ISSN 1120-1770

# ITALIAN JOURNAL OF FOOD SCIENCE

*Rivista italiana di scienza degli alimenti*

IJFS

*Volume XXI*
*Number 4*
*2009*

Spedizione in ab. post. comma 26 - art. 2 - legge 549/95 n. 4/2009 - Torino

CHIRIOTTI EDITORI

# ITALIAN JOURNAL OF FOOD SCIENCE

(RIVISTA ITALIANA DI SCIENZA DEGLI ALIMENTI)

Property 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 - Telex 662078 UNIPG - Telefax +39 075 5857939-5852067
E-mail: paolofan@unipg.it

**Scientific Editor:**
S. Mary F. Traynor, F.S.E., Ph.D.
Dipartimento di Scienze Economico-Estimative e degli Alimenti, Università di Perugia,
S. Costanzo, I-06126 Perugia, Italy
Tel. +39 075 5857912 - Telex 662078 UNIPG - Telefax +39 075 5857939-5852067
E-mail: ijfs@unipg.it

**Publisher:**
Alberto Chiriotti
Chiriotti Editori s.a.s., Viale Rimembranza 60, I-10064 Pinerolo, Italy
Tel. +39 0121 393127 - Telefax +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 in food science (chemistry, analysis, microbiology), food technology (engineering, processing) and related areas (nutrition, safety, toxicity, physiology, dietetics, economics, etc.). 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 Advisory Board 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:** 0.518 published in the 2007 Journal of Citation Reports, Institute for Scientific Information

**Subscription Rate:** 2010: Volume XXII

| | |
|---|---|
| PDF version | € 40.00 |
| Supporting | € 1,000.00 |

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 € 20.00 up to 5 pages; extra pages are € 30.00.
Reprints (100) will be sent free of charge.

# ITALIAN JOURNAL OF FOOD SCIENCE

IJFS

## ADVISORY BOARD


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

**G. Andrich**
Dip. di Chimica
e Biotecnologie Agrarie
Università di Pisa
Pisa, Italy

**F. Angerosa**
Ist. Sperim. per la Elaiotecnica
Città S. Angelo, Pescara, Italy

**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

**E. Carnovale**
Ist. Nazionale di Ricerca
per gli Alimenti e la Nutrizione
Unità Chimica degli Alimenti
Roma, Italy

**E. Marconi**
Dip. di Scienza e Tecnologia
Agro-Alimentare e Microbiologia
Università del Molise
Campobasso, Italy

**A. Curioni**
Dip. di Biotecnologie Agrarie
Università di Padova
Legnaro (PD), Italy

**G. Dall'Aglio**
Staz. Sperim. per l'Industria
delle Conserve Alimentari
Parma, Italy

**M. Dalla Rosa**
Dip. di Protezione e
Valorizzazione Agro-Alimentare
Università di Bologna
Bologna, Italy

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

**M. Di Matteo**
Dip. di Ingegneria Chimica
ed Alimentare
Università di Salerno
Fisciano (SA), Italy

**P.F. Fox**
Department of Food Chemistry
University College
Cork, Ireland

**G. Gandemer**
INRA – Centre Poitou-Charentes
Lusignan, France

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

**C. Finoli**
Dip. Economia, Ingegneria e
Tecnologie Agro-Forestali
Università di Palermo
Palermo, Italy

**R. Giangiacomo**
Istituto Sperim. Lattiero-Caseario
Lodi, Italy

**M. Gobbetti**
Dip. di Protezione delle Piante e
Microbiologia Applicata
Università di Bari
Bari, Italy

**T. Gomes**
Dip. di Progettazione
e Gestione dei Sistemi
Agro-Zootecnici e Forestali
Università di Bari
Bari, Italy

**M. Karel**
Dept. of Food Science
Rutgers University
New Brunswick, NJ, USA

**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

**M. Lucisano**
Dip. di Scienze e Tecnologie
Alimentari e Microbiologiche
Sez. Tecnologie Alimentari
Università di Milano
Milano, Italy

**R. Massini**
Dip. di Ingegneria Industriale
Università di Parma
Parma, Italy

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

**L. Moio**
Dip. di Scienze degli Alimenti
Università di Foggia
Foggia, Italy

**M. Moresi**
Ist. di Tecnologie Agro Alimentari
Università della Tuscia
Viterbo, Italy

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

**M. O'Keeffe**
Food Safety Department
The National Food Centre
Dublin, Ireland

**C. Peri**
Dip. di Scienze e Tecnologie
Alimentari e Microbiologiche
Sez. Tecnologie Alimentari
Università di Milano, Milano, Italy

**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

**E. Senesi**
Istituto Sperim.
per la Valorizzazione
Tecnologica dei Prodotti Agricoli
(I.V.T.P.A.)
Milano, Italy

**A. Sensidoni**
Dip. di Scienze degli Alimenti
Università di Udine
Udine, Italy

**S. Spagna-Musso**
Dip. di Scienze degli Alimenti
Università di Napoli
Portici (NA), Italy

**L. Stepaniak**
Dept. of Food Science
Agricultural University of Norway
Ås, Norway

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

**G. Versini**
Dip. Lab. Analisi e Ricerche
Ist. Agrario di S. Michele a/Adige
S. Michele all'Adige (TN), Italy

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

Dear Subscribers,

As the year 2009 draws to a close, I would like to take this opportunity to share the news about upcoming changes for the Italian Journal of Food Science. As you know, in addition to the standard printed version, the Journal was published for the first time in electronic form on the internet starting with n. 1, volume 20 in 2008. On January 1, 2010 the printed version will be discontinued and the journal will be published exclusively on the internet. Hence, prospective authors should carefully consult the new Guide to Authors to comply with the requirements.

With the new year, there will also be a new organization of the editorial board and of the reviewing process. A team of five co-editors, proposed by the Italian Society of Food Science and Technology (SISTAL) will assist me in the selection and evaluation of manuscripts submitted to IJFS, the official journal of SISTAL. In an effort to facilitate a more rapid turn-around time, the evaluation and publication process will be done on-line. It goes without saying that Italian and foreign scientists will be called upon to serve as referees for the manuscripts. To access the Journal, all paying subscribers will be issued a personal Username and Password. This will give the user access to all issues of the Journal. Hopefully this new format will allow a greater distribution of the Journal to a wider range of scientists, both authors and readers.

Due to these changes, IJFS will no longer maintain the secretariat in Perugia and because of this we will be losing our Scientific Editor, Dr. Sister Mary F. Traynor. However, she will stay with us to complete the work on all of the papers submitted up to December 31, 2009. I am profoundly grateful to Sister Mary for the highly professional, untiring and dedicated service that she has given to the Journal since its inception twenty-one years ago. I will always be indebted to her and cannot thank her sufficiently for helping to bring the journal to its present stage.

As we enter into the New Year of 2010 and this new phase of the Italian Journal of Food Science, I wish you a fruitful 2010.

*Paolo Fantozzi*
Editor-in-Chief

REVIEW

# STABILITY OF THE SENSORY QUALITY OF VIRGIN OLIVE OIL DURING STORAGE: AN OVERVIEW

PANORAMICA SULLE VARIAZIONI SENSORIALI DELL'OLIO VERGINE DA OLIVE DURANTE LA CONSERVAZIONE

**A. BENDINI*, L. CERRETANI, M.D. SALVADOR[1], G. FREGAPANE[1], and G. LERCKER[2]**
Dipartimento di Scienze degli Alimenti, Università di Bologna,
P.zza Goidanich 60, 47023 Cesena, FC, Italy
[1]Departamento de Tecnología de Alimentos, Universidad de Castilla-La Mancha,
Avda Camilo José Cela 10, 13071 Ciudad Real, Spain
[2]Dipartimento di Scienze degli Alimenti, Università di Bologna,
V.le Fanin 40, 40127 Bologna, Italy
*Corresponding author: alessandra.bendini@unibo.it

ABSTRACT

The storage conditions of bottled or tank-stored virgin olive oil, as well as all the agronomical and technological variables of the processing stages, are particularly relevant for preserving the highly valued organoleptic quality of this product. All the efforts made in the olive grove and in the oil mill to produce virgin olive oil with good sensory characteristics can be undermined if improper storage conditions are used. In particular, it is necessary to protect

RIASSUNTO

Le condizioni di conservazione dell'olio vergine di oliva in bottiglia o in cisterne, così come le variabili agronomiche e tecnologiche attuate durante le fasi che vanno dalla produzione delle olive alla loro trasformazione in olio, sono da considerarsi con particolare attenzione se si vuole mantenere elevata la qualità sensoriale di questo prodotto. Infatti, tutti gli sforzi fatti sia in campo che in frantoio per produrre un olio vergine di oliva con buone caratteristiche organolet-

- Key words: Minor compounds, oil storage, phenols, sensory stability, virgin olive oil, volatiles -

the oil against lipid oxidation which can have many deleterious effects on the quality of virgin olive oil, including the formation of unpleasant organoleptic characteristics, the drastic reduction of the bouquet and taste notes as well as the reduction of naturally-occurring antioxidant compounds and therefore shortening of its the shelf life. The oxidation process during storage is known and involves changes to both the major and minor components of virgin olive oil. To slow down the oxidation rate during storage, certain factors such as the presence of oxygen and traces of metals, exposure to light and the binomial storage time/temperature, must be kept under control. This overview will present the importance of reducing these pro-oxidizing effects, as well as the main changes which may occur during the storage of virgin olive oil at the expense of the minor constituents that are more relevant for the olfactive and gustative characteristics and its impact on the aroma and taste of the oil, particularly the phenolic and volatile compounds.

tiche possono essere vanificati qualora si attuino improprie condizioni di conservazione del prodotto. In particolare, è necessario proteggere l'olio dall'ossidazione lipidica che può avere effetti deleteri sulla sua qualità, come ad esempio la formazione di sentori sgradevoli, la drastica riduzione delle tipiche note percepibili all'olfatto ed al gusto, il depauperamento degli antiossidanti naturalmente presenti, con conseguente accorciamento della shelf life. È noto come il processo di ossidazione, che inevitabilmente avviene durante la conservazione, porti a variazioni dei componenti sia maggioritari che minoritari dell'olio vergine di oliva. Per rallentare la velocità delle modificazioni ossidative durante la conservazione dell'olio, alcuni fattori come la presenza dell'ossigeno, i metalli in tracce, l'esposizione alla luce ed il binomio tempo/temperature devono essere attentamente controllati. In questa panoramica si discuterà sull'importanza di ridurre l'effetto di questi fattori pro-ossidanti, così come delle più importanti modificazioni che, nel corso dello stoccaggio dell'olio, avvengono a spese dei componenti minori responsabili delle caratteristiche olfattive e gustative quali, rispettivamente, i componenti volatili e fenolici.

## INTRODUCTION

The assurance of the stability and quality of food products is a matter of great concern for producers and sellers. In the case of fats and oils, oxidation (one of the most fundamental reactions in lipid chemistry) is the main cause of quality deterioration and its reaction rate determines the shelf-life of this type of food product. To maintain the phenol and volatile molecule content responsible for the highly appreciated organoleptic and nutritional properties in newly produced virgin olive oil during storage, it is absolutely essential to control all the factors that promote lipid oxidation.

The high oxidative stability of virgin olive oil with respect to other vegetable oils is mainly due to its fatty acid composition, in particular, to the high monounsaturated-to-polyunsaturated ratio, and to the presence of minor compounds that play a major role in preventing oxidation. In spite of its high stability, virgin olive oil is also susceptible to oxidative process-

es, such as enzymatic oxidation, which occurs when the oil is in the fruit and during the extraction process, photo-oxidation, when the oil is exposed to light, and autoxidation which mainly occurs during processing and storage when the oil is in contact with oxygen (FRANKEL, 1985). This review is mainly concerned with the autoxidation process, the main cause of deterioration of virgin olive oil during its shelf life.

Lipid oxidation occurs through the interaction of the triacylglycerol fatty acids with molecular oxygen which yields hydroperoxides by a free radical mechanism. The activation energy of this reaction is high and the initiation of lipid oxidation requires traces of transition metals or exposure to light; the reaction is accelerated by an increase in temperature. These factors can catalyse the decomposition of hydroperoxides, the primary oxidation products. The unstable hydroperoxides decompose to produce a range of volatile and non-volatile products. Some volatile components, mainly aldehydes, are the major cause of the sensory perception of the rancid defect in vegetable oils (ANGEROSA, 2000). During the autoxidation reaction, a series of compounds are formed in virgin olive oil (VOO), while minor components are degraded, causing rancidity and off-flavours, loss of nutritional value and finally consumer rejection.

Some major factors influencing lipid oxidation are: the amount of oxygen dissolved in the oil that cannot be removed, the oxygen permeability of the packaging materials, the storage temperature, exposure to light and the fatty acid composition. Lipid oxidation is accelerated by the presence of free fatty acids, mono- and diacylglycerols, thermally-oxidized compounds and metals such as iron. In contrast, phenolic compounds and carotenoids decrease autoxidation in oil, while tocopherols, chlorophylls and phospholipids demonstrate both antioxidant and pro-oxidant activity depending on the oil system and storage conditions (CHOE and MIN, 2006).

## THE IMPORTANCE OF CONTROLLING THE PRO-OXIDIZING FACTORS

Key facts regarding the major external factors and olive oil constituents that influence lipid oxidation are shown in Table 1.

### Physical factors

As mentioned above, several physical factors play a key role in controlling oxidation in virgin olive oil during storage. All of these must be carefully monitored to prevent alterations in the oil and to extend the shelf-life.

#### Oxygen availability

The great effect that oxygen availability has on the oxidation reaction rate is directly related to its partial pressure. The level of oxygen in the oil depends on the conditions used in some technological operations such as centrifugation, and/or decanting and filtration. In the case of bottled or tank-stored olive oil, in which the surface-volume ratio in contact with the atmosphere is relatively small, the diffusion of oxygen into the bulk oil is a limiting parameter, and therefore the oxidation rate is controlled by diffusion (YANISHLIEVA, 2001). The head-space in the container and the oxygen permeability of the packaging material are two variables to be considered since they play a major role in oil stability during storage (GUTIÉRREZ *et al.*, 1988).

The basic factors that affect the shelf life of olive oil in different packaging systems and the main oxidative degradation mechanisms for them have been reported. Published results on plastic packaging material permeability show the following ranking of oil sample stability: PVC (polyvinyl chloride) ≥ PET (polyethyleneterephthalate) > PP (polypropylene)

≥ PS (polystyrene) (TAWFIK and HUYGHEBAERT, 1999). Moreover, the differences in the shelf life observed in oils bottled in PET or in glass are attributable to differences in the initial dissolved oxygen content in the oils (SACCHI *et al.*, 2008). While the decay kinetics of bottled virgin olive oil depend on the shape and size of the bottle, it mainly depends on the material used to make the bottle, and on the initial value of the oxygen partial pressure in the headspace (DEL NOBILE *et al.*, 2003). The shelf life of packaged olive oil under various storage conditions can be predicted by applying mathematical modeling and simulations (KANAVOURAS and COUTELIERIS, 2006a; KANAVOURAS *et al.*, 2006b). The feasibility of improving the stability of extra virgin olive oil by using inert gases, mainly nitrogen or argon in the head-space of the container, as a conditioner gas during storage to reduce dissolved oxygen, has been studied. The use of nitrogen as conditioner gas helped to avoid the risk of oxidation during storage (DI GIOVACCHINO *et al.*, 2002).

Table 1 - Key points of major external factors and olive oil constituents influencing lipid oxidation.

| Factors | Key points | References |
|---|---|---|
| Oxygen availability | - Partial pressure and diffusion<br>- Permeability of packaging materials<br>- Use of inert gases in the container | GUTIÉRREZ *et al.*, 1988<br>YANISHLIEVA, 2001<br>DI GIOVACCHINO *et al.*, 2002<br>SACCHI *et al.*, 2008 |
| Storage temperature | - Increases the reaction constant<br>- Improves the formation and decomposition rates of hydroperoxides<br>- Decreases oxygen solubility | RAGNARSSON and LABUZA, 1977<br>FRANKEL, 1998<br>VELASCO and DOBARGANES, 2002<br>GÓMEZ-ALONSO *et al.*, 2004 |
| Exposure to light | - Initiates auto-oxidation<br>- Produces photo-oxidation<br>- The oil must contain photosensitizers<br>- Opacity to light of packaging material | GUTIÉRREZ *et al.*, 1988<br>JADHAV *et al.*, 1996<br>CAPONIO *et al.*, 2005<br>MÉNDEZ and FALQUÉ, 2007 |
| Triacylglycerols | - Unsaturation degree of their fatty acids | HOLMAN and ELMER, 1947 |
| Free fatty acids | - Pro-oxidant effect exercised by carboxylic group | MIYASHITA and TAKAGI, 1986<br>KIRITSAKIS *et al.*, 1992<br>FREGA *et al.*, 1999 |
| Traces of metals | - Catalyze the decomposition of hydroperoxides | BENJELLOUN *et al.*, 1991<br>ANGEROSA *et al.*, 1993 |
| Phenols | - Direct relationship between content and oxidative stability<br>- Hydroxytyrosol and its secoiridoid derivatives are the most active antioxidants<br>- Tyrosol and its derivatives show a very low or no antioxidant activity | VÁZQUEZ-RONCERO *et al.*, 1973<br>GUTIÉRREZ *et al.*, 1977<br>MONTEDORO *et al.*, 1992<br>BALDIOLI *et al.*, 1996<br>SALVADOR *et al.*, 1999<br>MATEOS *et al.*, 2002<br>CARRASCO-PANCORBO *et al.*, 2005 |
| Tocopherols | - Lower antioxidant activity than hydroxytyrosol<br>- Antioxidant polarity paradox | PORTER *et al.*, 1989<br>FRANKEL *et al.*, 1994<br>BALDIOLI *et al.*, 1996<br>MATEOS, 2002 |
| Pigments | - Carotenoids: efficient protectors against photo-oxidation<br>- Chlorophylls: very active in lipid photo-oxidation but also low antioxidants during oxidation in dark | CUPPETT *et al.*, 1997<br>RAHMANI *et al.*, 1998<br>ENDO *et al.*, 1984<br>GUTIÉRREZ *et al.*, 1992<br>PSOMIADOU and TSIMIDOU, 2002 |

Storage temperature

It is well known that storage temperature is one of the most relevant factors affecting lipid oxidation. A kinetic study of the autoxidation reaction in olive oil triacylglycerols stored in darkness at different temperatures (25°, 40°, 50°, 60° and 75°C), in the absence of pro- and antioxidant compounds to avoid confounding effects, confirmed that the reaction constant increases exponentially with temperature (GÓMEZ-ALONSO *et al.*, 2004). The effect of temperature on the oxidation rate is quite complex; it increases the oxidation rate improving the formation rate of hydroperoxides, while it decreases the oxygen solubility in the bulk oil and increases the decomposition of hydroperoxides, changing the profile of the products formed (RAGNARSSON and LABUZA, 1977; FRANKEL; 1998; VELASCO and DOBARGANES, 2002). Therefore, it is very relevant to establish the relationship between storage temperature and VOO oxidation rate. The temperature-dependent kinetics of the oxidation indices and the unsaturated fatty acids are described well by the linear Arrhenius equation between 25° and 60°C ($0.960 \leq R^2 \leq 0.999$, $p \leq 0.05$). The time required to reach the upper limits for PV, $K_{232}$ and $K_{270}$ established for the extra virgin olive oil category in the current EU legislation, correlated well with temperature using an exponential equation (MANCEBO-CAMPOS *et al.*, 2008).

Exposure to light

Luminous radiation is another external factor to be considered in the lipid oxidation process during storage, since it initiates auto-oxidation and produces photo-oxidation. To observe these effects, the oil must contain photosensitizers, like chlorophylls, that are excited by light absorption. Prevention of light exposure during storage of virgin olive oil is absolutely necessary to extend its shelf life (JADHAV *et al.*, 1996); oils exposed to light are less stable than those kept in the dark (CAPONIO *et al.*, 2005). However, VOO is usually protected from exposure to light radiation from the time of its production until it is exposed as bottled oil on the supermarket shelves. From that time onwards the opacity to light of the packaging material is of fundamental importance for its preservation (GUTIÉRREZ *et al.*, 1988; MÉNDEZ and FALQUÉ, 2007). It has been observed that even small doses of UV radiation can induce oxidation in virgin olive oil (LUNA *et al.*, 2006).

Chemical factors

Apart from the external physical factors described above, the susceptibility of fats and oils to oxidation is influenced by their chemical composition, both major and minor constituents, including the oxidation products formed during the oxidation reaction itself, all of which can possess pro- or antioxidant activities. The well known high oxidative stability of VOO is related not only to the high monounsaturated/polyunsaturated fatty acid ratio of its lipid matrix, but also to the presence of minor components that have great antioxidant activity, particularly the phenolic compounds.

Triacylglycerols (TAG)

The susceptibility of lipids to oxidation increases as the unsaturation level of its fatty acids increases. As shown in a previous study (HOLMAN and ELMER, 1947), linoleate is 40 times more reactive than oleate, whereas linolenate is 2.4 times more reactive than linoleate. The stability of the triacylglycerol matrix of olive oil has been demonstrated in experiments conducted in the presence of different antioxidants and compared to a mixture of fatty acid methyl esters (FAME) with the same composition (MARINOVA and YANISHLIEVA, 1996). The position of the fatty acid within the triacylglycerol moiety also affects its susceptibility to oxidation (MIYASHITA *et al.*, 1990; MATEOS, 2002); they are slightly

less stable to oxidation when linoleate is in the 1,2- rather than the 1,3-triacylglycerol position. It has also been reported that the presence of mono- and diacylglycerols and of oxidized triacylglycerols has only a low pro-oxidant effect (MISTRY and MIN, 1988).

Free fatty acids (FFA)

Free fatty acids have a pro-oxidant effect when they are added to a purified lipid substrate (MIYASHITA and TAKAGI, 1986; MISTRY and MIN, 1987). Therefore, it is very important to have low levels of free fatty acids and hydroperoxides in newly produced oil as they may accelerate the oxidation of fats. The pro-oxidant action of free fatty acids seems to be exercised by a carboxylic group, which speeds up the decomposition rate of hydroperoxides (MIYASHITA and TAKAGI, 1986; KIRITSAKIS, 1992; FREGA *et al.*, 1999). In a recent work SCARPELLINI *et al.* (2005) confirmed the pro-oxidant effect shown by free fatty acids (FFA) added to refined peanut oil, as previously reported by other authors using different vegetable oils (CATALANO and DE FELICE, 1970; MIYASHITA and TAKAGI, 1986; FREGA *et al.*, 1999). In fact, the oxidative stability values of a neutralized oil decreased in proportion to increasing percentages of oleic acid. They already observed a substantial reduction of stability of oil samples at concentrations lower than 0.5% oleic acid.

Traces of metals

Transition metals, mainly iron and copper, can catalyze the decomposition of hydroperoxides according to their oxidation-reduction potential to yield lipid peroxyl and alkoxyl radicals that initiate free radical chain oxidation (BENJELLOUN *et al.*, 1991; ANGEROSA and DI GIACINTO, 1993). ANGEROSA and DI GIACINTO (1993) studied the catalytic effect of Mn and Ni on the oxidation of VOO. They measured the peroxide value (PV) and the $K_{232}$ as indices of primary oxidation compounds and *E*-2-pentenal and *E*-2-heptenal as markers of secondary oxidation products and showed a significant increase in the oxidation of VOO in the presence of metals. In the experiments carried out by POLVILLO *et al.* (1994), contamination with iron was detected in olive oil that had been in contact with carbon steel. The stability of VOO measured through the peroxide, $K_{270}$, and *p*-anisidine values was less after storage for one month in contact with a carbon steel sheet than when stored in the absence of the metal. BENDINI *et al.* (2006) measured the primary and secondary oxidation products in VOOs stored in the presence and absence of copper; drastic increases of these products were observed in samples with traces of metal. These results clearly demonstrate the ability of copper to promote autoxidation.

Phenols

The direct relationship between the content in phenolic compounds in VOO and its oxidative stability has been well known for a long time (VÁZQUEZ-RONCERO *et al.*, 1973; GUTIÉRREZ *et al.*, 1977; GUTFINGER, 1981; MONTEDORO *et al.*, 1992; BALDIOLI *et al.*, 1996; LITRIDOU *et al.*, 1997; SALVADOR *et al.*, 1999). In the case of hydroxytyrosol and its secoiridoid derivatives, which show a similar antioxidant activity (BALDIOLI *et al.*, 1996; MATEOS *et al.*, 2002; CARRASCO-PANCORBO *et al.*, 2005), the formation of a stable radical has been proposed to explain their antioxidant activity (VISIOLI and GALLI, 1998). In contrast, tyrosol and its derivatives show a very low or non antioxidant activity (BALDIOLI *et al.*, 1996; MATEOS *et al.*, 2002; CARRASCO-PANCORBO *et al.*, 2005), due to the absence of an electron donor group that has a high transition energy and a limitation in the formation of the phenoxyl radical. The presence of phenolic compounds in VOO is therefore extremely important, because they combat lipid oxidation in its initial stages. Howev-

er, they cannot block the autocatalytic mechanism. This effect is clearly visible from the data described by BENDINI *et al.* (2006) for an accelerated oxidation test (60°C) on virgin olive oils which differed only with respect to phenol contents. During the six weeks of testing, the peroxide and oxidized fatty acid (OFA) values increased in all the oils. There was a contemporary decrease of oxidative stability (OSI time), but with different rates that were proportional to the initial content in phenolic antioxidants. This effect was amplified when copper was added as a catalyst of lipid oxidation. MANCEBO-CAMPOS *et al.* (2007) studied the oxidation process of several virgin olive oils with different contents of natural antioxidants under accelerated oxidation conditions (Rancimat) and compared to long-term storage at room temperature. All the samples reached the peroxide number limit (20 meq $O_2$ $kg^{-1}$) but in very different times (28-56 hours vs 96-167 weeks) depending on the temperature (100°C for the Rancimat test and 25°C for the prolonged storage) and the initial content in antioxidants.

Tocopherols

Although α-tocopherol is considered to be the most relevant antioxidant in vegetable oils, as well as in the protection of the lipid structures in vivo, several researchers have reported a lower antioxidant activity than hydroxytyrosol (LE TUTOUR and GUEDON, 1992; BALDIOLI *et al.*, 1996; MATEOS, 2002). This may be explained by the "antioxidant polarity paradox" which states that hydrophilic antioxidants are often less effective in oil-in-water emulsions than lipophilic antioxidants, whereas lipophilic antioxidants are less effective in bulk oils than hydrophilic antioxidants (PORTER *et al.*, 1989; FRANKEL *et al.*, 1994). Moreover, it has also been reported that in the presence of *o*-diphenols, α-tocopherol gives rise to a synergic effect (SERVILI *et al.*, 1996).

Pigments

Carotenoids, especially β-carotene are efficient VOO protectors against photooxidation, since they are capable of deactivating the oxygen singlet giving back its triplet status (CUPPETT *et al.*, 1997; WAGNER and ELMAFDA, 1999). On the other hand, the capacity of the chlorophyll molecule to absorb light energy and transfer it to chemical substances, makes it very active in lipid photooxidation in VOO (RAHMANI and SAARI-CSALLANY, 1998). Chlorophylls may also act as low antioxidants during oxidation in the dark – absence of light – probably due to its capacity to donate hydrogen (ENDO *et al.*, 1984; GUTIÉRREZ *et al.*, 1992; PSOMIADOU and TSIMIDOU, 2002).

Filtered vs. unfiltered VOO

The results of some studies have hown a gradual loss in stability during the storage of filtered oils mainly due to a significant decrease in the phenolic components. When unfiltered and the corresponding filtered virgin olive oils were stored for nine months at ambient temperature in the dark, a loss in oxidative stability in the latter was observed due to a lower total phenolic content (TSIMIDOU *et al.*, 2005). Other researchers (GÓMEZ-CARAVACA *et al.*, 2007) have reported that eight virgin olive oils after filtration through cotton in the laboratory showed a significant loss of hydroxytyrosol, a simple phenol endowed with high antioxidant activity. Consequently there was lower oxidative stability of the filtered oils than of the unfiltered ones. Generally, the formation of simple phenols, such as hydroxytyrosol and tyrosol, was greater in unfiltered olive oils due to the hydrolysis rate of their secoiridoid derivatives. These reactions appear linked to the presence of a higher content of dispersed water droplets that maintain a partial enzymatic activity. On the other hand, it is well known that the filtration step which removes the organic sediments, prevents anaerobic fermenta-

tion which produces unpleasant volatile components responsible for the muddy defect. At the same time it has also been reported that filtration and dehydration decrease the hydrolysis rate of the triacylglycerol matrix, especially during storage at the higher temperature (40°C) and in oils with a higher initial free acidity (e.g. free acidity > 0.6%). Moreover, the formation rate of simple phenols due to hydrolysis of their secoiridoid derivatives was also greater in unfiltered olive oils. Thus, from this point of view, filtration and especially dehydration could help prolong the shelf life of some high-quality but less stable virgin olive oils, (e.g. Arbequina and Colombaia varieties) (FREGAPANE *et al.*, 2006).

### Degradation of minor constituents in VOO

Another consequence of the autoxidation reaction is the degradation of the naturally-occurring minor components in VOO (GÓMEZ-ALONSO *et al.*, 2007). The rates of degradation of α-tocopherol, squalene and phenolics in olive oil under different storage conditions have been reported. The main changes in the concentrations of these compounds are associated with the higher oxygen level in the empty portion of the glass bottles. α-tocopherol is the first molecule to be oxidized, whereas squalene and *o*-diphenols are protected in the first months due to the presence of α-tocopherol and their content decreases significantly only after 6 and 8 months, respectively (RASTRELLI *et al.*, 2002). The secoiridoid aglycones, namely, the oleuropein and ligstroside derivatives, and α-tocopherol decreased following pseudo-first-order kinetics during 8 months of storage in closed bottles in the dark, at 40 and 25°C. In all VOOs, the oleuropein derivatives were consumed faster than the corresponding ligstroside derivatives and α-tocopherol (LAVELLI *et al.*, 2006). Moreover, the α-tocopherol content decreased slightly and apparently linearly during its shelf-life, although there may be a lag phase at the beginning of storage (GÓMEZ-ALONSO *et al.*, 2007). Recently, there has been an increased interest in oxidized minor compounds (e.g. phenols, sterols, pigments), especially in relation to determining the freshness/aging status of VOO. The natural antioxidants in VOO are important not only for their *in vitro* protection against rancidity, and therefore the shelf life of the product, but also for their *in vivo* biological activity which enhances the nutritional value of this oil. Moreover, some individual phenolic compounds play an important role in positive sensory attributes, like bitterness, and hence in consumer preference.

## EFFECTS OF OIL STORAGE ON VOLATILE COMPOUNDS: IMPACT ON AROMA

Oxidation, an inevitable process that may start after the harvesting of the olives but certainly during the extraction of the virgin olive oil leads to a progressive deterioration of the product that becomes more serious during oil storage. Initially, lipids are oxidised to hydroperoxides, which are odourless and tasteless (FRANKEL, 1982) and do not account for sensory changes. However, they are susceptible to further oxidation or decomposition into products of secondary reactions, which, are responsible for the typical unpleasant sensory characteristics, identified on the whole as a rancid defect. This disagreeable sensory note is especially perceptible in oils that are strongly oxidized due to incorrect or excessively long storage. Decomposition occurs through a homolytic cleavage of the hydroperoxide group which produces various compounds, including aldehydes, ketones, acids, alcohols, hydrocarbons, lactones, furans and esters (FRANKEL, 1985).

The $C_6$ and $C_5$ compounds are enzy-

matically produced from polyunsaturated fatty acids through the so-called lipoxygenase (LOX) pathway. Quantitatively, linear $C_6$ unsaturated and saturated aldehydes are the most important fraction of volatile compounds in high quality virgin olive oils (ANGEROSA *et al.*, 2004). The quali-quantitative profiles of these volatile compounds depend on the level and activity of each enzyme involved in this LOX pathway (APARICIO and MORALES, 1998; ANGEROSA *et al.*, 2001).

The main contributors to virgin olive oil aroma are not necessarily the volatile compounds present in the highest concentrations. In fact, several factors, such as volatility, hydrophobicity, conformational structure of the molecules and type and position of functional groups may affect the odour intensity more than the concentration due to their capacity to establish bonds with olfactory receptor proteins. Therefore, the contribution of each volatile compound to the whole aroma and flavour is related to their concentration in the oil with respect to their sensory threshold (MORALES *et al.*, 1997).

The concentration and odour threshold of the volatile compounds are indeed crucial to virgin olive oil quality. Several researchers (SOLINAS *et al.*, 1987; ANGEROSA, 2000; 2002) have reported that during oxidation the drastic reduction of the $C_6$ aldehydes, alcohols and esters from the LOX pathway and the increase of many saturated and unsaturated aldehydes ($C_5$-$C_{11}$) from chemical oxidation, including hexanal, reduces the perception of the positive attributes and pleasant sensory notes leading to the kind of off-flavour in virgin olive oil recognized as a rancid defect by assessors. In a recent paper, CERRETANI *et al.* (2008) discussed the results relative to correlations between the chemical and sensory analysis. Assessors belonging to four panels (two Italian and two Spanish) analyzed 16 monocultivar VOOs produced in Italy and Spain. They assessed pleasant flavours related to different perception routes (orthonasal, retronasal and gustative) such as green and ripe notes from olive and other fruits as well as negative attributes. The four panels were in agreement on the presence of a slight rancid defect in only two samples characterized by a higher content of saturated aldehydes such as heptanal, nonanal and decanal.

The most advanced oxidation stages were characterised by the complete disappearance of compounds arising from the LOX cascade and by very high concentrations of the above-mentioned aldehydes. They contribute mainly to the undesirable defect perceptions due to their low odour thresholds (GUTH and GROSCH, 1990). Other contributors are the unsaturated hydrocarbons, furans and ketones.

It should be noted that although the *E*-2-hexenal content, which gives the typical "green note" to extra virgin olive oil, is by far the major $C_6$ aldehyde compound in all fresh oils, hexanal seems to contribute more to the green odour than *E*-2-hexenal because of its lower odour threshold (75-300 vs. 420-1,125 $\mu g\ kg^{-1}$) (REINERS and GROSCH, 1998; ANGEROSA, 2002; APARICIO and LUNA, 2002; MORALES *et al.*, 2005; KALUA *et al.*, 2007).

In addition to fruity, the "green" sensation reminiscent of freshly cut grass, leaf, tomato, artichoke, walnut husk, apple or other fruits generally contribute to the aroma of high quality oil. "Green" notes include *Z*-3-hexenal, and *Z*-3-hexenyl acetate whereas alcohols such as *E*-2-hexen-1-ol, *Z*-3-hexen-1-ol and hexan-1-ol have less sensory significance than aldehydes due to their higher odour threshold values. Their sensory descriptions are associated with ripe fruity, soft green and aromatic sensory notes (LUNA *et al.*, 2006; BENDINI *et al.*, 2007).

Esters are compounds associated with fruity nuances (APARICIO and LUNA, 2002; LUNA *et al.*, 2006). Hexyl acetate

and Z-3-hexenyl acetate, are present in the aroma of all fresh virgin olive oils but are minor components compared with aldehydes or alcohols. Different authors (REINERS and GROSCH, 1998; APARICIO and LUNA, 2002) indicate that Z-3-hexenyl acetate is linked to the pleasant green and banana notes (odour threshold 200-750 μg $kg^{-1}$).

Among the $C_5$ compounds, 1-penten-3-one has been mostly associated with fruity, sweet and pleasant attributes such as tomato and strawberry (ANGEROSA, 2000; APARICIO and LUNA, 2002; LUNA *et al.*, 2006; MORALES *et al.*, 1995). It has a very low odour threshold (0.7-50 μg $kg^{-1}$) so its contribution to the whole aroma can be considered important.

SOLINAS *et al.* (1987) found that the concentrations of the aldehydes *E*-2-pentenal, hexanal and *E*-2-heptenal increase considerably in the oxidised oils. The authors suggested using *E*-2-heptenal as a marker for oxidation rather than *E*-2-pentenal and hexanal, since these two compounds are already present in the aroma of extra virgin olive oils.

MORALES *et al.* (1997) monitored the oxidation stages of a virgin olive oil during an accelerated thermoxidation process by determining the concentration of nonanal. The hexanal/nonanal ratio was found to be an appropriate way to detect the beginning of oxidation and follow its evolution, even if the hexanal is present in the original flavour (MORALES *et al.*, 1997).

In an interesting work VICHI *et al.* (2003) investigated the oxidative changes in virgin olive oil headspace by using SPME/GC-MS technique to select some compounds as possible markers of the oxidation process. Samples, analyzed weekly, were oxidized at 60°C for 4 months. The peaks corresponding to nonanal and hexanal increased most rapidly during oxidation. This demonstrated that whereas the amount of hexanal is due to both autoxidation and the lipoxygenase cascade (through the formation of 13-LOOH), the concentration of nonanal may be solely attributed to the autoxidation of oleic acid.

2-Alkenals, such as *E*-2-pentenal, *E*-2-heptenal and *E*-2-decenal were also formed. These unsaturated aldehydes originate from secondary reactions of the primary autoxidation products (13-LnOOH, 9-LOOH and 9-OOOH, respectively). Moreover, the concentrations of 2,4-heptadienal isomers and *E,E*-2,4-decadienal (from 12-LnOOH and 9-LOOH decomposition, respectively) were positively correlated with the time of oxidation. Among the alkanes, octane (formed from 10-OOOH) increased the fatest. The hexanoic acid content also increased and was due to the secondary decomposition of hexanal and 2,4-decadienal from fatty acid oxidation. During the oxidation process, 2-pentylfuran and 2-ethylfuran are formed due to the degradation reactions of linoleate and linolenate hydroperoxides, respectively. These substituted furan compounds, in particular 2-pentylfuran, increased rapidly from traces to considerable amounts during oxidation. VICHI *et al.* (2003) proposed using them as markers for distinguishing oils in the late stages of oxidation.

KANAVOURAS *et al.* (2004) carried out a storage study based on three major contributors to the oxidative degradation in packaged olive oil: temperature, availability of light, and presence of oxygen. They selected a group of volatiles such as hexanal, 2-pentyl furan, *E*-2-heptenal, nonanal, and E-2-decenal, highly correlated with oxidation in packaged extra virgin olive oil under various storage conditions for one year (glass/PET/PVC bottles; 15/30/40°C temperature; light or dark conditions).

KALUA *et al.* (2006) carried out a real-time shelf life study for one year; virgin olive oil samples were stored under different conditions: in the light at ambient temperature, in the dark at ambient temperature, at low temperature in the dark, with or without headspace.

All volatile compounds found in fresh oil decreased during storage in the light in the presence of oxygen, particularly *E*-2-hexenal. Under the non-accelerated conditions used in this study, neither nonanal nor 2-pentenal or 2-heptenal were identified as oxidation markers. Octane was the marker for storage in the light, whereas hexanal discriminated virgin olive oil stored in the light in the presence of oxygen. Only pentanal discriminated low-temperature storage. The major difference between oils stored with or without headspace was the appearance of longer chain volatile compounds, such as octanal and *E*-2-nonen-1-ol.

LUNA *et al.* (2006) evaluated the effect of ultraviolet radiation on the production of off-flavours in bottled virgin olive oil. This study showed that it is possible to predict the value of the rancid attribute of a sample submitted to UV radiation based on its nonanal concentration. The intensity of positive attributes (green, fruity, bitter, and pungent) decreased along the process in both varieties under study (Arbequina and Picual), while the intensity of the rancid attribute increased greatly. During irradiation treatment with UV lamp for 12 days, *E*-2-hexenal was the volatile that decreased the most. On the other hand, 2-butenal, and 2-pentenal formed from hydroperoxides of linoleic acid and octane, octanal, nonanal, and 2-decenal formed from hydroperoxides of oleic acid were the compounds that showed the greatest variation.

The major compounds indicated by different researchers as markers of virgin olive oil oxidation, their sensory characteristics and odour thresholds are reported in Table 2.

In conclusion, *E*-2-heptenal, nonanal and 2-decenal are the most frequently used volatile markers of oxidation of virgin olive oil during storage. These three compounds are characterized by low odour threshold (5, 150 and 100 μg $kg^{-1}$, respectively) and by negative off-flavours namely oxidized, fatty and fish, that strongly contribute to the rancid defect perceived by assessors.

## THE EFFECTS OF OIL STORAGE ON PHENOLIC COMPOUNDS: THE IMPACT ON TASTE

Virgin olive oil contains minor compounds that are of great sensory and biological importance; the molecules that

Table 2 - Compounds indicated as markers of virgin olive oil oxidation, their sensory characteristics, odour thresholds and related references.

| Compounds | Sensory characteristics | Odor threshold (mg $kg^{-1}$) | References |
|---|---|---|---|
| octane | sweet | 0.940 | MORALES *et al.*, 2005 |
| octanal | fatty, soapy | 0.320 | MORALES *et al.*, 2005 |
| nonanal | waxy, paint, soapy | 0.150 | GUTH and GROSCH, 1991; MORALES *et al.*, 2005 |
| *E*-2-pentenal | paint, apple | 0.300 | SERVILI *et al.*, 2001; MORALES *et al.*, 2005 |
| *E*-2-heptenal | oxydized, tallowy | 0.050 | MORALES *et al.*, 2005 |
| *E*-2-decenal | paint, fishy, fatty | 0.010 | MORALES *et al.*, 2005 |
| 2,4-heptadienal | fatty, nutty, rancid | 3.62 | ULLRICH and GROSCH, 1988; MORALES *et al.*, 2005 |
| *E,E*-2,4-decadienal | fatty, deep-fried | 0.180 | REINERS and GROSCH, 1998 |
| 2-ethylfuran | sweet, rancid | n.d. | MORALES *et al.*, 1997 |
| hexanoic acid | sweaty, rancid | 0.700 | MORALES *et al.*, 1997 |

have a phenolic structure are noted for their antioxidant properties. Among these the tocopherols are related to lipids due to the presence of a hydrophobic side chain in the molecule and the hydrophilic phenols characterised by greater polarity. The former are important for the antiradical and nutritional (vitamin E) properties, whereas the latter greatly influence the taste quality (bitter and pungency attributes) as well as the beneficial biological activity and oxidative stability of virgin olive oil (SERVILI, 2004; BENDINI *et al.*, 2007). Few individuals, except for trained tasters of VOO, know that bitterness and pungency perceived by taste are positive attributes of VOO. These two sensory characteristics are strictly connected by the quali-quantitative phenolic profile of the product. Some phenols mainly elicit the taste perception of bitterness, while other phenolic molecules stimulate the free endings of the trigeminal nerve located in the palate and in the gustative buds giving rise to the chemesthetic perceptions of pungency and astringency attributes.

The major phenolic compounds identified and quantified in olive oil belong to five different classes: phenolic acids (especially derivatives of benzoic and cinnamic acids), flavons (luteolin and apigenin), lignans ((+)-pinoresinol and (+)-acetoxypinoresinol), phenyl-ethyl alcohols (hydroxytyrosol, tyrosol) and secoiridoids (aglycon derivatives of oleuropein and ligstroside) the latter are peculiar to virgin olive oil.

Several authors have associated some phenols with bitterness and have obtained models and determined relationships between individual phenols and bitterness intensity measured by a panel test or calculated from spectrophotometric absorbance at 225 nm known as the bitterness index (GUTIÉRREZ *et al.*, 1989; GUTIÉRREZ-ROSALES *et al.*, 2003; MATEOS *et al.*, 2004). A clear example of the different sensory properties of secoiridoid derivatives was reported by ANDREWES *et al.* (2003). They assessed the relationship between phenols and olive oil pungency; the dialdehydic form of ligstroside aglycon was found to be responsible for the burning sensation present in many olive oils. In contrast, the dialdehydic form of the oleuropein aglycon, tasted at an equivalent concentration, produced very little burning sensation. Another study confirmed that the dialdehydic form of ligstroside aglycon is the principal agent in VOO responsible for throat irritation. ANDREWES *et al.* (2003) deduced that the pungent intensity could be measured by isolating this molecule from different VOO. Starting from this last work BEAUCHAMP *et al.* (2005) compared the effect of the synthetic form (named "oleocanthal", with oleo- for olive, -canth- for sting, and -al for aldehyde) to that of the purified compound from VOO.

Regarding bitterness, after isolation and water solubilization of the major peaks of the phenolic profile of VOO separated by preparative HPLC and purified, GUTIÉRREZ-ROSALES *et al.* (2003) concluded that aldehydic and dialdehydic forms of oleuropein were mainly responsible for the bitter taste of VOO. One year later, MATEOS *et al.* (2004) reported a better correlation between the aldehydic form of oleuropein aglycon and bitterness. Using a trained olive oil sensory panel SINESIO *et al.* (2005) studied the temporal perception of bitterness and pungency utilizing a time-intensity (TI) evaluation technique. They showed that the bitterness curves had a faster rate of rising and declining than the pungency curves. The different kinetic perception is linked to the slower (2-30 m/sec) signal transmission of thermal nociceptors compared to other neurones (up to 100 m/s). During oil storage secoiridoids undergo modifications (decomposition such as hydrolysis and oxidation reactions) that result in their decline and, consequently, to a reduced intensity of the typical bitter taste and

pungent note. ESTI *et al.* (2009) recently carried out a study on intensity changes of bitterness and pungency perception over time in seven samples stored up to 18 months under two different temperature conditions (10° and 28°C). The results showed that all of the oleuropein and ligstroside derivatives considered, except for hydroxytyrosol and tyrosol, were relevant predictors of the static and dynamic analysis for bitterness and pungency. In contrast, the dialdehydic form of the ligstroside aglycon was only effective for predicting the pungency decrease in relation to storage time and temperature, but it was not correlated to changes in bitterness.

Even in small quantities, phenols in virgin olive oil are fundamental for protecting glycerides from oxidation; in fact, by virtue of their favourable oxidation potential, they exert an intense protective action by exposing themselves to oxidation in the place of the lipid substrate. Phenolic compounds can inhibit oxidation through a variety of mechanisms based on radical scavenging, hydrogen atom transfer and metal-chelating. As chain breakers, they act by donating a hydrogen radical to alkylperoxyl radicals that are formed during the initiation step of lipid oxidation. The important role that phenolic compounds play particularly the *o*-diphenol structure, in protecting lipids from autoxidation, also when catalysed by metal such as copper, was evidenced in several works that, in particular, even when catalysed by metal such as copper has been reported in several works that, in particular, deal with antioxidant activity of phenols in bulk oil and in oil-in-water emulsion.

PAIVA-MARTINS *et al.* (2006) studied the antioxidant activity and interactions with copper of oleuropein, hydroxytyrosol, monoaldehydic and dialdehydic forms of oleuropein, in virgin olive oil and oil-in-water emulsions stored at 60°C (BONOLI-CARBOGNIN *et al.*, 2008). They concluded that the formation of a copper complex with radical scavenging activity is a key step in the antioxidant action of the olive oil phenolic compounds in an emulsion containing copper ions. On the other hand, BENDINI *et al.* (2006) working with a bulk oil system, reported a more rapid consumption of the tocopherols in samples that had the lowest *o*-diphenols content and these latter were able to chelate copper.

The storage of virgin olive oils at low temperature may have a positive effect by slowing the kinetics of the oxidative reactions; even if at the same time it decreases the solubility of the phenolic fraction that combats the lipid oxidation. The effects of storage at incorrect temperatures, whether too high or too low, may have significant repercussions on phenolic substances and indirectly on both shelf life and sensory characteristics of virgin olive oils. These excesses in temperatures may occur during the winter or summer and lead to changes in the quality of the oil stored in tanks without a temperature regulator or in bottles kept in warehouses. Data obtained in a study by CERRETANI *et al.* (2005) and BONOLI *et al.* (2005), illustrate an important technological consequence related to the storage conditions of virgin olive oils. If the storage temperature is subjected to a marked temperature decreasing (e.g. close to 0°C), the oil will change its physical state due to the crystallization of more saturated triacylglycerols and waxes. This could result in a destabilization of both micro-droplets of water and polar phenolic molecules and a loss of the total availability of the antioxidant compounds which would reduce its natural protection against lipid oxidation. Some interesting changes were found in defrosted oils after the freezing process. The oils showed a significant decrease in the oxidative stability value (by OSI test) that corresponded to the loss in the phenolic fraction. The loss of phenols, particularly the *o*-diphenols which are known to be the most ac-

tive compounds for the shelf life of the oil, depends on their structure-activity relationship. A certain fraction of phenols, solubilised into the micro-droplets of water in the oil probably precipitated during the freezing/defrosting processes with a proportionate reduction in its oxidative resistance. Destabilization and precipitation of phenols also cause an unavoidable loss in positive taste attributes of the virgin olive oil.

The main effects observed in the phenolic fraction during oil storage are: hydrolysis of secoiridoids and oxidation of some phenolic molecules. The first mechanism, due to enzymatic or chemical reactions, leads to a breaking of ester bonds into molecules of oleuropein and ligstroside aglycons and to an increase in the amounts of elenolic acid, hydroxytyrosol and tyrosol. Furthermore, the oleuropein and ligstroside aglycons in their monoaldehydic forms generally lose the carboxymethyl group that causes an increase in concentrations of the respective dialdehydic forms. These trends have been well documented in several studies. CINQUANTA *et al.* (1997) studied the evolution of simple phenols during 18 months of storage in the dark. The tyrosol and hydroxytyrosol contents increased notably due to hydrolysis of their complex derivatives in a first stage, and a rapid loss of hydroxytyrosol compared with that of tyrosol at the end of the storage period, due to a higher antioxidant activity of the former. The partial transformation of secoiridoid derivatives into simple forms such as hydroxytyrosol, tyrosol and elenolic acid, leads

Table 3 - Correlations between phenolic compounds and taste perceptions and related references.

| Short name | Common name | Sensory description | References |
|---|---|---|---|
| 3,4-DHPEA-EDA | decarboxymethyl oleuropein aglycon | main compound responsible for bitter taste | KIRITSAKIS, 1998; |
| 3,4-DHPEA-EA | oleuropein aglycon | main compound responsible for bitter taste | GARCIA *et al.*, 2001 |
| *p*-HPEA-EDA | decarboxymethyl ligstroside aglycon | main compound responsible for bitter and pungent notes | TOVAR *et al.*, 2001 |
| 3,4-DHPEA-EDA | decarboxymethyl oleuropein aglycon | high positive correlation | GUTIÉRREZ- |
| 3,4-DHPEA-EA | oleuropein aglycon | between these compounds | ROSALES *et al.*, 2003 |
| *p*-HPEA-EDA | decarboxymethyl ligstroside aglycon | and bitterness intensity of olive oil | |
| *p*-HPEA-EDA | decarboxymethyl ligstroside aglycon | main compound responsible for the pungent sensation on back of the tongue | ANDREWES *et al.*, 2003 |
| 3,4-DHPEA-EA | oleuropein aglycon | a highly significant correlation with bitter taste of olive oil | MATEOS *et al.*, 2004 |
| 3,4-DHPEA-EA | oleuropein aglycon | positive correlation between this compound and bitterness and pungency intensity of several Spanish and Italian olive oils | CERRETANI *et al.*, 2008 |
| Secoiridoids | all oleuropein and ligstroside derivatives considered except for hydroxytyrosol and tyrosol | relevant predictors of the static and dynamic analysis for bitterness and pungency | ESTI *et al.*, 2009 |
| *p*-HPEA-EDA | decarboxymethyl ligstroside aglycon | effective only for predicting pungency | |

to a decrease in bitterness and pungent intensity.

The formation of new compounds due to phenol oxidation has been proposed by some researchers. According to ARMAFORTE *et al.* (2007) it may be feasible to use the ratio of fresh phenols/oxidized phenols to determine the freshness/aging ratio of a virgin olive oil. This ratio appeared to decrease rapidly in samples that had an increased content of oxidized phenols. Oxidized phenols are produced by thermic and forced oxidative stress, as in the case of extended conservation (ROVELLINI and CORTESI, 2002; RÍOS *et al.*, 2005; CARRASCO-PANCORBO *et al.*, 2007).

Table 3 shows the major compounds reported by different researchers to be the major components responsible for taste perceptions such as bitterness and pungency.

In conclusion, phenols, particularly secoiridoids generally decrease during VOO storage, due to the hydrolysis of secoiridoid derivatives in the hydroxytyrosol, tyrosol and elenolic acid and formation of oxidized phenols. These reactions lead to a decrease in bitterness and pungent intensity, positive attributes that are characteristic of a fresh VOO.

## REFERENCES


Andrewes P., Busch J.L.H.C., De Joode T., Groenewegen A. and Alexandre H. 2003. Sensory properties of virgin olive oil polyphenols: Identification of deacetoxy-ligstroside aglycon as a key contributor to pungency. J. Agric. Food Chem., 51: 1415.

Angerosa F. and Di Giacinto L. 1993. Oxidation des huiles d'olive vierges par les metaux: Manganese et nickel. Note I. ReV. Fran. Corps Gras, 40: 41.

Angerosa F. 2000. Sensory quality of olive oils. In "Handbook of Olive Oil: Analysis and Properties". J. Harwood & R. Aparicio (Eds.), Aspen Publications Inc Gaithersburg, MD, USA.

Angerosa F. 2002. Influence of volatile compounds on virgin olive oil quality evaluated by analytical approaches and sensor panels. Eur. J. Lipid Sci. Technol. 104: 639.

Angerosa F., Mostallino R., Basti C. and Vito R. 2001. Influence of malaxation temperature and time on the quality of virgin olive oils. Food Chem. 72: 19.

Angerosa F., Servili M., Selvaggini R., Taticchi A., Esposto S. and Montedoro G.F. 2004. Volatile compounds in virgin olive oil: occurrence and their relationship with the quality. J. Chrom. A, 1054: 17.

Aparicio R. and Morales M.T. 1998. Characterization of olive ripeness by green aroma compounds of virgin olive oil. J. Agric. Food Chem. 46: 1116.

Aparicio R. and Luna G. 2002. Characterization of monovarietal virgin olive oils. Eur. J. Lipid Sci. Technol. 104: 614.

Armaforte E., Mancebo-Campos V., Bendini A., Salvador M.D., Fregapane G. and Cerretani L. 2007. Retention effects of oxidized polyphenols during analytical extraction of phenolic compounds of virgin olive oil. J. Sep. Sci. 30: 2401.

Baccouri O., Bendini A., Cerretani L., Guerfel M., Baccouri B., Lercker G., Zarrouk M. and Daoud Ben Miled D. 2008. Comparative study on volatile compounds from Tunisian and Sicilian monovarietal virgin olive oils. Food Chem. 111: 322.

Baldioli M., Servili M., Perretti G. and Montedoro G.F. 1996. Antioxidant activity of tocopherols and phenolic compounds of virgin olive oil. J. Am. Oil Chem. Soc. 73: 1589.

Beauchamp G.K., Keast R.S.J., Morel D., Lin J., Pika J., Han Q., Lee C-H., Smith A. B. and Breslin P.A.S. 2005. Ibuprofen-like activity in extra-virgin olive oil. Nature 437: 45.

Bendini A., Cerretani L., Vecchi S., Carrasco-Pancorbo A. and Lercker G. 2006. Protective effects of extra virgin olive oil phenolics on oxidative stability in the presence or absence of copper ions. J. Agric. Food Chem. 54: 4880.

Bendini A., Cerretani L. and Lercker G. 2007. Panoramica sulla qualità degli oli vergini di oliva prodotti in Romagna. Riv. It. Sost. Grasse. 84: 191.

Bendini A., Cerretani L., Carrasco-Pancorbo A., Gómez-Caravaca A.M., Segura-Carretero A., Fernández-Gutiérrez A. and Lercker G. 2007. Phenolic molecules in virgin olive oils: a survey of their sensory properties, health effects, antioxidant activity and analytical methods. An overview of the last decade. Molecules, 12: 1679.

Benjelloun B., Talou T., Delmas M. and Gaset A. 1991. Oxidation of rapeseed oil: Effect of metal traces. J. Am. Oil Chem Soc. 68: 210.

Bonoli-Carbognin M., Cerretani L., Bendini A., Gallina Toschi T. and Lercker G. 2005. The case of monovarietal olive oil: storage test at different temperature. Ind. Alim. 44: 1135.

Bonoli-Carbognin M., Cerretani L., Bendini A., Pilar Almajano M. and Gordon M.H. 2008. Bovine serum albumin produces a synergistic increase in the antioxidant activity of virgin olive oil phenolic compounds in oil-in-water emulsions. J. Agric. Food Chem. 56: 7076.

Caponio F., Bilancia M.T., Pasqualone A., Sikorska E. and Gomes T. 2005. Influence of the ex-

posure to light on extra virgin olive oil quality during storage. Eur. Food Res. Technol. 221: 92.

Carrasco-Pancorbo A., Cerretani L., Bendini A., Segura-Carretero A., Del Carlo M., Gallina-Toschi T., Lercker G., Compagnone D., and Fernández-Gutiérrez A. 2005. Evaluation of the antioxidant capacity of individual phenolic compounds in virgin olive oil. J. Agric. Food Chem. 53: 8918.

Carrasco-Pancorbo A., Cerretani L., Bendini A., Segura-Carretero A., Lercker G. and Fernández-Gutiérrez A. 2007. Evaluation of the influence of thermal oxidation in the phenolic composition and in antioxidant activity of extra-virgin olive oils. J. Agric. Food Chem. 55: 4771.

Catalano M. and De Felice M. 1970. L'autossidazione delle sostanze grasse. nota 1 – Influenza degli acidi grassi liberi. Riv. It. Sost. Grasse 47: 484.

Cerretani L., Bendini A., Biguzzi B., Lercker G. and Gallina Toschi T. 2005. Freezing storage can affect the oxidative stability of not filtered extra-virgin olive oils. J. Commodity Sci. 44: 3.

Cerretani L., Salvador M.D., Bendini A. and Fregapane G. 2008. Relationship between sensory evaluation performed by italian and spanish official panels and volatile and phenolic profiles of virgin olive oils. Chem. Percept. 1: 258.

Choe E. and Min D.B. 2006. Mechanisms and factors for edible oil oxidation. Comprehensive Rev. Food Sci. Food Safety. 5: 169.

Cinquanta L., Esti M. and La Notte E. 1997. Evolution of phenolic compounds in virgin olive oil during storage. J. Am. Oil Chem. Soc. 74: 1259.

Cuppett S., Schnepf M. and Hall III C. 1997. Natural antioxidants-Are they reality? In "Natural Antioxidants: Chemistry, Health Effects and Applications". Shahidi, F. Ed. p 12. AOCS Press, Champaign, IL,.

Del Nobile M.A., Bove S., La Notte E. and Sacchi R. 2003. Influence of packaging geometry and material properties on the oxidation kinetic of bottled virgin olive oil. J. Food Eng. 57: 189.

Di Giovacchino L., Mucciarella M.R., Costantini N., Ferrante M.L. and Surricchio G. 2002. Use of nitrogen to improve stability of virgin olive oil during storage. J. Am. Oil Chem. Soc. 79: 339.

Endo Y., Usuki R. and Kaneda T. 1984. Prooxidant activities of chlorophylls and their decomposition products on the photoxidation of methyl linoleate. J. Am. Oil. Chem. Soc. 61: 781.

Esti M., Contini M., Moneta E. and Sinesio F. 2009. Phenolics compounds and temporal perception of bitterness and pungency in extra-virgin olive oils: Changes occurring throughout storage. Food Chem. 113: 1095.

Frankel E.N. 1982. Volatile lipid oxidation products. Prog. Lipid Res. 22: 1.

Frankel E.N. 1985. Chemistry of autoxidation: mechanism, products and flavor significance. In: "Flavor Chemistry of Fats and Oils". D. B. Min, T. H. Smouse Eds. p 1. AOCS Press, Champaign, IL (USA).

Frankel E.N., Huang S.W., Kanner J. and German J.B. 1994. Interfacial phenomena in the evaluation of antioxidants: bulk oils vs. emulsions. J. Agric. Food Chem. 42: 1054.

Frankel E.N., Huang S.W., Prior E. and Aeschbach R. 1996. Evaluation of antioxidant activity of rosemary extracts, carnosol and carnosic acid in bulk vegetable oils and fish oil and their emulsions. J. Sci. Food Agric. 72: 201.

Frankel N. 1998. "Lipid Oxidation". The Oily Press Ltd, Dundee, Scotland.

Frega N., Mozzon M. and Lercker G. 1999. Effects of free fatty acids on oxidative stability of vegetable oil. J. Am. Oil Chem. Soc. 76: 325.

Fregapane G., Lavelli V., Leon S., Kapuralin J. and Salvador M.D. 2006. Effect of filtration on virgin olive oil stability during storage. Eur. J. Lipid Sci. Technol. 108: 134.

Garcia J.M., Yousfi K., Mateos R., Olmo M. and A. Cert. 2001. Reduction of bitterness by heating of olive (*Olea europea*). J. Agric. Food Chem. 49: 4231.

Gómez-Alonso S., Mancebo-Campos V., Salvador M.D. and Fregapane G. 2004. Oxidation kinetics in olive oil triacylglycerols under accelerated shelf-life testing (25-75°C). Eur. J. Lipid Sci. Technol. 106: 369.

Gómez-Alonso S., Mancebo-Campos V., Salvador M.D. and Fregapane G. 2007. Evolution of major and minor components and oxidation indices of virgin olive oil during 21 months storage at room temperature. Food Chem. 100: 36.

Gómez-Caravaca A.M., Cerretani L., Bendini A., Segura-Carretero A., Fernández-Gutiérrez A. and Lercker G. 2007. Effect of filtration systems on the phenolic content in virgin olive oil by HPLC-DAD-MSD. Am. J. Food Technol. 2: 671.

Gutfinger T. 1981. Polyphenols in olive Oil. J. Am. Oil Chem. Soc. 58: 966.

Guth H. and Grosch W. 1990. Deterioration of soya-bean oil: quantification of primary flavour compounds using a stable isotope dilution assay. Lebensm. Wiss. u. Technol. 23: 513.

Guth H. and Grosch W. 1991. A comparative-study of the potent odorants of different virgin olive oils. Fat Sci. Technol. 93: 335.

Gutiérrez R., Janer del Valle C., Janer del Valle M.L. and Gutiérrez F. 1977. Relación entre los polifenoles y la calidad y estabilidad del aceite de oliva virgen. Grasas y Aceites. 28: 101.

Gutiérrez-Rosales F., Gómez-Herrera C. and Gutiérrez-González-Quijano R. 1988. Estudios de la cinética de evolución de los índices de calidad del aceite de oliva virgen durante su conservación en envases comerciales. Grasas y Aceites. 39: 245.

Gutiérrez F., Albi M.A., Palma R., Ríos J.J. and Olias J.M. 1989. Bitter taste of virgin olive oil: Correlation of sensory evaluation and instrumental HPLC analysis. J. Food Sci. 54: 68.

Gutiérrez-Rosales F., Garrido-Fernández J., Gallardo-Gerrero L., Gandul-Rojas B. and Mínguez-Mosquera M.I. 1992. Action of chlorophylls on the stability of virgin olive oil. J. Am. Oil. Chem. Soc. 69: 866.

Gutiérrez-Rosales F., Ríos J. J. and Gómez-Rey MA. L. 2003. Main polyphenols in the bitter taste of virgin olive oil. Structural confirmation by online high-performance liquid chromatography electrospray ionization mass spectrometry. J. Agric. Food Chem. 51: 6021.

Holman R.T. and Elmer O.C. 1947. The rates of oxidation of unsaturated fatty acids and esters. J. Am. Oil Chem. Soc. 24: 127.

Jadhav S.J., Nimbalkar S.S., Kulkami A.D. and Madhavi D.L. 1966. Lipid oxidation in biological and food systems. In: "Food Antioxidants" Madhavi, D.L., Deshpande, S.S., Salunkhe, D.K., Marcel Dekker Eds. P. 5. New York.

Kalua C.M., Bedgood D.R., Bishop A.G. and Prenzler P.D. 2006. Discrimination of storage conditions and freshness in virgin olive oil. J. Agric. Food Chem. 54: 7144.

Kalua C.M., Allen M.S., Bedgood D.R., Bishop A.G., Prenzler P.D. and Robards K. 2007. Olive oil volatile compounds, flavour development and quality: A critical review. Food Chem. 100: 273.

Kanavouras A., Hernandez-Munoz P. and Coutelieris F.A. 2004. Shelf life predictions for packaged olive oil using flavor compounds as markers. Eur. Food Res. Technol. 219: 190.

Kanavouras A. and Coutelieris F.A. 2006a. Shelf-life predictions for packaged olive oil based on simulations. Food Chem. 96: 48.

Kanavouras A., Hernandez-Munoz P. and Coutelieris F.A. 2006b. Packaging of olive oil: Quality issues and shelf life predictions. Food Rev. Intern. 22: 381.

Kiritsakis A.K. 1992. "El Aceite de Oliva". Ediciones A. Madrid Vicente. Madrid, Spain.

Kiritsakis A.K. 1998. Flavor components of olive oil. A review. J. Am. Oil Chem. Soc. 75: 673.

Lavelli V., Fregapane G. and Salvador M.D. 2006. Effect of storage on secoiridoid and tocopherol contents and antioxidant activity of monovarietal extra virgin olive oils. J. Agric. Food Chem. 54: 3002.

Le Tutour B. and Guedon D. 1992. Antioxidative activities of *Olea europaea* leaves and related phenolic compounds. Phytochem. 31: 1173.

Litridou M., Linssen J., Schols H., Bergmans M., Posthumus M., Tsimidou M. and Boskou D. 1997. Phenolic compounds in virgin olive oils: Fractionation by solid phase extraction and antioxidant activity assessment. J. Sci. Food Agric. 74: 169.

Luna G., Morales M.T. and Aparicio R. 2006. Changes induced by UV radiation during virgin olive oil storage. J. Agric. Food Chem. 54: 4790.

Mancebo-Campos V., Fregapane G. and Salvador M.D. 2008. Kinetic study for the development of an accelerated oxidative stability test to estimate virgin olive oil potential shelf life. Eur. J. Lipid Sci. Technol. 110: 969.

Mancebo-Campos V., Salvador M.D. and Fregapane G. 2007. Comparative study of virgin olive oil behavior under rancimat accelerated oxidation conditions and long-term room temperature storage. J. Agric. Food Chem. 55: 8231.

Marinova E.M. and Yanishlieva N.V. 1996. Antioxidative activity of phenolic acids on triacylglycerols and fatty acid methyl esters from olive oil. Food Chem. 56: 139.

Mateos R. 2002. Caracterización de componentes fenólicos del aceite de oliva y su relación con la estabilidad oxidativa y el amargo. PhD Thesis, Universidad de Sevilla.

Mateos R., Cert A., Pérez-Camino C.M. and García J.M. 2004. Evaluation of virgin olive oil bitterness by quantification of secoiridoid derivatives. J. Am. Oil Chem. Soc. 81: 71.

Méndez A.I. and Falqué E. 2007. Effect of storage time and container type on the quality of extra-virgin olive oil. Food Control. 18: 521.

Mistry B.S. and Min D.B. 1987. Effects of fatty acids on the oxidative stability of soybean oil. J. Food Sci. 52: 831.

Mistry B.S. and Min, D.B. 1988. Prooxidants effects of monoglycerides and diglycerides in soybean oil. J. Food Sci. 53: 1896.

Miyashita K., Frankel E.N. and Neff W.E. 1990. Autoxidation of polyunsaturated triacylglycerols. III. Synthetic triacylglycerols containing linoleate and linolenate. Lipids. 25: 48.

Miyashita K. and Takagi T. 1986. Study on the oxidative rate and prooxidant activity of free fatty acids. J. Am. Oil Chem. Soc. 63: 1380.

Montedoro G.F., Servili M., Baldioli M. and Miniati E. 1992. Simple and hydrolyzable phenolic compounds in olive oil. Note 1. Their extraction, separation, and quantitative and semi-quantitative separation and evaluation by HPLC. J. Agric. Food Chem. 40: 1571.

Morales M.T., Ríos J.J. and Aparicio R. 1997. Changes in the volatile composition of virgin olive oil during oxidation: flavors and off-flavors. J. Agric. Food Chem. 45: 2666.

Morales M.T., Luna G., and Aparicio R. 2005. Comparative study of virgin olive oil sensory defects. Food Chem. 91: 293.

Paiva-Martins F., Santos V., Mangericao H. and Gordon M.H. 2006. Effects of copper on the antioxidant activity of olive polyphenols in bulk oil and oil-in-water emulsions. J. Agric. Food Chem. 54: 3738.

Polvillo M.M., Albi T. and Guinda A. 1994. Determination of trace elements in edible vegetable oils by atomic absorption spectrophotometry. J. Am. Oil Chem. Soc. 71: 347.

Porter W.L., Black E.D. and Drolet A.M. 1989. Use of polyamide oxidative fluorescence test on lipid emulsions: contrast on relative effectiveness of

antioxidants in bulk versus dispersed systems. J. Agric. Food Chem. 37: 615.

Psomiadou E. and Tsimidou M. 2002. Stability of virgin olive oil. 1. Autoxidation studies. J. Agric. Food Chem. 50: 716.

Ragnarsson J.O. and Labuza T.P. 1977. Accelerated shelf-life testing for oxidative rancidity in foods - A review. Food Chem. 2: 291.

Rahmani M. and Saari-Csallany A. 1998. Role of minor constituents in the photoxidation of virgin olive oil. J. Am. Oil Chem. Soc., 75: 837.

Rastrelli L., Passi S., Ippolito F., Vacca G. and De Simone F. 2002. Rate of degradation of alpha-tocopherol, squalene, phenolics, and polyunsaturated fatty acids in olive oil during different storage conditions. J. Agric. Food Chem. 50: 5566.

Reiners J. and Grosch W. 1998. Odorants of virgin olive oils with different flavor profiles. J. Agric. Food Chem. 46: 2754.

Ríos J.J., Gil M.J. and Gutiérrez-Rosales F. 2005. Solid-phase extraction gas chromatography-ion trap-mass spectrometry qualitative method for evaluation of phenolic compounds in virgin olive oil and structural confirmation of oleuropein and ligstroside aglycons and their oxidation products. J. Chromatogr. A 1093: 167.

Rovellini P. and Cortesi N. 2002. Liquid chromatography-mass spectrometry in the study of oleuropein and ligstroside aglycons in virgin olive oil: aldehydic, dialdehydic forms and their oxidized products. Riv. Ital. Sost. Grasse 79: 1.

Sacchi R., Savarese M., Del Regno A., Paduano A., Terminiello R. and Ambrosino M.L. 2008. Shelf life of vegetable oils bottled in different scavenging polyethyleneterephthalate (PET) containers. Packaging Technol. Sci. 21: 269.

Salvador M.D., Aranda F. and Fregapane G. 1999. Contribution of chemical components of "Cornicabra" virgin olive oils to oxidative stability. A study of three successive crop seasons. J. Am. Oil Chem. Soc. 76: 427.

Scarpellini A., Cerretani L., Bendini A. and Gallina Toschi T. 2005. Effect of free acidity on the oxidative stability of a neutralized oil. Ind. Alim. 44: 22.

Servili M., Baldioli M., Miniati E. and Montedoro G.F. 1996. Antioxidant activity of new phenolic compounds extracted from virgin olive oil and their interaction with α-tocopherol and β-catotene. Riv. Ital. Sostanze Grasse. 73: 55.

Servili M., Selvaggini R., Taticchi A. and Montedoro G.F. 2001. In: "Food Flavors and Chemistry" A.M. Spanier, F. Shahidi, T.H. Parliment, C. Mussiman, C.-T. Ho, E. Tratras Contis (Eds.) p 236, The Royal Society of Chemistry Publishers, Cambridge, U.K.

Servili M., Selvaggini R., Esposto S., Taticchi A., Montedoro G.F. and Morozzi G. 2004. Health and sensory properties of virgin olive oil hydrophilic phenols: agronomic and technological aspects of production that affect their occurrence in the oil. J. Chrom. A. 1054: 113.

Sinesio F., Moneta E. and Esti M. 2005. The dynamic sensory evaluation of bitterness and pungency in virgin olive oil. Food Qual. Pref. 16: 557.

Solinas M., Angerosa F. and Cucurachi A. 1987. Connessione tra I prodotti di neoformazione ossidativa delle sostanze grasse e insorgenza del difetto di rancidità all'esame organolettico, Nota II. Determinazione quantitativa. Riv. It. Sost. Grasse 64: 137.

Tawfik M.S. and Huyghebaert A. 1999. Interaction of packaging materials and vegetable oils: oil stability. Food Chem. 64: 451.

Tovar M.J., Motilva M.J. and Romero M.P. 2001. Changes in the phenolic composition of virgin olive oil from young trees (*Olea europaea* L. cv. *Arbequina*) grown under linear irrigation strategies. J. Agric. Food Chem. 49: 5502.

Tsimidou M.Z., Georgiou A., Koidis A. and Boskou D. 2005. Loss of stability of "veiled" (cloudy) virgin olive oils in storage. Food Chem. 93; 377.

Ullrich F. and Grosch W. 1988. Identification of the most intense odor compounds formed during autoxidation of methyl linolenate at room temperature. J. Am. Oil Chem. Soc. 65: 1313.

Vázquez-Roncero A., Janer del Valle C. and Janer del Valle M.L. 1973. Determinación de los polifenoles totales del aceite de oliva. Grasas y Aceites. 24: 350.

Velasco J. and Dobarganes C. 2002. Oxidative stability of virgin olive oil. Eur. J. Lipid Sci. Technol. 104: 661.

Vichi S., Pizzale L., Conte L.S., Buxaderas S. and López-Tamames E. 2003. Solid-phase microextraction in the analysis of virgin olive oil volatile fraction: Modifications induced by oxidation and suitable markers of oxidative status. J. Agric. Food Chem. 51: 6564.

Visioli F. and Galli C. 1998. Olive oil phenols and their potential effects on human health. J. Agric. Food Chem. 46: 4292.

Wagner K.H. and Elmafda I. 1999. Nutrient antioxidants and stability of frying oils: tocochromanols, β-carotene, phylloquinone, ubiquinone 50, In "Frying Foods". Boskou, D. and Elmafda, I. Eds.p 163. Technomic Publishing, Lancaster, PE.

Yanishlieva-Maslarova N.V. 2001. Inhibiting oxidation. In "Antioxidants in Food". Pokorny, J., Yanishlieva and N., Gordon, M. Eds. p 22. Woodhead Publishing Ltd., Cambridge.

Paper received December 2, 2008 Accepted June 3, 2009

PAPER

# CROSS-NATIONAL SEGMENTATION FOR MESSAGING ABOUT CHEESE: TOWARDS A NEW APPROACH TO CONSUMER UNDERSTANDING, DIRECTED DEVELOPMENT, AND TARGETED MARKETING

## CROSS-SEGMENTAZIONE NAZIONALE PER LA MESSAGGISTICA SUL FORMAGGIO: VERSO UN NUOVO APPROCCIO ALLA COMPRENSIONE DEL CONSUMATORE, ALLO SVILUPPO DIRETTO E AL MARKETING MIRATO

**G. GABAY[1], K. PAULUS[2], P. AARTS[3], J. BECKLEY[4], H. ASHMAN[4], and H.R. MOSKOWITZ[5]***

[1] College of Management Academic Studies, School of Behavioral Studies, Rishon Letzion, Israel
[2]ProGes Food GmbH, Ringstr. 1, D - 55245 Waldalgesheim, Germany
[3]1A, Route de Marche, 5377 Somme-Leuze, Belgium
[4]The Insight & Understanding Group, Denville, New Jersey, USA
[5]Moskowitz Jacobs Inc., 1025 Westchester Ave., 4th fl., White Plains, NY 10604, USA
*Corresponding author: Tel. 914-421-7400, Fax 914-428-8364, e-mail: mjihrm@sprynet.com

ABSTRACT

Questions regarding the existence of one appropriate message across global markets for the same cheese

RIASSUNTO

Fino ad oggi, le questioni riguardanti l'esistenza di un messaggio appropriato in tutti i mercati mondiali per lo stes-

- Key words: cheese, conjoint analysis, consumer mindset, cross-nation segmentation -

do not seem to have been explored. Three parallel, linked conjoint analysis studies were conducted in Germany, France and the United Kingdom to identify which features and communications about cheese products drive consumer interest. In each study, consumers evaluated 60 unique concepts comprising 2-4 elements dealing with 'types of cheese, emotional benefits, and country of export, respectively. The elements varied in the degree to which they drove interest. Three mind-set segments emerged when the respondents were segmented by the pattern of their individual utility values, independent of country. The segmentation transcended countries and conventional classification groups, such as gender and age. Segment 1 comprised individuals responsive to flavour messages about *cheese with something* (46%), segment 2 comprised individuals responsive to flavour messages about cheese, i.e., *cheese as the focus* (32%), and segment 3 comprised individuals responsive to experiential messages about the occasions when cheese is served, i.e., the *cheese experience* (22%). Since these segments transcend conventional demographics, to find these segments in the population requires an intervention method, i.e., a 'consumer interactive typing tool'. This study revealed that a 3-phrase questionnaire could correctly classify about 2/3 of the respondents into the appropriate segments. The results are discussed in terms of how knowing these segments can guide product developers and marketers through the creation of inbound 'micro-sites' for development and outbound 'targeted selling messages' for sales.

so tipo di formaggio non sembrano essere state esplorate. Tre paralleli e fra loro collegati studi di analisi congiunta sono stati condotti in Germania, Francia e Regno Unito, per identificare quali caratteristiche e comunicazioni sui prodotti caseari determinano gli interessi dei consumatori. In ciascuno studio, i consumatori hanno valutato 60 concetti unici comprendenti da 2 a 4 elementi riguardanti rispettivamente i tipi di formaggi e benefici emozionali e il paese di esportazione. Gli elementi variano dell'interesse sollevato. Tre segmenti di atteggiamento mentale sono emersi quando gli intervistati sono stati segmentati in base al modello dei loro valori di utilità individuale, indipendente dal paese. La segmentazione trascende dai paesi e dai gruppi di classificazione convenzionale, come sesso ed età. Il segmento 1 comprende risposte individuali sui messaggi del gusto come *formaggio con qualcosa* (46%), il segmento 2 comprende risposte individuali sui messaggi del gusto come formaggio per se (32%) e il segmento 3 comprende risposte individuali su messaggi empirici circa i momenti di consumo del formaggio, es. *l'esperienza del formaggio* (22%). Poiché questi segmenti trascendono le demografie convenzionali, per trovare questi segmenti nella popolazione è richiesto un metodo di intervento come, ad esempio, "uno strumento interattivo per i consumatori". Questo studio rivela che un questionario con tre frasi potrebbe correttamente classificare circa 2/3 degli intervistati dentro il segmento appropriato. I risultati sono discussi in termini di come questi segmenti possano guidare i progettisti e venditori dei prodotti attraverso la creazione di un "micro-sito" in arrivo per lo sviluppo e uno in partenza "messaggi di vendita mirato" per i venditori.

## INTRODUCTION

Firms use branding as a key strategy to attract new consumers and to retain current consumers with original brands, thereby improving the firms' market share (ARNADE *et al.*, 2009). Consumers' brand loyalty and desire for brand varieties play an important role in the demand for cheese products and the profitability of firms which market foods. The study of TREVISIOL (2005) for the European Dairy Industry Model forecasted an optimistic growth rate for the consumption of cheese. According to these estimates the consumption of cheese will increase in each country in Europe. Furthermore, the consumption of cheese and processed cheese in countries from the European Union-11 will increase by the highest annual average.

Since country borders have a porous nature, manufacturers of packaged goods need to develop a strategy beyond branding in order to maintain the attractiveness of those brands that are exported from a region of strong brand development to another region where the brand is unknown (BONETTI, 2004). One such strategy is *messaging* (KOTLER, 1997). Messaging is defined as marketing-derived 'words and phrases' that a company uses to describe a product to a consumer, in order to convince the consumer to buy it and to consume it. Messaging carries with it a tonality beyond description. It is description motivated by the desire to convince consumers in a positive way, either about the product itself, the brand, or the experience surrounding the product. Messaging precedes both brand and product (NEDRIECH and SWAIN, 2008).

From the standpoint of business economics, the number of foreign and domestic competitors has increased enormously (BONETTI, 2004), requiring that a company become increasing customer-focused in order to maintain a competitive edge. Consumers represent the ultimate but yet the most important component within the food chain. The study of consumers helps firms and organizations to improve their marketing strategies by understanding consumers' attitudes and shopping behavior. With this knowledge, marketers adapt and improve their marketing messaging strategies in order to reach the consumers' needs in the most appropriate way.

It is important to keep in mind that when one works with consumers, simple descriptions of products, their benefits, and the experiences with those products, do not necessarily suffice to motivate purchase. And, in the creation of a product, simple descriptions alone do not necessarily capture the unique features of products to which the consumer is attuned, and which the developer must capture in the prototype to be created. The messaging adds that bit of individuality and flair to the product, which in turn guides the developer, helping to identify specific 'extras' to be incorporated into the product.

Various cheese studies have investigated responses to both products and to messages regarding specific tastes, texture, and emotional experiences connected with cheese. For example, LIGGETT, DRAKE and DELWICHE (2005) reported that in Switzerland consumer acceptance of commercial Swiss cheeses is a function of their flavor characteristics. PATTISSON and LINDGREEN (2004) reported that the best development potential for the dairy industry in southwest England is mature cheddar, specialty cheeses, suitable territorial cheese varieties and other fresh cheeses.

Cheeses are also subject to economic factors, and to evolving economic conditions. For instance, in the Slovakian dairy industry, in the past production focused on cheap bulk commodities. Nowadays increasingly sophisticated consumers and more competitive markets push companies to segment their offerings (KAPSDORFERO-

VA and NAGYOVA, 2007). These within-country segmentations have been the topic of a number of research projects. In Norway, WESTAD, HERSLETH and LEA (2005) segmented consumers based on their cheese preferences. MURPHY *et al.* (2004) segmented consumers in Ireland based on preferences for a strong flavor, a hard texture, a wax packaged wheel, made with pasteurized milk, nutritional information present on the package, a white colored cheese and a price. They reported two segments with different ideal product profiles. Finally, BOGUE and RITSON (2005) identified the extrinsic and intrinsic attributes which determined end-users' preferences for a range of full-fat and lighter dairy products in the United Kingdom. They identified clusters of end-users with similar preferences for both the full-fat and for the lighter dairy products.

With the increasing globalization of the business world, cognition, responses to messaging, and thus international messaging segmentation is evolving into an evermore important strategy for marketing (STEENKAMP and HOFSTEDE, 2002). Messaging can also play a role in product development. By knowing what consumers want, and by identifying unmet needs, product developers can better focus their efforts. The key then is to better discover the *specific messages*, a task that can become difficult when a product is to be developed for, or sold in several countries, having different cultures, different food habits, and different arrays of products against which the new product must compete.

There is a body of information about messaging regarding cheese, but not to the level of specificity which exists for response to actual products. Studies regarding the cheese country of origin (LUOMALA, 2007) showed that consumers attach meaning to foods of various origins. Finnish consumers associate partly overlapping, partly, distinct cognitive, affective and normative meanings with Swedish, German and French foods.

To date, questions regarding the existence of one appropriate message across global markets for the same cheese do not seem to have been explored. Does each market require its own messaging to interest consumers in that country? Does the same message perform differently in different countries, and if so then how can the marketer customize the message? These are questions which move beyond product to marketing, but depend vitally on the sensory characteristics of the product. People buy foods for sensory pleasure as well as for health and nutrition, so an understanding of what messaging works for foods may help the product find acceptance in new markets. In some cases, the messaging may also show what has to be changed in the product to appeal to the new consumer.

A typical consumer may see several thousand messages about products and services in a single day (SCHULTZ *et al.*, 1993). In light of the ongoing, ever-increasing bombardment of consumers by information, a key business objective is to identify the specific messaging which can break through, and then which can enhance product acceptance. Communication of food messaging demands knowing what to say about the product, what not to say, how to say it, and to whom one should communicate. The style of message, the content, and even the time of message, may all influence a consumer's receptivity (KOTLER, 1997). Consumers are continually confronted with communications about products and services. How do we know what particular elements reinforce a message regarding a specific food, and how can the marketer and the company use that information to sell their products more effectively?

Conventional methods to collect data regarding food, such as in depth interviews, or focus groups, typically consider a reduced set of test messages. Such

research settings are artificial versus an external world where multiple factors may affect consumer choice and subsequent consumption. Matters become even more difficult when the data collection process is long, expensive, and implemented in a global environment characterized by different, market-specific issues hampering research execution.

To cope with these issues researchers have developed other methods in order to understand the mind of the consumer. One of these, featured in this paper, uses experimental design of messages executed through web-based conjoint analysis. Conjoint analysis, a branch of experimental design with a long, 45+ year history (LUCE and TUKEY, 1964), and thousands of published papers, uses systematically varied test stimuli (concepts), comprising different messages, much as one might work with small test advertisements. In the conjoint experiment the researcher exposes respondents to an array of interacting elements, i.e., the test concepts. Consumers respond to the compound communication, in a way similar to what they might respond to in the external world. The components or elements of these mixtures must compete with each other to drive the consumer response to the test concept. By mixing and matching these messages in many different combinations and deducing what drives the response through *post-hoc* regression modelling, the researcher identifies that part-worth contribution of each element. For the food industry, conjoint analysis in particular, but experimental design in general, identifies the important elements. Conjoint analysis increases the scope of the message research, revealing 'what works, for whom, and where'. Furthermore, conjoint analysis provides the 'raw material' for segmentation based upon patterns of responses to actual test communications, rather than relying on questionnaire methods where the respondent can give 'politically correct answers' (GREEN and KRIEGER, 1991).

When applied to mixtures of communications rather than to single communication points, experimental design has been extraordinarily popular among both academics and business professionals, and for both theory of consumer behaviour as well as specific tactical issues for the business venture (WITTINK *et al.*, 1994). The foundational work using conjoint analysis, published 45 years ago, originally designed to identify how consumers 'trade off' features of products, was quickly applied to marketing issues (GIL and SANCHEZ, 1997; LUCE and TUKEY, 1964; GREEN and SRINIVASAN, 1980). Conjoint analysis reveals how consumers in markets respond to the array of actual messaging currently in the market (MOSKOWITZ *et al.*, 2003; MOSKOWITZ *et al.*, 2002). More recently, conjoint analysis has been used to identify what emotions and product features drive acceptance (MOSKOWITZ and GOFMAN, 2007). Finally, focus in the academic community is now recognizing the importance of individual-level modelling (e.g., GREEN and KRIEGER, 1996).

This paper presents country-level information, gathered by aggregating individual level models. Thus, experimental design of messaging may help the researcher, product developer, and marketer in a company to identify the specific messaging for a product, both within a country, and across countries. In the process of design and analysis, we developed three specific hypotheses to guide the design, the empirical data acquisition, and the analysis:

Hypothesis 1: There are distinct segments of cheese consumers. Some segments are based on product features and others on the experience of cheese.

Hypothesis 2: Segments transcend countries.

Hypothesis 3: Segments are based on responses to test stimuli rather than on people's self explicated attitudes, necessitating a different type of discovery mechanism.

This study uses experimental design, focusing on responses to concepts about cheese in three countries, and makes four contributions to data basing and global segmentation.

1. It shows how to create a database across countries for a single product, in order to understand the mind of the consumer in each country with respect to the same food product, cheese.

2. In contrast to traditional 'one-stimulus-at-a-time' methods used to identify powerful messaging, the research details a more advanced approach using experimental design of mixtures. Experimental design allows the more impactful elements to emerge, even if the respondent cannot articulate what is important.

3. The study extends previous methods for looking at common segmentation across countries, as part of an initial attempt to create general, 'world-rules' for specific products (STEENKAMP and HOFSTEDE, 2002).

4. The study shows how to assign new respondents to one of the three mind-set segments for cheese, based upon a short 'intervention', i.e., a typing questionnaire, lasting no more than one minute. The typing tool provides the opportunity to classify new individuals, making it possible to create micro-sites appropriate for each mind-set segment, with these micro-sites either devoted to product design using in-bound interactions with consumers, or devoted to product messaging using out-bound communications designed by marketers.

## MATERIALS AND METHODS

The original database from which these results are drawn comprised 20 studies per country dealing with response to foods (AARTS *et al.*, 2002). The studies were all of the same type – conjoint analysis of messaging for a particular product, followed by an extensive classification study. One study per product category was conducted in each of three countries: France, Germany, and the United Kingdom. The database from the 20 studies provides a large-scale cross-section of responses to messages for a variety of common foods, such as colas, pizza, etc. Each country had its own set of 20 studies. When a product was assessed across each of the three countries, the elements and the classification questionnaire were the same (MOSKOWITZ *et al.*, 2005). The approach to consumer understanding using multiple, linked studies using conjoint analysis is relatively new, having begun in 2001, with the availability of easy-to-execute, self-authored conjoint analysis on the internet (FOLEY *et al.*, 2009; (MOSKOWITZ *et al.*, 2001; MOSKOWITZ *et al.*, 2005a).

The elements for each conjoint study were organized into silos and elements, for a total of 36 elements. The silos and elements were created by a multi-country task force which designed the studies for three countries (France, Germany, United Kingdom) based upon previous studies of the same type conducted in the United States (BECKLEY and MOSKOWITZ, 2002). The elements were chosen in order to reveal how consumers in the different countries would react to different types of messaging, what type of country-to-country variations existed, whether the respondents reacted in a manner similar to emotional elements, and whether there existed a segmentation that transcended one country. The existence of such transnational segmentation simplifies mul-

ti-national product development and innovation.

The basic experimental design comprised four silos or buckets of elements, with nine elements per silo. Each silo comprised elements that were similar in qualitative nature. Silo 1 (elements 1-9) comprised phrases describing the type of cheese. Silo 2 (elements 10-18) comprised phrases dealing with uses and benefits of cheese, silo 3 (elements 19-27) comprised elements that promised emotional rewards; and finally, silo 4 (elements 28-36) comprised elements dealing with the cheese country of export. The division of elements into silos was done as a bookkeeping device to ensure that a concept would not contain two elements that could contradict each other. In the analytical phase all elements were treated as independent variables, so that the different silos are only relevant as control devices for the creation of the test concept. Across the three countries, 34 of the 36 elements were identical. Only two of the elements, corresponding to country of origin, differed across the three studies.

The 60 test concepts per respondent were developed by experimental design. Each respondent evaluated a unique set of combinations. The test concepts were short, easy to understand descriptions of a cheese product. A single concept comprised 2-4 concept elements, one or no element from each of the four silos. Each concept element appeared three times against different backgrounds among the 60 combinations evaluated by the respondent.

The experimental design was a main effects design, so that each of the 36 elements was statistically independent of every other element. This independence was achieved by having some silos entirely absent from a concept. Such independence has the statistical property of allowing an estimation of absolute level of utility, not just relative level as is typically the case when concepts are designed to comprise one element from each of the silos (BOX *et al.*, 1978). Furthermore, the unique set of 60 combinations was created for each respondent based upon a permutation scheme that used the basic design, but renumbered the elements. Thus each respondent evaluated a unique set of combinations. This scheme (MOSKOWITZ and GOFMAN, 2004) ensured that no bias could occur due to a specific pair of elements appearing unduly frequently with each other, a problem that plagues conventional conjoint studies using one basic set of test stimuli evaluated by many respondents.

The respondent was invited to participate in the survey by an e-mail invitation, which offered a sweepstakes reward, so that there was an incentive to participate. Respondents received their invitations from a local provider, specific to the country. The e-mail link simply specified that the respondents were being invited to a study of food and drink, but gave no more information. All three countries had reasonably well-developed internet penetration, so finding respondents was no problem. One internet panel provider in each country, specializing in such field work, was selected to provide the panel. There was an approximately 2 to 4% response from the different countries, slightly lower than the US response rate of about 6% for that same time frame.

Respondents who received the email invitation and clicked on the link were then guided to a 'wall'. The wall presented the available research studies, of which there were 20 per country. Each respondent who chose to participate was instructed to click on the study, which most interested him, at which point the study began. This strategy increases the probability that a respondent participates in a study that is personally relevant. After a study quota (>125 respondents) was already filled the study 'disappeared from the wall' and was automati-

cally suspended until reactivated by the researcher. This strategy ensured adequate numbers of respondents for each study. Each study began with a short introduction about the project. The introduction, one screen long, told the respondent that the study was about the degree to which respondents 'craved' or 'liked' a product very much. The introductory screen then gave the respondent a brief description of the scale and described the sweepstakes. Nothing else was mentioned. The study then moved to the test concepts.

The respondent completed the basic interview comprising 60 screens which presented the test concepts for cheese. The test concepts were created according to a basic experimental design, which was permuted. Every respondent evaluated each concept element three times in the set of 60 combinations, testing concepts that comprised 2-4 elements. The nature of the experimental design dictated that some concepts would be absent from a particular silo. After evaluating the 60 concepts, the respondent completed a short classification questionnaire which instructed the respondent to indicate gender, age, region where he/she lived, reasons for buying cheese, the specific times when cheese was desired, as well as self-rated hunger. Finally, at the end of the interview the respondent logged out of the study.

The respondent could not change the rating to any concept, once the rating had been assigned. As soon as a rating was typed it was sent to the server. The respondent could, however, change the self-profiling classification question before submitting the answer. The interview lasted 12-16 minutes, depending upon the speed of the respondent. This period falls within the range that has been shown to generate a pleasant respondent experience with an Internet-based interview, and is considered an appropriate time for best practices in internet-based interviews (MAC ELROY, 2000).

## RESULTS

The panel for the cheese study comprised 328 individuals, with more participants from France and fewer from the United Kingdom (Table 1). All three countries began with the same number of e-mail invitations sent out to prospective respondents at the start of the project. The United Kingdom required an additional email invitation to new respondents to boost the sample.

### Identifying drivers of concept acceptance through modelling

The ratings for each respondent were incorporated into a respondent-level model relating the presence/absence of the 36 concept elements to the ratings. Since the elements were systematically varied by experimental design, standard regression methods can be used to create the models. The simplest method generating the most understandable results is ordinary least-squares, using 'dummy variable' predictors. In the regression analysis, each of the 36 predictors,

Table 1 - Distribution of respondents across classification questions dealing with gender, age, and self-reported hunger level. Numbers in the body of the table are actual numbers of respondents in each classification group.

| | France | Germany | United Kingdom | Total |
|---|---|---|---|---|
| **Gender** | | | | |
| **Female** | 63 | 67 | 65 | 195 |
| **Male** | 80 | 36 | 17 | 133 |
| **Age (Upper)** | | | | |
| **18** | 16 | 7 | 2 | 25 |
| **25** | 43 | 33 | 11 | 87 |
| **35** | 33 | 53 | 42 | 128 |
| **45** | 33 | 4 | 12 | 49 |
| **55** | 14 | 3 | 11 | 28 |
| **65** | 4 | 3 | 4 | 11 |
| **Total** | **143** | **103** | **82** | **328** |

or independent variables, take on the value 0 if the element is absent from the concept and 1 if the element is present in the concept. This binary nature rather than metric nature of the independent variables is the reason for the name 'dummy variable'. Each respondent generates 60 such observations or cases in regression terminology, with each case comprising 2-4 concept elements, depending upon the specific combination called for by the design.

Two models were created, both using ordinary least squares regression, using an individual level model, and so-called dummy variables (SYSTAT, 2002). The only difference between the models was the specific dependent variable.

The persuasion model

Here the dependent variable was the actual 9-point rating assigned to the combination. The persuasion model can be written as: Rating = $k_0$ + $k_1$(Element 1).. $k_{36}$(Element 36).

The interest model

This model is developed after the ratings are converted to a binary scale, using criteria chosen by the consumer researcher to define the cut-point on the rating scale. For example, many consumer researchers working a five-point purchase scale choose the top two ratings on which to focus. For a 9-point scale the authors have typically focused on the top three ratings (ratings of 7-9) to represent acceptance of the particular concept being evaluated. This focus on membership in a group (concept acceptors; ratings 7-9) follows the convention in consumer research, which studies membership in a group, i.e., accept a concept, thus being a concept acceptor, rather than studying intensity of feeling. Intensity of feeling, rather than membership in a group, is left as a topic to research psychologists.

The interest model is also written as: Binary Rating = $k_0$ + $k_1$(Element 1)... $k_{36}$(Element 36). The dependent variable, i.e., the binary rating, is now either a 0 for any particular concept or a 100 for the concept, depending upon the original rating assigned by the respondent for that concept, using the 9-point scale. Ordinary least squares regression estimates the 36 coefficients and the additive constant, again at the level of the individual respondent.

For both regression models a very small random number (magnitude < 0.0001) is added to all of a respondent's ratings to ensure that the regression analysis would work. This statistical modification of the rating is prophylactic, ensuring no regression failures. The statistical correction has no perceptible impact on the parameters emerging from the regression modelling.

Although the original assignment of the elements to concepts was done within the framework of the silos, the regression analysis takes no account of the silos when estimating the model, nor does it need to do so. The experimental design makes the silo irrelevant for statistical analysis. The silos would be relevant when the design requires one element from each silo had to be present in the concept. By moving away from this so-called effects model (one element from each silo present) to true zero conditions for silos (a silo may be entirely absent), we avoid the problem faced by traditional research. Certainly smaller concepts with some silos missing may generate incomplete concepts. Yet, the incompleteness is outweighed by the benefits. The stratagem produces databases whose utilities have ratio scale properties, and can be compared across studies with different elements, done at different times. Thus the current approach produces data that can be used as the foundation for a science, rather than simply relative numbers that have meaning only within the limited world of the single study.

All discussion of modelling derives from the interest model, except for

the specific steps used in the development of segments. Analyses of variance were done for individual-level modelling, as well as for the large database which combined the data from all respondents within a single country. Estimates of significance with the full model suggest that coefficient differences for the interest model greater than 5 are highly significant ($p<0.01$). The precise cut-off value for statistical significance at the 95 and 99% confidence levels vary by element, however. A good and quite conservative rule of thumb considers differences of 6 or more to be statistically significant across all elements.

How the elements perform

The results from the modelling appear in Table 2. Each respondent generates an individual model. The table shows the average coefficient across respondents for each of the 36 elements, for each of the three countries. The model comprises two parts; the additive constant and the 36 terms. The additive constant for the interest model shows the conditional probability of a person being interested in cheese, in the absence of any particular communication element. The additive constant is an estimated value, based upon the pattern of ratings assigned by an individual to the 60 concepts. At a more interpretive level, the additive constant may be considered a baseline for acceptance of cheese. It is important to note that for the data in Table 2, the utility values are shown by country.

The individual utility for an element is the conditional probability or odds that a person will switch the rating from not interested (rating 1-6) to interested (rating 7-9) if the element were to be introduced into the concept. The statistical meaning of the individual utility is the coefficient for the element for the particular individual.

Do respondents in the three countries show the same pattern of interest in cheese?

The additive constant suggests that basic interest in cheese varies across countries. The constant has the lowest value for Germany (19, i.e. 19% of the respondents would give a rating of 7-9 for a cheese concept without any elements), an intermediate yet still low value of 31 for the United Kingdom (i.e. 31% of the respondents would give a rating of 7-9 for a cheese concept), and a high of 45 (i.e. 45% of the respondents would give a rating of 7-9 for a cheese concept) for France. For comparison purposes, a product such as pizza evaluated in the same type of study generated additive constants of 35 for the United Kingdom, 29 for Germany and 39 for France, respectively. The additive constant combines both a response bias (magnitude of the numbers used in a country) with actual country-to-country differences in the basic acceptance of a product idea.

The elements also differ in their impact. Table 2 shows a fairly wide range of these utilities in each country. For example, in the United Kingdom, the element "*Cheddar with a typical strong flavour*" generates a utility of +20, "*Cheese with a glass of wine or a soft drink of your choice*" generates a utility of +10, and finally "*Soft light cream coloured Brie - perfect with crusty bread*" generates a utility of +12. This means that when these elements are introduced into the concept an additional 20, 10 and 12% respectively of the respondents change their rating from 1-6, going up to 7-9, i.e., changing from indifferent/disinterested to interested.

There are clear country-to-country and within-country differences across the common elements. Yet, no simple pattern emerges to reveal why some concept elements perform well and others perform poorly. It would be incorrect to

conclude that concept elements dealing with the description of the product tend to perform well, whereas emotional benefits, as part of concepts, tend to perform poorly. However, 'puffery' emotional statements tend to perform poorly. Thus, elements such as "*Cheers you up*" and "*Celebrate special occasions with cheese - escape from the routine*" perform poorly across all three countries. Country of cheese export also generally perform poorly, except in some instances such as France, which does slightly better in Germany (utility +6) than it does in France (+4) and is irrelevant in the United Kingdom (-1).

## Do trans-national segments exist for cheese?

Individual differences in preference functions for actual products can be seen by visiting any supermarket (MOSKOWITZ *et al.*, 1985). These preference differences are profound, existing even at the level of 'model systems' of water and a simple tastant such as sugar or salt (EKMAN and AKESSON, 1964; PANGBORN, 1970).

One key benefit of running a multi-country study with the same elements is the ability to identify groups of like-minded respondents based upon their responses to the concept elements. The search for these segments focused on the 36 elements. The segmentation method has already been described in detail (MOSKOWITZ *et al.*, 2005b).

Segmentation follows the steps enumerated below. It is important to note that the segmentation does not begin with any preconceived notion either about the number of segments or the names of the segments. Rather, the segmentation partitions the respondents into groups such that the variability within a group or the respondent-to-respondent distance is small, whereas the variability across group or the group-to-group distance is large.

Step 1: Use the persuasion model at the individual respondent level to define the utilities for the 328 respondents, independent of country. This first step requires that the persuasion model be run at the individual respondent level, which is straightforward since the design created combinations appropriate for individual-level modelling. The persuasion model provides metric information about the degree of interest generated by a specific element.

Step 2: There may be redundancies in the utility values across the respondents for elements, i.e., specific elements on which the clustering will be performed. Remove the redundancy, and then perform a principal components analysis of the utilities on the identical elements across the 328 respondents, independent of country. This generated 11 factors, extracted by principal component factor analysis, whose solution can be rotated to a simple solution by quartimax rotation. Table 3 shows the strongest loading element(s) on each dimension or factor, defined as a correlation > 0.6 with the dimension.

Step 3: Each respondent corresponds to a single point in this 11-dimension factor space. The respondent is identified as a point having 11 values, one per dimension. Cluster the respondents based on their 11-dimensional factor scores, using k-means clustering. The measure of distance between respondents is defined as the geometric or Euclidean distance between the two respondents computed on their corresponding 11-dimensional factor scores. Table 4 presents the sum of squares within factors, the sum of squares between factors, and the F ratios. It is clear from the order in the table that those factors emerging from the principal components factor analysis and explaining most of the variability in the attribute ratings are not necessarily the same as

Table 2 - Average utility values for cheese type, cheese use benefits, cheese emotional benefits, and country of sponding coefficients from the individual models. The utilities come from the 'interest' model, so the numbers in the concept. Strong interest drivers (utility or coefficient > 10) are shown in bold. The coefficients are sorted right of every utility. (Note: Elements whose meaning differ across the three countries are shown at the bottom

| | France<br>Constant | MN<br>45 | SE | Germany<br>Constant |
|---|---|---|---|---|
| | **SILO A: Types of Cheese** | | | |
| A2 | Du fromage bien fait à la coupe | 8 | 2.3 | Stuecke von gereiftem Kaese |
| A9 | Un Brie fait à coeur, avec un morceau de pain frais et croustillant | **20** | 3.3 | Cremiger, hellgelber Brie, der auf dem Brot fast verlaeuft |
| A4 | Du fromage doux et crémeux | 1 | 2.8 | Cremiger Weichkaese |
| A7 | Un bleu traditionnel avec son arômeIntense | **11** | 3.8 | Blauschimmelkaese mit typischem und intensivem Geschmack |
| A5 | Des cubes de Feta pour une salade Grecque ou avec des légumes | -7 | 2.6 | Brocken von Fetakaese fuer einen griechischen Salat oder mit Gemuese |
| A3 | Du fromage à tartiner | -18 | 2.1 | Streichfertiger Kaese |
| A1 | Du fromage en tranches | -14 | 3.5 | Kaesescheiben |
| | **SILO B: Use Benefits of Cheese** | | | |
| B2 | Avec un verre de vin rouge | 5 | 3.4 | Mit dem passenden Getraenk, z.B. Rotwein |
| B9 | Si bon qu'on en savoure chaque bouchée | 2 | 1.8 | Jeder Biss so schmackhaft, dass man mit der Zunge ueber die Lippen faehrt |
| B5 | Y penser, c'est déjàle savourer! | 1 | 2.1 | Schon bei dem Gedanken daran laeuft einem das Wasser im Mund zusammen! |
| B6 | 100% naturel, frais et affiné avec soin | 6 | 2.3 | Natuerlich, frisch und schonend hergestellt |
| B8 | Le voir c'est le désirer! | 3 | 1.9 | Beim Betreten des Geschaeftes ist der Geschmack schon zu empfinden |
| B4 | Excellente qualité, ce grand classique du goût comme autrefois | 2 | 3.5 | Topqualitaet mit absolut typischem Geschmack |
| B3 | Avec du pain et du raisin | 2 | 3.5 | Mit Weissbrot und Trauben |
| B1 | Pas de fête sans fromage! | 4 | 3.8 | Es macht Spass, Kaese in Gesellschaft zu essen! |
| B7 | Avec tous les accompagnements souhaités | 1 | 2.2 | Mit selbst ausgewaehlten Extras, ganz ohne Einschraenkung |
| | **SILO C: Emotional Benefits of Cheese** | | | |
| C2 | Y penser, c'est le désirer le manger, ce n'est ne plus s'arrêter! | 3 | 1.8 | Schon beim Gedanken muss man ihn haben, und wenn man ihn hat, kann man nicht mehr aufhoeren! |
| C6 | Une fête pour vos sens...voir, sentir, goùter! | 4 | 2.0 | Sinnesfreuden pur: Sehen, Riechen, Schmecken! |
| C5 | Echappez à la routine avec du fromage... une manière de célébrer les moments uniques! | 5 | 2.2 | Fuer besondere Gelegenheiten! |
| C8 | Pur Plaisir! | 1 | 1.9 | Pure Begeisterung! |
| C7 | Une expérience unique.. a partager en famille ou entre amis! | 2 | 2.0 | Eine aussergewoehnliche Erfahrung, am schoensten mit lieben Menschen! |
| C9 | Le fromage satisfait vos petites faims! | 7 | 1.9 | Macht einfach satt und befriedugt das Verlangen! |
| C1 | Rapide et sympa... manger seul devient un plaisir | -2 | 2.2 | Macht einfach Spass, auch alleine! |
| C3 | Pour vos petit creux, au moment où il faut | 1 | 1.9 | Hilft gegen den aufkommenden Hunger! |
| C4 | Vous redonne le sourire...!! | 2 | 1.8 | Heitert auf und tut gut! |
| | **SILO D: Country of Export for Cheese, and emotional assurance** | | | |
| D8 | Por moi, simplementle meilleur fromage! | 0 | 1.9 | Einfach der beste Kaese fuer mich! |
| D9 | Avec les meilleurs standards de qualité auxquels vous faites confiance! | 1 | 2.0 | Ein absolut sicheres Produkt, vertrauenswuerdig und mit hoher Qualitaet! |
| D1 | De France | 4 | 1.9 | Aus Frankreich |
| D2 | De Suisse | -7 | 1.9 | Aus der Schweiz |
| D3 | D'Italie | -3 | 1.9 | Aus Italien |
| D4 | De Hollande | **-14** | 2.0 | Aus Holland |
| D5 | De Grèce | **-10** | 1.7 | Aus Griechenland |
| | **Elements 'unique' to a country** | | | |
| A8 | Du chèvre avec son goût typique | **12** | 3.4 | Schweizerkaese mit kraeftigem Geschmack |
| D6 | De Corse | -5 | 2.3 | Aus Daenemark |
| D7 | Des Pyrenées | -1 | 2.3 | Aus Deutschland |

export for cheese. Elements from the three countries are based on total panel results, aggregating the corre-
reflect the proportion of respondents who would rate a concept as interesting (7-9) if the element were present
with a silo by the utility values for the United Kingdom, to show country effects. Standard errors appear to the
of the table. MN = average utility for all respondents from that country; SE = standard error of the mean).

| MN 19 | SE | United Kingdom Constant | MN 31 | SE |
|---|---|---|---|---|
| 8 | 3 | Chunks of matured cheese | **12** | **3.6** |
| 7 | 3.7 | Soft, light cream coloured Brie, perfect with crusty bread | **12** | **4.1** |
| 5 | 2.9 | Creamy soft cheese | 6 | 3.6 |
| -5 | 4 | Blue cheese with typical and intense flavour | 1 | 3.6 |
| **17** | 3.2 | Cubes of Feta cheese for a greek salad or with vegetables | -1 | 3.8 |
| -3 | 3.0 | Spreadable cheese | -5 | 4.1 |
| 2 | 3.6 | Sliced cheese | -6 | 4.2 |
| **11** | 2.4 | With a glass of wine, or a soft drink of your choice | **10** | **2.7** |
| 5 | 1.9 | So tasty you have to lick your lips twice after each bite | 5 | 2.8 |
| 3 | 2.0 | You can just savour it, when you think about it | 4 | 2.3 |
| 8 | 2.0 | 100% natural, fresh and carefully prepared | 4 | 2.6 |
| 1 | 2.0 | You can imagine the taste as you see it in front of you | 3 | 2.6 |
| 1 | 2.4 | Premium quality...that great traditional taste | 1 | 3.5 |
| **12** | 2.4 | With bread and pickle | 0 | 3.0 |
| 2 | 4.0 | Cheese is great for parties | -2 | 5.4 |
| 1 | 4.0 | With all the accompaniments you want | -3 | 3.6 |
| 3 | 2.0 | When you think about it, you have to have it... and once you have it you want more | **7** | **2.2** |
| 4 | 2.1 | A joy for your senses...seeing, smelling, tasting | 5 | 2.4 |
| 2 | 2.3 | Celebrate special occasions with cheese 4 - escape from the routine | 2.6 | |
| 5 | 2.4 | Pure enjoyment | 4 | 2.4 |
| 0 | 2.3 | A real experience...shared with friends or family | 3 | 2.5 |
| -1 | 2.2 | It satisfies your hunger | 3 | 2.4 |
| 3 | 2.5 | Quick and fun ... eating alone doesn't have to be boring | 1 | 2.8 |
| -2 | 2.8 | It fills you up – just when you need it | 0 | 2.9 |
| -2 | 2.3 | Cheers you up | -2 | 2.2 |
| 1 | 2.2 | Simply the best cheese for me | 3 | 2.9 |
| 2 | 2.0 | With highest quality and standards that you trust | -1 | 2.2 |
| 6 | 2.2 | From France | 1 | 2.4 |
| 2 | 2.3 | From Switzerland | -3 | 2.6 |
| 4 | 2.7 | From Italy | -3 | 2.7 |
| 4 | 2.4 | From Holland | -7 | 2.6 |
| 3 | 2.5 | From Greece | -7 | 2.5 |
| **11** | 3.6 | Cheddar with a typical strong flavour | **20** | **4.9** |
| -7 | 2.7 | From Spain | -9 | 2.4 |
| 2 | 2.7 | From Britain | 2 | 2.9 |

the factors that drive the segmentation into three clusters.

Step 4: Extracting the relevant number of segments is both a statistical task and an interpretive art. The objective of clustering to generate segments which ensure a minimum variability within a segment versus maximum variable across segments, as well as an interpretable segmentation with the clusters 'making sense'. In this spirit we extracted thee segments, which was the minimum number of segments which seemed to 'tell a story'.

Step 5: Compute the average utility value for each element by segment, independent of country, using those respondents who are placed into the segment as the source of the utility values.

### Like-minded segments across countries

Segmentation divides the consumer population into worlds of individuals having different mind-sets. When segmentation is based on the response to type of cheese, use benefits of cheese, emotional benefits, and country of export for cheese, we work with a different type of segmentation than the conventional segmentation by attitudes so frequently developed (WELLS, 1975). Segmentation based on the utility patterns lies between actual behaviour on the one hand, and general attitudes towards the product on the other hand. We deal here with segmentation emerging from responses to actual stimuli, in the form of small 'advertisements' about the product, rather than in self-stated values and rather than products themselves. Table 5 shows the strongest elements for each of the three mind-set segments that emerged from analyzing what phrases most strongly 'drive' consumer responses.

### Where are the three segments found?

When respondents are segmented by the pattern of their responses to test stimuli, a behavioral response rather than attitudinal response, there are no clear correlates in the self-profiling classification which predict segment membership. Therefore, although the segments appear to be homogeneous with respect to the way they respond to messaging, they are not homogeneous with respect to other, easy-to-measure factors, such as age, gender, country, and so forth. This failure to find external correlates to issues about food and nutrition is pervasive (WARDLE *et al.*, 2004). We see an example of such heterogeneity of the segments when we classify the individuals by the country in which they reside (Table 6). Each country has respondents from each segment, albeit in different proportions.

### Discovering new members in a segment by intervention methods – 'the typing tool'

If membership in a concept-response segment cannot be easily predicted by the conventional methods of data-mining using variables such as age, gender, and even perhaps products bought recently, how then can the segmentation be made more 'actionable', for developers and marketers? The data strongly suggest three different groups of consumers, with different mind-sets, and thus product desires. The segmentation can guide new product development, but only when the individuals in the segments can be properly identified, and a line of communication opened up with them. Once the discovery is made that a specific individual belongs to a segment, the next steps in development and communication are simplified. The segments are homogeneous with respect to mind-sets, and thus look at the world

Table 3 - The eleven dimensions or factors underlying the common elements. The individual elements most highly correlated with each of the 11 dimensions are shown.

| Dimension | Element(s) that correlate highest with the dimension | % Variance |
|---|---|---|
| **Dim1** | It fills you up - just when you need it | |
| **Dim1** | Celebrate special occasions with cheese - escape from the routine | 13.6 |
| **Dim2** | Chunks of matured cheese | 7.9 |
| **Dim3** | From Italy | |
| **Dim3** | From France | |
| **Dim3** | From Holland | |
| **Dim3** | From Greece | 6.9 |
| **Dim4** | You can imagine the taste as you see it in front of you | |
| **Dim4** | So tasty you have to lick your lips twice after each bite | 5.4 |
| **Dim5** | Sliced cheese | |
| **Dim5** | Spreadable cheese | 4.6 |
| **Dim6** | With bread and pickle | 4.1 |
| **Dim7** | Cubes of Feta cheese for a Greek salad or with vegetables | 3.9 |
| **Dim8** | 100% natural, fresh and carefully prepared | 3.5 |
| **Dim9** | A joy for your senses...seeing, smelling, tasting | 3.5 |
| **Dim10** | Quick and fun ... eating alone doesn't have to be boring | |
| **Dim10** | Pure enjoyment | 3.2 |
| **Dim11** | Soft, light cream coloured Brie, perfect with crusty bread | |
| **Dim11** | Creamy soft cheese | 2.9 |

Table 4 - Results of k-means splitting cases into 3 groups.

| FACTOR used in clustering | Sum of Squares between factors | Degrees of freedom | Sum of squares within factors | Degrees of freedom | F-ratio |
|---|---|---|---|---|---|
| **FACTOR(1)** | 66.41 | 2 | 260.58 | 325 | 41.42 |
| **FACTOR(2)** | 38.17 | 2 | 288.82 | 325 | 21.48 |
| **FACTOR(3)** | 1.01 | 2 | 325.98 | 325 | 0.50 |
| **FACTOR(4)** | 14.65 | 2 | 312.34 | 325 | 7.63 |
| **FACTOR(5)** | 51.84 | 2 | 275.15 | 325 | 30.63 |
| **FACTOR(6)** | 20.72 | 2 | 306.27 | 325 | 11.00 |
| **FACTOR(7)** | 59.13 | 2 | 267.86 | 325 | 35.87 |
| **FACTOR(8)** | 5.15 | 2 | 321.84 | 325 | 2.60 |
| **FACTOR(9)** | 28.40 | 2 | 298.59 | 325 | 15.46 |
| **FACTOR(10)** | 30.72 | 2 | 296.27 | 325 | 16.21 |
| **FACTOR(11)** | 76.96 | 2 | 250.03 | 325 | 50.03 |
| **Total** | 393.21 | 22 | 3203.78 | 3575 | |

of cheese similarly, at least within the limits of this study.

Although conventional geo-demographics and even behaviors do not predict one's 'mind-set' for specific messaging, for any particular food, at the granular level, it is quite possible to create a simple 'intervention', a small and easily-administered survey', that can identify a person as belonging to a segment. The notion here is that instead of predicting segment membership of the individual without knowing the person, there is interaction with the person using a short interview. This way of thinking mirrors today's medical practice, where in order to understand the patient the doctor takes a blood sample, and from the pattern of the scores from various tests performed on the patient's blood, understands what problems and even illnesses the patient might have.

Table 5 - The strongest performing elements for the total panel and for each of the three concept-response (mind-set) segments. The table shows the coefficients for the interest model, for total panel, and for each of the three mind-set segments. The segmentation transcends the three countries. Only common elements across countries were used to develop the segments.

| | **Total** 33 | **Seg1** 34 | **Seg2** 28 | **Seg3** 39 |
|---|---|---|---|---|
| **Winning elements - total panel** | | | | |
| Soft, light cream coloured Brie, perfect with crusty bread | **14** | 0 | **26** | **26** |
| Chunks of matured cheese | **9** | **9** | **13** | 3 |
| With a glass of wine, or a soft drink of your choice | **8** | 3 | **9** | **17** |
| **Winning elements - Segment 1 of 3 – Cheese with something** | | | | |
| Cubes of Feta cheese for a Greek salad or with vegetables | 2 | **10** | 3 | -14 |
| Chunks of matured cheese | **9** | **9** | **13** | 3 |
| When you think about it, you have to have it...and once you have it you want more | 4 | **8** | 0 | 0 |
| Pure enjoyment | 3 | **8** | -6 | 5 |
| With bread and pickle | 5 | **7** | 5 | 0 |
| It satisfies your hunger | 3 | **7** | -4 | **7** |
| A joy for your senses...seeing, smelling, tasting | 4 | **7** | -1 | 5 |
| **Winning elements - Segment 2 of 3 – Cheese is the focus** | | | | |
| Soft, light cream coloured Brie, perfect with crusty bread | **14** | 0 | **26** | **26** |
| Chunks of matured cheese | **9** | **9** | **13** | 3 |
| Creamy soft cheese | 4 | -5 | **12** | **8** |
| 100% natural, fresh and carefully prepared | 6 | 3 | **11** | 4 |
| So tasty you have to lick your lips twice after each bite | 4 | 2 | **10** | -2 |
| With a glass of wine, or a soft drink of your choice | **8** | 3 | **9** | **17** |
| Blue cheese with typical and intense flavour | 3 | 0 | **9** | 1 |
| **Winning elements - Segment 3 of 3 – The cheese experience** | | | | |
| Soft, light cream coloured Brie, perfect with crusty bread | **14** | 0 | **26** | **26** |
| With a glass of wine, or a soft drink of your choice | **8** | 3 | **9** | **17** |
| Celebrate special occasions with cheese - escape from the routine | 4 | 3 | 0 | **11** |
| From Switzerland | 2 | -1 | -1 | **11** |
| A real experience...shared with friends or family | 2 | 2 | -5 | **11** |
| Cheese is great for parties | 2 | 3 | -4 | **9** |
| Creamy soft cheese | 4 | -5 | **12** | **8** |
| It satisfies your hunger | 3 | **7** | -4 | **7** |

Discriminant function analysis (SYSTAT, 2002) uncovers the elements for a typing test. The test itself comprises a few elements, presented as direct phrases. Since the persuasion model shows how each individual element 'drives' the 9-point rating scale, one can create 33 different stand alone phrases, from the 33 common elements across the three countries. For each respondent, we know both how each of the phrases would score on the 9-point rating scale if presented as a stand-alone concept, and the segment to which the respondent belongs. Discriminant function analysis identifies a limited set of such phrases, i.e., one-element concepts which together maximize the likelihood of assigning a person to the correct segment. Discriminant function analysis provides the classification rule, which can be used to assign a new individual to one of the three segments (i.e., scoring a new person).

A worked example of the classification rule appears in Table 7. Not all elements need be used. In fact, only responses to three elements are needed (A1, A9, C2) to generate almost 2/3 correct assignment. These elements generate the highest F ratios for ability to classify respondents correctly. The table shows the parameters of the three classification functions, hypothetical ratings by four new respondents (Res1-4), the value of the classification function corresponding to each segment for each respondent (bottom of the table), and then the assignment of the respondent to the segment whose classification function shows the highest numerical value.

Table 6 - The heterogeneous nature of each segment, when it comes to country in which the respondents reside. Each segment has consumers from all countries. Each country has consumers from all segments. Thus, the segmentation transcends country.

| How segments distribute by segment and country | Seg1 Cheese with something | Seg2 Cheese as the focus | Seg3 The cheese experience | Total% | Total No. |
|---|---|---|---|---|---|
| **France** | 56 | 45 | 42 | | 143 |
| **Germany** | 57 | 29 | 17 | | 103 |
| **United Kingdom** | 38 | 31 | 13 | | 82 |
| **Total** | 151 | 105 | 72 | | 328 |
| **Distribution of the three segments within each country (look across columns for each row)** | | | | | |
| **France** | 39% | 31% | 29% | 100% | 143 |
| **Germany** | 55% | 28% | 17% | 100% | 103 |
| **United Kingdom** | 46% | 38% | 16% | 100% | 82 |
| **Total** | 46% | 32% | 22% | 100% | |
| **Distribution of the three countries within each segment (look down across rows for each column)** | | | | | |
| **France** | 37% | 43% | 58% | 44% | 143 |
| **Germany** | 38% | 28% | 24% | 31% | 103 |
| **United Kingdom** | 25% | 30% | 18% | 25% | 82 |
| **Total** | 100% | 100% | 100% | 100% | |

Sometimes it is difficult to assign a person to only one segment, because the classification functions are very close. In that case, two segments are shown. The assignment is shown by the shaded cell. For example, Respondent 1 is assigned to Segment 2. In contrast, Respondent 4 assigns ratings to the three elements such that it is probably difficult to assign the respondent to a segment since the classification functions are negative. When, however, the respondent must be assigned, both Segment 1 and Segment 2 show approximately equal functional values for the classification function and so the respondent would be assigned to both Segment 1 and to Segment 2.

Finally, discriminant function analysis does not perfectly classify all of the respondents. It does the best possible, given noise in the responses. Table 8 shows the performance of the typing tool for the data taken from the 328 respondents. The typing tool was created using the 'jacknife' procedure, which estimated the parameters of the model, but left out certain cases in order to use them for validation testing. The classification function generates about 2/3 correct assignment, double what might be the case for random allocation of respondents to segments.

## DISCUSSION

To increase the odds of an item being purchased, the marketer must shape the product messaging. The marketer needs insights as to what to say, what not to say, how to say it and to whom it should be said. We identified winning elements of the messaging and examined them across countries. We found that there are three mindsets of consumers. It is important to note that these mindsets pertain to one product, cheese, and

Table 7 - Classification functions to assign new respondents to a segment, and example of the function applied to four respondents (Res1 - Res4).

| | | Seg1 | Seg2 | Seg3 | Res1 | Res2 | Res3 | Res4 |
|---|---|---|---|---|---|---|---|---|
| | Constant | -4.13 | -4.72 | -5.37 | | | | |
| A1 | Sliced cheese | | | | | | | |
| F=25.83 | | 0.30 | 0.42 | -0.23 | 7 | 5 | 1 | 2 |
| | SOFT, light cream coloured Brie, | | | | | | | |
| A9 | perfect with | | | | | | | |
| F=38.39 | crusty bread | 0.31 | 0.85 | 0.77 | 5 | 5 | 8 | 2 |
| | When you think about it, you have to have it...and | | | | | | | |
| C2 | once you have it | | | | | | | |
| F=30.75 | you want more | 0.73 | 0.07 | 0.76 | 1 | 8 | 4 | 2 |
| **Results: Assignment of new respondents to one of the three segments** | | | | | | | | |
| | | | | Seg1 | 0.26 | **4.78** | 1.58 | **-1.44** |
| | | | | Seg2 | **3.09** | 2.77 | **3.36** | -1.49 |
| | | | | Seg3 | -2.36 | 3.47 | **3.66** | -2.75 |

Table 8 - Assignment successes and failures by the discriminant function, working on the 238 respondents. The correct assignments in the training session are shown in the shaded cells.

| | And classified as Seg1 | And classified as Seg2 | And classified as Seg3 | % correct |
|---|---|---|---|---|
| Respondent is really in Seg1 | **119** | 23 | 9 | 79 |
| Respondent is really in Seg2 | 41 | **53** | 11 | 50 |
| Respondent is really in Seg3 | 21 | 18 | **33** | 46 |
| Total | 181 | 94 | 53 | **63** |

that they reveal differences at the very granular level of communication. Further, the approach represents a departure from the traditional macro-segmentation, done for food in general, where the difficulty then becomes one of determining how to apply the segmentation to the particular food (e.g., WELLS, 1975). Our approach begins from the ground up, and at all times maintains granularity of information, insight, and direction.

For each of these identified mindsets, the marketer may use the winning elements to shape an effective message that will enhance the acceptance of cheese. Each of the three major mindset segments for cheese responds to a different set of messages. The cross-national segmentation suggests a common type of message for the segment, independent of country. That is the first step. The second step is to fine tune the message for the segment, taking into account the sensibilities of the country, but remaining faithful to the general segmentation which exists. This strategy provides guidance both for marketing, but also for future product development, where the thrust can be on the basis of segments that exist across countries.

### Making segmentation actionable – finding these respondents for sales and product development

The segmentation of respondents by patterns of utilities creates groups that are intuitively meaningful. However, there are no clear methods by which to link membership in a segment to some demographic, attitudinal, or even need-state variable. Certainly there are differences between the segments. If one somehow 'knew' an individual's likelihood of being a member of Segment 1 or 2, or 3 one could craft a strongly appealing sales message using these data along with other messaging relevant for the consumers in the particular segment. However, knowing the country, gender, age, income, etc. does not predict segment membership. Additional analyses of the segments by gender and age (not shown) did not reveal any strong predictors of segment membership.

### Opportunities for improved product development and marketing

The lack of clear predictors of membership in segments coupled with the evidence that these segments appear to have strongly held preferences suggests that other methods must be developed to classify an individual as belonging to a concept-response segment. These other methods will not necessarily be simply statistical analyses of databases, but rather experimental interventions with prospects, as shown here with the three-question typing tool. The intervention or personalized typing should present the prospect with a stimulus or set of stimuli, get the response of the prospect, and then assign the pros-

pect to a segment based on the pattern of responses.

Instead of the segmentation being problematic because it does not directly co-vary with country, or any other conventional geo-demographic, or even other behaviors, the segmentation may provide new opportunities for targeted product development and marketing. Conventional thinking looks for external factors to predict segment membership. Once these factors are shown not to work, it becomes clear that a different way is required when looking at segments. That different way uses an active intervention with the respondent as the way to establish segment membership for a particular individual. Furthermore, the intervention is quite easy; a three-question interview followed by a classification function.

From this simple approach follows three immediate opportunities for the food industry:

Opportunity 1 – 'sequence the genome of the consumer mind'

This study shows how to understand the consumer mind with respect to one product, cheese, across different countries. If instead of one product being tested, one could do this same cross-national segmentation with 20+ products. For each product in turn, in a study run with the appropriate respondents, one would segment the respondents for that product, and then develop a product-specific typing test. At the end of the process, the result would be 20+ short, easy to administer questionnaires, or typing tests, each test each comprising 3-6 questions. Each typing questionnaire would correspond to a specific product. The nature of the segmentation need not be the same for each product.

At this point the typing tool becomes a way to understand entire populations, and effects of acculturation, and so forth. Any new respondent would then 'type' himself on each of the products, with the short, product-specific typing questionnaire. The result would be equivalent to sequencing a genome, only this time it would be sequencing the genome of the mind with respect to the different foods. Further understanding of consumer responses to food and beverage would emerge from understanding the mind-set of many individuals to a large array of different foods.

Opportunity 2 – 'targeted development' to individuals with the same mind-set

Current practice in product development uses consumers who identify themselves either as users, or non-users, or lapsed users. The underlying thinking is that these different groups of individuals have different needs to be satisfied. With the typing tool, one can quickly identify an individual as belonging to a mind-set segment, and work with groups of people with like mind-sets. The product development task then speeds up because of the coherence of the mind-set. Development efforts would work with individuals of similar mind-sets, rather than of known user groups or demographic groups. Such individuals would be easy to identify by the short typing questionnaire, which in turn could be embedded in a 'mind-typing' website. Such development efforts would be more focused, and presumably far more productive, because the individuals for whom the product is being developed are more likely to have homogeneous preferences with respect to the specific products.

Opportunity 3 – 'targeted sales communication'

Current advertising is purchased on the basis of media habits (magazines read, television viewing patterns, etc.). With the advent of the new media, and with an understanding of the mind-set of any particular individual available through a rapid typing exercise, one can created targeted, individualized messaging for cheese, or any oth-

er product, appropriate for an individual's known mind-set. The intervention exercise identifies the mind-set as well as referring back to the database, which shows what messaging is appropriate for that mind-set.

## CONCLUSIONS

Segments that emerge here come from actual responses to concepts, rather than from more easily measured, but perhaps less clearly related variables such as those that might be purchased from a third-party database. In dozens of studies of this type, whether with products or service variables or even social attitudes, there do not seem to be clear, a priori predictors of membership in a concept-response segment that might be gleaned from standard socio-demographic information. Yet the segmentation makes intuitive sense. The data are too compelling, since the segmentation is based upon the very response to concepts about the specific product, namely cheese. First, the immediacy of the segmentation, its clarity, and its compelling nature sense suggest that the segments are real. Second, the segmentation is based on response patterns to actual communication phrases of the type a person would see in advertisements. Third, when we do these types of studies with many products, we find similar concept-response segments emerging again and again (MOSKOWITZ *et al.*, 2005). We conclude that we are dealing with a real division of people in the population which allows us to shape the message content. However, we still do not have the necessary rules by which to predict membership in that division, except by behavioral responses, i.e., reactions to the stimulus itself.

Identifying a person as belonging to a mind-set or concept-response segment comes from a short intervention interview, the typing test, rather than coming from data-mining. The ingoing assumption is that the assignment of new individuals to segments is going to be far more accurate than a statistical, data-mining approach which looks for easy-to-purchase exogenous variables that are then combined to create a predictive model. With the stronger segmentation due to mind-set segmentation, it may be possible to speed up and more precisely focus the process of developing products, selling products, and in turn understanding consumers at a profound level.

## REFERENCES


Aarts P., Paulus K., Beckley J. and Moskowitz H.R. 2002. Food craveability and business implications: The 2002 EuroCrave database. Presented at the ESOMAR Congress, Barcelona, Spain.

Arnade C., Munisamy G. and Pick D. 2009. Consumers Show Strong Brand Loyalty in Cheese Purchases. Rept. Amber Waves, June. Available online from http://www.ers.usda.gov/AmberWaves/June09/Findings/CheesePurchases.htm

Ashman H. and Beckley J.H. 2004. Experiencing: *Kool-Aid* Ice *Cool*...with emphasis on cool. Food Processing July, 65 (7): 22.

Beckley J. and Moskowitz H. R. 2002. Databasing the consumer mind: The Crave it!, Drink It!, Buy It! & Healthy You! Databases. Institute of Food Technologists Vol. 74, Anaheim, CA.

Bonnetti E. 2004. The effectiveness of meta-brands in the typical product industry: mozzarella cheese. Brit. Food., 106: 746.

Bogue J. and Ritson C. 2005. Integrating consumer information with the new product development process: the development of lighter dairy products. Int. J. Consumer Studies 30: 44.

Box G.E.P., Hunter J. and Hunter S. 1978. "Statistics For Experimenters," John Wiley and Sons, New York.

Ekman G., and Akesson C. 1964. Saltiness, sweetness and preference: A study of quantitative relations in individual subjects. Rept. 177. Psychological Laboratories, University of Stockholm, Stockholm, Sweden.

Foley M., Beckley J., Ashman H. and Moskowitz H.R. 2009. The mind-set of teens towards food communications revealed by conjoint measurement and multi-food databases. Appetite. [Accessed online 01-06-09 from doi:10.1016/j.appet.2009.01.006].

Gil J. and Sanchez M. 1997. Consumer preferences for wine attributes: A conjoint approach. British Food J. 99: 3.

Green P.E. and Krieger A.M. 1991. Segmenting

markets with conjoint analysis. J. Marketing 55(S), 20.

Green P.E. and Krieger A.M. 1996. Individualized hybrid models for conjoint analysis. Management Sci. 42(6): 850.

Green P.E. and Srinivasan V. 1980. A general approach to product design optimization via conjoint measurement. J. Marketing 17: 45.

Kapsdorferove Z. and Nagyova L. 2007. Consumer behavior at the Slovak dairy market. Agricultural Economics.

Kotler P. 1997. "Marketing Management: Analysis, Planning, Implementation, and Control". Prentice Hall, New Jersey.

Liggett R.E., Drake M.A. and Delwiche J.F. 2005. Determining what consumers desire in Swiss cheese using partial-least squares regression of descriptove and consumer data. Presented at Annual Meeting, Institute of Food Technologists, New Orleans, LA, July 15-20.

Luce R.D. and Tukey J.W. 1964. Conjoint analysis: A new form of fundamental measurement. J. Math Psych. 1:1.

Luomala H.T. 2007. Exploring the role of food origin as a source of meanings for consumers and as a determinant of consumers' actual food choices. J. Business Res. 60(2): 122.

Mac Elroy B. 2000. Variables influencing dropout rates in Web-based surveys. Quirk's Marketing Research Review, July/Aug.

Moskowitz H. 2003. From psychophysics to the world…data acquired, lessons learned. Food Quality Pref. 15(7-8): 633.

Moskowitz H.R., Beckley J.H., Ashman H., Luckow T., Paulus K. and Aarts P. 2008. You're IT! Cross-National Segments for a Food Product: Defining Them and a Strategy for Finding Them in the Absence of "Mineable" Databases. J. Database Marketing 15(3): 191.

Moskowitz H.R., German J.B. and Saguy I.S. 2005a. Unveiling health attitudes and creating good for you foods: The genomics metaphor, consumer innovative web-based technologies. Crit. Rev. Food Sci. Nutr. 45:165.

Moskowitz H.R. and Gofman A. 2004. System and method for performing conjoint analysis. Provisional patent application, 60/538,787, filed January 23.

Moskowitz H. and Gofman A. 2007. "Selling Blue Elephants: How to Make Great Products Before Your Customers Even Know They Want Them", Wharton School Publishing, Upper Saddle River, New Jersey.

Moskowitz H.R., Gofman A., Ittay B., Katz R., Manchaiah M. and Ma Z. 2001. Rapid, inexpensive, actionable concept generation and optimization – the use and Promise of self-authoring conjoint analysis for the foodservice industry. Food Service Technol. 1: 149.

Moskowitz H., Itty B., Fink C. and Ewald J. 2003. Deconstructing automobile messaging: Clues to brand strategy. J. Database Marketing 10: 200.

Moskowitz H.R., Itty B., Shand A. and Krieger B. 2002. Understanding the consumer mind through a concept category appraisal: Toothpaste. Canadian J. Market Res. 20: 3.

Moskowitz H. R., Jacobs B.E. and Lazar N. 1985. Product response segmentation and the analysis of individual differences in liking. J. Food Quality, 8: 168.

Moskowitz H.R., Porretta S. and Silcher M. 2005b. "Concept Research in Food Product Design and Development", Blackwell Professional, Ames, Iowa.

Moskowitz H., Silcher M., Beckley J., Minkus-Mckenna D. and Mascuch T. 2005c. Sensory benefits, emotions and usage patterns for olives: using Internet-based conjoint analysis and segmentation to understand patterns of response. Food Quality Pref. 16(4): 369.

Murphy M., Cowan C. Meehan H. and O'Reilly S. 2004. A conjoint analysis of Irish consumer preferences for farmhouse cheese. British Food J. 106(4): 288.

Niedrich R.W. and Swain S.D. 2008. The effects of exposure-order and market entry-information on brand preference: a dual process model. J. Acad. Mark. Sci., 36: 3.

Pangborn R.M. 1970. Individual variations in affective responses to taste stimuli. Psychonomic Sci. 21: 125.

Pattisson N. and Lindgreen, A. 2004. Successes and failures in the dairy industry: South west England and northwest France. Brit. Food J. 106(6): 422.

Schultz D.E., Tannenbaum S.I. and Lauterborn R.F. 1993. "Integrated Marketing Communications, Pulling it Together and Making it Work", NTC Publications, Chicago, Illinois.

Steenkamp J. and Hofstede F.T. 2002. International marketing segmentation issues and perspectives. International J. Res. Marketing 19: 185.

Systat. 2004. "SYSTAT for Windows User Manual", Version 11. SYSTAT Software Inc., Chicago, Illinois.

Trevisiol A. 2005. The European Dairy Industry Model: Consumption of Dairy Products in the EU-15. 1960-2000 and 2010 Forecasts, 1: 23.

Wardle J., Haase A.M., Steptoe A., Nillapun M., Jonwutiwes K. and Bellisle F. 2004. Gender differences in food choice: the contribution of health beliefs and dieting. Annual Behavior of Medicine 27(2): 107.

Wells W.D. 1975. Psychographics: A critical review. J. Marketing Res. 12: 196.

Westad F., Hersleth M. and Lea P. 2005. Strategies for consumer segmentation with applications on preference data. Food Quality Preference 15(7-8): 681.

Wittink D.R., Vriens M. and Burhenne W. 1994. Commercial use of conjoint analysis in Europe: Results and critical reflections. Int. J. Res. Mark. 11(8): 41.

Revised paper received June 4, 2009 Accepted June 30, 2009

PAPER

# FREQUENCY OF FUNCTIONAL FOOD CONSUMPTION AND AWARENESS AND PERCEPTIONS OF HEALTH RISKS TOWARDS METABOLIC SYNDROME AMONG POLES AGED 15 AND ABOVE (LIPGENE PROJECT)

FREQUENZA DEL CONSUMO DI CIBI FUNZIONALI E CONSAPEVOLEZZA E PERCEZIONE DEI RISCHI PER LA SALUTE ATTRAVERSO LA SINDROME METABOLICA TRA I POLACCHI DI 15 ANNI E PIÙ (LIPGENE PROJECT)

**L. WĄDOŁOWSKA*, M. DANOWSKA-OZIEWICZ, B. STEWART-KNOX[1], and M. DANIEL VAZ DE ALMEIDA[2]**
Department of Human Nutrition, University of Warmia and Mazury,
10-718 Olsztyn, ul. Sloneczna 44a, Poland
[1]School of Biomedical Science,
University of Ulster at Coleraine, Coleraine, Northern Ireland, UK
[2]Faculty of Nutrition and Food Sciences, Porto University, Porto, Portugal
*Corresponding author: Fax +48 89 5234353,
e-mail: lidia.wadolowska@uwm.edu.pl

ABSTRACT

Adults in Poland have a high risk of metabolic syndrome due to increasing obesity and high blood pressure, cholesterol and blood sugar levels. Some people, especially those who are aware of health risks, assume that consuming functional foods may help decrease dis-

RIASSUNTO

Tra la popolazione polacca adulta si riscontra un alto rischio di sindrome metabolica a seguito dell'aumento crescente dell'obesità, della pressione sanguigna alta, di un elevato tasso di colesterolo e di un elevato tasso di glucosio nel sangue. Alcuni soggetti, in par-

- Key words: diet-related diseases, food consumption, functional food, health perceptions, metabolic syndrome -

ease risks and promote better health. The aim of this paper was to find specific clusters of Polish adults who consume certain functional foods and characterize them as functional food consumers or non-consumers in relation to their awareness of disease risk conditions and perception of the risk of metabolic syndrome.

ticolare quelli consapevoli delle minacce alla propria salute, ritengono che il consumo di alimenti funzionali possa svolgere un ruolo importante nella riduzione del rischio di malattie e nella promozione della salute. Lo scopo di questo studio è stato quello di trovare dei gruppi di Polacchi adulti i quali consumassero o non consumassero alimenti funzionali e di descriverli in base alla consapevolezza del proprio stato di salute e alla percezione del rischio di una sindrome metabolica.

## INTRODUCTION

Adults in Poland have a high risk of metabolic syndrome due to the increasing occurrence of obesity, high blood pressure, high blood cholesterol and high blood glucose level (NCEP, 2001; BUTTRISS, 2006; STAN ZDROWIA LUDNOŚCI POLSKI W, 2006; ZDROJEWSKI *et al.*, 2006; WYSOCKI and ZEJDA, 2007). According to the World Health Organisation (WHO), in Poland 25% of the disability-adjusted life years (DALYs) is due to high blood pressure, 13% to high cholesterol and 12% is due to being overweight and obese (POWLES *et al.*, 2005). The health status in Poland is very similar to other developed countries, although some health indices are less favourable to life and health quality than in other European countries. In 2006, the life expectancy in Poland was 70.9 years for men and 79.6 years for women, a few years less than in many Western European countries (WORLD HEALTH STATISTICS, 2006). Such statistics of major health risks have increased interest in prevention by understanding the possible roles that specific foods could play in improving health in Poland.

Food traditionally plays a nutritive role in providing energy and nutrients, but food is now becoming a way to decrease disease risk and improve health. This type of food is called functional food. There are many definitions of functional food. DOYON and LABRECQUE (2008), after consultation with a group of North American and European experts, proposed the following working definition: "A functional food is, or appears similar to, a conventional food. It is a part of a standard diet and is consumed on a regular basis, in normal quantities. It has proven health benefits that reduce the risk of specific chronic diseases or beneficially affects target functions beyond its basic nutritional function".

Some consumers believe that eating functional foods and/or supplements may be an easy way to be healthier and compensate for an unhealthy lifestyle (DE JONG *et al.*, 2003; SCHRYVER and SMITH, 2006). Among the Dutch population, 17% of a national representative sample aged 19-91 years thought that eating cholesterol-lowering margarine was an easy way to stay healthy (DE JONG *et al.*, 2003); 31% had similar expectations in relation to yoghurt with lactic acid bacteria and 39% in relation to foods with extra calcium. Recent studies have found that, de-

pending on the type of product, functional foods are consumed at least once a week by up to 50% of Europeans (DE JONG *et al.*, 2003; DE ALMEIDA *et al.*, 2006a). Positive attitudes towards functional foods are held by an even higher percentage of people in comparison to those who eat such food (DE JONG *et al.*, 2003). This indicates the potential of increasing functional food consumption.

Consumer attitudes towards functional foods may be stronger among people with a positive interest in health and greater awareness of disease risk. MOSCA *et al.* (2006) showed that awareness of increased personal health risk in women was positively associated with preventative actions for themselves and for their family members. Attitudes, beliefs and behaviour towards health differ; they depend on social and demographic factors and vary from country to country (KAFATOS *et al.*, 1999; PAMPALON *et al.*, 1999). Previous studies have focused on a set of individual predictors of health perception reflecting gender, lifestyle, socio-economic conditions, marital status and social support (PAMPALON *et al.*, 1999; DE JONG *et al.*, 2003). However, there is no information about adult consumers in Poland and the similarities and differences between them in relation to health risk perception towards metabolic syndrome. The association between the consumption of functional foods and consumer health status and perceived metabolic syndrome risk is not known.

It is our hypothesis that Polish people differ in perceived metabolic syndrome risk and this awareness affects the frequency of functional food consumption. The aim of this work was to find specific clusters of Polish adults who consume certain functional foods and characterize them as functional food consumers or non-consumers in relation to their awareness of disease risk conditions and metabolic syndrome risk perception, as well as various demographic factors.

## METHODS

The study was carried out as part of the LIPGENE project (FOOD-CT-2003505944). This is an integrated project (2004-2009) funded under the European Union's 6th Framework Programme for Research and Technology Development, entitled "Diet, genomics, and the metabolic syndrome: an integrated nutrition, agro-food, social and economics analysis". One of the goals of the project was to determine consumer attitudes in six European countries (France, Germany, Great Britain, Italy, Poland, Portugal) towards metabolic syndrome, genetics in nutrition and potential agro-food technologies (DE ALMEIDA *et al.*, 2006b).

### Sample selection

Data collection took place in Poland in June 2005. Recruitment, data gathering and entry were done by the market research company Ipsos Market and Opinion Research International (Ipsos-MORI), in each member state, including Poland. Details concerning sampling and questionnaire development are described by DE ALMEIDA *et al.* (2006b).

A sample of people aged 15 years and older (n=1005) was recruited in Poland. The objective of the LIPGENE project was focused on adult attitudes and was the first cross-cultural survey to investigate metabolic syndrome in six European countries. In several countries consumer studies and market research consider persons aged 15 and above as "adults"; therefore this lower age boundary was used in this study. Ethical approval was granted by the Office of Research Ethics Committee (OREC), UK. The market research company in charge of the fieldwork ensured that there were no ethical problems in participant selection.

As demographic information routinely collected by market research organisations can vary among member states,

care was taken to ensure that standardized information was obtained from all subjects in order to facilitate comparison. The subjects were initially classified according to the following characteristics:

(i) gender = male or female;

(ii) age group (years) = 15-24, 25-34, 35-44, 45-54, 55-64, 65-79, 80+;

(iii) education = highest level of education achieved by respondent – illiterate, primary, secondary, higher education, still studying;

(iv) working status = working, not working;

(v) region = country region.

No data regarding social class was collected because the criteria for defining social class differ among countries and it is difficult to standardize. Instead, the variable education (highest education level attained) was used as an indirect measure of social class (BLAKELY *et al.*, 2004; WAŚKIEWICZ and SYGNOWSKA, 2006). For the same reason, other detailed demographic data were not collected, *e.g.* religion. Furthermore, none of the Polish studies on food consumption have considered sample representativeness in the context of religion. For this reason, the current study made no reference to the issue.

The main demographic factors for sampling were the same across all member states, although there were small differences associated with country-specific factors. Demographic factors influencing the Polish sample selection were: sex, age, region and size of place of inhabitancy. In Poland 200 research areas were chosen for further analysis, complying with their geographical location and size of city/town/village. Respondents were chosen in a systematic drawing based on date of birth from a database of the State Registrar Department of the Ministry of Internal Affairs and Administration (using PESEL identity card number) and were visited in their own homes. Quotas were drawn from selected research areas, and then divided according to age and gender criteria. This was a quota-controlled sample so no refusal rates were recorded. To ensure that the samples from each participating country were as nationally representative as possible, responses were weighted by demographic factors for each sampling point based on the official statistics (in Poland: sex, age, region and size of place of inhabitancy). The sampling procedure ensured that the sample matched census statistics for the pre-defined characteristics. There were no reimbursements for people participating in the study.

The Polish sample included 48.2% men and 51.8% women aged from 15 years to 79 years. Working and non-working (including students) people made up 43.4 and 56.6% of the total sample, respectively. The sample characteristics are presented in Table 1.

Data collection

The survey was included in an omnibus research study in which subjects complete questionnaires on separate topics from different clients in a single session. The advantages of carrying out this type of survey are that interviewers are fully trained, the costs of sample selection are shared between the participating clients and surveys can be completed within a very short time period. However one drawback in the use of the omnibus research method was that the length of the questionnaire could not exceed 13 questions. Interviews were carried out by trained pollsters *face-to-face* using the closed question questionnaire. Pollsters presented special sheets (so-called "show cards") with an answer menu for each question and then filled in the questionnaire according to consumer responses. The sequence of answer presentation to subsequent readers was constantly altered (e.g. from A to E or from E to A).

The questionnaire was developed on

Table 1 - Sample characteristic (N=1005).

| Category | Sample percentage (%) |
|---|---|
| Gender | |
| Men | 48.2 |
| Female | 51.8 |
| Age | |
| 15-24 y | 23.0 |
| 25-34 y | 18.0 |
| 35-44 y | 16.3 |
| 45-54 y | 17.0 |
| 55-64 y | 15.1 |
| 65-79 y | 10.6 |
| Age (years*) | 40±29 |
| Employment status | |
| Working | 43.4 |
| Not working | 56.6 |
| Education level | |
| Primary | 21.4 |
| Secondary | 30.2 |
| Higher | 48.4 |

*Expressed as median and quartile deviation.

the basis of prior qualitative research (DE ALMEIDA *et al.*, 2006b) comprising a series of consumer focus group and stakeholder interviews. The survey was focused on metabolic syndrome, genetically modified products, food habits and health conditions. The fourteen focus groups were carried out with 50 consumers in Portugal and 50 in Northern Ireland (100 in total). Based on this qualitative research, several themes were identified and used to create the questionnaire. The questionnaire was designed by specialists (nutritionists, statisticians, psychologists and sociologists) from the six countries taking part in the study. The research centres taking part in the study and creating the questionnaire were: The Faculty of Nutrition and Food Sciences, Porto University (Portugal), The University of Ulster (Northern Ireland), Instituto Nazionale di Ricerca per gli Alimenti e la Nutrizione (Italy), The University of Warmia and Mazury (Poland), UNILEVER (The Netherlands) and Ipsos Market and Opinion Research International (Ipsos-MORI, Great Britain). The questionnaire was developed in English and then translated into the mother language of each country. The translations were verified by the researchers before administration to ensure that the original meaning had been maintained. The questionnaire was tested in a pilot study among about 30 people in each country (in total about 180 consumers). This helped to verify whether the questions and the terms used in the questionnaire were properly understood and whether the respondents found it difficult to provide an answer.

The final questionnaire included 12 close-ended questions and one open-ended item. The study used three closed questions only, which were related to: (i) past and present personal history of health risks, (ii) health risk perception and (iii) consumption of functional food at least once a week. Each of the three questions related to 5 or 7 separate statements; a total of 17 characteristics were analysed. This was done to simplify the process for a large number of samples and reduce time (and cost) for questionnaire completion. It was not possible to use a long version of a food frequency questionnaire in this study.

Data were gathered regarding the past and present personal history of health risks with respect to the following conditions:

(i) central obesity (described as "a higher than normal amount of fat around your waist"),

(ii) high blood cholesterol level,

(iii) high blood glucose level,

(iv) high blood pressure (or hypertension),

(v) high stress level (described as "stress resulting in difficulty concentrating, sleeping or relaxing at least once a week").

Respondents were asked to judge their health conditions (responses: yes/no/I don't know) separately for each of the five conditions mentioned above. Terms and phrases were used in the study as word-

ed above. The terms used were properly understood by respondents as they had been carefully prepared as described above. Doubts, if any, were additionally explained by pollsters, who were thoroughly instructed and well trained.

In addition, respondents were asked to assess their health risk perception of central obesity, high blood cholesterol level, high blood glucose level, high blood pressure and high stress level (currently or in the future). Each of the risks were measured on a scale from 0 to 10, where 0 meant "total lack of health risk", and 10 "very high health risk". Respondents could give the response: "I don't know".

Next, the frequency of consumption of the following foods at least once a week (responses: yes/no) was assessed (separately for each food):

(i) cholesterol lowering spreads or drinks (e.g. Benecol, Flora pro.activ),

(ii) yoghurt probiotic drinks (e.g. Yakult, Actimel),

(iii) high fibre foods (e.g. wholegrain breads, cereals with high fibre content),

(iv) weight loss products (e.g. meal replacements used for weight maintenance and/or obesity treatment),

(v) food with added vitamins and/or minerals,

(vi) energy drinks (e.g. sport drinks, Red Bull),

(vii) vegetables and/or fruit.

Respondents could also answer: "none of these" or "I don't know". Proper understanding of the questions was verified by the pollsters and doubts, if any, were explained orally, using specific examples of foods commercially available in Poland. A binary scale (yes/no) was used for the assessment of functional food consumption with a frequency at least once a week. The same or similar frequency of functional food consumption was used in previous studies (de JONG et al., 2003; URALA and LAHTEENMAKI, 2004; 2007). More detailed data regarding consumption was not required because the aim of this paper was to identify clusters of functional food consumers and non-consumers and characterize them.

The functional food list was created after a focused group interview analysis. It was observed that European consumers consider vegetables and fruit as functional foods (DE ALMEIDA et al., 2006b). For this reason they were included as "functional food" in the six-country studies. Although the frequency of vegetable and fruit consumption described as "once a week" did not quite match the description of dietary habits, it was the same as the frequency of consumption of other functional foods. This approach enabled further analysis and comparisons between functional food items. The use of energy drinks was also included in the survey. According to DOYON and LABRECQUE (2008) energy drinks should not be classified as a functional food, while other researchers disagree (URALA and LATHTEENMAKI, 2007). In summary, energy drinks and vegetables and/or fruit were classified as typical functional foods because they are: (i) marketed as such and/or (ii) perceived by consumers as such and/or (iii) investigated in similar papers as such.

Statistical analysis

Principal components analysis (PCA) and cluster analysis were carried out to determine characteristic clusters of people with certain food consumption patterns and their awareness of disease risk (HILL and LEWICKI, 2006; ARMITAGE *et al.*, 2008). Both analyses are a multi-dimensional method and permit the simultaneous analysis of several different factors, including qualitative and quantitative ones. These types of analyses are frequently used to describe dietary patterns in nutritional epidemiology (HU, 2002; DIXON *et al.*, 2004; HOFFMAN *et al.*, 2004; NEWBY *et al.*, 2004). The characteristic clusters of people were determined in two stages, first with the PCA method followed by a cluster analysis.

Principal components analysis

The PCA method was used to reduce the multi-dimensional factor set and find a lower number of new complex dimensions created by various input factors.

The PCA and clusters analysis were only based on data obtained from respondents who gave complete answers and answered yes or no. Individuals responding "I don't know" were excluded. In this way, a total of 52 individuals (5% of total sample) were excluded. This procedure did not change the consumer distribution in demographic categories. The input data included a continuous variable (perceived health risk) or binary variables (yes/no: self-reported health condition, frequency of functional food consumption). For binary answers, numeric values 0 and 1 were assigned. Before the PCA, the input data were standardized, *i.e.* transformed so that their mean value amounted to 0 and the standard deviation was 1. This transformation has a wide variety of applications because it makes the distributions of values easy to compare across variables and/or subsets (HILL and LEWICKI, 2006; ARMITAGE *et al.*, 2008). If applied to the input data, standardization also makes the results of a variety of statistical techniques entirely independent of the range of values or the units of measurement. Data standardization is also recommended for input data in PCA (HILL and LEWICKI, 2006; ARMITAGE *et al.*, 2008).

Seventeen variables concerning self-reported health conditions, health risk perception and frequency of weekly consumption of functional food were entered into the PCA. The factors were rotated by the orthogonal transformation (Varimax option), resulting in uncorrelated factors, to achieve a simpler structure with greater interpretability. In determining the number of factors to retain, three criteria were considered: (i) components with eigenvalue >1, (ii) the Scree test and (iii) the interpretability of the factors (HILL and LEWICKI, 2006; ARMITAGE *et al.*, 2008). The percentage of variance explained by each factor was not used because this criterion depends largely on the total number of variables included in the analyses. For factor loadings such as the cutoff point a value of 0.7 was used. However, in order to better illustrate the results, more data were provided; only the least significant values were omitted, i.e. factor loadings with an absolute value <0.3. The PCA generated five non-correlated principal components. These dimensions explained a total of 67.0% of the variance differentiation.

Cluster analysis

The cluster analysis allowed the creation of separate groups (clusters) of individuals using mathematically defined characteristics. Individuals belonging to the same group have a strong similarity but there are strong differences between individuals belonging to separate groups (ARMITAGE *et al.*, 2008). Using cluster analysis, characteristic clusters of Polish consumers were separated and each cluster contained subjects with similar health conditions, health risk perception and frequency of weekly functional food consumption.

In the cluster analysis, the input data were 5 principal components that had been separated in the PCA. For subject grouping, the k-means clustering was used (HILL and LEWICKI, 2006; ARMITAGE *et al.*, 2008). In turn, a varied number of clusters starting with two clusters were tested. Finally, six non-overlapping clusters were found. The correctness of cluster number choice was verified by a one-way variance analysis (ANOVA). Clusters were named using a combination of principal factors from 2 to 5 because there were significant differences between clusters (excluding factor 1). No significant differences were found in Eu-

clidean distances between clusters for factor 1. For this reason, factor 1 was not considered a determinant characteristic of any cluster. Finally, cluster names were created according to which features contributed either the highest or lowest values of sample percentage with respect to heath risk perception and frequency of weekly functional food consumption.

### Method of results analysing

The results are displayed as a sample percentage and/or a median and a quartile deviation, because factor distribution did not correspond to a normal distribution. Health risk perception was presented as a percentage of people with: no risk (0 point), low risk (1-3 points), medium risk (4-6 points), high risk (7-9 points) and extremely high risk (10 points). Factor distributions were compared using the $\chi^2$ test for comparison in pairs as well as for comparisons between six clusters. Differentiation between clusters regarding the health risk perception level was evaluated by the Kruskal-Wallis test. A post-hoc analysis for multiple comparisons in pairs was performed by a median test, except for age (continuous variable expressed in years), which was compared by a Kruskal-Wallis test. The statistical analysis was carried out using the Statistica PL v.7.1 software.

## RESULTS

### Self-reported health conditions and health risk perception

About 16% of Poles reported a high stress level resulting in difficulty concentrating, sleeping or relaxing at least once a week (Table 2). About 13% of the subjects declared having central obesity and about 11% declared high blood pressure. High blood cholesterol and high blood sugar were declared by about 6% and about 4% of the subjects, respectively.

The perception of health risk of high blood cholesterol, high blood pressure, central obesity, high blood sugar and high stress level were assessed as average (median 5 on a scale from 0 to 10). But some individuals had high or extremely high metabolic syndrome risk perception. High/extremely high health risk perception (>7 points) of high blood pressure was stated by over 40% of the subjects and high blood cholesterol or high stress level were declared by over 35% of the subjects (Fig. 1).

### Frequency of functional food consumption

In total, vegetables and/or fruit were consumed at least once a week by over 80% of the subjects while probiotic yoghurt drinks or high fibre foods were consumed by almost 40% of the subjects (Table 2). Around 20% of the subjects consumed cholesterol-lowering spreads or drinks and foods with added vitamin and/or minerals at least once a week. Energy drinks or weight loss products were consumed by 4-7% of the subjects at least once a week.

### PCA analysis and relationship between self-reported health conditions, health risk perception and frequency of functional food consumption

In the PCA, five main components were found that linked factors (Table 3). Main factor 1 was "health condition" because it included all factors concerning self-reported health conditions (factor loadings from 0.83 to 0.92). Main factor 2 linked all of the features concerning health risk perception (factor loadings from 0.76 to 0.91). The frequency of functional food consumption created three separate main factors. Main factor 3 included weekly consumption of weight loss products (0.67), foods with

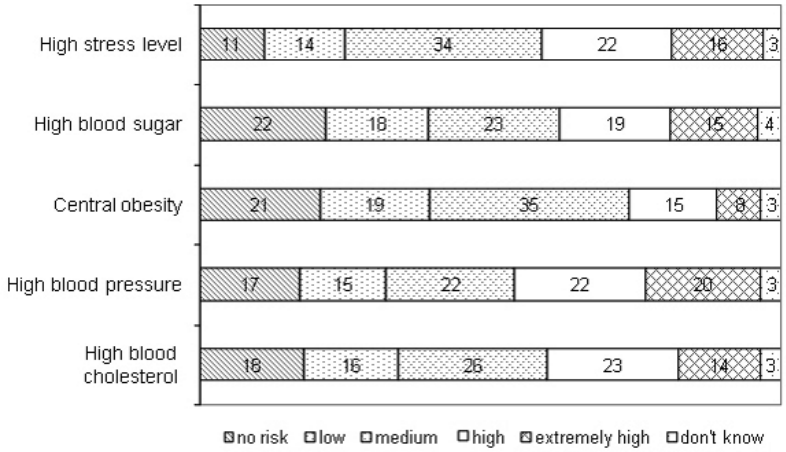



Fig. 1 - Distribution of health risk perception in relation to metabolic syndrome (N=1005). Health risk level defined from 0 to 10 points; no risk=0 point; low risk=1-3 points; medium risk=4-6 points; high risk=7-9 points; extremely high risk=10 points.

added vitamin and/or minerals (0.66) and high fibre foods (0.63); Main factor 4 included weekly consumption of cholesterol lowering spreads or drinks (0.70) and energy drinks (0.63). Main factor 5 included only one feature, the weekly consumption of fruit and/or vegetables (0.79).

## Clusters of Polish functional food consumers

Six clusters of subjects were identified which differed with respect to health risk perception and frequency of functional food consumption ($p<0.001$; Table 4-5). These consumers were: (I) aware of low health risk (23.5% of the subjects), (II) consuming cholesterol-lowering spreads or drinks and aware of low health risk (10.2%), (III) consuming cholesterol-lowering spreads or drinks and aware of high health risk (12.5%), (IV) eating vegetables and/or fruit and were aware of relatively high health risk (24.1%), (V) less-likely to eat functional food (25.5%) and (VI) eating functional food including weight loss products (4.2%). Self-reported health conditions (main fac-

Table 2 - Self-reported health conditions, health risk perception and weekly* consumption of some food products (N=1005).

| Category | Sample percentage (%) |
|---|---|
| Self-reported health conditions | |
| high blood cholesterol | 6.4 |
| high blood pressure | 11.2 |
| central obesity | 13.3 |
| high blood sugar | 3.7 |
| high stress level | 16.3 |
| Health risk perception** | |
| high blood cholesterol | 5±6 |
| high blood pressure | 5±7 |
| central obesity | 5±5 |
| high blood sugar | 5±7 |
| high stress level | 5±5 |
| Weekly* consumption of | |
| cholesterol-lowering spreads or drinks | 19.9 |
| probiotic yoghurt drinks | 37.1 |
| high fiber food | 38.1 |
| weight-loss products | 3.7 |
| foods with added vitamin and/or minerals | 19.6 |
| energy drinks | 7.0 |
| fruit and/or vegetables | 83.4 |
| None of these | 3.4 |

*Assessed as at least once a week;
**expressed as median and quartile deviation (on scale from 0 to 10).

Table 3 - Factor loadings matrix for the major factors extracted in the PCA method.

| Input variables | Factor 1 | Factor 2 | Factor 3 | Factor 4 | Factor 5 |
|---|---|---|---|---|---|
| Self-reported health conditions | | | | | |
| high blood cholesterol | 0.83 | | | | |
| high blood pressure | 0.87 | | | | |
| central obesity | 0.88 | | | | |
| high blood sugar | 0.92 | | | | |
| high stress level | 0.89 | | | | |
| Health risk perception | | | | | |
| high blood cholesterol | | 0.91 | | | |
| high blood pressure | | 0.91 | | | |
| central obesity | | 0.79 | | | |
| high blood sugar | | 0.90 | | | |
| high stress level | | 0.76 | | | |
| Weekly* consumption of | | | | | |
| cholesterol-lowering spreads or drinks | | | | 0.70 | |
| probiotic yoghurt drinks | | | 0.34 | 0.49 | 0.43 |
| high fiber food | | | 0.63 | | 0.38 |
| weight-loss products | | | 0.67 | | -0.38 |
| foods with added vitamin and/or minerals | | | 0.66 | | |
| energy drinks | | | | 0.63 | |
| fruit and/or vegetables | | | | | 0.79 |
| Share in explained variance** (%) | 23.1 | 21.8 | 8.5 | 7.0 | 6.6 |

*Assessed as at least once a week;
**share in the explained variance of five main factors amounts 67.0%;
The cut-off point in results interpretation was at 0.7, however, the table contains more data to better illustrate the results, without factor loadings with the absolute value <0.3.

Table 4 - Cluster number and characteristics (N=953).

| Cluster | Number | Sample percentage (%) | Cluster description* |
|---|---|---|---|
| I | 224 | 23.5 | Consumers with low health risk perception |
| II | 97 | 10.2 | Consumers consuming cholesterol-lowering spreads or drinks and with a low health risk perception |
| III | 119 | 12.5 | Consumers consuming cholesterol-lowering spreads or drinks and with a high health risk perception |
| IV | 230 | 24.1 | Consumers consuming fruit and/or vegetables and with a relatively high health risk perception |
| V | 243 | 25.5 | Less-likely consumers of functional food |
| VI | 40 | 4.2 | Consumers consuming functional food including weight-loss products |

*Cluster descriptions were formed by principal factors in combinations from 2 to 5 excluding main factor 1, because there were no differences in Euclidean distances between clusters for factor 1.

Table 5 - Health risk perception and weekly* consumption of some food products in clusters (N=953).

| Category | Sample percentage (%) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Total N=953 | Cluster I N=224 | Cluster II N=97 | Cluster III N=119 | Cluster IV N=230 | Cluster V N=243 | Cluster VI N=40 | p $\chi^2$ test |
| Self-reported health conditions | | | | | | | | |
| high blood cholesterol | 7.1 | 3.3 | 6.7 | 7.7 | 10.0 | 7.3 | 11.4 | 0.133 |
| high blood pressure | 12.3 | $3.8^{a,b,c}$ | $9.1^{e}$ | $12.2^{a}$ | $20.3^{b,e}$ | $13.6^{c}$ | $13.5^{d}$ | <0.001 |
| central obesity | 14.2 | $8.9^{a,b}$ | $6.8^{c,d,e}$ | $15.7^{c}$ | $19.8^{a,d}$ | $13.6^{f}$ | $29.0^{b,e,f}$ | <0.001 |
| high blood sugar | 4.0 | 1.4 | 1.1 | 7.6 | 4.2 | 5.5 | 5.3 | 0.059 |
| high stress level | 17.4 | 17.0 | 23.6 | 15.7 | $23.9^{a}$ | $10.1^{a}$ | 19.4 | 0.003 |
| Health risk perception** | | | | | | | | |
| high blood cholesterol | 5±6 | $0±3^{a,b,c,d}$ | $1±3^{a,e,f}$ | $8±4^{b,e,g,h}$ | $7±4^{f,g,i,j}$ | $6±3^{c,i,k}$ | $5±6^{d,h,j,k}$ | <0.001*** |
| high blood pressure | 5±7 | $1±3^{a,b,c,d}$ | $2±5^{a,e,f,g}$ | $9±3^{b,e,h,i}$ | $8±4^{f,h,j,k}$ | $7±4^{c,g,j,l}$ | $5±6^{d,i,k,l}$ | <0.001*** |
| central obesity | 5±5 | $1±3^{a,b,c,d}$ | $0±3^{a,e}$ | $5±4^{b,f}$ | $5±3^{e,f,g,h}$ | $5±3^{c,g}$ | $5±4^{d,h}$ | <0.001*** |
| high blood sugar | 5±7 | $0±2^{a,b,c,d,e}$ | $1±3^{a,f}$ | $9±4^{b,f,g,h,i}$ | $7±4^{c,g,j,k}$ | $6±3^{d,h,j,l}$ | $5±6^{e,i,k,l}$ | <0.001*** |
| high stress level | 5±5 | $2±5^{a,b,c}$ | $4±5^{a,d,e}$ | $8±5^{b,d,f}$ | $7±4^{e,f,g,h}$ | $6±3^{c,g}$ | $5±6^{h}$ | <0.001*** |
| Weekly* consumption of | | | | | | | | |
| cholesterol-lowering spreads or drinks | 19.9 | $1.3^{a,b,c}$ | $65.0^{a,d,e,f,g}$ | $86.6^{b,d,h,i,j}$ | $2.2^{e,h,k}$ | $2.5^{f,i,l}$ | $25.0^{c,g,j,k,l}$ | <0.001 |
| probiotic yoghurt drinks | 36.9 | $38.0^{a,b,c,d,e}$ | $54.6^{a,f}$ | $51.3^{b,g}$ | $51.3^{c,h}$ | $4.9^{d,f,g,h,i}$ | $57.5^{e,i}$ | <0.001 |
| high fiber food | 38.9 | $40.6^{a,b,c}$ | $40.2^{d,e,f}$ | $30.2^{g,h,i}$ | $68.7^{a,d,g,j}$ | $6.6^{b,e,h,j,k}$ | $77.5^{c,f,i,k}$ | <0.001 |
| weight-loss products | 3.7 | $0.0^{a}$ | $0.0^{b}$ | $0.8^{c}$ | $0.0^{d}$ | $0.0^{e}$ | $85.0^{a,b,c,d,e}$ | <0.001 |
| foods with added vitamin and/or minerals | 19.5 | $13.8^{a,b,c,d}$ | $27.8^{a,e,f}$ | $19.3^{g,h,i}$ | $32.2^{b,g,j}$ | $2.5^{c,e,h,j,k}$ | $62.5^{d,f,i,k}$ | <0.001 |
| energy drinks | 7.0 | $0.0^{a,b,c}$ | $43.3^{a,d,e,f,g}$ | $16.0^{b,d,h,i}$ | $0.0^{e,h,j}$ | $0.0^{f,i}$ | $15.0^{c,g,j}$ | <0.001 |
| fruit and/or vegetables | 83.7 | $97.3^{a,b,c,d,e}$ | $80.4^{a,f,g,i}$ | $84.9^{b,h,j,k}$ | $100.0^{c,f,i,l,m}$ | $59.7^{d,g,j,l}$ | $65.0^{e,k,m}$ | <0.001 |

*Assessed as at least once per week; **expressed as median and quartile deviation (in scale from 0 to 10);
Cluster names: I – consumers with low health risk perception, II – consumers consuming cholesterol-lowering spreads or drinks and with a low health risk perception, III – consumers consuming cholesterol-lowering spreads or drinks and with a high health risk perception, IV – consumers consuming fruit and/or vegetables and with a relatively high health risk perception, V – less-likely consumers of functional food, VI – consumers consuming functional food including weight-loss products; N – number of the sample;
a-a, b-b,... – significant differences in pairs at p<0.05; ***significance level of the Kruskal-Wallis test for health risk perception.

tor 1) were not characteristic features of the clusters. However, it was stated that clusters differed in the percentage of people with self-reported health conditions in relation to high blood pressure ($p<0.001$), central obesity ($p<0.001$) and high stress level ($p=0.003$; Table 5). Employment status did not influence the clusters (Table 6).

Subjects in cluster I perceived their health risk as very low (median from 0 to 2; Table 5). Only a few of them declared poor health conditions (central obesity <9% and high stress level, 17%). Almost all of them (about 97%) consumed fruit and/or vegetables at least once a week, but none consumed energy drinks or weight loss products. There were more women (57.6%) and young adults (median: 36 years; Table 6). This cluster contained the lowest number of people with a primary education (about 14%).

Subjects from cluster II perceived their health risk as low (median from 0 to 4; Table 5). This cluster included the fewest people who reported high blood sugar (about 1% of the sample) or central obesity (about 7%) and many subjects reported high stress level (about 24%). The cluster included the most subjects who consumed energy drinks (about 43%) at least once a week. Many consumers from this cluster reported eating cholesterol-lowering spreads or drinks (65%) or probiotic yoghurt drinks (about 55%) at least once a week. This cluster had the most men (57.7%), students aged 15-24 years (32%) and people with a higher level of education (about 53%; Table 6).

Subjects from cluster III perceived their health risk as high due to high blood cholesterol, high blood pressure, high blood sugar and high stress level (median from 8 to 9; Table 5). Their self-reported health conditions were similar to the total sample. This cluster included the most people who reported eating cholesterol-lowering spreads or drinks (about 87%) at least once a week. There were more men (55.5%) than women and consumers had a higher level of education (about 54%; Table 6).

Subjects from cluster IV perceived their health risk as relatively high due to high blood pressure, high blood cholesterol, high blood sugar and high stress level (median from 7 to 8; Table 5). This cluster contained the most people who self-reported high blood pressure (over 20%) and a high stress level (about 24%). All subjects from cluster IV reported consuming fruit and/or vegetables with a frequency of at least once a week. Many consumers from this cluster reported eating high fibre foods (about 70%) or probiotic yoghurt drinks (about 52%) or foods with added vitamin and/or minerals (about 32%) at least once a week. There were more women (57.4%) than men and fewer people had a primary education level (about 17%; Table 6).

Subjects from cluster V perceived their health risk as slightly high (median from 5 to 7; Table 5). The cluster included the fewest people who reported a high stress level (about 10%). Subjects from this cluster were the least likely to eat functional food. The lowest number of subjects (<60%), compared to the other clusters, reported eating fruit and/or vegetables at least once a week. Less than 7% reported eating other functional food products at least once a week (Table 5). There were more men (54.3%) and older people (median: 46 years) and the most people had a primary education (about 32%).

Subjects from cluster VI perceived their health risk as moderate (median 5; Table 5). This cluster included the most people who reported central obesity (about 30%) and who declared consuming weight-loss products (85%) at least once a week, as compared to other clusters. Many subjects from cluster VI reported eating high fibre food (about 78%) or foods with added vitamin and/or minerals (about 62%) at least once a week. Only 65% declared eating fruit and/or vegetables at least once a week. The cluster included mostly women (70%; Table

Table 6 - Sample distribution in clusters (N=953).

| Category | Sample percentage (%) | | | | | | | p $\chi^2$ test |
|---|---|---|---|---|---|---|---|---|
| | Total N=953 | Cluster I N=224 | Cluster II N=97 | Cluster III N=119 | Cluster IV N=230 | Cluster V N=243 | Cluster VI N=40 | |
| Gender | | | | | | | | 0.001 |
| Men | 48.2 | 42.4[a,b,c] | 57.7[a,d,e] | 55.5[b] | 42.6[d] | 54.3[c] | 30.0[e] | |
| Female | 51.8 | 57.6[a,b,c] | 42.3[a,d,e] | 44.5[b] | 57.4[d] | 45.7[c] | 70.0[e] | |
| Age | | | | | | | | 0.001 |
| 15-24 y | 23.0 | 24.1 | 32.0[a,b] | 18.5[a] | 24.4 | 18.5[b] | 27.5 | |
| 25-34 y | 18.0 | 22.8[a,b] | 22.7 | 17.6 | 15.2[a] | 14.8[b] | 17.5 | |
| 35-44 y | 16.3 | 17.4 | 11.3[a] | 21.8[a] | 17.4 | 14.8 | 7.5 | |
| 45-54 y | 17.0 | 14.3 | 17.5 | 16.0 | 17.8 | 20.2 | 10.0 | |
| 55-64 y | 15.1 | 13.8[a] | 10.3[b] | 16.8[c] | 10.4[d] | 18.5[e] | 35.0[a,b,c,d,e] | |
| 65-79 y | 10.6 | 7.6[a,b] | 6.2[c] | 9.2 | 14.8[a,c,d] | 13.2[b] | 2.5[d] | |
| Age (years*) | 40±29 | 36±27[a,b,c] | 31±26[a,d] | 41±28[b] | 40±29[d] | 46±28[c] | 42±34 | 0.001** |
| Working status | | | | | | | | NS |
| Working | 43.4 | 45.5 | 47.4 | 42.4 | 43.7 | 41.2 | 37.5 | |
| Not working | 56.6 | 54.5 | 52.6 | 57.6 | 56.3 | 58.8 | 62.5 | |
| Education level | | | | | | | | <0.001 |
| Primary | 21.4 | 14.4[a,b] | 15.5[c] | 24.6[a] | 17.4 | 32.5[b,c] | 22.5 | |
| Secondary | 30.2 | 34.5[a,b] | 30.9 | 22.0[a] | 31.3 | 25.9[b] | 47.5 | |
| Higher | 48.4 | 51.1[a,b] | 53.6[c,d] | 53.4 | 51.3 | 41.6[a] | 30.0[b,d] | |

*Expressed as median and quartile deviation;
Cluster names: I – consumers with low health risk perception, II – consumers consuming cholesterol-lowering spreads or drinks and with a low health risk perception, III – consumers consuming cholesterol-lowering spreads or drinks and with a high health risk perception, IV – consumers consuming fruit and/or vegetables and with a relatively high health risk perception, V – less-likely consumers of functional food, VI – consumers consuming functional food including weight-loss products; N – number of the sample;
a-a, b-b,… – significant differences in pairs at $p<0.05$; **significance level of the Kruskal-Wallis test.

6). There were more people aged from 55 to 64 years and with a secondary education level (about 48%), but higher education was quite rare (30%).

## DISCUSSION

The study showed that the functional food consumer group did not exceed 40% of Poles aged 15 years and above. Nearly 40% of subjects consumed high fibre food and probiotic yoghurt drinks at least once a week and about 20% of subjects consumed cholesterol-lowering spreads or drinks and foods with added vitamin and/or minerals at least once a week. Only a few consumers reported eating weight loss products. No studies have been found in the Polish literature in which the results are directly comparable to these, especially with reference to specific functional foods. Some background may be provided by food product market research. An analysis performed by an expert from Pentor Research International (http://www.poradnikhandlowca.com.pl/) found an annual growth of yoghurt sales of 6.9% (data from 2003-2004) and an increased interest in so-called "bioyoghurts". About 47% of the Polish market share is held by Danone, which produces increasingly popular probiotic yoghurts. In general, nearly 75% of Poles declared consuming yoghurt, including 70% who consumed it at least twice a week (http://www.poradnikhandlowca.com.pl/). The analysis cited above shows that nearly 23% of Poles consumed high-fibre crispy bread ("Wasa") with a varied frequency. Consumption of margarines and spread mixes were declared by nearly 70% of the Poles, including 60% who declared daily consumption. However, it is difficult to determine the percentage of overall consumption of cholesterol-lowering spreads and other high-fibre food. It may be supposed that the increased consumption of cholesterol-lowering spreads was greater than that of total margarines (annual sales growth in 2003-2004 was 2.7%). The results related to high fibre food, probiotic yoghurt drinks and cholesterol-lowering spreads seem to be consistent with the market analysis results.

In comparison to six other European countries, including Poles, the proportion of Polish functional food consumers was similar with respect to cholesterol-lowering spreads or drinks (17 vs. 20%, respectively) as well as foods with added vitamins and/or minerals (18 vs. 20%, respectively) and weight-loss products (5 vs. 4%, respectively) (de ALMEIDA *et al.*, 2006a). More Europeans consume high fibre food at least once a week (50 vs. 40%, respectively) and fewer consume probiotic yoghurt drinks (26 vs. 37%, respectively). A lower proportion of functional food consumers was noted by de JONG *et al.* (2003) in the Dutch population aged 19-91 years. The same frequency (at least once a week) was noted for yoghurt with lactic acid bacteria (8%), foods with extra calcium (10%) and cholesterol lowering-margarine (4%). These results show some similarities and differences between Poles and Western Europeans with respect to eating such products. The results confirmed that the consumption of functional foods depends on the type of product. The current results and analysis of the food product market in Poland indicate that Poles consume more probiotic yoghurt drinks than other Europeans.

It was found that only about 83% of the respondents reported consuming vegetables and/or fruit at least once a week. A similar low percentage was found for people eating vegetables and/or fruit (86%) with the same frequency among residents of the six European countries, including Poland (de ALMEIDA *et al.*, 2006a). It could not be ascertained how many times per week or per day these people ate vegetables and/or fruit. Although it can be stated that 15-20% of the Europeans were non-consumers of vegetables and/or fruit be-

cause they consumed vegetables and/or fruit less frequently than once a week. Thus, indirectly it can be stated that this group of Poles (and Europeans) consumes less than the recommended level of these products. This view is supported by numerous qualitative studies (EZZATI *et al.*, 2004; POMERLEAU *et al.*, 2005; SYGNOWSKA *et al.*, 2005). This negative dietary habit lowers the chances of preventing many diet-related diseases, *e.g.* metabolic syndrome.

Polish consumers differed in frequency of functional food consumption and perceived health risk. The occurrence of these features depended on gender, age and education level but not on working status. There were no clear differences between Polish consumers and less-likely consumers of functional foods in metabolic syndrome risk perception and demographic characteristics. The frequency of functional food consumption by Poles aged 15 years and older was weakly dependent on their awareness of health conditions.

Two consumer groups, those in cluster VI and cluster V, were generally classified as functional food consumers and less-likely consumers of functional food. The lowest probability of functional food consumption was found among older consumers, especially men and subjects with a low level of education and slightly high health risk perception (cluster V). Older people were less interested in novel food consumption and changing their dietary habits, especially people with a low level of education (MAYNARD *et al.*, 2005). The small group of general functional food consumers was made up of middle-aged women with a secondary level of education and with central obesity (cluster VI). Such consumers were the most likely to eat weight-loss products, food with added vitamins and/or minerals, high fibre food and probiotic yoghurt drinks. Nearly 30% of the consumers in this group self-reported obesity but, surprisingly, they saw it as a moderate threat to their health, currently and in future. Undoubtedly, there is strong link between self-reported obesity and declared eating of weight-loss products in consumers from cluster VI. Obese women could have a strong motivation to reduce their body mass and could be searching for different ways to lower body mass, *e.g.* by using special foods. Furthermore, it could be assumed that they were not interested in changing their dietary habits. Thirty-five percent of the individuals from cluster VI declared eating vegetables and/or fruit with a frequency of at least once a week. Men were more likely to be consumers of cholesterol-lowering spreads or drinks (cluster II and III), although the current study has provided no information on the amount of food consumed. Many papers have found a higher frequency and amount of fat/fat spreads consumed by Polish men than women (SZPONAR *et al.*, 2003; SYGNOWSKA *et al.*, 2005; WAŚKIEWICZ and SYGNOWSKA, 2006; WĄDOŁOWSKA *et al.*, 2008). Men often ate popular foods and preferred more tasty foods, *e.g.* fatty food or with added fat. It may be supposed that men who consumed cholesterol-lowering spreads or drinks were taking care of their health by making small changes in their dietary habits.

The consumption of cholesterol-lowering spreads or drinks was related to low health risk perception, as well as to high risk perception. The non-consumers of cholesterol-lowering spreads or drinks had the lowest health risk perception (cluster I) in comparison to the others. Subjects who were more likely to eat cholesterol-lowering spreads or drinks made up two separate clusters (II and III). While the most likely consumers of cholesterol-lowering spreads or drinks and probiotic yoghurt drinks were men and subjects with a higher level of education, some were younger and had a low health risk perception (cluster II) but others had a higher health risk perception (cluster III) in comparison to the others. There are at least three possible fac-

tors involved in the consumption of these functional products: age, gender and education. All of these factors have been identified as very important in differentiating health perception and food consumption (JOHANSSON *et al.*, 1999; KIRKLAND *et al.*, 1999; MARGETTS *et al.*, 1999; PAMPALON *et al.*, 1999; ZANDSTRA *et al.*, 2001; IDZIK, 2006; MOSCA *et al.*, 2006; WAŚKIEWICZ and SYGNOWSKA, 2006; WĄDOŁOWSKA *et al.*, 2008). Most studies show that women and younger people with a higher level of education are more aware of potential health risks, eat more frequently products that are beneficial for health, including functional foods, and avoid unhealthy foods. However, this relationship is unclear because the associations with health risk perception are very complex. JONG *et al.* (2003) stated that health-conscious individuals were not only ones who consumed functional foods to compensate for an unhealthy lifestyle. The consumption of functional food by Dutch adults strongly depended on the type of products. The current study also supports these findings.

Women were the most likely consumers of vegetables and/or fruit (cluster I and IV). Numerous studies have found that women consume vegetables and/or fruit more frequently than men (SYGNOWSKA *et al.*, 2005; WĄDOŁOWSKA *et al.*, 2008). This may be because they are more interested in their health and have more pro-health dietary habits (DE JONG *et al.*, 2003; MOSCA *et al.*, 2006). ZANDSTRA *et al*. (2001) affirmed that among adults general health interest was associated with an increased consumption of vegetables and fruit and a lower fat intake. According to MOSCA *et al*. (2006), women are more interested in their health and that of their families.

One limitation in the current study was that the actual amount of functional food consumed was not assessed; this makes it difficult to differentiate between the occasional functional food consumers and those who consume larger amounts regularly. For example, this study cannot show whether consumers from cluster IV really ate more vegetables and/or fruit (100% of them declared consuming those products at least once a week) than consumers from cluster V (about 60% of them declared consuming vegetables and/or fruit at least once a week), because the data related to frequency of consumption could not be interpreted quantitatively. A qualitative analysis could provide more detailed information, but according to DE JONG *et al*. (2003) only a few adults (<10%) were daily functional food consumers. A qualitative analysis of functional food consumption would require a larger sample size. Furthermore, a simple tool was used to distinguish between the two large groups: the consumers and non-consumers of certain food items. However, it was not an objective of the study to quantify food intake.

There is a certain limitation to the study related to sample choice. Initially, the sample was collected by quota controlling. Next, to ensure that the sample was as nationally representative as possible, responses were weighted. Due to the procedure, the sample matched census statistics on the pre-defined characteristics, but, the sample was not representative of Poles aged 15 and above. Another limitation could have been refusals, but they were not recorded because it was a quota-controlled sample. A literature review by GROVES *et al*. (2002) indicated that those who refused are more likely to be among those who are least likely to have meaningful attitudes towards the topic of interest, knowledge of the topic, exposure to information on the topic and a perceived need to form an opinion on the topic. Thus, this study probably had an over-representation of healthier people, those who were more interested in their health and subjects who were more aware of their health problems. However, it is very difficult to confirm or contradict this presumption.

## CONCLUSIONS

Non-consumers of functional food accounted for 4% of the respondents. Generally, the consumption of functional foods depended on the type of product. Factors that differentiated the Poles included metabolic syndrome risk perception and frequency of functional food consumption, but there was also a weak relationship to self-reported health conditions. The occurrence of these characteristics depended on gender, age and education. There were no clear differences between consumers and less-likely consumers of functional foods in metabolic syndrome risk perception and demographic characteristics. Men were more likely to be consumers of cholesterol-lowering products while women were more likely to consume vegetables and/or fruit. The consumption of cholesterol-lowering products was related to low health risk perception as well as high risk perception. Generally, those who were less-likely to be consumers of functional foods were older subjects, especially men with a low level of education and a slightly high health risk perception. Further studies should be aimed at understanding how to improve the interest of health risks among middle-aged Poles, particularly men with a lower level of education.


## ACKNOWLEDGMENTS

This work was completed on behalf of the LIPGENE Consortium and funded under the EU 6th Framework Food Quality and Safety Programme, code FOOD-CT-2003-505944.


## REFERENCES


Armitage P., Berry G. and Matthews J.N.S. 2008. "Statistical Methods in Medical Research" 4th ed. Blackwell Science, Oxford.

Blakely T., Hales S. and Woodward A. 2004. Poverty: assessing the distribution of health risks by socioeconomic position at national and local levels. WHO Reports, Nutrition for health and development protection of the human environment. Environmental Burden of Disease Series, No. 10, WHO Reports, Geneva.

Buttriss J.L. 2006. Perspective hanging on the coat tails of the obesity epidemic: will metabolic syndrome be the next public health crisis and does nutrition have a role in its prevention? J. Sci. Food Agric. 86: 2285.

de Almeida M.D.V., Pinhao S., Steward-Knox B., Parr H.J. and Gibney M.J. 2006a. An overview of findings from a six-country European survey on consumer attitudes to the metabolic syndrome, genetics in nutrition, and potential agro-food technologies. Brit. Nutr. Found. Nutr. Bull. 31: 239.

de Almeida M.D.V., Pinhao S., Steward-Knox B., Parr H.J. and Gibney M.J. 2006b. A six-country European survey on consumer attitudes to the metabolic syndrome, genetics in nutrition and potential agro-food technologies: questionnaire design methodology. Brit. Nutr. Found. Nutr. Bull. 31:138.

de Jong N., Ocke M.C., Branderhorst H.A.C. and Friele R. 2003. Demographic and lifestyle characteristic of funkcional food consumers and dietary supplement users. Brit. J. Nutr. 89: 273.

Dixon L.B., Balder H.F., Virtanen M.J., Rashidkhani B., Mannisto S., Krogh V., van den Brandt P.A., Hartman A.M., Pietinen P., Tan F., Virtamo J., Wolk A. and Goldbohm R.A. 2004. Dietary patterns associated with colon and rectal cancer: results from the Dietary Patterns and Cancer (DIETSCAN) Project. Am. J. Clin. Nutr. 80: 1003.

Doyon M. and Labrecque J. 2008. Functional foods: a conceptual definition. Brit. J. Nutr. 110: 1133.

Ezzati M., Lopez A.D., Rodgers A. and Murray C.J.L. (Eds). 2004. "Comparative Quantification of Health Risks: Global and Regional Burden of Disease Attributable to Selected Major Risk Factors". WHO Reports, Geneva.

Groves R.M., Dollman D.A., Eltinge J.L. and Little R.J.A. 2002. "Survey Non Response". p. 37. John Wiley & Sons Inc. New York.

Hill T. and Lewicki, P. 2006. "Statistics: Methods and Applications. A Comprehensive Reference for Science, Industry, and Data Mining". Wyd. Statsoft, Tulsa.

Hoffmann K., Schulze M.B., Schienkiewitz A., Nothlings U. and Boeing H. 2004. Application of a new statistical method to derive dietary patterns in nutritional epidemiology. Am. J. Epidemiol. 159: 935.

http://www.poradnikhandlowca.com.pl/bezcms/archiwum/online05/.

Hu FB. 2002. Dietary pattern analysis: a new direction in nutritional epidemiology. Curr. Opin. Lipidol. 13: 3.

Idzik A. 2006. Zdrowie Polaków. Zdrowie Publiczne 116: 223.

Johansson L., Thelle D.S., Solvoll K., Bjorneboe G-E.A. and Drevon C. 1999. Healthy dietary habits in relation to social determinants and lifestyle factors. Brit. J. Nutr. 81: 211.

Kafatos A., Manios Y., Markatji I., Giachetti I., de Almeida M.D.V. and Engstrom M. 1999. Regional, demographic and national influences on attitudes and beliefs with regard to physical activity, body weight and health in a nationally representative sample in the European Union. Pub. Health Nutr. 2: 87.

Kirkland S.A., MacLean D.R., Langille D.B., Joffres M.R., MacPherson K.M. and Andreou P. 1999. Knowledge and awareness of risk factors for cardiovascular disease among Canadians 55 to 74 years of age: results from the Canadian Heart Health Surveys, 1986-1992. Canad. Med. Assoc. J. 161(8 Suppl.): S10.

Margetts B.M., Rogers E., Widhal K., Remaut de Winter A-M. and Zunft H-J.F. 1999. Relationship between attitudes to health, body weight and physical activity in a nationally representative sample in the European Union. Pub. Health Nutr. 2: 97.

Maynard M., Gunnell D., Ness A.R., Abraham L., Bates C.J. and Blane D. 2005. What influences diet in early old age? Prospective and cross-sectional analyses of the Boyd Orr cohort. Eur. J. Pub. Health 16: 315.

Mosca L., Mochari H., Christian A., Berra K., Taubert K., Mills T., Burdick K.A. and Simpson S.L. 2006. National study of women's awareness, preventive action, and barriers to cardiovascular health. Circulation 113: 525.

NCEP. 2001. National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adults Treatment Panel III). Third Report. Bethesda, MD. NCEP NHLBI NIH, USA.

Newby P.K., Muller D. and Tucker K.L. 2004. Associations of empirically derived eating patterns with plasma lipid biomarkers: a comparison of factor and cluster analysis methods. Am. J. Clin. Nutr. 80: 759.

Pampalon R., Duncan C., Subramanian S.V. and Jones K. 1999. Geographies of health in Quebec: a multilevel perspective. Social Sci. Med. 8: 1483.

Pomerleau J., Lock K. and McKee M. 2005. The burden of cardiovascular disease and cancer attributable to low fruit and vegetable intake in the European Union: differences between old and new Member States. Pub. Health Nutr. 9: 575.

Powles J.W., Zatoński W., Vander Hoorn S. and Ezzati M. 2005. The contribution of leading diseases and risk factors to excess losses of healthy life in eastern Europe: burden of disease study. BMC Pub. Health 5: 116.

Schryver T. and Smith C. 2006. Participants' willingness to consume soy foods for lowering cholesterol and receive counselling on cardiovascular disease by nutrition professionals. Pub. Health. Nutr. 9: 866.

Stan zdrowia ludności Polski w 2004 r. 2006. Wyd. GUS, Departament Statystyki Społecznej, Warszawa.

Sygnowska E., Waśkiewicz A., Głuszek J., Kwaśniewska M., Biela U., Kozakiewicz K., Zdrojewski T. and Rywik S. 2005. Spożycie produktów spożywczych przez dorosłą populację Polski. Wyniki programu WOBASZ. Kardiologia Polska 63(6, Suppl. 4): S1.

Szponar L., Sekula W., Rychlik E., Ołtarzewski M. and Figurska K. 2003. "Badania Indywidualnego Spożycia Żywności i Stanu Odżywienia w Gospodarstwach Domowych". Wyd. IŻŻ, Warszawa.

Urala N. and Lathteenmaki L. 2004. Attitudes behind consumers' willingness to use functional foods. Food Quality Pref. 15: 793.

Urala N. and Lathteenmaki L. 2007. Consumer's changing attitudes towards functional foods. Food Quality Pref. 18: 1.

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.

Waśkiewicz A. and Sygnowska E. 2006. Wpływ poziomu wykształcenia na zachowania zdrowotne i czynniki żywieniowe związane z powstawaniem otyłości - badanie Pol-MONICA bis Warszawa. Zdrowie Publiczne 116: 227.

World health statistics 2006. 2006. WHO Reports, Geneva.

Wysocki M.J. and Zejda J.E. 2007. Epidemiologia chorób niezakaźnych w Polsce w drugiej połowie dwudziestego wieku. Przegl. Epidemiol. 61: 615.

Zandstra E.H., de Graaf C. and van Staveren W.A. 2001. Influence of health and taste attitudes on consumption of low- and high-fat foods. Food Quality Pref. 12: 75.

Zdrojewski T., Wyrzykowski B., Wierucki L., Grodzicki T., Januszewicz A., Narkiewicz K., Zarzeczna-Baran M., Gluszek J., Opolski G., Zatonski W., Tykarski A. and Drygas W. 2006. Attempt to eliminate heath inequalities in Poland arising at the time of political and economic transformation: Polish 400 Cities Project. Eur. J. Card. Prev. Rehabil. 13: 832.

Revised paper received April 7, 2009 Accepted June 4, 2009

PAPER

# EFFECTS OF ENRICHMENT WITH POLYPHENOLIC EXTRACTS FROM SARDINIAN PLANTS ON PHYSICO-CHEMICAL, ANTIOXIDANT AND MICROBIOLOGICAL PROPERTIES OF YOGURT

EFFETTO DELL'AGGIUNTA DI ESTRATTI POLIFENOLICI DI PIANTE DELLA FLORA SARDA SULLE PROPRIETÀ FISICO-CHIMICHE, ANTIOSSIDANTI E MICROBIOLOGICHE DEGLI YOGURT

**M. COSSU*, C. JULIANO, R. PISU, and M.C. ALAMANNI**
Dipartimento di Scienze del Farmaco, Via Muroni 23/a, Università di Sassari, 07100 Sassari, Italy
*Corresponding author: Tel. +39 079 228732, Fax +39 079 228733, e-mail: m.cossu@uniss.it

ABSTRACT

The effect of adding artichoke (*Cynara scolymus* L.), strawberry-tree fruit (*Arbutus unedo* L.) and cherry (*Prunus avium* L.) extracts was evaluated on the physico-chemical, antioxidant and microbiological properties of home-made yogurts. Yogurts, enriched with artichoke or red ripe strawberry-tree fruit extract, demonstrated high *in vitro*

RIASSUNTO

In questo lavoro è stato valutato l'effetto dell'aggiunta di estratti di carciofo (*Cynara scolymus* L.), frutti di corbezzolo (*Arbutus unedo* L.) e ciliegia (*Prunus avium* L.) sulle proprietà fisico-chimiche, antiossidanti e microbiologiche di yogurt preparati in laboratorio. Gli yogurt con estratti di parte edibile di carciofo o con frutti maturi di corbezzo-

- Key words: antioxidant properties, microbiological characteristics, vegetable extracts, yogurt -

antioxidant activity. Ten days after their preparation, no variations in the antioxidant components and antioxidant activity of the yogurts were observed. The enriched yogurts showed an increase in lactic acid and acetaldehyde. As regards microbial characteristics, there was a notable increase in the microbial count of Lactobacilli in yogurts with edible artichoke parts ($4.5x10^9$ cfu/g) and with strawberry-tree fruit extracts ($1.8x10^9$ cfu/g) during the three-week experimental period. During the same period persistence of Streptococci in yogurt with artichoke edible parts was observed until the end of the first week ($5.2x10^7$ cfu/g). Our results indicate that the enrichment of yogurts with Sardinian plant extracts can significantly improve the antioxidant activity of yogurt and in some cases the survival of its microbial flora.

lo dimostravano un'elevata attività antiossidante *in vitro*. Dieci giorni dopo la loro preparazione, gli yogurt mantenevano inalterato il contenuto in polifenoli e l'attività antiossidante e mostravano un significativo aumento nel contenuto di acido lattico e acetaldeide. Dopo sette giorni è stato osservato un incremento di Lactobacilli negli yogurt contenenti estratto di parte edibile di carciofo ($4,5x10^9$ cfu/g) e estratti di frutti di corbezzolo ($1,8x10^9$ cfu/g) ed una persistenza di Streptococchi negli yogurt contenenti estratto di parte edibile di carciofo ($5,2x10^7$ cfu/g). I risultati indicano che la preparazione di yogurt contenenti estratti di carciofo può migliorare significativamente la sopravvivenza della flora batterica solo per i primi 7 giorni e può potenziarne l'attività antiossidante.

## INTRODUCTION

Yogurt is a well-known fermented dairy product obtained by the lactic acid fermentation of milk by the action of yogurt starter bacteria, *Streptococcus salivarius* subsp. *thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*. The role of these bacterial strains in yogurt production is primarily milk acidification by D- and L-lactic acid and synthesis of flavour compounds that contribute to the final aroma of the yogurt, such as volatile and non-volatile acids, carbonyl compounds (acetaldehyde or diacetyl) and miscellaneous products (SERRA *et al.*, 2009). Yogurt has long been recognised as a healthy food since the associated bacterial metabolism is responsible for the development in a yogurt of compounds that make it beneficial for human health. For example, 3-hydroxy-3-methylglutaric acid is known to be an antihypercholesterolemic product (HEPNER *et al.*, 1979) and β-galactosidase is indispensable to lactose digestibility, and antihypertensive peptides inhibiting angiotensin-I-converting enzyme (ACE) derived from casein hydrolysis (TAKANO, 2002; ASHAR and CHAND, 2003). Moreover, the contribution of yogurt bacteria to the improvement of intestinal microflora is widely recognised and yogurt consumption is associated with health benefits: for lactose intolerance, diarrhoeal diseases, inflammatory bowel diseases, *Helicobacter pylori* infection and allergies (ADOLFSSON *et al.*, 2004).

To meet consumer demands, yogurt is also produced in flavoured, supplemented and enriched forms. Flavouring can be carried out by addition of natural ingredients i.e. "berry" fruits such as strawberry, raspberry, bilberries, black-

berry, blackcurrant and cherry, well known sources of polyphenolic antioxidants (COÏSSON *et al.*, 2005). Supplementation with functional components (APOSTOLIDIS *et al.*, 2006) is carried out for various reasons: probiotic strains, such us *Lactobacillus* and *Bifidobacterium*, are added for their therapeutic properties in gastrointestinal disorders and their ability to improve immune functions (ISOLAURI, 2001; SAAVEDRA, 2001). Prebiotic oligosaccharides enhance the growth of probiotics in the yogurt environment; ω-3 long-chain fatty acids are precursors of important anti-thrombotic and anti-inflammatory molecules (MARCHIOLI *et al.*, 1999; ALBERT *et al.*, 2002); phytosterols and phytostanols help lower the absorption of cholesterol (RAMJIGANESH *et al.*, 2000); and, finally, plant polyphenols are added because of their well-known antioxidant activity (FUKUMOTO and MAZZA, 2000; SZETO *et al.*, 2002).

In terms of natural polyphenolics from dietary plants, there is an increasing evidence that a diet with an increased intake of these compounds is associated with reduced risk of cardiovascular diseases (YOCHUM *et al.*, 1999) and certain types of cancer (YANG *et al.*, 2001). The characterization and quantification of phenolic compounds present in typical Sardinian plants such as artichoke (*Cynara scolymus* L.) Spinoso Sardo variety, strawberry-tree (*Arbutus unedo* L.) fruits and cherries (*Prunus avium* L.) Bonnannaro variety and the *in vitro* evaluation of their benefits have been the focus of recent research (ALAMANNI and COSSU, 2003; ALAMANNI *et al.*, 2006). The results showed a high content of antioxidant polyphenolics whose activity was comparable, and in some cases higher, than well-known antioxidants such as beta-carotene, ascorbic acid and catechins.

The effect of adding strawberry-tree fruit and artichoke extracts on the viability of fermenting bacterial strains and a probiotic strain was evaluated in a previous investigation (PISU *et al.*, 2006); the results showed that the extracts had a positive action on the development of bacterial flora.

The aim of this research was to investigate the effect of enrichment with artichoke, strawberry-tree fruit and cherry extracts from Sardinian plants on the physico-chemical, antioxidant and microbiological characteristics of homemade yogurt, as compared to commercially produced yogurts.

## MATERIALS AND METHODS

### Extract preparation and determination of polyphenolic content

Fresh artichokes (*Cynara scolymus* L.), Spinoso Sardo variety, purchased from the local market with stalk and leaves, were subdivided into the edible part (EP) (consisting of internal bracts, cup, and the edible part of the stem) and the non-edible part (NEP) (consisting of the fibrous portions rich in chlorophyll, i.e. outer bracts, stem bark and leaves). Strawberry-tree (*Arbutus unedo* L.) fruits at two different stages of ripening, ripe (red fruit) and unripe (yellow fruit), were collected in the Sardinian mountains (Tempio Pausania, Sassari, Italy). Cherries (*Prunus avium* L.), Bonnannaro variety, were harvested in the Bonnannaro area (Sassari, Italy). Plants and fruits were stored at -20°C. The extracts were prepared by suspending 300 g of each plant, finely homogenized in a blender, in 300 mL of Milli-Q water and boiling them for 30 min. The suspensions were filtered and the filtrate was concentrated up to ~30 mL at 70°C and at reduced pressure. The residue obtained was dissolved in water to a final volume of 50 mL. All plant extracts were stored at 5°C until use.

The amount of total phenolics in the extracts was determined according to the Folin-Ciocalteu spectrophotometric

method (SINGLETON and ROSSI, 1965). The results are expressed as mg/mL of gallic acid equivalent (GAE) (Acros, Milan, Italy). The calibration curve was prepared using concentrations of gallic acid ranging from 5 to 50 μg/mL.

Preparation of yogurts containing polyphenolic extracts

Commercial whole fresh pasteurised bovine milk was mixed with a commercial natural bovine yogurt as a starter culture at 5, 10 and 20% (v/v) in order to determine the best conditions required to obtain a "home-made" yogurt. The mixtures were incubated at 45°C and the yogurt was taken out of the incubator when the pH dropped to approximately 4.5 (~6-7 hours). The yogurt was cooled to room temperature and stored at 4°C. Samples were collected after 1 day at 4°C and analysed for the viable counts of both *L. bulgaricus* and *S. thermophilus*.

To prepare yogurts enriched with vegetable and fruit extracts, aliquots of 45 mL of whole milk inoculated with natural yogurt as a starter (10% v/v) (percentage chosen on the basis of microbiological results) were transferred to sterile plastic containers. Appropriate amounts of each extracts were added to obtain the following polyphenolic concentrations: 0.05% (w/w) for artichoke yogurt (edible and non-edible parts), 0.05% (w/w) for strawberry-tree fruit yogurt (ripe and unripe) and 0.02% (w/w) for cherry yogurt (Table 1). Controls consist of unsupplemented yogurt samples.

Table 1 - Commercial and home-made yogurts assayed.

| Home-made yogurt samples | Characteristics | Polyphenolic content % (w/w) |
|---|---|---|
| LW | unsupplemented yogurt (ctr) | |
| LE1 | with edible artichoke part (EP) extract | 0.05 |
| LE2 | with non edible artichoke part (NEP) extract | 0.05 |
| LE3 | with ripe strawberry-tree fruit (red) extract | 0.05 |
| LE4 | with unripe strawberry-tree fruits (yellow) extract | 0.05 |
| LE5 | with cherry extract 0.02 | |
| **Commercial yogurt samples** | | |
| CW1 | natural yogurt | |
| CW2 | natural yogurt | |
| CW3 | natural yogurt | |
| CW4 | natural yogurt | |
| CW5 | natural yogurt | |
| CW6 | natural yogurt | |
| CW7 | natural yogurt | |
| CW8 | natural yogurt | |
| CE1 | with Vit. C, $D_3$, E | |
| CE2 | with Q10 factor and Vit. A, $D_3$, E | |
| CE3 | with blueberry/currant extract | 0.07* |
| CE4 | with papaya/guava extract | 0.07* |

CW: Commercial natural; CE: Commercial enriched; LW: home-made natural; LE: home-made enriched; * reported on labels.

Commercial yogurt samples

Eight samples of natural bovine yogurt, and four samples of yogurt enriched with different antioxidant compounds and polyphenolic extracts, produced from whole bovine milk in different factories, were purchased one month before the expiry date at a local supermarket and stored at 4°C until used (Table 1).

Physico-chemical properties of yogurts

The pH of different yogurt samples was measured at 20°C by using a pH meter equipped with a specific electrode for semi-solid substances (Hanna FC200, Hanna Instruments, Milan, Italy).

The acidity of the yogurt was measured suspending 10 g of yogurt in 100 mL of water and titrating with a solution of 0.25 N NaOH, with phenolphthalein solution as indicator. Yogurt acidity is expressed in Soxhlet-Henkel degrees (SH°) and is reported for 100 g of yogurt according to the equation:

SH° = milliliters NaOH consumed/yogurt weight;

D- and L-lactic acid and acetaldehyde were dosed in yogurts using rapid UV-Kit enzymatic conventional methods (Megazyme International Ireland Limited, Bray, Co. Wicklow, Ireland) based on spectrophotometric detection of specific compounds.

Total polyphenolic content and antioxidant activity of the yogurt samples

One gram of yogurt was placed in a volumetric flask and diluted in a methanol/water (1:1 ratio) solution to 10 mL, it was then transferred to a plastic tube (15 mL) with a screw cap, shaken by vortex for 15 s and centrifuged at 4,000 rpm for 10 min. The total polyphenolic content was determined by the Folin-Ciocalteu method previously described and the results are expressed as micrograms of gallic acid equivalent (GAE) per gram of yogurt.

The antioxidant activity of the different yogurt samples was evaluated in terms of "free-radical-linked scavenging ability" by measuring the decoloration of a methanolic solution of the stable radical 1,1-diphenyl-2-picryl-hydrazyl (DPPH•) (Sigma-Aldrich, Milan, Italy) with a spectrophotometric test (VON GADOW *et al.*, 1997). The percentage of scavenging of the radical in the presence of the sample was calculated according to the following formula:

$$\text{Inhibition \%} = [(At_0 - At_{60})/At_0]x100$$

where $At_0$ is the absorbance of the control at 517 nm at time = 0 min and $At_{60}$ is the absorbance of the DPPH-sample solution at the same wavelength after 60 min. The percentage inhibition data are expressed as micrograms of Trolox equivalents (TE) per gram of yogurt. The calibration curve had been prepared using concentrations of Trolox (Acros, Milan, Italy) between 1.0 and 10.0 μg/mL ($r^2 = 0.999$, $y = 2.351x + 0.443$).

The determination of the reducing ability of yogurt was carried out by using a method based on the ferric reducing ability power (FRAP) (BENZIE and STRAIN, 1996). At low pH, when a ferric-tripyridyltriazine ($Fe^{3+}$-TPTZ) (Acros, Milan, Italy) complex is reduced to the ferrous ($Fe^{2+}$) form, an intense blue colour develops with an absorption maximum at 593 nm. The data of absorbance obtained are expressed as micrograms of Trolox equivalents (TE) per gram of yogurt by calibration curve in a range from 1.0 to 12.0 μg/mL ($r^2 = 0.999$, $2.337x + 0.0098$).

Microbiological analysis

Aliquots of 1.0 g of each yogurt sample were weighed in sterile tubes and mixed with 100 mL of sterile phosphate saline buffer (pH 7.4; PBS) (Oxoid, Milan, Italy); serial tenfold dilutions were prepared using the same buffer. The selective enumeration of yogurt microorganisms was

performed by plating the dilutions of yogurt samples onto appropriate media. Viable counts of *Streptococcus thermophilus* were carried out on M17 agar (final pH 6.9±0.2) (Oxoid Milan, Italy) plates incubated aerobically for 48 h (TERZAGHI and SANDINE, 1975), while *Lactobacillus delbrueckii* subsp. *bulgaricus* was enumerated on MRS agar, final pH 6.2±0.2 (Oxoid, Milan, Italy) plates, incubated anaerobically for 48 h in the Gas Generating Kit Anaerobic System (Oxoid, Milan, Italy) (DAVE and SHAH, 1996). Bacterial counts of yogurt samples and controls were carried out at $T_0$ (24 h after yogurt manufacturing) and after 1, 2 and 3 weeks of refrigerated storage (4°C).

The percentile survival rate was calculated for each time with the following formula:

$$\% = (\text{bacterial number at T/bacterial number at } T_0) \times 100$$

Statistical analysis

Data reported in the tables and figures are the means of experiments repeated three times. Statistical analyses were conducted by GraphPad Prism 4 software (GraphPad Software Inc., San Diego, CA, USA). Correlations were obtained by Pearson correlation coefficient in bivariate correlations. Differences between means at the 5% ($p<0.05$) level were considered significant.

## RESULTS

Total polyphenolic content of plant extracts

The total polyphenolic contents of the different fruits and plant extracts, expressed as mg/mL of gallic acid equivalents are reported in Table 2. As expected on the basis of the available literature (ALAMANNI and COSSU, 2003; ALARCAO-E-SILVA *et al.*, 2001; USENIK *et al.*, 2008), the polyphenolic content of the assayed extracts are quite different, ranging from 4.0 mg/mL (cherry extract) to 15.3 mg/mL (yellow strawberry-tree fruit extract).

Physico-chemical properties and antioxidant activity of natural yogurts

The baseline values of some chemical characteristics of commercial and homemade natural yogurts are reported in Table 3. L-lactic acid concentration and pH were comparable between the two types of yogurt; the L-lactic acid concentration was 3-5 times higher than D-lactic acid. Acetaldehyde levels were higher in commercial yogurts even though the high standard deviation (data not shown) demonstrates a great variability of this parameter (0.05 to 1.73 mg/g), probably due to the different geographical origins of the milk and/or the production process.

Good baseline correlations (r) were obtained between the polyphenolic content and the antioxidant activity of all the examined yogurts without any plant-based supplementation ($r = 0.771$ for DPPH test and $r = 0.827$ for FRAP test; $p<0.05$).

Physico-chemical properties and antioxidant activity of enriched yogurts

Enriched yogurts were prepared in the laboratory and analyzed 24 h after preparation, as previously described in

Table 2 - Total polyphenolic contents of artichoke, strawberry tree and cherry extracts, expressed as gallic acid equivalents (mg/mL).

| Extract | Polyphenolic content (mg/mL±SD) |
|---|---|
| Artichoke (EP) | 8.0±0.01 |
| Artichoke (NEP) | 6.7±0.04 |
| Red strawberry-tree fruits | 11.3±0.06 |
| Yellow strawberry-tree fruits | 15.3±0.03 |
| Cherry | 4.0±0.01 |

Mean±SD (standard deviation) of 3 determinations for each sample.

Table 3 - General features of commercial and home-made natural yogurts.

| | | | Commercial* mean±SD | Home-made* |
|---|---|---|---|---|
| pH | | | 4.3±0.26 | 4.4±0.11 |
| Acidity | | SH° | 52.8±8.1 | 40.5±3.5 |
| Lactic acid | D | mg/g | 3.29±3.3 | 1.3±0.1 |
| | L | | 9.47±2.2 | 9.8±0.14 |
| Acetaldehyde | | mg/g | 0.54±0.53 | 0.34±0.04 |
| Polyphenolic content | | GAE (µg/g) | 73.2±17.6 | 101.4±12.6 |
| Antioxidant activity | DPPH test | TE (µg/g) | 39.6±11.1 | 35.7±5.8 |
| | FRAP test | | 31.1±21.9 | 50.6±6.7 |

*Mean±SD of 8 samples.

the experimental section. In Table 4 the results of home-made enriched yogurts (LE 1-5) are compared with four selected commercial enriched yogurts (CE 1-4).

Compared to the unsupplemented yogurt (LW), all of the home-made enriched yogurts had a lower pH and consequently a higher SH° acidity value, no variation in D- and L-lactic acid contents and a lower concentration of acetaldehyde.

pH and acidity values of enriched commercial yogurt were comparable to those of the unsupplemented yogurt, while in the CE2 sample (yogurt with Q10 factor and vitamin A, D, E) the L-lactic acid and acetaldehyde contents were higher.

The polyphenolic content and the antioxidant activities of enriched yogurt samples, commercial and home-made yogurts are compared in Fig. 1. In general, the antioxidant activities were quite similar for all of the yogurts examined with the exception of the LE3 and LE4 samples (enriched with red strawberry-tree fruit and yellow fruit extract, respectively) that showed the highest activity in the DPPH radical scavenging assay. Moreover, yogurt enriched with edible parts of artichoke (LE1) contained

Table 4 - Physico-chemical characteristics of commercial and home-made yogurts enriched with polyphenolic substances (mean±SD of 3 determinations for each sample).

| Samples | pH | Acidity (SH°) | Lactic acid (mg/g) | | Acetaldehyde (µg/g) |
|---|---|---|---|---|---|
| | | | D | L | |
| CE1 | 4.45±0.08 | 46.20±1.3 | 0.0±0.00 | 9.3±0.34 | 88.1±3.4 |
| CE2 | 4.17±0.06 | 55.40±1.8 | 1.4±0.06 | 30.7±2.9 | 387.6±20.3 |
| CE3 | 4.23±0.07 | 40.20±1.1 | 0.2±0.01 | 5.7±0.19 | 141.0±7.7 |
| CE4 | 4.36±0.02 | 33.50±0.8 | 0.3±0.01 | 5.3±0.24 | 176.2±8.4 |
| LW | 4.4±0.03 | 40.5±1.5 | 1.3±0.04 | 9.1±0.39 | 343.9±18.6 |
| LE1 | 3.8±0.04 | 76.6±3.7 | 1.4±0.05 | 9.2±0.41 | 121.8±6.8 |
| LE2 | 3.8±0.07 | 69.9±2.4 | 1.7±0.08 | 9.6±0.65 | 103.6±5.5 |
| LE3 | 3.8±0.08 | 70.0±3.1 | 1.4±0.10 | 9.4±0.55 | 172.6±7.0 |
| LE4 | 3.7±0.05 | 63.7±2.1 | 1.1±0.09 | 9.6±0.47 | 246.7±13.8 |
| LE5 | 3.8±0.06 | 63.9±2.9 | 1.9±0.11 | 8.9±0.38 | 105.7±7.1 |

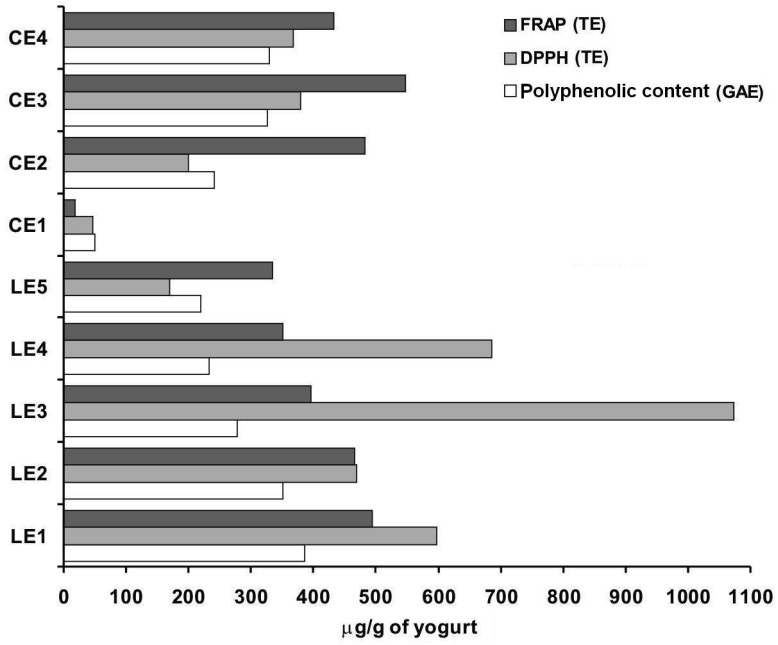



Fig. 1 - Antioxidant activities (expressed as Trolox equivalents, TE) and total polyphenolic content of commercial enriched yogurts and home-made enriched yogurts at $T_0$.

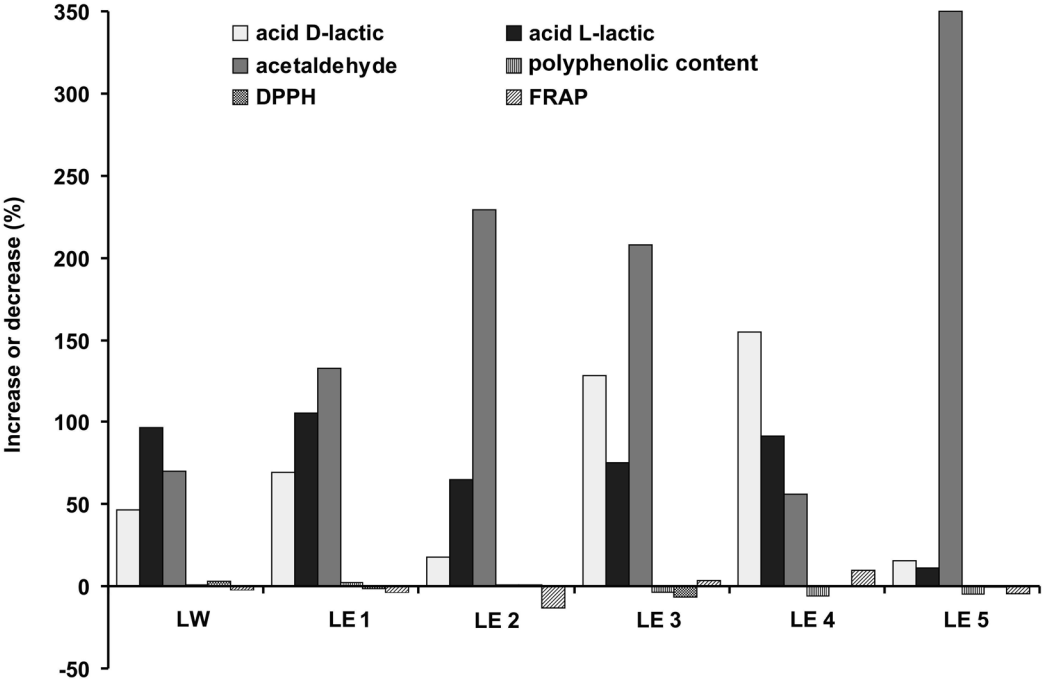



Fig. 2 - Characteristics of home-made yogurts: per cent increase or decrease of values after ten days.

a greater quantity of polyphenols and showed a higher antioxidant activity than commercial yogurts without any plant extract enrichment.

The variations in the physico-chemical characteristics, polyphenolic content and antioxidant activities of all of the home-made yogurts (both unenriched and enriched) were observed over a period of ten days. Results of these observations are reported in Fig. 2, where the increases or decreases (minus sign) in the values with respect to $t_0$ are reported as percentages. Notable increases were observed particularly in lactic acid (D and L) and acetaldehyde production; in particular there were marked increases in the D-lactic acid and L-lactic acid in yogurt containing strawberry-tree fruit extracts (LE4) and in yogurt enriched with edible artichoke parts (LE1). Acetaldehydes increased remarkably in several yogurts and especially in the sample enriched with cherry extract (LE4). After ten days, the antioxidant component levels were almost unchanged, and the antioxidant activity showed negligible variations.

Microbiological analysis

Bacteria were counted periodically in both the unsupplemented commercial and home-made yogurts. All the experiments were performed in triplicate and

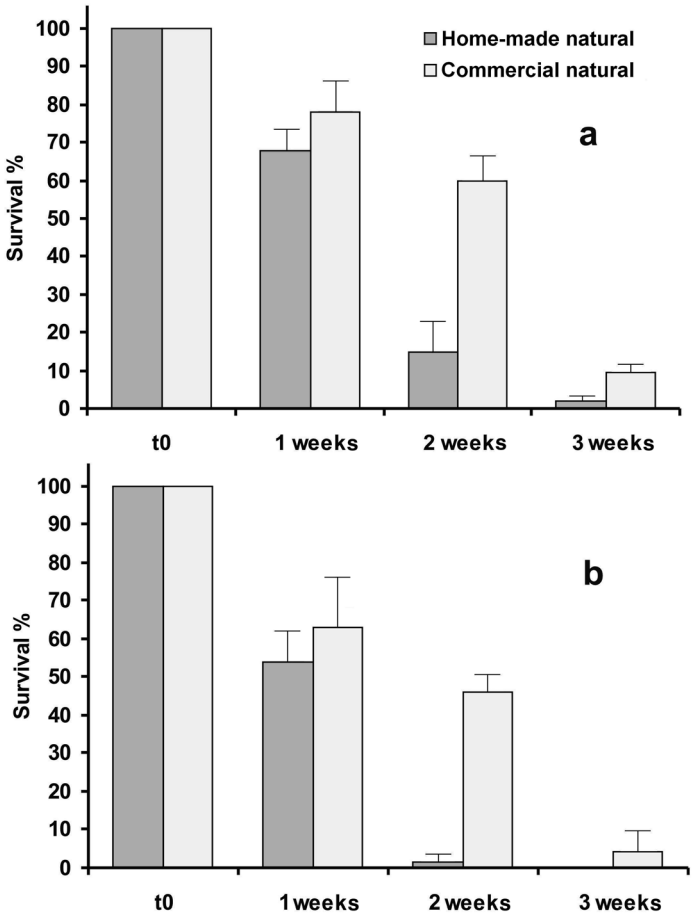



Fig. 3 - Viability of *L. bulgaricus* (a) and *S. thermophilus* (b) in commercial and home-made natural yogurt.

bacteria were also counted in triplicate for each sample. The results, expressed as % survival, are reported in Fig. 3.

In commercial natural yogurts, the initial cell counts were in the range of $2.6x10^9$ and $5.0x10^9$ cfu/g for *L. bulgaricus* and *S. thermophilus* respectively, and these numbers decreased steadily over the three-week experimental period.

Home-made yogurt samples were characterised by lower initial counts of both *L. bulgaricus* and *S. thermophilus* (ranges $3.5x10^8$ and $1.1x10^8$ cfu/g, respectively) and by a more marked decrease after the second week of storage. This might be due to the quality of milk used, to the yogurt used as an inoculum to produce home-made yogurt, which most likely was quite different if compared to the industrial starter, and also to the equipment and methodology of preparation.

The enriched yogurts showed an increasing trend in microbial counts when enriched with the edible artichoke extract (LE1) (Fig. 4): at time 0 both *L. bulgaricus* and *S. thermophilus* showed significantly higher numbers compared to

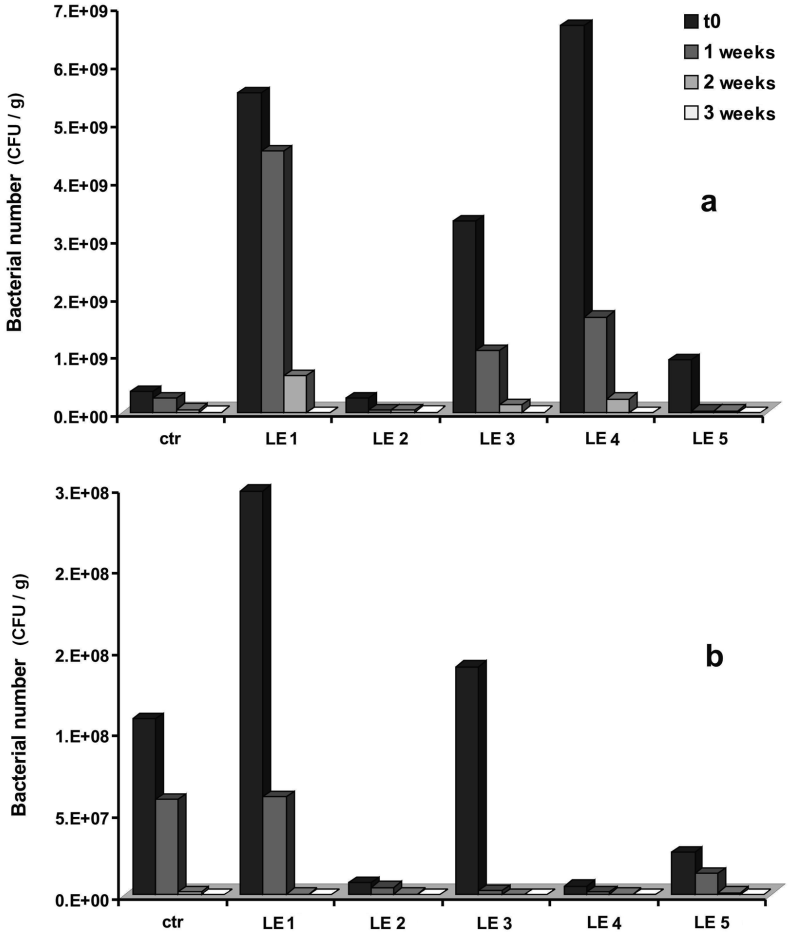



Fig. 4 - Survival of *L. bulgaricus* (a) and *S. thermophilus* (b) in home-made yogurt enriched with plant extracts over 3 weeks.

the controls. It is also noteworthy that this effect was not observed after the addition of non-edible artichoke parts (LE2). This result may be partly attributed to the presence of inulin, a well-known prebiotic polysaccharide (KRUSE *et al.*, 2005) in artichoke ED extracts. Moreover, at time zero a notable improvement of viable counts of lactobacilli was observed in yogurt enriched with unripe strawberry-tree extract (LE4) and an enhanced effect was observed after the addition of ripe strawberry-tree extract (LE3).

Fig. 5 shows the bacterial survival (expressed as percentage) in yogurts enriched with polyphenolic extracts in comparison with the control (LW) over the established period of time. As far as lactobacilli are concerned, the values for the survival after the first week were higher than the controls only in the presence of LE1 extract (85% and 70%, respectively), while the other extracts did not improve their viability. On the other hand, streptococci survival was positively affected by the presence of LE2 extract (65% viability vs 55%) after one week. The viabili-

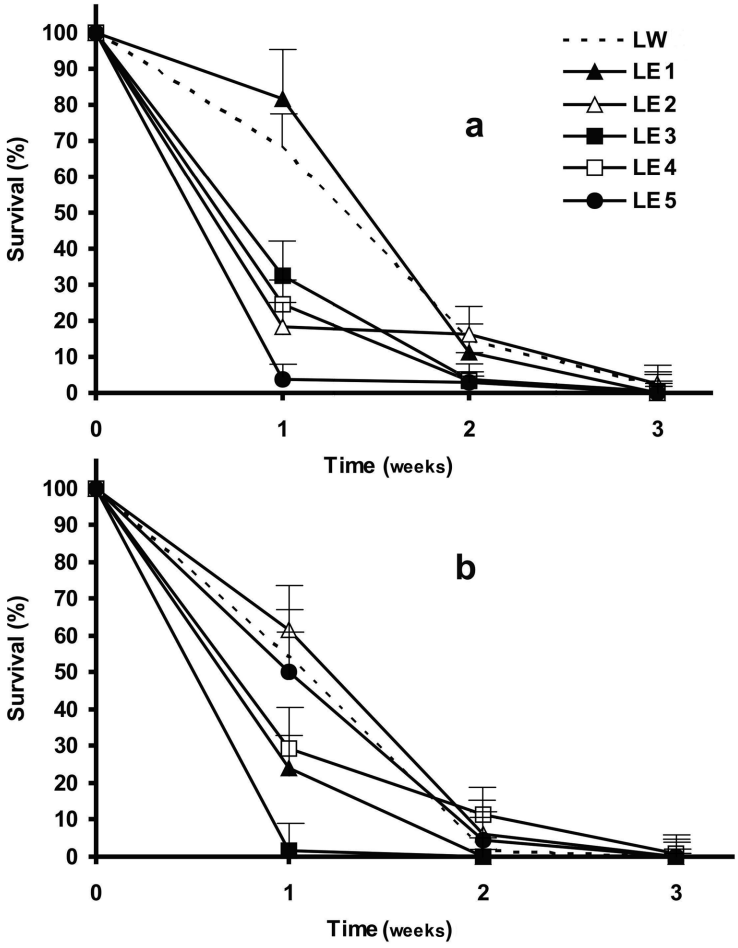



Fig. 5 - Evolution of *L. bulgaricus* (a) and *S. thermophilus* (b) in yogurts in the presence of polyphenolic extracts.

ty was not improved due to the extract at later times. At the second week, survival of the lactobacilli and streptococci was always less than 20%.

## CONCLUSIONS

Commercial dairy yogurts and home-made dairy yogurts were quite comparable in terms of chemical-physical characteristics. Moreover, these properties were not significantly modified by supplementation with different edible plant extracts just before fermentation, except for a slight decrease of pH and increase of acidity in all the samples.

Enriched home-made yogurts showed antioxidant activities quantitatively comparable to those of commercial enriched yogurts; the yogurt with red strawberry-tree fruit extract was the most active in the DPPH assay.

After ten days, an increment in the lactic acid (D and L) and acetaldehyde production was observed in some samples of home-made yogurt, while variations in the antioxidant components were not observed and antioxidant activities were almost unchanged.

In terms of microbial activity over the experimental period of three weeks, a high microbial count was observed at time 0 after fermentation with lactobacilli and streptococci in yogurts enriched with strawberry-tree extract and with edible artichoke extract. Under these storage conditions these counts did not change significantly in yogurt enriched with edible artichoke parts for the first week of the experiment.

On the basis of these results the incorporation of selected edible polyphenolic extracts did not affect the chemical and microbiological characteristics of yogurts. Further considering that a regular intake of these dietary polyphenolic compounds is regarded as beneficial for human health, it can be concluded that the yogurts produced as in this study could be of potential interest for the design of probiotic yogurts with additional bioactive metabolites from edible sources and therefore more effective functional foods.

## REFERENCES


Adolfsson O., Meydani S.N. and Russell R.M. 2004. Yogurt and gut function. Am. J. Clin. Nutr. 80: 245.

Alamanni M.C. and Cossu M. 2003. Antioxidant activity of the extracts of the edible part of artichoke (*Cynara scolymus* L.) var. Spinoso sardo. Ital. J. Food Sci. 2: 187.

Alamanni M.C., Cossu M. and Coloru G.C. 2006. Piccoli frutti colorati della flora selvatica sarda: composizione polifenolica e attività antiossidante. Riv. Sci. Alim. 1/2: 41.

Alarcao-E-Silva M.L.C.M.M., Leitao A.E.B., Azinheira H.G. and Leitao M.C.A. 2001. The arbutus berry: studies on its color and chemical characteristics at two mature stages. J. Food Comp. Anal. 14: 27.

Albert C.M., Campos H., Stampfer M.J., Ridker P.M., Manson J.E. and Willet W.C. 2002. Blood levels of long-chain ω-3 fatty acids and the risk of sudden death. N. Engl. J. Med. 346: 1113.

Apostolidis E., Kwon Y.I. and Shetty K. 2006. Potential of select yogurts for diabetes and hypertension management. J. Food Biochem. 30: 699.

Ashar M.N. and Chand R. 2003. ACE-Inhibitory activity of lactic acid bacteria in fermented milk. Milchwissenschaft 58: 59.

Benzie Iris 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.

Coïsson J.D., Travaglia F., Piana G., Capasso M. and Arlorio M. 2005. *Euterpe oleracea* juice as a functional pigment for yogurt. Food Res. Int. 38: 893.

Dave R.I. and Shah N.P. 1996. Evaluation of media for selective enumeration of *Streptococcus thermophilus*, *Lactobacillus delbrueckii* ssp. *bulgaricus*, *Lactobacillus acidophilus*, and Bifidobacteria. J. Dairy Sci. 79: 1529.

Fukumoto L.R. and Mazza G. 2000. Assessing antioxidant and prooxidant activities of phenolic compounds. J. Agric. Food Chem. 48: 3597.

Hepner G., Fried R., St. Jeor S., Fusetti L. and Morin R. 1979. Hypocholesterolemic effects of yoghurt and milk. Am. J. Clin. Nutr. 32: 19.

Isolauri E. 2001. Probiotics in human disease. Am. J. Nutr. 73: 1142.

Kruse H.P., Kleessen B. and Blaut M. 1999. Effects of inulin on faecal bifidobacteria in human subjects. Br. J. Nutr. 82: 375.

Marchioli R., Barzi F., Bomba E., Chieffo D., Di Gregorio D. and Mascio R. 1999. Dietary supplementation with ω-3 polyunsaturated fatty acids and vitamin E after miocardial infarction: results of (Gruppo italiano per lo studio delle soppravivenze nell'infarto), Gissi-Prevenzione. Lancet 354: 447.

Pisu R., Juliano C., Alamanni M.C., Coloru G.C. and Cossu M. 2006. Incorporation of different natural polyphenol extracts into a yogurt and their effect on viability of a selected probiotic strain. In Proceedings Book of the Congress Sardiniachem 2006: 109. Cagliari (Italy), June 5th 2006.

Ramjiganesh T., Roy S., Nicolosi R.J., Young T.L., Mc Intyre J.C. and Fernandez M.L. 2000. Corn husk oil lowers plasma LDL cholesterol concentrations by decreasing cholesterol absorption and altering hepatic cholesterol metabolism in guinea pigs. J. Nutr. Biochem. 11: 358.

Saavedra J.M. 2001. Clinical applications of probiotic agents. Am. J. Nutr. 73: 1147.

Serra M., Trujillo A.J., Guamis B. and Ferragut V. 2009. Flavour profiles and survival of starter cultures of yogurt produced from high-pressure homogenized milk. Int. Dairy J. 19: 100.

Singleton V.L. and Rossi J. 1965. Colorimetry of total phenolic with phosphomolybdic-phosphotungstic acid agent. Am. J. Enol. Vit. 16: 144.

Szeto Y.T., Tomlinson B. and Benzie I.F.F. 2002. Total antioxidant and ascorbic acid content of fresh fruits and vegetables: implications for dietary planning and food preservation. Br. J. Nutr. 87: 55.

Takano T. 2002. Anti-hypertensive activity of fermented dairy products containing biogenic peptides. Antonie Van Leeuvenhoeck 82: 333.

Terzaghi B.E. and Sandine W.E. 1975. Improved medium for lactic streptococci and their bacteriophages. Appl. Microbiol. 29: 807.

Usenik V., Fabcic J. and Stampar F. 2008. Sugars, organic acids, phenolic composition and antioxidant activity of sweet cherry (*Prunus avium* L.). Food Chem. 107: 185.

Von Gadow A., Joubert E. and Hansmann C.F. 1997. Comparison of the antioxydant activity of aspalathin with that of other plant phenols of Rooibos Tea (*Aspalatus linearis*), of α-tocoferolo, BTH and BHA. J. Agric. Food Chem. 45: 632.

Yang C.S., Landau J.M., Huang M. and Newmark H.L. 2001. Inhibition of carcinogenesis by dietary polyphenolic compounds. Annu. Rev. Nutr. 21: 381.

Yochum L., Kushi L.H., Meyer K. and Folsom A.R. 1999. Dietary flavonoid intake and risk of cardiovascular disease in postmenopausal women. Am. J. Epidemiol. 149: 943.

Revised paper received April 8, 2009 Accepted July 14, 2009

PAPER

# CAROTENOID CONTENT DURING TOMATO (*SOLANUM LYCOPERSICUM* L.) FRUIT RIPENING IN TRADITIONAL AND HIGH-PIGMENT CULTIVARS

VARIAZIONI NEL CONTENUTO DI CAROTENOIDI DURANTE LA MATURAZIONE DI BACCHE DI POMODORO (*SOLANUM LYCOPERSICUM* L.) DI CULTIVAR TRADIZIONALI E HIGH-PIGMENT

**M.S. LENUCCI, A. CACCIOPPOLA, M. DURANTE, L. SERRONE, M. DE CAROLI, G. PIRO, and G. DALESSANDRO***
Dipartimento di Scienze e Tecnologie Biologiche ed Ambientali (DiSTeBA), Università del Salento, Via Prov.le Lecce-Monteroni, 73100 Lecce, Italy
*Corresponding author: Tel. +39 0832 298611, Fax +39 0832 298858, e-mail: giuseppe.dalessandro@unisalento.it

ABSTRACT

The amounts of lycopene, β-carotene and lutein were monitored in tomato fruit of traditional and high-pigment cultivars (cvs) collected at the green, green-orange, orange-red and red stages of ripening. All data are expressed as mg/kg fresh weight (f.w.) of tomato fruit homogenates.

Each homogenate was centrifuged at 27,000 g and the pellets obtained, constituted of tomato cells, were lyophilised and ground to obtain a variety of homo-

RIASSUNTO

Il contenuto in licopene, β-carotene e luteina è stato monitorato in bacche di pomodoro di cultivar (cv) tradizionali e high-pigment raccolte a diversi stadi di maturazione (verde, verde-arancio, arancio-rosso e rosso). Tutti i dati sono espressi come mg/kg di peso fresco (f.w.) di bacche omogenate. Ciascun omogenato è stato centrifugato a 27.000; i pellet così ottenuti, costituiti da cellule parenchimatiche di pomodoro, sono stati liofilizzati e macinati per

- Key words: β-carotene, freeze-dried matter, lutein, lycopene, ripening, *Solanum lycopersicum* L., tomato -

geneous powders. The content of carotenoids in the powders, prepared at the different ripening stages, was measured and expressed as mg/kg freeze-dried matter (f-d.m.). At the green stage, the f-d.m. of each cv was rich in β-carotene and lutein (>95% as compared to the total carotenoid content). At the red stage, the f-d.m. was composed of more than 96.2% lycopene in each cv. However, there was twice as much lycopene in the high-pigment cvs compared to the traditional ones. The data confirm dramatic changes in carotenoid content during tomato fruit ripening and show that it could be possible to prepare tomato matrices with defined carotenoid mixes that could be extracted with supercritical $CO_2$.

ottenere una gamma di polveri a granulometria omogenea. Il contenuto di carotenoidi nelle polveri, preparate a differenti stadi di maturazione, è stato determinato ed espresso come mg/kg di peso liofilizzato (f-d.m.). Allo stadio verde, i liofilizzati di ciascuna cv sono ricchi in β-carotene e luteina (>95% rispetto al contenuto totale in carotenoidi). Allo stadio rosso, il licopene rappresenta più del 96,2% del contenuto totale in carotenoidi nei liofilizzati di ciascuna cv. La quantità di licopene nelle cv high-pigment è doppia rispetto a quelle tradizionali. I risultati confermano i cambiamenti drammatici nel contenuto di carotenoidi durante la maturazione delle bacche ed evidenziano la possibilità di preparare matrici di pomodoro con un definito contenuto di carotenoidi da estrarre con $CO_2$ supercritica.

## INTRODUCTION

Carotenoids are the most important pigments found in nature. More than 600 carotenoid molecules have been chemically characterized with an estimated annual yield of 100 million tonnes (FRASER and BRAMLEY, 2004; BRITTON *et al.*, 2004; BOUVIER *et al.*, 2005). In all organisms that photosynthesize, carotenoids are involved in numerous biological functions such as photosynthesis, photoprotection, phytohormone precursors, membrane stability and environmental adaptation. In addition, they are important components in pigmentation of flowers and fruits to attract animals that facilitate seed dispersion (NIYOGI *et al.*, 1997; PAULSEN, 1999; DEMMING-ADAMS and ADAMS, 2002; KOPSELL and KOPSELL, 2006).

Carotenoids are $C_{40}$ isoprenoids that are generally made up of eight isoprene units. These molecules are characterized by a long polyene chain consisting of 3 to 15 conjugated double bonds, which are responsible for the characteristic adsorption spectrum and, as such, for the colour of the carotenoids.

Animals do not synthesize carotenoids so they need to obtain them from their daily diet. Dietary carotenoids play important biological functions in human and animal nutrition mainly due to their antioxidative potential. β-carotene and other carotenoids with a β-ionone ring have provitamin A activity and a deficiency causes xerophthalmia, blindness and premature death (MAYNE, 1996). Lutein and zeaxanthin prevent macular degeneration and age-related eye diseases, whereas lycopene protects against chronic diseases, certain types of cancers and cardiovascular diseases and

enhances the immune system (GIOVANNUCCI, 2002; GARCIA *et al.*, 2003; OMONI and ALUCO, 2005; RAO, 2006).

The characteristic red colour of ripe tomato fruit and tomato products is due to the synthesis and accumulation of high concentrations of lycopene during ripening. Among other physiological events, chloroplasts are changed into chromoplasts (COOKSON *et al.*, 2003). Thus, fresh ripe red tomatoes are the major source of lycopene in the human diet (ALEXANDER and GRIERSON, 2002); they also contain minor amounts of α- and β-carotene, and lutein. In addition, tomatoes are a good source of vitamin C, α-tocopherol, flavonoids, phenols, sugars, inorganic ions and organic acids (LONG *et al.*, 2006).

The carotenoid biosynthetic pathway has been elucidated and there has been progress in gene cloning. Thus, stable transgenic plants with improved nutritional traits due to an enhanced synthesis of high value carotenoids such as lycopene and β-carotene have been obtained (RÖMER *et al.*, 2000; FRASER and BRAMLEY, 2004; DAVULURI *et al.*, 2005; ENFISSI *et al.*, 2005; BOTELLA-PAVÍA and RODRIGUEZ-CONCEPCIÓN, 2006). At the same time, tomato cultivars (cvs) with enhanced synthesis and accumulation of high value carotenoids has been the result of conventional plant breeding. As such, several high-pigment tomato hybrids having the characteristic of producing tomato fruit with a high carotenoid content, in particular lycopene, are commercially available (LEVIN *et al.*, 2004; BINO *et al.*, 2005; GIOVANNONI, 2006; SCHAUER *et al.*, 2006). The cultivation of these high-pigment tomato hybrids is important not only when ripe red fruit is eaten raw but also when the tomatoes are processed because the high lycopene content compensates the loss due to processing. It is well known that processing and the storage of tomato products cause lycopene degradation and, consequently, the loss of its biological activity (SHI and LE MAGUER, 2000). The aim is to put tomato fruit and/or tomato products on the market that have high carotenoid levels (in particular lycopene, β-carotene and lutein) in order to protect against long-term diseases. In addition, these high-pigment hybrids are a good source for innovative technologies such as the preparation of a tomato matrix enriched with lycopene or other carotenoids that is suitable for the extraction of these natural compounds by supercritical $CO_2$ (VASAPOLLO *et al.*, 2004; DIAZ-REINOSO *et al.*, 2006).

The commercial demand for carotenoids from natural sources has increased markedly due to their widespread industrial use for nutrient supplementation, pharmaceutical purposes, food additives and animal feeds (SANDMANN, 2001; FRASER and BRAMLEY, 2004). It is also well established that the use of chemopreventive natural products is important for human health (RAO, 2006).

This study reports biochemical data regarding the level of the most important carotenoids (lycopene, lutein and β-carotene) in two tomato cvs (cv Kalvert and cv Hly18) sold as high-pigment tomato hybrids which are supposed to produce plants with fruit having a higher content of lycopene and in two typical industrial cvs (cv Donald and cv Incas). The carotenoid content was determined by measuring the content over a time course during tomato ripening stages corresponding to the green, green-orange, orange-red and red colours. Moreover, at the different stages of ripening, a freeze-dried tomato matrix was prepared that was suitable for extracting natural carotenoids by supercritical $CO_2$.

## MATERIALS AND METHODS

### Tomato cultivation

Seeds of two different cvs of high-pigment tomato hybrids (Kalvert and Hly 18,

gifts from Esasem Spa, Casaleone (VR) - Italy and COIS '94, Belpasso (CT), Italy, respectively) and two traditional cvs (Donald and Incas) were germinated in alveolar boxes. One-month-old tomato seedlings were transplanted in an open field in the province of Lecce (Southern Italy) and grown to maturity during the 2006 season. Standard agronomical practices were used for plant nutrition and pathogen prevention. Briefly the field was ploughed (30-40 cm) and 1,000 kg/ha of a basic organomineral fertiliser (Fertil agreste start - Scam) was spread. Post-transplant nitric nutrition with ammonium nitrate (fertilizer Leon - Hydro Agri, Milan, Italy) 600 kg/ha was given when required. The fungicide propamocarb hydrochloride, the insecticide Confidor Supra (Bayer, Leverkusen, Germany) and metallic copper were used for pest control.

Healthy tomatoes uniform in size and colour were harvested and collected at the four ripening stages corresponding to the green, green-orange, orange-red and red fruit colour. The fruit was sterilized with sodium hypochlorite, washed with several changes of distilled water, photographed and processed.

### Preparation of fresh tomato fruit homogenate

Approximately 1 kg (5 replicates) of uniform randomly harvested tomatoes of each cv at each stage of ripening were cut into small pieces and then homogenized in a Waring blender for 2 min. Aliquots of the homogeneous suspension were used for carotenoid analyses.

### Carotenoid analyses on fresh tomato fruit homogenate

Carotenoid content was determined on triplicate aliquots of the homogeneous suspension (0.5 g) by the method of SADLER *et al.* (1990) modified by PERKINS-VEAZIE *et al.* (2001). Carotenoids were extracted with 0.05% (w/v) butylated hydroxytoluene (BHT) in acetone and 95% ethanol (1:1 v/v). Lycopene, β-carotene and lutein were separated by partitioning in hexane and directly assayed. A Dionex HPLC (Dionex s.r.l., Milan, Italy) with an AD 25 UV-Vis detector was used, the separation was performed at 31°C on an Acclaim HPLC column $C_{18}$ (5 μm, 250x4.6 mm) by using a linear gradient of acetonitrile (A), hexane (B) and methanol (C): 0→35 min A:B:C 70:7:23→70:4:26 v/v/v, at a flow rate of 1.5 mL/min. Standard solutions of lycopene, β-carotene and lutein (Sigma-Aldrich, Milan) were prepared by dissolving 1 mg of powder in a suitable amount of hexane. The concentration of standard solutions was calculated using the molar extinction coefficient $17.2x10^4$ for lycopene, $13.9x10^4$ for β-carotene and $14.3x10^4$ for lutein in hexane. Peaks were detected at 503 nm.

### Freeze-dried matter preparation

For each cv at each stage of ripening 100 g triplicate aliquots of the homogenated suspension were centrifuged at 27,000 g for 10 min by using a J2-21 Beckman centrifuge (Beckman Coulter s.p.a., Cassina De' Pecchi (MI), Italy) to remove water-soluble substances (average mass loss due to centrifugation was approximately 80%). The pellet, essentially tomato cell clumps, was lyophilized by using a Christ ALPHA 2-4 LSC freeze-dryer (Martin Christ GmbH, Osterode, Germany) for 24 h (average mass loss due to lyophilisation was approximately 85%). The freeze-dried matter (f-d.m.) was ground at 500 μm by using a laboratory mill (Retsch Italia s.r.l., Torre Boldone (BG) Italy) to obtain a homogeneous powder.

### Carotenoid analyses on freeze-dried matter

Triplicate aliquots of f-d.m. (50 mg) were used to extract lycopene, β-caro-

tene and lutein by the method of SADLER *et al.* (1990) modified by PERKINS-VEAZIE *et al.* (2001). The extracted carotenoids were separated and quantified by using HPLC in the same way as for the carotenoid determination on fresh tomato fruit homogenate.

### Micro- and macro-element determination on freeze-dried matter

For metallic element assays, f-d.m. aliquots (3 g) were dissolved in an acid solution (7 mL of 70% $HNO_3$ and 1 mL of 30% $H_2O_2$) in a microwave mineralizator. The assay was conducted by using inductively coupled plasma atomic emission spectroscopy (Varian Liberty 110, Varian Inc., Palo Alto, USA). Calibration of the spectrometer was conducted by using a dilution of the standard solution of the metal being examined.

### Statistical analysis

Results are presented as the mean value ± standard deviation of five independent replicated experiments.

## RESULTS AND CONCLUSIONS

The amount of carotenoids including lycopene, β-carotene and lutein in different cvs of tomato fruits harvested at the ripening stages corresponding to the green, green-orange, orange-red and red fruit colours are reported in Table 1 as mg/kg fresh weight (f.w.) of tomato fruit homogenates and as mg/kg freeze-dried matter (f-d.m.) in Table 2. In all of the cvs investigated, the amount of lutein decreased markedly during the tomato ripening stages as well as on the f-d.m. basis. At the green stage, lutein was the major carotenoid; there was much more lutein in the high-pigment cvs (Kalvert and Hly 18) than in the traditional ones (Donald and Incas). Cv Hly 18 had the most lutein (14.6 mg/kg f.w., 199 mg/kg f-d.m.) followed by cv Kalvert (8.4 mg/kg f.w., 188 mg/kg f-d.m.), cv Incas (5.6 mg/kg f.w., 114 mg/kg f-d.m.) and cv Donald (3.2 mg/kg f.w., 86 mg/kg f-d.m.). The fact that lutein was the major carotenoid at the green tomato stage shows that this typical chloroplast pigment is closely linked to the photosynthetic membranes as part of the light-harvesting complex (NIYOGI *et al.*, 1997; PAULSEN *et al.*, 1999). The sharp decrease of lutein during tomato ripening, in particular from the green to the green-orange stage, seems to be due to the progressive loss of functionality of the photosynthetic machinery during the transition from chloroplasts to chromoplasts. This indicates that, at the early stages of tomato ripening there was a sharp decrease in the activity of the lycopene ε-cyclase (LCYE) and lycopene β-cyclase (LCYB) enzymes that are involved in cycling of lycopene to α-carotene. By the action of β-ring hydroxylase (CHYB) and ε-ring hydroxylase (CHYE) α-carotene is converted into lutein (Fig. 1). Lycopene ε- and β-cyclase activities decrease and then disappear during tomato ripening (PECKER *et al.*, 1996; RONEN *et al.*, 1999). The presence of lutein in young red pepper and orange fruit cvs has also been demonstrated; it then disappears during ripening (HORNERO-MÉNDEZ *et al.*, 2000; RODRIGO *et al.*, 2003, 2004).

At the green stage, the lowest amount of β-carotene was detected in cv Donald (0.6 mg/kg f.w.; 17 mg/kg f-d.m.) followed by cv Incas (1.0 mg/kg f.w.; 21 mg/kg f-d.m.) whereas the highest amount of β-carotene was detected in cv Kalvert (8.3 mg/kg f.w.; 185 mg/kg f-d.m.) followed by cv Hly 18 (10.4 mg/kg f.w.; 167 mg/kg f-d.m.). Thus, the amount of β-carotene was much higher in the high-pigment tomato hybrids than in the traditional cvs. This also suggests that cycling of lycopene to β-carotene by the action of LCYB is more active in high-pigment hybrids than in the traditional cvs. The amount of β-carotene, on a f.w.

Table 1 - Lycopene, β-carotene and lutein in fruit of different tomato cvs harvested at different stages of ripening (season 2006). The determinations were made by HPLC. Data are the means ± standard deviation of three replicates.

| Carotenoids | Stage of ripening | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Green | | Green-orange | | Orange-red | | Red | |
| | mg/kg f.w. | % | mg/kg f.w. | % | mg/kg f.w. | % | mg/kg f.w. | % |
| **Cv Donald** | | | | | | | | |
| lycopene | 0.2±0.01 | 5.0 | 4.9±0.2 | 66.2 | 50.0±4.0 | 95.0 | 95.0±11.0 | 96.3 |
| β-carotene | 0.6±0.01 | 15.0 | 2.1±0.3 | 28.4 | 2.3±0.1 | 4.4 | 3.4±0.2 | 3.4 |
| lutein | 3.2±0.3 | 80.0 | 0.4±0.0 | 5.4 | 0.3±0.1 | 0.6 | 0.3±0.0 | 0.3 |
| Total | **4.0±0.32** | | **7.4±0.5** | | **52.6±4.2** | | **98.7±11.2** | |
| **Cv Incas** | | | | | | | | |
| lycopene | 0.2±0.01 | 2.9 | 8.0±0.6 | 74.8 | 51.0±2.0 | 93.4 | 93.0±5.0 | 97.2 |
| β-carotene | 1.0±0.02 | 14.7 | 1.4±0.1 | 13.1 | 2.4±0.2 | 4.4 | 2.3±0.4 | 2.4 |
| lutein | 5.6±0.6 | 82.4 | 1.3±0.2 | 12.1 | 1.2±0.0 | 2.2 | 0.4±0.0 | 0.4 |
| Total | **6.8±0.63** | | **10.7±0.9** | | **54.6±2.2** | | **95.7±5.4** | |
| **Cv Kalvert** | | | | | | | | |
| lycopene | 0.4±0.01 | 2.3 | 28.0±2 | 77.1 | 58.0±4.0 | 92.2 | 202.0±8.0 | 97.9 |
| β-carotene | 8.3±0.7 | 48.6 | 5.8±0.5 | 16.0 | 3.2±0.4 | 5.1 | 3.6±0.2 | 1.8 |
| lutein | 8.4±0.6 | 49.1 | 2.5±0.4 | 6.9 | 1.7±0.2 | 2.7 | 0.7±0.0 | 0.3 |
| Total | **17.1±1.31** | | **36.3±2.9** | | **62.9±4.6** | | **206.3±8.2** | |
| **Cv Hly18** | | | | | | | | |
| lycopene | 0.4±0.01 | 1.6 | 61.0±4 | 89.2 | 122.0±9.0 | 95.5 | 195.0±12.0 | 97.4 |
| β-carotene | 10.4±0.4 | 41.0 | 5.2±0.4 | 7.6 | 3.9±0.5 | 3.0 | 3.4±0.2 | 1.7 |
| lutein | 14.6±3 | 57.4 | 2.2±0.1 | 3.2 | 1.8±0.1 | 1.4 | 1.8±0.1 | 0.9 |
| Total | **25.4±3.41** | | **68.4±4.5** | | **127.7±9.6** | | **200.2±12.3** | |

basis, increased markedly between the green stage and the red stage of tomato ripening in both cv Donald and Incas by 5.6- and 2.3-fold respectively whereas, calculated on f-d.m. basis, it was 8.8 and 5.6-fold, respectively. On the contrary, it decreased in both cv Kalvert and cv Hly 18 by 57 and 67%, respectively, calculated on f.w. basis. When calculated on f-d.m, this decrease was approximately 18.4 and 12.5% in Kalvert and Hly18 cv, respectively. However, at the red stage, the amount of β-carotene was very similar in cv Donald (3.4 mg/kg f.w., 150 mg/kg f-d.m.), cv Kalvert (3.6 mg/kg f.w.; 151 mg/kg f-d.m.), cv Hly 18 (3.4 mg/kg f.w.; 146 mg/kg f-d.m.) with the exception of cv Incas (2.3 mg/kg f.w.; 118 mg/kg f-d.m.) in which it was approximately 21 and 33% lower than the other cvs on a f.w. and f-d.m. basis, respectively. Therefore, although the traditional tomato cvs had a much lower level of β-carotene than the high-pigment ones, at the green stage, during ripening, the differential activity of LCYB resulted in an equal amount of β-carotene in cv Donald, Kalvert and Hly 18, at the red stage.

It is important to consider the ratio of lutein to β-carotene since they are synthesised starting from the cycling of lycopene through two different branches of the same pathway (BOTELLA-PAVÍA and

Table 2 - Lycopene, β-carotene and lutein in the lyophilised matrices obtained from fruit of different tomato cvs harvested at different stages of ripening (season 2006). The determinations were made by HPLC. Data are the means ± standard deviation of three replicates.

| Carotenoids | Stage of ripening | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Green | | Green-orange | | Orange-red | | Red | |
| | mg/kg f-d.m. | % | mg/kg f-d.m. | % | mg/kg f-d.m. | % | mg/kg f-d.m. | % |
| **Cv Donald** | | | | | | | | |
| lycopene | 5±0.4 | 4.6 | 120±12 | 61.9 | 1517±96 | 93.9 | 4209±283 | 96.2 |
| β-carotene | 17±2 | 15.7 | 52±4 | 26.8 | 79±7 | 4.9 | 150±7 | 3.4 |
| lutein | 86±9 | 79.7 | 22±1 | 11.3 | 19±2 | 1.2 | 15±1 | 0.4 |
| Total | **108±11.4** | | **194±17** | | **1615±105** | | **4374±291** | |
| **Cv Incas** | | | | | | | | |
| lycopene | 5±0.6 | 3.6 | 372±31 | 76.7 | 2160±38 | 93.4 | 4546±286 | 97.0 |
| β-carotene | 21±1 | 15.0 | 54±4 | 11.1 | 103±14 | 4.5 | 118±13 | 2.5 |
| lutein | 114±9 | 81.4 | 59±8 | 12.2 | 49±3 | 2.1 | 22±1 | 0.5 |
| Total | **140±10.6** | | **485±43** | | **2312±55** | | **4686±300** | |
| **Cv Kalvert** | | | | | | | | |
| lycopene | 9±0.7 | 2.4 | 895±53 | 78.5 | 2055±183 | 89.9 | 8398±321 | 97.9 |
| β-carotene | 185±10 | 48.4 | 184±16 | 16.1 | 172±9 | 7.5 | 151±10 | 1.8 |
| lutein | 188±15 | 49.2 | 62±6 | 5.4 | 60±4 | 2.6 | 30±1 | 0.3 |
| Total | **382±25.7** | | **1141±75** | | **2287±196** | | **8579±332** | |
| **Cv Hly18** | | | | | | | | |
| lycopene | 8±0.7 | 2.1 | 2207±157 | 91.0 | 5056±417 | 95.8 | 8083±220 | 97.5 |
| β-carotene | 167±9 | 44.7 | 154±13 | 6.3 | 154±12 | 2.9 | 146±4 | 1.7 |
| lutein | 199±19 | 53.2 | 65±4 | 2.7 | 67±2 | 1.3 | 65±2 | 0.8 |
| Total | **374±28.7** | | **2426±174** | | **5277±431** | | **8294±226** | |

RODRIGUEZ-CONCEPCIÓN, 2006; KIM and DELLAPENNA, 2006; LI *et al.*, 2006). At the green stage the lutein/β-carotene ratio, evaluated on a f-d.m. basis, was approximately 5.1, 5.4, 1.0 and 1.2 for the cv Donald, Incas, Kalvert and Hly 18, respectively (Table 2). During tomato ripening, this ratio decreased markedly in the cv Donald (from 5.1 at the green stage to 0.1 at red stage) and Incas (from 5.4 at the green stage to 0.19 at the red stage) and to a lesser degree, in the cv Kalvert (from 1.0 at the green stage to 0.20 at the red stage) and Hly 18 (from 1.2 at the green stage to 0.45 at the red stage). A similar pattern was observed when the lutein/β-carotene ratio was calculated on a f.w. basis (Table 1). At the green stage, the biosynthesis of lutein prevailed markedly over that of β-carotene in cvs Donald and Incas and much less in cv Hly 18; it remained the same in cv Kalvert. On the contrary, starting from the green-orange stage of ripening, the biosynthesis of β-carotene prevailed over that of lutein which reached the lowest level at the red stage in all the cvs. This result indicates that a differential gene expression operates at the level of lycopene cycling which leads to the synthesis of β-carotene and lutein by the two branches of the same pathway. It has been demonstrated that the accumulation of β-carotene in fruit of a

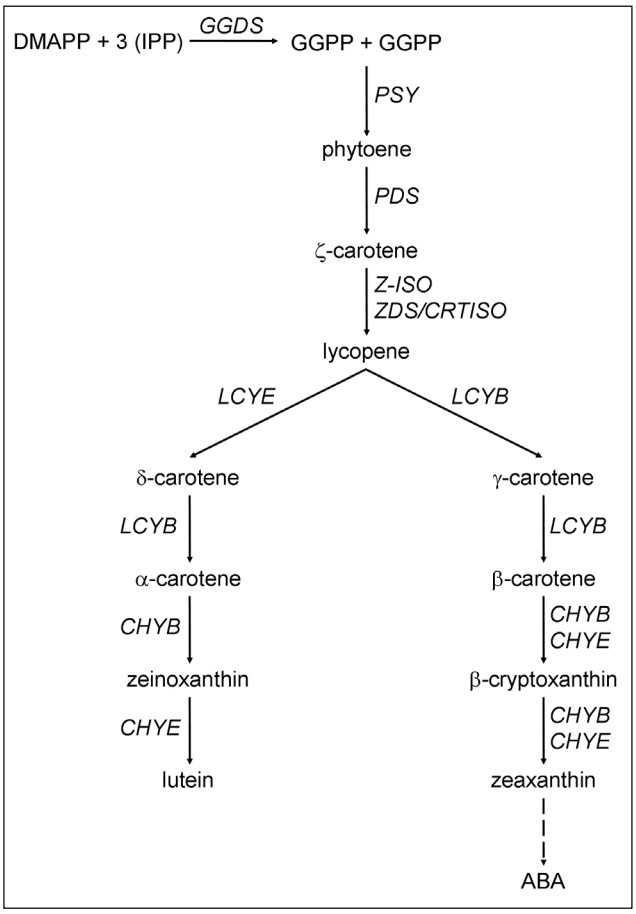



Fig. 1 - Simplified carotenoid biosynthetic pathway in plants. IPP, isopentenyl diphosphate; DMAPP dimethylallyl diphosphate; GGPP, geranylgeranyl diphosphate; ABA, abscisic acid. Enzymes indicated are: GGDS, GGPP synthase; PSY, phytoene synthase; PDS, phytoene desaturase; Z-ISO 15-cis zetacarotene isomerase; ZDS, zetacarotene desaturase; CRTISO, carotene isomerase; LCYB, lycopene β-cyclase; LCYE, lycopene ε-cyclase; CHYB, carotenoid β-ring hydroxylase; CHYE, carotenoid ε-ring hydroxylase (Modified from: HARJES *et al.*, 2008).

δ-tomato mutant is due to a differential gene expression (CUNNINGHAM *et al.*, 1996; RONEN *et al.*, 1999, 2000). An independent regulation of the two branches of the carotenoid cycling pathway has recently been confirmed (KIM and DELLAPENNA, 2006; HARJES *et al.*, 2008).

In all of the tomato cvs investigated, the amount of lycopene in fresh tomato fruit calculated as mg/kg f.w. (Table 1) and as mg/kg f-d.m. (Table 2), increased dramatically from the green to the red stage of ripening. This increase was 475-, 465-, 505- and 488-fold, when referred to f.w., and 842-, 909-, 933- and 1,010-fold, when referred to f-d.m., for the cv Donald, Incas, Kalvert and Hly 18, respectively. An approximate 500-fold increase occurs in the synthesis and accumulation of lycopene during tomato ripening, when evaluated on a f.w. basis (FRASER *et al.*, 1994). The results of this study confirm previous results on traditional tomato cvs and show that similar results are found with high-pigment tomato hybrids. In addition, there was an accumulation of lycopene during tomato ripening, referred to f-d.m. (Table 2), of approximately 842- and 1,010-fold in all of the cvs. The values calculated on f-d.m. are extremely important in view of the industrial uses of these lycopene-enriched matrices as well as in other carotenoids or mixtures of them. These

matrices, appropriately processed, can be directly used for different industrial preparations, as well as for the extraction of biologically active natural compounds by using innovative technologies such as supercritical carbon dioxide ($CO_2$) (VASAPOLLO *et al.*, 2004). $CO_2$ is an ideal solvent for extracting natural bioactive molecules because it is non-toxic, non-explosive, readily available, easily removable from the products and environmentally compatible. In addition, it can be used at low-to-moderate operating temperature and pressure (VASAPOLLO *et al.*, 2004; DÍAZ-REINOSO *et al.*, 2006; VÁGI *et al.*, 2007).

Lycopene was the lowest carotenoid determined at the green stage, in all cvs, even though the amount was higher in cvs Kalvert (0.4 mg/kg f.w.; 9 mg/kg f-d.m.) and Hly 18 (0.4 mg/kg f.w.; 8 mg/kg f-d.m.) than in the traditional cvs Donald (0.2 mg/kg f.w.; 5 mg/kg f-d.m.) and Incas (0.2 mg/kg f.w.; 5 mg/kg f-d.m.). The accumulation of lycopene was very evident when the tomatoes passed from the green stage to the green-orange stage, commonly referred to as the "breaker" stage. The lycopene content increased 24-, 74-, 99-, and 276-fold in cv Donald, Incas, Kalvert and Hly 18, respectively, between these two ripening stages.

At the red stage, cv Kalvert had the highest lycopene content (202 mg/kg f.w., 8,398 mg/kg f-d.m.) followed by cv Hly 18 (195 mg/kg f.w., 8,083 mg/kg f-d.m.), cv Donald (95 mg/kg f.w., 4,209 mg/kg f-d.m.) and cv Incas (93 mg/kg f.w., 4,546 mg/kg f-d.m.). Thus, the tomato fruit of cv Kalvert and cv Hly 18 accumulated twice as much lycopene, calculated on a f.w. and a f-d.m. basis, than cv Donald and cv Incas. The capability of cvs Kalvert and Hly 18 to accumulate more lycopene in tomato fruit chromoplasts with respect to cvs Donald and Incas is probably the net result of an increased biosynthesis and sequestration of the end products. With regard to this, it has been demonstrated that, after the genes for phytoene synthase (PSY) and phytoene desaturase (PDS) were cloned, the mRNA levels for these two genes increased significantly during the "breaker" stage (PECKER *et al.*, 1992; GIULIANO *et al.*, 1993; FRASER *et al.*, 1994). A similar increase was found for the mRNAs of geranylgeranyl diphosphate synthase (GGDPsynthase), PSY and PDS during the ripening of bell pepper (CAMARA *et al.*, 1995). Carotenoid accumulation, however, does not depend solely on the increase of its biosynthesis but also on the control of other metabolic processes. Previous studies have reported that the sequestration of carotenoid end products plays an important role in carotenoid accumulation (DURUERE *et al.*, 1994, VISHNEVETSKY *et al.*, 1999). This is supported by the fact that plastoglobules, present in chromoplasts, have recently been recognised as being the main storage and remodelling site for carotenoids (VIDI *et al.*, 2006; YTTERBERG *et al.*, 2006; AUSTIN II *et al.*, 2006; BRÉHÉLIN, 2007). It has recently been reported that the Or gene encodes a plastid membrane protein (LU *et al.*, 2006) which might be involved in the sequestration of carotenoids (LI *et al.*, 2006). Lycopene accumulation during tomato ripening also depends on the progressive reduction of lycopene cycling (RONEN *et al.*, 1999; BRAMLEY, 2002). The current data, expressed on a f.w. and f-d.m. basis, confirm that the percentages of β-carotene plus lutein decreased sharply during tomato fruit ripening from a value higher than 95% at the green stage to a value of less than 3.8% at the red stage.

At all stages of ripening, the total amounts of carotenoids including lycopene, β-carotene and lutein were much higher in cvs Kalvert and Hly 18 than cvs Incas and Donald when calculated on f.w. and f-d.m. basis (Tables 1 and 2). This confirms that in the selected high-pigment tomato hybrids the metabolic pathway of carotenoids is over-expressed and controlled at all stages of ripening. It

Table 3 - Micro- and macro-elements, expressed as mg/kg freeze-dried matter (f-d.m.), in the lyophilised matrices obtained from fully ripe (red stage) fruit of one traditional (Donald) and two high-pigment (Kalvert and Hly18) tomato cvs. Data are the means ± standard deviation of three independent replicates.

| Cultivar | Micro-elements | | | | | | | | | | | | Macro-elements | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | As | Cd | Co | Cr | Cu | Fe | Mn | Ni | Pb | Se | V | Zn | Ca | K | Mg | Na |
| Donald | <20 | 0.13 | 1.09 | 0.07 | 10.0 | 30.6 | 7.94 | 2.19 | 0.65 | <10 | <0.5 | 17.2 | 1.35 | 31.6 | 1.11 | 0.77 |
| Kalvert | <20 | 0.11 | <0.50 | 0.12 | 10.1 | 23.2 | 8.48 | 1.43 | <0.50 | <10 | <0.5 | 17.4 | 1.35 | 36.7 | 1.35 | 0.94 |
| Hly 18 | <20 | 0.09 | <0.50 | 0.47 | 9.7 | 20.0 | 0.99 | 1.40 | 0.50 | <10 | <0.5 | 15.5 | 1.20 | 31.7 | 1.10 | 0.29 |
| Standard deviation | --- | ±0.01 | ±0.04 | ±0.01 | ±0.4 | ±0.3 | ±0.04 | ±0.02 | ±0.05 | ±5 | --- | ±0.4 | ±0.05 | ±0.1 | ±0.04 | ±0.04 |

has been well documented that the high pigment (hp) locus in tomato also affects the total amount of carotenoids (LEVIN *et al.*, 2004; BINO *et al.*, 2005).

At the red stage in all of the tomato cvs investigated lycopene accounted for more than 96.2% of all the carotenoids, evaluated as f.w. and f-d.m. However, there was twice as much lycopene in the high-pigment tomato hybrids (cv Kalvert and cv Hly 18) compared to the traditional cvs (Donald and Incas). Thus, the f-d.m. of the red stage lycopene-enriched tomato can be directly used by the industries or processed by supercritical $CO_2$ to extract lycopene. The prepared f-d.m. of cv Donald, Kalvert and Hly 18 contained very low amounts of micro- and macro-elements (Table 3). Preliminary experiments have shown that this f-d.m. can be efficiently used to extract a natural lycopene-rich product in an oleoresin form. The lycopene recovery was more than 80% efficient and could be improved by further optimization of the process. Twice as much lycopene has been extracted by supercritical $CO_2$ using f-d.m. prepared from high-pigment hybrids (LENUCCI *et al.*, 2008). This natural, concentrated lycopene-rich product can be used for different pharmaceutical health-promoting mixtures. The beneficial effects of lycopene in preventing chronic diseases have been well documented (RAO, 2006). At the green stage, lutein plus β-carotene, evaluated as f.w. and f-d.m., made up more than 95.0% of all the carotenoids in all the cvs. Even in this case, preliminary experiments have shown that the f-d.m. extracted with supercritical $CO_2$ produces mixtures that are essentially composed of lutein and β-carotene (data not shown). The role of lutein in protecting against macular degeneration (SOMMERBURG *et al.*, 1998; JOHNSON *et al.*, 2000) has been well-documented as has the synergistic effect of a mixture of carotenoids in preventing chronic diseases (RAO, 2006).

Abbreviations

BHT, butylated hydroxytoluene; CHYB, carotenoid β-ring hydroxylase; CHYE, carotenoid ε-ring hydroxylase; cv, cultivar; f-d.m., freeze-dried matter; LCYB, lycopene β-cyclase; LCYE, lycopene ε-cyclase; PDS, phytoene desaturase; PSY, phytoene synthase.

ACKNOWLEDGMENTS

The authors thank Alessandra Iannizzotto (COIS '94 s.r.l.) for supplying the Hly seeds, Sergio Montemurro (ESASEM) for the Kalvert seeds, Antonello and Giovanna Russo (Agricultural farm "Le Lame") for the Donald tomatoes, Leonardo Rescio (Pierre Chimica s.r.l.) for preliminary extractions of lycopene with supercritical $CO_2$, Alessandro Buccolieri and Angelo Dell'Atti for micro- and macro-element analyses, and Gaetano Carrozzo for his technical assistance in cultivating the tomato plants. Financial assistance was received from project MIUR 7885/55 PAR 2001.

REFERENCES


Alexander L. and Grierson D. 2002. Ethylene biosynthesis and action in tomato: a model for climacteric fruit ripening. J. Exp. Bot. 53: 2055.

Austin II J.R., Frost E., Vidi P.A., Kessler F. and Staehelin L.A. 2006. Plastoglobules are lipoprotein subcompartments of the chloroplast that are permanently coupled to thylakoid membranes and contain biosynthetic enzymes. Plant Cell 18: 1693.

Bino R.J., Ric de Vos C.H., Lieberman M., Hall R.D., Bovy A., Jonker H.H., Tikunov Y., Lommen A., Moco S. and Levin I. 2005. The light-hyperresponsive *high pigment-2*$^{dg}$ mutation of tomato: alterations in the fruit metabolome. New Phytol. 166: 427.

Botella-Pavía P. and Rodriguez-Concepción M. 2006. Carotenoid biotechnology in plants nutritionally improved foods. Physiol. Plantarum 126: 369.

Bouvier F., Isner J.C., Dogbo O. and Camara B. 2005. Oxidative tailoring of carotenoids: a prospect towards novel functions in plants. Trends Plant Sci. 10: 187.

Bramley P.M. 2002. Regulation of carotenoid formation during tomato fruit ripening and development. J. Exp. Bot. 53: 2107.

Bréhélin C., Kessler F. and van Wijk K.J. 2007. Plastoglobules: versatile lipoprotein particles in plastids. Trends Plant Sci. 12: 260.

Britton G., Liaaen-Jensen S. and Pfander H. 2004. "Carotenoid Handbook". Birkhäuser Verlag, Basel.

Camara B., Hugueney P., Bouvier F., Kuntz M. and Moneger R. 1995. Biochemistry and molecular biology of chromoplast development. Int. Rev. Cytol. 163: 175.

Cookson P.J., Kiano J.W., Shipton C.A., Fraser P.D., Romer S., Schuch W., Bramley P.M. and Pyke K.A. 2003. Increase in cell elongation, plastid compartment size and phytoene synthase activity underlie the phenotype of the *high pigment-1* mutant of tomato. Planta 217: 896.

Cunningham F.X., Pogson B., Sun Z.R., McDonald K.A., DellaPenna D. and Gantt E. 1996. Functional analysis of the beta- and epsilon-cyclase enzymes of *Arabidopsis* reveals a mechanism for control of cyclic carotenoid formation. Plant Cell 8: 1613.

Davuluri G.R., van Tuinen A., Fraser P.D., Manfredonia A., Newman R., Burgees D., Brummel D.A., King S.R., Palys J., Uhlig J., Bramley P.M., Pennings H.M. and Bowler C. 2005. Fruit-specific RNAi-mediated suppression of DET1 enhances carotenoid and flavonoid content in tomatoes. Nature Biotechnol. 23: 890.

Demmig-Adams B. and Adams W.W. 2002. Antioxidants in photosynthesis and human nutrition. Science 298: 2149.

Díaz-Reinoso B., Moure A., Domínguez H. and Parajó J.C. 2006. Supercritical $CO_2$ extraction and purification of compounds with antioxidant activity. J. Agric. Food Chem. 54: 2441.

Duruere J., Romer S., d'Harlingue A., Backhaus R.A., Kuntz M. and Camara B. 1994. Fibril assembly and carotenoid overaccumulation in chromoplasts: a model for supramolecular lipoprotein structures. Plant Cell 6: 119.

Enfissi E.M.A., Fraser P.D., Lois L.M., Boronat A., Schuch W. and Bramley P.M. 2005. Metabolic engineering of the mevalonate and non-mevalonate isopentenyl diphosphate-forming pathways for the production of health-promoting isoprenoids in tomato. Plant Biotechnol. J. 3: 17.

Fraser P.D. and Bramley P.M. 2004. The biosynthesis and nutritional uses of carotenoids. Prog. Lipid Res. 43: 228.

Fraser P.D., Truesdale M.R., Bird C.R., Schuch W. and Bramley P.M. 1994. Carotenoid biosynthesis during tomato fruit development. Plant Physiol. 105: 405.

Garcia A.L., Rühl R., Herz U., Koebnick C., Schweigert F.J. and Worm M. 2003. Retinoid- and carotenoid-enriched diets influence the ontogenesis of the immune system in mice. Immunology 110: 180.

Giovannoni J.J. 2006. Breeding new life into plant metabolism. Nature Biotechnol. 24: 418.

Giovannucci E. 2002. A review of epidemiologic studies of tomatoes, lycopene, and prostate cancer. Exp. Biol. Med. 227: 852.

Giuliano G., Bartley G.E. and Scolnik A. 1993. Regulation of carotenoid biosynthesis during tomato development. Plant Cell 5: 379.

Harjes C.E., Rocheford T.R., Bai L., Brutnell T.P., Kandianis C.B., Sowinski S.G., Stapleton A.E., Vallabhaneni R., Williams M., Wurtzel E.T., Yan J. and Buckelr E.S. 2008. Natural genetic variation in lycopene epsilon cyclase tapped for maize biofortification. Science 319: 330.

Hornero-Méndez D., Gómez-Ladrón de Guevara R. and Mínguez-Mosquera M.I. 2000. Carotenoid biosynthesis changes in five red pepper (*Capsicum annuum* L.) cultivars during ripening. Cultivar selection for breeding. J. Agr. Food Chem. 48: 3857.

Kim J. and DellaPenna D. 2006. Defining the primary route for lutein synthesis in plants: the role of *Arabidopsis* carotenoid β-ring hydroxylase CYP97A3. Proc. Natl Acad. Sci. USA 103: 3474.

Kopsell D.A. and Kopsell D.E. 2006. Accumulation and bioavailability of dietary carotenoids in vegetable crops. Trends Plant Sci. 11: 499.

Johnson E.J., Hammond B.R., Yeum K.J., Qin J., Wang X.D., Castaneda C., Snodderly D.M. and Russell R.M. 2000. Relation among serum and tissue concentrations of lutein and zeaxanthin and macular pigment density. Am. J. Clin. Nutr. 71: 1555.

Lenucci M.S., Caccioppola A., Durante M., Serrone L., Piro G. and Dalessandro G. 2008. Preparation of biological matrices from red-ripe tomato fruits suitable for lycopene extraction with supercritical $CO_2$. Acta of the Italian-Japanese Joint Symposium "Natural Products and Functional Foods", Salerno, Italy.

Levin I., Lalazar A., Bar M. and Schaffer A.A. 2004. Non GMO fruit factories. Strategies for modulating metabolic pathways in the tomato fruit. Ind. Crop Prod. 20: 29.

Li L., Lu S., Cosman K.M., Earle E.D., Garvin D.F. and O'Neill J. 2006. β-Carotene accumulation induced by the cauliflower *Or* gene is not due to an increased capacity of biosynthesis. Phytochem. 67: 1177.

Long M., Millar D.J., Kimura Y., Donovan G., Rees J., Fraser P.D., Bramley P.M. and Bolwell G.P. 2006. Metabolite profiling of carotenoid and phenolic pathways in mutant and transgenic lines of tomato: identification of a high antioxidant fruit line. Phytochem. 67: 1750.

Lu S., Van Eck J., Zhou X., Lopez A.B., O'Halloran D.M., Cosman K.M., Conlin B.J., Paolillo D.J., Garvin D.F., Vrebalov J., Kochian L.V., Küpper H., Earle E.D., Cao J. and Li L. 2006. The cauliflower *Or* gene encodes a DnaJ cisteine-rich domain-containing protein that mediates high levels of β-carotene accumulation. Plant Cell 18: 3594.

Mayne S.T. 1996. β-Carotene, carotenoids and disease prevention in humans. FASEB J. 10: 690.

Niyogi K.K., Bjorkman O. and Grossman A.R. 1997. The roles of specific xanthophylls in photoprotection. Proc. Natl. Acad. Sci. USA 94: 14162.

Omoni A.O. and Aluko R.E. 2005. The anti-carcinogenic anti-atherogenic effects of lycopene: a review. Trends Food Sci. Technol. 16: 344.

Paulsen H. 1999. Carotenoids and the assembly of light-harvesting complexes. In "The Photochemistry of Carotenoids" (edited by H.A. Frank, A.Y. Young, G. Britton, R.J. Cogdell). p. 123. Kluwer Academic Publishers, Dordrecht, Netherlands.

Pecker I., Chamovitz D., Linden H., Sandmann G. and Hirschberg J. 1992. A single polypeptide catalyzing the conversion of phytoene to ζ-carotene is transcriptionally regulated during tomato fruit ripening. Proc. Natl. Acad. Sci. USA 89: 4962.

Pecker I., Gabbay R., Cunningham F.X. and Hirschberg J. 1996. Cloning and characterization of the cDNA for lycopene beta-cyclase from tomato reveals decrease in its expression during fruit ripening. Plant Mol. Biol. 30: 807.

Perkins-Veazie P., Collins J.K., Pair S.D. and Roberts W. 2001. Lycopene content differs among red-fleshed watermelon cultivars. J. Sci. Food Agric. 81: 983.

Rao A.V. 2006. "Tomatoes, Lycopene & Human Health. Preventing Chronic Diseases" (A.V. Rao ed.). Caledonian Science Press, Badalona, Spain.

Rodrigo M.J., Marcos J.F., Alférez F., Mallent M.D. and Zacarías L. 2003. Characterization of pinalate, a novel *Citrus sinensis* mutant with a fruit-specific alteration that results in yellow pigmentation and decreased ABA content. J. Exp. Bot. 54: 727.

Rodrigo M.J., Marcos J.F. and Zacarias L. 2004. Biochemical and molecular analysis of carotenoid biosynthesis in flavedo of orange (*Citrus sinensis* L.) during fruit development and maturation. J. Agric. Food Chem. 52: 6724.

Römer S., Fraser P.D., Kiano J.W., Shipton C.A., Misawa N., Schuch W. and Bramley P.M. 2000. Elevation of the provitamin A content of transgenic tomato plants. Nature Biotechnol. 18: 666.

Ronen G., Cohen M., Zamir D. and Hirschberg J. 1999. Regulation of carotenoid biosynthesis during tomato fruit development: expression of the gene for lycopene epsilon-cyclase is down-regulated during ripening and is elevated in the mutant *Delta*. Plant J. 17: 341.

Ronen G., Carmel-Goren L., Zamir D. and Hirschberg J. 2000. An alternative pathway to beta-carotene formation in plant chromoplasts discovered by map-based cloning of *Beta* and *old-gold* colour mutations in tomato Proc. Natl. Acad. Sci. USA 97: 11102.

Sadler G., Davis J. and Dezman D. 1990. Rapid extraction of lycopene and β-carotene from reconstituted tomato paste and pink grapefruit homogenates. J. Food Sci. 55: 1460.

Sandmann G. 2001. Genetic manipulation of carotenoid biosynthesis: strategies, problems and achievements. Trends Plant Sci. 6: 14.

Schauer N., Semel Y., Roessner U., Gur A., Balbo I., Carrari F., Pleban T., Perez-Melis A., Bruedigam C., Kopka J., Willmitzer L., Zamir D. and Fernie A.R. 2006. Comprehensive metabolic profiling and phenotyping of interspecific introgression lines for tomato improvement. Nature Biotechnol. 24: 447.

Shi J. and Le Maguer M. 2000. Lycopene in tomatoes: chemical and physical properties affected by food processing. Crit. Rev. Food Sci. Nutr. 40: 1.

Sommerburg O., Keunen J.E.E., Bird A.C. and van Kuijk F.K.G.M. 1998. Fruit and vegetables that are sources for lutein and zeaxanthin: the macular pigment in human eyes. Br. J. Ophthalmol. 82: 907.

Vági E., Simándi B., Vásárheliné K.P., Daood H., Kéry Á., Doleschall F. and Nagy B. 2007. Supercritical carbon dioxide extraction of carotenoids, tocopherols and sitosterols from industrial tomato by-products. J. Supercrit. Fluids 40: 218.

Vasapollo G., Longo L., Rescio L. and Ciurlia L. 2004. Innovative supercritical $CO_2$ extraction of lycopene from tomato in the presence of vegetable oil as co-solvent. J. Supercrit. Fluids 29: 87.

Vidi P.A., Kanwischer M., Baginsky S., Austin J.R., Csucs G., Dörmann P., Kessler F. and Bréhélin C. 2006. Tocopherol cyclase (VET1) localization and vitamin E accumulation in chloroplast plastoglobule lipoprotein particles. J. Biol. Chem. 281: 11225.

Vishnevetsky M., Ovadis M. and Vainstein A. 1999. Carotenoid sequestration in plants: the role of carotenoid-associated proteins. Trends Plant Sci. 4: 232.

Ytterberg A.J., Peltier J.B. and van Wijk K.J. 2006. Protein profiling of plastoglobules in chloroplasts and chromoplasts. A surprising site for differential accumulation of metabolic enzymes. Plant Physiol. 140: 984.

Revised paper received April 3, 2009 Accepted May 29, 2009

PAPER

# PROBIOTIC PROPERTIES OF *BIFIDOBACTERIUM ANIMALIS* SUBSP. *LACTIS* BB-12 IN CEREAL FLAKES ENRICHED WITH INULIN

PROPRIETÀ PROBIOTICHE DI *BIFIDOBACTERIUM ANIMALIS* SUBSP. *LACTIS* BB-12 NEI FIOCCHI DI CEREALI ARRICCHITI CON INULINA

**G. HAJDUK*, B. KOS[1], J. ŠUŠKOVIĆ[1], J. FRECE[1], A. LEBOŠ PAVUNC[1], and J. BEGANOVIĆ[1]**
Podravka Inc., Food Industry, Ante Starčevića 32, HR-48000 Koprivnica, Croatia
[1]Faculty of Food Technology and Biotechnology, Pierottieva 6, HR-10000 Zagreb, Croatia
*Corresponding author: Tel. +385 48 651 969, Fax +385 622 268, e-mail: gordana.hajduk@podravka.hr

ABSTRACT

Production of functional food supplemented with probiotic bifidobacteria is of growing interest because of the health-promoting effects in humans. In this study a novel probiotic product was prepared: cereal flakes enriched with inulin and a lyophilized probiotic culture of *Bifidobacterium animalis* subsp. *lactis* BB-12, intended to be used as an infant formula. The influence of this food matrix on probiotic properties of *B. animalis* subsp. *lactis* BB-12

RIASSUNTO

La produzione di cibi funzionali supplementati con bifidobatteri probiotici è in aumento grazie ai loro effetti benefici per la salute umana. In questo lavoro è stato sviluppato un nuovo prodotto probiotico: fiocchi di cereali arricchiti con inulina e con una coltura liofilizzata di *Bifidobacterium animalis* subsp. *lactis* BB-12. È stata studiata l'influenza della matrice di questo cibo sulle proprietà probiotiche di *B. animalis* subsp. *lactis* BB-12. La tolleranza del

- Key words: adhesion, *Bifidobacterium*, bile salt hydrolase (BSH) activity, competitive exclusion, gastrointestinal tract, immunomodulation -

was examined. The tolerance to simulated gastric and small intestinal juices was much higher when the *B. animalis* subsp. *lactis* BB-12 probiotic culture was applied in combination with cereal flakes, inulin and skim milk with respect to the use of the pure lyophilized probiotic culture. Bile salt resistance and bile salt hydrolase activity (BSH) of *B. animalis* subsp. *lactis* BB-12 was also monitored *in vitro*. Furthermore, the influence of cereal flakes and inulin on the functional probiotic properties of *B. animalis* subsp. *lactis* BB-12 was examined *in vivo* in Swiss albino mice. The higher viable cell count of lactic acid bacteria and lower viable cell count of enterobacteria and sulphite-reducing clostridia in the large intestine of mice were observed after feeding the mice with the novel probiotic product compared to results obtained when mice were fed probiotic alone. The induction of serum IgA, IgG and IgM antibody production in Swiss albino mice by *B. animalis* subsp. *lactis* BB-12 was not influenced by the addition of cereal and inulin.

succo gastrico e del succo dell'intestino tenue simulati era molto più elevata dopo l'aggiunta di *B. animalis* subsp. *lactis* BB-12 assieme con i fiocchi cereali, inulina ed latte, di quanto lo era con la sola coltura probiotica liofilizzata. È stata valutata la resistenza *in vitro* ai sali biliari e all'attività di idrolisi dei sali biliari di *B. animalis* subsp. *lactis* BB-12. È stata esaminata inoltre, l'influenza di fiocchi cereali ed inulina sulle proprietà probiotiche funzionali di *B. animalis* subsp. *lactis* BB-12 tramite degli esperimenti *in vivo* sui topi albino svizzeri. È stata osservata una migliore capacità di adesione di batteri lattici e la riduzione degli enterobatteri e dei clostridi solfito riduttori nell'intestino crasso dei topi, dopo che sono stati nutriti con il nuovo prodotto probiotico rispetto all'adesione e all'attività antibatterica della sola coltura probiotica. L'attività immunomodulante di *B. animalis* subsp. *lactis* BB-12, saggiata per la produzione sierica di anticorpi IgA, IgG e IgM nei topi albino svizzeri, non è stata influenzata dall'aggiunta di cereali ed inulina.

## INTRODUCTION

The most common food matrices for probiotic bacteria are different kinds of fermented milk products, especially yoghurts (GUEIMONDE, 2005; TEMMERMAN, 2003). In the past few years many probiotic foods have become available on the market. Probiotics can be found in non-fermented milk, in fruit and berry juices and in cereal-based products. However, relatively little information is available on the stability and functionality of probiotics in non-fermented food matrices, while the stability of probiotics in fermented dairy products has been widely studied (KAILASAPATHY and RYBKA, 1997; KRASAEKOOPT *et al., 2003).*

Bifidobacteria are Gram-positive, anaerobic, non-motile, non-sporeforming organisms that are mainly present in the gut and constitute a major part of the natural microflora of the human intestine. When present in sufficient numbers, bifidobacteria create a healthy equilibrium between beneficial and harmful microorganisms in the human gut (SHAH and LANKAPUTHRA, 2002). In order to be active in the gastrointestinal tract (GIT) and to positively influence the intestinal microflora, these bacteria should be resistant to the de-

fence mechanisms of the host (JONSON and CONWAY, 1992). Due to the rigorous conditions present in the GIT, the survival of probiotic bacteria, in sufficient numbers during transit through the human GIT remains a major challenge for effective delivery of these beneficial bacteria. Survival of probiotics during the processing and storage of functional food products is of concern for the development of products with a guaranteed content of viable bacterial cells (ANAL and SINGH, 2007). The colonization of the intestine by exogenous probiotics is influenced by many factors including inoculum size, physiological state of the bacteria, buffering capacity of the delivery food and the capacity of the microorganisms to resist the acid and bile concentrations encountered in the upper segments of the GIT (CONWAY *et al.*, 1987). Probiotics must be resistant to the enzymes of the intestine, e.g. lysosyme and the toxic metabolites produced during digestion (HOIER, 1992). *Bifidobacterium* strains vary greatly in their sensitivity to the harsh acidic environment of the stomach and some foods (CHARTERIS *et al.*, 1998; TRUELSTRUP *et al.*, 2002). The success of functional foods containing bifidobacteria depends on the viability of bifidobacteria in the product during storage as well as on the resistance of these bacteria to the conditions present in the GIT. PÉREZ-CONESA *et al.* (2006) have shown that the composition of the fecal microbiota in rats and human infants changed after feeding with follow-up formula supplemented with *Bifidobacterium* spp. These results have demonstrated that bifidobacteria make up a significant portion of the anaerobe group in faecal matter. Moreover, SU *et al.* (2007) have shown that fructo-oligosaccharide, inulin and soybean oligosaccharide enhanced the survival and prolonged the retention of *Lactobacillus acidophilus* L10, *Bifidobacterium lactis* B94 and *Lactobacillus casei* L26 *in vivo* in the GIT of mice.

The objective of this study was to evaluate probiotic properties of the strain *B. animalis* subsp. *lactis* BB-12, in wheat grits cereal flakes enriched with inulin, intended to be used as an infant formula. Survival in the novel probiotic product during storage at two different temperatures, capacity to deconjugate bile salts, as well as survive in simulated GIT conditions, of this probiotic strain, were investigated *in vitro*. Furthermore, the functional properties of *B. animalis* subsp. *lactis* BB-12 in the novel probiotic product, intended to be used as an infant formula, were examined *in vivo*, namely the survival and adhesion ability in the GIT of mice, the effect on the intestinal and faecal microflora and changes in the immune system.

## MATERIALS AND METHODS

### Preparation of the novel probiotic product with *B. animalis subsp. lactis BB-12*

The commercial strain *B. animalis* subsp. *lactis* BB-12 used in this study is available as Probio-Tec BB-12 Powder (Chr. Hansen A/S, Denmark). The inulin is commercially available as Raftilose Synergy 1 (Remy Industries n.v., Belgium). The novel probiotic product was prepared by mixing dehydrated wheat grits cereal flakes (Podravka, Croatia) with 0.25% (w/w) of a lyophilized culture of *B. animalis* subsp. *lactis* BB-12 and 2% (w/w) inulin in a laboratory mixer type M5R MK (Lödige, Germany) at 290 rpm/min for 5 min. For mouse feeding experiments, 20 g of probiotic product were dissolved in 200 mL of skim milk.

### Enumeration of *B. animalis* subsp. *lactis* BB-12

Survival of *B. animalis* subsp. *lactis* BB-12 in the novel probiotic product during 12 months of storage at 6° and

22°C, was determined by the MRS agar plate method. The plates were incubated anaerobically for 24 h at 37°C.

### The survival of *B. animalis* subsp. *lactis* BB-12 in simulated gastric and small intestinal juices

The simulated gastric and small intestinal juices were prepared according to KOS *et al.* (2000). Simulated gastric juice was prepared by suspending pepsin (3 g/L) in a sterile sodium chloride solution (0.5%) and the pH was adjusted to 2.5 with concentrated HCl. Pepsin (from porcine stomach mucosa) was obtained from Sigma Chemical Co (St. Louis, MO, USA). Simulated small intestinal juice was prepared by suspending pancreatin (1 g/L) and bile salts (1.5 g/L oxgall) in a sterile sodium chloride solution (0.5%) and the pH was adjusted to 8.0 with 0.1 mol/L NaOH. Pancreatin (from hog pancreas, 165 U/mg) was obtained from Fluka Biochemica (Buchs, Switzerland). Oxgall (dehydrated fresh bile) was obtained from Difco Laboratories (Sparks, MD, USA).

Lyophilized culture of *B. animalis* subsp. *lactis* BB-12 (0.005 g) was exposed to simulated gastric juice (50 mL). In parallel, probiotic product (2 g) and milk powder (2.8% milk fat; 2 g) (Dukat, Croatia) were also exposed to simulated gastric juice (50 mL). After exposure to simulated gastric juice (pH = 2.5, incubation time 2 h) the contents were transferred to simulated small intestinal juice (pH = 8.0, incubation time 4 h). The changes in the total viable count were monitored during the treatment with gastric (2 h) and small intestinal juice (4 h), respectively, by the pour plate method on MRS agar. Samples were taken every hour during the treatment with gastric juice and every 2 hours during treatment with small intestinal juice. The viable cell count was determined after 24 h of anaerobic incubation at 37°C. The results (log CFU/mL) are expressed as the mean of 3 replicates on MRS agar.

### Screening of bile salt deconjugation activity

To determine the bile salt hydrolase activity the strain *B. animalis* subsp. *lactis* BB-12 was maintained in MRS broth (Bio Life, Milan, Italy) at 37°C, under anaerobic conditions (Anaerocult A System, Merck, Darmstadt, Germany).

The ability of *B. animalis* subsp. *lactis* BB-12 to deconjugate bile salts was determined according to TARANTO *et al.* (1995). Bile salt plates were prepared by adding 5 mg/mL sodium salt of taurodeoxycholic acid (Fluka Biochemica, Buchs, Switzerland) to MRS agar (Biolife, Milan, Italy). One mL of the overnight *B. animalis* subsp. *lactis* BB-12 culture was spread on MRS agar and incubated for 48 h. In the presence of precipitated bile acids the colonies turn white, and this was considered a positive result.

### Quantitative determination of bile salt hydrolase (BSH) activity

BSH activity was determined by quantifying the amount of cholic acid released from the conjugated bile salt as indicated by WALKER and GILLILAND (1993), with minor modifications by ŠUŠKOVIĆ *et al.* (2000). Samples (3 mL) were taken every 2 h for the first 8 h and after 24 h of incubation in MRS broth with sodium salt 2 mg/mL taurocholic acid. Samples were mixed with 4 M NaOH (0.6 mL) and ethyl acetate (3.6 mL) and left for 15 min at room temperature in order to separate the phases. Aliquots of 2 mL of the upper ethyl acetate layers were transferred to clean tubes and evaporated until dryness at 60°C under a flow of nitrogen gas. One mL of 0.01 M NaOH was added to each tube to dissolve the residue. Six mL of 8 M $H_2SO_4$ were then added to each tube, followed by the addition of 1 mL of 1% furfuraldehyde. The content in the tubes was

mixed, heated for 13 min at 65°C in a water bath, and cooled at room temperature. Five mL of glacial acetic acid were added to each tube and mixed. The absorbance at 600 nm was measured, against a reagent blank, and compared with a standard curve to determine the concentration of free cholic acid. Growth of *B. animalis* subsp. *lactis* BB-12 during 24 h of cultivation was determined by the plate method using MRS agar. The results were expressed as a percentage of growth inhibition after 8 h of cultivation (end point of exponential growth phase of control culture) calculated as follows:

$$\text{Growth inhibition (\%)} = \\ = [(\log N_c - \log N_{bs}) / \log N_c] \times 100$$

$N_c$ viable cell count in the control after 8 h/viable cell count at the beginning of cultivation;

$N_{bs}$ viable cell count in medium with bile salt after 8 h/viable cell count at the beginning of cultivation.

Animals

Four-month-old female Swiss albino mice weighing from 22 to 24g were used after a month-long quarantine period. Each experimental group consisted of 4 mice, housed in a cage, kept in a controlled atmosphere (temperature 22±2°C; humidity 55±2%) with a 12 h light/dark cycle. Mice had continuous access to water and were fed *ad libitum* with standard rodent feed (Lab-feed, Sydney, Australia). All experimental procedures were carried out according to the standards set in the "Guide for the Care and Use of Laboratory Animal's of the National Research Council" (1996).

The influence of novel probiotic product on faecal and intestinal microflora of mice

Mice were fed orally with 200 μL of lyophilized culture of *B. animalis* subsp. *lactis* BB-12 alone, suspended in sterile 0.5% saline solution ($8x10^6$ CFU/200 μL) and 200 μL of the novel probiotic product suspended in skim milk ($8x10^6$ CFU/200 μL) for 4 consecutive days. The control group was fed 200 μL of sterile saline solution (0.5%). Survival of *B. animalis* subsp. *lactis* BB-12 during transit through the GIT of mice and the influence of novel probiotic product on the faecal microflora of mice were determined in faecal samples on days 1 and 8 after the feeding period.

For faecal bacterial count, 1 g of fresh faecal sample was collected *per* mouse and resuspended in 1 mL of sterile 0.5% saline solution. The suspension was plated with an appropriate dilution on non-selective medium: peptone yeast extract glucose agar (Biolife, Milan, Italy) and selective media: MRS agar (Biolife, Milan, Italy) for lactic acid bacteria (LAB) count, violet red bile glucose agar (Biolife, Milan, Italy) for *Enterobacteriaceae* count and sulfite agar (Biolife, Milan, Italy) for sulphite-reducing clostridia count. Plates were incubated for 48 h at 37°C, under aerobic conditions, with the exception of MRS agar plates and sulphite agar plates, which were incubated anaerobically (Anaerocult A System, Merck, Darmstadt, Germany). In addition, plates of non-selective medium were also incubated under anaerobic conditions.

The adhesion ability of the examined probiotic strain and the influence of the novel probiotic product on intestinal microflora of mice was determined in homogenates of the large intestine of Swiss albino mice on days 1 and 8 after the mice were fed for 4 consecutive days with probiotic strain of *B. animalis* subsp. *lactis* BB-12 alone, and in combination with infant cereal flakes and inulin. The length (5 cm) of the large intestine was gently rinsed with sterile 0.5% saline solution and homogenized using a Teflon homogenizer (1 g of tissue samples per mL of sterile 0.5% saline solu-

tion), and serially diluted before plating in non-selective and selective media as described in this section.

### Immunization

The oral immunization of mice with the probiotic bacteria and the novel probiotic product was performed 4 times over a period of 4 consecutive days. Mice were fed *ad libitum* as described in the previous section. On days 3, 7, 11 and 21, after the first immunization, blood samples were collected by bleeding the tail vein with heparinized capillaries into the tubes; the blood was allowed to clot at room temperature for 1 h and then left overnight at 4°C. Blood samples were centrifuged at 3,000 rpm/min for 20 min. The serum samples were kept at -20°C until used.

### Serum antibody determination (ELISA method)

The total antibody sera titres were determined in polystyrene microtitre plates (NUNC), using the method of ROGELJ *et al.* (1999), with some modifications by FRECE *et al.* (2005). Plates were first incubated with antimouse IgA (α-chain specific, Sigma M-1272; 200 ng/well), or antimouse IgG (γ-chain specific, Sigma M-1397; 200 ng/well), or antimouse IgM (μ-chain specific, Sigma M-8644; 200 ng/well) overnight at 4°C. To saturate the remaining binding sites, plates were incubated with 200 μL of 3% bovine serum albumin in phosphate-buffered saline (0.01 M, 0.15 M NaCl, pH = 7.2) (PBS) for 2 h at 37°C. After washing four times for 10 min with 0.05% Tween in PBS (T-PBS) (200 μL/well) the plates were incubated for 2 h at 37°C with dilutions of the sera (100 μL/well) in T-PBS. The plates were then washed 4 times in 0.05% T-PBS for 10 min and incubated in peroxidase-conjugated goat antimouse IgA (α-chain specific, Sigma A-4789), or antimouse IgG (Fc-specific, Sigma A-9309), or antimouse IgM (μ-chain specific, Sigma A-8786) antibodies diluted 1:10,000 in PBS (50 μL/well) for 2 h at 37°C. The plates were then washed 4 times with 0.05% T-PBS for 10 min (200 μL/well). The total antibody sera titres were estimated after the reaction with horse-radish peroxidase substrate o-phenylenediamine (ODP) dihydrochloride (Sigma P-8287) diluted in phosphate-citrate buffer (0.05 M, pH = 5) with 0.03% sodium perborate (100 μL/well). After 40 min the reaction was stopped with 0.2 M $H_2SO_4$ (50 μL/well) and absorption was determined at 450 nm with a Microplate rider (LKB 5060-006, "GDV", Rome, Italy).

### Statistical methods

All experiments were carried out in triplicate. The results are expressed as the mean±S.D. (standard deviation). The SAS statistical computer package was used to analyze the experimental data (SAS Institute, Cary, NC, USA). Values marked with asterisks are significantly different from the control group, according to the student's test ($P \leq 0.05$).

## RESULTS AND DISCUSSION

The viability and activity of probiotic bacteria during preparation and storage are important prerequisites for their industrial application. Probiotics can be added to functional products as fresh or lyophilized cells. High population levels, ranging from $10^6$ to $10^8$ microbial cells per mL or per g, should be present in probiotic products (SANDERS and HUIS IN'T VELD, 1999). The initial number of *B. animalis* subsp. *lactis* BB-12 in cereal flakes enriched with 2% inulin, was $4.45 \times 10^8$ CFU/g (Fig. 1). The viable cell count of *B. animalis* subsp. *lactis* BB-12 in this novel probiotic product, during 12 months of storage at 6°C was stable, but when stored at room temperature, the viable cell count decreased by 1.5 log units after 2 months of storage, and 2 log units after 12 months of stor-

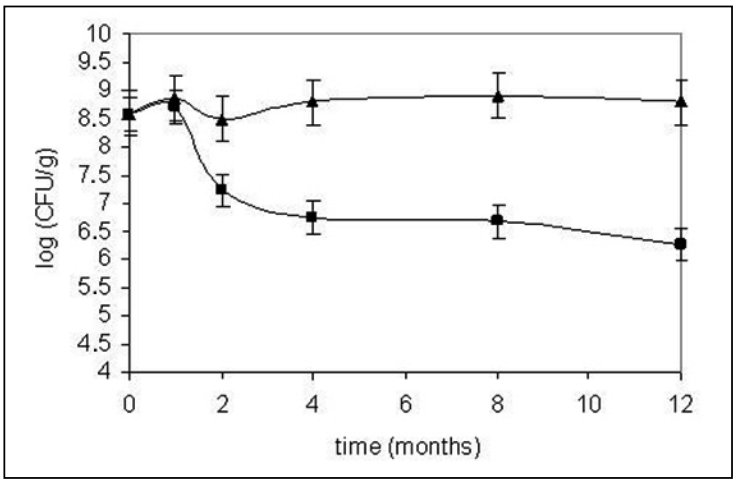



Fig. 1 - Viability of *B. animalis* subsp. *lactis* BB-12 in cereal flakes during 12 months of storage at: ▲ 6°C and ■ room temperature (22°C). Error bars represent standard deviations of the mean values of results from three replicates.

age. However, at the end of the storage period, the number of viable probiotic bacteria was still sufficient to satisfy the requirements of the probiotic product (SANDERS and HUIS IN'T VELD, 1999).

The influence of the applied food matrix on probiotic properties of *B. animalis* subsp. *lactis* BB-12 was examined. The probiotic strain was exposed to simulated gastrointestinal conditions as pure

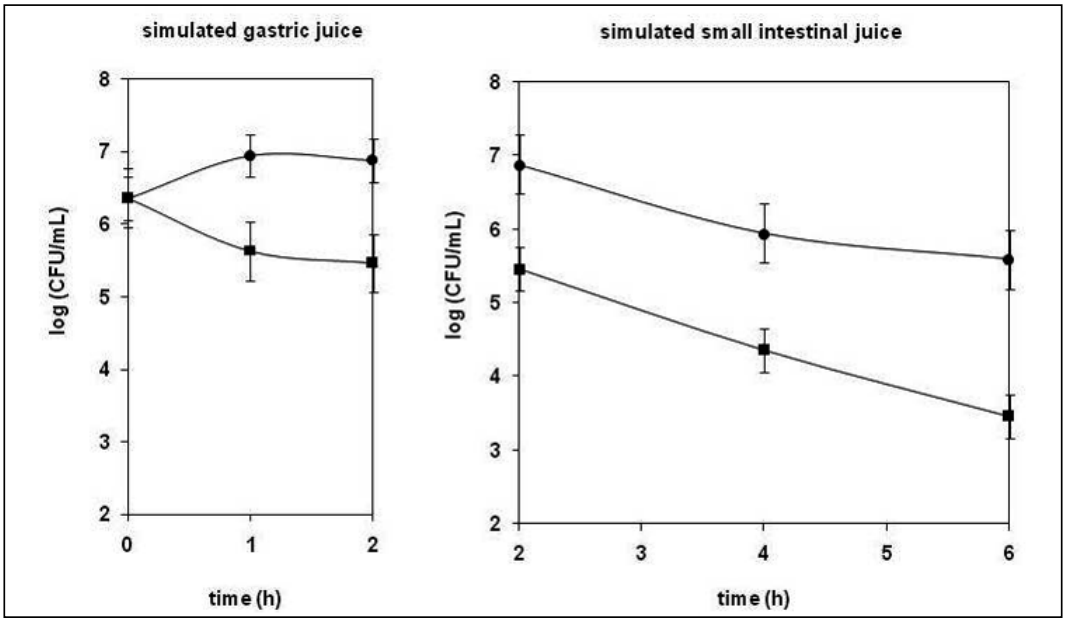



Fig. 2 - Cumulative effect of: a) – simulated gastric juice (pH = 2.5, incubation time 2 h) and b) – small intestinal juice (pH = 8.0, incubation time 4 h) on survival of *B. animalis* subsp. *lactis* BB-12; ● pure lyophilized culture of *B. animalis* subsp. *lactis* BB-12 (control); ■ *B. animalis subsp. lactis* BB-12 in cereal flakes enriched with inulin (2 g) + skim milk powder (2 g). Error bars represent standard deviations of the mean values of results from three replicates.

culture and incorporated in the novel probiotic product. The survival of probiotic culture was first investigated in simulated gastric juice (pH=2.5, residence time 2 h) (Fig. 2A). After 1 h of exposure to simulated gastric juice, the survival of *B. animalis* subsp. *lactis* BB-12 was 88% and after 2 h of exposure the viable cell count decreased 14% with respect to the initial viable cell count. In contrast, the number of viable *B. animalis* subsp. *lactis* BB-12 cells applied in the novel probiotic product, increased 9% after 1 h of exposure to simulated gastric juice, and decreased 1% after 2 h of exposure. The increase in the viable cell count of *B. animalis* subsp. *lactis* BB-12 in the novel probiotic product compared to the pure lyophilized culture of *B. animalis* subsp. *lactis* BB-12, in simulated gastric juice, can be explained by a symbiotic effect of probiotic bacteria and the food matrix, composed of cereal flakes, skim milk and prebiotic inulin. This effect could be attributed to the prebiotic activity of inulin. Some authors have recently reported that this prebiotic substrate can stimulate the bifidobacterial growth in the intestine of healthy volunteers (PEREZ-CONESA *et al.*, 2006; SU *et al.*, 2007; BOUHNIK *et al.*, 2007). The protective capacity of skim milk could be due to its function as a buffering agent and as an inhibitor of digestive protease activity *in vivo* (KOS *et al.*, 2000; LÖNNERDAL, 2003; DOLEYRES and LACROIX, 2005).

After 2 h of exposure to the simulated gastric juice, the survival of *B. animalis* subsp. *lactis* BB-12 was continuously examined in the simulated small intestinal juice (pH = 8.0, residence time 4 h). The conditions in the duodenum, where bile salts and pancreatic juices are secreted, have an even stronger inhibitory effect on the viability of bacteria. According to Sanders and Huis in't Veld (1999) the survival of probiotics during gastrointestinal transit is estimated to be 10-40%. The majority of Lactobacillus and Bifidobacterium strains are resistant to the simulated pancreatic juice, but have lower tolerances to the bile salts (CHARTERIS *et al.*, 1998). According to the general probiotic requirements, to be effective, probiotic bacteria should be resistant to 0.15-0.3% oxgall (ŠUŠKOVIĆ, 1996).

During the direct transit from simulated gastric juice (pH = 2.5) to simulated small intestinal juice (pH = 8.0, 0.15% (w/v) oxgall), only 54% of *B. animalis* subsp. *lactis* BB-12 cells of pure lyophilized culture survived, compared to the 88% survival of *B. animalis* subsp. *lactis* BB-12 in the novel probiotic product (Fig. 2A, B). The total number of viable cells after the transit through simulated gastrointestinal juices was approximately $4x10^5$ CFU/mL. This number of viable probiotic cells is sufficient to exert probiotic effect *in vivo* (SANDERS and HUIS IN'T VELD, 1999; CHARTERIS *et al.*, 1998). The results suggest that cereal flakes, inulin and skim milk act as a protective food matrix for *B. animalis* subsp. *lactis* BB-12.

The main barrier that prevents LAB from colonizing the GIT is the high bile salt concentration. Hence, tolerance to the bile salts is a prerequisite for colonization and metabolic activity of bifidobacteria in the small intestine of the host. Bile salt hydrolase (BSH) plays an important role in bile salt resistance because deconjugated cholic acid precipitates under acidic conditions (ŠUŠKOVIĆ *et al.*, 2000). The World Health Organization (WHO) has stated that the deconjugation of bile salts is one of the major activities of intestinal microbiota that is used to consider them as probiotic microorganisms (FAO/WHO, 2002). Therefore, *B. animalis* subsp. *lactis* BB-12 was screened for BSH on MRS agar plates supplemented with 0.5% (w/v) TDCA (sodium salt of taurodeoxycholic acid). There was no inhibition of bacterial growth compared to the control plates which had no TDCA added. The bacterial colonies of B. animalis subsp. lactis BB-12 became white because of the decon-

jugated deoxycholic acid precipitated as a consequence of the bacterial bile salt hydrolase activity (data not shown).

Previous studies have shown that the bile tolerance of bifidobacteria depended on the strain and bile, the resistance levels varies as bile concentrations ranged from 0.125 to 2.0% (MARGOLLES *et al., 2003;* LIAN *et al., 2003).* Some studies (NORIEGA *et al.*, 2006; VINDEROLA and REINHEIMER, 2003) have indicated that most of the examined bifidobacteria strains were not able to deconjugate primary bile salts, while other authors have reported BSH activity of bifidobacterial strains against the primary bile salts GCA (sodium salt of glycocholic acid) and TCA (LIONG and SHAH, 2005). In this perspective, the level of BSH activity of *B. animalis* subsp. *lactis* BB-12 was examined by growing the bacterial cells in MRS broth supplemented with 0.2% (w/v) TCA (sodium salt of taurocholic acid). As reported in Fig. 3, the growth inhibition of *B. animalis* subsp. *lactis* BB-12 after 8 h of incubation was 81%. This indicates that there is a higher toxicity of the primary bile salt TCA than the secondary salt TDCA (sodium salt of taurodeoxycholic acid) to *B. animalis* subsp. *lactis* BB-12 since the probiotic strain grew well in medium supplemented with 0.5% (w/v) TDCA (data not shown). More free cholic acid was produced during the stationary phase than in the exponential phase of growth in the presence of taurocholic acid. About 30% of taurocholate added to the growth medium was deconjugated after 24 h of incubation (Fig. 3).

Modulation of the composition and metabolic activity of the intestinal microflora are considered the major health benefits of probiotics. According to the literature (LIEVIN *et al.*, 2000; SHU *et al.*, 2000; BIBILONI *et al.*, 2001) bifidobacteria can colonize the intestinal tract and protect the host against pathogen infection. In this study the mouse was used as the animal model to study interactions between the gut microbes and the host. Survival of *B. animalis* subsp. *lactis* BB-12 during transit through the GIT of mice was determined in the faeces. The viable cell counts of LAB, determined in faecal samples of mice fed with the probiotic strain *B. animalis* subsp. *lactis* BB-12, were higher than in the control group of mice, and were the highest when mice were fed with the novel probiotic product (Fig. 4). In contrast, there was a significant decline in the viable cell count of enterobacteria and sulphite-reducing

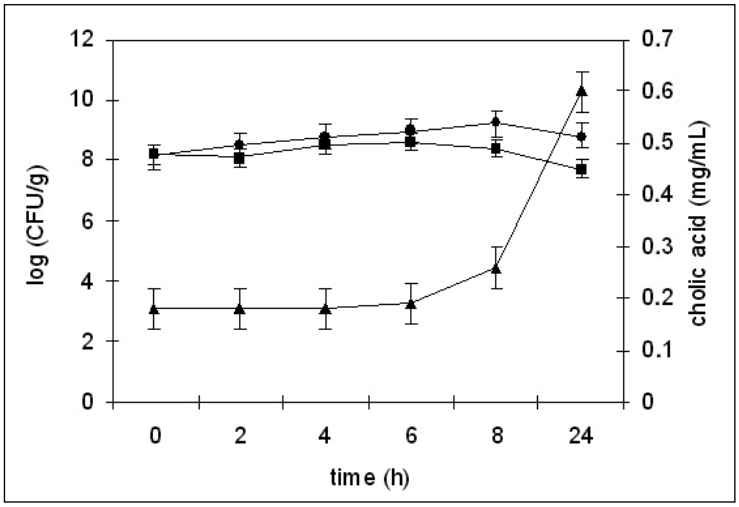



Fig. 3 - Bile salt hydrolase activity of *B. animalis* subsp. *lactis* BB-12 during 24 h of cultivation in: ● MRS-broth (control), ■ MRS – broth supplemented with 2 mg/mL TCA (sodium salt of taurocholic acid) and ▲ cholic acid concentration. Error bars represent standard deviations of the mean values of results from three replicates.

clostridia in faeces after the mice were fed with the probiotic strain *B. animalis* subsp. *lactis* BB-12 alone or in the novel probiotic product. These results demonstrate that *B. animalis* subsp. *lactis* BB-12 can interact and compete with other microorganisms within the gut environment and has a better effect on faecal microflora when applied in combination with inulin and cereal flakes.

To determine the ability of *B. animalis* subsp. *lactis* BB-12 to inhibit pathogens, and its influence on intestinal microflora, changes were observed in the composition of intestinal microflora when the mice were fed pure culture of *B. animalis* subsp. *lactis* BB-12 or with the novel probiotic product (Fig. 5). The number of enterobacteria and sulphite-reducing clostridia in the large intestine of mice was reduced when the probiotic strain was administered. Furthermore, the viable cell count of LAB increased in the large intestine even 8 days after *B. animalis* subsp. *lactis* BB-12 was administered, while the viable cell count of enterobacteria and sulphite-reducing clostridia decreased. These results could be due to the antibacterial activity of *B. animalis* subsp. *lactis* BB-12 that was also shown by in vitro experiments against different Gram-positive and Gram-negative microorganisms (data not shown). Better results were observed when *B. animalis* subsp. *lactis* BB-12 was applied in the novel probiotic product. This result could be due to the prebiotic effect of the inulin, on autochthonous LAB in the mouse intestine. Inulin probably selectively influenced the growth of LAB in the intestine and these bacteria could inhibit the enterobacteria by competitive exclusion in the mouse large intestine (Fig. 5).

Some possible competitive exclusion mechanisms of probiotic action include the direct attack of probiotic cells by producing antibacterial substances, competition for nutrients and receptors on the gut enterocytes and immune stimulation of the non-specific immune system (FRECE *et al.*, 2005). Therefore, the

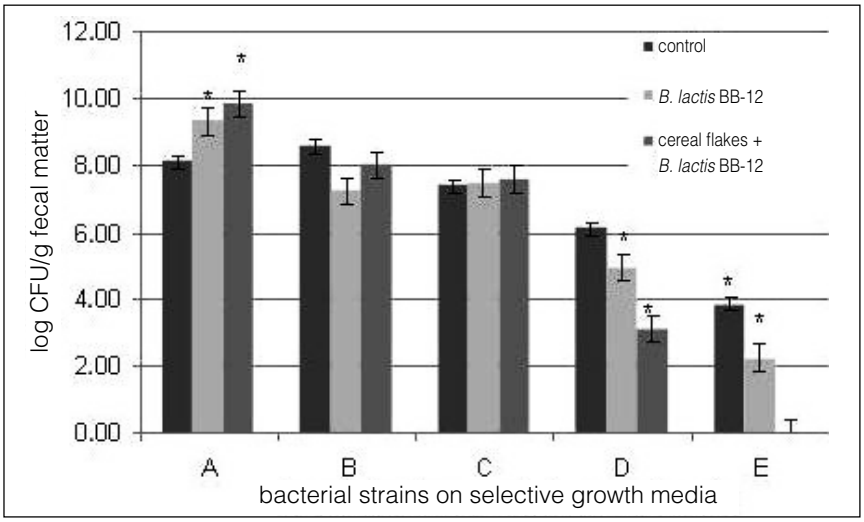



Fig. 4. Bacterial cell counts in faeces of mice on day 1 after feeding with *B. animalis* subsp. *lactis* BB-12 alone and in combination with cereal flakes and inulin: A – total lactic acid bacteria on MRS Agar (anaerobically); total aerobic (B) and anaerobic bacteria (C) on peptone yeast extract glucose agar; D – *Enterobacteriaceae* on violet red bile glucose agar; E – sulfite-reducing clostridia on sulfite agar. Error bars represent standard deviations of the mean values of results from three replicates.

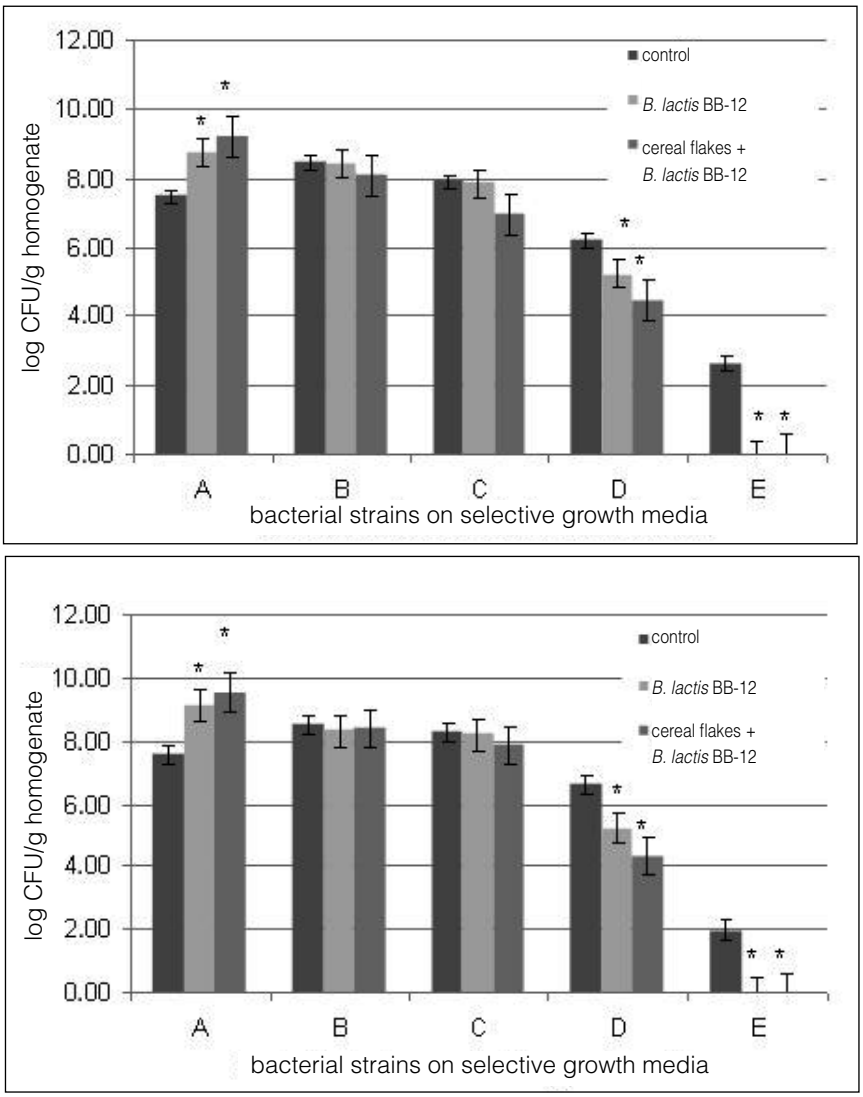
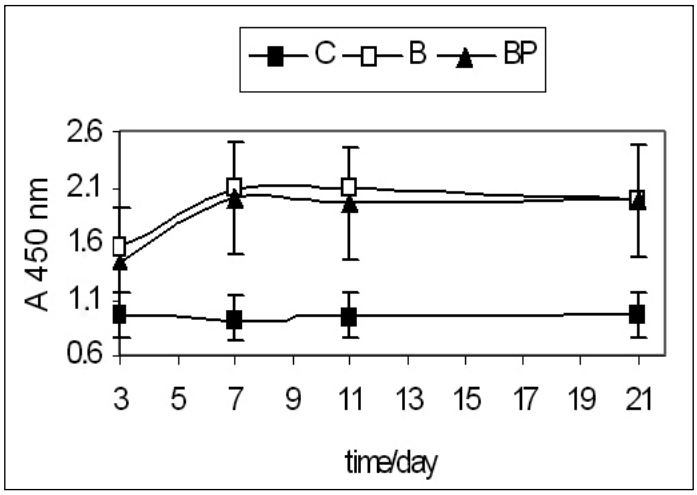
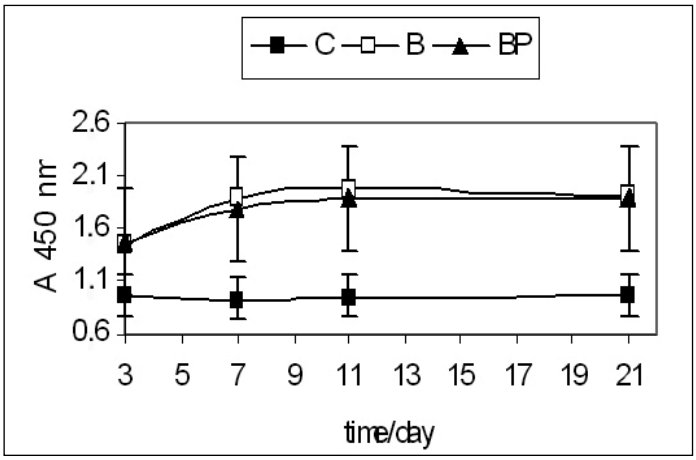



Fig. 5 - Bacterial cell counts in large intestine of mice on day 1 (a) and day 8 (b) after feeding with *B. animalis* subsp. *lactis* BB-12 alone and in combination with cereal flakes and inulin: A – total LAB on MRS Agar (anaerobically); total aerobic (B) and anaerobic bacteria (C) on peptone yeast extract glucose agar; D – *Enterobacteriaceae* on violet red bile glucose agar; E – sulfite-reducing clostridia on sulfite agar. Values marked with asterisks are significantly different from the control group, according to the student's test (P≤0.05). Error bars represent standard deviations of the mean values of results from three replicates.

immunomodulating capacity of *B. animalis* subsp. *lactis* BB-12 was examined by considering an induction of antibody production in Swiss albino mice by this strain. Oral immunization of mice with viable cells of *B. animalis* subsp. *lactis* BB-12 alone and in the novel probiotic product stimulated the humoral immune response (Fig. 6).

The levels of serum IgA, IgG and IgM



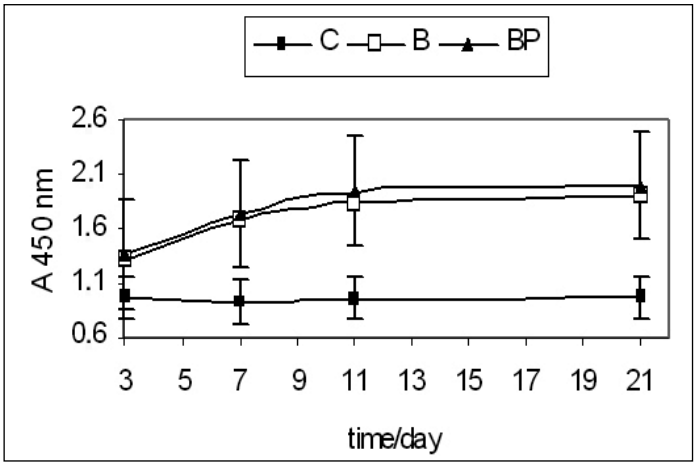




Fig. 6 - Determination of IgA (a), IgG (b) and IgM (c) antibodies in sera diluted 1:100 after oral immunization of mice with viable cells of *B. animalis* subsp. *lactis* BB-12 (B), and with viable cells of *B. animalis* subsp. *lactis* BB-12 in cereal flakes enriched with inulin (BP) by ELISA method. Control (C). Error bars represent standard deviations of the mean values of results from three replicates.

antibodies in all the groups of mice were significantly higher compared to the control groups. The cereal flakes enriched with inulin did not influence the immunomodulating capacity of the probiotic strain *B. animalis* subsp. *lactis* BB-12 (Fig. 6). These results are in agreement with those reported by FUKUSHIMA *et al.* (1999) who demonstrated that mice fed with bifidobacteria had significantly higher levels of total feacal IgA compared to those of the control group. In contrast, FRECE *et al.* (2009) showed that inulin positively influenced the imunomodulating capacity of the probiotic strain by increasing the levels of faecal and total serum IgA antibodies in mice. However, further_investigations based on human *in vivo* trials will be carried out to determine the influence of food matrix on the probiotic activity of *B. animalis* subsp. *lactis* BB-12 in the novel probiotic product.

## CONCLUSIONS

The results of this study suggest that the cereal flakes enriched with inulin positively influenced the functionality of *B. animalis* subsp. *lactis* BB-12. When this probiotic strain was applied in the novel probiotic product, the viable cell count of LAB was higher and the number of enterobacteria and sulphite-reducing clostridia was lower in the faecal and intestinal microflora of mice compared to the results obtained with pure lyophilized probiotic culture. Lyophilized probiotic cells remained viable (above $10^6$ CFU/g of probiotic product) during 12 months of storage at 6° and 22°C in the novel probiotic product. Cereal flakes, skim milk and inulin play a protective role and foster the survival of probiotic strain as was shown under simulated gastrointestinal conditions. The confirmed BSH activity of *B. animalis* subsp. *lactis* BB-12 could be a protective mechanism against the toxicity of bile salts in the human GIT.

## REFERENCES


Anal A. and Singh H. 2007. Recent advances in microencapsulation of probiotics for industrial applications and targeted delivery. Trends Food Sci. Technol. 18: 240.

Bibiloni R., Perez P.F., Garrote G.L., Disalvo E.A. and De Antoni G.L. 2001. Surface characterization and adhesive properties of bifidobacteria. Methods Enzymol. 336: 411.

Bouuhnik Y., Raskine L., Champion K., Andrieux C., Penven S., Jacobs H. and Simoneau G. 2007. Prolonged administration of low-dose inulin stimulates the growth of bifidobacteria in humans. Nutr. Res. 27: 187.

Charteris W.P., Kelly P.M., Morelli L. and Collins J.K. 1998. Development and application of an *in vitro* methodology to determine the transit tolerance of potencialy probiotic *Lactobacillus* and *Bifidobacterium* species in the upper human gastrointestinal tract. J. Appl. Microbiol. 84: 759.

Conway P.L., Gorbach S.L. and Goldin B.R. 1987. Survival of lactic acid bacteria in the human stomach and adhesion to intestinal cells. J. Dairy Sci. 70: 1.

FAO/WHO 2002. Drafting guidelines for the evaluation of probiotics in foods. Report of a joint FAO/WHO Working Group, London Ontario, Canada, April 30 and May 1, from http://www.who.int/foodsafety/fs_management/en/probiotic_guide-lines.pdf

Doleyres Y. and Lacroix C. 2005. Technologies with free and immobilized cells for probiotic bifidobacteria production and protection. Int. Dairy J. 15: 973.

Frece J., Kos B., Svetec I. K., Zgaga Z., Beganović J., Leboš A. and Šušković J. 2009. Synbiotic effect of *Lactobacillus helveticus* M92 and prebiotics on the intestinal microflora and immune system of mice. J. Dairy Res. 76: 98.

Frece J., Kos B., Svetec I.K., Zgaga Z., Mrša V. and Šušković J. 2005. Importance of S-layer proteins in probiotic activity of *Lactobacillus acidophilus* M92. J. Appl. Microbiol. 98: 285.

Fukushima Y., Kawata Y., Mizumachi K., Kurisaki J. I. and Mitsuoka T. 1999. Effect of bifidobacteria feeding on fecal flora and production of immunoglobulins in lactating mouse. Int. J. Food Microbiol. 46: 193.

Gueimonde M., Delgado S., Mayo B., Ruas-Madeido P., Margolles A., de los Reyes-Gavilán C.G. and Salminen S. 2005. Ability of *Bifidobacterium* strains with acquired resistance to bile to adhere to human intestinal mucus. Int. J. Food Microbiol. 101: 342.

Hoier E. 1992. Use of probiotic starter strains in dairy products. Food Aust. 44: 418.

Jonson E. and Conway P. 1992. Probiotics for Pigs. In: "Probiotics, The Scientific Basis", p. 259, R. Fuler (Ed.), Chapman & Hall, London.

Kailasapathy K. and Rybka S. 1997. *Lactobacillus*

*acidophilus* and *Bifidobacterium* spp. their therapeutic potential and survival in yoghurt. Aust. J. Dairy Technol. 52: 28.

Kos B., Šušković J., Goreta J. and Matošić S. 2000. Effect of protectors on the viability of *Lactobacillus acidophilus* M92 in simulated gastrointestinal conditions. Food Technol. Biotechnol. 38: 121.

Krasaekoopt W., Bhandari B. and Deeth H. 2003. Evaluation of encapsulation techniques of probiotics for yoghurt. Int. Dairy J. 13: 1.

Lian W.C., Hsiao H.C. and Chou C.C. 2003. Viability of microencapsulated bifidobacteria in simulated gastric juice and bile solution. Int. J. Food Microbiol. 86: 293.

Lievin V., Peiffer I., Hudault S., Rochat F., Brassart D., Neeser J.R. and Servin A.L. 2000. *Bifidobacterium* strains from resident infant human gastrointestinal microflora exert antimicrobial activity. Gut 47: 646.

Liong M.T. and Shah N.P. 2005. Acid and bile tolerance and the cholesterol removal ability of bifidobacteria strains. Biosci. Microflora 24: 1.

Lönnerdal B. 2003. Nutritional and physiological significance of human milk proteins. Am. J. Clin. Nutr. 77:1537S.

Margolles A., García L., Sánchez B., Gueimonde M. and de los Reyes-Gavilán C.G. 2003. Characterization of a *Bifidobacterium* strain with acquired resistance to cholate – a preliminary study. Int. J. Food Microbiol. 82: 191.

Noriega L., Cuevas I., Margolles A. and de los Reyes-Gavilán C.G. 2006. Deconjugation and bile salts hydrolase activity by *Bifidobacterium* strains with acquired resistance to bile. Int. Dairy J. 16:850.

National Research Council. 1996. "Guide for the Care and Use of Laboratory Animals". Institute of Laboratory Animal Resources, National Academy Press, Washington DC.

Pérez-Conesa D., López G., Abellán P. and Ros G. 2006. Fecal microbiota changes with the consumption of follow-up formulas containing *Bifidobacterium* spp. and/or galactooligosaccharides by rats and a follow-up infant formula containing *Bifidobacterium* spp. by human infants. J. Food Sci. 71: M7.

Rogelj L., Narat M. and Hočevar I. 1999. The immune responses in mice immunized with *Lactobacillus acidop*hilus LF221 – a potential probiotic strain. Food Technol. Biotechnol. 37: 153.

Sanders M.E. and Huis in't Veld J. 1999. Bringing a probiotic-containing functional food to the market: Microbiological, product, regulatory and labeling issues. Antonie van Leeuwenhoek 76: 293.

Shah N.P. and Lankaputhra W.E.V. 2002. *Bifidobacterium* spp: Morphology and physiology. In: "Encyclopedia of Dairy Sciences", p. 141, H. Roginski, J. W. Fuquay, P. F. Fox (Eds.), Academic Press, London.

Shu Q., Lin H., Rutherfurd K.J., Fenwick S.G., Prasad J., Gopal P.K. and Gill H.S. 2000. Dietary *Bifidobacterium lactis* (HN019) enhances resistance to oral *Salmonella typhimurium* infection in mice. Microbiol. Immunol. 44: 213.

Su P., Henriksson A. and Mitchell H. 2007. Prebiotics enhance survival and prolong the retention period of specific probiotic inocula in an *in vivo* murine model. J. Appl. Microbiol. 103: 2392.

Šušković J. 1996. The growth and probiotic effect of chosen lactic acid bacteria. Ph.D. Thesis, Faculty of Food Technology and Biotechnology, University of Zagreb, Croatia.

Šušković J., Kos B., Matošić S. and Besendorfer V. 2000. The effect of bile salts on survival and morphology of a potential probiotic strain *Lactobacillus acidophilus* M92. World J. Microbiol. Biotechnol. 16: 673.

Taranto M.P., de Ruiz Holgado A.P. and de Valdez G.F. 1995. Bile salt hydrolase activity in *Enterococcus faecium* strains. Microbiol. Alim. Nutr. 13: 375.

Temmerman R., Pot B., Huys G. and Swings J. 2003. Identification and antibiotic susceptibility of bacterial isolates from probiotic products. Int. J. Food Microbiol. 81: 1.

Truelstrup Hansen L., Allan-Wojtas P. M., Jin Y.L. and Paulson A.T. 2002. Survival of Ca-alginate microencapsulated *Bifidobacterium* spp. in milk and simulated gastronitestinal conditions. Food Microbiol. 19: 35.

Vinderola C.G. and Reinheimer J.A. 2003. Lactic acid starter and probiotic bacteria: A comparative *in vitro* study of probiotic characteristics and biological barrier resistance. Food Res. Int. 36: 895.

Walker D.K. and Gilliland S.E. 1993. Relationship among bile tolerance, bile salt deconjugation, and assimilation of cholesterol by *Lactobacillus acidophilus*. J. Dairy Sci. 76: 956.

Revised paper received February 27, 2009 Accepted May18, 2009

PAPER

# CHANGES IN PHYSICAL PROPERTIES OF CHITOSAN FILMS AT SUBZERO TEMPERATURES

CAMBIAMENTI NELLE PROPRIETÀ FISICHE DEI FILM DI CHITOSANO A TEMPERATURE SOTTOZERO

**Y. DENG[1,2,*], L.W. ZHU[2], W. LUO[2], C.L. XIAO[2], X.Y. SONG[2], and J.S. CHEN[2]**
[1]SJTU-Bor Luh Food Safety Center, Shanghai Jiao Tong University,
800 Dongchuan Road, Shanghai, 200240, P.R.China
[2]Department of Food Science and Engineering, Shanghai Jiao Tong University,
800 Dongchuan Road, Shanghai, 200240, P.R.China
*Corresponding author: Tel. + 86-21-34206613, Fax + 86-21-34206918
e-mail: y_deng@sjtu.edu.cn

ABSTRACT

The influence of frozen storage conditions (temperature and relative humidity) on water vapor permeability coefficients (WVPC), mechanical properties, color, transparency, thickness and moisture content of chitosan films were evaluated after 20 and 40 days of storage. Chitosan films (2% w/v) were prepared by casting using 25% glycerol (v/g chitosan) as plasticizer and 0.2% Tween 80 (v/v) as emulsifier. The films were stored in freezers at -20°C

RIASSUNTO

L'influenza delle condizioni di conservazione a temperature di surgelazione (temperatura e umidità relativa) sui coefficienti di permeabilità del vapor acqueo (WVPC), le proprietà meccaniche, il colore, la trasparenza, la durezza e l'umidità dei film di chitosano sono state valutate dopo 20 e 40 giorni di conservazione. I film di chitosano (2% p/v) sono stati preparati per colata usando 25% di glicerolo (v/g chitosano) come plasticizzante e 0,2% Tween 80 (v/v) come emul-

- Key words: chitosan, edible film, mechanical properties, subzero temperature, water vapor permeability -

and 82% relative humidity or at -50°C and 62% relative humidity. WVPC values decreased gradually from 2.4 to $1.5\times10^{-5}$ g•cm$^{-1}$•day$^{-1}$•kPa$^{-1}$ for the films stored at -20°C, however, WVPC of the films at -50°C declined from 8.2 to $4.1\times10^{-5}$ g•cm$^{-1}$•day$^{-1}$•kPa$^{-1}$ in about 34 days then increased to $18.5\times10^{-5}$ g•cm$^{-1}$•day$^{-1}$•kPa$^{-1}$. Tensile strength decreased after 20 days and then increased sharply after 40 days. Elongation (50.3-23.2%) decreased with storage time. No significant differences occurred between tensile strength and elongation values of the films stored at -20° and -50°C during the same test period (P<0.05). Elastic modulus evolution was similar to the changes observed in tensile strength change. The films became more yellowish as the temperature decreased and storage time increased. There were no differences in the color between films kept at -20° and -50°C (P<0.05). The transparency of the films kept at -50°C was about six times greater than that of the control and films kept at -20°C up to 40 days.

sionante. I film sono conservati in freezers a -20°C e 82% di umidità relativa o a 50°C e 62% di umidità relativa. I valori di WVPC sono diminuiti gradualmente da 2,4 a $1,5\text{x}10^{-5}$ g•cm$^{-1}$•day$^{-1}$•kPa$^{-1}$ per i film conservati a -20°C, mentre, il WVPC dei film a -50°C è diminuito da 8,2 a $4,1\text{x}10^{-5}$ g•cm$^{-1}$•day$^{-1}$•kPa$^{-1}$ in circa 34 giorni poi aumentato a $18,5\text{x}10^{-5}$ g•cm$^{-1}$•day$^{-1}$•kPa$^{-1}$. La forza tensile è diminuita dopo 20 giorni e poi aumentata bruscamente dopo 40 giorni. L'allungamento del film (50,3-23,2%) è diminuito con il tempo di conservazione. Non ci sono differenze significative tra la forza tensile e i valori di allungamento dei film conservati a -20° e -50°C durante lo stesso periodo di test (P<0,05). La valutazione del modulo elastico è stata simile ai cambiamenti osservati nel cambiamento della forza tensile. I film diventano più gialli con il diminuire della temperatura e l'aumento del tempo di conservazione. Non c'erano differenze nel colore tra i film mantenuti a -20° e -50°C (P<0,05). La trasparenza dei film mantenuti a -50°C era circa sei volte più grande di quella del controllo e dei film mantenuti a -20°C fino a 40 giorni.

## INTRODUCTION

Chitosan is an edible, biodegradable polymer derived from chitin, which is the major organic skeletal substance in the exoskeleton of arthropods, including insects, crustaceans, and some fungi (BUTLER *et al.*, 1996; CANER *et al.*, 1998). Chitosan is composed of N-acetyl-D-glucosamine units with β(1-4) glycosidic bonds and could be an ideal food preservative coating due to its good film-forming and mechanical properties. It is also nontoxic, biodegradable, and has antimicrobial action (NO *et al.*, 2007). The low cost and the multiple physical properties of chitosan are the major factors motivating studies on how it can be used to improve fresh and processed meat, sea food, poultry, and fruit and vegetables (LAZARIDOU and BILIADERIS, 2002; KILINCCEKER *et al.,* 2009).

All of the physical properties of films and coatings depend on the film formulation and forming procedure as well as the products to which it is applied and storage conditions (relative humidity and temperature). A considerable number of papers have been devoted to the study of development and preparation, preservative mechanisms and film

physical properties of the coating and films kept at room temperature (BUTLER *et al.*, 1996; OSÉS *et al.*, 2005; NO *et al.*, 2007). It is well known that temperature is an important variable affecting the physical and mechanical properties of films and coatings. The coating and film properties also generally vary over the same storage time due to the intrinsic instability of their raw materials (OSÉS *et al.*, 2005). In addition, at higher relative humidity (RH) conditions, physical strength decreases and oxygen permeability increases substantially. Changes in the temperature and relative humidity could affect film functionality on foods. Therefore, a high degree of stability of film properties over a long period storage is generally desired. However, there is limited information about the effect of storage conditions (especially a subzero temperature environment) on the physicochemical properties of the coating and films.

Several researchers have investigated the effects of storage conditions on water vapor permeability, mechanical properties, color, etc. at above-zero temperature. BUTLER *et al.* (1996) stated that no significant differences were found in the mechanical properties of chitosan films plasticized with glycerol after 9 weeks of storage at 23°C and 50% RH. In addition, there were no significant deviations in the tensile strength of chitosan films plasticized with polyethyleneglycol after a 12-week storage period at 23°C and 50% RH (CANER *et al.*, 1998). During a 16-week storage time, the mechanical and water barrier properties of glutenin films plasticized with glycerol changed markedly, while those of films plasticized with sorbitol or triethanolamine remained stable at 23°C and 50% RH (HERNÁNDEZ *et al.*, 2004). Whey protein isolate films plasticized with sorbitol became harder and less flexible during a 6-month storage period at room temperature and 50 or 75% RH, while storage time did not influence the appearance or mechanical properties of films plasticized with glycerol (OSÉS *et al.*, 2005). The effect of low temperature on the properties of coating and films has been reported by a few authors. KAMPER and FENNEMA (1984) stated that a bilayer film consisting of stearic and palmitic acids and hydroxypropyl methylcellulose had water vapor permeability values of $5.73\times10^{-7}$ g g•cm$^{-1}$•day$^{-1}$•kPa$^{-1}$ at 25°C and $1.15\times10^{-5}$ g•cm$^{-1}$•day$^{-1}$•kPa$^{-11}$ at -19°C. This group also noted that the stearic acid-palmitic acid emulsion film, at -20°C, essentially stopped the transfer of moisture during the 70-day storage period (KAMPER and FENNEMA, 1985). An edible film of lipids and cellulose ethers displayed good resistance to fracture at low temperatures, and the water vapor transmission values measured at 25°C showed only a slight increase after 3 and 9 weeks of storage at -40°C, compared to no low-temperature storage (KESTER and FENNEMA, 1989).

Although published studies indicate that the chitosan coatings can prolong the storage life of long-term frozen strawberries (HAN *et al.*, 2004), skinless pink salmon fillets (SATHIVEL, 2005; SATHIVEL *et al.*, 2007) and Atlantic cod and herring (JEON *et al.*, 2002), to date it is still not clear what influence the subfreezing conditions have on the physical properties of the chitosan coatings. Therefore, a more complete understanding of the influence that subzero temperature environments have on the film properties should help food scientists to further design and optimize film and coating formulations as well as film application systems suitable for frozen foods.

The objective of this work was to investigate the effects of subzero temperature conditions on the physical properties of chitosan coating including the mechanical properties, water vapor permeability, opacity, color parameters, film thickness and moisture content.

## MATERIALS AND METHODS

### Materials

Shrimp chitosan with a molecular weight of $1.5\times10^5$~$1.7\times10^5$ (90% deacetylation, viscosity of 200 cps, food-grade, water-soluble, odorless, and tasteless powder, moisture content ≤10% as stated by the manufacturer) was purchased from Nantong Xincheng Biological lndustrial Co. Ltd., Nantong, China. The chemicals used in this study were glycerol, Tween 80, glacial acetic acid and anhydrous calcium chloride (Sinopharm Chemical Reagent Co. Ltd., Shanghai, China), potassium bromide (Shanghai Jufeng Chemical Technology Co. Ltd., Shanghai, China), and NaOH (Shanghai Feida Co. Ltd., Shanghai, China).

### Film preparation

To prepare 2% (w/v) chitosan solution, 20 g chitosan were dispersed in 980 g distilled water and stirred for 10 min, and 20 g glacial acetic acid (at 4°C) were added to the mixture. After stirring overnight at room temperature, 25% glycerol (based on the weight of chitosan) and 0.2% Tween 80 (v/v) were added to the mixture which was then homogenized (SENCO S312, Shanghai SENCO Technology Co. Ltd, China) at 1,000 rpm for 10 min. To guarantee the stability of the emulsions, the pH was adjusted to 5.6 with 1.0 mol/L NaOH (VARGAS *et al.*, 2006). Finally, the solution was strained through 8 layers of cheesecloth and degassed under vacuum of 100 mmHg at room temperature. Chitosan film solutions were stored under refrigeration (4°C) for 1-2 days.

Film-forming solutions (ca. 200 g) were cast over level glass plates (280×280 mm). The castings were placed in a fume hood, which was maintained at 50 ± 5% RH and 23 ± 2°C for approximately 50 h. After drying the films were peeled from plates and cut into pieces for property analysis. Film sheets (27×80 mm) were used for the mechanical analysis, 12×44 mm sheets were used for transparency tests and moisture determination, 50×50 mm sheets for color evaluation, and 106×106 mm sheets for water vapor permeability tests.

### Frozen storage

Prior to testing or frozen storage trials, all of the cut film pieces were conditioned at 25°C and 50% RH for 48 h in a controlled temperature-humidity chamber (Binder, Im Mittleren Ösch 5 D-78532 Tuttlingen, Germany). The conditioned film specimens (except those for WVPC) were put into unsealed plastic bags and then stored in two freezers one at -20°C and the other at -50°C for 40 days. At 0, 20 and 40 days of storage, film samples from each treatment were removed from the freezer and the properties were analyzed immediately.

### Film thickness determination

The film thickness was determined using a manual digital micrometer (500-196-20, Mitutoyo, Japan) with a resolution of 0.01mm and an accuracy of 0.02 mm. The measurements were taken at 10 randomly chosen positions of each specimen and the mean values were used to estimate the cross-sectional area of the sample. Ten film samples per treatment were analyzed.

### Moisture determination analysis

Film samples were sampled at various time intervals, weighed (±0.0001 g) in aluminum dishes, then dried in an oven at 105°C until constant weight (for ca. 24 h). Moisture content was determined as percentage of initial film weight lost during drying and results are reported on wet basis. Determinations were performed on three film specimens stored

at each condition, the average and standard deviation are reported.

Film transparency analysis

The transparency of the chitosan film was measured using a spectrophotometric test cell (UV 1601, model CPS-240, Shimadzu, Kyoto, Japan) according to the method of CHOI and HAN (2002). The rectangular film piece (12×44 mm) was placed on the test cell, using an empty test cell as the reference. The film transparency was determined by the equation: Transparency = A600/ΔX, where, A600 is the value of absorbance at 600 nm, and ΔX is the average film thickness in mm.

Mechanical properties test

To obtain a broad understanding of the changes in mechanical characteristics of chitosan films under subzero temperature conditions, the puncture and tensile tests were performed using a texture analyzer TA-XT plus Texture Analyzer (Stable Micro Systems Ltd., Surrey, England, UK).

Tensile tests: Tensile strength (TS) at breaking and percentage of elongation (%E) at break were performed according to the ASTM (1996) D882-91 method. The frozen film strips (27×80 mm) were taken from the freezers and immediately mounted between the grips of the texture analyzer. The initial grip separation and the cross-head speed were set at 50 mm and 1.0 mm/s, respectively.

Puncture test: A 90 mm-diameter disk film was fixed on a plate with a hole of 10 mm diameter using a tape. A cylindrical probe of 1.0 mm diameter was moved downward perpendicular to the film surface at a speed of 0.8 mm/s until the probe penetrated through the film.

The tensile strength (MPa) for the tensile and puncture test was calculated by dividing the maximum force by the film cross-section area and by dividing the force at break by the probe cross-section, respectively. Maximum breaking force (N), percent elongation at break (deformation divided by initial probe length and multiplying by 100%), elastic modulus (slope of force–deformation curve, N/mm), breaking factor (maximum breaking force/film thickness, N/mm) and deformation at break (extension at the moment of rupture, mm) were obtained from force vs. deformation curves. Ten replicates were made per treatment.

Color measurement

Film color was determined using a Color Difference Meter (WSC-S, Shanghai Precise Scientific Apparatus, Shanghai, China). CIE-*Lab* coordinates were obtained from the reflection spectra of the samples using a D65 illuminant and the observer at 10°. Color parameters range from L = 0 (black) to $L$ = 100 (white); -$a$ (greenness) to +$a$ (redness), and -$b$ (blueness) to +$b$ (yellowness). Measurements were performed by placing the film sample over the standard. Samples were analyzed in triplicate, recording four measurements per sample.

Total color differences ($\Delta E$) were also calculated by the following equation:

$$\Delta E = \sqrt{(\Delta L)^2 + (\Delta a)^2 + (\Delta b)^2} \qquad (1)$$

where: $\Delta L = L - L_0$, $\Delta a = a - a_0$, $\Delta b = b - b_0$, with $L_0$, $a_0$, $b_0$ bing the color parameter values of the standard and $L, a, b$, the color parameter values of the sample.

Water vapor permeability analysis

Water vapor permeability (WVP) of the film specimens was determined according to a modified ASTM method (ASTM, 1983; KAMPER and FENNEMA, 1984). The circular opening of a permeation cell had an internal diameter of 8.42 cm, an external diameter of 9.22 cm and a depth of 1.26 cm with an exposed area of 55.68 cm$^2$. The film was cut circu-

larly with a 10.6 cm diameter and then sealed over the opening cell containing anhydrous calcium chloride (0% RH, 0 Pa water vapor partial pressure) using vacuum grease and two rubber O-rings. The film-covered cells were weighed with their contents and placed in three glass laboratory desiccators. One desiccator containing a saturated solution of potassium bromide was put in a temperature and RH controlled chamber (Binder, Im Mittleren Ösch 5 D-78532 Tuttlingen, Germany) providing a RH of 83% at 25°C (ca. 2632 Pa vapor partial pressure). Another one whose bottom was covered with a layer of ice was kept in a freezer (Sanyo MDF-135, Sanyo Electric Co. Ltd., Japan) maintaining a RH of 82.3% at -20°C (ca. 103 Pa vapor partial pressure). The last one whose bottom was covered with a layer of ice was placed in another freezer (REVCO ULT1386-3-V35, Kendro Laboratory Products, NC, USA) affording a RH of 62% at -50°C (ca. 4 Pa vapor partial pressure). The water vapor transferred through the films at different time intervals was determined from the weight gain of the cells. Cell weight, to the nearest 0.0001 g, was recorded at various times. All tests were conducted four times. The water vapor transmission rate (WVTR) and water vapor permeability coefficient (WVPC) were calculated according to KAMPER and FENNEMA (1984). The equations were as follows:

$$WVTR=\Delta W/(A\cdot\Delta t) \quad (2)$$

$$WVPC=(\Delta W\cdot\Delta X)/[A\cdot\Delta t\cdot(P_2-P_1)] \quad (3)$$

Where, $\Delta W$ is the weight of water absorbed in the cell (g), $\Delta t$ is the time for weight change (day), A is the area of film ($cm^2$), $P_1$ is the partial pressure inside the cell (kPa), $P_2$ the water vapor partial pressure at the film outer surface in the system (kPa), and $\Delta X$ is the average film thickness in cm.

Statistical analysis

The data were analyzed using ANOVA ($P<0.05$). Mean differences were established by the Duncan's multiple range test. The data were analyzed using SAS 8.0 statistical data analytical software.

## RESULTS AND DISCUSSION

### Water vapor permeability

Water vapor permeability (WVP) is a rather crucial issue because most natural biopolymers are very prone to plasticization of water which can modify the characteristics of the polymer matrix. The amount of water vapor that permeates per unit of area and time under specified temperature and humidity conditions is quantified by the water vapor permeability coefficient (WVPC). The effects of storage temperature and time on the mean WVPC of chitosan films are shown in Fig.1. During 40 days of storage, WVPC values decreased gradually from 1.6 to $0.3\times10^{-5}$ $g\bullet cm^{-1}\bullet day^{-1}\bullet kPa^{-1}$ for the films at 25°C and from 2.4 to $1.5\times10^{-5}$ $g\bullet cm^{-1}\bullet day^{-1}\bullet kPa^{-1}$ for the films stored at -20°C, respectively. For the films stored at -50°C, the WVPC values decreased rapidly from 8.2 to $5.8\times10^{-5}$ $g\bullet cm^{-1}\bullet day^{-1}\bullet kPa^{-1}$ in about 6 days, decreased slowly from the sixth to the thirty-fourth day, and then increased from 4.1 to $18.5\times10^{-5}$ $g\bullet cm^{-1}\bullet day^{-1}\bullet kPa^{-1}$. WVPC values for the chitosan films prepared in the present work are of the same order of magnitude as those of WONG *et al.* (1992) (chitosan 2%, $3.2\text{-}4.1\times10^{-5}$ $g\bullet cm^{-1}\bullet day^{-1}\bullet kPa^{-1}$), CANER *et al.* (1998) (chitosan 3%, $5.4\text{-}13.2\times10^{-5}$ $g\bullet cm^{-1}\bullet day^{-1}\bullet kPa^{-1}$) and GARCÍA *et al.* (2004) (chitosan 2%, $6.3\times10^{-5}$ $g\bullet cm^{-1}\bullet day^{-1}\bullet kPa^{-1}$). The higher WVPC values of the forty day samples can not be explained due to the limited data. BUTLER *et al.* (1996) found that WVPC of chitosan films that contained 0.50 mL/g glycerol decreased during storage (23°C and 50%

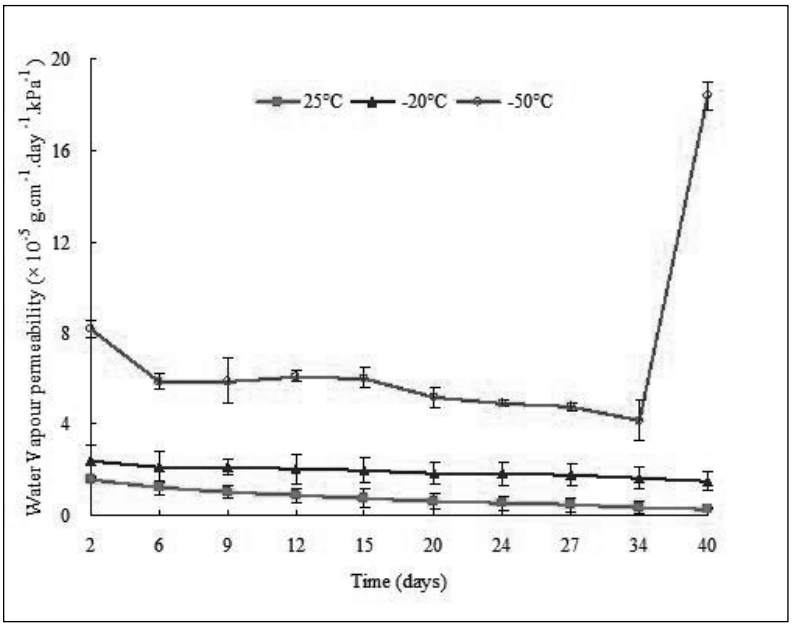



Fig. 1 - Effects of storage temperature and time on water vapor permeability of chitosan film. Data are presented as mean ± SD (n=4).

RH), while there were no changes in the films prepared with 0.25 mL/g glycerol. CANER *et al.* (1998) also observed that WVPC values of chitosan films made with acetic, formic, lactic, propionic acid and polyethylene glycol were independent on storage time at 22°C and 40-60% RH. No changes in water barrier properties of round scad (*Decapterus maruadsi*) protein-based film were observed throughout 4-weeks of storage at 54% RH and 28°-30°C (P>0.05) (ARTHARN *et al.*, 2009). These results confirmed that water vapor permeability of edible films is mainly influenced by chemical and structural factors, such as the composition and geometry of the films, and experimental conditions that can reduce molecular mobility (YOSHIDA *et al.*, 2003). As seen from the results, the storage temperature had a negative effect on the water vapor barrier property of the chitosan film, which may be dependent on and associated with the level of hydration around the polar groups and on the occurrence of low temperature fracturing in the film (NOGUCHI, 1981; KAMPER and FENNEMA, 1984).

At almost the same relative humidity, as the temperature decreased from 25° to -20°C, the WVPC values of the chitosan films increased approximately 1.5-5.2 times during storage. When the temperature was lowered to -50°C, WVPC values were 4.9-64 and 2.5-12.4 times higher than those of the films at 25° and at -20°C, respectively. Similarly, KAMPER and FENNEMA (1984) stated that the WVPC value of the C18-C16 E-Film increased as the temperature was raised above or lowered below 25°C. LABUZA and CONTRERAS-MEDELLIN (1981) observed that polyethylene packaging films are less permeable to water vapor at 35° than at -30°C. This increased permeability of the film at low temperatures may be due to increased hydration around polar groups in the films and from the occurrence of low temperature fractur-

Table 1- Changes in mechanical properties of chitosan films stored for 40 days at -20° and -50°C*.

| Treatments | Tensile properties | | | Puncture properties | |
|---|---|---|---|---|---|
| | Tensile strength (TS)(MPa) | Elongation at break (%) | Elastic modulus (N/mm) | Deformation (mm) | Elastic modulus (N/mm) |
| Initial | 21.2±7.2$^{b}$ | 50.3±4.9$^{a}$ | 2.43±0.69$^{b}$ | 4.72±0.19$^{a}$ | 4.36±0.03$^{b}$ |
| After 20 days | | | | | |
| -20°C storage | 18.3±3.6$^{bc}$ | 23.2±3.7$^{c}$ | 2.08±0.36$^{b}$ | 3.35±0.52$^{bc}$ | 3.02±0.99$^{c}$ |
| -50°C storage | 11.9±8.7$^{c}$ | 35.6±2.6$^{bc}$ | 0.47±0.39$^{c}$ | 3.28±0.18$^{c}$ | 3.11±1.01$^{bc}$ |
| After 40 days | | | | | |
| -20°C storage | 49.2±5.5$^{a}$ | 40.1±1.8$^{b}$ | 5.63±0.14$^{a}$ | 3.76±0.44$^{bc}$ | 7.89±0.22$^{a}$ |
| -50°C storage | 53.5±9.2$^{a}$ | 36.5±3.6$^{bc}$ | 5.96±1.23$^{a}$ | 4.08±0.71$^{b}$ | 7.45±0.94$^{a}$ |

*Data are presented as mean ± SD (n=10).
Values within a column followed by the same letter are not significantly different (P<0.05).

ing in the film (NOGUCHI, 1981; KAMPER and FENNEMA, 1984). Such an increase in permeation rates as a function of time is usually a sign of film deterioration.

Mechanical properties

The effects of storage temperature and time on the mechanical properties are compared in Table 1. It can be observed that the mean TS value of chitosan film prior to storage was ca. 21.2 MPa. While the values of the present results were higher than those obtained in previous research, the differences may be ascribed to chitosan composition and suppliers, plasticizer type and content, and film preparation method (BUTLER *et al.*, 1996 (14.6-18.7MPa); CANER *et al.*, 1998 (19.6MPa); GARCÍA *et al.*, 2004 (10MPa). The mean TS of frozen chitosan films decreased from 21.2 to 11.9-18.3 MPa after 20 days of storage, then increased significantly to 49.2-53.5 MPa after 40 days. The changes in TS may be associated with a change in density and reversibility of intermolecular interactions that occurred in the edible films from the chitosan network that was formed (BOURTOOM *et al.*, 2006). There were no significant differences in TS values between the temperatures of -20° and -50°C. In addition, elongation at break of these films decreased from 50.3% before storage to 23.2-40.1% after 40 days. No significant differences occurred in elongation values of the films stored at -20° and -50°C during the same test period. Elastic modulus evolution behaved similar to TS evolution for all films treated. With regard to puncture tests, the elastic modulus showed the same trend as in the tensile test. BUTLER *et al.* (1996) noted that the TS of films with 0.25 mL/g glycerol showed a slight increase during the fourth and eighth weeks of storage but fell again in the twelfth week, and no significant change occurred in elongation as a function of time. However, the films with 0.5 mL/g glycerol indicated a reduction in mean elongation as storage time increased, and no significant change in mean TS was observed over 12 weeks of storage at 23°C and 50% RH. CANER *et al.* (1998) stated that WVPC and TS of chitosan films with different acids were not dependent on time, but elongation (14-70%) decreased linearly with time during 9 weeks of storage. These different conclusions may be associated with chitosan composition, film-making conditions, storage temperature and humidity, etc.

The mechanical properties of films depend on the polymer architecture, average molecular weight and molecular ar-

rangement which is related to temperature, moisture, crystallinity, molecular weight, water diffusion, etc. Therefore, it is necessary to assess the mechanical performance and stability of biopolymers under subzero temperature conditions. The brittle characteristic of chitosan film may be responsible for an increment in tensile strength and tensile modulus of the films. Compared with the conditions at 25°C, the subzero temperature conditions decreased the degree of molecular mobility and caused cryo-concentrated effects that increased the chitosan concentration. On the other hand, water plasticization of chitosan film leads to increased intermolecular distance, reduced local viscosity and increased back-bone chain segmental mobility. These phenomena that occurred at subzero temperatures may be responsible for the property evolution of frozen chitosan films mentioned above. The reasons need to be investigated through structural and thermal property analysis.

### Color, transparency, thickness and moisture content

Color attributes are of prime importance because they directly affect consumer acceptability. The effects of storage temperature and time on *L*, *a* and *b* values of chitosan films were compared (Table 2). In relation to the control, the frozen films became darker as shown by the decreases in *L* values. The *a* values increased within the first 20 days and then no changes were observed in the control after 40 days ($P<0.05$). The *b* values increased were noted in all of the frozen films. These results indicate that the films became more yellowish as the temperature decreased and storage time increased. On day 20, there were no significant fluctuations in the total color difference ($\Delta E$) between the control and the films stored at -20°C. However, by day 60 the $\Delta E$ values decreased significantly. The changes in color could be ascribed to non-enzymatic browning reactions. The increase in free amino groups in chitosan may cause increased browning as shown by the increased yellowness of the film. On the other hand, glycerol in chitosan may be oxidized, to produce various products such as aldehydes which can react with amino group through the Maillard reaction. SOLOMON *et al.* (1995) reported that aldehydes from lipid oxidation might combine with amino acids and contribute to the non-enzymatic browning. During the same storage period, no significant differences in color parame-

Table 2 - Changes in color, transparency, thickness and moisture content of chitosan films stored for 40 days at -20° and -50°C*.

| Treatments | Color | | | | Transparency | Thickness (mm) | Moisture content (%) |
|---|---|---|---|---|---|---|---|
| | *L* | *a* | *b* | $\Delta E$ | | | |
| Initial | 88.91±0.59$^{a}$ | -2.33±0.09$^{c}$ | 10.98±0.14$^{c}$ | 18.05±1.65$^{ab}$ | 2.42±0.85$^{b}$ | 0.014±0.0083$^{a}$ | 31.26±1.45$^{a}$ |
| After 20 days | | | | | | | |
| -20°C storage | 86.71±0.41$^{ab}$ | -1.08±0.23$^{b}$ | 11.13±1.23$^{b}$ | 18.48±0.18$^{a}$ | 2.89±0.32$^{b}$ | 0.011±0.0010$^{a}$ | 31.81±0.74$^{a}$ |
| -50°C storage | 86.04±0.13$^{b}$ | -0.41±0.07$^{a}$ | 11.95±0.39$^{ab}$ | 17.92±0.45$^{ab}$ | 3.25±0.58$^{b}$ | 0.012±0.0003a | 30.60±0.45$^{a}$ |
| After 40 days | | | | | | | |
| -20°C storage | 85.87±0.28$^{b}$ | -2.36±0.01$^{c}$ | 12.45±0.36$^{a}$ | 16.76±0.19$^{b}$ | 12.86±1.38$^{a}$ | 0.011±0.0004$^{a}$ | 31.94±1.52$^{a}$ |
| -50°C storage | 85.59±0.55$^{b}$ | -1.87±0.11$^{c}$ | 11.84±0.69$^{ab}$ | 16.79±0.63$^{b}$ | 12.10±2.97$^{a}$ | 0.010±0.0008$^{a}$ | 31.68±0.74$^{a}$ |

*Data are presented as mean ± SD.
Values within a column followed by the same letter are not significantly different ($P<0.05$).

ters, except for the *a* value, were found between -20° and -50°C ($P<0.05$).

Compared to the control, the transparency values of frozen films increased slightly (but not significantly, $P<0.05$) during the first 20 days and then rose sharply. The transparency of films kept at -50°C were about six-times greater than that of the control and films kept at -20°C up to 60 days. Increased transparency values showed an increase in opacity, that was probably due to the orientation of polymers in the film matrix during storage. The transparency values of frozen films at the same test point were not affected by the different storage temperatures.

The thickness of films varied between 0.01 and 0.014 mm, and the moisture content ranged from 30.6 to 31.94% (Table 1). As expected, changes in frozen storage conditions did not significantly affect film thickness and moisture content ($P<0.05$).

## CONCLUSIONS

This study has shown that the physical properties of chitosan films can be modified by the temperature and relative humidity used in frozen storage. In general, the present data show a decrease in WVPC, elongation, *L* value and total color difference and an increase in b values and opacity with respect to storage time. Compared with the control, the subzero-temperature films had a higher water vapor permeability coefficient, tensile strength and modulus, and transparency after 40 days of storage. During the first 20 days, there were no significant differences in tensile strength, elastic modulus, *L* and $\Delta E$ values and transparency between the controls and the films stored at- 20°C. No significant deviations were observed in tensile strength, elongation, modulus, color parameters and transparency between storage temperature of -20° and -50°C. The higher WVPC values of the forty-day films stored at -50°C could not be explained due to the limited data. In order to clearly explain this phenomenon, further studies of oxygen permeability and microstructure and thermal properties of chitosan films at subzero temperature are needed.


## ACKNOWLEDGMENTS

This research was supported by the Science Foundation for The Youth Scholars of the School of Agriculture and Biology of SJTU.


## REFERENCES


Artharn A., Prodpran T. and Benjakul S. 2009. Round scad protein-based film: storage stability and its effectiveness for shelf-life extension of dried fish powder. Lebensm.-Wiss.Technol. 42:1238.

ASTM. 1983. "Standard Test Methods for Water Vapor Transmission of Materials". Annual Book of ASTM Standards. American Society for Testing and Materials, Philadelphia, PA.

ASTM 1996. "Standard Test Methods for Tensile Properties of Thin Plastic Sheeting". Annual Book of ASTM Standards. American Society for Testing and Materials, Philadelphia, PA.

Bourtoom T., Chinnan M.S., Jantawat P. and Sanguandeekul R. 2006. Effect of plasticizer type and concentration on the properties of edible film from water-soluble fish proteins in surimi wash-water. Food Sci. Technol. Int. 12:119.

Butler B.L., Vergano P.J., Testin R.F., Bunn J.M. and Wiles J.L. 1996. Mechanical and barrier properties of edible chitosan films as affected by composition and storage. J. Food Sci. 61: 953.

Caner C., Vergano P.J. and Wiles J.L. 1998. Chitosan film mechanical and permeation properties as affected by acid, plasticizer, and storage. J. Food Sci. 63:1049.

Choi W.S. and Han J.H. 2002. Film-forming mechanism and heat denaturation effects on the physical and chemical properties of pea-protein-isolate edible films. J. Food Sci. 67: 1399.

García M.A., Pinotti A., Martino M.N. and Zaritzky M.E. 2004. Characterization of composite hydrocolloid films. Carbohydrate Polym. 56: 339.

Han C., Zhao Y., Leonard S.W. and Traber M.G. 2004. Edible coatings to improve storability and enhance nutritional value of fresh and frozen strawberries (*Fragaria* × *ananassa*) and raspberries (*Rubus ideaus*). Postharvest Biol. and Technol. 33: 67.

Hernández P., Rubio A., Del Valle V., Almenar E. and Gavara R. 2004. Mechanical and water barrier properties of glutenin films influenced by storage time. J. Agric. Food Chem. 52:79.

Jeon Y.I., Kamil J.Y.V.A. and Shahidi F. 2002. Chitosan as an edible invisible film for quality preservation of herring and Atlantic cod. J. Agric. Food Chem. 20: 5167.

Kamper S.L and Fennema O. 1984. Water vapor permeability of an edible, fatty acid, bilayer film. J. Food Sci. 49: 1482.

Kamper S.L. and Fennema O. 1985. Use of an edible film to maintain water vapor gradients in foods. J. Food Sci. 50: 382.

Kester J.J. and Fennema O. 1989. An edible film of lipids and cellulose ethers: barrier properties to moisture vapor transmission and structural evaluation. J. Food Sci. 54: 1383.

Kilincceker O., Dogan S. and Kucukoner E. 2009. Effect of edible coatings on the quality of frozen fish fillets. Lebensm.-Wiss.Technol.42: 868.

Labuza T.P. and Contreras-Medellin R. 1981. Prediction of moisture protection requirements for foods. Cereal Food World, 26: 335.

Lazaridou A. and Biliaderis C.G. 2002. Thermophysical properties of chitosan, chitosan-starch and chitosan-pullulan films near the glass transition. Carbohydrate Polym**.** 48: 179.

No H.K., Meyers S.P., Prinyawiwatkul W. and Xu Z. 2007. Applications of chitosan for improvement of quality and shelf life of foods: a review. J. Food Sci. 72: R87.

Noguchi H. 1981. Hydration around hydrophobic groups. In "Water Activity: Influences on Food Quality". Rockland L.B. and Stewart G.F. (Ed.). p. 281. Academic Press, Inc. New York.

Osés J., Fernández-Pan I., Mendoza M. and Maté J.I. 2009. Stability of the mechanical properties of edible films based on whey protein isolate during storage at different relative humidity. Food Hydrocolloid. 23: 125.

Sathivel S. 2005. Chitosan and protein coatings affect yield, moisture loss, and lipid oxidation of pink salmon (*Oncorhynchus gorbuscha*) fillets during frozen storage. J. Food Sci. 70: 455.

Sathivel S., Liu Q., Huang J. and Prinyawiwatkul W. 2007. The influence of chitosan glazing on the quality of skinless pink salmon (*Oncorhynchus gorbuscha*) fillets during frozen storage. J. Food Eng. 83: 366.

Solomon O., Svanberg H. and Sahlstr M.A. 1995. Effect of oxygen and fluorescent light on the quality of orange juice during storage at 8°C. Food Chem. 53: 363.

Vargas M., Albors A., Chiralt A. and Gonźalez-Martínez C. 2006. Quality of cold-stored strawberries as affected by chitosan-oleic acid edible coatings. Postharvest Biol. Technol. 41: 164.

Wong D.W.S., Gastineau F.A., Gregorski K.S., Tillin S.J. and Pavlath A.E. 1992. Chitosan lipid films: Microstructure and surface energy. J. Agric. Food Chem. 40: 540.

Yoshida C.M.P., Antunes A.C.B., Antunes L.J. and Antunes A.J. 2003. An analysis of water vapour diffusion in whey protein films. Int. J. Food Sci. Technol. 38: 595.

Revised paper received April 5, 2009 Accepted June 3, 2009

PAPER

# APPLICATION OF FOOD SAFETY AND QUALITY MANAGEMENT SYSTEMS TO BULGUR PROCESSING

APPLICAZIONE DI SISTEMI DI SICUREZZA ALIMENTARE E GESTIONE QUALITÀ AL PROCESSO DI PRODUZIONE

**K.B. BELİBAĞLI*, H. VARDİN[1], and A.C. DALGIÇ**
Faculty of Engineering, Department of Food Engineering, University of Gaziantep, 27310 Gaziantep, Turkey
[1]Faculty of Agriculture, Department of Food Engineering, University of Harran, Şanlıurfa, Turkey
*Corresponding author: Fax +90 342 3601105, e-mail belibagli@gantep.edu.tr

ABSTRACT

Bulgur is precooked whole wheat which has a high level of energy nutrients and is a natural source of vitamins and minerals. Bulgur is generally produced in small- and medium-sized factories. Some health and safety problems arise with respect to the raw material, contamination, and production environment. Implementation of the quality and safety management system in the production of bulgur is a valuable tool for improving the safety and quali-

RIASSUNTO

Il Bulgur è un alimento precotto a base di frumento integrale che possiede nutrienti ad alto potere energetico ed è una fonte naturale di vitamine e minerali. Il Bulgur è prodotto generalmente in piccole e medie aziende. Alcuni problemi di salute e sicurezza insorgono per quanto riguarda la materia prima, l'ambiente di produzione e la produzione stessa. L'implementazione di un sistema di gestione della qualità e della sicurezza nella produ-

- Key words: bulgur, food safety management system, HACCP, ISO 9000, ISO 22000, quality management system -

ty characteristics of bulgur. Hazard assessment is considered as a five-class hazard score matrix. Prerequisite programs, critical control points, critical limits, preventive, corrective actions and quality control points of bulgur processing are discussed.

zione del Bulgur è un prezioso strumento per migliorarne la sicurezza e la qualità. Nella valutazione del rischio la matrice è considerata come rischio di classe 5. Vengono discussi i prerequisiti, i punti critici di controllo, le azioni preventive e correttive e i punti di controllo della qualità della lavorazione del Bulgur.

## INTRODUCTION

The hazard analysis critical control points (HACCP) system is a systematic approach used to determine the hazards related to food, identify critical control points (CCP) and get them under control. In the food industry, the HACCP program combined with prerequisite programs (PrPs) is currently recognized as the best approach to control food safety (ARVANITOYANNIS and VARZAKAS, 2009). In addition, some processes (documentation and record processes, internal auditing processes, etc.) applied with the ISO 9000-Quality Management System (QMS) standard can be used with the HACCP system. The ISO 22000-Food Safety Management System (FSMS) standard is being introduced as a way to organize all of these requirements. The goal is to use it as a single standard throughout the world (ISO, 2005).

Bulgur is a cleaned, washed, parboiled, debranned, crushed, and sifted wheat product. Bulgur is an excellent ingredient in salads, soups, baked goods, stuffings, casseroles, and as a meat substitute in vegetarian dishes. It is widely consumed in Central Asia, the Middle East, North Africa and eastern European countries (YU and KIES, 1993, CERTEL, 1998).

The ancient method of producing bulgur, which is referred to as Arisah in the Old Testament, consists of boiling whole wheat in open vessels until it becomes tender. The cooked wheat is then spread out in thin layer to dry in the sun. The outer bran layers are removed by sprinkling with water and rubbing by hand. This is followed by cracking the grains with a stone or in a crude mill (KENT, 1990). Modern mechanized bulgur processing has been developed on the basis of the ancient steps of bulgur-making. The bulgur processing methods are nearly the same in all countries around the world. The technologies used and the properties of the raw material affect the quality of the end product. There are two kinds of problems in bulgur processing, quality and safety hazards. Quality hazards include color, texture, flavor and size, while safety hazards are toxins, metals, pathogens, etc.

Food safety and quality are affected by some problems related to administration, supplier, production technologies, working environment, human resources, and control activities. All of these affect the complete production process starting from the raw material to the final product. To have a successful operation to obtain products with the desired quality and safety assurance it is necessary to understand the critical control points, the processing factors, and their effects on product quality. To date no information has been published con-

cerning the safety and quality assurance of bulgur processing. Therefore, this study was undertaken to investigate the quality and safety assurance of bulgur production based on the HACCP concept. This study also focused on the following; safety hazard analysis, cause analysis and hazard assessment, CCPs and HACCP plan for "Food Safety Management System" standard, and quality hazard analysis, quality control points and quality plans for the "Quality Management System" standard. The integration of prerequisite programs and quality processes is also discussed for HACCP and quality plans in bulgur processing.

## QUALITY AND SAFETY CHARACTERISTICS OF BULGUR PROCESSING

### Product characteristics

Bulgur is a nutritious, versatile wheat product. The nutritive composition of wheat and bulgur is shown in Table 1. The nutritive value of bulgur is similar to that of wheat. However, the fat, ash and crude fiber levels are slightly lower than those of wheat. The cooking process affects the mineral content, and there is a 70% retention of riboflavin, thiamine and niacin levels (HAYTA, 2001; ÖZBOY and KÖKSEL, 1998; KOCA and ANIL, 1996; TSE, 2001, 2003).

### Bulgur processing

Bulgur is a pre-cooked wheat product that has been cleaned, cooked, dried, ground into particles and sifted into distinct sizes. It has good storage properties due to enzyme inactivation during cooking. Bulgur has gained importance as a substitute for rice in the western countries. Bulgur is generally produced from *Triticum durum* because of its good milling properties. Foreign grain seeds in the raw material could be addressed as the first problem in bulgur processing. It is still produced using traditional ways. In most cases, it is not produced under hygienic conditions and has a lower quality criteria in color, size, shape and taste.

Modern bulgur processing lines are slowly replacing these old methods. A flow diagram for basic bulgur processing is shown in Fig. 1. The basic system for processing bulgur takes place in three stages. The first stage involves pre-cleaning; foreign materials such as weed seeds, non-grain materials, damaged and shriveled kernels, etc. are removed by sieves, washers, and separators. Washing removes dust and dirty materials and centrifuging removes excess water. In this step the moisture content of the wheat can be increased up to 15-17%.

The second stage involves tempering, cooking, and drying. Tempering is the controlled addition of water to the wheat in order to soften the endosperm, facilitate separation of the bran, and toughen the bran. The basic reason for cooking the wheat is to convert the starch into a digestible form by gelatinization. The cooking process varies from using atmospheric cooking (95°C) in small enterprises to using pressurized cookers in larger facilities. During cooking, temperatures below 95°C are preferred in order to minimize the loss of nutritional compounds. There should be no white spots on the cooked wheat and gelatinization of starch has to be completed by the cooking process. The amount of water that is added is important. The moisture content must be grater than 40%. If the moisture content of the kernel is less than 40%, white spots appear in the kernel, which are ungelatinized starch. If an insufficient amount of water added is added, complete gelatinization will not be achieved. If too much water is added there will be a loss of nutritional compounds, especially vitamin B, that will occur during decantation of water. Drying an important step in bulgur produc-

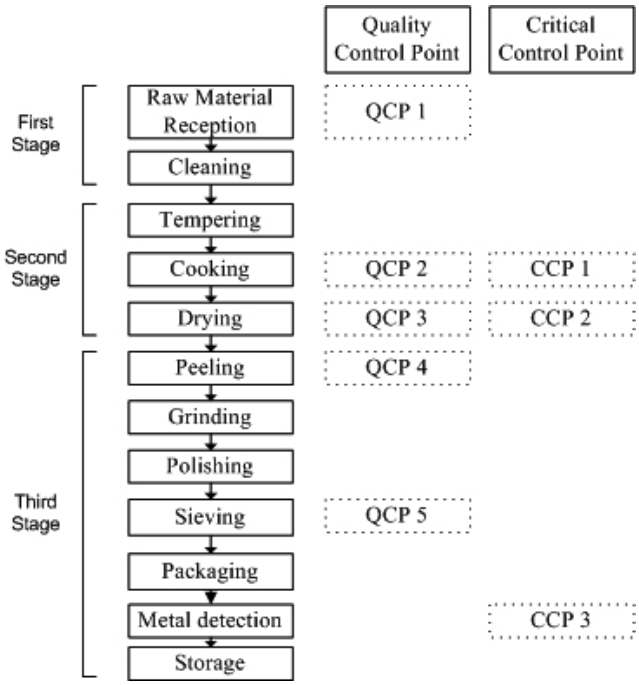



Fig. 1 - Flow diagram of bulgur processing.

tion is carried out by fluidized bed, rotary dryer, sun drying, tower, and tunnel dryers (HAYTA, 2001). Cooked bulgur is usually dried in a tunnel drier with a hot air stream at 60°C until a 10-12% of moisture content is achieved (YILDIRIM, 2004; BAYRAM, 2005; BAYRAM and ÖNER, 2002). Mechanical dryers are more sanitary than the traditional sun drying method.

The last stage consists of dehulling, grinding, polishing, and classification. In the dehulling process, bran is removed from the cooked wheat by abrasives or stone mills. The grains are than ground to produce small particles; the use of roller milling equipment has become widespread, but color and shape problems sometimes occur. The size and shape of bulgur is always important. Oval and smooth shapes are preferred. Screening and classification are the next steps. Color sorting equipment may be applied. The color of bulgur should be light yellow and homogeneous. The polishing step is not always used since bulgur is nutritionally damaged during polishing when a cylindrical system is used to polish the product to obtain a good yellow color.

There could be mold growth if the wheat has gotten wet many times or if it was wet for an extended time (FAO/WHO, 2001; GIRAY *et al.*, 2007). A study indicated that *Aspergilus* spp., mostly *Aspergilus flavus,* was detected in wet samples. In another report low levels *Aspergilus flavus* and detectable aflatoxin levels were found (ÇOKSÖYLER *et al.*, 1993).

## FOOD SAFETY SYSTEM FOR THE BULGUR PROCESSING

Food safety is becoming increasingly important in the food industry. The main reason is the susceptibility of food products to microbiological, physical,

and chemical hazards. For the food industry, the HACCP program is currently recognized as the best approach for food safety control (MORTIMORE and WALLACE, 1998; ILSI EUROPE, 2004; CODEX ALIMENTARIUS COMMISSION, 2003). ISO 22000 focuses exclusively on food safety and tells food producers how to build a food safety system.

The HACCP system had been required in the operational applications such as Good Manufacturing Practice (GMP), Good Hygienic Practice (GHP), and Sanitation Standard Operating Procedures (SSOP) before the ISO 22000-FSMS Standard was published. The ISO 22000-FSMS Standard has included and organized the definition and contents of these applications. The prerequisite programs, in this study, are classified as strategic, operational and supportive programs (Table 2). ISO 22000-FSMS and ISO 9000-QMS have the same framework properties. The standards have been built on customer focus, continuous improvement, and process approach. Fig. 3 shows the properties and similarities of the standards. The ISO 22000-FSMS standard includes HACCP principles with PrPs (ISO, 2005). The prerequisite programs and processes that are required by both ISO 9000-QMS and ISO 22000-FSMS are shown in Table 2. The documentation and record-keeping processes (QP 3), which are the fundamental items in both standards, are listed in article number 4. The planning (QP 4) and internal communication (QP 2) are in article number 5. Resource management process (QP 5) is in article number 6. Bulgur production (QP 7) and purchasing processes (QP 8) are in article number 7. Internal audit (QP 9) and data analysis processes (QP 10) are in article number 8. Therefore, these processes are common to both standards.

Table 1 - Nutrient composition of wheat and bulgur (value per 100 g).

| | Wheat (durum) | Bulgur |
|---|---|---|
| Energy, kcal | 339.00 | 342.00 |
| Water, g | 10.94 | 9.00 |
| Protein, g | 13.68 | 12.29 |
| Fat, g | 2.47 | 1.33 |
| Carbohydrate, g | 71.13 | 75.87 |
| Fiber, total dietary, g | No data | 18.30 |
| Ash, g | 1.78 | 1.51 |
| Mineral | | |
| Calcium, mg | 34.00 | 35.00 |
| Phosphorus, mg | 508.00 | 300.00 |
| Iron, mg | 3.52 | 2.46 |
| Zinc, mg | 4.16 | 1.93 |
| Vitamin | | |
| Thiamin, mg | 0.419 | 0.232 |
| Riboflavin, mg | 0.121 | 0.115 |
| Niacin, mg | 6.738 | 5.114 |
| Pantothenic acid, mg | 0.935 | 1.045 |

Source: Anon, 2005.

In this study, hazard analysis, hazard assessment, and HACCP plans are determined according to the ISO 22000-FSMS standard. The details of all of the processes required and prerequisite programs are not given. Only the most important ones which could affect bulgur processing are discussed. Bulgur processing is mostly affected by the (PR 1), (PR 2), and (PR 3) programs.

## Hazard analyses and hazard assessment

Hazard analysis begins with identification of the food safety hazards associated with the raw material, and establishment of a priority list that is given in Table 3. First, a complete list of hazards that could potentially be of concern is drawn up. Cause analysis is based on determining potential hazard sources and classifying the causes (ARVANITOYANNIS and TRAIKOU, 2005). The failure mode and effect analysis (FMEA) model has been applied for the risk assessment of potato chip manufacturing (ARVANITOYANNIS and VARZAKAS, 2007a). The cause and effect diagram (also known as Ishikawa,

Table 2 - Some quality processes and prerequisite programs in food processing plants.

| | Strategic | Operational | Support |
|---|---|---|---|
| ISO 9000:2000-QMS | QP.1 Market Research and Customer Relation<br>QP.2 Internal Communications<br>QP.3 Document and record Control<br>QP.4 Planning<br>QP.5 Resources Management | QP.6 Product Design<br>QP.7 Bulgur Processing | QP.8 Purchasing<br>QP.9 Internal Audit<br>QP.10 Data Analysis<br>QP.11 Maintenance of measurement and process equipment<br>QP.12 Calibration of measurement equipment |
| ISO 22000:2005-FSMS | PR.1 Construction and lay-out of buildings and associated utilities<br>PR.2 Lay-out of premises, including workspace and employee facilities<br>PR.3 The suitability of equipment and its accessibility for cleaning, maintenance and preventative maintenance | PR.4 Supplies of air, water, energy and other utilities<br>PR.5 Supporting services, including waste and sewage disposal<br>PR.6 Cleaning and sanitizing<br>PR.7 Pest control<br>PR.8 Personnel hygiene<br>PR.9 Measures for the prevention of cross contamination | PR.10 Management of purchased materials (e.g. raw materials, ingredients, chemicals and packaging), and supplies |

tree diagram and fishbone diagram) has been studied in order to show the underlying causes of failures in strudel manufacturing (ARVANITOYANNIS and VARZAKAS, 2007b). The cause analysis for bulgur processing has been summarized in a fish bone diagram (Fig. 2) and the results are given in Table 3. All hazard sources (not detailed or shown in the fishbone diagram) in bulgur processing were compiled from regional bulgur processors.

Next to identify significant hazards, a number of questions reported in Table 3 have to be answered for each hazard that could be of concern at each step in the bulgur production process (ILSI, 2004). Hazard analysis starts questioning the presence of a potential hazard whether in the raw material or in the processing steps. Questioning determines whether the hazard is significant or not. This type of questioning has been reported by many authors (HORCHNER *et al.*, 2006; SERRA *et al.*, 1999). The results are shown in Table 3.

Bulgur processing basically consists of cleaning, cooking-drying and grinding-sieving steps. One of the main hazards comes from the properties of the raw material. Raw material may contain pesticides and mycotoxins (such as ocratoxin and/or aflatoxin). These hazards, as shown in Table 3, have to be tested and controlled in every batch. The hazard in the cooking and drying processes is the inefficient control of the survival and growth of microorganisms. Metal contamination is a hazard during grinding and sieving because machines are used in these processes.

The results obtained from the hazard analysis indicate the magnitude of the hazard in a next step, and whether it is a CCP or not.

The ISO 22000-Food Safety Management System standard requires

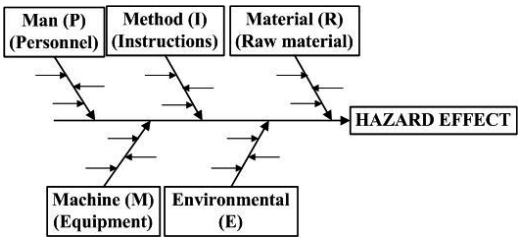



Fig. 2 - Cause and effect analysis (Fish bone diagram).

that all hazards be identified starting from the raw materials to the finished products in the processing plant (clause 7.4.2. of ISO 22000-FSMS). In the meantime, the possible severity of unfavorable health effects and the likelihood of their occurrence have to be evaluated (clause 7.4.3. of ISO 22000-FSMS). After assessing the hazards, control measures have to be considered for preventing, eliminating, or reducing these food safety hazards to an acceptable level (clause 7.4.4. of ISO 22000-FSMS) (ISO, 2005).

Hazard assessment following the hazard analysis must be quantified. It has been stated that hazard assessment is a mixture of probability and severity (ISO, 2005). In this study, the probability and severity of the hazards are considered based on a five-class hazard score matrix. This is shown in Table 4. The significant hazards found in the hazard analysis (Table 3) are used to determine the hazard assessment. Mycotoxin content in bulgur was found to be the 4$^{th}$ most important risk class (Table 5), while the cooking and drying processes were classified as the 2$^{nd}$ risk class. The packaging process was classified as the 3$^{rd}$ risk class.

## Critical control points in the bulgur processing

Critical control points include location, operation, procedure, or process that can be checked and if found, the food safety hazard can be removed or brought to an acceptable level. Critical control points of bulgur processing were determined. A decision tree was used to determine the steps which could be designated as critical control points (ILSI EUROPE, 2004; BOLAT, 2002; LEE and HATHAWAY, 1998; SANDROU and ARVANITOYANNIS, 2000a, b; ARVANITOYANNIS and MAUROPOULOS, 2000). Critical control points of bulgur processing are listed in Table 5.

After questioning the cooking, drying, and metal detection steps of bulgur processing were found to be in the CCP structure. Significant hazards with in these processes have to be checked by special monitoring systems.

Critical limits are the minimum and/or maximum values of the biological, chemical, or physical parameters that must be monitored at a CCP to prevent, or a food safety hazard eliminate, or reduce it to an acceptable level. These limits indicate if the identified hazards can be brought under control or not. Critical limits may be determined for factors such as temperature, time, physical dimensions, etc. Critical limits for each critical control point for bulgur processing were evaluated and are reported in Table 6. These were taken from the literature, legal provisions, or from comparable standard procedures (TSE, 2001, 2003). The identified CCPs have to be checked in the HACCP plan as defined in clause 7.6.2. of ISO 22000-FSMS.

## Implementation of HACCP System-HACCP Plan

Ideally, a HACCP study should be carried out as part of product and process development, so that potential hazards can be "designed out" at the earliest stage possible. In any case, a HACCP study results in a HACCP plan that should be correctly implemented to ensure that the appropriate control measures are put in place before products are put on the market.

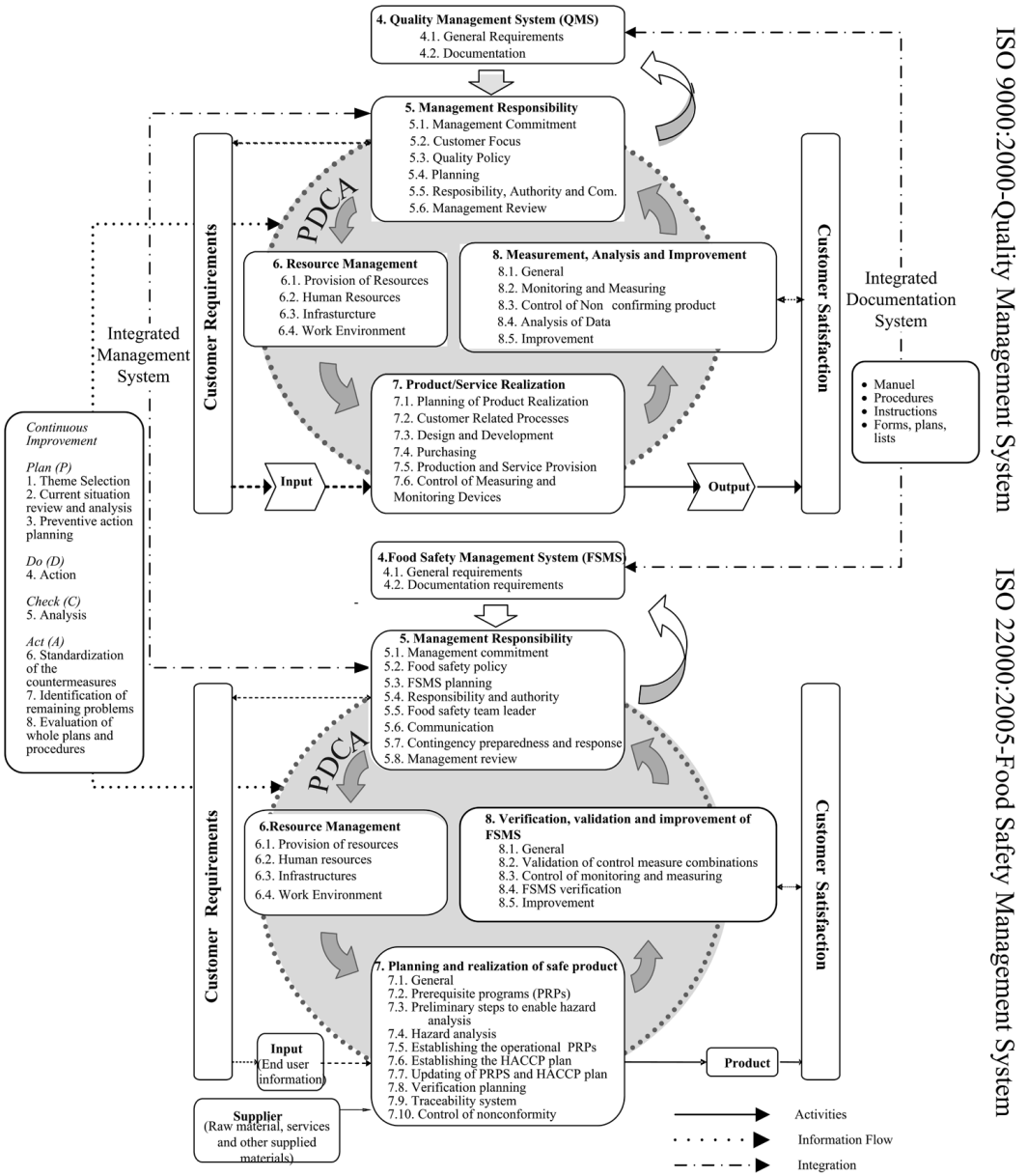



Fig. 3 - Continuous Improvement Models in ISO 22000:2005-Food Safety Management System and ISO 9000:2000-Quality Management System.

The HACCP plan is a document which consists of a list of significant hazards, critical control points, critical limits for each hazard at each CCP, monitoring procedures for each hazard at each CCP, and corrective actions if critical limits are exceeded. Some hazards are considered to be control points (CPs) because the hazards can be controlled by prerequisite programs (DEROSIER *et al.*, 2002).

Table 3 - Hazard and cause analysis for bulgur production. (Continue)

| Process name/step | Process descriptions | Potential Hazards | Cause analysis* | | Hazard Analysis (Y/N)** | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Causes (Sources and reasons) | Cause class | Q1 | Q2 | Q3 | Q4 | Q5 | Significant Hazard |
| Raw Material Reception | Durum wheat (*Triticum durum*) is received and analyzed (foreign matter, insects, pesticide) | B: Insects, rats, pests, microorganisms | Agricultural environment, storage. | R, E | Y | - | - | Y | Y | N |
| | | C: pesticide residues, mycotoxins | Pesticides used in the growing of vegetables; Mycotoxins produced by microorganisms under undesirable storage and processing conditions. | R, E | Y | - | - | Y | N | Y |
| Cleaning | Foreign materials and broken kernels are separated by using separators. | P: Foreign material | Insufficient cleaning | I, M | Y | - | - | N | - | N |
| Tempering | The milling process starts with the tempering of wheat in order to moisten the bran to enhance the separation of the bran from the endosperm and germ separation. (at room temperature for 12 to 48 h)<br><br>Tempering helps to uniform the water content of the wheat. | B: Survival of pathogens | The temperature and/or time of tempering and amount of water used. | I, M | - | Y | Y | Y | Y | N |
| Cooking | Starch is gelatinized and kernel is almost cooked during processing. Enzymes and microorganisms are also inactivated. (97°C for 40 min) | B: Survival of pathogens | The temperature and/or time of cooking | I, M | - | Y | Y | Y | N | Y |
| Drying | The cooked kernels dried (about 14 h) in a tray drier at 55°C to a moisture content of 10% (wb). | B: Survival of microorganisms | The temperature and/or time of drying | I, M | - | Y | Y | Y | N | Y |

Continue table 3

| Process name/step | Process descriptions | Potential Hazards | Cause analysis* | | Hazard Analysis (Y/N)** | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Causes (Sources and reasons) | Cause class | Q1 | Q2 | Q3 | Q4 | Q5 | Significant Hazard |
| Peeling | Removing of bran from endosperm by using bran separators. | P: Metal contamination | Metals contaminate during peeling | M | - | Y | Y | Y | Y | N |
| Grinding and polishing | After peeling, bulgur is ground and polished. | P: Metal contamination | Metals contaminate during grinding and sieving | M | - | Y | Y | Y | Y | N |
| Sieving | Bulgur is sieved to reduce particle size. | P: Metal contamination | Metals contaminate during grinding and sieving | M | - | Y | Y | Y | Y | N |
| Packaging | Classified bulgur is packed with suitable packing materials against environmental agents. | P: Dirt, and foreign materials with packaging material | Recontamination by packaging material | M | Y | - | - | Y | N | Y |
| Metal Detection | Detection of metal particles by metal detectors | P: Metal residue | Improper metal detector | M | - | Y | Y | Y | N | Y |
| Storage | Packed bulgur is stored in dry and suitable places. | B: Microbial growth | Inappropriate storage conditions | I, E | - | Y | Y | Y | N | Y |

* Cause and effect analyses (Fig. 2.);
** Hazard analysis-Questions (ILSI, 2004);
For raw material:
Q1. Is the presence of a potential hazard in raw material probable? No: No hazard; Yes: Go to Q4.
For individual process steps:
Q2. Is the presence of a potential hazard in the line or the environment probable? No: No hazard; Yes: Go to Q3;
Q3. Is an unacceptable contamination at this step probable? No: No hazard; Yes: Go to Q4;
Q4. Is an unacceptable level, survival, persistence or increase at this step? No: No hazard; Yes: Go to Q5; Q5. Is reduction, if any, at a further step adequate? Yes: No hazard; No: Significant hazard.

Table 4 - Five-class hazard scoring matrix.

| | | | | Risk Classes | | | | |
|---|---|---|---|---|---|---|---|---|
| Severity | Catastrophic | Death or lasting damage | E | 3 | 4 | 4 | 4 | 4 |
| | Critical | Many concerned people and lasting or continuous damage | D | 3 | 3 | 4 | 4 | 4 |
| | Serious | Many concerned people, no lasting damage | C | 2 | 3 | 3 | 4 | 4 |
| | Low | Single case, no lasting damage or minimal concentration | B | 2 | 2 | 3 | 3 | 4 |
| | Ignorable | Hazard is discovered prior to consumption or minimal unwillingness | A | 1 | 2 | 2 | 3 | 3 |
| | | | | I | II | III | IV | V |
| | | | | Unlikely (< per 1 years) | Rare (per year) | Occasional (per semester) | Frequent (per month) | Very frequent (per week) |
| | | | | Probability | | | | |

| Risk class | Control measures |
|---|---|
| 1 | No measure necessary. |
| 2 | Periodic measures are measures which often cover a one-time activity. |
| 3 | General control measures, such as proper hygiene facilities, procedures for cleaning and disinfecting personal hygiene instructions and maintenance, vermin control, maintenance and calibration, purchasing procedures and raw material specifications, complaint handling and recall procedures, etc. (of course, many of them are prerequisite programs) |
| 4 | Specific control measures are developed and used to control the risk. |

Table 5 - Hazard assessment and CCPs in the processing of bulgur.

| Process name/step | Significant hazard | Hazard assessment* | | | Preventive actions/control measures | CCP analysis (Y/N)** | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Severity | Probability | Risk class | | Q1 | Q2 | Q3 | Q4 | CCP no: |
| Raw Material Reception | C: Pesticides, mycotoxins | E | IV | 4 | Certified suppliers with HACCP program, mycotoxin analysis | Y | N | N | - | CP |
| Cooking | B: Survival of pathogens | B | II | 2 | Pathogens cannot be killed following the steps. Measurement parameters are temperature and time. | Y | Y | - | - | CCP1 |
| Drying | B: Survival of microorganisms | B | II | 2 | Time-temperature profile. | Y | N | Y | N | CCP2 |
| Packaging | P: Dirt, and foreign materials with packaging material | A | IV | 3 | Check if the cleaning schedule of the equipment is correctly applied | Y | N | N | - | CP |
| Metal Detection | P: Metal residue | A | II | 2 | Calibration | Y | N | Y | N | CCP3 |
| Storage | B: Microbial growth | B | II | 2 | Good storage practices | Y | N | N | - | CP |

* Hazard assessment (Table 4).
** CCP analysis-Questions (Mortimer and Wallace, 1998).
Q1. Do one or more effective preventive measures exist? No: not CCP; Modify step, process or product, Yes: Go to Q2.
Q2. Is the step specifically designed to eliminate or reduce the likely occurrence of a hazard to an acceptable level? No: Go to Q3. Yes: This step is CCP.
Q3. Could contamination with identified hazard(s) occur in excess of acceptable level(s) or could this increase to unacceptable levels? No: not CCP. Yes: Go to Q4.
Q4. Will a subsequent step eliminate identified hazard(s) or reduce likely occurrence to acceptable level(s)? No: This step is CCP. Yes: not CCP.

Table 6 - HACCP plan for the processing of bulgur.

| CCP N. | Process name/step | Significant hazard | Critical limits | Monitoring | | | Corrective action |
|---|---|---|---|---|---|---|---|
| | | | | Method | Frequency | Responsible | |
| CCP1 | Cooking | B: Survival of pathogens | No tolerance (TSE, 2003) | Time/Temp. | Each run | Operator | Check/repair the machine, reprocess if necessary |
| CCP2 | Drying | B: Survival of Microorganisms | No tolerance (TSE, 2003) | Time/Temp., Moisture content | Each run | Operator | Check/repair the machine, reprocess if necessary |
| CCP3 | Metal detection | P: Metal residue | No tolerance (TSE, 2003) | Metal detection | Per month | Operator | Calibration |

Monitoring involves a systematic regularly scheduled observation to make sure that CCPs are under control. When monitoring results show that criteria are not being met, appropriate and immediate measures should be taken to correct the deviations. For example, corrective actions may include re-heating or reworking, increasing the operation temperature, extending operation time, decreasing pH, changing monitoring equipment, changing equipment of process and maintaining of equipment, extra training for employees, revising HACCP documents, changing the process flow, etc. Corrective procedures should be continuously improved (ARVANITOYANNIS and TRAIKOU, 2005; EFSTRATIADIS and ARVANITOYANNIS, 2000).

Aflatoxin analysis of the wheat at the time of receiving the raw material could be done by using rapid test kits for each batch. Packaging materials can also be tested for each batch. Temperatures during the cooking, cooling, and drying processes have to be monitored. Monitoring results should be continuously recorded. The problems revealed in the raw materials and in the bulgur processing steps should be reprocessed and corrective actions must be applied. Metal detectors must be frequently calibrated and checked for accuracy (Table 6).

Verification is the effort to determine whether the HACCP plan is valid and whether the system is operating as planned; the HACCP plan is scientifically and technically evaluated. All stages in the process should be documented in order to enable retrospective observation, verification, and validation of the HACCP system. Verification should be implemented through internal audits which involve reviewing the pre-requisites, the hazards and risk assessment, critical control points, and critical limits.

The following should be included in the HACCP plan according to clause 7.6.2. of ISO 22000-FSMS: food safety hazards to be checked at the CCPs, control measures, critical limits, monitoring procedures, corrective actions to be taken if critical limits are exceeded, responsibilities, authority, and monitoring records.

## QUALITY SYSTEM FOR BULGUR PROCESSING

ISO 9000:2000-QMS is quality system that focuses on documenting all of the quality assurance and improvement processes in a company (ISO, 1999). Although the ISO 9000:2000-QMS was originally developed for the manufacturing sector, it has also been applied to many service organizations and has been gaining some acceptance in the food industry.

The ISO 9000:2000-QMS includes all the management, production, distribution, product design and service activities. The ISO 9000:2000-QMS standard describes a basic set of 8 elements by which quality management system can be developed and implemented.

As seen in Fig. 3, ISO 9000-QMS standard consists of five main parts: quality management system, management responsibility, resource management, product realization, measurement, analysis and improvement. The standard articles in Deming's cycle (PDCA) are being continuously improved. The standard is worked out by essential processes that are classified as strategic, operational and supportive processes (Table 2). As seen in Fig. 3, these processes cover all of the standard articles as well as some procedures along with the mandatory processes. In order to hygienically produce bulgur, the producers have to apply all of the quality processes. The interaction of bulgur production and these processes are given in Table 7. Bulgur production is technically affected by maintenance (QP 11), production planning (QP 4), resource management (QP 5), internal audits (QP 9) and data anal-

Table 7 - The effects of quality processes and prerequisite programs on bulgur processing.

| ISO 9000:2000-QMS Quality Processes (QP) | | | | | | | | | | | | ISO 22000:2005-FSMS Prerequisite Programs (PR) | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| QP:1 | QP:2 | QP:3 | QP:4 | QP:5 | QP:6 | QP:8 | QP:9 | QP:10 | QP:11 | QP:12 | QCP | QP:7 Bulgur Processing Process name/step | PR.1 | PR.2 | PR.3 | PR.4 | PR.5 | PR.6 | PR.7 | PR.8 | PR.9 | PR.10 | CCP |
| ○ | ○ | □ | ○ | ○ | | ○ | ○ | □ | | □ | 1 | Raw Material Reception | | △ | | | | △ | □ | □ | □ | ○ | |
| | □ | △ | △ | | | | △ | △ | ○ | | | Cleaning | □ | | □ | ○ | △ | ○ | △ | | | | | |
| | □ | △ | △ | | | | △ | △ | ○ | □ | | Tempering | △ | | □ | □ | △ | | | | □ | | | |
| | □ | □ | ○ | □ | □ | | ○ | ○ | ○ | ○ | 2 | Cooking | □ | | ○ | ○ | | | | □ | △ | | | 1 |
| △ | □ | □ | ○ | □ | □ | | ○ | ○ | ○ | ○ | 3 | Drying | □ | | ○ | ○ | | | | □ | △ | | | 2 |
| | | | | | | | △ | | ○ | | 4 | Peeling | △ | | □ | | | | | | □ | | | |
| | | | | | | | | | ○ | | | Grinding and polishing | △ | | ○ | | | | | | □ | | | |
| □ | □ | | | | □ | | | | ○ | ○ | 5 | Sieving | △ | | ○ | | | | | | □ | | | |
| △ | □ | □ | ○ | □ | | | ○ | ○ | ○ | □ | | Packaging | □ | △ | ○ | | | ○ | △ | □ | △ | | | |
| | | | | □ | | | ○ | | ○ | ○ | | Metal Detection | △ | | □ | | | | | | | | | 3 |
| △ | □ | □ | ○ | □ | | | ○ | ○ | ○ | □ | | Storage | ○ | □ | | ○ | | □ | ○ | □ | □ | | | |

○: High; □: Medium; △: Low.

ysis (QP 10). Bulgur processing is affected little by design and market surveying processes. The market is usually local and the process is not significantly affected. For example, the size and color of the bulgur are usually determined by market-consumer interaction. There is almost no design in small-scale factories.

Bulgur processing is not a long-chain process, but some physical processes are usually involved. Quality parameters of bulgur processing in small-scale plants are affected by technological and administrative applications. Quality parameters of bulgur processing and quality hazards in the process are given in Table 6.

The key points for a quality bulgur processing system include: (1) how to avoid the browning reaction and maintain good product appearance; (2) how to avoid degradation and loss of biological activities in the bulgur to insure a nutritious product; and (3) how to have bulgur with a long shelf life (HAYTA, 2001; GIANNOU *et al.*, 2003; BAYRAM and ÖNER, 2002). Quality control points (QCP) are determined by quality hazards. Quality control point involves a procedure where a control can be applied and a quality hazard (for example, taste, appearance, nutrition, color) can be prevented, eliminated, or reduced to an acceptable level.

Receipt of raw materials, cooking, drying, peeling, and sieving processes are analyzed as quality control points (Table 8). Other processes are determined such as control points on quality parameters. Foreign matter is removed from raw materials in the sieving process. Defects in wheat particles that directly affect quality can not be eliminated through this process. Color is an irreversible quality parameter during

Table 8 - QCPs in the processing of bulgur.

| Process name/step | Quality Hazard | Preventive actions/control measures | QCP analysis (Y/N)* | | | | |
|---|---|---|---|---|---|---|---|
| | | | Q1. | Q2. | Q3. | Q4. | QCP no: |
| Raw Material Reception | Foreign substances and grain seeds in wheat | Effective supplier assurance | Y | N | Y | N | QCP1 |
| | Moisture content of wheat | | | | | | |
| Cleaning | Foreign contaminants in water and air | Air filter, and water softening | Y | N | N | - | CP |
| Tempering | Moisture content | Time and temperature control | Y | N | N | - | CP |
| Cooking | Color and white spots | Time and temperature control | Y | N | Y | N | QCP2 |
| Drying | Color and moisture content | Time and temperature control | Y | Y | - | - | QCP3 |
| Peeling | Unpeeled grains | Adjusting tempering and milling process | Y | Y | - | - | QCP4 |
| Grinding and polishing | Amount of broken/undersized grains | Adjusting operation conditions | Y | N | N | - | CP |
| Sieving | Amount of undersized/oversized grains | Adjusting operation conditions | Y | Y | - | - | QCP5 |
| Packaging | Defective packaging | Adjusting operation conditions | Y | N | N | - | CP |
| Storage | Decreasing of the shelf life | Control of the storage conditions | Y | N | N | - | CP |

* QCP analysis-Questions. Q1. Do one or more effective preventive measures exist? No: not QCP; Modify step, process or product, Yes: Go to Q2. Q2. Is the step specifically designed to eliminate or reduce the likely occurrence of a quality hazard to an acceptable level? No: Go to Q3. Yes: This step is QCP. Q3. Could contamination with identified hazard(s) occur in excess of acceptable level(s) or could this increase to unacceptable levels? No: not QCP. Yes: Go to Q4. Q4. Will a subsequent step eliminate identified hazard(s) or reduce likely occurrence to acceptable level(s)? No: This step is QCP. Yes: not QCP.

the cooking and drying processes. Broken and defective wheat grains received with the raw materials should not exceed 1% of the total and should be analyzed in every batch. Cooking and drying directly affect the final product color. The preferred bulgur color is light yellow. The critical limits, monitoring and corrective actions of the quality parameters determined through the QCPs are given in Table 9.

## CONCLUSIONS

Bulgur is one of the most important key ingredients in traditional Middle Eastern foods. Currently there is a great market demand for bulgur. It is usually produced in small- to medium-sized companies that are not hygienic and have many safety hazards. In this work, a safety and quality parameter analysis in bulgur production has been presented. The incoming hazards in every processing stage have been described and outlined starting from the raw materials to the final product. Hazard analysis, cause analysis, and hazard assessment for bulgur production have the following CCPs; cooking, drying and metal detection processes. Raw material reception, cooking, drying, peeling, and sieving processes were determined to be the quality control points. The importance of strategic, operational and support processes during bulgur production have been emphasized.

## REFERENCES

Anon, 2005. USDA National Nutrient Database for Standart Reference, Release 18, Beltsville, MD, USA.

Arvanitoyannis I.S. and Mauropoulos A.A. 2000. Implementation of the hazard analy-

Table 9 - Quality plan for the processing of bulgur.

| Process name/step | Quality control points | Quality parameter/hazard | Quality parameter limits | Monitoring | | | Corrective action |
|---|---|---|---|---|---|---|---|
| | | | | Method | Frequency | Responsible | |
| Raw Material Reception | QCP1 | Foreign substances and grain seeds in wheat, moisture content of wheat | 1% (max.) foreign substances, 14% (max.) moisture content (TSE, 2001) | Percent foreign substances content moisture content | Each Run | Operator | Reject |
| Cooking | QCP2 | Color and white spots | Amber color, 1% (max.) | Colorimeter, (TSE, 2003) | Each Run | Operator | Reject |
| Drying | QCP3 | Color and moisture content | Amber color, 10-12% Moisture content | Colorimeter, (TSE, 2003) | Each Run | Operator | reprocess if necessary |
| Peeling | QCP4 | Unpeeled grains | No tolerance (TSE, 2003) | (TSE, 2003) | Each Run | Operator | reprocess if necessary |
| Sieving | QCP5 | Amount of undersized/ oversized grains | Customer requirements, classifications | Sieving analysis (TSE, 2003) | Each Run | Operator | reprocess if necessary |


sis critical control point (HACCP) system to Kasseri/Kefalotiri and Anevato cheese production lines. Food Control 11:31.

Arvanitoyannis I.S. and Traikou A. 2005. A comprehensive review of the implementation of hazard analysis critical control point (HACCP) to the production of flour and flour-based products. Crit. Rev. Food Sci. Nut. 45:327.

Arvanitoyannis I.S. and Varzakas T.H. 2009. Application of ISO 22000 and HACCP to octopus Part I. Int. J. Food Sci. Technol. 44:58.

Arvanitoyannis I.S. and Varzakas T.H. 2007a. Application of failure mode and effect analysis (FMEA), cause and effect analysis and Pareto diagram in conjunction with HACCP to a potato to chips manufacturing plant. Int. J. Food Sci. Technol. 42:1424.

Arvanitoyannis I.S. and Varzakas T.H. 2007b. A conjoint study of quantitative and semi-quantitative assessment of failure in a strudel manufacturing plant by means of FMEA and HACCP, Cause and effect and Pareto diagram . Int. J. Food Sci. Technol. 42:1156.

Bayram M. 2005. Modelling of cooking of wheat to produce bulgur. J. Food Eng. 71:179.

Bayram M. and Öner M.D. 2002. The new old wheat: convenience and nutrition driving demand for bulgur. World Grain (November):51.

Bolat T. 2002. Implementation of the hazard analysis critical control point (HACCP) system in a fast food business. Food Rev. Int. 18:337.

Certel M. 1998. Effects of different variety and cooking methods on the gelatinization properties of corn bulgurs. Gıda 23:267.

Codex Alimentarius Commission. 2003. Hazard Analysis and Critical Control Point (HACCP) system and guidelines for its application. In "Food Hygiene Basic Texts". Food and Agriculture Organization of the United Nations, World Health Organization, Rome.

Çoksöyler N., Özkaya N. and Boncuk H. 1993. Investigation of possibility of aflatoxin occurrence in bulgur. Gıda 18:89.

Derosier J., Stalhandske E., Bagian J.P. and Nudell, T. 2002. Using health care failure mode and effect analysis: the va national center for patient safety's prospective risk analysis system. The joint commission. J. Qual. Imp. 27:248.

Efstratiadis M.M. and Arvanitoyannis I.S. 2000. Implementation of HACCP to large scale production line of Greek ouzo and brandy: a case study. Food Control 11:19.

Food and Agriculture Organization/World Health Organization (FAO/WHO). 2001. Manuel on the application of the HACCP system in mycotoxin prevention and control. Food and Agriculture Organization of the United Nations, Rome.

Giannou V., Kessoglou V. and Tzia C. 2003. Quality and safety characteristics of bread made from frozen dough. Trends Food Sci. Technol. 14:99.

Giray B., Girgin G., Engin A.B., Aydin S. and Sahin G. 2007. Aflatoxin levels in wheat samples consumed in some regions of Turkey. Food Control 18: 23.

Hayta M. 2006. Bulgur quality as affected by drying methods. J. Food Sci. 67:2241.

Horchner P.M., Brett D., Gormley B., Jenson I. and Pointon A.M. 2006. HACCP-based approach to the derivation of an on-farm food safety program for the Australian red meat industry. Food Control 17:497.

ILSI Europe. 2004. A simple guide to understanding and applying the hazard analysis critical control point concept. Concise monograph series. ILSI Press, Washington, DC.

ISO 9000:2000-Quality Management Systems: Requirements. 1999. International Organization for Standardization. Geneva, Switzerland.

ISO 22000:2005-Food Safety Management Systems: Requirements. 2005. International Organization for Standardization, Geneva, Switzerland.

Kent N.L. 1990. "Technology of Cereals". Third Edition, Pergamon Press. Oxford, UK.

Koca A.F. and Anıl M. 1996. The effects of different wheat kinds and cooking methods on bulgur quality. Gıda 21:369.

Lee J.A. and Hathaway S.C. 1998. The challenge of designing valid HACCP plans for raw food commodities. Food Control 9:111.

Mortimore S.E. and Wallace C.A. 1998. "HACCP: A Practical Approach", 2nd edition, Aspen Publishers Inc, Gaithersburg, MD, USA.

Özboy Ö. and Köksel H. 1998. The effects of bulgur production on various chemical properties of wheats. Gıda 23:449.

Sandrou D.K. and Arvanitoyannis I.S. 2000a. Application of hazard analysis critical control point (HACCP) system to the cheese-making industry: A review. Food Rev. Int. 16:327.

Sandrou D.K. and Arvanitoyannis I.S. 2000b. Implementation of hazard analysis critical control point (HACCP) to the dairy industry: current status and perspectives. Food Rev. Int. 16:77.

Serra J.A., Domenech E., Escriche I. and Martorell S. 1999. Risk assessment and critical control points from the production perspective. Int. J. Food Microb. 46:9.

TSE. 2001. Wheat. Turkish Standard Institute 2974, Ankara, Turkey.

TSE. 2003. Bulgur (Boiled and pounded wheat). Turkish Standard Institute 2284, Ankara, Turkey.

Yıldırım A. 2004. Effect of different milling systems on selected quality parameters of bulgur. MSc Thesis in Gaziantep University, Turkey.

Yu B.H. and Kies C. 1993. Wheat products as acceptable substitutes for rice. plant foods for human Nutrition. 44:79.

Revised paper received March 3, 2009 Accepted April 21, 2009

PAPER

# IN-HOUSE PESTICIDE RESIDUE MONITORING OF TOMATOES FROM SOUSS-MASSA (MOROCCO) AND PESTICIDE RESIDUE LEVELS IN TOMATOES GROWN IN A GREENHOUSE AFTER MULTIPLE APPLICATIONS OF DICOFOL AND DIFENOCONAZOLE

MONITORAGGIO DEI RESIDUI DI ANTIPARASSITARI DEL POMODORO DA SOUSS-MASSA (MAROCCO) E DEI LIVELLI DI RESIDUI DI ANTIPARASSITARI IN POMODORI COLTIVATI IN SERRA DOPO APPLICAZIONI MULTIPLE DI DICOFOL E DIFENOCONAZOLO

**O. ID EL MOUDEN, M. ZOUGAGH[1]*, A. LEMERHYERATTE[2], R. SALGHI, L. BAZZI[3], A. HORMATALLAH[4], A. CHAKIR[5], and A. RÍOS[1]**
Laboratoire d'Ingénieries des Procédés de l'Energie et de l'Environnement, Ecole Nationale des Sciences Appliquées, BP 1136 Agadir, Morocco
[1]Department of Analytical Chemistry and Food Technology, Faculty of Chemistry, University of Castilla-La Mancha, Av. Camilo José Cela s/n, E-13004 Ciudad Real, Spain
[2]Laboratoire Régionale de l'Etablissement Autonome de Contrôle et de Coordination des Exportations d'Agadir, Morocco
[3]Laboratoire de Matériaux et de l'Environnement, Faculté des Sciences d'Agadir Morocco
[4]Laboratoire des pesticides, Institut Agronomique et Vétérinaire Hassan II, Complexe Horticole d'Agadir, Morocco
[5]Département de Chimie, Laboratoire de Chimie-Physique, Faculté des Sciences, Université de Reims, UMR 6089, Moulin de la housse, BP 1039, FR-51687 Reims Cedex 2, France
*Corresponding author: Tel. +34 926 29 53 00, Fax +34 926295318
e-mail: mohammed.zougagh@uclm.es

- Key words: dicofol, difenoconazole, pesticide residues, tomato -

ABSTRACT

A study was undertaken to evaluate the rate of decline of the residues of dicofol and difenoconazole in a Daniela tomato variety grown in an experimental plastic greenhouse. These two pesticides were selected on the basis of previous excesses of 22 and 25%, respectively, found in tomato samples cultivated in 2005 in eastern Morocco. Each group of tomatoes was subjected to a single chemical treatment when close to ripeness; pesticides were applied at the doses recommended by the manufacturers. Fruit samples were periodically collected until the end of the preharvest interval (p.i.). Residue levels of dicofol and difenoconazole were determined by liquid-liquid extraction (LLE), solid phase extraction (SPE) and gas chromatography with electron capture detection (GC-ECD). Residue levels of dicofol ranged from 1.54 to 0.26 mg/kg and the levels of difenoconazole ranged from 2.00 to 0.28 mg/kg, with median values of 0.82 and 0.99 mg/kg, respectively. The residual concentrations after the p.i. were below the legal limits. The difenoconazole value was high in the tomato plants of group VI, which were also treated with copper sulfate. This result indicates that extreme care must be taken when spraying doses of this fungicide, particularly on tomato varieties that are to be used fresh.

RIASSUNTO

È stato condotto uno studio per la valutazione della velocità di riduzione dei residui di dicofol e difenoconazolo in un pomodoro di varietà Daniela cresciuto in una serra sperimentale di plastica. Questi due pesticidi sono stati selezionati sulla base di precedenti elevati residui, del 22 e del 25% rispettivamente, trovati in campioni di pomodoro coltivati nell'est del Marocco nel 2005. Ogni gruppo di pomodori veniva sottoposto ad un singolo trattamento chimico poco prima della stagionatura; i pesticidi venivano applicati alle dosi raccomandate dai produttori. Campioni di frutto venivano raccolti periodicamente fino al termine della fase di pre-raccolta. I livelli dei residui di dicofol e difenoconazolo venivano determinati per estrazione liquido-liquido (LLE), estrazione in fase solida (SPE) e gascromatografia con rivelatore a cattura di elettroni (GC-ECD). I livelli di residui di dicofol variavano da 1,54 a 0,26 mg/kg e i livelli di difenoconazolo variavano da 2,00 a 0,28 mg/kg, con valori medi di 0,82 e 0,99 mg/kg, rispettivamente. Le concentrazioni residue dopo la fase di pre-raccolta erano al di sotto dei limiti legali. I residui di difenoconazolo erano più alti nelle piante di pomodoro appartenenti al gruppo VI, che erano state trattate anche con solfato di rame. Questi risultati indicano che andrebbe osservata un'attenzione estrema quando si spruzzano dosi di tali fungicidi, in particolare su varietà di pomodori che vengono consumate fresche.

## INTRODUCTION

In recent decades the increased demand for food safety has stimulated research regarding the risk associated with the consumption of pesticide-contaminated foodstuffs. It is well known that the level of risk is closely related to the daily intake of contaminated food and that the risk is even higher for certain products that are consumed more frequently (MATTERN *et al.*, 1991). Tomato is a basic component of the Mediterranean diet and is used almost daily in several European countries. Tomatoes are consumed raw, home-cooked or processed as a canned product, juice or paste. A significant oncogenic risk has been associated with this crop by the National Academy of Sciences, but only the possibility of fungicide contamination has been considered (PRECHEUR *et al.*, 1992).

In eastern Morocco open field and/or greenhouse cultivation is widespread for the production of tomatoes to be processed or consumed as a fresh product. Chemical protection of processing tomatoes is commonly carried out with scheduled treatments using different kinds of pesticides. Dicofol [2,2,2-trichloro-1,1-bis(4-chlorophenyl)ethanol] and difenoconazole {cis-trans-3-chloro-4-[4-methyl-2-(1H-1,2,4-triazol-1-ylmethyl)-1,3-dioxolan-2-yl]phenyl 4-chlorophenyl ether} are among the most extensively used pesticides in this area. Dicofol is a miticide that is very effective against red spider mite, while difenoconazole is effective in controlling a range of agronomic pests, particularly Alternaria solani in tomatoes. The molecular structures of dicofol and difenoconazole are given in Fig. 1.

The application of pesticides depends on the nature of the pest and the pathogen. In general, in Moroccan orchards

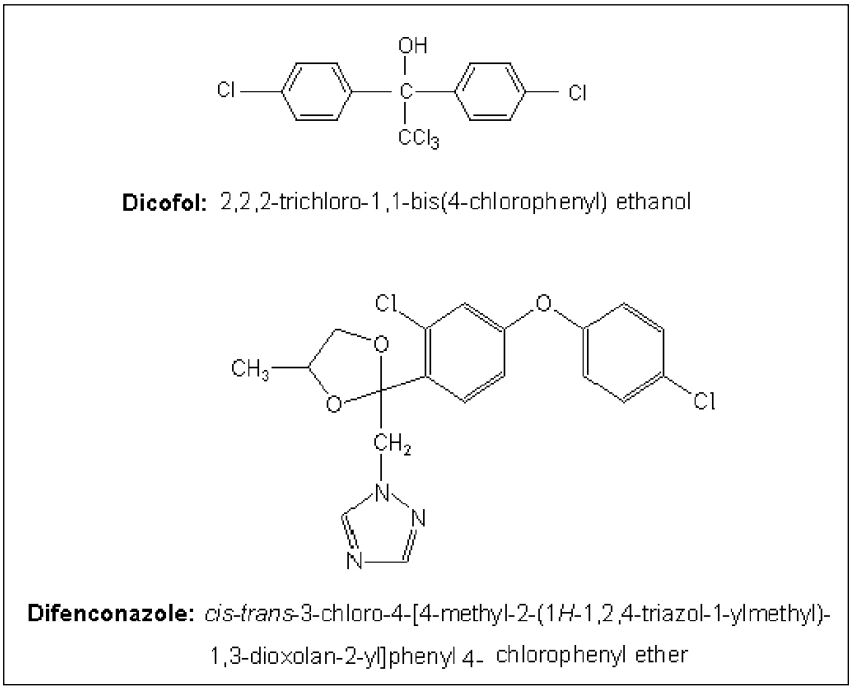



Fig. 1 - Molecular structures of dicofol and difenoconazole.

several pesticide applications are needed annually to protect the crop from heavy damage. For example, medfly may require more than 10 applications per year (SASMA, 2002). Despite the heavy application of pesticides, Moroccan exporters must comply with and fulfil the conditions imposed by Autonomous Establishment for Export Control and Coordination (EACCE) in terms of respecting the Maximum Residue Levels for all exported commodities (SASMA, 2002). Several reports have been published in the literature concerning the detection of residues of such active ingredients (a.i.) and their derivatives in different kinds of vegetables, including tomatoes that were randomly purchased from local markets (MATTERN *et al.*, 1991; AHMED and ISMAIL, 1995; DELRIO *et al.*, 1989; ROSSO *et al.*, 2000; STENSVAND, 2000). However, very few studies have been carried out on the decline kinetics in tomatoes and only products grown in greenhouses have been analysed in this way (CABRAS *et al.*, 1985; GIL-GARCIA *et al.*, 1997).

In view of the importance of this crop and considering that the maximum residue levels in the European Union of the aforementioned pesticides were recently revised (DIRECTIVE 2005/396/CE., 2005), further study is necessary.

We report here the results of an in-depth investigation in which the levels of pesticide residues in tomatoes produced in the Souss Massa area were determined. The residue behaviour of two selected active ingredients (a.i.), dicofol and difenoconazole, applied in an experimental greenhouse to protect tomatoes grown for processing purposes is described.

## METHODS AND MATERIALS

### Monitoring programme for all pesticides residues (2006-2007)

The investigation was conducted on 20 farms in 2006-2007. The sampling was carried out in collaboration with the Regional Office of Agricultural Development Souss-Massa (ORMVAS) and the Association of Fruit and Vegetable Producers (APEFEL). A survey questionnaire was prepared and given to selected farmers. The survey included data on operations and the approach taken by producers to manage pesticides. Visits were made to the producers surveyed as well as to the packing plants in order to supplement the information concerning pesticide traceability.

In order to investigate the presence, frequency and type of pesticides found in tomatoes destined for export from the area of the Souss Valley, a sample of 96 growers-exporters from 20 different packing houses out of the existing 21 in the area were randomly selected. Each sample corresponded to a grower-exporter. In large packing houses, 5 growers were selected and 4 were selected in 17 packing houses. The samples were collected between 15/12/06 and 30/04/07 and each sample consisted of 120 tomatoes. All samples were transported to the laboratory and stored in a cold room at 4°C prior to the study. From the sample of 120 tomatoes per grower, only a sub-sample of 30 tomatoes was chosen for the analysis of pesticide residues according to the method described by DGCCRF (1998).

### Experimental design used to study dicofol and difenoconazole

The investigation was conducted in 2007-2008 in a plastic greenhouse located in Massa (Agadir, Morocco). The plastic greenhouse was 500 m2 in area and the tomato planting density (variety Daniela) was 2 plants/m2. Residue levels of dicofol and difenoconazole were determined in tomatoes of commercial size (110-130 g) over a seven-week period, during which six consecutive treatments with dicofol or difenoconazole pesticide

were applied to the different plantations (treatments I, II, III, IV, V and VI) at intervals of 0, 1, 5, 22 and 22 days between each application. Samples were collected at 0, 4, 8, 12, 16 and 20 days after treatment I (samples I+0, I+4, I+8, I+12, I+16 and I+20); at 0, 2, 5, 8 and 11 days after treatment II (samples II+0, II+2, II+5, II+8 and II+11); at 0, 4, 8, 12, 16 and 20 days after treatment III (samples III+0, III+4, III+8, III+12, III+16 and III+20); at 0, 2, 5, 8 and 11 days after treatment IV (samples IV+0, IV+2, IV+5, IV+8 and IV+11); at 0, 4, 8, 12, 16 and 20 days after treatment V (samples V+0 V+4, V+8, V+12, V+16 and V+20) and at 0, 2, 5, 8, 11, 14 and 17 days after treatment VI (samples VI+0, VI+2, VI+5, VI+8, VI+11, VI+14 and VI+17). In all cases, the greenhouse samples consisted of 30 pieces of tomato taken at random according to the method described by the DGCCRF (1998). The treatments and sampling are summarized in Table 1.

The daily maximum/minimum temperatures in the greenhouse throughout the study were 12° and 32°C, respectively, and the daily mean relative humidity was 88%. The irrigation flow was 34 $m^3$/ha.

Reagents, standards and samples

All chemicals and solvents were of analytical and chromatographic grade. All standards were greater than 99% pure and were purchased from Dr. Ehrenstorfer, GmbH (AUGSBURG, GERMANY). The organic solvents were obtained from Panreac (Barcelona, Spain). Each standard was dissolved in acetone to obtain stock solutions of approximately 200 mg/L and these were stored in the dark at 4°C until further use. The fresh working standard solutions were obtained by dilution with n-hexane. Florisil adsorbent (16-30 mesh) was obtained from Sigma-Aldrich (St. Louis, MO, USA).

Immediately after picking, the green-

Table 1 - Treatment and sampling details.

| Day | Treatment | Samples collected | Day | Treatment | Samples collected |
|---|---|---|---|---|---|
| 0 | I: 03/03/08) | I+0 | 0 | IV: 09/03/08 | IV+0 |
| 4 | Dicofol | I+4 | 2 | Difenoconazole | IV+2 |
| 8 | | I+8 | 5 | | IV+5 |
| 12 | | I+12 | 8 | | IV+8 |
| 16 | | I+16 | 11 | | IV+11 |
| 20 | | I+20 | | | |
| 0 | II: 3/03/08 | II+0 | 0 | V: 01/04/08 | V+0 |
| 2 | Difenoconazole | II+2 | 4 | Dicofol | V+4 |
| 5 | | II+5 | 8 | | V+8 |
| 8 | | II+8 | 12 | | V+12 |
| 11 | | II+11 | 16 | | V+16 |
| | | | 20 | | V+20 |
| 0 | III: 04/03/08 | III+0 | 0 | VI: 23/04/08 | VI+0 |
| 4 | Dicofol | III+4 | 2 | Difenoconazole | VI+2 |
| 8 | | III+8 | 5 | and copper | VI+5 |
| 12 | | III+12 | 8 | sulphate | VI+8 |
| 16 | | III+16 | 11 | | VI+11 |
| 20 | | III+20 | 14 | | VI+14 |
| --- | --- | --- | 17 | | VI+17 |

house tomato samples were put into polyethylene bags and transported to the laboratory, where they were washed with tap water, dried with absorbent paper and stored at -20°C in the dark prior to analysis.

Instruments and chromatographic conditions

Greenhouse tomato samples were ground using a food processor. Analysis of all pesticides was carried out with a Hewlett-Packard 6890 gas chromatograph (Palo Alto, CA, USA) equipped with an ECD Detector, split/splitless injection port, and fused silica capillary HP-5 column (5% diphenyl and 95% dimethylpolysiloxane, 25 m × 0.32 mm ID, 0.52 μm film thickness); and temperature programming from 80° to 250°C (15°C/min); injector temperature 250°C and detector temperature 300°C. Carrier gas (helium) flow rate, 2.6 mL/min; makeup gas (nitrogen) flow rate, 60 mL/min; injection volume, 1 μL; and splitless time, 0.1 min. Data were acquired and the equipment was controlled using HP (Palo Alto, CA, USA) Chem-Station software, which was run under Microsoft Windows NT on an HP compatible personal computer. The identification of the a.i. peaks and the absence of interfering substances were assessed by comparing the sample chromatograms with those of a pesticide standard mixture and of an untreated sample. The internal standard method was used to quantify the residues by measuring the peak areas vs. concentrations.

Liquid-liquid and solid-phase extraction procedure

The method used to extract the pesticides from tomatoes was adapted from CHARLES and RAYMOND (1991). Each 50 g of sample was ground using a food processor and 150 mL of acetone was added. The mixture was homogenised for 2 min and then mixed for 2 h. The mixture was filtered through glass wool and the acetone residues were partitioned using saturated aqueous sodium chloride (30 mL) and dichloromethane (70 mL) in a separating funnel. The extraction was repeated with another 70 mL of dichloromethane and the combined extracts were dried over anhydrous sodium sulfate. The dichlo-

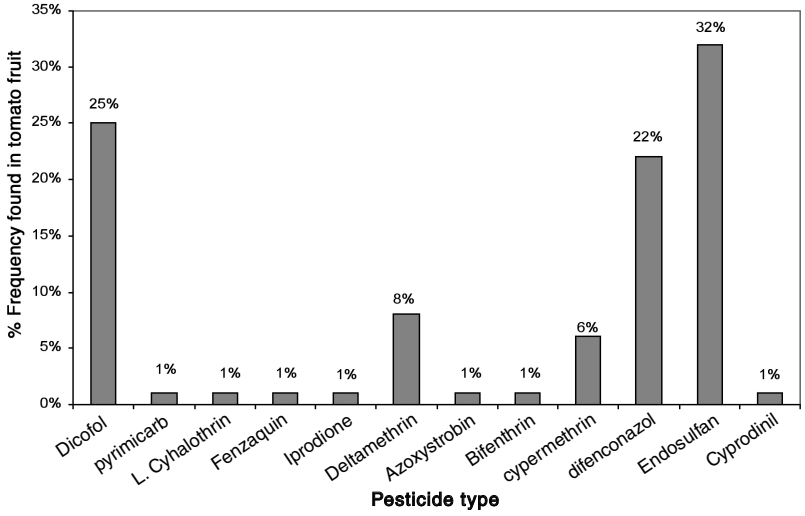



Fig. 2 - Percentage frequency of pesticides found in tomato fruit.

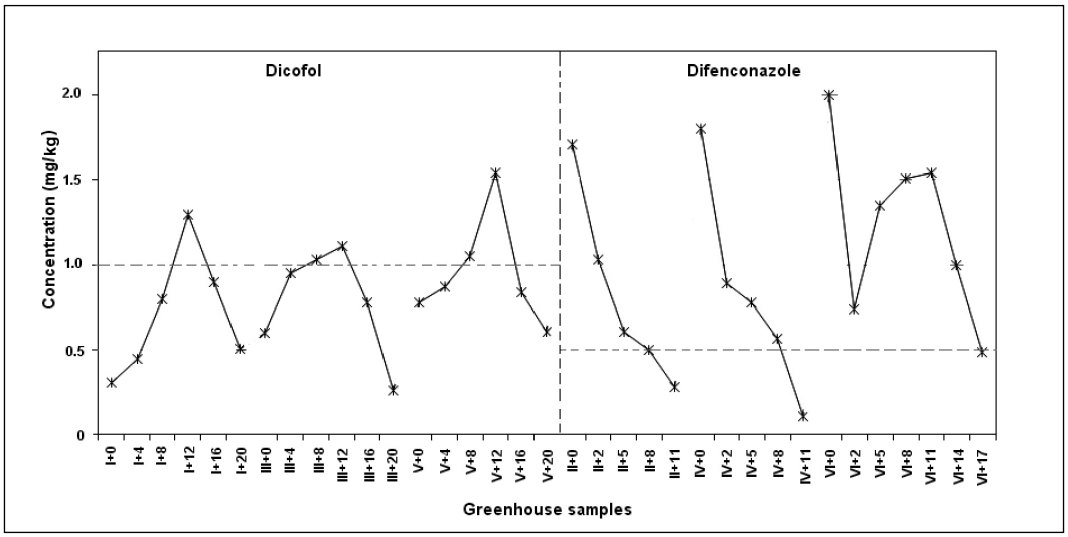



Fig. 3 - Residue levels of dicofol and difenoconazole in tomato samples.

romethane fraction was collected and evaporated on a rotatory vacuum evaporator at 40°C and the residues were dissolved in an acetone/hexane (1:9) mixture (10 mL). In the clean-up step, 1 mL of the extract was passed through a florisil column previously conditioned with 5 mL of acetone/diethyl ether (6:4) and 5 mL of diethyl ether. The pesticide residues were eluted with acetone/diethyl ether (6:4) (4 mL). Samples were analysed by gas chromatography.

## RESULTS AND DISCUSSION

### Performance of the analytical method

The use of combined GC-ECD with liquid-liquid extraction (LLE) and solid phase extraction (SPE) seemed to be an excellent way to determine the types and levels of pesticides in tomato samples with high selectivity and sensitivity. The linear dynamic range, precision (as relative standard deviation) and sensitivity (as limit of detection) values are reported in Table 2.

- Linear range: Individual calibration graphs were run with mixtures of all the pesticides studied at concentrations ranging from 10 to 200 ng $mL^{-1}$. Each solution was injected five times. The linear range, intercept and slope of the curve are given in Table 2 along with the regression coefficient for each pesticide.

- Sensitivity: The theoretical limit of detection, defined as the concentration of analyte that gives a signal equivalent to the blank signal plus three times its standard deviation, was calculated for each individual pesticide (MILLER and MILLER, 2004). In this work, the limit of detection (LOD) was taken to be the amount of analyte that gave a signal that was clearly distinguishable from the background noise of the instrument. The LODs calculated in this way ranged from 0.181 to 1.297 ng $mL^{-1}$ for dicofol and difenoconazole, respectively. The limit of quantification (LOQ), defined as the concentration of analyte that gave a signal equivalent to the blank signal plus ten times its standard deviation, ranged from 0.604 to 1.989 ng $mL^{-1}$ for the pesticides studied (MILLER and MILLER, 2004).

Table 2 - Data obtained for the method under investigation (see text for details).

| Analyte (%) | Linear range (ng mL$^{-1}$) | Y = a X + b | $R^2$ | $S_{y/x}$ | LOD (ng mL$^{-1}$) | LOQ (ng mL$^{-1}$) | RSD (n=5) |
|---|---|---|---|---|---|---|---|
| Dicofol | 25-200 | (15.498±0.194)X–(46.272±2.088) | 0.9995 | 26.310 | 0.404 | 1.348 | 4.4 |
| Difenoconazole | 25-200 | (9.368±0.1653)X–(4.413±1.781) | 0.9991 | 22.435 | 0.570 | 1.901 | 5.1 |
| pyrimicarb | 20-200 | (27.797±0.511)X–(84.826±5.527 | 0.9990 | 71.749 | 0.597 | 1.989 | 6.5 |
| L. Cyhalothrin | 10-100 | (20.421±0.220)X–(70.490±1.340) | 0.9997 | 17.068 | 0.197 | 0.6563 | 5.3 |
| Fenzaquin | 50-500 | (9.878±0.137)X–(68.827±4.271) | 0.9994 | 52.578 | 1.297 | 4.324 | 9.3 |
| Deltamethrin | 25-200 | (18.38±0.222)X+(60.334±2.387) | 0.9996 | 30.071 | 0.390 | 1.299 | 5.0 |
| Azoxystrobin | 20-200 | (27.798±0.511)X–(84.826±5.527) | 0.9990 | 71.7491 | 0.597 | 1.989 | 7.2 |
| Bifenthrin | 25-200 | (9.374±0.167)X+(0.875±1.796) | 0.9991 | 22.616 | 0.575 | 1.915 | 4.7 |
| cypermethrin | 25-200 | (15.498±0.194)X–(40.2721±2.088) | 0.9995 | 26.310 | 0.404 | 1.348 | 2.6 |
| Endosulfan | 10-100 | (42.174±1.047)X+(61.320±6.512) | 0.9982 | 76.466 | 0.464 | 1.546 | 3.9 |
| Cyprodinil | 10-100 | (52.120±0.517)X–(292.263±3.146) | 0.9997 | 40.058 | 0.181 | 0.604 | 4.8 |

*a*; slope a; b, Intercept; *R*, regression coefficient; $S_{y/x}$, standard deviation of residuals; LOD, limit of detection; LOQ, limit of quantification, RSD, relative standard deviation.

- Precision: The precision of the method for solvent standards, expressed as relative standard deviation (RSD), for the determination of 100 ng mL$^{-1}$ of each studied pesticide ranged from 2.6 to 7.2% (n = 5).

Analytes were extracted by LLE, retained in florisil, eluted with acetone/diethyl ether and injected into the chromatographic system for separation and quantification of individual pesticides.

Untreated samples were fortified by the addition of an intermediate pesticide mixture solution. Samples were allowed to equilibrate for 2 h prior to extraction and were processed according to the procedure described above. The "practical value of the limit of quantification", expressed in mg/kg, and the mean recovery values of each pesticide from tomato are reported in Table 3 along with the corresponding coefficients of variation. The values of spiking levels at which the recovery tests were carried out was 0.003 ppm.

## Analysis of greenhouse tomato samples

Based on the results of surveys conducted in 2006-2007 in the area around Souss Massa regarding the greenhouse crop, 22 pesticides were identified and these included 22 different active ingredients. Dithiocarbamate (27%) was the most commonly used pesticide, while organochlorine pesticides (11%) – including organophosphorus (11%), organochlorine (11%), pyrethroids (11%) and carbamates (23%) – were among the least used chemical class.

The most widely used formulations were emulsifier concentrates (EC) at 63% and wettable powder (WP) at 37%. The most widely used chemical group was insecticides at 46%, fungicides at 50% and acaricides at 4%. The pesticide used is 65.3 kg/hectare of active ingredient.

Table 3 - Limit of quantification, average recovery and coefficient of variation values for the pesticides analysed.

| Analyte | LOQs* (mg/kg) | N. of samples | Average recovery (%) | R.S.D (%) |
|---|---|---|---|---|
| Dicofol | 0.007 | 20 | 81 | 4.7 |
| Difenoconazole | 0.003 | 15 | 85 | 6.1 |
| Pyrimicarb | 0.003 | 16 | 89 | 4.5 |
| L. Cyhalothrin | 0.008 | 13 | 87 | 6.3 |
| Fenzaquin | 0,005 | 11 | 85 | 7.3 |
| Deltamethrin | 0.002 | 21 | 84 | 5.0 |
| Azoxystrobin | 0.003 | 12 | 89 | 6.2 |
| Bifenthrin | 0.003 | 10 | 95 | 3.2 |
| Cypermethrin | 0.003 | 15 | 88 | 2.8 |
| Endosulfan | 0.002 | 17 | 98 | 4.9 |
| Cyprodinil | 0.008 | 23 | 89 | 5.8 |

*LOQ was defined as the minimum level at which a pesticide can be quantified with acceptable accuracy (>80%) and precision (<10%).

Table 4 - Details of pesticides and doses used.

| Active ingredient | Commercial formulation | Dose | Pre-harvest interval (days) | MLR (mg/kg) |
|---|---|---|---|---|
| Dicofol | Kelthan 480g/L | 150 cc/hL | 15 | 1 |
| Difenocozole | Score 250 g/L | 50 cc/hL | 7 | 0.5 |

Data from the 96 tomato samples analysed indicate that some chemicals are more persistent than others. The most frequently found compounds included endosulfan (32%), dicofol (25%), difenoconazole (22%), deltamethrin (8%) and cypermethrin (6%).

The ranges of residue levels found in the tomatoes were: dicofol (0.01 to 1.06 mg $kg^{-1}$); difenoconazole (0.08 to 0.80 mg $kg^{-1}$); endosulfan (0.01 to 0.40 mg $kg^{-1}$); deltamethrin (0.01 to 0.05 mg $kg^{-1}$) and cypermethrin (0.04 to 0.79 mg $kg^{-1}$). If the MRLs of the pesticides established by EU regulations (DIRECTIVE N. 2005/396/CE) are considered, the values for endosulfan (32%), dicofol (25%) and difenoconazole (22%) are very high. Thus, the persistence of dicofol and difenoconazole residues in tomato samples must be studied. The persistence of endosolfan was discussed in a previous paper (ZEROUALI *et al.*, 2006).

## Evaluation of the decrease in dicofol and difenoconazole residues in greenhouse tomato samples

The two pesticides under investigation differed in terms of dosage, preharvest interval and MRL (Table 4). The preharvest times for dicofol and difenoconazole are 15 and 7 days, respectively, if they are sprayed as pure formulas.

In the case of samples taken after treatments, the "safety period" was extended to 14 days because they were in

Table 5 - Dicofol and difenoconazole residue levels in the tomato samples.

| Dicofol | | Difenoconazole | |
|---|---|---|---|
| Samples | Concentration (mg kg$^{-1}$) | Samples | Concentration (mg kg$^{-1}$) |
| I+0 | 0.31 | II+0 | 1.71 |
| I+4 | 0.45 | II+2 | 1.03 |
| I+8 | 0.80 | II+5 | 0.61 |
| I+12 | 1.30 | II+8 | 0.50 |
| I+16 | 0.90 | II+11 | 0.28 |
| I+20 | 0.51 | IV+0 | 1.80 |
| III+0 | 0.60 | IV+2 | 0.89 |
| III+4 | 0.95 | IV+5 | 0.78 |
| III+8 | 1.03 | IV+8 | 0.57 |
| III+12 | 1.11 | IV+11 | 0.11 |
| III+16 | 0.78 | VI+0 | 2.00 |
| III+20 | 0.26 | VI+2 | 0.74 |
| V+0 | 0.78 | VI+5 | 1.35 |
| V+4 | 0.87 | VI+8 | 1.51 |
| V+8 | 1.05 | VI+11 | 1.54 |
| V+12 | 1.54 | VI+14 | 1.00 |
| V+16 | 0.84 | VI+17 | 0.49 |
| V+20 | 0.61 | ------ | ----- |
| Maximum level | 1.54 | | 2.00 |
| Minimum level | 0.26 | | 0.28 |
| median | 0.82 | | 0.99 |

association with copper sulfate. The levels of dicofol and difenoconazole residues determined in all of the tomato samples during the study period are given in Table 5 together with the maximum, minimum and mean values.

As expected, the residue values at time 0 were in proportion to the dosages applied. The values for dicofol ranged from 1.54 to 0.26 mg/kg whereas the difenoconazole residues ranged from 2.00 to 0.28 mg/kg.

In the case of dicofol, the maximum residual concentrations of 1.30, 1.11 and 1.54 mg/kg were found after 12 days of treatment of plants I, III and V, respectively. The values decreased markedly after 16 days (0.90, 0.78 and 0.84 mg/kg, respectively). After the p.i. (15 days), the residual level was below the permitted value in all of the samples.

Plants treated with pure difenoconazole (samples taken after treatments II and IV) had residual concentrations of 1.71 and 1.80 mg/kg, respectively, immediately after treatment, but a marked decrease was already detected after 8 days (0.57 mg/kg).

Considering the residue levels after the p.i. established by the EU Regulations for the pure a.i., the difenoconazole value seems to be very high in the tomatoes in planting VI. This could be due to the formation of chelates with copper ions, which could slow down pesticide degradation. At 17 days the MRL was not exceeded.

The results of this study indicate that if tomatoes are destined to be sold as a fresh product, it would be advisable to reduce the dose of the formulate containing difenoconazole. Washing the fruit does not guarantee a significant de-

crease in residue levels (ZEROUALI *et al.*, 2006; VALVERDE *et al.*, 2002). Different considerations should be made for the product destined for the food industry. In fact, the dramatic decrease of pesticide residues, mainly due to peeling and cooking, should markedly reduce the risk in tomato derivatives (ABOU-ARAB, 1999). Unfortunately, little information is available about the behaviour of defenconazole after tomato processing and an in-depth investigation is therefore required. Further studies are also needed to better assess the behaviour of this pesticide after scheduled treatments, considering different varieties of tomato that are commonly used as fresh food.

## CONCLUSIONS

- From different samples collected in the packing houses of the Souss area, it appears that the compounds frequently found are dicofol, difenoconazole, endosulfan, deltamethrin and cypermethrin at levels of 25, 22, 32, 8 and 6%, respectively.

- During the study, residue levels in the plantation were between 1.54 and 0.31 mg/kg for dicofol and between 1.74 and 0.28 mg/kg for difenoconazole, with median values of 0.82 and 0.99 mg/kg, respectively.

- The residual concentrations after the p.i. were below the legal limits; nevertheless, the value for difenoconazole in the samples taken after treatments with copper sulfate seemed to be rather high.

## ACKNOWLEDGEMENTS

The Authors wish to thank the NATO program (CBP.MD.CLG 983108) for supporting this Work. The Spanish Ministry of Education and Science and JJCC Castilla-La Mancha are gratefully acknowledged for funding this work with Grants CTQ2007-61830 and PCC08-0015-0722, respectively.

## REFERENCES

Abou-Arab A.A.K. 1999. Behaviour of pesticides in tomatoes during commercial and home preparation. Food Chem. 65: 509.

Ahmed M.T. and Ismail S.M.M. 1995. Residues of methomyl in strawberries, tomatoes and cucumbers. Pest Sc. 44:197.

Cabras P., Meloni M., Pirisi F.M. and Cabitza F. 1985. Behavior of acylanilide and dicarboximidic fungicide residues on greenhouse tomatoes. J. Agric. Food Chem. 33: 86.

Charles R. W and Raymond T.H.T. 1991. "The Pesticide Manual". 9th Ed. P. 212, British Crop Prot. Council, Surrey, UK.

Crisippi T., Zini G. and Fabbrini R. 1993. Gas chromatographic determination of benalaxyl residues in different crops and water. JAOAC. 76: 650.

Delrio G., Luciano P., Floris I., Cabitza F., Cubeddu M. and Cabras P. 1989. Prove di lotta contro i fitofagi del pomodoro da industria in Sardegna. La difesa delle piante 12: 97.

Directive N° 2005/396/CE, February 23, 2005. EU Official Bulletin. http://ec.europa.eu/sanco_pesticides/public/index.cfm

Direction Générale de la Concurrence et de la Consommation des Répressions de la France (DGCCRF) (1998), Méthode d'échantillonnage des fruits et légumes. Paris.

Gil-Garcia M.D., Martinez-Vidal J.L., Martinez-Galera M., Rodriguez-Torreblanca C. and Gonzalez C. 1997. Determination and degradation of methomyl in tomatoes and green beans grown in greenhouses. JAOAC. 80: 633.

Mattern G.C., Liu C.H., Louis J.B and Rosen J.D. 1991. GC/MS and LC/MS determination of 20 pesticides for which dietary oncogenic risk has been estimated. J. Agric. Food Chem. 39: 700.

Miller J.N. and Miller J.N. 2004. "Statistics and Chemometrics for Analytical Chemistry", 4th Ed., Pearson Prentice Hall, England.

Precheur R.J., Bennett M.A., Riedel R.M., Wiese K.L. and Dudek J. 1992. Management of fungicide residues on processing tomatoes. Plant Disease. 76: 700.

Rosso I., Giraudi G., Gamberini R., Baggiani C. and Vanni A. 2000. Application of an ELISA to the determination of benalaxyl in red wines. J. Agric. Food Chem. 48: 33.

Société Agricole de Développement Marocaine (SASMA). Contribution à l'étude de certains pesticides organophosphorés sur tomate. Document interne. 2002. 1. Casa, Morocco.

Stensvand A. 2000. Investigation on fungicide residues in greenhouse-grown strawberries. J. Agric. Food Chem. 48: 917.

Valverde A., Aguilera A., Rodriguez M., Boulaid M. and El Begrani M. 2002. Pesticide residue levels in peppers grown in a greenhouse after multiple applications of pyridaben and tralomethrin. J. Agric. Food Chem. 50: 7303.

Zerouali E., Salghi R., Hormatallah A., Hammouti B. and Bazzi L. 2006. Pesticide residues in tomatoes grown in greenhouses in Souss Massa valley in Morocco and dissipation of endosulfan and Ddltamethrin. Environ. Bull. 15: 267.

Paper received March 24, 2008 Accepted July 10, 2009

SHORT COMMUNICATION

# MOISTURE CONTENT DETERMINATION OF COLORED MALTS

DETERMINAZIONE DELL'UMIDITÀ NEI MALTI COLORATI

**L. MARTE, S. FLORIDI, O. MARCONI[1], and G. PERRETTI[1]***
Italian Brewing Research Center, Centro di Eccellenza per la Ricerca sulla Birra (CERB), Università degli Studi di Perugia,
Via San Costanzo, n.c.n., 06126 Perugia, Italy
[1]Department of Economics and Food Sciences, Università degli Studi di Perugia,
Via San Costanzo, n.c.n., 06126 Perugia, Italy
*Corresponding author: e-mail: perretti@unipg.it

ABSTRACT

The present paper deals with the validation of a method to determine the moisture content of roasted and caramel colored malts. The data agree with those reported in the literature, but differ with respect to the tested validation range.

RIASSUNTO

Il presente lavoro consiste nella validazione di un metodo per la determinazione del contenuto di umidità nei malti colorati tostati e di tipo caramello. I dati concordano con quanto riportato in letteratura, ma differiscono rispetto agli intervalli riportati per la validazione del metodo.

- Key words. Analytica-EBC, caramel malt, colored malt, moisture, roasted malt -

## INTRODUCTION

Colored barley malts are commonly used in brewing to add color and particular flavors to beer (O'SHAUGHNESSY, 2003; COGHE *et al.,* 2004a). They are produced by subjecting malt to special heat treatments after kilning, which favor the formation of Maillard reaction products (COGHE *et al.,* 2004b). Moreover, the addition of these special malts can enhance the antioxidant activity (WOFFENDEN *et al.*, 2001) and xanthohumol content (WUNDERLICH *et al.,* 2005).

In this study, a method for determining the moisture content of colored malts was validated. This method was validated in a collaborative trial carried out by the EUROPEAN BREWERY CONVENTION (EBC) Analysis Committee in 1996, and is presently included in the official Analytica-EBC methods of the EUROPEAN BREWERY CONVENTION (2006a),

Caramel and roasted malts were tested in the collaborative trial (BÉNARD, 1997a; b), and data are summarized in the Analytica-EBC method 5.4 Colored Malts: Moisture (EUROPEAN BREWERY CONVENTION, 2006a). This method is specific for this kind of malt and differs from the generic method used for all malts, i.e., Analytica-EBC 4.2 (EUROPEAN BREWERY CONVENTION, 2006b) with respect to the validation parameters. The generic method tested in a collaborative trial, showed the following parameters (BÉNARD 1997a; b): range = 3.8-7.3%; $r_{95}$ = 0.13%, and $R_{95}$ = 0.6%. The validation parameters obtained for colored malts were: range = 4.1-7.7%; $r_{95}$ = 0.07%, and $R_{95}$ = 0.32%. By comparing these two data sets, it can be seen that the moisture in colored malts is similar to, or slightly higher than that of the other kinds of malt.

There is considerable evidence that colored malts, due to the high temperature treatments used to develop the colored compounds have a lower moisture content. BRIGGS (1988) reported that the moisture content of crystal and caramel malts ranged from 3 to 7.5%, and was ≤2% for chocolate and black-roasted malts. KUNZE (2004) reported that the water content in dark malt was 1.0 to 4.5%, and even less (0.5-4%) in freshly kilned malts. SOUNIER (1988) reported that the water content in dark malts ranged from 1 to 2%. According to the "Dictionary of Beer & Brewing" (RABIN and FORGET, 1998), moisture content of dark malts is 2-5% after kilning, but before roasting. Moreover, the MEBAK (2006a; b) method for determining moisture content in malt (method 3.1 Gerstenmalz) reports reference values of 3-5% for pale malt and 1-4% for dark malt, while in the method specific for special malts (method 3.2.1 Wasser), the reference value for caramel and roasted malt is <6%.

The variation in these values suggests that the validation reported in the EBC method was conducted on a set of samples that was not representative of roasted and caramel colored malts and therefore, the repeatability and reproducibility values obtained from this set are probably not reliable.

In this study the color (visual method) and moisture contents in a set of roasted and caramel colored malts was analyzed to determine the range of values corresponding to this kind of malt. The repeatability of the moisture content measurement was also tested on roasted colored malt. Measurements were performed in the same laboratory.

## MATERIALS AND METHODS

The major Italian brewers supplied samples of the colored and caramel malt samples so they are representative of the raw materials used in beer production.

The color of malts was determined by the Analytica-EBC method for colored

malts (EUROPEAN BREWERY CONVENTION, 2006c), in which there is a visual comparison between congress worts and glass slide discs.

Moisture content of the colored malts was measured by following the Analytica-EBC method for malts (EUROPEAN BREWERY CONVENTION, 2006b).

Color and moisture content measurements were carried out at the same time, immediately after opening the sealed boxes where the samples were stored at room temperature for few days before analyses.

The repeatability factor was tested by measuring the moisture content of roasted colored malt 10 times, under the same conditions. The distribution was previously reported to be normal according to Shapiro-Wilks, and the absence of anomalous data was verified by the Huber test; both tests were related to a 95% confidence level (HAHN and SHAPIRO, 1994; UNICHIM, 2001).

Apparatus: Sample divider (VLB, Berlin, Germany); DLFU type disc mill (Bhüler, Uzwil, Switzerland), set to a gap of 0.2 mm; aluminum dishes (VLB, Berlin, Germany); FD115 electrically heated ventilated oven, capable of maintaining the temperature within ±0.5°C (Binder, Tuttlingen, Germany); AB204-S balance, accuracy ±0.0005 g (Mettler-Toledo, Greifensee, Switzerland); PB-1502-S/FACT balance, accuracy ±0.1 g (Mettler-Toledo, Greifensee, Switzerland); Elix 3 System (Millipore, Billerica, MA, USA); Kongress Mash LB 12 Electronic (Lochner, Bayreuth, Germany); 3000 Comparator (Tintometer, Dortmund, Germany).

## RESULTS AND CONCLUSIONS

In 2007 and 2008, 27 samples of caramel and roasted colored malts where analyzed with respect to color and moisture content, according to the Analytica-EBC methods 4.2, and 5.6 (EUROPEAN BREWERY CONVENTION, 2006b; c). The data are reported in Tables 1 and 2.

The moisture content in caramel

Table 1 - Color values for colored malts.

| Kind of malt | Number of samples | Color (EBC unit) | | | sd |
|---|---|---|---|---|---|
| | | Average | Minimum | Maximum | |
| all | 27 | 731 | 128 | 1,400 | 402 |
| roasted | 19 | 963 | 750 | 1,400 | 202 |
| caramel | 8 | 181 | 128 | 263 | 49 |
| sd: standard deviation. | | | | | |

Table 2 - Moisture content (%) for colored malts.

| Kind of malt | Number of samples | Average | Minimum | Maximum | sd |
|---|---|---|---|---|---|
| all | 27 | 2.2 | 0.5 | 4.7 | 14 |
| roasted | 19 | 1.4 | 0.5 | 2.9 | 06 |
| caramel | 8 | 3.9 | 3.2 | 4.7 | 05 |
| sd: standard deviation. | | | | | |

Table 3 - Repeatability analysis on roasted colored malt (%) (n=19).

| | |
|---|---|
| Average value | 0.94 |
| Minimum value | 0.88 |
| Maximum value | 0.98 |
| Standard deviation ($s_r$) | 0.033 |
| Repeatability limit ($r_{95}$) | 0.107 |

malts, which have less color intensity was higher than in the roasted ones, which are more intensely colored. These data are in agreement with the literature data cited above. The heat treatments that are responsible for colored-molecule development (by means of the Maillard reaction), definitely have a drying effect on the malts.

The results of the repeatability study carried out on roasted colored malt show that the data distribution is normal, according to the Shapiro-Wilks test. No anomalous data were detected according to the Huber test. Both tests were carried out at the 5% confidence level (Table 3).

The repeatability limit determined in this study lies between that reported in Analytica-EBC 4.2 ($r_{95}$ = 0.13), and that reported in the Analytica-EBC 5.4 ($r_{95}$ = 0.07) (EUROPEAN BREWERY CONVENTION, 2006a; b). The standard deviation of the repeatability trial ($s_r$) is comparable in both methods: $\sigma_r$ = 0.046 and 0.025 for the validation of Analytica-EBC 4.2 and Analytica-EBC 5.4, respectively.

The data obtained in this study are in agreement with those already reported in the literature regarding the expected moisture content values in colored malts. These results highlight the need to carry out a collaborative trial in order to validate the method used to determine the moisture content in colored malts, to confirm the applicability of the official method to the samples that are commonly available on the market. The new validation should be carried out giving particular attention to the choice and management of the colored malt samples used for the collaborative trials to have new reference values for moisture mainly corresponding to the characteristics of the colored malt samples available on the market.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the financial support of the Italian Ministry of University and Research, the Italian Ministry of Agriculture, and the Italian Ministry of Industry.

REFERENCES

Bénard M. 1997a. Determination of repeatability and reproducibility of EBC accepted methods. IV. Coloured malts. J. Inst. Brew. 103: 163.

Bénard M. 1997b. Determination of repeatability and reproducibility of EBC accepted methods. IV. Coloured malts. Monatsschr. Brauwiss. 50: 114.

Briggs D.E. 1998. "Malts and Malting". London: Blackie Academic & Professional. p 716.

Coghe S., Martens E., D'Hollander H., Dirinck P.J. and Delvaux F.R. 2004a. Sensory and instrumental flavour analysis of wort brewed with dark speciality malts. J. Inst. Brew. 110: 94.

Coghe S., Adriaenssons B., Leonard S. and Delvaux F.R. 2004b. Fractionaction of coloured Maillard reaction products from dark speciality malts. J. Am. Soc. Brew. Chem. 62: 79.

European Brewery Convention. 2006a. 5.4 "Colored Malts: Moisture". European Brewery Convention, ed.. Analytica-EBC. Fachverlag Hans Carl, Nürnberg, Germany.

European Brewery Convention. 2006b. 4.2 "Moisture Content of Malt". European Brewery Convention, ed.. Analytica-EBC. Fachverlag Hans Carl, Nürnberg, Germany.

European Brewery Convention. 2006c. 5.6 "Coloured Malts: Colour, Visual Method". European Brewery Convention, ed. Analytica-EBC. Fachverlag Hans Carl, Nürnberg, Germany.

Hahn G.H. and Shapiro S.S. 1994. "Statistical Models in Engineering". John Wiley & Sons, New York.

Kunze W. 2004. "Technology of Brewing and Malting". p 176, 3RD int. ed. VLB, Berlin, Germany.

MEBAK. 2006a. 3.1 Gerstenmalz. In: "Brautechnische Analysebmethoden, Band Rohstoffe". Heinz-Michael Anger, ed. Mitteleuropäische Brautechnische Analysenkommission, Freising-Weihenstephan, Germany.

MEBAK. 2006b. 3.2.1 Wasser (EBC-Methode). In: "Brautechnische Analysebmethoden, Band Rohstoffe". Heinz-Michael Anger, ed. Mitteleuropäische Brautechnische Analysenkommission, Freising-Weihenstephan, Germany.

O'Shaughnessy C.L., Chandra G.S., Fryer P.J., Robbins P.T. and Wedzicha B.L. 2003. Monitoring flavor development during the roasting of cereals. Tech. Q. Master Brew. Assoc. Am. 40: 98.

Rabin D. and Forget C. 1998. "The Dictionary of Beer & Brewing". p 44, Brewers Publications, Boulder, CO, USA.

Sunier J. 1988. "La Fabbricazione del Malto e della Birra". p 90, Unione Italiana Fabbricanti Birra e Malto, Rome, Italy.

Unichim. 2001. Valutazione della precisione (ripetibilità ristretta) di un metodo analitico eseguito in un unico laboratorio da un solo operatore su di un unico strumento in un breve intervallo di tempo. In: Unichim. "Linee Guida per la Validazione di Metodi Analitici nei Laboratori Chimici". Vol. 179/1, Milan, Italy.

Woffenden H.M., Ames J.M. and Chandra S. 2001. Relationships between antioxidant activity, color, and flavor compounds of crystal malt extracts. J. Agr. Food Chem. 49: 552.

Wunderlich S., Zuercher A. and Back W. 2005. Enrichment of xanthohumol in brewing process. Mol. Nutr. Food Res., 49: 874.

Revised paper received December 5, 2008 Accepted January 22, 2009

# AUTHOR INDEX

VOLUME XXI (2009)


Name page

Aarts, P. 407
Adam, M. 89
Akalin, A.S. 357
Alamanni, M.C. 477
Ambrosoli, R. 287
Amendola, G. 325
Aquilanti, L. 269
Ashman, H. 407
Ayuso, C. 17
Ballabio, D. 149
Barbagallo, R. 3
Barroso, J.G. 183
Battaglini, L.M. 287
Bazzi, L. 517
Beckley, J. 407
Beganović, J. 473
Belibağli, K.B. 499
Bendini, A. 389
Bentes, J. 183
Bernalte, J. 17
Biondi, P.A. 149
Borowska, E.J. 197
Bravi, E. 211
Bucchini, A. 337
Caccioppola, A. 461
Canese, S. 325
Cantoni, C. 149
Carcea, M. 241
Carcione, F. 149
Carnì, A. 375
Cattaneo, P. 149
Cerretani, L. 389
Cervenka, L. 89
Chakir, A. 517
Chen, J.S. 487
Chiesa, L.M. 149
Chisari, M. 3
Clementi, F. 269
Comitini, F. 269
Cossu, M. 447
Costa, M. 183
Cukelj, N. 123
Curic, D. 123
Curioni, A. 97
Curko, N. 123
Czaplicki, S. 197
Dalay, M.C. 357
Dalessandro, G. 461
Dalgiç, A.C. 499
D'Andrea, M. 365
Daniel Vaz de Almeida, M. 429
Danowska-Oziewicz, M. 429
Das, A.K. 81
De Caroli, M. 461
Deng, Y. 487
Deschamps, F.C. 231
Drainas, C. 135
Durante, M. 461
Ercişli, S. 65
Erdoğrul, O. 107
Erzetti, M. 211
Evrendilek, G.A. 171, 347
Ezer, M. 107
Fantozzi, P. 211
Ferreira, I.M.P.L.V.O. 255
Figueiredo, A.C. 183
Floridi, S. 529
Fraternale, D. 337
Frece, J. 473
Fregapane, G. 389
Gabay, G. 407
Gabric, D. 123

Gangemi, S. ..... 375
García, J. ..... 17
Garda, A. ..... 287
Giamperi, L. ..... 337
Giannou, E. ..... 135
Giovanelli, G. ..... 305
Gonçalves, F.J. ..... 51
González, J.A. ..... 17
Hajduk, G. ..... 473
Hernández, T. ..... 17
Hormatallah, A. ..... 517
Id El Mouden, O. ..... 517
Isik, H.B. ..... 37
Jordão, A.M. ..... 51
Juliano, C. ..... 447
Kakouri, A. ..... 135
Kallimanis, A. ..... 135
Kandarakis, I. ..... 73
Kos, B. ..... 473
Kralovsky, J. ..... 89
Leboš, A. ..... 473
Lemerhyeratte, A. ..... 517
Lenucci, M.S. ..... 461
Lercker, G. ..... 389
Lianou, A. ..... 135
Lovino, R. ..... 325
Lozano, M. ..... 17
Luo, W. ..... 487
Lussiana, C. ..... 287
Mainente, F. ..... 97
Mannazzu, I. ..... 269
Marconi, O. ..... 211, 529
Marte, L. ..... 529
Melini, F. ..... 241
Merigioli, A. ..... 365
Miguel, M.G. ..... 183
Montanari, L. ..... 211
Monteiro, I. ..... 183
Moschopoulou, E. ..... 73
Moskowitz, H.R. ..... 407
Novotni, D. ..... 123
Oliveira, G.F. ..... 231
Onoufriou, E. ..... 73
Osimani, A. ..... 269
Özgen, M. ..... 65
Ozogul, Y. ..... 317
Pagliarini, E. ..... 305
Pasini, G. ..... 97
Paulus, K. ..... 407
Pedro, L.G. ..... 183
Perretti, G. ..... 211, 529
Pessatti, M.L. ..... 231
Pilla, F. ..... 365
Pinho, O. ..... 255
Piro, G. ..... 461
Pisu, R. ..... 447
Polat, S. ..... 317
Ramos, B. ..... 255
Renna, M. ..... 287
Rezkova, S. ..... 89
Ricci, D. ..... 337
Ricciardi, L. ..... 375
Ríos, A. ..... 517
Saitta, S. ..... 375
Saja, A. ..... 375
Salghi, R. ..... 517
Salvador, M.D. ..... 389
Samelis, J. ..... 135
Scarano, M-T. ..... 365
Serçe, S. ..... 65
Serrone, L. ..... 461
Seyis Bilkay, I. ..... 29
Sharma, R.B. ..... 81
Simonato, B. ..... 97
Soncin, S. ..... 149
Song, X.Y. ..... 487
Soylu, E.M. ..... 171, 347
Soylu, S. ..... 171, 347
Spagna, G. ..... 3
Speciale, A. ..... 375
Stewart-Knox, B. ..... 429
Suglia, I. ..... 97
Šušković, J. ..... 473
Szajdek, A. ..... 197
Tok, F.M. ..... 171
Topcu, Y. ..... 37, 157
Torun, A.A. ..... 65
Tuffi, R. ..... 325
Ünal, G. ..... 357
Uzundumlu, A.S. ..... 37, 157
Vardin, H. ..... 499
Ventura, K. ..... 89
Vidal-Aragón, C. ..... 17
Wądołowska, L. ..... 429
Xiao, C.L. ..... 487
Zannini, E. ..... 269
Zhu, L.W. ..... 487
Zougagh, M. ..... 517

# SUBJECT INDEX

VOLUME XXI (2009)


Abomasum
Parts and contents from lamb, effect on enzymic properties of rennet ..... 73

Allergy
To a para-amino compound ..... 375

*Alternaria alternata*
Characterization of beta-galactosidase ..... 29

Amino acid
Composition of bread from extruded corn and soybean flour blend ..... 123

Analytica-EBC
Moisture determination in colored malts ..... 529

*Anethum graveolens* (Dill)
Antibacterial activity of essential oil ..... 347

Anthocyanin
Content in chokeberry juice, effect of heat and enzyme treatment ..... 197
In *Prunus spinosa* fruit juice ..... 337

Antibacterial
Activity of essential oils of bitter fennel and dill ..... 347

Antioxidant
Composition of tomato products consumed in Italy ..... 305
Activity in red wine aged in contact with Portuguese and American wood ..... 51
Activity of *Arbutus andrachne* fruit at different maturation stages ..... 65
Activity of chokeberry juice, effect of heat and enzyme treatment ..... 197
Activity of essential oils and extracts of *Thymbra capitata* and *Thymus mastichina* 183
Activity of *Prunus spinosa* L. fruit juice ..... 337
Activity of yogurt enriched with polyphenolic plant extracts ..... 447

*Arbutus andrachne* (Greek Strawberry Tree)
Chemical composition, antioxidant activity, total phenols at different maturity stages ..... 65

Artisanal
Rennet from abomasum parts of lambs, effect on enzymic properties ..... 73

Ascorbic acid
In tomato products consumed in Italy ..... 305

Azo dyes
Allergic response to food wrapping ..... 375

Bakery products
High protein bread from extruded corn and soybean flour blend ..... 123
Sourdoughs of Marche region, lactic acid bacteria and yeasts ..... 269
Volatile profile and sensory characteristics of creams ..... 255

B-carotene
In tomato products consumed in Italy ..... 305
In traditional and high-pigment tomato cultivars during ripening ..... 461

Beta-galactosidase
Characterization from *Alternaria alternata* on solid-state cultivation ..... 29

*Bifidobacterium animalis*
In probiotic yogurt, influence of *Spriulina platensis* ..... 357
Subsp. *lactis* BB-12, probiotic properties in cereal flakes enriched with inulin ..... 473

Biogenic amines
In malting and brewing, HACCP in production chain ..... 211

Blackthorn or sloe (*Prunus spinosa*)
Fruit juice, antioxidant activity ..... 337

*Botrytis cinerea*
Post harvest control with ozone and NAI ..... 325

Bread
High protein, using extruded corn and soybean flour blend ..... 123

Breast muscle
Of capons, fatty acid composition of lipid classes as marker of dietary fat source ..... 149

Brewing
Moisture content of coloured malts ..... 529
Use of HACCP to evaluate risks in production chain ..... 211

Broccoli
Productive characteristics and volatile compounds in cultivars ..... 17

Browning
Enzymatic, in vegetable crops ..... 3

Bulgur
Processing, food safety and quality systems ..... 499

By-products
Of Brazilian fishery, fatty acid composition ..... 231

Capons
Fatty acid composition of lipids in breast muscle ..... 149

Carotenoid
Content in traditional and high-pigment tomato cultivars during ripening ..... 461
In tomato products consumed in Italy ..... 305

Cattle
Traceability of breed, in meat and cheese using SnaPshot method ..... 365

Cereal
Flakes enriched with inulin, probiotic properties of *Bifidobacterium animalis* ..... 473

Cheese
Consumer mindset and cross-national segmentation ..... 407
DNA polymorphisms, using SnaPshot methods for cattle breed traceability ..... 365
Graviera, identification and biopreservation of *Enterococcus* in ..... 135
Organic Fontina PDO, chemical, nutritional, microbiological characterization ..... 269

Chemical composition
Of *Arbutus andrachne* fruit at different maturation stages ... 65
Of essential oils of bitter fennel and dill ... 347
Of organic Fontina PDO cheese ... 287

Chevon nuggets
Effect of goat milk whey on quality characteristics ... 81

Chitosan
Films, changes in physical properties at subzero temperatures ... 487

Chokeberry
Juice, effect of heat and enzyme treatment on phenolic content and antioxidant capacity ... 197

Cobalt
Levels in honey from Turkey ... 107

Conjoint analysis
To analyze consumer features driving consumer interest in cheese ... 407

Conjugated linoleic acid
In Organic PDO Fontina cheese ... 269

Consumer
Attitudes toward food products in Erzurum, Turkey ... 37
Mindset about cheese and cross-national segmentation ... 407
Retailer loyalty in the Turkish food market ... 157

Contamination
Heavy metals, of honey in Turkey ... 107

Corn
Extruded, and soybean flour in high protein bread ... 123
Zeins, fining efficiency in red wine ... 97

Creams
As fillers in bakery products, volatile profile and sensory characteristics ... 255

Cross-national segmentation
For messaging about cheese ... 407

Cultivars
Broccoli, productive characteristics and volatile compounds ... 17
Carotenoid content during ripening in traditional and high-pigment tomato ... 461

Dairy products
Fontina PDO cheese, chemical, nutritional and microbiological characterization ... 269
Graviera cheese, identification and biopreservation of *Enterococcus* in ... 135
Identification of cattle breed using cheese and DNA polymorphisms ... 365

Dermatitis
Systemic contact, allergic response to a para-amino compound ... 375

Dicofol
Monitoring in greenhouse grown tomatoes in Morocco ... 517

Diet
Fatty acid composition in capon breast meat as marker of fat source ... 149

Of lambs, effect on enzymic properties of liquid rennet ..... 73
Related diseases, metabolic syndrome in Poles 15 years and above ..... 429

Difenoconazole
Monitoring in greenhouse grown tomatoes in Morocco ..... 517

Dill (*Anethum graveolens*)
Antibacterial activity of essential oil ..... 347

DNA
Polymorphisms by the SnaPshot method for cattle breed traceability ..... 365

Dye
Para-amino in plastic packaging, food allergy to ..... 375

Egg creams
In bakery products, home made vs industrial ..... 255

*Enterococcus*
Isolated from Graviera Greek cheese, identification and biopreservation of ..... 135

Enzymatic
Browning in vegetable crops ..... 3
Properties of liquid lamb rennet, effect of diet and abomasums parts ..... 73

Enzyme
Beta-galactosidase from *Alternaria alternata* by solid state cultivation ..... 29
Endogenous in fresh-cut vegetables ..... 3
Treatment of chokeberry mash, effect on yield, phenols and antioxidant capacity ..... 197

Essential oil
Of *Foeniculum vulgare* and *Anethum graveolens* as antibacterial agents ..... 347
Of *Thymbra capitata* and *Thymus mastichina*, antioxidant activity of ..... 183

Extracts
From Portuguese *Thymbra capitata* and *Thymus mastichina*, antioxidant activity of ..... 183
From Sardinian plants, effect on antioxidant and microbiological properties of yogurt ..... 447

Extrusion
Of corn and soybean flour to produce high protein bread ..... 123

Factor analysis
To evaluate consumer food choices in Erzurum, Turkey ..... 37
To evaluate customer retailer loyalty in Erzurum, Turkey ..... 157

Fatty acid composition
Of Brazilian fishery by-products ..... 231
Of lipid classes in capon breast muscle as marker of dietary fat source ..... 149
Of organic PDO Fontina cheese ..... 269
Of seaweeds from Northeastern Mediterranean coast ..... 317

Fennel (*Foeniculum vulgare*)
Bitter, antibacterial activity of essential oil ..... 347

Films
Chitosan, changes in physical properties at subzero temperatures ..... 487

Fining
Of red wine, efficiency of corn zeins ..... 97

Fishery
By-products, fatty acid composition ..... 231

*Foeniculum vulgare* (Fennel)
Antibacterial activity of essential oil ..... 347

Fontina
PDO cheese, chemical, nutritional, microbiological characterization ..... 269

Food
Choices, of consumers in Erzurum, Turkey ..... 37
Functional, perceptions of health risks towards metabolic syndrome among Poles ..... 249
New approach to consumer understanding and market development of cheese ..... 407
Safety and quality management in bulgur processing ..... 499

Fresh-cut
Vegetables, enzymatic browning ..... 3

Fruit juice
Chokeberry, effect of heat and enzyme treatment on phenolic content and antioxidant capacity ..... 197
Inactivation of *Botrytis cinerea* using pulsed electric fields ..... 171
Of *Prunus spinosa*, antioxidant activity ..... 337

Functional food
Consumption and awareness of health regarding metabolic syndrome of Poles ..... 429
Probiotic properties of *Bifidobacterium animalis* in inulin-enriched cereal flakes ..... 473

Fungistat
Ozone and NAI to control post-harvest mould development ..... 325

Germination tube elongation
Of *Botrytis cinerea* inoculated into fruit juice, effect of pulsed electric fields ..... 171

Goat milk
Whey, effect on quality characteristics of chevon nuggets ..... 81

Graphite furnace atomic absorption spectrometry
To determine heavy metal levels in honey ..... 107

Graviera cheese
Identification and biopreservation of *Enterococcus* in ..... 135

Greek Strawberry Tree (*Arbutus andrachne*)
Chemical composition, antioxidant activity, total phenols at different maturity stages ..... 65

Greenhouse,
Tomatoes, monitoring pesticide levels in Morocco ..... 517

HACCP
In bulgur processing ..... 499
In malting and brewing production, for mycotoxins, nitrosamines and biogenic amines ..... 211

Health
Perceptions of risks by Poles, metabolic syndrome ..... 429

Honey
From Kahramanmaraş, Turkey, levels of heavy metals ..... 107

Hydrodisillation-aqueous phase
Antioxidant activity of extracts from Portuguese *Thymbra capitata* and *Thymus mastichina* ..... 183

Inactivation
Of *Botrytis cinerea* in sour cherry, apricot and peach nectars using pulsed electric fields ..... 171

Intellectual Property Rights
In MoniQA Network of Excellence ..... 241

Inulin
Enrichment of cereal flakes, effect on properties of *Bifidobacterium animalis* ..... 473

Karl Fischer titration
To determine water content in meat pâté ..... 89

Lactic acid bacteria
From wheat sourdoughs from Marche region ..... 269

Lamb rennet
Effect of diet and abomasum parts on enzymic properties ..... 73

Lead
Levels in honey from Turkey ..... 107

Lipgene project
Functional food consumption and metabolic syndrome among Poles ..... 429

Lipid
Classes, fatty acid composition in capon breast muscle as marker of dietary fat source ..... 149
In Brazilian fishery by-products, fatty acid composition ..... 231

*Listeria monocytogenes*
Bio-protective role of *Enterococcus* in Graviera cheese ..... 135

Lutein
In traditional and high-pigment tomato cultivars during ripening ..... 461

Lycopene
In traditional and high-pigment tomato cultivars during ripening ..... 461

Lysine score
Of bread from extruded corn and soybean flour blend ..... 123

Maceration
Of chokeberry mash, effect of heat and enzyme treatment ..... 197

Majoram (*Thymus mastichina*)
Antioxidant activity of essential oil of ..... 183

Malt
Moisture content determination ..... 529

Malting
Use of HACCP to evaluate risks in production chain ..... 211

Market segmentation
Consumer food choices evaluated by k-means cluster analysis ..... 37
Consumer retailer loyalty evaluated by k-means cluster analysis ..... 157
Cross-national, consumer mindset about cheese ..... 407

Maturity
Stages of *Arbutus andrachne* fruit, changes in antioxidant activity and total phenols ..... 65

*MC1R*
For molecular breed traceability of cattle ..... 365

Meat
Chevon nuggets, effect of goat milk whey on quality characteristics ..... 81
DNA polymorphisms by SnaPshot method for cattle breed traceability ..... 365
Fatty acid composition of lipid classes in capon breast muscle ..... 149
Pâté, determination of water content by Karl Fischer titration ..... 89

Metabolic syndrome
Perceptions of health risks among Poles ..... 429

Methodology
GC-ECD and LLE for pesticide residue determination in tomato ..... 517
GC/MS to determine volatile compounds in broccoli ..... 17
Graphite furnace atomic absorption spectrometry to determine heavy metals in honey ..... 107
Karl Fischer titration to determine water content in meat pâté ..... 89
Pulsed electric fields on germination tube elongation of *Botrytis cinerea* in fruit juice ..... 171
SnaPshot method for meat and cheese DNA polymorphisms for cattle breed traceability ..... 365
Solid state cultivation of *Alternaria alternate* for beta-galactosidase ..... 29
SPME and GC-MS for volatile profile of bakery creams ..... 255
Thin layer chromatography for fatty acid composition in capon breast muscle ..... 149

Microbiological properties
Of organic PDO Fontina cheese ..... 269
Of probiotic yogurts during refrigerated storage, influence of *Spirulina platnesis* ..... 357
Of yogurt enriched with polyphenolic plant extracts ..... 447

Mineral elements
In *Arbutus andrachne* fruit at different maturation stages ..... 65
In seaweeds from Northeastern Mediterranean coast ..... 317

Mobility
Of researchers, legal constraints and limiting factors ..... 241

Moisture content
Determination in colored malts ..... 529
Determination in meat pâté ..... 89

MoniQA network
Legal constraints and factors limiting researcher mobility ..... 241

Mould
Post-harvest control with ozone and NAI ..... 325

Mycotoxins
In malting and brewing, HACCP in production chain ..... 211

Negative air ions (NAI)
And ozone against moulds ..... 325

Network
MoniQA, overview of legal constrains and researcher mobility and integration ..... 241

Nickel
Levels in honey from Turkey ..... 107

Nitrosamines
In malting and brewing, HACCP in production chain ..... 211

Oil
Essential, antibacterial activity of *Anethum graveolens* and *Foeniculum vulgare* .....347
Essential, antioxidant activity of *Thymbra capitata* and *Thymus mastichina* ..... 183
Virgin olive, stability of sensory quality during storage ..... 389

Olive oil
Stability of sensory quality during storage ..... 389

Organic
Acids in *Arbutus andrachne* fruit at different maturation stages ..... 65
Fontina PDO cheese, chemical, nutritional and microbiological characterization ..... 269

Ozone
And negative air ions against moulds ..... 325

Packaging
Coloured plastic, para-amino dyes and allergic response ..... 375

Pathogens
Food-borne and seed-borne, antibacterial activity of essential oils against ..... 347

*Penicillum expansum*
Post harvest control with ozone and NAI ..... 325

Pesticide
Monitoring residue levels in tomatoes in Moroccan greenhouses ..... 517

Phenols
Content in chokeberry mash juice, effect of heat and enzyme treatment..... 197
Effect of storage on sensory quality of olive oil ..... 389
Extracts from Sardinian plants, effect on antioxidant and microbiological properties of yogurt ..... 447
In tomato products consumed in Italy..... 305
Total, in *Arbutus andrachne* fruit at different maturation stages ..... 65

Polymorphisms
DNA, by the SnaPshot method for cattle breed traceability..... 365

Polyphenol oxidase
And enzymatic browning in vegetables..... 3

Post-harvest
Control of moulds with ozone and NAI ..... 325
Enzymatic browning and softening in vegetable crops..... 3

Proanthocyanidins
In red wine aged in contact with Portuguese and American wood..... 51

Probiotic
Properties of *Bifidobacterium animalis* in cereal flakes enriched with inulin ..... 473
Yogurt, influence of *Spirulina platensis* on microbiological viability during storage ..... 357

Processing
Of bulgur, food safety and quality management systems ..... 499

Protein
Digestibility, of bread produced from extruded corn and soybean flour blend ..... 123
Plant, for red wine fining ..... 97

*Prunus spinosa* (blackthorn or sloe)
Fruit juice, antioxidant activity ..... 337

Pulsed electric fields
On germination tube elongation and spore germination of *Botrytis cinerea* in fruit juice ..... 171

Puree
Tomato, antioxidant composition ..... 305

Quality
And food safety, HACCP in Bulgur processing ..... 499
Characteristics of chevon nuggets using whey from goat milk ..... 81
HACCP in malting and brewing, mycotoxin, biogenic amine risks ..... 211
Management systems and food safety in bulgur processing ..... 499
Productive characteristics and volatile compounds in broccoli ..... 17
Sensory, of virgin olive oil during storage ..... 389

*Quercus alba* (American oak) chips
In contact with red wine, effect on antioxidant activity and proanthocyanidin fraction ..... 51

*Quercus pyrenaica* (Portuguese oak) chips
In contact with red wine, effect on antioxidant activity and proanthocyanidin fraction ..... 51

Rennet
Liquid lamb, effect of diet and abomasums parts on enzymic properties ..... 73

Residue
Monitoring pesticide levels in tomatoes in Moroccan greenhouses ..... 517

Ripening
Of traditional and high-pigment tomatoes, carotenoid content ..... 461

Rutin
In tomato products consumed in Italy ..... 305

Safety
And quality management systems in bulgur processing ..... 499
HACCP in malting and brewing, mycotoxin, biogenic amine risks ..... 211

Seasonal
Variation, effect on organic PDO Fontina cheese ..... 269

Seaweeds
From Northeastern Mediterranean coast, fatty acid, mineral and proximate composition ..... 317

Sensory
Attributes of chevon nuggets, effect of fluid goat milk whey ... 81
Characteristics of bakery creams, home made vs industrial ... 255
Quality of virgin olive oil during storage ... 389

SnaPshot method
Meat and cheese DNA polymorphisms for cattle breed traceability ... 365

Softening
Enzymatic browning in vegetables ... 3

*Solanum lycopersicum* (Tomato)
Carotenoid content during ripening in traditional and high-pigment cultivars ... 461
Products consumed in Italy, antioxidant composition ... 305

Solid Phase Microextraction
With GC/MS for volatile profile of bakery creams ... 255

Solid-state cultivation
Beta-galactosidase from *Alternaria alternata* ... 29

Soluble sugars
In *Arbutus andrachne* fruit at different maturation stages ... 65

Sourdough
From Marche region, lactic acid bacteria and yeasts ... 269

Soybean flour
And extruded corn to produce high protein bread ... 123

*Spirulina platensis*
Influence on microbiological viability in probiotic yogurts
during refrigerated storage ... 357

Spore germination rate
Of *Botrytis cinerea* inoculated into fruit juice, effect of pulsed electric fields ... 171

Stability
Of beta-galactosidase form *Alternaria alternata* ... 29
Of sensory quality of virgin olive oil during storage ... 389

Starter
Cultures, LAB and yeasts in wheat sourdough from Marche region ... 269

Storage
Effect on stability of sensory quality of virgin olive oil ... 389
Frozen, on physical properties of chitosan films ... 487
Of probiotic yogurt, influence of *Spirulina platensis* on microbiological viability ... 357

Temperature
Subzero, effect of physical properties of chitosan films ... 487

Thyme (*Thymbra capitata*)
Antioxidant activity of essential oil of ... 183

*Thymus mastichina* (Majoram)
Antioxidant activity of essential oil of ... 183

Thin layer chromatography
For fatty acid composition of lipid classes in capon breast muscle ... 149

Tomato (*Solanum lycopersicum*)
Carotenoid content during ripening in traditional and high-pigment cultivars ... 461
Pesticide residue monitoring in Moroccan greenhouses ... 517
Products consumed in Italy, antioxidant composition ... 305

Traceability
Of cattle breeds, use of DNA polymorphisms by SnaPshot method ... 365

Urticaria
Chronic, to a para-amino compound ... 375

Vegetables
Broccoli cultivars and volatile compounds ... 17
Enzymatic browning in ... 3

Viability
Microbiological, in probiotic yogurts during refrigerated storage, influence of *Spirulina platensis* ... 357

Volatile
Compounds in broccoli cultivars ... 17
Profile of creams in bakery products, home made vs industrial ... 255
Stability of sensory quality of virgin olive oil during storage ... 389

Water
Content in meat pâté, determination by Karl Fischer titration ... 89
Vapour permeability of chitosan films at subzero temperatures ... 487

Wheat
Bran, beta-galactosidase by *Alternaria alternata* produced on ... 29
Sourdoughs from Marche region, LAB and yeasts ... 269

Whey
From goat milk, effect on quality characteristics of chevon nuggets ... 81

Wine
Red, aged in contact with wood chips, antioxidant activity and proanthocyanidin fraction ... 51
Red, fining efficiency of corn zeins ... 97

Wood chips
Portuguese and American, in contact with red wine, effect on antioxidant activity ... 51

Yeasts
In wheat sourdoughs from Marche region ... 269

Yogurt
Enriched with polyphenolic extracts, antioxidant and microbiological properties ... 447
Traditional and probiotic, influence of *Spirulina platensis* on microbiological viability ... 357

Yield
Effect of heat and enzyme treatment on chokeberry mash juice ... 197

Zeins
Corn, efficiency in fining red wine ... 97

# CONTENTS OF VOLUME XXI - 2009

REVIEW

Enzymatic Browning and Softening in Vegetable Crops: Studies and Experiences.....**3**
***R.N. Barbagallo, M. Chisari and G. Spagna***

PAPERS

Productive Characteristics and Volatile Compounds of Seven Broccoli Cultivars.....**17**
***C. Vidal-Aragón, M. Lozano, J. Bernalte, C. Ayuso, I. García, T. Hernández, J. García and J.A. González***

Characterization of Beta-Galactosidase from *Alternaria alternata* on Solid-State Cultivation.....**29**
***I. Seyis Bilkay***

Turkish Consumer Attitudes Toward Food Products: The Case of Erzurum
***Y. Topcu, H.B. Isik and A.S. Uzundumlu*.....37**

Changes in Antioxidant Activity and the Proanthocyanidin Fraction of Red Wine Aged in Contact with Portuguese (*Quercus pyrenaica* Willd.) and American (*Quercus alba* L.) Oak Wood Chips.....**51**
***F.J. Gonçalves and A.M. Jordão***

Changes in Chemical Composition, Antioxidant Activities and Total Phenolic Content of *Arbutus andrachne* Fruit at Different Maturation Stages.....**65**
***M. Özgen, A.A. Torun, S. Ercişli and S. Serçe***

Effect of Diet and Abomasum Parts on Enzymic Properties of Liquid Lamb Rennet.....**73**
***E. Moschopoulou, E. Onoufriou and I. Kandarakis***

SHORT COMMUNICATIONS

The Effect of Fluid Goat Milk Whey on Some Quality Characteristics of Chevon Nuggets.... **81**
***A.K. Das and R.B. Sharma***

Determination of Water Content in Meat Pâté by Karl Fischer Titration and Its Moisture Sorption Characteristics.....**89**
***M. Adam, L. Cervenka, S. Rezkova, K. Ventura and J. Kralovsky***

Evaluation of Fining Efficiency of Corn Zeins in Red Wine: A Preliminary Study.....**97**
***B. Simonato, F. Mainente, I. Suglia, A. Curioni and G. Pasini***

Cobalt, Lead and Nickel Levels in Honey Produced in Kahramanmaraş, Turkey.....**107**
***O. Erdoğrul and M. Ezer***

ERRATA CORRIGE .....**113**
BOOKS - NEWS.....**117**

PAPERS

Production of High Protein Bread Using Extruded Corn and Soybean Flour Blend.....**123**
***D. Novotni, D. Curic, D. Gabric, N. Cukelj and N. Curko***

Identification and Biopreservation Potential of *Enterococcus* spp. Isolated from Fully Ripened Graviera, a Traditional Hard Greek Cheese .....**135**
***E. Giannou, A. Lianou, A. Kakouri, A. Kallimanis, C. Drainas and J. Samelis***

Fatty Acid Composition of Lipid Classes in Commercial Breast Muscle of Capons as a Marker of the Dietary Fat Source.....**149**
***F. Carcione, L.M. Chiesa, D. Ballabio, S. Soncin, P.A. Biondi, P. Cattaneo and C. Cantoni***

Analysis of Factors Affecting Customer Retailer Loyalty in the Turkish Food Market: The Case Study of Erzurum .....**157**
***Y. Topcu and A.S. Uzundumlu***

Effect of Pulsed Electric Fields on Germination Tube Elongation and Spore Germination of *Botrytis cinerea* Inoculated into Sour Cherry Juice, Apricot and Peach Nectars .....**171**
***G. Akdemir Evrendilek, F.M. Tok, E.M. Soylu and S. Soylu***

Antioxidant Activities of the Essential Oils and Extracts of Portuguese *Thymbra capitata* and *Thymus mastichina* ..... **183**
***J. Bentes, M.G. Miguel, I. Monteiro, M. Costa, A.C. Figueiredo, J.G. Barroso, L.G. Pedro***

Effect of Heat and Enzyme Treatment on Yield, Phenolic Content and Antioxidant Capacity of Juices from Chokeberry Mash .....**197**
***E.J. Borowska, A. Szajdek and S. Czaplicki***

HACCP in the Malting and Brewing Production Chain: Mycotoxin, Nitrosamine and Biogenic Amine Risks.....**211**
***M. Erzetti, O. Marconi, E. Bravi, G. Perretti, L. Montanari and P. Fantozzi***

SHORT COMMUNICATIONS

The Fatty Acid Composition of Brazilian Fishery By-Products.....**231**
***G.F. Oliveira, F.C. Deschamps and M.L. Pessatti***

OVERVIEW

The MoniQA Network: An Overview on Legal Constraints and Factors Limiting Researcher Mobility and Integration within the Network.....**241**
***M. Carcea and F. Melini***

GUIDE FOR AUTHORS .....**247**

CONTRIBUTORS.....**250**

PAPERS

Volatile Profile and Sensory Characteristics of Creams Used as Fillers of Bakery Products: Home Made Egg Creams vs Industrial Creams ..... **255**
***B. Ramos, O. Pinho and I.M.P.L.V.O. Ferreira***

Lactic Acid Bacteria and Yeasts from Wheat Sourdoughs of the Marche Region ..... **269**
***A. Osimani, E. Zannini, L. Aquilanti, I. Mannazzu, F. Comitini and F. Clementi***

Chemical, Nutritional and Microbiological Characterization of Organic Fontina PDO Cheese ..... **287**
***M. Renna, A. Garda, C. Lussiana, R. Ambrosoli and L.M. Battaglini***

Antioxidant Composition of Tomato Products Typically Consumed in Italy ..... **305**
***G. Giovanelli and E. Pagliarini***

Fatty Acid, Mineral and Proximate Composition of Some Seaweeds from the Northeastern Mediterranean Coast..... **317**
***S. Polat and Y. Ozogul***

SHORT COMMUNICATIONS

Effects of Ozone and Negative Air Ions (NAI) on the *in vitro* Development of *Botrytis cinerea* and *Penicillum expansum* Moulds..... **325**
***R. Tuffi, R. Lovino, S. Canese and G. Amendola***

Antioxidant Activity of *Prunus spinosa* L. Fruit Juice..... **337**
***D. Fraternale, L. Giamperi, A. Bucchini and D. Ricci***

Chemical Composition and Antibacterial Activity of Essential Oils of Bitter Fennel (*Foeniculum vulgare* Mill. Var. *Vulgare*) and Dill (*Anethum graveolens* l.) Against the Growth of Food-borne and Seed-borne Pathogenic Bacteria ..... **347**
***S. Soylu, E.M. Soylu and G.A. Evrendilek***

Influence of *Spirulina platensis* Biomass on Microbiological Viability in Traditional and Probiotic Yogurts during Refrigerated Storage..... **357**
***A.S. Akalin, G. Ünal and M.C. Dalay***

Cattle Breed Traceability in Meat and Cheese using DNA Polymorphisms Detected by the Snapshot Method ..... **365**
***M. D'Andrea, A. Merigioli, M-T. Scarano and F. Pilla***

Allergic Response to a Para-Amino Compound ..... **375**
***S. Saitta, L. Ricciardi, A. Carnì, A. Speciale, A. Saja and S. Gangemi***

GUIDE FOR AUTHORS ..... **379**

CONTRIBUTORS..... **382**

REVIEW

Stability of the Sensory Quality of Virgin Olive Oil during Storage: An Overview
***A. Bendini, L. Cerretani, M.D. Salvador, G. Fregapane and G. Lercker*** ..... **389**

PAPERS

Cross-National Segmentation for Messaging about Cheese:
Towards a New Approach to Consumer Understanding, Directed Development
and Targeted Marketing
***G. Gabay, K. Paulus, P. Aarts, J. Beckley, H. Ashman and H.R. Moskowitz*** ..... **407**

Frequency of Functional Food Consumption and Awareness and Perceptions
of Health Risks Towards Metabolic Syndrome Among Poles Aged 15+. Lipgene Project
***L. Wądołowska, M. Danowska-Oziewicz, B. Stewart-Knox***
***and M. Daniel Vaz de Almeida*** ..... **429**

Effects of Enrichment with Polyphenolic Extracts from Sardinian Plants
on Physico-Chemical, Antioxidant and Microbiological Properties of Yogurt
***M. Cossu, C. Juliano, R. Pisu and M.C. Alamanni*** ..... **447**

Carotenoid Content During Tomato (*Solanum lycopersicum* L.)
Fruit Ripening in Traditional and High-Pigment Cultivars
***M.S. Lenucci, A. Caccioppola, M. Durante, L. Serrone,***
***M. De Caroli, G. Piro and G. Dalessandro*** ..... **461**

Probiotic Properties of *Bifidobacterium animalis* subsp. *Lactis* BB-12
in Baby Cereal Flakes Enriched with Inulin
***G. Hajduk, B. Kos, J. Šušković, J. Frece, A. Leboš and J. Beganović*** ..... **473**

Changes in Physical Properties of Chitosan Films at Subzero Temperatures
***Y. Deng, L.W. Zhu, W. Luo, C.L. Xiao, X.Y. Song and J.S. Chen*** ..... **487**

Application of Food Safety and Quality Management Systems to Bulgur Processing
***K.B. Belibağli, H. Vardin and A.C. Dalgiç*** ..... **499**

In-House Pesticide Residue Monitoring of Tomatoes from Souss-Massa (Morocco)
and Pesticide Residue Levels in Tomatoes Grown in a Greenhouse
after Multiple Applications of Dicofol and Difenoconazole
***O. Id El Mouden, M. Zougagh, A. Lemerhyeratte, R. Salghi, L. Bazzi,***
***A. Hormatallah, A. Chakir and A. Ríos*** ..... **517**

SHORT COMMUNICATION

Moisture Content Determination of Colored Malts
***L. Marte, S. Floridi, O. Marconi and G. Perretti*** ..... **529**

AUTHOR INDEX ..... **534**
SUBJECT INDEX ..... **536**
CONTENTS OF VOLUME XXI ..... **547**
GUIDE FOR AUTHORS ..... **551**
CONTRIBUTORS ..... **554**

# GUIDE FOR AUTHORS

ITALIAN JOURNAL OF FOOD SCIENCE -IJFS

## 1. Manuscript Submission

Manuscripts must submitted as an electronic version by e-mail to ijfs@unipg.it or paolofan@unipg.it (www.chiriottieditori.com/ijf/papersub.htm). 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:
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 (ini-

tials 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 Ruber 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 preparation 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. I. 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 2009.

Si ringraziano i seguenti Enti, Ditte ed Istituti per aver voluto contribuire fattivamente alla realizzazione della Rivista, sottoscrivendo un abbonamento sostenitore per il 2009.

## ASSOCIATIONS

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

## INDUSTRIES

Soremartec Italia srl - Alba Fax +39-0173-313966

Tecnoalimenti scpa - Milano Fax +39-02-67077405

# 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 € 6.00

ISSN 1120-1770 © 2009

CHIRIOTTI EDITORI sas - 10064 Pinerolo - Italy

publishes the technical magazines:

INDUSTRIE ALIMENTARI
INDUSTRIE DELLE BEVANDE
TECNICA MOLITORIA
MANGIMIFICI - PASTIFICI - MOLINI - SILI
AROMI ADDITIVI SEMILAVORATI INGREDIENTI ALIMENTARI
Italian Food Technology
Italian Beverage Technology
PASTICCERIA INTERNAZIONALE
IJFS

VOLUME XXI IJFS No. 4, 2009

# CONTENTS

LETTER FROM THE EDITOR ..... **387**

REVIEW

Stability of the Sensory Quality of Virgin Olive Oil during Storage: An Overview
***A. Bendini, L. Cerretani, M.D. Salvador, G. Fregapane and G. Lercker*** ..... **389**

PAPERS

Cross-National Segmentation for Messaging about Cheese:
Towards a New Approach to Consumer Understanding, Directed Development and Targeted Marketing
***G. Gabay, K. Paulus, P. Aarts, J. Beckley, H. Ashman and H.R. Moskowitz*** ..... **407**

Frequency of Functional Food Consumption and Awareness and Perceptions of Health Risks Towards Metabolic Syndrome Among Poles Aged 15+. Lipgene Project
***L. Wądołowska, M. Danowska-Oziewicz, B. Stewart-Knox and M. Daniel Vaz de Almeida*** ..... **429**

Effects of Enrichment with Polyphenolic Extracts from Sardinian Plants on Physico-Chemical, Antioxidant and Microbiological Properties of Yogurt
***M. Cossu, C. Juliano, R. Pisu and M.C. Alamanni*** ..... **447**

Carotenoid Content During Tomato (*Solanum lycopersicum* L.) Fruit Ripening in Traditional and High-Pigment Cultivars
***M.S. Lenucci, A. Caccioppola, M. Durante, L. Serrone, M. De Caroli, G. Piro and G. Dalessandro*** ..... **461**

Probiotic Properties of *Bifidobacterium animalis* subsp. *Lactis* BB-12 in Baby Cereal Flakes Enriched with Inulin
***G. Hajduk, B. Kos, J. Šušković, J. Frece, A. Leboš and J. Beganović*** ..... **473**

Changes in Physical Properties of Chitosan Films at Subzero Temperatures
***Y. Deng, L.W. Zhu, W. Luo, C.L. Xiao, X.Y. Song and J.S. Chen*** ..... **487**

Application of Food Safety and Quality Management Systems to Bulgur Processing
***K.B. Belibağli, H. Vardin and A.C. Dalgiç*** ..... **499**

In-House Pesticide Residue Monitoring of Tomatoes from Souss-Massa (Morocco) and Pesticide Residue Levels in Tomatoes Grown in a Greenhouse after Multiple Applications of Dicofol and Difenoconazole
***O. Id El Mouden, M. Zougagh, A. Lemerhyeratte, R. Salghi, L. Bazzi, A. Hormatallah, A. Chakir and A. Ríos*** ..... **517**

SHORT COMMUNICATION

Moisture Content Determination of Colored Malts
***L. Marte, S. Floridi, O. Marconi and G. Perretti*** ..... **529**

AUTHOR INDEX ..... **534**
SUBJECT INDEX ..... **536**
CONTENTS OF VOLUME XXI ..... **547**
GUIDE FOR AUTHORS ..... **551**
CONTRIBUTORS ..... **554**