_{a}24.11^{c}$	$_{a}22.79^{d}$	$_{a}24.82^{b}$	$_{a}25.84^{a}$	$_{a}21.51^{e}$

Each value is the mean of two replicates.
*Values within the same column with different subscripts are significantly ($p \leq 0.05$) different according to LSD.
**Values within the same row with different superscripts denote significant differences ($p \leq 0.05$) according to LSD.

Burgers stored for three months showed higher ($P \leq 0.05$) loss in moisture and fat than those at the start and one month of storage.

Sensory evaluation

The mean sensory scores for the various burger treatments are reported in Table 5. A 9-hedonic scale test was used which ranged from 9 to 1, where 9, 8, 7, 6, 5, 4, 3, 2 and 1 means: like extremely, like very much, like moderately, like slightly, neither like nor dislike, dislike slightly, dislike moderately, dislike very much and dislike extremely, respectively.

Appearance

Regarding the appearance of the freshly prepared samples, no significant ($P > 0.05$) differences were found among the treatments. They ranged from like slightly to like moderately. After one month of storage, the appearance scores of the mixed, chicken and beef treatments remained within the same range, while chicken and beef treatments with olive oil declined from neither like nor dislike to like slightly. After three months of storage, the mixed treatment had the same score, whereas beef with olive oil showed a remarkable decline to dislike slightly. Beef,

Table 5 - Effect of formulation and storage time on sensory evaluation scores of the burger samples.

Characteristic	Time of storage (month)	Treatment				
		Beef	Chicken	Mixed	Beef with olive oil	Chicken with olive oil
Appearance	0	$_{a}6.11^{a}$	$_{a}6.83^{a}$	$_{a}6.94^{a}$	$_{a}6.00^{a}$	$_{a}6.56^{a}$
	1	$_{a}6.06^{ab}$	$_{ab}6.22^{ab}$	$_{a}7.00^{a}$	$_{a}5.33^{b}$	$_{a}5.61^{b}$
	3	$_{a}5.94^{ab}$	$_{b}5.50^{b}$	$_{a}6.83^{a}$	$_{b}4.16^{c}$	$_{a}5.72^{b}$
Color	0	$_{a}5.67^{b}$	$_{a}6.61^{ab}$	$_{a}6.89^{a}$	$_{a}5.88^{b}$	$_{a}6.67^{ab}$
	1	$_{a}5.56^{bc}$	$_{ab}6.00^{ab}$	$_{a}7.00^{a}$	$_{a}5.39^{c}$	$_{a}5.61^{bc}$
	3	$_{a}6.33^{a}$	$_{b}5.11^{b}$	$_{a}6.44^{a}$	$_{b}4.00^{c}$	$_{a}5.61^{ab}$
Tenderness	0	$_{a}4.44^{b}$	$_{a}6.56^{a}$	$_{a}6.10^{a}$	$_{a}4.27^{b}$	$_{a}6.44^{a}$
	1	$_{a}4.55^{b}$	$_{a}6.33^{a}$	$_{a}6.72^{a}$	$_{a}4.50^{b}$	$_{a}6.50^{a}$
	3	$_{a}4.60^{b}$	$_{a}6.22^{a}$	$_{a}6.67^{a}$	$_{a}4.33^{b}$	$_{a}6.17^{a}$
Flavor	0	$_{a}4.94^{b}$	$_{a}6.33^{a}$	$_{a}5.78^{ab}$	$_{a}5.06^{b}$	$_{a}6.06^{a}$
	1	$_{a}5.06^{bc}$	$_{a}5.88^{ab}$	$_{a}6.28^{a}$	$_{ab}4.72^{c}$	$_{a}5.12^{bc}$
	3	$_{a}4.83^{bc}$	$_{a}5.50^{ab}$	$_{a}5.94^{a}$	$_{b}3.94^{c}$	$_{a}5.63^{ab}$
Juiciness	0	$_{a}4.17^{b}$	$_{a}6.44^{a}$	$_{a}6.11^{a}$	$_{a}4.44^{b}$	$_{a}6.17^{a}$
	1	$_{a}4.27^{b}$	$_{a}5.61^{a}$	$_{a}5.67^{a}$	$_{a}4.22^{b}$	$_{a}5.44^{a}$
	3	$_{a}4.44^{b}$	$_{a}5.61^{a}$	$_{a}5.83^{a}$	$_{a}3.56^{c}$	$_{a}5.50^{ab}$
Overall acceptability	0	$_{a}5.38^{b}$	$_{a}6.52^{a}$	$_{a}6.39^{ab}$	$_{a}5.39^{b}$	$_{a}6.10^{ab}$
	1	$_{a}5.00^{c}$	$_{ab}6.06^{ab}$	$_{a}6.50^{a}$	$_{ab}4.44^{c}$	$_{a}5.17^{bc}$
	3	$_{a}5.22^{a}$	$_{b}5.51^{a}$	$_{a}6.00^{a}$	$_{b}3.72^{b}$	$_{a}5.83^{a}$

Means in the same column with the same subscripts denote no significant differences among treatments ($p>0.05$) according to LSD.
Means in the same row with different superscripts denote significant differences among treatments ($p\leq0.05$) according to LSD.
Means are the average of 18 determinations.

chicken and chicken with olive oil ranged from neither like nor dislike to like slightly.

Color

The color scores of the freshly prepared samples determined by the panelists showed that the mixed sample had the highest value (6.89), while beef had the lowest value (5.67). The color of beef and beef with olive oil ranged from neither like nor dislike to like slightly, and no significant ($P>0.05$) difference was found between them. On the other hand, mixed, chicken and chicken with olive oil treatments had higher color scores than either beef treatment, where they ranged from like slightly to like moderately, and no significant ($P>0.05$) differences were found between them. Storage significantly ($P\leq0.05$) affected the color scores of chicken treatment and beef with olive oil treatment at the third month, while the color scores of the other treatments were not ($P>0.05$) affected by storage.

Tenderness

The tenderness scores for the freshly prepared samples showed that the chicken sample had the highest ($P\leq0.05$) score followed by chicken with olive oil and mixed samples which ranged from like slightly to like moderately. Beef and beef with olive oil samples had the lowest ($P\leq0.05$) tenderness scores and ranged from dislike slightly to neither like nor dislike. Storage time did not affect ($P>0.05$) the tenderness in the various treatments.

Flavor

Flavor evaluation of the burger samples immediately after formulation showed that chicken, chicken with olive oil and mixed treatments had significantly ($P\leq0.05$) higher flavor scores, which were close to like slightly. While the scores of beef and beef with olive oil treatments were not significantly ($P>0.05$) different and were close to neither like nor dislike. There was no significant ($P>0.05$) effect of storage on the flavor scores of all the treatments, except for beef with olive oil, which decreased during storage. However the flavor range of some treatments changed during storage, chicken and chicken with olive oil samples which had a flavor range of like slightly to like moderately at the beginning of storage which then changed to the range like slightly to neither like nor dislike. Beef with olive oil also had a remarkable ($P\leq0.05$) decrease in flavor scores at the end of storage time.

Juiciness

The juiciness scores of the freshly prepared burger samples showed that both chicken samples and the mixed sample had significantly ($P\leq0.05$) higher values that ranged from like slightly to like moderately. Both beef samples had significantly ($P\leq0.05$) lower scores that were

close to dislike slightly. The storage period affected the juiciness range of both chicken samples and the mixed sample, since their range decreased to like slightly to neither like nor dislike. Statistically, they were not significantly (P>0.05) different. However, storage period did not affect the range of either beef sample, but the range decreased to dislike slightly to dislike moderately for the beef with olive oil at the third month.

Overall acceptability

The overall acceptability evaluation of the freshly prepared burger samples showed that both chicken samples and the mixed sample which ranged from like slightly to like moderately had significantly higher (P≤0.05) overall acceptability scores, while both beef samples that ranged from neither like nor dislike to dislike slightly were not significantly (P>0.05) different from chicken with olive oil and the mixed treatments. During the storage period, beef, mixed and chicken samples remained in the same range, except for chicken which declined to the range of neither like nor dislike to like slightly at the end of storage. Overall acceptability of the chicken with olive oil declined to the range of neither like nor dislike to like slightly, whereas beef with olive oil declined significantly (P≤0.05) to the range of dislike slightly to dislike moderately at the third month of storage.

Correlation between the sensory scores

Table 6 shows the Pearson's correlation coefficients between the appearance, color, tenderness, flavor, juiciness and overall acceptability for the burger formulations. There were significant (P≤0.05) correlations between all of the sensory scores. High correlations were observed between appearance and color, appearance and overall acceptability, color and overall acceptability, tenderness and juiciness, flavor and juiciness, flavor and overall acceptability, as well as juiciness and overall acceptability. Moderate correlations were observed between appearance and flavor, appearance and juiciness, color and flavor, color and juiciness, tenderness and flavor, as well as tenderness and overall acceptability, whereas low correlations were observed between appearance and tenderness, also color and tenderness.

DISCUSSION

Cooking loss

Tables 3 and 4 illustrate the cooking loss, fat and moisture loss of burger treatments respectively. Burger treatments showed high cooking loss ranging between 43-51%, for the freshly prepared treatments. This is in agreement with results obtained by RODRIGUEZ-ESTRADA *et al.* (1997) and DREELING *et al.* (2000) who found that cooking loss was highest for grilled or deep fat fried burgers. PAN and PAUL SINGH (2001) reported that more than 30% water was lost when the meat was heated to 70°C, and the starting temperature for fat loss was assumed to be 30°C, since the fat usually starts to melt at a temperature above 30°C.

The chicken sample with olive oil showed lower cooking loss when compared to the corresponding samples without olive oil. This result shows the ability of the protein matrix to bind monounsaturated fat. This result is in agreement with those of LURUEÑA-MARTÍNEZ *et al.* (2004) who found that frankfurters prepared with olive oil had a lower percentage of fat separated during cooking than those made with the same animal fat. This difference was statistically significant, showing an important ability of the mixture used to retain monounsaturated fat in the protein matrix.

KAYAARDı and GŐK (2003) studied four formulations of Turkish soudjouks; one control using 85% beef and 15% beef fat and three treatments by replacing 20, 40 and 60% of beef fat with virgin olive oil incorporated as pre-emulsified fat with soy protein isolates. They found that weight loss was lower for soudjouks containing olive oil. SEVERINI *et al.* (2003) studied the partial substitution of pork backfat with extra-virgin olive oil in salami products. After 30 days of storage, the products with olive oil had a higher water activity than the basic formulation. The slow rate of dehydration of the samples containing oil was due to the production of water in oil emulsion in the samples with oil.

The chicken sample with olive oil had lower cooking loss when compared to beef samples which is due to a higher water holding capacity, lipid capacity and lipid stability of the chicken meat rather than beef meat (ZORBA and KURT, 2006; DZUDIE *et al.*, 2004). In the mixed treatment the cooking loss value was expected to be between the beef and chicken sample values, but an unexpected result was obtained. The outcome showed that the mixed treatment had the highest cooking loss. More investigation is needed to explain these results.

The highest cooking loss was found after three months of storage which can be attributed to the weakness of the protein matrix to entrap moisture and fat during storage. Moreover, this weakness of the protein matrix results in a decrease in the water and lipid holding capacity and stability, which could be due to the denaturation of protein during frozen storage.

Sensory evaluation

As shown in Table 5, none of the sensory characteristics exceeded the range of like moderately, while some of the sensory characteris-

Table 6 - Pearson's correlation coefficients among the sensory scores of the burger formulations.

Sensory scores	Appearance	Color	Tenderness	Flavor	Juiciness	Overall acceptability
Appearance	1.00	0.95*	0.60*	0.79*	0.72*	0.90*
Color	0.95*	1.00	0.59*	0.71*	0.70*	0.85*
Tenderness	0.60*	0.59*	1.00	0.78*	0.96*	0.79*
Flavor	0.79*	0.71*	0.78*	1.00	0.84*	0.92*
Juiciness	0.72*	0.70*	0.96*	0.84*	1.00	0.88*
Overall acceptability	0.90*	0.85*	0.79*	0.92*	0.88*	1.00

*Correlation is significant at the 0.05 level.

tics declined to dislike slightly. These low sensory scores for all the treatments could be attributed to the fact that the burgers were free of additives that are usually added to these types of products such as spices, salt, protein derivatives of vegetable origin, dietary fibers, antioxidants, flavor enhancers and other additives which enhance the sensory characteristics and stability of the meat products.

Another factor which might have affected the sensory characteristics in this study is the fat content; it was about 15% in all burger treatments, and these products should contain 20-30% fat to give the desirable succulence and texture (WILSON *et al.*, 1981). BERRY and LEDDY (1984) demonstrated that higher tenderness and juiciness values of ground beef patties were related to higher fat contents. Consequently, panfrying yielded higher juiciness and flavor ratings than broiling, since broiling resulted in more fat loss. SOFOS and ALLEN (1977) reported that reducing the fat content may alter meat product quality, since fat acts as a reservoir for flavor compounds and contributes to product texture. However, WARRISS (2000) attributed the reductions in juiciness as a result of cooking to the myofibrillar protein denaturation which leads to water loss from the meat.

Mixing of chicken with beef meat enhanced the sensory characteristics of the beef. In general, mixed samples had higher sensory scores than beef samples, and were close to the chicken samples. Furthermore, the mixed treatment was the most stable with respect to the sensory evaluation during the storage period. The freshly prepared mixed treatment had higher appearance and color scores (6.94 and 6.89, respectively) than those of beef and chicken treatments (6.11 and 6.83, respectively for appearance) and (5.67 and 6.61, respectively for color). These high scores for appearance and color of the mixed treatment, compared to those scores of beef and chicken treatments, may have been due to a reduction in redness of the beef treatment and of yellowness in the chicken treatment. The appearance and color of the mixed treatment was between the beef and chicken treatments (between redness and yellowness), since beef contains more myoglobin than chicken.

The sensory qualities of appearance and color are related as shown in Table 6, so this modification in color in the mixed treatment affected the appearance, which in turn affected the panelist's evaluation. FERNÁNDEZ-LÓPEZ *et al.* (2006) showed that appearance and color are the most important attributes among the sensory characteristics which influence customer choice. In the meat industry, color also plays a very important part in quality evaluation, as it is one of the main appearance attributes that determine the purchase decisions regarding meat (CARPENTER *et al.,* 2001).

The evaluation of tenderness of meat and meat products by panelists is correlated mainly with juiciness (Table 6), so the scores for tenderness and juiciness of beef (4.44 and 4.17, respectively), chicken (6.56 and 6.44, respectively) and mixed treatments (6.10 and 6.11, respectively) were very close. However, tenderness and juiciness scores of the mixed treatment were significantly higher than those of the beef treatment, and very close to those of the chicken treatment. This result indicates that tenderness and juiciness are more strongly related to the type of meat than other factors.

These results confirm those obtained by ABDULLAH (2007) who studied the sensory properties of five canned luncheon meat formulations, and reported that juiciness is related to the type of meat used in the formulations, rather than the chemical composition of the luncheon meat. Although, reduction of the fat content in frankfurters had been reported to decrease juiciness (PARK *et al.,* 1989), in our study, as shown in Table 2, the fat content of the mixed treatment (11.79%) was very close to that of the beef treatment (11.83%), but the juiciness scores for the mixed treatment were higher than those of the beef treatment. This might be attributed to the fact that the taste panelists tended to not like juiciness that is associated with an increased fat content (LEE *et al.,* 1987).

Overall acceptability of the mixed treatment (6.39) was between those of the beef and chicken treatments (5.38 and 6.52, respectively). This overall acceptability of each treatment was the result of the other sensory characteristics, which were evaluated by the panelists, since there were

high correlations between the overall acceptability and all the other sensory attributes (Table 6).

Substitution of meat fat in beef and chicken samples with olive oil, in general, did not affect the sensory characteristics, since no significant differences were found between the sensory scores of the samples with and without olive oil (Table 5). Beef with olive oil showed lower sensory scores after three months of storage compared to the beef sample with tallow, whereas the sensory characteristics of the chicken with olive oil remained stable during the storage period. These results agree with those of BLOUKAS *et al.* (1997) who reported that color of fermented pork sausage in which animal fat was replaced with olive oil was lighter and more yellowish than the control one, while there were no significant differences between treatments with respect to redness, odor and taste. BLOUKAS and PANERAS (1993) reported that low-fat frankfurters (10%) made with olive oil showed no significant difference in color means and storage time compared to the control sample.

CROSS *et al.* (1976) found a direct correlation between the juiciness and weight loss of steaks during cooking. The juiciest steaks were those which sustained the least weight loss. In contrast, in this study chicken with olive oil treatment showed a lower cooking loss compared with the chicken treatment (Table 3), but the tenderness and juiciness scores of these two treatments were not significantly different. These results agree with those obtained by SUNDARARAJAN *et al.* (1999) who found no direct correlation between weight loss and juiciness indicators of cooked hamburgers.

Storage time did not significantly affect the sensory evaluation scores of each treatment, except for chicken in which the appearance, color and overall acceptability at the end of storage were lower than the initial values. Appearance, color, flavor and overall acceptability of beef with olive oil were also affected by storage time. This decline in sensory parameters of these samples might be attributed to the oxidation which could deteriorate those parameters.

However, McMILLIN *et al.* (1991) reported that the sensory rancid flavor scores of beef patties did not agree strongly with the TBARS values. AWONORIN (1993) found that the measured increased in both peroxide value (PV) and TBARS values of smoked chicken sausage did not affect the panelist assessment for flavor. Tenderness, flavor and color of frozen samples were not affected by storage time, while juiciness and overall acceptability decreased as the period of cold storage increased.

Changes in color of stored ground beef were found by HUNT *et al.* (1999). They reported that the longer meat was stored, the more predominant either oxymyoglobin (OMb) or metmyoglobin (MMb) was as opposed to deoxymyoglobin (DMb), which in turn, would predispose the meat to a faster browning rate. FERNÁNDEZ-LÓPEZ *et al.* (2006) reported that the redness of burgers decreased as storage time increased, since redness values declined very rapidly during the first 6 days of storage. PHILLIPS *et al.* (2001) also reported a decrease in redness values of ground beef linked to longer storage time.

REFERENCES

Abdullah B.M. 2007. Properties of five canned luncheon meat formulations. Inter. J. Food Sci. Technol. 42: 30.

AOAC. 1995. "Official Methods of Analysis" (16th Ed.). Association of Official Analysis Chemists. Washington, DC.

Awonorin S.O. 1993. Quality of smoked chicken-Guinea-Fowl sausage as affected by processing conditions and cold storage. Lebensm.-Wiss. u.-Technol. 26: 285.

Berry B.W. and Leddy K.F. 1984. Effects of fat level and cooking method on sensory and textural properties of ground beef patties. J. Food Sci. 49: 870.

Bloukas J.G. and Paneras E.D. 1993. Substituting olive oil for pork backfat affects quality of low-fat frankfurters. J. Food Sci. 58: 705.

Bloukas J.G., Paneras E.D. and Fournitzis G.C. 1997. Effect of replacing pork backfat with olive oil on processing and quality characteristics of fermented sausages. Meat Sci. 45: 133.

Carpenter C.E., Cornforth D.P. and Whittier D. 2001. Consumer preference for beef colour and packaging did not affect eating satisfaction. Meat Sci. 57: 359.

Cross H., Stanfield M. and Koch J. 1976. Beef palatability as affected by cooking rate and final center temperature. J. Animal Sci. 43: 114.

Dreeling N., Allen P. and Butler F. 2000. Effect of cooking method on sensory and instrumental texture attributes of low-fat beef burgers. Lebensm.-Wiss.u.-Technol. 33: 234.

Dzudie T., Kouebou C., Essia-Ngang J. and Mbofung C. 2004. Lipid sources and essential oils effects on quality and stability of beef patties. J. Food Eng. 65: 67.

Fernández-López J., Jiménez S., Sayas-Barberá E., Sendra E. and Pérez-Alvare J.A. 2006. Quality characteristics of ostrich (*Struthio camelus*) burgers. Meat Sci. 73: 295.

Hunt M., Sorheim O. and Slinde E. 1999. Color and heat denaturation of myoglobin forms in ground beef. J. Food Sci. 64: 847.

International Olive Oil Council. 1983. International trade standards applying to olive oil and olive-residues oils. COI/T. 15 NC NO 1. Madrid, Spain.

Jordanian Institute for Standards and Metrology, The Hashemite Kingdom of Jordan. 2002. Standard No. JS: 1334/2002, (2nd Ed.). Meat and Meat Products: Poultry-Chilled and/or Frozen Chicken /or Burger. Amman, Jordan. (In Arabic)

Kayaardı S. and Gök V. 2003. Effect of replacing beef fat with olive oil on quality characteristics of Turkish soudjouk (sucuk). Meat Sci. 66: 249.

Larmond E. 1991. "Laboratory Methods for Sensory Evaluation of Food", (2nd Ed.). Canadian Department of Agriculture Publication. Ottawa, Canada.

Lee C.M., Whiting R.C. and Jenkins R.K. 1987. Texture and sensory evaluation of frankfurters made with different formulations and process. J. Food Sci. 52: 896.

Lurueña-Martínez M.A., Vivar-Quintana A.M. and Revilla I. 2004. Effect of locust bean/xanthan gum addition and replacement of pork fat with olive oil on the quality characteristics of low-fat frankfurters. Meat Sci. 68: 383.

Mattson F.H. and Grundy S.M. 1985. Comparison of dietary saturated, monounsaturated and polyunsaturated fatty acids on plasma lipids and lipoproteins in man. J. Lipid Res. 26: 194.

McMillin K. W., Bidner T.D., Felchle S.E., Dugas S.M. and Koh K.C. 1991. Flavor and oxidative stability of ground

beef patties as affected by source and storage. J. Food Sci. 56: 899.

Pan Z. and Paul Singh R. 2001. Physical and thermal properties of ground beef during cooking. Lebensm.-Wiss. u.-Technol. 34: 437.

Papadina S.N. and Bloukas J.G. 1999. Effect of fat level and storage conditions on quality characteristics of traditional Greek sausage. Meat Sci. 51: 103.

Park J., Rhee K.S., Kccton J.T. and Rhee K.C. 1989. Properties of low-fat frankfurters containing monounsaturated and omega-3 polyunsaturated oils. J. Food Sci. 54: 500.

Phillips A.L., Mancini R., Sun Q., Lynch M.P. and Faustman C. 2001. Effect of erythorvic acid on cooked color in ground beef. Meat Sci. 57: 31.

Quiles J.L., Ramirez-Tortosa M.C. and Yaqoob P. 2006. "Olive Oil and Health". CABI head office. Oxford, UK.

Rodriguez-Estrada M.T., Penazzi G., Caboni M.F., Bertacco G. and Lercker G. 1997. Effect of different cooking methods on some lipid and protein components of hamburgers. Meat Sci. 45: 365.

SAS Institute. 2000. "SAS User's Guide in Statistics", (8th Ed.). Cary, NC., U.S.A.: SAS Institute, Inc.

Severini C., De Pilli T. and Baiano A. 2003. Partial substitution of pork backfat with extra-virgin olive oil in 'salami' products: effects on chemical, physical and sensorial quality. Meat Sci. 64: 323.

Sofos J.N. and Allen C.E. 1977. Effects of lean meat source and levels of fat and soy protein on the properties of wiener-type products. J. Food Sci. 42: 875.

Sundararajan N., Ndife M., Basel R. and Green S. 1999. Comparison of sensory properties of hamburgers cooked by conventional and carcinogen reducing "safe grill" equipment. Meat Sci. 51: 289.

Viola P. 1997. "Olive Oil and Health". International Olive Oil Council. Madrid, Spain.

Warriss P. D. 2000. "Meat Science" (an introductory text). CABI Publishing. ,Oxford, UK.

Wilson N., Dyett E., Hughes R. and Jones C. 1981. "Meat and Meat Products: Factors Affecting Quality Control". Applied Science Publishers. London.

Zanardi E., Novelli E., Ghiretti G.P. and Chizzolini R. 2000. Oxidative stability of lipids and cholesterol in salame Milano, coppa and Parma ham: dietary supplementation with vitamin E and oleic acid. Meat Sci. 55: 169.

Zorba Ö. and Kurt Ş. 2006. Optimization of emulsion characteristics of beef, chicken and turkey meat mixtures in model system using mixture design. Meat Sci. 73: 611.

Paper received March 11, 2009 Accepted August 3, 2009

PAPER

EVOLUTION OF MECHANICAL VARIABLES OF WINEGRAPES FOR ICEWINE PRODUCTION DURING ON-VINE DRYING

L. ROLLE*, F. TORCHIO, E. CAGNASSO and V. GERBI
DIVAPRA - Settore di Tecnologie Alimentari, Università degli Studi di Torino,
Via L. da Vinci 44, 10095 Grugliasco, Torino, Italy
*Corresponding author: Tel. +39 0116708558, Fax +39 0116708549
e-mail: luca.rolle@unito.it

ABSTRACT

The evolution of some mechanical properties of the berry skin and pedicel during on-vine drying of wine-grapes was studied, in order to assess the suitability of two alpine cultivars, Becuét and Fumin, for the production of icewines. This overripening process on the vine, which lasts until complete freezing of the grapes, results in marked changes in the mechanical characteristics of the grapes. During grape dehydration, the skin thickness increased in both cultivars (Becuét +73 μm, Fumin +85 μm). The skin hardness also increased, particularly in the Becuét berries, in which the break skin force and break skin energy values increased of 0.148 N and 0.823 mJ, respectively. In contrast, the peduncle detachment force decreased during the drying process; in Becuét grapes, this occurred after only 15 days. While the two cultivars have different mechanical properties, both proved to be suitable for the production of icewines ("Vini del ghiaccio"); the changes that occur during on-vine drying do not preclude the resistance of the berries to shattering and of the berry skin to splitting.

- Key words: berry skin, grape dehydration, grape withering, peduncle, texture analysis -

INTRODUCTION

Many varieties of wines world-wide are produced by partial or total use of overripe and/or dehydrated grapes. Among these wines, there are those characterized by the absence of sugar ("dry"), and other, ("sweet" or dessert wines), which contain the presence more or less elevated levels (10-250 g/L) of residual sugar. The latter are generally very well-known and are commercially more important. These include the "Passiti" wines, the "icewines", the Sauternes, the Tokaij and the Jerez, as well as many other lesser known wines that are produced in almost all viticultural areas of the world (BARBANTI *et al.*, 2008, SOLEAS and PICKERING, 2007; GENOVESE *et al.*, 2007; MIKLÓSY *et al.*, 2000).

Various withering procedures are used in the production of these wines. Dehydration of harvested grapes (off-vine grape drying) can be performed directly in the sun, as in the production of Passito di Pantelleria or in sweet Greek wines (ASTORINO and DI STEFANO, 2003; KARAGIANNIS *et al.*, 2000), or indirectly in ventilated rooms, tunnel-dried (BELLINCONTRO *et al.*, 2004) with or without use of ventilation systems and/or thermohygrometric control (artificial overripening). On-vine grape drying is the withering technique that is used in the production of Sauternes and icewines (RIBÉREAU-GAYON *et al.*, 2000).

The various withering techniques influence the evolution of many compounds such as glycerol, aromatic substances (BAILLY *et al.*, 2006; COSTANTINI *et al.*, 2006), and polyphenols (AMATI *et al.*, 1983; FRANGIPANE *et al.*, 2007) that contribute directly to the sensory characteristics of the wine, as well as to the development and selection of the microflora present on the grapes (CHAMBERLAIN *et al.*, 1997; RIBÉREAU-GAYON *et al.*, 2000; URSO *et al.*, 2008). While the chemical and microbiological knowledge is available in the scientific literature, information about the evolution of the mechanical behavior of winegrapes during the different withering processes is very limited. In particular, there are no studies related to on-vine grape drying. Knowledge of the evolution of some mechanical variables such as the ease of pedicel detachment, or the hardness and thickness of the skin, is important because these aspects are directly related to the phenomena of shattering, resistance to splitting and plant diseases (GABLER *et al.*, 2003; LANG and DURING, 1990).

Canada, Germany and Austria are the main icewine producers followed by many other European countries including France, Hungary, Slovenia and Switzerland. Icewines ("Eiswein") are produced according to strict production standards and regulations. Harvesting and winemaking are monitored by public and/or private institutions. In Canada the grapes must be naturally frozen on the vine at -8°C or colder and this temperature must be maintained throughout the pressing process without artificial refrigeration (CLIFF *et al.*, 2002; NURGEL *et al.*, 2004). According to German regulations grapes must be frozen naturally at -7°C or lower and the must is required to have an Oechsle value of 125 degrees (ZIRALDO, 2000). The Vidal, Riesling, Traminer, Chardonnay and Muscat Ottonel cultivars are traditionally used to produce icewines, but some countries use local cultivars.

In Italy the climatic conditions for producing icewines ("Vini del ghiaccio") are only present in mountainous grape-producing environments. This study was conducted on two cultivars, Becuét and Fumin, that are mainly distributed in the Western Alps in Europe (SCHNEIDER *et al.*, 2001). These cultivars can adapt to a shorter growing season and freezing temperatures in those areas. One of the key factors in the economic development of viticulture and the wine industry in mountainous areas is the exploitation of ancient, local grape cultivars. In this context, the production of icewines could provide a major opportunity for companies operating in alpine regions. The climatic conditions suitable for the production of these wines are only found in such areas.

The purpose of this study was to assess the evolution of some mechanical characteristics during withering on the vine of different winegrapes cultivars used to produce icewines.

MATERIALS AND METHODS

Grapes, sampling and chemical analyses

Two red grapes varieties were studied: Becuét and Fumin. The university experimental vineyard is located at 750 m asl on the sun-exposed side of the Susa Valley in the Province of Turin (Piedmont, Italy). The study was carried-out in 2007.

Four hundred berries, with attached pedicels, were sampled for each cultivar on six dates, in a random manner, during the overripe period (20 September-to 19 December), according to the sampling method described by CARBONNEAU *et al.* (1991). Cluster fragments were randomly picked in the middle part of the cane or cordon; the first rows of the vineyard were excluded.

The climatic conditions (temperature, relative humidity, rain) in the vineyard were monitored by a Vantage PRO2 weather station (Davis Instruments, Hayward, CA, USA).

For each sampling date, the analytical variables of grape ripeness (soluble solids concentration, titratable acidity, pH) were estimated using EEC methods (1990). Malic acid was monitored by HPLC (SCHNEIDER *et al.*, 1987).

Texture analysis

The mechanical properties of grapes were measured on sub-samples of 30 berries each from the 400 berries described above, for each cultivar and for each test. The remaining berries were used for chemical determinations.

To avoid any alteration, the tests were performed on the day that the berries were picked. Prior to testing, the berries, arranged in a single layer, were thermally conditioned at 20°C in a thermostatically controlled chamber.

A Universal Testing Machine TAxT2i Texture Analyzer (Stable Micro Systems - SMS, Surrey, UK) equipped with a HDP/90 platform (with and without perforations) and a 5 kg load cell was used. All the acquisitions were made at 400 Hz; data were evaluated using the Texture Expert Exceed software package (vers. 2.54 in Windows 2000). The test conditions applied, the probe and the mechanical parameters measured (Table 1), were based on previous work (LETAIEF *et al.*, 2008a,b; ROLLE *et al.* 2007).

Statistical analysis

Analysis of variance (ANOVA) was applied to all the variables studied. Statistical analyses were performed using the statistical software SPSS version 17.0 (SPSS Inc., Chicago, IL, USA). All statistical analysis results are reported in Table 2.

RESULTS AND DISCUSSION

After 90 days of withering on the vine, both cultivars reached the sugar level required for the production of icewines in Europe (Fig. 1). The early-morning temperatures (from -7° to -14°C) met the icewine production requirements. The sugar concentration increased regularly throughout the period of overripening in parallel with berry dehydration and relative weight loss (Fig. 2).

The decrease in acidity during slow dehydration is also observed in long-term drying under uncontrolled environmental conditions and has been attributed to malic acid depletion (BELLINCONTRO *et al.*, 2002). This aspect was particularly evident in Fumin grapes in which the titratable acidity and malic acid concentration decreased markedly (Fig. 1).

Fig. 3 shows the evolution of the berry skin mechanical variables. The F_{sk} and W_{sk} values, in spite of the elevated dispersion of the data, increased progressively during grape overripening on the vine, while E_{sk} showed the opposite trend which was more evident in the Becuét grapes than in the Fumin ones. Throughout the period of drying on-vine the Becuét berry skin was characterized by higher values for both skin break force and skin break energy. These factors are important because they allow the grapes to resist fungal diseases, as well as strong winds.

In parallel with the hardening of the skin tis-

Table 1 - Operating conditions used in the texture analysis of Becuét and Fumin grapes.

Test	Probe - Platform	Test speed	Force	Mechanical properties
Berry skin hardness	SMS P/2N Needle; HDP/90 platform	1 mm s^{-1}	compression 3 mm	F_{sk} = Berry skin break force (N) W_{sk} = Berry skin break energy (mJ) E_{sk} = Skin Young's modulus (N/mm)
Berry skin thickness	SMS P/2 Ø 2 mm; HDP/90 platform	0.2 mm s^{-1}	-	Sp_{sk} = Berry skin thickness (µm)
Peduncle detachment resistance	SMS A/PS modified with rigid arm; HDP/90 perforated (Ø 5 mm) platform	1 mm s^{-1}	traction 10 mm	F_{ped} = Peduncle detachment force (N) W_{ped} = Peduncle detachment Energy (mJ)

Table 2 - Statistical analysis results. Latin letters (a, b, c) in the same column are used to compare the influence of overripening time within each cultivar. Greek letters (α, β) in the same column are used to compare the cultivar differences. The same letterare not significantly different (P≤0.05).

Over ripening	Berry weight		F_{sk}		W_{sk}		E_{sk}		Thickness		F_{ped}		W_{ped}	
days	Becuét	Fumin	Becuét	Fumin	Becuét	Fumin	Becuét	Fumin	Becuét	Fumin	Becuét	Fumin	Becuét	Fumin
0 (20 sept)	a	a	b,α	a,β	b,α	b,β	a,α	a,α	c,α	d,α	a,α	a,α	ab,α	ab,α
14 (4 oct)	a	a	ab,α	a,β	ab,α	b,β	b,α	b,α	b,α	bc,α	b,β	a,α	a,α	ab,α
29 (19 oct)	ab	ab	b,α	a,β	b,α	b,β	b,β	ab,α	ab,β	a,α	b,α	ab,α	bc,α	b,α
48 (7 nov)	ab	ab	ab,α	a,β	ab,α	a,β	c,α	c,β	ab,α	cd,α	b,β	ab,α	c,β	ab,α
66 (25 nov)	b	b	ab,α	a,β	ab,α	a,β	d,α	c,α	a,β	a,α	b,β	ab,α	bc,β	a,α
90 (19 dec)	b	b	a,α	a,β	a,α	a,β	d,α	c,α	a,α	ab,α	b,β	b,α	bc,β	a,α

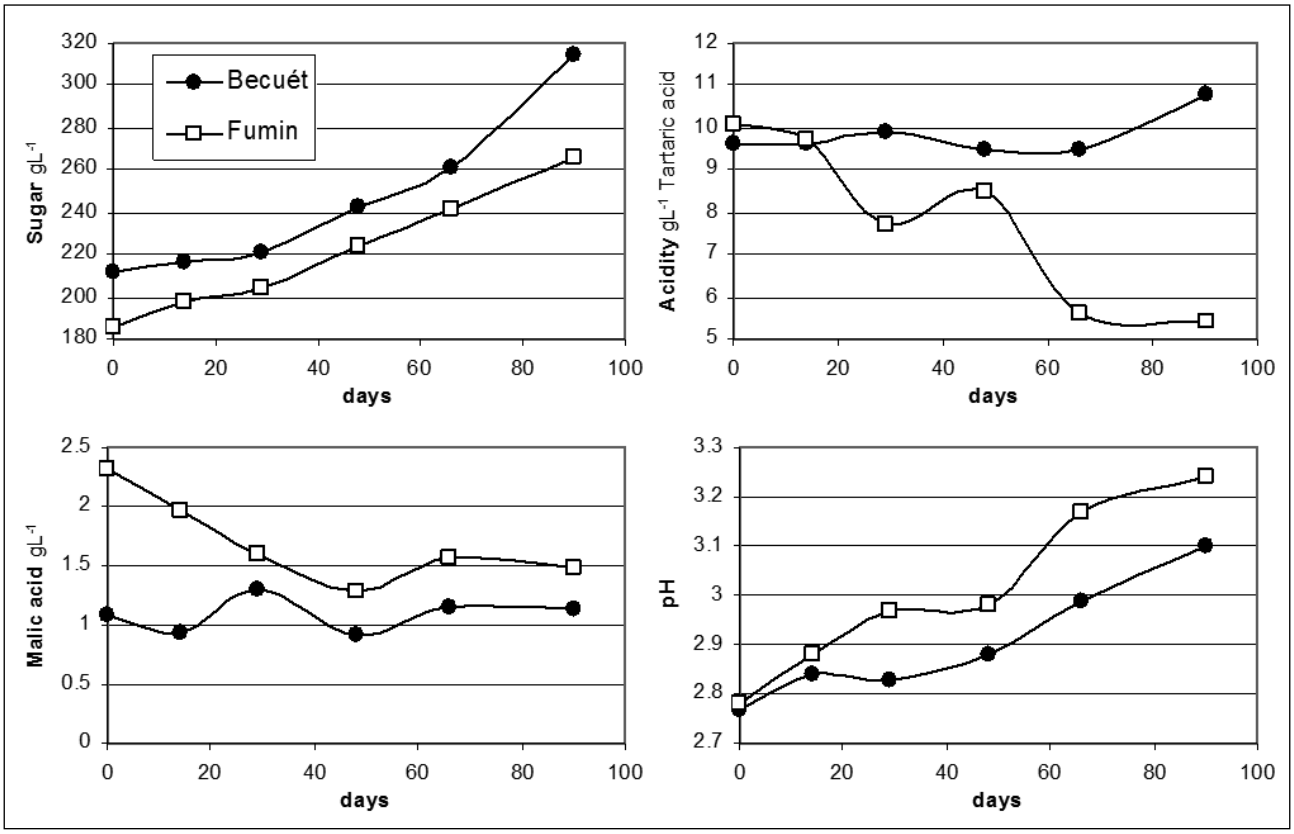


Fig. 1 - Monitoring variables used for assessing overripening of grapes on the vine for the Becuét and Fumin cultivars.

sues, the average skin thickness also increased (Fig. 4), which partially explains the higher F_{sk} and W_{sk} values discussed above. The increase in thickness was particularly rapid in the first 30 days. At the end of the withering period, the skin thickness in both cultivars had increased about 35%. There were no differences between Becuét and Fumin with respect to Sp_{sk}, although the latter generally had higher average values.

On 1 November 2007, there were 32 mm of rainfall in the vineyard after a prolonged period of drought (75 days). Some of the abnormal data related to Sp_{sk}, and the titratable acidity in the Fumin grapes on 7 of November can be attributed to this rainfall. During the rest of the experimental period there was no rain, but 30 cm of snow fell on 12 December. At the time of the last sampling (19 December), the clusters carried a considerable amount of snow, the weight of which could have caused a significant loss of grape berries if the detachment force was low. The evolution of the strength and energy of detachment of the pedicel showed that the Becuét grapes had a greater propensity to fall during the entire wither-

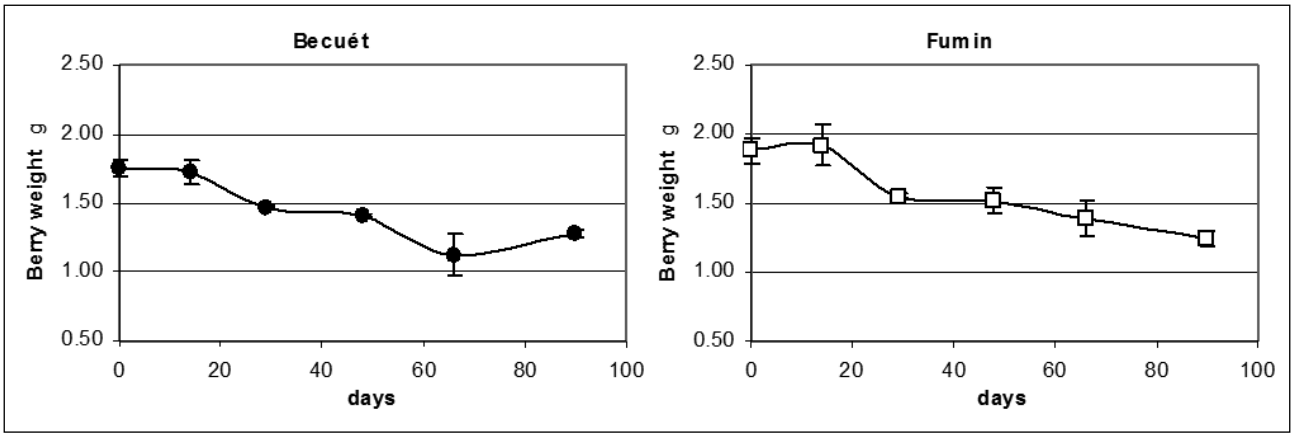


Fig. 2 - Evolution of Becuét and Fumin grape berry weight (g) during overripening on the vine. Average value ± standard deviation (*n*=30).

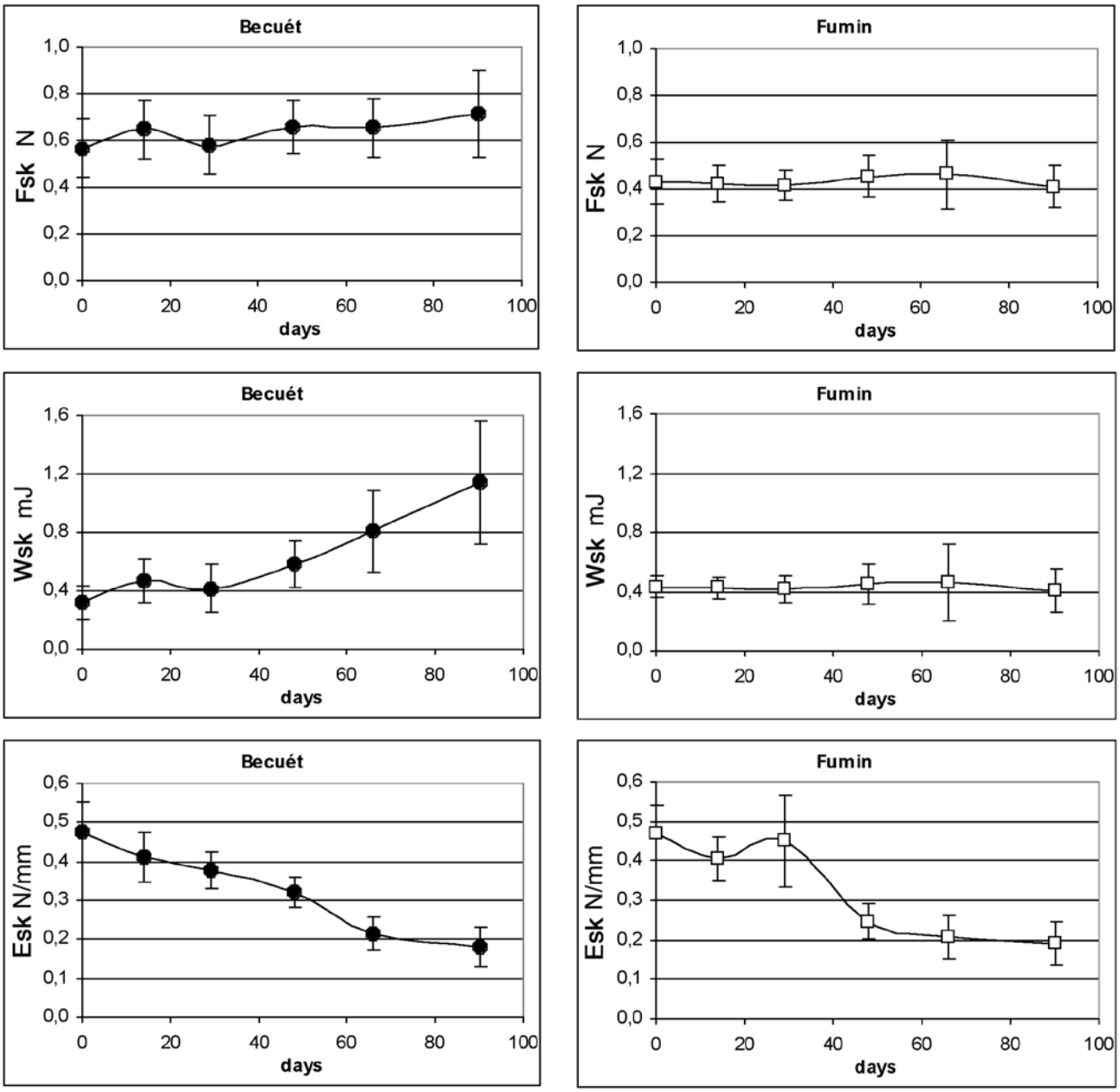


Fig. 3 - Trends in the mechanical properties of Becuét and Fumin grape berry skin during overripening on the vine. F_{sk} = Berry skin break force; W_{sk} = Berry skin break energy; E_{sk} = Skin Young's modulus. Average value ± standard deviation (*n*=30).

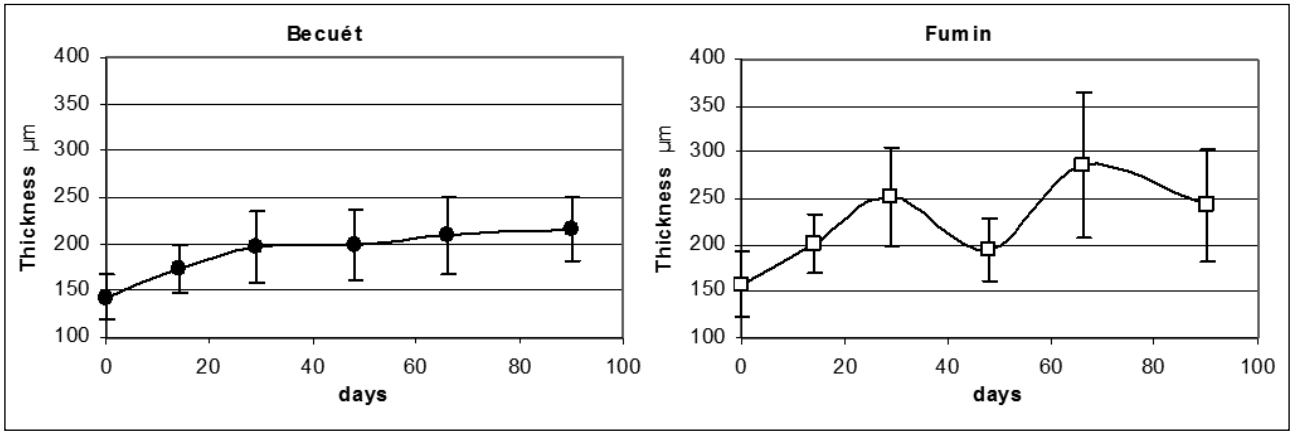


Fig. 4 - Variation of the thickness of Becuét and Fumin grape berry skin (µm) during overripening on the vine. Average value ± standard deviation (*n*=30).

ing period (Fig. 5). This tendency could already be noted after 15 days of overripening and the lower F_{ped} and W_{ped} values occur when the chance of snow is higher. At the end of the dehydration period, the Fumin grapes had average F_{ped} values that were considerably higher (+57%), and displayed a more gradual decrease during the drying process. This cultivar also showed a progressive W_{ped} increase; during the traction test, before breaking away, the peduncle tends to deform the apical part of the berry and stretches the skin. This increasing elasticity of the Fumin grape skin tissues explains the higher energy of the detachment values that were recorded.

The mechanical characteristics of the grapes showed a high variability which increased for almost all of the variables in the final stages of overripening due to non-homogeneous dehydration of the grapes. Despite this variability, the evolution of the mechanical variables was evident. Even during the normal process of grape ripening from veraison to harvest, some changes in the mechanical properties occur, particularly regarding firmness (LEE and BOURNE, 1980; ROBIN *et al.*, 1997). However, these changes cannot be used as a criterion for determining the time of grape harvest. However, they can be used to discriminate the different production areas (LE MOIGNE *et al.*, 2008). Mechanical properties can also be used to characterize the different cultivars, even if the variables are strongly conditioned by the annual climatic trends (LETAIEF *et al.*, 2008b). The values for many of the variables, including F_{sk}, W_{sk} and F_{ped} differed in the Becuét and Fumin cultivars.

The skin break force is an important technological variable for red winegrapes because it is related to the extractability of phenolic substances from the skin (ROLLE *et al.*, 2008). Regarding the extraction of anthocyanins, there is an interaction between F_{sk} and the stages of ripening (ROLLE *et al.*, 2009). Harder skins lead to a more complete extraction of anthocyanins, but with slower extraction kinetics. F_{sk} and Sp_{sk} are directly related to the cell permeability index (EA%) (RIO SEGADE *et al.*, 2008), which provides information relative to the ease of transfer of these compounds. Higher EA% values indicate a slower dissolution of anthocyanins in the must (CAGNASSO *et al.*, 2008).

In the production of icewines, phenolic compounds are only extracted during the pressing of the frozen grapes because there is no maceration during fermentation. Due to the brevity of contact between the skin and must, varieties with hard skins could have a reduced anthocyanin content in the wine. This aspect is particularly important for cultivars with a low anthocyanin content. The Becuét cultivar, has highest F_{sk} values and is rich in anthocyanins (> 1,300 mg/kg grapes) (GERBI *et al.*, 2005), so even a

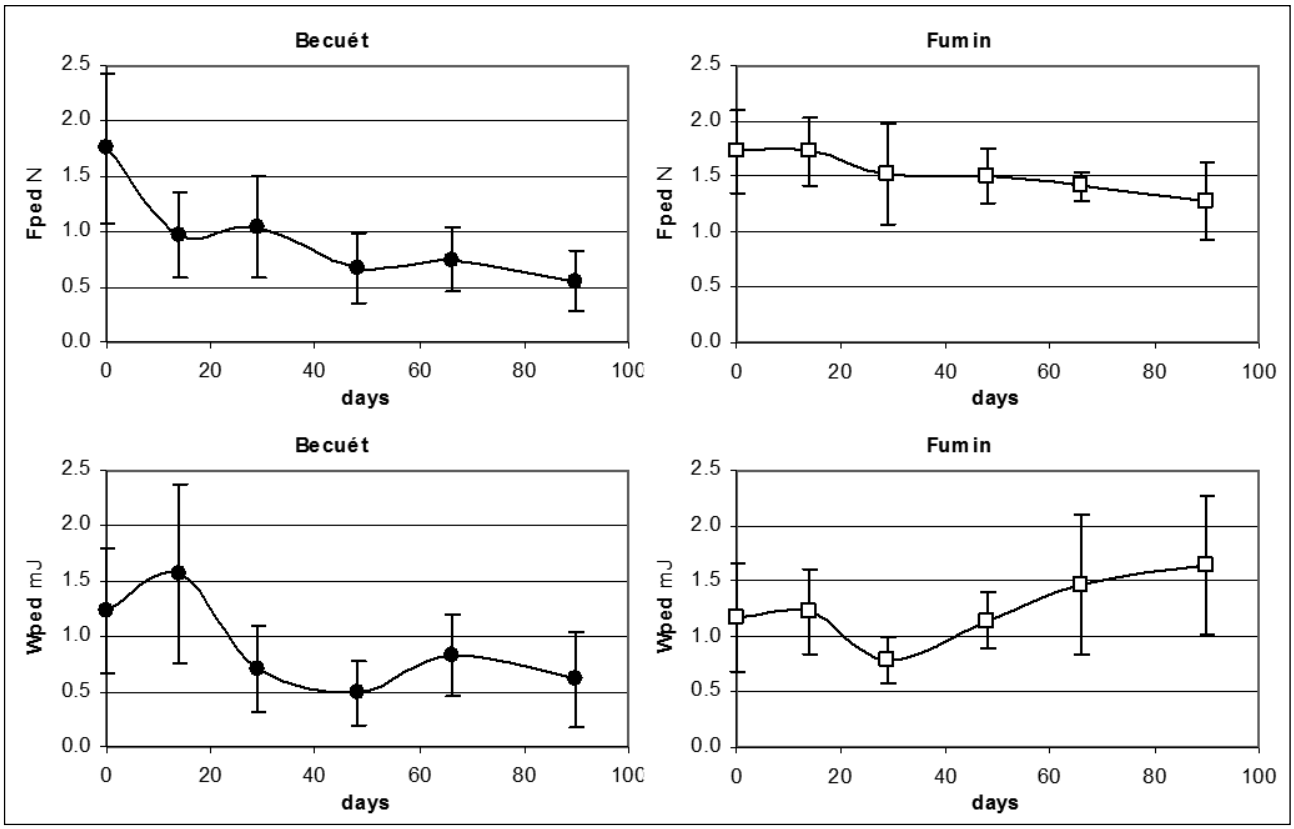


Fig. 5 - Trends in resistance to peduncle detachment in Becuét and Fumin berries during overripening on the vine. F_{ped} = Peduncle detachment force; W_{ped} = Peduncle detach energy. Average value ± standard deviation (n=30).

brief contact time is sufficient to ensure adequate color intensity in the wines.

CONCLUSIONS

The evolution of some mechanical variables of grapes during withering on the vine were shown. Icewines or "Vini del ghiaccio" cannot be produced with grape varieties that do not perform well in the strict environmental conditions required for withering. The main characteristic required is high resistance to shattering and detachment force of the pedicel is an effective parameter for monitoring this characteristic. The hardness and thickness of the skin are important variables for resistance to plant diseases; they also play an important role in must preparation.

The cultivars Becuét and Fumin have different mechanical properties but both proved to be suitable for the production of icewine. In addition to these mechanical variables, cultivars must be carefully selected to ensure that at technological ripeness, the sugar levels are high in order to reduce the time on the vine. To avoid losing the product, the grapes should reach the desired sugar level as early as possible so that harvest of the dried grapes can be carried out as soon as the ambient temperature (<-7°C) permits.

REFERENCES

Amati A., Ferrarini R., Riponi C. and Zironi R. 1983. Una nuova tecnologia per l'appassimento delle uve. Vigne Vini. 9: 27.

Astorino S. and Di Stefano R. 2003. Composizione di uve passe Zibibbo ottenute con processi di disidratazione diversi. L'Enologo. 39: 99.

Bailly S., Jerkovic V., Marchand-Brynaert J. and Collin S. 2006. Aroma extraction dilution analysis of Sauternes wines.0 Key role of polyfunctional thiols. J. Agric. Food Chem. 54: 7227.

Barbanti D., Mora B., Ferrarini R., Tornielli G.B. and Cipriani M. 2008. Effect of various thermo-hygrometric conditions on the withering kinetics of grapes used for the production of "Amarone" and "Recioto" wines. J. Food Eng. 85: 350.

Bellincontro A., De Santis D., Mencarelli F. Nardin C. and Villa I. 2002. Nuova tecnologia di appassimento di uve Trebbiano e Malvasia. Caratteristiche qualitative ed aromatiche in confronto con il sistema tradizionale. Industrie delle Bevande. 31: 538.

Bellincontro A., De Santis D., Botondi R., Villa I. and Mencarelli F. 2004. Different postharvest dehydration rates affect quality characteristics and volatile compounds of Malvasia, Trebbiano and Sangiovese grapes for wine production. J. Sci. Food Agric. 84: 1791.

Cagnasso E., Rolle L., Caudana A. and Gerbi V. 2008. Relations between grape phenolic maturity and red wine phenolic composition. Ital. J. Food Sci. 20: 365.

Carbonneau A., Moueix A., Leclair N. and Renoux. J.L. 1991. Proposition d'une méthode de prélèvement de raisins à partir de l'analyse de l'hétéréogénéité de maturation sur un cep. Bull. OIV. 64: 679.

Chamberlain G., Husnik J. and Subden R.E. 1997. Freeze-desiccation survival in wild yeasts in the bloom of icewine grapes. Food Res. Int. 30: 435.

Cliff M., Yuksel D., Girard B. and King M. 2002. Characterization of Canadian ice wines by sensory and compositional analyses. Am. J. Enol. Vitic. 53: 46.

Costantini V., Bellincontro A., De Santis D., Botondi R. and Mencarelli F. 2006. Metabolic changes of Malvasia grapes for wine production during postharvest drying. J. Agric. Food Chem. 54: 3334.

EEC. 1990. Commission Regulation N. 2676 of 17 September 1990. Determining Community methods for analysis of wines. OJ L272, 3.10.1990.

Frangipane M.T., Ceccarelli A., Mencarelli F. and Anelli G. 2007. Study of phenolic compounds in Aleatico grapes dried in a forced air tunnel. Ital. J. Food. Sci. 19: 203.

Gabler F.M., Smilanick J.L., Mansour M., Ramming D.W. and Mackey B.E. 2003. Correlations of morphological, anatomical, and chemical features of grape berries with resistance to *Botrytis cinerea*. Phytopathology. 93: 1263.

Genovese A., Gambuti A., Piombino P. and Moio L. 2007. Sensory properties and aroma compounds of sweet Fiano wine. Food Chem. 103: 1228.

Gerbi V., Rolle L., Zeppa G., Guidoni S. and Schneider A. 2005. Indagine sul profilo antocianico di vitigni autoctoni piemontesi. Industrie delle Bevande, 34: 23.

Karagiannis S., Economou A. and Lanardis P. 2000. Phenolic and volatile composition of wines made from *Vitis vinifera* cv. Muscat Lefko grapes from the Island of Samos. J. Agric. Food Chem. 48: 5639.

Lang A. and During H. 1990. Grape berry splitting and some mechanical properties of the skin. Vitis., 29: 61.

Lee C.Y. and Bourne M.C.1980. Changes in grape firmness during maturation. J. Texture Stud. 11:163.

Le Moigne M., Maury C., Bertrand D. and Jourjon F. 2008. Sensory and instrumental characterization of Cabernet franc grapes according to ripening stages and growing location. Food Qual. Pref., 19: 220.

Letaief H., Rolle L., Zeppa G. and Gerbi V. 2008a. Assessment of grape skin hardness by a puncture test. J. Sci. Food Agric. 88: 1567.

Letaief H., Rolle L. and Gerbi V. 2008b. Mechanical behavior of winegrapes under compression tests. Am. J. Enol. Vitic. 59: 323.

Miklósy È., Kalmár Z., Pölös V. and Keréyi Z. 2000. Study of volatile aroma components in young Tokaji Aszu wines by GC-MS. Chromatographia. 51: S305.

Nurgel C., Pickering G.J. and Inglis D.L. 2004. Sensory and chemical characteristics of Canadian ice wines. J. Sci. Food Agric. 84: 1675.

Ribéreau-Gayon P., Dubourdieu D., Donèche B.J. and Lonvaud A. 2000. "Handbook of Enology". Vol. 1. The microbiology of wine and vinification. John Wiley & Sons Ltd, Chichester, UK.

Río Segade S., Rolle L., Gerbi V. and Orriols I. 2008. Phenolic ripeness assessment of grape skin by texture analysis. J. Food Comp. Anal. 21: 644.

Robin J.P., Abbal P. and Salmon J.M. 1997. Fermeté et maturation du raisin. Définition et évolution de différents paramètres rhéologiques au cours de la maturation. J. Int. Sci. Vigne Vin. 31: 127.

Rolle L., Zeppa G., Letaief H., Ghirardello D. and Gerbi V. 2007. Metodi per lo studio delle proprietà meccaniche delle uve da vino. Riv. Vitic. Enol. 60: 59.

Rolle L., Torchio F., Zeppa G. and Gerbi V. 2008. Anthocyanin extractability assessment of grape skins by texture analysis. J. Int. Sci. Vigne Vin. 42: 157.

Rolle L., Torchio F., Zeppa G. and Gerbi V. 2009. Relations between break skin force and anthocyanin extractability at different stages of ripening. Am. J. Enol. Vitic. 60: 93.

Schneider A., Carra A., Akkak A., This P., Laucou V. and Botta R. 2001. Verifying synonymies between grape cultivars from France and Northwestern Italy using molecular markers. Vitis., 40: 197.

Schneider A., Gerbi V. and Redoglia M. 1987. A rapid HPLC method for separation and determination of major organic acids in grape musts and wines. Am. J. Enol. Vitic., 38:151.

Soleas G.J. and Pickering G.J. 2007. Influence of variety, wine style, vintage and viticultural area on selected chemical parameters of Canadian icewine. J. Food Agric. Environ. 5: 97.

Urso R., Rantsiou K., Dolci P. Rolle L. Comi G. and Cocolin L. 2008. Yeast biodiversity and dynamics during sweet wine production as determined by molecular methods. FEMS Yeast Res. 8: 1053.

Ziraldo D.J.P. 2000. "Anatomy of a Winery", Key Porter Books, Toronto, ON.

Revised paper received September 10, 2009 Accepted September 21, 2009

PAPER

LEAD AND CADMIUM IN SOME MILK AND MILK-BASED MOZZARELLA CHEESES FROM APULIA, ITALY

M. FACCIA*, G. GAMBACORTA, M. QUINTO[1] and A. DI LUCCIA[2]
Dipartimento di Progettazione e Gestione dei Sistemi Agro-Forestali, Università di Bari,
Via Amendola 165/A, 70126 Bari, Italy
[1]Dipartimento di Scienze Agroambientali, Chimica e Difesa Vegetale, Università di Foggia,
Via Napoli 25, 71100 Foggia, Italy
[2]Dipartimento di Scienze degli Alimenti, Università di Foggia,
Via Napoli 25, 71100 Foggia, Italy
*Corresponding author: Tel. +39 80 5443012, Fax +39 80 5442942
e-mail: michele.faccia@agr.uniba.it

ABSTRACT

The content of lead and cadmium in milk produced near a rural area subjected to illegal dumping and the transfer of the two metals from milk to Mozzarella cheese were investigated. The concentrations found in milk (from 2.00 to 19.17 for Pb and from 0.04 to 1.43 mg L^{-1} for Cd) did not exceed the maximum limits established by European Community regulations. The cheese-making process was responsible for the transfer of the metals into the cheese but the level of contamination differed, also depending on the dairy, the production technology and the extent of exogenous contamination. On the whole, the contribution that Mozzarella makes to the daily intake of lead and cadmium appears to be toxicologically negligible.

- Key words: cadmium, lead, milk, Mozzarella cheese -

INTRODUCTION

The ingestion of contaminated foodstuffs is one of the possible causes of chronic lead and cadmium poisoning. This type of food contamination is ascribed mainly to environmental pollution caused by uncontrolled human activities. For this reason the periodical monitoring of Pb and Cd levels is planned in the HACCP of food industries, in particular those involved in the production of widely consumed foodstuffs. Among these are milk and cheese, in which, fortunately, very low concentrations are usually detected (KOOPS and WESTERBEEK, 1983; BALDINI *et al.*, 1988; LARSEN and RASMUSSEN, 1991; CABRERA *et al.*, 1995; GARCIA *et al.*, 1999; TRIPATHI *et al.*, 1999; YUZBASI *et al.*, 2003). Nevertheless, episodes of milk contamination have been reported, mainly in association with farms situated very close to highways (BHATIA and CHOUDHRI, 1996, SIMSEK *et al.*, 2000), those that are subjected to environmental contamination from industrial waste, or due to feeding animals with contaminated foodstuffs (BAARS *et al.*, 1990; OKADA *et al.*, 1997).

Pb and Cd in milk are mainly associated with casein, and their distribution is not affected by the heating or freezing processes, with the exception of very strong treatments, such as desiccation (JENG *et al.*, 1994; MATA *et al.*, 1996; MORENO-ROJAS *et al.*, 1999). As regards the fate of these metals during cheesemaking, the information available in the literature is scarce and generally indicates that the metal concentration increase on going from milk to cheese. MATA *et al.* (1995) reported having recovered almost all the lead and cadmium that had been experimentally added to milk in the casein fraction obtained by enzymatic coagulation; ZURERA-COSANO *et al.* (1994) reported an increased lead concentration in the curd of Manchego-type cheese when compared to milk; CONI *et al.* (1996) reported that curdling resulted in an increased concentration of the two elements. The evaluation of the transfer of lead and cadmium from milk to cheese can be complicated because of the processing equipment used and the cheesemaking procedure (YUZBASI *et al.*, 2003), which may considerably change in connection with the type of cheese that is produced. The problem is far from being clearly defined, and the tolerance levels in dairy products have not yet been established, with the exception of the lead concentration in raw matter which may be 20 mg/kg^{-1} (EUROPEAN UNION COMMISSION, 2001).

In the last decade increasing attention has been given to soil contamination caused by illegal dumping of toxic substances in isolated rural areas, where police surveillance is difficult. This is a particularly serious problem in the areas in which dairy cattle are reared. Episodes of soil contamination by heavy metals due to illegal dumping were recently discovered in a small area within a wide rural zone in Apulia, southern Italy. These episodes raised concerns about the safety of the milk products, which prompted this investigation of the Cd and Pb content in milk from farms located in this area, and about the fate of these metals during the manufacture of Mozzarella cheese.

MATERIALS AND METHODS

Assessment of milk contamination

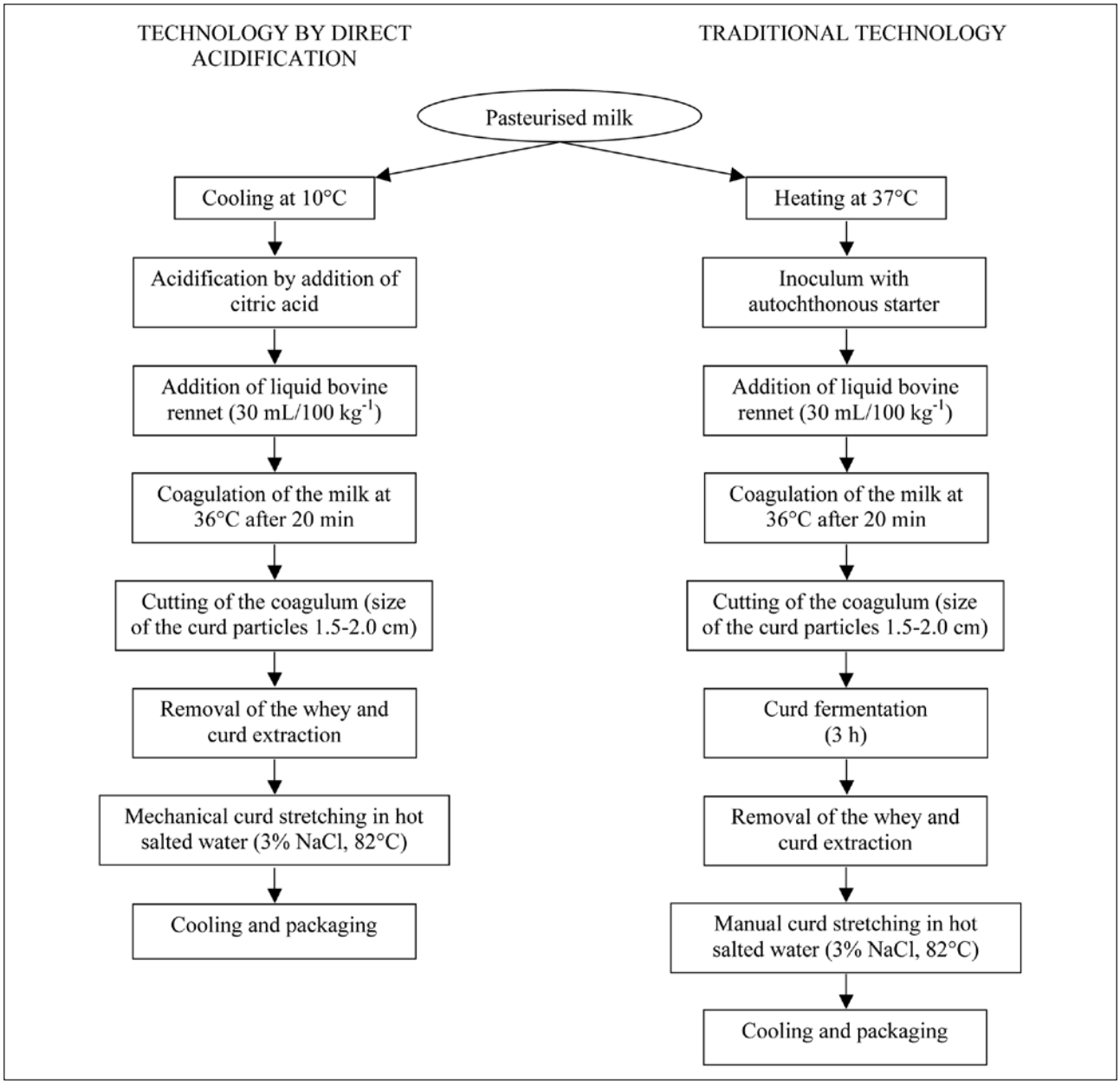
Milk samples were collected from eighteen farms located in the territory under study: nine farms were in the vicinity of the contaminated zone (less than 10 km, Group A), and the remaining nine farms were far away (more than 50 km, Group B). The milk samples (about 2 L each) were collected directly from the storage tanks and transported in refrigerated bags to the lab, where they were immediately analyzed. Precautions were taken to minimize exogenous contamination.

Production of Mozzarella cheese

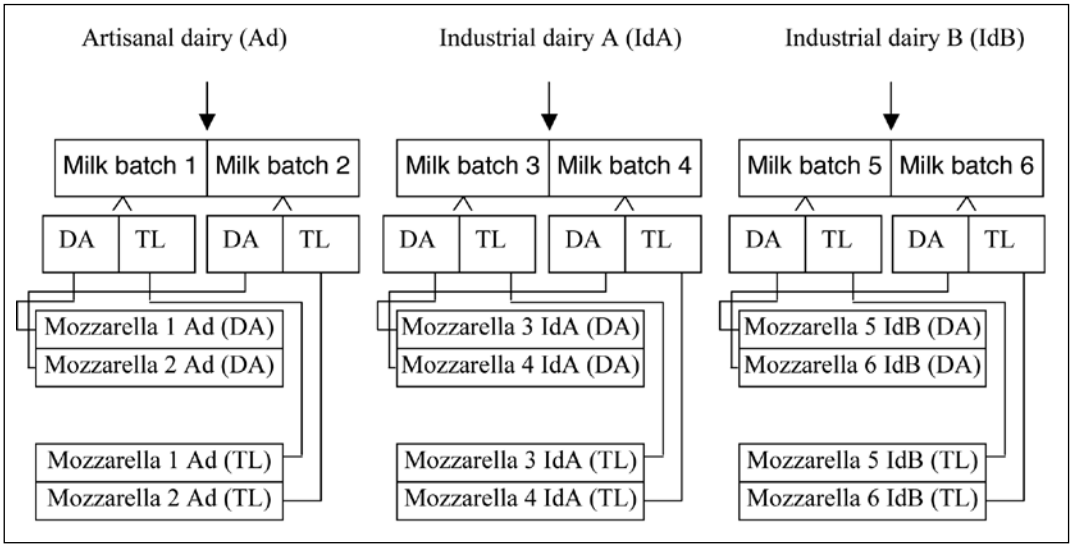
Six batches of bulk milk were used to produce Mozzarella cheese using two different technologies, direct acidification (DA) and traditional lactic fermentation (TL), at three different dairy plants: one artisanal (Ad), and the other two industrial (IdA and IdB). The manufacturing protocols and experimental design are reported in Schemes 1 and 2. Two replicates (500 kg each) were performed for each cheesemaking trial.

Lead and cadmium determination by atomic absorption spectrometry (AAS)

Standard solutions of cadmium and lead were purchased from Sigma Aldrich (St. Louis, MO, USA), nitric acid for AAS analyses and hydrogen peroxide for the mineralization process were purchased from J.T. Backer (Phillipsburg, NJ, USA). The matrix modifier used in the analysis was obtained by dissolving 0.104 g of $Mg(NO)_3.6H_2O$ (Sigma-Aldrich) and 1.21 g of $NH_4H_2PO_4$ (Fluka, Buchs, SG, Switzerland) in a 100 mL flask with 2% HNO_3 . All other reagents were of analytical grade. For the mineralization process, a CEM MDS 2100 (CEM, Matthews, NC, USA) was used. The milk samples (4 g) were accurately weighed in Advanced Composite Vessels (ACV), then 3 mL of 70% HNO_3 (purity level: [Pb] ≤ 0.05 ppb, [Cd] ≤ 0.01 ppb) and 3 mL of 30% H_2O_2 were added. The same procedure was used for the mozzarella samples (1.5 g), but 5 mL of 30% H_2O_2 were used. Autosampler cups, plastic bottles and glassware were cleaned by soaking them in 1.40 mol L^{-1} HNO_3 for 24 h and rinsing five times with Milli-Q water. They were dried and stored in a class 100 laminar flow hood.



Scheme 1 - Manufacturing procedures of Mozzarella cheese.



Scheme 2 - Experimental design for the cheesemaking trials. DA = direct acidification; TL = traditional lactic fermentation.

The samples were then subjected to microwave oven programs adjusted for the purposes of the study. The sample solutions were then diluted with water to a final volume of 20 mL, in order to reduce the HNO_3 concentration and to prolong the life of the graphite tube. An AAS Shimadzu model AA6701 (Shimadzu Ltd., Kyoto, Japan), equipped with flame and graphite atomiser and autosampler with wizAArd software for automatic dilution was used. Background correction was carried out with a deuterium lamp. Hallow cathode lamps operating at 8 mA and a spectral band width of 0.5 nm were used to determine cadmium and lead at their primary resonances of 228.8 and 283.3 nm, respectively. Argon at 0.1 mL min^{-1} was used as inert gas except during the atomization step, where the flow was stopped. Twenty mL were injected and pyrolytic graphite L'vov platforms (Shimadzu) were used. In order to avoid matrix interference, 4 mL of Zeeman matrix modifier (Sigma Aldrich, Cotati, CA, USA) were added to each sample and standard injection.

Statistical analysis

Mean values and standard deviations were calculated and analysed with the JMP package program (S.A.S. Ins., Cary, NC, USA) for a one-way analysis of variance (ANOVA) with the Cd and Pb contents in the milk being the source of variance.

RESULTS AND CONCLUSIONS

Analytical characteristics

The calculation of the detection limits in the sample was carried out according to IUPAC rules (LONG and WINEFORDNER, 1983). Accuracy of the method was checked with recovery assays, by adding known amounts of Pb and Cd to five milk samples and processing the mixtures in the same way as indicated for the experimental samples. Precision was determined by 10 replicate determinations on each of five samples. The results are reported in Table 1.

Assessment of milk contamination

The concentration of lead was higher than that of cadmium in all of the milk samples, in agreement with the data reported in the literature (Table 2). On the average, the lead contents were slightly higher than those commonly found in cow milk (GARCIA *et al.*, 1999; HERMANSEN *et al.*, 2005), but all of the values were below the maximum limit established by EU regulations. The concentration levels were close to the tolerance level in only in two cases (sample 3 from Group A and sample 6 from Group B). The results clearly indicate that cadmium was always present in very low amounts. To date, a maximum limit for this element in milk has not yet been established by law, but the concentrations were similar to those reported in the literature (CABRERA *et al.*, 1995; TOKUSOGLU *et al.*, 2004).

Table 2 - Concentrations of lead and cadmium (mg/kg± SD, mean values, n = 4) in milk samples from farms near the contaminated zone (less than 10 km, Group A) and from farms far away (more than 50 km, Group B).

Sample	Pb		Cd	
	Group A	Group B	Group A	Group B
1	3.04±0.05	6.76±1.15	0.23±0.05	0.53±0.06
2	6.47±0.02	3.15±0.12	0.24±0.04	0.12±0.03
3	19.17±2.48	10.70±0.26	1.43±0.01	0.35±0.02
4	3.48±0.21	2.00±0.16	0.04±0.00	0.09±0.01
5	4.96±0.15	4.43±0.28	0.15±0.02	0.43±0.09
6	8.76±0.5	15.54±1.62	0.38±0.02	0.76±0.14
7	7.92±0.24	4.77±0.35	0.57±0.02	0.30±0.03
8	10.93±0.77	9.15±0.80	0.06±0.01	0.06±0.01
9	12.40±0.87	7.56±0.52	0.07±0.01	0.06±0.02

The relationships between milk contamination and the geographical area of origin are reported in Figs. 1 and 2. The statistical analysis did not show any significant difference between the two groups of farms regarding the lead and cadmium levels which demonstrates an absence of a significant impact of soil contamination on milk quality. This finding confirms the results already obtained by other authors (SHARMA *et al.*, 1982; VREMAN *et al.*, 1986; OSKARSSON *et al.*, 1992), who reported that only prolonged and acute contamination of soil and fodder can cause a significant increase in the concentration of heavy metals in milk. Moreover, in this geographical area farmers are resorting less and less to grazing and therefore the cattle have less exposure to this type of pollution.

Table 1 - Analytical characteristics for lead and cadmium determination.

	Detection limit (μg kg^{-1})	Characteristic mass, pg	Recovery assays (%)	Precision (RSD, %)
Lead	0.08	10.0	97.3 ± 0.8	6.2
Cadmium	0.03	0.4	95.2 ± 0.5	5.8

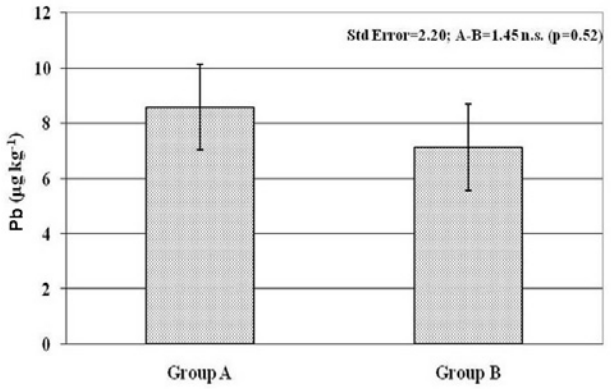


Fig. 1 - One-way analysis of variance (ANOVA) of lead concentrations in the milk samples from the two groups of farms. Group A= farms near the contaminated zone (less than 10 km); Group B = farms far away from the contaminated zone (more than 50 km).

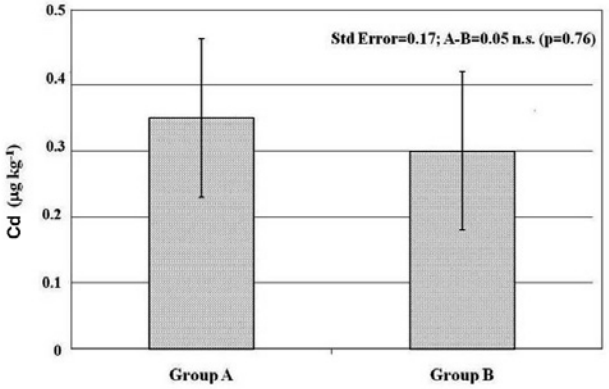


Fig. 2 - One-way analysis of variance (ANOVA) of cadmium concentrations in the milk samples from the two groups of farms. Group A= farms near the contaminated zone (less than 10 km); Group B = farms far away from the contaminated zone (more than 50 km).

Pb and Cd during the production of Mozzarella cheese

As previously observed for milk from the individual farms, the lead content was greater than the cadmium in the six batches of milk used for cheesemaking (Table 3). The extent of the transfer of the metals from milk to cheese can be deduced from the data in Table 4, where the levels of Pb and Cd in the samples of Mozzarella and the corresponding "concentration factor" (cf, metal content in cheese/metal content in milk), are shown. The results confirm that the cheesemaking process has a concentration effect, but the extent of the transfer of the two metals was different. The cf for Cd ranged from 3.41 to 7.74, whereas that for Pb ranged from 2.43 to 35.26. The reasons for the wide variations can be inferred from the data of Table 5. First, the type of plant in which the cheesemaking trials were carried out had a great influence. The concentration factor in the artisanal dairy was always higher than those of the two industrial plants which were similar to each other. As regards the production technology, the lactic fermentation seems to have favoured the transfer of lead but, in the case of the artisanal dairy, the cf was very high and exceeded the Pb values found in the raw materials. This abnormal data can only be explained by some exogenous contamination; the source could not be ascertained in the present study and will be the objective of a future investigation. In view of this, it should be noted that traditional and hand-craft production technology involves a more complex series of operations than industrial technology based on direct acid-

Table 3 - Cadmium and lead (μg/kg± SD, mean values, n = 4) in the milk batches used for cheesemaking.

Milk Sample	Pb	Cd
Batch1	3.17± 0.38	0.30±0.02
Batch2	15.59±3.21	1.72±0.09
Batch3	5.05±0.07	2.07±0.03
Batch4	9.38±0.21	0.58±0.09
Batch5	5.98±0.12	0.97±0.02
Batch6	11.81±0.17	2.23±0.13

Table 4 - Cadmium and lead (μg/kg ± SD, mean values, n = 4) in Mozzarella cheese manufactured from the milk batches of Table 6. Ad = artisanal dairy; IdA and IdB = industrial dairies; DA = direct acidification; TL = traditional lactic fermentation; cf = concentration factor from milk to cheese.

Mozzarella Sample	Pb	cf	Cd	cf
1 Ad (DA)	14.70±0.87	4.64	2.06±0.14	6.87
2 Ad (DA)	91.43±10.04	5.86	12.47±0.56	7.25
3 IdA (DA)	14.55±0.36	2.88	7.50±0.29	3.62
4 IdA (DA)	22.79±1.17	2.43	2.38±0.07	4.10
5 IdB (DA)	19.17±2.69	3.21	4.84±0.64	4.99
6 IdB (DA)	32.83±5.69	2.78	8.59±0.48	3.85
1 Ad (TL)	111.77±14.38	35.26	2.26±0.30	7.53
2 Ad (TL)	217.84±19.04	13.97	13.32±1.60	7.74
3 IdA (TL)	20.17±0.95	3.99	7.05±0.63	3.41
4 IdA (TL)	29.27±0.32	3.12	2.64±0.11	4.55
5 IdB (TL)	26.97±3.97	4.51	3.85±0.23	3.97
6 IdB (TL)	48.42±2.55	4.10	11.60±0.94	5.11

Table 5 - Concentration factors (cf) of Pb and Cd from milk to cheese: comparison among the different dairies.

Dairy	Pb cf DA	Pb cf TL	Cd cf DA	Cd cf TL	Pb mean	Cd mean
Ad	5.25	24.61	7.06	7.63	14.93	7.34
IdA	2.65	3.55	3.86	3.98	3.10	3.92
IdB	2.99	4.30	4.42	4.54	3.64	4.48
mean	3.63	10.82	5.11	5.38		

ification. Among these operations, the production and addition of the autochthonous lactic starter (the so-called "sieroinnesto"), the prolonged fermentation in open vats, curd-shredding and hand-stretching, involve the use of several tools and containers which could be a potential source of contamination. Finally, some comments about the contribution that mozzarella makes to the daily intake of the two metals should be made. The average per capita consumption of mozzarella in Italy is 5 kg per year (AMBROSI, 2002; AGRISOLE, 2005); this corresponds to about 100 g per week. In the area under study, these data are greatly underestimated, since mozzarella is consumed almost daily. Nevertheless, considering the Provisional Tolerable Intakes (PTI) of 50 mg kg^{-1} body weight per week for lead and of 7 mg kg^{-1} for cadmium (NOEL *et al.*, 2003), the data from this investigation excludes any significant risk for human health. Even if the calculations are made from the highest contents observed (217.84 and 13.32 mg kg^{-1} for Pb and Cd, respectively), a person of average weight would have to eat more than 20-40 kg Mozzarella per week in order to approach the PTI values, and much more in the case of the lowest concentrations.

REFERENCES

Ambrosi G. 2002. Vendite di Mozzarella in volume e valore nell'ultimo anno e dinamica dei consumi. Available from: http://www.milkonline.com/fiera/settegiorni/assolatte150402.htm#3

Agrisole, 2005. Industria alimentare: latte e formaggi. Supplemento ad Agrisole del 17/07/2005. Avalilable from: http://www.agrisole.it/casearia05.pdf.

Baars A.J., Van Beek H., Visser I.J., Vos G., Van Delft W., Fennema G., Lieben G.W., Lautenbag K., Nieuwenhuijs J.H. and De Lezenne Coulander P.A. 1990. Lead poisoning in cattle in north Netherlands. October 1989-January 1990. Tijdschr Diergeneeskd. 115: 882.

Baldini M., Bocca A. and Mosca M. 1988. Investigation of microelement content of bulk milk from different regions of Italy. Food Add. Contam. 5: 45.

Bhatia I. and Choudhri C.N. 1996. Lead poisoning of milk - the basic need for the foundation of human civilization. Indian J. Pub. Healt. 40: 24.

Cabrera C., Lorenzo M.L. and Lopez M.C. 1995. Lead and cadmium contamination in dairy products and its repercussion on total dietary intake. J. Agr. Food Chem. 43: 1605.

Coni E., Bocca A., Coppolelli P., Caroli S., Cavallucci C. and Trabalza Marinucci M. 1996. Minor and trace element content in sheep and goat milk and dairy products. Food Chem. 57: 253.

European Union Commission, 2001. Regulation n. 466/2001 of 8 March 2001 setting maximum levels for certain contaminants in foodstuffs. Official J. Europ. Union L 77 of 16.03.2001.

Garcia E.M., Lorenzo M.L., Cabrera C., Lopez M.C. and Sanchez J. 1999. Trace element determination in different milk slurries. J. Dairy Res. 66: 569.

Hermansen J.E., Badsberg J.H., Kristensen T. and Gundersen V. 2005. Major and trace elements in organically or conventionally produced milk. J. Dairy Res. 72: 362.

Koops J. and Westerbeek D. 1983. Flameless atomic absorption spectrometric determination of the lead and cadmium contents in Gouda and Edam cheese produced in the Netherlands. Neth. Milk Dairy J. 37: 21.

Jeng S.L., Lee S.J. and Lin S.Y. 1994. Determination of cadmium and lead in raw milk by graphite furnace atomic absorption spectrophotometer. J. Dairy Sci. 77: 945.

Larsen E.H and Rasmussen L.Z. 1991. Chromium, lead and cadmium in Danish milk products and cheese determined by Zeeman graphite furnace atomic absorption spectrometry after direct injection or pressurized ashing. Zeitschrift Lebensmitt. Forsch. 192: 136.

Long G.L. and Winefordner J.D. 1983. Limit of detection. A closer look at the IUPAC definition. Anal. Chem. 55: 712.

Mata L., Perez M.D., Puyol P. and Calvo M. 1995. Distribution of added lead and cadmium in human and bovine milk. J. Food Prot. 58: 305.

Mata L., Sanchez L., Puyol P. and Calvo M. 1996. Changes in the distribution of cadmium and lead in human and bovine milk induced by heating or freezing. J. Food Prot. 59: 46.

Moreno-Rojas R., Sanchez-Segarra P.J., Amaro-Lopez M.A. and Zurera-Cosano G. 1999. Influence of the pasteurisation, sterilisation and desiccation processes of milk on its lead and cadmium content. Milchwissensch. 54: 210.

Noel L., Leblanc J.C. and Guérin T. 2003. Determination of several elements in duplicate meals from catering establishments using closed vessel microwave digestion with inductively coupled plasma mass spectrometry detection: estimation of daily dietary intake. Food Add. Contam. 20: 44.

Okada I.A., Sakuma A.M., Maio F.D., Dovidauskas S. and Zenebon O. 1997. Evaluation of lead and cadmium levels in milk in environmental contamination in the Vale do Paraiba Region, southeastern Brazil. Rev. Saude Publ. 31: 140.

Oskarsson A., Jorhem L., Sundberg J., Nilsson N.G. and Albanus L. 1992. Lead poisoning in cattle - transfer of lead to milk. Sci. Tot. Env. 111: 83.

Sharma R.P., Street J.C., Shupe J.L. and Bourcier D.R. 1982. Accumulation and depletion of cadmium and lead in tissues and milk of lactating cows fed small amounts of these metals. J. Dairy Sci. 65: 972.

Simsek O., Gultekin R., Oksuz O. and Kurultay S. 2000. The effect of environmental pollution on the heavy metal content of raw milk. Nahrung. 44: 360.

Tokuşoğlu O., Aycan Ş., Akalyn S., Koçak S. and Ersoy N. 2004. Simultaneous differential pulse polarographic determination of cadmium, lead, and copper in milk and dairy products. J.Agric. Food Chem. 52: 1795.

Tripathi R.M., Raghunath R., Sastry V.N. and Krisnamoorthy T.M. 1999. Daily intake of heavy metals by infants through milk and milk products. Sci. Tot. Env. 227: 229.

Vreman K., Van Der Veen N.G., Van Der Moler E.J. and De Ruig W.G. 1986. Transfer of cadmium, lead, mercury and arsenic from feed into milk and various tissues of grazing cows. Neth. J. Agric. Sci. 34: 129.

Yuzbasi N., Sezgin E., Yildirim M. and Yildirim Z. 2003. Survey of lead, cadmium, iron, copper and zinc in Kasar cheese. Food Add. Contam. 20: 464.

Zurera-Cosano G., Moreno-Rojas R. and Amaro-Lopez M.A. 1994. Effects of processing on the concentration of lead in Manchego type cheese. Food Add. Contam. 11: 91.

Paper received May 29, 2009 Accepted September 17, 2009

PAPER

ANTIOXIDANT ACTIVITY AND PHENOLIC CONTENT OF WATER EXTRACTS OF *BORAGO OFFICINALIS* L.: INFLUENCE OF PLANT PART AND COOKING PROCEDURE

C. GARCÍA-HERREROS[2], M. GARCÍA-IÑIGUEZ[1], I. ASTIASARÁN[1] and D. ANSORENA[1]*
[1]Department of Nutrition and Food Science, Physiology and Toxicology, Phaculty of Pharmacy, University of Navarra, C/ Irunlarrea s/n 31008, Pamplona (Navarra), Spain
[2]Olus Tecnologia S.L. Travesía de Etxesakan, nº28 Edificio AZYSA. Oficina 6, 31185 Zizur Mayor (Navarra), Spain
*Corresponding author: Tel. +34 948425600 (Ext 6263), Fax +34 948425649
e-mail: dansorena@unav.es

ABSTRACT

Water extracts of borage (*Borago officinalis* L.) were prepared from raw stems and leaves and from cooked (boiled and steamed) stems. Antioxidant activity (AA) was determined by ABTS and DPPH after their respective calibration with Trolox as standard and the results of both assays are expressed as μg Trolox/g fresh plant. Total phenolic compounds (TPC) were also determined for each extract. The DPPH and ABTS values in leaf extracts were similar and approximately 3.5 times higher than the values obtained for stem extracts. The high antioxidant activity of leaf extracts might be attributed to the high amount of phenolic compounds (2.36 mg GAE/g fresh plant for leaves and 0.57 mg GAE/g fresh plant for stems). Boiling significantly decreased the antioxidant activity (51-52% decrease) and total phenolic compounds (67% decrease). Steaming had no significant effect on the antioxidant activity values, whereas the total phenolic compound values showed approximately half of the decrease found for boiling (35%). Borage water extracts, particularly those of their by-products (leaves), showed high antioxidant activity, that could potentially be used for different applications in the food industry.

- Key words: ABTS, boiling, borage, DPPH, steaming -

INTRODUCTION

Borage (*Borago officinalis* L.) is an herbaceous plant that belongs to the *Boraginaceae* family. It is consumed in many Mediterranean countries, and in some countries is considered to be a luxury product because of its characteristic taste. The stems are the edible part of the plant and are cooked prior to consumption, whereas the leaves are discarded. Borage seeds are traditionally known for their oil, which has certain beneficial effects associated with the high γ-linolenic acid content (KHAN and SHAHIDI, 2000). However, the non-lipidic fraction of borage has received less attention and little information is available about it. Defatted borage seeds were subjected to ethanol extractions in order to evaluate the presence of antioxidant phenolic compounds, which were further evaluated in a meat model system (WETTASINGHE and SHAHIDI, 1999). BANDONIENE and MURKOVIC (2002) analyzed the presence of radical scavenging compounds in crude methanolic extracts of borage leaves and observed a high radical-quenching ability due to the presence of rosmarinic acid, along with other phenolic compounds.

Antioxidant compounds protect against harmful free radicals and have been strongly associated with the reduced risk of chronic diseases, such as cardiovascular disease, cancer, diabetes, Alzheimer´s disease, cataracts and age-related functional decline, as well as other health benefits (GUNDGAARD *et al.*, 2003). Vegetable waste materials have been successfully explored as sources of natural antioxidants because, like other desirable compounds, higher concentrations are available in the residues (SCHEIBER *et al.*, 2001; OKONOGI *et al.*, 2007). In relation to borage leaves, BANDONIENE *et al.* (2002) were the first to demonstrate a strong antioxidant activity in crude acetone extracts of borage.

Information on the antioxidant components, antioxidant activity and their changes during cooking is still limited (ZHANG and HAMAUZU, 2004). In general it is known that thermal processing leads to losses in the bioactive compounds of plant products due to their heat-induced instability. However, if water is used as the heat-transfer medium, different and sometimes contradictory effects have been seen in the antioxidant activity of plant material. Nevertheless, it is very important to find the best way to preserve the bioactive compounds and antioxidant activities of processed plant materials (GORINSTEIN *et al.*, 2009).

The total antioxidant capacity of a given food is not the sum of each single compound, but rather may result from the integrated and synergistic action of different compounds (DANESI and BORDONI, 2008). No single assay can be considered a "total antioxidant capacity assay" even though it could be performed in both an aqueous solution and a lipophilic environment (PRIOR *et al.*, 2005). Different methods have been used to perform DPPH or ABTS assays in plant products, so it is often difficult to compare the results from different papers. Furthermore, different ways of expressing concentrations also complicate the comparison of results (dry or fresh weight, molar or mass units, etc.). For DPPH, the results can be shown as the remaining DPPH, (PRIOR *et al.*, 2005), % Inhibition (PESCHEL *et al.*, 2006), IC_{50} (concentration that causes a 50% decrease in the initial DPPH concentration), (OKONOGI *et al.*, 2007) or as the antiradical efficiency AE = $1/(IC_{50}*T_{IC})$ (SANCHEZ-MORENO *et al.*, 1998). ABTS results are usually expressed as Trolox equivalents (either μM or μg) per amount of sample (RIVERO-PEREZ *et al.*, 2007) using either absorbance values at a certain time or measuring the area under the curve resulting from the absorbance decrease during that time (PEREZ-JIMENEZ and SAURA-CALIXTO 2006; RE *et al.*, 1999). Trolox, a water soluble analogue of vitamin E, has been the choice reference standard in most cases due to its effectiveness in both lipophilic and hydrophilic systems (NENADIS *et al.*, 2007). A more objective comparison of results could be possible by applying the same interpretation procedure with the same common standard and a unified standardization procedure (STRATIL *et al.*, 2006).

The objective of this paper was to evaluate the antioxidant activity and total phenolic content of different parts of borage (stems and leaves) and to study the changes induced by different cooking treatments of the edible part of this plant. Furthermore, a standardization procedure for expressing these results was attempted.

MATERIAL AND METHODS

Reagents

ABTS (2,2-Azino-*bis*(3-ethylbenzothiazoline-6-sulfonicacid) diammonium salt), DPPH (2,2-Diphenyl-1-picryl-hydrazyl) and Folin-Ciocalteu reagent were purchased from SIGMA-ALDRICH Chemie GmbH (Steinheim, Germany), Trolox (6-hydroxy-2,5,7,8 tetramethylchromon-2-carboxylic acid) was purchased from SIGMA-ALDRICH Chemie GmbH (Steinheim, Germany). Gallic acid (Gallic acid 1-hydrate) and methanol were purchased from PANREAC Quimica SAU (Barcelona, Spain) and ethanol from OPPAC, S.A. (Noain, Spain).

Plant material and sample preparation

Fresh borage (*Borago officinalis* L.) (5 kg) was purchased from a local supermarket. The plants were separated into leaves and stems; they were cleaned, washed, and comminuted separately. The moisture content of the raw samples was measured according to the Official AOAC Meth-

od. The water extraction procedure was carried out as follows: 100 g of raw leaves were weighed and put into 100 mL of distilled water, preheated at 96°C. The mixture was subjected to sonication for 30 min at room temperature, and filtered with a metallic filter. The extraction process was repeated with another 100 mL of distilled water, and the two extracts were united and adjusted with distilled water to a final volume of 250 mL. The same procedure was used for the raw stem extracts, using Whatman n. 3 filter paper instead of a metallic filter.

Two different cooking procedures were applied to borage stems: a) 100 g of fresh cut stems were boiled with 1000 mL of distilled water for 30 minutes at 96°C to simulate domestic processing; b) 100 g of fresh stems were steam cooked with a conventional steam pot using 1,000 mL of distilled water for 30 min at 96°C. The cooked samples were subsequently subjected to an extraction procedure using the same method as that used for the raw stem samples.

Each extract was diluted with distilled water to obtain different solutions with concentrations ranging from 0.0009 to 0.4 g of fresh plant/mL, which were subsequently used to evaluate the antioxidant activity and the total phenolic content. All extractions were performed in duplicate.

Determination of total phenolic content (TPC)

TPC was determined spectrophotometrically following the Folin-Ciocalteu colorimetric method (SINGLETON and ROSSI, 1965). Dilutions of borage extracts ranging from 0.004 to 0.4 g/mL were chosen in order to obtain readings within the standard calibration curve made from dilutions between 0.005 and 2 mg of gallic acid. The reaction mixture consisted of 0.1 mL of suitably diluted sample, 7.9 mL of distilled water, 0.5 mL of Folin-Ciocalteu reagent, and 1.5 mL of a 20% sodium carbonate anhydrous solution (added 2 min after the Folin-Ciocalteu reagent). After the initial mixing, the tubes were allowed to stand at room temperature for 2 h in the dark. The optical density of the blue-colored resulting solution was measured at 765 nm using a Lambda 5-UV-VIS spectrophotometer (Perkin Elmer, Paris, France). The total phenolic content is expressed as mg of gallic acid equivalents (mg GAE)/g fresh plant, using the corresponding calibration curve and taking into account the concentration of the diluted extracts. Absorbance measurements were made in duplicate for each diluted solution.

DPPH method

The DPPH assay was performed according to the method of BLOIS (1958) with some modifications. Briefly, a DPPH solution of approximately 20 mg/mL was prepared in methanol and subsequently diluted to obtain an absorbance of 0.8 at 516 nm (working solution). Two mL of diluted water extracts of borage of different concentrations (0.0009 g/mL - 0.2 g/mL) were allowed to react with 2 mL of DPPH working solution for 30 min in the dark, at room temperature. A control sample was prepared with 2 mL of methanol. The final absorbance of the reaction mixture was measured at 516 nm with a Lambda 5 UV-VIS Spectrophotometer (Perkin Elmer, Paris, France). The radical scavenging capacity of each dilution was calculated as the percent of inhibition (% I), calculated according to the formula:

$$\% I = (Abs_{control} - Abs_{sample})/Abs_{control} * 100$$

Where $Abs_{control}$ was the absorbance of the control after 30 min of reaction and Abs_{sample} was the absorbance of the sample after 30 min of reaction. The percent of inhibition was plotted versus the concentration of the extracts. A calibration curve with Trolox (0.1-200 μg/mL) was used to calculate the antioxidant capacity in μg Trolox/mL. Results were finally expressed as μg Trolox/g fresh plant. Absorbance measurements were made in duplicate for each diluted solution.

ABTS method

For the ABTS assay, the procedure described by RE *et al.* (1999) was used with some modifications. Briefly, the $ABTS^{\cdot+}$ chromogenic radical was generated by a chemical reaction mixing an aqueous solution of ABTS with $K_2S_2O_4$ (140 mM) to reach a 7 mM final concentration of ABTS. The mixture was kept in the dark for 12-16 h at room temperature (stock solution). Before use, 1 mL of $ABTS^{\cdot+}$ stock solution was diluted with ethanol (50%) to an absorbance of 0.70 (+/-0.02) at 741 nm (working solution). Three mL of $ABTS^{\cdot+}$ working solution were allowed to react with 300 μL of suitably diluted water borage extracts (0.0009 g/mL - 0.2 g/mL) or control (ethanol-50%) for 6 min, and then the absorbance was measured at 741 nm (Lambda 5 UV-VIS Spectrophotometer, Perkin Elmer, Paris, France). The decrease in absorbance was recorded as percent of inhibition (% I) and was calculated according to the formula:

$$\% I = (Abs_{control} - Abs_{sample})/Abs_{control} * 100$$

Where $Abs_{control}$ was the absorbance of the control after 6 min of reaction and Abs_{sample} was the absorbance of the sample after 6 min of reaction. The percent of inhibition was plotted versus the concentration of the extracts. A calibration curve with Trolox (0.1-60 μg/mL) was used to calculate the antioxidant capacity. Results were finally expressed as μg Trolox/g fresh plant. Absorbance measurements were made in duplicate for each diluted solution.

Statistical analysis

Differences between raw, boiled and steamed stems were evaluated by a one-way ANOVA and the Tukey b Post hoc test was applied when appropriate. A Student-t test was used ($p>0.05$) to evaluate differences between raw samples (leaves and stems). A Pearson correlation test was performed to study the correlation between phenolic compounds and both ABTS and DPPH. The correlation between ABTS and DPPH was also evaluated (SPSS 15.0, Inc., Chicago, IL, U.S.A.).

RESULTS AND DISCUSSION

Methodology (ABTS versus DPPH)

Calibration curves were plotted for both ABTS and DPPH assays using Trolox as the reference compound (Fig. 1 a, b) in order to express the results of the antioxidant activity of the samples using the same units for both methods. Within the range of Trolox concentrations that nearly reached 100% of% I, ABTS fit to a linear model (R^2=0.999), whereas DPPH fit an exponential model (R^2=0.994).

Increasing concentrations of different diluted borage extracts (g fresh plant/mL) were subjected to both assays, and plotting the results of % I. vs. concentration (Figs. 2 and 3) were plotted. As occurred with Trolox, ABTS fit a linear model and DPPH fit an exponential model. These% I were transformed to their corresponding μg Trolox/mL values after applying the required calibration equations (shown in Figs. 1 a, b). Taking into account the concentration (g fresh plant/mL) the antioxidant activity was then given for each diluted extract (μg Trolox/g fresh plant). After a careful examination of these data, only percentages of inhibition values between 20% and 80% were considered representative of each extract and were used to calculate the final mean value expressed as μg Trolox/g fresh plant. RE *et al.* (1999) also selected this range for the ABTS evaluation of different antioxidant solutions. At the concentrations tested in this study, in general the ABTS results did not reach 80%. For the DPPH results, the concentration of samples that had a % I greater than 80% could not be reproduced and consequently were not used to calculate the mean antioxidant activity of the borage extracts. To our knowledge, this is the first time that this approach has been used to obtain the antioxidant activity value of a plant extract. Most papers in the literature report only evaluation of one or two extract concentrations in order to make comparisons with fixed amounts of standard compounds.

The analysis of different borage extracts using these methodologies revealed similar values of μg Trolox/g fresh plant for ABTS and DPPH ($p>0.05$); the coefficients of variation were low in both cases (Table 1). In wines, the results for both tests, expressed in mM Trolox, gave higher values for ABTS than for DPPH. This was attributed to the capacity of ABTS to measure lipophilic and hydrophilic activity, whereas DPPH seems to be more specific for lipophilic antioxidants (RIVERO-PEREZ *et al.*, 2007). However, THAIPONG *et al.* (2006) found that the data for ABTS and DPPH were comparable in guava methanol extracts. This finding shows that the solvent used for the extractions plays a critical role in allowing comparisons between tests, as reported by VENSKUTONIS *et al.* (2007). STRATIL *et al.* (2006) obtained nearly the same results for TEAC and DPPH, while the values obtained with the FRAP method were about 50% higher when testing the antioxidant activity of fruits and cereals. In that paper, significant correlations were found between total phenolic compounds and the total

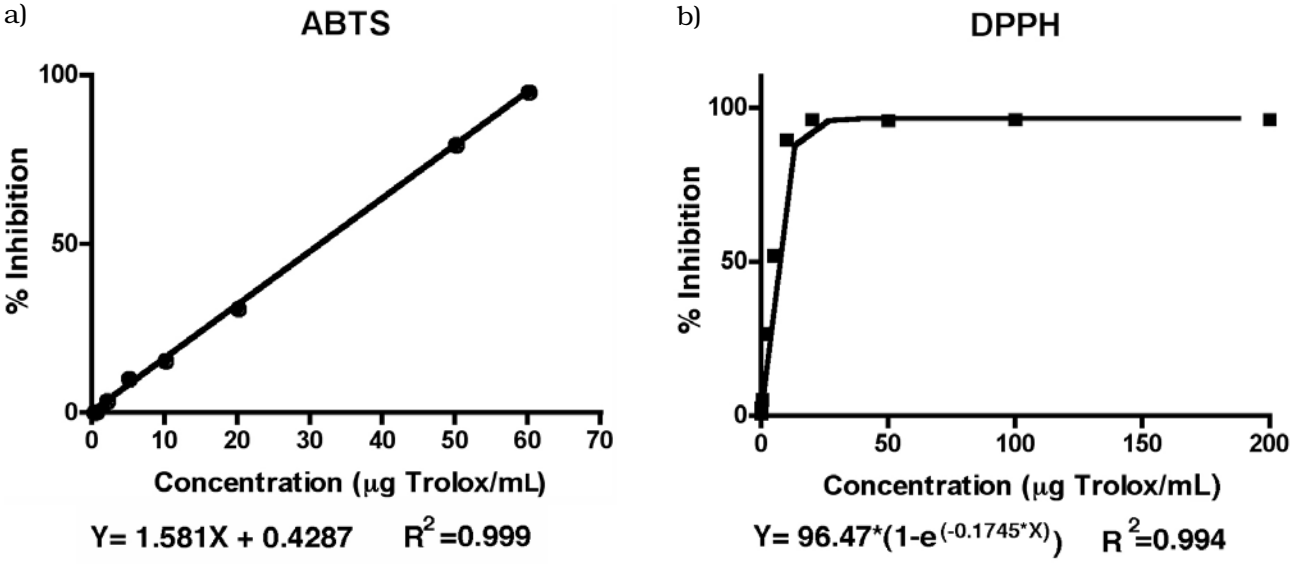


Fig. 1 - Calibration curves for ABTS and DPPH assays using Trolox as the reference compound.

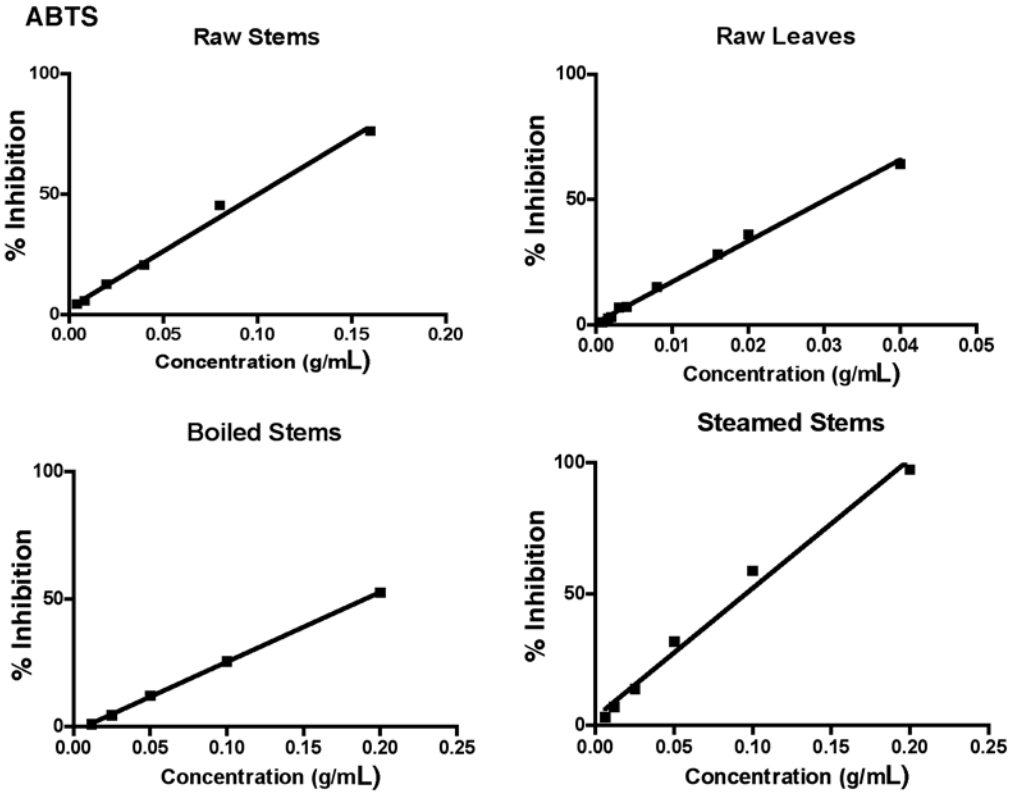


Fig. 2 - ABTS% Inhibition *vs* extract concentration (g fresh plant/mL).

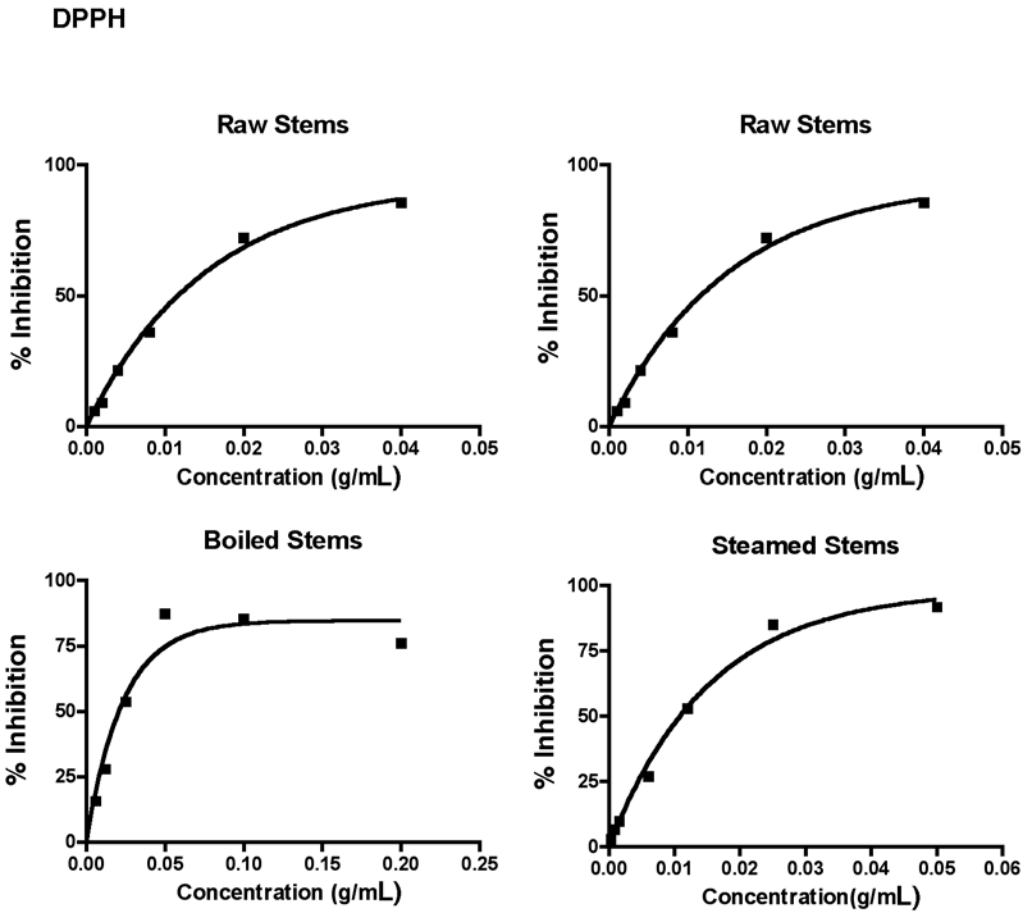


Fig. 3 - DPPH% Inhibition *vs* extract concentration (g fresh plant/mL).

Table 1 - Antioxidant activity measured by ABTS and DPPH.

		Antioxidant activity		
		ABTS (μgT/g fresh plant)	DPPH (μgT/g fresh plant)	Student t test
Raw	Leaves	1128.38±52.23^{A} (4.63)	1236.71±117.19^{A} (9.47)	ns
	Stems	324.53±27.97^{Bb} (8.62)	342.34±29.51^{Bb} (8.62)	ns
Cooked stems	Boiled	158.21±7.72^{a} (4.88)	164.60±14.81^{a} (8.99)	ns
	Steamed	368.19±30.04^{b} (8.16)	341.19±36.96^{b} (10.83)	ns

Results are expressed as means ± standard deviation (Coefficient of Variation).
^{AB}In the same column for raw samples denotes significant differences between leaves and stems (student t-test) ($p<0.05$).
^{ab}In the same column for stems denotes significant differences (1 way ANOVA), among raw, boiled and steamed samples.
ns: not significant ($p>0.05$) differences between DPPH and ABTS values.

antioxidant activity of plants measured by different methods. In the present study, although some discrepancies were found in the decrease of TPC and AA as a consequence of steaming, high correlations were found between each of the AA parameters and TPC (R^2=0.971 for ABTS and TPC; R^2=0.979 for DPPH and TPC). Good correlation values were also obtained between the DPPH and ABTS results (R^2=0.985).

Antioxidant activity: leaves and stems

As shown in Table 1, raw borage leaf extracts showed significantly higher antioxidant activity than raw stem extracts ($p<0.05$). Values for both DPPH and ABTS in leaf extracts were 1236.71 μg Trolox/g fresh plant and 1128.38 μg Trolox/g fresh plant respectively; approximately 3.5 times higher than the values obtained for stem extracts (342.34 μg Trolox/g fresh plant and 324.53 μg Trolox/g fresh plant). These results suggest that, since borage leaves are not used for human consumption and are usually discarded before cooking, they could be a by-product used as a good source of bioactive compounds. BANDONIENE *et al.* (2002) showed that an acetone extract of borage leaves efficiently reduced the oxidation rate of rapeseed oil at 80°C in terms of peroxides. PYO *et al.* (2004) also found significantly higher antioxidant activity in the leaves of Swiss chard compared to the stems, although in that case both plant

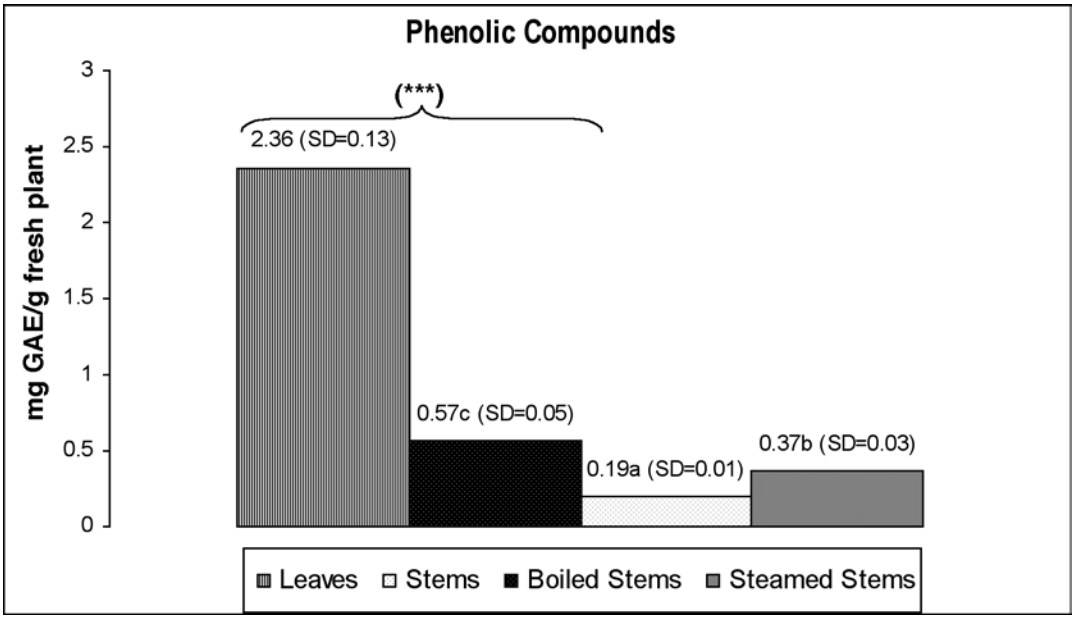


Fig. 4 - Phenolic compounds (mg GAE/g fresh plant).
Different small letters denote significant differences (one way ANOVA) among raw, boiled and steamed stems.
***Student-t test ($p<0.01$): significant differences between leaves and stems.

parts are consumed. Significant differences in the antioxidant activity and the levels of phenolics between leaves and stems or other plant parts of other vegetables have also been reported (PESCHEL *et al.*, 2006).

The high antioxidant activity of borage leaf extracts could be due to the high levels of phenolic compounds (2.36 mg GAE/g fresh plant for leaves and 0.57 mg GAE/g fresh plant for stems) (Fig. 4). Although there is an overestimation of total phenolic compounds when the plants are analyzed by the Folin-Ciocalteu method due to interference with some reducing components such as ascorbic acid and sugars (PADDA and PICHA, 2007), a linear correlation between radical scavenging activity and polyphenolic concentration has been found in different vegetables and fruits (KAHKONEN *et al.*, 1999; MEZADRI *et al.*, 2008). This correlation was also found in this study, especially in raw extracts. It can therefore be concluded that the antioxidant activity of each extract is mostly related to the concentration of phenolic hydroxyl groups, as also reported by PYO *et al.* (2004). TURKMEN *et al.* (2005) reported that total phenolic content of different fresh vegetables (pepper, squash, green beans, peas, leek broccoli and spinach) ranged from 183.2 to 1344.7 mg GAE/100 g on dry weight basis. These values are three times lower than those found for borage extracts in this work (602 mg GAE/100 g dry weight for stems and 4,262 mg GAE/100 g dry weight for leaves).

Many studies report the phenolic content of different vegetables in mg GAE/g or 100 g (WU *et al.*, 2004; ZHANG and HAMAUZU, 2004; PATTAHAMAKANOKPORN *et al.*, 2008). In these studies different mixtures of solvents were used; acetone/water/acetic acid gave the highest values for fresh broccoli (337 mg GAE/100 g fresh plant) (PYO *et al.*, 2004). The other results in those papers did not exceed 236 mg GAE/100 g fresh plant, the value obtained with the borage leaf water extracts in the current work.

LLORACH *et al.* (2003) evaluated water extracts of cauliflower by-products as a possible source of antioxidant phenolics; 1.8 g of total phenolic compounds per kg of cauliflower by-product were obtained. Water extracts showed total phenolic values that were more similar than other conventional solvent extracts used with by-products (PESCHEL *et al.*, 1999). The advantages obtained were an easier manipulation with a lower cost and a safer extraction process. PESCHEL *et al.* (1999) found that the total phenolic content of extracts from different plants obtained with organic solvents were: 48.6, 251.4 and 514.2 mg GAE/g dry extract, for apple, golden rod and artichoke, respectively. However, these authors pointed out that more nutritional studies are needed to determine the amount of extracts needed to obtain beneficial effects.

Cooking effect

Clear differences in the antioxidant activity and phenolic compounds were observed depending on the type of thermal process applied to the borage stems. When stems were boiled, both ABTS and DPPH decreased significantly (51-52%) (Table 1), and the total phenolic compounds decreased about 67% (Fig. 4). It can be concluded that boiling reduces the potential health benefits associated with the antioxidant compounds present in fresh borage. The water used in this type of cooking was probably enriched with, at least, some of these compounds due to a dilution effect. This aspect was confirmed by GLISZCZYNSKA-SWIGLO *et al.* (2006) in a similar work carried out with broccoli. These authors also found that cooking in water significantly decreased most of the health-promoting compounds of broccoli. They also confirmed that the loss of both vitamin C and polyphenols was mainly due to being leached into the cooking water.

When steam cooking of borage stems was applied, no effect was observed in the antioxidant activity values. The ABTS and DPPH showed results that were similar to those obtained for raw stems (Table 1). Total phenolic compounds decreased with this treatment (around 35%, approximately half of the decrease found for boiling (Fig. 4). In comparison to boiling, this milder heat treatment, with no leaching-effect, resulted in a higher antioxidant value and phenolic content in the edible portions of borage. In both cooking methods, the relative loss of phenolic compounds in the treated samples compared to the fresh ones was more evident than that observed for the antioxidant activity. TURKMEN *et al.* (2005) also reported different results for the phenolic compounds and antioxidant activity depending on the vegetables being analyzed and the cooking treatments applied. Some authors suggest that there is the possibility of formation of new antioxidant compounds (e.g. Maillard reaction products) during heat treatment (NICOLI *et al.*, 1999).

The literature shows that the effect of thermal treatment on the potential antioxidant activity of vegetables is different depending on several factors such as species, intensity and modality of the treatment. Analyzing the influence of heat treatment on the antioxidant activities and polyphenolic compounds of Shiitake mushroom extracts CHOI *et al.* (2006) found that they increased as heating temperature and time increased. These results are in agreement with those found by DEWANTO *et al.* (2002), who showed that thermal processing enhanced the nutritional value of tomatoes by increasing the bio-accessible lycopene content and total antioxidant activity.

On the contrary, some studies on broccoli and cauliflower showed that heat treatments

(blanching and cooking) had a detrimental effect on the polyphenols and antioxidant activity in comparison with the raw products (GEBCZYNSKI and KMIECIK, 2007). ZHANG and HAMAUZU (2004) also found that the antioxidant components (including phenolic compounds) and antioxidant activity in broccoli decreased markedly during conventional and microwave cooking. RACCHI *et al.* (2002) found that boiling had different effects on the antiradical activity of water-soluble components depending on the type of vegetable. Testing the effect of thermal treatment on the water-soluble fraction of different vegetables, ROY *et al.* (2007) concluded that normal cooking temperatures (75°-100°C, 10-30 min) were detrimental to the phenolic content and to the antiradical and antiproliferative activities of many vegetable juices; however mild heating (50°C, 10-30 min) preserved 80-100% of the phenolic content.

All of these findings confirm that more research is needed to determine the effects of cooking on total phenolics, as stated by RICKMAN *et al.* (2007) in a recent review of the nutritional value of different fresh and processed fruits and vegetables.

In summary, borage water extracts, particularly, those of their by-products (leaves) showed great antioxidant activity, that could potentially be used for different applications in the food industry. Steam cooking of borage was a better technology than traditional boiling for preserving the antioxidant activity and total phenolic content of borage.

ACKNOWLEDGEMENTS

The authors are grateful to CONSOLIDER-INGENIO 2010 CARNISENUSA CSD2007-00016, to project AGL 2008-01099/ALI (Ministerio de Ciencia y Tecnología, Spain), to the Government of Navarra (Plan Tecnológico), to PIUNA (Plan de Investigación de la Universidad de Navarra) and to OLUS Tecnología for their financial support of this research project.

REFERENCES

AOAC 2000. AOAC Official Method 930.04 Moisture in plants. Loss on drying.

Bandoniene D. and Murkovic M. 2002. The detection of radical scavenging compounds in crude extract of borage (*Borago officinalis L.*) by using an on-line HPLC-DPPH method. J. Biochem. Biophys. Methods 53: 45.

Bandoniene D., Venskutonis P.R., Gruzdiene D. and Murkovic M. 2002. Antioxidant activity of sage (*Salvia officinalis* L.), savory (*Satureja hotensis* L.) and borage (*Borago officinalis* L.) extracts in rapeseed oil. Eur. J. Lipid. Sci. Technol. 104: 286.

Blois M.S. 1958. Antioxidant determinations by the use of a stable free radical. Nature 181: 1199.

Choi Y., Lee S.M., Chun J., Lee H.B. and Lee J. 2006. Influence of heat treatment on the antioxidant activities and polyphenolic compounds of Shiitake (*Lentinus edodes*) mushroom. Food Chem. 99: 381.

Danesi F. and Bordoni A. 2008. Effect of home freezing and Italian style of cooking on antioxidant activity of edible vegetables. J. Food Sci. 73: H109.

Dewanto V., Wu X.Z., Adom K.K. and Liu R.H. 2002. Thermal processing enhances the nutritional value of tomatoes by increasing total antioxidant activity. J. Agr. Food Chem. 50: 3010.

Gebczynski P. and Kmiecik W. 2007. Effects of traditional and modified technology, in the production of frozen cauliflower, on the contents of selected antioxidative compounds. Food Chem. 101: 229.

Gliszczynska-Swiglo A., Ciska E., Pawlak-Lemanska K., Chmielewski J., Borkowski T. and Tyrakowska B. 2006. Changes in the content of health-promoting compounds and antioxidant activity of broccoli after domestic processing. Food Addit. Contam. 23: 1088.

Gorinstein S., Jastrzebski Z., Leontowicz H., Leontowicz M., Namiesnik J., Najman K., Park Y.S., Heo B.G., Cho J.Y. and Bae J.H. 2009. Comparative control of the bioactivity of some frequently consumed vegetables subjected to different processing conditions. Food Control 20: 407.

Gundgaard J., Nielsen J.N., Olsen J. and Sorensen J. 2003. Increased intake of fruit and vegetables, estimation of impact in terms of life expectancy and healthcare costs. Public Health Nutr. 6: 25.

Kahkonen M.P., Hopia A.I., Vuorela H.J., Rauha J-P., Pihlaja K., Kujala T.S. and Heinonen M. 1999. Antioxidant activity of plant extracts containing phenolic compounds. J. Agr. Food Chem. 47: 3954.

Khan M.A. and Shahidi F. 2000. Oxidative stability of stripped and nonstripped borage and evening primrose oils and their emulsions in water. J. Am. Oil Chem. Soc. 77: 963.

Llorach R., Espin J.C., Tomas-Barberan F.A. and Ferreres F. 2003. Valorization of cauliflower (*Brassica oleracea L. var. botrytis*) by-products as a source of antioxidant phenolics. J. Agr. Food Chem. 51: 2181.

Mezadri T., Villano D., Fernandez-Pachón M.S., García-Parrilla M.C. and Troncoso A.M. 2008. Antioxidant compounds and antioxidant activity in acerola (*Malpighia emarginata DC.*) fruits and derivates. J. Food Compos. Anal. 21: 282.

Nenadis N., Lazaridou O. and Tsimidou M.Z. 2007. Use of reference compounds in antioxidant activity assessment. J. Agr. Food Chem.55: 5452.

Nicoli M.C., Anese M. and Parpinel M. 1999. Influence of processing on the antioxidant properties of fruit and vegetables. Trends Food Sci. Tech. 10: 94.

Okonogi S., Duangrat C., Anuchpreeda S., Tachakittirungrod S. and Chowwanapoonpohn S. 2007. Comparison of antioxidant capacities and cytotoxicities of certain fruit peels. Food Chem. 103: 839.

Padda M.S. and Picha D.H. 2007. Methodology optimization for quantification of total phenolics and individual phenolic acids in sweetpotato (*Ipomoea batatas L.*) roots. J. Food Sci. 2: C412.

Pattahamakanokporn O., Puwastien P., Nitithamyong A. and Sirichakwal P.P. 2008. Changes of antioxidant activity and total phenolic compounds during storage of selected fruits. J. Food Compos. Anal. 21: 241.

Perez-Jimenez J and Saura-Calixto F. 2006. Effect of solvent and certain food constituents on different antioxidant capacity assays. Food Res. Int. 39: 791.

Peschel W., Sanchez-Rabaneda F., Diekmann W., Plescher A., Gartzia I., Jimenez D., Lamuela-Raventos R., Buxaderas S. and Codina C. 2006. An industrial approach in the search of natural antioxidants from vegetable and fruit wastes. Food Chem. 97: 137.

Prior R.L., Wu X.L. and Schaich K. 2005. Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. J. Agr. Food Chem. 53: 4290.

Pyo Y.H., Lee T.C., Logendra L. and Rosen R.T. 2004. Antioxidant activity and phenolic compounds of Swiss chard (*Beta vulgaris subspecies cycla*) extracts. Food Chem. 85: 19.

Racchi M., Daglia M., Lanni C., Papetti A., Govoni S. and Gazzani G. 2002. Antiradical activity of water soluble components in common diet vegetables. J. Agr. Food Chem. 50: 1272.

Re R., Pellegrini N., Proteggente A., Pannala A., Yang M. and Rice-Evans C. 1999. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Rad. Biol. Med. 26: 1231.

Rickman J.C., Barrett D.M. and Bruhn C.M. 2007. Nutritional comparison of fresh, frozen and canned fruits and vegetables. Part 1. Vitamins C and B and phenolic compounds. J. Sci. Food Agric. 87: 930.

Rivero-Perez M.D., Muniz P. and Gonzalez-Sanjose M.L. 2007. Antioxidant profile of red wines evaluated by total antioxidant capacity, scavenger activity, and biomarkers of oxidative stress methodologies. J. Agr. Food Chem. 55: 5476.

Roy M.K., Takenaka M., Isobe S. and Tsushida T. 2007. Antioxidant potential, anti-proliferative activities, and phenolic content in water-soluble fractions of some commonly consumed vegetables: effects of thermal treatment. Food Chem. 103: 106.

Sanchez-Moreno C., Larrauri J.A. and Saura-Calixto F. 1998. A procedure to measure the antiradical efficiency of polyphenols. J. Sci. Food Agric. 76: 270.

Schieber A., Stintzing F.C. and Carle R. 2001. By-products of plant food processing as a source of functional compounds - recent developments. Trends Food Sci. Tech. 12: 401.

Singleton V.L. and Rossi J.A. 1965. Colorimetry of total phenolics with phospho-molybdic-phosphotungstic acid reagents. Am. J. of Enol. Viticult. 16: 144.

Stratil P., Klejdus B. and Kuban V. 2006. Determination of total content of phenolic compounds and their antioxidant activity in vegetables - Evaluation of spectrophotometric methods. J. Agr. Food Chem. 54: 607.

Thaipong K., Boonprakob U., Crosby K., Cisneros-Zevallos L. and Byrne D.H. 2006. Comparison of ABTS, DPPH, FRAP, and ORAC assays for estimating antioxidant activity from guava fruit extracts. J. Food Compos. Anal. 19: 669.

Turkmen N., Sari F. and Velioglu Y.S. 2005. The effect of cooking methods on total phenolics and antioxidant activity of selected green vegetables. Food Chem. 93: 713.

Venskutonis P.R., Dvaranauskaite A. and Labokas J. 2007. Radical scavenging activity and composition of raspberry (*Rubus idaeus*) leaves from different locations in Lithuania. Fitoterapia 78: 162.

Wettasinghe M., and Shahidi F. 1999. Antioxidant and free radical-scavenging properties of ethanolic extracts of defatted borage (*Borago officinalis L.*) seeds. Food Chem. 67: 399.

Wu X.L., Beecher G.R., Holden J.M., Haytowitz D.B., Gebhardt S.E. and Prior R.L. 2004. Lipophilic and hydrophilic antioxidant capacities of common foods in the United States. J. Agr. Food Chem. 52: 4026.

Zhang D.L. and Hamauzu Y. 2004. Phenolics, ascorbic acid, carotenoids and antioxidant activity of broccoli and their changes during conventional and microwave cooking. Food Chem. 88: 503.

Paper received June 10, 2009 Accepted October 2, 2009

PAPER

AGRONOMIC AND QUALITY PARAMETERS OF FLAT PEACH UFO3, UFO4 AND SWEETCAP®

FCA. HERNÁNDEZ*, J.J. MARTÍNEZ and P. LEGUA
Plant Science and Microbiology Department, Universitas Miguel Hernández. Ctra Beniel 3.2, 03312 Orihuela (Alicante), Spain
*Corresponding author: Tel. +34 966749702, Fax +34 966749693
e-mail: francisca.hernandez@umh.es

ABSTRACT

The agronomic and fruit quality characteristics of the peach cultivars 'Ufo3', 'Ufo4' and 'Sweetcap®' cultivars grown in Murcia, Spain are reported. The cultivar 'Sweetcap®' is a mid-season flat peach while 'Ufo3' and 'Ufo4' are early flat peaches. 'Sweetcap®' displayed good plantation uniformity, had an average yield of 5.1 kg/m^2, a high calibre fruit and weight (162.5 g), high soluble solids content (12.7°Brix), high pulp yield (97.0%) and high quality taste. The cultivars 'Ufo3' and 'Ufo4' also displayed good plantation uniformity, with a higher average yield than 'Sweetcap®' (5.2 and 5.3 kg/m^2, respectively), an average fruit weight that was slightly less, 90.6 g for 'Ufo3' and 103.3 g for 'Ufo4', flatter fruit, and a soluble solids content ranging from 11.4° to 12.2°Brix. The 'Ufo4' cultivar had lower acidity and very high-quality tasting fruit (high SSC/TA ratio).

- Key words: flat peach, fruit quality, fruit size, *Prunus persicae,* total acidity, yield -

INTRODUCTION

Peach [*Prunus persicae* (L.) Batsch] is the third most important fruit grown in the world, after apples and pears. Peach production comes mainly from China, the Mediterranean area (Italy and Spain) and the United States (FAOSTAT, 2007). In Spain, peach is one of the most important fruit species cultivated and it produces around 26% of the total EU production. Spain is the second largest European producer after Italy (FAOSTAT, 2007). In 2007 the Spanish Murcia region (CARM, 2008), the third most important peach producing region, produced 241,292 tons: 85.5% peaches, 9.5% nectarines and 5.0% flat peaches.

Traditionally, production and marketing of flat peaches in Spain has been limited both in quantity and quality. The main drawbacks of traditional cultivars are their lack of appealing colour, low consistency of the fruit and excessive cracking of the pistil which means the product does not meet market quality demands. In addition the traditional varieties do not sufficiently supply year-round demands. Just over a decade ago new flat peach cultivars, which have more attractively coloured fruit and good consistency, were introduced into Spain and peach production and marketing have increased. The newly introduced flat peach cultivars came mainly from France (Agro Selection Fruits) and Italy (Istituto Sperimentale per la Frutticoltura, Rome), but these countries have not yet experienced the production increase that has occurred in Spain.

In the 1980s, the Istituto Sperimentale per la Frutticoltura in Rome began a breeding program to obtain a cultivar with a higher yield, improved organoleptic quality, better consistency of flesh and increased fruit size and without split pits. All these objectives were set to create a number of cultivars that ripened at different times, so as to avoid an oversupply at any one time. To fulfil these goals, crosses were made using parental genotypes with flat fruit and genotypes with standard or globe-shaped fruit (NICOTRA *et al.*, 2001a,b). The result was a new type of peach, called UFO. The UFO series contains 9 *cvs*: from 'Ufo1' to 'Ufo9'.

In the 1990s, Arsène and Laurence Maillard began a genetic improvement program to obtain new flat peach varieties. The 'Sweetcap®' Maillarflat cultivar and the 'Regalcake®' and 'Nectacake®' series of cultivars were developed and these have been greatly strengthened in recent years with the acquisition of many varieties that virtually cover the whole fruit production calendar, from mid- to late-season.

The basic difference between the cultivars originating in Maillard (France) and those in Italy, is that the cultivars from Maillard are mid-season (second half of June) and late-season varieties (mid September), while those originating in Rome are mostly early varieties with some mid-season and one or two late-season varieties. The results of the two programs are complementary in terms of providing new flat peach cultivars that cover a wider harvesting period.

In this study the agronomic behaviour and organoleptic quality parameters were evaluated in two Italian Ufo flat peach cultivars ('Ufo3' and 'Ufo4') and the 'Sweetcap®' cultivar (Maillarflat). The cultivars were all grafted onto the GF-677 hybrid rootstock, widely used for peach. While literature reports numerous studies on the agronomic performance of this rootstock when grafted with different peach cultivars (GIORGI *et al.*, 2005, HUDINA *et al.*, 2006, SPORNBERGER *et al.*, 2005 and ZARROUK *et al.*, 2006). The characterization of morphological, physical or organoleptic properties of the cultivars under assay have not been reported.

MATERIALS AND METHODS

The peach cultivars [*Prunus persicae* (L.) Batsch] 'Ufo3' and 'Ufo4' (originating from crossing Maybelle x Saturn, of Italian origin) and 'Sweetcap®' (obtained by Maillard of French origin) were studied. All cultivars were grafted onto the hybrid rootstock GF-677 (*Prunus persicae* x *Prunus amygdalus*).

The trial was conducted at an experimental orchard located near Cieza, Murcia, Spain (Latitude 38° 17´N – 1° 27´W). Soil conditions were poor and stony in the first layer (0-10 cm) and clayish below this level (clay loam texture). Soil was highly calcareous with a 15% content of active lime and a pH of 7.8. The 'Ufo3' and 'Ufo4' planting density was 4 x 1.5 m, and that for 'Sweetcap® was 5 x 2.5 m, trained to a central leader system. The study was conducted during the period 2005-2008. Conventional commercial summer pruning and drip irrigation treatments were performed. A randomised block design was used with five single-tree replications for each variety. Guard rows were used to preclude edge effects.

The onset of bud sprouting, full bloom and petal fall were recorded on five trees per variety and on four branches per tree. Branches were homogeneous in all cases with a similar length (30-60 cm), located at three different heights in the tree (low, medium, high) and marked at the phenological stage A (BAGGIOLINI, 1952). Full bloom was recorded when more than 50% of the flowers were open.

The number of fruits/m^2 and yield (kg/m^2) were measured. The number of fruits/m^2 was determined as the ratio between the total number of fruits per tree and the tree spacing. Yield (kg/m^2) was determined as the ratio between the total kilograms of fruit per tree and the tree spacing.

Samples of 50 fruits per variety (10 fruits per tree) were hand-harvested at random at the commercial maturity stage, on the basis of

their skin colour (fully-coloured). Immediately after harvest, fruits were transported in an air-conditioned car to the laboratory. Fresh weight, equatorial, suture and polar diameter, flesh thickness, flesh firmness, stone weight, width and length, pulp yield, pH, soluble solid content (SSC), titratable acidity (TA), SSC/TA ratio and colour were measured. Fruit and stone weight were measured with a Sartorius digital balance (model BL-600, 0.01 g accuracy, Goettingen, Germany). Fruit equatorial, suture and polar diameter and flesh thickness, as well as stone width and length were measured with Mitutoyo electronic digital slide gauge (model CD-15DC, 0.01 mm accuracy, England, U.K.). Pulp yield percentage was calculated as (fruit weight-stone weight)/fruit weight x100.

Flesh firmness (FF) was measured with a Bertuzzi Penetrometer (model FT-327, Facchini, Alfonsine, Italy) with an 8-mm cylindrical plunger and measurements were made on the two opposite faces in the equatorial zone. The CIELAB L^* (brightness or lightness; 0= black, 100 = white), a^* ($-a^*$ = greenness, $+a^*$ = redness) and b^* ($-b^*$ = blueness, $+b^*$ = yellowness) colour variables were measured on the skin on the four opposite faces in the equatorial zone, using a chromatometer (CR-300, Minolta Co., Osaka, Japan).

The chemical analyses were carried out on four juice samples (10 peaches each). The juice was extracted using a blender and filtered through cheesecloth. Juice pH was calculated by a Crison pH-meter (model MicropH 2001, Barcelona, Spain). Soluble solids content (SSC) was measured at 20°C with a refractometer (Atago Co., Tokyo, Japan). Titratable acidity (TA) was determined with an acid-base potentiometer (0.1N NaOH up to pH 8.1) and is expressed as grams per liter (g/L) of malic acid. The ripeness index (RI) was calculated as the ratio of soluble solids content and total acidity.

Statistical analysis

Statistical analyses were performed using SPSS 16.0 for Windows. A basic descriptive statistical analysis was followed by an analysis of variance test for mean comparisons, with standard errors. To discriminate among the means (multiple range test), the Fisher´s least significant difference (LSD) procedure was used at the 95.0% confidence level. Pearson correlation coefficients were determined.

RESULTS AND DISCUSSION

Agronomic parameters

During the three-year study, the onset of bud sprouting in 'Ufo3' and 'Ufo4' cultivars occurred around the first two weeks of February, two weeks ahead of the 'Sweetcap ®'cultivar (late February) (Table 1). 'Ufo3' and 'Ufo4' can be considered early harvest cultivars: (late May-early June). The fruit was harvested in 8-12 days. 'Sweetcap ®' is clearly a mid-season harvest cultivar, (mid July). The fruit was harvested in 15-21 days. Under the tested cultivation conditions, 'Ufo3' was harvested 26 days earlier than 'Big Top' and 21 days earlier than 'Ufo4'.

There was no significant difference in yield among the cultivars studied (mean value 5.2 kg/m^2). The number of fruits per m^2 in cultivars 'Ufo3' and 'Ufo4' were similar (53.6 and 49.2 fruit/m^2, respectively) and were greater than that of 'Sweetcap ®' (39.5 fruits/m^2).

Fruit quality parameters

The fresh fruit weight for cultivar 'Ufo3' (90.6 g) was 7.8% greater than that obtained by NICOTRA *et al.* (2001a), while the average fruit weight for

Table 1 - Dates of onset of bud sprouting, full bloom, petal fall and harvesting (2005-2008) of the three peach cultivars.

	Onset of bud sprouting	Full bloom	Petal fall	Harvesting
Ufo3	7-9 February	25 February	12-16 March	27 May-10 June
Ufo4	12-14 February	1 March	16-19 March	2 June-15 June
Sweetcap®	20 February	7-10 March	30 March	3 July-25 July

Table 2 - Fresh weight, equatorial, suture and polar diameter and flesh thickness of the three peach cultivars.

Cultivar	Fruit weight (g)	Equatorial diameter (mm)	Suture diameter (mm)	Polar diameter (mm)	Flesh thickness (mm)
Ufo3	90.66±1.87 a	64.17±0.46 a	65.05±0.49 a	35.99±0.26 a	21.54±0.32 a
Ufo4	103.73±1.70 b	67.87±0.39 b	68.42±0.41 b	36.62±0.30 a	23.10±0.27 b
Sweetcap®	162.54±2.14 c	78.50±0.51 c	75.81±0.43 c	44.92±0.28 b	24.95±0.33 c

Values with different letters within columns indicate statistically significant differences (95% LSD). Mean values ± S.E. for the 3-year study (sample size for each parameter is 50 fruits).

cultivar 'Ufo4' (103.4 g) was 9.75% lower than that obtained by NICOTRA *et al.* (2001a). These differences are probably due to the different environmental conditions. The cultivar 'Sweetcap ®' had the heaviest fruit (162.5 g), probably due to being a later ripening variety compared to the Ufo cultivars tested (Table 2).

'Sweetcap ®' produced the largest but the least flattened fruit (largest polar diameter) compared with the 'Ufo3' and 'Ufo4' cultivars. 'Ufo3' and 'Ufo4' had a greater fruit diameter compared with those obtained by NICOTRA *et al.* (2001b, 2002) but lower than that of 'Sweetcap ®' (Table 2). Flesh thickness was significantly greater for 'Sweetcap ®' (24.9 mm) than for 'Ufo3' and 'Ufo4'. 'Ufo3' had the lowest flesh thickness values.

Fruit from the three cultivars had low-acidity with pH values greater than 4.0 (DIRLEWANGER *et al.* 2006) (Table 3). 'Sweet cap®' had a greater TA compared to 'Ufo3' and 'Ufo4' (Table 3).

The total soluble solids content (Table 3) for the early cultivars 'Ufo3' and 'Ufo4' was 11.4° and 12.2°Brix, respectively; the values were lower than those obtained by NICOTRA *et al.* (2001b). The mid-season cultivar 'Sweetcap ®' had a value of 12.7° Brix, which was not significantly different from cultivar 'Ufo4'. The highest SSC/TA ratio was recorded for the 'Ufo4' cultivar (high quality taste).

Table 4 shows that the 'Ufo3' fruit had lighter, smaller stones (3.2 g), while the 'Sweetcap ®' cultivar produced fruit with larger heavier stones (4.6 g). The highest pulp yield (97%) was recorded for 'Sweetcap®'; despite having the largest stone, the fruit was the largest and heaviest.

Flesh firmness (Table 5) was very similar among the three cultivars, although cultivars 'Sweetcap ®' and 'Ufo3' had the most consistent fruit flesh.

Fruit colour is an important factor for marketability and consumer acceptance and can also be indicative of the ripeness index. Cultivars 'Ufo3' and 'Ufo4' produced the reddest (high a* values) skin, while cultivar 'Sweetcap®' had the yellowest skin (high b* and low a* values) (Table 5). The a*/b* ratio was the lowest for the 'Sweetcap®' cultivar. The L values of fruit of 'Ufo3', 'Ufo4' and 'Sweetcap®' did not differ significantly.

Table 3 - pH, soluble solids content (SSC), titratable acidity (TA) and SSC/TA ratio of the three peach cultivars.

Cultivar	pH	SSC (°Brix)	TA (g/L)	SSC/TA
Ufo3	4.48±0.07 b	11.45±0.29 a	0.32±0.02 a	37.08±2.98 b
Ufo4	4.32±0.08 ab	12.25±0.35 ab	0.29±0.03 a	47.00±4.25 c
Sweetcap®	4.15±0.04 a	12.74±0,15 b	0.53±0.02 b	25.04±1.42 a

Values with different letters within columns indicate statistically significant differences (95% LSD). Mean values ± S.E. for the 3-year study (sample size for each parameter is n=12).

Table 4 - Stone weight, width and length of stone and pulp yield of the three peach cultivars.

Cultivar	Stone weight (g)	Stone width (mm)	Stone length (mm)	Pulp yield (%)
Ufo3	3.22±0.08 a	19.49±0.17 a	20.49±0.18 a	96.01±0.098 a
Ufo4	3.59±0.05 b	20.05±0.14 b	22.00±0.26 b	96.48±0.063 b
Sweetcap®	4.64±0.06 c	21.62±0.11 c	22.73±0.18 c	97.04±0.064 c

Values with different letters within columns indicate statistically significant differences (95% LSD). Mean values ± S.E. for the 3-year study (sample size for each parameter is 50 fruits).

Table 5 - Colour and flesh firmness of the three peach cultivars.

Cultivar	L	a*	b*	a*/b*	Flesh firmness (kg/cm^2)
Ufo3	54.12±1.01 a	21.66±0.53 b	28.71±0.41 a	0.772±0.023 b	5.71±0.19 ab
Ufo4	51.47±1.06 a	21.00±0.55 b	28.08±0.44 a	0.771±0.026 b	5.37±0.18 a
Sweetcap®	54.59±1.27 a	19.16±0.60 a	30.79±0.53 b	0.652±0.028 a	5.93±0.15 b

Values with different letters within columns indicate statistically significant differences (95% LSD). Mean values ± S.E. for the 3-year study (sample size for each parameter is 50 fruits).

Table 6 - Pearson product moment correlations and (P value) among the variables studied.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1. Fruit weight	1																	
	0.8698																	
2. ø Equatorial	**(0.0000)**	1																
	0.7922	**0.8572**																
3. ø Suture	**(0.0000)**	**(0.0000)**	1															
	0.8508	**0.9371**	**0.9048**															
4. ø polar	**(0.0000)**	**(0.0000)**	**(0.0000)**	1														
	0.6132	**0.6451**	**0.6824**	**0.6081**														
5. Pulp thickness	**(0.0000)**	**(0.0000)**	**(0.0000)**	**(0.0001)**	1													
	0.6237	**0.6402**	**0.6270**	**0.7442**	0.2846													
6. Stone weight	**(0.0000)**	**(0.0000)**	**(0.0000)**	**(0.0000)**	(0.0925)	1												
	0.0547	0.1333	0.1632	0.1680	-0.1294	**0.5192**												
7. Stone width	(0.7514)	(0.4382)	(0.3415)	(0.3272)	(0.4501)	**(0.0012)**	1											
	0.4404	**0.5577**	**0.5028**	**0.5756**	0.2613	**0.5496**	0.0656											
8. Stone length	(0.0072)	**(0.0004)**	**(0.0018)**	**(0.0002)**	(0.1238)	**(0.0005)**	(0.7038)	1										
	0.6280	**0.7076**	**0.7130**	**0.6059**	**0.6540**	0.0065	-0.2267	0.2104										
9. Pulp yield	**(0.0000)**	**(0.0000)**	**(0.0000)**	**(0.0001)**	**(0.0000)**	(0.9701)	(0.1837)	(0.2180)	1									
	-0.4939	-0.4157	-0.4258	-0.4033	-0.1470	-0.2887	-0.0926	-0.1748	-0.3041									
10. pH	(0.0022)	(0.0117)	(0.0096)	(0.0147)	(0.3923)	(0.0878)	(0.5911)	(0.3077)	(0.0713)	1								
	0.6871	**0.6576**	**0.5631**	**0.6839**	0.3178	**0.5222**	0.0218	0.4344	0.3800	**-0.6818**								
11. TA	**(0.0000)**	**(0.0000)**	**(0.0004)**	**(0.0000)**	(0.0589)	**(0.0010)**	(0.8996)	(0.0081)	(0.0223)	**(0.0000)**	1							
	0.4320	0.4543	**0.6078**	**0.5220**	0.3823	0.2848	0.0940	0.3255	0.4970	-0.0508	0.1495							
12. SSC	(0.0085)	(0.0054)	**(0.0001)**	**(0.0011)**	(0.0214)	(0.0923)	(0.5855)	(0.0527)	(0.0020)	(0.7687)	(0.3843)	1						
	-04672	-0.4108	-0.3215	-0.4423	-0.1223	-0.3734	-0.0156	-0.2993	-0.1663	**0.6982**	**-0.9139**	0.0950	1					
13. SSC/TA	(0.0041)	(0.0128)	(0.0559)	(0.0069)	(0.4774)	(0.0249)	(0.9281)	(0.0762)	(0.3325)	**(0.0000)**	**(0.0000)**	(0.5814)						
	-0.0280	-0.0268	-0.1744	-0.0308	-0.0336	0.0371	0.1597	-0.1297	-0.1563	0.0102	0.1774	-0.2766	-0.2586	1				
14. Flesh	(0.9710)	(0.8765)	(0.3090)	(0.8584)	(0.8456)	(0.8301)	(0.3523)	(0.4510)	(0.3626)	(0.9531)	(0.3007)	(0.1025)	(0.1278)					
firmness	0.1087	0.1516	0.0555	0.1261	0.1088	0.1772	0.3408	-0.0095	0.0030	0.2369	-0.0424	-0.1338	0.0965	0.2754	1			
15. *L*	(0.5281)	(0.3775)	(0.7478)	(0.4635)	(0.5276)	(0.3011)	(0.0420)	(0.9562)	(0.9863)	(0.1641)	(0.8060)	(04365)	(0.5757)	(01040)				
	-0.3555	-0.4510	-0.3365	-0.3791	-0.3487	-0.2305	-0.0681	-0.1800	-0.3140	0.0369	-0.1264	-0.0157	0.0425	-0.2039	**-0.7088**	1		
16. *a**	(0.0333)	(0.0058)	(0.0448)	(0.0226)	(0.0372)	(0.1761)	(0.6931)	(0.2934)	(0.0622)	(08307)	(0.4626)	(0.9274)	(0.8054)	(0.2330)	**(0.0000)**			
	0.5398	**0.5068**	0.4466	**0.5355**	0.3471	0.3084	-0.0692	0.4116	0.3750	-0.1787	0.4328	0.0411	-0.3386	0.0985	**0.6135**	**-0.6471**	1	
17. *b**	(0.0007)	**(0.0016)**	(0.0063)	**(0.0008)**	(0.0381)	(0.0672)	(0.6884)	(0.0126)	(0.0242)	(0.2972)	(0.0084)	(0.8120)	(0.0434)	(0.5676)	**(0.0001)**	**(0.0000)**		
	-0.4118	-0.4780	-0.3681	-0.4262	-0.3794	-0.2615	-0.0411	-0.2454	-0.3321	0.0505	-0.1855	-0.0054	0.0999	-0.2092	**-0.7442**	**0.9818**	-0.7668	1
18. *a/b****	(0.0126)	(0.0032)	(0.0272)	(0.0095)	(0.0225)	(0.1234)	(0.8121)	(0.1491)	(0.0478)	(0.7698)	(0.2786)	(0.9750)	(0.5620)	(0.2208)	**(0.0000)**	**(0.0000)**	**(0.0000)**	

Absolute linear correlations ≥ 0.50 are marked in bold type.

Correlation analyses

The interdependence of the variables was assessed by a correlation analysis in which forty-two pairs of variables were correlated (Table 6).

Correlation analyses showed that the greatest correlation was between fruit weight and equatorial diameter (r = 0.87), suture diameter (r = 0.79) and polar diameter (r = 0.85), as well as fruit weight and flesh thickness (r =0.61) and fruit weight and stone weight (r = 0.62). The pH was negatively correlated with TA (r = -0.68) and positively correlated with the SSC/TA ratio (r = 0.69), while the TA was negatively correlated with the SSC/TA ratio (r = -0.91).

Skin brightness was negatively correlated with skin redness (*a** values) *r* = -0.70 and a*/b* ratio (r = -0.77). Similar results have been reported for other clingstone peaches (DROGOUDI and TSIPOURIDIS, 2007). Skin redness (a* value) was positively correlated with the a*/b* ratio (r = 0.98), while the skin yellowness was negatively correlated with a*/b* ratio (r = -0.76).

We conclude that the 'Sweetcap ®' cultivar is a good mid-season flat peach due to its potential size, production uniformity and very high-quality taste. Cultivars 'Ufo3' and 'Ufo4' are two good options for early flat peach cultivars for marketing. They may weigh less and be smaller than 'Sweetcap®'. This is compensated by their early harvest because they can get very high prices, since they are the first on the market. The size of cultivars 'Ufo3' and 'Ufo4' improved under our growing conditions (Murcia, Spain), compared to reports by NICOTRA *et al.* (2001b). 'Ufo4' was proven to be more regular compared to 'Ufo3', which was more variable.

Given the remarkable variability in flat peaches, this study must be extended to other cultivars present on the market and to promote those with a high organoleptic, nutritional and nutraceutical profile. The results of this study confirm that these three flat peach cultivars are highly recommended and of interest to the Murcia area and other Mediterranean countries with similar edaphoclimatic conditions.

REFERENCES

Baggiolini M. 1952. Stade repères du pêcher. Rev. Rom. Agric. Vit. Arb. 4: 29.

CARM. 2008. http://www.carm.es.

Dirlewanger E., Cosson P., Boudehri K., Renaud C., Capdeville G., Tauzin Y., Laigret F. and Moing A. 2006. Development of a second-generation genetic linkage map for peach (*Prunus persica* (L.) Bastch) and characterization of morphological traits affecting flower and fruit. Tree Genet. Genome 3:1.

Drogoudi P.D. and Tsipouridis C.Gr. 2007. Effects of cultivar and rootstock on the antioxidant content and physical characters of clingstone peaches. Sci. Hortic. 115: 34.

FAOSTAT. 2007. http://www.faostats.

Giorgi M., Capocasa F., Scalzo J., Murri G., Battino M. and Mezzetti B. 2005. The rootstock effects on plant adaptability, production, fruit quality, and nutrition in the peach (*cv.* 'Suncrest`). Sci. Hortic. 107: 36.

Hudina M., Fajt N. and Stampar F. 2006. Influence of rootstock on orchard productivity and fruit quality in peach *cv.* 'Redhaven'. J. Hort. Sci. Biotech. 81: 1064.

Nicotra A., Conte L., Moser L. and Fantechi P. 2001a. New peach varieties. Italus Hortus, 8: 48.

Nicotra A., Conte L., Moser L. and Fantechi P. 2001b. The ufo series, six new cultivars of peach with flat fruit. Informatore Agrario, 57: 61.

Nicotra A., Conte L., Moser L. and Fantechi P. 2002. New types of high quality peaches: flat peaches (*P. persica var. platicarpa*) and ghiaccio peach series with long on tree fruit life. Acta Hort. (ISHS) 592:131.

Spornberger A., Pieber K. and Modl P. 2005. Suitability of rootstocks for commercial peach production under pannonic climatic conditions with the cultivars 'Suncrest' and 'Redcal'. Mitteilungen Klosterneuburg, Rebe und Wein, Obstbau und Fruchteverwertung, 55: 156.

Zarrouk O., Gogorcena Y., Moreno M.A. and Pinochet J. 2006. Graft compatibility between peach cultivars and *Prunus* rootstocks. HortScience, 41: 1389.

Revised paper received July 31, 2009 Accepted November 4, 2009

SHORT COMMUNICATION

DIFFERENT SENSITIVITY TO BROWNING IN FRESH-CUT PINEAPPLE, APPLE AND PEAR: THE ROLE OF ENDOGENOUS VITAMIN C

S. TAVARINI, L. TRINCI, E. DEGL'INNOCENTI and L. GUIDI*
Dipartimento di Biologia delle Piante Agrarie, University of Pisa, Viale delle Piagge 23, 56124 Pisa, Italy
*Corresponding author: Tel. +39 050 2216613, Fax +39 050 2216630
e-mail: guidilu@agr.unipi.it

ABSTRACT

Pineapple, apple and pear were studied as fresh-cut fruit compared to the whole ones upon storage at 4°C. Pineapple did not show any symptom related to enzymatic browning even after 216 h of storage, in comparison symptoms were evident in apple and pear as soon as 24 h after cutting. The different sensitivity of the three types of fruit was linked to the high content in ascorbic acid in pineapple as compared to apple and pear.

- Key words: apple, enzymatic browning, pear, phenylalanine ammonia lyase, pineapple, polyphenol oxidase -

INTRODUCTION

In recent years, the demand for fresh-cut (or minimally processed) fruit has increased in relation to their convenience as ready-to-eat and fresh-like quality products that improve human health (LIU, 2003). However, it is known that fresh-cut fruit and vegetables are more perishable in comparison to intact fruit and vegetables (GIL *et al.*, 2006). Fresh-cut fruit deteriorates faster because of internal and external browning of the cut surfaces (MARTINEZ and WHITAKER, 1995; GIL *et al.*, 2006; TOIVONEN and BRUMMEL, 2008) that alters the quality and, consequently, the marketing of the products. Surface browning is caused by oxidative enzymes, in particular polyphenol oxidase (PPO), which oxidizes phenols to quinones, which are slightly coloured. The quinones condense and react non-enzymatically with amino acids and proteins, leading to brown melanin pigments (MARTINEZ and WHITAKER, 1995; BRECHT *et al.*, 2004). This phenomenon has been well studied in lettuce and other salad vegetables (CAMPOS-VARGAS and SALTVEIT, 2002). Research has shown that in some cases, like rocket salad, resistance to browning is associated with higher activities of phenylalanine ammonia lyase (PAL) and PPO, as compared with lettuce (DEGL'INNOCENTI *et al.*, 2007). However, rocket salad is characterized by a higher content of ascorbic acid (ASA), which can probably prevent the occurrence of browning by acting on PPO activity. It is well known that ASA is the key component of the commercial anti-browning formulations available on the market (RUPASINGHE *et al.*, 2005). ASA controls PPO activity through its ability to reduce quinones to the native diphenols (NICOLAS *et al.*, 1994) and typically it is used as an anti-browning agent in fresh-cut fruit and vegetables (ALTUNKAYA and GOKMEN, 2008; ROJAS-GRAU *et al.*, 2008). However not many studies have been carried out on the role of endogenous ascorbic acid content on preventing browning (MDLULI and OWUSU-APENTEN, 2007).

It has been reported that the nutrient content of fresh-cut fruit can decrease as a consequence of the cutting process (BRECHT *et al.*, 2004). Nutrient losses may also be accelerated when plant tissues are wounded (KLEIN, 1987). However, literature on this topic is controversial. In fact, stresses such as light, temperature, water-stress and wounding affect the physiology of fresh produce by triggering responses that could induce the accumulation of phenolic compounds (KAYS, 1997) and induce the activation of the enzyme PAL. REYES *et al.* (2007) reported that the antioxidant capacity of some fruit and vegetables was positively influenced by cutting being that the levels of ascorbic acid and phenolic compounds were much higher in cut tissue as compared with intact tissues. Other research has demonstrated that wounding increases the antioxidant capacity of lettuce (KANG and SALTVEIT, 2003) and purple-flesh potatoes (REYES and CISNEROS-ZEVALLOS, 2003).

The objective of this study was to investigate the role of endogenous ASA in the sensitivity to browning of fresh-cut pineapples, apples and pears upon storage. Changes in the content of soluble solids content, flesh firmness and antioxidant capacity were evaluated in the fresh-cut fruit.

MATERIALS AND METHODS

Fruit material and storage conditions

Pineapples (*Ananas comosus* L. *cultivar* Smooth Cayenne), apples (*Malus domestica* Borkh *cultivar* Golden Delicious) and pears (*Pyrus communis* L. *cultivar* Coscia) were purchased at a local market. The pears were obtained between June and August 2006, while the apples and pineapples between October and December 2006. All fruit was at an acceptable ripening stage based on eating quality. They were carefully selected for regular shape, uniform size and absence of external defects (for each species). The fruit was gently washed with chlorinated drinking water and excess water was removed with cheesecloth. All fruit was divided into two lots of 30 pieces for pineapple and 20 for apple and pear. One lot was processed as fresh-cut fruit pieces, prepared as reported below for the different species; they were randomly divided and put into clear oval PET (Polyethylene Terephthalate) hinged containers (1.5 L) (Elipack EL390, BonSai Plastics, London, UK) that were not gas tight when closed. The other lot was stored as whole fruit and on the day of sampling was processed in the same way as the fresh-cut samples. All fruit, fresh-cut and whole, was maintained at 4°C in the dark for the entire period of the experiments which were repeated three times and results were pooled together.

In the laboratory, at room temperature, 30 pineapples were peeled and the cores were removed with a sharp stainless steel knife. The peeled pineapples were cut into 2 cm thick slices and the fruit slices were cut into 2 x 2 x 2 cm pieces. On each sampling day, three whole pieces of fruit were cut and processed as described for fresh-cut samples. Pineapple as fresh-cut or whole fruit was stored for 216 h.

Apples and pears were cored and cut into 2-cm thick slices with a sharp knife. The fruit slices were cut into 2 x 2 x 2 cm pieces. On each sampling day, three whole apples and three whole pears were cut and processed as described for the fresh-cut samples. Apples and pears as fresh-cut or whole fruit were stored for 48 h.

Visual symptoms

The fruit was scored to estimate visually the extent of browning development. The rating scale reported in our previous report (DEGL'INNOCENTI *et al.*, 2005) was used to estimate visually the extent of browning in the fresh-cut fruit: the score ranged from 0 (no injury) to 9 (severe injury, i.e., complete browning of the cut).

Firmness and soluble solids determination

The penetration test consists of measuring the strength necessary to penetrate the flesh-fruit with a metal point of a known diameter to a pre-established distance. The measure was performed by using a digital penetrometer installed on a driving column equipped with an 8 mm probe (Model 53205, TR, Forlì, Italy). Measurements were carried out on a flat surface, from opposite sides of the fruit in the centre of each piece of pineapple, apple and pear samples. Flesh Firmness (FF) is expressed in kg.

Soluble solids concentration (SSC) was estimated using the mean of two refractometer readings taken on juice obtained from each sample, using a digital refractometer (Model 53011, TR, Forlì, Italy). Measurements are expressed as °Brix

Analysis of vitamin C

Ascorbic acid was assayed by using a starch-iodine titration following the methods reported by SIGMANN and WHEELER (2004). Twenty millilitres of juice sample per replicate were diluted with 80 mL of distilled water and titrated with an iodine solution (0.5 g in 400 mL distilled water). Ascorbic acid is a weak reductant, which reacts stoichiometrically and rapidly with the generated iodine to give dehydroascorbic acid. Iodine is quantified with starch and the complex formed is typically blue. The amount of ascorbic acid in the fruit juices is proportional to the amount of iodine required to turn the solution blue. To determine the amount of ascorbic acid, a control titration was carried out: a solution of ascorbic acid (0.1 g in 200 mL of distilled water) was titrated with iodine. The iodine volume used to turn the solution blue corresponded to 25 mg of ascorbic acid. Vitamin C is expressed as mg Ascorbic Acid/100 g fresh weight.

Extraction and analysis of phenols

For analysis of phenolic compounds, a 4 g sample was homogenized with 10 mL of HPLC grade methanol, and the homogenates were filtered and centrifuged at 15,000 *g* at 20°C for 15 min. The content of phenols in the extract was determined according to the method reported by DEWANTO *et al.* (2002) based on the method of Folin-Ciocalteau. The values are expressed as mg gallic acid (GA)/100 g FW.

Analysis of antioxidant capacity

The method used to test the antioxidant capacity of the fruit extracts measured the iron-reducing capacity of a pool of antioxidant substances (FRAP) (BENZIE and STRAIN, 1996). This assay is quick and simple to perform and linearly related to the molar concentration of antioxidant(s) present. The assay was carried out on 4 g samples of fruit homogenized with 10 mL of HPLC grade methanol; the homogenates were filtered and centrifuged at 15,000 *g* at 20°C for 15 min. The FRAP reagent contains 1 mM 2,4,6-tripyridyl-2-triazine (TPTZ) and 2 mM ferric chloride in 0.25 M sodium acetate (pH 3.6). The Fe^{2+} forms a complex with 2,2'-dipyridyl. An intense blue coloration is formed when the ferric-tripyridyltriazine (Fe^{3+}-TPZ) complex is reduced to the ferrous form at 593 nm. The final value of FRAP content is expressed as mmol Fe^{2+}/100 g FW.

Enzyme extraction and assay

PAL (E.C. 4.3.1.5) activity was determined evaluating the accumulation of *trans*-cinnamic acid at 290 nm, using phenylalanine as PAL substrate, according to the method reported in DEGL'INNOCENTI *et al.* (2005). PAL activity is expressed as mmol *trans*-cinnamic acid $h^{-1}g^{-1}$FW.

For PPO (E.C. 1.10.3.2) analysis, fruit samples were extracted according to the method reported by CAMPOS-VARGAS and SALTVEIT (2002). Two grams of fruit tissue were homogenized with 6 mL of 50 mM phosphate buffer (pH 6.5), 0.5 mL of 2 mM β-mercaptaethanol and an aliquot of polyvynilpyrrolidone. The homogenate was filtered and centrifuged at 20,000 *g* at 4°C for 20 min. After centrifugation, an aliquot of the supernatant was applied to a Sephadex G25 column, previously equilibrated with 50 mM phosphate buffer (pH 6.5). PPO activity was assayed as reported by COUTURE *et al.* (1993): 0.2 mL of 0.1 mM caffeic acid ethanolic solution was added to a volume of pineapple and pear fruit extract. For apple, chlorogenic acid was used. The absorbance was recorded at 480 and 400 nm for 5 min, respectively, for caffeic or chlorogenic acid. PPO activity is expressed as units g^{-1} fresh weight, 1 unit being the amount of enzyme that increases the absorbance by 0.001 during reaction kinetics.

Statistical analysis

Data are the means of three or more replicates depending on the analysis. Data were subjected to two-way ANOVA, with storage and post-harvest treatments as variability factors. Comparison between the means was carried out using a Least Significant Difference (LSD) test at P=0.05. To correlate the antioxidant capacity and phenols or vitamin C content, a regression analysis was carried out.

RESULTS

Fresh-cut pineapples were stored at 4°C for 216 h when visual quality was judged to be unmarketable and the appearance of the slices was compromised by flaccidity and turgor loss. However, at this time of storage, fresh-cut pineapples did not showed any color alterations, attributable to enzymatic browning (Fig. 1). In comparison, evident symptoms of browning were observed in apple and pear slices as soon as 24 h after cutting. The score in apple was 5.5 after 24 h of cold storage, which then increased significantly ($P<0.05$), reaching a value of 8.5 by 48 h. A similar pattern was evidenced in fresh-cut pears with values of browning of 6 after 24 h of cold storage and a value of 9 at the end of storage. This difference in sensitivity to browning between pineapples, pears and apples was used to characterize a model of the browning process in fruit. From the analysis of visual aspects of the three types of fruit, it is evident that fresh-cut pineapples appear resistant to browning as compared with apples and pears.

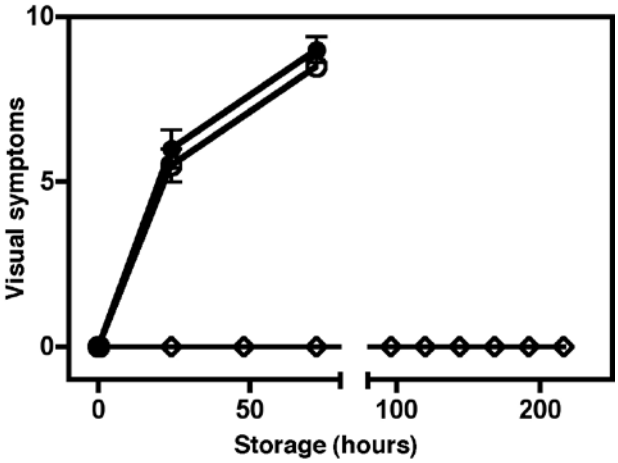


Fig. 1 - Estimate of browning in fresh cut pineapple (open rhomb), apple (open circle) and pear (closed circle). Values are the means of six replicates. Bars indicate standard deviation.

In pineapples flesh firmness diminished after cutting because there were significant differences in FF between fresh-cut and whole fruit (Table 1). Also soluble solids content decreased as an effect of storage in pineapple. In fact, in whole as in the fresh-cut fruit SSC diminished significantly. Stored whole apples were firmer than the fresh-cut apples (Table 1). No changes in SSC were observed in fresh-cut apples, while an increase was recoded in whole stored apples. Finally, firmness decreased in pear pieces over the 3-day storage period, while no changes were reported in the whole fruit. There were no differences in SSC among pieces from whole or fresh-cut pears up to 3 days.

PAL is the most important enzyme involved in enzymatic browning because it catalyzes the step by which primary metabolism is diverged to the phenylpropanoid pathway. In whole pineapple PAL activity did not change during storage (Fig. 2), while a significant increase was observed in fresh-cut pieces after 120 h of storage even if at the end the values of PAL activity were similar to the ones obtained at the beginning of the experiment. In whole apples PAL activity increased slightly after 24 h of storage at 4°C but at 48 h, at the end of the experiment, the enzymatic activity was similar to values recorded at the beginning (Fig. 2). In comparison, PAL activity in fresh-cut apples increased significantly upon storage. In addition, in fresh-cut pear pieces PAL activity increased upon storage, while no changes were observed in pieces from whole fruit.

In whole pineapple, PPO activity increased after 120 h of storage however, on the following days the PPO activity decreased to values lower than the ones reported at the beginning of storage (Fig. 3). Fresh-cut pieces of pineapples also presented a decrease in PPO activity. At the end of storage (216 h) no differences in PPO activity were recorded between whole and fresh-cut pineapples. PPO did not change in whole apple, while a significant increase was observed in fresh-cut pieces 48 h after cutting (Fig. 3). Finally, in pear the activity of PPO increased in whole and fresh-cut fruit (Fig. 3). In fact, an increase

Table 1 - Changes in organoleptic characteristics of several fresh-cut processed fruit determined at the start and at the end of the storage.

		Flesh Firmness (kg)		Soluble Solids Content (°Brix)	
Storage		Start	End	Start	End
Pineapple	whole	2.3±0.07 a	2.1±0.02 a	16.9±0.61 a	11.6±0.02 c
	fresh-cut	2.4±0.06 a	1.6±0.64 b	17.0±0.63 a	14.9±1.29 b
Apple	whole	4.7±0.06 a	4.7±0.55 a	13.5±1.20 b	15.3±1.05 a
	fresh-cut	4.8±0.08 a	2.6±0.44 b	13.8±1.22 b	13.1±0.25 b
Pear	whole	5.0±0.52 a	4.9±0.20 a	12.5±0.50 a	12.4±0.38 a
	fresh-cut	5.1±0.81 a	1.1±0.23 b	12.7±0.22 a	11.5±0.45c

Each value represents the mean of three replicates ± one standard deviation. Mean values followed by the same letters are not significantly different for P = 0.05 following a two way ANOVA test with storage and post-harvest treatment as variability factors.

was observed after 24 of storage at 4°C but in fresh-cut pieces PPO activity increased 12-fold as compared with the initial levels, while only 2 times in whole fruit. At the end of the storage (48 h) in fresh-cut pears PPO activity remained higher than whole fruit.

The content of phenols in pineapples and apples did not present changes between whole and fresh-cut fruit when stored 4°C for 216 h and 48 h, respectively (Fig. 4). Even in pears, no significant differences were observed in whole stored and fresh-cut fruit, while slightly changes were observed after 24 and 48 h of storage when phenol content values in fresh-cut pieces were higher than in whole pears (Fig. 4).

The most evident difference among the three

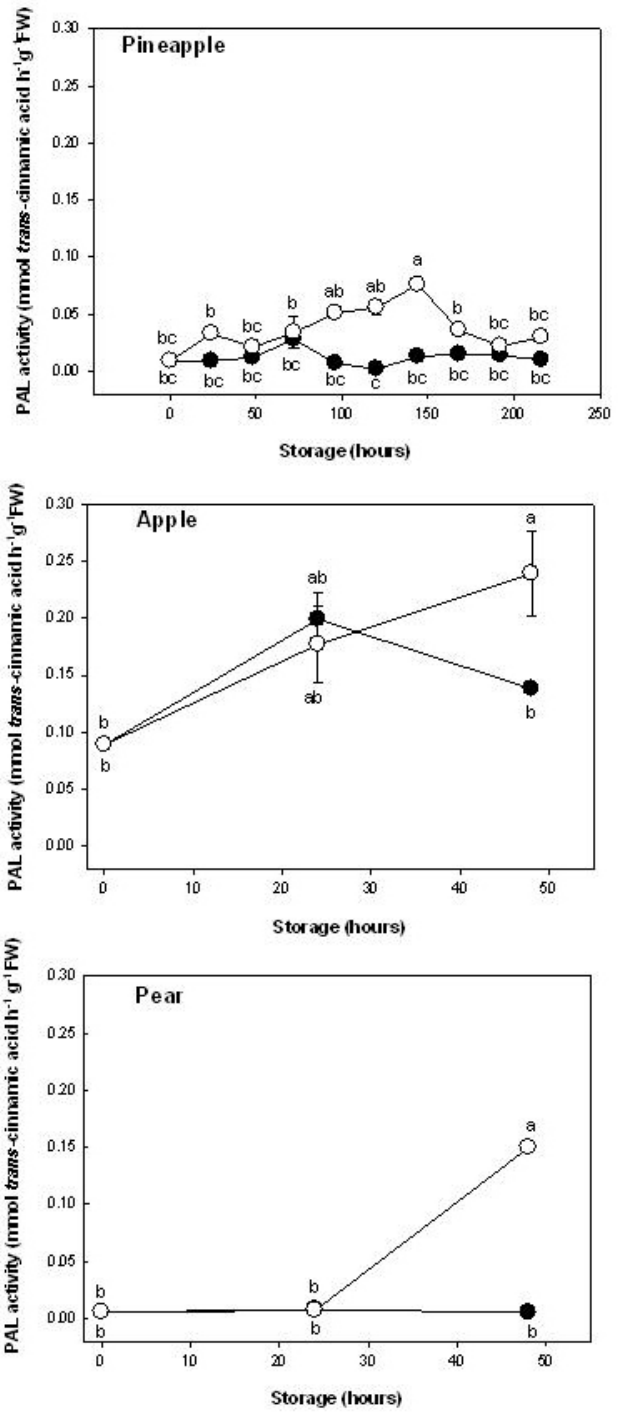


Fig. 2 - PAL activity in pieces of whole (closed circle) or fresh cut (open circle) stored fruit. Values are the means of three replicates. Bars indicate standard deviation. Means flanked by the same letters are not significantly different for P=0.05 following a two way ANOVA test with storage and post-harvest treatment as variability factors.

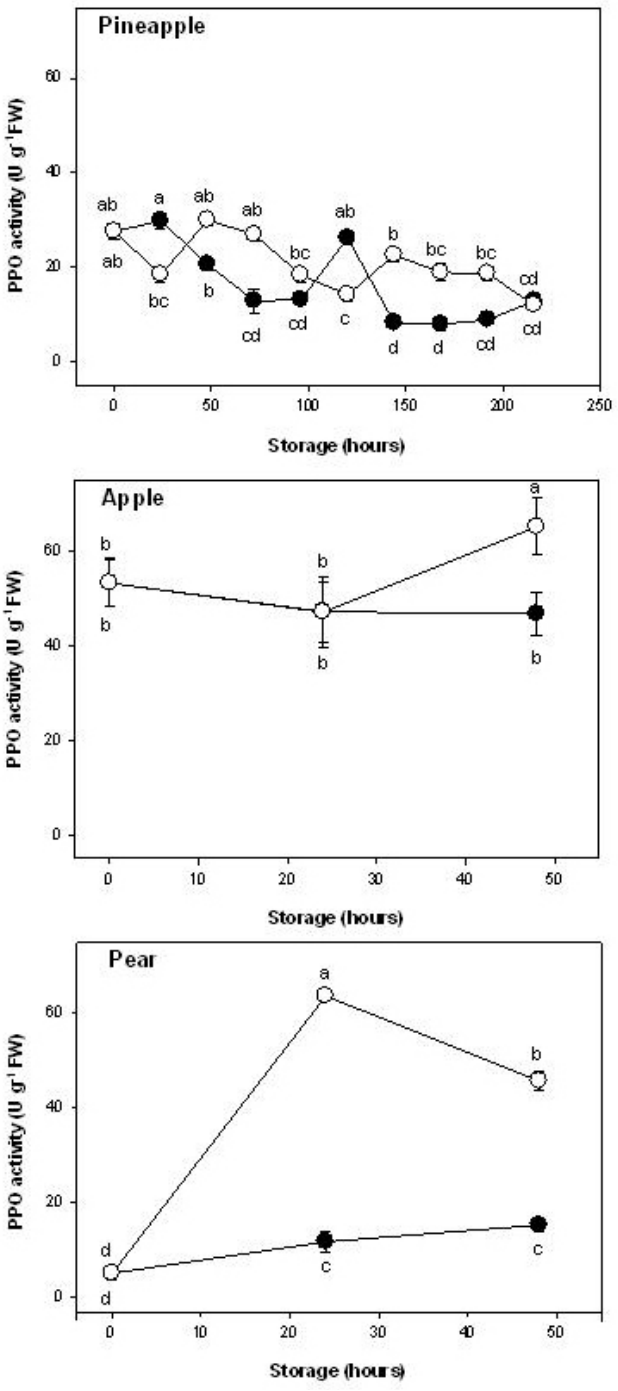


Fig. 3 - PPO activity in pieces of whole (closed circle) or fresh cut (open circle) stored fruit. Values are the means of three replicates. Bars indicate standard deviation. Means flanked by the same letters are not significantly different for P=0.05 following a two way ANOVA test with storage and post-harvest treatment as variability factors.

fruit species was the vitamin C content. In fact, the vitamin C content in pineapple was significantly higher compared to apples and pears ($P<0.05$) (Fig. 5). In whole and minimally processed pineapples a significant loss in vitamin C was observed, and at the end of storage, the values decreased and were similar in fresh-cut and whole fruit (-59 and -63%, respectively) (Fig. 5). A decrease in vitamin C was recorded in whole apples after 48 h, reaching values lower than the initial ones. Vitamin C significantly diminished upon storage in fresh-cut apples. In pears, a de-

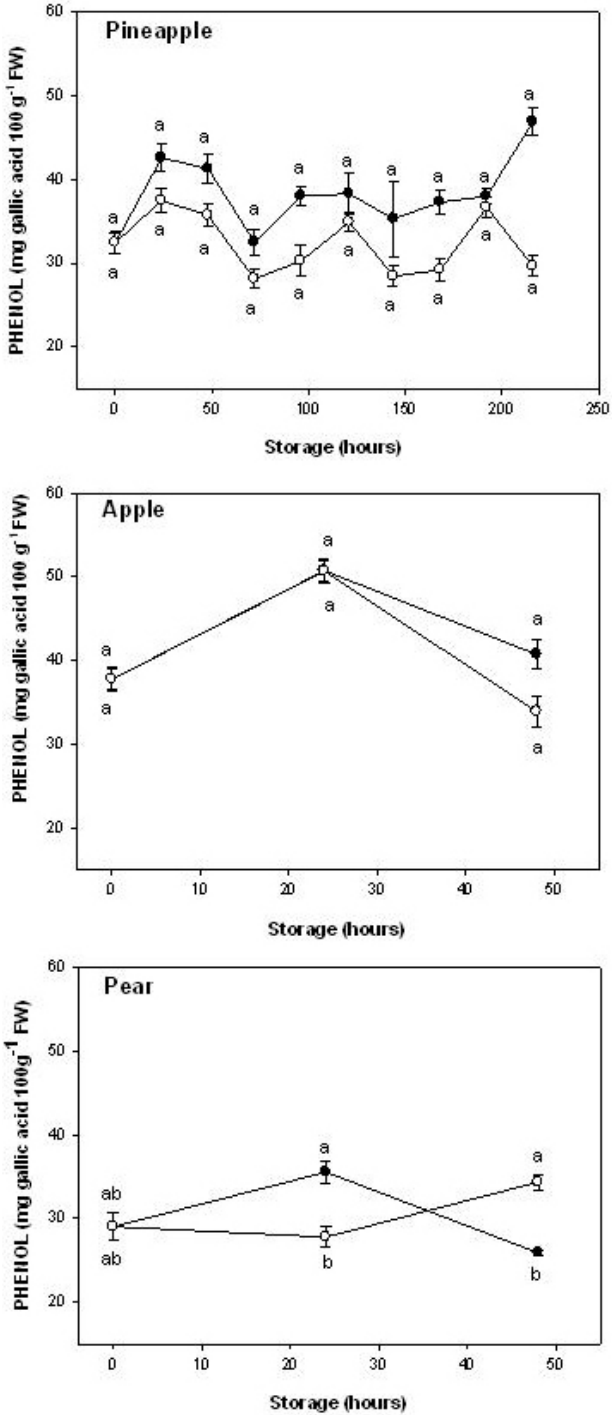


Fig. 4 - Phenol content in pieces of whole (closed circle) or fresh cut (open circle) stored fruit. Values are the means of three replicates. Bars indicate standard deviation. Means flanked by the same letters are not significantly different for P=0.05 following a two way ANOVA test with storage and post-harvest treatment as variability factors.

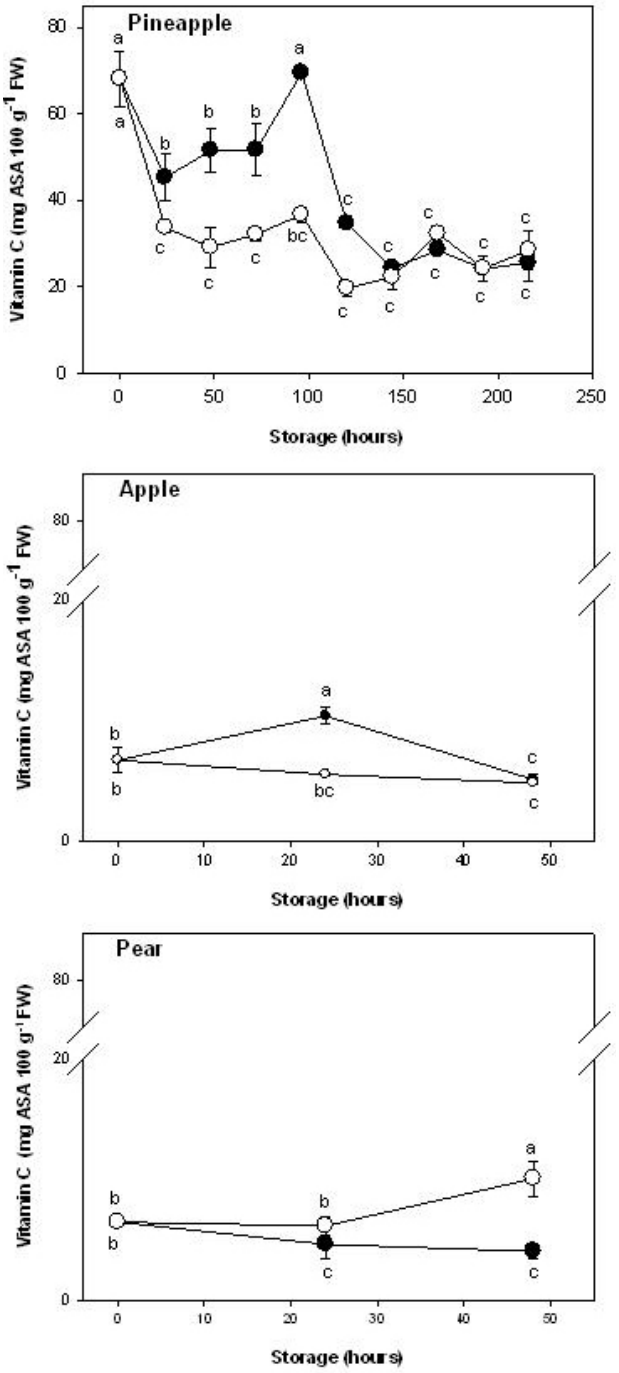


Fig. 5 - Vitamin C content in pieces of whole (closed circle) or fresh cut (open circle) stored fruit. Values are the means of three replicates. Bars indicate standard deviation. Means flanked by the same letters are not significantly different for P=0.05 following a two way ANOVA test with storage and post-harvest treatment as variability factors.

crease in ascorbic acid was observed in whole fruit, while an increase was recorded in fresh-cut fruit after 48 h (Fig. 5).

The antioxidant capacity in pineapple, determined by FRAP assay, did not change, in whole or in fresh-cut fruit (Fig. 6). Pears and apples showed values of antioxidant capacity lower than pineapples ($P<0.05$). In whole apples only a slight increase was observed at 24 h of storage which was noticed as well, in a more pronounced way, in fresh-cut fruit (Fig. 6). At the end of storage (48 h), the antioxidant capacity decreased and reached values similar to the ones found in whole and fresh-cut apple. There was an interesting difference in the FRAP assay for pears, which presented an increase in whole fruit and a decrease in fresh-cut fruit after 24 h of storage (Fig. 6). At the end of storage at 4°C (48 h) values of antioxidant capacity of whole fruit remained higher than fresh-cut pears.

DISCUSSION

The three types of fruit considered in this study differed in their sensitivity to browning; in fact, pineapples did not show any symptoms attributable to this disorder even 216 h after storage as fresh-cut products, while apples and pears showed brown areas as soon as 24 h after cutting. Another important difference was the vitamin C content which was very much higher in pineapple as compared to apple or pear.

Browning of fresh cut fruit or vegetables is attributable to the combined action of enzymes PAL and PPO (TOIVONEN and BRUMMEL, 2008). PAL, activated by cutting, increased the concentration of phenolic substances which is the substrate utilized by PPO. Browning reactions have generally been assumed to be a direct consequence of PPO action on phenols (MARTINEZ and WHITAKER, 1995, TOIVONEN and BRUMMEL, 2008) and this phenomenon has been well defined for lettuce by DEGL'INNOCENTI *et al.* (2007). PAL increased upon storage in the three types of fruit, while PPO activity increased only in apple and pear, but the phenol content increased significantly only in pear.

The biochemical pattern in apples was different. The content of phenols did not change, probably due to the increase in PAL and the parallel increase in PPO activity, which typically used phenols as substrates. Similar results were found by other authors in apple and apple products (NICOLAS *et al.*, 1994). In fresh-cut pears, different results were obtained and an increase in PPO action was observed after 24 h of storage, while the PAL activity showed a significant increase at 48 of storage. It seems that in this species the first enzyme stimulated by cutting was PPO, which, by using phenols, determined a decrease in their content. Later, PAL action increased and induced an accumulation of phenols. It is well known that in pears PPO activity is not a limiting factor in enzymatic browning (LARRIGUADIERE *et al.*, 1998). Since PPO is inside the cytoplasm or plastids and its substrates in the vacuole (NICOLAS *et al.*, 1994), enzymatic browning is the direct consequence of membrane disintegration caused by cutting operation.

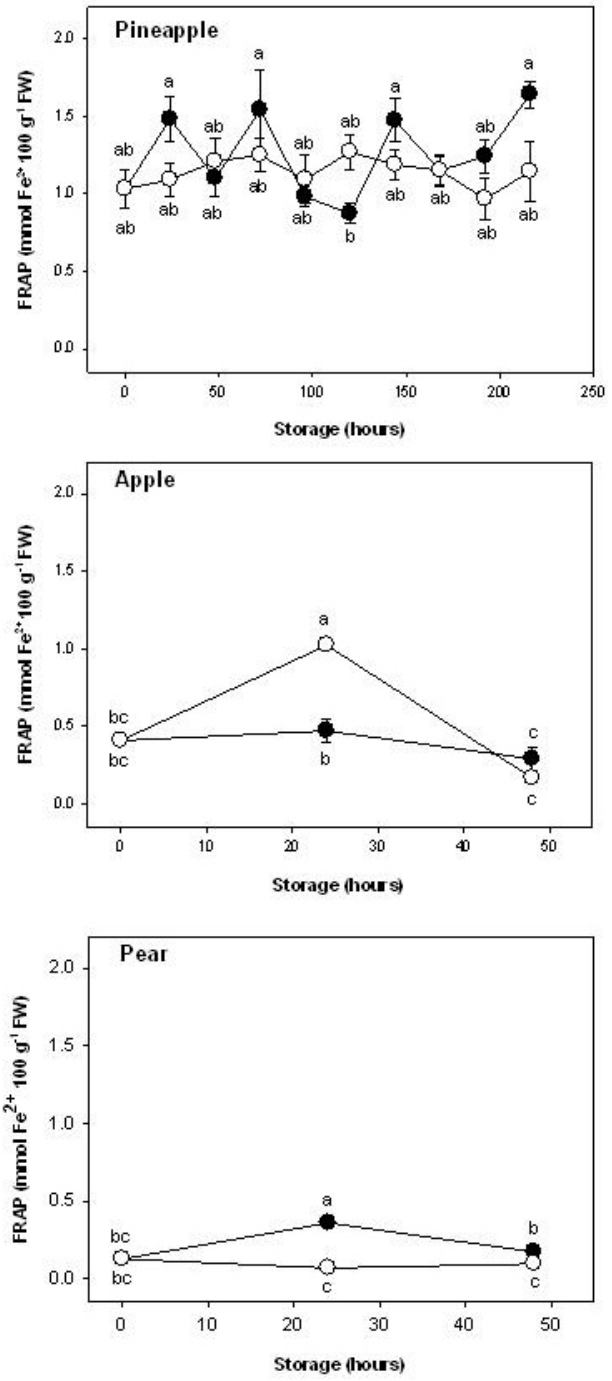


Fig. 6 - Antioxidant capacity in pieces of whole (closed circle) or fresh cut (open circle) stored fruit. Values are the means of three replicates. Bars indicate standard deviation. Means flanked by the same letters are not significantly different for $P=0.05$ following a two way ANOVA test with storage and post-harvest treatment as variability factors.

The resistance to browning showed by fresh-cut pineapple is related to different factors. PAL increased only after 120 and 144 hours of storage, while the behaviour of PPO activity upon storage was characterized by values lower than those recorded at the time of cutting after 122 and 216 hours. The decrease in PPO activity observed in fresh-cut pineapple is presumably linked to the high ascorbic acid content found in this fruit. Other authors have reported a strong correlation between browning of fruit tissue and ASA levels (POINTING and JOSLYN, 1947). Browning of fresh fruit occurs when ASA drops below a certain threshold. It seems that browning does not occur until ASA in the tissue is oxidised (VELTMAN *et al.*, 1999). On the other hand, VELTMAN *et al.* (1999) reported that the low level of ascorbic acid in the pear core is strictly related to the start of browning. A similar patter to those recorded in fresh-cut pineapple was also found in minimally processed spinach, a vegetable characterized by a high content of ascorbic acid (BOTTINO *et al.,* 2009). In spinach the availability of endogenous ASA reduced a great part of PPO enzymatic reaction products, thus inhibiting enzymatic browning. In other words, ASA did not stop the enzymatic reaction itself, but delayed the subsequent polymerization events; in fact, no decrease in PPO activity was observed. In pineapple, PPO activity diminished in parallel with the decrease of ascorbic acid content to indicate a possible important role of the antioxidant ascorbic acid in the prevention of browning.

The antioxidant capacity of fruit is very important (KAUR and KAPOOR, 2001). The resistance of pineapples to enzymatic browning confers to this fruit optimal quality for storage as fresh-cut product. Pineapple also represents a significant source of ascorbic acid, which is one of the most important vitamins for human nutrition and it also acts as an antioxidant (GONZALEZ-AGUILAR *et al.*, 2005). However, when pineapples are stored as fresh-cut fruit the content of ascorbic acid significantly decreases. The decrease of ascorbic acid during storage and processing in fruit and vegetables is a common phenomenon (WRIGHT and KADER, 1997; COCCI *et al.*, 2006) and similar results were obtained in pineapple by GIL *et al.* (2006). It is important to emphasize, however, that even if the ascorbic acid content in pineapple slices decreases, the antioxidant capacity does not change, indicating that it is not just this organic compound that contributes to the total antioxidant capacity.

Different results were obtained for apples and pears in which the higher sensitivity to browning certainly altered their marketability as fresh-cut products. In addition, the ascorbic acid content, which was significantly lower than that recorded in pineapples, decreased upon storage, negatively influencing the fruit quality.

In conclusion, fresh-cut processing results in stress factors for fruit which determines a shorter shelf-life in comparison to whole fruit independent of differences of fruit species. Biological variation and species differences add to the complexity and make it difficult to provide a single solution to this scenario. Enzymatic browning is certainly a deleterious process that negatively influences the storage of fresh-cut fruit and understanding of the biochemical mechanisms involved is the key to improving the shelf-life.

REFERENCES

Altunkaya A. and Gokmen V. 2008. Effect of various inhibitors on enzymatic browning, antioxidant activity and total phenol content of fresh lettuce (*Lactuca sativa*). Food Chem. 107: 1173.

Benzie I.F.F. and Strain J.J. 1996. The ferric reducing ability of plasma (FRAP) as a measure of "Antioxidant Power": The FRAP assay. Anal. Biochem. 239: 70.

Bottino A., Degl'Innocenti E., Guidi L., Graziani G. and Fogliano V. 2009. Bioactive compounds during storage of fresh-cut spinach: the role of endogenous ascorbic acid in the improvement of product quality. J. Agr. Food Chem. 57: 2925.

Brecht J.K., Saltveit M.E. Talcott S.T., Schneider K.R., Felkey K. and Bartz J.A. 2004. Fresh-cut vegetables and fruits. Hort. Rev. 30: 185.

Campos-Vargas R. and Saltveit M.E. 2002. Involvement of putative chemical wound signals in the induction of phenolic metabolism in wounded lettuce. Physiol. Plant. 114: 73.

Cocci E., Rocculi P., Romani S. and Dalla Rosa M. 2006. Changes in nutritional properties of minimally processed apples during storage. Postharvest Biol. Technol. 39: 265.

Couture R., Cantwell M.I., Ke D. and Saltveit M.E. 1993. Physiological attributes related to quality attributes and storage life of minimally processed lettuce. HortSci. 58: 609.

Degl'innocenti E., Guidi L., Pardossi and A. Tognoni F. 2005. A biochemical study of leaf browning in minimally processed leaves of lettuce (Lactuca sativa L. var. acephala). J. Agric. Food Chem. 53: 9980.

Degl'innocenti E., Pardossi A., Tognoni F. and Guidi L. 2007. Physiological basis of sensitivity to enzymatic browning in 'lettuce', 'escarole' and 'rocket salad' when stored as fresh-cut products. Food Chem. 104: 209.

Dewanto V., Wu X., Adom K.K. and Liu R.H. 2002. Thermal processing enhances the nutritional value of tomatoes by increasing total antioxidant activity. J. Agric. Food Chem. 50: 3010.

Gil M.I., Aguayo E. and Kader A.A. 2006. Quality changes and nutrient retention in fresh-cut versus whole fruits during storage. J. Agric. Food Chem. 54: 4284.

Gonzalez-Aguilar G.A., Ruiz-Cruz S., Soto-Valdez H., Vàzquez-Ortiz F., Pacheco-Aguilar R. and Ang C. 2005. Biochemical changes of fresh-cut pineapple slices treated with antibrowning agents. Int. J. Food Chem. 40: 377.

Kang H. and Saltveit M.E. 2003. Antioxidant capacity of lettuce leaf tissue increases after wounding. J. Agric. Food Chem. 50: 7536.

Kaur C. and Kapoor H.C. 2001. Antioxidant in fruits and vegetables. The millenium's health. Int. J. Food Sci. Technol. 36: 703.

Kays, S.J. 1997. Stress in harvested products. In "Postharvest Physiology of Perishable Plant Products". Kays, S.J. (Ed.), p. 335. Exon Press, Athens.

Klein J.D. 1987. Relationship of harvest date, storage conditions and fruit characteristics to bruise susceptibility of apple. J. Am. Soc. Hort. Sci., 112: 113.

Larriguadiere C., Lentheric I. and Vendrell M. 1998. Relationship between enzymatic browning and internal disorder in controlled-atmosphere stored pears. J. Sci. Food Agr. 78: 232.

Liu R.H. 2003. Health benefits of fruit and vegetables are from additive and synergistic combinations of phytochemicals. Am. J. Clin. Nutr. 78: 517S.

Martinez M.V. and Whitaker J.R. 1995. The biochemistry and control of enzymatic browning. Trends Food Sci. 6: 195.

Mdluli K.M. and Owusu-Apenten R. 2007. Enzymatic browning in marula fruit 1: effect of endogenous antioxidants on marula fruit polyphenol oxidase. J. Food Biochem., 27: 67.

Nicolas J.J., Richard-Forget F.C., Goupy P.M., Amiot M.J. and Aubert S.Y. 1994. Enzymatic browning in apple and apple products. Critical Rev. Food Sci. Nutr, 34: 109.

Pointing J.D. and Joslyn M.A. 1947. Ascorbic acid oxidation and browning in apple tissue extracts. Arch. Biochem. 19: 47.

Reyes L.F. and Cisneros-Zevallos L. 2003. Wounding stress increases the phenolic content and antioxidant capacity of purple-flesh potatoes. J. Agric. Food Chem., 51: 5296.

Reyes L.F., Villareal J.E. and Cisneros-Zevallos L. 2007. The increase in antioxidant capacity after wounding depends on the type of fruit or vegetable tissue. Food Chem. 101: 1254.

Rojas-Grau M.A., Soliva-Fortuny R. and Martin-Belloso O. 2008. Effect of natural antibrowning agents on color and related enzymes in fresh-cut Fuji apples as an alternative to the use of ascorbic acid. J. Food Sci. 73: S267.

Rupasinghe H.P.V., Murr D.P., DeEll J.R. and Odumeru J. 2005. Influence of 1-methylcyclopropene and natural seal on the quality of fresh-cut "Empire" and "Crispin" apples. J. Food Qual. 28: 289.

Sigmann S.B. and Wheeler D.E. 2004. Quantitative determination of citric and ascorbic acid in powdered drink mixes. J. Chem. Educ. 81: 1479.

Toivonen P.M.A. and Brummel D.A. 2008. Biochemical bases and texture changes in fresh-cut fruit and vegetables. Postharvest Biol. Technol. 48: 1.

Veltman R.H., Sanders M.G., Persijn S.T., Peppelenbos H.W. and Oosterhaven J. 1999. Decreased ascorbic acid levels and brown core development in pears (*Pyrus communis cv.* Conference). Physiol. Plant. 154: 697.

Wright K.P. and Kader A.A. 1997. Effect of slicing and controlled-atmosphere storage on the ascorbate content and quality of strawberries and persimmons. Postharvest Biol. Technol. 10: 39.

Revised paper received October 19, 2009 Accepted January 28, 2010

SHORT COMMUNICATION

EFFECT OF A TOMATO EXTRACT-SUPPLEMENTED DIET ON EGG YOLK PIGMENTATION AND LYCOPENE TRANSFER EFFICIENCY

L. ROTOLO, G. STRAZZULLO[1], M. PAGELLA[2], A. BRUGIAPAGLIA, L. POZZO[2] and A. SCHIAVONE[2]*

Università degli Studi di Torino, Dipartimento di Scienze Zootecniche
[1]Consiglio Nazionale delle Ricerche, Istituto di Scienze delle Produzioni Alimentari
[2]Università degli Studi di Torino, Dipartimento di Produzioni Animali Epidemiologia ed Ecologia,
Via Leonardo da Vinci 44, 10095 Grugliasco (TO), Italy
*Corresponding author: Tel. +39 011 6709208, Fax +39 011 6709240
e-mail: achille.schiavone@unito.it

ABSTRACT

The aim of this research was to measure the ability of laying hens fed a tomato extract-supplemented diet to deposit dietary lycopene into the egg yolk, and to investigate the effects of lycopene on yolk pigmentation. Twenty Isa Brown hens were individually caged and fed two dietary treatments: 1) control diet (C) and 2) control diet + 0.08% (w/w) tomato extract dissolved in soybean oil (2% w/w) (TE). The tomato extract-supplemented diet provided a calculated lycopene level of 13 mg/g. Feed and water were provided *ad libitum*. Colorimetrical and HPLC analysis confirmed that the dietary lycopene was incorporated into the egg yolk at values ranging from 0.12 to 0.16 µg/g yolk (as is). Dietary tomato extract affected L*, a* and b* values of yolk and approximately 0.13% lycopene was transferred from the feed to the yolk. The data suggest that the TE diet resulted in a significant lycopene carry over and the intensity of egg yolk colour was influenced by dietary lycopene supplementation.

- Key words: carotenoids, egg, laying hen, lycopene, tomato extract, yolk pigmentation -

INTRODUCTION

Diet optimisation is the most important issue for many human nutritionists. Due to its nutritive excellence, egg is an ideal food and allows nutritional imbalances in modern diets to be overcome (SURAI, 2002).

Life-expectancy is increasing in developed countries and advances are being made in food technology, so consumers are paying more attention to diet as part of a healthy lifestyle. In an effort to re-evaluate animal-derived products, several studies have been performed to increase the enhanced levels of functional substances such as lutein, selenium and n-3 fatty acids in egg. Results have shown that eggs are not only a good nutritional product but are also a good vector for delivering essential nutrients for human health (SURAI, 2000; SURAI *et al.*, 2000; LESKANICH and NOBLE, 1997).

Carotenoids, the most conspicuous and widespread group of pigments in nature, have many physiological functions. Most carotenoids found in food have antioxidant activity, acting as free-radical scavengers. Many authors have studied the dietary carotenoid intake of humans, but there are no data on the optimal levels (HAZELS MITMESSER *et al.*, 2000). Even if no dietary references have been proposed for carotenoids, some authors have described the carotenoid plasma and serum response after tomato product intake (STAHL and SIES, 1996). Lycopene is one of the main components of the carotenoid fraction in tomato and the mean value ranges from 2 to 14 mg/100 g of fresh tomato (ZANFINI *et al.*, 2007). Lycopene exerts peculiar biological effects; in particular, it has the greatest ability to quench singlet oxygen (DI MASCIO *et al.*, 1989). Moreover, it has been shown that the dietary intake of lycopene from tomatoes is inversely related to the risk of certain types of cancer, such as prostate, digestive-tract and lung cancers (GIOVANNUCCI *et al.*, 1995; FRANCESCHI *et al.*, 1994; LE MARCHAND *et al.*, 1989).

The purpose of this work was to investigate the effect of lycopene supplementation on egg yolk pigmentation and to determine the amount of lycopene that crossed over in laying hens fed with a tomato extract-supplemented diet in a view of designing lycopene enriched eggs.

MATERIALS AND METHODS

Chemicals

A lycopene standard was obtained from Sigma (St. Louis, MO, USA). Diethyl ether, hexane, acetone, ethanol and dichloromethane were p.a. grade, while acetonitrile and isopropanol were HPLC grade. All solvents were purchased from Merck (Whitehouse Station, NJ, USA).

Tomato extract

The tomato extract was obtained from *San Marzano* canned peeled tomatoes (10.8 kg). The *San Marzano* cultivar was chosen due to its high carotenoid, lycopene and β-carotene contents (STRAZZULLO *et al.*, 2007). Canned tomatoes were homogenised in a blender for 5 min. The puree was extracted with diethyl ether (w/v 1:2) under stirring, in the dark overnight in order to obtain a high yield of carotenoids in the lipophilic fraction. The upper red layer containing the carotenoids was filtered, concentrated in a rotary evaporator in vacuum (T <35°C) and dried under N_2 as suggested by STRAZZULLO *et al.* (2007). The extract was stored at -30°C pending diet formulation.

Animals and diets

Twenty Isa Brown laying hens (20 weeks old) were individually caged (1,925 cm^2/bird) in two double-deck cage batteries; the mean ambient temperature during the experimental period was 20°-25°C. The birds were randomly divided into two groups, of ten hens each. The control group (C) was fed with a standard layer diet (containing 2% oil w/w) and the test group (TE, tomato extract) was fed the same diet supplemented with 0.08% (w/w) tomato extract (equivalent to 13 μg of lycopene/g diet) previously dissolved in the oil fraction. Soybean oil was used to incorporate the tomato lypophilic extract in the feed. The composition of the diet is reported in Table 1. The trial lasted 14 days and the animals were monitored daily. Feed and water were provided *ad libitum*.

On days 1, 7 and 14 of feeding, the eggs laid in each experimental unit were collected (n=10), weighed and cracked. Shells, albumen and yolks were separated and weighed and the egg yolk colour was measured. Egg yolks and albumens were then lyophilized and stored at -20°C for further analyses.

Yolk measurement and analysis

The yolk colour was determined (n=10) at room temperature (20°C) by using a portable Minolta Colorimeter CR-331C (Minolta Camera, Osaka, Japan) with D_{65} illuminant and the 2° standard observer. The results are expressed in terms of lightness (L*), redness (a*) and yellowness (b*) in the CIELAB colour space model (CIE, 1978). Additional reflectance data such as chroma (C*), and hue angle (h*) were also calculated. The colorimeter was calibrated throughout the study using a standard white ceramic tile. Colour difference (ΔE*) was calculated as follows: $\Delta E^* = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2}$, where the ΔL*, Δa*, and Δb* values are the differences in colour co-ordinates between treatments.

Yolk and albumen dry matter, ether extract

Table 1 - Ingredients and calculated composition of basal diet[1].

Ingredients	g/kg	
Corn		590
Wheat roughage		15
Soybean meal		180
Alfalfa meal		30
Corn gluten		40
Oil		20
Calcium carbonate		82
Dicalcium phosphate		23
NaCl		5
DL-Methionine		3
L-Lysine		2
Vitamin and mineral premix[2]		10
Calculated composition		
ME	MJ/kg	11.51
Crude protein %		16.60
Ether extract %		4.55
Crude fiber %		4.19
Ash %		13.00
Dry matter %		87.06
Calcium %		3.79
Phosphorus %		0.74
Methionine %		0.57
Lysine %		0.85

[1]Diet was fed at 120 g/bird/day.
[2]Premix provided (mg/kg of diet): vitamin A, 1500 IU; vitamin D, 800 IU; vitamin E, 10 mg; vitamin K, 0.5 mg; riboflavin, 3.6 mg; pantothenic acid, 10 mg; vitamin B_{12}, 0.1 mg; choline chloride, 200 mg; niacin, 17.0 mg; manganese sulfate, 66 mg; zinc sulfate, 60.5 mg; iron sulfate, 49.5 mg; copper sulfate, 49.5 mg; calcium iodate, 0.26 mg; sodium selenite, 0.10 mg.

and ash were assessed by AOAC methods (1990) (n=5). Crude protein was determined by using "Rapid N" (FARINA and BEDETTI, 2007) (n=5).

The yolk lycopene value was determined by HPLC (n=5). Lyophilized yolk (600 mg) was extracted with 40 mL of hexane-acetone-95% ethanol (50:25:25 v/v/v). The mixture was kept on ice and agitated continuously on a magnetic stirrer plate for 15 min at 150 RPM until all the lycopene was completely extracted. Agitation was continued at room temperature for another 5 min after adding 6 mL of deionised water. After decanting, the upper non-polar layer (NPL) containing lycopene was filtered and then, concentrated in a rotary evaporator in vacuum (T < 35°C) and dried under N_2.

The HPLC apparatus consisted of a Dionex P680 pump (Dionex, Sunnyvale, CA,USA) equipped with a Rheodyne Model 7725i injection valve (Rheodyne, Rohnert Park, CA, USA), a Dionex UVD-170/U UV-vis detector (λ=470 nm), a Dionex thermostatted column compartment TCC-100, and a Chromeleon® 6 data handling system (Dionex, Sunnyvale, CA, USA).

An Ascentis™ C18 column (25 cm × 4.6 mm, 5 μm particles) (Supelco, Bellefonte, PA, USA) was used for the analysis. Twenty μL of NPL dissolved in 1 mL of dichloromethane were injected into the chromatographic system by a full loop injection system. The system was run isocratically with a mobile phase containing acetonitrile-isopropanol (90:10 v/v) for 25 min at a flow rate of 2.5 mL/min. The column temperature was kept at 30°C for all of the chromatographic runs. The identification and quantification of lycopene were obtained through the combined use of the retention time, and co-chromatography with standard.

The lycopene yield (expressed in terms of percentage) from feed to egg yolk were calculated by the following equation:

Lycopene transfer efficiency = Lycopene deposition in egg yolk (A) × 100 / Lycopene consumption by feed (B)

where:

A = yolk weight (g) × × yolk lycopene concentration (μg/g)

B = feed consumption (g/d/bird) × × feed lycopene concentration (μg/g).

Statistical analysis

Data were analyzed by one-way ANOVA using SPSS (SPSS, 2008) considering the dietary treatment as the only factor for each sampling day. Statistical significance was accepted at $P < 0.05$. Results are presented as mean ± standard deviation (SD).

RESULTS AND DISCUSSION

Diets were isonitrogenous and isoenergetic (Table 1) and satisfied the energy requirements of the laying hens

The quality traits of eggs on days 1, 7 and 14 are reported in Table 2. Dietary treatment did not affect these parameters. This supports the findings of LEESON and CASTON (2004), who observed production parameters were not influe ced by diet in laying hens fed with different inclusion levels of lutein in corn-soy diets.

The chemical composition of yolk and albumen was not influenced by dietary treatment as illustrated in Table 3; the dry matter, crude protein, ether extract and ash values were in agreement with the literature (CEROLINI *et al.*, 2008; THAPON and BOURGEOIS, 1994).

Colorimetrical data (Table 4) showed that yolks from hens fed with the supplemented tomato extract diet were darker than those of the control group. On days 7 and 14, the values of yolk colour parameter a* of the TE group were significantly higher ($P < 0.01$), and the L* values were lower ($P < 0.05$ and < 0.01, respectively) as were the b* values ($P < 0.05$). As expected, tomato extract contributed to egg yolk colour because lycopene, a red pigment, is darker than the yel-

Table 2 - Effects of incorporating tomato extract in the diet for 2 weeks on egg quality (n=10, mean±SD).

		Day 1			Day 7			Day 14		
		C	TE	P	C	TE	P	C	TE	P
Egg weight	g	55.74±4.78	59.06±4.17	NS	58.99±5.01	56.40±2.70	NS	60.95±8.12	57.12±3.40	NS
Shell weight	g	7.09±0.79	6.70±0.50	NS	7.19±0.42	6.95±0.31	NS	6.71±0.69	6.89±0.69	NS
Yolk weight	g	13.48±1.45	13.93±1.32	NS	14.35±1.08	12.91±1.72	NS	15.15±0.85	14.51±1.21	NS
Albumen weight	g	34.10±2.93	37.22±2.84	NS	37.79±4.02	36.36±2.25	NS	38.97±4.65	36.32±2.13	NS

C= Control group; TE = Tomato extract group.

Table 3 - Proximate analysis of egg yolks and albumen (n=5, mean±SD).

		Day 1			Day 7			Day 14		
		C	TE	P	C	TE	P	C	TE	P
Yolk										
Dry matter	%	51.15±0.87	51.27±0.43	NS	52.07±0.68	51.41±0.77	NS	51.32±0.56	51.69±0.60	NS
Ash	%	1.50±0.36	1.51±0.33	NS	1.51±0.15	1.35±0.63	NS	2.16±0.95	1.82±0.35	NS
Ether extract	%	29.58±0.76	29.49±0.40	NS	27.82±2.18	26.27±0.75	NS	34.20±9.02	30.21±1.67	NS
Crude protein	%	16.58±0.59	16.37±0.65	NS	16.21±1.48	16.27±0.40	NS	16.49±4.79	16.11±0.82	NS
Albumen										
Dry matter	%	21.14±2.47	21.51±1.13	NS	21.03±2.16	20.14±1.20	NS	20.83±1.90	20.30±2.01	NS
Ash	%	0.60±0.07	0.73±0.16	NS	0.55±0.12	0.48±0.37	NS	0.64±0.26	0.53±0.10	NS
Ether extract	%	0.04±0.02	0.05±0.02	NS	0.03±0.01	0.04±0.12	NS	0.04±0.01	0.04±0.01	NS
Crude protein	%	11.84±2.01	12.52±2.43	NS	9.29±1.24	9.28±0.39	NS	11.31±3.90	9.90±1.56	NS

C= Control group; TE = Tomato extract group.

low lutein and zeaxanthin pigments normally present in egg yolk (OLSON *et al.*, 2008).

In a previous study with Isa Brown laying hens, FERRANTE *et al.* (2003) showed that a tomato extract-supplemented diet resulted in a higher a* value in egg yolk. The difference in the h* co-ordinate value ($P < 0.01$) between groups C and TE increased from day 7 to 14. Moreover, tomato extract addition resulted in a detectable colour difference; the variation was higher on day 7 than on day 14 ($\Delta E^* = 4.93$ and 3.71, respectively). Compared to a* and b*, the L* parameter, influenced the calculated colour variation (ΔE) the most. In fact, on days 7 and 14 the ΔL^* values were 4.08 and 2.77; Δa^* were 2.39 and 2.06 and Δb^* were 1.38 and 1.35, respectively.

The influence of lycopene on egg yolk colour was also observed by KARADAS *et al.* (2006). In their trial on adult Japanese quails fed a diet containing tomato powder, the Roche colour fan showed a well defined orange colour in the egg yolk which was the result of a combination of yellow xanthophylls and lycopene.

The carotenoid content in egg yolk is a reflection of their dietary provision, as previously reported by SURAI *et al.* (1998), SURAI and SPEAKE (1998) and SURAI (2002). In this study, lycopene transfer was further confirmed by HPLC, measuring the lycopene incorporated in the egg yolk. No lycopene content was detected in the eggs on day 1. The lycopene content in the yolk, increased in the TE group: 0.12 μg/g on day 7 and 0.16 μg/g on day 14. Therefore assuming a feed intake of 120 g/bird/d and a 15 g daily yolk mass production, the transfer efficiency of lycopene calculated from the tomato extract-supplemented diet to the egg yolk was about 0.13% (w/w). This value is in accord with that reported by KNOBLICH *et al.* (2005). Recently, OLSON *et al.* (2008) ex-

Table 4 - CIELAB parameters (n=10, mean±SD) and lycopene content (n=5, mean±SD) in egg yolks.

	Day 1			Day 7			Day 14		
	C	TE	P	C	TE	P	C	TE	P
L*	60.92±1.58	60.90±1.58	NS	62.13±2.04	58.05±3.78	< 0.05	63.48±1.46	60.71±1.99	< 0.01
a*	14.02±1.08	13.98±1.09	NS	14.46±1.31	16.85±0.90	< 0.01	14.45±1.25	16.51±1.32	< 0.01
b*	34.84±0.85	34.85±0.86	NS	35.38±1.31	34.00±0.94	< 0.05	35.90±1.04	34.55±1.37	< 0.05
C*	37.57±0.90	37.57±0.90	NS	38.24±1.28	37.96±1.08	NS	36.14±10.03	38.31±1.28	NS
h*	68.12±1.58	68.12±1.58	NS	67.80±2.00	63.67±1.10	< 0.01	68.09±2.03	64.49±2.11	< 0.01
Lycopene µg/g (fresh matter basis)	*N.D.	*N.D.		*N.D.	0.12±0.03		*N.D.	0.16±0.06	

C= Control group;
TE = Tomato extract group;
*Limit of detection of yolk lycopene concentration was <20 ng of lycopene/g.

amined the effect of lycopene addition on egg yolk in laying hens, testing at much higher levels; the result was much greater yolk lycopene deposition (0.6-4.5%). The Authors showed that the transfer efficiency of lycopene declined as its dietary intake increased.

CONCLUSIONS

The results of this study confirm that the intensity of egg yolk colour is influenced by a lycopene-supplemented diet. Lycopene is a good natural pigmenting compound, especially for the redness index. During the trial an average of 0.13% lycopene crossed over into the egg yolk; the lycopene transfer occurred even at low dietary concentrations.

The use of tomato extract introduced directly into the diet through a lipid phase (soybean oil) is relevant in terms of hen feedstuff. Compared to tomato peel or tomato supplemented diet (as proposed by KNOBLICH *et al.* 2005), the method described here to obtain a supplemented diet resulted in an increase of lycopene intake by hens, because no fibrous ingredients were used and digestibility was not affected.

REFERENCES

AOAC. 1990. "Official Methods of Analysis", 15th Ed. Association of Official Analytical Chemists, Arlington, VA, USA.

Cerolini S., Marzoni Fecia di Cossato M., Romboli I., Schiavone A. and Zaniboni L. 2008. "Avicoltura e Coniglicoltura". Point Veterinaire, Milan, Italy.

C.I.E. 1978. International Commission on Illumination, Recommendations on uniform color spaces, color-difference equations, psychometric color terms. Supplement no. 2 to CIE publication no. 15 (E.-1.3.1) 1971/(TC-1.3.) 1978. Bureau de la CIE, Paris, France.

Di Mascio P., Kaiser S. and Sies H. 1989. Lycopene as the most efficient biological carotenoid singlet oxygen quencher. Arch. Biochem. Biophys. 274: 532.

Farina A. and Bedetti C. 2007. "Microanalisi Elementare Organica Collezione di Strumenti". Istituto Superiore di Sanità, Rome, Italy.

Ferrante V., Baroli D., Marelli S., Mangiagalli M.G. and Cavalchini L.G. 2003. Effect of tomato by-product diet supplementation on egg yolk colour. Ital. J. Anim. Sci. 2: 459.

Franceschi S., Bidoli E., Vecchia C., Talamini R., D'Avanzo B. and Negri E. 1994. Tomatoes and risk of digestive-tract cancers. Int. J. Cancer. 59: 181.

Giovannucci E., Ascherio A., Rimm E.B., Stampfer M.J., Colditz G.A. and Willett W.C. 1995. Intake of carotenoids and retino in relation to risk of prostate cancer. J. Nat. Cancer Inst. 87: 1767.

Hazels Mitmesser S., Giraud D.W. and Driskell J.A. 2000. Dietary and plasma levels of carotenoids, vitamin E, and vitamin C in a group of young and middle-aged nonsupplemented women and men. Nutr. Res. 20: 1537.

Karadas F., Grammenidis E., Surai P.F., Acamovic T. and Sparks N.H. 2006. Effects of carotenoids from lucerne, marigold and tomato on egg yolk pigmentation and carotenoid composition. Br. Poult. Sci. 47: 561.

Knoblich M., Anderson B. and Latshaw D. 2005. Analyses of tomato peel and seed byproducts and their use as a source of carotenoids. J. Sci. Food Agric. 85: 1166.

Le Marchand L., Yoshizawa C.N., Kolonel L.N., Hankin J.H. and Goodman M.T. 1989. Vegetable consumption and lung cancer risk: a population-based case-control study in Hawaii. J. Nat. Cancer Inst. 81: 1158.

Leeson S. and Caston L. 2004. Enrichment of eggs with lutein. Poult. Sci. 83: 1709.

Leskanich C.O. and Noble R.C. 1997. Manipulation of the n-3 polyunsaturated fatty acid composition of avian egg and meat. Worlds Poult. Sci. J. 53: 155.

Olson J.B., Ward N.E. and Koutsos E.A. 2008. Lycopene incorporation into egg yolk and effects on laying hen immune function. Poult. Sci. 87: 2573.

SPSS. 2008. SPSS release 17.0. Chicago, IL, USA.

Stahl W. and Sies H. 1996. Lycopene: a biologically important carotenoid for humans? Arch. Biochem. Biophys. 336: 1.

Strazzullo G., De Giulio A., Tommonaro G., La Pastina C., Poli A., Nicolaus B., De Prisco R. and Saturnino C. 2007. Antioxidative activity and lycopene and β-carotene contents in different cultivars of tomato (*Lycopersicon esculentum*). Int. J. Food Prop. 10: 321.

Surai P.F. 2000. Organic Selenium: benefits to animals and humans, a biochemist's view. In: "Biotechnology in the feed industry. Proceedings of Alltech's 16th Annual Symposium: the future of food" T.P. Lyons and K.A.

Jacques (Ed.), p. 205. Nottingham University Press, Nottingham, UK.

Surai P.F. 2002. "Natural Antioxidants in Avian Nutrition and Reproduction" Nottingham University Press, Nottingham, UK.

Surai P.F and Speake B.K. 1998. Distribution of carotenoids from the yolk to the tissues of the chick embryo. J. Nutr. Biochem. 9: 645.

Surai P.F., Ionov I.A., Kuklenko T.V., Kostjuk I.A., MacPherson A., Speake B.K., Noble R.C. and Sparks N.H. 1998. Effect of supplementing the hen's diet with vitamin A on the accumulation of vitamins A and E, ascorbic acid and carotenoids in the egg yolk and in the embryonic liver. Br. Poult. Sci. 39: 257.

Surai P.F., MacPherson A., Speake B.K. and Sparks NHC. 2000. Designer egg evaluation in a controlled trial. Eur. J. Clin. Nutr. 54: 298.

Thapon J.L. and Bourgeois C.M. 1994. "L'Oeuf et les Ovoproduits". Lavoisier, Paris, France.

Zanfini A., Dreassi E., La Rosa C., D'Addario C. and Corti P. 2007. Quantitative variations of the main carotenoids in Italian tomatoes in relation to geographic location, harvest time, varieties and ripening stage Ital. J. Food Sci. 19: 181.

Revised paper received September 29, 2009 Accepted November 5, 2009

SHORT COMMUNICATION

QUALITY OF TOMATO FERTILIZED WITH NITROGEN AND PHOSPHOROUS

L.F. DI CESARE, C. MIGLIORI, D. VISCARDI and M. PARISI[1]
C.R.A.-I.A.A. Unità di ricerca per i processi dell'Industria Agro-Alimentare,
Via G. Venezian 26, 20133 Milano, Italy
[1]C.R.A.- Centro di Ricerca per l'Orticoltura, Azienda Sperimentale, S.S. 18, 84091 Battipaglia (SA)
*Corresponding author: Tel. +39 02 239557215
e-mail: luigi.dicesare@entecra.it

ABSTRACT

The influence of different levels of nitrogen (N1, N2), phosphorous (P1, P2) and N+P (N1P1, N1P2, N2P1, N2P2) mixed fertilizers was investigated on volatile substances, soluble sugars, organic acids, titratable acidity, dry matter and lycopene of "Corbarino" cherry-like tomatoes. The flavour volatiles reached the highest concentrations in red-ripe cherry-like tomatoes treated with N1. Glucose, fructose, dry matter and titratable acidity increased only in N+P fertilized samples. Among the organic acids, citric increased, malic decreased and oxalic was constant in all the fertilized samples. Lycopene reached the highest values in N2 and N2P2 samples.

- Key words: GC/MS and HPLC analysis, flavour volatiles, lycopene, organic acids, soluble sugars -

INTRODUCTION

Tomato, a key vegetable in the Italian Mediterranean diet, has recently gaining been attention in relation to the prevention of some human diseases. This interest is due to the presence of carotenoids and particularly lycopene, which is an unsaturated alkylic compound, that appears to be an active compound in the prevention of cancer and cardiovascular risk and in slowing down cellular aging, owing to its high antioxidant and antiradical power (GERSTER 1997; GIOVANNUCCI *et al.* 1995). Lycopene is found in fresh, red-ripe tomatoes as all-*trans* (79-91%) and *cis*- (9-21%) isomers (STAHH and SIES, 1992; SHI *et al.* 1999; BOILEAU *et al.* 2002).

Lycopene and vitamins give a measure of the nutraceutical quality, while flavour volatiles, soluble sugars, organic acids, etc. express the nutritional quality.

In red-ripe tomato fruit, many volatile compounds have been identified [2(E)-hexenal, hexanal, 3(Z)-hexen-1-ol, β-ionone, 2(E)4(Z)-decadienal, 2-isobutylthiazole, 3(Z)-hexenol, linalool, methylsalicylate, 2-methoxyphenol, 6-methyl-5-hepten-2-one, 6-methyl-5-hepten-2-ol, 2,3-epoxygeranial, neral, geranial, nerylacetone, β-damascenone, α-terpineol etc.] (DI CESARE *et al.* 2003).

Hexanal, 2(E)-hexenal, 3(Z)-hexen-1-ol, 2-isobutylthiazole, are considered to be responsible for the fresh tomato flavour (DIRINCK *et al.* 1976). While, according to other authors (BUTTERY *et al.* 1989; BUTTERY, 1993; BUTTERY and LING, 1993 a,b) 3(Z)-hexenal, β-ionone, hexanal, β-damescenone, 2(E)-hexenal, 1-penten-3-one, 2 and 3-methylbutanols, 2-isobutylthiazole, 2-isobutylcyanide, 2(E)-heptenal, phenylacetaldehyde, 6-methyl-5-hepten-2-one, 3(Z)-hexenol, 2-phenylethanol, methylsalycilate are considered to be important contributors to tomato flavour.

Soluble sugars and organic acids play an important role in the characterization of the tomato taste. Sugars (glucose, fructose and traces of sucrose) and organic acids (citric, malic and oxalic) represent half of the total dry matter of tomato fruit (SILVESTRI and SIVIERO, 1991).

In tomato cultivation, fertilizer application regimes can play an important role both to increase the yield and size and to improve the nutritional and nutraceutical quality of the fruit.

A large amount of N is required for optimum production as the tomato root absorbs a great deal from the soil. N-deficiency can cause a decrease in production (number and size of tomatoes), a reduction in storage quality, colour, and tomato taste (SAINJU *et al.*, 2003).

Secondary plant metabolites which lack N in their structure such as lycopene, β-carotene, phenolics and flavonols are favoured under N-limiting conditions, although photosynthetic activity is not simultaneously reduced. However nitrogen-containing compounds are favoured when N is readily available and not limiting to growth (DORAIS *et al.*, 2008).

As far as nutritional quality and suitability for processing are concerned, many authors have highlighted the negative effects of excessive N, such as an increase in pH and a decrease in soluble sugars, soluble solids, dry matter, sugars/dry matter ratio and firmness (Kaniszewski *et al.*, 1987; KOONER and RANDHAWA, 1990; PARISI *et al.*, 2006).

XU *et al.* (2001) found that by using an application of nitrogen within the proper range increased the concentration of the volatile compounds in tomato, while an excessive N application decreased their concentration.

YU-TAO *et al.* (2007) studied the effects of applications of N levels on volatile substances, taste compounds and firmness of fresh tomato fruits. The results pointed out that increasing N application increased the concentrations of 1-penten-3-one, hexanal, 3(Z)-hexenal, 2-methyl-4-pentenal, 2(E)-hexenal, 6-methyl-5-hepten-2-one, titratable acidity, soluble sugars and soluble solids; under the same conditions the concentrations of phenylacetaldehyde decreased, whereas the concentration of 2(E)4(E)-hexadienal and firmness of fresh tomato fruit initially increased and then decreased.

WRIGHT and HARRIS (1985) pointed out that flavour scores indicated that increasing N and K fertilization had a detrimental effect on tomato flavour. An increase in titratable acidity and soluble solids was found with increasing fertilization. Concentrations of hexanal, 2-hexanone, benzaldehyde, phenylacetaldehyde, β-ionone and 6-methyl-5-hepten-2-one increased with increasing N+K levels.

Phosphorous fertilization is a key component in the metabolism and regulation of several pathways involved in the biosynthesis of secondary plant metabolites, many of which are nutraceuticals. P may increase the level of some phytochemicals such as ascorbic acid, anthocyanins, flavonoids and lycopene, although interaction with climatic factors and the growing season may occur (BRUULSEMA *et al.* 2004). SAITO AND KANO (1970) found that by increasing P-fertilization from 0 to 100 mg L^{-1} nutrient solution under hydroponic growth conditions, the colour of the tomato fruit greatly improved and consequently lycopene content was increased.

OKE *et al.* (2005) studied the effect of P fertilizer on the quality of tomato under field conditions for three consecutive years, evaluating the pH, titratable acidity, lycopene, vitamin C, flavour volatiles etc. They noted that the influence of P application on several of the quality parameters mentioned above was marginal, while climatic features had a more predominant effect.

In this study, the effects of two different levels of N and P and their mixture (N+P) on the nutritional and nutraceutical quality of "Corbarino" cherry-like tomatoes was evaluated.

MATERIALS AND METHODS

Plant source: The study was performed on "Corbarino" cherry-like tomatoes (accession PC05), a traditional ecotype originating in southern Italy (Campania region), characterized by small fruits (13-20 g) having a strong fruity odour and flavour, excellent health and nutritional properties and highly suitable for canning (GIORDANO *et al.*, 1999, 2000; ANDREAKIS *et al.*, 2004; SINESIO *et al.*, 2007).

The unfertilized tomatoes were grown in soil with a high content of organic matter (1.3% carbon) and available P (85 ppm), but low in available N (N Kjeldhal = 0.091%; NO_3 = 7.0 ppm; NH_4 = 11.5 ppm).

Fertilization was applied at two different levels of N (100 kg ha^{-1}= N_1; 200 kg ha^{-1}= N_2), P (150 kg ha^{-1}= P_1; 300 kg ha^{-1}= P_2) and their mixture (N_1P_1=100 kg ha^{-1} N+150 kg ha^{-1} P; N_1P_2= 100 kg ha^{-1} N+300 kg ha^{-1}P; N_2P_1= 200 kg ha^{-1} N+150 kg ha^{-1} P and N_2P_2= 200 kg ha^{-1} N+300 kg ha^{-1} P). Each level of P (P_2O_5) was applied before transplanting, while the amount of N for each treatment was applied in four doses during the crop cycle: 25% before transplanting as $(NH_4)_2SO_4$, 25% at 30 DAT (days after transplanting), 25% at 60 DAT and 25% at 80 DAT as NH_4NO_3.

All the samples were cultivated, at Battipaglia (Sele Valley, SA) in open fields and harvested at the red-ripe stage between 20-24 August 2008.

The average yield of tomatoes (t/ha) according to the treatment applied is reported in Table 1. Each test was repeated three times.

Extraction-concentration and analysis of volatile substances: The volatile compounds were extracted and concentrated by microwave-resin-solvent combined method and the extracts obtained were analyzed quali-quantitatively by GC/MS. The method and the operating conditions were reported in a previous note (DI CESARE *et al.*, 2003).

Soluble sugars and organic acids: They were extracted with double distilled water and analyzed by HPLC (FORNI *et al.*, 1992).

Lycopene: It was extracted using an hexane: acetone: ethanol mixture (2:1:1) and the hexane layer was analyzed by HPLC (MIGLIORI *et al.*, 2008).

Dry matter: It was determined using a laboratory oven at 85°C up to a constant weight (AOAC, 1980).

Statistical analysis: Tukey test was used to evaluate the differences among the different levels of fertilization. Mean values were considered significantly different when $\rho \leq 0.05$.

Table 1 - Tomato yield (t/ha) according to the treatment applied. Different letters indicate significant difference ($\rho \leq 0.05$).

Treatment	Average yield (t/ha)
Unfertilized	36.8 a
N_1	60.4 ab
N_2	64.7 ab
P_1	39.0 a
P_2	44.3 a
N_1P_1	59.0 ab
N_1P_2	59.9 ab
N_2P_1	69.6 b
N_2P_2	73.4 b

RESULTS AND CONCLUSIONS

Volatile substances: Table 2 shows the volatile compounds identified in unfertilized and fertilized cherry-like tomatoes (alcohols, carbonyl compounds (aldehydes and ketones), phenolic derivatives, heterocyclic compounds and terpenes). Results are in agreement with what was found in fresh tomato fruits by BUTTERY *et al.* (1989), BUTTERY (1993), BUTTERY and LING (1993 a,b).

As for alcohols, 3-methyl-1-butanol decreased in all the fertilized samples and this trend was more evident in N_2P_1 and N_2P_2. All the other alcohols increased only in N_1 samples, and they decreased in all the other fertilized samples, with respect to the unfertilized one.

Among the carbonyl compounds, 1-penten-3-one, 2(E)-hexenal, 2(E)-heptenal, 2(E)4(E)-decadienal increased with all the fertilized samples. Hexanal and 6-methyl-5-hepten-2-one increased in both levels of N, but decreased or were similar in the other fertilizations, with respect to the unfertilized sample.

Methyl salicylate increased with both levels of N and in the N+P samples, but decreased with both levels of P.

2-isobutylthiazole increased in N1 samples, but decreased in all the other fertilized samples.

Concerning terpenes, linalool increased in N_1, N_2, P_1 and P_2 samples, but decreased in the N+P samples. All the other terpenes increased in N_1 and N_2 samples and decreased or were constant in P_1 and P_2 and N+P fertilized samples.

As reported above, it can be seen that most of the contributor volatile compounds showed the highest concentration in N fertilized samples especially at the lower level. As N supply increased, so did the similar behaviour of increasing concentrations of several volatile compounds, in agreement with YU-TAO *et al.* (2007). A lot of studies have demonstrated that the concentrations of amino acids and fatty acids increased with increasing N supply (MOZAFAR, 1993). S-amino acids (cysteine and methionine) are precursor of thiazole, as 2-isobutylthiazole. Aromatic amino acids are precursors of phenolic volatile compounds (ESCHIN, 1990). Leucine and valine are precursors of 2-methyl-1-butanol, 3-methyl-1-butanol and lycopene whose conversion gives 6-methyl-5-hepten-2-one, 6-methyl-5-hep-

Table 2 - Quali-quantitative composition (μg/100 g d.m.) of volatile compounds identified in "Corbarino" cherry-like tomatoe samples, treated with N and P fertilizers. Different letters indicate significant difference (ρ≤ 0.05).

COMPOUNDS	Unfertilized	Fertilized							
		N_1	N_2	P_1	P_2	N_1P_1	N_1P_2	N_2P_1	N_2P_2
ALCOHOLS									
3-methyl-1-butanol	409.62 c	373.52 b	264.15 ab	270.03 ab	299.40 ab	287.96 ab	301.79 ab	230.16 a	220.12 a
2-methyl-1-butanol	28.63 bc	55.32 e	20.90 d	11.95 a	25.83 b	23.32 c	21.16 b	24.72 b	20.67 c
6-methyl-5-hepten-2-ol	8.17 d	17.92 d	7.16 c	7.47 c	6.33 b	4.41 a	5.21 ab	5.58 ab	4.15 a
phenethyl alcohol	112.91 d	255.79 e	109.00 d	100.33 d	43.65 b	63.48 bc	29.43 a	72.07 c	71.42 c
CARBONYL COMPOUNDS									
1-penten-3-one	4.21 b	13.10 c	16.78 d	11.85 a	13.16 c	14.03 c	16.01 d	11.81 c	15.06 d
hexanal	214.90 b	256.43 c	284.60 c	198.09 a	204.48 ab	213.32 b	224.52 b	186.90 a	176.16 a
2(E)-hexenal	28.86 b	47.62 c	43.71 c	37.37 a	41.91 ab	38.04 bc	46.58 c	33.59 ab	28.86 b
2(E)-heptenal	22.04 b	40.43 c	51.10 d	29.68 bc	38.94 c	43.66 c	40.25 c	21.83 b	24.67 a
6-methyl-5-hepten-2-one	212.43 ab	333.52 c	262.32 b	191.46 a	173.16 a	195.81 a	183.16 a	159.62 a	162.13 a
2(E).4(E)-decadienal	35.45 a	70.26 c	75.37 c	34.69 a	41.42 ab	54.15 b	67.81 bc	36.65 a	58.53 b
PHENOLIC DERIVATIVES									
methyl salicylate	79.32 b	227.06 e	173.04 d	67.38 a	72.40 ab	146.27 cd	151.34 cd	124.95 c	131.66 c
HETEROCYCLIC COMPOUND									
2-isobutylthiazole	242.93 b	288.35 c	215.22 b	200.86 b	182.81 b	204.82 b	201.11 b	100.13 a	115.86 a
TERPENES									
linalool	51.04 b	91.49 c	87.84 c	180.98 d	163.65 d	44.80 b	8.47 a	55.77 b	52.31 b
neral	30.07 b	50.39 c	39.13 ab	24.14 a	25.66 a	24.31 b	27.00 ab	20.43 a	18.92 a
geranial	54.19 c	95.44 e	67.31 d	44.63 b	38.18 ab	36.29 a	47.71 b	32.11 a	44.37 b
β-damascenone	25.86 b	52.13 d	42.93 c	17.39 a	18.12 a	16.59 a	27.50 b	23.99 b	24.56 b
neryl acetone	26.29 b	37.86 cd	40.42 d	21.16 b	20.09 c	11.97 a	29.50 bc	19.53 b	24.91 b
β(Z)-ionone	41.41 c	48.22 c	51.33 d	24.25 a	31.99 b	19.53 a	25.13 a	21.99 a	18.75 a

ten-2-ol and terpenes. Fatty acids are precursors of lipids, whose degradation gives aldehydes and ketones (WRIGHT and HARRIS, 1985; CARBALLO *et al.*, 1994; WESTON and BARTH, 1997). This could explain why the concentration of all classes of volatile compounds increased with increasing N supply.

Except for linalool and 2(E)-hexenal, which showed the highest concentrations in P_1 and P_2 samples, all the other volatile compounds showed lower values with respect to unfertilized and/or N_1 and N_2 ones. In N+P samples, the values were always lower than the unfertilized sample, apart from some carbonyl compounds and methyl salycilate. These results agree with OKE *et al.* (2005) who observed no major differences in the volatile concentrations vs. various phosphorous supplementations.

Chemico-physical and nutraceutical parameters: The values of soluble sugars, organic acids, titratable acidity, dry matter and lycopene are reported in Table 3. The concentrations of glucose and fructose were quite comparable with the unfertilized sample in the cherry-like tomatoes fertilized with N or P. On the contrary, in the samples fertilized with both N + P, there was an increase of glucose in N_2P_1 and N_2P_2, and fructose in N_2P_1. No effects on sugar contents of N fertilization up to 200 kg/ha were found in peeled tomato by PARISI *et al.* (2006).

Table 3 - Effect of N and P fertilizers on chemical-physical and nutraceutical parameters of "Corbarino" cherry-like tomatoes. Soluble sugars are expressed in g/100 g f.w., organic acids and lycopene in mg/100 g f.w., titratable acidity in meq/100 g f.w. Different letters indicate significant difference (ρ≤ 0.05).

	Unfertilized	Fertilized							
		N_1	N_2	P_1	P_2	N_1P_1	N_1P_2	N_2P_1	N_2P_2
SOLUBLE SUGARS									
Glucose	2.37 a	2.37 a	2.27 a	2.19 a	2.39 a	2.54 a	2.50 a	2.91 b	2.64 b
Fructose	2.64 a	2.43 a	2.38 a	2.59 a	2.63 a	2.64 a	2.62 a	2.91 b	2.64 a
ORGANIC ACIDS									
Oxalic	5.72 b	5.41 b	6.04 b	5.88 b	5.90 b	5.44 b	6.10 b	4.84 a	6.15 b
Citric	346.28 b	422.63 c	466.74 c	317.90 a	407.91 c	442.82 c	411.18 c	508.33 d	472.54 d
Malic	55.11 b	43.27 a	51.03 ab	43.51 a	51.73 c	49.34 b	45.77 a	46.72 ab	44.02 a
TITATRABLE ACIDITY	8.06 a	10.31 c	10.39 c	9.13 b	8.69 a	8.02 a	9.84 b	12.28 d	11.43 d
D.M. %	9.28 b	9.60 b	9.88 b	8.68 a	8.73 a	10.12 c	9.90 b	9.93 b	10.29 c
LYCOPENE	6.84 a	10.49 bc	15.15 d	8.96 b	12.68 c	9.38 b	9.17 b	11.56 c	14.62 d

For organic acids, oxalic acid was constant in all the samples except N_2P_1, where it decreased in comparison to the unfertilized sample. Citric acid showed the lowest value only in the unfertilized sample, while malic acid decreased in all samples, except in N_2 where it was similar to the unfertilized sample.

Dry matter decreased in P_1 and P_2 samples and increased in N_1P_1 and N_2P_2, while it remained constant in the other samples, compared to the unfertilized one. Taking into account the effects of N on this quality trait, these results are in agreement with those of PARISI *et al.* (2006).

Titratable acidity increased in all the fertilized samples, and the highest values were in N_2P_1 and N_2P_2.

WRIGHT and HARRIS (1985) and LI *et al.* (1997) reported that organic acids, soluble sugars and titratable acidity increased with increasing rates of N and K application. In our research, a different response of soluble sugars, organic acids and titratable acidity can be observed with respect to the cited literature. In fact, the highest concentrations of glucose, fructose and titratable acidity were achieved by mixing N and P.

The unfertilized sample showed the lowest concentration (6.84 mg/100 g f.w.) of lycopene whereas it increased up to 10.49 mg/100 g f.w. in N_1 and 15.15 mg/100 g f.w. in N_2 in the fields fertilized with N.

In the fields fertilized with P, lycopene increased up to 8.96 mg/100 g f.w. in P_1, and 12.68 mg/100 g f.w. in P_2. These values were higher than the unfertilized samples, but lower than the samples treated with N.

When the fields were treated with both N + P fertilizations, in N_1P_1 and N_1P_2, lycopene ranged 9.17 to 9.38 mg/100 g f.w. But in N_2P_1 and N_2P_2, the values were 11.56 and 14.62 mg/100 g f.w., respectively. The latter value and that of N_2 were the highest ones observed among all the samples.

Similar behaviour was observed by KOBRYN *et al.* (2004) in three tomato types (cluster, cherry and large-fruit), cultivated on rockwool and fertilized with different levels of nitrogen. The N supply increased the content of lycopene in all tomato types, but this effect was more evident in the cluster one.

The same results were obtained by ELKNER *et al.* (2004) in two tomato types (Fanny F1 and Dual plus F1) fertilized with N+K and drip irrigated, fertigated and broadcast irrigated. Drip irrigation and fertigation with N+K and complex fertilizers had a positive effect on ascorbic acid, pectin and hemicelluloses and in particular on lycopene content in Dual plus F1.

DE PASCALE *et al.* (2008) studied the effects of three different rates of N supply (0, 100, 200 kg/ha) on the nutritional value of organically and conventionally grown tomatoes. They observed that carotenoids and antioxidant activity increased in organic tomatoes, even if the yields were lower than conventional ones.

Recent studies have pointed out that lycopene, as well as other carotenoids, are synthesized from isoprenoids that are products of mevalonic acid (MVA) and methylerythritol-4-phosphate (MEP) pathway (BOTELLA-PAVIA and RODRIGUEZ-CONCEPCION, 2006). Increasing of N fertilization could most likely enhance the action of enzymes involved in these pathways such as deoxyxylulose 5-phosphate synthase.

Our results indicate that the effect of N supplementation is more evident than P and mixing (N+P) fertilizations in carotenoid biosynthesis, even if phosphorous is a key component (ATP, NADP(H)2). However OKE *et al.* (2005) found that P supplementation may not significantly affect the processing quality parameters of tomato fruit. This may depend on the starting P content in the soil, which is already adequate for complete synthesis of nutraceutical compounds (Vegetable Production Recommendations, 1999), as reported above.

Under these experimental conditions, besides the total yield (Table 1), N fertilization positively influenced the physico-chemico-organoleptic and nutraceutical parameters, compared to P fertilization and N+P mixings. In fact, cherry-like tomatoes fertilized only with N had a higher concentration of volatile substances and lycopene in their tissues. These compounds improve the organoleptic characteristics and, perhaps most importantly, prevent chronic diseases, such as cancer, cardiovascular and neurodegenerative diseases.

Our data clearly shows that, even if P fertilization in combination with nitrogen increases the nutraceutical quality, it can be limited or avoided if the P content in the soil is enough to regulate the cellular metabolism of tomatoes.

REFERENCES

Andreakis N., Giordano I., Pentangelo A., Fogliano V., Graziani G., Monti L.M. and Rao R. 2004. DNA fingerprinting and quality traits of Corbarino cherry-like tomato landraces. J. Agric. Food Chem. 52:3366.

AOAC. 1980. In: "Official Methods of Analysis" No. 22.018-46.024-22.061, 13th Ed. Association of Official Analytical Chemists, Washington DC.

Boileau T.W.M., Boileau A.C. and Erdman J.W. Jr. 2002. Bioavailability of all-*trans* and *cis*-isomers of lycopene. Exp. Biol. Med. 227:914.

Botella-Pavia P. and Rodriguez-Conception M. 2006. Carotenoid biotechnology in plants for nutritionally improved foods. Physiologia Plantarum 126:369.

Bruulsema T.W., Paliyath G., Schofield A. and Oke M. 2004. Phosphorous and phytochemicals. Better Crops 88:6.

Buttery R.G., Teranishi R., Flath R.A. and Ling L.C. 1989. Fresh tomato volatiles: Composition and sensory studies, in Teranishi R., Buttery R.G., Shahidi F. Flavor Chemistry: Trends and Developments. Am. Chem. Soc., Washington D.C.:213.

Buttery R.G. and Ling L.C. 1993a. Enzymatic production of volatiles in tomatoes. In "Flavor Precursors", P. Schreier and P. Winterhalter, (Ed.). p. 137, Allured Publ., Wheaton, IL.

Buttery R.G. and Ling L.C. 1993b. Volatiles components of

tomato fruit and plant parts, Relationship and biogenesis. In: "Volatile Compounds from Plants". R. Teranishi, R. Buttery and H. Sugisawa (Ed.), p. 23.Bioactive ACS Books, Washington D.C.

Buttery R.G. 1993. Quantitative and sensory aspects of flavour of tomato and other vegetables and fruits, In: "Flavour Science: Sensible Principles and Techniques". T.E. Acree and R. Teranishi (Ed.), p. 259. Chem. Soc., Washington D.C.

Carballo S.J., Blankenship S.M. and Sanders D.C. 1994. Drip fertigation with nitrogen and potassium and postharvest susceptibility to bacterial soft rot of bell peppers. J. Plant Nutr. 17:1175.

De Pascale S., Tamburrino R., Maggio A., Barbieri G., Fogliano V. and Pernice R. 2008. Effects of nitrogen fertilization on the nutritional value of organically and conventionally grown tomatoes. Acta Hort. 700: International symposium towards ecologically sound fertilization strategies for field vegetables production.

Di Cesare L.F., Forni E., Viscardi D. and Ferrari V. 2003. Valutazione della composizione aromatica e chimico-fisica di alcune cultivar di pomodoro da impiegare per il consumo diretto o per la trasformazione. Ind. Conserve 78:195,

Dirinck P., Schreyen L., van Wassenhove F. and Schamp N. 1976. Flavour quality of tomatoes. J. Sci. Food Agric. 27:499.

Dorais M., Ehret D.L., and Papadopoulos A.P. 2008. Tomato (*Solanum lycopersicum*) health components: from the seed to the consumer. Phytochem. Rev. 7:231.

Elkner K., Kaniszewski S. and Dysko J. 2004. Effect of fertigation on the content of ascorbic acid, carotenoids and dietary fiber in tomato fruits. Vegetable Crops Research Bulletin 61:69.

Eschin MNA. 1990. Biochemical changes in raw foods: Fruits and vegetables. In "Biochemistry of Food", p. 134. 2nd, Academic Press. Inc. New York.

Forni E., Erba M.L., Maestrelli A. and Polesello A. 1992. Sorbitol and free sugar contents in plums. Food Chem. 44:269.

Gerster H. 1997. The potential role of lycopene for human health. J. Am. College Nutr. 16:109.

Giordano I., Pentangelo A., Carboni A., Castaldo D. and Villari G. 1999. Biomorphological and productive characterization of several accession of small "Pomodorino di Corbara" tomatoes. Acta Hort. 487:343.

Giordano I., Pentangelo A., Villari G., Fasanaro G. and Castaldo D. 2000. Caratteristiche bio-agronomiche e idoneità alla trasformazione di pomodori dell'ecotipo "Corbarino". Ind. Conserve 75:317.

Giovannucci E., Ascherio A., Rimm E.B., Stampfer M.J., Colditz G.A. and Willett W.C. 1995. Intake of carotenoids and retinol in relation to risk of prostate cancer. J. Nat. Canc. Inst. 87:1767.

Kaniszewski S., Elkner K. and Rumpel J. 1987. Effect of nitrogen fertilization on yield, nitrogen status in plants and quality of fruits of direct seeded tomatoes. Acta Hort. 200:195.

Kobryn J. and Hallmann E. 2004. The effect of nitrogen fertilization on the quality of three tomato type cultivated on rockwool. Acta Hort. 691:341.

Kooner K.S. and Randhawa K.S. 1990. Effect of varying levels and sources of nitrogen on yield and processing qualities of tomato varieties. Acta Hort. 267:120.

Li Y.X., Li J.H., He L.L., Gong Y.G. and Li T.L. 1997. The effect of N, P, K mixed application on yields and quality of tomato in solar green house. China Vegetables 4:10.

Migliori C., Viscardi D., Di Cesare L.F., Campanelli G., Ferrari V., Quinto G. and Mennone C. 2008. Pomodoro "Cherry": effetti di concimazione e di micorrizzazione. Colture Protette 4:79.

Mozafar A. 1993. Nitrogen fertilizers and the amount of vitamins in plants: a review. J. Plant Nutr. 16:2479.

Oke M., Ahn T., Schofield A. and Paliyath G. 2005. Effects of phosphorous fertilizer supplementation on processing quality and functional food ingredients in tomato. J. Agric. Food Chem. 56:1531.

Parisi M., Giordano I., Pentangelo A., D'onofrio B. and Villari G. 2006. Effects of different levels of nitrogen fertilization on yield and fruit quality in processing tomato. In Proceedings of ISHS Symposium "Toward ecologically sound fertilization strategies for field vegetable production", Perugia, June, 7-10. Acta Hort., 700: 129.

Sainju U.M., Dris R. and Singh B. 2003. Mineral nutrition of tomato. Food, Agriculture & Environment 1(2):176.

Saito S. and Kano F.J. 1970. Influence of nutrients on growth of solanaceous vegetable plants, quality and chemical composition in their fruits. (part 1). On the effect of different phosphate levels on the lycopene content of tomatoes. J. Agric. Sci. Tokyo 14:233.

Shi J., Le Maguer M., and Niekamp F. 1999. Lycopene degradation and isomerization in tomato dehydration. Food Res. 32:15.

Silvestri G. and Siviero P. 1991. La coltivazione del pomodoro da industria. Edizioni l'informatore Agrario:39.

Sinesio F., Paparario M. and Moneta E. 2007. Sensory characteristics of traditional field grown tomato genotypes in southern Italy. J. Food Quality 30:878.

Stahl W. and Sies H. 1992. Uptake of lycopene and its geometrical isomers is greater from heat-processed than from unprocessed tomato juice in humans. J. Nutr. 122:2161.

Vegetable Production Recommendations. 1999. Ontario Ministry of Agriculture and Food. p. 135, Ottawa, Canada.

Weston L.A. and Barth M.M. 1997. Preharvest factor affecting post-harvest quality of vegetables. HortSci. 32:812.

Wright D.H. and Harris N.D. 1985. Effect of nitrogen and potassium fertilization on tomato flavour. J. Agric. Food Chem. 33:355.

Xu K., Zhong G.S. and Wang X.F. 2001. Effects of nitrogen rates on colonial photosynthesis yield and qualities of ginger (in Chinese). Plant Nutr. Fert. Sci. 9:189.

Yu-Tao W., Shao-Wen H., Rong-Le L. and Ji-Yun J. 2007. Effects of nitrogen application on flavour compounds of cherry tomato fruits. J. Plant Nutr. Soil Sci. 170:461.

Revised paper received October 2, 2009 Accepted December 16, 2009

SHORT COMMUNICATION

NUTRITIONAL EVALUATION OF FILLETS, PULP AND CROQUETTES OF WILD CAUGHT LAKE TRASIMENO GOLDFISH (*CARASSIUS AURATUS* L.)

A. DAL BOSCO*, E. MOURVAKI, C. MUGNAI, S. RUGGERI and C. CASTELLINI
Dipartimento di Biologia Applicata, Università di Perugia, Borgo XX Giugno 74, 06121 Perugia, Italy
*Corresponding author: dalbosco@unipg.it

ABSTRACT

Processing is required to render the products of some fish species edible, thus enhancing their economic value. The aim of the present study was to evaluate the nutritional value of goldfish (*Carassius auratus* L.) products from Lake Trasimeno (Umbria, Italy). The same number of fillets (n=20), raw and pre-cooked pulp samples, as well as of croquettes were used to evaluate the proximate composition, total lipid fatty acid profile and the protein and lipid oxidative stability. The water holding capacity (WHC) and the percentage of cooking loss (CL) were also assessed. Despite the low amount of lipids (1.85%), fresh goldfish fillets were characterized by a high PUFA level (54.30%), with DHA being the most abundant long-chain fatty acid (15.60%) followed by EPA (9.45%). Fillet processing reduced both WHC and CL of the final products, while the production of TBARS and carbonylated proteins increased, especially in croquettes (+ 59 and + 77%, respectively). Thrombogenic and atherogenic indexes also increased due to the PUFA reduction, reaching statistical significance only for the croquettes (0.53 *vs* 0.21 and 0.46 *vs* 0.34, respectively). These results suggest that goldfish fillets are characterized by a high nutritional value and a good oxidative stability. However, more efforts are needed to preserve these characteristics throughout processing.

- Key words: croquette, carbonylated proteins, fillet, goldfish, nutritional quality, PUFA, pulp, TBARS -

INTRODUCTION

Trasimeno is one of the largest and shallowest lakes of the Italian peninsula with an average surface area of about 122 km^2 and a maximum depth of less than 6 m. It is located about twenty kilometres west of Perugia, in central Italy. It is a closed lake with no natural outlets. Its catchment basin is located entirely on lithotypes with a low permeability and almost corresponds to a hydro-geological basin (DRAGONI *et al.*, 2003). The water, seeping through the pleistocenic and recent sediments in the basin, probably reaches the lake via the aquifer surrounding it. Because of these characteristics, the water level of the lake undergoes considerable variations in accordance with the rate of local rainfall and, thus, it strictly depends on meteorological and climatic conditions (BURZIGOTTI *et al.*, 2003). Despite the high variability in the water level, Lake Trasimeno is very rich in fish such as chub (*Leuciscus cephalus* L.), rudd (*Scardinius erythrophthalmus* L.), eel (*Anguilla anguilla* L.), pike (*Exos lucius*), carp (*Ciprinus carpio*), tench (*Tenca tenca*), goldfish (*Carassius auratus* L.) and herbivore carp (*Ctenopharyngodon idellus Val.*) (NATALI, 1989).

Goldfish is one of the most common autocthonous fish species in Lake Trasimeno (MEARELLI *et al.*, 1990; GHETTI *et al.*, 2007; LORENZONI *et al.*, 2007). The high invasive capacity of this fish is due to its notable tolerance to extreme environmental conditions (low temperatures, high rate of pollution, murky water with a low percentage of oxygen), high sexual fertility and a wide feeding regimen (LUSHCHAK *et al.*, 2001, 2005, 2007; FORD *and* BEITINGER, 2005). It usually lives in shallow ponds, lakes rich in vegetation and slow moving rivers. During the dry season or winter it burrows into the mud. Goldfish feed on plants, insect larvae and plankton. Because of these characteristics, the goldfish population, as well as other similar autocthonous fish species, such as carp and tench, has greatly increased over the last years to the detriment of the autochthonous fish species, causing an alteration in the equilibrium of the Lake Trasimeno fish community (MEARELLI *et al.*, 1990; LORENZONI *et al.*, 2007). For this reason, it is of great importance to find solutions to reduce the number of goldfish and simultaneously benefit the local fishermen's yearly income.

One of the major disadvantages of goldfish is the presence of a high number of fine inter-muscular bones which limits its direct consumption. Moreover, consumers' perception of goldfish, which is generally considered as a pet animal, should also be taken into account. Raw goldfish fillets could be a solution to the first problem, as they can be easily processed into more edible products and these products could be a further stimulus for goldfish fishing and marketing. The second limitation could be more difficult to overcome.

The goals of this study were to determine the nutritional value of the fillets of the Lake Trasimeno goldfish, taking into account the proximate and fatty acid composition and to investigate potential modifications of the chemical composition, oxidative stability and physical properties of the goldfish products (pulp and croquettes). Differences between pulp samples before and after pre-cooking were also evaluated.

MATERIAL AND METHODS

The study was carried out in collaboration with the "Albatrasimeno" Fisherman Cooperative of S. Feliciano (Perugia, Italy). Forty goldfish were caught by net in April 2008 and taken to the laboratory of the cooperative for processing as described below.

Fillets, pulp samples and croquette preparation

Goldfish, of an average live weight of 550 g, were eviscerated after washing with running water and the heads and tails were removed, dorsal and ventral fillets were dissected and twenty of them (mean weight of 100 ± 13 g) were taken for analysis. The others were double minced (∅ 4.5 and 2.5 mm) and the pulp samples obtained were used for nutritional evaluation before (mean weight of 100 ± 3 g, each) and after cooking (weight of 100 ± 2 g, each). In detail, forty pulp samples were pre-cooked at 72° C for 6 min and twenty were used for the croquette preparation (25 ± 1 g, each). The following ingredients were used to prepare the goldfish croquettes: pre-cooked goldfish pulp (65%), red potatoes (16%), grated bread (6%), parmesan cheese (6%), egg (5%), parsley (1%) and salt (1%).

All fillet samples, raw and pre-cooked pulp and croquettes (n=20) were taken to the laboratory of the Department of Applied Biology for nutritional evaluation.

Proximate analyses and fatty acid composition

All samples were analysed in duplicate to determine the proximate composition. In detail, moisture, ash, and total nitrogen were assessed using the AOAC methods (1995. N. 950.46B, 920.153, and 928.08, respectively). Total protein was calculated by Kjeldahl nitrogen using 6.25 as the conversion factor. Total lipids were extracted in duplicate from 5 g of each homogenised sample and calculated gravimetrically (FOLCH *et al.*, 1957).

Fatty acids were determined by gas chromatography after lipid extraction according to the method of FOLCH *et al.* (1957). One mL of lipid

extract was evaporated under a stream of nitrogen and the residue was derivatized by adding 3 mL of sulfuric acid (3% in methanol). After incubating at 80°C for 1 h, methyl esters were extracted with petroleum ether and 1 μL was injected into the gas chromatograph (Fisions Mega 2 Carlo Erba Gas Chromatograph, model HRGC Milano, Italy), equipped with a flame ionization detector. Separation of fatty acid methyl esters (FAME) was carried out on an Agilent (J&W) capillary column (30 m x 0.25 mm I.D, CPS Analitica, Milan, Italy) coated with a DB-Wax stationary phase (film thickness of 0.25 μm). The operating conditions were those described by DAL BOSCO *et al.* (2007). Individual fatty acid methyl esters (FAME) were identified by reference to the retention time of FAME authentic standards. The relative proportion of each fatty acid in the samples was expressed as a percentage.

The mean value of each fatty acid was used to calculate the sum of the saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids and to calculate the peroxidability index (PI) according to the equation proposed by ARAKAWA *and* SAGAI (1986):

PI = (% monoenoic x 0.025) + (% dienoic x 1) + (% trienoic x 2) + (% tetraenoic x 4) + (% pentaenoic x 6) + (% hexaenoic x 8).

The concentration of each fatty acid (mg/100 g of fish) was calculated from the lipid content of the fish using 0.91 as the conversion factor according to JOHANSSON *et al.* (2000).

The amount of each fatty acid was also used to calculate the atherogenicity and thrombogenicity indexes as proposed by ULBRICHT *and* SOUTHGATE (1991):

- atherogenic index = (C12:0 + 4xC14:0 + C16:0)/[(ΣMUFA + Σ (n-6) + Σ (n-3)];
- thrombogenic index = (C14:0 + C16:0 + C18:0)/[(0.5 x ΣMUFA + 0.5 x (n-6) + 3 x (n-3) + (n-3)/(n-6)].

Evaluation of pH, water holding capacity and cooking loss

The pH was measured with a Knick digital pH-meter (Broadly Corp., Santa Anna, CA, USA) after homogenizing the samples with iodoacetate (KORKEALA *et al.,* 1986).

The Water Holding Capacity (WHC) was evaluated (NAKAMURA and KATOH, 1985) by centrifuging one gram of sample, placed on a tissue paper inside a tube, for 4 min at 1,500 x g. The water remaining after centrifugation was quantified by drying the samples at 70°C overnight. WHC was calculated as follows: (weight after centrifugation - weight after drying)/initial weight x 100.

To evaluate cooking loss, samples of about 20 g were placed in open aluminium pans and cooked in an electric oven (pre-heated at 200°C) for 15 min to an internal temperature of 80°C (CYRIL *et al.*, 1996). Cooking loss was estimated as the percentage of weight of the cooked samples (cooled for 30 min at 15°C) with respect to the weight of the raw samples.

Assessment of oxidative stability

The extent of lipid oxidation was quantified by spectrophotometry (Hitachi U-2000) as thio-barbituric acid reactive substances (TBARS) according to the method of KE *et al.* (1977) and using a molar extinction coefficient of 156 x 10^3 M/cm at 532 nm. Results are expressed as malondialdehyde (MDA) equivalents (mg MDA/kg).

The amount of the products of oxidative damage to proteins (protein carbonyl groups, PCGs) were measured spectrophotometrically by reaction with 2,4-dinitrophenylhydrazine (DNPH) according to the method proposed by LUSHCHAK *et al.* (2005). In detail, samples were homogenized (1:10 w/v) using a Potter-Elvehjem glass homogenizer in 50 mM potassium phosphate buffer, pH 7.0, containing 0.5 mM EDTA. A few crystals of phenylmethylsulfonyl fluoride were added prior to homogenization to inhibit proteases. A 250 mL aliquot of this homogenate was then mixed with 0.5 mL of 10% (final concentration) trichloroacetic acid and centrifuged for 5 min at 13,000 g. The resulting pellets were mixed with 1 mL of 10 mM DNPH in 2 M HCl and incubated for 1h at room temperature. Control samples were mixed with 1 mL of 2 M HCl. After washing three-times with 1ml of ethanol-butylacetate (1:1 v/v), pellets were dissolved in 1.5 mL urea (6M) and PCGs were measured by means of a spectrophotometer (Hitachi U-2000) set at 370 nm, using a molar extinction coefficient of 22 x 10^3 M/cm. The values are expressed as nanomoles of PCGs per milligram protein in the urea solution. Protein concentration was measured by the BRADFORD (1976) method with Coomassie Brilliant Blue G-250 and using bovine serum albumin as a standard.

The sum of thiol groups (SH) was spectrophotometrically measured at 412 nm (molar extinction coefficient of 13,6 x 10^3 M/cm) in supernatants after reaction with 5,5'-dithio-bis (2-nitrobenzoic acid) as described by LUSHCHAK and BAGNYUKOVA (2006). Sample homogenates were prepared as described for the PCG assay and supernatants were obtained after centrifugation at 4°C for 15 min at 15,000 *g*. The thiol levels are expressed as micromoles of SH-groups per gram.

Reagents

Unless otherwise noted, all chemicals were analytical and were purchased from Sigma Chemical Co (St Louis, MO, USA).

Statistical evaluation

The data were analysed with a one way linear model (Statacorp®, 2005) evaluating the effect of technological treatment. Differences among goldfish products were evaluated by t-test, reporting the mean and standard error of the mean (SEM), at P<0.05.

RESULTS AND DISCUSSION

Proximate composition and physical traits of goldfish fillets, pulp and croquettes

The proximate composition of natural goldfish (fillets) and processed products (pulp and croquettes) is reported in Table 1. The amount of protein in the raw fillets (17.15%) was similar to the levels observed by TAKEUCHI *et al.* (1979) and by HOSSIAN and JAUNCEV (1989) in carp (*Cipri-nus carp*; 16-19%), whereas it was much higher with respect to the protein content of sutchi catfish (*Pangasius hypophthalmus*; 12.6%, ORBAN, 2007). The latter fish species is commonly used for croquettes or other processed products because of its very low cost.

The lipid level of the goldfish fillets (1.85%) was much lower than that of brown (2.8%) and of rainbow trout (4.20%), which are both freshwater fish (DAL BOSCO *et al.,* 2007). It was also lower than the crucian carp (*Carassius carassius* L.), which represents about 4.10% of the total weight, including the skin (KOLAKOWSKA *et al.*, 2000). On the other hand, the lipid level of goldfish fillets was very similar to the lipid content of other lean freshwater fish species, such as burbot (*Lota lota* L.), zander (*Stizostedion lucioperca* L.) and roach (*Rutilus rutilus* L.) and marine garfish (*Belone belone* L.), having a lipid content less than 2% (KOLAKOWSKA *et al.*, 2000).

Grinding did not cause any significant modification in the proximate composition of goldfish fillet, while cooking of raw pulp caused a significant reduction in the moisture content and a consequent increase in the amounts of protein and lipids. The addition of ingredients to the pre-cooked pulp for croquette preparation negatively affected (P<0.05) all of the aforementioned parameters.

The pH value of fillets was also affected by processing. The pH was significantly increased after cooking, whereas it was reduced after the addition of various ingredients to the cooked pulp (Table 1). Heat is known to cause variations in the electric charge of acid groups, separation of peptide chains and production of new compounds, reducing the concentration of hydrogen-ions (GRAU, 1978). SHERMAN (1961) has also attributed the increased pH of cooked pork meat to the fission of protein chains at labile linkages involving imidazole, -SH and -OH groups, followed by hydrogen bonding between carboxyl and amino groups. On the contrary, addition of various ingredients to the cooked pulp probably increased the number of acid groups, decreasing the pH of the croquettes.

Regarding the physical traits of goldfish fillet and processed products, it was found that grinding reduced (P<0.05) the WHC of fillets/pulp most probably by breaking down the muscle cell membranes (Table 1). This parameter was further reduced by thermal treatment, increasing the amount of water lost by cooking. It is well known that protein denaturation and coagulation, induced by heat, may reduce the space within the myofibril protein resulting in water loss (HAMM, 1969) and reduction in WHC (HONIKEL, 1998). The lower cooking loss of the cooked pulp, despite the lower WHC, could be justified by the reduced moisture level.

Nutritional characteristics

The fatty acid profile of the total lipids of fillets, pulp and croquettes is reported in Table 2. The percentage of total SFA in the goldfish fillet (29.00%) is similar to that of crucian carp (29.55%) (KOLAKOWSKA *et al.*, 2000), but much lower than that of sutchi catfish (41.1%). On the contrary, the total PUFA (54.30%) of goldfish was 1.6 and 4.3 times higher than that

Table 1 - Proximate composition and physical traits of goldfish fillets, pulp and croquettes.

		Fillet	Pulp		Croquette	SEM
			Raw	Cooked		
Moisture	%	79.86^{c}	78.57^{c}	75.93^{b}	72.05^{a}	2.11
Protein		17.15^{a}	18.20^{b}	20.11^{c}	24.10^{d}	0.31
Lipids		1.85^{a}	1.95^{a}	2.15^{ab}	2.54^{b}	0.42
Ash		1.14	1.28	1.82	1.31	0.36
pH		6.46^{b}	6.40^{b}	6.68^{c}	6.16^{a}	0.12
WHC*	”	54.41^{c}	50.51^{b}	47.10^{a}	48.69^{a}	2.07
Cooking loss	”	30.49^{b}	31.44^{b}	22.55^{a}	23.58^{a}	1.86

N=20 per group; a..d: P<0.05.
Legend: *WHC stands for water holding capacity.

Table 2 - Fatty acid profile (%) of goldfish fillets, pulp and croquettes.

	Fillet	Pulp		Croquette	SEM
		Raw	Cooked		
C14:0	1.08[a]	1.78[a]	1.73[a]	2.89[b]	0.92
C16:0	19.19[a]	18.67[a]	18.64[a]	24.01[b]	2.14
C18:0	6.30[a]	5.65[a]	7.71[b]	8.94[c]	1.01
Others	2.43[a]	2.62[a]	2.00[a]	3.16[b]	0.87
Total SFA*	29.00[a]	28.72[a]	30.08[a]	39.00[b]	2.65
C14:1n-6	0.03[a]	0.11[ab]	0.06[a]	0.21[b]	0.12
C16:1n-7	3.55[a]	5.97[b]	5.09[b]	5.08[b]	0.45
C18:1n-9	9.28[a]	10.94[a]	11.44[a]	19.12[b]	1.89
Others	3.82[b]	4.64[b]	5.08[c]	2.78[a]	0.85
Total MUFA**	16.68[a]	21.66[b]	21.67[b]	27.19[c]	1.80
C18:2n-6	5.57[a]	6.86[a]	7.05[a]	12.64	2.03
C20:3n-6	0.73[c]	0.60[b]	0.66[bc]	0.07[a]	0.10
C20:4n-6	9.05[c]	7.41[b]	9.07[c]	4.56[a]	0.57
C18:3n-3	5.84[c]	8.57[d]	4.18[b]	3.09[a]	0.41
C20:3n-3	0.58[c]	0.66[d]	0.44[b]	0.22[a]	0.02
C20:5n-3	9.45[b]	7.83[c]	7.20[b]	3.40[a]	0.32
C21:5n-3	0.68[b]	0.40[a]	0.63[b]	0.14[a]	0.31
C22:5n-3	1.33[b]	2.49[c]	3.08[d]	1.46[a]	0.29
C22:6n-3	15.60[d]	10.17[b]	11.63[c]	5.94[a]	0.79
Others	5.47[d]	4.61[c]	4.29[b]	2.29[a]	0.27
Total PUFA***	54.30[c]	49.60[b]	48.23[b]	33.81[a]	3.12
Total PUFA n-3	36.22[b]	32.43[b]	29.31[b]	15.40[a]	9.08
Total PUFA n-6	18.09[b]	17.18[a]	18.93[c]	18.42[b]	0.74

N=20 per group; a..d: P<0.05.
Legend: *SFA stands for saturated fatty acids; **MUFA stands for monounsaturated fatty acids; ***PUFA stands for polyunsaturated fatty acids.

found in crucian carp (32.98%, KOLAKOWSKA *et al.*, 2000) and sutchi catfish (12.5%, ORBAN, 2007), respectively. Goldfish was poorer in total MUFA than crucian carp (16.68 *vs* 32.98%, KOLAKOWSKA *et al.*, 2000).

The major fatty acids of goldfish fillet were: palmitate (16:0) for SFA, oleate (C18:1n-9) for MUFA, arachidoneate (C20:4n-6), eicosapentoneate (C20:5n-3, EPA) and docosahexaeonate (C22:6n-3, DHA) for PUFA. A similar fatty acid profile was also observed in two other fish species from lakes in central Italy, such as the Common whitefish (*Coregonus lavaretus*) and the European perch (*Perca fluviatilis*) (Lazio Region, PRAL 1999-2001). In particular, it was found that the fat of European perch, fished in Lake Trasimeno, contains about 33.32% PUFA, of which 9.78 and 19.94% are represented by EPA and DHA, respectively (POLI *et al.*, 1994). Compared with other fish species, the level of these n-3 PUFA in goldfish fillet was much higher than in crucian carp (4.98 *vs* 9.45% and 9.93 *vs* 15.60% for EPA and DHA, respectively KOLAKOWSKA *et al.*, 2000). Moreover, the percentage of EPA in goldfish fillet was similar to that reported for gilthead sea bream (*Dicentrarchus labrax*, 10.91%) and European sea bass (*Aurata sparus*, 10.94%), while the percentage of DHA was even higher (15.60 *vs* 10.69% and 13.99%, respectively; TURCHETTO *et al.*, 1994). In sutchi catfish, both of these long-chain PUFAs are lower, while the amount of linoleic acid is very high, representing about 44% of the total PUFA (ORBAN, 2007). Differences in the fatty acid profile among fish species are due to the dietary habits, phytoplacton and zooplacton availability and other environmental factors, such as temperature and oxygen availability, as well as endogenous factors, like age and behaviour/metabolism/physiology.

The fatty acid profile of the fillet was affected in various ways by the three processing steps. Grinding of fillet increased the percentage of MUFA, by increasing the amount of palmitoleic acid (C16:1n-7), while reduced the percentage of PUFA, especially arachidonic acid (C20:4n-6), EPA and DHA. Consequently, the amounts of total n-3 and n-6 PUFA in the raw pulp decreased, reaching a statistical significance only for the n-6 series. The reduction of PUFA in raw pulp could be ascribed to exposure of a higher surface area of meat to oxygen after mincing, which was also confirmed by the higher TBARS level as discussed below (Table 3). PUFA are known to be more susceptible to oxidation than the other fatty acid groups because of the higher number in double bonds.

Apart from the reduction in EPA and DHA levels, cooking did not produce any further modification in the main fatty acid of the pulp.

On the contrary, addition of ingredients to the cooked pulp for croquette preparation resulted in a considerable increase in SFA and MUFA, especially oleic acid (C18:1n-9), and a concomitant decrease in PUFA (P<0.05), especially the n-3 group. Among the ingredients used, Parmesan cheese is very rich in oleic acid and might have contributed to the final concentration of this fatty acid in the croquettes. Eggs are also rich in various fatty acid groups, chiefly C16:0, C18:1n-9 and C18:2n-6.

The quantitative levels of the main fatty acid groups are reported in Table 3. It is worth noting that 100 g of goldfish fillet, containing only 1.85 g of total lipids, may provide a considerable amount of n-3 fatty acids, approximately 610 mg, of which 159 mg are represented by EPA and 263 mg by DHA. These findings are in agreement with those reported by HEPBURN *et al.* (1986), WESTRE *et al.* (1993) and HYVONEN *and* KOIVISTOINEN (1994). HEPBURN *et al.* (1986) found that crucian carp fillet provides the same level of PUFA as goldfish, but it contains a higher amount of lipid (5%).

The n-6/n-3 ratio of fresh goldfish fillet, an acknowledged nutritional index for human health, was lower than that reported for the rainbow trout living in the Valnerina Lake, Central Italy (0.50 *vs* 0.62, DAL BOSCO *et al.*, 2007), but higher than in crucian carp (0.22, KOLAKOWSKA *et al.*, 2000). When fillets were processed into croquettes, the value was doubled (P<0.05). Also the other nutritional indexes (atherogenic and trombogenic) changed during processing, reaching their highest value for goldfish croquettes (P<0.05). These changes are in accord with the changes in the fatty acid composition (SFA increased to the detriment of PUFA) which occurred after each treatment (mincing, cooking, addition of ingredients).

Finally, the thrombogenic index of fresh goldfish fillets was identical to that of Valnerina rainbow trout, whereas the atherogenic index was much lower (0.34 *vs* 0.45, DAL BOSCO *et al.*, 2007), most probably due to the lower level of SFA.

Oxidative stability

The Peroxidability Index of goldfish fillet, measuring the relationship between the fatty acid composition of a tissue and its susceptibility to oxidation, was quite high (273.12), in agreement with its high content in long-chain polyunsaturated fatty acids (Table 3). These results are similar to the findings of TESTI *et al.* (2006), but higher with respect to the value reported for the Valnerina rainbow trout (273.12 *vs* 230.03, DAL BOSCO *et al.*, 2007). This index tended to drop after grinding and cooking, reaching statistical significance only after the addition of ingredients for croquette production. This trend was similar to that observed for PUFA during transformation.

The extent of lipid and protein oxidation of goldfish fillet, pulp and croquette were evaluated by measuring the end products of lipid peroxidation (TBARS) and protein carbonylation (PCGs). Accordingly, it was found that both mechanical and thermal treatments seriously compromised the oxidative stability of processed products, as verified by the increase in production either of TBARS or PCGs (Table 3). Food products rich in PUFA (the primary targets for free radical attack), as the Lake Trasimeno goldfish, are more prone to undergo oxidation if no precau-

Table 3 - Quantitative fatty acid contents, oxidative status and nutritional indexes of goldfish fillets, pulp and croquettes.

		Fillet	Pulp		Croquette	SEM
			Raw	Cooked		
SFA*	mg/100 g	488.21^{a}	509.64^{a}	588.51^{a}	901.45^{b}	54.62
MUFA**	”	280.81^{a}	384.36^{b}	423.97^{b}	628.47^{c}	65.47
Σ n-3	”	609.68^{b}	575.38^{b}	573.35^{b}	355.84^{a}	47.81
Σ n-6	”	304.46^{a}	304.77^{a}	370.27^{ab}	425.64^{b}	56.69
EPA	mg/100 g	159.09^{c}	138.94^{b}	140.87^{b}	78.59^{a}	12.49
DHA	”	262.63^{b}	180.47^{ab}	227.54^{b}	137.30^{a}	53.24
PUFA***	”	914.14^{b}	880.15^{b}	943.62^{b}	781.48^{a}	87.98
n-6/n-3		0.50^{a}	0.53^{a}	0.63^{a}	1.19^{b}	0.35
Thiols	µmol/g	0.27^{a}	0.22^{a}	0.10^{b}	0.08^{b}	0.11
PCGs§	nmol/mg protein	1.43^{a}	3.13^{b}	4.22^{b}	6.23^{c}	1.21
TBARS¥	mg MDA/kg	0.54^{a}	0.79^{b}	0.83^{b}	1.33^{c}	0.42
Peroxidability index		273.12^{b}	216.98^{b}	226.78^{b}	80.27^{a}	39.87
Atherogenic index		0.34^{b}	0.37^{b}	0.23^{a}	0.46^{c}	0.10
Trombogenic index		0.21^{a}	0.38^{b}	0.27^{a}	0.53^{c}	0.13

N=20 per group; a..d: P<0.05.
Legend: *SFA stands for saturated fatty acids; **MUFA stands for monounsaturated fatty acids; ***PUFA stands for polyunsaturated fatty acids; §PCGs stands for protein carbonylated groups; ¥TBARS stands for thiobarbituric acid reactive species.

tion is taken during processing. In fact, the level of TBARS significantly increased after grinding, as a consequence of a greater oxygenation of the minced fish fillet. On the other hand, heat shock has been demonstrated to increase lipid oxidation, and the rate of TBARS production depends on the temperature of cooking and duration (DAL BOSCO *et al.*, 2001). The conditions used in the present study for the cooking of raw pulp of goldfish only slightly elevated the TBARS level. In contrast, addition of ingredients like salt and parmesan cheese during croquette preparation resulted in a considerable increase in both lipid and protein oxidation products ($P<0.05$), also because of higher amounts of protein (24.10 *vs* 17.15%) and lipids (2.54 *vs* 1.85%). However, TBARS production never exceeded the threshold level (2.5 mg MDA/kg, CASTELLINI *et al.*, 2006), confirming no sign of sensorial deterioration in the processed products. Finally, it was found that PCG levels were significantly correlated with TBARS concentration ($r=0.89$, $P<0.05$, data not shown), throughout goldfish processing, suggesting a possible relationship between these two oxidative stress markers. Hence, it is likely that production of toxic aldehydes, such as MDA, during lipid oxidation, might have triggered protein carbonylation.

In addition to carbonylation, involving protein-NH_2 groups, processing of goldfish fillet modified the protein-SH groups as well (Table 3). Thiols are known to play a key role in oxidative stress by donating a hydrogen atom to the reactive free radicals and may protect, along with other antioxidants, biomacromolecules against oxidative damage (DRÖGE, 2002). The chief low-molecular weight thiol is glutathione, which plays a key role also in the maintenance of high levels of other protein-related thiols by reducing the formation of disulfides in collaboration with thioredoxin and glutaredoxin (SCHAFER and BUETTNER, 2001). As for the other two oxidative stress indexes, the level of total thiols underwent a noticeable reduction during cooking, underlying the necessity of additional antioxidants, such as vitamin E or C, to preserve the oxidative stability of goldfish proteins in the processed products. Finally, transformation of NH groups into carbonyls and oxidation of SH, might also have contributed to the changes in pH, WHC and CL values, during processing.

CONCLUSIONS

The data of the present study, taken together, suggest that goldfish from Lake Trasimeno are characterized by a high nutritional quality (high levels of EPA and DHA) and good oxidative stability, both comparable to that of other valuable fish species. Nevertheless, the presence of a large number of bones limits direct consumption of goldfish. For this reason, it is necessary to process them into more easily edible products using various technological processes which may alter the original nutritional quality. Among these, grinding and pre-cooking seem to slightly reduce the nutritional value, while addition of salt, parmesan cheese and eggs to the pre-cooked pulp results a significant reduction of PUFA, mainly of the n-3 series and a reduction of the oxidative stability of the final product with a consequent increase in both atherogenic and thrombogenic indexes. Even so, the goldfish croquettes can be considered as a good source of n-3 PUFA, providing an amount of approximately 78.59 mg of EPA and 137.30 mg of DHA per 100 g of consumed product; this latter value is higher than the daily recommended value for adult men (120 mg day^{-1} of DHA) as specified by NRF (2001). Further studies are needed to optimize the processing in order to preserve the nutritional characteristics of goldfish fillets.

ACKNOWLEDGMENTS

Authors are grateful to Dr. Mauro Natali (Servizio Programmazione e Gestione Faunistica, Provincia Perugia), Mr. Aurelio Cocchini (Cooperativa Pescatori Alba Trasimeno, S. Feliciano, Perugia) and Mr. Francesco Gonnelli for technical assistance.

REFERENCES

AOAC 1995. "Official Methods of Analysis". 15th Ed. Association of Official Analytical Chemists, Washington, DC.

Arakawa K.and Sagai M. 1986. Species differences in lipid peroxide levels in lung tissue and investigation of their determining factors. Lipids 21: 769.

Bradford M.M. 1976. A rapid and sensitive method for quantization of microgram quantities of protein utilizing the principle of protein-dye-binding. Anal. Biochem. 72: 248.

Burzigotti R., Dragoni W., Evangelisti C. and Gervasi L. 2003. The Role of Lake Trasimeno (central Italy) in the History of Hydrology and Water Management. 3rd Internat. Wat. Hist. Ass. Conf., Alexandria, Egypt, 11-14 December 2003.

Castellini C., Dal Bosco A., Mugnai C. and Pedrazzoli M. 2006. Oxidative stability and sensory attributes of poultry meat during display according to age and rearing system. Ital. J. Anim. Sci. 5: 355.

Cyril H.W., Castellini C. and Dal Bosco A. 1996. Comparison of three cooking methods of rabbit meat. Ital. J. Food Sci. 8: 337.

Dal Bosco A., Castellini C. and Bernardini M. 2001. Nutritional quality of rabbit meat as affected by cooking procedure and dietary vitamin E. J. Food Sci. 66: 1047

Dal Bosco A., Mourvaki E., Rossetti E., Mugnai C., Cecconi M. and Castellini C. 2007. Oxidative stability and health assessment index of rainbow trout (*Oncorhynchus mykiss* L.) farm reared in the Valnerina. Ital. J. Food Sci. 19: 391.

Dragoni W., Evangelisti C., Gnucci L. and Valigi D. 2003. Impact of climatic changes on hydrological systems : the case of Lake Trasimeno (Central Italy). Geophysical Research Abstracts, Vol. 5, 2003 - EGS-AGU-EUG Joint Assembly.

Dröge W. 2002. Free radicals in the physiological control of cell function. Physiol. Rev. 82: 47.

Folch J., Lees M. and Sloane-Stanley G.H. 1957. A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. 226: 497.

Ford T. and Beitinger T.L. 2005. Temperature tolerance in the goldfish, *Carassius auratus*. J. Therm. Biol. 30: 147.

Ghetti L., Carosi A., Lorenzoni M., Pedicillo R. and Dolciami R. 2007. L'Introduzione delle Specie Esotiche nelle Acque Dolci. Il Caso del Carassio Dorato nel Lago Trasimeno. Litograf Ed.

Grau R. 1978. Denaturazione, trattamento con il calore. In "Carne e Prodotti Carnei" Grau R. (Ed.). p 110. Edagricole, Bologna.

Hamm R. 1969. Properties of meat proteins. In "Proteins as Human Food" Lawrie RA (Ed.). p. 167. Avi Publ. Co., Westport, CT.

Hepburn N., J. Exler and Weihrauch J.L. 1986. Provisional tables on the content of omega-3 fatty acids and other fat components of selected foods. J. Am. Dietetic Assoc. 6: 788.

Honikel K.O. 1998. Reference methods for the assessment of physical characteristics of meat. Meat Sci. 49: 447.

Hossain M.A. and Jauncey K. 1989. Nutritional evaluation of some Bangladeshi oil seed meals as partial substitutes for fish meal in the diets of common carp, *Cyprinus carpio* L. Aquacult. Fish. Manag. 20:255.

Hyvonen L. and Koivistoinen P. 1994. Fatty acid analysis, TAG equivalents as net fat value, and nutritional attributes of fish and fish products. J. Food Camp. Anal. 7: 658.

Johansson L., Kiessling A., Kiesling KH. and Berglund L. 2000. Effect of altered ration levels on sensory characteristics, lipid content and fatty acid composition of rainbow trout (*Oncorhynchus mikyss*). Food Qual. Pref. 11: 247.

Ke P.J., Ackman R.G., Linke B.H. and Nash D.M. 1977. Differential lipid oxidation products in various parts of frozen mackerel. J. Food Techn. 12: 37.

Kołakowska A., Szczygielski M., Bienkiewicz G. and Zienkowicz L. 2000. Some fish species as a source of n-3 polyunsaturated fatty acids. Acta Ichthyol. Piscat. 30: 59.

Korkeala H., MÄki-Petais O., Alanko T. and Sorvettula O. 1986. Determination of pH in meat. Meat Sci. 18: 121.

Lorenzoni M., Corboli M., Ghetti L., Pedicillo G. and Carosi A. 2007. Growth and reproduction of the goldfish *Carassius auratus*: a case study from Italy. In "Biological Invaders in Inland Waters: Profiles, Distribution, and Threats" Gherardi F (Ed.) p. 259. Springer, Milano, Italy.

Lushchak V.I., Lushchak L.P., Mota A.A. and Hermes-Lima M., 2001. Oxidative stress and antioxidant defenses in goldfish *Carassius auratus* during anoxia and reoxygenation. Am. J. Physiol. 280: 100.

Lushchak V.I., Bagnyukova T.V., Husak V.V., Luzhna L.I., Lushchak O.V. and Storey K.B. 2005. Hyperoxia results in transient oxidative stress and an adaptive response by antioxidant enzymes in goldfish tissues. Int. J. Biochem. Cell Biol. 37: 1670.

Lushchak, V.I. and Bagnyukova T.V. 2006. Temperature increase results in oxidative stress in goldfish tissues. 1. Indices of oxidative stress. Comp. Biochem. Physiol. C 143: 30.

Lushchak V.I., Bagnyukova T.V., Lushchak O.V., Storey J.M. and Storey K.B. 2007. Diethyldithiocarbamate injection induces transient oxidative stress in goldfish tissues. Chem. Biol. Int. 170: 8.

Mearelli M., Lorenzoni M., Mantilacci L. 1990. Il Lago Trasimeno. Riv. Idrobiol. 29: 353.

Nakamura M. and Katoh K. 1985. Influence of thawing method on several properties of rabbit meat. Bull. Ishikawa Pref. Coll. Agric. Japan 11: 45.

Natali M. 1989. La fauna ittica del Lago Trasimeno: Aggiornamento al 1988. Riv. Idrobiol. 28: 33.

Nutritional Recommendations for the French Population (NRF). 2001. 3e éd. coordonnateur Ambroise Martin. CNERNA-CNRS, Paris, Technique & documentation. Sci. Alim. 4:21.

Orban E. 2007. Le specie emergenti del mercato ittico ed i prodotti dell'acquacoltura nazionale: qualità alimentare a confronto. Acquacoltura, XI, 11.

Poli B.M., Parisi G., Dell'Agnello M., Guidotti P., Bonelli A. and Geri G. 1994. Caratteristiche chimiche e qualità dei lipidi di pesci di diversa origine (tipo genetico, allevamento e cattura, acque naturali o riscaldate). Atti convegno int. "Parliamo di ...acquacoltura". PRAL, Programma Triennale di Ricerca Agricola. Agroambientale ed Agroindustriale della Regione Lazio1999-2001.

Schafer F.Q. and Buettner G.R. 2001. Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple. Free Radic. Biol. Med. 30: 1191.

Sherman P. 1961. The water binding capacity of fresh pork. 3. The influence of cooking temperature on the water binding capacity of lean pork. Food Technol. 15: 90.

StataCorp 2005. Stata Statistical Software: Release 9. Stata Corp L.P., College Station, TX.

Takeuchi T., Watanabe T. and Ogino C. 1979. Digestibility of hydrogenated fish oils in carp and rainbow trout. Bull. Jpn. Sot. Sci. Fish. 45: 1521.

Testi S., Bonaldo A., Gatta P.P. and Badiani A. 2006. Nutritional traits of dorsal and ventral fillets from three farmed fish species. Food Chem. 98: 104.

Turchetto E., Pregolato P., Barzanti V., Mmaranesi M., Mordenti T. and Rogato F. 1994. Caratteristiche qualitative e pregi nutrizionali dei prodotti ittici di allevamento. Realtà e Prospettive dell'acquacoltura. La situazione italiana: Produzione, Qualità, Schede informative 3-4/94, Istituto Scotti Bassani per la ricerca e l'informazione scientifica e nutrizionale, Milano, 31-39.

Ulbricht T.L. and Southgate D.A.T. 1991. Coronary heart disease: seven dietary factors. The Lancet 338: 985.

Westre K., Hoist J., Rimestad A.H., Paulsen D. and Lie O. 1993: "Facts About Fish", p. 1-27. Norwegian, Seafood Export Council, Tromsø, Norway.

Revised paper received October 20, 2009 Accepted October 30, 2009

SHORT COMMUNICATION

CHANGES IN CYTOCHROME C OXIDASE (CCO) AND LACTATE DEHYDROGENASE (LDH) ENZYME ACTIVITY OF THE WHITE EPAXIAL MUSCLE TISSUE OF SEA BASS (*DICENTRARCHUS LABRAX* L.) AS SHELF-LIFE PREDICTIVE INDICES DURING ICE-STORAGE

C. NATHANAILIDES*, O. LOPEZ-ALBORS[1], D. LENAS, F. ATHANASSOPOULOU[2] and K. GRIGORAKIS[3]
Dept. Aquaculture & Fisheries, Epirus Technological Educational Institute, Igoumenitsa, Greece
[1]Dept Anatomía y Anatomía Patológica Comparadas,
University of Murcia, Veterinary Faculty, 30100 Murcia, Spain
[2] Laboratory of Ichthyology & Aquatic Animal Health, University of Thessaly,
Faculty of Veterinary Medicine, School of Health Sciences, 43100 Karditsa, Greece
[3]Institute of Aquaculture, Hellenic Centre for Marine Research (HCMR), Athens, Greece
*Corresponding author: cosmasfax@yahoo.com

ABSTRACT

Fish freshness can be evaluated by sensory, physical and biochemical parameters. The purpose of the present work was to evaluate the potential utilisation of enzyme activity changes as indicators of shelf life in ice-stored sea bass. Changes in the enzyme content and organoleptic score of ungutted ice-stored sea bass were monitored over a two-week period. Organoleptic score and activity of CCO and LDH decreased gradually with a 50% reduction after 10 days of storage in ice. The results show that monitoring the activity of the mitochondrial and cytoplasmic enzymes of fish muscle can be used as an indicator of fish freshness.

- Key words: freshness, quality, refrigeration, seafood, spoilage -

INTRODUCTION

Fish and seafood are highly perishable foods. Spoilage of chilled, fresh and minimally processed fish is a function of endogenous enzyme and bacterial activity (GRAM and HUSS, 1996; SHIMADA *et al.*, 2000, EIE *et al.*, 2007). Compared to land animals (AGUIAR *et al.*, 2009), fish flesh has a higher water content, a high free amino acid pool and contains less connective tissue (BUCHTOVÁ *et al.*, 2009, DUMAS *et al.*, 2010). These lead to rapid biochemical changes during cold-storage and shorten the shelf-life of fish (HUSS, 1988). The development of unpleasant odours of raw fish correlates well with bacterial spoilage and storage time (PONS-SANCHEZ-CASCADO *et al.*, 2006).

The demand for sea bass in Europe constitutes a significant element of the European fish market; sea bass is widely cultivated in the Mediterranean Sea. Consumers of European sea bass frequently buy this product fresh, within a few days of ice-cold storage. The shelf-life of ice-stored fish varies according to several parameters and can be extended depending on the initial bacterial and lipid content, as well on the season, killing method and handling conditions (HUSS, 1988; GRIGORAKIS *et al.*, 2004; LAMBOOIJ *et al.*, 2008). An initial autolytic enzymatic process, followed by bacterial spoilage in the later stages of shelf-life, are the post-mortem changes that occur during ice-storage of sea bass (KYRANA and LOUGOVOIS, 2002; PAPADOPOULOS *et al.*, 2003).

Several methods exist to assess fish quality. Sensory, microbial, chemical and physical post-mortem changes of sea bass have been well reviewed (ALASALVAR *et al.*, 2002; KYRANA and LOUGOVOIS, 2002; KOUTSOUMANIS *et al.*, 2002; PAPADOPOULOS *et al.*, 2003; GRIGORAKIS *et al.*, 2004; OZOGUL *et al.*, 2005; CAKLI *et al.*, 2006; HOWGATE, 2006).

Recent developments of electronic devices such as the electronic nose systems offer new, rapid methodologies for assessing the quality of seafood. Using an electronic nose apparatus, LIMBO *et al.* (2009) reported a significant reduction of sea bass freshness after 8 days in cold storage at 2°C.

In general, the release of proteolytic enzymes from muscle cell lysosomes occurs immediately after death (HUSS, 1988). The decomposition of structural and enzymatic muscle proteins results in a progressive protein structural loss and degradation of metabolic enzymes. As a result, enzyme activity can influence the postharvest quality of seafood (HAARD, 2000). During storage, the rate of protein degradation is proportional to the storage time (BAUCHART *et al.*, 2007). Thus, the level of protein decomposition during ice-storage should be related to the shelf-life of fish during ice-cold storage. This is a well-known phenomenon observed during storage of terrestrial animal meat (COLLINS *et al.*, 1991; GODIKSEN and JESSEN, 2001). Among the various endogenous enzymes, the roles and post-mortem activities of specific proteinases such as caplain and cathepsins have been studied in sea bass (LADRAT *et al.*, 2003; DELBARRE-LADRAT *et al.*, 2004; CHÉRET *et al.,* 2006). However, there is a lack of data on the activity and changes in other endogenous enzymes that occur during ice-storage of sea bass. Such information would be useful in providing new alternative indexes for evaluating sea bass freshness.

The aim of the present work was to monitor post-mortem changes in a cytoplasmic and a mitochondrial enzyme during ice-storage of sea bass. Lactate dehydrogenase (LDH) is located in the cytoplasm and catalyses the interconversion of lactate and pyruvate. Muscle tissue is rich in this enzyme and its presence is related to the capacity for anaerobic glycolysis. Cytochrome *c* oxidase (CCO) is a membrane-bound mitochondrial enzyme which catalyses the reduction of molecular oxygen to water in the last step of the mitochondrial respiratory chain. The post-mortem changes of these two basic metabolic enzymes have been studied in ice- or frozen-stored meat of terrestrial origin (COLLINS *et al.*, 1991; GODIKSEN and JESSEN, 2001) and frozen fish (DAMODARAN NAMBUDIRI and GOPAKUMAR, 1992), but not ice-stored fish.

MATERIALS AND METHODS

Fish samples

Maricultured sea bass of commercial size (average weight 364.06±19.07 g) were obtained from a commercial fish farm (Sagiada, NW Greece) in April 2008. Standard killing methodology by immersion in ice / water (at 2/1 ratio) was followed, and ice-stored fish were transported to the laboratory within a day after killing. Fish were washed, and whole, ungutted samples were stored in insulated polystyrene boxes filled with ice in cold storage at 3°±1°C. Boxes had perforated bottoms to allow melting ice to drain. Samples of the epaxial muscle tissue were obtained for analysis (three fish per sampling day, on the 1st, 3rd, 7th, 10th and 15th day of ice-storage).

Assay of enzymes

All enzyme assays were performed in duplicate according to methods previously described (TYLER and NATHANAILIDES, 1995; NATHANAILIDES *et al.*, 2010). Duplication in analysis was chosen to ensure repeatability of the method for each fish. Each enzyme assay was performed on three fish on each sampling day. Homogenates were prepared and the CCO activity was assayed at 20°C by monitoring the reduction of ferrocytochrome c absorbance at 550 nm, in a

medium containing 0.075 M potassium phosphate buffer, pH 6.8 and 0.025 mM ferrocytochrome c. The activity of the glycolytic enzyme lactate dehydrogenase (LDH) was assayed (by the rate of NADH oxidation at 340 nm when LDH catalyses the reversible oxidation of pyruvate to lactate) in a medium of 50 mM potassium phosphate buffer (pH 7.0), containing 0.15 mM NADH and 0.60 mM sodium pyruvate (omitted from the control). Enzyme activity is reported in units per mg protein. Protein content was estimated using the Folin-Lowry method (LOWRY *et al.*, 1951). All reagents were obtained from Sigma-Aldrich (St Louis, MO, USA). The use of Triton X-100 in the homogenisation buffer together with the absence of high-speed centrifugation of the muscle homogenates ensured that the total CCO activity was assayed (TYLER and NATHANAILIDES, 1995; GODIKSEN and JESSEN, 2001).

Sensory evaluation of fish quality

A trained sensory panel of four persons evaluated the odour of raw fish. Organoleptic evaluation included gill and vent odours. These two characteristics were scored according to the modified TFRU scheme according to ALASALVAR *et al.* (2002) using a four-point scale (0= fresh, 3= spoiled).

Statistics

One Way Analysis of Variance (ANOVA), and Tuckey test were used to make multiple comparisons of the means for the enzyme studied and odour scores (ZAR, 1984). In all cases, the confidence level was 95%. A two-tailed Pearson correlation was used to correlate the enzyme changes with the respective values previously found (GRIGORAKIS *et al.*, 2004) K-values of ice-stored sea bass. The SPSS 10.0 software was used for the statistical analysis.

RESULTS AND CONCLUSIONS

During ice-storage, significant changes were observed in the lactate dehydrogenase (LDH) and cytochrome c oxidase (CCO) activities in the white epaxial muscle of sea bass (Figs. 1 and 2, respectively). The LDH activity decreased significantly already after day 3 of ice-storage. On day 3, the average value was 767 μmoles min^{-1} mg $protein^{-1}$, compared to an initial value of 858 μmoles min^{-1} mg $protein^{-1}$ on day 1 of storage. The value continued to decrease until it reached a value of 503 μmoles min^{-1} mg $protein^{-1}$ on the 10^{th} day of ice-storage (Fig. 1). The CCO activity remained almost unchanged (no significant changes) until the 7^{th} day of storage and decreased thereafter. Thus, the non-significant change from 6.86 to 5.57 μmoles min^{-1} mg $protein^{-1}$ in the first seven days was followed by a rapid decrease to 2.61 μmoles min^{-1} mg $protein^{-1}$ on the 15^{th} day of storage (Fig. 2). The differences in the loss of activity between the two enzymes may be the result of different post-mortem conditions in the cytoplasm and inner-mitochondrial membrane environment. Nevertheless, both enzymes exhibited an initial reduction after 3 (LDH) or 7 (CCO) days of storage in ice, this reduction was more marked after 10 days of storage.

The panellists reported a significant reduction in the organoleptic quality of odour in ice-stored sea bass. On the 15^{th} day of ice-storage, the fish exhibited deterioration in odour and the smell was characterized as "unpleasant" to "unacceptable" by the panellists. The odour changes observed during ice storage are presented in Fig. 3.

There was a very good correlation between the LDH and CCO activities and the K-values, according to GRIGORAKIS *et al.* (2004). The respective two-tailed Pearson correlations for LDH and CCO were -0.977 ($p<0.001$) and -0.846 ($p<0.001$), respectively.

Similar to the present results, the LDH activi-

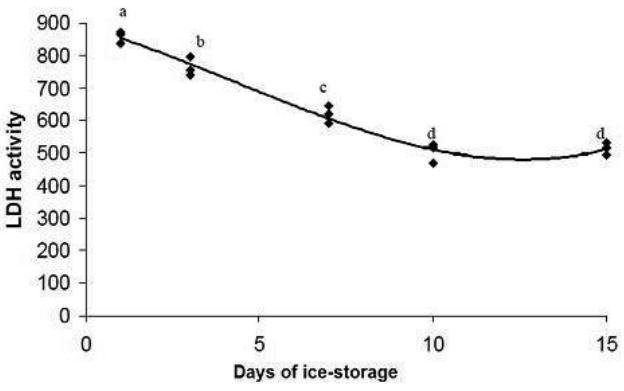


Fig. 1 - Changes of LDH activity (μmoles min^{-1} mg $protein^{-1}$) of white epaxial muscle tissue of sea bass during ice-cold storage. Best-fit curve is third order polynomial, with regression value 0.97. Different letters (a,b,c,d) denote statistically significant changes of activity over storage time.

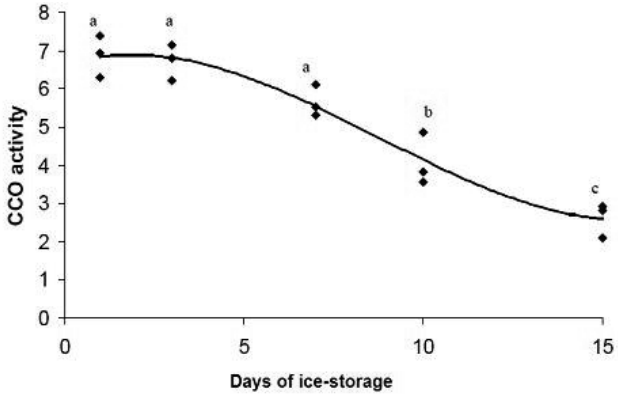


Fig. 2 - Changes of CCO activity (μmoles min^{-1} mg $protein^{-1}$) of white epaxial muscle tissue of sea bass during ice-cold storage. Best-fit curve is third order polynomial, with regression value 0.93. Different letters (a,b,c) denote statistically significant changes of activity over storage time.

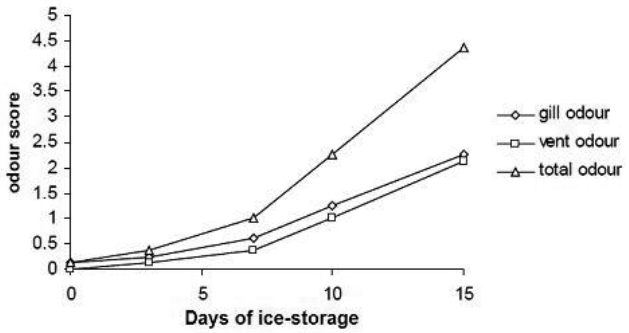


Fig. 3 - Organoleptic changes in sea bass during ice storage, evaluated by a four-member panel, as represented by the sum of the average gill (0= fresh, 3= spoiled) and vent (0= fresh, 3= spoiled) odour scores (total odour score).

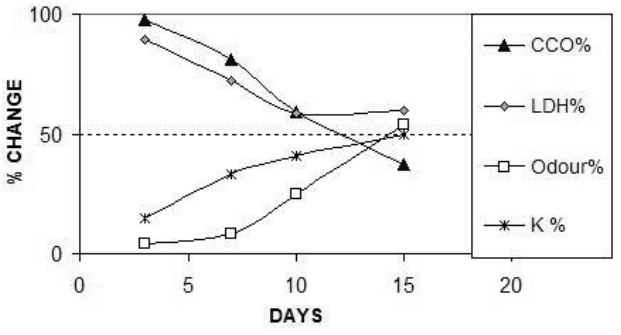


Fig. 4 - Changes (%) in the activity of CCO and LDH, total odour scores and K value (K-value data obtained from GRIGORAKIS *et al.*, 2004) of white epaxial muscle tissue of sea bass during ice-cold storage.

ty decreased in frozen-stored fish muscle in five fish species (DAMODARAN NAMBUDIRI and GOPAKUMAR, 1992); these results indicate that this enzyme is suitable for evaluating freshness in ice-stored or frozen-stored fish. The LDH activity was significantly reduced even after the 3rd day of ice storage, and this indicates that the respective enzyme can be a very good freshness indicator for sea bass. The correlation of LDH activity with initial quality changes, and its suitability as a freshness index has been previously proposed for frozen mullet, pearlspot milkfish and tilapia (DAMODARAN NAMBUDIRI and GOPAKUMAR, 1992).

On the other hand, CCO activity seems to be a more suitable spoilage indicator, because it indicates the degree of spoilage rather than the freshness stage of the fish. It remains rather stable during the first seven days of ice-storage and then decreases significantly. Both enzymes can be useful indexes for assessing the shelf-life of ice-stored sea bass. This is confirmed by the strong negative correlation of both enzymes with the K-value and organoleptic scores.

The spoilage rate during ice-storage of sea bass varies according to the initial handling and storage conditions and the killing method used (OZOGUL *et al.*, 2005; CAKLI *et al.*, 2006). In the present work, after 10 days of storage, sea bass received lower organoleptic scores due to the development of unpleasant odours and after 15 days the fish were characterized by strong unpleasant odours (Fig. 3); this period also coincides with the acceptability limit provided in the literature (KYRANA and LOUGOVOIS, 2002; TALIADOUROU *et al.*, 2003; GRIGORAKIS *et al.*, 2004; PALEOLOGOS *et al.*, 2004; CASTRO *et al.*, 2006). These researchers reported the initial development of an off-flavour and moderate sensory scores after 9 days in ice-storage. The results did not vary between gutted and ungutted ice-cold stored sea bass (ÖZOGUL *et al.*, 2005; POLI *et al.*, 2006). In agreement with the aforementioned literature, the present results also indicate that unpleasant odours develop within 10 days. This is a critical period for the quality of ice-stored sea bass, and can be characterized as the high quality shelf-life of the fish, since significant quality reduction occurs thereafter. A 50% reduction in CCO and LDH activity, coincides with the previously reported (GRIGORAKIS *et al.*, 2004) 50% increase in the respective K value in ice-stored sea bass (Fig. 4).

The results of the present work show that proteolytic activity in the white muscle of sea bass flesh during ice-cold storage coincides with a reduction of muscle CCO and LDH contents. In conclusion, the monitoring of CCO and LDH activities during ice-cold storage provides reliable indexes of the shelf-life of this product. The changes in these two enzymes indicate that LDH may be a useful index of freshness, since it exhibited alterations even at the early stages of ice-storage, while CCO appears to be more suitable for determining the degree of spoilage, since the levels changed significantly in the later stage of shelf-life.

REFERENCES

Aguiar A.P.S., Contreras-Castillo C.J., Baggio S.R. and Vicente E. 2008. Meat quality of broilers from different rearing systems. Ital. J. Food Sci. 20: 213.

Alasalvar C., Taylor K.D.A., Öksuz A., Shahidy F. and Alexis M.N. 2002. Comparison of freshness quality of cultured and wild sea bass (*Dicentrarchus labrax*). J. Food Sci. 67: 3220.

Bauchart C., Chambon C., Mirand P.P., Savary-Auzeloux I., Remond D. and Morzel M. 2007. Peptides in rainbow trout (*Oncorhynchus mykiss*) muscle subjected to ice storage and cooking. Food Chem. 100: 1566.

Buchtová H., Svobodová Z., Kocour M. and Velísek J. 2009. Amino acid composition in fillets of mirror crossbreds common carp (*Cyprinus carpio*). Acta Vet. Brno. 78: 337

Cakli S., Killing B., Cajun A., Dinkier T. and Tolls S. 2006. Effects of gutting and ungutting on microbiological, chemical, and sensory properties of aquacultured sea bream (*Sparus aurata*) and sea bass (*Dicentrarchus labrax*) stored in ice. Crit. Rev. Food. Sci. 46: 519.

Castro P., Padron J.C.P., Cansino M.J.C., Velazquez E.S. and Larriva R.M.D. 2006. Total volatile base nitrogen and its use to assess freshness in European sea bass stored in ice. Food Control. 17: 245.

Chéret R., Hernández-Andrés A., Delbarre-Ladrat C., de Lamballerie M. and Verrez-Bagnis V. 2006. Proteins and proteolytic activity changes during refrigerated storage in sea bass (*Dicentrarchus labrax*) muscle after high-pressure treatment. Eur. Food Res. Technol. 222: 527.

Collins S.S., Keeton J.T. and Smith S.B. 1991. Lactate dehydrogenase enzyme activity in raw, cured, heated porcine muscle. J. Agric. Food. Chem. 39: 1294.

Damodaran-Nambudiri D. and Gopakumar K. 1992. ATPase and lactate dehydrogenase activities in frozen stored fish muscle as indices of cold storage deterioration. J. Food Sci. 57: 72.

Delbarre-Ladrat C., Verrez-Bagnis V., Noël J. and Fleurence J. 2004. Relative contribution of calpain and cathepsins to protein degradation in muscle of sea bass (*Dicentrarchus labrax* L.). Food. Chem. 88: 389.

Dumas A., France J. and Bureau D. 2010. Modeling growth and body composition in fish nutrition: Where have we been and where are we going? Aquac. Res. 41: 161.

Eie T., Larsen H., Sorheim O., Pettersen M.K., Hansen A.A., Wold J.P., Naterstad K. and Mielnik M. 2007. New technologies for extending shelf life. Ital. J. Food Sci. 19: 127.

Godiksen H. and Jessen F. 2001 Cytochrome oxidase as an indicator of ice storage and frozen storage. J Agric. Food. Chem. 49: 4488.

Gram L. and Huss H.H. 1996. Microbiological spoilage of fish and fish products. Int. J. Food. Microbiol. 33: 121.

Grigorakis K., Alexis M., Gialamas I. and Nikolopoulou D. 2004. Sensory, microbiological, and chemical spoilage of cultured common sea bass (*Dicentrarchus labrax*) stored in ice: a seasonal differentiation. Eur. Food Res. Technol. 219: 584.

Haard N.F. 2000. Seafood enzymes: The role of adaptation and other intraspecifc factors. In "Seafood Enzymes, Utilization and Influence on Postharvest Seafood Quality", p. 365, Haard N.F. and Simpson B.K. (Eds.). Marcel Dekker Inc., N.Y.

Howgate P. 2006. A review of the kinetics of degradation on inosine monophosphate in some species during chilled storage. Int. J. Food Sci. Technol. 41: 341.

Huss H.H. 1988. Quality and Quality Changes in Fresh Fish. FAO Fisheries Technical Paper, vol. 348. p. 195 FAO, Rome, Italy.

Koutsoumanis K., Giannakourou M.C., Taoukis P.S. and Nychas G.J.E. 2002. Application of shelf life decision system (SLDS) to marine cultured fish quality. Int. J. Food. Microbiol. 73: 375.

Kyrana V.R. and Lougovois V.P. 2002. Sensory, chemical and microbiological assessment of farm-raised European sea bass (*Dicentrarchus labrax*) stored in melting ice. Int. J. Food Sci. and Technol. 37: 319.

Ladrat C., Verrez-Bagnis V., Noël J. and Fleurence J. 2003. In vitro proteolysis of myofibrillar and sarcoplasmic proteins of white muscle of sea bass (*Dicentrarchus labrax* L.): Effects of cathepsins B, D and L. Food Chem. 81: 517.

Lambooij B., Gerritzen M.A., Reimert H., Burggraaf D., André G. and Van De Vis H. 2008. Evaluation of electrical stunning of sea bass (*Dicentrarchus labrax*) in seawater and killing by chilling. Welfare aspects, product quality and possibilities for implementation. Aquac. Res. 39: 50.

Limbo S., Sinelli N., Torri, L. and Riva M. 2009. Freshness decay and shelf life predictive modelling of European sea bass (*Dicentrarchus labrax*) applying chemical methods and electronic nose. LWT - Food Sci. Technol. 42: 977.

Lowry O., Rosebrough H., Farr N.T. and Randall RJ. 1951. Protein measurement with the folin phenon reagent. J. Biol. Chem. 193: 265.

Nathanailides C., Paschos I., Tsoumani M., Perdikaris C. and Kapareliotis A. 2010. Capacity for thermal acclimation and winter mortality of sea bass (*Dicentrarchus labrax*) in freshwater earthen ponds. Ital. J. Zool. 77: 23-28.

Özogul F., Gökbulut C., Özyurt G. and Özogul Y. 2005. Quality assessment of gutted wild sea bass stored in ice, cling film and aluminium foil. Eur. Food. Res. Technol. 220: 292.

Paleologos E.K., Savvaidis I.N. and Kontominas M.G. 2004. Biogenic amine formation and its relation to microbiological and sensory attributes in ice-stored whole, gutted and filleted Mediterranean sea bass (*Dicentrarchus labrax*). Food Microbiol. 21: 549.

Papadopoulos V., Chouliara I., Badeka A., Savvadis I.N. and Kontominas M.G. 2003. Effect of gutting on microbiological, chemical, and sensory properties of aquacultured sea bass (*Dicentrarchus labrax*) stored in ice. Food Microbiol. 20: 411.

Poli M.B, Messini A., Parisi G., Scappini F., Vigiani V. and Giorni G. 2006. Sensory, physical, chemical and microbiological changes in European sea bass (*Dicentrarchus labrax*) fillets packed under modified atmosphere/air or prepared from whole fish stored in ice. Int. J. Food Sci. Technol. 41: 444.

Pons-Sánchez-Cascado S., Vidal-Carou M.C., Nunes M.L. and Veciana-Nogués M.T. 2006. Sensory analysis to assess the freshness of Mediterranean *anchovies (Engraulis encrasicholus*) stored in ice. Food Control. 17: 564.

Shimada, R., Ushio H. and Yamanaka H. 2000. Initial changes in ATP equivalents in the post mortem muscles of three species of lobster. Fisher. Sc. 66: 755.

Taliadourou D., Papadopoulos V., Domvridou E., Savvaidis I.N. and Kontominas M.G. 2003. Microbiological, chemical and sensory changes of whole and filleted Mediterranean aquacultured sea bass (*Dicentrarchus labrax*) stored in ice. J. Sc. Food. Agric. 83: 1373.

Tyler D.D. and Nathanailides C. 1995. Assaying for maximal activity of cytochrome c oxidase in fish muscle. Basic. Appl. Myol. 5: 99.

Zar J.H. 1984. "Biostatistical Analysis". Prentice-Hall International Editions, New Jersey, USA.

Paper Received March 16, 2009 Accepted June 30, 2009

SHORT COMMUNICATION

INFLUENCE OF SEASON ON MICROBIOLOGICAL LOADS IN READY-TO-EAT VEGETABLES

V. CORONEO*, A. SANNA, F. DANJOU, P. CABONI[1] and S. DESSÌ
Dipartimento di Sanità Pubblica, Via Porcell 4, 09100, Università di Cagliari, Italy
[1]Dipartimento di Tossicologia, Via Ospedale 72, 09124
Università di Cagliari, Italy
*Corresponding author: Tel. +39 070 6758379
e-mail: coroneo@unica.it

ABSTRACT

The safety of ready-to-eat vegetables was evaluated in relationship to microbiological risk to human health associated with their consumption through the study of hygiene-health requirements. Vegetable quality was tested using standard methods to enumerate *E. coli* and *Enterobacteriaceae*, that are indicators of contamination in food processing. The presence of other microorganisms such as *Salmonella spp.* and *Listeria monocytogenes* was also ascertained. During the warm seasons (spring and summer), the ready-to-eat products had high aerobic plate counts at 30°C ($8.9x10^6$ cfu/g); *Enterobacteriaceae* were also recorded ($2.4x10^4$ cfu/g). Therefore, these products should be transported and stored according to the strict requirements of the cold chain cycle. An information campaign targeting both the demand and supply sides should be carried out. Consumers should be made aware of the necessity to preserve the food cold-chain in order to minimize the risk of microbial contamination (e.g., by using thermal bags to transport food). Likewise, ready-to-eat products should be properly labelled.

- Key words: food safety, microbiological characterization, microbiological contamination, ready-to-eat foods -

INTRODUCTION

It is estimated that 30% of the population in developed countries is affected annually by pathogens associated with food consumption (SALMASO and TOZZI, 2001; GILLI and CARRARO, 2002). Roughly 50% of the cases occur in domestic environments. The consequences of this situation are unacceptable, first, because they seriously affect social health and, secondly, from an economic viewpoint, because they imply higher health care costs as well as loss of work-hours. Furthermore, food-borne diseases jeopardize the image of market products.

Consumer demand for freshly prepared and minimally processed fruit, vegetables and salads is increasing. Industry has responded to consumer demand by creating a wide range of fresh products (DE SIMONE,1995; FRAZZOLI, 2005). These products include raw fruit and vegetables that have been processed so as to be ready for consumption without further operations (washing or cooking). These products are used in public catering (hospitals, schools, canteens), restaurants and for family consumption (MASSANTINI and SALCINI, 2003). The production and consumption of these products is affected by limited shelf-life, potential microbial contamination and proliferation during product preparation and high market prices (SAGOO *et al.*, 2003; VENTURA *et al.*, 2005). As a consequence, good manufacturing practices are required to guarantee the safety of these freshly prepared products; particular attention must be given to potential microbial contamination pathways.

In fact, food-borne pathogens and spoilage flora can arise in food products, thus enhancing the risk of food-borne diseases and reducing product shelf-life (ALBENZIO *et al.*, 1998; LEONI and LEGNANI, 2003; BORIOLI, 1996; SCOCOZZA *et al.*, 2003; PRANZETTI and GALLI, 2005).

Recent EU regulations concerning food hygiene (EC 852/2004) refer to the control and prevention of microbiological hazards in food, especially *Salmonella* spp. and *Listeria monocytogenes*. The aim of this study was to evaluate the safety and potential microbiological risks to human health of ready-to-eat food through the study of hygiene-health requirements. *E. coli* and *Enterobacteriaceae* were enumerated using standard control methods to test the microbiological quality of vegetables. These microorganisms are indicators of contamination during food processing. In the framework of a comprehensive approach to food safety, tests were also carried out to ascertain the presence of microorganisms such as *Salmonella* spp and *Listeria monocytogenes*.

Table 1 - Commodities classified by produce type.

Sample type	Commodity	Number of samples
herbs	parsley	38
	basil	36
salad	tomatoes	40
	lettuce	47
	carrots	23
mushroom	champignons	11
vegetable soup		11
Total		206

MATERIALS AND METHODS

Sampling

Sampling was carried out between May 2007 and August 2008. Samples of ready- to-eat food were taken from refrigerated units (temperatures between 6°-8°C), in local retail shops in Sardinia. A total of 206 samples were collected. Produce commodities included herbs, salads, mushrooms and vegetable soups (Table 1). Samples were transported to the laboratory in thermal bags at a temperature of 4°±3°C, measured with a primary thermometer.

Microbiological assay

Ten g of sample were suspended in buffered peptone water at a ratio of 1:10. After homogenization in a Stomacher (PBI, Milan), serial dilutions of the suspension and cultures were prepared in agar medium according to the inclusion method and surface plated in keeping with ISO 6887. The aerobic colony counts were taken after culturing the samples at 30°C (ISO 4833/2004).

The quantitative analysis of *Enterobacteriaceae* was carried out following the ISO procedure (ISO 21528-2:2004); API 20E was used to identify the *Enterobacteriaceae* groups. The quantitative analysis of *E. coli* was carried out following the ISO procedure (ISO 16649-2:2001). For the qualitative determination of *Salmonella* (UNI EN ISO 6579:2004), 25 g of sample were taken and then homogenized with 225 mL of buffered peptone water. The homogenized sample was kept in the pre-enrichment stage at 37°C for 18 h. The selective enrichment step was carried out in Muller-Kauffmann broth at 37°C for 24 h and, in Rappaport-Vassiliadis broth at 41.5°C for 24 h. Cultures were transferred to specific media, Agar XLD (Xylose Lysine Deoxycholate) and SS (*Salmonella* and *Shigella*), which were incubated at 37°C for 24 h.

To detect the presence of *Listeria monocytogenes* (UNE EN ISO 11290-1:2005), the enrichment step was carried out using 25 g of the homogenized sample in Listeria Fraser broth.

Table 2 - Microbiological requirements for ready-to-eat vegetables (Ministerial Decree of the French Republic of 22/03/93).

Microbiological parameters	n	c	minimum	maximum
Aerobic colony count				
Production[(a)]	5	2	$5x10^5$	$5x10^6$
Consumption				$5x10^7$
E. coli	5	2	10	10^2
Salmonella	5	0	absent	absent
Listeria monocytogenes	5	0	absent	absent

Notes: (a) except for parsley, cress, aromatic herbs whose values are m=$5x10^4$/g M= $5x10^7$/g
n= number of units which form the sample; c= number of units that give the values between m and M; m= conformed values;
M= Maximum acceptable study.

The homogenized material was kept at 37°C for 48 h and subsequently cultured on Aloa agar and incubated at 37°C for 48 h. Finally, a biochemical profile of the isolated strains was carried out using API Listeria in order to indentify the Listeria species. The analysis of the test results is based upon the limits set by the European Community (EC 2073/2005) and the French regulations (Table 2).

Statistical assay

The total bacterial load and *Enterobacteriaceae* data were treated as continuous variables, while differences in *E. coli* levels in different samples were elaborated as dichotomous variables (absent below 100 cfu/g, present over 100 cfu/g).

The presence of other microorganisms such as *Salmonella* spp. and, *Listeria monocytogenes* were calculated as discrete variables. Differences between groups were assessed through the χ^2 Pearson test for dichotomous variables and the Mann-Whitney test or Student's t-test for continuous variables considering two hot and two cold seasons.

The distribution of potentially pathogenic and spoilage microorganisms were studied in relation to seasonal and monthly temperatures. EPI Info (Epi info2006) and SPSS (Rel. 15.0.1.2006, Chicago, USA) were used to analyze the data.

Table 3 - Median values of aerobic colony count in ready-to-eat products (cfu/g) in different seasons.

	Summer	Spring	Autumn	Winter
mushroom	$6.4x10^3$	$2.5x10^3$	1	2
seasoning	$3.0x10^3$	3	2	2
salad	$1x10^3$	12	1	1
vegetable soup	32	$1.2x10^2$	1	1

Table 4 - Number of samples from all ready-to-eat products, with *E. coli* contamination according to season (χ^2, p=0.014)

	E. Coli		
	<100 cfu/g	>100 cfu/g	Sample size
Autumn-Winter	39	5	44
Spring-Summer	114	48	162
Sample size	153	53	206

RESULTS

In 2007-2008, the temperature data were recorded at the Meteorological Station of Cagliari Sardinia (Italy). The highest temperatures (25.2°, 26.2° and 22.6°C) were recorded in July, August and September, respectively.

The median values of the aerobic colony counts at 30°C expressed in cfu/g are reported in Table 3. The aerobic counts decreased markedly in the colder seasons. The median values for mushrooms decreased from 6.4 $x10^3$ cfu/g in summer to 1 cfu/g in autumn and 2 cfu/g in winter. Likewise, the colony count in vegetable soups dropped from $1.2x10^2$ cfu/g in the spring to 1 cfu/g in the autumn and winter.

The results from two six-month periods show a significant difference in the *E. coli* contamination levels (Table 4) for all types of ready-to-eat products (p=0.014). In particular, a median value of $6.9x10^2$ cfu/g was recorded in July, $1.1x10^3$ cfu/g in August and $1.2x10^3$ cfu/g in September.

The *Enterobacteriaceae* counts for May, June, August and September 2007-2008 were: 2.9 $x10^3$ cfu/g, 1.1 $x10^3$ cfu/g, 9.7 $x10^3$ cfu/g, and 1.1 $x10^3$ cfu/g, respectively. The difference between *Enterobacteriaceae* detected in autumn-winter ($1.3x10^3$cfu/g) and in spring-summer (7.9 $x10^3$ cfu/g) was significant (p<0.001 – Mann Whitney U test).

The *Enterobacteriaceae* contamination values were lower during the cold months of January, February, October and December. A value of 1.4 $x10^3$ cfu/g was detected in January, 1.7 $x10^3$ cfu/g in February, 1.8 $x10^3$ cfu/g in October and 1.8 $x10^3$cfu/g in December (Fig. 1). Likewise, the aerobic colony contamination

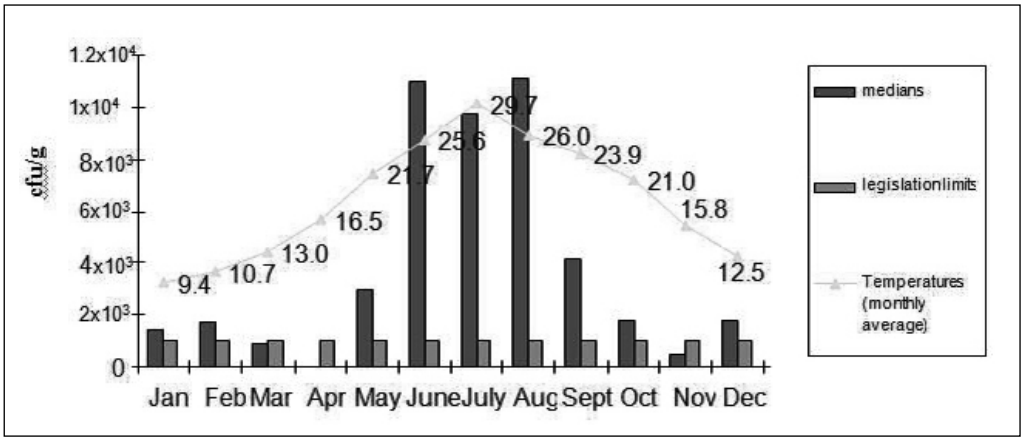


Fig. 1 - Monthly *Enterobacteriaceae* contamination values in ready-to-eat products.

levels detected in mushrooms were higher in spring and summer; the *Enterobacteriaceae* values were 1.3 x10^3 cfu/g in spring and 1.4. x10^3 cfu/g in summer. Microbiological identification was carried out for all the different typologies and in different seasons of ready-to-eat products (Table 5). *Salmonella* spp. was only found in one sample of salad; the contamination level was 0.49%.

DISCUSSION AND CONCLUSIONS

The results of the total bacterial load in ready-to-eat products revealed that the risk of microorganism contamination was the highest in the spring and summer (Table 3).

These products have an average shelf-life of seven days due to the high microbial load of the raw material that was not sufficiently reduced during processing. The quality of ready-to-eat products depends on six factors: quality of raw materials, selection of suppliers, careful sanitization of tools and equipment, processing, staff training according to good manufacturing practices and temperature control in work rooms and storage areas without interruption of the cold chain. All of these factors should be fulfilled in order to guarantee product quality for five to eight days.

The results of this study confirm that seasonal differences in temperature can influence the productive cycle of ready-to-eat products thereby affecting the average levels of microbial contamination and shelf-life limits. In spring and summer, processing, delivery and storage temperatures should be carefully observed.

Based on the results of this study the expiration dates currently shown on packages are too optimistic, especially during the summer. To guarantee consumer health especially during spring and summer, every type of product should be washed before being eaten.

During spring and summer ready-to-eat products should be transported and stored according to the requirements of the cold chain. Consumers should be encouraged to preserve the cold chain by transporting fresh foods in thermal bags. Ready-to-eat products should be correctly labelled with accurate consumer information concerning transportation and storage temperatures.

REFERENCES

Albenzio M., Corbo M.R. and Sinigaglia M. 1998. Influenza del confezionamento sulla shelf-life di ortaggi di IV gamma. Ind. Alim. 37: 31.

Borioli A. 1996. I vegetali di IV gamma. Ch. 30. In "Microbiologia dei Prodotti di Origine Vegetale". (Ed.) Ottaviani F. p. 611 Chiriotti Editori, Pinerolo, Italy.

Commission Regulation No 2073/2005 of 15 novembre 2005. Microbiological criteria for foodstuff. Official Journal of the European Union L 338:1.

Commission Regulation No 852/2004 of 30 April 2004, on the hygiene of foodstuffs. Official Journal of the European Union L 139:1.

Table 5 - Percent of microorganisms isolated in all seasons from ready-to-eat products.

Microorganism	%	Microorganism	%
Citrobacter freundii	12.50	*Enterobacter cloacae*	21.16
Mucor pilori	2.43	*Salmonella* spp.	0.49
Escherichia coli	15.73	*Aeromonas* spp.	1.94
Klebsiella oxytoca	16.27	*Proteus vulgaris*	7.74
Serratia marcescens	11.96	*Pseudomonas aeruginosa*	8.06

Contatto R. 2003. Linee guida per la IV gamma. Ind. Alim. 62: 624.

De Simone R. 1995. Quarta e quinta gamma: dal campo alla tavola. Rist. coll. – catering, 11:30.

Dgccrf. Direction générale de la concurrence, de la consommation et de la répression des frauds. http://www.minefi.gouv.fr/directions_services/dgccrf.

Donati V. 2003. Solo se si mantiene la catena del freddo. Colt. Prot. 32:39.

Francis G.A., Thomas C. and O'Beirne D. 1999. The microbiological safety of minimally processed vegetables. Int. J. Food Sci. Tecnol. 34:1.

Frazzoli R. 2005. Innovazione nel mercato di IV gamma. Ind. Alim. 64:21.

Gilli G. and Carraro E. 2002. Gli "strumenti" della "sicurezza alimentare". Intervento alla Tavola Rotonda "Sicurezza alimentare nelle filiere produttive: novità attese e riflessi applicativi"; Alessandria, 15 novembre 2002.

ISO 2004. Microbiology of Food and Animal Feedeing Suffs. Horizontal Methods for the Detection and Enumeration of *Enterobacteriaceae*.21528-2. Geneva. Switzerland.

ISO 2001. Microbiology of Food and Animal Feeding Stuffs. Horizontal Methods for the Enumeration of β-glucuronidase-positive *Escherichia coli*, Colony Counts Technique at 44°C. ISO 16649-2. Geneva. Switzerland.

Leoni E., Legnani P.P. and Corradini N. and Lucano A. 2003. Analisi di un punto critico di controllo nella filiera di lavorazione di prodotti orticoli freschi. Ind. Alim. 62: 361.

Massantini R. and Salcini M.C. 2003. Innovazione nelle tecnologie per la preparazione dei prodotti di IV gamma. Ind. Alim. 62: 953.

Montagna M.T., De Donno A., Sanapo S., Erroi R., Bagordo F. and Caggiano G. 1997. Microbiological considerations on vegetables of various origins. J. Prev. Med. Hyg. 38: 25.

Pranzetti L. and Galli A. 2005. Qualità microbiologica e igienica degli ortaggi di IV gamma. Ind. Alim. 4:21.

Sagoo S.K., Little C.L., Word L. and Gillespie I.A. 2003. Microbiological study of ready-to-eat salad vegetables from retail establishments uncovers a national outbreak of salmonellosi. J. Food Prot. 66:403.

Salmaso S. and Tozzi A.E. 2001. Epidemiologia delle tossinfezioni alimentari. Ch 3. In: "Recenti Sviluppi di Igiene e di Microbiologia degli Alimenti". (Ed.) De Felip G. p. 42. Tecniche Nuove, Milan, Italy.

Scocozza F., Chiancone I., De Luca I., Ventura M. and Caponigro V. 2003. *Aeromonas hydrophila*, *Salmonella* sp. e *Listeria monocytogenes* su insalate di IV gamma.. XIV Conf. Naz., Bologna, Italy.

Tiecco G. 1997. Pulizia e disinfezione degli impianti di produzione. Ch. 9 In: "Igiene e Tecnologia Alimentare". p. 457. (Ed.) Calderini Edagricole, Bologna, Italy.

UNE 2004. Microbiology of Food and Animal Feeding Stuffs. Horizontal Method for the Enumeration of Microorganism. Colony Count Technique at 30°C. UNI EN ISO 4833. Milan, Italy.

UNE 2004. Microbiology of Food and Animal Feeding Stuffs. Horizontal Method for the Detection of *Salmonella spp* . UNI EN ISO 6579. Milan, Italy.

UNE 2005. Microbiology of Food and Animal Feeding Stuffs Horizontal Method for the Detection and Enumeration of *Listeria monocytogenes*. UNI EN ISO 11290-1. Milan, Italy.

Ventura M., Chiancone I., De Luca F., Scocozza I. and Caponigro V. 2005. Biofilm batterici e recupero microbico sulle insalate di IV gamma. XIV Conferenza nazionale, Bologna, Italy.

Revised paper received July 3, 2009 Accepted December 15, 2009

SHORT COMMUNICATION

IN VITRO SYNERGISTIC ANTIBACTERIAL ACTIVITY OF *HELICHRYSUM ARENARIUM, INULA HELENIUM, CICHORIUM INTYBUS* AND SOME PRESERVATIVES

D. STANOJEVIĆ*, L.J. ĆOMIĆ, O. STEFANOVIĆ and S. SOLUJIĆ SUKDOL0AK[1]
Department of Biology
[1]Department of Chemistry
Faculty of Science, University of Kragujevac, 34 000 Kragujevac, Serbia
*Corresponding author: stanojevicd@ptt.rs

ABSTRACT

The aim of this study was to investigate the antibacterial activity of aqueous extracts of the species *Helichrysum arenarium* L., *Inula helenium* L. and *Cichorium intybus* L. and their synergistic action with the preservatives sodium nitrite, sodium benzoate and potassium sorbate *in vitro* against selected food-spoiling bacteria, for potential use in the food industry. Synergism was noticed for all aqueous extracts and in relation to: *Agrobacterium radiobacter* var. *tumefaciens*, *Bacillus subtilis*, *Bacillus mycoides*, *Erwinia carotovora*, *Enterobacter cloaceae* and *Pseudomonas fluorescens.* Synergism was established at plant extract and preservative concentrations corresponding to 1/4 and 1/8 MIC values, which indicates the possibility of avoiding the use of higher concentrations of the preservatives tested.

- Key words: aqueous extract, *Cichorium intybus* L., *Helichrysum arenarium* L., *Inula helenium* L., preservatives, synergism -

INTRODUCTION

Helichrysum arenarium L., *Inula helenium* L., *Cichorium intybus* L. (Asteraceae family) are aromatic, perennial plants widely distributed in Serbia. They have been known in European herbal medicine for long time for their beneficial effects (LEMBERKOVICS *et al.*, 2002; CZINNER *et al.*, 1999; KONISHI *et al.*, 2002; NASSIRIAN and ESLAMI, 2008). Traditional herbal medicines offer a novel source of antioxidants for the food industry for boosting both the shelf-life and nutritional content of food (HINNEBURG *et al.*, 2006). According to reviews by KALOGEROPOULOS *et al.* (2009) and KOSTAKI *et al.* (2009), the antioxidant properties of plants could provide alternatives to synthetic preservatives.

C. intybus L., best known as a caffeine-free substitute for coffee which can also be used in winter salads and in cooking, is regarded as a food source of nutritionally relevant phytochemicals (STEVENS *et al.*, 2001; FIGUEIRA, *et al.*, 2004). A steam distilled produce of *H. arenarium* L with a yellow-reddish essential oil, popular in fragrance for its unique scent, is best described as a mixture of burnt sugar and ham. The beneficial effects of *H. arenarium* L. are attributed mainly to the presence of flavonoids which may be related to their antioxidant activity (KERY *et al.*, 2001), but they are also influenced by other organic (phenolics: coumarins, phenolic acids, etc.) and inorganic (macro- and micro-elements) ingredients (CZINNER *et al.*, 2001).

I. helenium L. leaves are used in cooking and their taste is bitter and aromatic; the root is candied and eaten as a sweetmeat. Bioactive compounds isolated from *I. helenium* L. are responsible for its antimicrobial activity (VAJS *et al.*, 1989).

There is a major drive towards natural rather than chemical or synthetic additives in food, as they are perceived by consumers to be safe (BAGAMBOULA *et al.*, 2003). Use of plant extracts as natural preservatives has been especially highlighted since the European Food Safety Authority (EFSA, 2008) said that rosemary extract was safe to use as an antioxidant in food. Different kinds of preservatives are used to prevent biodeterioration of food products. In our test we chose three preservatives most commonly used in the food industry: sodium benzoate, sodium nitrite, potassium sorbate. Sodium benzoate is a controversial additive, as recent studies have highlighted health concerns regarding its use (HAWS *et al.*, 2007; McCANN *et al.*, 2007) and the commonly used preservative sodium nitrite has been under the spotlight since 2007 (JIANG *et al.*, 2007).

Nowadays plant extracts are widely used as food preservatives since plant matrices possess natural antimicrobial products to protect themselves from microbial infection, thereby expanding the possibilities for more effective and safe preservation of food. The aim of this study was to determine the antibacterial activity of *H. arenarium* L., *I. helenium* L. and *C. intybus* L. grown in Serbia and to estimate the efficiency of the combined actions of the plant extracts and selected preservatives against food spoilers and thereby expanding the possibilities for more effective and safe preservation of food.

MATERIAL AND METHODS

Plant material

H. arenarium L., *I. helenium* L., *C. intybus* L. were collected during the summer of 2006 on Mt. Suvobor (Serbia). Identification and classification of the plant material was performed at the Faculty of Science, University of Kragujevac. The voucher specimen of the plant was deposited in the Herbarium of the Faculty of Science, University of Kragujevac.

Preparation of plant extracts

Aqueous extracts were obtained by boiling dry ground plant material (50 g) in a water bath for 2-3 h at 80°C. Following filtration, the aqueous extract was evaporated in a water bath and redissolved in a defined volume of solvent (5% dimethylsulfoxid). The DMSO solvent was purchased from Merck (Germany). Solvent control (DMSO) was maintained throughout the experiment.

Preparation of preservatives

The preservatives used in the experiment was as follows: sodium benzoate (C Product, Belgrade, 2007); sodium nitrite (Laboratory of Biochemistry, Faculty of Science, University of Kragujevac); and potassium sorbate (C Product, Belgrade, 2007). Different concentrations of preservatives were created by dissolving them in liquid Mueller-Hinton broth (Torlak, Belgrade). Before testing, preservatives were heat treated at 80°C for 15 min.

Test microorganisms

The antibacterial activity of aqueous extracts was tested *in vitro* against the Gram-positive bacteria: *Bacillus mycoides* (PMFKg-B1), *Bacillus subtilis* (PMFKg-B2) and *Staphylococcus aureus* (PMFKg-B30); and the Gram-negative bacteria: *Agrobacterium radiobacter* var. *tumefaciens* (PMFKg-B11), *Enterobacter cloacae* (PMFKg-B22), *Erwinia carotovora* (PMFKg-B31), *Escherichia coli* (PMFKg-B26), *Pseudomonas fluorescens* (PMFKg-B28), *Proteus* sp.(PMFKg-B20). All microorganisms were obtained from stock cultures of

the Laboratory of Microbiology (Faculty of Science, University of Kragujevac).

Minimal inhibitory concentrations (MIC)

MIC was determined by the tube dilution method (NCCLS, 1997).A solution of each extract was serially diluted twofold in Mueller-Hinton broth so the final concentrations of the extracts in the medium ranged from 40 mg/mL to 1.25 mg/mL. Initial inocula were prepared by suspending growth in sterile saline and turbidity was adjusted to correspond to the 0.5 McFarland standard and was then diluted (1:10). 0.1 mL of prepared inocula was added into each tube to obtain final turbidity (approximately 10^4 CFU/mL). The MIC was defined as the lowest concentration of the plant extract at which visible growth is inhibited. The test tubes were incubated at 27°C/ 24 h. Each test included controls consisting of the medium with the solvent (5% DMSO), medium with bacterial suspension and sterility control. All tests were performed in duplicate. MIC of preservatives was determined in the same way (NCCLS, 1997). The final concentrations of the preservatives ranged from 10 mg/mL to 0.3 mg/mL.

Synergism

Synergism between aqueous extracts with chosen preservatives was assessed by the checkerboard assay method (SATISH *et al.*, 2005). The following combinations were tested: extract of each tested plant+ (sodium benzoate, sodium nitrite, potassium sorbate). From the first to the sixth horizontal column, each extract of the combination was doubly diluted in Mueller- Hinton broth (MIC values up to 32), while each of the tested preservatives of the combination was doubly diluted (MIC values up to 32) and added in a quantity of 0.1 mL from the first to the sixth vertical row. Each test tube contained a different concentration of plant extract/ preservative combination. Test tubes were inoculated with 0.1 mL prepared inocula and incubated 27°C/ 24 h. The MIC was defined as the lowest concentration of tested agents in combination at which visible bacterial growth was inhibited. Each test included two controls consisting of the substrate with and without the solvent.

The synergism between plant extracts and preservatives was determined by calculating the fractional inhibitory index (FIC) according to the formula: $\sum FIC = FICA + FIC\ B = [A]/\ MIC_A + [B]/\ MIC_B$. Where FIC A is the MIC of drug A in the combination/ MIC of drug A alone, and FIC B is the MIC of drug B in the combination/ MIC of drug B alone. The interaction was defined as synergistic if the FIC index was less than or equal to 0.5, additive if the FIC index was greater than 0.5 and less than or equal to 1.0, indifferent if the FIC index was greater than 1.0 and less than or equal to 2.0 and antagonistic if the FIC index was greater than 2.0 (CLIMO *et al.*, 1999; KITAHARA *et al.*, 2006).

RESULTS

The antibacterial activity of the aqueous extracts tested was quantitatively estimated by MIC values and the results are presented in Table 1. The minimal inhibitory concentration varied, depending on the type and concentration of plant extracts and taxonomic characteristics of the species of microorganism tested. All plant extracts exerted an antimicrobial effect. The negative control (5% DMSO) did not inhibit the growth of the bacteria tested.

Considering the range of MIC values the strongest antibacterial activity was exhibited by the extract of *C. intybus*. MIC values fluctuated from 5-20 mg/mL. *Escherichia coli* and *Staphylococcus aureus* were the most resistant (MIC 20 mg/mL), while the most sensitive was *Pseudomonas fluorescens*. MIC for the aqueous extract of *H. arenarium* and *I. helenium* fluctuat-

Table 1 - MIC values of tested plant extracts and preservatives.

	MIC(mg/mL)					
	aqueous extracts			preservatives		
Bacterial species	*C. int.*	*H. are.*	*I. hel.*	S. benz.	S. nit.	P. sor.
Agrobacterium tumefaciens	10	5	10	10	1	10
Bacillus mycoides	10	5	5	10	2	10
Bacillus subtilis	10	5	10	10	1	10
Enterobacter cloaceae	10	10	10	10	2	10
Erwinia carotovora	10	5	10	10	2	5
Escherichia coli	20	40	40	5	2	5
Proteus sp.	10	10	20	10	2	10
Pseudomonas fluorescens	5	10	10	5	0.5	10
Staphylococcus aureus	20	20	40	10	1	10

S. nit. - sodium nitrite, S. benz. - sodium benzoate, P. sor. - potassium sorbate;
C. int. - Cichorium intybus; H. are. - Helichrisum arenarium; I. hel. - Inula helenium.

Table 2 - FIC values of tested bacterial species and types of interaction between plant extracts and preservatives.

	∑FIC* index								
	H. arenarium +			*C. intybus* +			*I. helenium* +		
Bacterial species	S. nit.	S. benz.	P. sor.	S. nit.	S. benz.	P. sor	S. nit.	S. benz.	P. sor.
Agrobacterium tumefaciens	0.50(S)	0.50(S)	0.75(A)	1.00(A)	0.375(S)	0.75(A)	1.00(A)	1.00(A)	1.00(A)
Bacillus mycoides	1.00(A)	0.75(A)	1.00(A)	0.75(A)	1.00(A)	0.50(S)	1.00(A)	0.75(A)	1.50(I)
Bacillus subtilis	0.50(S)	0.375(S)	0.75(A)	0.50(S)	0.50(S)	0.375(S)	0.50(S)	0.75(A)	1.50(I)
Enterobacter cloaceae	1.00(A)	1.00(A)	1.50(I)	1.00(A)	0.375(S)	1.00(A)	2.00(I)	2.00(A)	0.75(A)
Erwinia carotovora	0.375(S)	1.50(I)	0.75(A)	0.75(A)	0.75(A)	1.00(A)	0.75(A)	0.75(A)	0.75(A)
Escherichia coli	2.00(I)	2.00(I)	2.00(I)	1.50(I)	1.00(A)	1.50(I)	0.75(A)	1.50(I)	0.75(A)
Proteus sp.	0.75(A)	1.00(A)	1.00(A)	0.75(A)	0.75(A)	0.75(A)	0.75(A)	0.75(A)	1.25(I)
Pseudomonas fluorescens	1.00(A)	0.75(A)	1.00(A)	1.00(A)	0.75(A)	0.75(A)	0.375(S)	1.00(A)	0.375(S)
Staphylococcus Aures	2.00(I)	1.50(I)	2.00(I)	1.25(I)	1.50(I)	1.50(I)	1.25(I)	1.50(I)	2.00(I)

∑FIC*- most effective combination; (S)-synergistic effect, (A)-additive effect, (I)-indifferent effect; S. nit. - sodium nitrite, S. benz. - sodium benzoate, P. sor. - potassium sorbate.

Table 3 - FIC values showing synergism for combination of *H. arenarium*/ preservatives.

	MIC(mg/mL)			MIC(mg/mL) *H. arenarium*+			
Bacterial species	Extr.	S. n.	S. b.	Sodium nitrite	FIC values	Sodium benzoate	FIC values
Agrobacterium tumefaciens	5	1	10	1.25+0.25	**0.50**	1.25+2.5	**0.50**
Bacillus subtilis	5	1	10	1.25+0.25	**0.50**	1.25+1.25	**0.375**
Erwinia carotovora	5	2	10	1.25+0.25	**0.375**		

S. n. sodium nitrite; S. b. sodium benzoate.

ed from 5-40 mg/mL. *Escherichia coli* (MIC 40 mg/mL) was the most resistant in relation to both aqueous extracts The MIC values of preservatives ranged from 5 to10 mg/mL for sodium benzoate, from 0.5 to 2 mg/mL for sodium nitrite and from 5 to 10 mg/mL for potassium sorbate. A synergistic effect was determined for all the aqueous extracts tested. Regarding synergism, types of interactions between plant extracts and preservatives, as deduced from calculation of the FIC index were, additive and had an indifferent effect (Table 2).

For the *H. arenarium* L. extract, synergism was established in combination with sodium benzoate and sodium nitrite, in relation to *Bacillus subtilis* and *Agrobacterium tumefaciens*. FIC values ranged from 0.375 to 0.50.The MIC values of plant extracts were reduced up to ¼ MIC, while MIC of preservatives was reduced up to 1/8 MIC (Table 3). According to the FIC index this combination showed additive and indifferent effect.

C. intybus L. extract showed synergism with all the tested preservatives. Synergism was detected against *Agrobacterium tumefaciens, Bacillus subtilis, Bacillus mycoides* and *Enterobacter cloacea*. FIC values were from 0.375 to 0.50. MIC values of plant extracts and preservatives were reduced up to 1/8 MIC values (Table 4). Additive and indifferent effects were established.

Synergism of *I. helenium* L. extract was established with sodium nitrite and potassium sorbate in relation to *Bacillus subtilis* and *Pseudomonas fluorescens*. FIC ranged from 0.375 to 0.50. MIC values of plant extracts was reduced up to ¼ MIC and MIC of preservatives up to 1/8 MIC. In addition to synergism for this combination, additive and indifferent effects were noticed. The results are presented in Table 5.

DISCUSSION

H. arenarium L., *I. helenium* L., *C. intybus* L. are plants which are used in a variety of food preparations. In agreement with earlier studies (DERIU *et al.*, 2008; PETROVIC *et al.*, 2004; CHINOU *et al.*, 1997), this work also showed significant antibacterial activity of the aqueous extracts tested. The most resistant species against all tested plant extracts were *Escherichia coli* and *Staphylococcus aureus*. CHINOU *et al.* (2004) showed antibacterial activity of *Helichrysum*

Table 4 - FIC values showing synergism for combination of *C. intybus*/preservatives.

Bacterial species	MIC(mg/mL) Extr.	S. b.	P. s	S. n.	MIC(mg/mL) *C. intybus*+ Sodium benzoate	FIC values	Potassium sorbate	FIC values	Sodium nitrite	FIC values
Agrobacterium tumefaciens	10	10	10	1	1.25+2.5	**0.375**				
Bacillus mycoides	10	10	10	2			2.5+2.5	**0.50**		
Bacillus subtilis	10	10	10	1	2.5+2.5	**0.50**	1.25+1.25	**0.375**	2.5+0.25	**0.50**
Enterobacter cloaceae	10	10	10	2	2.5+1.25	**0.375**				

S. n. - sodium nitrite, S. b. - sodium benzoate, P. s. - potassium sorbate.

Table 5 - FIC values showing synergism for *I. helenium*/ preservatives.

Bacterial species	MIC(mg/mL) Extr.	Sodium Nitrite	Potassium sorbate	MIC(mg/mL) *I. helenium*+ Sodium nitrite	FIC values	Potassium sorbate	FIC values
Bacillus subtilis	10	1	10	2.5+0.25	**0.50**		
Pseudomonas fluorescens	10	0.5	10	2.5+0.06	**0.375**	2.5+2.5	**0.50**

amorginum L. (MIC values 6.50-20 mg/mL). *Inula racemosa* L. inhibited the growth of bacterial species at a concentration of 10 mg/mL (LOKHANDE *et al.*, 2007).

Earlier studies showed that aqueous extracts of *C. intybus* L. possess antialergic effects (MIN KIM *et al.*, 1999) and aqueous extracts of *H. arenarium* L. antiviral effects (GUARINO and SCIARRILLO, 2003).

Due to the results of our work, along with recent studies which have highlighted health concerns from use of artificial preservatives (HAWS *et al.*, 2007; JIANG *et al.*, 2007; McCANN *et al.*, 2007), the potential synergism between plant extracts and preservatives was tested. Synergistic effect of plant extracts with preservatives was tested by ROMANO *et al.* (2009). They proved that plant extracts enhanced the antibacterial and antioxidant efficiency of preservatives. GARCIA-IÑIGUEZ de CIRIANO *et al.* (2009) demonstrated that aqueous plant extracts are a valuable alternative for food preservation.

To our knowledge there have been no studies that tested the potential synergism between aqueous extracts of *H. arenarium* L., *I. helenium* L. and *C. intybus* L. with preservatives. We established synergism for the combination of all three aqueous extracts.

Ethanol and methanol extracts of *C. intybus* L. have been reported to exert significant activity (MUTHUSAMY *et al.*, 2008; PUSHPARAJ *et al.*, 2007). In the present study the aqueous extract showed significant activity. INNOCENTI *et al.* (2005) reported Quercetin 3-*O*-glucuronide as the main phenolic compound in *C. intybus* L, one of the most potent antioxidants, also presenting antibacterial effect. Synergism was established in relation to 4 bacterial species and with all preservatives tested. MIC values of sodium benzoate and potassium sorbate in combination were reduced up to four times and the MIC of sodium nitrite in combination was reduced up to three times. Compared to *C. intybus* L., *H. arenarium* L. and *I. helenium* L. extracts were less effective. Synergism was detected in relation to a fewer number of species tested. These two extracts in combination with the preservatives tested reduced the MIC values of sodium nitrite and sodium benzoate up to four times, while there was no synergism with potassium sorbate. SROKA *et al.* (2004) demonstrated that some of the phenolic compounds identified in *H. arenarium* possess antiradical and antioxidant properties. Among them quercetin (flavonoid) and caffeic acid (phenolic acid) are known as strong antioxidants. 10-Isobutyryloxy-8, 9-epoxythymol isobutyrate, a major compound from *I. helenium* L. is responsible for its antibacterial activity, as shown by STOJAKOWSKA *et al.* (2005).

Bacillus subtilis, a common food spoilage bacteria, was the most sensitive on combinations of tested agents. In relation to this species, synergism was established in different combinations of three aqueous extracts with all tested preservatives. Also *Agrobacterium radiobacter* var. *tume-*

faciens showed significant sensitivity. Synergism was detected for combinations *H. arenarium* L. / sodium nitrite, sodium benzoate and *C. intybus* L. / sodium benzoate.

According to MIC values and FIC index *Escherichia coli* and *Staphylococcus aureus* exerted the greatest resistance when plant extracts were tested alone or in combination with preservatives.

The combination of tested extracts with sodium nitrite, sodium benzoate and potassium sorbate inhibited the growth of a significant number of bacterial species at a lower concentration than when the single agents were assayed separately. Synergism was recorded at 1/4 and 1/8 MIC values of preservatives, which indicated the possibility of avoiding the use of higher concentrations of tested preservatives that could lead to accumulation of toxic products in preserved food. Aqueous extract did not decrease the activity of preservatives because there was no antagonism.

CONCLUSIONS

The results confirmed that the aqueous extracts of *Helichrysum arenarium* L., *Inula helenium,* L., *Cichorium intybus* L. possess antimicrobial activity and according to the synergism exhibited with sodium benzoate, potassium sorbate and sodium nitrite, suggest that these aqueous extracts may be used in biotechnological fields as natural preservative ingredients in food.

ACKNOWLEDGEMENTS

This study was supported by the Ministry of Science and Technological Development of the Republic of Serbia.

REFERENCES

Bagamboula C.F., Uyttendaele M. and Debevere J. 2003. Antimicrobial effects of spices and herbs on *Shigella sonnei* and *S. flexneri*. J. Food Prot. 66: 668.

Climo M.W., Patron R.L. and Archer G.L. 1999. Combinations of vancomycin and beta-lactams are synergistic against staphylococci with reduced susceptibilities to vancomycin. Antimicrob. Agents. Chemother. 43: 1747.

Chinou I.B., Roussis V., Perdetzoglou D., Tzakou A. and Loukis A. 1997. Chemical and antibacterial studies of two Helichrysum species of Greek origin. Planta Med. 63:181.

Chinou I.B., Bougatsos C. and Perdetzoglou D. 2004. Chemical composition and antimicrobial activities of *Helichrysum amorginum* cultivated in Greece. J. Essent. Oil Research. 16: 243.

Czinner E., Kéry A., Hagymási K., Blázovics A., Lugasi A., Szöke E. and Lemberkovics E. 1999. Biologically active compounds of *Helichrysum arenarium* (L.) Moench. Eur. J. Drug Metab. Pharmacokinet. 24: 309.

Czinner E., Hagymasi K., Blazovics A., Kery A., Szoke E. and Lemberkovics E. 2001. The *In vitro* effect of *Helichrysi flos* on microsomal lipid peroxidation. J. Ethnopharmacol. 77: 31.

Deriu A., Zanetti S., Sechi L. A., Marongiu B., Piras A., Porcedda S. and Tuveri E. 2008. Antimicrobial activity of *Inula helenium* L. essential oil against Gram-positive and Gram-negative bacteria and *Candida* spp. Int. J. Antimicro. Ag. 31: 588.

EFSA, 2008. Scientific opinion of the panel on food additives, flavourings, processing aids and materials in contact with food on a request from the Commission on the use of rosemary extracts as a food additive. 2008. The EFSA J. 721:1.

Figueira G.M., Park K.J., Reis Brod F.P. and Honório S.L. 2004. Evaluation of desorption isotherms, drying rates and inulin concentration of chicory roots (*Cichorium intybus* L.) with and without enzymatic inactivation. J. Food. Eng. 63: 273.

García-Iñiguez de Ciriano M., García-Herreros C., Larequi E., Valencia I., Ansorena D. and Astiasarán I. 2009. Use of natural antioxidants from lyophilized water extracts of *Borago officinalis* in dry fermented sausages enriched in ω-3 pufa. Meat Sci. 83: 271.

Guarino C. and Sciarrillo R. 2003. Inhibition of herpes simplex virus type 1 by aqueous extracts from leaves of *Helichrisum litoreum* Guss. Boll. Chim. Farm.142: 242.

Haws L. C., Tachovsky J.A., Williams E.S., Scott L.F., Paustenbach D.J. and Harris M.A. 2007. Assessment of potential human health risks posed by benzene in beverages. J. Food Sci. 73: T33.

Hinneburg I., Damien Dorman H.J. and Hiltunen R. 2006. Antioxidant activities of extracts from selected culinary herbs and spices. Food Chem. 97:122.

Innocenti M., Gallori S., Giaccherini C., Ieri F., Vincieri F.F. and Mulinacci N. 2005. Evaluation of the phenolic content in the aerial parts of different varieties of *Cichorium intybus* L. J. Agric. Food Chem. 53: 6497.

Jiang R., Paik D.C., Hankinson J.L. and Barr R.G. 2007. Cured meat consumption, lung function, and chronic obstructive pulmonary disease among United States adult. Am. J. Respir. Crit. Care Med.175: 798.

Kalogeropoulos N., Konteles J.S., Troullidou E., Mourtzinos I. and Karathanos V.T. 2009. Chemical composition, antioxidant activity and antimicrobial properties of propolis extracts from Greece and Cyprus. Food Chem. 116: 452.

Kery A., Blazovics A., Fejes S., Nagy E., Lugasi A., Kursinszki L., Czinner E., Kristo Sz. T., Apati R., Balazs A. and Szoke E. 2001. Antioxidant activity of medical plants used in phytotherapy. Int. J. Hortic. Sci. 7: 28.

Kitahara T., Aoyama Y., Hirakata Y., Kamihira S., Kohno S., Ichikawa N., Nakashima M., Sasaki H. and Higuchi S. 2006. *In vitro* activity of lauric acid or myristylamine in combination with six antimicrobial agents against methicillin-resistant *Staphylococcus aureus* (MRSA). Int. J. Antimicro. Ag. 27: 5Konishi T., Shimada Y., Nagao T., Okabe H. and Konoshima T. 2002. Antiproliferative sesquiterpene lactones from the roots of *Inula helenium*. Biol. Pharm. Bull. 25:1370.

Kostaki M., Giatrakou V., Savvaidis I.N. and Kontominas M.G. 2009. Combined effect of MAP and thyme essential oil on the microbiological, chemical and sensory attributes of organically aquacultured sea bass (*Dicentrarchus labrax*) fillets. Food Microbiol. 26 (5): 475.

Lemberkovics E., Czinner E., Szentmihalyi K., Balazs A. and Szoke E. 2002. Comparative evaluation of *Helichrysi flos* herbal extracts as dietary sources of plant polyphenols, and macro- and micro-elements. Food Chem. 78:119.

Lokhande P.D., Gawai K.R., Kodam K.M., Kuchekar B.S., Chabukswar A.R. and Jagdale S.C. 2007. Antibacterial activity of isolated constituents and extract of roots of *Inula racemosa*. Res. J. Med. Plant.1: 7.

McCann D., Barrett A., Cooper A., Crumpler D., Dalen L., Grimshaw K., Kitchin E., Lok K., Porteous L., Prince E., Sounga-Barke E., Warner J.O. and Stevenson J. 2007. Food additives and hyperactive behaviour in 3-year-old and 8/9 year-old children in the community a randomised, double-blinded, placebo-controlled trial. Lancet. 370: 1560.

Min Kim H., Won Kim H., Su Lyu Y., Hee Won J., Ki Kim D., Mi Lee Y., Morii E., Jippo T., Kitamura Y. and Hyoung N. 1999. An inhibitory effect of mast cell-mediated immediate-type allergic reactions by *Cichorium intybus*. Pharmacol. Res. 40: 61.

Muthusamy V.S., Anand S., Sangeetha K.N., Sujatha S., Balakrishnan A. and Lakshmi B. S. 2008. Tannins present in *Cichorium intybus* enhance glucose uptake and inhibit adipogenesis in 3T3-L1 adipocytes through PTP1B inhibition. Chem. Biol. Interact.174: 69.

Nassirian H. and Eslami S.T. 2008. Effects of *Chichorium intybus* on bilirubin. Indian J. Pediatr. 75:331.

NCCLS. 1997. Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically, 4th Ed. Approved Standard M7-A4: Wayne, PA.

Petrovic J., Stanojkovic A., Comic L.J. and Curcic S. 2004. Antibacterial activity of *Cichorium intybus*. Fitoterapia. 75: 737.

Pushparaj P.N., Low H.K., Manikandan J., Tan B.K.H. and Tan C.H. 2007. Anti-diabetic effects of *Cichorium intybus* in streptozotocin- induced diabetic rats. J. Ethnopharmacol. 111: 430.

Romano C.S., Abadi K., Repetto V., Vojnov A.A. and Moreno S. 2009. Synergistic antioxidant and antibacterial activity of rosemary plus butylated derivatives. Food Chem. 115: 456.

Satish K.P., Moellering R.C. and Eliopoulos G.M. 2005. Antimicrobial combinations. In: Lorian V (ED)." Antibiotics in Laboratory Medicine". Lippincott Williams & Wilkins. Philadelphia.

Sroka Z., Kuta I., Cisowski W. and Drys A. 2004. Antiradical activity of hydrolyzed and non-hydrolyzed extracts from *Helichrysi inflorescentia* and its phenolic contents. Zeit. Naturforsch. 59c: 363.

Stevens C.V., Meriggi A. and Booten K. 2001. Chemical modification of inulin, a valuable renewable resource, and its industrial applications. Biomacromolecules. 2:1.

Stojakowska A., Kędzia B. and Kisiel W. 2005. Antimicrobial activity of 10-isobutyryloxy-8, 9-epoxythymol isobutyrate. Fitoterapia. 76:687.

Vajs V., Jeremić D., Milosavljević S. and Slobodan Macura S. 1989. Sesquiterpene lactones from *Inula helenium*. Phytochemistry. 28:1763.

Revised paper received August 30, 2009 Accepted September 17, 2009

SHORT COMMUNICATION

MICROBIOLOGICAL EVALUATION OF THE DRY-SLAUGHTER PROCESS FOR SMALL SCALE POULTRY MEAT PRODUCTION

L. VALNEGRI, M. FRANZONI, L. VERCELLOTTI[1] and G. SONCINI*
Dipartimento di Scienze e Tecnologie Veterinarie per la Sicurezza Alimentare,
Via Celoria 10, 20133 Milano, Italy
[1]Unità Sanitaria Locale "VC", 13100 Vercelli, Italy
*Corresponding author: Tel. +39 02 50317872, Fax +39 02 50317870
e-mail: gabriella.soncini@unimi.it

ABSTRACT

The dry-slaughter process for poultry includes mechanical defeathering followed by a waxing phase, without scalding. The aim of the present work was to study the impact of dry-slaughter procedures on the microbiological quality of poultry carcasses. A total of 161 laying hens were examined. Sponge swab samples were collected from different carcass sites at three successive processing stages (i.e. from the neck after stunning and from the breast after defeathering and after waxing). Each carcass sample was analyzed for counts of total aerobic mesophilic bacteria, coliforms, *Escherichia coli*, *Staphylococcus aureus* and the incidence of *Salmonella* spp. In addition, fecal swab samples collected from each bird were analyzed for the presence of *Salmonella* spp. The microbiological analysis results showed low levels of carcass contamination for each microorganism, at all stages of the dry-slaughter process. However, an increase in the levels of *Staphylococcus aureus* was recorded in the post-waxing phase. *Salmonella* spp. was not found in any of the samples tested.

- Key words: carcass quality, dry-slaughter, microbial contamination, poultry -

INTRODUCTION

The microbiology of carcass meats depends greatly on the conditions under which animals are reared, slaughtered and processed. The main microorganisms on poultry meat are: *Pseudomonas* spp., *Micrococcus* spp., *Staphylococcus* spp., *Flavobacterium* spp., *Acinetobacter* spp., *Moraxella* spp., *Escherichia coli*, coliforms, *Listeria* spp., *Salmonella* spp., *Campylobacter* spp., *Streptococcus* D, *Clostridium perfringens*, yeast and molds (JAMES *et al.*, 2006; NYCHAS and DROSINOS, 2000). Along with saprophytic and spoilage microorganisms, some of the bacteria found on poultry meat can cause foodborne diseases in humans, specifically *Salmonella* spp., *C. jejuni and C. coli*, *Cl. perfringens*, *L. monocytogenes*, *S. aureus* and *E. coli* (JAMES *et al.*, 2006; SUN and OCKERMAN, 2005). Some of these microorganisms originate from the bird's intestinal tract, and others from the environment with which the bird had contact at some point before or during slaughter (NYCHAS and DROSINOS, 2000).

The traditional poultry slaughtering process may cause extensive microbial contamination of carcasses, especially because of cross-contamination that occurs during scalding and defeathering (ABU-RUWAIDA *et al.*, 1994; CLOUSER *et al.*, 1995; GEORNARAS *et al.*, 1997; MEAD *et al.*, 1994). After bleeding, the birds are usually immersed in tanks of scalding hot water maintained at about 51°/52°C to facilitate the subsequent operation of defeathering. Depending on the capacity of the tanks and the frequency of water replacement, after a couple of hours of operation, levels of aerobic mesophilic bacteria in the water normally reach 10^6 to 10^7 CFU/mL, 10^4 to 10^5 CFU/mL of which are *Enterobacteriaceae*. These contamination levels remain constant throughout the day because the water temperature can kill microorganisms such as psychrophiles and psychrotrophs, but most of the bacteria introduced by live birds are mesophiles and these conditions favour their survival (ANONYMOUS, 2007). Particularly dangerous at this stage is the survival of pathogenic bacteria such as *Salmonella* and *Campylobacter* that may be transferred from carcass to carcass through cross-contamination (CASON *et al.*, 2000; HINTON *et al.*, 2004; MULDER *et al.*, 1978; PEREIRA *et al.*, 2008). CASON *et al.* (2000) reported that these bacteria may be a source of contamination later in the process, and could persist in the finished product.

The hygiene deficiencies inherent in the above-described process justify an evaluation of poultry slaughtering techniques that use alternative methods to scalding. At the European Union (EU) level there are no approved chemical treatments for decontaminating carcasses during slaughter, but the EU Standing Veterinary Committee recently upheld the ban on the importation of chicken meat from the United States that is decontaminated with chlorine. The system of dry-slaughter of poultry considered in this study is based on the exclusion of water from the entire process, particularly the absence of scalding tanks. The poultry carcasses are defeathered mechanically by means of rotating disks, then waxed by immersion in a liquid paraffin bath maintained at about 60°C, and then peeled and hand-finished. The application of this system is currently limited to small scale slaughtering of poultry, but it could be used on a larger scale to reduce cross-contamination and to improve the microbiological condition of poultry carcasses (CHIESA and VERCELLOTTI, 1992).

In this work the impact of poultry dry-slaughter procedures on the microbiological quality of the product was studied and the microbial profiles of carcasses during different stages of the slaughter process were evaluated.

MATERIALS AND METHODS

Processing plant

The work was conducted at a poultry slaughterhouse located in the Province of Vercelli, northern Italy. The slaughterhouse is licensed to process 1,000 birds a week according to Italian and European Union legislation. About 200 animals of several avian species (chickens, pigeons, guinea fowl, pheasants, red partridges, peacocks and germani) are slaughtered daily, three days a week. The number varies depending on the market demand.

The facility is functionally and hygienically divided into five main areas; the carcasses proceed from an "unclean zone" (areas n. 1 to n. 3) to a "clean zone" (areas n. 4 and n. 5). The slaughter process includes the following stages: in area n. 1 (ante-mortem), electrostunning by means of a clamp apparatus, bleeding by internal jugular severing and crop cleaning; in area n. 2, machine plucking; in area n. 3, pneumatic gun evisceration; in area n. 4, waxing and hand peeling and in area n. 5, hand-finishing of carcasses.

Sampling design and sampling sites

Between January and June 2009, a total of 161 laying hens (naked-neck breed) were examined as follows: (i) carcass samples were taken from three different carcass sites at three successive processing stages, i.e. from the neck region after stunning, from the right side of the breast after defeathering and from the left side of the breast after waxing, for a total of 483 samples; and (ii) faecal samples were collected from each bird immediately after stunning, for a total of 161 samples.

Moreover, in an intermediate stage of the study samples were taken from the hands of the

workers throughout one working day in order to assess their hygienic status during the performance of tasks.

Sample collection

Carcass samples were taken with a non-destructive sponge swabbing method using commercial kits, specifically the sterile "Speci-Sponge" (International Pbi S.p.A., Milan, Italy), which consists of an airtight plastic bag containing a cellulose acetate sponge premoistened with 10 mL of phosphate buffer solution. When sampling, the sponge was removed from the bag with a gloved hand and rubbed, for no less than 20 s, on an area (100 cm^2) outlined by a sterile template, at each site selected for the carcass sampling. After swabbing, the sponge was returned to its original bag.

The faecal samples were collected using cloacal swabs. After sampling, each swab was placed in a sterile plastic test tube containing 10 mL of Buffered Peptone Water (Oxoid S.p.A., Milan, Italy).

The hands of the workers were sampled by sponge swabbing ("Speci-Sponge" sterile kit, International Pbi S.p.A.), by rubbing the sponge over the surface of the palms of both hands, tested separately.

All samples were placed in refrigerated containers (4°±2°C), transported to the laboratory; the microbiological analyses were performed within 24 h of sampling.

Microbiological analyses

Each carcass sample was analyzed for counts of total aerobic mesophilic bacteria, coliforms, *Escherichia coli*, *Staphylococcus aureus* and the incidence of *Salmonella* spp.; the faecal samples were examined for *Salmonella* spp.; and the samples from the hands of the workers were examined for *Staphylococcus aureus*.

Mesophilic aerobic bacteria counts were determined on Petrifilm Aerobic Count Plate (3M Microbiology, MN, USA) and incubated at 30°C for 48 h (AFAQ/AFNOR 3M-01/1-09/89).

Total coliform and *E. coli* counts were determined on Petrifilm Coliform Count Plate (3M Microbiology) and Petrifilm Select E. coli Count Plate (3M Microbiology) and incubated at 30°C for 24 h (AFNOR 3M-01/2-09/89A) and at 42°C for 24 h (AFAQ/AFNOR 3M-01/08-06/01), respectively.

Staphylococcus aureus counts were determined with Petrifilm Staph Express Count Plate (3M Microbiology) and incubated at 37°C for 24 h and for an additional 3 h at 37°C if the Petrifilm Staph Express Disk (3M Microbiology) was used (AFNOR 3M-01/09-04/03).

Salmonella spp. determination was carried out following the ISO 6579:2002/Cor.1:2004 instructions. There was a pre-enrichment of the sample in Buffered Peptone Water (Oxoid S.p.A.), incubated at 37°C for 18 h, followed by enrichment in Rappaport-Vassiliadis Enrichment Broth (Oxoid S.p.A.) and Muller-Kaufmann Tetrathionate Broth (Oxoid S.p.A.), incubated at 41.5°C for 24 h and at 37°C for 24 h, respectively. The isolation and identification were done by spreading the enrichment broths on XLD Medium (Oxoid S.p.A.) and Brilliant Green Agar (Oxoid S.p.A.) and incubating at 37°C for 24 h and at 35°C for 18-24 h, respectively. In the case of presence of suspect colonies of *Salmonella* spp., the methodology calls for biochemical confirmation and further serotyping.

RESULTS AND DISCUSSION

The trial was based on constant microbiological monitoring, during the process of dry-slaughtering of poultry to evaluate the trend of bacterial contamination of carcasses. The following carcass sampling points were chosen: an early stage of the slaughter process (samples taken from the neck after stunning), which gave an indication of the general bacteriological state of the bird; at the stages of defeathering and waxing (samples taken from the breast after each of the two processing steps), these stages were considered unique to the dry-slaughter process.

As reported by GOKSOY *et al.* (2004), LILLARD (1990) and TSOLA *et al.* (2008), the choice of the microbial groups to examine on poultry carcasses was focused on the total aerobic mesophilic bacteria. These were identified as the main indicators of variability of bacterial contamination of carcasses along the slaughter process, and total coliforms, *E. coli*, *Salmonella* spp. and *S. aureus* were identified as the most common among the microorganisms on poultry meat that are responsible for spoilage (coliforms and *E. coli*) and potentially responsible for foodborne disease in humans (*E. coli*, *Salmonella* and *S. aureus*). Faecal samples were also tested for *Salmonella* spp. to have a complete picture of the presence of the pathogens present at slaughter. *Salmonella* spp. is the only one that must be tested for by law on poultry carcasses (EC, 2005; 2007).

The distribution of total aerobic mesophilic bacteria, total coliform, *E. coli* and *S. aureus* counts (Log CFU/cm^2) for carcass samples from each sampling site is presented in Table 1. These data show: a decrease in the contamination levels of total aerobic mesophilic bacteria at the different stages of slaughter considered; a decrease in the contamination levels of total coliforms and *E. coli* and a decrease in the number of samples contaminated by any of them throughout the stages of slaughter and an increase in the number of samples contaminated with *Staphylococcus aureus* following the waxing process.

These findings indicate a reduction in carcass contamination along the different stages of

Table 1 - Distribution of counts (Log CFU/cm²) of total aerobic mesophilic bacteria, total coliforms, *E. coli* and *S. aureus* for carcass samples.

Microbial group	Sample sites[a]	Log CFU/cm² 0-1	1-2	2-3	3-4	4-5	Absent	Positive samples (%)
total aerobic mesophilic bacteria	NPS	0	1	24	103	33	0	161/161(100)
	BPD	0	22	77	58	4	0	161/161(100)
	BPW	0	44	97	20	0	0	161/161(100)
total coliforms	NPS	15	20	26	7	0	93	68/161(42.2)
	BPD	6	23	10	0	0	122	39/161(24.2)
	BPW	10	16	0	0	0	135	26/161(16.1)
E. coli	NPS	4	33	15	5	0	104	57/161(35.4)
	BPD	13	27	12	1	0	108	53/161(32.9)
	BPW	9	6	3	0	0	143	18/161(11.1)
S. aureus	NPS	42	49	17	0	0	53	108/161(67)
	BPD	78	21	4	0	0	58	103/161(63.9)
	BPW	56	63	1	0	0	41	120/161(74.5)

[a]NPS = neck post-stunning; BPD = breast post-defeathering; BPW = breast post-waxing.

slaughtering by all the microorganisms examined, except for *Staphylococcus aureus* which was found at levels of 10^3 CFU/cm² in all of the samples taken from the hands of the workers. This result is indicative of poor hand hygiene by the staff while performing their duties and could explain the increased contamination of carcasses with *S. aureus* after waxing. In fact, after the immersion of carcasses in the liquid paraffin bath, the waxing phase requires removal of the wax from each carcass by hand-peeling. Because of the intense handling, at this stage the operators could be an important source of carcass contamination.

Overall, the levels of carcass contamination for all of the microorganisms considered, including *S. aureus* were low, throughout all of the stages of the dry-slaughter process.

Salmonella spp. was not found in any of the carcass samples tested and was absent in all 161 faecal samples. These data exclude the presence of the microorganisms in the animals tested and serve as proof of the wholesome quality of their meat. However, in order to evaluate the quality of the product for consumer safety, the study will be extended to include testing for other pathogenic bacteria, particularly *Campylobacter* spp. on poultry carcasses.

CONCLUSIONS

The results demonstrate that the dry-slaughter process used for poultry reduces microbial contamination in the carcasses and highlight the importance of applying Good Manufacturing Practice (GMP), especially in a context of slaughtering where the procedures require hand work. Given their crucial role, operators, must be fully aware of slaughterhouse procedures and must follow hygienic practices in order to exclude being a potential source of contamination.

The dry-slaughter process is most suitable for a small-scale production unit, as an alternative to traditional slaughtering of poultry. Slaughterhouses like the one described in this study could be constructed by several producers in the Italian poultry sector. The system is particularly well suited to marginally disadvantaged areas, or for work on a continuous supply chain because of its simplicity, low cost of equipment (low-tech equipment) and ease of management (small number of personnel are required for the operation). Finally this method could satisfy the need to ensure quality products that meet specific consumer protection requirements through improved production techniques applied to the entire production chain.

REFERENCES

Abu-Ruwaida A.S., Sawaya W.N., Dashti B.H., Murad M. and Al-Othman H.A. 1994. Microbiological quality of broilers during processing in a modern commercial slaughterhouse in Kuwait. J. Food Protect. 57:887.

Anonymous. 2007. SAN-PEL – Coadiuvante tecnologico per i programmi GMP e HACCP. Available at http://www.chimiba.com/IT/product.html. Accessed 03 December 2007.

Cason J.A., Hintor A. Jr. and Ingram K.D. 2000. Coliform, *Escherichia coli*, and salmonellae concentrations in a multiple-tank, counterflow poultry scalder. J. Food Protect. 63:1184.

Chiesa F. and Vercellotti L. 1992. Controllo igienico-sanitario e ambientale in un macello per la selvaggina allevata. Ingegneria Aliment. 4:24.

Clouser C.S., Doores M., Mast M.G. and Knabel S.J. 1995. The role of defeathering in the contamination of turkey skin by *Salmonella* species and *Listeria monocytogenes*. Poultry Sci. 74:723.

EC 2005. Commission Regulation (EC) No. 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs. Official Journal of the European Union.

EC 2007. Commission Regulation (EC) No. 1441/2007 of 5 December 2007 amending Regulation (EC) No. 2073/2005 on microbiological criteria for foodstuffs. Official Journal of the European Union, 2073/2005 of 15 November 2005.

Geornaras I., de Jesus A.E., van Zyl E. and von Holy A. 1997. Bacterial populations of different sample types from carcasses in the dirty area of a South African poultry abattoir. J. Food Protect. 60:551.

Goksoy E.O., Kirkan S. and Kok F. 2004. Microbiological quality of broiler carcasses during processing in two slaughterhouses in Turkey. Poultry Sci. 83:1427.

Hinton A. Jr., Cason J.A., Hume M.E. and Ingram K.D. 2004. Use of MIDI-fatty acid methyl ester analysis to monitor the transmission of *Campylobacter* during commercial poultry processing. J. Food Protect. 67:1610.

James C., Vincent C., de Andrade Lima T.I. and James S.J. 2006. The primary chilling of poultry carcasses – a review. Int. J. Refrig. 29:847.

Lillard H.S. 1990. The impact of commercial processing procedures on the bacterial contamination and cross-contamination of broiler carcasses. J. Food Protect. 53:202.

Mead G.C., Hudson W.R. and Hinton M.H. 1994. Use of a marker organism in poultry processing to identify sites of cross-contamination and evaluate possible control measures. Brit. Poultry Sci. 35:345.

Mulder R.W.A.W., Dorresteijn L.W.H. and van der Broek J. 1978. Cross-contamination during the scalding and plucking of broilers. Brit. Poultry Sci. 19:61.

Nychas G.-J.E. and Drosinos E.H. 2000. Meat and poultry/Spoilage of meat. In "Encyclopedia of Food Microbiology" vol. 2. R.K. Robinson, C.A. Batt and P.D. Patel (Ed.), p. 1253. Academic Press, London, UK.

Pereira M.K., Muratori M.C.S., Pereira M.M.G., de Macedo N.A., Costa A.P.R., de S. Santos H., Macedo C.M., de Azevedo L.M. and Neves R.A. 2008. Bacteriological analysis of chicken scald water. Hygiene Alimentar. 22:60.

Sun Y.M. and Ockerman H.W. 2005. A review of the needs and current applications of hazard analysis and critical control point (HACCP) system in foodservice areas. Food Control. 16:325.

Tsola E., Drosinos E.H. and Zoiopoulos P. 2008. Impact of poultry slaughter house modernisation and updating of food safety management systems on the microbiological quality and safety of products. Food Control. 19:423.

Paper received July 10, 2009 Accepted December 15, 2009

SHORT COMMUNICATION

BACTERICIDAL ACTIVITY OF ELECTROLYZED OXIDIZING WATER ON FOOD PROCESSING SURFACES

A. SERRAINO, G. VERONESE, S. ALONSO, R. MATERA, B. LUGOBONI and F. GIACOMETTI*

Dipartimento di Sanità Pubblica Veterinaria e Patologia Animale. Alma Mater Studiorum, Università di Bologna, Via Tolara di Sopra 50, Ozzano dell'Emilia 40064, Bologna, Italy

*Corresponding author: Tel. +39 051 2097332, Fax +39 051 2097346

e-mail: federica.giacometti3@unibo.it

ABSTRACT

The efficacy of electrolyzed oxidizing water (EOW) as a disinfectant of food processing surfaces was evaluated. Bacterial inactivation was first tested by direct contact of EOW with bacterial suspensions. Then three different surfaces (teflon, stainless steel and ceramic) were artificially contaminated and bacterial survival was determined after treatment with water and EOW. Efficacy was tested for mesophilic bacteria and for *Salmonella Typhimurium*, *Listeria monocytogenes*, verotoxigenic *Escherichia coli* O157:H7 and *Staphylococcus aureus*. Contact test (30 s) of EOW with bacterial suspensions resulted in an 8 log reduction in bacterial populations. Spraying treatment of artificially contaminated surfaces revealed different degrees of disinfectant activity of EOW on different bacterial species. No differences in efficacy were detected between different surfaces. Changes in the physico-chemical properties (pH, oxidation-reduction activity (ORP), free CHLC) of EOW were measured during contact with different surfaces. After low-pressure spraying both ORP activity and free-chlorine content rapidly decreased with time. Low-pressure spraying does not seem to affect EOW disinfectant activity. EOW has a very strong disinfectant activity which, along with its easy and safe use, makes it a good alternative to many other more widely used disinfectants.

- Key words: bacterial inactivation, electrolyzed oxidizing water, food, surfaces -

INTRODUCTION

Problems related to food safety are a major public health issue. Foodborne pathogens are currently estimated to be responsible for one-third of human diseases in the developed world. Although animals and humans are a major source of bacterial food contamination, indirect contamination caused by processing plant environment and production equipment plays a significant role. Disinfecting food processing surfaces is a key procedure for reducing and even eliminating bacterial contamination which might cause changes to the sensory characteristics of foodstuffs or be responsible for foodborne disease outbreaks (PARK *et al.,* 2002). Cross contamination via inanimate surfaces is an important factor in food-borne infections (DEZA *et al.*, 2005) and a wide variety of surfaces such as plastic, ceramic, stainless steel, glass, etc. can carry pathogenic microorganisms. Equipment in food processing plants, as well as protective clothing and gloves worn during working activities, have been shown to be carriers of foodborne pathogens (LIU and SU, 2006). Moreover, surfaces in public areas (toilets, laboratories, hospital instruments) carry high levels of bacteria and pathogens (PARK *et al.,* 2002). Procedures to reduce or even eliminate pathogens from surfaces is one of the key points of an effective HACCP program in the food industry and in controlling food contamination in homes, food markets, restaurants, health facilities and public areas (VENKITAANARAYANAN *et al.,* 1999).

Electrolyzed oxidizing water is obtained from the electrolysis of a NaCl solution (ca 2 g/L). When electricity flows through the NaCl solution it generates two types of water: the cathode produces alkaline electrolyzed water containing sodium hydroxide (pH 116, ORP ≈ -795 mV), while the anode produces acidic electrolyzed water containing hypochlorous acid (pH 2.4-2.7, ORP ≈ 1,150 mV); the concentration of the residual chlorine depends on the EO water machine setting.

The disinfectant activity of EOW lies in its low pH, its ORP activity and the presence of free chlorine and hypochlorous acid (SHARMA and DEMIRCI, 2003; STEVENSON *et al.*, 2004). Hypochlorous acid inactivates the bacterial cell by oxidation of sulphydric components of the membrane and by inactivation of respiratory enzymes, inhibition of ATP production and restraint of transport systems of the bacterial cell (PARK *et al.*, 2002). The strong oxidation-reduction activity of the EOW sequesters the membrane electrodes, rendering them unstable and helping antibacterial compounds to enter the bacterial cell. The low EOW pH influences the permeability of the bacterial membrane, affecting metabolic and reproduction activities of microorganisms (FABRIZIO *et al.*, 2002; LIAO *et al.*, 2007). EOW possesses antimicrobial activity against a variety of microorganisms on crops, bacteria and fungi. In recent years, EOW has gained interest as a disinfectant used in agriculture, dentistry, medicine and the food industry (HUANG *et al.*, 2007). EOW is an effective antimicrobial agent for cut vegetables like lettuce, alfalfa seeds, sprouts, pears, apples, peaches, tomatoes and strawberry, for cutting boards (VENKITANARAYANAN *et al.,* 1999), poultry carcasses (FABRIZIO *et al.*, 2002; PARK *et al.,* 2002; HINTON *et al.,* 2007), eggs (RUSSELL, 2003) and food processing equipment: for various surface materials commonly found in food processing facilities (AYEBACH and HUNG, 2005), stainless steel and glass surfaces (DEZA *et al.*, 2005), surfaces in food service areas (GUENTZEL *et al.,* 2007) and on *Listeria monocytogenes* biofilms formed on stainless steel (AYEBACH *et al.*, 2005; KIM *et al.*, 2001), as a sanitizer for treating different surfaces (PARK *et al.,* 2002b) and for use in abattoirs (BACH *et al.,* 2006), on seafood processing surfaces (LIU *et al.,* 2006) and gloves (LIU and SU, 2006) and for cleaning and disinfecting materials used in milking systems (WALKER *et al.*, 2005a, b).

The aim of this study was to evaluate the efficacy of electrolyzed oxidizing water (EOW) as a disinfectant for food processing surfaces to define its possible use in operating conditions in a slaughterhouse. The antibacterial characteristics of EOW and its changes with time after use were also tested to define the most appropriate ways of application.

MATERIALS AND METHODS

All tests were performed at the experimental slaughterhouse of the Veterinary Medicine Faculty of the University of Bologna (Italy).

EOW Solution

Electrolyzed oxidizing water was generated with a Vn 1000M EO water generator (Akuatech S.r.l® Treviso, Italy) through electrolysis of a dilute salt solution (3% NaCl). The current passing through the EO water generator was 90 amperes, the voltage between the electrodes was 24 volts and the flow rate was about 12 L/min. The EO water was used within a week after being produced. Chemical characteristics, like pH and oxidation-reduction activity (ORP), were measured on arrival at the laboratory with a pH metre (HI 98240, Hanna Instrument, Padova, Italy), using R334 and FC201D electrodes to measure ORP and pH, respectively. The direct iodometric titration method was used to determine free chlorine in the EOW solution (ISO7393-3:1990).

The principle of direct iodometric titration is the oxidation reaction under acidic conditions (pH 4 or less) of potassium iodide by chlorine with the release of free iodine: one mL of a saturated solution of potassium iodide was added

to a known volume of EOW solution. The free iodine was immediately titrated with a sodium thiosulfate standard solution until the yellow colour was almost discharged. Then 1 mL of a starch solution was added as indicator and the titration continued until the blue colour was completely discharged.

The concentration of free chlorine was then calculated by the volume of thiosulfate solution consumed.

Bacterial cultures

Two bacterial cultures were used to test the EOW solution: mesophilic bacterial suspension (MBS) and pathogen suspensions (i.e. *Salmonella Typhimurium*, *Staphylococcus aureus*, verotoxigenic *Escherichia coli* O157:H7 and *Listeria monocytogenes*).

The mesophilic bacterial suspension was obtained from samples collected at the experimental slaughterhouse of the Veterinary Medicine Faculty of the University of Bologna (Italy). After slaughtering activities and before cleaning and disinfecting the working areas, 100 cm^2 areas from three different types of surfaces (stainless steel viscera slip, teflon sectioning area and ceramic tile skinning area) were sampled with a sterile cotton swab. The collected material was suspended in 10 mL of saline solution and later 10 μL were plated on Petri dishes containing Standard Plate Count Agar (PCA, Oxoid) and incubated at 30°C for 72 h to allow the growth of the microorganisms. Bacterial growth was collected using a sterile plastic loop and then suspended in 100 mL of sterile saline solution. Turbidity was adjusted to obtain a bacterial suspension equal to number 3 on the MacFarland scale (about $9x10^8$ CFU/mL). To determine the exact concentration of the bacterial suspension 100 μL of serial dilutions of the original mesophilic suspension were seeded and incubated in PCA Petri dishes at 30°C. A colony count was done after 72 h. The suspension was diluted once more before use.

Suspensions of the above-mentioned pathogens were also prepared. Detailed characteristics and origin of strains used in this study are listed in Table 1. Strains were seeded on Tryptone Soya Sgar (TSA, Oxoid, Cambridge, UK) supplemented with Bacto Yeast Extract (Difco, Maryland, USA) and incubated at 37°C. Bacterial growth was collected after 24 h and suspended in a 10 mL saline solution and turbidity was adjusted to number 3 on the MacFarland scale (about $9x10^8$ CFU/mL). Each pathogen suspension used for the inactivation test was obtained by mixing equal parts of the prepared suspensions of the three available strains (Table 1). The exact titration of each pathogen

Table 1 - Bacterial strains used and their origin.

Microorganism species	Strain	Origin
Salmonella Typhimurium	ATCC 14028	
29RA1	Wild strain from poultry	Isolated from infectious disease laboratory of the Department
6221/2155	Wild strain from pork	Isolated from infectious disease laboratory of the Department
Staphylococcus aureus	ATCC 25923	
270/5	Wild strain from cow's milk	Isolated from infectious disease laboratory of the Department
B/122/1	Wild strain from poultry	Isolated from food hygiene laboratory of the Department
Verotoxigenic E. coli O157: H7	ATCC 700927	
ED166	Wild strain from beef	Isolated from food hygiene laboratory of the Department
ED933	Wild strain from bovine faeces	Isolated from food hygiene laboratory of the Department
Listeria monocytogenes	ATCC 7644	
B/122/4	Wild strain from pork	Isolated from food hygiene laboratory of the Department
B/122/7	Wild strain from bovine faeces	Isolated from food hygiene laboratory of the Department

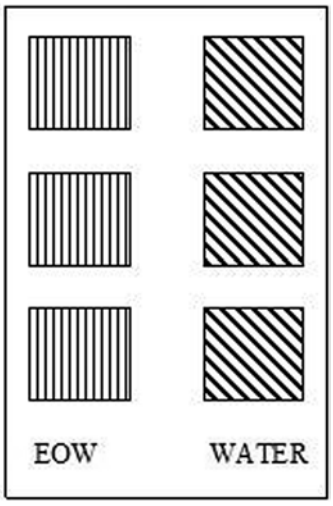


Fig. 1 - Artificially contaminated areas.

solution was checked by seeding 100 μL of serial dilutions of the initial suspensions in PCA Petri dishes and incubating them at 37°C for 24-48 h. All suspensions were diluted once more before use.

Test 1 or "contact test"

One mL of each bacterial suspension (mesophilic suspension, *Salmonella Typhimurium*, *Staphylococcus aureus*, verotoxigenic *Escherichia coli* O157:H7 and *Listeria monocytogenes*) was added to both 9 mL of acidic EOW and 9 mL of tap water. After incubation at room temperature for 30 s, 1 mL of each inoculated liquid was serially diluted using tubes containing 9 mL of neutralization buffer (Difco) and 0.L mL of each dilution was seeded in PCA Petri dishes to count mesophilic bacteria and each pathogen and then incubated at 30°C for 72 h and 37°C for 24-48 h, respectively.

To identify low numbers of bacteria or injured bacterial cells after treatment, 1 mL of the bacterial suspensions in contact with EOW (see above) was added to 20 mL of Tryptone Soya Broth supplemented with yeast (TSB-Y, Oxoid) and incubated at 37°C for 48 h to allow multiplication of bacteria. These broths (0.1 mL) were seeded in TSA-Y and incubated at 37°C. A colony count was done after 48 h of incubation.

Test 2: Efficacy on artificially contaminated surfaces

All tests were performed on equipment surfaces in the experimental slaughterhouse of the Veterinary Medicine Faculty of the University of Bologna (Italy) which are regularly used for slaughtering cattle, swine and goat-like animals. Tests were performed on three different surfaces: ceramic, stainless steel and teflon.

Six 100 cm^2 areas near one another were identified on the surfaces as illustrated in Fig 1. Before contamination, each area was disinfected by direct application of pure ethyl alcohol and then flamed with a portable Bunsen burner.

Contamination by mesophilic bacterial suspension (MBS): six 100 cm^2 areas were contaminated with 1 mL of a 10^7 CFU/mL suspension of MBS, for a total contamination surface area of about 10^5 CFU/cm^2.

Contamination by MBS and pathogen suspension: 5 mL of each pathogen suspension (*Salmonella Typhimurium*, *Staphylococcus aureus*, verotoxigenic *Escherichia coli* O157:H7 and *Listeria monocytogenes*) were mixed with 5 mL of MBS and diluted to obtain 10^7 CFU/mL suspensions. Each of these suspensions was used to contaminate six 100 cm^2 areas, that is, 1 mL of suspension was spread over each of the six 100 cm^2 areas.

After complete drying of the contaminated surfaces, treatment was performed by spraying tap water on three 100 cm^2 of the six contaminated areas for five seconds and spraying EOW on the remaining three areas for five seconds, using a low pressure pump with a 1.2 mm diameter outlet.

Once the surfaces had dried by unaffected evaporation to simulate the operating conditions, a sample was collected from each area and bacteria were isolated and counted. In detail, a sterile gauze was rubbed over the whole treated area 10 times and then put in a sterile plastic bag containing 100 mL of sterile saline solution. After mixing in a stomacker (Blender Eco 400, PBI, Milan, Italy) 1 mL of the sample obtained and 1 mL of five serial 10-fold dilutions were seeded in plastic Petri dishes containing PCA for mesophilic bacteria count (after incubation at 30°C for 72 h), and selective agar medium for each type of pathogen (37°C for 24-48 h).

Each of these experiments was repeated twice.

Test 3: EOW disinfectant activity after application on surfaces

In order to test the two materials with higher and lower conductivity, ORP, pH and free chlorine ion quantity, modifications were tested in EOW after contact with two different surface materials (teflon and stainless steel). EOW (200 mL) was placed in containers to form 2mL EOW films at the bottom of the containers. The physico-chemical characteristics above were measured after 0, 2, 4, 6 and 24 h of treatment.

The pH and ORP were measured with a Hanna Instrument HI 98240 pH metre with R334 and FC201D electrodes for measuring ORP and pH, respectively. Quantification of free chlorine ion was determined by iodometric titration following the procedure described by GARY (2003) and FABRIZIO *et al.* (2002).

Test 4: Influence of spraying on EOW chemical characteristics

EOW was sprayed in Teflon and stainless steel

containers by a low pressure pump with a 1.2 mm diameter outlet. Immediately after spraying, the liquid was collected and tested for pH and ORP.

RESULTS AND DISCUSSION

EOW solution

On delivery, EOW had a pH of 2.69, an ORP of 1,135 mV and the free chlorine concentration was about 110 mg/L.

Test 1 or "contact test"

The culture of tubes containing tap water had the same number of colonies as the amount inoculated (Table 2). No colony was isolated from the samples treated with EOW by direct plating. A statistically significant 8 log reduction ($p<0.05$) in the bacterial population was recorded comparing EOW with tap water.

Further enrichment of all samples confirmed total inactivation of bacterial cells in the tubes containing EOW.

Test 2: Efficacy on artificially contaminated surfaces

Table 3 shows the bacterial counts on each type of surface after treatment with either tap water or EOW. Significant differences ($p<0.001$) on bacterial inactivation were found between treatments with EOW and tap water for each microorganism under study. Complete inactivation was always obtained for *Salmonella Typhimurium*, verotoxigenic *Escherichia coli* and *Listeria monocytogenes*, especially after treatment with EOW. However, no differences were found when comparing the disinfectant activity on the three different surfaces.

Treatment with EOW for 5 seconds on artificially contaminated surfaces led to a significantly greater reduction of bacterial concentration than that obtained by treatment with tap water ($p<0.001$) for each microorganism. In detail, mesophilic bacteria were reduced by 2.28 log on the average, while pathogens showed an average reduction of 1.77 log, with a maximum reduction of 2.12 log for *E. coli* O157:H7 and a minimum reduction for *S. Typhimurium* of 1.34 log.

No statistically significant difference in inactivation was found between the different surfaces for either mesophilic or pathogen bacteria. Only one test on ceramics showed an extremely high level of inactivation (3.23 log). However, the smooth nature of ceramics might be partly responsible for the greater efficacy of EOW. While stainless steel and teflon are more vulnerable to degradation with use and time, ceramics tend to maintain a smooth surface that facilitates activity of disinfectants against bacteria.

A major result was that complete inactivation of bacteria was reached by treatment with EOW only when the level of contamination after treatment with water was equal to or less than 2.1 log. This proves the higher efficacy of EOW compared to water and suggests that the final result may depend on initial contamination. Moreover, all tests were performed on artificially contaminated surfaces resulting in highly contaminated areas (10^5 CFU/cm^2). Future research should focus on the dynamics of inactivation at different initial bacterial concentrations.

The activity of EOW was compared with that of another disinfectant (iodophor, IOD), resulting in a greater efficacy of EOW especially in reducing total aerobic bacterial contamination of surfaces (BACH *et al.*, 2006).

Besides its greater efficacy as a disinfectant, other advantages of EOW compared with other well-known disinfectants include: i) no risk for users, ii) highly concentrated disinfectant solutions are avoided since EOW can be delivered ready to use or produced in the place of use (PARK *et al.*, 2002 a), iii) easy and relatively cheap to produce (FABRIZIO *et al.*, 2002) and iv) very low environmental impact (FABRIZIO *et al.*, 2002), whereas organic acid disinfectants may be very harmful to the environment and even modify the sensory characteristics of food products (FABRIZIO *et al.*, 2002).

Hypochlorite solution is one of the most widely used disinfectants, mainly because of its wide bactericidal spectrum and its relatively low price (LIU *et al.*, 2006). EOW has a higher bactericidal activity than an equally concentrated hypochlorite solution, mainly because of its low pH and high ORP (PARK *et al.*, 2002 a), and a 200 mg/L hypochlorite solution (LIU *et al.*, 2006; PARK *et al.*, 2005).

The bactericidal efficacy of EOW is reduced when relatively large amounts of organic material are present. This is characteristic of many other disinfectants, including hypochlorite (BACH *et al.*, 2006; LIU *et al.*, 2006). This effect can be reduced by using the alkaline solution derived from the production of EOW before applying the acidic EOW. The alkaline solution contains a high concentration of sodium hydroxide that acts as a detergent by reacting against fats and

Table 2 - EOW efficacy on bacterial suspensions.

Microorganism species	Bacterial population (log_{10} CFU/mL)[a]	
	Tap water 30"	EOW 30"
Mesophilic bacteria	8.83±0.14	N.G.[b]
Salmonella Typhimurium	8.53±0.16	N.G.[b]
Staphylococcus aureus	8.89±0.01	N.G.[b]
Verotoxigenic E. coli O157:H7	8.60±0.09	N.G.[b]
Listeria monocytogenes	8.67±0.07	N.G.[b]

[a] average of six tests ± standard deviation;
[b] no growth of viable colonies in TSA Petri dishes.

Table 3 - Bacterial counts after treatment with either tap water or EOW on each type of surface.

Microorganism species	Bacterial population (log_{10} CFU/ cm^2)[a]					
	Ceramics		Stainless steel		Teflon	
	Tap water	EOW	Tap water	EOW	Tap water	EOW
Mesophilic bacteria	2.77±0.09	0.9±0.4	2.31±0.15	0.15±0.27[c]	2.51±0.10	1.03±0.47
Salmonella Typhimurium	1.23±0.22	NG [b]	1.5±0.09	NG[b]	1.3±0.45	NG[b]
Staphylococcus aureus	2.30±0.06	0.38±0.40[c]	2.03±0.15	NG[b]	2.47±0.31	0.23±0.40[c]
Verotoxigenic E. coli O157:H7	2.18±0.22	NG [b]	2.09±0.21	NG[b]	2.09±0.21	NG[b]
Listeria monocytogenes	2.27±0.56	NG [b]	1.57±0.56	NG[b]	1.09±0.19	NG[b]

[a] average of six tests ± standard deviation;
[b] no growth of viable colonies in TSA Petri dishes in all tests;
[c] no growth of viable colonies in TSA Petri dishes in one or two tests.

proteins and dissolving and breaking the polymeric compounds outside the bacterial membrane. This enhances the subsequent activity of the acidic components (AYEBAH *et al.*, 2005).

Test 3: EOW disinfectant activity after application on surfaces (pH, ORP and free chlorine)

The pH did not undergo marked changes over time, except for a small decrease over 6 h, whereas modifications in the ORP activity and free chlorine content of EOW occurred. As seen in Table 4, both parameters showed a fast reduction with time, reaching half of the initial value after 24 h in the case of ORP activity and almost a two-fold reduction in free chlorine content after a 6 h treatment.

Test 4: Influence of spraying on EOW chemical characteristics (pH and ORP)

Low-pressure spraying slightly influenced EOW characteristics and its physico-chemical properties (Table 4). The pH remained between 2.69 and 2.83 and ORP was reduced from 1,135 to 1,109 mV.

On the other hand, after application on two types of surfaces, the free chlorine concentration dropped rapidly over time, along with a parallel decrease of ORP which, in 24 h, reached the levels regularly present in tap water. The hypochlorous acid is the most active form of chlorine. It penetrates the bacterial cell and inhibits essential respiratory enzymes (FABRIZIO *et al.*, 2002); it is 80 times more efficient than hypochlorite ion (KIM *et al.*, 2000). When hypochlorite is added to water, it generates undissociated hypochlorous acid. The hypochlorous acid dissociates into the anion hypochlorite in an alkaline environment and into chlorine gas in an acid environment (IZUMI, 1999). Thus, the chorine level in EOW tends to decrease when EOW is in contact with air due to the evaporation of chlorine gas or self-decomposition (LEN *et al.*, 2002).

CONCLUSIONS

The results of this study proved the efficacy of EOW in reducing the mesophilic bacterial contamination in operating conditions using short treatments (5 min) and using partially contaminated working equipment. The contamination levels in food industry environments are usually lower than that used in our study, especially when considering pathogenic bacteria. The efficacy of EOW to inactivate the entire population of pathogens present on surfaces might be even higher under natural working conditions. EOW can be considered a good alternative to hypochlorite, which must be used in large amounts and may be harmful to humans by intake via food (BACH *et al.*, 2006; FABRIZIO *et al.*, 2002; LIU *et al.*, 2006; SHARMA and DEMIRCI, 2003). EOW has been proven to be very unstable after application on surfaces which might make

Table 4 - Changes in EOW characteristics with time after spraying on steel and teflon surfaces.

Time	pH		ORP (mV)		Free chlorine (mg/L)	
	Steel	Teflon	Steel	Teflon	Steel	Teflon
0 min	2.69	2.69	1139	1139	110.76	110.76
1 h 30 min	2.81	2.67	1123	1150	31.8	33.7
3h 30 min	2.85	2.71	1127	1134	8.9	11.0
6 h	2.55	2.56	688	925	1.8	0.71
24 h	2.37	2.44	542	550	1.9	0.35

it unnecessary to rinse surfaces after its application and before the initiation of working activities, depending on the length of time between disinfection and onset of working activities. This would save time and, hence be more profitable.

REFERENCES

Ayebah B. and Hung Y.C. 2005. Electrolyzed water and its corrosiveness on various surface materials commonly found in food processing facilities. J. Food Process Eng. 28: 247.

Ayebah B., Frank J.F. and Hung Y.C. 2005. Enhancing the bactericidal effect of oxidizing water on *Listeria monocytogenes* biofilms formed on stainless steel. J. Food Prot. 68: 1375.

Bach S.J., Jones S., Stanford K., Ralston B., Milligan D., Wallins G.L., Zahiroddini H., Stewart T., Giffen C. and McAllister T.A. 2006. Electrolyzed Oxidizing Anode Water as a sanitizer for use in abattoirs. J. Food Prot. 69: 1616.

Deza M.A., Araujo M. and Garrido M.J. 2005. Inactivation of *Escherichia coli, Listeria monocytogenes, Pseudomonas aeruginosa* and *Staphylococcus aureus* on stainless steel and glass surfaces by neutral electrolysed water. Lett. Appl. Microbiol. 40: 341.

Fabrizio K.A., Cutter C.N., Demirci A. and Sharma R.R. 2002. Comparison of elecrolyzed oxidizing water with various antimicrobial interventions to reduce Salmonella species on poultry. Poultry Sci. 81: 1598.

Gary D.C. 2003. "Analytical Chemistry". 6th ed. John Wiley and Sons, (WIE). New York, USA.

Guentzel J.L., Lam K.L., Callan M.A., Emmons S.A. and Dunham V.L. 2007. Reduction of bacteria on spinach, lettuce, and surfaces in food service areas using neutral electrolyzed oxidizing water. Food Microbiol. 25: 36.

Hinton A., Northcutt J.K., Smith D.P., Mushgrove M.T. and Ingram K.D. 2007. Spoilage microflora of broiler carcasses washed with electrolyzed oxidizing or chlorinated water using an inside-outside bird washer. Poultry Sci. 86:123.

Huang Y.R., Hung Y.C., Hsu S.Y., Huang Y.W. and Hwang D.F. 2007. Application of electrolyzed water in the food industry. Food Control. 19:329.

ISO7393-3:1990. Water quality – Determination of free chlorine and total chlorine – Part 3: iodometric titration method for the determination of total chlorine. International Organizaztion for Standardization, Geneva, Switzerland.

Izumi H. 1999. Electrolyzed oxidizing water as a disinfectant for fresh-cut vegetables. J. Food Sci. 64: 536.

Kim C., Brackett R.E. and Hung H.C. 2000. Efficacy of electrolyzed oxidizing water and chemically modified water on different types of foodborne pathogens. Int. J. Food Microbiol. 61: 199.

Len S.V., Hung Y.C., Chung D., Anderson J.L., Erickson M.C. and Morita K. 2002. Effects of storage conditions and pH on chlorine loss in electrolyzed oxidizing (EO) water. J. Agric. Food Chem. 50: 209.

Liao L.B., Chen W.M. and Xiao X.M. 2007. The generation and inactivation mechanism of oxidation-reduction potential of electrolyzed oxidizing water. J. Food Eng. 78: 1326.

Liu C., Duan J. and Su Y.C. 2006. Effect of electrolyzed oxidizing water on reducing *Listeria monocytogenes* contamination on seafood processing surfaces. Int. J. Food Microbiol. 86: 231.

Liu C. and Su Y.C. 2006. Efficiency of electrolyzed oxidizing water on reducing *Listeria monocytogenes* contamination on seafood processing gloves. Int. J. Food Microbiol. 110: 149.

Park H., Brakett R.E. and Hung Y.C. 2002a. Antimicrobial effect of electrolyzed oxidizing water for inactivating *Campylobacter jejuni* during poultry washing. Int. J. Food Microbiol. 72: 77.

Park H., Hung Y.C. and Kim C. 2002b. Effectiveness of electrolyzed water as a sanitizer for treating different surfaces. J. Food Prot. 65: 1276.

Park C.M., Brakett R.E, Hung Y.C. and Lin C.S. 2005. Efficacy of electrolyzed oxidizing water in inactivating *Salmonella enteritidis* and *Listeria monocytogenes* on shell eggs. J. Food Prot. 68: 986.

Russell S.M. 2003. The effect of electrolyzed oxidative water applied using electrostatic spraying on pathogenic and indicator bacteria on the surface of eggs. Poultry Sci. 82:158.

Sharma R.R. and Demirci A. 2003. Treatment of *Escherichia coli* O157:H7 inoculated alfalfa seeds and sprouts with electrolyzed oxidizing water. Int. J. Food Microbiol. 86: 231.

Stevenson S.M., Bach S.J., Cook S.R. and McAllister T.A. 2004. Effects of water source, dilution, storage and bacterial and fecal loads on the efficacy of electrolyzed oxidizing water for the control of *Escherichia coli* O157:H7. J. Food Prot. 67: 1377.

Venkitaanarayanan K., Doyle M.P., Ezeike G.O. and Hung Y.C. 1999. Efficacy of electrolyzed oxidizing water for inactivating *Escherichia coli* O157:H7, *Salmonella enteritidis* and *Listeria monocytogenes*. Appl. Environ. Microbiol. *65* : 4276.

Walker S.P., Demirci A., Graves R.E., Spencer S.B. and Roberts R.F. 2005. Cleaning milking systems using electrolyzed oxidizing water. Transactions of ASAE. 48: 1827.

Walker S.P., Demirci A., Graves R.E., Spencer S.B. and Roberts R.F. 2005. CIP cleaning of a pipeline milking system using electrolyzed oxidizing water. Int. Dairy Tech. 58: 65.

Revised paper received December 11, 2008 Accepted July 20, 2009

SHORT COMMUNICATION

PRESENCE OF BIOGENIC AMINES IN RICOTTA FORTE, A TRADITIONAL ITALIAN DAIRY PRODUCT FROM THE PUGLIA REGION

S. REA*, N. MASCARO, M. RICCIUTELLI[1], N. CAMMERTONI, S. CECCHINI, A.R. LOSCHI and R. STOCCHI

Scuola di Scienze Mediche Veterinarie, Sezione di Scienze di Base e Applicate per la Produzione e la Sanità Animale e per la Sicurezza Alimentare, Università di Camerino, Via Circonvallazione 93/95, 62024 Matelica (MC), Italy

[1]Laboratorio di Cromatografia Liquida e Spettrometria di Massa, Università di Camerino, Via S. Agostino 1, 62032 Camerino (MC), Italy

*Corresponding author: Tel. +39 0737 403443, Fax +39 0737 403402

e-mail: stefano.rea@unicam.it

ABSTRACT

Ricotta Forte is a niche dairy product belonging to the traditional Italian agroindustrial products of the Puglia region. It is produced from sheep, cow or goat whey proteins as "classic" ricotta, but is characterized by a prolonged shelf life. The intense fermentation process that is the result of a unique manufacturing technology that requires a considerable amount of handling and prolonged ripening makes the product a favourable substratum for biogenic amine (BA) production. The aim of the present study was to determine the presence of the most important BAs, such as cadaverine, putrescine, histamine, tyramine, β-phenylethylamine, spermine and spermidine, in samples of different kinds of Ricotta Forte produced and purchased within the traditional area. High levels of BAs were detected in all the samples, especially in those to which no preservative (sorbic acid) was added. Since Ricotta Forte is used mostly in small quantities (one-two teaspoons) as a flavouring ingredient in pasta, meatballs, appetizers, etc. to enrich and enhance their taste, the amount of BAs eaten is not alarming. However its consumption is not recommended for persons belonging to categories at risk for such compounds.

- Key words: biogenic amines, food safety, HPLC, Ricotta Forte, traditional products -

INTRODUCTION

Ricotta Forte is a creamy, spreadable dairy product with a china-white colour; it has a hot and savoury taste and a sharp but pleasant smell (RESMINI *et al.*, 1999). It was included among the traditional Italian agroindustrial products of the Puglia region by the Ministerial Decree of 18 July 2000 (M.D., 2000). It is mostly produced in the Salento area in the provinces of Brindisi and Bari, especially between May and October. Sheep whey is usually used but cow and/or goat whey is also used. Its particular hot, sharp taste makes Ricotta Forte a niche product used by a rather narrow segment of consumers, mostly made of estimators of "ancient tastes" influenced by a food culture based on strong, sharp flavours. Ricotta Forte originated from the need to preserve for an extended time the part of the ricotta that was produced but not immediately eaten. However, current production is based on both unsold and specifically prepared ricotta (TANTILLO, 2007). Only about 10% of all sheep milk produced in the Puglia region is used to manufacture ricotta (including Ricotta Forte) and the yield is very low (about 5%). Therefore the quantity of Ricotta Forte produced is rather small (about 40 q per year) (RESMINI *et al.*, 1999).

In the early stages, the processing technology is the same as that used for "classic ricotta", a typical Italian dairy product obtained by acid and thermal curdling of whole sheep whey from cheese-making, sometimes supplemented with milk but with no starters added.

The whey is heated slowly in a copper or steel boiler over direct heat to a temperature of about 80°C. Once the curdled whey proteins have completely risen to the surface, the mass is collected by a skimmer and put in rush or plastic baskets to drain. At this stage the manufacturing process of the two types of ricotta diversifies. The draining time for classic ricotta is short (1-4 h) followed by packaging and fast marketing. The draining time for Ricotta Forte lasts for 3-4 days. It is then put in glazed terracotta, wooden or plastic truncated conical containers. Traditionally, the mass is kept in such containers in underground cellars at a constant temperature (around 10°C) and RH (65 to 85%) for 2-6 months (sometimes 18-24 months). The mass is periodically mixed using a wooden spoon to prevent mould growth and to help the acidifying activity of fermenting microorganisms. In industrial processing this stage is carried out in steel tanks equipped with blades that provide a slow, continuous mixing of the product (TANTILLO, 2007). At the end of ripening, the ricotta has acquired the typical smell, dark ivory colour and sour taste. At this stage it is removed from the container, salted (0.2-1.5% with NaCl) and packed in glass jars. The product can be stored for 10-12 months at temperatures lower than 15°C but during this period the colour changes to brown-grey and a yellow oily layer forms on the surface which increases its shelf life (RESMINI *et al.*, 1999).

The particular organoleptic features and the manufacturing process are based on ancient practices. Research is needed to increase knowledge about this product, not only to enhance it but also to ensure quality standards and a good level of hygiene and safety that are not always achieved in local and artisan processes.

The long ripening process characterized by strong fermentative phenomena and the prolonged shelf life of Ricotta Forte could favour the production of biogenic amines (BAs). These compounds are formed by the decarboxylation of amino acids due to tissue and/or microbial enzymes especially in fermented or long-term stored food (REA *et al.*, 2005). The presence of BAs is important due to the toxic effects that some of them (particularly histamine and tyramine) may have on the human body due to their vasoactive action. Consumers who are particularly at risk include children, the elderly, subjects suffering from digestive tract pathologies, alcoholics and drug users. People with deficiencies or defects of natural detoxifying mechanisms mostly at the gastrointestinal level, such as monoamine oxidase (MAO), diamino oxidase (DAO) and histamine methyltranferase (HMT), could experience the onset of symptoms even at low BA doses.

Besides the above-described health concerns, BAs may also be indicators of the quality and hygiene of the raw materials, compliance with good manufacturing practices and food conditions (CANTONI, 1995; REA *et al.*, 2008b).

The aim of the work was to qualitatively and quantitatively determine the most important BAs present in samples of Ricotta Forte purchased on the market. This was done to acquire better knowledge about the hygienic and safety features of this particular traditional Italian product about which little information is available in the literature.

MATERIALS AND METHODS

Samples of three brands of Ricotta Forte packaged in glass jars taken from different selling units were purchased from dealers located in the province of Lecce (Puglia region) and analyzed. Two brands were produced by artisan factories: one was made with mixed whey from cow, sheep and goat without preservatives; while the other was prepared with sheep whey with sorbic acid added as preservative. The third Ricotta Forte was purchased from a street trader during a village festival; the label said it was produced from sheep whey without preservatives, by the dealer himself.

The concentrations of the most impor-

tant BAs (histamine, putrescine, cadaverine, tyramine, β-phenylethylamine, spermine and spermidine) were determined according to REA *et al.* (2005). For each sample 10 g of Ricotta Forte were homogenized in 35 mL of 5% trichloroacetic acid, centrifuged at 3,000 rpm for 10 min and filtered in a 100 mL flask. The extraction process was repeated once and both filtrates were collected. Working solutions containing 10 mg/mL of each amine (Sigma, St. Louis, MO, USA) in distilled water were prepared as standards and used for arranging the calibration curves. A 10 mg/mL solution of 1,7-diaminoheptane (Sigma, USA) in water was prepared and added (960 μL) as internal standard (I.S.) to each sample during the extraction step. Extracts and standard solutions were derivatized by dansyl chloride (Sigma, USA) just before injection onto an HP 1090 Liquid Chromatograph (Hewlett Packard, Avondale, PA, USA) equipped with an HP diode array detector (DAD). An HP Spherisorb C 18 ODS 2 (250x4 mm, particle size 5 μm) column mounted with a guard column LiChroCART 4-4 LiChroSPHER 100 RP-18 (particle size 5 μm) (Merck, Darmstadt, GE) was used. BAs were identified by comparing the HPLC profile of samples to the pattern obtained from standard amines at known concentration. Quantitative determinations were carried out using the ratio of each BA area to the I.S. area of the same sample and reporting such a value on the calibration curve of standards. All samples were analysed in triplicate.

The recovery percentage was determined by comparing the concentration of each amine detected in a sample extract without standards to the concentration of the corresponding amine in another extract from the same sample with a known amount of standard added (500 μL of each working solution), according to the following formula:

$$R(\%) = (BA_2\text{-}BA_1)/BA_3$$

Where:
BA_1 = BA content before addition;
BA_2 = BA content after addition;
BA_3 = BA added.

RESULTS AND DISCUSSION

Recovery percentages of BAs ranged from 79.30% for cadaverine to 111.49% for β-phenylethylamine, with intermediate values of 94.61% for putrescine, 91.78% for histamine, 105.05% for tyramine, 92.15% for spermidine and 111.43% for spermine. With the exception of cadaverine, recoveries were all higher than 88% which is considered to be the acceptable limit (HERNANDEZ-JOVER *et al.*, 1996). These data confirm the extreme versatility of the analytical method and its suitability for a wide variety of food products of animal origin including dairy products (REA *et al.*, 2008a).

The BA contents in the samples are reported in Table 1; the BA concentrations were much lower in sheep ricotta with sorbic acid added compared to the products without the preservative.

In all samples the highest concentrations of BAs were cadaverine, tyramine and putrescine in a decreasing order, with the exception of sheep Ricotta Forte, with preservative added, in which putrescine was slightly higher than tyramine.

The very high concentration of cadaverine in all three types of products could be responsible for the slightly lower efficacy of the analytical method in recovering this compound, anyway the recovery was close to 80%. Cadaverine and putrescine are responsible for off-flavours (KRAUSE *et al.*, 1997) and are present in many foods of animal and plant origin, while tyramine is typically in cheese and other dairy products because the proteinic component is particularly rich in tyrosine. Tyrosine is quantitatively the third most abundant amino acid both in caseins and whey proteins from cow milk after glutamine and lysine that are precursors of pu-

Table 1 - BA content (ppm) in samples of different types of Ricotta Forte (mean±S.D.).

Biogenic amines	Types of Ricotta Forte		
	Mixed without preservative	Sheep with preservative	Sheep without preservative
β-Phenylethylamine	491.33±76.18	n.d.	721.80±6.48
Putrescine	1469.10±236.25	431.68±10.61	1468.51±39.15
Cadaverine	3759.50±610.34	1044.61±28.80	4641.05±140.49
Histamine	1256.96±207.40	201.44±5.29	1273.51±38.92
Tyramine	2797.78±473.75	387.77±8.97	2142.08±65.33
Spermidine	12.14±7.72	n.d.	17.27±1.24
Spermine	19.34±5.60	21.12±1.00	29.79±1.28
Total	*9806.14±1426.21*	*2089.98±363.11*	*10294.01±1596.36*

n.d. = not detected.

trescine and cadaverine, respectively (CANTARELLI, 1984; SALVADORI DEL PRATO, 2001). Several cases of "cheese reaction" have been reported in the literature (SHALABY, 1996; VALE and GLORIA, 1998; MCCABE-SELLERS *et al.*, 2006) in which tyrosine causes peripheral vasoconstriction, increase of cardiac output, lacrimation, salivation, dyspnea, migraine and hyperglycemia.

The histamine concentration observed in the samples of both mixed and sheep Ricotta Forte without preservatives was very high. The histamine level is of concern because it is known that 100-180 mg of this substance can induce symptoms in subjects weighing 60 kg (STRATTON *et al.*, 1991; RODRIGUEZ-JEREZ *et al.*, 1994; LEHANE and OLLEY, 1999). Furthermore, cadaverine, tyramine and putrescine, the most abundant BAs in the present study, act as potentiators of histamine toxicity; they may interfere with enzymatic detoxification mechanisms in the body and thus potentiate the effects of this substance (STRATTON *et al.*, 1991; HERNÀNDEZ-JOVER *et al.*, 1996; VALSAMAKI *et al.*, 2000). The BA content was high in the tested samples, especially histamine and tyramine that are considered to be the most dangerous compounds. However, it must be emphasized that Ricotta Forte is eaten in very small amounts; it is used to flavour pasta and as an ingredient of meatballs, appetizers and potato croquettes to enrich and enhance their taste. From interviews carried out within the area of traditional production and consumption, it was found that the average amount of product used in the different preparations is one or two teaspoons (about 9-18 g), due to its particularly strong taste. If Ricotta Forte is eaten in such low doses, the present data are not alarming because the highest amounts of histamine and tyramine eaten would be 22.9 and 50.4 mg, respectively, considering the samples containing the highest levels of BAs.

Finally, as for the hygienic significance of BAs, the high total concentration of cadaverine, tyramine, putrescine, histamine and β-phenylethylamine detected in samples of both mixed and sheep Ricotta Forte without preservatives could be due to the growth of lactic acid microbial flora with strong decarboxylating activity (JOOSTEN AND NORTHOLT, 1987; HALASZ *et al.*, 1994; BOVER-CID and HOLZAPFEL, 1999; SUZZI and GARDINI, 2003; AYMERICH *et al.*, 2006) due to contamination during manufacturing. TANTILLO (2007) reported that the manufacturing process of Ricotta Forte hardly meets hygienic standards of modern technology although the products showed good microbiological quality at the end of ripening having no pathogens and being rich enough in lactic acid bacteria and yeasts to have high stability and long shelf-life even at room temperature. However, some species among these contaminant microorganisms can be responsible for BA production in several types of food including dairy products (SHALABY, 1996; GARDINI *et al.*, 2006). These findings could help to explain why much lower BA concentrations were detected in samples of Ricotta Forte with sorbic acid added. The sorbic acid favours lactic acid fermentation and has antimicrobial activity especially against yeasts and moulds as well as some contaminant bacteria (STRATFORD and ANSLOW, 1998; CERUTTI, 1999; JAY *et al.*, 2009).

CONCLUSIONS

The results concerning the BA content in samples of Ricotta Forte suggest moderate use by subjects belonging to categories at risk, although the data are not alarming due to the generally small quantities eaten.

Besides observing strict production hygiene, the addition of authorized preservatives would undoubtedly limit BA production during ripening. This would enable an indispensable compromise between the pursuit of "ancient" or "traditional" tastes and food safety. Nevertheless, the real effect of using preservatives in this product must still be studied in detail, because this aspect was not one of the aims of the present paper. It was an accidental finding, even though expected. Further investigations are needed to characterize the microbiological profile and conduct an in-depth study of the bacterial and fungal flora (moulds and yeasts) responsible for BA production. Other technological practices such as the inoculation of microbial cultures with reduced decarboxylating activity or those able to degrade BAs should also be investigated (BOVER-CID *et al.*, 2001), even though the isolation and use of appropriate starters are probably not applicable in this product (TANTILLO, 2007).

The implementation of further studies aimed at improving the knowledge and increasing the awareness of traditional products, the development of a suitable technology in which the production criteria meet current health standards while respecting ancient traditions, and the establishment of an appropriate marketing policy, could enable this niche product to appear on new markets.

REFERENCES

Aymerich T., Martin B., Garriga M., Vidal-Carou M.C., Bover-Cid S. and Hugas M. 2006. Safety properties and molecular strain typing of lactic acid bacteria from slightly fermented sausages. J. Appl. Microbiol. 100: 40.

Bover-Cid S. and Holzapfel W.H. 1999. Improved screening procedure for biogenic amine production by lactic acid bacteria. Int. J. Food Microbiol. 53: 33.

Bover-Cid S., Hugas M., Izquierdo-Pulido M. and Vidal-Carou M.C. 2001. Amino acid-decarboxylase activity of bacteria isolated from fermented pork sausages. Int. J. Food Microbiol. 66: 185.

Cantarelli C. 1984. "Principi di Tecnologia delle Industrie Agrarie". Edagricole, Bologna, Italy.

Cantoni C. 1995. Amine biogene di prodotti carnei nazionali. Ind. Aliment. 34: 9.

Cerutti G. 1999. "Residui, Additivi e Contaminanti degli Alimenti". Edizioni Tecniche Nuove, Milano, Italy.

Gardini F., Tofalo R., Belletti N., Iucci L., Suzzi G., Torriani S., Guerzoni M.E. and Lanciotti R. 2006. Characterization of yeasts involved in the ripening of Pecorino Crotonese cheese. Food Microbiol. 23: 641.

Halasz A., Barath A., Simon-Sakardi L. and Holzapfel W. 1994. Biogenic amines and their production by microorganisms in food. Trends Food Sci. Tech. 5: 42.

Hernàndez-Jover T., Izquierdo-Pulido M., Veciana-Nogués M.T. and Vidal-Carou M.C. 1996. Ion-pair high-performance liquid chromatographic determination of biogenic amines in meat and meat products. J. Agric. Food Chem. 44: 2710.

Jay J.M., Loessner M.J. and Golden D.A. 2009. "Microbiologia degli Alimenti". Springer-Verlag Italia S.r.l., Milano.

Joosten H.M.L.J. and Northolt M.D. 1987. Conditions allowing the formation of biogenic amines in cheese. 2. Decarboxylative properties of some non-starter bacteria. Neth. Milk Dairy J. 41: 259.

Krause I., Bockhardt A. and Klostermeyer H. 1997. Characterization of cheese ripening by free amino acids and biogenic amines and influence of bactofugation and heat-treatment of milk. Lait 77: 101.

Lehane L. and Olley J. 1999. "Histamine (Scombroid) Fish Poisoning: A Review in a Risk-Assessment Framework". National Office of Animal and Plant Health, Camberra, Australia.

McCabe-Sellers B.J., Staggs C.G. and Bogle M.L. 2006. Tyramine in foods and monoamine oxidase inhibitor drugs: A crossroad where medicine, nutrition, pharmacy, and food industry converge. J. Food Compos. Anal. 19 S58.

M.D. 2000. Ministerial Decree of 18 July 2000. Elenco nazionale dei prodotti agroalimentari tradizionali. Gazzetta Ufficiale della Repubblica Italiana of 21 August 2000, N. 194 - Supplemento Ordinario N. 130.

Rea S., Ricciutelli M., Cecchini S., Pacifici L., Stocchi R. and Loschi A.R. 2005. Biogenic amine concentration in "Lardellato" salami, a traditional product of central Italy, during ripening. It. J. Food Sci. 17: 211.

Rea S., Mascaro N., Cecchini S., Stocchi R., Loschi A.R. and Ricciutelli M. 2008a. Metodo di estrazione delle amine biogene e valutazione della capacità di recupero da matrici alimentari diverse. Ind. Aliment. 47: 752.

Rea S., Mascaro N., Cecchini S., Stocchi R., Loschi A.R. and Ricciutelli M. 2008b. Indagine sulla presenza di amine biogene in differenti prodotti alimentari di origine animale. Ind. Aliment. 47: 983.

Resmini P., Pompei C., Pellegrino L., Volonterio G., Lembo P., Lodi R., Riva M. and Spedicato E. 1999. "I Prodotti Caseari del Mezzogiorno". In: Cannatà M. 1999. C.N.R. Collana Multimediale "Progetti strategici". Progetto strategico n°3. Thera S.r.l., Roma, Italy.

Rodriguez-Jerez J.J., Mora-Ventura M.T. and Civera T. 1994. Istamina e prodotti ittici: un problema attuale. Parte II: relazione fra alterazione del pesce e formazione d'istamina. Ind. Aliment. 33: 393.

Salvadori del Prato O. 2001. "Trattato di Tecnologia Casearia". Calderini Edagricole, Bologna, Italy.

Shalaby A.R. 1996. Significance of biogenic amines to food safety and human health. Food Res. Int. 29: 675.

Stratford M. and Anslow P.A. 1998. Evidence that sorbic acid does not inhibit yeast as a classic "weak acid preservative". Lett. Appl. Microbiol. 27: 203.

Stratton J.E., Hutkins R.W. and Taylor S.L. 1991. Biogenic amines in cheese and other fermented foods: a review. J. Food Prot. 54: 460.

Suzzi G. and Gardini F. 2003. Biogenic amines in dry fermented sausages: a review. Int. J. Food Microbiol. 88: 41.

Tantillo G.M. 2007. "Carta d'Identità dei Prodotti Tradizionali Pugliesi". Ed. Infosei, Bari, Italy.

Vale S. and Gloria M.B.A. 1998. Biogenic amines in Brazilian cheeses. Food Chem. 63: 343.

Valsamaki K., Michaelidou A. and Polychroniadou A. 2000. Biogenic amine production in Feta cheese. Food Chem. 71: 259.

- Revised paper received November 5, 2009 Accepted December 21, 2009 -

SHORT COMMUNICATION

DEVELOPMENT OF A FLOTATION METHOD FOR DETECTING INSECT FRAGMENTS IN BRAN AND FINE BRAN

G.S. GERMINARA*, A. DE CRISTOFARO[1] and G. ROTUNDO[1]
Dipartimento di Scienze Agro-ambientali, Chimica e Difesa Vegetale,
Università degli Studi di Foggia, Via Napoli 25, 71100 Foggia, Italy
[1]Dipartimento di Scienze Animali, Vegetali, Università degli Studi di Molise,
Via De Sanctis, 86100 Campobasso, Italy
*Corresponding author: s.germinara@unifg.it

ABSTRACT

A simple and rapid flotation method for separating insect fragments in bran and fine bran was developed. A 25 g sample is properly hydrated by immersion and stirring (10 min) in hot (90°C) distilled water (600 mL) and then sieved (mesh sieve n. 140) under jets of cold tap water. The trapping is quantitatively transferred to a Wildman trap flask, dissolved in ethanol (60%) and floated with light mineral oil. The material floated is paper-filtered with a Buchner funnel under vacuum. Material obtained from three consecutive flotations and filtrations are microscopically examined and the insect fragments are counted. The new method which requires low input of facilities and non-toxic reagents could be useful to detect insect fragments in bran and fine bran used for the production of whole food or animal feed.

- Key words: animal feed, filth-test, hygienic quality, whole food -

INTRODUCTION

Cereals are an important source of food for humans and animals worldwide (Aisbitt *et al.*, 2008; Larson *et al.*, 2008). A large number of insect species attack and reproduce almost continuously in stored cereals and cereal-derived products (Domenichini, 1997; Süss and Locatelli, 2001; Buntin *et al.*, 2007). Insect infestation generally results in substrate contamination directly through body fragments, frass and cuticle and indirectly by microorganism dissemination, heat damage and molds (Magan *et al.*, 2003). As a consequence, insects are one of the main causes of biological contamination of cereal products and play an important role in the hygienic quality of raw materials and cereal products.

Several techniques have been developed to measure insect contamination in wheat (Quinn *et al.*, 1992; Schatzki *et al.*, 1993; Rotundo *et al.*, 2000; Perez-Mendoza *et al.*, 2003). While the use of bran and fine bran is increasing in the preparation of a wide variety of whole foods for human consumption and animal feed (Matz, 1991; Leelavathi and Rao, 1993; Sudha *et al.*, 2007), a specific method for isolating insect fragments in these wheat milling fractions is not currently available.

The test currently used to determine the presence of insect fragments is the same method used to assess insect contamination in flour and processed cereal products. This method involves acid hydrolysis of a sample, followed by extracting (flotation and filtration) and counting (microscopically) of the insect fragments. This method is not very suitable for assaying bran and fine bran. Given that the digestion time of plant fragments is very similar to that of insects, some insect fragments will be digested during sample hydrolysis. To avoid this problem, the hydrolysis must be stopped before the plant fragments are completely digested. This leads to the floating of a conspicuous amount of undigested plant fibre which makes it very difficult to count the insect fragments.

A simple flotation method was developed which exploits the hygroscopic nature of fibre-rich substrates, that causes a rapid weight increase through water adsorption, in contrast to the hydrophobic character of insect fragments, due to the lipid content of the cuticle layers. To this end, the hydration and flotation steps were optimised by studying various parameters such as the time necessary for an adequate hydration of the sample, the amount of material floated and the recovery of insect fragments from bran and fine bran samples to which a known number of insect legs had been added.

Finally, the accuracy of the method was evaluated by analysing the milled bran and fine bran fractions of wheat samples that had been previously contaminated with an increasing number of dead adult granary weevils, *Sitophilus granarius* (L.) (Coleoptera: Dryophthoridae).

MATERIALS AND METHODS

Insects

S. granarius were reared on whole wheat kernels in cylindrical glass containers (Ø 10 x 18 cm) covered with a fine wire screen (mesh sieve n. 18) and kept at 25°±2°C, 60±5% R.H. with a L12:D12 photoperiod. Dead adults were dried at 120°C for 4 h and their legs were used to contaminate bran and fine bran samples that were to be analysed in order to optimize the method. Whole dried insects were used to contaminate intact wheat kernel samples that were then employed to evaluate the method.

Chemicals

Denatured ethanol (purity 95%) was purchased at a local market and diluted (v:v) in distilled water to obtain different ethanol solutions (20, 40, 60, 80%). Petroleum ether (purity >95%, product n. 77379), light mineral oil (purity >95%, product n. M8410) and diethyl ether (purity > 99%, product n. 346136) were supplied by Sigma-Aldrich (Milan, Italy).

Preparation of bran and fine bran samples

Durum wheat (*Triticum durum* L., var. Simeto) samples (3 kg) were milled in a cereal mill (Buhler AG, MLU 202 model, Uzwil, Switzerland) to reproduce large-scale processing conditions. To exclude any insect contamination, wheat kernels were incubated at 25°±2°C and 60±5% R.H. for two months and sieved (mesh sieve n. 7) before milling.

Uninfested wheat samples (n = 8) were milled to obtain bran and fine bran that were to be used for the hydration and flotation steps. To test the accuracy of the method, bran and fine bran contaminated by insect fragments were prepared by adding increasing numbers of dried *S. granarius* adults (0, 9, 18, 36, 54, 72 insects/3 kg wheat sample) to the wheat samples before milling.

Development of the method

To determine the time needed to ensure an adequate hydration of the bran and fine bran, samples (25 g) from each fraction were placed in individual flasks (1 L) containing hot (90°C) distilled water (600 mL) and kept on a heated stirring plate. For each sample, the time needed for the bran and fine bran to be completely settled on the bottom of the flask was measured.

To establish the optimal conditions for separating the insect fragments from the plant fragments, the samples were diluted in different ethanol solutions (20, 40, 60 or 80%) in combination with petroleum ether or light mineral oil used as a flotation means. Samples of bran or fine bran to which 10 adult insect legs had been added were properly hydrated, transferred to a fine mesh stainless steel sieve (mesh sieve n. 140) and washed repeatedly with jets of cold tap water. The material retained by the sieve was transferred to a Wildman flask (1 L), dissolved in an ethanol solution and subjected to three consecutive flotations with petroleum ether or mineral oil (40 mL each time) following the procedure described in the Italian Official Method of Filth-test (G.U., 1994).

The material that had floated to the neck of the flask was skimmed off and filtered through a disc (Ø 110 mm) of lined filter paper (Schleicher & Schuell, 0858, Dassel, Germany) in a Buchner funnel connected to a water vacuum pump. The content on the filters (n = 3 per sample) was checked under a stereomicroscope and the number of insect legs was counted. When mineral oil was used, the filters with the retained materials were rinsed with diethyl ether to remove the oil. After solvent evaporation, filters were dried (105°C) to a constant weight. The percent recovery of insect legs, after arcsin $\sqrt{x}$ transformation, and the dry weight of the floated material in the various flotation tests were then subjected to analysis of variance (ANOVA) followed by Student *t*-test or Least Significant Difference (LSD) test for the separation of means. The SPSS (Statistical Package for the Social Sciences) version 10.0.7 for Windows (SPSS Inc., Chicago, IL) was used for the analyses.

Evaluation of the method

Each bran and fine bran fraction from the wheat kernel samples that had been contaminated with varying numbers of adult insects before milling was spread out on a flat surface and divided into 4 equal portions. After mixing and scattering the opposite quarters, small aliquots of test material were collected from 5 different points to get 4 samples (25 g each) for analysis. These were analysed using the optimal conditions established for sample hydration and flotation; filtration and microscopic examination of the filters were performed according to the Italian Official Method of Filth-test (G.U., 1994).

A linear regression analysis was performed between the mean number of insect fragments found in the bran and fine bran samples and the number of insects added to the wheat samples before milling. The coefficient of determination (R^2) and the significance (P) of the angular coefficients of the regression lines were calculated (Alder and Roessler, 1977).

RESULTS

Development of the method

Bran and fine bran samples settled completely to the bottom of the flask within 10 min. Dissolving the bran samples in 60 or 80% ethanol solution significantly reduced the quantities of floating material compared with samples dissolved with lower alcohol concentrations using either petroleum ether or mineral oil (Table 1). Using 60 or 80% ethanol, the amount of floating material was significantly less in mineral oil compared with petroleum ether.

Fine bran samples dissolved in 60% ethanol had significantly less floating material than those dissolved in lower alcohol concentrations, with both petroleum ether and mineral oil. From 60 to 80% ethanol, petroleum ether significantly reduced the amount of floating material (Table 1). From 20 to 60% ethanol, the amount of floating material was significantly less in mineral oil compared with petroleum ether.

Table 1 - Mean quantity (± S.D.) of material (plant fragments and insect legs) floated by petroleum ether or mineral oil in bran and fine bran samples (n = 5) dissolved in different percentages of ethanol solutions.

Ethanol (%)	Material floated (g)			
	Bran		Fine bran	
	Petroleum ether	Mineral oil	Petroleum ether	Mineral oil
20	3.00±1.28 **a**	3.03±0.78 **a**	4.21±1.80 **a**	2.77±0.45 **a***
40	2.52±0.96 **b**	2.41±0.19 **b**	2.36±0.89 **b**	1.62±0.38 **b***
60	1.72±0.51 **c**	0.19±0.10 **c***	1.31±0.32 **c**	0.42±0.09 **c***
80	1.32±0.25 **c**	0.18±0.04 **c***	0.54±0.13 **d**	0.34±0.14 **c**

Values in the same column with different letters are significantly different at P = 0.05 (LSD test).
For a set substrate and an ethanol solution, the asterisk indicates significant differences at P = 0.05 (Student's *t*-test) between the mean quantities of material floated by petroleum ether or mineral oil.

Table 2 - Mean percent recovery (± S.D.) of insect legs from bran and fine bran samples (n = 5) dissolved in different percentages of ethanol solutions and floated with petroleum ether or mineral oil.

Ethanol (%)	Insect legs (%)			
	Bran		Fine bran	
	Petroleum ether	Mineral oil	Petroleum ether	Mineral oil
20	58.00±13.04 **a**	78.00±4.47 **a***	34.00±5.48 **a**	70.00±7.07 **a***
40	64.00±13.42 **a**	84.00±5.48 **ab***	44.00±5.48 **a**	76.00±5.48 **a***
60	68.00±13.04 **a**	92.00±8.37 **b***	78.00±14.83 **b**	90.00±7.08 **b**
80	72.00±19.24 **a**	92.00±4.47 **b**	88.00±4.47 **b**	90.00±7.08 **b**

Values in the same column with different letters are significantly different at P = 0.05 (LSD test).
For a set substrate and an ethanol solution, the asterisk indicates significant differences at P = 0.05 (Student's *t*-test) between the mean percent recovery of insect legs using petroleum ether or mineral oil.

The mean percent recovery of insect legs from bran samples with petroleum ether flotation ranged from 58 to 72% and did not change significantly with increasing ethanol concentrations (Table 2). Using mineral oil, the mean percent recovery of insect legs at 20% ethanol (78%) was significantly lower than that at 60 and 80% ethanol (92%). With ethanol solutions from 20 to 60%, the recovery of insect legs was significantly higher with mineral oil than with petroleum ether.

The mean percent recovery of insect legs from fine bran samples dissolved in 60 or 80% ethanol was significantly higher than those obtained at lower ethanol concentrations when floated with either petroleum ether or mineral oil (Table 2). At 60 and 80% ethanol, the mean recovery of insect legs did not vary significantly between the two flotation means. Using 20 and 40% ethanol, the mean percent recovery of insect legs was significantly higher when mineral oil was used compared to petroleum ether.

Evaluation of the method

The angular coefficients of the regression lines calculated between the mean number of insect fragments per bran or fine bran sample and the number of insects added to the corresponding wheat sample before milling were 0.05 for bran and 0.57 for fine bran (Fig. 1) and significant at the P = 0.05 and P = 0.01 levels, respectively.

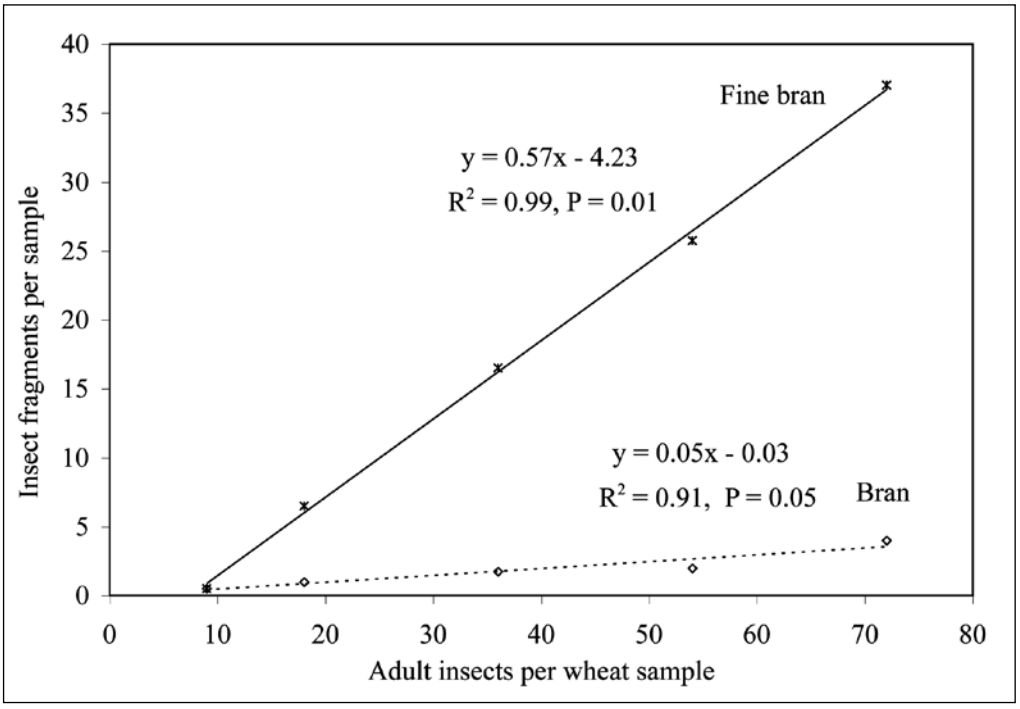


Fig. 1 - Linear regression analysis between the mean number of insect fragments in bran and fine bran samples (25 g; n = 4) and the number of adult insects (9, 18, 36, 54, 72) added to wheat samples before milling, coefficients of determination (R^2) and significance (P) of the angular coefficients.

The linear regression R^2 values were 0.91 and 0.99 for bran and fine bran, respectively.

CONCLUSIONS

The hydration of 25 g bran or fine bran sample in hot (90°C) distilled water (600 mL) and stirring for 10 min, resulted in a complete settling on the bottom of a glass flask (1 L).

Dissolving the hydrated bran or fine bran sample in increasing concentrations of ethanol resulted in a significant reduction of the floating material in the neck of the Wildman flask when either petroleum ether or mineral oil was used as a flotation mean.

A 60% ethanol solution was shown to ensure a small amount of plant fragments on the filters which allowed for easy detection and quick counting of the insect fragments. Mineral oil provided the best results both for a smaller amount of plant fragments floated and a larger number of insect legs recovered (≥ 90%). In addition, mineral oil is less volatile and less harmful than petroleum ether, so its use reduces the health risks for operators. Therefore, for a simple, accurate detection of insect fragments in bran and fine bran samples, a properly hydrated sample should be dissolved in a 60% ethanol solution followed by three consecutive flotations with mineral oil.

In this study, the high coefficient of determination (R^2) values and the significance (P) of the angular coefficient values indicate positive, significant correlations between the insect fragments found in bran and fine bran and the level of insect contamination in wheat. Consequently, the method developed provides a suitable way to measure the number of insects present in wheat before milling. Since regression analysis for fine bran showed the highest R^2 value and the lowest P value, this milling fraction is more suitable than the bran fraction for estimating the insect infestation level in cereal grains with a high accuracy.

The different values for the slopes of the regression lines indicate that different amounts of insect fragments were found in bran and fine bran samples. This result suggests that a different distribution of insect fragments occurs during the milling process.

The method here developed requires very simple equipment and inexpensive, non-toxic chemical reagents. Therefore, it can be used in the whole food and animal feed processing industries to assess the insect contamination in bran and fine bran.

REFERENCES

Aisbitt B., Caswell H. and Lunn J. 2008. Cereals - current and emerging nutritional issues. Nutrition Bulletin 33:169.

Alder H.L. and Roessler E.B. 1977. "Introduction to Probability and Statistics", p. 426. Freeman WH. & Co., San Francisco, CA.

Buntin G.D., Pike K.S., Weiss M.J. and Webster J.A. 2007. "Handbook of Small Grain Insects", p. 144. Entomological Society of America & APS Press, Lanham, MD.

Domenichini G. 1997. "Atlante delle Impurità Solide negli Alimenti" p. 408. Chiriotti Editori. Pinerolo (To), Italy.

G.U. 1994. Gazzetta Ufficiale della Repubblica Italiana n. 186, 10 agosto 1994, supplemento n. 114. "Approvazione dei metodi ufficiali di analisi dei cereali e derivati". Supplemento n. 4. "Determinazione delle impurità solide (Filth-Test) negli sfarinati e nei prodotti di trasformazione": p. 29.

Larson Z., Subramanyam B. and Herrman T. 2008. Stored-product insects associated with eight feed mills in the midwestern United States. J. Econ. Entomol. 101: 998.

Leelavathi K. and Rao P.H. 1993. Development of high fibre biscuits using wheat bran. J. Food Sci. Technol. 30: 187.

Magan N., Hope R., Cairns V. and Aldred D. 2003. Postharvest fungal ecology: impact of fungal growth and mycotoxin accumulation in stored grain. Eur. J. Plant Pathol. 109: 723.

Matz S.A. 1991. "The Chemistry and Technology of Cereals as Food and Feed", p. 751. Van Nostrand Reinhold, New York, Usa.

Perez-Mendoza J., Throne J.E., Dowell F.E. and Baker J.E. 2003. Detection of insect fragments in wheat flour by near-infrared spectroscopy. J. Stored Prod. Res. 39: 305.

Quinn F.A., Burkholder W. and Kitto G.B. 1992. Immunological technique for measuring insect contamination of grain. J. Econ. Entomol. 85: 1463.

Rotundo G., Germinara G.S. and De Cristofaro A. 2000. Immuno-osmophoretic technique for detecting *Sitophilus granarius* (L.) infestation in wheat. J. Stored Prod. Res. 36: 153.

Schatzki T.F., Wilson E.K., Kitto G.B., Behrens P. and Heller I. 1993. Determination of hidden *Sitophilus granarius* (Coleoptera: Curculionidae) in wheat by miosin ELISA. J. Econ. Entomol. 86: 1584.

Sudha M.L., Vetrimani R. and Leelavathi K. 2007. Influence of fibre from different cereals on the rheological characteristics of wheat flour dough and on biscuit quality. Food Chem. 100: 1365.

Süss L. and Locatelli D.P. 2001. "I Parassiti delle Derrate", p. XIV-364. Calderini Edagricole, Bologna, Italy.

Paper received May 6, 2009 Accepted September 8, 2009

ANALYSIS OF FACTORS AFFECTING TURKISH SUNFLOWER OIL CONSUMER BEHAVIOR: THE CASE OF ERZURUM PROVINCE

Y. TOPCU*, B. TURHAN[1] and A.S. UZUNDUMLU
Department of Agricultural Economics, College of Agriculture, Ataturk University, 25240-Erzurum, Turkey
[1]Hospital of Egirdir Bone and Joint Infections, Isparta, Turkey
*Corresponding author: Tel. +90 442 231 1393, Fax +90 442 231 2678
e-mail: ytopcu@atauni.edu.tr

ABSTRACT

Sunflower oil consumption in Turkey has been increasing for the past decade due to dietary and health concerns. However, consumption of sunflower oil exceeds its production. Efforts have been made to overcome the production deficit for the last decade by importing the oil, but this deficit has continued to increase. The aim of this study was to explore Turkish consumer behaviour with respect to sunflower oil consumption in the city of Erzurum. Factor and regression analyses were used to determine significant factors related to sunflower oil attributes and the socioeconomic and demographic characteristics of the consumers. To this end, factor and multiple linear regression analyses using the SPSS statistical program were carried out on the data from a 250-household survey. The results show that consumer satisfaction with sunflower oil and its price play an important role in consumer buying decisions and the amount bought is related to sunflower oil attributes. With regard to consumer buying power and frequency, vegetable oil expenditures are directly affected by the stage of the life-cycle and job position. These factors also play an important role in consumer buying attitudes and behaviour. These findings are also beneficial to marketers, manufacturers and retailers for product design and developing new marketing tactics and strategies.

- Key words: consumer behaviour, factor analysis, regression analysis, sunflower oil -

INTRODUCTION

Fats are usually divided into two groups: animal fats and vegetable oils. Consumption of animal fats, such as butter and edible tallow which are high in saturated fats has remained stable. In contrast, vegetable oils, such as sunflower, corn, soybean, olive and cottonseed oil which are low in saturated fats are covering an increasing percentage of the total fat and oil intake (GRIGG, 1999; BAYAR and YILMAZ, 2004; GOKSU, 2007).

Fats and oils improve the texture and mouthfeel of foods and are a source of energy. If consumed in the correct proportion to other foods, they are a primary source of energy. In addition, fats and oils help humans absorb fat-soluble vitamins, including vitamins A, D, E and K, and they are necessary building blocks of cellular membranes. Sterols found in fats and oils also help hormone production and regulation and the absorption of calcium (GRIGG, 1999; DEMIRCI and BOLUKBASI, 2003; AYAZ, 2008; NSA, 2009; URE, 2009).

To consume the right kind of fats or oils is very important for health and dietary concerns. Sunflower oil is light in taste and appearance and supplies more Vitamin E than any other vegetable oil. In addition, it is a combination of monounsaturated and polyunsaturated fats with low saturated fat levels. Therefore, consumers have shown a strong preference for sunflower oil because about 90% of the fat found in sunflower oil is unsaturated, so cholesterol levels are lower (GRIGG, 1999; NSA, 2009). Consumers prefer sunflower oil because of the taste, frying performance and health benefits. The vitamin E in sunflower is a required nutrient that functions as an antioxidant and decreases the risk of heart disease and cancer and bolsters immune function (GRIGG, 1999; NSA, 2009).

Spurred by increased income and a 1.3% population growth in Turkey, as well as rapidly expanding food processing industries and socioeconomic changes, the overall growth in consumption of sunflower oil is outpacing that of most other vegetable oils. While the average annual consumption of sunflower oil (sunflower oil production plus import minus export) in the world, EU and Turkey increased by 2.1, 3.3 and 6.4%, respectively, between 2000 and 2007; that of corn, soybean and olive oils in the world, EU and Turkey changed as follows: 0.9, 5.4 and 2.0%; -3.4, 8.8 and 1.8%; 4.5, -6.1 and -2.9%, respectively (Table 1).

When sunflower oil consumption in Turkey is compared with that in the EU and the world, it is clear that Turkish sunflower oil consumption is much higher. While sunflower oil production and importation have been increasing in the last decade, its exportation has been steadily decreasing. This is due to the domestic consumption of sunflower oil; about 50% of all vegetable oil consumed in Turkey is sunflower oil (KOLSARICI *et al.*, 2000; GOKSU, 2007). Sunflower oil consumption per capita in 2000 and 2007[1] was 1.6 and 1.7 kg in the World, 5.4 and 6.7 kg in the EU, and 7 and 9.7 kg, in Turkey, respectively (Table 1). This shows that sunflower oil consumption per capita increased more in Turkey than in the EU and throughout the world.

Considering sunflower oil consumption and production levels in Turkey, it is clear that sunflower oil production can not meet the national demand. While total consumption of sunflower oil was 467,782 and 707,520 tons, in 2000 and 2007, respectively, production in 2000 and 2007 was 481,370 and 577,260 tons, respectively (FAOSTAT, 2009). These figures show that the sunflower oil deficit has increased gradually each year. To offset this deficit, the importation of sunflower oil has increased. This has also caused an important foreign currency outflow.

Given these trends, the following questions arise: Why do people tend to prefer more sunflower oil? What salient attributes of sunflower oil attract consumers to purchase it? To answer these questions, it is important to analyze Turkish sunflower oil consumers' attitudes. Today consumers have more choices, so purchasing decisions are more complex and many factors influence purchasing decisions. These factors can be classified as marketing-related, psychological and sensorial factors (TOPCU *et al.*, 2009). Consumer attitudes and behaviour in relation to the purchase of vegetable oil have been widely studied (NIELSEN *et al.*, 1998; DA COSTA *et al.*, 2000; MURRAY and DELAHUNTY, 2000; WĄDOŁOWSKA *et al.*, 2002; ZENNER *et al.*, 2003; KRYSTALLIS and NESS, 2005; NEWBY *et al.*, 2006; AKBAY, 2007; HADDAD *et al.*, 2007; TOPCU *et al.*, 2008, 2009; TOPCU and ISIK, 2008; TOPCU, 2009; TOPCU and UZUNDUMLU, 2009). These studies have shown that the most important factors affecting consumer decisions were price, quality, oil type, brand-name, nutritional value, taste, freshness, health and dietary concerns.

Consumers have limited processing capacity and so they can use only a certain part of the available information when choosing a product in order to reduce the complexity of selection (LAROCHE *et al.*, 2001). Brand-name can make this process less complex since consumers believe that brand, price and quality are closely correlated. Consumers create a link between brand-names and product attributes. If the product is a well-known, trusted brand-name then, without further questioning of the price or quality, the consumer decides to purchase the product. The brand benefits the decision-making process by identifying major attributes and the quality image of the product which reduces the feeling

Table 1 - Total annual vegetable oil consumption and per capita consumption*.

Oil types		World				EU				Turkey			
		2000	2005	2006	2007	2000	2005	2006	2007	2000	2005	2006	2007
Sunflower	**C**	9893	10387	11325	11527	2601	2857	3419	3290	468	711	927	708
	P	**1.6**	**1.6**	**1.7**	**1.7**	**5.4**	**5.8**	**7.0**	**6.7**	**7.0**	**10.0**	**12.9**	**9.7**
Corn	**C**	2049	2108	2081	2195	260	210	212	189	112	124	140	152
	P	**0.3**	**0.3**	**0.3**	**0.3**	**0.5**	**0.4**	**0.4**	**0.4**	**1.7**	**1.7**	**2.0**	**2.1**
Soybean	**C**	25249	33557	33506	36143	1947	2507	3112	3310	231	388	301	119
	P	**4.1**	**5.2**	**5.1**	**5.4**	**4.0**	**5.1**	**6.3**	**6.7**	**3.5**	**5.5**	**4.2**	**1.6**
Olive	**C**	2645	2707	2877	3064	1790	1821	2138	2043	171	23	92	132
	P	**0.4**	**0.4**	**4.4**	**0.5**	**3.7**	**3.7**	**4.4**	**4.1**	**2.6**	**0.3**	**1.3**	**1.8**

Source: (FAOSTAT, 2009).
C: Annual consumption (1,000 tons); P: Annual consumption per capita (kg).
*Consumption and per capita consumption for all years were calculated as (production+import/export) and (production+import/export)/population from FAOSTAT data, respectively.

of risk. Moreover, the brand-name can give the consumer prestige in social settings, and it allows them to learn and evaluate the results of different shopping experiences.

The aim of this study was to analyze Turkish sunflower oil consumer behaviour and purchasing decisions in Erzurum, Turkey. Due to the increasing consumer demand for sunflower oil and the growing competition between manufacturers and retailers, this study could be important for determining variations in consumer trends due to different sunflower oil attributes.

MATERIAL AND METHODS

The preliminary data used in this study were obtained from a survey conducted in Erzurum[2], Turkey. In order to determine the sample size, while minimizing sample bias and representing the population correctly, the city centre was divided into three districts: the west-side Aziziye district with 6,562 households, the south-side Palandoken district with 30,022 households, the nord and east-sides Yakutiye district with 44,077 households (ANONYMOUS, 2007).

To determine the sample size for each district, the following formula was used (TOPCU, 2009):

$$n = \frac{Z^2 * p * (1 - p)}{c^2} = 250$$

Where:
n = sample size
Z = Z value, (1.96 for 95% confidence level)
p = percentage making a choice, (0.8 used for sample size needed)
c = confidence interval, (used 0.05 = ±5).

Then, based on the population of each district, the weighted sample size and distribution of the surveys for each district were determined proportionally. A total of 250 surveys were distributed with 20, 93 and 137, questionnaires allocated to the Aziziye, Palandoken and Yakutiye districts, respectively.

Each district was divided into sub-districts and then a face-to-face survey was conducted with randomly selected heads of households (either the wife or husband) in various parts of the sub-districts. The survey was conducted either in their home or at shopping centres.

Participants in the survey were asked to respond to each statement, indicating the significance level of the sunflower oil attributes for them. A Likert-format 1-5 scale was used (where 1 refers to the least important and 5 refers to the most important attribute). Of the 35 attributes, five are related to physical attributes (packaging material and appearance, product weight (litre (*L*)), shelf life, ease-of-use), nine are related to sensory and real quality (food safety and hygiene, brand name, loyalty and individuality, health and dietary concern, nutrition and energy value, colour and viscosity, taste and flavour, quality), eight are related to the sale-increasing activities (ISO registration certificate, promotion/discount, price, brand penetration, new design, product variety, outreach to customer, brand/retailer advertisement), ten are related to individual preferences (specialized product line, trust of manufacturer and retailer, manufacturer experience, use of environment-friendly material, price-quality relation (perceived value of food product), product origin, natural/ecological oil, retailer loyalty, consumer experience), three are related to social status (reference groups and influence of customs, roles and status). Each question was reduced to a single statement to which responses were collected using the Likert scale described above.

Demographic and socioeconomic information about the consumer was also gathered (gender, age and occupation group, education, vegetable oil, food and other expenditures, social influence, income, etc.) in the survey. These data consist

of string variables (i.e. gender: 1: male and 2: female; occupation: 1: businessman, 2: blue-collar state worker, 3: white-collar state employee, 4: small-scale retailer, 5: pensioner, 6: unemployed, 7: housewife and 8: student education: 1: literate, 2: elementary school, 3: high school and 4: college) and numeric variables (i.e. age, monthly vegetable oil expenditure (€/*L*), food and other expenditures) that are measured by scale and nominal measures.

After editing and coding, the data were first subjected to factor analysis to determine the main factors related to sunflower oil attitudes and the demographic and socioeconomic characteristics that affect sunflower oil buying behaviours. Regression analysis was then carried out to measure the effectiveness of target consumers' buying behaviour. The SPSS statistical software was used to perform these two analyses.

These techniques have been widely used in marketing studies that analyze food product attributes (O'CONNOR *et al.,* 2006; TOPCU, 2006; ENNEKING *et al.,* 2007; HADDAD *et al.,* 2007; TOPCU and ISIK, 2008; WADOLOWSKA *et al.,* 2008; TOPCU and UZUNDUMLU, 2009; TOPCU *et al.*, 2009).

Factor analysis is a data reduction technique that reduces the number of variables used in an analysis by creating new variables (called factors) that combine redundancy in the data (SPSS 15.0, 2006).

The first step in factor analysis is to determine the number of relevant factors. The factor analysis in this study reduced the number of sunflower oil attributes and consumers' demographic and socioeconomic characteristics from 35 to 9 and the number of factors from 16 to 5 (total of 14 factors) having Eigen-values greater than 1.0. This was done by principal component analysis[3] using the varimax rotation method[4]. Factor analysis was used initially to identify underlying aspects that could explain a correlation among a set of food attributes and the socioeconomic and demographic attitudes and the associated consumption values. The purpose of the factor analysis was to identify those attributes and consumers' demographic and socioeconomic characteristics that account for a relatively large proportion of the variance in the sample.

In the second step of the analysis, the data obtained from the factor analyses were used for multiple linear regression analysis. For this end, linear functional form and enter regression selection technique were chosen to estimate the relationship between a dependent variable and the independent variables. SPSS 15.0 statistical software program was used for both factor and regression analyses. The multiple linear regression model could be written as follows:

$$SFC = f(F1, F2, F3, F4, F5, F6, F7, F8, F9, F10, F11, F12, F13, F14, \text{å}_i)$$

Dependent Variable

SFC: Monthly sunflower oil consumption (L/month)

Independent Variables

F1 : Social status
F2 : Brand loyalty
F3 : Food reliability
F4 : Product freshness
F5 : Manufacturer image
F6 : Retailer loyalty
F7 : Satisfaction with food quality
F8 : Product price (€/L)
F9 : Consumer experiences
F10 : Current expenditure (€)
F11 : Family life-cycle stage
F12 : Vegetable oil expenditure (€/L)
F13 : Job position
F14 : Fixed expenditure (€)

Coefficient estimates were obtained using ordinary least squares (OLS). Individual and group significance of these coefficients were tested using t and F tests, respectively. Multicollinearity among variables was detected by calculating the variance-inflating factor (VIF). The presence of auto-correlation was assessed using the Durbin-Watson d statistic (GUJARATI, 2005; SPSS 15.0, 2006).

RESULTS AND DISCUSSION

Kaiser Normalization (KMO) which compares partial correlation coefficients with observed ones was calculated as 0.83 for sunflower oil attributes and as 0.69 for consumer demographic and socioeconomic characteristics, which means that the data sets are at a perfect level for factor analyses since the test score is greater than 0.50 (Tables 2 and 3).

The principal component analysis, using varimax rotation, first grouped 35 variables related to sunflower oil attributes into nine factors with Eigen-values greater than 1.0. These nine factors explained 62.29% of the total variance. F1 accounted for 24.82% of the total variance and was dominated by attributes and customs of the reference group. Therefore, F1 is associated with social status. F2 accounted for 8.94% of the total variance, and is information about brand and usage. Hence, F2 is more associated with brand loyalty. F3 reported 6.15% of the total variance, and was dominated by food safety, hygiene and food content. So, it could be named as food reliability. F4 explained 4.59% of the total variance and related to product freshness due to natural or ecological product with shelf-life, taste and flavour attributes. F5, F6, F7, F8 and F9 accounted for 4.25, 3.70, 3.54, 3.29 and 3.02% of the total variance, respectively, on the other hand; and dealt with manufacturers and retailers attitudes, the product sensory quality

Table 2 - Factors and correlated variable loadings related to sunflower oil attributes.

Variables	Factor loadings*								
	F1	**F2**	**F3**	**F4**	**F5**	**F6**	**F7**	**F8**	**F9**
Social status (F1)									
Reference group influence	**0.795**	-0.013	0.112	0.114	0.005	0.085	0.050	-0.078	0.111
Roles and status	**0.729**	0.142	0.048	0.126	-0.065	0.210	0.065	0.063	0.044
Brand personality	**0.728**	0.269	0.074	-0.003	0.276	0.176	-0.021	0.013	-0.138
Influence of customs	**0.582**	0.114	0.034	-0.160	-0.011	-0.127	0.311	0.039	0.272
Brand loyalty (F2)									
Product origin	-0.013	**0.721**	0.211	0.031	0.091	0.077	0.145	0.076	-0.049
Brand penetration	0.174	**0.612**	0.150	0.317	0.226	0.108	0.102	-0.091	0.187
New design	0.242	**0.586**	0.122	0.150	0.199	0.099	0.020	0.104	-0.161
Brand name	0.155	**0.553**	0.106	0.117	0.026	0.325	0.290	-0.015	0.146
Packaging appearance	0.238	**0.541**	-0.060	0.062	0.008	0.341	-0.055	0.203	0.218
Ease-of-use	0.090	**0.508**	0.339	0.009	-0.111	-0.030	0.103	0.192	0.366
Brand loyalty	0.187	**0.507**	0.160	-0.289	0.203	0.197	0.392	0.132	0.128
Food reliability (F3)									
Health and dietary concern	0.066	0.050	**0.706**	0.200	0.146	-0.139	0.069	0.095	-0.054
Food safety and hygiene	0.019	0.041	**0.651**	0.107	0.035	0.076	0.300	0.028	0.166
Nutrition and energy value	0.074	0.242	**0.619**	0.289	0.074	0.218	-0.042	0.122	0.011
Oil colour and viscosity	-0.056	0.292	**0.600**	0.003	0.167	0.099	0.279	-0.060	0.063
Packaging material	0.042	0.083	**0.538**	0.058	0.073	0.288	-0.066	0.290	0.214
Weight	-0.101	0.354	**0.504**	0.223	0.273	0.015	-0.104	0.139	0.357
Product freshness (F4)									
Natural/ecological oil	0.103	0.366	0.084	**0.738**	0.119	0.178	0.020	-0.103	-0.012
Shelf-life	0.056	-0.015	0.266	**0.706**	-0.012	-0.102	0.282	0.008	-0.025
Taste and flavour	0.174	0.038	0.227	**0.568**	0.269	0.025	0.049	-0.035	0.234
ISO registration certificate	0.208	0.261	0.312	**0.407**	0.094	0.273	-0.142	0.066	0.045
Manufacturer image (F5)									
Specialized at a product line	0.097	-0.057	0.086	0.174	**0.775**	0.154	-0.095	0.123	0.062
Trust to manufacturer	0.077	0.140	0.189	0.154	**0.762**	0.047	0.133	0.030	0.203
Manufacturer experience	0.083	0.116	0.217	0.044	**0.703**	0.351	-0.002	0.067	0.047
Retailer loyalty (F6)									
Using environment-friendly material	0.163	0.235	0.049	0.111	0.184	**0.691**	0.227	0.138	0.039
Product variety	0.172	0.053	0.325	0.020	0.218	**0.675**	0.111	0.134	-0.057
Trust to retailer	-0.389	0.333	-0.062	0.314	0.473	**0.589**	0.134	-0.018	-0.034
Retailer loyalty	-0.175	0.259	-0.306	0.031	0.114	**0.547**	0.317	0.060	-0.052
Reach to customers	0.273	0.329	0.009	0.055	0.382	**0.508**	0.118	0.054	-0.140
Brand/retailer advertisement	0.334	0.115	-0.186	-0.166	-0.248	**0.506**	-0.053	-0.076	0.313
Satisfaction with food quality (F7)									
Quality	0.073	0.015	0.290	0.211	0.062	0.073	**0.679**	-0.011	-0.024
Quality-price relation	-0.015	-0.046	-0.015	0.270	0.114	0.075	**0.577**	0.496	0.250
Product price (F8)									
Price	0.013	0.073	0.119	-0.040	0.070	0.075	0.064	**0.868**	-0.100
Promotion/discount	0.144	0.246	0.180	-0.182	-0.052	0.111	-0.123	**0.587**	0.141
Consumer experience (F9)									
Consumer experience	0.068	0.014	0.226	0.096	0.194	0.060	0.139	0.045	**0.769**
Eigen-value	**8.686**	**3.130**	**2.153**	**1.606**	**1.486**	**1.293**	**1.238**	**1.151**	**1.056**
Share of explained variance (%)	**24.817**	**8.944**	**6.152**	**4.589**	**4.245**	**3.696**	**3.537**	**3.289**	**3.018**
Cumulative share of explained variance (%)	**24.817**	**33.762**	**39.913**	**44.502**	**48.747**	**52.442**	**55.979**	**59.268**	**62.286**
KMO (Kaiser-Meyer-Olkin) Statistic									**0.831**
Bartlett's test of Sphericity									**(Chi-square, df: 595): 3555.608 (p: 0.000)**

*Bold numbers indicate the largest loading for each variable.

Table 3 - Factors and correlated variable loadings related to consumer demographic and socioeconomic characteristics.

Variables	Factor loadings*				
	F10	F11	F12	F13	F14
Current Expenditure (F10)					
General food expenditure	**0.903**	0.024	0.099	-0.038	-0.045
Food expenditure in markets	**0.897**	0.040	-0.047	0.002	0.046
Clothing expenditure	**0.596**	0.001	0.410	0.027	0.037
Family life-cycle stage (F11)					
Lifestyle	-0.009	**0.856**	0.027	-0.094	0.315
Marital status	-0.037	**0.813**	-0.005	0.005	0.232
Age	0.014	**0.806**	0.082	-0.182	-0.026
Gender	-0.064	**0.767**	0.101	-0.295	0.067
Vegetable oil expenditure (F12)					
Store-brand oil expenditure	-0.165	-0.091	**0.805**	-0.197	0.085
Manufacturer-brand expenditure	0.165	-0.154	**0.695**	-0.139	0.215
Job position (F13)					
Income	-0.004	-0.048	0.117	**0.791**	0.224
Education	-0.034	0.114	-0.068	**0.790**	0.020
Occupation	0.065	0.535	-0.002	**0.581**	0.143
Fixed expenditure (F14)					
Education expenditure	0.378	0.073	0.040	0.121	**0.545**
Sheltering expenditure	0.331	-0.052	-0.002	0.368	**0.540**
Health expenditure	0.214	0.176	-0.144	0.299	**0.526**
Entertainment expenditure	0.329	0.100	0.184	0.227	**0.419**
Eigen-value	**3.616**	**2.459**	**1.764**	**1.299**	**1.127**
Share of explained variance (%)	**22.601**	**15.368**	**11.027**	**8.119**	**7.042**
Cumulative share of explained variance (%)	**22.601**	**37.969**	**48.996**	**57.115**	**64.157**
KMO (Kaiser-Meyer-Olkin) Statistic					**0.690**
Bartlett's test of Sphericity					**(Chi- square, df: 120): 1373.407 (p:0.000)**

*Bold numbers indicate the largest loading for each variable.

and price and consumer experiences. The factors were therefore called manufacturer image, retailer loyalty, satisfaction with food quality, product price and consumer experience, respectively (Table 2).

For the second factor analysis based on principal component analysis, using varimax rotation, 16 variables were grouped into five factor groups related to consumers' demographic and socioeconomic characteristics with Eigenvalues greater than 1.0. These five factors explained 64.16% of the total variance. F10, F11, F12, F13 and F14 accounted for 22.60, 15.37, 11.03, 8.12 and 7.04% of the total variance, respectively. Therefore, these factors were named current expenditure, family life-cycle stage, vegetable oil expenditure, job position and fixed expenditure, respectively (Table 3).

The overall multicollinearity in the model was measured by calculating the variance inflating factor (VIF) (Table 3). The calculated VIF values were between 1.006 and 1.086, indicating acceptable levels of multicollinearity since the values were between 1.00 and 2.50. The Durbin-Watson d statistic was used to determine the auto-correlation. The computed d value (2.46) lies between d_u (1.92) and 4-d_u (1.62). Therefore, in the regression model there is no problem related to auto-correlation or multicollinearity (KALAYCI, 2005). So, this data set could be used in regression analysis.

In the regression model, the regression coefficient *(Adj.R^2)* was calculated to be 0.81. The OLS estimates of the model coefficients and other statistical measurements are shown in Table 4. The F-statistic was calculated to be 75.61 (p: 0.000) and the null hypothesis was rejected; all coefficients were equal to zero. On the other hand, the partial regression coefficients of *F4, F7, F8, F10, F11, F12, F13, F14* and *F3, F9* were found to be meaningful at the 1 and 5% significance levels, respectively, an addition, all regression coefficient signs were found to be correct and complied with economic theory.

Two factors, the coefficients of sunflower oil price (*F8)* and fixed expenditure *(F14)* have negative signs (Table 4). As *F8* and *F14* among consumer expenditures increased, the amount of

sunflower oil consumption decreased noticeably. These results show that, if food prices on the competitive market and fixed expenditures among expendable income of consumers increase, sunflower oil consumption decreases with respect to the demand. These results are similar to those reported in previous studies conducted for vegetable and olive oils (MTIMET *et al.*, 2008; TOPCU *et al.*, 2008; TOPCU, 2009).

The other factor coefficients have positive signs; when their effects increased, sunflower oil consumption increased significantly. Sunflower oil attributes, consist of food reliability *(F3)*, product freshness *(F4)*, satisfaction with food quality *(F7)* and consumer experiences *(F9)*, while consumers' socioeconomic and demographic characteristics, include current expenditure *(F10)*, family life-cycle stage *(F11)*, vegetable oil expenditure *(F12)* and job position *(F13)*. All of these factors affect consumer buying behaviour and exert an important influence on sunflower oil consumption (Table 4).

Consumer satisfaction with sunflower oil included the attributes of food reliability and freshness, satisfaction with food quality and consumer experiences which were the most important factors in the decision to purchase it. These findings are supported by previous research related to product satisfaction (MTIMET *et al.*, 2008; TOPCU *et al.*, 2008, 2009; TOPCU and UZUNDUMLU, 2009).

The results of this study also provide improved information about how consumer demographic and socioeconomic characteristics affect Turkish consumer attitudes toward sunflower oil. Based on current expenditures and associated with job position, vegetable oil expenditures are significantly affected by family life-cycle stage. As family life-cycle stage increased and job position improved, vegetable oil consumption increased (i.e. in Turkey more sunflower oil has been consumed in the last decade than vegetable oils such as corn, soybean and olive oil) (FAOSTAT, 2009).

In particular, sunflower oil has the highest vitamin E concentration among vegetable oils. It is widely used for meal sector, and is consumed more than other vegetable oils in Turkey (GOKSU, 2007). Consumption has increased yearly and expenditure for sunflower oil has steadily increased compared with other vegetable oils. This increase could be due to increased dietary and health concerns. It is also cheaper than the other oils because it is the most widely produced oil in Turkey. These results were obtained from previous research based on consumer demographic and socioeconomic characteristics (MTIMET *et al.*, 2008; TOPCU *et al.*, 2008, 2009; TOPCU, 2009; TOPCU and UZUNDUMLU, 2009).

CONCLUSION

The results of this study indicate that food reliability and freshness, satisfaction with food quality and consumer experiences (consumer satisfaction with sunflower oil and its price) play an important role in sunflower oil purchasing decisions and consumption. Considering consumer buying power and frequency, demographic and socioeconomic characteristics play an important

Table 4 - Multiple linear regression analysis results.

n: 250	**R^2: 0.82**		**Adj R^2: 0.81**	**F (14,235): 75.61***	**d_L=1.62**		**d_u=1.92**		**DW d_h=2.46**
Variables	**Multiple linear regression model**				**Collinearity statistics**		**Correlations**		
	Coefficients	Std.error	t_h-value	p-value	Tolerance	VIF	Zero-order	Partial	Part
Constant	50.956	1.259	40.472	0.000*	-	-	-	-	-
F1	0.003	1.287	0.114	0.909	0.921	1.085	0.091	0.679	0.088
F2	0.025	1.271	0.885	0.377	0.967	1.034	0.074	0.604	0.072
F3	0.052	1.313	2.573	0.011**	0.944	1.059	0.780	0.795	0.753
F4	0.047	1.286	2.656	0.010*	0.905	1.104	0.107	0.733	0.102
F5	0.029	1.277	1.032	0.303	0.944	1.060	0.043	0.389	0.040
F6	0.037	1.277	1.297	0.196	0.978	1.022	0.058	0.526	0.059
F7	0.025	1.281	2.882	0.008*	0.950	1.053	0.058	0.140	0.013
F8	-0.012	1.288	-3.054	0.003*	0.921	1.086	-0.160	0.078	0.007
F9	0.056	1.283	1.986	0.048**	0.992	1.008	0.069	0.084	0.008
F10	0.003	1.312	3.324	0.001*	0.941	1.062	0.018	0.020	0.002
F11	0.151	1.293	5.312	0.000*	0.971	1.030	0.017	0.038	0.004
F12	0.866	1.330	29.552	0.000*	0.958	1.044	0.136	0.043	0.004
F13	0.187	1.293	6.549	0.000*	0.994	1.006	0.064	0.025	0.002
F14	-0.168	1.289	-5.904	0.000*	0.930	1.075	-0.021	0.019	0.002

*p<0.01
**p<0.05.

role in consumer buying attitudes and level of consumption. Vegetable oil expenditures are affected by life-cycle stage and job position; about 50% of all vegetable oil consumption is sunflower oil. Sunflower oil consumption has continuously increased in parallel with the increase of all the factors considered except for fixed expenditure. These findings could also be beneficial for marketers, manufacturers and retailer for designing a product and developing new marketing tactics and strategies.

While this study has some scientific merit for the academic community and food manufacturing sector, it is not without some limitations. The results of this research have limited generalizability since the data were collected in a single city. If the survey was conducted on a national scale the larger data base would give more objective results about target consumer attitudes. In future studies, this model could be expanded to include more factors and other vegetable oils, and sunflower oil consumers could be divided into groups based on demographic and socioeconomic characteristics.

NOTES

[1] Total annual amounts of vegetable oil consumption and their consumption per capita in 2007 were calculated as (production+import/export) and (production+import/export)/population from FAOSTAT data, respectively.

[2] Erzurum (39°45'N latitude and 41°15'E longitude) is located in the north-east region of Turkey and is on the Silk Road. Erzurum is one of the biggest provinces in the region with a total population; 962,000 and the city centre population is 402,000.

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

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

REFERENCES

Anonymous 2007. Annual report, the data of documentation centre of Erzurum metropolitan municipality, Erzurum, Turkey.

Akbay C. 2007. Urban households' cooking oil and fat consumption patterns in Turkey: quality vs. quantity. Quality and Quantity 41: 851.

Ayaz A. 2008. Importance for diet of seed oils. Hacettepe University, Health Science Institutes, Ankara, Turkey.

Bayar R. and Yılmaz M. 2004. Importance of soybean oil for Turkey economy. ISSN: 1303-5134, J. Inter. Human Sci., 4:1.

Da Costa M.C., Deliza R., Rosenthal A., Hedderley D. and Frewer L.J. 2001. Non-conventional technologies and impact on consumer behavior. Trends Food Sci. Technol., 11: 188.

Demirci M. and Bolukbasi B. 2003. Olive oil importance in Mediterranean diet types. Turkey 1st Olive Oil and Diet Olive Congress, Izmir, Turkey.

Enneking U., Neumann C. and Henneberg S. 2007. How important intrinsic and extrinsic product attributes affect purchase decision. Food Qual. Prefer. 18: 133.

FAOSTAT 2009. Food and Agriculture Organization of the United Nations (FAOSTAT): Production, Consumption and trade. Cited 3 March 2009, available on the internet: http://faostat.fao.org/site/609/default.aspx#ancor.

Goksu C. 2007. Report of vegetable oils. Cited 30 December 2007, available on the internet: www.igeme.org.tr/tur/rapor/sektor/bitkisel_yaglar.

Grigg D. 1999. The fat of the land: a geography of oil fat consumption. Geojournal 48: 259.

Gujarati D.N. 2005. Basic econometrics p. 540. Reference Publish: 33, ISBN 975-7860-99-9, Istanbul.

Haddad Y., Haddad J., Olabi A., Shuayto N., Haddad T. and Toufeili I. 2007. Mapping determinants of purchase intent of concentrated yogurt (Labneh) by conjoint analysis. Food Qual. Pref. 18: 795.

Kalaycı S. 2005. SPSS Applications, Multi-Variables Statistics Techniques. p. 273. Asil Publish, First Edition, ISBN 975-9091-14-3, Ankara.

Kolsarici O., Basalma D., Isler N., Arıoglu H., Gur A., Olhan E. and Saglam C. 2000. Production of vegetable oils. Cited 30 December 2007. Available on the internet http://www.zmo.org.tr/etkinlikler/5tk02/20.pdf.

Krystallis A. and Ness M. 2005. Consumer preference for quality foods from a South European Perspective: A Conjoint Analysis implementation on Greek olive oil. Int. Food Agribus. Manage. Rev., 8: 62.

Laroache M., Teng L. and Kalamas M. 2001. Consumer evaluation of net utility: effect of competition on consumer brand selection. Japan. Psych Res. 43: 168.

Mtimet N., Kashiwagi A.K., Zaibet L. and Masakazu N. 2008. Exploring Japanese olive oil consumer behavior. 12th EAAE Congress, people, food, environments: global trends and European strategies, Gent (Belgium), 26-29 August 2008.

Murray J.M. and Delahunty C.M. 2000. Mapping consumer preference for the sensory and packaging attributes of Cheddar cheese. Food Qual. Pref. 11: 419.

Newby P.K., Weismayer C., Åkesson A., Tucker K.L. and Wolk A. 2006. Long-term stability of food patterns identified by use of factor analysis among Swedish women. J. Nutr. 136: 626.

Nielsen N.A., Bech-Larsen T. and Grunert K.G. 1998. Consumer purchase motives and product perceptions: a laddering study on vegetable oil in three countries. Food Quality Prefer. 9 : 455.

NSA 2009. Sunflower oil a health way to eat. National Sunflower oil Assosiation (NSA), Cited 3 March 2009. Available on the internet: http://www.sunflowernsa.com/oil.

O'Connor E., Cowan C., Williams G., O'Connell J. and Boland M.P. 2006. Irish consumer acceptance of a hypothetical second-generation GM yogurt product. Food Qual. Pref. 17: 400.

SPSS Base 15.0 2006. SPSS Base 15 User's Guide 11, p. 161. Chicago, IL.

Topcu Y. and Isik H.B. 2008. Determining the best product quality set for packed yogurt in Turkey. Ital. J. Food. Sci. 20 : 401.

Topcu Y., Isik H.B. and Uzundumlu A.S. 2008. Manufacturing brands vs. store brands: A case of liquid vegetable oil market. International Conference on Business Management and Economics, 26 January 2008, Erzurum, Turkey.

Topcu Y. 2009. Exploring Turkish olive oil consumer behavior using conjoint analysis. J. Applied Biological Sci. (JABS), 3: 12.

Topcu Y., Isik H.B. and Uzundumlu A.S. 2009. Turkish consumer attitudes toward food products: The case of Erzurum. Ital. J. Food. Sci. 21 : 37.

Topcu Y. and Uzundumlu A.S. 2009. Analysis of factors af-

fecting customer retailer loyalty in Turkish food market: The case study of Erzurum. Ital. J. Food. Sci. 21 : 149.

Ure S. 2009. Vegetable Oil. Cited 03 December 2009, available on the internet: http://www.diyetinfo.com/details.

Wądołowska L., Babicz-Zielińska E., Schlegel-Zawadzka M., Przysławski J. and Czarnocińska J. 2002. Preferences, consumption and choice factors for milk and its products among school children. Pol. J. Food Nutr. Sci. 11 : 81.

Wądołowska L., Babicz-Zielińska E. and Czarnocińska J. 2008. Food choice models and their relation with food preferences and eating frequency in the Polish population: POFPRES study. Food Policy, 33 : 122.

Zenner S., Wirthgen B. and Altmann M. 2003. Analysis of the purchase and consumer behavior towards direct purchase of food. Schriften der Gesellschaft für Wirtschafts und Sozialwissenschaften des Landbaus 39: 1.

Revised paper received December 19, 2009 Accepted March 5, 2010

GUIDE FOR AUTHORS

ITALIAN JOURNAL OF FOOD SCIENCE -IJFS

1. Manuscript Submission

Manuscripts must submitted as an electronic version by e-mail paolofan@unipg.it. The word processor used to generate the file should be indicated and the files should be saved in format "Text only"; **graphs, pictures and diagrams must be saved at 300 dpi in TIF, JPG or EPS formats** (not included in MsWord documents).

Manuscripts must be typed, double-spaced and pages should be in A4 format using Times New Roman 12 pt as the advised font. Top, bottom and side margins should be 25 mm. Pages and lines on all pages, including those for References and figure legends, **must be electronically numbered in the left margin.**

English is the official language. The Editor-in-Chief and/or Co-Editors reserve the right to make literary corrections and to make suggestions to improve brevity, but **the paper must be previously revised for English by the authors**. Authors who are not fluent in written English **must seek** help from one of the following agencies (or other similar official agencies):

www.journalexperts.com
www.sciencedocs.com
www.internationalscienceediting.com
www.writescienceright.com
www.genedits.com

2. Manuscript Preparation

(1) The paper should be divided under the following headings in this order:

Title. Informative of the content of the article (<50 characters + spaces).

Author(s). Initials and Surname, omit professional and official titles. The institute and address where the research was carried out and the current address of each author should be given on the title page.

Abstract. Clearly state the objective of the study, give a concise description of experiment(s), observations, results and conclusions. No references should be cited. **Do not exceed 100 words**.

Key words. Up to six words, in alphabetical order, which describe the document must be given to aid data retrieval and indexing.

Introduction. Review pertinent previous work and cite appropriate references. State the purpose of the investigation.

Materials and Methods. Indicate apparatus, instruments, reagents, etc., giving sufficient detail to allow the work to be repeated.

Results and Conclusions. Results and Conclusions may be presented together or separately. Concisely present results using tables and figures to help justify conclusions (do not present the same information in both forms). Use statistical analysis when appropriate. Unsupported hypotheses should be avoided. Conclusions should point out the significance of the findings and, if possible, relate the new findings to some problem in Food Science and Technology.

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

Units. A list of units particular to the paper may be included.

References. References in the Reference list should be arranged alphabetically (initials of first name, only), and, for the same author, should be arranged consecutively by year, typed double-spaced. Each individual reference should begin flush left (no indentation). Refer to attached examples taken from "Style Guide for Research Papers" by the Institute of Food Technologists (Chicago - I1linois - USA). Literature citations in the text should be referred to by Surname and year in parentheses. If there are more than two authors, give the surname of the first author and add et al. and the year in parentheses. Examples: (SMITH, 2007), (SMITH and JONES, 2008) (SMITH et al., 2008).

(2) Tables should be as few and as simple as possible and include only essential data. Each table must be saved and printed on a separate sheet and have an Arabic number, e.g. Table 4 NOT Tab.

4. Legends must be self-explanatory and on a separate sheet. Use lower-case letters for footnotes in tables and explain below the table in the order in which they appear in the table.

(3) Figures must be prepared and saved separately in **TIF, JPEG, EPS (300 dpi resolution).** They should be prepared so that on 50% reduction, lines, figures and symbols will be clearly legible and not overcrowded. All figures must be given Arabic numbers, e.g. Fig. 3. Legends for figures must be self-explanatory and should be typed on a separate sheet under "Legends to Figures".

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

Common species names should be followed by the Latin binomial (italics) at the first mention. For subsequent use, the generic name should be contracted to a single letter if it is unambiguous.

3. Editorial and Review Policy

Scientific contributions in one of the following forms may be submitted:

Reviews –They can be submitted directly to the Editor-in-Chief or articles can be requested directly by the Editor-in-Chief.

Short Communications, Surveys and Opinions – They do not need to have the formal organization of a research paper; they will receive priority in publication; maximum of five pages allowed.

Papers – The paper must follow the guidelines as specified under the section Manuscript Preparation.

Reviews, Papers, Short Communications and Surveys will be **subjected to critical review by referees.**

(1) Manuscripts will be processed in the order received. The Editor-in-Chief will select papers to enter into the reviewing system based on originality and innovation. A letter will be sent to the authors acknowledging receipt of the manuscript along with a Declaration form stating that it has NOT been previously published, accepted or submitted for publication elsewhere and agreeing to the page charges upon acceptance of the paper. On receipt of the signed Declaration form, the Editor-in-Chief will send the manuscript to a Co-Editor and/or referees for evaluation.

(2) Referees may not be from the same institution as the author. Referees should make their comments and questions in detail and return the paper to the Editor-in-Chief and/or Co-Editor as soon as possible, usually within two weeks. The identity and report of the referees are made know to the Editor-in-Chief, but only the anonymous referee report is sent to the author(s). If all referees recommend acceptance or rejection, the decision stands. If the opinions of the referees tie, the Editor-in-Chief and/or Co-Editors have the freedom to decide upon acceptance or rejection of the paper.

(3) The results of the refereeing process, accompanied by a letter from the Editor-in-Chief or the Co-Editor, will be sent to the author(s). Papers needing revision must be returned to the Co-Editor within the timeframe suggested, otherwise the paper will be considered as

withdrawn. A letter announcing acceptance of the manuscript will be sent to the author(s) upon acceptance by the referees.

(4) The authors **will receive galley proofs** of the manuscript along **with the invoice** for the page charges (stated on the first page of each issue) which **must be paid in order to allow for publication**. The proofs will be sent to the corresponding author as a PDF file by e-mail, only. A hard copy will be sent by mail only if the author makes this request when the paper is accepted for publication.

The revised galley proofs must be returned by fax or mail to Chiriotti Editori – 10064 Pinerolo (TO) – Italy –Fax: +39 0121 794480; e-mail: info@chiriottieditori.it

REFERENCE EXAMPLES

EXAMPLES of use in a Reference list are given below. The bold-faced parenthetical type of citation above the example is indicated ONLY for information and is NOT to be included in the reference list.

(Anonymous)

Anonymous. 1982. Tomato product invention merits CTRI Award. Food Technol. 36(9): 23.

(Book)

AOAC. 1980. "Official Methods of Analysis" 13th ed. Association of Official Analytical Chemists, Washington, DC.

Weast, R.C. (Ed.). 1981 "Handbook of Chemistry and Physics" 62nd ed. The Chemical Rubber Co. Cleveland, OH.

(Bulletin, circular)

Willets C.O. and Hill, C.H. 1976. Maple syrup producers manual Agric. Handbook No. 134, U.S. Dept. of Agriculture, Washington, DC.

(Chapter of book)

Hood L.F. 1982. Current concepts of starch structure. Ch. 13. In "Food Carbohydrates". D.R. Lineback and G.E. Inglett (Ed.), p. 217. AVI Publishing Co., Westport, CT.

(Journal)

Cardello A.V. and Maller O. 1982. Acceptability of water, selected beverages and foods as a function of serving temperature. J. Food Sci. 47: 1549.

IFT Sensory Evaluation Div. 1981a. Sensory evaluation guide for testing food and beverage products. Food Technol. 35 (11): 50.

IFT Sensory Evaluation Div. 1981b. Guidelines for the prepaation and review of papers reporting sensory evaluation data. Food Technol. 35(4): 16.

(Non-English reference)

Minguez-Mosquera M.I., Franquelo Camacho A, and Fernandez Diez M.J. 1981. Pastas de pimiento. Normalizacion de la medida del color. Grasas y Aceites 33 (1): 1.

(Paper accepted)

Bhowmik S.R. and Hayakawa, K. 1983. Influence of selected thermal processing conditions on steam consumption and on mass average sterilizing values. J. Food Sci. In press.

(Paper presented)

Takeguchi C.A. 1982. Regulatory aspects of food irradiation. Paper No. 8, presented at 42nd Annual Meeting of Inst. of Food Technologists, Las Vegas, NV, June 22-25.

(Patent)

Nezbed R.I. 1974. Amorphous beta lactose for tableting U.S. patent 3,802,911, April 9.

(Secondary source)

Sakata R., Ohso M. and Nagata Y. 1981. Effect of porcine muscle conditions on the color of cooked cured meat. Agric. & Biol. Chem. 45 (9): 2077. (In Food Sci. Technol. Abstr. (1982) 14 (5): 5S877).

Wehrmann K.H. 1961. Apple flavor. Ph. D. thesis. Michigan State Univ., East Lansing. Quoted in Wehrmann, K.H. (1966). "Newer Knowledge of Apple Constitution", p. 141, Academic Press, New York.

(Thesis)

Gejl-Hansen F. 1977. Microstructure and stability of Freeze dried solute containing oil-in-water emulsions Sc. D. Thesis, Massachusetts Inst. of Technology, Cambridge.

(Unpublished data/letter)

Peleg M. 1982. Unpublished data. Dept. of Food Engineering., Univ. of Massachusetts, Amherst.

Bills D.D. 1982. Private communication. USDA-ARS. Eastern Regional Research Center, Philadelphia, PA.

CONTRIBUTORS

Gratitude is expressed to the following entities for contributing to the realization of the Journal by being supporting subscribers for 2010.

ASSOCIATIONS and COMPANIES

Associazione Italiana di Tecnologia Alimentare (A.I.T.A.) - Milano	Fax +39-02-2365015 www.aita-nazionale.it
Società Italiana di Scienze e Tecnologie Alimentari (S.I.S.T.Al) - Perugia	Fax +39-075-5857939 www.sistal.org
Soremartec Italia srl - Alba	Fax +39-0173-313966

RESEARCH INSTITUTES

Dipartimento di Scienze Tecnologie Agroalimentari (D.I.S.T.A.),
Facoltà di Agraria,
Università degli Studi della Tuscia, Viterbo Fax +39-0761-357498

Dipartimento di Ingegneria e Tecnologie Agro-Forestali,
Università di Palermo, Palermo Fax +39-091-484035

Dipartimento di Scienze degli Alimenti,
Università di Udine, Udine Fax +39-0432-558130

Dipartimento di Scienze e Tecnologie Agroalimentari e
Microbiologiche (DI.S.T.A.A.M.),
Università del Molise, Campobasso Fax +39-0874-404652

Dipartimento di Scienze e Tecnologie Alimentari
e Microbiologiche (DI.S.T.A.M.), Università di Milano, Milano Fax +39-02-50316601

Dipartimento di Valorizzazione e Protezione delle Risorse
Agroforestali (DI.VA.P.R.A.), Sezione Microbiologia
ed Industrie Agrarie, Università di Torino, Grugliasco Fax +39-011-6708549

ITALIAN JOURNAL OF FOOD SCIENCE
Rivista Italiana di Scienza degli Alimenti
DIRETTORE RESPONSABILE: Giovanni Chiriotti
AUTORIZZAZIONE: n. 3/89 in data 31/1/1989
del Tribunale di Perugia
Proprietà dell'Università di Perugia
TIPOGRAFIA Giuseppini - Pinerolo
Una copia € 20.00

ISSN 1120-1770 © 2010

CHIRIOTTI EDITORI sas - 10064 Pinerolo - Italy

publishes the technical magazines:

INDUSTRIE ALIMENTARI
CARNI & TECNOLOGIE
INDUSTRIE DELLE BEVANDE
INGREDIENTI ALIMENTARI
ALIMENTI FUNZIONALI
TECNICA MOLITORIA
ITALIAN FOOD TECHNOLOGY
ITALIAN BEVERAGE TECHNOLOGY
PASTICCERIA INTERNAZIONALE
ITALIAN JOURNAL OF FOOD SCIENCE

VOLUME XXII IJFS No. 2, 2010

CONTENTS

REVIEW

Succinic Acid and its Derivatives: Fermentative Production using Sustainable Industrial Agro-Food By-Products and its Applications in the Food Industry
F. Chimirri, F. Bosco, R. Ceccarelli, A. Venturello and F. Geobaldo **119**

PAPERS

Microstructure of Natural, Extruded and Co-Extruded Collagen Casings Before and After Heating
S. Barbut **126**

Evaluation of Some Sensory Properties and Cooking Loss of Different Burger Formulations
K.M. Al-Mrazeeq, B.M. Al-Abdullah and K.M. Al-Ismail **134**

Evolution of Mechanical Variables of Winegrapes for Icewine Production during On-Vine Drying
L. Rolle, F. Torchio, E. Cagnasso and V. Gerbi **143**

Lead and Cadmium in Some Milk and Milk-Based Mozzarella Cheeses from Apulia, Italy
M. Faccia, G. Gambacorta, M. Quinto and A. Di Luccia **150**

Antioxidant Activity and Phenolic Content of Water Extracts of *Borago officinalis* L.: Influence of Plant Part and Cooking Procedure
C. García-Herreros, M. García-Iñiguez, I. Astiasarán and D. Ansorena **156**

Agronomic and Quality Parameters of Flat Peach UFO3, UFO4 and Sweetcap®
FCA. Hernández, J.J. Martínez and P. Legua **165**

SHORT COMMUNICATIONS

Different Sensitivity to Browning in Fresh-Cut Pineapple, Apple and Pear: The Role of Endogenous Vitamin C
S. Tavarini, L. Trinci, E. Degl'Innocenti and L. Guidi **171**

Effect of a Tomato Extract-Supplemented Diet on Egg Yolk Pigmentation and Lycopene Transfer Efficiency
L. Rotolo, G. Strazzullo, M. Pagella, A. Brugiapaglia, L. Pozzo and A. Schiavone **180**

Quality of Tomato Fertilized with Nitrogen and Phosphorous
L.F. Di Cesare, C. Migliori, D. Viscardi and M. Parisi **186**

Nutritional Evaluation of Fillets, Pulp and Croquettes of Wild Caught Lake Trasimeno Goldfish (*Carassius Auratus* L.)
A. Dal Bosco, E. Mourvaki, C. Mugnai and C. Castellini **192**

Changes in Cytochrome C Oxidase (CCO) and Lactate Dehydrogenase (LDH) Enzyme Activity of the White Epaxial Muscle Tissue of Sea Bass (*Dicentrarchus labrax* L.) as Shelf-Life Predictive Indices during Ice-Storage
C. Nathanailides, O. Lopez-Albors, D. Lenas, F. Athanassopoulou and K. Grigorakis **200**

Influence of Season on Microbiological Loads in Ready-To-Eat Vegetables
V. Coroneo, A. Sanna, F. Danjou, P. Caboni and S. Dessì **205**

In Vitro Synergistic Antibacterial Activity of *Helichrysum arenarium, Inula helenium, Cichorium intybus* and Some Preservatives
D. Stanojević, L.J. Čomić, O. Stefanović and S. Solujić Sukdolak **210**

Microbiological Evaluation of the Dry-Slaughter Process for Small Scale Poultry Meat Production
L. Valnegri, M. Franzoni, L. Vercellotti and G. Soncini **217**

Bactericidal Activity of Electrolyzed Oxidizing Water on Food Processing Surfaces
A. Serraino, G. Veronese, S. Alonso, R. Matera, B. Lugoboni and F. Giacometti **222**

Presence of Biogenic Amines in Ricotta Forte, a Traditional Italian Dairy Product from the Puglia Region
S. Rea, N. Mascaro, M. Ricciutelli, N. Cammertoni, S. Cecchini, A.R. Loschi and R. Stocchi **229**

Development of a Flotation Method for Detecting Insect Fragments in Bran and Fine Bran
G.S. Germinara, A. De Cristofaro and G. Rotundo **234**

SURVEYS

Analysis of Factors Affecting Turkish Sunflower Oil Consumer Behavior: The Case of Erzurum province
Y. Topcu, B. Turhan and A.S. Uzundumlu **239**

GUIDE FOR AUTHORS **248**

CONTRIBUTORS **251**