ISSN 1120-1770

ITALIAN JOURNAL OF FOOD SCIENCE

Rivista italiana di scienza degli alimenti

IJFS

Volume XXIV
Number 1
2012

CHIRIOTTI EDITORI

ITALIAN JOURNAL OF FOOD SCIENCE

(RIVISTA ITALIANA DI SCIENZA DEGLI ALIMENTI) 2nd series

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

Editor-in-Chief:

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

Co-Editors:

Bruno Zanoni - Università degli Studi di Firenze, e-mail: bruno.zanoni@unifi.it
Carlo Pompei - Università degli Studi di Milano, e-mail: carlo.pompei@unimi.it
Lanfranco Conte - Università degli Studi di Udine, e-mail: lanfranco.conte@uniud.it
Lina Chianese - Università degli Studi di Napoli Federico II, e-mail: chianese@unina.it
Milena Sinigaglia - SIMTREA - Università degli Studi di Bari, e-mail: m.sinigaglia@unifg.it

Publisher:

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

Aim: The Italian Journal of Food Science is an international journal publishing original, basic and applied papers, reviews, short communications, surveys and opinions on food science and technology with specific reference to the Mediterranean Region. Its expanded scope includes food production, food engineering, food management, food quality, shelf-life, consumer acceptance of foodstuffs. food safety and nutrition, and environmental aspects of food processing.

Reviews and surveys on specific topics relevant to the advance of the Mediterranean food industry are particularly welcome.

Upon request and free of charge, announcements of congresses, presentations of research institutes, books and proceedings may also be published in a special "News" section.

Review Policy:

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

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

Impact Factor: 5-Year Impact Factor: 0.587 published in 2010 Journal of Citation Reports, Institute for Scientific Information; Index Copernicus Journal Master List 2009 (ICV): 13.19

IJFS is abstracted/indexed in: Chemical Abstracts Service (USA); Foods Adlibra Publ. (USA); Gialine - Ensia (F); Institut Information Sci. Acad. Sciences (Russia); Institute for Scientific Information; CurrentContents®/AB&ES; SciSearch® (USA-GB); Int. Food Information Service - IFIS (D); Int. Food Information Service - IFIS (UK); EBSCO Publishing; Index Copernicus Journal Master List (PL).

IJFS has a page charge of € 25.00 each page.

Subscription Rate: IJFS is available on-line in PDF format only.
2012: Volume XXIV: PDF for tablet € 60.50 (€ 50.00 + € 10.50 VAT) - Supporting € 1,210.00 (€ 1,000.00 + 210.00 VAT)

ITALIAN JOURNAL OF FOOD SCIENCE



ADVISORY BOARD

SCIENTISTS

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

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

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

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

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

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

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

A. Leclerc
Institut Pasteur
Paris, France

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

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

J. O'Brien
Head, Quality and Safety Dept.
Nestle Research Centre
Lausanne, Switzerland

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

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

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

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

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

REPRESENTATIVES of CONTRIBUTORS

R. Coppola
Dipartimento di Scienze
e Tecnologie Agroalimentari e Microbiologiche
(DI.S.T.A.A.M.), Università del Molise,
Campobasso, Italy

M. Fontana
Soremartec Italia, Ferrero Group
Alba, Italy

V. Gerbi
Dipartimento di Valorizzazione
e Protezione delle Risorse Agroforestali (DI.VA.P.R.A.)
Sezione Microbiologia ed Industrie Agrarie,
Università di Torino, Torino, Italy

M. Moresi
Dipartimento di Scienze
e Tecnologie Agroalimentari (D.I.S.T..A.)
Facoltà di Agraria, Università
degli Studi della Tuscia, Viterbo, Italy

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

M. Rossi
Dipartimento di Scienze e Tecnologie Alimentari e
Microbiologiche (DI.S.T.A.M.)
Università di Milano, Milano, Italy

A. Sensidoni
Dipartimento di Scienze degli Alimenti
Università di Udine, Udine, Italy

PAPER

SPE-IMS AS A NEW ANALYSIS TECHNIQUE FOR IDENTIFICATION AND QUANTIFICATION OF METALAXYL RESIDUE IN CUCUMBER

M. SOLEIMANI[1]*, M. AZAM[2], M. AZIMI[2] and K. BORHANI[2]
[1]Department of Chemistry, Imam Khomeini International University (IKIU), Qazvin, Iran
[2]Engineering Research Center of Isfahan, Isfahan 81395/619, Iran
*Corresponding author: Tel./Fax +98 2813780040,
email: m-soleimani@hotmail.com

ABSTRACT

Detection and determination of metalaxyl residue in cucumber by using SPE-ion mobility spectrometer (IMS) with ^{63}Ni as ionization source was evaluated for the first time. Pretreatment included extraction of the pigments from the sample by liquid–liquid extraction and extraction of metalaxyl by solid phase extraction (SPE) using C_{18} sorbent. The limit of detection (LOD) and the limit of quantification (LOQ) for the metalaxyl were obtained 0.24 and 3.75 ng/g in positive mode, respectively. At optimum effective parameters, the concentration factor 133.3 was obtained. The working range of this compound was about two orders of magnitude and the relative standard deviation (RSD) of repeatability at the 0.5 µg/g level was 5.5%.

- Key words: metalaxyl, pesticide, ion mobility spectrometry, cucumber -

INTRODUCTION

Pesticide poisoning is a serious health problem that disproportionately affects infants and children. New plant disease control has become available with the discovery of systematic fungicides (ARIAS-ESTEVEZ *et al.,* 2008).

Although the use of pesticides provide benefits essential in providing a plentiful, low-cost supply of high-quality fruits and vegetables, their incorrect application may leave harmful residues which involve possible health risks (TORRES *et al.,* 1996). As a consequence, governments and international organizations have established maximum residue limits (MRLs) in fruits and vegetables to ensure their levels (WHO/FAO Codex Committee, 2007).

A great portion of the pesticide residues is fungicides and herbicides, which are used primarily to control spoilage of fruits and vegetables through fungal attack lingcontrolling weeds. The most frequently used methods for analysis of the fungicides and herbicides in fruits and vegetables are gas chromatography (GC) with nitrgen-phosphorous (NPD), electroncapture (ECD), a mass spectrometric (MS) detection (BERMUDEZ-COUSO *et al.,* 2007; GARRIDO-FRENICH *et al.,* 2003; MARTINEZ-VIDAL *et al.,* 2002; BASA-CESNIK *et al.,* 2006; GONZALEZ-RODRIGUEZ *et al.,* 2008; RASTRELLI *et al.,* 2002; REY-SALGUEIRO *et al.,* 2008; RIAL-OTERO *et al.,* 2005; FERNANDES-GONZALES *et al.,* 2003) and liquid chromatography (LC) with ultraviolet (UV) and diode-array detection (DAD) (RIAL-OTERO *et al.,* 2003; PICO *et al.,* 2000; BLASCO *et al.,* 2002; MARK-LEE *et al.,* 1991). These methods ar based on liquid-liquid extraction (LLE) (MAHARA *et al.,* 1998), solid-phase extraction (SPE) (SIMPLICO and VILAS-BOAS, *1999*), matrix solid-phase dispersion (TADEO *et al.,* 2000) and supercritical fluid extraction (SFE) (KIM *et al.,* 1998).

Fruits are often treated close to their harvest or post harvest to ensure that whole some products reach the consumer. The presence of pesticide residues in canned pure fruit juices can be a significant route to human exposure. Only a few procedures have been reported for the analysis of pesticide residues in fruit juices (WALORCZYK, 2004; BUSHWAY *et al.,* 1989; TOPUZ *et al.,* 2004; SANNINO *et al.,* 2004). The development of easy analytical methods to monitor these pesticides in fruit juices is very important.

Metalaxyl [DL-N-(2, 6-dimethylphenyl)-N-(2-methoxyacetyl) alanine methyl ester] is one of these compounds which allows the control of one of the most serious cucumber diseases as problem. This pesticide exhibits fairly high acute toxicity, so that sensitive, rapid and reliable detection and identification of this toxic substance is very important for the protection of the environment and human health. Metalaxyl is reported to be practically nontoxic to birds, freshwater fish (U.S. Environmental Protection Agency, 1988) and bees (KIDD and JAMES, 1991). In humans and animals: Studies with rats and goats showed rapid metabolism and excretion via the urine and feces. Metalaxyl is metabolized to a variety of products before excretion. Forty-day feeding studies with dairy cattle at 15 ppm/day, showed that less than 0.01 ppm was stored in the muscle and fat. The liver contained 0.13 to 0.20 ppm and the kidney 0.26 to 0.83 ppm. Chickens fed for 28 days at 5 ppm in the diet had less than 0.05 ppm in the eggs, skin, fat, breast, and thigh, and less than 0.1 ppm in the liver (CIBA-GIEGY, 1992). Metalaxyl is a systemic fungicide that is widely used in agriculture and horticultural crops to control root and stem diseaes and damp off seedlings (SINGH *et al.,* 2003). The determination of metalaxyl residue in real samples can be performed by various methods such as SPE-GC (VAQUERO-FERNANDEZ *et al.,* 2009), GC-MS (ARIAS *et al.,* 2006; RIAL-OTERO and GONZALEZ-RODRIGUEZ, 2004; STAJNBAHER and ZUPANCIC-KRALJ, 2003), HPLC (GARRIDO-FERENICH *et al.,* 2004; ZHANG *et al.,* 2010) LC-UV and GC-MS (POSE-JUAN *et al.,* 2009) etc. However, these methods are known to be expensive and quite laborious. Ion mobility spectrometry (IMS) has become one of the best methods to analyze pesticides (TUOVINEN *et al.,* 2000). The major advantages of this method are low detection limit, fast response, simplicity and portability. Available laboratory and field studies indicate that metalaxyl is stable to hydrolysis under normal environmental pH values. It is also photolytically stable in water and soil when exposed to natural sunlight. Its tolerance to a wide range of pH, light, and temperature leads to its continued use in agriculture. Metalaxyl is photo decomposed in UV light (SUKUL and SPITELLER, 2001). Metalaxyl is very soluble in water and is stable to photo degradation in soil. In aquatic systems, Metalaxyl degrades moderately rapidly (GARDNER and BRANHAM, 2001). Very little of the chemical is lost through volatilization. Metalaxyl is persistent and mobile (FERNANDES *et al.,* 2003).

Ion mobility spectrometry (IMS) is one of the most promising analytical techniques currently investigated for the detection of pesticides. The major advantages of IMS are its fast response times and its sensitivity to many of the commonly used pesticides due to their proton affinity (TUOVINEN *et al.*, 2001).

In this work, we described a method based on SPE-IMS to determine metalaxyl in cucumber tissue. The developed method has been tested on fresh cucumber in Isfahan, Iran.

MATERIALS AND METHODS

Materials

The metalaxyl standard (CGA-48988) of 99.6% purity was purchased from the Plant Protection Organization (Tehran). All solvents (Spectroscopy grade) were obtained from Merck (Darmstadt, Germany). C_{18} SPE cartridges (Bond EluLRC-C18, 500 mg/10 mL, Varian, CA, USA) were used in the extraction step. Filtration was otained using a 0.2 µm membrane filter (Phenomenex, CA, USA).

Preparation of solutions

The stock standard solutio o mtalaxyl (100 µg/mL) was prepared in methanol. The working standard solutions of pesticide at the concentration of 0.1-10 µg/mL were obtained by dilution with methanol. The standard solutions were stored at -20°C and used for 1 month.

Extraction procedure

40 g of spiked and non-poisoned cucumber (skin and tissue of the sample) was placed into a mortar and blended to obtain a homogeneous mixture. Then the result paste was filtered through a vacuum Buckner funnel with paper filter. In eac etraction about 20 (±1.5) mL cucumber juice was obtained. The obtained cucumber juice was included interference green pigments that exist excess peaks in IMS. therefore, the pigments were extracted by liquid-liquid xtaction two times with 20 mL diethyl-ether. For more effective extraction, in the second extraction 0.4 g NaCl was added into the samples. The sample solutions were stirred in room temperature in order to complete the elimination of Diethyl-ether. Finally, the sample solutions were filtered through a Filter holdr with membrane filter 0.2 µm.

For solid phase extraction of metalaxyl from the sample solutions, C_{18} cartridges were washed with 10 mL of dichloromethane, 10 mL methanol to remove impurits and were conditioned with 5 mL of water. The sample solutions were passed through the cartridgs ung vacuum at the rate of 5-7 mL/min. After being washed with 5 mL of solvent (methanol-water, 20:80, v/v) to eliminate interferences, the sorbent bed was dried under vacuum for 10 min. The analyte was eluted with 2 mL of methanol as elution solvent into a vial. The elute w concentrated by evaporating the solvent under a gentle stream of nitrogen at 40° The residue was dissolved in 0.3 mL methanol to obtain cocntration factor of 133.3 and 10 µL sample was injected into the IMS system.

Detection procedure

The Ion mobility spectrometer used in this work has been made in Engineering Research Center of Isfahan by Detector group. A 17 cm long IMS cell was made from 15 stainless steel rings with 2.8 cm inner diameter separated from each other by thin PTFE sheets. The schematic diagram of the apparatus is shown in Fig. 1. A 12 mCi-^{63}Ni foil was used as an ionization source in this cell. The rings were connected by a series of resistors to form the electric field. Air flow as drift gas was introduced from the end of the cell, near the collector. The injection port was a T shape union made of brass alloy. The carrier gas was passed through the injection port and carried the analyte vapor to the IMS cell. The injection port was equipped with a heating element and a digital temperature controller (Lae, Italy). A steel cap was used to introduce samples. The sample solutions were placed on a cap and after evaporating the solvent, the cap was inserted into the injection port. A flow of air carrier gas was used to introduce the sample vapor to ionization source. The gases were filtered with a 13X molecular sieves (Fluka) trap before they

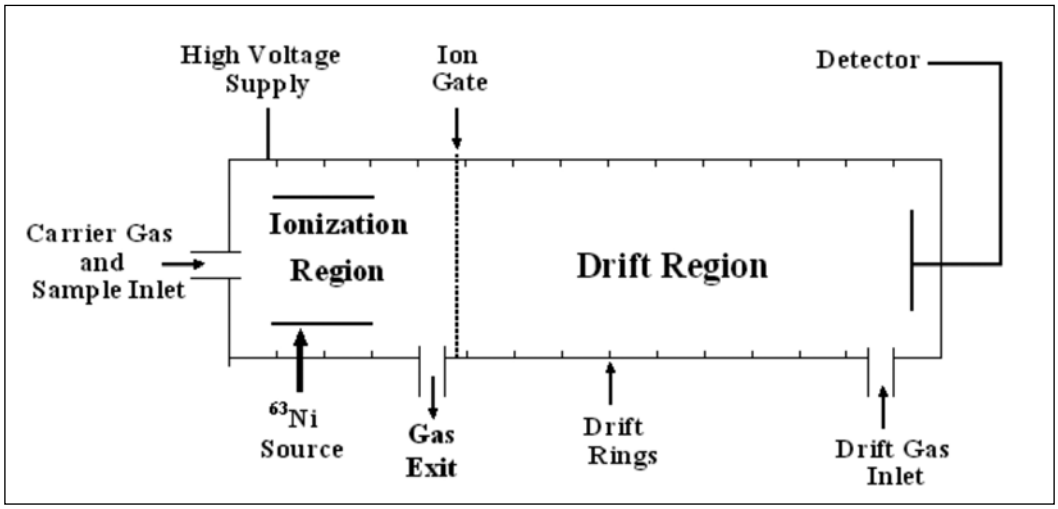


Fig. 1 - Schematic diagram of ion mobility spectrometer instrument.

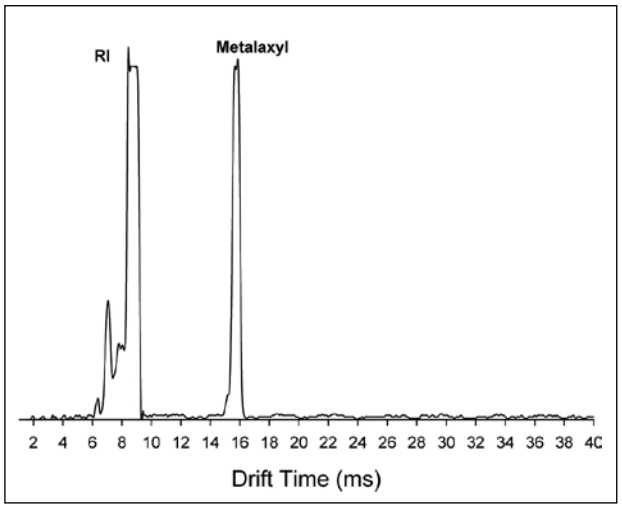


Fig. 2 - Ion mobility spectrum of metalaxyl.

Tab. 1 - IMS operation parameters.

Parameters	Setting
Drift field (V cm^{-1})	250
Drift gas flow (air) (mL min^{-1})	600
Carrier gas flow (air) (mL min^{-1})	350
Drift tube temperature (°C)	160
Injection temperature (°C)	150
Drift tube length (cm)	10
Pressure (torr)	670
Shutter grid pulse (µs)	350

entered to the IMS in order to remove water vapor or other contamination.

RESULTS AND DISCUSSION

The ion mobility spectrum of standard metalaxyl is shown in Fig. 2. This spectrum was obtained at the optimized IMS conditions given in Table 1. The spectrum shows one peak for metalaxyl. The reduced mobility of reactant ions in this work was 2.43±0.04 $cm^2V^{-1}s^{-1}$ which was close to 2.37±0.10 $cm^2V^{-1}s^{-1}$ for $(H_2O)_nH^+$ as reported by JAFARI *et al.* (2007) and for metalaxyl was 1.37±0.02 which was equal to 1.37±0.07 (obtained by JAFARI and AZIMI, 2006).

In this work, Air was used as a reagent gas to create alternate reactant ions that provided additional selectiviyin response. A sample volume 10 µL was placed on the cap and after the evaporation of the solvent, the cap was inserted into the injection port. The signal appeared after a short time and reached the maximum level and decayed exponentially. It was noticed that during the acquisition time (from the injection time until the sample peaks disappear) the relative peak area changed. The integration of thi plot was considered as a response.

The presence of natural pigments made the analysis of fruit juices difficult. Clean-up on C_{18} cartridges eliminated most interfering peaks and allowed good recoveries at low fortification levels. Complete separation of all compounds was achieved using a concave gradient elution with methanol and water on a C_{18} column. This afforded good resolution and short analysis time.

The procedure is illustrated in Fig. 3, which shows the peaks of unspiked cucumber juice and spiked samples with pesticide. As can be seen, the spectra were clear without the interference peaks over the entire drift time range.

SPE with C_{18} cartridges was used in order to achieve suitable sensitivity and detection of limit. The various extraction solvents studied for the elution of the pesticide retained on the SPE cartridges. The elution of pesticide with different solvent compositions was carried out and the best extraction condition and less interference was the composition of 20:80 (v/v) from methanol-water. Cucumber juices containing no detectable amount of this pesticide were spike at concentrations 0.5, 0.7, and 1 µg/g. Average recoveries obtained for cucumber juice samples spiked with metalaxyl at three concentration levels were 85.94±3.11%.

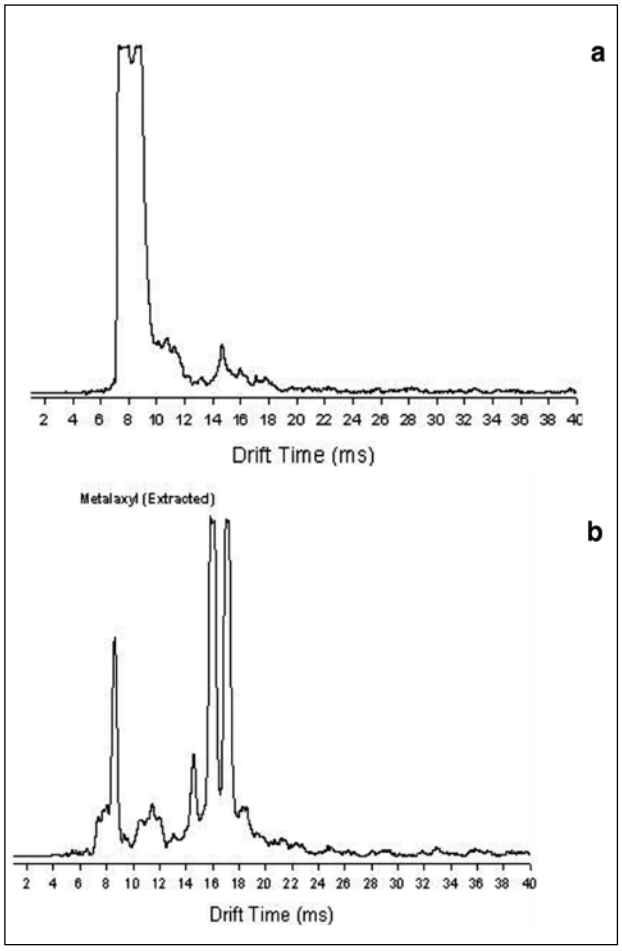


Fig. 3 - Ion mobility spectrum of (a) unspike cucumber juice sample, (b) cucumber juice spiked with metalaxyl.

ANALYTICAL PERFORMANCE

Quantification of this pesticide in the extracted sample was performed using integrated peak area with their known external standard. The linerit was observed in the range of 0.1-10 µg/g (n=3) in cucumber juice, with the correlation coefficient of 0.9980. The limit of detection (LOD) was calculated as the lowest concentration giving a response of three-times the average of the baseline noise defined from unspiked juices. The LOD for the metalaxyl investigated was 0.24 ng/g.

The limit of quantification (LOQ) of proposed method was defined as the lowest concentration of compound in a sample that could be quantitatively determined with suitable precision and accuracy. The LOQ of metalaxyl was found to be 3.75 ng/g with relative standard deviation (RSD) of repeatability (n=5) 5.5%. Differences between the matrices tested were not observed. The LOQ was lower tan the MRL set by the European Union (0.5 µg/g for the Metalaxyl in cucumber).

Using the improved method, metalaxyl was analyzed in cucumber juice (Fig. 4). Result showed that the cucumber juice analyzed contained more than 1 µg/g detectable residue.

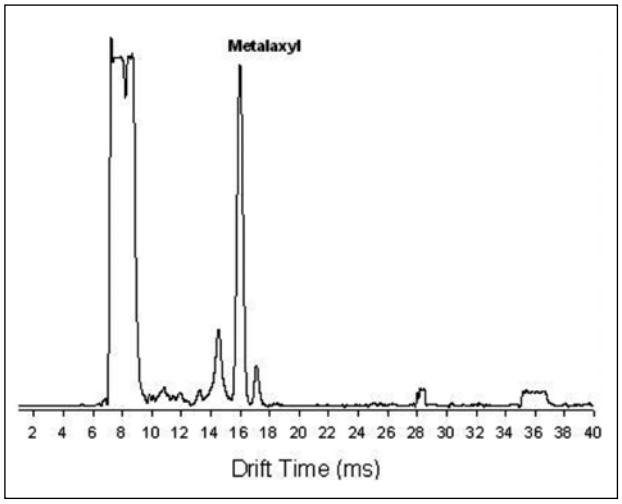


Fig. 4 - Ion mobility spectrum of metalaxyl residue in real sample.

Tab. 2 - Characteristic ions for reactant and metalaxyl ion mobility spectra and Reactant and metalyl ion mobility spectra obtained by Jafari, Khayamian, Shaer and Zarei (2007) and Jaari and Azimi (2006) respectively.

Peak	Reduced mobility K_o ($cm^2 V^{-1} s^{-1}$)	Drift time (ms)
RI	2.37±0.10	5.16
M	1.37±0.07	14.96
RI	2.43±0.04	8.80
M	1.37±0.02	15.66

From a practical point of view, the method described in this research allowed simple, rapid and sensitive determination of metalaxyl residue in cucumber juice using IMS. The total analysis time, including SPE and IMS, was about 3-4 hours.

CONCLUSIONS

The ion mobility spectrum of metalaxyl was obtained using ^{63}Ni-IMS in positive mode. The extraction, clean-up step and the analysis of the metalaxyl were established. The method was used in the analysis of metalaxyl in real samples. It was demonstrated that IMS can also be used successfully in identification of metalaxyl, whilst with respect to the other methods its simplicity, rapidity and low amounts of waste of solvents which are hazardous for the environment, were considered.

ACKNOWLEDGEMENTS

The authors are extremely grateful to the Isfahan Engineering Research Center for their financial support. Mr Saeed Hajialigol is also acknowledged for his valuable discussions.

REFERENCES

Arias M., Paradelo M., López E. and Simal-Gándara J. 2006. Influence of pH and soil copper on adsorption of metalaxyl and penconazole by the surface layer of vineyard soils. J. Agric. Food Chem. 54 (21): 8155.

Arias-Estévez M., López-Periago E., Martínez-Carballo E., Simal-Gándara J., Mejuto J.C. and García-Río L. 2008. The mobility and degradation of pesticides in soils and the pollution f groundwater resources. Agr. Ecosyst. Environ. 123: 247.

Basa-Cesnik H., Gregorcic A. and Velikonja-Bolta S. 2006. Pesticide residues in agricultural products of slovene origin in 2005. Acta Chim. Slov. 53: 95.

Bermúdez-Couso A., Arias-Estévez M., Nóvoa-Muñoz J.C., ópez-Periago E., Soto-González B. and Simal-Gándara J. 2007. Seasonal distributions of fungicides in soils and sediments of small river basin partially devoted to vineyards. Water Res. 41: 4515.

Blasco C., Pico Y., Manes J. and Font G. 2002. Determination of fungicide residus in fruits and vegetables by liquid chromatography-atmospheric pressure chemical ionization mass spectrometry. J. Chromatogr. A 947: 227.

Bushway R.J., Perkins B., Savage S.A., Lekousi S.L. and Ferguson B.S. 1989. Determination of atrazine residues in food by enzyme immunoassay. Bull. Environ. Contamin. & Toxicol. 42: 899.

Ciba-Giegy 1992. Agricultural Division. Letter of April 20, 10-20.

Fernandes M.C., Cox L., Hermosin M.C. and Cornejo J. 2003. Adsorption- desorption of metalaxyl as affectiong dissipation and leaching in soils: role of mineral and organic components. Pest Manag. Sci. 59: 545.

Fernandes-Gonzales C., Rial-Otere R., Cancho-Grande B. and Simal-Gandara J. 2003. Determination of fungicide residues in white grapes for winemaking by gas chromatography with mass spectrometric detection and assessment of matrix effects. J. AOAC Int. 86(5): 1008.

Gardner D.S. and Branham B.E. 2001. Effect of turfgrass

cover and irrigation on soil mobility and dissipation of mefenoxam and propiconazole. J. Environ. Qual. 30: 1612.

Garrido-Frenich A., Arrebola-Liebanas F.J., Mateu-Sanchez M. and Martinez-Vidal J.L. 2003. Multicomponent determination of pesticides in vegetables by gas chromatography with mass spectrometric detection and multivariate calibration. Talanta 60: 765.

Garrido-Frenich A., Martınez-Vidal J.L., Lopez T., Cortes-Aguado S. and Martınez-Salvador I. 2004. Monitoring multi-class pesticide residues in fresh fruits and vegetables by liquid chromatography with tandem mass spectrometry. J. Chromatogr. A 1048: 199.

González-Rodríguez R.M., Rial-Otero R., Cancho-Grande B. and Simal-Gándara J. 2008. Occurrence of fungicide and insecticide residues in trade samples of leafy vegetables. Food Chem. 107(3): 1342.

Jafari M.T. and Azimi M. 2006. Analysis of sevin, amitraz and metalaxyl pesticides using ion mobility spectrometry. Anal. Lett. 39: 2061.

Jafari M.T., Khayamian T., Shaer V. and Zarei N. 2007. Determination of veterinary drug residues in chicken meat using corona discharge ion mobility spectrometry. Anal. Chim. Acta 581: 147.

Kidd H. and James D.R., 1991. Eds. The Agrochemicals Handbook, Third Edition. Royal Society of Chemistry Information Services, Cambridge, UK, (As Updated)10-2.

Kim D.H., Heo G.S. and Lee D.W. 1998. Determination of organophosphorus pesticides in wheat flour by supercritical uid extraction and gas chromatography with nitrogen-– phosphorus detection. J. Chromatogr. A 824: 6.

Mahara B.M., Borossay J. and Torkos K. 1998. Liquid-liquid extraction for sample preparaton prior to gas chromatography and gas Chromatography-mass spectrometry determination of herbicide and pesticide compounds. Microchem. J., 58: 31.

Mark-Lee S., Papathakis M.L., Feng H.C., Hunter G.F. and Carr J.E. 1991. Multipesticide residue method for fruits and vegetables: California Department of Food and Agriculture. J. Anal. Chem., 339: 376.

Martinez-Vidal J.L., Arrebola F.J. and Mateu-Sanchez M. 2002. Application to routine analysis of a method to determine multiclass pesticide residues in fresh vegetables by gas chromatography-tandem mass spectrometry. Rapid Commun. Mass Sp. 16: 1106.

Pico Y., Font G., Molto J.C. and Manes J. 2000. Pesticide residue determintion in fruit and vegetables by liquid chromatography-mass spectrometry. J. Chromatogr. A 882: 153.

Pose-Juan E., Rial-Otero R., Martínez-Carballo E., López-Periago E. and Simal-Gándara J. 2009. Determination of metalaxyl and identification of adjuvants in wettable powder pesticide technical formulas. Anal. Bioanal. Chem., 394: 1535.

Rastrelli L., Totaro K. and De-Simone F. 2002. Determination of organophosphorus pesticide residues in Cilento (Campania, Italy) virgin olive oil by capillary gaschromatography Foohem. 79: 303.

Rey-Sagueiro L., García-Falcón M.S., Martínez-Carballo, E. and González-Barreiro C. 2008. Determination of 23 pesticide residues in leafy vegetables using gas chromatography-ion trap mass spectrometry and analyte protectants. J. Chromatogr. A 100: 1196.

Rial-Otero R. and Gonzalez-Rodriguez R.M. 2004. Parameters affecting extraction of selected fungicides from vineyard soils. J. Agr. Food Chem. 52(24): 7227.

Rial-Otero R., Arias-Estévez M., López-Periago E., Cancho-Grande B. and Simal-Gándara J. 2005. Variation in concentrations of the fungicides tebuconazole and dichlofluanid following successive applications to greenhouse-grown lettuces. J. Agr. Food Chem. 53 (11): 4471.

Rial-Otero R., Cancho-Grande B. and Simal-Gándara J. 2003. Multiresidue method for fourteen fungicides in white grapes by liquid-liquid and solid-phase extraction followed by liquid chromatography-diode array detection. J. Chromatogr. A 992: 121.

Sannino A., Bolzoni L. and Bandini M. 2004. Application of liquid chromatography with electrospray tandem mass spectrometry to the determination of a new generation of pesticides in processed fruits and vegetables. J. Chromatogr. A 1036: 161.

Simplıcio A.L. and Vilas-Boas L. 1999. Validation of a solid-phase microextraction method for the determination of organophosphorus pesticides in fruits and fruit juice. J. Chromatogr. A 833: 35.

Singh M., Mersie W. and Brlansky R. H. 2003. Phytotoxicity of the fungicide metalaxyl and its optical isomers. Plant Dis. 87: 1144.

Stajnbaher D. and Zupancic-Kralj L. 2003. Multiresidue method for determination of 90 pesticides in fresh fruits and vegetables using solid-phase extraction and gas chromatography-mass spectrometry. J. Chromatogr. A 1015: 185.

Sukul P. and Spiteller M. 2001. Influence of biotic and abiotic factors on dissipating in soil. Chemosphere 45: 941.

Tadeo J.L., Sanchez-Brunete C., Perez R.A. and Fernandez M.D. 2000. Analysis of herbicide residues in cereals, fruits and vegetables. J. Chromatogr. A 882: 175.

Topuz S., Ozhan G. and Alpertunga B. 2004. Simultaneous determination of various pesticides in fruit juices by HPLC-DAD. Food Control 15: 335.

Torres C.M., Pico Y. and Manes J. 1996. Determination of pesticides residues in fruit and vegetables. J. Chromatogr. A 754: 301.

Tuovinen K., Kolehmainen M. and Paakkanen H. 2001. Determination and identification of pesticides from liquid matrices using ion mobility spectrometry. Anal. Chim. Acta 429: 257.

Tuovinen K., Paakkanen H. and Hanninen O. 2000. Detection of pesticides from liquid matrices by ion mobility spectrometry. Anal. Chim. Acta 404: 7.

U.S. Environmental Protection Agency 1988. Pesticide fact sheet: Metalaxyl. office of pesticides and toxic substances, Washington, DC 10-47.

Vaquero-Fernández L., Sanz-Asensio J., López-Alonso M. and Martínez-Soria M.T. 2009. Analysis of pyrimethanil, metalaxyl, dichlofluanid, and penconazol in must and wine from red grapes by solid-phase extraction and gas chromatography. Anal. Lett., 42(12): 1761.

Walorczyk S. 2004. Fast identification of trace- level pesticide residues in agricultural crops applying low- pressure gas chromatography/ mass spectrometry. J. Proteome Research, 44(2): 117.

WHO/FAO Codex Committee, FAO/ WHO Food Standards, Last update 30 March 2009, last access 22 Jan. 2011, http://www.codexalimentarius.net/mrls/pestdes/jsp/pest_q-e.jsp.

Zhang X., Xia T., Chen J., Huang L. and Cai X. 2010. Direct chiral resolution of metalaxyl and metabolite metalaxyl acid in aged mobile phases: the role of trace water. J. Agric. Food Chem., 58 (8): 500.

Paper receveid January 24, 2011 Accepted March 14, 2011

PAPER

PREVALENCE OF MAJOR PATHOGENS ON SHEEP CARCASSES SLAUGHTERED IN ITALY

S. BILEI, E.M. FLORES RODAS, R. TOLLI, P. DE SANTIS, I. DI DOMENICO, S. DEL FRATE, P. PALMIERI and R. CONDOLEO
Istituto Zooprofilattico Sperimentale delle Regioni Lazio e Toscana,
Via Appia Nuova 1411 - 00178 Roma (Capannelle), Italy
Corresponding author: stefano.bilei@izslt.it

ABSTRACT

Sheep are known to be a major vector of the most common zoonoses such as *Salmonella* spp., *Campylobacter* spp., *Listeria monocytogenes*, *Escherichia coli* O157, and *Clostridium perfringens*. The aim of the present study is to detect the presence of these pathogens in ovine carcasses immediately after slaughtering, in order to collect useful data for risk assessment. Ovine carcasses (n=320) from different European countries were sampled using surface swabs and a destructive method. *Campylobacter* spp. was detected on 10 carcasses (3.12%), *E. coli* O157 on 13 (4.06%), *Salmonella* spp. on 2 (0.62%), *Clostridium perfringens* on 6 out of 150 carcasses sampled (4%). No *Listeria monocytogenes* was detected.

- Key words: *Campylobacter*, sheep, *E. coli* O157, *Clostridium perfringens*, foodborne pathogens, *Salmonella* -

INTRODUCTION

Livestock which is bred to produce foodstuffs intended for human consumption can be potential vectors of pathogens. Sheep have been reported to be potential carriers of the most common zoonoses such as *Salmonella* spp., *Campylobacter* spp., *Listeria monocytogenes*, *Escherichia coli* O157 and *Clostridium perfringens*. Such pathogens can be part of the intestinal micro-flora or be present on ovine skin and fleece (DUFFY *et al.*, 2010; MILNES *et al.*, 2008; ZWEIFEL *et al.*, 2004; WESLEY, 1999). These bacteria may enter into the food chain during the slaughtering process; the lack of adequate slaughter hygiene in meat production can be responsible for microbial carcass contamination. During the slaughtering phases and in particular, during the process of skinning and evisceration, there is a possibility of bacteria spreading to the surface of the carcass through contact with the contents of the intestine and other animal-derived contaminated materials (eg. fleece) (NØRRUNG and BUNCIC, 2008; BYRNE *et al.*, 2007).

As a result, data collected from various national surveys carried out in some European countries and from national and international literature reports the presence of these pathogens on the surface of sheep carcasses and on ovine meat products (EFSA, 2007; WONG *et al.*, 2007a; WONG *et al.*, 2007b; CHAPMAN *et al.*, 2001; MACGOWAN *et al.*, 1994). The consumption of ovine meat-based food contaminated by pathogens may be responsible for human disease outbreaks. National surveys on foodborne human infections have notified outbreaks due to ovine meat consumption, relating to VTEC and *Salmonella*, even if they proved to be sporadic (CDC, 2007; OZFOODNET WORKING GROUP, 2006). With regard to human salmonellosis, there have been at least three reported cases associated with the consumption of mutton and lamb (PURVIS *et al.*, 2005; EVANS *et al.*, 1999; SYNNOTT *et al.*, 1993). On the contrary, in Italy, despite the large consumption of such products (in 2004, 28.145 tons per family; ISMEA – ACNIELSEN, 2006), no foodborne human infections have been reported.

The aim of the present study was to collect data concerning the presence of *Salmonella* spp., *Campylobacter* spp., *Listeria monocytogenes*, *Escherichia coli* O157 and *Clostridium perfringens* on sheep and lamb carcasses originating from six different countries.

MATERIALS AND METHODS

Study design

This study was carried out in a slaughterhouse in the Latium region (central Italy) with EU authorization. This plant has two different slaughtering lines and a capacity of slaughtering up to 480 head/hour, especially lambs (about 500,000 head/year) and sheep (about 70,000 head/year). The abattoir slaughters animals not only of Italian origin but also from several European countries in order to meet the high demand for sheep meat. The plant also has cutting rooms where ovine meat is prepared for large-scale retail trade.

Ovine carcasses (n=320) belonging to 35 different slaughtering batches were randomly sampled, taking from 5 to 13 carcasses from each batch. Carcasses sampled were 252 adult sheep and 68 lambs, of which 135 came from Italy, 110 from Spain, 32 from France, 28 from Romania, 10 from Poland and 5 from Germany.

The National Health Veterinary Service, operating within the plant, carried out the sampling immediately after dehinding and evisceration, in order to estimate the effective contamination of the carcasses before their introduction into the refrigeration tunnel. All the carcasses were sampled in two different areas (brisket and leg), with the exception of 10 subjects, which were sampled only on the leg, for a total of 630 samples. Sampling was performed according to ISO 17604:2003 "Microbiology of food and animal feeding stuffs - Carcass sampling for microbiological analysis", using a non-destructive method (double swabbing method) on 171 carcasses or a destructive method (excision method) on the remaining 149. The double swabbing method consists in the rubbing of an area of 100 cm^2 with a cotton-tipped swab, which has been moistened with a peptone solution and then with a dry cotton-tipped swab, both placed in a sterile tube containing 10 mL of Buffered Peptone Water (BPW, Conda Pronadisa). The excision method consists in the incision of an area of 25 cm^2, about 2 mm deep, using a scalpel and subsequently removing it with the aid of sterile forceps.

All samples were examined for the detection of *Campylobacter* spp., *Salmonella* spp., *Escherichia coli* O157, *Listeria monocytogenes*. The detection of *Clostridium perfringens* was performed only on 150 animals (130 sheep and 20 lambs).

Detection of *Listeria monocytogenes*

The detection of *Listeria monocytogenes* was conducted according to ISO 11290-1:2005 (ISO, 2005) after an immunoassay screening with VIDAS LMOII (BioMérieux, Marcy l'Etoile, France, valid. AFNOR BIO 12/11 – 03/04). A test portion of 25 g was inoculated into a selective pre-enrichment Half Fraser Broth (HFB, Liofilchem, Teramo, Italia). After incubation for 24 h at 30°C, a portion of 0.1 mL of HFB was inoculated into 10 mL of Fraser Broth (FB, Biorad, Milan, Italy) then incubated for 48 h at 37°C. Agar *Listeria* Ottaviani Agosti (ALOA, Conda Pronadisa, Madrid, Spain) and *Listeria* Selective Medium (LSM, Conda), two solid selective media were inoculated with the HFB. Both solid media were incubated for 24-48 h at 37°C. After 24 h of incubation,

a portion of 1 mL of FSB was transferred into an empty sterile tube and heat-treated for 15 s at 98°C. A portion of 500 µL of the heat-treated FSB was tested by the Enzyme Linked Immunosorbent Assay VIDAS LMOII (BioMérieux). In case of a positive result, after incubation for 48 h at 37°C, FB was plated onto ALOA and LSM, then incubated for 24-48 h at 37°C. After incubation, at least 5 typical colonies were picked from each plate and plated onto Tryptone Soya Yeast Extract (TSYEA, Conda Pronadisa), incubated for 24 h at 37°C, then tested for biochemical identification in micromethod (API *Listeria*, BioMérieux). Strains identified as *Listeria monocytogenes* were serotyped with polyvalent O:1 and O:4 sera (Difco).

Detection of *Salmonella* spp.

The detection of *Salmonella* spp. was performed according to ISO 6579:2002 (ISO, 2002) after an immunoassay screening method, VIDAS SLM (BioMérieux , valid. AFNOR BIO 12/1- 04/94). A 25 g test portion was inoculated in Buffered Peptone Water (BPW, Conda Pronadisa). After 24 h of incubation at 37°C, 0.1 mL of the enrichment broth was inoculated into 10 mL of Rappaport Vassiliadis Soy broth (RVS, Liofilchem) incubated for 24 h at 41.5°C and 1 mL of BPW was inoculated into 10 mL of Muller Kauffmann Tetrathionate Novobiocin broth (MKTTn, Biolife, Milan, Italy) incubated for 24 h at 37°C. After 6 h of incubation, 1 mL aliquots from RVS broth and 0,1 mL aliquots from MKTTn were each inoculated into one tube of M broth (BioMérieux), which is a specific medium used to perform VIDAS SLM test, and both incubated for 18 h at 41.5°C. After incubation, 500 µL from each M broth was transferred into an empty sterile tube and heat-treated for 15 s at 98°C. A volume of 500 µL from the heat-treated sample was tested by Enzyme Linked Fluorescent Assay VIDAS SLM (BioMérieux). In the case of a positive result, after 24 h of incubation, RVS and MKTTn were plated onto Xylose Lysine Deoxycholate agar (XLD, Conda Pronadisa) and *Salmonella Shigella* agar (SS, Conda Pronadisa) and incubated for 24 h at 37°C. After incubation, from each plate at least five typical colonies were picked to be confirmed. These colonies were plated onto Nutrient Agar (NA, Conda Pronadisa) and incubated for 24 h at 37°C. The colonies on NA were confirmed by the biochemical micromethod (API 20E, BioMérieux) and serotyped by polyvalent anti-*Salmonella* sera and monovalent anti-flagellar and anti-somatic sera (Sifin, Berlin, Germany; Difco, Franklin Lakes NJ, USA; SSI, Copenhagen, Denmark).

Detection of *Campylobacter* spp.

The detection of *Campylobacter* spp. was conducted according to ISO 10272:2006 (ISO, 2006). A test portion of 25 g was inoculated into a selective pre-enrichment medium, Bolton broth (Oxoid) and incubated in a microphilic atmosphere (Campygen, Oxoid, Cambridge, UK) at 37°C for 6 h and then at 41.5°C for 48 h. After incubation, the Bolton Broth was plated onto different selective solid media, Modified Charcoal Cefoperazone Deoxycholate Agar (MCCDA, Oxoid) and *Campylobacter* Selective Medium - Skirrow (SK, Biolife) and incubated for 48 h at 41.5°C in a microphilic atmosphere. After incubation, at least five typical colonies were plated onto Columbia Blood Agar (ASC; Biolife), incubated for 48 h at 41.5°C in a microphilic atmosphere. After incubation, the cultures were confirmed by microscopic observation using the oxidase test (Oxoid), the mobility test, a double growth test at 25°C in a microphilic atmosphere and at 41.5°C in aerobiosis.

The isolates were confirmed by using two different qualitative PCR assays. The first is a commercial real time PCR kit (Adiafood Detection System Campylobacter, AES Chemunex Canada Inc., Canada) and it is used as a screening method by following the manufacturer's instructions and using a Stratagene Mx3500P (Agilent technologies, Italy) thermal cycler. The target pathogen DNA was amplified using specific primers and molecular beacons, designed to detect common sequences of the three thermotolerant *Campylobacter* species: *C. coli*, *C. jejuni* and *C. lari*. All the samples found positive at the first screening, were further analysed to distinguish the three thermo-tolerant *Campylobacter* species, using a second multiplex PCR, which is able to differentiate between *C. coli* and *C. jejuni*, by employing two pairs of primers. The first pair amplifying a *C. jejuni*-specific *hipO* gene sequence of 735 bp (LINTON *et al.*, 1997) and the second a 500 bp fragment of a *C. coli* putative aspartokinase gene (LINTON *et al.*, 1997). This multiplex PCR was performed in a GeneAmp® PCR System 9700 (Applied Boisystems, Italy) in a final volume of 50 µL containing 1X AmpliTaq Gold® PCR Master Mix (Applied Biosystems, Italy), 1.5 mM $MgCl_2$, dNTPs 0.2 mM each, 25 pmoles of each primer and 2Uts of Hot Start *Taq* polymerase. The thermal profile consisted of an initial denaturation step at 95°C for 10 minutes, 30 cycles of 30 seconds at 95°C, 30 seconds at 60°C, 30 seconds at 72°C and a final extension at 72°C for 7 minutes. The product of the reaction was analysed in agarose electrophoresis gel. Positive and negative controls, as well as a no template control, were included in each assay.

Detection of *Escherichia coli* O157

The detection of *E. coli* O157 was conducted according to ISO 16654:2001 (ISO, 2001) after an immunoassay screening with VIDAS ECO (BioMérieux valid. AFNOR BIO 12/8 – 07/00). A test portion of 10 g was inoculated into the pre-enrichment medium Modified Tryptone

Soya Broth (mTSB, Biolife) and incubated for 24 h at 415°C. After 6 h of incubation, a first immunomagnetic separation with Dynabeads® anti-*E. coli* O157 (Dynal, Milan, Italy) was performed; 50 µL of the immunoseparated sample was plated onto 2 solid selective media, Cefixime Tellurite Sorbitol Mc Conkey agar (CT-SMAC, Biokar) and Sorbitol Mc Conkey agar (SMAC, Biokar, DID spa, Milan, Italy) and incubated for 24 h at 37°C. At the same time, a portion of 1 mL of mTSB was inoculated into Mc Conkey broth (MCB, Conda Pronadisa) in order to perform the VIDAS ECO test. After 18 h of incubation at 37°C, 500 µL of enrichment broth was tested, after being heat-treated at 98°C for 15 s, and, in the case of a positive result, the microbiological method was used. After 24 h of incubation, a second immunoseparation from the mTSB medium was performed by plating and incubating the immunoseparated sample as described before. After incubation, at least 5 colonies were picked and plated onto Nutrient Agar (NA, Conda Pronadisa) and incubated for 24 h at 37°C, in order to perform the indole test and the agglutination test with *E. coli* O157 Latex Test (Oxoid). All *E. coli* O157 were tested to verify the presence of virulence factors with multiplex PCR in order to detect the genes encoding for *vtx1* and *vtx2* verocytotoxin and the *eae* gene responsible for the "attaching and effacing" mechanism. A single colony, sampled from a solid media Petri dish containing a pure *E. coli* culture, was picked using a sterile disposable plastic loop and resuspended in 100 µL of PCR grade water. The suspension was boiled for 10 minutes and immediately cooled in ice. A 5 µL aliquote was used as template for the multiplex PCR assay. The PCR was performed in a 50 µL reaction containing 1X AmpliTaq Gold® PCR Master Mix (Applied Biosystems, Italy), 1.5 mM $MgCl_2$, dNTPs 0.2 mM each, 50 pmoles of each primer and 2Uts of Hot Start *Taq* polymerase. The primers used were derived from literature (PATON and PATON, 1998) and targeted a fragment of 384 bp of the *eae* gene, 180 bp of the *vtx1* gene and 255 bp of the *vtx2* gene. The *vtx2* primers recognise all variants of the *vtx2* gene except *vtx2ff*. The reactions were incubated in a thermal cycler (GeneAmp® PCR System 9700, Applied Boisystems, Italy) by using the thermal profile as described by PATON and PATON (1998). The product of the multiplex PCR reaction was analysed in agarose electrophoresis gel. Positive and negative controls, including a no template control, were included in each assay.

Detection of *Clostridium perfringens*

The detection of *C. perfringens* was conducted according to ISO 7937:2004 (ISO, 2004). A sample portion of 10 g was inoculated into Peptone Tryptone Water (PTW, Conda Pronadisa). Subsequently, tenfold diluitions in PTW were made and then plated onto *Perfringens* egg-free Cycloserine agar (TS, Biolife). The plates were incubated for 20 h at 37°C in anaerobiosis (Anaerogen, Oxoid). After incubation, 5 typical black colonies were selected from each plate and inoculated into Thioglycollate medium (THIOL, Biokar) then incubated in anaerobiosis for 24 h at 37°C. After incubation, from each THIOL, 5 drops were rapidly transferred into Lactose Sulfite Medium with sodium metabisulfite and ammonium ferric citrate and a Durham's bell (LS, Conda Pronadisa) and then incubated in anaerobiosis for 24 h at 46°C. After incubation, the tubes, which contained gas in the Durham's bell for more than 0.25% of the volume and had a black precipitate on the bottom, were considered positive for the presence of *C. perfringens*.

Statistical analysis

The incidence of pathogens was calculated by dividing the number of positive cases by the total. The Chi-square test or the Fisher exact test (for values less than five) were used to compare the difference between proportions in dissimilar groups. For all statistical tests, differences were considered significant if $P<0.05$. Data was analysed using STATA/SE 9 statistical software.

RESULTS AND DISCUSSION

Listeria monocytogenes was detected with the ELFA method on a sheep leg swab but its presence was not confirmed by the microbiological method.

Salmonella was detected with the ELFA method on 5 briskets (4 using the destructive method and 1 the non destructive one) from 5 different sheep carcasses. The following microbiological test led to its isolation in 2 out of 5 samples (2/320 0.62%, 95% CI 0% - 1.47%) from two different production batches. Serological identification identified the isolates as *S. enterica subspecies diarizonae* IIIb (61:k1,5,7) Gr O:61 and *Salmonella Enteritidis* (9,12:g,m:-) Gr O:9 (D1).

Campylobacter spp. was detected with the ELFA method (VIDAS (R) BioMérieux) on a total of 16 individual samples (3 brisket and 13 leg) (16/630 2.53%, 95% CI 1.30% - 3.75%), 13 of which were confirmed by the microbiological method (3 brisket and 10 leg) (13/630 2.06%, 95% CI 0.93% - 3.19%). Brisket and leg tested positive simultaneously on 3 carcasses so the positive results using the ELFA method concerned only 13 different carcasses (4.1% of those studied); 10 out of these 13 carcasses have been confirmed microbiologically (10/320, 3.12%, 95% CI 1.22% - 5.02%). The results of the *Campylobacter* spp. detection are showed in detail in Table 1.

The following identification of strains by bio-

Table 1 - Number of analysed and positive samples for *Campylobacter* spp. and number of analysed and positive carcasses confirmed by cultural exam.

Carcass	Origin	Samples				Carcasses	
		Briskets	Positive briskets	Legs	Positive legs	Number	Positive[&]
Lamb	Local farm	40	-	40	-	40	-
	Foreign farm	28	3	28	8	28	8
	Total	68	3	68	8	68	8
Sheep	Local farm	95	-	95	-	95	-
	Foreign farm	147	-	157	2	157	2
	Total	242	-	252	2	252	2
Total		310	3	320	10	320	10

[&]Carcasses positive for one sampling site (brisket or leg) or both.

molecular methods, performed on 12 of the 13 strains, has given the following results: 5 *C. coli* strains, 4 *C. jejuni* strains, and 3 strains of species, which are different from *C. jejuni*, *coli* and *lari* (Table 2). There were 4 out of 35 *Campylobacter* contaminated batches.

The presence of *Escherichia coli* O157 was detected with the ELFA method in 19 individual samples (3 briskets and 16 legs) (3.11% of the examined samples); 14 out of 19 were confirmed by a microbiological test (14/630 2.22% 95% CI 1,07% - 3,35%). The total number of contaminated carcasses, which belonged to 3 different slaughtering batches (Table 3), was 13 (13/320 4.06, 95%% CI 1.98% - 6.14%).

PCR revealed the presence of the *eae* gene in only 5 isolates. In one of these isolates the *vtx2* gene has also been identified (Table 4).

Clostridium perfringens was isolated on 7 destructive samples (6 briskets and 1 leg) (7/300 2.33%, 95% CI 0.63% - 4.02%) from 6 sheep carcasses (2 positive samples found on the same carcass) (6/150 4%, 95% CI 0.87% - 7.3%). Positive carcasses belonged to one single production batch of the 15 examined. Counts of bacterial cells produced varied results ranging from 0.69 $\log_{10}$ CFU/cm^2 to 2.57 $\log_{10}$ CFU/cm^2. No further investigation was performed on those isolates to verify toxin production or toxin gene presence.

Statistical analysis of the number of con-

Table 2 - *Campylobacter* spp. species of isolated (n=13) strains after biomolecular identification.

No. of isolates	Animal's country of origin	Site of sampling	Carcass	Species
5	Romania	leg	lamb	*C. coli*
2	France	leg	sheep	*C. jejuni*
2	Romania	brisket	lamb	*C. jejuni*
2	Romania	leg	lamb	Negative[&]
1	Romania	brisket	lamb	Negative[&]
1	Romania	leg	lamb	-*

[&]Different from *C. coli*, *C. jejuni,* and *C. lari.*
*Biomolecular identification was not performed for this *Campylobacter* isolate.

Table 3 - Number of analysed and positive samples for *Escherichia coli* O157 and number of analysed and positive carcasses confirmed by cultural exam.

Carcass	Origin	Samples				Carcasses	
		Briskets	Positive briskets	Legs	Positive legs	Number	Positive[&]
Lamb	Local farm	40	-	40	-	40	0
	Foreign farm	28	2	28	6	28	7
	Total	68	2	68	6	68	7
Sheep	Local farm	95	0	95	1	95	1
	Foreign farm	147	1	157	4	157	5
	Total	242	1	252	5	252	6
Total		310	3	320	11	320	13

[&]Carcasses positive for one sampling site (brisket or leg) or both.

Table 4 - Virulence profiles of *Escherichia coli* O157 isolates from positive samples (n=14).

No. of isolates	Animal's country of origin	Site of sampling	Carcass	Virulence gene*		
				eae	*vtx1*	*vtx2*
1	Romania	leg	lamb	+	-	+
4	Romania	leg	lamb	+	-	-
4	France	leg	sheep	-	-	-
2	Romania	brisket	lamb	-	-	-
1	Romania	leg	lamb	-	-	-
1	France	brisket	sheep	-	-	-
1	Italy	leg	sheep	-	-	-

* -, gene absent; +, gene present.

taminated carcasses and their country of origin, showed a statistically significant difference in the presence of *Campylobacter* (Fisher's exact test p-value = 0.003) and *E. coli* O157 (Chi-square = 6.61 p-value= 0.01) in ovine carcasses from foreign countries compared to those of local farm origin. The same analysis for *Salmonella* was not significant due to the low rate of positive findings.

Considering the age of individual animals, the difference in isolation of *Campylobacter* spp. and *E. coli* O157 was statistically significant: lamb carcasses were significantly more contaminated with *Campylobacter* spp. (Fisher's exact test p-value= 0.0000916) and with *E. coli* O157 (Fisher's exact test p-value= 0.0085) than sheep (Table 5).

CONCLUSION

The prevalence of *Salmonella*, *Campylobacter* and *E. coli* O157 on ovine carcasses has been studied by many authors, while less information is available regarding *Listeria monocytogenes* and especially *Clostridium perfringens* (DUFFY *et al.*, 2010; LENAHAN *et al.*, 2007; GARCIA *et al.*, 2010; EDRINGTON *et al.*, 2009; MARTINELI *et al.*, 2009; KALCHAYANAND *et al.*, 2007; SMALL *et al.*, 2006; PHILLIPS *et al.*, 2006; ANTONIOLLO *et al.*, 2003; DUFFY *et al.*, 2001; PHILLIPS *et al.*, 2001; VANDERLINDE *et al.*, 1999; MADDEN *et al.*, 1998; SIERRA *et al.*, 1995; HUDSON and MOTT, 1994; POCIECHA *et al.*, 1991). The only pathogen that was not isolated during the present study is *Listeria monocytogenes*, whose absence on the surface of ovine carcasses has been also reported in other studies (MARTINELI *et al.*, 2009; HUDSON and MOTT, 1994; POCIECHA *et al.*, 1991). However, the presence of this pathogen has been described in literature (ANTONIOLLO *et al.*, 2003). According to these authors, the lack of *Listeria monocytogenes* isolated from ovine carcasses may be related to the feeding in natural pastures instead of silage or to the absence of carriers among the groups of animals selected. In the present study, it was not possible to verify these hypotheses, owing to the lack of information. BORCH *et al.* (1996) reported that, in comparison with other pathogens, the main source of contamination by *Listeria monocytogenes* during the slaughtering process seem to be as a result of environmental contamination, especially when inadequate cleaning and disinfection measures are used. The general hygienic conditions of the slaughterhouse may have influenced the level of contamination of carcasses in the present study. However, no environmental sample was collected to investigate the presence of *Listeria* in the plant.

Table 5 - Difference comparison between proportions in dissimilar groups.

	Number of carcasses	*Campylobacter* positive (%)	p-value	*E. coli* O157 positive (%)	p-value
Animal provenience					
Local farm	135	0	0.003*	1 (0.7)	0.01
Foreign farm	175	10 (5.4)		12 (6.5)	
Carcass					
Lamb	68	8 (11.8)		7 (10.3)	
Sheep	252	2 (0.8)	0.0000916*	6 (2.4)	0.0085*
Total	320	10 (3.1)	13 (4.1)		

* Fisher's exact test.

The percentage of positive carcasses for *Salmonella* (0.62% of the total examined carcasses) is consistent with data reported in studies performed in the USA and Brazil (between 0 and 1.80%) (MARTINELI *et al.*, 2009; EDRINGTON *et al.*, 2009; KALCHAYANAND *et al.*, 2007; DUFFY *et al.*, 2001). Three baseline surveys conducted in Australia reported a similar prevalence (PHILLIPS *et al.*, 2006; PHILLIPS *et al.*, 2001; VANDERLINDE *et al.*, 1999). The first survey reported a slightly higher value compared to the present study (5.74%) but the tissue excision technique was used to collect samples from all carcasses (VANDERLINDE *et al.*, 1999). This method allows a higher recovery of bacteria than the sponge or swabbing sampling technique (PEPPERELL *et al.*, 2005). Three different studies on the prevalence of *Salmonella* on ovine carcasses were performed in Europe (LENAHAN *et al.*, 2010; SMALL *et al.*, 2006; SIERRA *et al.*, 1995). While the prevalence of positive lamb carcasses in Ireland, detected by Lenahan *et al.*, agreed with our study (0.25%), the UK survey reported a higher isolation rate (9.6% of lamb carcasses) compared to other cited reports (SMALL *et al.*, 2006). The authors hypothesized that the United Kingdom had a higher isolation rate for *Salmonella* in comparison with other countries, or that the sampling technique used allowed a greater recovery rate (unlike other studies, the entire surface of the carcasses was sampled). Smart *et al.* (2006) also supposed that the results obtained in their study could be an artifact of the limited size of the study. SIERRA *et al.* (1995) reported that in Spain there was a high percentage of lamb carcasses contaminated with *Salmonella* just after slaughtering (10%) but in our opinion the number of animals tested (30) is too small to draw any conclusions.

The two identified *Salmonella* serotypes (*S. enterica subspecies diarizonae* and *Salmonella Enteritidis*) are not frequently isolated from sheep at the stage of primary production. Various studies reported the presence of different strains on sheep carcasses from those isolated in this survey (DUFFY *et al.*, 2010; KALCHAYANAND *et al.*, 2007; LENAHAN *et al.*, 2010; SMALL *et al.*, 2006; SIERRA *et al.*, 1995). *S. Enteritidis* is frequently associated with human salmonellosis outbreaks (WOLLIN, 2007). Although ovine raw meat or ovine meat products do not seem to be a major source of *S. Enteritidis*, this serotype was detected on retail lamb meat (YANG *et al.*, 2010). However, foodborne outbreaks linked with contaminated ovine meat with this serotype were not reported in literature, but many authors demonstrated that ovine meat products can be responsible for human salmonellosis outbreaks (PURVIS *et al.*, 2005; EVANS *et al.*, 1999; SYNNOTT *et al.*, 1993).

The prevalence of *Campylobacter* spp. on ovine carcasses was 3.12% but considering only *C. jejuny* and *C. coli,* it was 2.18%. Most studies agree with our data, reporting a similar low percentage of contaminated carcasses. Two surveys conducted in Australia, revealed a prevalence of 0.40% for *Campylobacter* spp. and 1.29% for *C. coli* and *C. jejuny* (PHILLIPS *et al.*, 2006; VANDERLINDE *et al.*, 1999). In other studies conducted in Europe and the USA, the presence of *Campylobacter* spp. was not detected (DUFFY *et al.*, 2001; SIERRA *et al.*, 1995; MADDEN *et al.*, 1998). In a recent survey, Garcia *et al.* (2010) found a very high prevalence of *Campylobacter* (90% of 80 carcasses). The authors suggested that laboratory procedures could explain the high rate of recoveries: samples were processed only 3 hours (average time) after sampling at the abattoir. Another hypothesis was that the abattoir practices may also have influenced the increased contamination rates from carcasses.

After biomolecular identification, 75% of strains of *Campylobacter* isolates belongs to species *coli* and *jejuni* (9 out of 12 strains). Similar results were reported by GARCIA *et al.* (92%) on 72 strains recovered from ovine carcasses (GARCIA *et al.*, 2010). *C. jejuni* and *C. coli* are the species more commonly responsible for human campylobacteriosis (NØRRUNG and BUNCIC, 2008). KRAMER *et al.* (2000) reported that a significant proportion of *Campylobacter* isolates from lamb meat shared identical subtypes with human isolates. According to these authors, we point out the role of ovine carcasses and meat as a potential source of infection, but the outbreak data and the international literature does not provide strong evidence that ovine meat is a major transmission route for campylobacteriosis.

The isolation rate of *E. coli* O157 on ovine carcasses (4.06%) was slightly higher than that reported in other studies. DUFFY *et al.* (2010) and CHAPMAN *et al.* (2001) reported a prevalence on ovine carcasses of 0.60 and 0.70% respectively. The higher prevalence reported in this study may be due to the presence of healthy-carrier animals within the slaughtering groups included in the sampling plan. This may also have allowed an "*ante-mortem*" contamination of animals and increased the percentage of contamination of carcasses during slaughtering operations (NØRRUNG and BUNCIC, 2008; SMALL *et al.*, 2002). In fact, the 13 positive carcasses in our study belonged to only 3 out of 35 slaughtering batches. However, no samples to confirm this hypothesis were taken from animals before slaughtering and no further analysis were performed to verify whether the isolates belonged to the same clonal strains.

Many studies reported data about *E. coli* O157:H7 contamination on ovine carcasses. In the USA, two researches reported a prevalence on lamb carcasses of 1.79 and 2.90% (EDRINGTON *et al.*, 2009; KALCHAYANAND *et al.*, 2007). Several surveys performed in Australia, report-

ed a very similar prevalence in sheep (between 0.60% and 0.70%) (PHILLIPS *et al.*, 2006; PHILLIPS *et al.*, 2001; VANDERLINDE *et al.*, 1999). However, the cited data results refer only to *E. coli* O157:H7, while in the present study we have not considered the serotyped, flagellar antigen of isolates.

The majority (92.8%) of *E. coli* O157 isolates detected were negative for the presence of genes encoding verotoxins. This data is in contrast with the trend of other Australian studies, in which populations of STEC obtained from sheep, predominantly carried both *vtx1* and *vtx2* (DUFFY *et al.*, 2010). The *E. coli* O157 isolated from sheep in the USA (96%), Ireland (72.7%) and Scotland (97.7%) were more likely to carry *vtx2* alone (DUFFY *et al.*, 2010).

An Italian study on the prevalence of fecal carriage of *E. coli* O157 in lambs (BATTISTI *et al.*, 2006), reported only one strain positive to PCR for the *vtx2* gene out of five isolates. This survey was designed in the same manner of our study and similarly all animals tested positive came from Eastern Europe (8 out 14 strains of this study came from Romanian carcasses).

There are no particular production hygiene and procedures in these countries but the sheep to be slaughtered in Italy have to take a journey significantly longer than that of animals from other country concerned. The results reported by two studies regarding the virulence factors of isolated strains are very similar, suggesting that the presence and type of genes encoding vtx in *E. coli* O157 strains may be linked to the origin of animals.

Outbreaks of *E. coli* O157:H7 infection after contact with sheep have been described previously (STIRLING *et al.*, 2008; HEUVELINK *et al.*, 2002; CHAPMAN *et al.*, 2000) and at least two VTEC foodborne outbreaks linked to consumption of ovine meat have been reported in literature (SCHIMMER *et al.*, 2008; ESPIE *et al.*, 2006). Our study confirms that ovine carcasses are potential sources of *E. coli* O157 human infection.

Referring to *Clostridium perfringens*, in literature, we could not find a similar study with which we could compare our results. This microorganism has been isolated from the perianal region of ovine carcasses (SMART *et al.*, 1979). The present study shows that *Clostridium perfringens* can also be found in other regions of the carcass when this has been contaminated with fecal material. The importance of *Cl. perfringens* as a foodborne disease agent in the ovine species is not proved only by its isolation in processed ovine meat (NØRRUNG and BUNCIC, 2008; HALL and ANGELOTTI, 1965) but also by many outbreaks due to culinary preparations based on contaminated lamb meat (CENTERS FOR DISEASE CONTROL AND PREVENTION, 2007; SMART *et al.*, 1979; NEW ZEALAND FOOD SAFETY AUTHORITY, 2007).

The European Community law does not establish microbiological criteria regarding the presence of pathogens considered on sheep carcasses with the exception of *Salmonella*. Regulation 2073/2005 requires that no more than 2 out of 50 ovine carcasses can be positive for *Salmonella* before chilling (EUROPEAN COMMISSION, 2005). In the present study, the results are within these limits, because we obtained only two isolates. A result, any mandatory corrective action, such as the improvement of slaughter hygiene and the review of process controls, required to be performed in the slaughterhouse.

Ovine carcasses from foreign countries were more contaminated than those of local origin for *Campylobacter* spp. and *E. coli* O157. The transport-lairage phase usually increases the occurrence and/or levels of foodborne pathogens in animals (NØRRUNG and BUNCIC, 2008) but there are many different factors that influence fresh meat hygiene and data in the current study are not adequate to draw a conclusion.

The higher level of contamination detected in lamb carcasses could be due to many factors, animal husbandry as well as the transport and the slaughtering phases. Even the analysis of EU data on the occurrence of any microbial pathogen is very difficult to interpret. However, it is known that the occurrence of pathogens in raw meat products can vary significantly depending on the operators and even on the different manufacturing stages of the same operator (NØRRUNG and BUNCIC, 2008). The primary sources of microbial contamination in the abattoir are the hair and skin of animals, the alimentary tract and the nasopharyngeal cavities. For this reason, the slaughtering of pathogen-free animals should be performed before pathogen-carrying animals to reduce the possibility of meat contamination or, at least, after preslaughter washing of dirty sheep that can help reducing the prevalence of bacteria on carcasses (BYRNE *et al.*, 2007; NØRRUNG and BUNCIC, 2008).

This study confirms, in agreement with other studies in the bibliography, that ovine carcasses can be contaminated with the most common pathogens responsible for foodborne infections. For sheep, no similar research has been carried out in Italy for the cited pathogens.

Different factors can affect the prevalence rate, such as the hygiene conditions of the slaughterhouse, the different practices of slaughtering, the use of decontamination techniques and the presence of carrier animals. Further investigation should be conducted to assess the role of these variables and how much they influence the contamination level of carcasses.

For an efficient risk assessment, further studies should be carried out to assess the contamination of carcasses with quantitative techniques because in literature there are few studies that provide such information (DUFFY *et al.*, 2010).

REFERENCES

Antoniollo P.C., Bandeira F.d.S., Jantzen M.M., Duval E.H. and Silva W.P. 2003. Prevalence of *Listeria* spp. in feces and carcasses at a lamb packing plant in Brazil. J. Food Prot. 66:328.

Battisti A., Lovari S., Franco A., Di Egidio A., Tozzoli R., Caprioli A. and Morabito S. 2006. Prevalence of *Escherichia coli* O157 in lambs at slaughter in Rome, central Italy. Epidemiol. Infect. 134:415.

Borch E., Nesbakken T. and Christensen H. 1996. Hazard identification in swine slaughter with respect to foodborne bacteria. Int. J. Food Microbiol. 30:9.

Byrne B., Dunne G., Lyng J. and Bolton D.J. 2007. The development of a 'clean sheep policy' in compliance with the new Hygiene Regulation (EC) 853/2004 (Hygiene 2). Food Microbiol. 24:301.

Centers for Disease Control and Prevention. 2007. 2006 Annual Listing of Foodborne Disease Outbreaks, United States. http://www.cdc.gov/foodborneoutbreaks/documents/2006_line_list/2006_line_list.pdf Last access: 03/21/2008.

Chapman P.A., Cerdán Malo A.T., Ellin M., Ashton R. and Harkin M.A. 2001. *Escherichia coli* O157 in cattle and sheep at slaughter, on beef and lamb carcasses and in raw beef and lamb products in South Yorkshire, UK. International Journal of Food Microbiology 64:139.

Chapman P.A., Cornell J. and Green C. 2000. Infection with verocytotoxin-producing *Escherichia coli* O157 during a visit to an inner city open farm. Epidemiol. Infect. 125:531.

Duffy E.A., Belk K.E., Sofos J.N., LeValley S.B., Kain M.L., Tatum J.D., Smith G.C. and Kimberling C.V. 2001. Microbial contamination occurring on lamb carcasses processed in the United States. J. Food Prot. 64:503.

Duffy L.L., Small A. and Fegan N. 2010. Concentration and prevalence of *Escherichia coli* O157 and *Salmonella* serotypes in sheep during slaughter at two Australian abattoirs. Aust. Vet. J. 88:399.

Edrington T.S., Long M., Ross T.T., Thomas J.D., Callaway T.R., Anderson R.C., Craddock F., Salisbury M.W. and Nisbet D.J. 2009. Prevalence and antimicrobial resistance profiles of *Escherichia coli* O157:H7 and *Salmonella* isolated from feedlot lambs. J. Food Prot. 72:1713.

EFSA. 2007. The Community Summary Report on Trends and Sources of Zoonoses, Zoonotic Agents, Antimicrobial Resistance and Foodborne Outbreaks in the European Union in 2006. The EFSA Journal. 130:30/05/2010.

Espie E., Grimont F., Vaillant V., Montet M.P., Carle I., Bavai C., de Valk H. and Vernozy-Rozand C. 2006. O148 Shiga toxin-producing *Escherichia coli* outbreak: microbiological investigation as a useful complement to epidemiological investigation. Clin. Microbiol. Infect. 12:992.

European Commission. 2005. COMMISSION REGULATION (EC) No 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs. 2073:1.

Evans M.R., Salmon R.L., Nehaul L., Mably S., Wafford L., Nolan-Farrell M.Z., Gardner D. and Ribeiro C.D. 1999. An outbreak of *Salmonella typhimurium* DT170 associated with kebab meat and yogurt relish. Epidemiol. Infect. 122:377.

Garcia A.B., Steele W.B. and Taylor D.J. 2010. Prevalence and carcass contamination with *Campylobacter* in sheep sent for slaughter in Scotland. J. Food Saf. 30:237.

Hall H.E. and Angelotti R. 1965. *Clostridium Perfringens* in Meat and Meat Products. Appl. Microbiol. 13:352.

Heuvelink A.E., van Heerwaarden C., Zwartkruis-Nahuis J.T., van Oosterom R., Edink K., van Duynhoven Y.T. and de Boer E. 2002. *Escherichia coli* O157 infection associated with a petting zoo. Epidemiol. Infect. 129:295.

Hudson J.A. and Mott S.J. 1994. A survey for *Listeria* species, motile aeromonads and Yersinia enterocolitica on bovine and ovine carcasses. N. Z. Vet. J. 42:33.

Ismea – AcNielsen. 2006. Panel Consumi N.4 2000-2004 Acquisti domestici: Carne. Panel Consumi.

ISO. 2001. ISO 16654:2001 "Microbiology of food and feeding stuffs – Horizontal method for the detection of *Escherichia coli* O157".

ISO. 2002. ISO 6579:2002 "Microbiology of food and feeding stuffs – Horizontal method for the detection of *Salmonella* spp.".

ISO. 2004. ISO 7937:2004 "Microbiology of food and animal feeding stuffs – Horizontal method for the enumeration of *C. perfringens* – Colony count technique".

ISO. 2005. ISO 11290-1:2005 "Microbiology of food and feeding stuffs – Horizontal method for the detection and enumeration of *Listeria monocytogenes*. Part 1: detection method".

ISO. 2006. ISO 10272:2006 "Microbiology of food and feeding stuffs – Horizontal method for the detection and the enumeration of *Campylobacter* spp. part 1: detection method".

Kalchayanand N., Arthur T.M., Bosilevac J.M., Brichta-Harhay D.M., Guerini M.N., Shackelford S.D., Wheeler T.L. and Koohmaraie M. 2007. Microbiological characterization of lamb carcasses at commercial processing plants in the United States. J. Food Prot. 70:1811.

Kramer J.M., Frost J.A., Bolton F.J. and Wareing D.R.A. 2000. *Campylobacter* contamination of raw meat and poultry at retail sale: identification of multiple types and comparison with isolates from human infection. J. Food Prot. 63:1654.

Lenahan M., O'Brien S.B., Kinsella K., Sweeney T. and Sheridan J.J. 2010. Assessment of lamb carcass hygiene before and after chilling at five Irish abattoirs Food Control. 21:313-318.

Linton D., Lawson A.J., Owen R.J. and Stanley J. 1997. PCR detection, identification to species level, and fingerprinting of *Campylobacter jejuni* and *Campylobacter coli* direct from diarrheic samples. J. Clin. Microbiol. 35:2568.

MacGowan A.P., Bowker K., McLauchlin J., Bennett P.M. and Reeves D.S. 1994. The occurrence and seasonal changes in the isolation of *Listeria* spp. in shop bought foodstuffs, human faeces, sewage and soil from urban sources. Int. J. Food Microbiol. 21:325.

Madden R.H., Moran L. and Scates P. 1998. Frequency of occurrence of *Campylobacter* spp. in red meats and poultry in Northern Ireland and their subsequent subtyping using polymerase chain reaction-restriction fragment length polymorphism and the random amplified polymorphic DNA method. J. Appl. Microbiol. 84:703.

Martineli T.M., Rossi Junior O.D., Cereser N.D., Cardozo M.V., Fontoura C.L. and Perri S.H.V. 2009. Microbiological counting in lamb carcasses from an abattoir in Sao Paulo, Brazil. Ciencia Rural. 39:1836.

Milnes A.S., Stewart I., Clifton-Hadley F.A., Davies R.H., Newell D.G., Sayers A.R., Cheasty T., Cassar C., Ridley A., Cook A.J., Evans S.J., Teale C.J., Smith R.P., McNally A., Toszeghy M., Futter R., Kay A. and Paiba G.A. 2008. Intestinal carriage of verocytotoxigenic *Escherichia coli* O157, *Salmonella*, thermophilic *Campylobacter* and *Yersinia enterocolitica*, in cattle, sheep and pigs at slaughter in Great Britain during 2003. Epidemiol. Infect. 136:739.

New Zealand Food Safety Authority. 2007.RISK PROFILE: CAMPYLOBACTER JEJUNI/COLI IN RED MEAT. http://www.foodsafety.govt.nz/elibrary/industry/Risk_Profile_Campylobacter_Jejuni_Coli-Science_Research.pdf Last access: 06/04/2007.

Nørrung B. and Buncic S. 2008. Microbial safety of meat in the European Union. Meat Sci. 78:14.

OzFoodNet Working Group. 2006. Burden and causes of foodborne disease in Australia: Annual report of the OzFoodNet network, 2005. Commun. Dis. Intell. 30:278.

Paton A.W. and Paton J.C. 1998. Detection and characterization of Shiga toxigenic *Escherichia coli* by using multiplex PCR assays for stx1, stx2, eaeA, enterohemorrhagic *E. coli* hlyA, rfbO111, and rfbO157. J. Microbiol. 36:598.

Pepperell R., Reid C.A., Solano S.N., Hutchison M.L., Walters L.D., Johnston A.M. and Buncic S. 2005. Experimental comparison of excision and swabbing microbiological sampling methods for carcasses. J. Food Prot. 68:2163.

Phillips D., Jordan D., Morris S., Jenson I. and Sumner J. 2006. Microbiological quality of Australian sheep meat in 2004. Meat Sci. 74:261.

Phillips D., Sumner J., Alexander J.F. and Dutton K.M. 2001. Microbiological quality of Australian sheep meat. J. Food Prot. 64:697.

Pociecha J.Z., Smith K.R. and Manderson G.J. 1991. Incidence of *Listeria monocytogenes* in meat production environments of a South Island (New Zealand) mutton slaughterhouse. International Journal of Food Microbiology 13:321.

Purvis G.M., Hullah K., Pascoe S.J., Evans S.J. and Davies R.H. 2005. Persistence of *Salmonella Typhimurium* DT120 in abattoir paddocks holding sheep Vet.Rec. 157:165.

Schimmer B., Nygard K., Eriksen H.M., Lassen J., Lindstedt B.A., Brandal L.T., Kapperud G. and Aavitsland P. 2008. Outbreak of haemolytic uraemic syndrome in Norway caused by stx2-positive *Escherichia coli* O103:H25 traced to cured mutton sausages BMC Infect. Dis. 8:41.

Sierra M.L., GonzalezFandos E., Garcia Lopez M.L., Garcia Fernandez M.C. and Prieto M. 1995. Prevalence of *Salmonella*, *Yersinia*, *Aeromonas*, *Campylobacter*, and cold-growing *Escherichia coli* on freshly dressed lamb carcasses. J. Food Prot. 58:1183.

Small A., James C., James S., Davies R., Liebana E., Howell M., Hutchison M. and Buncic S. 2006. Presence of *Salmonella* in the red meat abattoir lairage after routine cleansing and disinfection and on carcasses. J. Food Prot. 69:2342.

Small A., Reid C.A., Avery S.M., Karabasil N., Crowley C. and Buncic S. 2002. Potential for the spread of Escherichia coli O157, Salmonella, and Campylobacter in the lairage environment at abattoirs. J. Food Prot. 65:931.

Smart J.L., Roberts T.A., Stringer M.F. and Shah N. 1979. The incidence and serotypes of *Clostridium perfringens* on beef, pork and lamb carcasses. J. Appl. Bacteriol. 46:377.

Stirling J., Griffith M., Dooley J.S., Goldsmith C.E., Loughrey A., Lowery C.J., McClurg R., McCorry K., McDowell D., McMahon A., Millar B.C., Rao J., Rooney P.J., Snelling W.J., Matsuda M. and Moore J.E. 2008. Zoonoses associated with petting farms and open zoos. Vector Borne Zoonotic Dis. 8:85.

Synnott M., Morse D.L., Maguire H., Majid F., Plummer M., Leicester M., Threlfall E.J. and Cowden J. 1993. An outbreak of *Salmonella mikawasima* associated with doner kebabs Epidemiol. Infect. 111:473.

Vanderlinde P.B., Shay B. and Murray J. 1999. Microbiological status of Australian sheep meat. J. Food Prot. 62:380.

Wesley I.V. 1999. Listeria in animals. In: *Listeria*, Listeriosis, and Food Safety. Second edition. Elliot T. Ryser, Elmer H. Marth (Ed.), p. 39. Marcel Dekker Inc., New York.

Wollin R. 2007. A study of invasiveness of different *Salmonella* serovars based on analysis of the Enter-net database. Euro Surveill. 12:E070927.3.

Wong T.L., Hollis L., Cornelius A., Nicol C., Cook R. and Hudson J.A. 2007a. Prevalence, numbers, and subtypes of *Campylobacter jejuni* and *Campylobacter coli* in uncooked retail meat samples. J. Food Prot. 70:566.

Wong T.L., Nicol C., Cook R. and MacDiarmid S. 2007b. *Salmonella* in uncooked retail meats in New Zealand. J. Food Prot. 70:1360.

Yang B., Qu D., Zhang X., Shen J., Cui S., Shi Y., Xi M., Sheng M., Zhi S. and Meng J. 2010. Prevalence and characterization of *Salmonella* serovars in retail meats of marketplace in Shaanxi, China. Int. J. Food Microbiol. 141:63.

Zweifel C., Zychowska M.A. and Stephan R. 2004. Prevalence and characteristics of Shiga toxin-producing *Escherichia coli*, *Salmonella* spp. and *Campylobacter* spp. isolated from slaughtered sheep in Switzerland. Int. J. Food Microbiol. 92:45.

Paper received May 6, 2011 Accepted June 27, 2011

PAPER

CHEMICAL COMPOSITION AND ANTIMICROBIAL ACTIVITIES OF THE ESSENTIAL OILS FROM FLOWERS, LEAVES, AND STEMS OF *WEDELIA URTICIFOLIA*

L. ZHU*, Y.J TIAN, Y.C YIN and S.M. ZHU
School of Light Industry and Food Sciences, South China University of Technology,
510641 Guangzhou, China
*Corresponding author: Tel. +86 20 87113849, Fax +86 20 87113849
email: zhuliang@scut.edu.cn

ABSTRACT

The composition of essential oils from the flowers, leaves, and stems of *Wedelia urticifolia* were analysed. α-Pinene (19.80%) and d-limonene (17.31%) were the most abundant compounds in flower oils, while α-pinene (15.57%), d-limonene (11.19%), α-phellandrene (9.69%), γ-terpinene (9.01%), and germacrene D (9.68%) were the main constituents of leaf oil. In stems oils, the major compounds were D-limonene (18.11%), γ-terpinene (13.89%), germacrene D (12.80%), and α-pinene (9.21%). Oils from flowers, leaves, and stems exhibited antimicrobial activity against a variety of bacteria, yeasts, and moulds as indicated by the diameter of zones of inhibition (flower oil: 20.8-23.2 mm, leaf oil: 19.4-21.2 mm, stem oil: 18.6-20.6 mm) and their respective MIC values (flower oil: 6.25-25.0 μg/mL, leaf oil: 12.5-50.0 μg/mL, stem oil: 25.0-50.0 μg/mL).

- Key words: antimicrobial activity, chemical composition, essential oils, *Wedelia urticifolia* -

INTRODUCTION

The genus *Wedelia* comprises about 60 species distributed in tropical and warm temperate regions, including India, Burma, Sri Lanka, China, and Japan (LI *et al.*, 2007). A number of plants in this genus are employed in the folk medicine of many countries to treat a variety of diseases, such as headaches, fevers, infections and pathologies of the respiratory tract (ROQUE *et al.*, 1987; HOWARD *et al.*, 1990).

It was reported that many plants from the genus *Wedelia* displayed various activities, such as hepatoprotection effects (MEOTTI *et al.*, 2006), antipyretic-analgesic effects, molluscicidal effects, antibacterial effects, hypoglycemic effects, anti-osteoporotic effects, and antitumor effects (GUPTA *et al.*, 2007). The main chemical constituents of *Wedelia* extracts were terpenes, steroids, flavonoids, coumarins, cyclitols, and organic acids (LI *et al.*, 2007).

Growing consumer demand for natural food preservatives and concerns over the microbial resistance towards conventional synthetic preservatives (GOULD, 1995) have sparked greater interest in new plant-derived antimicrobial agents. The essential oils from several *Wedelia* species have broad antimicrobial activities. Indeed, essential oils from *W. trilobata* possessed significant antifungal (JIANG *et al.*, 2008) and antibacterial activities against *Bacillus subtilis* and *Staphylococcus aureus* (NIRMAL *et al.*, 2005). In addition, the essential oil of *W. chinensis* was effective against gram positive bacteria and fungi, while the essential oils of *W. chinensis* and *W. biflora* exhibited significant antibacterial and antifungal activity compared to ciproflacin (SURESHKUMAR *et al.*, 2007).

Wedelia urticifolia (Chinese name-Dixuegen) is an erect, weak, perennial herb with elliptic leaves, bright yellow flowers, terminal heads, and a light, camphor-like odor and luxuriantly grows in many districts of China. In East Asia *W. urticifolia* is used to treat fractures, muscles injuries, and infected wounds. The aim of this study was to determine the chemical composition of the hydrodistilled essential oils of *W. urticifolia* using gas chromatography (GC)/mass spectroscopy (MS), and to evaluate their antimicrobial properties.

MATERIALS AND METHODS

Pant material

Aerial parts of *W. urticifolia* at flowering stage were collected from the Danxia Mountains, Guangdong Province, China in June 2009. The flowers, leaves and stems were separated by hand. Samples were dried in the shade. Plant was identified and authenticated by a plant taxonomist, Dr. Xun Gong. A voucher specimen (No. 52935) was deposited in the Kuming Institute of Botany, Chinese Academy of Sciences.

Isolation of the essential oils

Dried aerial parts (flowers, leaves and stems) of the plants (200 g) were ground separately and submitted to hydrodistillation for 4 h using a Clevenger-type apparatus. The oils obtained were dried over anhydrous sodium sulfate and preserved in sealed vials at 4°C until further analysis.

Gas chromatography-mass spectrometry (GC-MS) analysis

Quantitative and qualitative analyses of the essential oils were performed using a GC-MS (Model 6890-5975 GC-MS, Agilent, America) equipped with a HP-5 MS fused silica capillary column (30 m × 0.25 mm i.d., film thickness 0.25 μm). For GC-MS detection, an electron ionization system with ionization energy of 70 eV was used. Helium gas was used as the carrier gas at a constant flow rate of 1 mL/min. Injector and mass transfer line temperature were set to 250 and 280°C, respectively. Essential oil solution (1 μL) in hexane was injected and analyzed with the column held initially at 40°C for 1 min and then increased to 250°C with a 3°C/min heating ramp and subsequently kept at 250°C for 20 min. The Kovats indices were calculated for all volatile constituents using a homologous series of n-alkanes C_8-C_{25}. The major components of oils were identified by co-injection of known standards (wherever possible) and confirmed with Kovats indices using the Wiley (V.7.0) and the National Institute of Standards and Technology (NIST) V.2.0 GC-MS library. The relative concentration of each compound in the essential oils was quantified using the analysis software.

Antimicrobial activity

Test microorganisms: The *in vitro* antimicrobial activities of the essential oils were evaluated against panel which included laboratory control strains obtained from the China Center for Type Culture Collection (CCTCC): two Gram-negative bacteria (*Pseudomonas aeruginosa* CCTCC AB93066 and *Escherichia coli* CCTCC AB91112), two Gram-positive bacteria (*Bacillus subtilis* CCTCC AB92068 and *Staphylococcus aureus* CCTCC AB91053), two yeasts (*Hansenula anomala* CCTCC AY92046 and *Saccharomyces cerevisiae* CCTCC AY92042), and four moulds (*Aspergillus niger* CCTCC AF91004, *Chaetomium globosum* CCTCC AF 200039, *Mucor racemosus* CCTCC AF 93209, and *Monascus anka* CCTCC AF93208). All microorganisms were maintained on an agar slant at 4°C. Bacterial strains were cultured on MHB (Muller-Hinton Broth) at 37°C for 24 h, yeasts were cultured on SDA (Sabour-

aud Dextrose Agar) at 28°C for 48 h and fungal strains were cultured on SDA at 28°C for 120 h before testing.

Inhibitory effect by Agar-diffusion method

Agar-diffusion method was employed to determine antimicrobial activities of the essential oils (NCCLS, 1999). Petri plates were prepared by pouring 20 mL of MBH or SDA and allowed the media to solidify. Plates were dried and 0.1 mL of standardized inoculum containing 10^{6-7} CFU/mL bacterial suspension was poured, spread uniformly, and the inoculum was allowed to dry for 5 min. A Whatman No. 1 sterile filter paper disc (6 mm diameter) was impregnated with 1,000 µg/disc of essential oils. Negative controls were prepared using the same solvent employed to dissolve the samples. The standard reference antibiotics, streptomycin and tetracycline (10 µg/disc) were used as positive controls for the tested bacteria. The plates were incubated at 37°C for 24 h, at 28°C for 48 h, or at 28°C for 120 h, respectively for bacteria, yeasts and fungi. Antimicrobial activity was evaluated by measuring the diameter of the zones of inhibition against the tested organisms. The experiments were repeated in triplicate and the results were expressed as average values.

Determination of minimum inhibitory concentration (MIC)

MICs of the essential oils against the test microorganisms were determined by the broth microdilution method (NCCLS, 1999). Briefly, dilutions of the essential oil were prepared in MHB or SDA in the range 0.05 to 200 µL/mL. Exactly 0.5 MacFarland standard suspensions of the test microorganisms were inoculated in the tubes. A control test was also performed containing inoculated broth or agar supplemented with only DMSO under identical conditions with gentamicin as reference. The bacteria were incubated at 37°C for 24 h, yeasts at 28°C for 48 h, and fungi at 28°C for 96 h. The minimum inhibitory concentrations (MICs) were defined as the lowest concentrations of extract at which no visible growth appeared during the incubation.

Statistical analysis

Tests were carried out in triplicate and the results were calculated as mean ± SD.

RESULTS AND DISCUSSION

The yields of essential oil from the flowers, leaves and stems were 0.8, 1.1 and 0.1% (v/w), respectively. Altogether, 69 compounds were identified in the three essential oils, accounting for 98.77, 94.58 and 95.33% of the whole oils, respectively. The components identified in the essential oils are listed in Table 1 ordered on the basis of their experimental retention indices. All the volatiles compounds were mono- and sesquiterpenes, both hydrocarbons and oxygenated derivatives, together with some non-terpene compounds.

In the flowers, monoterpene hydrocarbons (12 compounds) were the most abundant class of volatile compounds, accounting for 85.7% of the total oil. Amongst these derivatives, the main ones were α-pinene (19.80%), d-limonene (17.31%), α-phellandrene (5.55%), γ-terpinene (4.55%), β-pinene (3.66%) and β-myrcene (3.56%). Sesquiterpene hydrocarbons were the second most abundant class of chemicals identified (10 compounds, 15.01%). Ten oxygenated

Table 1 - Essential oil composition of flowers, leaves and stems of *W. urticifolia* (%).

RI[a]	Components	Parts			Identification[b]
		Flowers	Leaves	Stems	
854	3-Hexen-1-ol	1.32	2.01	2.10	MS, RI
862	(E)-2-Hexen-1-ol	1.12	2.07	3.04	MS, RI
902	Heptanal	0.28	0.26	0.42	MS, RI, Co
928	α-Thujene	1.04	0.18	0.28	MS, RI
936	α-Pinene	19.80	15.57	9.21	MS, RI
949	Camphene	2.47	1.11		MS, RI
973	Sabinene	1.31			MS, RI
978	β-Pinene	3.66	1.69	1.94	MS, RI
982	1-Octen-3-ol	1.33	1.57		MS, RI
986	β-Myrcene	3.56	1.19	0.92	MS, RI, Co
998	α-Phellandrene	5.55	9.69	1.90	MS, RI
1012	3-Carene	0.61	2.39	2.26	MS, RI
1017	α-Terpinene		0.16		MS, RI
1020	p-Cymene	2.83	1.35	2.36	MS, RI
1028	β-Phellandrene	3.33	0.91		MS, RI

(continues)

RI[a]	Components	Parts			Identification[b]
		Flowers	Leaves	Stems	
1030	d-Limonene	17.31	11.19	18.11	MS, RI, Co
1033	Eucalyptol	0.24			MS, RI
1040	(Z)-β-Ocimene	1.39			MS, RI
1059	γ-Terpinene	4.55	9.01	13.89	MS, RI
1078	cis-Linalool oxide		0.18		MS, RI, Co
1099	Linalool	0.38			MS, RI
1137	trans-Pinocarveol	0.89		0.24	MS, RI
1170	Lavandulol	0.29			MS, RI
1178	Terpinene-4-ol	0.57			MS, RI
1191	α-Terpineol	1.38	0.23		MS, RI
1193	Hexyl butanoate	0.15		0.23	MS, RI, Co
1214	Octyl acetate	0.23			MS, RI
1228	Citronellol	0.32	0.14		MS, RI
1235	Methyl thymyl ether	0.88			MS, RI
1242	Carvone	0.36			MS, RI
1246	Carvacrol methyl ether	0.42			MS, RI
1270	Geranial	0.38			MS, RI
1286	Bornyl acetate		0.21		MS, RI, Co
1307	Thymol		0.18		MS, RI, Co
1351	α-Cubebene		0.94		MS, RI
1357	Eugenol	0.25	0.50		MS, RI
1375	α-Copaene		0.10		MS, RI
1390	β-Elemene		0.96		MS, RI
1402	Methyl eugenol		0.91		MS, RI
1405	α-Cedrene		1.05		MS, RI
1413	β-Panasinsene	0.54			MS, RI
1418	β-Caryophyllene	2.22	3.10	6.11	MS, RI
1437	γ-Elemene	2.29	2.91	5.85	MS, RI
1448	Nerylacetone	0.25			MS, RI, Co
1454	α-Humulene	1.24			MS, RI
1458	(E)-β-Farnesene	0.74	5.51	5.69	MS, RI
1475	γ-Muurolene	2.81	3.29		MS, RI
1486	Germacrene D	2.59	9.68	12.80	MS, RI
1502	α-Muurolene	1.51			MS, RI
1508	β-Bisabolene			0.23	MS, RI
1524	D-Cadinene	0.68			MS, RI
1543	Selina-3,7 (11)-diene	0.39			MS, RI
1562	(trans)-Nerolidol	1.43			MS, RI
1578	Spathulenol	1.73	1.45	1.91	MS, RI
1578	Caryophyllene oxide	0.64	0.99	1.91	MS, RI, Co
1608	Guaiol	0.31			MS, RI
1639	Isospathulenol			0.59	MS, RI
1646	α-Muurolol			0.77	MS, RI
1650	γ-Eudesmol			0.14	MS, RI, Co
1652	α-Cadinol		0.91	0.33	MS, RI
1654	β-Eudesmol	0.53			MS, RI
1724	(Z,E)-Farnesol	0.19			MS, RI, Co
1762	Benzyl benzoate			0.22	MS, RI
1791	Cyclocolorenone			0.25	MS, RI
1845	Hexahydrofarnesyl acetone			0.26	MS, RI
1905	(Z)-7-Hexadecenoic acid, methyl ester			0.28	MS, RI
1948	Isophytol	0.17	0.99	0.79	MS, RI, Co
1971	n-Hexadecanoic acid	0.31			MS, RI
2122	(Z)-9-Octadecenoic acid			0.30	MS, RI
	Total identified (%)	98.77	94.58	95.33	
	Monoterpene hydrocarbons	64.58	53.09	45.81	
	Oxygenated monoterpenes	5.69	0.55	0.24	
	Sesquiterpene hydrocarbons	15.01	27.54	30.68	
	Oxygenated sesquiterpenes	4.83	3.35	5.65	
	Others	8.66	10.05	10.25	
	Oil yield (%) (v/w)	0.8	1.1	0.1	

Percentages are the mean of three runs and were obtained from electronic integration measurements using a selective mass detector.
[a]Retention index relative to n-alkanes C8-C25 on HP-5 MS capillary column;
[b]RI is the retention index, MS is mass spectrum, Co is co-injection with authentic compound.

monoterpenes (5.69%) and six oxygenated sesquiterpenes (4.83%) were also detected. In total, monoterpene and sesquiterpene hydrocarbons constituted almost all the emission of volatiles compounds (79.59%) in the flowers oil, and oxygenated compounds only 10.55%.

Eleven monoterpene hydrocarbons (53.09%) were found in the oil of the leaves, with α-pinene (15.57%), d-limonene (11.19%), α-phellandrene (9.69%) and γ-terpinene (9.01%) as the main ones. Sesquiterpene hydrocarbons increased in percentage (from 15.01 to 27.54%) with respect to the flowers, with germacrene D(9.68%), (E)-β-farnesene (5.51%), γ-muurolene (3.29%) and β-caryophyllene (3.10%) as the main ones. Oxygenated monoterpene decreased both in number (from 11 to 4 compounds) and, particularly, in percentage (from 5.69 to 0.55%) with compared to the flowers. Three oxygenated sesquiterpenes were detected, which constituted the 3.35% of the total.

In stems, terpene hydrocarbons accounted for 86.49% of the whole essential oil. Monoterpene hydrocarbons (45.81%) were lower than in flower or leaf oil, but sesquiterpene hydrocarbons (30.68%) were more abundant. D-limonene (18.11%), γ-terpinene (13.89%), germacrene D(12.80%), α-pinene (9.21%), β-caryophyllene (6.11%), (E)-β-farnesene (5.69%) and γ-elemene (5.85%) were the main components of stems oil.

The essential oil of *Wedelia* were analysed previously (CRAVEIRO *et al.*, 1993; KOHEIL, 2000) and monoterpene hydrocarbons were found to be the major compounds in the essential oils. *Wedelia paludosa* oils contained β-pinene (10.3%), limonene (21.3%) and γ-muurolene (11.8%), while the major constituents of *W. trilobata* oils were α-phellandrene (17.4%) and limonene (16.3%) (CRAVEIRO *et al.*, 1993). Major components of the essential oils obtained from the flower-heads of *W. trilobata* were β-phellandrene (25.65%), limonene (8.93%), γ-terpinene (5.90%), trans-β-caryophyllene (4.83%), and α-pinene (4.72%) (KOHEIL, 2000). Our results indicate that the essential oil of *W. urticifolia* contain some relatively similar compounds shared by these other species of *Wedelia*.

Synthetic preservatives are used to prevent the growth of food borne bacteria, and to increase the storage shelf-life of foods. Although they are effective, the potential harmful effects of these chemical preservatives are a growing concern (DEBA *et al.*, 2008). Consumer demand has increased for safe natural products that inhibit food spoilage without chemical residues. Much effort has been expended in the search for new, effective, and nontoxic antimicrobial compounds from natural sources. One such possibility is the use of plant essential oils as antimicrobial additives (SOYLU *et al.*, 2009). Plant essential oils are aromatic volatile products of plant secondary metabolism and have been used for thousands of years. These compounds have formed the basis of many applications in the food flavoring and preservation industries (RAHMAN and KANG, 2009) and generally show selective toxicity towards various pathogens but are relatively safe to humans.

The diameters of the zones of inhibition and the MICs of the oils from flowers, leaves and stems of *W. urticifolia* are shown in Tables 2 and 3. These results indicate that the oils have definite antimicrobial activity against all test organisms. In every case, tetracycline showed the highest antimicrobial activity, but the flowers oil exhibited higher antimicrobial activity than streptomycin and the other oils in every cases. As negative control, the solvent did not affect the growth of the sample strains at the concentration used in this study.

Table 2 - Antimicrobial activity of essential oils of *W. urticifolia* against the growth of microbes.

	Diameter of zones of inhibition				
	[a]Essential oils			[b]Standard	
	Flowers	Leaves	Stem	SM	TC
Pseudomonas aeruginosa	20.9±0.9	19.4±0.7	18.6±0.8	20.6±0.6	21.6±0.5
Escherichia coli	21.2±0.8	20.3±0.9	19.5±1.0	21.1±0.5	22.3±0.8
Bacillus subtilis	22.8±0.9	20.7±0.8	20.1±0.7	20.5±0.4	23.4±0.5
Staphylococcus aureus	21.7±0.7	20.8±0.7	20.3±0.6	20.4±0.6	23.6±0.7
Hansenula anomala	20.8±0.7	20.4±0.7	20.0±0.5	20.7±0.8	23.4±0.5
Saccharomyces cerevisiae	21.3±0.8	20.7±0.6	20.6±0.8	20.8±0.5	22.9±0.5
Aspergillus niger	23.2±0.7	21.2±0.8	20.5±1.0	21.6±0.6	22.0±0.7
Chaetomium globosum	21.8±0.8	20.7±0.7	20.2±0.7	20.6±0.6	22.5±0.6
Mucor racemosus	21.4±0.8	20.2±0.8	19.8±0.7	20.9±0.6	21.8±0.7
Monascus anka	21.5±0.8	20.4±0.6	20.1±0.5	20.9±0.6	22.4±0.6

Diameter of inhibition zones (mm) including the diameter of disc (6 mm), values are given as mean ± SD of triplicate experiment.
[a]Diameter of inhibition zones of essential oil (tested volume 1,000 μg/disc);
[b]Standard antibiotics: SM, streptomycin; TC, tetracycline (tested volume 10 μg/disc).

Table 3 - Minimum inhibitory concentrations of essential oils of *W. urticifolia* against the growth of microbes.

	[a]MICs of essential oils		
	Flowers	Leaves	Stem
Pseudomonas aeruginosa	25.0	50.0	50.0
Escherichia coli	25.0	25.0	50.0
Bacillus subtilis	12.5	25.0	25.0
Staphylococcus aureus	12.5	25.0	25.0
Hansenula anomala	25.0	25.0	50.0
Saccharomyces cerevisiae	25.0	50.0	50.0
Aspergillus niger	6.25	12.5	25.0
Chaetomium globosum	12.5	25.0	50.0
Mucor racemosus	12.5	25.0	50.0
Monascus anka	12.5	12.5	25.0

[a]MIC, Minimum inhibitory concentration (values in μL/mL).

Previous papers on the analysis and antibacterial properties of the essential oils of *W. trilobata* have demonstrated that it has a varying degree of growth inhibitory effects against some bacterial due to its chemical compositions such as limonene, β-phellandrene, α-phellandrene, γ-terpinene, β-caryophyllene, and α-pinene (JIANG *et al.*, 2008; NIRMAL *et al.*, 2005; CRAVEIRO *et al.*, 1993; KOHEIL, 2000).

In our opinion, the antimicrobial activity of the oils from *W. urticifolia* are also due, at least in part, to these major constituents such as α-pinene, limonene, γ-terpinene, α-phellandrene, β-caryophyllene, and germacrene D. Pinene, one of the major components of *W. urticifolia* oils, has been shown to possess significant antimicrobial activity (BAKKALI *et al.*, 2008; SOKOVIC and GRIENSVEN, 2006; MAGIATIS *et al.*, 1999; WU *et al.*, 2010). Limonene, another major components of the oils, has been demonstrated to have bacteriostatic activity against several microorganisms (BAKKALI *et al.*, 2008; SOKOVIC and GRIENSVEN, 2006; GNAN and RANA, 1982; DONSÌ *et al.*, 2011; SINGH *et al.*, 2010). In addition, γ-terpinene also has relatively strong antimicrobial activities (BAKKALI *et al.*, 2008) due to its possible synergistic actions with other compound (VARDAR-UNLU *et al.*, 2003). α-Phellandrene, has proven *in vitro* activity against *Bacillus* sp, *Candida albicans*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* (PEREZ *et al.*, 1999; COSTA *et al.*, 2000). β-Caryophyllene is critical for play a critical part in plant defense (ULUBELEN *et al.*, 1994), and antimicrobial activity of β-Caryophyllene has been reported (AZAZ *et al.*, 2000; KALODERA *et al.*, 1997; SIMIC *et al.*, 2002). Germacrene-D, the main constituents of some medicinal plants, have significant antibacterial and antifungal activities (KALODERA *et al.*, 1997; SIMIC *et al.*, 2002).

In addition, lower amounts of components such as γ-elemene, caryophyllene oxide, γ-murolene, β-pinene, (E)-β-farnesene, spathulenol, β-pinene, linalool, eugenol, *p*-cymene, and α-terpineol could also contribute to the antimicrobial activity of the oils. It is also possible that the minor components might be involved in some type of synergism with the other active compounds (MARINO *et al.*, 2001; GILES *et al.*, 2010). In fact, the synergistic effect of the diversity of major and minor constituents present in the essential oils should be taken into account for their biological activity (BURT, 2004).

The mechanism of action of these compounds has not been completely elucidated, but it is speculated that these chemical components exert their toxic effects against these microorganisms through the disruption of bacteria or fungal membrane integrity (COWAN, 1999; FILIPOWICZ *et al.*, 2003). Indeed, α-Pinene, and β-pinene are able to destroy cellular integrity and thereby inhibit respiration and ion transport processes. They also increase the membrane permeability in yeast cells and isolated mitochondria (ANDREWS *et al.*, 1980; URIBE *et al.*, 1985). CONNER and BEUCHAT (1984) suggested that the antimicrobial activity of essential oils of herbs and spices or their constituents could be the result of damage to or disturbance in several enzymatic systems, including those involved in energy production and synthesis of structural components of the cell.

CONCLUSIONS

The antimicrobial properties of the essential oils from many plants are of great interest in medical research and in the food, cosmetic, and pharmaceutical industries due to their potential to replace synthetic antimicrobials. In this study, the bioactivities and chemical compositions of the essential oils from *W. urticifolia* were reported for the first time. The results obtained in the present study are in general agreement with the traditional uses of *W. urticifolia*. These oils are valuable sources for antimicrobials for the food industry, where pathogens cause spoilage and present a risk to human health. These compounds could meet consumer demand for natural antimicrobials, but further studies are needed to evaluate the safety and efficacy of these oils.

ACKNOWLEDGEMENTS

The Authors are grateful for financially supported by the National Natural Science Foundation of China (20676041) and by the Fundamental Research Funds for the Central Universities (2011ZM0106).

REFERENCES

Andrews R.E., Parks L.W. and Spence K.D. 1980. Some effects of Douglas fir terpenes on certain microorganisms. Appl. Environ. Microb. 40: 301.

Azaz D., Demirci F., Satil F., Kürkçüoğlu M. and Başer K.H. 2002. Antimicrobial activity of some *Satureja* essential oils. Z. Naturforsch C. 57: 817.

Bakkali F., Averbeck S., Averbeck D. and Idaomar M. 2008. Biological effects of essential oils. A review. Food Chem. Toxicol. 46: 446.

Burt S. 2004. Essential oils: their antibacterial properties and potential applications in foods. A review. Int. J. Food Microbiol. 94: 223.

Conner D.E. and Beuchat L.R. 1984. Effects of essential oils from plants on growth of food spoilage yeasts. J. Food Sci. 49: 429.

Costa T.R., Fernandes O.L.F., Santos S.C., Oliveria C.M.A., Liao L.M., Ferri P.H., Paulo J.R., Ferreira H.D., Sales B.H.N. and Silva M.R.R. 2000. Antifungal activity of volatile constituents of *Eugenia dysenterica* leaf oil. J. Ethnopharmcol. 72: 111.

Cowan M.M. 1999. Plant products as antimicrobial agents. Clin. Microbiol. Rev. 12: 564.

Craveiro A.A., Matos F.J.A., Alencar J.W., Machado M.I.L., Krush A. and Silva M.G.V. 1993. Volatile constituents of two *Wedelia* species. J. Essent. Oil Res. 5: 439.

Deba F., Xuan T.D., Yasuda M. and Tawata S. 2008. Chemical composition and antioxidant, antibacterial and antifungal activities of the essential oils from *Bidens pilosa* Linn. Var. *Radiata*. Food Control 19(4): 346.

Donsì F., Annunziata M., Sessa M. and Ferrari G. 2011. Nanoencapsulation of essential oils to enhance their antimicrobial activity in foods. LWT-Food Sci. Technol. doi:10.1016/j.lwt.2011.03.003.

Filipowicz N., Kaminski M., Kurlenda J. and Asztemborska M. 2003. Antibacterial and antifungal activity of juniper berry oil and its selected components. Phytother. Res. 17: 227.

Giles M., Zhao J., An M. and Agboola S. 2010. Chemical composition and antimicrobial properties of essential oil of three *Australian Eucalyptus* species. Food Chem. 119: 731.

Gnan S.O. and Rana M.S. 1982. Antimicrobial activity of limonene. Libyan J. Agric. 11: 99.

Gould G.W. 1995. "Homeostatic mechanisms during food preservation by combined methods". In: Barbosa-Cánovas G.V. and Welti-Chanes J. (Ed.), Food preservation by moisture control. Techromic Publishing Co, Lancaster.

Gupta M., Mazumder U.K., Haldar P., Kandar C.H., Manikandana L. and Senthil G. 2007. Anticancer activity of *indigofera aspalathoides* and *Wedelia calendulaceae* in swiss albino mice. Iran. J. Pharm. Res. 6 (2):141.

Howard M.D., Chittawong V. and Payne A.M. 1990. Cotton boll weevil antifeedant activity and antifungal activity (*Rhizoctonia solani* and *Pythium ultimum*) of extracts of the stems of *Wedelia biflora*. J. Agri. Food Chem.38: 1591.

Jiang G.B., Chen S. and Zeng R.S. 2008. Identification and fungitoxicity of volatiles of invasive plant *Wedelia trilobata* L. Chin. J. Eco-Agr. 16: 905.

Kalodera Z., Pepeljnjak S., Blazevic C.N. and Petrac T. 1997. Chemical composition and antimicrobial activity of *Tanacetum parthenium* essential oil. Pharmazie 52(11): 885.

Koheil M.A. 2000. Study of the essential oil of flower-heads of *Wedelia* (L.) Hitch. Al-Azhar. J. Pharm. Sci. 26: 288.

Li X., Dong M., Liu Y., Shi Q.W. and Kiyota H. 2007. Structures and biological properties of the chemical constituents from the genus *Wedelia*. Chem. Biodivers. 4: 823.

Magiatis P., Melliou E., Skaltsounis A.L., Chinou I. and Mitaku S. 1999. Chemical composition and antimicrobial activity of the essential oils of *Pistacia lentiscus* var. chia. Planta Med. 65: 749.

Marino M., Bersani C. and Comi G. 2001. Impedance measurements to study the antimicrobial activity of essential oils from *Lamiaceace* and *Compositae*. Int. J. Food Microbiol. 67: 187.

Meotti F.C., Rosa J.M., Brocardo P.S., Balz D., Waltrick A.P., Bagio A., Goulart E.C., Dafre A.L., Rodrigues A.L. and Santos A.R. 2006. Protective effect of crude extract from *Wedelia paludosa* (Asteraceae) on the hepatotoxicity induced by paracetamol in mice. J. Pharm. Pharmacol. 58: 137.

NCCLS (National Committee for Clinical Laboratory Standards). 1999. Performance standards for antimicrobial susceptibility testing; 9th International Supplement, Wayne Pa. M100-S9.

Nirmal S.A., Chavan M.J., Tambe V.D., Jadhav R.S., Ghogare P.B., Bhalke R.D. and Girme A.S. 2005. Chemical composition and antimicrobial activity of essential oil of *Wedelia trilobata* leaves. Indian J. Nat. Prod. 21(3): 33.

Perez C., Agnese A.M. and Cabrera J.L. 1999. The essential oil of *Scenecio graveolans* (compositae)-chemical composition and antimicrobial activity tests. J. Ethnopharmacol. 66: 91.

Rahman A. and Kang S.C. 2009. *In vitro* control of foodborne and food spoilage bacteria by essential oil and ethanol extracts of *Lonicera japonica* Thunb. Food Chem. 116: 670.

Roque N.F., Giannella T.L., Giesbrecht A.M. and Barbosa R. de C.S.B.C. 1987. Kaurenes diterpenes from *Wedelia paludosa*. Rev. Latinoamer. Quím.18: 110.

Simic N., Palic R., Vajs V., Milosavljevic S. and Djkovic D. 2002. Composition and antibacterial activity of *Achillea asplenifolia* essential oil. J. Essent. Oil Res. 14(1): 76.

Singh P., Shukla R., Prakash B., Kumar A., Singh S., Mishra P.K. and Dubey N.K. 2010. Chemical profile, antifungal, antiaflatoxigenic and antioxidant activity of *Citrus maxima* Burm. and *Citrus sinensis* (L.) Osbeck essential oils and their cyclic monoterpene, DL-limonene. Food Chem. Toxicol. 48: 1734.

Sokovic M. and Griensven L.J.L.D. 2006. Antimicrobial activity of essential oils and their components against the three major pathogens of cultivated button mushroom *Agaricus bisporus*. Eur. J. Plant Pathol. 116: 211.

Soylu S., Soylu E.M. and Evrendilek G.A. 2009. 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 foodborne and seed-borne pathogenic bacteria. Ital. J. Food Sci. 21(3): 347.

Sureshkumar S., Kanagasabail R., Sivakumar T., Chandrasekar M.J.N., Thiruvenkatasubramaniam R. and Thenmozhi S. 2007. Antimicrobiological studies on different essential oils of *Wedelia* species (*W. chinensis*, *W. trilobata*, and *W. biflora*) and *Eclipta alba* (Asteraceae). Asian J. Chem. 19: 4674.

Ulubelen A., Topcu G. and Eris C. 1994. Terpenoids from *Salvia sclarea*. Phytochemistry 36: 971.

Uribe S., Ramirez J. and Peña A. 1985. Effects beta-pinene on yeast membrane functions. J. Bacteriol. 161: 1195.

Vardar-Unlu G., Candan F., Sokmen A., Daferera D., Polissiou M., Sokmen M., Donmez E. and Tepe B. 2003. Antibacterial and antioxidant activity of the essential oil and methanol extracts of *Thymus pectinatus* Fisch. et Mey var. pectinatus (Lamiaceae). J. Agr. Food Chem. 51: 63.

Wu N., Fu Y.J., Kong Y., Zu Y.G. and Liu X.L. 2010. Chemical composition and antimicrobial activity of the essential oil of Rosemary. Environ Toxicol Phar. doi:10.1016/j.etap.2011.03.011.

Received paper January 31, 2011 Accepted July 4, 2011

PAPER

EFFECT OF DRYING PROCESS IN CHAMBER AT CONTROLLED TEMPERATURE ON THE GRAPE PHENOLIC COMPOUNDS

M.T. FRANGIPANE*, S. TORRESI, D. DE SANTIS and R. MASSANTINI
Dipartimento per l'Innovazione nei sistemi Biologici Agroalimentari e Forestali,
Università degli Studi della Tuscia, Via San Camillo De Lellis, 01100 Viterbo, Italy
*Corresponding author: mtfrangi@unitus.it

ABSTRACT

The aim of this paper was to study the phenolic compounds evolution during the off-vine drying process in chamber at controlled temperature and to assess whether this dehydration technique might be used to avoid strong oxidations. Grapes were placed in perforated plastic boxes inside a chamber; fans were installed to ensure humidity and temperature control. Phenolic compounds were characterized by HPLC. Dehydration was fast and regular. This technique ensures phenols protection, artificially dried grapes maintained a higher phenolic concentration than those just harvested. This grape drying technique may ensure the production of sweet wines in which phenols content is preserved.

- Key words: grapes, HPLC, off-vine drying process, phenolic compounds, sweet wines -

INTRODUCTION

Grape drying is a technique used in the production of sweet wines (MORENO *et al.*, 2007). The off-vine grape drying process is in many ways similar to the natural on-vine drying process established by twisting each bunch stalk. Physically, berries gradually shrink, deteriorating their skin as consequence of the loss of water. The drying process has a concentration effect on sugars and a lesser effect on acids (it even decreases their contents), (RIBÉRAU-GAYON *et al.*, 2000; FRANCO *et al.*, 2004). Dried grapes have a high sugar concentration which gives a wine that is rich in flavour, extractive substances and glycerine, conferring body and aromatic qualities to the wines (FERRARINI, 1982). Grapes can be dehydrated by drying in the sun or shade or with more recent artificially methods (PANGAVHANE and SAWHNEY, 2002). Sun-drying is the most traditional and widely used grape drying method; with this system grape drying process is done by direct exposure to sun, but it is only feasible in some warm grape-growing regions (FADHEL *et al.*, 2005). Considerable product loss may occur during natural sun drying due to several factors, such as rodents, birds, insects, rain and microorganisms (PANGAVHANE *et al.*, 2002). The quality of dried product in this case may also be significantly low. Grape deterioration caused by the above mentioned factors in recent years has encouraged the development of drying methods involving grape protection (ESMAIILI *et al.*, 2007). Some studies reported the effect of dipping treatments on the grape drying rate (PANGAVHANE *et al.*, 1999a). In the sunless drying method, instead, grapes are placed in pans or on racks, in ventilated tunnels; this drying process is based on an air speed of 1 m/sec which ensures a continuous control of relative humidity and temperature, using suitable detectors (BELLINCONTRO *et al.*, 2004). Dehydration time and intensity affect several qualitative components of grapes (TONUTTI and MENCARELLI, 2005; PANGAVHANE *et al.*, 1999b) such as sugars, tartaric acid and malic acid (CANCELLIER and MICHELET, 1994). Moreover, grapes contain a large amount of different phenolic compounds in skins, pulp and seeds, that are partially extracted during winemaking (RIBÉREAU-GAYON *et al.*, 2000). Phenolics have positive health effects because of their antioxidant activity (PEINADO *et al.*, 2009). In fact, it is now widely accepted that antioxidants play a crucial role in the prevention of many diseases, thanks to their capacity for capturing, de-activating or repairing the damage caused by the free radicals that are implicated in such diseases (ALONSO *et al.*, 2004). Anyway, little information is available about dehydration effects on the secondary metabolic compounds, such as polyphenols. DI STEFANO *et al.* (1995) reported a decrease of polyphenols in skins and juice caused by oxidative phenomena connected to the high temperature reached during the sun drying process (DI STEFANO *et al.*, 1997; DI STEFANO and MAGGIOROTTO, 1995; BORSA and DI STEFANO, 2000). High temperature in fact influences the enzymatic systems and therefore it may affect the preservation of aromatic compounds during dehydration (BELLINCONTRO *et al.*, 2002; FRANGIPANE *et al.*, 2007). The drying process based on artificial control of relative humidity and temperature fosters the dehydration of grapes in ideal conditions and also the protection of polyphenols from strong oxidations (FRANGIPANE *et al.*, 2005).

The aim of this paper is to study the phenolic compounds evolution during the off-vine drying process in chamber at a controlled temperature and to assess whether this dehydration technique could be used to avoid strong oxidations. This work also aims at searching for alternatives to sun drying in order to avoid the problems related to traditional grape dried method.

MATERIALS AND METHODS

The solutions for samples preparation and analysis were prepared fresh prior to use with ethanol/water/HCl (70:30:1); methanol/water (80/20) and Na_2CO_3 (10% w/v).

All solvents used were HPLC gradient grade.

Drying the experiments over-ripe "*Roscetto*" grapes, grown around the Bolsena lake in 2009 were harvested on the 11th September with soluble solids at 22,54°Brix. Grapes were carefully placed in perforated plastic boxes with an average of 6 kg (± 500 g) fruit per box. The boxes were immediately placed inside a chamber where fans and cooler were installed in order to ensure humidity and temperature control. During dehydration, temperature, humidity and air speed were continuously monitored.

Air speed was constant at 1m/s, temperature was kept between 18° and 21°C and relative humidity was kept at 68%. The experiment was carried out for sixteen days; clusters were weighed and samples were regularly and randomly taken for titratable acidity, pH and sugar analysis. For each analysis the experiment was carried out in triplicate.

During the experiment, samples were periodically collected and the weight loss of grapes was measured. The reducing sugar content (measured as °Brix) was used as a tracking criterion of the grape dehydration process. The drying was ended when the sugar concentration was around 45°Brix.

During dehydration time, grape samples composed by 100 berries each one were randomly taken. Grape berries were processed following the DI STEFANO and CRAVERO method (1991) suitably modified. Each berry was weighted and peeled, skins were dried with blotting paper and put into 250 mL of a ethanol/water/hydrogen

chloride (70/30/1) solution for 21 hours. The obtained extracts were carefully separated from the skins and freezed and stored at -20°C until analysis. Pulps were crushed with 50 mg of $K_2S_2O_5$, centrifuged at 10,000 rpm for 10 minutes, the surnatant was then freezed at -20°C until analysis.

Grape-stones were washed, wiped and weighted. Each 10 g of seeds, 100 mL of a methanol/water (80:20) solution was added, this preparation was homogenized with Ultra Turrax® at 9,500 rpm for 2 minutes and then left in rest for 24 hours. The extract obtained after filtration with 0,45 μm filters was then freezed at -20°C until analysis.

Sugars were calculated according to the corresponding °Brix, which was measured with a hand rifractometer. Titratable acidity, pH and buffer capacity (data not shown) were quantified by using the European Union recommended methods (E.E.C., 1990).

Berry samples processed as above described following the DI STEFANO and CRAVERO method (1991), were submitted to phenolic analysis. Total polyphenols were determined on skin and grape-stone extracts (DI STEFANO *et al.*, 1989).

In a 20 mL flask, 0,1 mL of sample, 5 mL of distilled water and 1 mL of Folin-Ciocalteau reagent have been mixed. After 4 minutes, 4 mL of 10% Na_2CO_3 solution have been added and the flask has been filled with distilled water. After 90 minutes of incubation, the absorbance at 700 nm has been measured.

The total polyphenols content has been expressed as mg/1,000 berries of Gallic acid, because the number of berries is not as variable as the weight during the drying process.

Total flavonoids were determined in skins and grape stones extracts prepared as above reported. The sample has been opportunely diluted with an ethanol/water/hydrogen chloride (70/30/1) solution. The absorbance spectrum was registered between 230 and 400 nm. Subsequently, the absorbance at 540 nm has been marked and then the tangent at the absorbance curve from the UV minimum and the parallel to the absorbance axis passing through the maximum at 280 nm (M), until crossing the tangent in the T point, have been drawn. The length of the MT segment has been then transformed in absorbance unit (E'280) and finally used to determine the total flavonoids content expressed as mg/1,000 berries.

Hydroxycinnamic acids were determined in the juice extracts (DI STEFANO and MAGGIOROTTO, 1995). The juice has been diluted with H_2SO_4 10N (9:1) and then further diluted 10 fold with distilled water. Samples were filtered through a 45 μm filters and the absorbance spectrum were registered between 230 and 400 nm, the maximum of the absorbance were fixed at 320 nm. Hydroxicinnamic acids were expressed as mg/L of caffeic acid.

Phenolic acids were characterized by HPLC, according to the MOUTOUNET *et al.* method (1989), suitably modified. After filtration on Millipore filters (0,22 μm diameter) samples were directly injected in a Dionex chromatograph (loop 20 μL) with a photodiode detector. A C18 column, 300 mm x 3,9 mm (Waters), kept at 22°C was used as stationary phase, with a mobile phase flow rate of 0.5 mL/min. Solvent A = 50mM dihydrogen ammonium phosphate adjusted to pH 2.6 with orthophosphoric acid; solvent B = 20% A with 80% acetonitrile; solvent C = 0.2 M orthophosphoric acid adjusted with NaOH to pH 1.5. The wavelength was monitored at 280 nm using a Perkin-Elmer lambda 3-b spectrophotometer.

Peaks identification was carried out by comparing the retention time of the unknown component to that of each standard (Sigma-Aldrich, Milan, Italy).

All the above mentioned results were expressed in mg/1,000 berries, because the number of berries is not as variable as the weight during the drying process.

All the analyses were done in triplicates and a statistical analysis was carried out on the results obtained. Variance homogeneity was analysed with Student-Newman-Keuls test. All pair wise multiple comparison procedures were performed at the $p < 0,05$ significance level (UNDERWOOD, 1997).

RESULTS AND DISCUSSION

The grape drying process in a conditioned chamber contributes to a faster berry dehydration (SERRATOSA *et al.*, 2008) more than any other traditional technique and it ensures a high final sugar concentration.

Table 1 shows the results of weight loss and sugar increase monitored throughout the process. At harvest time, berries had a sugar concentration of 22,54°Brix. At the end of the process this parameter reached a value of 45°Brix with a percentage value of 99,64%. During the drying process, a progressive weight loss occurs, with an end value of 43,36%.

Changes in berry weight and soluble solids during dehydration can be explained as evaporative weight loss and concentration (MORENO *et al.*, 2008). In Table 1, temperature monitoring in shown and during the overall process, it was kept between 18° and 21°C. Fast and regular dehydration gives a quality sweet wines faster than traditional techniques (BELLINCONTRO *et al.*, 2002).

Total polyphenols and flavonoids determinations in skins during the drying process are shown in Fig. 1. At the end of the process, phenolic compounds maintained high values similarly to fresh grapes. The artificial dehydration system may ensure a greater protection of the phenolic fraction than traditional techniques

Table 1 - Mean values of temperature, sugars and weight loss of grapes during the drying process.

Sampling date	Temperature °C	Sugars °Brix	Sugar increase %	Weight loss %
11 Sept	20	22.54±1.00	0.00	0.00
24 Sept	21	27.45±1.50	21.78	16.95
05 Oct	18	30.78±2.00	36.56	26.24
18 Oct	18	35.56±3.00	57.76	34.49
03 Nov	18	42.90±4.00	90.33	41.24
10 Nov	18	45.00±5.00	99.64	43.36

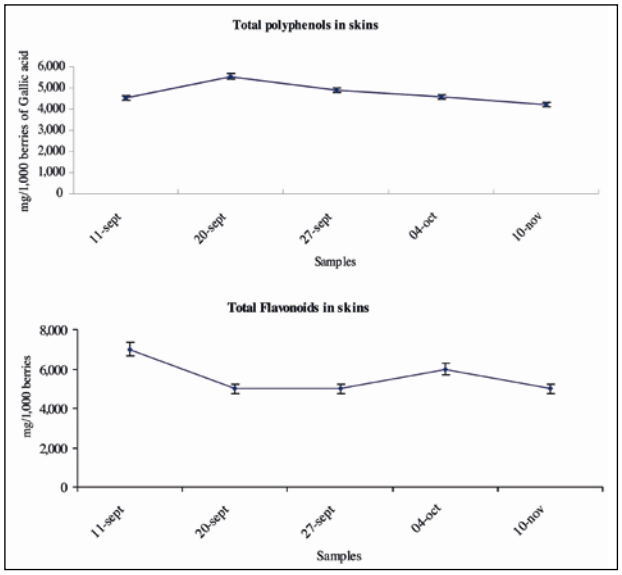


Fig. 1 - Evolution of total polyphenols and flavonoids in skins during the grape drying process.

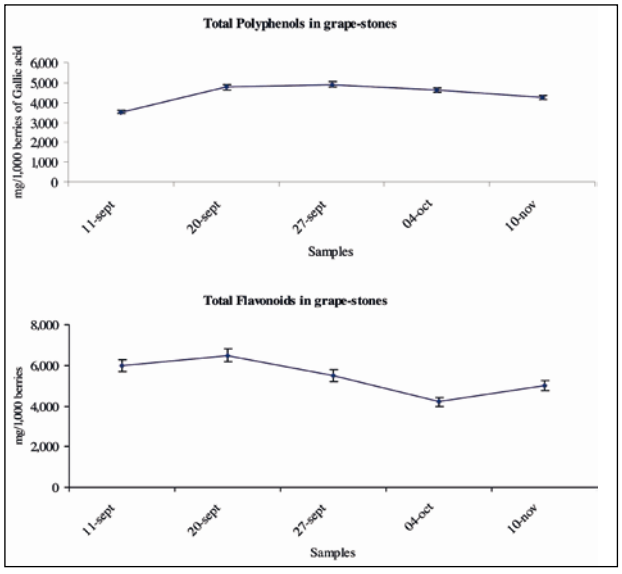


Fig. 2 - Evolution of total polyphenols and flavonoids in grape-stones during the grape drying process.

thus producing a low oxidation of these compounds. Total flavonoids evolution, showed in Fig. 1, slightly decreases during the drying process.

Total polyphenol and flavonoid determination in grape-stones is shown in Fig. 2. Both polyphenols and flavonoids maintained a final value similar to that of fresh grapes. This evolution is probably dues to the grape drying technology used in this study. Artificial dehydration, in fact, ensures a protection of these compounds.

Fig. 3 shows hydroxycinnamic acids evolution during the dehydration process. A slight decrease of these compounds during dehydration time can be observed. This probably indicates that inside the chamber slow oxidation processes may always occur. Preservation of these compounds is, however, better granted than with other traditional drying techniques (FRANGIPANE *et al.*, 2007). This is due to the fact that the parameters inside the chamber slowed down generally occurring oxidative phenomena.

All the above mentioned results were expressed in mg/1,000 berries, because the number of berries is not as variable as the weight during the drying process.

Figg. 4a and 4b show HPLC determinations of phenolic acids. As to these compounds, a grater presence of protocatechuic, ferulic vanillic and siringic acids was observed. Moreover, the preservation of p-coumaric acid was good though not as obvious as in the above mentioned compounds.

Finally gallic, caffeic and p-hydroxybenzoic ac-

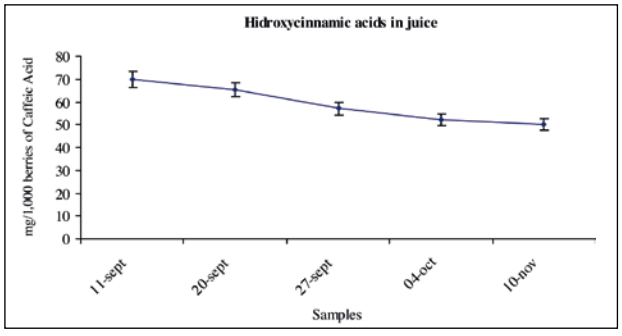


Fig. 3 - Evolution of hidroxycinnamic acids in juice during the grape drying process.

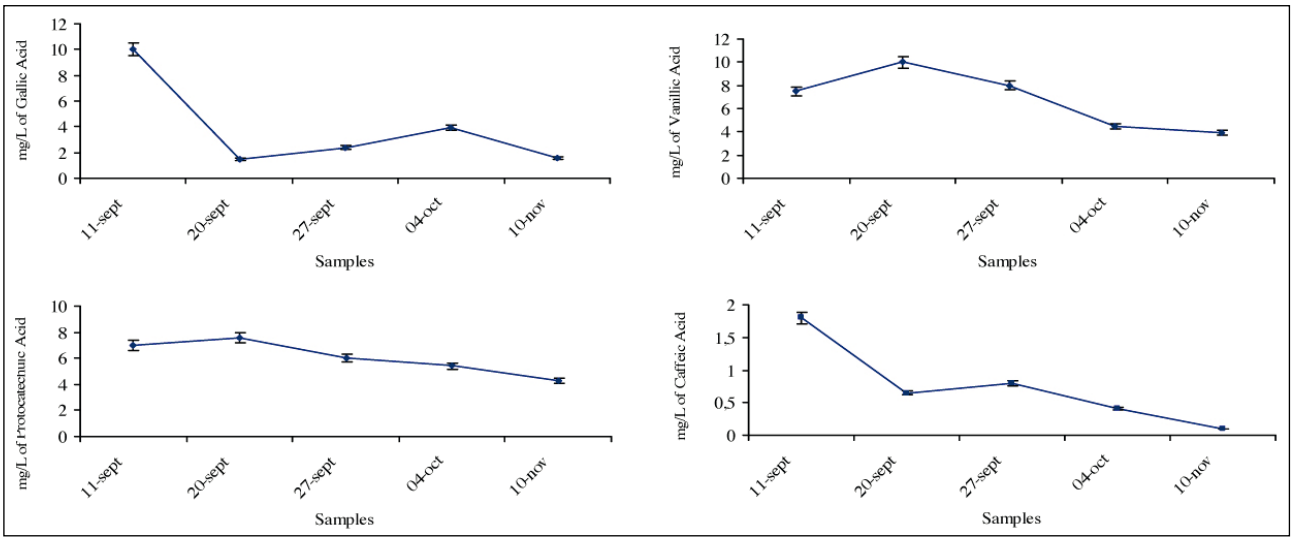


Fig. 4a - Evolution of phenolic acids in juice during the grape drying process.

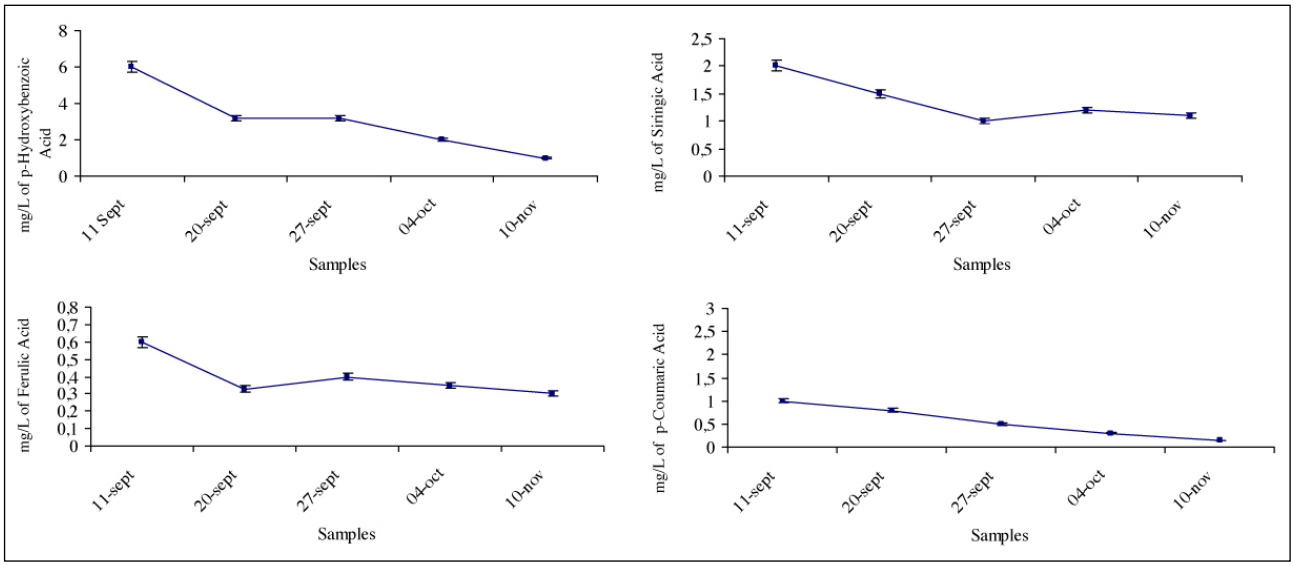


Fig. 4b - Evolution of phenolic acids in juice during the grape drying process.

ids showed the largest decrease, probably due to the oxidations usually occurring during the dehydration process.

CONCLUSIONS

Overall, the results showed that grape drying technology in chamber at controlled temperature and humidity contributes to obtain a dried grape with a high sugar concentration.

The phenolic compounds evolution study, showed that this drying process ensures grapes protection against strong oxidations. In fact, artificially dried grapes maintained a higher phenolic concentration than those just harvested. In light of these considerations, this grape drying technique may ensure the production of a good-quality sweet wine.

ACKNOWLEDGEMENTS

This work was supported by an Italian winemaker dr. Riccardo Cotarella through his investment body and his grape. The work was conducted by the Department of Food Science and Technology in the University of Tuscia, Viterbo (Italy). The Authors appreciate the ongoing support provided by "Falesco" winery in Umbria, Italy.

REFERENCES

Alonso Á.M., Castro R., Rodríguez M.C., Guillén D.A. and Barroso C.G. (2004). Study of the antioxidant power of brandies and vinegars derived from Sherry wines and correlation with their content in polyphenols. Food Res. Int. 37: 715-721.

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

Bellincontro A., De Santis D., Mencarelli F., Nordin C. and Villa I. (2002). Nuova tecnologia di appassimento di uve Trebbiano e Malvasia. Industrie delle Bevande. 182: 538.

Borsa D. and Di Stefano R. (2000). Evoluzione dei polifenoli durante l'appassimento di uve a frutto colorato. Rivista di Viticoltura ed Enologia. 4: 25.

Cancellier S. and Michelet E. (1994). Osservazioni di alcuni componenti durante la conservazione in fruttaio di uve Boschera, Prosecco, Verdino. L'Enotecnico. 10: 87.

Di Stefano R. and Cravero M.C. (1991). Metodi per lo studio dei polifenoli dell'uva. Rivista di Viticoltura ed Enologia. 2: 37.

Di Stefano R. and Maggiorotto G. (1995). Antociani, acidi idrossicinnamici e flavoni del frutto, delle foglie e dei tralci della vite. Rivista di Viticoltura ed Enologia. 48 (2): 51-65.

Di Stefano R., Borsa D., Gentilizi N., Corino L. and Tronfi S. (1997). Evoluzione degli zuccheri, degli acidi fissi e dei composti fenolici dell'uva durante l'appassimento in fruttaio. Rivista di Viticoltura ed Enologia. 1: 33.

Di Stefano R., Cravero M.C. and Gentilizi N. (1989). Metodi per lo studio dei polifenoli nei vini. L'Enotecnico. 5: 83.

Di Stefano R., Maggiorotto G., Melia V., Di Bernardi D., Sparacio A., Fina B. and Sparla S. (1995). Evoluzione dei composti terpenici durante il processo di appassimento dell'uva Zibibbo di Pantelleria. L'Enotecnico. 10: 73.

Esmaiili M., Sotudeh-Gharebagh R., Cronin K., Mousavi M.A.E. and Rezazadeh G. (2007). Grape drying: a review. Food Rev. Int. 23: 257-280.

Fadhel A., Kooli S., Farhat A. and Bellghith A. (2005). Study of the solar drying of grapes by three different processes. Desalination. 185: 535-541.

Ferrarini R. (1982). Nuova tecnologia per la surmaturazione delle uve. Vignevini. 4: 37.

Franco M., Peinado R.A., Medina M. and Moreno J. (2004). Off-vine grape drying effect on volatile compounds and aromatic series in musts from Pedro Ximénez grape variety. J. Agric. Food Chem. 52: 3905-3910.

Frangipane M.T., Ceccarelli A. and Cotarella R. (2005). Nuovo sistema di appassimento artificiale delle uve – evoluzione dei composti fenolici. Industrie delle Bevande. XX-XIV: Febbraio.

Frangipane M.T., Ceccarelli A., Mencarelli F. and Anelli G. (2007). Study of phenolic compounds in Aleatico grapes dried in a forced air tunnel. Ital. J. Food Sci. 19 (2).

Moreno J., Cerpa-Calderón F., Cohen S.D., Fang Y., Qian M. and Kennedy J.A. (2008). Effect of postharvest dehydration on the composition of pinot noir grapes (*Vitis vinifera* L.) and wine. Food Chem. 109: 755-762.

Moreno J., Peinado J. and Peinado R.A. (2007). Antioxidant activity of musts from Pedro Ximénez grapes subjected to off-vine drying process. Food Chem. 104: 224-228.

Moutounet M., Rabier P., Puech J.L., Verette E. and Barillere J.M. (1989). Analysis by HPLC of extractable substances in oak wood. Application to a Chardonnay wine. Sci. Aliment. 9: 35-51.

Pangavhane D.R. and Sawhney R.L. (2002). Review of research and development work on solar dryers for grape drying. Energ. Convers. Manag. 43: 45-61.

Pangavhane D.R., Sawhney R.L. and Sarsawadia P.N. (1999a). Effect of various dipping pretreatment on drying kinetics of Thompson seedless grapes. J. Food Eng. 39: 211-216.

Pangavhane D.R., Sawhney R.L. and Sarsawadia P.N. (1999b). Comparative studies on drying time of different methods for grapes dehydration. Renewable energies and energy efficiency for sustainable development. Proceeding of the 23rd National Renewable Energy Convention-99, Devi Ahilya Vishwavidyalaya, Indore, 260-263.

Pangavhane D.R., Sawhney R.L. and Sarsawadia P.N. (2002). Design, development and performance testing of a new natural convection solar dryer. Energy: 27 (6), 579-590.

Peinado J., Lopez De Lerma N., Moreno J. and Peinado R.A. (2009). Antioxidant activity of different phenolic fractions isolated in musts from Pedro Ximenez grapes at different stages of he off-vine drying process. Food Chem. 114: 1050-1055.

Ribérau-Gayon P., Dubourdieu D., Donèche B. and Lonvaud A. (2000). Handbook of enology (Vol.1). New York, Wiley.

Serratosa M.P., Lopez-Toledano A., Medina M. and Merida J. (2008). Drying of Pedro Ximenez grapes in chamber at controlled temperature and with dipping pretreatments. Changes in the color fractions. J. Agric. Food Chem. 56: 10739-10746.

Tonutti P. and Mencarelli F. (2005). Aspetti fisiologici di disidratazione appassimento e sovramaturazione delle uve da vino. L'Informatore Agrario Supplemento. 14 (1).

Underwood A.J. (1997). Experimental ecology. Their logical design and interpretation using analyses of variance. Cambridge Univ. Press, UK.

Paper received March 16, 2011 Accepted July 19, 2011

PAPER

PHYSICO-CHEMICAL AND ELECTRONIC NOSE MEASUREMENTS ON THE STUDY OF BISCUIT BAKING KINETICS

S. ROMANI*, F. BALESTRA, A. ANGIOLONI, P. ROCCULI and M. DALLA ROSA
Alma Mater Studiorum, University of Bologna, Department of Food Science,
Campus od Food Science, Piazza Goidanich 60, 47521 Cesena, FC, Italy
*Corresponding author: Tel. +39 0547 636120, Fax +39 0547 382348
email: santina.romani2@unibo.it

ABSTRACT

The main physico-chemical modifications during the cooking process of laboratory-made biscuits were monitored at different cooking times (0, 2, 4, 6, 8, 10, 12 min). Moisture content, surface colours and textures were measured. In addition the evolution of the flavour release was performed by means of an electronic nose equipped with 10 metal-oxide sensors. Multivariate statistical analyses were performed to distinguish samples as a function of their physico-chemical characteristics. The electronic nose permitted differentiation between raw, under cooked, well-cooked and over cooked products. Similar and complementary information was obtained considering both electronic nose data and traditional physico-chemical cooking indexes. The obtained results showed that it could be feasible to monitor the changes in the biscuit's aroma and cooking level directly during the process by using an electronic nose with a simplified gas sensor array, as well as allowing the optimization of the technological parameters.

- Key words: biscuit, colour, cooking process, electronic nose, texture -

INTRODUCTION

Biscuits are a popular food product, consumed by a wide range of people, due to their varied taste, long shelf-life and relatively low cost (VITALI *et al.*, 2009).

The main sequential operations in the production of plain, round biscuits are ingredient metering, dough mixing, dough sheeting, dough sheet relaxation, biscuit shape forming, baking, cooling and packaging. Each of the above mentioned steps in the process is of equal importance in determining the final character of the biscuit (CRONIN and PREIS, 2000).

Short doughs are characterised by their composition based upon three major ingredients: flour, sugar and fat. Physico-chemical changes occurring in biscuit dough during the baking stage are very complex and take place following specific kinetics depending on heating conditions. The main transformations induced by cooking are water evaporation, protein denaturation, starch gelatinisation/destruction, browning and surface colouration (principally due to the Maillard reactions), dough expansion during the production and the thermal expansion of gas cells formed during mixing (CHEVALLIER *et al.*, 2000; MANLEY, 2001).

In the baking process, the viscoelastic dough is transformed into a solid-like baked item. This process determines the biscuit's final physical characteristics including dimensions (diameter and thickness), weight and moisture content (CRONIN *et al.*, 2000). During the cooking process a typical evolution in the aroma profile takes place. In particular in the initial stages heating produces an increase in the volatility of the distinctive aromas of raw food, deriving from the ingredients used in the formulation. Thereafter, cooking involves the genesis and release of new volatile compounds that are a consequence of chemical reactions occurring in the food matrix. Finally pirolysis reactions (i.e. Maillard and Strecker degradation) take place on the food's surface with the formation of specific volatile compounds (WARD *et al.*, 2002). The biscuit's initial aromatic profile, due to the raw matrix composition, changes considerably becoming, at the end of cooking process, very complex in terms of the type and quantity of the numerous chemical substances.

Consumers consider texture and related flavour release as factors of primary importance in determining the profile of dry bakery products and assess texture by using a combinations of bites, particle decay and sound evaluations together with flavour release in the mouth (PIAZZA *et al.*, 2008).

The complexity of most food aromas makes them difficult to characterize with conventional flavour analysis techniques such as gas chromatography or gas chromatography olfactometry (PERIS and ESCUDER-GOLABERT, 2009).

Food organoleptic features are usually assessed by human sensory methods. The classical techniques of descriptive analysis, namely flavour profile, quantitative descriptive analysis and texture profile, are mainly useful for the sensory characterisation of food products. These sensory methods need a group of well-trained assessors and several established attributes in order to provide reliable results; requirements that are time-consuming and, in some cases, could cause serious problems to some industries and laboratories (SINESIO *et al.*, 2000). Hence, an instrument such as the electronic nose (e-nose), with recognised high sensitivity and the ability to provide data closely linked to results obtained from human sensory panels would be very useful for several specific applications in food control.

Because they are easy to build, cost-effective and as they provide analysis quickly, e-noses are becoming more and more popular as objective automated non-destructive techniques to characterize and/or monitor food flavours (PERIS *et al.*, 2009).

GARDNER and BARTLETT (1994) defined the e-nose as "an instrument which comprises an array of electronic chemical sensors with partial specificity and an appropriate pattern recognition (PR) system capable of recognizing simple or complex odours".

E-noses were proposed some years ago as a promising technology for odour detection and discrimination, and it has been successfully applied in different fields, such as food science, medicine or environmental pollution control.

Relevant recent literature shows that there are five major categories of use for e-noses in food control. These are (i) process monitoring, (ii) shelf-life investigation, (iii) freshness evaluation, (iv) authenticity assessment and (v) other quality control studies (PERIS *et al.*, 2009).

Detection of key aromas of bakery products during baking is of crucial importance at an industrial level with regard to the final food quality. Previous works showed the possibility of using an e-nose to detect and monitor the evolution of volatile compounds during the cooking process of some foods (WARD *et al.*, 2002) or key aromas of bread baking (PONZONI *et al.*, 2008). To our knowledge, there are no works in which the evolution of biscuit quality characteristics during baking has been monitored considering both traditional cooking indexes and a gas sensory array such as e-nose.

The aim of this work was to monitor the baking process of biscuits in terms of the main physico-chemical product characteristics (moisture content, colour and texture) together with the evolution of aromatic compounds during cooking time. The analysis of flavour release was performed by using of an e-nose equipped with 10 metal-oxide sensors. The obtained e-nose responses were subjected to a multivariate statistical analysis in order to classify the biscuits on the basis of their cooking level.

MATERIALS AND METHODS

Biscuit preparation

Biscuits were prepared using wheat flour, sugar, butter, eggs, baking powder and salt according to the formula given in Table 1. The recipe was based on previous trials performed on the same materials.

The ingredients were mixed in a household mixer (Kenwood, Major, Hampshire - UK) for 10 min, and, after mixing, the dough was kept for 20 min at room temperature (23° ± 1°C) in a closed food container in order to avoid dehydration. Afterwards, the dough was sheeted to a thickness of about 2.5 mm and then was cut by using a stainless steel circular mould (3.5 mm diameter) and placed on a tray. Biscuits were baked in an electric thermo-convection oven (FC61, ANGELO PO, Grandi Cucine S.p.A, Carpi, Italy) at 175°C for different cooking times: 2, 4, 6, 8, 10, and 12 min. The cooking times were chosen on the basis of preliminary experiments in order to obtain under and over baked products. The baking experiments were carried out in triplicate for each cooking time. For each test 16 biscuits were cooked in order to obtain enough samples for all the other measurements.

After cooking, the biscuits were removed from the oven and immediately set for the e-nose analysis. Conversely, before being used for the physico-chemical analyses (moisture content, texture and colour) the biscuits were cooled at room temperature for 1 hour.

Analyses of biscuits

After each baking test, the following analytical determinations were performed: moisture, colour, texture properties, and the flavour release.

Moisture

Biscuit moisture was determined by following the ICC Standard Method n° 110/1 (ICC 2004). For each sample three replicates were performed.

Table 1 - Biscuit dough formulation (1,000 g).

Ingredients	Amount (g)
Biscuit flour	544
Sugar	136
Butter	136
Egg	163
Baking powder (E450*; E500**, E470***)	19.6
Salt	1.4

* Disodium diphosphate; ** Sodium bicarbonate; *** Fatty acids calcium salts.

Colour analysis by computer vision system (CVS)

Six biscuits from each cooking experiment, representative of whole sample, were used for colour measurements. Biscuits were placed on a matte black background and images were captured by using the image acquisition system developed by MENDOZA and AGUILERA (2004) with slight modifications. Samples were illuminated by using two parallel lamps (with two fluorescent tubes by lamp, model TLD Deluxe, Natural Daylight, 18W/965, Philips, NY, USA) with a colour temperature of 6500 K (D65, the standard light source commonly used in food research) and a colour-rendering index (Ra) close to 90%. A colour digital camera (CDC) mod. PowerShot A70 (Canon, NY, USA) was located vertically over the sample at a distance of 12.5 cm. Lamps and CDC were inside a wooden box with internal walls painted black in order to avoid the light and reflection from the room. Images of biscuit surfaces were taken on the matte black background and saved in JPG format. The algorithms for pre-processing of full images, image segmentation and colour quantification were written in MATLAB 6.5 (The MathWorks, Inc., USA). The average value of the segmented pixels in CIE L*a*b* colour space was registered as the colour of the sample. The biscuits' colour were described in terms of: luminosity (L*), hue angle (h°) = $\tan^{-1}(b^*/a^*)$ and Chroma (C*) = $\sqrt{(a^*)^2 + (b^*)^2}$ (McGUIRE ,1992).

Texture

The mechanical and fracture properties of biscuit samples were determined by the three-point bending test (TYAGI *et al.*, 2007) using a TA.HDi 500 Texture Analyser (Stable Micro Systems, Godalming, UK) equipped with a 5 kg load cell.

For the analysis, each biscuit was placed on the aluminium platform situated on two supporting beams spaced at a distance of 35 mm. The blade was attached to the crosshead of the instrument and was brought down to break the biscuit at a crosshead speed of 3.0 mm/s. Care was taken to see that the point of contact was at an equivalent distance from both the supporting beams. This test simulates the biscuit hardness during consumer handling and the way it breaks by bending (TYAGI *et al.*, 2007). All the tests were carried out at room temperature. The blade was brought down at a constant speed under computer control and the applied force was recorded as a function of time. The absolute peak force from the resulting curve was considered as biscuit hardness, while the distance at the break (resistance of the sample to bending) was recorded as sample fracturability. Samples that break at a very short distance have a high fracturability. In order to better understand the re-

sults in this study the fracturability was expressed as 1/break point distance. Six biscuits for each cooking time were tested.

E-nose and test procedures

Samples were analyzed as follows: about 3 g of powdered biscuit sample was placed into a glass vials (capacity 40 mL); these vials were then sealed with a suitable lid equipped with a pierceable Silicon/Teflon disk in the cap. Preliminary experiments, carried out in order to obtain signals of suitable intensity and good reproducibility, showed that after 60 min at 25° ± 1°C of equilibration, the headspace reached a steady state. After this period, the headspace was analyzed with a commercial portable e-nose PEN2 (Airsense Analytics, Milano, Italy) composed of an array of 10 temperature-moderated metal-oxide sensors (MOS), a sampling system, a data acquisition device and a data processing system. The sensor response was expressed as relative conductivity (G/G_0) of a single sensor with time, G_0 is the initial conductivity taken as a reference.

The signal output of the sensors was digitized by recording, and normalized to a value of 1.0 prior to sampling; this arbitrary baseline value was subtracted from the sensor responses prior to enhancement determination. The signal output was measured at 1 s intervals for 60 s at a flow rate of 400 mL min^{-1}. The injection time was long enough for most of the sensors to reach a steady state condition. After each sample analysis the system was purged for 120 s with filtered air prior to the next sample injection to allow re-establishment of the instrument base line. No sensor drift was experienced during the measurement period. The sensor values from 40 to 60 s were used for the statistical analysis.

For each sample, 3 different vials were prepared and each vial was evaluated three times; an average of these nine measurements for each cooking time was used for the statistical analysis.

Statistical analysis

The pattern recognition techniques used in this work were Principal Component Analysis (PCA) and Cluster Analysis (CA). PCA was used to reduce the number of variables in the data matrix and to select the most discriminating parameters in order to classify the different samples. CA was applied on electronic-nose selected sensor responses. CA performs agglomerative hierarchical clustering of objects on the basis of distance measurements of dissimilarity or similarity.

The statistical package STSG statistica for Windows, version 6.0 (Stat-Soft Inc., Tulsa, OK, USA) was used.

RESULTS AND DISCUSSION

Baking is the key stage in baked good production; during this process the main quality characteristics of the final product such as size extension, brownness, texture and flavour are formed thanks to several physico-chemical changes in the product. During baking, transformations, which depend on the course of water content and temperature, are decisive for the final product quality. The majority of these transformations occur together and influence each other (HADIYANTO *et al.*, 2007).

In the first part of this study, the cooking kinetics of biscuits were analyzed on the basis of the main changes regarding the most important parameters involved in the phenomenon.

In Fig. 1, the changes of the biscuit's water content as a function of baking time are shown.

It can be observed that at the beginning of cooking there is a faster reduction in the level of moisture owing to the water migration from the inner towards the biscuit surface due to diffusion and capillarity phenomena.

After 8 min, the optimum cooking time on the basis of previous experiments, the moisture loss rate decreased reaching the minimum and constant value after 10 min.

Usually, in the last stage of baking, when most of water has evaporated, a dried and coloured crust starts to form.

Colour is one of the most important quality attributes for consumer acceptance of baked goods. In fact the surface colour of a baked product is one of the main criterion of acceptance by consumers, together with texture and taste.

Colour is influenced by dough composition, water content and process conditions, temperature and duration of baking. Sugars together with proteins will produce browning compounds, which give colour to the product that lead to irreversible changes. The required relative high temperature needed for these reactions is reached only in the outer zones, once the product is sufficiently dried to have a low water activity and corresponding high evaporation temperature. The duration of this phase depends on the initial water content of the dough and is ruled by heat and mass transfer phenomena (HADIYANTO *et al.*, 2007).

In Fig. 2 colour results (lightness (L*), hue angle (h°) and Chroma (C*)) measured on the biscuit samples at different cooking times are reported. Obtained variations of colour parameters during baking draw a characteristic trajectory, previously found in similar researches (BROYART *et al.*, 1998; CHEVALLIER *et al.*, 2002).

During the first 4 min of cooking the lightness (L*) and hue angle (h°) values increased respectively from about 70 to 80 and from 87 to 90, while chroma (C*) decreased from about 46 to 39. Subsequently, with the increase of the product's temperature, lightness started to de-

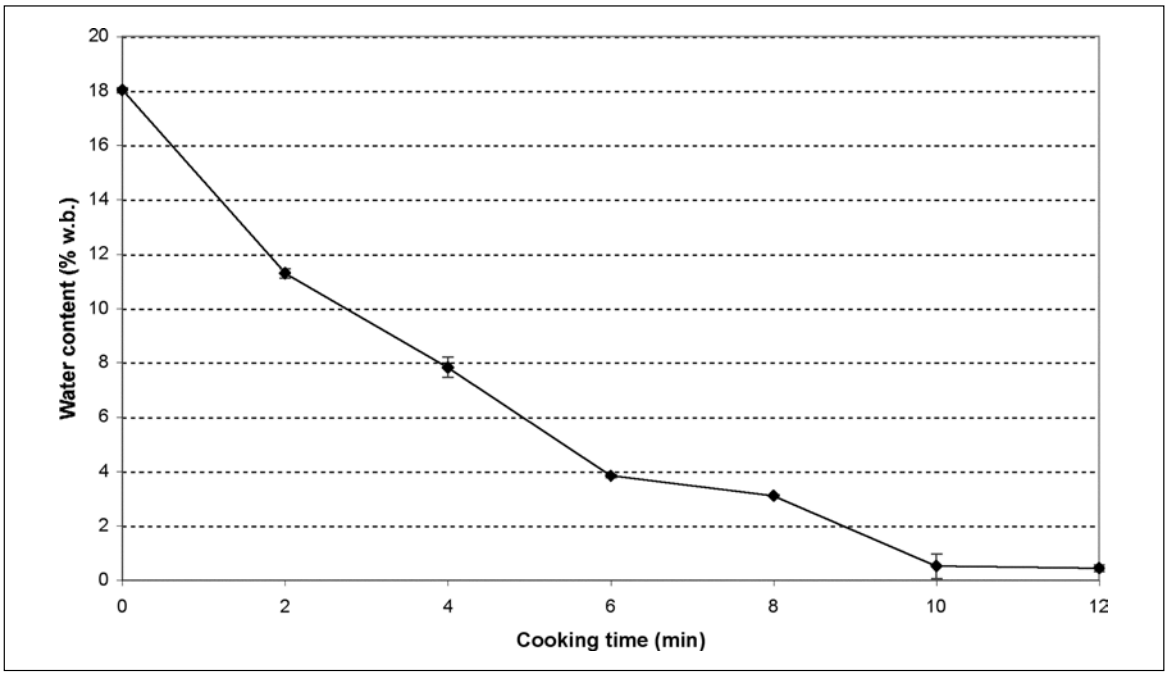


Fig. 1 - Changes of biscuit water content during cooking time.

crease and the biscuit surface started to brown. These findings suggest that the biscuit samples underwent initial whitening and a subsequent gradual intensification of colour, from a yellow to a brown hue. After 8 min of baking the considerable decrease in lightness, hue and chroma were concomitant with a dark browning of the biscuit surface (Fig. 3), that becomes unacceptable for consumers.

As is known and established by previous researches (SHIBUKAWA *et al.*, 1989; BROYART *et al.*, 1998; CHEVALLIER *et al.*, 2002) the overall biscuit colouring kinetics involve two stages. During the first stage of baking the increase in lightness, hue and decrease in chroma correspond to a brightening of the biscuit surface; this makes the product surface appear to be lighter. These colour chang-

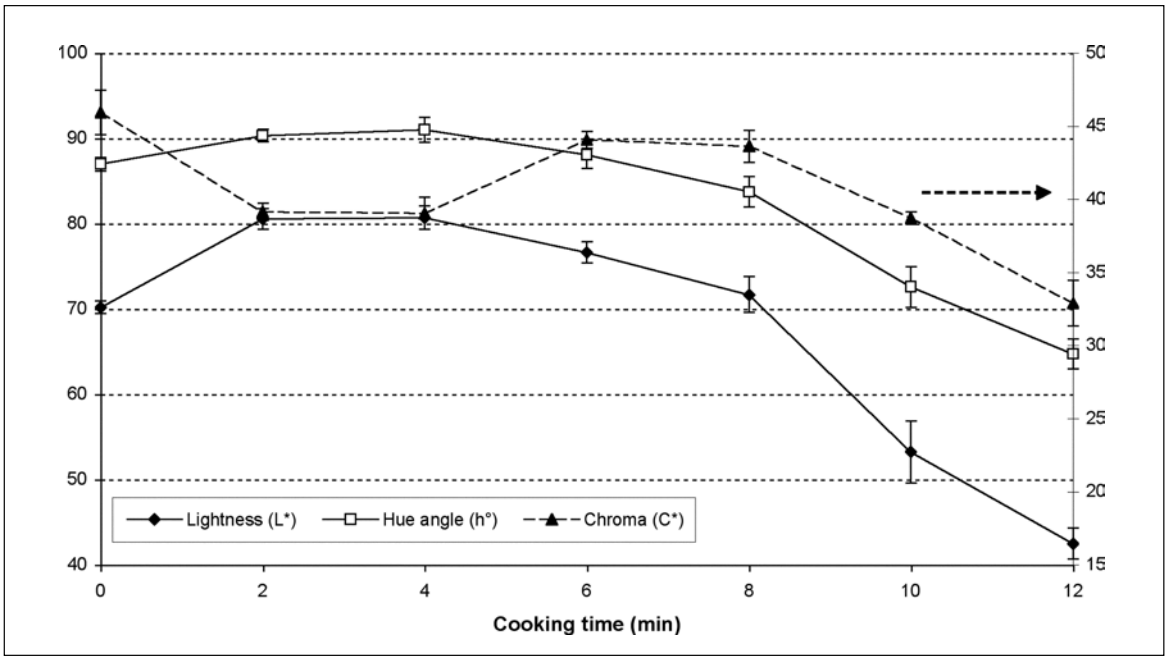


Fig. 2 - Changes of biscuit colour parameters (L*, h° and C*) during cooking time.

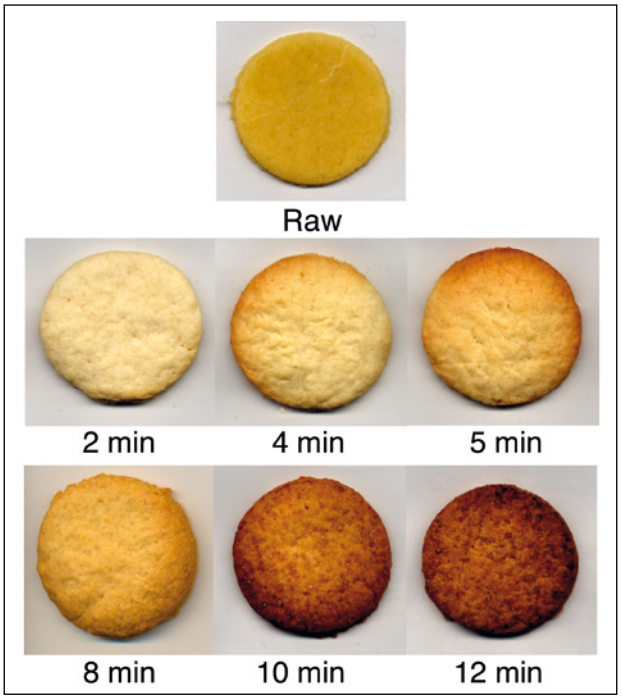


Fig. 3 - Images of biscuit subjected to different cooking times.

es are due to the water migration toward the surface and/or modification in the surface state attributable to the sample rise (CHEVALLIER *et al.*, 2002). In the second stage the progressive browning of the biscuit surface is mainly caused by chemical reactions (Maillard reactions, sugar caramelisation) activated by thermal treatment.

In Fig. 4 the changes of texture parameters of biscuit samples at different cooking times are shown.

As expected, the longer the cooking time the higher the hardness and the fracturability of the biscuits. During cooking, biscuit samples underwent an increase in hardness values from 0.46 kg after 2 min of cooking to 2.21 kg at the end of the process. Regarding the fracturability, the biscuits exhibited values between 1.29 (1/mm) after two min of cooking and 3.76 (1/mm) at the end of the process. The rate of fracturability increase was faster until the first 8 min of cooking; after this time, when the evaporation of water and the production of gases had ended, the fracturability increased more slowly.

These macroscopic changes of biscuit texture characteristics are caused by several phenomena that take place during baking: the production of gases from chemical liveners, the water vaporization, the gas thermal expansion associated with a biscuit's thickness increases and the product dries, resulting in a large decrease in product density and on the development of an open porous structure (CHEVALLIER *et al.*, 2002).

As previously reported, during cooking changes in the biscuit's colour and texture are concomitant to the evolution of a typical aroma profile.

In Fig. 5 the responses (relative conductivity mean data of six replicates) of the e-nose sensors (MOS 1 to 10) obtained at each considered biscuit cooking time are shown.

It can be observed that only some sensors were more sensitive to the evolution of biscuit aromatic profile during baking. In particular, the responses of 1, 3 and 5 sensors increased with the increasing of cooking time; the 2 and

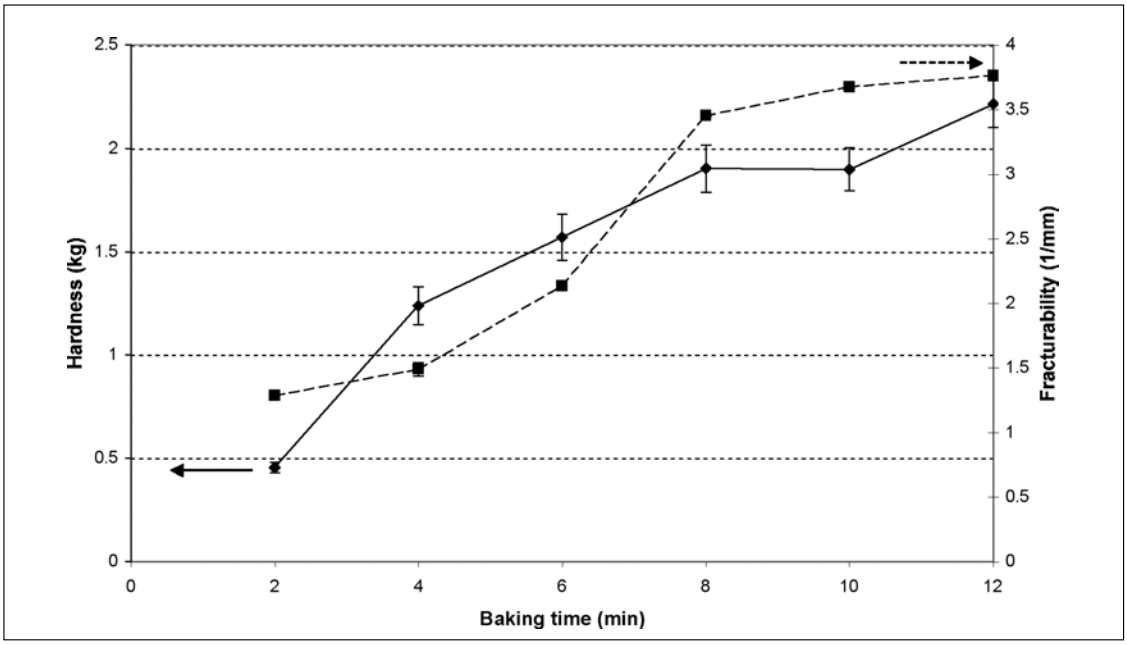


Fig. 4 - Changes of biscuit texture parameters (hardness and fracturability) during cooking time.

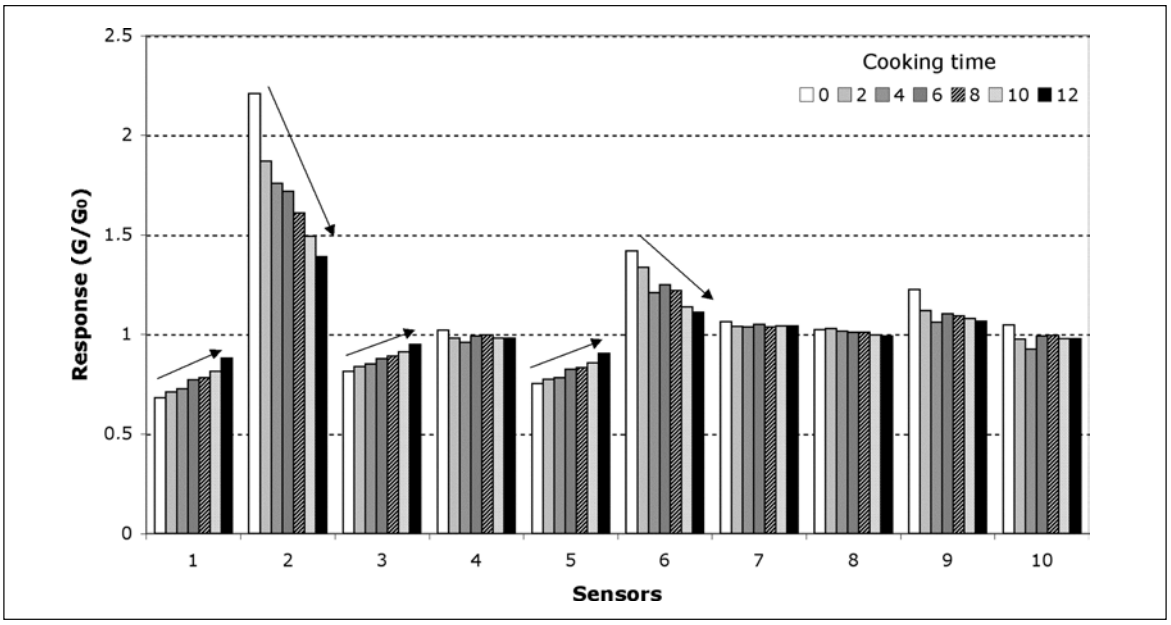


Fig. 5 - Sensor (MOS 1 to 10) responses (mean relative conductivity data: G/G0) obtained at the considered biscuit cooking times.

6 sensors displayed the highest relative conductivity signals. From these results, it seems that the responses of the 1, 3 and 5 sensors are most related to the aroma compounds that are formed and developed during the cooking process. Meanwhile, the 2 and 6 sensor responses seem to be related to the disappearance and/or conversion of others chemical volatile compounds. All the other sensor results were less sensitive to the aromatic profile developed during biscuit cooking process.

PCA was carried out by using the responses from the e-nose sensors collected at the end of each cooking time, in order to highlight and to evaluate whether the most sensible e-nose sensors were able to detect differences between the biscuit samples during cooking stages as a function of their volatile components. The PCA loading plot reported in Fig. 6 confirms results shown in Fig. 5.

Few sensors were able to explain most of the variance and in particular the MOS 1, 3, 5, 2 and 6 had the highest influence in the pattern

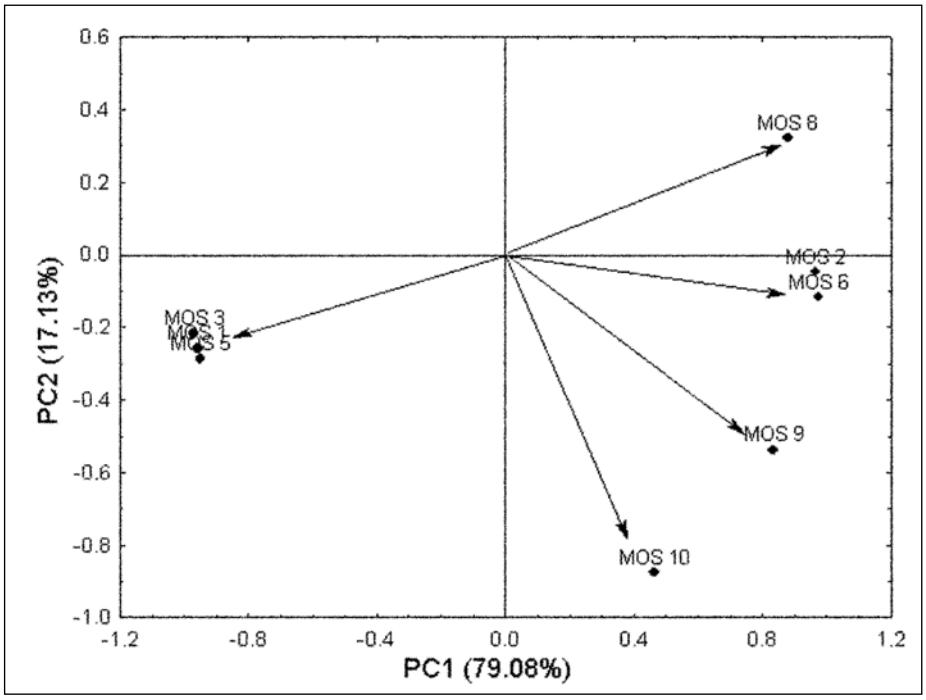


Fig. 6 - PCA loading plot in the plane defined by the first two principal components of the e-nose sensor responses.

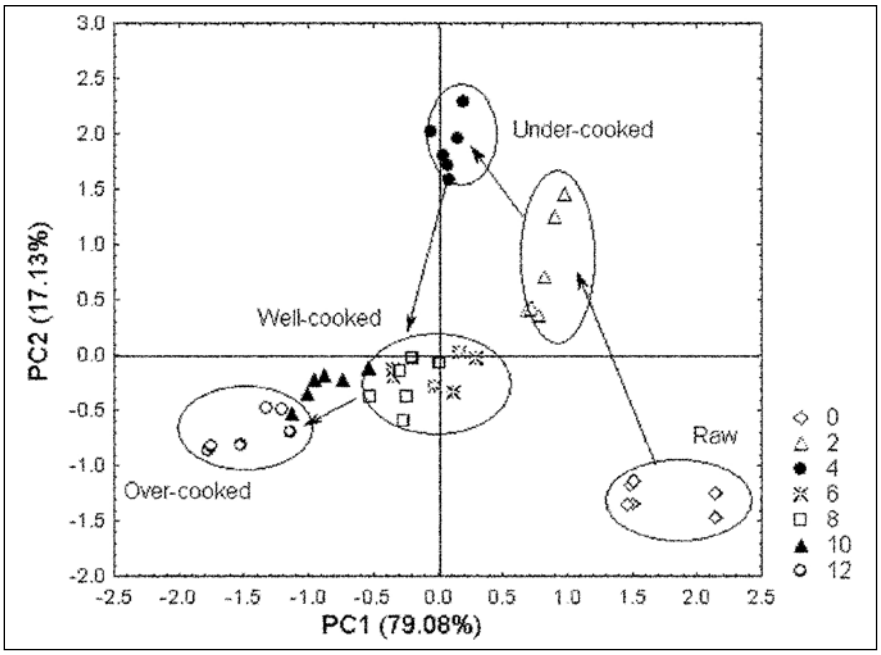


Fig. 7 - Score plot of biscuit samples in the plane defined by the first two principal components on the basis of the e-nose sensor responses during cooking process.

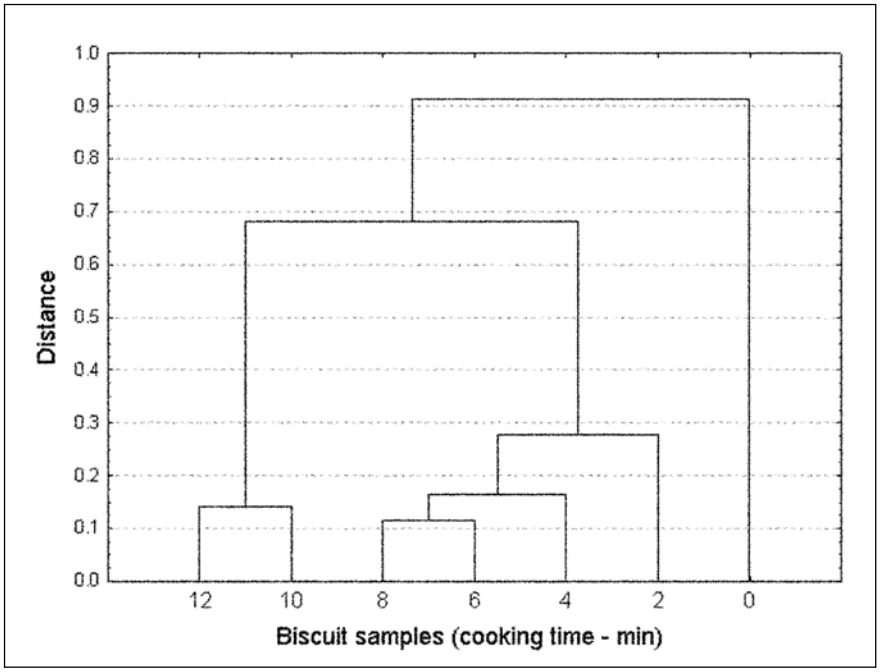


Fig. 8 - Classification dendogram (Cluster analysis) for group of biscuits on the bases of volatile composition: 0 to 12 are cooking time (min). The distance is the geometric distance in the multidimensional space.

file. Their responses were the most relevant in the discrimination of biscuits on the bases of the level of cooking. The 4 and 7 MOS sensor responses were not significant from the PCA. Examining the score plot of Fig. 7 in the area defined by the first two principal components (96.21% of the total variance) a clear separation of biscuit samples into different clusters, according to the cooking degree was found.

Moving from right to left along the PC1 axis (79.08% of the total variance), raw biscuit cluster is first encountered, then the biscuits were cooked for 2, 4, 6, 8, 10 and 12 min respectively. In particular, the group of sensors on the right of the loading plot better discriminate the raw and under cooked (0-4 min) biscuit aromatic compounds; while those on the left of the plot (MOS 1, 3 and 5) better discriminate the cooked and over cooked samples. The biscuit samples cooked for 6 and 8 min were grouped in the same cluster, probably because of their similar aromatic profiles. The biscuits cooked

for 10 min were positioned among them and the over cooked (10 to 12 min) samples. The obtained finger print (sequence) shows a behaviour similar to that obtained by PONZONI *et al.* (2008) who detected, with only four metal oxide e-nose sensors, key artificial aromas related to different stages of the bread baking process. In the same way at which biscuit samples were here distinguished and separated along the PC axes, they firstly obtained the separation of acetaldehyde and diacetyl, that are key odorants identifying the initial baking steps, and then of pyridines and pyrazines, that are flavouring compounds produced by Maillard reactions as the baking process continues. To verify results obtained from PCA, a cluster analysis was run with the same e-nose sensor selected responses (Fig. 8) in order to isolate groups of biscuits with similar aromatic profiles. The dendogram was obtained using Ward method and Euclidean distance.

Through this analysis, it was possible to subdivide the biscuit samples on the bases of their aromatic profiles, related to cooking times, into five groups: 0 (raw), 2, 4, 6 and 8 min, 10 and 12 min. The distances among biscuit groups increased proportionally to the increase of cooking time, being characterized by more and more different volatile aroma compounds.

The CA results were in agreement with those obtained from PCA and were able to better distinguish the biscuit samples cooked for 10 and 12 min from the others. These outcomes confirm further the suitability of the e-nose in monitoring the evolution of the biscuit aroma changes and cooking degree.

CONCLUSIONS

The potential of the e-nose, as an off-line system, for distinguishing the biscuits' cooking levels on the bases of their peculiar aromatic profiles was demonstrated. Multivariate statistical analysis of the e-nose data collected during the biscuit cooking process showed a clear separation between raw, under-cooked, well-cooked and over-cooked biscuits. The discrimination of biscuits according to the cooking time, obtained by analyzing the e-nose signals, confirmed the results provided from all the other considered traditional cooking indexes (moisture content, colour and texture), showing the interchangeable ability of both approaches.

In particular, only few sensor responses were able to explain most of the variance of the data in the PCA and consequently were relevant in the discrimination of the biscuits' cooking levels.

The use of e-nose would appear to have a great potential in the future to develop an on-line monitoring system of the baking process. For this purpose much more research is required, as well as many calibration tests to demonstrate its on-line application.

REFERENCES

Broyart B., Trystram G. and Duquenoy A. 1998. Predicting colour kinetics during cracker baking. J. Food Eng. 35: 351.

Chevallier S., Colonna P., Della Valle G. and Lourdin D. 2000. Contribution of major ingredients during baking of biscuit dough systems. J. Cereal Sci. 31: 241.

Chevallier S., Della Valle G.P., Colonna B., Broyart B. and Trystram G. 2002. Structural and chemical modifications of short dough during baking. J. Cereal Sci. 35: 1.

Cronin K. and Preis C. 2000. A statistical analysis of biscuit physical properties as affected by baking. J. Food Eng. 46: 217.

Gardner J.W. and Bartlett P.N. 1994. A brief history of electronic noses. Sensor Actuat B- Chem. 18: 211.

Hadiyanto H., Asselman A., Van Straten G., Boom R.M., Esveld D.C. and Van Boxtel A.J.B. 2007. Quality prediction of bakery products in the initial phase of process design. Innov. Food Sci. Emerg. Technol. 8: 285.

ICC. 2004. "International Association for Cereal Science and Technology". Determination of the moisture content of cereals and cereal products (Practical method). Standard Method 110. Approved 1960, revised 1976. Vienna.

Manley D. 2001. Baking Techniques. In: Manley D. (Ed.), "Biscuit, cracker and cookie recipes for the food industry", p. 27. Woodhead Publishing Ltd and CRC Press LLC, Cambridge England.

Mcguire R.G. 1992. Reporting of objective colour measurements. Hort. Sci. 27: 1254.

Mendoza F. and Aguilera J.M. 2004. Application of Image Analysis for classification of ripening Bananas. JFS E: Food Eng. Physical Prop. 69: E471.

Peris M. and Escuder-Golabert L. 2009. A 21st century technique for food control: Electronic noses. Anal. Chim. Acta 638: 1.

Piazza L., Gigli J. and Benedetti S. 2008. Study of structure and flavour release relationship in low moisture bakery products by means of the acoustic-mechanical combined technique and the electronic nose. J. Cereal Sci. 48: 413.

Ponzoni A., Depari A., Falasconi M., Comini E., Flammini A., Marioli D., Taroni A. and Sberveglieri G. 2008. Bread baking aromas detection by low-cost electronic nose. Sensor Actuat B-Chem. 130: 100.

Shibukawa S., Sugiyama K. and Yano T. 1989. Effect of heat transfer by radiation and convection on browning of cookies at baking. J. Food Sci. 54: 621.

Sinesio F., DI Natale C., Quaglia G.B., Bucarelli F.M., Moneta E., Macagnano A., Paolesse R. and D'Amico A. 2000. Use of electronic nose and trained sensory panel in the evaluation of tomato quality. J. Sci. Food Agric. 80: 63.

Tyagi S.K., Manikantan M.R., Oberoi H.S. and Kaur G. 2007. Effect of mustard flour incorporation on nutritional, textural and organoleptic characteristics of biscuits. J. Food Eng. 80: 1043.

Vitali D., Vedrina Dragojevic I. and Sebecic B. 2009. Effects of incorporation of integral raw materials and dietary fibre on the selected nutritional and functional properties of biscuits. Food Chem., 114: 1462.

Ward D., Benedetti S. and Riva M. 2002. Monitoring and controlling coking processes using an electronic olfaction device. pp. 292-8. Proceedings of the 9th International Symposium on Olfaction and Electronic Nose (ISOEN); 2002 Rome, Italy.

Paper received March 8, 2011 Accepted September 8, 2011

PAPER

SHELF-LIFE OF MINIMALLY PROCESSED BLOOD ORANGES AS AFFECTED BY MODIFIED ATMOSPHERE PACKAGING

P. RAPISARDA[1]*, C. CAGGIA[2], S.E. BELLOMO[1], P. PANNUZZO[1], C. RESTUCCIA[2], N. TIMPANARO[1] and C.M. LANZA[2]

[1]CRA-ACM - Centro di Ricerca per l'Agrumicoltura e le Colture Mediterranee, Corso Savoia 190, 95024 Acireale, Italy

[2]Dipartimento di Scienze delle Produzioni Agrarie e Alimentari (DISPA), Sezione Tecnologia e Microbiologia degli Alimenti, University of Catania, Via S. Sofia 98, 95123 Catania, Italy

*Corresponding author: Tel. +39 095 7653134, Fax +39 095 7653113, email: paolo.rapisarda@entecra.it

ABSTRACT

The present study evaluated the shelf-life of two minimally processed Tarocco genotypes (Gallo and Scirè) that were packaged in a modified atmosphere with three gas mixtures of different percentages of O_2, CO_2 and N_2. No important physicochemical differences were observed among the orange slices packaged under modified atmosphere and stored at 4°C for 15 ds. The low O_2 percentage in the atmosphere of trays may have caused a decrease in the oxidation process. In addition, the increasing amount of CO_2 due to the respiration process did not lead to fermentation phenomena. Sensory and microbiological changes, however, were observed among the slices packaged with the three gas mixtures, especially for the Tarocco Scirè.

- Key words: fresh-cut orange, MAP, microbial growth, physicochemical analysis, sensory evaluation, Tarocco orange -

INTRODUCTION

Minimal processing of raw fruits and vegetables is designed to maintain a sufficient shelf-life to make distribution feasible within a region of consumption and keep produce fresh without losing nutritional quality (HUXSON and BOLIN, 1989). Modified atmosphere packaging (MAP) has commonly been used in combination with refrigeration to increase the shelf-life of food products (MOLEYAR and NARASIMHAM, 1994). The MAP technique consists of a voluntary replacement of atmospheric gases, which are in the package head space, with specific gases or gas mixtures with various properties. Modified atmosphere packaging should be carefully designed (i.e., it should take into consideration not only steady-state conditions but also the dynamic process) because the package may have no benefit if the product is exposed to an unsuitable gas composition for a long time before reaching an adequate atmosphere (FONSECA *et al.*, 2002).

Generally, the replacement of air in the package is carried out using mixtures of O_2, CO_2 and N_2 (JACXSENS *et al.*, 1999; AHVENAINEN, 2000). Low levels of O_2 and high levels of CO_2 in MAP (JACXSENS *et al.*, 1999; SANDHYA, 2010) can potentially reduce the respiration rate, ethylene production, decay and physiological changes, which would extend the shelf-life of fresh produce (KADER, 1986; ZAGORY and KADER, 1988; WATADA *et al.*, 1990; FONSECA *et al.*, 2000; FONSECA *et al.*, 2002). Moreover, this atmosphere in minimally processed fruits and vegetables has also been shown to inhibit the growth of spoilage microorganisms, which was attributed to the low O_2 concentration because the elevated CO_2 percentage (3-10%) inside the package was not high enough to act against microorganisms (FABER, 1992; BENNIK *et al.*, 1998; FRANCIS *et al.*, 1999). It is important, however, to emphasize that MAP does not guarantee the desired effect, or at least not in the desired measure, if food is not produced in microbiologically optimal conditions.

Oranges are a non-climacteric fruit, and they are likely to be minimally transformed because of their morphological and physiological characteristics (PRETEL *et al.*, 1998). Previous research on minimally processed oranges studied the effects of different types of processing on fruits of common varieties. PRETEL *et al.* (1997 and 1998) studied the effects of enzymatic peeling and MAP on orange *cv* Salustiana, whereas ROCHA *et al.* (1995) evaluated sensory changes during storage at low temperatures of autochthon Portugal orange slides. Moreover, studies have examined shelf-life extension, microbiological stability and bitter substance development of peeled oranges (PAO and PETRACEK, 1997; PAO *et al.*, 1997). Finally, DEL CARO *et al.* (2004) have assessed the effect of minimal processing on flavanone glycoside, ascorbic acid contents and antioxidant capacity of cold-stored Salustiana and Shamouti orange segments.

Recently, research has been directed towards the possibility of processing blood oranges (Tarocco, Moro and Sanguinello varieties) in ready-to-eat products with the aim of finding a new market segment for blood oranges, which have excellent nutritional and sensory characteristics. Previous studies have evaluated the shelf-life of minimally processed fruits of different Tarocco genotypes packaged with films that have different oxygen permeability (RAPISARDA *et al.*, 2006a) and the changes in health-promoting components and antioxidant capacity in ready-to-eat Tarocco orange fruit (RAPISARDA *et al.*, 2006b). In addition, studies have showed the evolution of several enzymes that cause the qualitative decay in blood orange slices during chilled storage (CATALANO *et al.*, 2009).

The present study investigated the physicochemical, microbiological and sensory changes of fresh-cut orange slices of two Tarocco genotypes (Gallo and Scirè), the most extensively grown blood orange varieties in Italy, packaged under modified atmosphere. Since low O_2 and high CO_2 atmosphere is known to extend the shelf-life of fresh-cut fruits (ROJAS-GRAÜ *et al.* 2009), three different gas mixtures of O_2 (2.5, 5.0 and 10%) and CO_2 (8.0, 15 and 20%) were used. These studies were designed to identify the most suitable genotype to be processed and the best packaging conditions to extend the shelf-life and marketability of Tarocco products.

MATERIALS AND METHODS

Sample preparation

Fruits of two different Tarocco genotypes (Gallo and Scirè) were harvested at the experimental field "Palazzelli" of the CRA-Centro di Ricerca per l'Agrumicoltura e le Colture Mediterranee (Acireale, Italy). The samples were processed in a suitably equipped and hygienically controlled environment (UV lamps lighted before processing, disinfection of surfaces and machines by food grade solution of quaternary ammonium salt and room temperature at 15°±1°C). To reduce microbial contamination, the operators wore gloves and masks during all the sample preparation stages, and the cutting and packaging operations were done under a laminar flow hood.

Oranges with uniform size and no blemishes were harvested at maturity (TSS/TA > 10) and sanitized by immersion for 2 min in a solution containing 200 ppm of Cl_2 (420 ppm of sodium hypochlorite adjusted to a pH of 7.0±0.1 with citric acid). The oranges were then rinsed with distilled water, air-dried and manually peeled. Next, the fruits were sliced along the equatorial axis using a commercial slicing machine (ALA 430, Treviso, Italy). Fruit slices were handled by sanitized

steel pliers and packaged under modified atmosphere conditions with three gas mixtures containing different percentages of O_2, CO_2 and N_2 (Table 1). Crystal polyethylene terephthalate (CPET) trays were heat-sealed with the following film by a vacuum/gas machine (TecnoFoodpack, Pavia, Italy): 17 g/m^2 oriented nylon (OPA), 2 g/m^2 adhesive and 42 g/m^2 polyester (PET) with permeability to oxygen at 35 mL/(m^2·24 h), permeability to water vapor at 15 g/(m^2·24 h) and a sealing point at 140°/220°C. The thickness of each orange slice was approximately 1 cm, and each tray contained four orange slices (≈ 180 g). The trays were stored at 4°±1°C for approximately 15 ds. During the storage period, groups of five trays of each Tarocco genotype were sampled every 3-4 ds to determine the main physicochemical, sensory and microbiological parameters.

Table 1 - Gas mixtures percentages used for packaged fresh-cut blood oranges.

	O_2 (%)	CO_2 (%)	N_2 (%)
Gas mixture 1	2.5	8.0	89.5
Gas mixture 2	5.0	15	80
Gas mixture 3	10	20	70

Physicochemical analyses

The gas (O_2, CO_2 and N_2) percentages inside the trays were evaluated by a gas detector (PBI Dansensor mod. CheckMate 9900, Dansensor Italia srl, Segrate, Italy). The firmness of the orange slices was determined by a digital penetrometer (Turoni & C. snc, Forlì, Italy), and the color evaluation (CIE L* a* b*) was performed with a Minolta CR200 colorimeter (Minolta, Milano, Italy). The chemical analyses were carried out on juice obtained by squeezing the orange slices of each tray using a juice extractor (Moulinex, Milano, Italy). The pH value, total acidity (TA) and total soluble solids (TSS) were determined according to classic analytical methods (MAF, 1989; KIMBALL, 1991). The anthocyanins were determined using the method described by RAPISARDA *et al.* (2000), and expressed as mg/L of cyanidin-3-glucoside. The determination of ascorbic acid was carried out by HPLC (RAPISARDA and INTELISANO, 1996) using a Waters mod. 600-E liquid chromatograph (Milano, Italy) equipped with a PDA Waters 996 detector and managed by Millennium 3.2 Waters software. The column used was a C-18 Hypersil ODS 5-µm (Phenomenex, Torrance, CA), maintained at 35°C, and the elution was performed with a solution 0.02M of H_3PO_4 and a flow rate of 1 mL/min. The detection wavelength was set at 260 nm. The sugars (sucrose, fructose and glucose) were determined by HPLC using an aminic column (IB-SIL5-NH_2, Phenomenex, Torrence, CA) and the same liquid chromatograph described above equipped with a refractive index detector (Waters-410). The mobile phase consisted of an 80:20 mixture of acetonitrile:water with a column flow of 1.8 mL/min.

Sensory analyses

We used the profile method for the sensory evaluation of the slices during storage (ISO 13299, 2003), which involved the quali-quantitative description of the sample sensory attributes by a trained panel (ISO 8586-1, 1993) in a laboratory consistent with the standard UNI ISO 8589 (1990). Twenty-four judges between 25 and 28 years old were recruited among students from the University of Catania (Italy). The suitable descriptors (i.e., freshness, aroma, acidity, juiciness, sweetness, firmness, and off-flavor) for evaluating the product were defined by the judges in preliminary sessions. During the training period, the judges were required to estimate several samples of Tarocco oranges using fresh product as a reference. In the session, the judges evaluated the intensity of each chosen descriptor and assigned a score between one (absence of the descriptor) and nine (extreme intensity of the descriptor) on a numerical unipolar scale (UNI-ISO/WD 4121, 2001).

Microbiological analyses

The slices of each tray were aseptically weighed and placed in a sterile plastic bag to be blended in a Stomacher® 400 (Lab-Blender 400, Seward Medical, London, UK) for 3 min. The microbiological analyses of each trial were performed in triplicate until 12 ds of storage. Psychotropic viable counts (PVC) were performed on plate count agar (PCA, Oxoid, CM325) by spread plating after one week of incubation at 15°C. Mesophilic viable counts (MVC) were performed with the same medium by spread plating after 48 h of incubation at 32°C. Acetic acid bacteria counts (AABC) were determined by pour plating using GYCA medium (50 g/L glucose, 10 g/L yeast extract, 30 g/L calcium carbonate, and 25 g/L agar) after incubation at 32°C for 48 h. Yeast and mold counts (YMC) were determined by pour plating with Sabouraud dextrose agar (Oxoid, CM41) after incubation at 25°C for 4 ds (SAB). Acidophilic microorganisms counts (AMC) were performed by pour plating using orange serum agar (OSA, Oxoid, CM657) after incubation at 32°C for 48 h. Colonies were manually counted and expressed as Log_{10}CFUs.

Statistical analysis

Statistical analysis of the physicochemical results was carried out with the STATSOFT 6.0 program (Vigonza, Padova, Italy). The statistical differences at 0, 3, 7, 12 and 15 ds of storage were evaluated by analysis of variance (ANOVA), and the differences in the means were evaluat-

ed using Tukey's test. The sensory analysis scores were analyzed by ANOVA to verify significant differences among attributes during storage, and the differences between the means were evaluated by Tukey's test.

RESULTS AND DISCUSSION

Physicochemical results

Tables 2 and 3 show the physicochemical results of two Tarocco orange genotypes (Gallo and Scirè) that were packaged in modified atmosphere with three gas mixtures of different O_2, CO_2 and N_2 percentages and stored at 4°C for 15 ds. Analysis of the O_2, CO_2 and N_2 percentages in the tray head space showed that there was a significant decrease of O_2 after 3 ds of storage in both Tarocco Gallo and Scirè products, for the three tested mixtures. The CO_2 level was increased in all treatments, whereas the N_2 percentage in the trays decreased, which balanced the CO_2 production. Peeling and slicing severely injured the tissues and induced a high respiration rate, which created a gas atmosphere rich in CO_2 and poor in O_2 inside the trays (PRETEL *et al.*, 1998).

Regarding quality parameters, no important changes were observed for products packaged under modified atmosphere. These results were similar to a previous study that examined the same Tarocco genotypes (Gallo and Scirè) packaged with three films with different oxygen permeability (RAPISARDA *et al.*, 2006a). Only a change of firmness between the first and last sampling for Tarocco Scirè slices packaged with mixture 1 was observed, probably due to an increase of pectin viscosity (the gelling behavior) rather than metabolic processes. In addition, during the entire storage period, only a slight decrease in sugar concentration was noted for sucrose in the Tarocco Scirè slic-

Table 2 - Physicochemical parameters of Tarocco 'Gallo' slices packaged under modified atmosphere with three gas mixtures and stored for 15 days at 4°C.

	Days	Firmness (kg)	pH	TA[a] (%)	TSS[a] (%)	L*	a*	b*	AA[a] (mg/100 mL)	ANT[a] (mg/L)	FRU[a] (mg/100mL)	GLU[a] (mg/100 mL)	SUCR[a] (mg/100 mL)	O_2 (%)	CO_2 (%)	N_2 (%)
Gas mixture 1	0	0.32	3.82	0.65	11.73	47.50	10.40	14.92	61.53	8.15	2.15	2.12	4.83	2.38^{A}	9.06^{B}	88.56^{A}
	3	0.31	3.88	0.57	11.19	47.22	10.29	15.47	55.96	7.45	2.33	2.29	5.07	0.46^{B}	13.28^{AB}	86.26^{AB}
	5	0.33	3.89	0.57	11.40	47.46	9.84	14.78	56.72	7.49	2.41	2.45	5.04	0.12^{BC}	16.86^{A}	83.02^{BC}
	7	0.36	3.95	0.58	11.40	46.86	9.86	15.41	55.41	9.13	2.39	2.41	5.27	0.11^{BC}	17.93^{A}	81.96^{C}
	10	0.32	3.88	0.67	12.07	49.29	9.21	15.59	63.47	9.33	2.45	2.53	5.24	0.00^{C}	19.02^{A}	80.98^{CD}
	12	0.33	3.93	0.65	11.82	48.91	9.19	14.60	60.91	8.49	2.46	2.55	5.63	0.01^{C}	19.04^{A}	80.95^{CD}
	15	0.33	3.98	0.60	11.23	48.29	9.61	15.04	59.26	9.16	2.35	2.36	5.29	0.01^{C}	20.38^{A}	79.61^{D}
Gas mixture 2	0	0.35	3.90	0.64	11.64	46.47	10.77	14.63	66.06	8.33	2.54	2.43	5.33	5.58^{A}	14.42^{D}	80.00^{A}
	3	0.34	3.94	0.64	12.01	45.86	11.16	14.92	64.89	8.63	2.42	2.46	5.46	2.58^{B}	18.58^{CD}	78.84^{A}
	5	0.30	4.03	0.55	11.65	46.27	10.83	15.40	63.50	7.85	2.46	2.47	5.17	0.75^{C}	22.44^{BC}	76.81^{AB}
	7	0.29	4.01	0.57	12.11	45.05	11.15	16.07	57.15	11.09	2.59	2.72	5.96	0.34^{CD}	25.72^{AB}	73.94^{BC}
	10	0.32	4.00	0.61	11.79	47.26	10.52	15.68	69.29	10.06	2.50	2.56	5.53	0.00^{D}	26.76^{AB}	73.24^{BC}
	12	0.36	3.91	0.66	12.48	46.90	10.23	15.29	65.18	10.46	2.62	2.83	6.06	0.01^{D}	26.60^{AB}	73.39^{BC}
	15	0.32	4.02	0.63	12.61	47.27	10.53	15.33	66.04	8.74	2.78	3.01	5.64	0.00^{D}	29.40^{A}	70.60^{C}
Gas mixture 3	0	0.31	3.77	0.71	12.16	45.84	11.27	12.20	58.75	12.12	2.52	2.57	5.72	9.99^{A}	19.83^{D}	70.18^{AB}
	3	0.38	3.79	0.67	12.03	43.90	11.96	12.72	65.87	12.24	2.51	2.50	5.33	7.71^{B}	21.12^{D}	71.17^{A}
	5	0.30	3.92	0.59	12.16	43.83	11.83	12.68	57.56	13.01	2.66	2.59	5.74	4.37^{C}	26.51^{C}	69.12^{ABC}
	7	0.35	3.92	0.68	11.79	44.35	11.35	12.79	57.35	12.95	2.34	2.50	5.68	4.14^{C}	27.32^{BC}	68.54^{BCD}
	10	0.33	3.99	0.60	11.82	45.88	11.13	13.04	53.85	11.63	2.35	2.50	5.58	1.67^{D}	31.30^{ABC}	67.03^{CD}
	12	0.37	3.92	0.69	12.56	45.27	10.79	12.40	65.69	13.10	2.64	2.86	5.75	1.60^{DE}	31.72^{AB}	66.68^{D}
	15	0.32	3.96	0.65	11.53	45.29	10.41	12.07	64.41	11.47	2.51	2.67	5.29	1.37^{E}	35.00^{A}	63.63^{E}

Within a column for a given parameter and mixture, different letters indicate significant differences at p≤0.01; no letters no significant differences.
[a] TA, titratable acidity; TSS, total soluble solid; AA, ascorbic acid; ANT, anthocyanins; FRU, fructose; GLU, glucose; SUCR, sucrose.

Table 3 - Physicochemical parameters of Tarocco 'Scirè' slices packaged under modified atmosphere with three gas mixtures and stored for 15 days at 4°C.

	Days	Firmness (kg)	pH	TA[a] (%)	TSS[a] (%)	L*	a*	b*	AA[a] (mg/100 mL)	ANT[a] (mg/L)	FRU[a] (mg/100mL)	GLU[a] (mg/100mL)	SUCR[a] (mg/100mL)	O_2 (%)	CO_2 (%)	N_2 (%)
Gas mixture 1	0	0.20^{B}	3.90	0.73	11.99	48.62	6.83	7.32	58.64	26.32	2.66	2.76	5.23^{A}	2.07^{A}	9.00^{D}	88.93^{A}
	3	0.21^{B}	3.92	0.73	11.87	46.08	7.38	7.98	57.92	24.08	2.64	2.74	5.20^{A}	0.82^{B}	12.52^{C}	86.66^{AB}
	5	0.23^{AB}	3.91	0.70	11.91	46.57	6.98	7.40	58.88	25.17	2.63	2.73	5.22^{A}	0.16^{C}	14.76^{BC}	85.08^{BC}
	7	0.22^{AB}	4.02	0.65	11.98	47.04	6.68	7.04	56.33	28.43	2.67	2.80	5.12^{AB}	0.13^{C}	15.59^{B}	84.28^{BCD}
	10	0.23^{AB}	3.90	0.72	11.96	46.46	7.06	7.48	53.47	28.49	2.56	2.76	4.93^{AB}	0.08^{C}	17.14^{AB}	82.78^{D}
	12	0.22^{AB}	3.93	0.69	11.68	46.15	7.79	8.10	53.22	28.40	2.48	2.63	4.80^{AB}	0.00^{C}	19.04^{A}	80.96^{D}
	15	0.26^{A}	3.96	0.66	11.31	46.56	7.11	7.96	55.44	26.97	2.42	2.56	4.72^{B}	0.00^{C}	18.68^{A}	81.32^{CD}
Gas mixture 2	0	0.24	3.88	0.81	11.99	44.32	9.87	12.29	58.72	18.09	2.52	2.57	5.23^{A}	4.63^{A}	15.36^{D}	80.01^{A}
	3	0.24	3.86	0.79	11.92	44.72	9.52	11.63	56.39	18.02	2.57	2.68	5.00^{AB}	1.74^{B}	20.54^{C}	77.72^{AB}
	5	0.25	3.89	0.78	12.10	45.94	9.12	10.94	57.14	21.21	2.52	2.65	4.95^{AB}	1.35^{BC}	21.20^{BC}	77.45^{AB}
	7	0.25	3.79	0.83	12.12	46.94	9.09	11.08	60.25	20.85	2.52	2.66	5.11^{AB}	0.66^{CD}	23.68^{B}	75.66^{BC}
	10	0.24	3.91	0.77	12.04	45.83	9.38	11.17	57.14	19.27	2.34	2.45	4.29^{B}	0.02^{D}	27.00^{A}	72.98^{C}
	12	0.26	3.86	0.79	11.78	45.77	9.37	11.91	56.87	20.84	2.27	2.42	4.46^{AB}	0.01^{D}	27.08^{A}	72.91^{C}
	15	0.25	3.90	0.77	12.21	45.92	9.34	12.09	59.86	21.66	2.45	2.63	4.59^{AB}	0.00^{D}	26.98^{A}	73.02^{C}
Gas mixture 3	0	0.23	3.77	0.77	12.20	47.26	7.47	7.71	59.95^{A}	22.93	2.80	2.93	5.17	9.65^{A}	19.20^{E}	71.15^{A}
	3	0.24	3.89	0.64	12.29	45.79	7.45	7.28	56.89^{AB}	22.52	2.86	3.01	5.42	7.08^{B}	22.64^{DE}	70.28^{A}
	5	0.22	3.80	0.69	12.90	46.15	7.15	6.63	54.98^{AB}	21.95	2.99	3.15	5.61	6.06^{B}	23.82^{CD}	70.10^{A}
	7	0.26	3.80	0.67	12.47	46.67	6.85	6.78	54.45^{AB}	23.53	2.89	3.05	5.35	3.91^{C}	27.30^{BC}	68.79^{AB}
	10	0.25	3.91	0.63	12.42	45.77	7.03	6.59	50.75^{B}	23.53	2.77	2.93	5.29	3.89^{C}	28.53^{AB}	67.58^{BC}
	12	0.25	3.90	0.68	12.45	46.21	7.60	7.31	50.70^{B}	21.71	2.67	2.85	5.11	2.91^{CD}	29.72^{AB}	67.37^{BC}
	15	0.25	3.85	0.67	12.35	47.92	7.82	7.25	49.13^{B}	21.79	2.74	2.95	5.23	1.84^{D}	32.44^{A}	65.72^{C}

Within a column for a given parameter and mixture, different letters indicate significant differences at $p \leq 0.01$; no letters no significant differences.
[a] TA, titratable acidity; TSS, total soluble solid; AA, ascorbic acid; ANT, anthocyanins; FRU, fructose; GLU, glucose; SUCR, sucrose.

es packaged with mixtures 1 and 2. This decrease was probably associated with weak hydrolysis phenomena of the sugar, but the decrease was not directly observed through monosaccharide analyses.

Among the various orange varieties, *cv* Tarocco is the richest in vitamin C (RAPISARDA and INTELISANO, 1996; RAPISARDA *et al.*, 2001). The content of ascorbic acid in the juice of Tarocco genotypes has been reported to be between 60-90 mg/100 mL (RAPISARDA and RUSSO, 2000). Studies on fresh-cut blood oranges packaged in normal air with three films with different oxygen permeability (RAPISARDA *et al.*, 2004; RAPISARDA *et al.*, 2006a) have shown a decrease in ascorbic acid by the last day of storage; however, the decrease did not exceed 40% of the ascorbic acid content on the production day. In contrast, most of the products packaged in modified atmosphere containing a low percentage of O_2 have maintained the ascorbic acid content during the entire storage period. A loss of ascorbic acid was only observed in slices of Tarocco Scirè packaged with mixture 3 with significant differences after 7 ds of storage. The decrease of this component was probably due to the residual O_2 percentage in the packaging.

One of the most important objectives for minimally processed products is the maintenance of the original color (DORANTES-ALVAREZ and CHIRALT, 2000). The two Tarocco genotypes packaged with three gas mixtures showed no change in the parameters of the color (L*, a*, b*) during storage. These data provide further confirmation of the absence of browning phenomena of the slices during storage, which would be highlighted with a decrease in L* and an increase in b* values. Moreover, the maintenance of the red color parameter (a*) during the 15 ds of storage was confirmed by the absence of changes in the

Table 4 - Sensory evaluation of Tarocco 'Gallo' slices packaged under modified atmosphere with three gas mixtures and stored for 15 days at 4°C.

	Gas mixture 1							Gas mixture 2							Gas mixture 3						
	Days							Days							Days						
	0	3	5	7	10	12	15	0	3	5	7	10	12	15	0	3	5	7	10	12	15
Freshness	5.85	4.69	4.39	5.05	5.12	5.00	4.64	5.82	5.21	5.12	5.14	5.96	4.36	4.82	5.87	5.51	4.75	4.85	4.00	4.30	4.16
Aroma	5.55^{a}	4.22^{ab}	4.01^{ab}	3.22^{b}	3.56^{ab}	3.96^{ab}	4.53^{ab}	4.76	5.05	4.76	3.96	3.68	4.08	4.02	5.20^{a}	5.22^{a}	4.49^{ab}	3.48^{ab}	2.65^{b}	3.14^{ab}	3.26^{ab}
Acidity	2.67^{ab}	4.52^{a}	3.28^{ab}	2.72^{ab}	2.65^{ab}	2.38^{b}	3.59^{ab}	2.85^{ab}	4.32^{a}	3.07^{ab}	2.35^{b}	2.99^{ab}	2.88^{ab}	3.13^{ab}	3.27^{ab}	3.72^{ab}	2.62^{b}	2.40^{b}	4.69^{a}	4.17^{ab}	4.09^{ab}
Juiciness	5.68	4.53	4.24	5.42	5.31	5.35	4.85	5.31	5.64	5.07	5.31	6.38	4.66	5.45	5.77	5.00	5.15	5.72	4.79	5.16	5.02
Sweetness	6.32a	4.68^{ab}	4.74^{ab}	4.06^{b}	5.27^{ab}	4.20^{ab}	5.37^{ab}	5.18	5.20	4.70	5.96	5.48	4.15	5.13	6.04	5.68	4.97	4.54	4.35	4.58	5.29
Firmness	5.15	5.82	5.98	5.45	5.42	5.77	5.59	5.03	5.45	5.85	5.68	5.64	5.79	5.12	4.94	5.59	4.71	5.57	5.50	6.30	5.07
Off-flavor	3.94	3.13	3.04	2.78	3.05	3.28	3.55	3.11	3.28	3.88	2.87	3.15	2.77	3.79	4.14	3.02	3.26	4.36	4.54	4.69	4.88

Within a row for a given parameter and mixture, different letters indicate significant differences at p≤0.05; no letters no significant differences.

Table 5 - Sensory evaluation of Tarocco 'Scirè' slices packaged under modified atmosphere with three gas mixtures and stored for 15 days at 4°C.

	Gas mixture 1							Gas mixture 2							Gas mixture 3						
	Days							Days							Days						
	0	3	5	7	10	12	15	0	3	5	7	10	12	15	0	3	5	7	10	12	15
Freshness	7.25^{a}	6.25^{ab}	4.48^{bc}	4.73^{bc}	4.60^{bc}	4.74^{bc}	3.42^{c}	6.59^{a}	6.23^{a}	5.01^{a}	5.00^{a}	4.65^{ab}	4.42^{ab}	2.62^{b}	6.70^{a}	6.69^{a}	4.98^{ab}	4.91^{ab}	5.22^{ab}	4.05^{bc}	2.48^{c}
Aroma	6.00^{a}	5.19^{ab}	5.15^{ab}	5.14^{ab}	3.40^{bc}	3.27^{bc}	2.65^{c}	5.68^{a}	5.38^{a}	5.10^{a}	4.18^{ab}	4.38^{a}	3.65^{ab}	2.15^{b}	5.25^{a}	5.02^{a}	4.62^{ab}	3.72^{ab}	4.48^{ab}	2.98^{ab}	2.23^{b}
Acidity	2.85^{ab}	2.59^{ab}	4.20^{a}	3.36^{ab}	3.58^{ab}	2.61^{ab}	2.15^{b}	3.38	4.19	4.38	4.04	4.81	3.59	3.69	2.98^{ab}	2.64^{ab}	4.58^{a}	4.58^{a}	4.33^{ab}	3.16^{ab}	2.08^{b}
Juiciness	6.08^{a}	5.97^{ab}	4.67^{ab}	5.43^{ab}	5.53^{ab}	5.80^{ab}	3.65^{b}	6.28	5.18	4.96	4.83	4.88	5.22	4.77	6.20^{a}	6.27^{a}	5.40^{a}	5.29^{a}	5.27^{a}	5.41^{a}	2.31^{a}
Sweetness	5.40	5.64	3.98	5.25	3.95	4.68	4.41	4.69	4.27	4.82	4.67	4.65	4.22	3.95	4.98^{a}	5.31^{a}	5.08^{a}	4.69^{ab}	3.53^{ab}	3.10^{ab}	1.96^{b}
Firmness	4.51	5.81	5.39	4.32	4.97	4.42	3.65	4.38	5.88	5.95	4.77	5.62	5.21	3.81	5.30^{a}	5.80^{a}	6.28^{a}	4.85^{ab}	4.92^{ab}	4.48^{ab}	2.92^{b}
Off-flavor	1.87^{b}	3.42^{ab}	4.68^{a}	3.88^{ab}	3.15^{ab}	3.62^{ab}	2.35^{ab}	1.73^{b}	3.99^{ab}	5.44^{a}	2.92^{ab}	3.88^{ab}	3.79^{ab}	4.48^{a}	2.22^{b}	2.49^{b}	5.78^{a}	3.19^{ab}	4.75^{ab}	5.09^{ab}	2.82^{b}

Within a row for a given parameter and mixture, different letters indicate significant differences at p≤0.05; no letters no significant differences.

concentration of anthocyanins, which are the pigments responsible for the typical red flesh color for both genotypes.

Sensory results

In all the MAP treatments, the judges found that only a few descriptors were changed in the Tarocco Gallo slices (Table 4). A significant decrease in aroma was noted between the fresh and 7 ds stored products packaged with mixture 1. Moreover, a change in aroma was found between the fresh and 10 ds stored products packaged with mixture 3. The highest value of acidity was observed after 3 ds of storage for slices packaged with mixture 1 and mixture 2. A delay of perception of acidity was noted for slices packaged with mixture 3. Nevertheless, the observed trends of acidity were not comparable with the equivalent chemical parameters. No changes in pH or TA were found for slices packaged with any of the three mixtures. Considering the sweetness descriptor, only the Tarocco Gallo packaged with mixture 1 showed a decrease between fresh slices and slices stored for 7 ds. Freshness, juiciness, firmness and off-flavor remained unchanged in all treatments.

Tarocco Scirè slices had the worst results with changes observed for almost all the considered descriptors (Table 5). For slices packaged with mixture 1, a loss of freshness was already perceived after 3 ds of storage, but the other packaged products maintained acceptable levels of freshness up to 12-15 ds of storage. In slices packaged in mixture 1, the aroma descriptor was dif-

ferent by the 10th day, whereas the judges did not notice a difference in aroma for the other two mixtures until the 12th day. During the 15 ds of storage, changes in the acidity and juiciness parameters were observed in the slices packaged with gas mixtures 1 and 3, whereas the sweetness and firmness parameters had significant differences in slices packaged in gas mixture 3. Furthermore, the judges perceived an increase in the off-flavor at 5 ds of storage in all Tarocco Scirè packaged products.

Microbiological analysis

Table 6 shows the microbiological evolution of both Tarocco orange genotypes during storage.

None of the experimental samples of the Tarocco Gallo slices had microbiological values that exceeded the microbiological criteria (maximal level of 7.5 log CFU/g) recognized for minimally processed fruits and vegetables (CNERNA-CNRS, 1996). In the three different modified atmospheres, the highest microbiological values for the Tarocco Gallo slices were always lower than 4.0 log CFU/g, and the highest value was observed for mesophilic aerobes (MVC) that were cultured on PCA and incubated at 32°C (on the 9th day of storage with mixture 3). A different evolution of the bacterial growth in Tarocco Scirè slices packaged with the three mixtures was observed. Microbiological analyses of orange slices packaged with mixture 1, especially for AMC and MVC, showed high values (up to 6.0 log CFU/g) at 6th day and 9th day of storage, respectively. However, the highest values for all detected microbial groups were observed at 9th day of storage. A similar trend was also noted for the product packaged with mixture 3 (i.e., yeasts and molds had values up to 6.0 log CFU/g). Slices packaged with mixture 2 had an increased microbiological value up to the 3th day of storage, which was followed by a decrease from the 3th day to the 6th day and then another increase during the last days of storage (values reached 7.5 log CFU/g for all the considered microbial groups).

Compared with the normal air atmosphere, the microbial results obtained from the slices of Tarocco Gallo and Tarocco Scirè slices packaged in modified atmosphere did not demonstrate an increase of product stability. Previous studies have shown that Tarocco slices packaged in normal air with three different films have lower CFU/g values for all detected microbial groups than the values established by CNERNA-CNRS (1996), both at the production day and last day of storage (RAPISARDA *et al.*, 2006a).

CONCLUSIONS

The present study found that sliced oranges obtained from two Tarocco genotypes packaged in modified atmosphere with three different gas mixtures did not show marked physicochemical changes during 15 ds of storage. The low oxygen percentages of the three gas mixtures may have contributed to the maintenance of vitamin C (ascorbic acid) and anthocyanin concentrations. Moreover, the high acidity and maintenance of vitamin C content prevented enzymatic browning development and marked changes in the color of the slices. The sensory descriptors and estimated microbial group evolution during storage at 4°C

Table 6 - Microbiological evolution during storage of Tarocco 'Gallo' and 'Scirè' slices packaged under modified atmosphere with three different gas mixtures (log CFU/g).

	Gas mixture 1 Days					Gas mixture 2 Days					Gas mixture 3 Days				
	0	3	6	9	12	0	3	6	9	12	0	3	6	9	12
						Tarocco 'Gallo'									
PVC[a]	0.00	3.14	1.73	2.00	2.00	0.00	2.91	2.43	2.00	2.00	0.00	2.70	1.53	2.00	2.00
MVC[a]	0.00	3.72	2.03	2.60	2.00	0.00	2.82	2.56	2.85	2.00	0.00	3.06	2.40	4.00	2.60
AABC[a]	0.00	2.76	2.37	3.92	2.00	0.00	2.79	2.52	2.00	2.00	0.00	2.15	1.43	2.00	2.00
YMC[a]	0.00	2.80	1.87	2.00	2.00	0.00	2.93	2.32	2.00	2.00	0.00	2.06	1.01	2.00	2.60
AMC[a]	0.00	3.72	2.28	2.00	2.60	0.00	2.97	2.97	2.00	2.00	0.00	3.07	1.53	2.00	2.00
						Tarocco 'Scirè'									
PVC[a]	0.00	2.31	4.00	4.61	3.01	0.00	3.00	0.42	3.53	7.52	0.00	1.48	3.92	4.90	5.60
MVC[a]	0.00	2.26	5.52	6.57	3.14	0.00	2.69	1.73	3.31	7.53	0.00	1.33	4.52	5.12	2.60
AABC[a]	0.00	2.38	3.83	4.37	4.14	0.00	3.00	2.56	3.01	7.53	0.00	1.22	3.73	5.05	3.52
YMC[a]	0.00	2.25	4.53	4.17	2.00	0.00	3.19	1.56	2.00	7.53	0.00	1.78	5.52	6.07	2.00
AMC[a]	0.00	2.48	6.52	6.82	3.32	0.00	3.04	1.52	3.37	7.53	0.00	1.49	4.52	6.53	2.00

[a]PVC, psychotropic viable count; MVC, mesophilic viable count; AABC, acetic acid bacteria count; YMC, yeast and mold count; AMC, acidophilic microorganisms count.

revealed that Gallo was the best Tarocco genotype to be processed as a fresh-cut product. Slices of Tarocco Gallo oranges maintained good freshness, juiciness and firmness during storage. In addition, no development of off-flavors occurred, and the growth of microorganisms did not exceed acceptable limits (CNERNA-CNRS, 1996). In contrast, the Tarocco Scirè slices spoiled faster than the Gallo slices under the same conditions. The results of the present study suggest that the shelf-life of Tarocco Gallo slices is 12 ds, whereas Tarocco Scirè slices are not considered acceptable after 5 to 6 ds of storage.

REFERENCES

Ahvenainen R. 2000. Minimal processing of fresh produce. In: "Minimally processed fruits and vegetables". S.M. Alzamora, M.S. Tapia and A. Lopez-Mallo (Ed), p. 277. ASPEN Publishers Inc. Gaithersburg, Maryland.

Bennik M.H.J., Van Overbeek W., Smid E.J. and Gorris L.G. 1998. The influence of oxygen and carbon dioxide on the growth of prevalent Enterobacteriaceae and Pseudomonas species isolated from fresh and controlled atmosphere-stored vegetables. Food Microbiol. 15: 459.

Catalano A.E., Ingallinera B., Todaro A., Rapisarda P. and Spagna G. 2009. Degradative enzymatic activities in fresh-cut blood orange slices during chilled-storage. Int. J. Food Sci. Technol. 44: 1041.

CNERNA-CNRS. 1996. Products de la IV gamme. In: "La qualité microbiologique des aliments (maitris et critères)". Jouve P. Ed Polytechnica, Paris, France.

Del Caro A., Piga A., Vacca V. and Agabbio M. 2004. Changes of flavonoids, vitamin C and antioxidant capacity in minimally processed citrus segments and juices during storage. Food Chem. 84: 99.

Dorantes-Alvarez L. and Chiralt A. 2000. Color of minimally processed fruits and vegetables as affected by some chemical and biochemical changes. In: "Minimally processed fruits and vegetables". S.M. Alzamora, M.S. Tapia and A. Lopez-Mallo (Ed), p. 111. ASPEN Publishers Inc. Gaithersburg, Maryland.

Faber J.M. 1992. Microbiological aspects of modified atmosphere packaging technology: a review. J. Food Protect. 54: 58.

Fonseca S.C., Oliveira F.A.R., Lino I.B.M. and Brecth J.K. 2002. Modelling respiration rate of fresh fruits and vegetables for modified atmosphere packages: a review. J. Food Eng. 52: 99.

Fonseca S.C., Oliveira F.A.R., Lino I.B.M., Brecth J.K. and Chau K.V. 2000. Modelling O_2 and CO_2 exchange for development of perforation-mediated modified atmosphere packaging. J. Food Eng. 43: 9.

Francis G.A., Thomas C. and O'Beirne D. 1999. The microbiological safety of minimally processed vegetables. Int. J. Food Sci. Technol. 34: 1.

Huxson C.C. and Bolin H.R. 1989. Processing and distribution alternatives for minimally processed fruit and vegetables. Food Technol. 43, 124-128.

ISO 13299. 2003. Sensory analysis - Methodology - General guidance for establishing a sensory profile.

ISO 8586-1. 1993. Sensory analysis - General guidance for the selection, training and monitoring of assessors - Part 1: Selected assessors.

Jacxsens L., Devlieghere F. and Debevere J. 1999. Validation of a systematic approach to design equilibrium modified atmosphere packages for fresh-cut produce. LWT-Food Sci. Technol. 32: 425.

Kader A.A. 1986. Biochemical and physiological basis for effects of controlled and modified atmospheres on fruits and vegetables. Food Technol. 40: 99.

Kimball D. 1991. Citrus Processing. Quality Control and Technology. AVI Book, New York.

MAF. 1988. Metodi ufficiali d'analisi per le conserve vegetali (Official Analysis Methods for Plant Preserves). Parte generale, D.M.

Moleyar V. and Narasimham P. 1994. Modified atmosphere packaging of vegetables: an appraisal. J. Food Sci. Technol. 31: 267.

Pao S. and Petracek P.D. 1997. Shelf-life extension of peeled oranges by citric acid treatment. Food Microbiol. 14: 485.

Pao S., Widmer W.W. and Petracek P.D. 1997. Effects of cutting on juice leakage, microbiological stability and bitter substances of peeled citrus. Lebensm. Wiss. u.Technol. 30: 670.

Pretel M.T., Fernández P.S., Romojaro F. and Martínez A. 1998. The effect of modified atmosphere packaging on 'ready-to-eat' oranges. LWT-Food Sci. Technol. 31: 322.

Pretel M.T., Lozano P., Riquelme F. and Romojaro F. 1997. Pectic enzymes in fresh fruit processing: optimization of enzymic peeling of oranges. Proc. Biochem. 32: 43.

Rapisarda P. and Intelisano S. 1996. Sample preparation for vitamin C analysis of pigmented orange juice. Ital. J. Food Sci. 3: 251.

Rapisarda P. and Russo G. 2000. Fruit quality of five "Tarocco" selections grown in Italy. Proc. Intl. Soc. Citriculture IX: 1149.

Rapisarda P., Bellomo S.E. and Intrigliolo F. 2001. Anthocyanins in blood oranges: composition and biological activity. In: Recent Research Developments in Agricultural and Food Chemistry. S.G. Pandalai (Ed.), Vol. 5, p. 217. Research Signpost, Trivandrum, India.

Rapisarda P., Bellomo S.E., Pannuzzo P., Caggia C., Lanza C.M. and Restuccia C. 2004. Minimally processed blood oranges: physicochemical, microbiological and sensory evaluation of four 'Tarocco' clones slices. Proc. Intl. Soc. Citriculture III: 1149.

Rapisarda P., Caggia C., Lanza C.M., Bellomo S.E., Pannuzzo P. and Restuccia C. 2006a. Physicochemical, microbiological and sensory evaluation of minimally processed Tarocco clone oranges with 3 different permeability films. J. Food Sci.71: 299.

Rapisarda P., Fanella F. and Maccarone E. 2000. Reliability of analytical method for determining anthocyanins in blood orange juice. J. Agric. Food Chem. 48: 2249.

Rapisarda P., Pannuzzo P., Timpanaro N. and Lo Bianco M. 2006b. Changes in polyphenol, vitamin C content and antioxidant capacity in minimally processed Tarocco orange fruit. Special Issue Ital. J. Food Sci., 439.

Rocha A.M.C.N., Brochado C.M., Kirby R. and Morais A.M.M.B. 1995. Shelf-life of chilled cut orange determined by sensory quality. Food Control 6: 317.

Rojas-Graü M.A., Oms-Oliu G., Soliva-Fortuny R. and Martin-Belloso O. 2009. The use of packaging techniques to maintain freshness in fresh-cut fruits and vegetables: a review. Int. J. Food Sci. Technol. 44: 875.

Saltveit M.E. 2003. Is it possible to find an optimal controlled atmosphere? Postharvest Biol. Technol. 27: 3.

Sandhya. 2010. Modified atmosphere of fresh produce: current status and future needs. LWT- Food Sci. Technol. 43: 381.

UNI ISO 8589. 1990. Analisi sensoriale – Criteri generali per la progettazione di locali destinati all'analisi sensoriale.

UNI ISO/WD 4121. 2001. Analisi sensoriale – Linee guida per l'utilizzo di scale quantitative di risposta.

Watada A.E., Abe K. and Yamauchi N. 1990. Physiological activities of partially processed fruits and vegetables. Food Technol. 44: 116.

Zagory D. and Kader A.A. 1988. Modified atmosphere packaging of fresh produce. Food Technol. 42: 70.

Paper received March 16, 2011 Accepted September 19, 2011

PAPER

DETERMINATION OF CAPSAICINOIDS FROM DRIED PEPPER FRUITS BY FAST-GAS CHROMATOGRAPHY

M. BONONI and F. TATEO*
Dipartimento di Produzione Vegetale, Facoltà di Agraria, Università degli Studi di Milano,
Via Celoria 2, 20133 Milano, Italy
Corresponding author: Tel. +39 02 50316540, Fax +39 02 50316539,
email: fernando.tateo@unimi.it

ABSTRACT

The present paper describes the application of Fast-gas chromatography (GC) to quantify the capsaicinoid content in dried pepper fruit with the aim to calculate the Scoville heat values (SHVs) for routine quality control. Fast-GC was used to analyse various samples of dried pepper fruits with a wide distribution of SHVs. The calibration curves demonstrated a polynomial model in the range of 3.0-195.0 μg mL^{-1} for capsaicin and 2.6-105.0 μg mL^{-1} for dihydrocapsaicin and nordihydrocapsaicin, with correlation coefficients of 0.996 and 0.997, respectively. The data showed an intraday relative standard deviation (RSD) <5% and an interday relative repeatability standard deviation (RSDr) <6%.

- Key words: *Capsicum*, spice, fast-gas chromatography -

INTRODUCTION

Pepper fruits (*Capsicum* spp.) are among the most commonly consumed spices having significant pungency. They contain capsaicinoids, derivatives of phenylpropanoid compounds, which are largely responsible for the hot taste, pungency, and trade quality. Eleven capsaicinoids have been identified, including amide derivatives of vanillylamine and carboxylic acids with eight to eleven carbon atoms, some unsaturated (GARCÉS-CLAVER *et al.*, 2006; KOZUKUE *et al.*, 2005). The capsaicinoid content can be inferred by the heat value based on the Scoville organoleptic test, which was developed by the American Spice Trade Association (ASTA). However, the Scoville Heat Value (SHV) is not always reliable and various authors have attempted to replace this method with more accurate instrumental measurements of the capsaicinoid content. When evaluating pungency, capsaicin (*trans*-8-methyl-N vanillyl-6-nonenamide) and dihydrocapsaicin (8-methyl-N-vanillylnonanamide) constitute 90% or more of the total capsaicinoids, and they account for the majority of the "hotness" (LASKARIDOU-MONNERVILLE, 1999). Nevertheless, the AOAC Official Method 995.03 (AOAC, 1995) recommends calculating the total capsaicinoid content as the sum of capsaicin, dihydrocapsaicin, and nordihydrocapsaicin; then, the Scoville Heat Unit (SHU) for a given compound is determined by multiplying the total content of these capsaicinoids (expressed as $\mu g\ g^{-1}$ capsicum) by the pungency value for "pure capsaicin" (15 SHU). The SHV of the product is equal to the sum of the single SHU values. TODD *et al.* (1977) showed the capsaicinoid content could be converted to a more accurate SHU value by multiplying the content of each capsaicinoid by a coefficient: 16.1 for both capsaicin and dihydrocapsaicin, 9.3 for nordihydrocapsaicin, and lower coefficients for other capsaicinoids. The lowest coefficient was 6.9 for homocapsaicin.

Several varieties of *Capsicum* (Solanaceae family) have been distinguished and described; five are commonly recognised as domesticated: *Capsicum annuum* L. var. *Annuum*, *Capsicum baccatum* L. var. *Pendulum*, *Capsicum pubescens*, *Capsicum frutescens* L. (Cayenne pepper), and *Capsicum chinense* (PETER, 2001). Many different techniques are used to extract capsaicinoids from dried plant matrices, such as maceration (KIRSCHBAUM-TITZE *et al.*, 2002), magnetic stirring (CONTRERAS-PADILLA and YAHIA, 1998), enzymatic extraction (SANTAMARÍA *et al.*, 2000), a solvent and shake-flask extraction (Soxhlet) (KOREL *et al.*, 2002; KRAJEWSKA and POWERS, 1987), extraction by supercritical fluids (DAOOD *et al.*, 2002), extraction by pressurized liquids (BARBERO *et al.*, 2006a), and ultrasound assisted or microwave assisted extraction (BARBERO *et al.*, 2006a BARBERO *et al.*, 2008a; KARNKA *et al.*, 2002; NAZARI *et al.*, 2007; WILLIAMS *et al.*, 2004). In general, there is always demand for simple extraction techniques, reduced organic solvent consumption, and low toxicity solvents. In particular, the AOAC Official Method 995.03 (AOAC, 1995) suggests extraction with warm ethanol in a reflux condenser, liquid chromatography, and fluorimetric quantification. COLLINS *et al.* (1995) also suggested extraction with acetonitrile at reflux and high pressure liquid chromatography (HPLC) for quantification, but BARBERO *et al.* (2006b) studied the extraction of capsaicinoids with various solvents and found acetonitrile is a fairly efficacious solvent, but less so than ethanol and methanol.

In recent years, reverse phase HPLC was the most frequently used method for quantification of capsaicinoids (BARBERO *et al.*, 2008b; CHOI *et al.*, 2006; KAALE *et al.*, 2002; MAILLARD *et al.*, 1997; PERUCKA and OLESZEK, 2000; SARIA *et al.*, 1981; THOMPSON *et al.*, 2005; WEAVER and AWDE, 1986). HPLC followed by mass spectrometry (HPLC/MS) was proposed more recently (GARCÉS-CLAVER *et al.*, 2006; KOZUKUE *et al.*, 2005). However, some authors have suggested the use of gas chromatography (GC) followed by mass spectrometry (MS) (HAWER *et al.*, 1994; KRAJEWSHA and POWERS, 1987; KRAJEWSHA and POWERS, 1988; MANIRAKIZA *et al.*, 1999; THOMAS *et al.*, 1998) and a recent report described coupled GC/MS with solid phase microextraction (SPME) (PEÑA-ALVAREZ *et al.*, 2009).

This paper presents an alternative and simple method to detect and quantify capsaicin, dihydrocapsaicin, and nordihydrocapsaicin with a Fast-GC system, which is useful for routine analysis. This method has not previously been proposed for the instrumental quantification of capsaicinoids. The AOAC Official Method 995.03 was used as the extraction method. GC/MS was adopted to confirm the identity of capsaicinoids in the start-up phase. The Fast-GC application demonstrated good peak resolution and the run time was comparable with that of various conventional and rapid HPLC techniques and lower than that corresponding to other GC conditions.

MATERIALS AND METHODS

Samples

Ten samples of dried ground red pepper from different origins were collected from import operators of the Italian market.

Reagents

Analytical-grade ethyl alcohol (99.8%) was purchased from Fluka Chemika (Sigma Aldrich-Milan, Italy). Capsaicin (>97%) and dihydrocapsaicin (90%) were purchased from Sigma Aldrich (Milan, Italy). Stock solutions of these capsaici-

noids were prepared by weighing and dissolving each compound in ethyl alcohol. These solutions were stored at 4°C and used for preparing diluted working standard solutions in ethyl alcohol.

Instruments and programmes

GC/MS. GC/MS analyses were carried out with a Shimadzu 2010 gas chromatograph coupled to a Shimadzu 2010 MSD quadrupole mass spectrometer (Shimadzu, Italy). Helium was used as the carrier gas at a flow rate of 1 mL min^{-1}. Suitable identification of the analytes was achieved using an Equity™-5, a poly (5% diphenyl - 95% dimethylsiloxane) stationary phase column, 30 m × 0.25 mm i.d., 0.25 μm film thickness (Supelco-Milan, Italy). The oven temperature programme was 60°C (held for 1 min) to 240°C at a rate of 3°C min^{-1} (held for 5 min). The injector temperature was 230°C and the split injector mode (1:5) was used. The ion source and transfer line temperatures were 200° and 250°C, respectively. The MS operating conditions were: positive electron ionization mode (EI+) with automatic gain control, 70 eV of electron energy, and 250 mA of emission current. The mass spectrometer was operated in full scan mode from m/z 40 to 340. Capsaicin, dihydrocapsaicin, and nordihydrocapsaicin were identified by comparing the retention times and spectra of each capsaicinoid with those of standard compounds. The spectral data were obtained from the Di.Pro.Ve.-UNIMI homemade library.

Fast-GC. Fast-GC analyses were performed on a Shimadzu system composed of a GC 2010 gas chromatograph (Shimadzu Italia, Milan, Italy) equipped with a split/splitless injector, an autosampler AOC-20i, and a FID detector. Data acquisition was performed with GC Solution Software (Shimadzu Italia, Milan, Italy). Analyses were performed with a RTX®-5 (Crossbond® 5% diphenyl- 95% dimethyl polysiloxane) stationary phase column (Restek, Bellefonte, PA, USA). The column dimensions were 10 m × 0.10 mm i.d., 0.10 μm film thickness. The oven temperature programme was 180°C (held for 2 min) increased to 240°C at a rate of 8°C min^{-1}, then increased to 320°C at a rate of 14°C min^{-1} (held for 5 min). The pressure programme (336 kPa) was a constant linear velocity. The velocity of the carrier gas (H_2) was 78.5 cm sec^{-1}. The injector temperature was 280°C and the split mode (1:100) was used. The injection volume was 1 μL. The detector parameters (FID) were: T = 340°C; H_2, 40 mL min^{-1}; Air, 400 mL min^{-1}; Makeup (N_2), 30 mL min^{-1}; Base period, 8 ms; Filter time constant, 50 ms; Sampling rate, 8 ms.

Extraction of capsaicinoids

Data were collected to determine the usefulness of the instrumental response of Fast-GC for quantitative evaluation of capsaicinoids to calculate the SHVs for routine quality control. To prepare the extracts for Fast-GC evaluation, we used the AOAC Official Method 995.03 (1995). The filtered extract, produced by reflux of 25 g of ground or crushed red pepper with 200 mL of ethanol, was diluted 1:2 in ethyl alcohol prior to injection in Fast-GC.

Calibration and precision data

Once the Fast-GC procedure was optimised, calibration curves were created with standard solutions of the two standards of capsaicinoids commercially available, capsaicin and dihydrocapsaicin. Standard solutions were prepared in ethyl alcohol to cover concentrations up to 195 μg mL^{-1} for capsaicin (i.e.: 3.0, 6.1, 9.8, 24.4, 48.8, 97.5, 146.2, and 195.0 μg mL^{-1}) and up to 105 μg mL^{-1} for dihydrocapsaicin (i.e.: 2.6, 5.2, 13.1, 26.2, 52.5, 78.8, and 105.0 μg mL^{-1}). These concentration ranges were previously defined to be useful to calculate the content as mg kg^{-1} of considered capsaicinoids in dried matrices studied by the concentration data of the alcoholic extract. The previous ranges cover the possible concentration of the extracts deriving from natural matrices: the factor multiplicative 16, deriving from the extraction method parameters, permits to express the capsaicinoid content in the matrix as μg g^{-1} from the measured concentration μg mL^{-1} in the extract. Regression equations and correlation coefficients were calculated using Microsoft Office Excel 2007 and are reported in Figure 1. The standard distribution data demonstrated a polynomial model for both capsaicin and dihydrocapsaicin in the range 3.0 - 195.0 mg mL^{-1} for capsaicin and 2.6 - 105.0 mg mL^{-1} for dihydrocapsaicin in the extracts analysed by Fast-GC. The data were fit with an R^2 = 0.996 for capsaicin and R^2 = 0.997 for dihydrocapsaicin and nordihydrocapsaicin. The calibration curve of dihydrocapsaicin was also used for evaluating nordihydrocapsaicin, as conventionally adopted by others (BARBERO *et al.*, 2006b; ZIINO *et al.*, 2009).

The limit of detection (LOD) and limit of quantitation (LOQ) values were respectively: 1.2 and 3.5 μg mL^{-1} for capsaicin and 1.0 and 3.0 mg mL^{-1} for dihydrocapsaicin and nordihydrocapsaicin. The repeatability and reproducibility were studied in respect to the concentration of each capsaicinoid. The average intraday relative standard deviations (RSD%), calculated on five samples shown in Table 1, were 3.9, 4.2, and 4.3 for capsaicin, dihydrocapsaicin, and nordihydrocapsaicin, respectively. The average interday relative repeatability standard deviations (RSDr%), calculated on the ten samples shown in Table 1, were 5.4, 5.6, and 5.8 for the same three capsaicinoids. These data were determined with the NMKL procedure n.5 (NORDIC COMMITTEE ON FOOD ANALYSIS, 1997) for the estimation

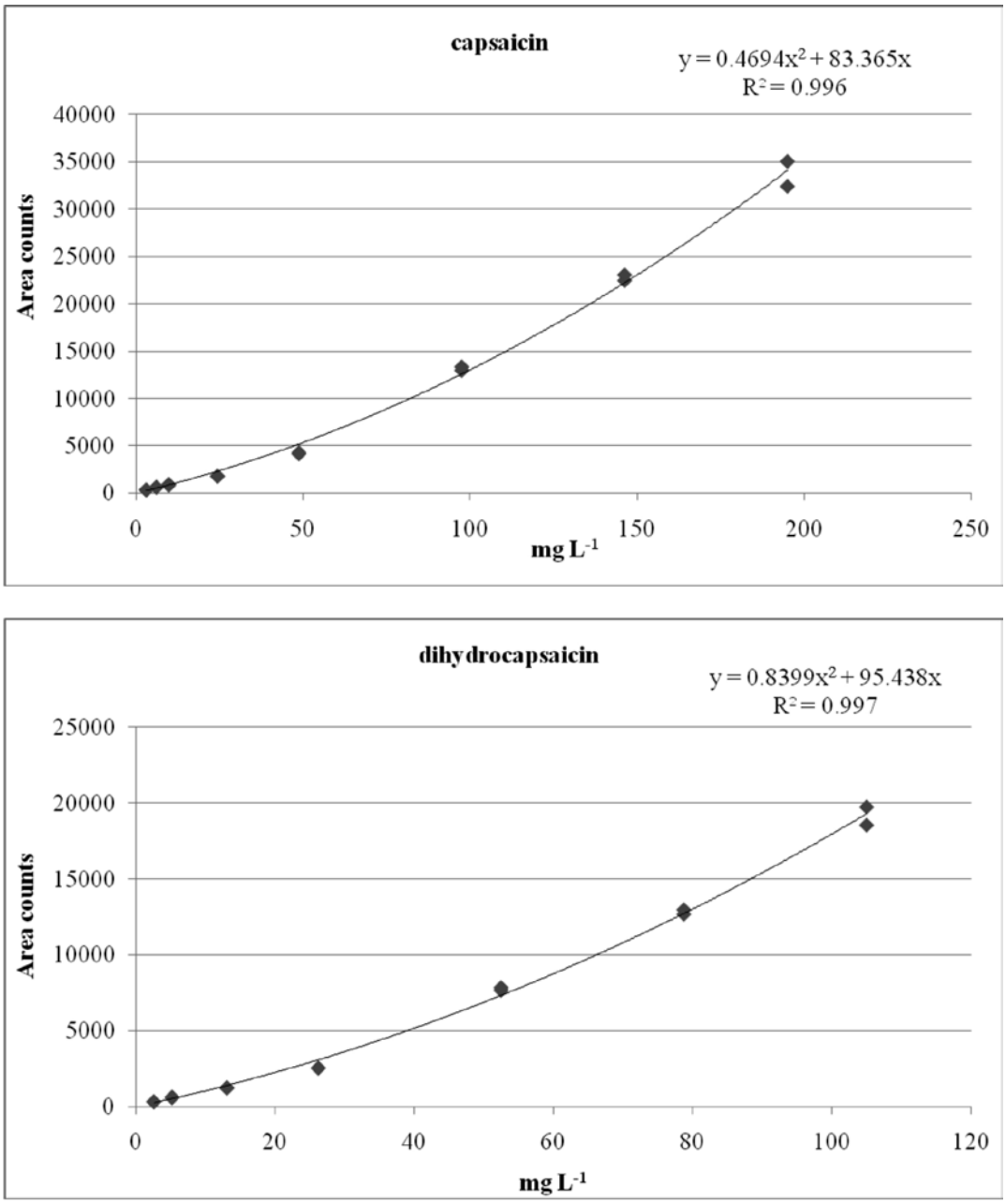


Fig. 1 - Calibration curves for capsaicinoids quantified with the Fast-GC method. The areas under the GC peaks were evaluated in counts (Y-axes) for each concentration of capsaicinoid (X-axes) in the extract. The data of both curves were fit (solid lines) with polynomial equations (upper right).

and expression of measurement uncertainty in chemical analysis.

Recovery data were estimated by spiking two samples (n. 4 and n. 5 in Table 1) having very different Scoville Heat Value, adding a calculated concentration of 500 mg kg^{-1} for capsaicin and 200 mg kg^{-1} for dihydrocapsaicin. The resulting recoveries were 98.6 and 97.7% respectively for capsaicin and dihydrocapsaicin for sample n.4 (mean of two determinations); the corresponding values for sample n.5 result 95,0 and 94,2%.

Statistical data performed by other GC methods cited in bibliography don't show to be improved in respect of the ones reported in this paper.

RESULTS AND DISCUSSION

The Fast-GC method was used for quality control measurements in one company that produces a primary brand in the retail spices market. Ten different samples of *Capsicum* were tested with standard conditions and showed a wide range of SHVs (Table 1). Fast-GC equipment permitted good resolution of the

Table 1 - Scoville heat values (mean of two determinations) obtained for 10 commercial samples of *Capsicum* (different market samples) under standard operative conditions optimised for the Fast-GC method. The results cover a wide range of SHV values (mean of two determinations).

Sample	Capsaicin (mg kg^{-1})	Dihydrocapsaicin (mg kg^{-1})	Nordihydrocapsaicin (mg kg^{-1})	Scoville Heat Value SHV
1	366.7	244.9	270.2	12,360
2	4095.4	1901.6	373.5	100,028
3	1195.7	908.3	185.7	35,602
4	3742.3	1354.0	347.5	85,282
5	1028.4	610.1	138.9	27,673
6	3848.1	1823.1	352.6	94,585
7	847.0	603.4	157.3	25,619
8	1161.0	557.0	141.0	28,970
9	1353.3	945.7	209.2	38,960
10	3632.5	1679.1	363.0	88,892

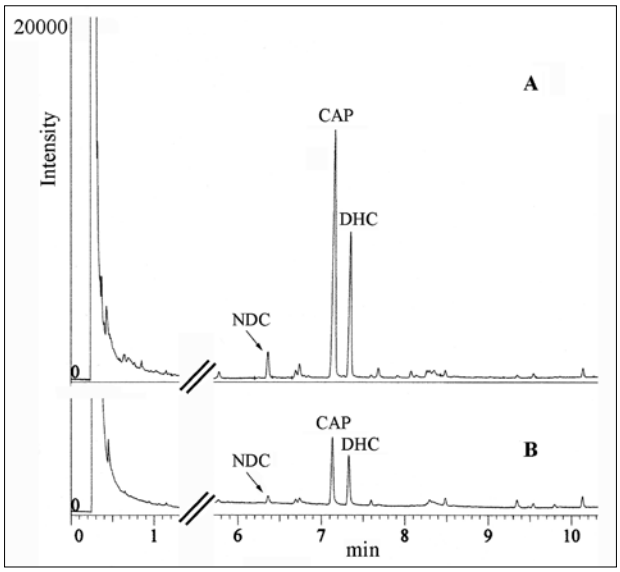


Fig. 2 - Fast-GC trace corresponding to the capsaicinoids, nordihydrocapsaicin (NDC), capsaicin (CAP), and dihydrocapsaicin (DHC), detected in pepper fruit samples. Samples 6 (A) and 7 (B) are characterised in Table 1. The SHV values are 94,585 and 25,619 for (A) and (B), respectively.

peaks of interest. Figure 2 shows two examples of resolution, A and B refer to samples 6 and 7, respectively, in Table 1 with SHVs of 94,585 and 25,619. Figure 2 shows that the identification and quantification of three capsaicinoids, using previously described standard operating conditions, were not difficult even for samples with very different SHVs. The primary advantage of Fast-GC quantitative determination was the rapidity of chromatographic elution. The time required for Fast-GC was comparable with the time required for other proposed HPLC methods, but shorter than that required by other currently proposed GC or GC/MS methods. These advantages were associated with RSD values below 5% and RSDr values below 6%.

CONCLUSION

We evaluated whether Fast-GC was useful for simultaneous analysis of three capsaicinoids commonly utilized for SHV determinations in pepper fruits. The results derived from this chromatographic system showed similar analysis times and good sensitivity and reproducibility compared to more frequently used HPLC-DAD methods. The Fast-GC technique, coupled to a proper extraction method, is particularly suitable for routine quality control. The total time of analysis depends on the preferred extraction technique but the time of Fast-GC analysis results very low (about 8 min) in comparison with the other corresponding GC chromatographicconditions.

REFERENCES

AOAC Official Method 995.03. 1995 Capsaicinoids in Capsicums and their extractives - Liquid Chromatographic Method. 43: 13-15.

Barbero G.F., Liazid A., Palma M. and Barroso C.G. 2008a. Ultrasound-assisted extraction of capsaicinoids from peppers. Talanta 75: 1332.

Barbero G.F., Liazid A., Palma M. and Barroso C.G. 2008b. Fast determination of capsaicinoids from peppers by high-performance liquid chromatography using a reversed phase monolithic column. Food Chem. 107: 1276.

Barbero G.F., Palma M. and Barroso C.G. 2006a. Pressurized Liquid Extraction of Capsaicinoids from Peppers. J. Agric. Food Chem. 54:3231

Barbero G.F., Palma M. and Barroso C.G. 2006b. Determination of capsaicinoids in pepper by microwave-assisted extraction-high-performance liquid chromatography with fluorescence detection. Anal. Chim. Acta, 578: 227.

Choi S.H., Suh B.S., Kozukue E., Kozukue N., Levin C.E. and Friedman M. 2006. Analysis of the Contents of Pungent Compounds in Fresh Korean Red Peppers and in Pepper-Containing Foods. J. Agr. Food Chem. 54:9024.

Collins M.D., Wasmund L.M. and Bosland P.W. 1995. Improved method for quantifying capsaicinoids in Capsicum using high-performance liquid chromatography. HortScience 30: 137.

Contreras-Padilla M. and Yahia E.M. 1998. Changes in capsaicinoids during development, maturation, and senescence of chile peppers and relation with peroxidase activity. J. Agr. Food Chem. 46:2057.

Daood H.G., Illés V. Gnayfeed M.H., Mészáros B., Horváth

G. and Biacs P.A. 2002. Extraction of pungent spice paprika by supercritical carbon dioxide and subcritical propane. J. Supercritic. Fluids 23:43.

Garcés-Claver A., Arnedo-Andreis M.S., Abadia J., Gil-Ortega R. and Alvarez-Fernandez A. 2006. Determination of capsaicin and dihydrocapsaicin in Capsicum fruits by liquid chromatography-electrospray/time-of-flight mass spectrometry. J. Agr. Food Chem. 54: 9303.

Hawer W.S., Ha J., Hwang J. and Nam Y. 1994. Effective separation and quantitative analysis of major heat principles in red pepper by capillary gas chromatography. Food Chem. 49: 99.

Kaale, E., Van Schepdael A., Roets E. and Hoogmartens J. 2002. Determination of capsaicinoids in topical cream by liquid- liquid extraction and liquid chromatography. J. Pharm. Biomed. Anal. 30: 1331.

Karnka R., Rayanakorn M., Watanesk S. and Vaneesorn Y. 2002. Optimization of high-performance liquid chromatographic parameters for the determination of capsaicinoid compounds using the simplex method.

Kirschbaum-Titze P., Hiepler C., Mueller-Seitz E and Petz M. 2002. Pungency in paprika (*Capsicum annuum*). 1. decrease of capsaicinoid content following cellular disruption. J. Agric. Food Chem. 50: 1260.

Korel F., Bağdatlioğlu N., Balaban M.Ö. and Hişil Y. 2002. Ground red peppers: capsaicinoids content, scoville scores, and discrimination by an electronic nose. J. Agric. Food Chem. 50: 3257.

Kozukue N., Han J.S., Kozukue E., Lee S.J., Kim J.A., Lee K.R., Levin C.E. and Friedman M. 2005. Analysis of eight capsaicinoids in peppers and pepper-containing foods by high-performance liquid chromatography and liquid chromatography-mass spectrometry. J. Agr. Food Chem. 53: 9172.

Krajewska A.M. and Powers J.J. 1987. Gas chromatography of methyl derivatives of naturally occurring capsaicinoids. J. Chromatogr. A. 409: 223.

Krajewska A.M. and Powers J.J. 1988. Pentafluorobenzylation of capsaicinoids for gas chromatography with electron-capture detection. J. Chromatogr. A., 457: 279.

Laskaridou-Monnerville A. 1999. Determination of capsaicin and dihydrocapsaicin by micellar electrokinetic capillary chromatography and its application tovarious species of *Capsicum*, Solanaceae. J. Chromatogr. A., 838: 293.

Maillard M.N., Giampaoli P. and Richard H.M.J. 1997. Analysis of eleven capsaicinoids by reversed-phase high performance liquid chromatography. Flavour Frag. J. 12: 409.

Manirakiza P., Covac A. and Schepens P. 1999. Solid-phase extraction and gas chromatography with mass spectrometric determination of capsaicin and some of its analogues from chili peppers (*Capsicum* spp.). J. AOAC Int. 82: 1399.

Nazari F., Ebrahimi, S.N., Talebi M., Rassouli A. and Bijanzadeh H.R. 2007. Multivariate optimization of microwave-assisted extraction of capsaicin from *Capsicum frutescens* L. and quantitative analysis by ^{1}H-NMR. Phytochem. Anal. 18:333.

Nordic Committee on Food Analysis (NMKL), 1997. Estimation and expression of measurement uncertainty in chemical analysis. NMKL Procedure No. 5.

Peña-Alvarez A., Ramirez-Maya E. and Alvarado-Suarez L.A. 2009. Analysis of capsaicin and dihydrocapsaicin in peppers and pepper sauces by solid phase microextraction-gas chromatography-mass spectrometry. J. Chromatogr. A. 1216: 2843.

Perucka I. and Oleszek W. 2000. Analytical, Nutritional and Clinical Methods Section Extraction and determination of capsaicinoids in fruit of hot pepper Capsicum annuum L. by spectrophotometry and high-performance liquid chromatography. Food Chem. 71: 287-291.

Peter K.V. 2001. Capsicum, Chillies, Paprika, Bird's Eye Chilli. Ch.8. In: "Handbook of Herbs and Spices, Vol. 1". K.V. Peter (Ed.) p. 111. Woodhead Publishing Limited, Boca Raton, FL.

Santamaría R.I., Reyes-Duarte M.D., Bárzana E., Fernando D., Gama F.M., Mota M., and López-Munguía A. 2000. Selective enzyme-mediated extraction of capsaicinoids and carotenoids from chili Guajillo Puya (*Capsicum annuum* L.) using ethanol as solvent. J. Agr. Food Chem. 48: 3063.

Saria A., Lembeck F. and Skofitsch G. 1981. Determination of capsaicin in tissue and separation of capsaicin analogues by high-performance liquid chromatography. J. Chromatogr. A. 208: 41-46.

Thomas B.V, Schreiber A.A. and Weisskopf C.P. 1998. Simple Method for Quantitation of Capsaicinoids in Peppers Using Capillary Gas Chromatography. J. Agric. Food Chem. 46: 2655.

Thompson R.Q., Phinney K.W., Sander L.C. and M.J. Welch. 2005. Reversed-phase liquid chromatography and argentation chromatography of the minor capsaicinoids. Anal. Bioanal. Chem. 381: 1432.

Todd P.H. Jr, Bensinger M.G. and Biftu T. 1977. Determination of pungency due to capsicum by gas-liquid chromatography. J. Food Sci. 42: 660.

Weaver K.M. and Awde D.B. 1986. Rapid high-performance liquid chromatographic method for the determination of very low capsaicin levels. J. Chrom. A. 367: 438.

Williams O.J., Viyaya-Raghavan G.S., Orsat V. and Dai J. 2004. Microwave-assisted extraction of capsaicinoids from capsicum fruit. J. Food Biochem. 28:113.

Ziino M., Condurso C., Romeo V., Tripodi G. and Verzera A. 2009. Volatile compounds and capsaicinoid content of fresh hot pepper (*Capsicum annum* L.) of different Calabrian varieties. J. Sci. Food Agr. 89: 774.

Paper received June 6, 2011 Accepted September 27, 2011

PAPER

ANTIOXIDANT ACTIVITY OF SESAME LIGNAN COMPOUNDS ON SOYBEAN OIL

M. BAHMAEI and H. PEYMAN
Department of Chemistry, Islamic Azad University, 9 Daftari Alley, Shariati Street, Tehran, Iran
Corresponding author: honey_peyman@yahoo.com

ABSTRACT

Sesame (*sesamum indicum L.*) seed and oil contain abundant lignans, including sesamol, sesamin, sesamolin and lignin glycosides. In this study we investigated the effects of extracted lignin compounds on soybean oil without antioxidant. Sesame cake was extracted with two different solvents (methanol and ethanol) to get a purified extract with higher antioxidant content and better activity. The qualitative analysis of antioxidant lignans present in the extract was carried out by reverse phase high performance liquid chromatography (HPLC) using a C_{18} column. Antioxidant activity of ethanolic extracts of sesame cake (EESC) and methanolic extract of sesame cake (MESC) was evaluated in soybean oil (SBO) using: Schaal Oven Test, Rancimat test. Results of peroxide value (PV) and *p*-anisidine value (p-AV) showed that EESC at concentrations of 1,500 and 2,000 mg/kg and MESC at concentration of 2,000 mg/kg in vegetable oils, could significantly (P <0.05) lower the peroxide value and *p*-anisidine value of oils during storage at 60°C. This is indicative of antioxidant activity in sesame cake extract (ESC) Butylated hydroxyl toluene (BHT) was used as control in this study.

- Key words: antioxidant activity, sesame cake extract, BHT, soybean oil, lignan -

INTRODUCTION

Lipid oxidation is an important chemical change that lowers the nutritional quality of food. The primary and secondary products of lipid oxidation are detrimental to health. In the body excess production of free radicals affects lipid cell membranes to produce lipid peroxides and reactive oxygen species (ROS) which lead to many biological changes such as DNA, protein and lipids damage, ageing, heart disease and cancer (1,2). Different environmental stress factors like pollution, drought, temperature, excessive light intensities, and nutritional limitation are able to increase the production of ROS, that have important roles in cell signaling and homeostasis(3). Sesame (*Sesamum indicum L.*) is an important source of edible oil. The oil shows remarkable stability despite being is highly unsaturated. Sesame seeds are used in confectionery and are considered in the orient, to be a health food (15). Lignans, and lignan glycosides, present in sesame seed, oil and cake, are responsible for the important properties of sesame**.** Sesame is one of the most important oilseed crops, cultivated in India, China and Iran. It is used in some traditional foods in Iran also.

The addition of antioxidants is a method for retarding the oxidation of lipids and lipid containing foods. Synthetic antioxidants such as BHT and BHA have restricted use in food as they are reported to be carcinogenic (4). In the present study, a by-product of sesame seed, containing unique antioxidant compounds such as sesamin, sesamolin and sesamol (called lignans) are studied as an antioxidant extract that can be used in the place of synthetic antioxidants in vegetable oils.

EXPERIMENTAL PROCEDURES

Materials

Seeds from Iranian variety were obtained from local markets in Darab, Bushehr. They were sealed in a bottle and stored at 4°C until used. Refined, bleached, deodorized (RBD) soybean oil, without antioxidant was supplied by Behshahr Industrial Company. p-Anisidine was purchased from Sigma. Sesamol (3,4 Methylendioxy phenol) was used as internal standard purchased from Merck. BHT was purchased from Jan Dekler company. All chemicals used were obtained from Sigma and Merck chemical company. All other regents were of analytical grade.

Extraction

Sesame seed was dried and powdered. One hundred grams of the sample was initially extracted with hexane (two times with a total of 300 mL of hexane) at room temperature. The defatted residue was washed with distilled water (two times with a total of 300 mL of water) to remove soluble sugars and proteins and dried below 70°C (7). 10 grams of the above purified residue was extracted with 150 mL ethanol and methanol with the Soxhlet method for 16 h. The extract was filtered, solvent removed under vacuum /N_2 flow to dryness, weighed and stored in a refrigerator until analyzed (6).

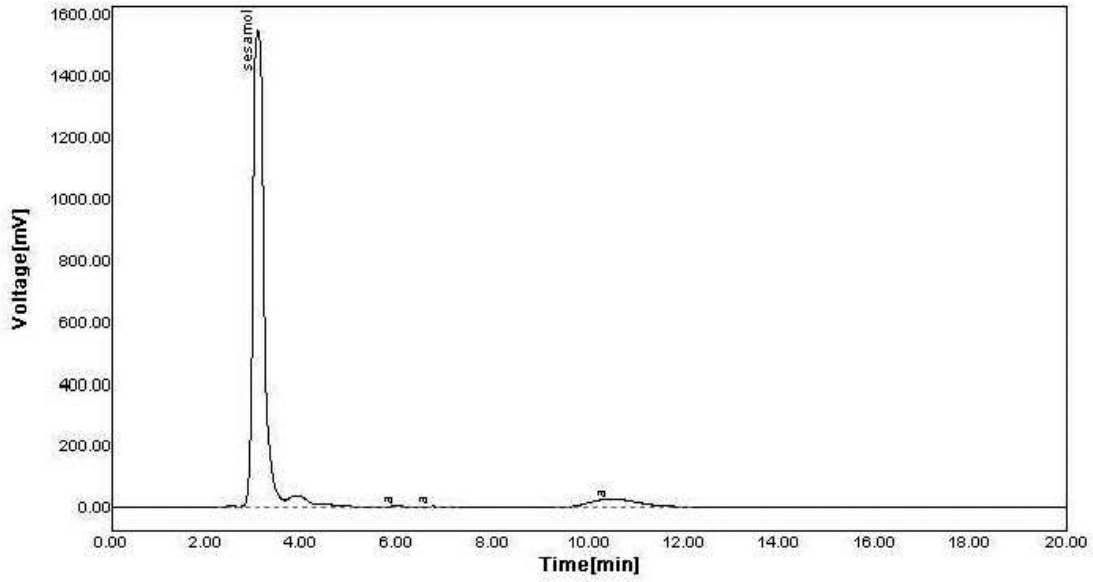


Fig. 1 - HPLC profile of Methanolic extract of sesame cake
a: other lignans (sesamin, sesamolin and lignin glycosides).

HPLC analysis of sesame cake extra

HPLC analysis was carried out in a Young Lin SP930D pump, CTS30 model oven fitted with a 20 μL sample loop, with 730D UV-Visible detector. The analysis was carried out using a Waters μ-bondapack C_{18} column (4.6 mm ID × 300 mm) in the reversed phase with a C_{18} guard column. The mobile phase used was methanol: water (70:30) with a flow rate of 1 mL/min. The UV-Visible detector was set at 288 nm. The extract was filtered through PTFE membrane before injection into HPLC. The quantification was done with the external standard method (7, 17). Standard sesamol was dissolved in methanol and solutions of different concentrations in the range of 500-1,000 mg/kg were prepared and injected into HPLC to check the linearity between concentration and peak areas. The values of sesamol are according to Bailey (Bailey's Industrial oil & fat products, 1996).

Oil storage studies

Schaal oven test was conducted to evaluate the effect of antioxidants against oxidation during the accelerated storage of oils. The storage test was carried out on soybean oil. Refined, bleached, deodorized soybean oil, without any added synthetic antioxidant supplied by soybean oil manufacture, was used for storage studies. Oil samples were stored at 60°C for a definite period in oven. Sesame cake extracts, at different concentrations (500, 1,000, 1,500, 2,000 mg/kg based on extract weight) were partly dried under nitrogen and then we weighed for example 2,000 mg/kg of extracts and added to 50 g of antioxidant free oil and mixed them under few temperature. Experiments were also carried out with synthetic antioxidants such as BHT at 75ppm level, and a control sample. The samples were analyzed after 3, 6, 9, 12, 15, 18, 21, 24 days for peroxide value and p-anisidine value to follow the oxidative changes (7).

Rancimat test

A rancimat instrument, model 743 from Metrohm was used in this study. The tests were carried out with 2.5 g of oil at 110°C and with an air flow of 20 L/h. The volatile degradation products were trapped in distilled water, increasing the water conductivity. The oxidative stability index was the time necessary to reach curve inflection point (AOCS Method number: cd 12b-92).

Analyses of soybean oil before oxidation

Chemical analyses

The Official Method of American Oil Chemists· society (AOCS) was used for determination of peroxide value (PV, method cd 8b-90), para anisidine value (p-AV, method Cd18-90), Karl Fisher titration (Ca2e-84), iodine value (Cd1-25) and free fatty acid content (Ca5a-40).

Gas chromatographic analysis

The fatty acid compositions of the oils were determined by gas chromatography (GC). After extraction of methyl esters into hexane (AOCS Method number Ce 1k-09), they were analyzed using a Agilent 6890A gas chromatograph (G15330A) SGE with a 120 m ×0.25 mm column (BPX70) with a flame ionization detector. Quantification was performed by computer control using area normalization.

Atomic absorption spectroscopy

A flame atomic absorption spectrometer (Avanta ε) was used with a hollow cathode lamp (GBC) for determining Cu-Fe-Pb-As in soybean oil. The experimental conditions and instrumental settings were used as recommended by the manufacturer. The bandwidth was set to 0.5 nm. The flame gases were air/acetylene the lamp current for As, Fe, Sb and Cu was 8, 7,5 and 5 mA respectively. The fuel flow rate for As was 1.25min^{-1} and for Sb, Cu and Fe was 2 min^{-1}.

Statistical analysis

All the experiments were carried out three times and analysis variance test were performed on statistical analysis system (IBM SPSS statistics version 19) at the level of $p<0.05$ and evaluated significant differences between means by Wilcoxon Signed Rank Test (NPar Tests).

RESULTS AND DISCUSSION

General

The oxidative stability studies were carried out at 60°C. Determination of PV of oils oxidized at 100°C is unrealiable because hydroperoxides decompose at elevated temperature. Therefore, the antioxidant efficacies of sesame cake extracts in soybean oil were evaluated at 60°C. Figure 1 shows the presence of sesamol in EESC and MSSC measured with HPLC.The sesamol content of the ethanolic extract is 681.14 + 15.31 mg/kg and the sesamol content of the methanolic extract is 714.6 + 10.21 mg/kg the other peaks indicate the rest of the lignans.

Many factors can accelerate oxidation, such as peroxide, free fatty acid, iodine value, moisture, metals. Therefore before the oxidation we measured these factors in soybean oil. The analysis showed 0.6 meq/kg peroxide, 0.08% acidity, 129.92 iodine value, 0.05% moisture, 0.2444

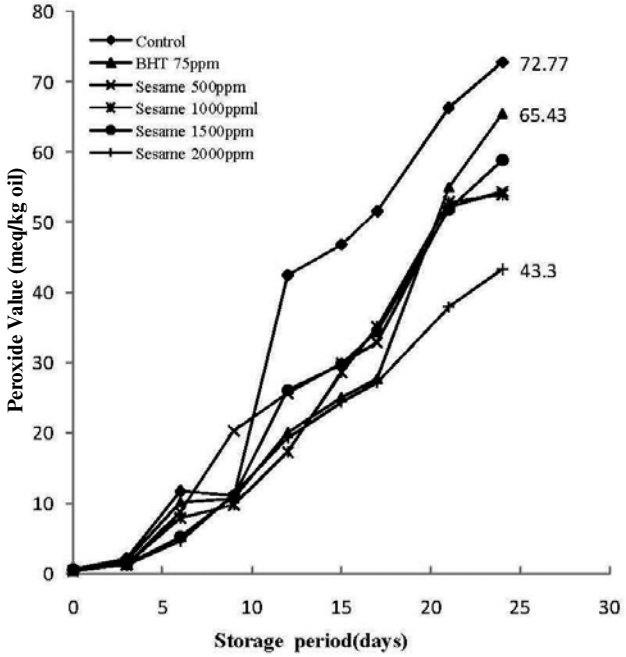


Fig. 2 - Changes of peroxide value (meq/kg) of soybean oil with MESC stored at 60°C for 24 days.

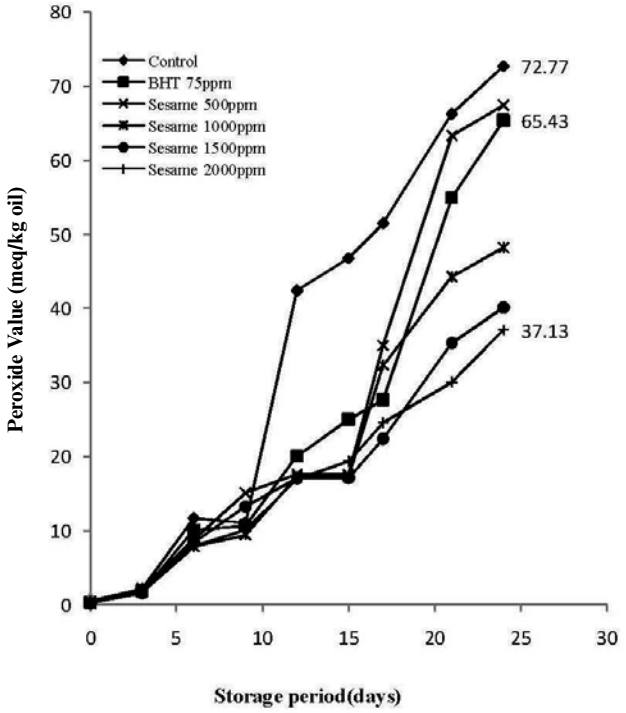


Fig. 3 - Changes of peroxide value (meq/kg) of soybean oil with EESC stored at 60°C for 24 days.

mg/kg Fe, 0.14 mg/kg Cu and the amount of Sb and As were ND.

Free acid composition

Before the oxidation we measured the fatty acids of soybean oil without antioxidant. In general all lipids groups had Oleic and Linoleic acids as the most predominant among the unsaturated fatty acids (C18:1:23.06+1.40, C18:2:54.71+1.26), and Palmitic and Stearic acids as the most predominant among the saturated fatty acids (C16:0:10.28+0.10, C18:0:4.45+0.06).

Peroxide value (PV)

The primary products of lipid oxidation are hydroperoxides which are generally referred to as peroxides. Therefore, the determination of peroxide value can be used as an oxidative index of lipid oxidation. We determined PV by means of an Automatic Potentiometric Titrator (Metrohm 751 GPD). The oxidative stability of EESC and MESC were studied by the accelerated storage method of Schaal (at 65°C for 24 days). Figures 3 and 4 show PV developments during storage of soybean oil (SBO) with various concentrations of MESC and EESC. SBO without the antioxidant (control) reached a maximum PV of 72.77 meq/kg after 24 days of storage. A significant difference ($P < 0.05$) in PV was observed between the control, SBO containing methanolic and ethanolic sesame cake extract and BHT which showed the rate of peroxide formation. The PV of SBO samples with 500, 1,000, 1,500, 2,000 mg/kg of MESC and 500, 1,000, 1,500, 2,000 mg/kg of EESC and 75 mg/kg of BHT were 54.38, 53.92, 58.86,43.30, 67.44, 48.23, 40.15, 37.13, and 65.43 respectively after 24 days. These results indicate that the antioxidant effects of MESC and EESC at 1,500 and 2,000 mg/kg are better than BHT at 75 mg/kg.

The antioxidant effect of EESC is seen to be better than MESC, because there is a significant difference between BHT and ESSC at 1,500 and 2,000 mg/kg, whereas for MSSC there is just a significant difference at 2,000 mg/kg. Therefore we can apply ESSC in lower concentration than MSSC.

p-Anisidine Value (p-AV)

The degree of secondary oxidation is usually reflected by the changes in p-anisidine value. Changes in p-Anisidine value are shown in Figures 3 and 4. The formation of secondary oxidation products also increased during storage. The p-anisidine value of the control reached a maxium of 29.25 after 24 days of storage. The p-AV of MESC at 500, 1,000, 1,500, 2,000 mg/kg, and EESC at 500, 1,000, 1,500, 2,000 mg/kg and BHT at 75 mg/kg were 15.23, 14.12, 14.10, 14.02, 18.87, 18.10, 15.25, 10.70 and 20.89 respectively. A significant difference was noted between the p-A values for control and experimental samples. EESC and MESC are more effective at 1,500, and 2,000 mg/kg, and the effects are more than that of the synthetic antioxidant BHT at 75 mg/kg. The results of the present study indicate the presence of com-

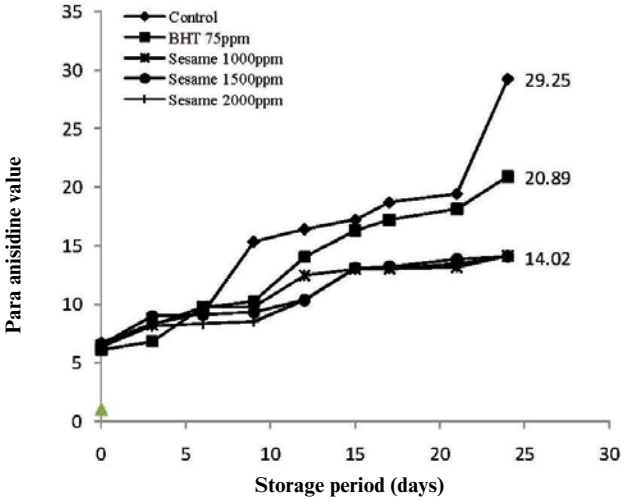


Fig. 4 - Changes of p-anisidine value of soybean oil with MESC stored at 60°C for 24 days.

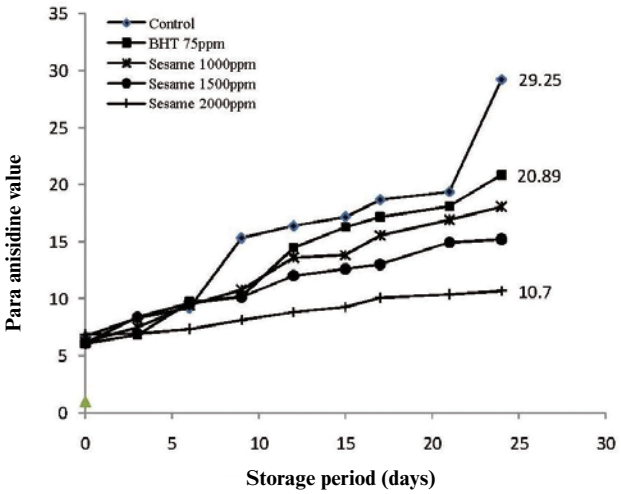


Fig. 5 - Changes of p-anisidine value of soybean oil with EESC stored at 60°C for 24 days.

pounds with high antioxidant activity in sesame cake extract.

Rancimat

Table 1 shows the results of Rancimat analysis. These results also show that MESC at concentration of 2,000 mg/kg and EESC at concentrations of 1,500 and 2,000 mg/kg are more effective than BHT at 75 mg/kg after 24 days. Even between extracts, ethanol extract of sesame cake proved to be better than methanol extract.

CONCLUSIONS

The effectiveness of two solvents on extraction of sesamol and lignin content from sesame cake was studied. Both ethanol and methanol solvents were used for Soxhlet extraction. Ethanol was found to be more efficient for extracting the antioxidant compounds, especially sesamol and the lignans.

In this study we used higher concentrations of extract and obtained results comparable with that of BHT. The results of our study demonstrated that sesame cake extract could be used as a substitute for synthetic antioxidants to protect vegetable oils such as soybean oil. The protection offered by the sesame cake extract at 2,000 mg/kg is comparable with, or in some cases better than, that of the widely used synthetic antioxidant BHT at 75 mg/kg and thought at much higher concentrations, sesame extracts could be a suitable for BHT, being safe and without any hazard.

ACKNOWLEDGMENTS

We are grateful to the Oil seed Research and Development company (ORDC) of Iran for financial support.

REFERENCES

1) Cosgrove J.P., Church D.F and Pryor W.A. 1987. The kinetics of the oxidation of polyunsaturated fatty acids. Lipids, 22: 229-304.
2) Labuza T.P. 1971. Kinetics of lipid oxidation in food. CRC Critical Review of food Technology, 2: 355-405.
3) Arora A., Sairam R.K and Srivastave G.C. 2002. Oxidation stress and oxidative systems in plants. Curr. Sci., 82: 1227-1238.
4) Ito N., Fukushima S. and Tsuda. 1986. Carcinogenicity

Table 1 - Results of rancimat with different concentration at 110°C and air flow of 20 L/h.

Blank	BHT at 75 ppm	MESC at 500 ppm	MESC at 1,000 ppm	MESC at 1,500 ppm	MSSC at 2,000 ppm	EESC at 500 ppm	EESC at 1,000 ppm	EESC at 1,500 ppm	EESC at 2,000 ppm
7.93 h	8.13 h	7.89 h	7.96 h	8.06 h	8.85 h	7.95 h	8.04 h	8.38 h	9.53 h

MESC: methanolic extract of sesame cake;
EESC: ethanolic extract of sesame cake;
BHT: Butylated hydroxyl toluene.

and modification of the carcinogenic response by BHA, BHT and other antioxidants. CRC Critical Reviews in Toxicology, 5: 109-150.

5) Koleva I.I., Van Beek T.A., Linssen J.P.H., de Groot A. and Evstatieva L.N. 2002. Screening of plant extracts for antioxidant activity: a comparative study on three testing methods. Phytochem. Analysis,13:8-17.

6) Suja K.P., Jayalekshmy A. and Arumughary C. 2005. Antioxidant activity of sesame cake extract. Food Chemistry, 9: 213-219.

7) Suja K.P., Abraham John T., Thamizh Selvam N., Jayalekshmy A. and Arumughan C. 2004. Antioxidant efficacy of sesame cake extract in vegetable oil protection. Food Chemistry, 84:393-400.

8) Hassel R.L. 1976. Thermal analysis, An alternative method of measuring oil stability. Journal of the American Chemical Society, 53: 179-181.

9) Simon P., Kolman L., Niklova I. and Schmidt S. 2000. Analysis of induction period of oxidation of edible oils by differential scanning calorimetry. Ibid., 77: 639-642.

10) Hemalatha S. and Ghafoorunissa. 2007. Sesame lignans enhance the thermal stability of edible vegetable oils. Food Chemistry, 105: 1076-1085.

11) Lee W.C., Wen J.Y., Shiow C.H. and Pin D.D. 2002. Antioxidant activity of sesame coat. Food Chemistry, 78: 347-354.

12) Jinyoung L., Yoosung L. and Eunok C. 2008. Effects of sesamol, sesamin, and sesamolin extracted from roasted sesame oil on the thermal oxidation of methyl linoleate. Food Science and Technology, 41: 1871-1875.

13) Hany A.A.G., A.Adel.Y Shehata and Fereidoon S. 2000. Effect of processing on oxidative stability and lipid classes of sesame oil. Food Research International, 33: 331-340.

14) Om P. Sharma and Tej K. Bhat. 2009. DPPH antioxidant assay revisited. Food Chemistry, 113: 1202-1205.

15) Namiki M. 1995. The chemistry and physiological functions of sesame. Food Reviews International, 11: 281-329.

16) Nuchanart R., Nanthanit P. and Chulabhorn M. 2010. Variation of sesamin, sesamolin and tocopherols in sesame seed and oil products in Thailand. Food chemistry, 122: 724-730.

Paper received June 21, 2011 Accepted September 27, 2011

SHORT COMMUNICATION

THE STABILITY OF FROZEN MINCED AFRICAN CATFISH

J.P. DURÃES[1], P.R.C. OLIVEIRA FILHO[1], J.C.C. BALIEIRO[2], C.R. DEL CARRATORE[3] and E.M.M. VIEGAS[1]*

[1]Department of Animal Science, Faculty of Animal Science and Food Engineering, University of São Paulo, Av. Duque de Caxias Norte 225, 13635-900 Pirassununga, SP, Brazil

[2]Department of Basic Science, Faculty of Animal Science and Food Engineering, University of São Paulo, Av. Duque de Caxias Norte 225, 13635-900 Pirassununga, SP, Brazil

[3]Department of Animal Science, University of Marília, Av. Higyno Muzzi Filho 1001, 17.525 Marília, SP, Brazil

*Corresponding author: emviegas@usp.br

ABSTRACT

The objective of the present study was to evaluate the stability (-18°C) of minced African catfish (MF) for 180 days. Microbiological aspects, lipid oxidation, and total volatile nitrogenous bases (TVB-N) of the MF were determined with and without washing. Washing of the MF caused an increase in moisture content, decrease in proteins, and leaching of the compounds responsible for lipid oxidation. The TVB-N remained stable during the storage period. The microbiological parameters of the MF remained within the legal limits. Thus, a 180-days storage period does not affect the quality of MF and could be a good alternative for the exploitation of this species in Brazil.

- Key words: minced fish, *Clarias gariepinus*, fishburger, stability, fish technology -

INTRODUCTION

The African catfish (*Clarias gariepinus*) belongs to the order Siluriformes of the family *Clariidae*, with a geographical distribution in Asia Minor to the south of Africa (RIBEIRO and PAVANELLI, 2001). It is a non-scaled, robust fish with an elongated body, a slightly flattened head, and the ability to breathe out of the water (OZÓRIO *et al.*, 2004). It shows omnivorous feeding habits and inhabits riverbeds and turbid lakes. Its meat shows good protein and mineral content and low saturated fat (ERSOY and OZEREN, 2009). In 2005, Brazil produced 224 tons of this fish. The State of Espirito Santo was the largest producer (115 tons), followed by Santa Catarina (97 tons) and Rio de Janeiro (12 tons) (IBAMA, 2007). In Brazil, industrial processing has involved obtaining frozen and/or smoked fillets, the latter being more appreciated by the European Market. However, new processing methods should be evaluated to better exploit this raw material.

Minced fish (MF) is a product obtained from a single fish species or mixture of fish species with similar sensory characteristics using a mechanized process to separate the edible parts, producing skeletal muscle particles absent of viscera, scales, bones, and skin (TENUTA-FILHO and JESUS, 2003). Residues from fish filleting and under-used species can be used to produce MF for use in the preparation of sausages (OLIVEIRA FILHO *et al.*, 2010; OLIVEIRA FILHO *et al.*, 2011), nuggets (KIRSCHNIK and VIEGAS, 2009), and fishburgers. According to MORAIS and MARTINS (1981), the process for obtaining MF has the potential for a greater recovery of meat compared to that obtained from the traditional methods of filleting, decreasing production costs and the amounts of residues generated while allowing for diverse products from the fisheries industry. However, the process of obtaining MF causes breakage of the muscular integrity of the fish, allowing for close contact between the cell components and environmental oxygen (RODRÍGUEZ-HERRERA *et al.*, 2006). This can lead to lipid oxidation, which affects the color, flavor, and texture of the MF, thus reducing its consumer acceptance (BENJAKUL *et al.*, 2005). One of the ways used to reduce this undesirable characteristic is by washing the MF, which removes pigments, soluble proteins, enzymes and part of the lipids and flavor components. This increases the stability and improves the sensory aspects of color and odor (PARK and LIN, 2005) but can also cause changes in the physicochemical characteristics of the MF.

The objective of this study was to make and characterize three types of MF from African catfish and evaluate their stabilities during storage at -18°C.

MATERIAL AND METHODS

African catfish (n=39; 2.43 ± 0.55 kg) was supplied by the fish-farming of the University of Marilia, SP, Brazil. They were carried alive to the laboratory, stunned by cold water, slaughtered by decapitation, and then placed in thermally insulated boxes covered with ice (1:1) (ice: fish) until the moment of processing (approximately 24 h later). The minced fish (MF) was obtained by passing the fish through a meat and bone separating machine (HT 250, High Tech, Chapecó, Brazil). After producing the MF, it was divided into three treatments: non-washed MF, once-washed MF, and twice-washed MF.

Ice water (±0°C) was used to wash the MF in the proportion of 3:1 (water to fish). This mixture was manually stirred for 2 min in a plastic container, left at rest for 3 min, and then filtered through nylon bags (45 x 70 cm with pore size of approximately 0.042 mm^2), which were pressed manually to remove the excess water. The process was repeated once more to obtain the twice-washed MF, according to OLIVEIRA FILHO *et al.* (2011). For all treatments, 0.5% cryoprotector (tripolyphosphate) (Acordini, Series 700, Ibrac, Rio Claro, Brazil) and 0.5% antioxidant (sodium erythorbate) (Ibracor, Series 500, Ibrac, Rio Claro, Brazil) were added. The MF samples were packed into 500-g plastic bags, frozen in an ultra-fast plate freezer (UCE - 20, Eco, São Paulo, Brazil) until the internal temperature reached -32°C (approximately 90 min) and stored frozen (-18°C) until the moment of analysis. Three samples were taken from each treatment for the chemical and microbiological treatments at the start of storage (zero time) and after 30, 60, 90, 120, 150, and 180 days.

The proximal composition (moisture, crude protein, lipids and ash) was determined at zero time (AOAC, 1999). During frozen storage, the samples were removed (zero time, 30, 60, 90, 120, 150 and 180 days) and analyzed for lipid oxidation (TBARS, according to VYNCKE, 1970) using TMP (tetramethoxypropane) to obtain the straight line equation (y= 65.73x + 0.0002) used to calculate the values. The other analyses carried out were total volatile nitrogenous bases - TVB-N (HOWGATE, 1976); pH was measured directly with a pH-meter equipped with an immersion electrode after the homogenization of 10 g sample with 40 mL distilled water and drip loss, according to KIRSCHNIK and MACEDO-VIEGAS (2009). The microbiological analysis of the MF was carried out by aerobic psychrotrophic microbial count at 7°C, according to APHA (1992).

At zero time and after 90 and 180 days of storage, the raw materials (non-washed MF, once washed MF and twice washed MF) were thawed in refrigerator (5°C) for approximately 24 h. To make the fishburgers (90 g each), the raw materials were weighed and then manually mixed with other ingredients (4% soy protein textured

- Bunge Alimentos, Esteio, Brazil, 2% cassava starch, 1% fishburger seasoning - Kerry, Rio Claro, Brazil, and 1% salt). Next, the products were frozen in an ultra-fast freezer (UCE - 20, Eco, São Paulo, Brazil) until the internal temperature reached -32°C (approximately 50 min) and stored frozen (-18°C) until microbiological and sensory analysis. The fishburger microbiological analyses were the same as those used for the MF.

The sensory evaluation of the fishburgers was carried out in individual booths with white fluorescent light. Affective acceptability tests were performed using the methodology described by MEILGAARD *et al.* (1999) at 0, 90, and 180 days of storage. Samples were evaluated using a 9-point hedonic scale (1. "disliked extremely" to 9. "liked extremely") for the attributes of flavor, texture, color, odor, and overall acceptance. Thirty untrained panelists of both sexes and various age groups participated in the acceptability test. Fishburger samples were presented to the panelists in a monadic way under a completely randomized blocks experimental design. The panelists represented different blocks, respectively. Fishburger were fried in hot oil, cut into six pieces, and served to the panelists in disposable plastic containers along with water and cream crackers.

For the statistical analysis, a completely randomized design (CRD) was used in 3x7 factorial and three repetitions. The three levels of factor 1 were 0, 1, and 2 washings of the MF and factor 2 were days of storage, which 7 intervals (0, 30, 60, 90, 120, 150, and 180 days of storage). The minimal square method was used to evaluate the chemical variables of stability. The data were analyzed by the analysis of variance (ANOVA) at a 5% level of significance. Since significant effects were observed for the double interactions, the developments were conducted using regression analysis with each washing level. All tests were performed with the aid of the Statistical Analysis System© (SAS, 2001), using the PROC GLM procedure.

RESULTS AND DISCUSSION

Table 1 shows the results for the processing yield of the African catfish MF. A yield for the carcass without the viscera and head of 57.98% was obtained for fish with a mean weight of 1.41 kg. In a similar study with African catfish with live weights of 1 to 2 kg, SOUZA *et al.* (1999) obtained a yield of 56.67% for the clean trunk.

In the present study, a yield of approximately 58% clean fish can be considered good, since this fish has a large head representing between 23.62 and 27.44% of the total weight (SOUZA *et al.*, 1999), which can result in a decrease in the processing yields. However, the present values were lower than for surubim, *Pseudoplatystoma spp*, another type of catfish (66.9 to 70.9% for fish weighing between 1.5 and 2.7 kg) (CREPALDI *et al.*, 2008). Despite this, the exploitation in the form of MF was high (85.85%), considering the clean fish without the head and viscera.

Table 1 - Values for total weight, mean weight and yield in meat obtained from the production of the African catfish MF.

Variable	Total weight (kg)	Mean weight[2] (kg)	Yield (%)
Whole fish	94.65	2.43±0.55	----
Clean fish[1]	54.88	1.41±0.29	57.98
Waste (H+V+S)	39.77	----	42.02
MF	47.12	----	49.78 (WF)
		----	85.85 (CF)
MF waste	5.13	----	5.41 (LF)

[1]Clean fish = without the head and viscera. Waste: H = head; V = viscera; S = skin. Yield: (WF) = whole fish. (CF) = clean fish. (LF) = live fish.
[2]Mean±standard deviation.

The twice-washed MF showed higher moisture content (84.26%, P<0.01) than the non-washed (78.42%) and once-washed (78.52%) samples (Table 2). GRYSCHEK *et al.* (2003) and KIRSCHNIK and MACEDO-VIEGAS (2009) also observed an increase in moisture content for the washing of Nile tilapia MF, from 78.50 to 86.70% and from 79.83 to 88.78%, respectively. The increase in moisture content of the MF with increasing numbers of washing cycles could be related to the fact that the moisture adjustment of the MF was carried out manually without the use of centrifuges or presses, thus making it more difficult to remove the excess water contained in the meat (NEGBENEBOR *et al.*, 1999). Moreover, according to ASGHARZADEH *et al.* (2010) the removal of water-soluble components such as blood, pigments, proteins, and salts may have led to an increasing hydration availability of the MF.

Washing the MF up to two times caused a 4.81% decrease (P<0.01) in the proteins (Table 2), which could have been caused by leaching of the sarcoplasmic proteins and non-protein nitrogen, which are soluble in water. GRYSCHEK *et al.* (2003) and WILLES *et al.* (2004) also observed decreases in the protein contents of washed MF from Nile tilapia and from the channel catfish *Ictalurus punctatus*, respectively. Washing of the MF also caused a decrease (P<0.01) in the lipids (3.88 and 2.75% for the non-washed and twice-washed MF, respectively). KIRSCHNIK and MACEDO-VIEGAS (2009) and OLIVEIRA-FILHO *et al.* (2011) also observed this with the MF from the filleting waste of Nile tilapia. According to ASGHARZADEH *et al.* (2010), fat content underwent an important decrease after washing. This can be explained as a result of the minced material floating to the surface, leading to an increasing availability to be lost during such a processing step.

The washing cycles of African catfish MF

Table 2 - Mean values ± standard error for the proximal composition of non-washed, once-washed and twice-washed African catfish MF and of fishburgers made from African catfish MF.

Product	Washing of minced fish	Proximal composition (%)			
		Moisture	Crude protein	Lipid	Ash
Minced fish	0	78.42±0.81B	15.86±0.57A	3.88±0.02A	1.51±0.08A
	1	78.52±0.81B	14.89±0.57A	3.18±0.02B	0.86±0.08B
	2	84.26±0.81A	11.05±0.57B	2.75±0.02C	0.77±0.08B
Fishburguer	0	72.68±0.81B	16.60±0.57A	3.46±0.02A	3.11±0.08A
	1	74.11±0.81AB	16.05±0.57A	2.75±0.02B	2.37±0.08B
	2	77.47±0.81A	13.49±0.57B	2.72±0.02B	2.44±0.08B

Different letters in the same column indicate significant difference (P<0.05).

decreased the ash contents (P<0.01). The ash contents found in the present study were close to the values found for the non-washed (1.35%) and washed (0.46%) MF from Nile tilapia (KIRSCHNIK and MACEDO-VIEGAS, 2009). This decrease in the ash contents can be explained by leaching of the soluble mineral content into the water during the MF washing process (OLIVEIRA FILHO *et al.*, 2011). As in the raw material (MF), the fishburgers also showed lower protein, lipid and ash contents (P<0.05) and an increase in moisture content with washing of the MF (Table 2). The protein and ash contents of the fishburgers were higher than those in the raw materials, probably due to the addition of soy protein, starch, salt and seasonings.

There was no difference (P>0.05) in the values obtained for the TVB-N of the non-washed MF during 180 days of storage (15.79 ± 0.22 mg/100 g). However in once- and twice-washed MF, there was an increase (P<0.05) in the values of TVB-N until the end of storage, varying from 5.09 to 5.82 and 2.24 to 2.98 mg/100 g, respectively (Fig. 1). In studies with pink perch (*Nemipterus japonicus*) MF and silver carp (*Hypophthalmichthys molitrix*) MF observed an expressive increase in TVB-N during 180 days of

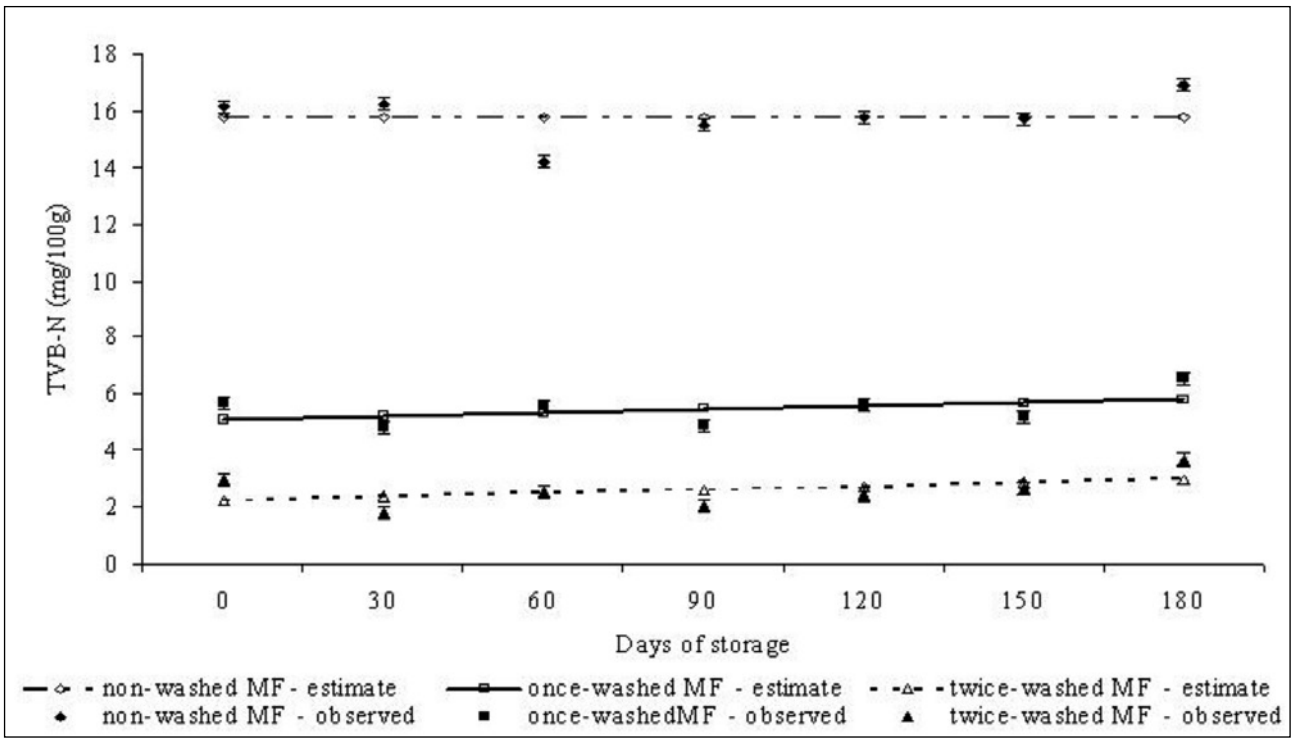


Fig. 1 - TVB-N values (mg/100 g) of non-washed (y=15.79), once-washed (y=5.093+0.004057x) and twice-washed (2.239+0.004106x) African catfish MF during 180 days of frozen storage (S.E. = 0.22).

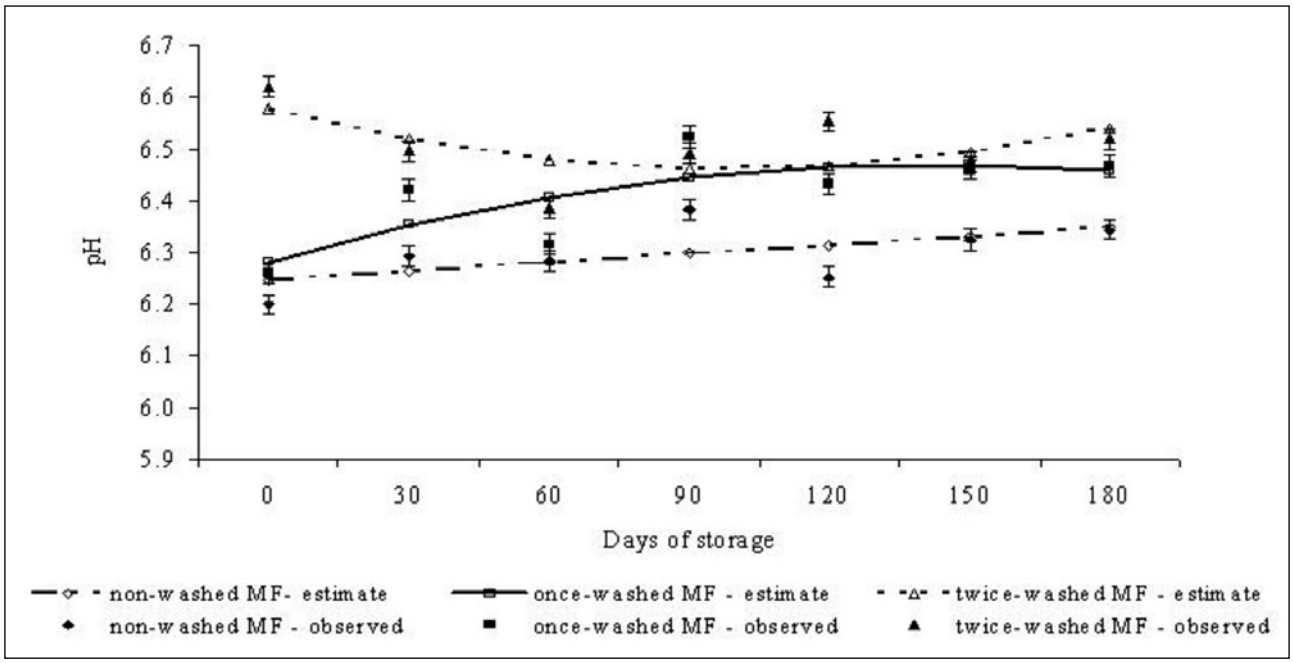


Fig. 2 - Values for the pH of non-washed (y=6.247+0.000556x), once-washed (y=6.282+0.002619x-0.0000091x^2) and twice-washed (y=6.579-0.0024x+0.000012x^2) African catfish MF during 180 days of frozen storage (S.E. = 0.02).

frozen storage (REDDY *et al.*, 1992; SIDDAIAH *et al.*, 2001). This small increase in MF TVB-N during storage indicates that the processes of freezing and storage were efficient to limit or inhibit the development of microorganisms and action of endogenous enzymes. TVB-N is built up by ammonia, trimethylamine, and dimethylamine originating from the breakdown of nucleotides and from the deamination of amino acids by microorganisms (CONTRERAS-GUZMÁN, 2002). There were differences (P<0.05) between the treatments as such for the TVB-N values; the non-washed MF showed higher values than the once- and twice-washed samples. Lower TVB-N values in washed MF can be explained as a result of partial removal of N-containing molecules susceptible to breakdown during the washing step, leading to volatile and basic compounds (ASGHARZADEH *et al.*, 2010). In a study with Nile tilapia, KIRSCHNIK and MACEDO VIEGAS (2009) also observed a decrease in TVB-N from 11.99 (unwashed MF) to 2.63 mg/100 g (washed MF). According to the Brazilian legislation, fresh fish for consumption should present TVB-N values of up to 30 mg/100 g (BRASIL, 1952). All treatments remained below this established limit during the 180-days storage period.

At zero time, the once- and twice-washed African catfish MF showed higher pH values (P<0.05) than the non-washed MF (Fig. 2), a fact also observed by KARTHIKEYAN *et al.* (2004), WILLES *et al.* (2004), and ASGHARZADEH *et al.* (2010). According to these Authors, the increase of pH values during washing process of MF could be attributed to the removal of lactic acid, amino acids and other water-soluble acids during the washing process.

On day zero, the pH value of the non-washed MF was 6.25, increasing to 6.35 (P<0.05) by the end of the storage period (Fig. 2). The once-washed MF showed an initial pH value of 6.28, increasing to 6.46 (P<0.05) after 180 days of frozen storage. Other studies with washed and non-washed MF tilapia observed an increase in pH values with the increase of days of frozen storage (GRYSCHEK *et al.* 2003; KIRSCHNIK and MACEDO-VIEGAS, 2009). This may have been due to protein degradation, producing basic compounds like ammonia and amines. On the other hand, the twice-washed MF showed an initial pH value of 6.58, which fell (P<0.05) to 6.46 after 90 days of frozen storage and then slightly increased, reaching 6.54 after 180 days. This decrease in pH value in twice-washed MF up to 90 days of storage was an unexpected effect that is difficult to explain.

The washing process of the MF caused most of the components responsible for lipid oxidation to leach (P<0.05), as evaluated by TBARS (0.216 mg malonaldehyde/kg for non-washed MF, 0.083 mg malonaldehyde/kg for once-washed MF and 0.099 mg malonaldehyde/kg for twice-washed MF) (Fig. 3). The same behavior was observed for Nile tilapia MF (KIRSCHNIK and MACEDO-VIEGAS, 2009) and silver carp MF (ASGHARZADEH *et al.*, 2010). For the three treatments, there was then a significant (P<0.05) fall in the values up to 60 days of storage (0.070, 0.038, and 0.041 mg malonaldehyde/kg for the non-washed, once-washed and twice-washed MF, respectively). The values in-

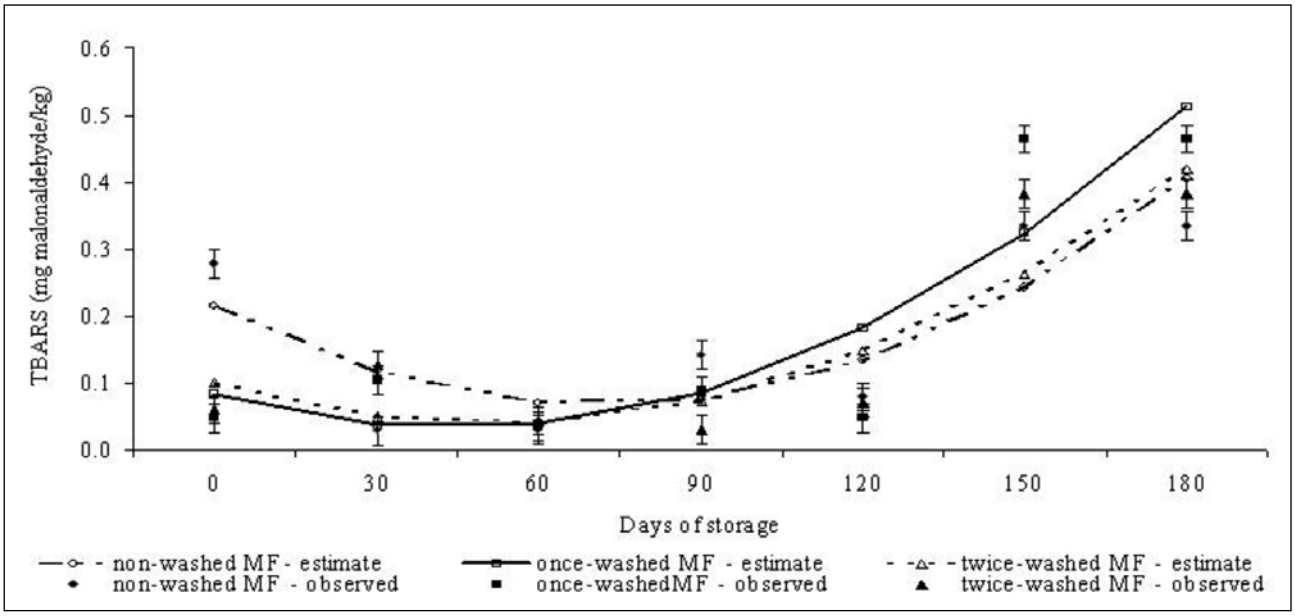


Fig. 3 - TBARS values (mg malonaldehyde/kg) for the non-washed ($y=0.216-0.00417x+0.00003x^2$), once-washed($y=0.083-0.00230x+0.00003x^2$) and twice-washed ($y=0.0998-0.00236x+0.00002x^2$) African catfish MF during 180 days of frozen storage (S.E. = 0.02).

creased significantly up to 180 days of storage, when they reached 0.405, 0.511, and 0.420 mg malonaldehyde/kg for the non-washed, once-washed, and twice-washed MF, respectively. This decrease in TBARS values after 60 days of storage was probably due to the protective effect of the antioxidant used in the MF. However, the process of obtaining MF can cause disruption of muscle membranes, facilitating interaction of the oxidizing agents with polyunsaturated fatty acids and resulting in the propagation of oxidative reactions (GRAY *et al.*, 1996; TOKUR *et al.*, 2004). The increase after 60 days was due to lipid oxidation of the raw material during frozen storage. Brazilian legislation does not indicate any maximum limit for the TBARS values of fish MF. However, according to AL-KAHTANI *et al.* (1996), the values for TBARS should be below 3 mg malonaldehyde/kg sample in order for the fish to be in good condition for consumption. Although the TBA values of the MF increase up to 180 days of storage, there were no decreases in the values of fishburger sensory attributes prepared with this raw material during the storage period (Table 4).

Table 4 - Means ± standard error of the sensory attributes for odor, color, flavor, texture and overall acceptance of fishburgers made with non-washed, once-washed and twice-washed African catfish MF during up to 180 days of frozen storage.

Sensory attributes	Washing of minced fish		
	0	1	2
Odor	7.06±0.17^{A}	7.03±0.17^{A}	6.81±0.17^{A}
Color	5.77±0.19^{B}	6.59±0.19^{A}	6.43±0.19^{AB}
Flavor	6.99±0.18^{A}	7.04±0.18^{A}	6.49±0.18^{A}
Texture	6.56±0.17^{AB}	6.78±0.17^{A}	6.18±0.17^{B}
Overall acceptance	6.59±0.17^{A}	6.89±0.17^{A}	6.43±0.17^{A}

Different letters in the same row indicate significant difference (P<0.05). Hedonic scale: 1 - dislike extremely; 2 - dislike very much; 3 - dislike moderately; 4 - dislike slightly; 5 - neither like nor dislike; 6 - like slightly; 7 - like moderately; 8 - like very much; 9 - like extremely.

The drip loss of the MF suffered no alterations (P>0.05) during the storage period (Fig. 4), showing that the use of a cryoprotector, quick freezing, and a low storage temperature were capable of reducing protein denaturation and improving the water holding capacity (BOBBIO and BOBBIO, 2001). To the contrary, REDDY and SRIKAR (1991) found an increase in drip loss for pink perch, *Nemipterus japonicus*, MF from 4.85 to 20% after 180 days of frozen storage (-18°C). The Authors explained that the water holding capacity decreased with the increase in storage time and that the amount of drip loss generally reflects a certain degree of protein denaturation, which could be due to surface drying and the rupture of cells by ice crystals. Nevertheless, the non-washed MF presented a greater (P<0.05) volume of drip loss (11.82%) than the once-washed (1.59%) and twice-washed (3.50%) MF (Fig. 4). This higher value of drip loss in non-washed MF compared to once-washed and twice-washed MF is probably due to the largest amount of sarcoplasmic proteins of this raw material in relation to washed MF. Thus, it causes low water holding capacity and, consequently, greater drip loss values (GRYSCHEK *et al.*, 2003; KIRSCHNIK, 2007). This higher drip loss value in twice-washed MF with respect to once-washed MF is probably due to higher moister of twice-washed MF (Table 2).

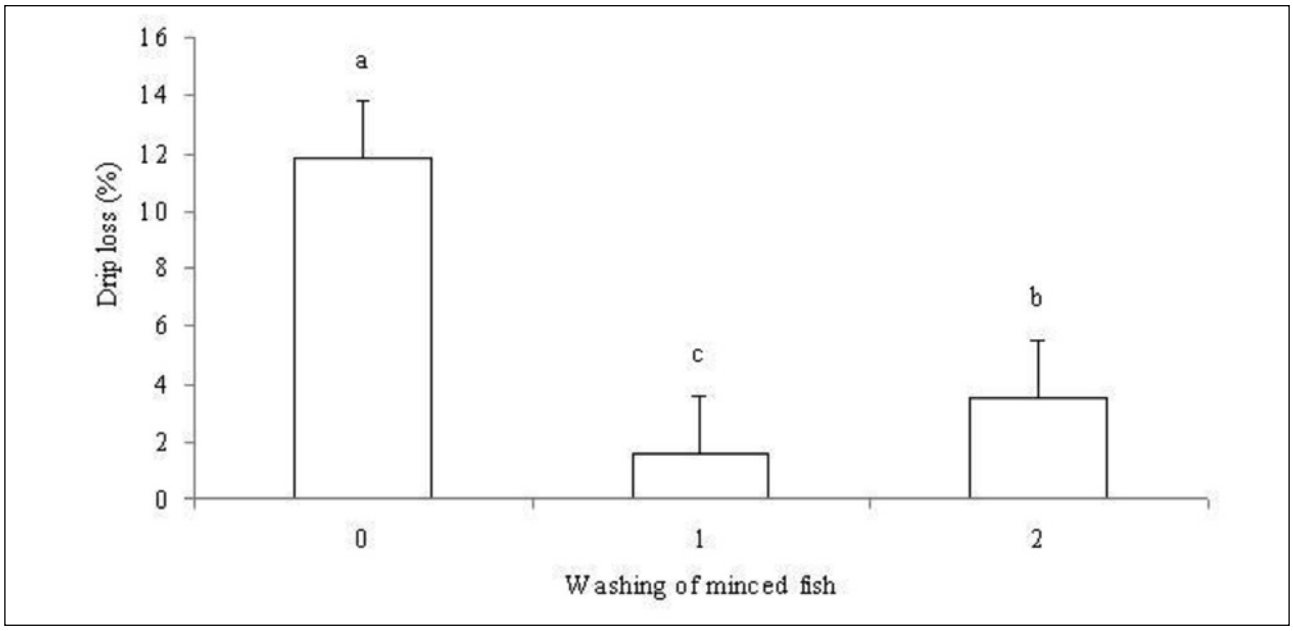


Fig. 4 - Drip loss (%) of the non-washed, once-washed and twice-washed African catfish MF during 180 days of frozen storage (S.E. = 0.25).

Moreover, according PARK and LIN (2005), the majority of sarcoplasmic proteins is fairly soluble and removed during the initial MF washing steps. Subsequent washing removes the residual sarcoplasmic proteins along with a small amount of myofibrillar proteins. After the sarcoplasmic proteins are completely removed, further washing causes a severe loss of myofibrillar proteins and consequently decreases the water holding capacity.

The ANOVA for the aerobic psychrotrophic bacterial counts detected significant interaction between MF and days of storage ($P<0.01$). One of the models had some negative values, as shown in Table 3. At zero time, the lowest counts ($P<0.05$) were found in the once-washed (0.86 log CFU/g) and twice-washed MF (0.47 log CFU/g) compared to non-washed MF (2.01 log CFU/g), indicating that the washing process had a positive influence on the removal of the aerobic psychrotrophic bacteria. Similar results were observed in a study of washed Nile tilapia MF (KIRSCHNIK and MACEDO-VIEGAS, 2009). During the time of storage, there was a decrease in MF aerobic psychrotrophic bacteria counts. Corroborating these results, Jesus *et al.* (2001) observed a decrease in the aerobic psychrotrophic bacterial counts for the MF of Amazonian fish, from log 5.89 CFU/g at the start of storage to log 4.32 CFU/g after 120 days of storage. According to these Authors, this is typical behavior for MF during frozen storage. Brazilian legislation does not establish limits for the presence of aerobic psychrotrophic bacteria in fish; however, for the ICMSF (1986), the maximum permitted limit is log 7 CFU/g. The values found in the present treatments were all below this limit.

In the microbiological analyses of the fishburgers, the absence of *Salmonella* and *Staphylococcus aureus* was confirmed and less than 3 MPN/g sample of coliforms at 45°C. According to Brazilian legislation (BRASIL, 2001), frozen fish-based products can present the following limits of bacterial contamination: maximum of $5x10^2$

Table 3 - Total aerobic psychrotrophic bacterial counts (log CFU/g) in the non-washed, once-washed and twice-washed African catfish MF during 180 days of frozen storage (S.E. = 0.18).

	Days of storage						
Washing of MF	**0**	**30**	**60**	**90**	**120**	**150**	**180**
0	2.01^{aA}	0.14^{bA}	0.19^{bA}	0.00^{bA}	0.00^{bA}	0.00^{bA}	0.00^{bA}
1	0.86^{aB}	0.33^{abA}	0.21^{bA}	0.09^{bA}	0.08^{bA}	0.00^{bA}	0.00^{bA}
2	0.47^{aB}	0.19^{aA}	0.24^{aA}	0.00^{aA}	0.00^{aA}	0.00^{aA}	0.00^{aA}

Different capital letters in the same column indicate significant difference ($P<0.05$).
Different lowercase letters in the same row indicate significant difference ($P<0.05$).

CFU/g of *Staphylococcus aureus*, absence of *Salmonella* in 25 g sample and a maximum of 10^2 MPN/g coliforms at 45°C. There are no limits for coliforms at 45°C or for total psychrotrophic bacteria. Thus the products obtained in the present study were within the limits demanded by the Brazilian legislation.

The ANOVA for sensory analysis detected only principal effects for washing MF. No differences (P>0.05) were found in the sensory attributes for odor, color, flavor, texture, and overall acceptance for the fishburgers (Table 4) during the 180 days of storage of the MF. In addition, acceptance of the products was good, with scores above 5 (neither liked nor disliked). Supporting the present findings, TOKUR *et al.* (2004) did not observe any differences in sensory attributes of Nile tilapia fishburger during 8 months of frozen storage, with acceptance scores varying from 8 (like very much) to 9 (like extremely).

For the odor attribute, there was no difference between the treatments (P>0.05). The scores remained above 6 (liked slightly), showing good acceptance. OLIVEIRA FILHO *et al.* (2011) did not find any differences in odor attribute between inclusion level and washing cycles of Nile tilapia MF in sausages, reaching values of 5.9 (neither liked nor disliked). For the attribute of color, fishburgers made with once- (6.59) or twice-washed MF (6.43) were better accepted than those made with non-washed MF (5.77). The panelists preferred the lighter colored fishburgers over the darker colored ones. According to FERREIRA *et al.* (2000), it is the appearance that most influences consumer opinion in the decision to buy and, consequently, to consume. This situation can be observed in the present study, since the panelists preferred the color of the fishburgers made with washed MF, probably because they were of the opinion that fishburgers should be lighter and similar in color to a fish fillet; the fishburgers from non-washed MF appeared more like bovine meat hamburgers.

For the flavor attribute, there was no difference (P>0.05) between the fishburgers made with non-washed MF, once-washed MF and twice-washed MF, ranging from 6.49 (liked slightly) to 7.04 (liked moderately). In sausages prepared with washed or non-washed MF Nile tilapia, no difference was observed in flavor (6.0 - liked slightly) (OLIVEIRA FILHO, 2011).

The texture acceptance of fishburger decreased (P<0.05) with the increase in washing cycles of MF. This could have been due to the higher moisture content of the twice-washed MF (Table 2) and its tenderness; even the addition of soy protein was not capable of providing a product with adequate texture. In another study with MF catfish, WILES *et al.* (2004) observed an increase in moisture content with washing MF and reduced mechanical hardness. The overall acceptance of fishburgers made with African Catfish MF was good, with scores around the 6 (liked slightly). However, there was no difference (P>0.05) in overall acceptance between the fishburgers made with washed or non-washed MF. Other Authors also observed good acceptability of products made with MF (KIRSCHNIK, 2007; OLIVEIRA FILHO *et al.*, 2010) and found no differences in overall acceptance of other products made with washed or non-washed MF (OLIVEIRA FILHO *et al.*, 2011), showing the feasibility of exploiting African Catfish MF in the use of fish products.

CONCLUSIONS

The washing of African catfish MF caused an increase in moisture content and losses of proteins, lipid, and ash. The African catfish MF remained stable during storage at -18°C for up to 180 days, indicating the viability of formulating products from this MF. Similar changes occurred in fishburgers made with the washed MF and moister products containing less protein, lipid, and ash compared to the non-washed raw material. The fishburgers made during storage of the MF at -18°C for up to 180 days were well accepted, the best overall evaluation being awarded to those produced with the once-washed African catfish MF.

REFERENCES

Al-Kahtani H.A., Abu-Tarboush H.M. and Bajaber A.S. 1996. Chemical changes after irradiation and post-irradiation storage in tilapia and Spanish mackerel. J. Food Sci. 61: 733.

AOAC. 1999. "Official Methods of Analysis" 16th Ed. Association of Official Analytical Chemists, Washington, DC.

APHA. 1992. "American Public Health Association" 3rd Ed. Compendium of Methods for the Microbiological Examination of Foods, Washington, DC.

Asgharzadeh A., Shabanpour B., Aubourg S.P. and Hosseini H. 2010. Chemical changes in silver carp (*Hypophthalmichthys molitrix*) minced muscle during frozen storage: Effect of a previous washing process. Grasas y Aceites, 61: 101.

Benjakul S., Visessanguan W., Phongkanpal V. and Tanaka M. 2005. Antioxidative activity of caramelisation products and their preventive effect on lipid oxidation in fish mince. Food Chem. 90: 239.

Bobbio P.A. and Bobbio F.O. (Eds.). 2001. Química do processamento de alimentos. 3rd Ed. Varela, São Paulo, SP.

Brasil 1952. Ministério da agricultura, pecuária e abastecimento. Departamento de Inspeção de Produtos de Origem Animal – DIPOA. Divisão de normas técnicas. Brasília, 1997. Decreto n. 30.691 de 29 de março de 1952. Regulamento da Inspeção Industrial e Sanitária de Produtos de Origem Animal. Título VII, capítulo VII, sessão I.

Brasil 2001. Ministério da Saúde. Agência Nacional de Vigilância Sanitária – ANVISA. Brasília, 2001. Resolução RDC n. 12 de 02 de janeiro de 2001 – Diário Oficial da União de 10 de janeiro de 2001, seção I.

Contreras-Guzmán E.S. (Eds.). 2002. Bioquímica de Pescados e Invertebrados. CECTA-USACH, Santiago, Chile.

Crepaldi D.V., Teixeira E.A., Faria P.M., Ribeiro L.P., Melo D.C., Oliveira D.A.A., Turra E.M. and Queiroz B.M. 2008. Rendimento de carcaça em surubim (*Pseudoplatysto-*

ma spp.) avaliado por ultra-som. Rev. Bras. Saúde Prod. An. 9: 824.

Ersoy B. and Özeren A. 2009. The effect of cooking methods on mineral and vitamin contents of African catfish. Food Chem. 115: 422.

Ferreira V.L.P. (Ed.). 2000. Análise sensorial: testes discriminativos e afetivos. 1st Ed. SBCTA, Campinas, SP.

Gray J.I., Gomaa E.A. and Buclkey D.J. 1996. Oxidative quality and shelf life of meats. Meat Sci. 43:123.

Gryschek S.F., Oetterer M. and Gallo C.R. 2003. Characterization and frozen storage stability of minced Nile tilapia (*Oreochromis niloticus*) and red tilapia (*Oreochromis spp.*). J. Aquat. Food Prod. Technol. 12: 69.

Howgate P. 1976. Determination of total volatile bases. Torry Research Station. Aberdeen, TD 564, Appendix 4.

IBAMA 2007. Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis. Estatística da Pesca 2005 Brasil: grandes regiões e unidades da confederação, Brasília, DF.

ICMSF 1986. "International Commission on Microbiological Specification for Foods". Microoganisms in foods 2. Sampling for microbiological analysis: principles and specific applications. 2nd Ed. University of Toronto Press, Toronto.

Jesus R.S., Lessi E. and Tenuta-Filho A. 2001. Estabilidade química e microbiológica de *minced fish* de peixes amazônicos durante o congelamento. Ciênc. Tecnol. Alim. 21: 148.

Karthikeyan M., Shamasundar B.A., Mathew S., Kumar P.R. and Prakash V. 2004. Physico-chemical and functional properties of proteins from pelagic fatty fish (*Sardinella longiceps*) as a function of water washing. Int. J. Food Proper. 7: 365.

Kirschnik P.G. 2007. Avaliação da estabilidade de produtos obtidos de carne mecanicamente separada de tilápia nilótica (*Oreochromis niloticus*) Sc. D. Thesis, Jaboticabal Centro de Aqüicultura, UNESP.

Kirschnik P.G. and Macedo-Viegas E.M. 2009. Efeito da lavagem e da adição de aditivos sobre a estabilidade de carne mecanicamente separada de tilápia do Nilo (*Oreochromis niloticus*) durante estocagem a -18°C. Ciênc. Tecnol. Aliment. 29: 206.

Meilgaard M., Civille G.V. and Carr T.B. (Eds.) 1999. Sensory evaluation techniques. 3rd Ed. CRC Press, New York, NY, USA.

Morais C. and Martins J.F.P. 1981. Considerações sobre o aproveitamento de sobras de industrialização de pescado na elaboração de produtos alimentícios. Bol. Inst. Tecnol. Aliment. 18: 281.

Negbenebor C.A., Godiya A.A. and Igene J.O. 1999. Evaluation of *Clarias Anguillaris* treated with spice *Piper guineense* for washed mince and kamaboko-type product. J. Food Comp. Anal. 12:322.

Oliveira Filho P.R.C., Fávaro-Trindade C. S., Trindade M. A., Balieiro J.C.C. and Viegas E.M.M. 2010. Quality of sausage elaborated using minced Nile tilapia submitted to cold storage. Scien. Agri. 67: 190.

Oliveira Filho P.R.C., Viegas E.M.M., Kamimura E.S. and Trindade M.A. 2011. Evaluation of physicochemical and sensory properties of sausages made with washed and unwashed mince from Nile tilapia by-products. J. Aquat. Food Prod. Technol. available on line 2 January 2012.

Ozório R.O.A., Avnimelech Y. and Castagnolli N. 2004. Sistemas intensivos fechados de produção de peixes. In: "Tópicos especiais em piscicultura de água doce tropical intensiva". J.E.P. Cyrino *et al.* (Eds.), p. 11-15, Tecart, São Paulo, SP.

Park J.W. and Lin T.M.J. 2005. Surimi: manufacturing and evaluation. In: "Surimi and surimi seafood". J.W. Park (Ed.), p. 33-106. Taylor and Francis Group, CRC, London.

Reddy G.V.S., Srikar L.N. and Sudhakara N.S. 1992. Deteriorative changes in pink perch mince during frozen storage. Intern. J. Food Sci. Technol. 27: 276.

Reddy G.V.S. and Srikar L.N. 1991. Preprocessing ice storage effects on functional properties of fish mince protein. J. Food Sci. 56: 968.

Ribeiro R.P. and Pavanelli C.S. 2001. Classificação sistemática dos peixes. In: "Fundamentos da moderna aqüicultura". H.L.M. Moreira *et al.* (Eds.), p. 11-14. Ed. ULBRA, Canoas, RS.

Rodríguez-Herrera J., Bernárdez M., Sampedro G., Cabo M.L. and Pastoriza L. 2006. Possible role for cryostabilizers in preventing protein and lipid alterations in frozen-stored minced muscle of Atlantic mackerel. J. Agri. Food Chem. 54: 3333.

SAS 2001. User's Guide: Basic and Statistic; SAS: Cary, NC.

Siddaiah D., Reddy G.V.S., Raju C.V. and Chandrasekhar T.C. 2001. Changes in lipids, proteins and kamaboko forming ability of silver carp (*Hypophthalmichthys molitrix*) mince during frozen storage. Food Res. Intern. 34:53.

Souza M.L.R., Lima S., Furuya W.M., Pinto A.A., Loures B.T.R.R. and Povh J.A. 1999. Estudo de carcaça do bagre africano (*Clarias gariepinus*) em diferentes categorias de peso. Act. Scient. 21: 644.

Tenuta-Filho A. and Jesus R.S. 2003. Aspectos da utilização de carne mecanicamente separada de pescado como matéria-prima industrial. Bol. SBCTA, 37: 64.

Tokur B., Polat A., Beklevik G. and Ozkutuk S. 2004. Changes in the quality of fishburger produced from Tilapia (*Oreochromis niloticus*) during frozen storage (18°C). Eur. Food Res. Technol. 218:423.

Vyncke W. 1970. Direct determination of the thiobarbituric acid value in trichloroacetic extracts of fish as a measure of oxidative rancity. Feete-Scifen Anstrichmettel, 72: 1087.

Willes J.L., Green B.W. and Bryant R. 2004. Texture profile analysis and composition of a minced catfish product. J. Text. Stud., 35: 337.

Paper received February 3, 2011 Accepted September 3, 2011

SHORT COMMUNICATION

PHYSICAL, CHEMICAL, ENZYMATIC AND MICROBIOLOGICAL CHARACTERISTICS OF ARTISANAL RENNET PASTES FROM THE CENTRE OF ITALY

C. TRIPALDI*, G. PALOCCI, S. BILEI[1], T. BOGDANOVA[1], M.F. SCINTU[2] and M. ADDIS[2]
Consiglio per la Ricerca e la Sperimentazione in Agricoltura,
Centro di Ricerca per la Produzione delle Carni e il Miglioramento Genetico,
Via Salaria 31, 00016 Monterotondo, Rome, Italy
[1]Istituto Zooprofilattico Sperimentale delle Regioni Lazio e Toscana,
Via Appia Nuova 1411, 00178 Rome, Italy
[2]Dipartimento per la Ricerca nelle Produzioni Animali, AGRIS,
Loc. Bonassai, 07040 Olmedo, SS, Italy
*Corresponding author: carmela.tripaldi@entecra.it

ABSTRACT

The safety and hygienic characteristics and enzymatic activities of artisanal rennet pastes were analysed to obtain a special derogation from regulation on the hygiene of foodstuffs that was necessary to continue making cheeses by traditional processes. We found great variability among samples coming from different cheese plants at the farm. The samples had good clotting (189±77 IMCU/g) and lipolytic activities (36,2±27,5 ILU/g). Pathogens and microorganisms, considered to be markers of hygienic conditions, were absent or less than the maximum count tolerated. Our results showed that the treatment performed on artisanal rennet pastes did not favour the growth of germs dangerous to consumer health and assured the enzymatic content necessary to achieve typical characteristics of traditional cheeses.

- Key words: artisanal rennet pastes, enzymatic activities, pathogens -

INTRODUCTION

There is recent interest in the safety and promotion of cheeses prepared according to local traditional processes. One subject of the renewed attention is artisanal rennet, which is considered one of the more important factors affecting the characteristics of some typical cheeses made in Mediterranean countries from sheep, goat, and bovine milk.

Previous research investigated some of the factors affecting coagulating and lipolytic activities of rennet pastes. It was concluded from these studies that the enzymatic activity is affected by different factors: age at slaughtering; diet (suckling or suckling and other feed); *abomasum* condition (empty or full); treatment of *abomasa* (drying with or without salting); and length of aging (PETTINAU *et al.*, 1977; PIREDDA and ADDIS, 1998; BUSTAMANTE *et al.*, 2000; ROSSANO *et al.*, 2003; ADDIS *et al.*, 2005).

Some studies were carried out to evaluate the health and hygienic characteristics of artisanal rennet, (COSSEDDU and PISANU, 1980; DEIANA *et al.*, 1980; PISANU and COSSEDDU, 1982; BARZAGHI *et al.*, 1997; CALANDRELLI *et al.*, 1997; IRIGOYEN *et al.*, 2001; MOATSOU *et al.*, 2004; MOSCHOPOULOU *et al.*, 2007) and there was good agreement in their results.

The principal aim of this study is to investigate the safety and hygienic characteristics of these rennets. It is necessary to determine these characteristics to obtain a special derogation from regulation on the hygiene of foodstuffs to continue making cheeses by traditional processes (Commission Decision 97/284/EC). The other aim is to study the clotting and lipolytic activity of artisanal rennets to understand their role in the specificity of such cheeses.

MATERIALS AND METHODS

Rennet sampling

Samples of artisanal rennet were collected from five small cheese plants at the farm localized in the Latium region. The samples were named A, B, C, D, and E. The shepherds described the process they used to produce rennet, which consisted of collecting the *abomasa* from lamb slaughtered immediately after suckling and then of submitting the *abomasa* to a drying phase that was sometimes preceded by a salting phase. The samples prepared in the laboratory for chemical and enzymatic analyses were frozen until analyses. The *abomasa* for the microbiological analyses were stored at 2° ± 3°C and analysed in a few hours. One repetition of each analysis was carried out.

Physico-chemical characteristics and enzymatic activities

Official methods for the analysis of cheese (MAF, 1986) were followed to evaluate pH, DM, and sodium chloride content.

Total milk clotting activities of the artisanal rennet pastes were determined according to ISO 23058 IDF 199: 2006 method known as REMCAT method. Milk clotting activity was also expressed as "Soxhlet Titre" as described by SAVINI (1946) and in UC (Unit Clotting) as described in the modified Berridge method (ALAIS, 1984).

To determine the chymosin and pepsin content, the test samples were prepared by dissolving 25 g of rennet paste in 100 g of buffer solution (CH_3COOH/CH_3COONa) at pH 5.5; the samples were centrifuged at 3,000 rpm (2189 *g*, refrigerated centrifuge ALC 4237R, ALC, Milano, Italy) for 30 min at 4°C. The supernatant was analysed as described in the International IDF Standard 110B: 1997. Chymosin and pepsin enzymes were expressed as a percentage of the sample's total milk clotting activity.

Lipase activity was analysed as described in the FOOD CHEMICAL CODEX (1981).

Microbiological analyses

All samples were subjected to qualitative tests for the detection of *Salmonella* and *Listeria monocytogenes*, and to the following bacterial enumerations: *Listeria monocytogenes*, sulphite reducing Clostridia, total microbial count at 30°C, coagulase-positive Staphylococci, and β-glucuronidase positive *E. coli*. The detection test for *Listeria monocytogenes* was carried out according to UNI EN ISO 11290-1:2005. The detection of *Salmonella spp* was carried out as reported in ISO 6579:2002. The enumeration of *Listeria monocytogenes*, sulfite reducing clostridia, total bacterial count and β-glucuronidase positive *E. coli* were performed respectively according to following standards: UNI EN ISO 11290-2:2005, ISO 15213:2003, ISO 4833:2003 and AFNOR - BIO 12/5-01/99.

The correlation between chemical and enzymatic characteristics of samples was performed using the CORR procedure (SAS, 2006).

RESULTS AND DISCUSSION

Physico-chemical characteristics

The pH, dry matter (DM), ash, and sodium chloride content of the rennet samples are reported in Table 1. The values of all parameters were variable. The mean pH value was 4.8 with a range from 4.0 to 5.5. The average content of DM was about 68 g/100 g with a minimum of 50 g/100 g and a maximum of 76 g/100 g. The salt content was very high (34.5 g/100 g) in sample

Table 1 - pH, dry matter, ash and salt content of artisanal rennet pastes.

Sample	pH	Dry matter (%)	NaCl (% of dry matter)	Ash (% of dry matter)
A	4.76	75.98	2.13	2.97
B	4.04	73.86	3.28	3.84
C	4.68	73.04	34.52	34.58
D	5.26	69.08	2.13	5.03
E	5.51	50.12	1.44	3.47
Mean ± sd	4.85±0.57	68.42±10.53	8.7±14.45	9.98±13.77

C and very low in the other samples, which had an average of 2.2 g/100 g. Sodium chloride is the major component of the ash content and it depends strictly on the salt added. The ash content of sample C was 34.6 g/100 g and the mean value of the other samples was 3.8 g/100 g.

The pH values of rennet pastes used by BUSTAMANTE *et al.* (2000) to process Idiazabal, an ovine cheese from Basque country, varied over a wide range (4.0 – 5.9). The great variability in DM content (39 g/100 g to 87 g/100 g) of artisanal rennets was also observed by FULCO *et al.* (1987). The variability in DM content could be ascribed to different temperatures and humidity levels in the aging room, to initial weight of the *abomasa* and to the content of *abomasa* (milk, grass, forages and other feeds).

The very different results in the sodium chloride content demonstrated that the treatment of rennets was managed in different ways and not all producers performed the salting phase. In fact, the very small amount of salt in four samples (2.2 g/100 g on average) could be attributed to the internal content of this compound and not to the sodium chloride added during the salting phase. In previous reports, higher values of salt content were found in salted *abomasa*. PETTINAU *et al.* (1977) found the sodium chloride content was about 13 g/100g in the internal content of *abomasa* and 36 g/100 g in the stomach wall in lamb rennets salted down seven days and then dried until 65-90 days. The minimum content of sodium chloride observed in Sicilian rennet pastes was 8 g/100 g (FULCO *et al.*, 1987).

Enzymatic activities

The milk clotting results and lipolytic activities of rennet pastes are reported in Table 2.

The average milk clotting activity, expressed as IMCU/g, was 189±77 and activity ranged from 114 to 316 IMCU/g. These values were similar to those (155 – 363 IMCU/g) found by BUSTAMANTE *et al.* (2000) and higher than those obtained from other artisanal lamb rennet pastes from the Sardinia region (48 – 95 IMCU/g) (ADDIS *et al.*, 2005). The milk clotting activity expressed as Soxhlet values ranged from 1:4,606 to 1:10,056. If evaluated by the Berridge method, it ranged from 7.42 to 10.5. The average Soxhlet value (1:7,210) was lower than that found on average (1:10,000) by CALANDRELLI *et al.* (1997) in artisanal kid rennet submitted first to drying and then to the salting process. The range reported in commercial rennet pastes by SALVADORI DEL PRATO (2001) was 1:8,000 – 1:15,000.

The chymosin activity of samples analysed was much higher (minimum 81% and maximum 96%) than the activities found by others. The chymosin activity in commercial rennet pastes ranged from 51 to 83% (BARZAGHI and RAMPILLI, 1996); in artisanal rennet pastes, activities ranged from 75 to 80% and from 64 to 87% (BUSTAMANTE *et al.*, 2000; ADDIS *et al.*, 2005).

The lipolytic activity of samples determined at pH 6.2 also largely varied between different samples (6.7 – 68.2 ILU/g) with an average value of 36.2±27.5 ILU/g. These results were higher if compared with those found in other studies utilising similar pH values of substrate. In fact

Table 2 - Milk clotting and lipolytic activity of artisanal rennet pastes.

Sample	Clotting activity			Chymosin and pepsin content		Lipolytic activity
	IMCU/g	Soxhlet	Berridge	Chymosin (%RU)	Pepsin (%RU)	ILU/g (original pH)
A	169.06	4,61	7.42	95.53	4.47	58.29
B	196.73	8,95	10.08	87.43	12.57	10.63
C	316.36	10,06	10.5	80.66	19.34	6.68
D	113.94	4,77	8.14	96.2	3.8	37.16
E	151.10	7,66	7.72	90.19	9.81	68.16
Mean ± sd	189.44±77.05	7.21±2.45	8.77±1.42	90.00±6.38	10.00±6.38	36.18±27.55

the lipolytic activity of artisanal rennet pastes ranged from 3.7 to 26.9 units/g when the samples were tested at pH 6.0 (BUSTAMANTE *et al.*, 2000) and from 0.6 to 2.0 units/g at pH 6.2 (ADDIS *et al.*, 2005). A very wide range from 0.1 to 94.3 units/g was detected in commercial rennet pastes analysed at pH 6.2 (BARZAGHI and RAMPILLI, 1996).

When compared with data from previous studies, the analysed samples had good coagulating and lipolytic activities, which are optimal for making cheeses with specific characteristics, especially with sensorial attributes as the release of free fatty acids during lipolysis is considered to influence the flavour of cheeses (WOO *et al.*, 1984). The typical characteristics of some Italian cheeses are probably due to the use of rennet paste containing a balanced amount of milk clotting and milk fat-hydrolysing enzymes (ADDIS *et al.*, 2005).

The correlations of all parameters examined are shown in Table 3. We observed that the relation between Soxhlet and Berridge methods was higher (r=0.85; P≤0.07) than those observed between Soxhlet (r=0.78; P≤0.11) and Berridge methods (r=0.79; P≤0.11) with respect to REMCAT method. The more strict relation between Soxhlet and Berridge methods probably is due to the principle on which both analyses are based.

The salt content of rennet seems to positively affect the clotting activity expressed as IMCU (r=0.93; P≤0.11). These results were in agreement with those of other studies. The negative effect of the lack of salting on clotting activity was observed by PETTINAU *et al.* (1977) in rennet pastes after a few months of storage. In contrast, BUSTAMANTE *et al.* (2000) found that the amount of salt added did not affect the clotting activity or its stability.

The pH of rennet pastes positively affects the lipolytic activity (r=0.74; P≤0.15). Similar results were obtained by FERRI (2007), who observed maximum lipolytic activity of artisanal rennet pastes at pH 6.2 and minimum activity at pH 5.5. The optimum pH range for the pregastric lipase activity is 4.5 – 6.0 with complete inhibition below pH 2.4 and above pH 7.8 (NELSON *et al.*, 1977). D'SOUZA and ORIEL (1992) found a less wide pH range for optimal lipolytic activity of lamb pregastric lipase which ranged from 5.5 to 6.0. The condition of the *abomasum* affects the pH of rennet pastes (BUSTAMANTE *et al.*, 2000). The pH of rennets originated from animals slaughtered after suckling was less (< 4.7) than those belonging to animals slaughtered on an empty stomach (> 5.2).

The enzymatic activities of artisanal rennet samples collected from already ground *abomasa* (unpublished data) were lower than that from whole *abomasa* from the five plants: 135 IMCU/g on average *vs* 189 IMCU/g and 15.2 *vs* 36.2 ILU/g respectively. These lower clotting and lipolytic activities could be attributed to the widespread practice of only utilizing the interior of the *abomasum*. In fact, as observed by PETTINAU *et al.* (1977), both enzymatic activities, particularly the clotting activity, are higher in the wall of the stomach, and when the wall is mixed with the interior content the enzymatic activities increases.

Microbiological characteristics

The results from microbiological analyses of the rennet paste samples are reported in Table 4.

Salmonella and *Listeria monocytogenes*, pathogens considered to be health markers (Reg. EC 2073/2005 and Reg. EC 1441/2007), were negative by qualitative analyses. The samples were also quantitatively analyzed for *Listeria monocytogenes*. The highest count found was 39 cfu/g, which is less than the maximum count tolerated for aged cheeses (100 cfu/g). The other samples contained less counts than the detection limit of this method (10 cfu/g). The microbiological results of rennet samples were compared with the limits fixed for cheese because there are no limits for rennet.

PISANU and COSSEDDU (1982) verified that *Salmonella* and *Brucella* survived in fresh and aged *abomasa*. In aged and salted rennets, *Brucella* survived only a few hours, while *Salmonella* was resistant for a long time to low temperature (4°C). No *Salmonella* were detected in the artisanal liquid rennets extracted from *abomasa* and utilized in the process of Roncal and Feta cheeses, respectively (IRIGOYEN *et al.*, 2001; MOSCHOPOULOU *et al.*, 2007).

Table 3 - Correlation between chemical and enzymatic characteristics of artisanal rennet pastes.

	Soxhlet	Berridge	IMCU/g	ILU/g (original pH)	Dry matter %	NaCl (% dry matter)	pH
Soxhlet	1.00						
Berridge	0.85	1.00					
IMCU/g	0.78	0.79	1,00				
ILU/g	-0.57	-0.92	-0.64	1.00			
Dry matter (%)	-0.07	0.40	0.34	-0.67	1.00		
NaCl (% dry matter)	0.66	0.71	0.93	-0.62	0.29	1.00	
pH	-0.42	-0.68	-0.47	0.74	-0.73	-0.22	1.00

Table 4 - Microbiological characteristics of artisanal rennet pastes.

Sample	*Salmonella* spp. (in 25 g)	*Listeria monocytogenes* (in 25 g)	*Listeria monocytogenes* (cfu/g)	Coagulase positive staphylococci (cfu/g)	*Escherichia coli* (cfu/g)	Sulphite reducing clostridia (cfu/g)	Total mesophilic count (cfu/g)
A	negative	negative	<10	<10	<10	<10	7.2x104
B	negative	negative	3.9x10	3.9x10	<10	1.4x10	5.7x107
C	negative	negative	<10	<10	<10	<10	3.3x106
D	negative	negative	<10	60	<10	<10	<10
E	negative	negative	<10	<10	<10	5.3x103	-

Escherichia coli and coagulase positive staphylococci, which include *Staphylococcus aureus*, are considered hygienic markers (Reg. EC 2073/2005). The maximum count for coagulase positive staphylococci in cheese made from raw milk is 10^5 cfu/g. Samples with higher counts must be submitted to analysis for the detection of staphylococci enterotoxins. The maximum number found in our samples was 60 cfu/g. The *E. coli* in all samples was lower than the detection limit of the method (10 cfu/g).

Staphylococcus aureus were absent in the artisanal rennet pastes prepared in different ways and analysed at different ages (COSSEDDU and PISANU, 1980) and also in the artisanal liquid rennets (IRIGOYEN *et al.*, 2001; MOSCHOPOULOU *et al.*, 2007). CALANDRELLI *et al.* (1997) found some *Staphylococcus aureus* (<100 cfu/g) in artisanal rennet pastes.

Coliforms were not detected in the artisanal rennet pastes (DEIANA *et al.*, 1980) and *E. coli* was not found in artisanal liquid rennets (IRIGOYEN *et al.*, 2001). Some coliforms (<10 cfu/g) were found by CALANDRELLI *et al.* (1997) and BARZAGHI *et al.* (1997) in artisanal rennet pastes; the latter one was utilized in the cheese making of Provolone. In the other artisanal liquid rennets, coliforms reached a maximum of 4.0×10^2 cfu/g (MOSCHOPOULOU *et al.*, 2007).

Another group of microorganisms considered to be hygienic markers is sulphite reducing clostridia, but legislation has not established the levels admitted for food consumption. Only one sample of the examined *abomasa* contained these germs at 5.3×10^3 cfu/g. This result suggests that more attention is needed during preparation of the rennet pastes, especially if the product is vacuum-packed because of the anaerobic demands of these microorganisms. These kinds of germs were not detected in artisanal liquid rennets (IRIGOYEN *et al.*, 2001).

In our samples, the mean total mesophilic count was 2.4×10^7 cfu/g. The total mesophilic counts reported for artisanal rennet pastes vary as follows: $1.1 \times 10^3 - 2.1 \times 10^5$ (DEIANA *et al.*, 1980), $1.0 \times 10^3 - 1.9 \times 10^4$ cfu/g (CALANDRELLI *et al.*, 1997), and $8.5 \times 10^3 - 9.1 \times 10^4$ (BARZAGHI *et al.*, 1997), while in artisanal liquid rennets they are $3.9 \times 10^2 - >3.0 \times 10^5$ cfu/g and $2.4 \times 10^4 - 7.2 \times 10^4$ cfu/g (IRIGOYEN *et al.*, 2001; MOSCHOPOULOU *et al.*, 2007). Therefore, the total mesophilic count of our samples was higher compared with results from above cited studies. These microorganisms are probably contaminants that originate from the environment of the room where the rennet was treated. However, the microbial quality as well as the hygienic characteristics of artisanal rennet pastes was not affected. Our results indicate that the treatments performed on the rennets analysed did not favour the growth of pathogens and microorganisms considered to be markers of hygienic conditions.

CONCLUSIONS

From our results, along with those from other studies where *abomasa* were prepared in different ways, it can be concluded that the use of artisanal rennet pastes should not compromise the hygiene of the process of traditional cheese making. There was good coagulating and lipolytic activities in our samples, which could contribute to obtaining cheeses with typical characteristics, especially flavour characteristics. Finally, it is noteworthy that the highly variable salt content between samples due to the different management of the salting phase, that sometimes is even avoided, did not affect either the microbiological or the enzymatic characteristics of the rennets.

ACKNOWLEDGEMENTS

This work was supported by funds from the Agenzia Regionale per lo Sviluppo e l'Innovazione dell'Agricultura del Lazio, Italy (ARSIAL).

REFERENCES

Addis M., Piredda G., Pes M., Di Salvo R., Scintu M.F. and Pirisi A. 2005. Effect of the use of three different lamb paste rennets on lipolysis of the PDO Pecorino Romano Cheese. Int. Dairy J. 15: 563.

AFNOR - BIO 12/5-01/99 – Enumeration of beta-D-glucuronidase positive Escherichia coli (coli ID).

Alais C. 1984. Scienza del Latte. Tecniche Nuove. 560-561.

Barzaghi S. and Rampilli M. 1996. Valutazione delle caratteristiche enzimatiche di cagli in pasta commerciali. Sci. Tecn. Latt.-Cas. 47: 171.

Barzaghi S., Davoli E., Rampilli M. and Contarini G. 1997. La lipolisi nel formaggio Provolone: ruolo del caglio in pasta. Sci. Tecn. Latt.-Cas. 48: 146.

Bustamante M., Chavarri F., Santisteban A., Ceballos G., Hernandez I., Miguelez M.J., Aranburu I., Barron L.J.R., Virto M. and De Renobales M. 2000. Coagulating and lipolytic activities of artisanal lamb rennet pastes. J. Dairy Res. 67: 393.

Calandrelli M., Rubino R., Masoero G., Clementi F., Morone G. and Pizzillo M. 1997. Effetto della tecnica di preparazione del caglio di capretto sulle sue caratteristiche microbiologiche e sulla composizione chimica del formaggio caprino semicotto. Sci. Tecn. Latt.-Cas. 46: 343.

Commission Decision 97/284/EC establishing the list of milk-based products in respect of which Member States are authorized to grant individual or general derogations pursuant to Article 8 of Directive 92/46/EEC and the nature of the derogations applicable to the manufacture of such products.

Cosseddu A.M. and Pisanu S. 1980. Sulla presenza di stafilococchi patogeni nel latte ovino industriale e nei cagli di agnello e capretto prodotti in Sardegna. Il Latte 5: 203.

Deiana P., Farris G.A., Fatichenti F., Carini S., Lodi R. and Todesco R. 1980. Impiego di caglio di agnello e di capretto nella fabbricazione di formaggio Fiore Sardo: aspetti microbiologici e tecnologici. Il Latte 5: 191.

D'Souza T.M. and Oriel P. 1992. Purification and characterization of lamb pregastric lipase. Appl. Biochem. Biotechn., 36: 183.

Ferri B. 2007. Caratteristiche chimiche ed enzimatiche del caglio in pasta artigianale d'agnello in relazione alle tecniche di produzione. Tesi di laurea.

Food Chemical Codex. 1981. Lipase/esterase for stomach activity, p. 493. Washington, DC.

Fulco A., Candido A., Nuccio M.G. and Imbrò M. 1987. Caratteristiche del caglio di agnello in pasta impiegato nelle produzioni casearie tipiche siciliane. Sci. Tecn. Latt.-Cas. 38: 146.

IDF Standard 110B: 1997. Calf rennet and Adult Bovine rennet – Determination of chymosin and bovine pepsin contents (chromatographic method).

ISO 23058 IDF 199: 2006. Milk and milk products – Ovine and caprine rennets – Determination of total milk – clotting activity.

Irigoyen, A., Izco, J.M., Ibàñez, F.C. and Torre P. 2001. Influence of rennet milk clotting activity on the proteolytic, and sensory characteristics of an ovine cheese. Food Chem. 72: 137.

ISO 6888-2:1999 - Microbiology of food and animal feeling stuff - Horizzontal method for the enumeration of coagulase-positive staphilococci - Part 2: Technique using rabbit plasma Fibrinogen agar medium.

ISO 6579:2002 - Microbiology of food and animal feeding stuffs -- Horizontal method for the detection of Salmonella spp.

ISO 4833 - 2003 - Microbiology of food and animal feeding stuffs - Microbiology of food and animal feeding stuffs - Horizontal method for the enumeration of microorganisms - Colony-count technique at 30°C.

ISO 15213:2003 – Microbiology of food and animal feeding stuffs – Horizontal method for the enumeration of sulfite-reducing bacteria growing under anaerobic conditions

MAF. 1986. D.M. 21/4. Official methods for cheese analyses. G.U. n. 229, 2 ottobre 1986.

Moatsou G., Moschopoulou E., Georgala A., Zoidou E., Kandarakis I., Kaminarides S. and Anifantakis E. 2004. Effect of artisanal liquid rennet from kids and lambs abomasa on the characteristics of Feta cheese. Food Chem. 72: 237.

Moschopoulou E., Kandarakis I. and Anifantakis E. 2007. Characteristics of lamb and kid artisanal liquid rennet used for traditional Feta cheese manufacture. Small Rum. Res. 72: 237.

Nelson J.H., Jensen R.G. and Pitas R.E. 1977. Pregastric esterase and other oral lipases. J. Dairy Sci., 60: 327.

Pettinau M., Nuvoli G., Podda F. and Ledda A. 1977. Influenza del metodo di preparazione del caglio di agnello sulla conservazione delle proprietà coagulanti, proteolotiche e lipolitiche. Sci. Tecn. Latt.-Cas. 28: 101.

Piredda G. and Addis M. 1998. Influenza dell'alimentazione sulle caratteristiche enzimatiche del caglio in pasta d'agnello. Sci. Tecn. Latt.-Cas. 49: 129.

Pisanu S. and Cosseddu A.M. 1982. Sopravvivenza di "Salmonella abortus ovis" e Brucella melitensis" nel caglio di capretto. Il Latte 7: 729.

Rossano R., D'Ambrosio A., Ferrara V., D'Elia A., Pizzillo M. and Riccio P. 2003. Influence of diet and age of kids on enzymatic activities of kid rennet pastes. Ital. J. Food Sci. 15: 585.

Salvadori Del Prato O. 2001. Trattato di Tecnologia Casearia. Calderini Edagricole.

Savini E. 1946. Analisi del Latte e dei Latticini. Ulrico Hoepli. Milano.

UNI EN ISO 11290-1:2005 - Horizontal method for searching and enumeration of Listeria monocytogenes - Part 1: Method for the research.

UNI EN ISO 11290-2:2005 - Microbiology of food and feed stuffs - Horizontal method for searching and enumeration of Listeria monocytogenes - Method for counting.

Woo A.H., Kollodge S. and Lindsay R.C. 1984. Quantification of major free fatty acids in several cheese varieties. J. Dairy Sci. 67: 874.

Received paper March 2, 2011 Accepted September 27, 2011

DIETARY HABITS OF CHILDREN LIVING IN THE ITALIAN AREA OF VERSILIA

D. FIORINI*, S. PERSEO and R. BALLINI
School of Science and Technology, Chemistry Division, University of Camerino,
Via Sant'Agostino 1, 62032 Camerino (MC), Italy
*Corresponding author: Tel. +39 0737402254, +39 0737402297,
email: dennis.fiorini@unicam.it

ABSTRACT

This study evaluates the implementation of the Italian dietary guidelines in children's diets. The results indicate only 52% of children in primary school eat breakfast daily, 92% in nursery school and 77% in middle school; 28-46% have a snack daily in the morning as compared to 50-81% in the afternoon. The consumption frequency of fruit as a snack or for breakfast is very low, with a maximum of 12% consuming fruit everyday. During lunch and dinner, consumption frequencies of vegetables are very low: 15-17% never eat vegetables. Furthermore, 7-14% of children never eat fish. More efforts are needed to educate children and induce them to adopt healthier dietary behaviours in order to prevent and to fight against childhood obesity and related diseases.

- Key words: children's diet, obesity, breakfast frequency, snack frequency -

INTRODUCTION

The problem of childhood obesity has received growing attention due to its spread in industrialised countries and to its recognised links to adult pathologies such as cardiovascular and digestive diseases, hypertension, and hyperlipidaemia (DEHGHAN *et al.*, 2005). Italy is among the European countries having the highest percentages of overweight and obese children (LOBSTEIN and FRELUT, 2003). Thus, in order to impact their dietary choices at an early stage, when diet plays a fundamental role in preventing obesity and related diseases (WASHINGTON, 2008; BURNIAT *et al.*, 2002; LUEPKER *et al.*, 1998; ALLAN, 1992), it is necessary to know the actual favourite meals and eating habits of children.

In this study, a survey was administered to the children attending nursery, primary and middle schools of the municipality of Forte dei Marmi (Tuscany, Italy) in order to ascertain the eating habits and possible dietary mistakes of the children residing there. Forte dei Marmi is a seaside town with high level of fish consumption. Moreover, it is located in a wealthy area where population has a high standard of living compared to other Italian seaside areas. Data presented in this study should, therefore, be considered as representative of areas with similar characteristics. Due to the lack of a comprehensive point of reference for Europe, the Italian dietary guidelines (IDG) were utilized (Italian Institute for Research in Food and Nutrition, INRAN, 2003) as a model for a balanced and healthy diet. Apropos of this, the European Food Safety Authority (EFSA) Panel on Dietetic Products, Nutrition, and Allergies has recently stated that establishing detailed and effective food-based dietary guidelines to be used at the EU level is not feasible (EFSA Panel on Dietetic Products, Nutrition, and Allergies, 2010).

The main dietary mistakes made by children, as described in the IDG, are considered in this study, as reported herein below:

- Children do not eat a healthy breakfast or do not eat breakfast at all;
- Children do not have morning and afternoon snacks or they consume only appealing foods, typically lacking good nutritional value;
- Children do not consume enough fruit and vegetables during lunch and dinner;
- Children frequently consume unhealthy, fatty, or sweetened foods such as sausages, chocolate, snack cakes, chips, sweets, and soda drinks;
- Children often consume "fast food" meals which are highly caloric and contain *trans* fatty acids, salt, sugars and low levels of dietary fiber and vitamins.

Table 1 - Weekly consumption frequency (d/w) percentages of breakfast, morning, and afternoon snack by children in nursery (N), primary (PS) and middle school (MS).

	Frequency (d/w)	N (%)	PS (%)	MS (%)
Breakfast	7	92	52	77
	4-6	4	37	12
	2-3	3	8	10
	0-1	1	3	1
Morning snack	7	28	33	46
	4-6	26	46	36
	2-3	22	17	12
	0-1	24	4	6
Afternoon snack	7	81	50	65
	4-6	15	40	29
	2-3	3	8	5
	0-1	1	2	1

MATERIALS AND METHODS

Once the project was approved by the schools involved and the city council and the parents were informed, anonymous questionnaires about the dietary behaviour of children were administrated to the parents of children from nursery, primary and middle schools. The study surveyed 932 children aged 3-14 years (203 in nursery school, 392 in primary school and 337 in middle school) living in the municipality of Forte dei Marmi (Tuscany, Italy). Two questionnaires were administrated. The first one investigated habits regarding breakfast, mid-morning and afternoon snacks, examining the weekly consumption frequency of each meal (Table 1), the food and beverages consumed during the meals (within a given list as indicated in Table 2) and the weekly consumption frequency of each food and drink. The second dealt with food and drinks usually consumed for lunch and dinner (Tables 3 and 4), investigating the weekly consumption frequency of each food (in a given list) and the consumption distribution between lunch and dinner. Consumption frequencies of sweets and chewing-gums and habits concerning the consumption of beverages were also surveyed (Table 5). The questionnaires were planned with paediatricians, teachers and dieticians. The children's parents filled them out (with the help of their children).

RESULTS

The first questionnaire regarding breakfast, morning and afternoon snacks, was filled out by 57, 76 and 52% of the parents interviewed, respectively for children attending nursery, primary, and middle school. The second questionnaire, concerning questions about the main

Table 2 - Weekly consumption frequency (Freq., d/w) percentages of foods for breakfast (B), morning snack (Msn), and afternoon snack (Asn) by children in nursery (N), primary (PS) and middle school (MS).

Food		Freq. (d/w)	N (%)			PS (%)			MS (%)		
			B	Msn	Asn	B	Msn	Asn	B	Msn	Asn
Milk		7	70	1	8	38	1	4	55	3	5
		4-6	11	0	5	33	1	5	13	2	8
		2-3	11	2	8	12	1	7	19	2	12
		0-1	8	97	79	17	97	84	13	93	75
with	Coffee	7	3	n.a.	n.a.	5	n.a.	n.a.	11	n.a.	n.a.
		4-6	0	n.a.	n.a.	2	n.a.	n.a.	3	n.a.	n.a.
		2-3	1	n.a.	n.a.	2	n.a.	n.a.	9	n.a.	n.a.
		0-1	96	n.a.	n.a.	91	n.a.	n.a.	77	n.a.	n.a.
	Barley coffee	7	2	n.a.	n.a.	3	n.a.	n.a.	5	n.a.	n.a.
		4-6	1	n.a.	n.a.	3	n.a.	n.a.	2	n.a.	n.a.
		2-3	0	n.a.	n.a.	2	n.a.	n.a.	2	n.a.	n.a.
		0-1	97	n.a.	n.a.	92	n.a.	n.a.	91	n.a.	n.a.
	Cacao	7	14	2	0	10	1	1	16	2	2
		4-6	5	1	0	9	0	2	6	2	3
		2-3	10	1	5	9	1	2	12	2	6
		0-1	71	96	95	72	98	95	66	94	89
	Sugar	7	11	n.a.	n.a.	17	n.a.	n.a.	20	n.a.	n.a.
		4-6	2	n.a.	n.a.	8	n.a.	n.a.	7	n.a.	n.a.
		2-3	4	n.a.	n.a.	4	n.a.	n.a.	11	n.a.	n.a.
		0-1	83	n.a.	n.a.	71	n.a.	n.a.	62	n.a.	n.a.
Fresh fruit		7	1	10	9	2	3	4	5	1	8
		4-6	3	20	11	1	3	5	3	3	8
		2-3	6	16	23	2	7	13	9	11	21
		0-1	90	54	57	95	87	78	83	85	63
Fresh fruit juice		7	1	n.a.	n.a.	4	n.a.	n.a.	3	n.a.	n.a.
		4-6	1	n.a.	n.a.	0	n.a.	n.a.	6	n.a.	n.a.
		2-3	7	n.a.	n.a.	5	n.a.	n.a.	14	n.a.	n.a.
		0-1	91	n.a.	n.a.	91	n.a.	n.a.	77	n.a.	n.a.
Packaged fruit juice		7	4	4	13	5	8	8	3	3	9
		4-6	2	4	9	2	7	7	7	3	7
		2-3	12	9	23	7	11	15	10	11	17
		0-1	82	83	55	86	74	70	80	83	67
Milk shake		7	0	n.a.	n.a.	1	n.a.	n.a.	1	n.a.	n.a.
		4-6	0	n.a.	n.a.	0	n.a.	n.a.	2	n.a.	n.a.
		2-3	1	n.a.	n.a.	1	n.a.	n.a.	1	n.a.	n.a.
		0-1	99	n.a.	n.a.	98	n.a.	n.a.	96	n.a.	n.a.
Yoghurt		7	3	2	7	3	2	5	5	1	4
		4-6	4	4	7	3	3	4	3	1	9
		2-3	14	10	33	10	4	19	11	3	26
		0-1	79	84	53	84	91	72	81	95	61
Ice cream		7	n.a.	n.a.	1	n.a.	n.a.	5	n.a.	n.a.	5
		4-6	n.a.	n.a.	4	n.a.	n.a.	2	n.a.	n.a.	8
		2-3	n.a.	n.a.	33	n.a.	n.a.	24	n.a.	n.a.	28
		0-1	n.a.	n.a.	62	n.a.	n.a.	69	n.a.	n.a.	59
Cereals		7	9	0	3	9	1	5	20	2	3
		4-6	8	0	7	17	3	1	12	6	7
		2-3	22	3	16	18	9	8	23	19	16
		0-1	61	97	74	56	87	86	45	73	74
Biscuits		7	33	2	6	19	2	3	24	3	5
		4-6	15	6	7	19	3	3	14	6	8
		2-3	26	20	36	20	9	16	24	9	16
		0-1	26	72	51	42	86	78	38	82	71
Biscuit bread		7	2	0	1	3	3	2	4	1	1
		4-6	1	0	0	4	0	1	4	1	2
		2-3	10	4	9	9	1	3	15	3	4
		0-1	87	96	90	84	96	94	77	95	93
Crackers		7	0	2	2	2	4	2	3	6	2
		4-6	0	2	3	1	8	2	1	11	3
		2-3	6	11	16	2	16	9	7	16	18
		0-1	94	85	79	95	73	87	89	67	77
Croissant		7	1	0	0	2	3	3	5	2	2
		4-6	0	0	0	1	2	0	4	2	2
		2-3	7	3	3	9	3	4	11	4	5
		0-1	92	97	97	88	92	93	80	92	91

(continues)

Food		Freq. (d/w)	N (%)			PS (%)			MS (%)		
			B	Msn	Asn	B	Msn	Asn	B	Msn	Asn
Snack cakes		7	1	3	2	4	6	5	4	9	3
		4-6	4	3	3	3	9	6	5	10	3
		2-3	12	2	19	7	16	12	10	18	21
		0-1	83	92	76	86	69	77	81	63	73
Chocolate snacks		7	n.a.	0	1	n.a.	1	5	n.a.	2	3
		4-6	n.a.	0	4	n.a.	3	1	n.a.	6	7
		2-3	n.a.	3	10	n.a.	9	8	n.a.	19	16
		0-1	n.a.	97	85	n.a.	87	86	n.a.	73	74
Home-made cakes		7	0	1	1	5	3	4	3	1	3
		4-6	0	0	0	1	2	1	5	3	3
		2-3	9	2	15	6	3	9	9	2	13
		0-1	91	97	84	88	92	86	83	94	81
Pizza		7	1	1	1	2	6	12	3	9	5
		4-6	0	4	4	1	15	6	2	12	5
		2-3	5	15	24	2	10	19	6	11	25
		0-1	94	80	71	95	69	63	89	68	65
Chips		7	n.a.	0	0	n.a.	1	2	n.a.	3	5
		4-6	n.a.	0	0	n.a.	0	1	n.a.	1	2
		2-3	n.a.	0	7	n.a.	4	6	n.a.	7	7
		0-1	n.a.	100	93	n.a.	95	91	n.a.	89	86
Cola drinks		7	n.a.	0	1	n.a.	1	2	n.a.	2	4
		4-6	n.a.	0	1	n.a.	0	2	n.a.	1	5
		2-3	n.a.	3	8	n.a.	2	7	n.a.	3	10
		0-1	n.a.	97	90	n.a.	97	89	n.a.	94	81
Tea		7	n.a.	2	2	n.a.	6	3	n.a.	5	5
		4-6	n.a.	0	1	n.a.	6	4	n.a.	4	7
		2-3	n.a.	2	9	n.a.	6	10	n.a.	9	17
		0-1	n.a.	96	88	n.a.	82	83	n.a.	82	71
Bread		7	2	n.a.	n.a.	4	n.a.	n.a.	5	n.a.	n.a.
		4-6	2	n.a.	n.a.	2	n.a.	n.a.	3	n.a.	n.a.
		2-3	7	n.a.	n.a.	6	n.a.	n.a.	0	n.a.	n.a.
		0-1	89	n.a.	n.a.	88	n.a.	n.a.	82	n.a.	n.a.
with	Butter	7	1	n.a.	n.a.	2	n.a.	n.a.	2	n.a.	n.a.
		4-6	1	n.a.	n.a.	1	n.a.	n.a.	2	n.a.	n.a.
		2-3	3	n.a.	n.a.	5	n.a.	n.a.	4	n.a.	n.a.
		0-1	95	n.a.	n.a.	92	n.a.	n.a.	92	n.a.	n.a.
	Jam	7	2	0	0	4	1	2	2	1	2
		4-6	3	2	2	2	1	1	3	1	1
		2-3	11	1	16	9	1	5	13	3	7
		0-1	84	97	82	85	97	92	82	95	90
	Chocolate	7	1	1	1	2	1	3	3	3	3
		4-6	2	0	2	1	2	4	5	3	11
		2-3	8	2	11	9	3	14	12	8	25
		0-1	89	97	86	88	94	79	80	86	61
	Honey	7	9	n.a.	n.a.	5	n.a.	n.a.	5	n.a.	n.a.
		4-6	3	n.a.	n.a.	3	n.a.	n.a.	2	n.a.	n.a.
		2-3	5	n.a.	n.a.	4	n.a.	n.a.	6	n.a.	n.a.
		0-1	83	n.a.	n.a.	88	n.a.	n.a.	87	n.a.	n.a.
	Ham	7	n.a.	1	3	n.a.	4	7	n.a.	8	10
		4-6	n.a.	1	1	n.a.	7	9	n.a.	7	9
		2-3	n.a.	3	24	n.a.	10	21	n.a.	17	30
		0-1	n.a.	95	72	n.a.	79	63	n.a.	68	51
	Sausages	7	n.a.	1	0	n.a.	3	2	n.a.	2	3
		4-6	n.a.	0	0	n.a.	3	1	n.a.	2	3
		2-3	n.a.	1	3	n.a.	2	6	n.a.	4	11
		0-1	n.a.	98	97	n.a.	92	91	n.a.	92	83
	Cheese	7	n.a.	1	0	n.a.	1	4	n.a.	2	2
		4-6	n.a.	0	0	n.a.	1	1	n.a.	2	2
		2-3	n.a.	1	4	n.a.	1	5	n.a.	3	9
		0-1	n.a.	98	96	n.a.	97	90	n.a.	93	87
Else		7	2	n.a.	n.a.	3	n.a.	n.a.	3	n.a.	n.a.
		4-6	1	n.a.	n.a.	2	n.a.	n.a.	2	n.a.	n.a.
		2-3	3	n.a.	n.a.	4	n.a.	n.a.	2	n.a.	n.a.
		0-1	94	n.a.	n.a.	91	n.a.	n.a.	93	n.a.	n.a.

n.a.: not asked.

meals (lunch and dinner), beverages, sweets, and chewing-gum, was answered by 45, 53 and 41% of the parents interviewed, respectively for children attending nursery, primary, and middle school. Tables 1-5 report the answers for each questionnaire as percentages calculated based on the number of questionnaires correctly filled in.

Table 3 - Weekly consumption frequency (d/w) percentages of the various food categories during lunch and/or dinner by children in nursery (N), primary (PS) and middle school (MS).

Food	Frequency (d/w)	N (%)	PS (%)	MS (%)
Pasta	7	61	58	51
	4-6	32	33	40
	1-3	7	9	8
	0	0	0	1
Bread	7	57	55	51
	4-6	18	20	17
	1-3	20	20	28
	0	5	5	4
Soup	7	1	2	1
	4-6	7	7	10
	1-3	80	78	69
	0	12	13	20
Potatoes	7	0	1	1
	4-6	4	8	6
	1-3	95	85	85
	0	1	6	8
Legumes	7	1	1	1
	4-6	7	6	10
	1-3	71	60	61
	0	22	33	28
Meat	7	1	8	11
	4-6	40	44	53
	1-3	57	49	34
	0	2	1	2
Fish	7	0	1	1
	4-6	5	4	12
	1-3	88	77	73
	0	7	18	14
Eggs	7	0	1	0
	4-6	1	3	5
	1-3	80	87	82
	0	19	9	13
Cheese	7	3	6	12
	4-6	21	12	23
	1-3	62	67	46
	0	14	15	19
Sausages	7	2	5	9
	4-6	11	24	27
	1-3	75	63	55
	0	12	8	9
Vegetables	7	34	18	19
	4-6	18	29	22
	1-3	33	37	42
	0	15	16	17
Fruit	7	53	37	43
	4-6	26	26	30
	1-3	13	28	17
	0	8	9	10

Table 4 - Consumption distribution percentage of the various food categories during lunch and dinner in nursery (N), primary (PS) and middle school (MS).

Food	Frequency	N (%)	PS (%)	MS (%)
Pasta	never	0	0	1
	lunch	56	61	64
	dinner	5	7	9
	lunch and dinner	39	32	26
Bread	never	5	5	4
	lunch	16	17	23
	dinner	13	15	17
	lunch and dinner	66	63	56
Soup	never	12	13	20
	lunch	20	21	17
	dinner	35	40	45
	lunch and dinner	33	26	18
Potatoes	never	1	6	8
	lunch	24	17	14
	dinner	38	46	52
	lunch and dinner	37	31	26
Legumes	never	22	33	28
	lunch	18	13	16
	dinner	26	28	35
	lunch and dinner	34	26	21
Meat	never	2	1	2
	lunch	10	9	14
	dinner	37	40	35
	lunch and dinner	51	50	49
Fish	never	7	18	14
	lunch	11	12	14
	dinner	33	31	32
	lunch and dinner	49	39	40
Eggs	never	18	9	13
	lunch	13	21	23
	dinner	34	40	35
	lunch and dinner	35	30	29
Cheese	never	14	15	19
	lunch	3	10	9
	dinner	26	29	24
	lunch and dinner	57	46	48
Sausages	never	12	8	9
	lunch	13	14	22
	dinner	24	26	21
	lunch and dinner	51	52	48
Vegetables	never	15	16	17
	lunch	3	7	6
	dinner	15	22	15
	lunch and dinner	67	55	62
Fruit	never	8	9	10
	lunch	12	12	13
	dinner	8	7	9
	lunch and dinner	72	72	68

Table 5 - Consumption of beverages and sweets during the day by children in nursery (N), primary (PS), and middle school (MS).

Food	Frequency	N (%)	PS (%)	MS (%)
Soda drinks	never	42	40	27
	during meals	28	30	28
	out of meals	30	30	41
	during and out of meals	0	1	4
Tap water	never	84	78	64
	during meals	2	6	11
	out of meals	5	8	15
	during and out of meals	9	8	10
Bottled water	never	5	1	5
	during meals	16	28	37
	out of meals	11	9	9
	during and out of meals	68	62	49
Sweets	never	19	21	18
	once	40	46	37
	twice	23	19	20
	more than twice	18	14	25

Breakfast

Breakfast is considered an essential part of the daily diet since it comes many hours after the previous meal and substantially contributes to daily nutrient intake and energy needs. Literature widely reports that skipping breakfast has adverse effects on the academic performance of children (MURPHY *et al.*, 1998; POLLITT and MATHEWS, 1988; WYON *et al.*, 1997). Moreover several cross-sectional studies demonstrate that skipping breakfast may lead to weight gain in children (BERKEY *et al.*, 2003). As can be seen in Table 1, the percentage of children who eat breakfast daily or almost everyday (4-7 days/week) decreases significantly from 96% in nursery school to 89% in primary and middle school. Moreover it is noteworthy to report that in primary school only 52% of the children eat breakfast everyday, compared to 92% in nursery school and 77% in middle school. It is clear that children aged 6 to 11 should be encouraged to eat breakfast more frequently.

Milk and yoghurt

Fig. 1 illustrates the foods most frequently consumed (4-7 days/week) for breakfast, expressed as percentages based on the total of the responses from each school. The consumption frequency of milk strongly decreases from nursery school (81%) to primary and middle school where consumption is about the same, 71 and 68% respectively. 70% of the children in nursery school drink milk everyday, while only 55% in middle school and only 38% in primary school.

Yoghurt is a valid alternative to milk as a source of calcium, and, furthermore, its lactic bacteria and low lactose content make it easily digestible (DEWITT *et al.*, 1988). Moreover the market offers many flavoured yoghurt which children may like more. Nevertheless, the consumption of yoghurt is quite low and about the same in each group, independent of age (Table 2, Fig. 1). The consumption of milk shakes is negligible, and other milk derivatives are not consumed for breakfast. It is clear that it would be very difficult to reach the daily recommended intakes of calcium consuming only milk, thus it is very important to recommend the consumption of other foods containing appreciable amounts of calcium, like cheese, or vegetables like beans, broccoli, and artichokes, or dried fruit, like figs, almond, and hazelnuts.

Starchy foods

Among the starchy foods consumed for breakfast, cereals, bread, biscuit bread and home baked cakes should be preferred over croissants, snack cakes, biscuits, and pizza which usually have a higher content of low quality fat (higher amounts of *trans* and saturated fatty acids). Among the children interviewed, biscuits are the starchy food most frequently consumed (Fig. 1), followed by cereals. While the consumption frequency of biscuits decreases from nursery to primary and middle school, the consumption of cereals follows the opposite trend. Among the other starchy foods consumed 4-7 days/week, it can be seen that in middle school, croissants, snack cakes, biscuit bread, home baked cakes, and bread with chocolate cream are consumed by similar percentages of children (8-9%). This is followed by pizza, which is consumed only by 5%. Thus, there is no preference for healthier food, such as bread with jam or home baked cakes. In primary school, after biscuits and cereals, the highest percentages of children consume bread with honey, biscuit bread, snack cakes, home baked cakes, bread with jam 4-7 days/week and, with lower percentages, pizza and croissants. In nursery school, after biscuits and cereals, bread with honey is consumed 4-7 days/week by 12% of children while the other starchy foods are consumed 4-7 days/week only by a percentage of children lower than 6%.

Considering the most fatty foods, croissants and pizza, which are often prepared with fats high in saturated fatty acids, their consumption frequency is almost negligible in nursery school, and very low in primary school, while it increases to significant percentages in middle school, demonstrating that children need to directed towards a healthy diet also when they grow up and have more freedom in their choices.

Fruit

Consumption of fruit (fresh fruit, fresh fruit juices, and packaged fruit juices) for breakfast is low, the percentage of children consuming this kind of food or beverages everyday or almost everyday is lower than 12%. The con-

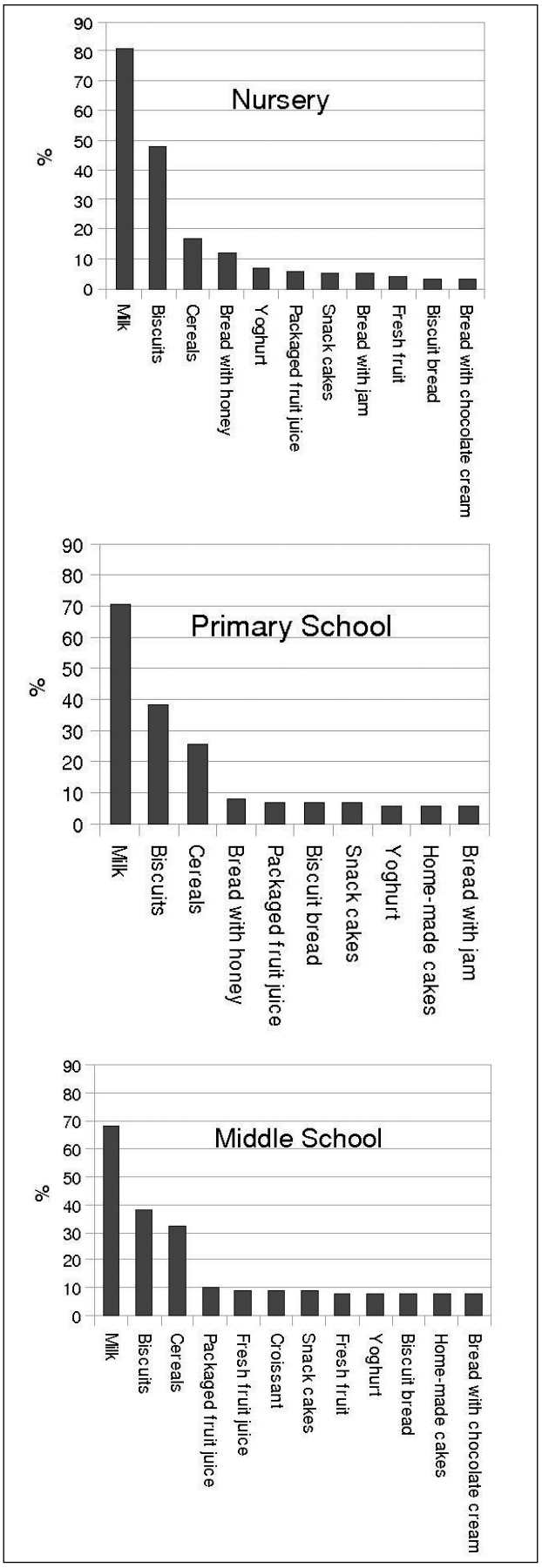


Fig. 1 - Top ten foods most frequently consumed for breakfast 4-7 days/week by children in nursery, primary and middle school.

sumption of fresh fruit should be preferred due to its higher amount of vitamins and fibre with respect to packaged fruit juices, which contain only 20-70% of the given fruit. The highest percentage of children that consume fresh fruit and fresh fruit juice everyday or almost everyday is in middle school, while the lowest percentages of fruit consumption frequency is in primary school children.

Morning and afternoon snacks

Insufficient intake of some nutrients is often found in children around twelve years of age since their nutrient requirements per body weight unit is higher with respect to adult nutrient requirements, and it is difficult to cover the nutrient requirements with only two or three meals a day. For this reason, two healthy snacks are recommended during the day (morning and afternoon snacks) between the three main meals (breakfast, lunch and dinner). These snacks should be balanced and such that they do not compromise the appetite during lunch or dinner (INRAN, 2003). Table 1 shows that the habit of having a morning snack increases as children get older. 46% of middle school children have a morning snack daily, compared to 33% of primary school children and 28% in nursery school. The trend is less defined for the afternoon snack, but the habit of having an afternoon snack is much more widespread independent of age. While the percentage of children in nursery school consuming a morning snack everyday is the lowest, the percentage of those that have an afternoon snack everyday is the highest. This may be due to the fact that children in nursery school usually eat less during lunch and dinner with respect to older children, and, thus, they feel the need more for an afternoon snack. Nevertheless, while this habit of children in nursery school is appreciable, their weekly frequency of morning snack consumption is clearly too low, considering that children should always have one.

In nursery school, the foods most frequently (4-7 days/week) consumed for morning snack are: fresh fruit (30%), biscuits (8%), packaged fruit juice (8%), yoghurt (6%), snack cakes (6%) and pizza (5%) (Fig. 2). The healthy habit of eating fresh fruit has the highest frequency, while it is quite lower among children in primary and middle school. It is also noteworthy that packaged fruit juice and yoghurt are also often consumed even if their percentages follow that of biscuits. In primary school, the foods most frequently consumed (4-7 days/week) for morning snacks are: pizza (21%), snack cakes (15%), packaged fruit juice (15%), crackers (12%), tea (12%) and bread with ham (11%). In this case, fatty foods are largely predominant in the diet and that starchy foods are the favourite. In middle school the foods most frequently consumed

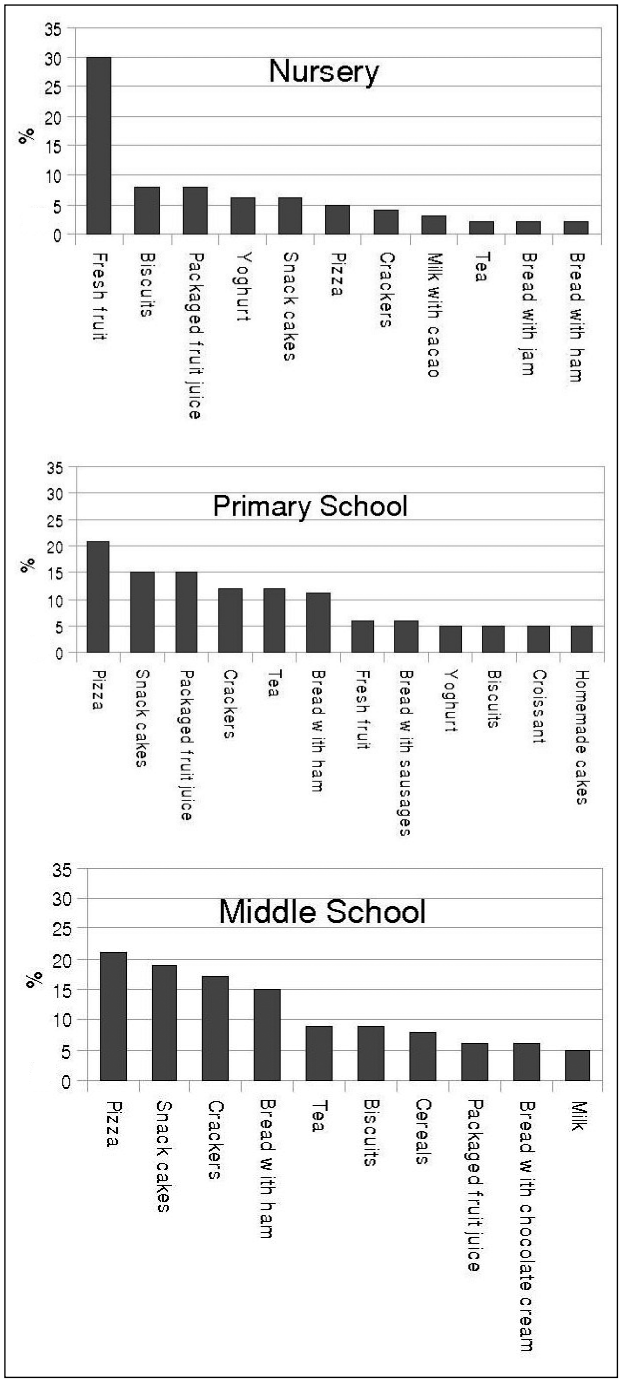


Fig. 2 - Top ten foods most frequently consumed as a morning snack 4-7 days/week by children in nursery, primary and middle school.

are: pizza (21%), snack cakes (19%), crackers (17%), bread with ham (15%) and biscuits (9%). Also in this case, first place belong to fatty and starchy foods, while the consumption frequencies of fruit and milk derivatives are very low.

For the afternoon snack, the foods most frequently consumed in nursery school are in descending order: packaged fruit juice, fresh fruit, yoghurt, milk, biscuits and cereals; in primary school they are: pizza, bread with ham, packaged fruit juice, snack cakes, milk, fresh fruit and yoghurt; in middle school: bread with ham, packaged fruit juice, fresh fruit, milk, yoghurt, ice cream and biscuits (Fig. 3).

The worst dietary habits are in primary school both in the morning and in the afternoon snack, where the consumption of pizza and snack cakes is more frequent than that of milk, fresh fruit and yoghurt. Even if the diet should be modified in primary school, in middle school and nursery school, afternoon snacks are much healthier, as the most frequently consumed snacks are fresh fruit, yoghurt, bread with ham and packaged fruit juice.

Lunch and dinner

Table 3 reports the consumption frequencies of the main food categories for lunch and dinner for children in nursery, primary and middle School. The consumption of fruit and vegetables is highly recommended due to their content of precious nutrients such as vitamins and minerals and their dietary fibre fraction which also plays an important role in helping with constipation, haemorrhoids and diverticular disease. Furthermore some studies demonstrate its role in the treatment and prevention of obesity (KIMM, 1995; LIU *et al.*, 2003). Regarding the consumption of vegetables during the main meals, it is alarming that 15, 16, and 17% of children respectively in nursery, primary, and middle school, never eat vegetables. These values are lower for fruit consumption (around 10%), but still too high, since they should be null if possible. The highest consumption frequencies of fruit and vegetables are among children in nursery school: 79% of them eat fruit 4-7 times a week and 52% of them eat vegetables 4-7 times a week. The consumption of legumes is also very low. Regarding the consumption of fish, the Italian Society for Human Nutrition (SINU) suggests eating fish at least 2-3 times a week (SINU, 1996). Only 58% of children in nursery school eat fish 1-3 times a week, while in primary and middle school, 77 and 73% of the children eat fish 1-3 times a week, respectively. Moreover the percentages of children who never eat fish are very high, above all in primary (14%) and middle school (18%). As expected, since it is typical of the Italian diet, the consumption frequency of pasta and bread is very high.

Table 4 provides data about the distribution of the various foods during the main meals. Pasta is the only food which is preferably eaten during one of the two main meals (lunch) by more than 55% of the children interviewed in each school, while the remaining percentage is almost completely represented by children who eat it both for lunch and for dinner. Bread is consumed by about the same percentage of children only for lunch or only for dinner and by the largest percentage (56-63%) both for lunch and for dinner. The percentage

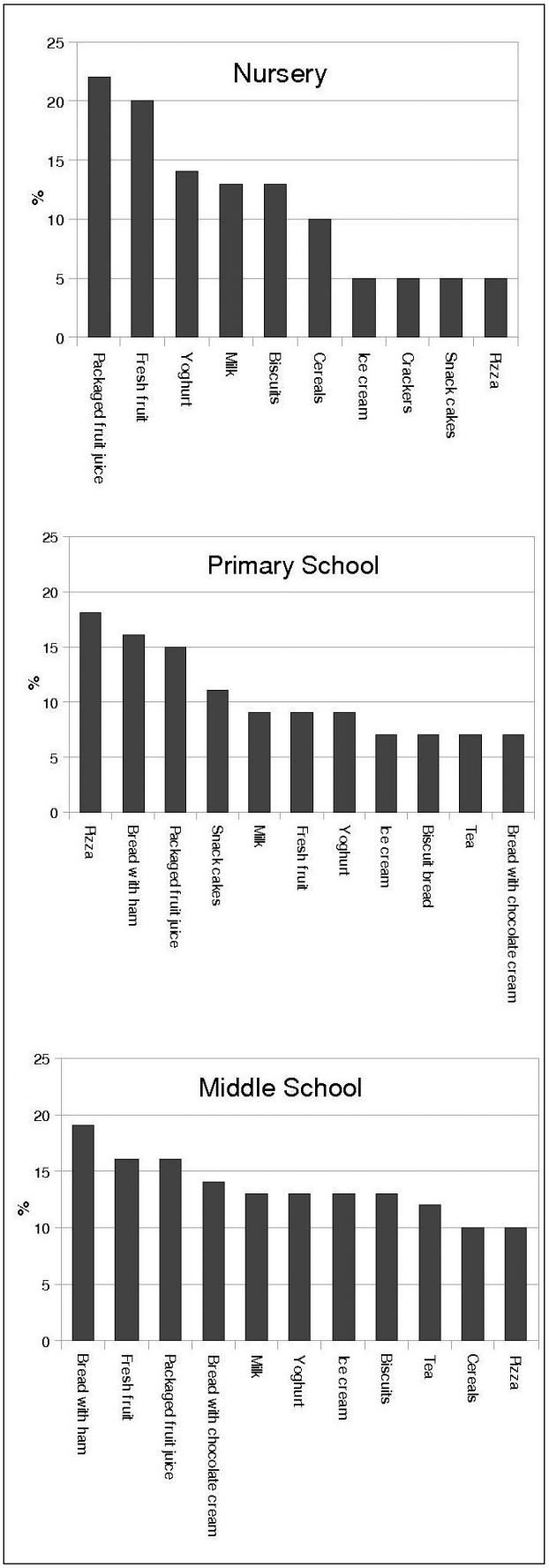


Fig. 3 - Top ten foods most frequently consumed as an afternoon snack 4-7 days/week children in nursery, primary and middle school.

of children who consume high protein foods, like meat, fish, eggs, and cheese only for dinner is significant and always higher than that of children who consume high protein foods only for lunch, with the sole exception of the consumption of sausages by children in middle school, which is about the same for lunch and for dinner. Fruit, vegetables, and bread are the only foods which are indistinctly eaten both for lunch and for dinner by the large majority of children, and the remaining children consume these kinds of food only for lunch or only for dinner with similar percentages.

Consumption of beverages and sweets

Among beverages consumed during snack breaks, children more frequently drink in descending order: packaged fruit juice, tea and soda drinks (Table 2). Soda drink consumption is approximately equally divided between children who drink them only during meals and those who drink them only between meals. Regarding the consumption of water, a large majority of children interviewed drink bottled water in preference to tap water (Table 5). The consumption of sweets is very widespread among children, with only 19, 21 and 18% who never eat sweets in nursery, in primary and middle school, respectively.

CONCLUSIONS

Taking into consideration the main dietary mistakes made by children, as reported in the IDG, this survey highlights that the percentage of children interviewed who do not eat breakfast everyday is very high in primary (48%) and middle school (23%), while in nursery school it is much lower (8%). For the morning snack, the percentages of children who eat one everyday is low independent of age, with 28, 33 and 47% in nursery, primary and middle school, respectively. For the afternoon snack, the percentages are significantly higher (50-81%). These results suggest that more efforts are needed to increase the consumption frequencies of the morning snack and this could be facilitated by the school personnel where kids can be taught about the importance of a morning snack and where teachers have the direct control over the children's activities and the meals they eat during the break.

Among the starchy foods eaten for breakfast, biscuits are largely preferred, but the consumption frequency of cereals is also high. The consumption of fruit (fresh fruit, fresh fruit juice or even packaged fruit juices) is too low. Referring to the quality of the foods consumed in the morning and the afternoon snacks, it is evident that the worst dietary habits are among children in primary school, where the foods consumed

most frequently are pizza and snack cakes, and the consumption of fruit and yoghurt is almost negligible.

Concerning the main meals, the data obtained show that the consumption of fruit, vegetables, and legumes is too low independent of the age of the children interviewed and that the consumption frequencies of fish are low above all among children in nursery, where the intake of the precious nutrients typically provided by fish is particularly important.

The results obtained through this study constitute a basis for the fundamental activity of promoting food education among children and their families. The main actors of this project are: i) the school personnel, who can direct children towards the right dietary choices, providing them with the fundamentals of food and nutrition science; ii) the children's parents, who could give direct examples of nutritionally balanced and varied diets, teach them how to read labels, direct them towards child-friendly meals and follow the basic principles of hygiene for storing foods at home and use the cooking methods most suitable for children; iii) local and national media, which can help children, parents and teachers by playing a supporting role in food education.

ACKNOWLEDGEMENTS

The authors are grateful to the Municipality of Forte dei Marmi (Italy) for collaboration. The authors acknowledge the Ministero Istruzione Università Ricerca (MIUR) (Italy) and the University of Camerino (Italy) for financial support.

REFERENCES

Allan R. 1992. Is coronary heart disease a lifestyle disorder? A review of psychologic and behavioural factors (Part I). Cardiovascular Reviews & Reports. 13:13.

Berkey C.S., Rockett H.R.H., Gillman M.W., Field A.E. and Colditz G.A. 2003. Longitudinal study of skipping breakfast and weightchange in adolescents. Int. J. Obesity 27: 1258.

Burniat W., Cole T., Lissau I. and Poskitt E. 2002. Child and adolescent obesity. Cambridge University Press ISBN 0521 652375

Dehghan M., Akhtar-Danesh N. and Merchant A.T. 2005. Childhood obesity, prevalence and prevention. Nutr. J. 4: 24.

Dewitt O., Pochart P. and Desjeux J.F. 1988. Breath hydrogen concentration and plasma glucose, insulin and free fatty acid levels after lactose, milk, fresh or heated yogurt ingestion by healthy young adults with or without lactose malabsorption. Nutr. 4: 131.

EFSA Panel on Dietetic Products, Nutrition, and Allergies. 2010. Scientific Opinion on establishing Food-Based Dietary Guidelines. EFSA Journal 8 (3): 1460.

INRAN, Italian Institute for Research in Food and Nutrition. 2003. Italian dietary guidelines ("Linee Guida per una Sana Alimentazione Italiana"), http://www.inran.it/648/linee_guida.html.

Kimm S.Y.S. 1995. The Role of Dietary Fiber in the Development and Treatment of Childhood Obesity. Pediatrics 96: 1010.

Liu S., Willett W.C., Manson J.E., Hu F.B., Rosner B. and Colditz G. 2003. Relation between changes in intakes of dietary fiber and grain products and changes in weight and development of obesity among middle-aged women. Am. J. Clin. Nutr. 78: 920.

Lobstein T. and Frelut M.-L. 2003. Prevalence of overweight among children in Europe. Obes. Rev. 4: 195.

Luepker R.V., Perry C.L., Osganian V., Nader P.R., Parcel G.S., Stone E.J. and Webber L.S. 1998. The child and adolescent trial for cardiovascular health (catch). J. Nutr. Biochem. 9: 525.

Murphy J.M., Pagano M.E., Machmani J., Sperling P., Kane S. and Kleinman R.E. 1998. The relationship of school breakfast and psychosocial and academic functioning. Arch. Pediatr. Adolesc. Med. 152: 899.

Pollitt E. and Mathews R. 1988. Breakfast and cognition: an integrative summary. Am. J. Clin. Nutr. 67: 804S.

SINU, Italian Society for Human Nutrition. 1996. Italian reference values for the recommended daily intake of nutrients (LARN). http://www.sinu.it/larn/tab_rias.asp.

Washington R. 2008. Overview of the expert committee's recommendations for prevention, diagnosis, and treatment of child and adolescent obesity. Prog. Pediatr. Cardiol. 25: 125.

Wyon D.P., Abrahamsson L., Jartelius M. and Fletcher R.J. 1997. An experimental study of the effects of energy intake at breakfast on the test performance of 10-year-old children in school. Int. J. Food Sci. Nutr. 48: 5.

Paper received April 14, 2011 Accepted June 27, 2011

SURVEY

FOOD SAFETY KNOWLEDGE AND PRACTICES OF NURSING STUDENTS

N. SANLIER[1]*, S. BILICI[1], B. ÇELIK[1] and E. MEMIŞ[2]
[1]Gazi University, Faculty of Health Sciences,
Department of Nutrition and Dietetics, Besevler, Ankara, Turkey
[2]Gaziosmanpasa University
Dincerler School of Tourism and Hotel Management
Tokat, Turkey
*Corresponding author: Tel. +90 312 2162604,
email: ntekgul@gazi.edu.tr, nevintekgul@gmail.com

ABSTRACT

The aim of the study was to evaluate the perception of food safety knowledge of 662 nursing students (629 females, 33 males) aged between 18 and 26. A statistically significant correlation was found between food hygiene, personal/kitchen hygiene knowledge, food handling practices, food safety attitudes, and total score ($p<0.01$). The female students' food safety knowledge, personal/kitchen hygiene knowledge, food handling practices, and total scores relating to food safety scored significantly higher than those of males ($p<0.01$). Results strongly emphasize the need for basic education in food safety throughout the nursing course.

- Key words: attitudes, food safety, hygiene, knowledge, nurse, practices -

INTRODUCTION

Food safety is a global health goal and foodborne diseases are a major health issue (VELUSAMY *et al.*, 2010). Foodborne diseases represent a widespread and growing public health problem, in both developed and developing countries. This problem, however, has more impact on health and economy in developing countries but no reliable data are available (WHO, 2007). The major food safety risks are not eating a healthy diet and failure to avoid foodborne diseases (CHASSY, 2010). Foodborne diseases caused by microbial pathogens, biotoxins, and chemical contaminants in food represent serious threats to the health of thousands of millions of people (FAO and WHO, 2008). It is difficult to estimate the incidence of foodborne diseases worldwide because most cases of the disease cannot be recorded. Within two decades, the human population is predicted to reach 8.5 billion, 80% of whom are expected to be in developing countries (WHO, 1997). In the industrialised countries up to 10% of the population may suffer annually from foodborne diseases (WHO, 1998; KAFERSTEIN and ABDULSSAULAM, 1999). WHO estimated that in 2005 1.8 million people worldwide died from the effects of foodborne diseases (WHO, 2005). It has been estimated that foodborne diseases cause approximately 76 million illnesses, 325,000 hospitalisations and 5000 deaths in the United States each year (MEAD and SLUTSKER, 1999). In Turkey, according to the Ministry of Health (2006), 26,772 people were hospitalised because of foodborne diseases. Some 23,901 *Salmonella typhii* infections, 429 *Salmonella paratyphii* infections, 21,068 cases of dysentery and 8,824 cases of hepatitis A occurred in 2004. Since the reporting of foodborne diseases to a specified agency is not obligatory in Turkey, data on foodborne infections are inaccurate (WHO, 2004).

Institutions and professions, which are in constant interaction with the public, are reliable resources to persuade people to adopt desirable habits as regards food safety. Therefore nurses working in the health sector can be regarded as a potential source to be used for this purpose, which in turn makes their information level in terms of food safety crucial for several reasons. The major task of public health professionals is to deal with public awareness. As health professionals, nurses should have appropriate knowledge about adequate and balanced nutrition as well as food safety (BUCCHERI *et al.*, 2007). The importance of safe food for hospitalised patients and the detrimental effect that contaminated food could have on their recovery has been widely emphasised. Food safety in hospital may have unique features: many patients are more vulnerable than healthy subjects to microbiological and nutritional risks; large numbers of people can be exposed to infections and possible complications; gastroenteritis can impair digestion and absorption of nutrients; and the perception or fear about poor food hygiene practices might result in patients rejecting the meals supplied by the hospital catering service (BARRIE 1996; DEREA *et al.*, 2008).

Further concern arises from the common use of nurses or domestic staff as food handlers, as they are not specifically trained about food hygiene and HACCP but can be engaged in the receipt, distribution and serving of readymade foods and supervision of these services (BUCCHERI *et al.*, 2007). In Turkey, owing to the small number of dieticians in hospitals nurses are sometimes responsible for the distribution and serving of meals and helping patients to consume their meals properly.

The prevention of foodborne diseases requires all food consumers, especially nurses, to be taught about food handling practices. Prior to such education, however, it is important to identify the food safety issues relevant to nurses as consumers (JEVSNIK *et al.*, 2008; OSAILI *et al.*, 2011). In addition, education about food and nutrition issues at an early age influences the attitudes of youngsters and enhances the knowledge and skills required for them to understand contemporary issues related to food (LYTLE, 1995).

There is no education programme in Turkey for improving the food safety knowledge and practices of consumers in any age group or in schools, and assessing the basic knowledge of young consumers is essential for developing effective food safety programmes. To the best of our knowledge, limited studies focusing on nursing students have been conducted and there is no study concerning food safety knowledge of nursing students in Turkey and the world. Therefore, this study aimed at evaluating the perception of food safety by nursing students and the influence of food, personal and kitchen hygiene and safety education on the food safety knowledge, attitude and practices of nursing students.

METHOD

Design, sample and data collection

A cross-sectional study was conducted and the questionnaire was administered between March and June 2010 to assess food safety knowledge, practice and attitudes of nursing students in Ankara, the capital of Turkey. The study population constituted final-year students from five typical public universities and consisted of 739 nursing students whose ages were between 18 and 26. A structured questionnaire was self-administered by 739 students in the classroom environment and 681 were returned (92% return rate). After questionnaires which

Appendix Assessment of the answers.

Questions	Assessment	Score range
Food safety knowledge (10 questions) Personal and kitchen hygiene knowledge (6 questions)	True = 1 point False = 0 points	Food safety knowledge: 0-10 points Personal and kitchen hygiene:0-6 points
Food handling practice (14 questions) Food safety attitude (8 questions)	Always=3 points Sometimes=2 points Never=1 point	Food handling practice: 14-42 points Food safety attitude: 8-24 points
Total		22-82 points (mean = 64.24±6.69)

had missing values were eliminated, analyses were carried out on 662 (629 – 95% female, 33 - 5% male) valid questionnaires. In Turkey, nursing is largely perceived as a woman's profession, although in recent years men have also entered this profession. Therefore, the number of female nursing students participating in the study was greater than the number of male nursing students. The participants were informed about the subject, purpose and rules of the research. Students" consent was required prior to commencement of the study. Each participant signed a voluntary participation form and filled in the questionnaires in strict accordance with the Declaration of Helsinki (World Medical Association). The participants were informed about the subject and the purpose of the research.

INSTRUMENT

A structured questionnaire was designed to collect the research data. The questionnaire was administered to assess the food safety knowledge, food safety practices and food safety beliefs of the participants. The questionnaire was based on those used in earlier studies (SAMMARCO *et al.*, 1997; HAAPALA and PROBART, 2004; SANLIER, 2009). The questionnaire was pilot-tested by 86 participants between March and April 2010, resulting in minor modifications to the wording. The revised questionnaire was divided into five sections: demographic section (three questions), food safety knowledge (ten questions), personal/kitchen hygiene knowledge (six questions), food handling practice (fourteen questions) and food safety attitude (eight questions).

The assessments of the answers were summarized as in the Appendix. Answers to the questions related to food safety knowledge were graded, one point being given for the right answer and zero for the wrong answer. The scale (which was related to safe food preparation practices) included a set of negative sentences (Table 2, statements, 1, 2, 5, 8, 10, and 11) in addition to the positive ones. Responses to the positive sentences were graded as follows: never 1, sometimes 2, always 3 s. In the negative sentences, the grades were assigned in reverse order.

The Cronbach alpha coefficient of internal consistency was used to estimate the reliability of the questionnaire. An alpha coefficient of 0.76 was considered to be acceptable for food safety knowledge.

A higher score represents a higher level of food safety, personal/kitchen hygiene knowledge, food handling practice and attitude. The questionnaire was given to the nursing students by the researchers in the face-to-face method and took approximately 15 min to complete. Students were informed about the purpose of the study and the importance of the reliability of their answers. To guarantee the anonymity of respondents and easy identification of questionnaires completed by individuals, identity numbers were randomly assigned to each questionnaire. Items in the questionnaire were explained when necessary and administered at one sitting as much as possible.

STATISTICAL ANALYSIS

All statistical analyses were performed with SPSS (Statistical Package for Social Sciences) Version 15.0 (SPSS, Inc., Chicago, IL, USA). Descriptive statistics (mean and standard deviation, or frequency) were used for all variables. The independent sample t test was used to assess the difference between gender and age. Correlation analysis with Pearson's correlation coefficient was conducted to explore the associations between knowledge, practice and attitude. In all analyses, 5% significance levels were used.

RESULTS

Ninety-five per cent of participants were female and 5% were male. The mean age of the study population was 20.9±1.6 years (male: 21.0±1.3 years, female: 20.9±1.7 years, respectively) and 55.4% of them were over 20 years old.

Whereas 77.2% of the participants replied

that the statement 'the smell/appearance of foods that cause food poisoning is bad' was false, 78.2% of them replied that the statement 'there is no objection to the use of broken or cracked eggs' was true. Also, the majority of the participants rated as true the statements 'eggs cooked well can be kept at room temperature for more than two days', 'incorrect storage of foods does not constitute a hazard' and 'foods are hazardous that prepared with raw eggs' (75.7, 84.3, 65.1%, respectively). As regards the distribution of participant responses relating to food and personal hygiene knowledge, 38.5% of the nursing students rated as false the statement 'cigarette smoke blowing into the air contaminates food and the air with saliva', whereas 46.8% of them rated as true the statement 'wiping the cutting board with a disposable towel after use prevents bacterial growth'. The majority of the respondents rated as true the statements 'bacteria passed by hands to foods will cause toxins', 'hands should be dried with disposable towels' and 'surfaces should be cleaned before preparing foods' (82.8, 83.5, and 90.5%, respectively), (Table 1).

It was found that 26.7% of the participants always/sometimes use dirty eggs without washing them carefully and 24.9% of them never touch cooked foods after handling raw foods. Sixty-nine per cent of the participants stated that they never tasted milk to test it instead of checking the expiry date and 65.4% of them do not allow raw chicken, fish and meat to come into contact with each other. It was also found that 62.6% of the participants sometimes/always use outdoor milk after boiling it for half an hour and 56.2% of them stated that they thawed frozen meat always/sometimes on countertops/radiators. The assessment of the attitudes concerning food safety practices showed that 44.5% of nursing students stated it was not important whether bought ready meals contained additives or not, 59.4% of them said shopping should always/sometimes be done at the supermarket because their products were of higher quality, 39.3% of them reported that sales promotions

Table 1 - Distribution of nursing student responses relating to food, personal and kitchen hygiene knowledge.

Statements	True		False	
	n	(%)	n	(%)
Food hygiene knowledge				
1. The taste, appearance and smell of foods that cause food poisoning are bad (**False**)	151	(22.8)	511	(77.2)
2. There is no objection to the use of cracked or broken eggs (**False**)	518	(78.2)	144	(21.8)
3. Eggs that are cooked well can be kept at room temperature for more than 2 days (**False**)	501	(75.7)	161	(24.3)
4. Freezing does not kill bacteria in foods, only stops their reproduction (**True**)	454	(68.6)	208	(31.4)
5. Canned foods can be stored on shelves in the original packaging (**True**)	406	(61.3)	256	(38.7)
6. Foods stored incorrectly do not constitute a health hazard (**False**)	558	(84.3)	104	(15.7)
7. The internal temperature of chicken and Turkey must be high for safe cooking (**True**)	311	(47.0)	351	(53.0)
8. Raw eggs or foods containing raw eggs are hazardous (**True**)	431	(65.1)	231	(34.9)
9. Meals suspected of being spoiled could be consumed again after boiling (**False**)	528	(79.8)	134	(20.2)
10. Fruit and vegetables that can be peeled should be washed (**True**)	574	(86.7)	88	(13.3)
Personal and kitchen hygiene knowledge				
11. Cigarette smoke blown into the air contaminates foods and the air with saliva (**True**)	407	(61.5)	255	(38.5)
12. Wiping the cutting board with a disposable towel after use prevents bacterial growth (**False**)	310	(46.8)	352	(53.2)
13. Bacteria passed by hands to foods will cause toxins (**True**)	548	(82.8)	114	(17.2)
14. Hands should be dried with disposable towels (**True**)	553	(83.5)	109	(16.5)
15. Surfaces should be cleaned before preparing foods (**True**)	599	(90.5)	63	(9.5)
16. Swabs can be used as cleaning equipment during the preparation of foods (**True**)	235	(35.5)	427	(64.5)

Table 2 - Distribution of nurse practices and attitudes related to food safety.

	Always		Sometimes		Never	
	n	(%)	n	(%)	n	(%)
Food handling practices						
1. Keep cooked foods at room temperature until they are cooled **(-)**	441	(66.6)	146	(22.1)	75	(11.3)
2. Use dirty eggs carefully without washing **(-)**	96	(14.5)	81	(12.2)	485	(73.3)
3. Do not touch cooked foods after handling raw foods	296	(44.7)	201	(30.4)	165	(24.9)
4. Keep raw and cooked food separate	485	(73.3)	106	(16.0)	71	(10.7)
5. Test the safety of milk by tasting instead of checking the expiry date **(-)**	116	(17.5)	89	(13.4)	457	(69.0)
6. Store pasteurised milk in the refrigerator up to 3 days	362	(54.7)	177	(26.7)	123	(18.6)
7. Do not allow raw chicken, fish and meat to touch each other	433	(65.4)	174	(26.3)	55	(8.3)
8. Taste the foods to see whether they are safe or not **(-)**	175	(26.4)	172	(26.0)	315	(47.6)
9. Do not freeze foods again after thawing	509	(76.9)	108	(16.3)	45	(6.8)
10. Use outdoor milk after boiling for half an hour **(-)**	217	(32.8)	197	(29.8)	248	(37.5)
11. Thaw frozen meat on countertops/ radiators **(-)**	193	(29.2)	179	(27.0)	290	(43.8)
12. Put leftover foods into the refrigerator within two hours	451	(68.1)	168	(25.4)	43	(6.5)
13. After purchasing foods that could go off in a short time put them into the refrigerator within two hours	476	(71.9)	132	(19.9)	54	(8.2)
14. Wash poultry like chicken, turkey, etc. before cooking	564	(85.2)	68	(10.3)	30	(4.5)
Attitudes and statements on the implementation of food safety						
15. It is not important whether bought ready meals contain additives or not	267	(40.3)	102	(15.4)	293	(44.3)
16. Branded products should be purchased	135	(20.4)	251	(37.9)	276	(41.7)
17. A clean environment of places selling	632	(95.5)	15	(2.3)	15	(2.3)
18. food products is important						
19. The nutrient value should be considered rather than food taste	390	(58.9)	180	(27.2)	92	(13.9)
20. Food shopping should be done at supermarkets that are considered to offer better quality products	102	(15.4)	291	(44.0)	269	(40.6)
21. Sales promotions have an important impact on the decision to buy certain foods	228	(34.4)	174	(26.3)	260	(39.3)
22. Bloated cans should not be consumed	552	(83.4)	67	(10.1)	43	(6.5)
23. When purchasing products one should read the information and instructions on packaging carefully	583	(88.1)	54	(8.2)	25	(3.8)

have an important impact on food purchasing decisions. The majority of the participants indicated that bloated cans should not be consumed (83.4%) and one should read the information and instructions on packaging (88.1%) (Table 2).

The comparison of scores according to various variables is given in Table 3. It was found that the female nursing students' food safety knowledge ($t = -2.920$, $p = 0.004$), personal/kitchen hygiene knowledge ($t = -3.327$, $p = 0.001$), food handling practices ($t = -3.095$, $p = 0.002$), attitudes towards food safety and total scores relating to food safety ($t = -3.579$, $p = 0.000$) were significantly higher than those of males ($p < 0.05$). In terms of age groups the differences were not statistically significant ($p > 0.05$) (Table 3).

As regards the correlation of food safety knowledge, personal/kitchen hygiene knowledge, food handling practice and food handling practice scores, the nursing students' personal/kitchen hygiene was found to be positively correlated with food safety knowledge score ($r = 0.514$, $p < 0.01$) and food handling practice ($r = 0.484$, $p < 0.01$) and food safety attitudes ($r = 0.178$, $p < 0.01$), food handling practices were found to be positively correlated with food safety knowledge ($r = 0.550$, $p < 0.01$) and food safety attitudes ($r = 0.249$, $p < 0.01$), food safety attitudes were correlated with food safety knowledge ($r = 0.202$, $p < 0.01$) and food handling practices ($r=0.249$, $p < 0.01$) and total score were correlated with all study variables ($p< 0.01$) (Table 4).

DISCUSSION

Although the public is increasingly concerned about food-related risks, the rise in food poisoning cases suggests that people still make decisions about food consumption, food storage and food preparation that are less than ideal from a health and safety perspective (MCCARTHY *et al.*, 2007). This study investigates the perception of food safety by nursing students and the impact of food, personal/kitchen hygiene and safety ed-

Table 3 - Comparison of scores according to various variables.

Variables		Food safety knowledge $\overline{X}$±SD	Personal/kitchen hygiene knowledge $\overline{X}$±SD	Food handling practice $\overline{X}$±SD	Food safety attitude $\overline{X}$±SD	Total score $\overline{X}$±SD
Gender						
Male (n=33)		5.76±2.41	3.27±1.35	32.61±3.23	18.58±2.00	60.21±6.61
Female (n=629)		6.74±1.86	4.04±1.29	34.68±3.78	18.98±1.96	64.45±6.64
	t	-2.920	-3.327	-3.095	-1.165	-3.579
	p	**0.004**	**0.001**	**0.002**	**0.245**	**0.000**
Age (year)						
≤ 20 (n=295)		6.82±1.78	4.02±1.19	34.63±3.52	18.89±1.91	64.37±6.18
> 20 (n=367)		6.59±1.99	3.99±1.40	34.53±3.97	19.01±2.01	64.14±7.09
	t	1.564	0.192	0.326	-0.768	0.447
	p	0.118	0.848	0.744	0.442	0.655

Table 4 - Correlation of food safety knowledge, personal/kitchen hygiene knowledge, food handling practice and food handling practice scores (r).

	Personal/kitchen hygiene knowledge	Food handling practice	Food safety attitude	Total score
Food safety knowledge	0.514**	0.550**	0.202**	0.754**
Personal/kitchen hygiene knowledge		0.484**	0.178**	0.667**
Food handling practice			0.249**	0.888**
Food safety attitude				0.526**

**P< 0.01.

ucation on the food safety knowledge, attitude and practices of nursing students. The food safety knowledge, attitude, and behaviour of people play a major role in the incidence of foodborne diseases. Foodborne diseases are a widespread public health problem all over the world and often seen in both developed and less developed countries although the incidence varies from one country to another, depending on economic conditions and forms of social life (UNUSAN, 2007; SANLIER, 2009 and 2010). In response to the increasing number of foodborne diseases, governments all over the world are intensifying their efforts to improve food safety (SUBBA *et al.*, 2007).

Foodborne diseases have been associated with improper storage or reheating, food stored inappropriately and cross-contamination. These contributory factors result from lack of food hygiene awareness or implementation (WALKER *et al.*, 2003; SANLIER, 2009; SCHAFER *et al.*, 1993). International studies have found that consumers often implement unsafe food-handling behaviours during domestic food preparation. Such malpractices need to be improved to reduce the risk and incidence of food poisoning (REDMOND and GRIFFITH, 2004; SANLIER *et al.*, 2010).

Consumers need knowledge of effective food handling skills and then need to be motivated to act on that knowledge as preconditions to behavioural change (BARANOWSKI *et al.*, 2002). Healthcare providers are a trusted source of information on health care and food safety practices. Health professions may play a substantial role in educating consumers on safe and appropriate behaviours (FEIN *et al.*, 1995). Nurses in particular are considered to lead the community and are responsible for the development and administration of education about preventative health services for the public.

In this study it was found that the majority of nurse consumers do not have right information about food, personal and kitchen hygiene (Table 1, Table 2). For example, they did not give the right answer to statements like 'freezing does not kill the bacteria but only stops their reproduction', 'the inner temperature of poultry while cooking must be high', 'foods prepared with raw eggs have a potential risk', 'wiping cutting boards with disposable towels inhibits bacterial growth'.

A number of studies have demonstrated a lack of correlation between food hygiene training and improvements in food hygiene behaviour (ANSARI-LARI *et al.*, 2009; SANLIER, 2009; SANLIER and TÜRKMEN, 2010). In this study nursing student practices and attitudes regarding food safety were found to be inappropriate (Table 2). Consumers' true perceptions of safe food, food safety knowledge, behaviour, attitudes and practices are important. The results of consumer studies concerning food safety knowledge and practic-

es have shown that consumers are aware of and are thinking about food safety, although there are also many gaps in food safety knowledge and practices that could result in foodborne diseases (BADRIE *et al.*, 2006; RASPOR *et al.*, 2006; JEVSNIK *et al.*, 2008).

Surveys have produced inconsistent results with regard to the relation between food safety behaviours and education level, some risky practices being more common at higher levels of education and income (SHIFERAW *et al.*, 2000; SANLIER *et al.*, 2010). According to the studies carried out in Turkey, food hygiene knowledge, attitude and perception scores are higher in women than men, higher in those aged 45 and older, higher in health areas than social areas, and college graduates have greater knowledge of food safety but lower scores at the same time (UNUSAN, 2007; SANLIER, 2009). The present study supports earlier findings (Tables 3 and 4). Studies have suggested that education and income are associated with attitudes towards food safety (BREWER and PRESTAT, 2007; STINSON *et al.*, 2008; SUBBA *et al.*, 2007; ÖZBILGEN, 2010). YARROW *et al.* (2009) stated that food safety beliefs, attitudes, knowledge and practices could change through educational intervention. SAMMARCO *et al.* (1997) indicated that consumers do not have enough knowledge and have improper practices related to personal hygiene, food handling and food storage and even basic and simple knowledge about detergents, disinfectants and sterilisation is inadequate. Findings from our study suggest that improper practices, such as using dirty eggs without careful washing and thawing frozen meat at room temperature, are widespread among the respondents. Similar behaviours are described in several previous studies and confirm that cross-contamination is a poorly perceived food safety issue (ANGELILLO *et al.*, 2001; ALTEKRUSE *et al.*, 1999; SHIFERAW *et al.*, 2000).

In this study, a statistically significant correlation was found between nursing students' food hygiene, personal/kitchen hygiene knowledge, food handling practices, food safety attitudes and the total score ($p<0.01$). Improper practices and inadequate knowledge and perception of nursing students' were identified in the study (Table 4). Results strongly emphasise the need for basic food safety education for nurses who are non-professional food handlers involved in hospital food service functions.

The results obtained from this study revealed the need for consumer education regarding safe food handling practices from the point of purchase to the home, as well as within the home. We have to educate nurse consumers so that they can cope with all eventualities in the field of food safety on a daily basis (RASPOR *et al.*, 2006). The attitudes of consumers towards food safety and their practices concerning food are themes of interest to food producers and retailers, public authorities and health educators (WILCOCKY *et al.*, 2004; JEVSNIK *et al.*, 2008) Nurses' consumer education should target safe practices in food hygiene, causative foodborne diseases and agents, food-handling concepts such as cross-contamination, and the principles of HACCP, ISO 22000 as applied to health and food education in nursing school programmes (WHO, 2000; BADRIE *et al.*, 2006). School and university settings would be the best places to reach young people and teach them key food safety concepts.

LIMITATIONS OF THE STUDY

Because the population of this study consisted of nursing students from central Turkey, the results cannot be generalized to all nurses or to all ages. Although the reliability coefficient was found to be high, interpretation of the results is limited owing to responses being self-reported and prone to bias by the subjects. Instrument validation for all university students through more testing and observational studies is needed.

ACKNOWLEDGEMENTS

The Authors would like to thank the participants for their honest completion of the questionnaires.

REFERENCES

Altekruse S.F., Yang S., Timbo B.B. and Angulo F.J. 1999. A multi-state survey of consumer food-handling and food-consumption practices. Am. J. Prev. Med. 16: 216.

Angelillo I.F., Foresta M.F, Scozzafava C. and Pavia M. 2001. Consumers and foodborne diseases: knowledge, attitudes, and reported behavior in one region of Italy. Int. J. Food Microbiol. 64: 161.

Ansari-Lari M., Soodbakhsh S. and Lakzadeh L. 2009. Knowledge, attitudes and practices of workers on food hygienic practices in meat processing plants in Fars, Iran. Food Control 21: 260.

Badrie N., Gobin A., Dookeran S. and Duncan R. 2006. Consumer awareness and perception to food safety hazards in Trinidad, West Indies. Food Control 17: 370.

Baranowski T., Perry C.L. and Parcel G.S. 2002. How individuals, environments, and health behaviour interact. Social Cognitive Theory. In: "Health Behaviour and Practice" 3rd Ed. Glanz, K., Rimer B.K. and Lewis, F.M. (Ed). p. 165. Jossey-Bass, San Francisco, CA.

Barrie D. 1996. The provision of food and catering services in hospital. J. Hosp. Infect. 33: 13.

Brewer M.S. and Prestat C. J. 2007. Consumer attitudes toward food safety issues. Journal of Food Safety 22 (2): 67.

Buccheri C., Casuccio A., Giammanco S., Giammanco M., Guardia M.L. and Mammina C. 2007. Food safety in hospital: knowledge, attitudes and practices of nursing staff of two hospitals in Sicily, Italy. BMC Health Services Research 7: 45.

Chassy B.M. 2010. Food safety risks and consumer health. New Biotechnology. In press.

Derea H.E., Salem E., Fawzi M. and Azeem A. 2008. Safety of patients' meals in 2 hospitals in Alexandria, Egypt be-

fore and after training food handlers. Eastern Mediterranean Health Journal 14: 4.

FAO & WHO. 2008. Assuring food safety and quality. Guidelines for strengthening national food control systems. Joint FAO/WHO publication.

Fein S.B., Lin C.T.J. and Levy A.S. 1995. Foodborne illness: perceptions, experience and preventive behaviors in the United States. J. Food Protect. 58: 1405.

Haapala I. and Probart C. 2004. Food safety knowledge, perceptions, and behaviors among middle school students. J. Nutr. Educ. Behav. 36: 71.

Jevsnik M., Hlebec V. and Raspor P. 2008. Consumers' awareness of food safety from shopping to eating. Food Control 19: 737.

Köferstein F. and Abdulssalam M. 1999. Food safety in the 21st century. Bulletin of the World Health Organization 77 (4): 347.

Lytle L. 1995. Nutrition education for school-age children. Journal of Nutrition Education 27: 298.

McCarthy M., Brennan M., Kelly A. L., Ritson C., de Boer M. and Thompson N. 2007. Who is at risk and what do they know? Segmenting a population on their food safety knowledge. Food Quality and Preference 18 (2): 205.

Mead P.S. and Slutsker L. 1999. Food-related illness and death in the United States. Emerg Infect. Dis. 5: 607.

Ministry of Health (Turkey). 2006. Basic health statistics of Turkey 2004. Ministry of Health Publications, Ankara.

Osaili T.M, Obeidat B.A., Abu Jamous D.O. and Bawadi H.A. 2011. Food safety knowledge and practices among college female students in north of Jordan. Food Control 22: 269.

Özbilgen S. 2010. Food safety education makes the difference: food safety perceptions, knowledge, attitudes and practices among Turkish university students. Journal of Consumer Protection and Food Safety. In press.

Raspor P., Jevsnik M. and Hlebec V. 2006. Consumers' awareness of food safety from shopping to eating. In: "Nutrition and food safety". D. Banati (Ed.), p.112. Consortium International Congress on Food Safety, The Safe Consortium, Brussels.

Redmond E.C. and Griffith C.J. 2004. Consumer perceptions of food safety risk, control and responsibility. Appetite 43: 309.

Sammarco M.L., Ripabelli G. and Grasso G.M. 1997. Consumer attitude and awareness towards food-related hygienic hazards. Journal of Food Safety 17: 215.

Sanlier N. 2009. The knowledge and practices of food safety by young and adult consumers. Food Control 20: 538.

Sanlier N. 2010. Food safety knowledge and the safe food handling behaviours of female and male consumers. Pakistan Journal of Medical Sciences 26 (3): 653.

Sanlier N., Cömert M. and Durlu Özkaya F. 2010. Hygiene perception conditions of hotel kitchen staff in Ankara, Turkey. Journal of Food Safety 30: 415.

Sanlıer N. and Turkmen F. 2010. Perception of hygiene among staff working in food companies. Research Journal of Medical Sciences 4 (3): 231.

Schafer E., Schafer R.B. and Bultena G.L. 1993. Safety of the United States food supply: consumer concerns and behaviour. J of Consumer Studies and Home Economics 17: 137.

Shiferaw B., Yang S., Cieslak P., Vugia D., Marcus R., Koehler J., Deneen V., Angulo F. and The Foodnet Working Group. 2000. Prevalence of high-risk food consumption and food-handling practices among adults: a multistate survey, 1996-1997. J. Food Prot. 63: 1538.

Stinson T.F., Gosh K., Kinsey J. and Degeneffe D. 2008. Do household attitudes about food defense and food safety change following highly visible national food recalls? Am. J. of Agri. Econ. 90: 1272.

Subba Rao G.M., Sudershan R.V, Rao P., Rao M.V.V. and Polasa K. 2007. Food safety knowledge, attitudes and practices of mothers: findings from focus group studies in South India. Appetite 49: 441.

Unusan N. 2007. Consumer food safety knowledge and practices in the home in Turkey. Food Control 18: 45.

Velusamy V., Arshak K., Korostynska O., Oliwa K. and Adley C. 2010. An overview of foodborne pathogen detection: In the perspective of biosensors. Biotechnology Advances 28: 232.

Walker E., Pritchard C. and Forsythe S. 2003. Food handlers' hygiene knowledge in small food businesses. Food Control 14: 339.

WHO. 1997. Unpublished data. Advisory Committee on Health Research. A research policy agenda for science and technology. World Health Organization, Geneva.

WHO. 1998. Unpublished data. Health education in food safety. World Health Organization, Geneva.

WHO. 2000. Foodborne disease: A focus for health education. World Health Organization, Geneva.

WHO. 2004. Surveillance programme for control of foodborne infections and toxications in Europe, 8th report, 1999-2000. Country reports: Turkey.

WHO. 2005. http://www.who.int/mediacentre/factsheets/fs237/en/.

WHO. 2007. http://www.who.int/whr/2007/en/index.html

Wilcocky M., Pun J. Khanonax and Aung M. 2004. Consumer attitudes, knowledge and behaviour: A review of food safety issues. Trends in Food Science and Technology 15: 56.

Yarrow L., Reming V.M. and Higging M.M. 2009. Food safety education intervention positively influences college students' food safety attitudes, beliefs, knowledge, and self-reported practices. J. of Environ. Health 71: 30.

Paper received March 30, 2011 Accepted July 19, 2011

GUIDE FOR AUTHORS
ITALIAN JOURNAL OF FOOD SCIENCE -IJFS

Publication Ethics and Publication Malpractice

Italian Journal of Food Science is committed to upholding the highest standards of publication ethics and takes all possible measures against any publication malpractices. All Authors submitting their works to Italian Journal of Food Science for publication as original articles attest that the submitted works represent their Authors' contributions and have not been copied or plagiarized in whole or in part from other works. The Authors acknowledge that they have disclosed all and any actual or potential conflicts of interest with their work or partial benefits associated with it. In the same manner, Italian Journal of Food Science is committed to objective and fair Editor(s) review of the submitted for publication works and to prevent any actual or potential conflict of interests between the editorial personnel and the reviewed material. Any departures from the above-defined rules should be reported directly to the Editor-in-Chief, who is unequivocally committed to providing swift resolutions to any of such a type of problems.

1. Manuscript Submission

Manuscripts must submitted as an electronic version by e-mail to paolofan@unipg.it. The word processor used to generate the file should be indicated and the files should be saved in format "Text only"; **graphs, pictures and diagrams must be saved at 300 dpi in TIF, JPG or EPS formats** (not included in MsWord documents).

Manuscripts must be typed, double-spaced and pages should be in A4 format using Times New Roman 12 pt as the advised font. Top, bottom and side margins should be 25 mm. Pages and lines on all pages, including those for References and figure legends, **must be electronically numbered in the left margin.**

English is the official language. The Editor-in-Chief and/or Co-Editors reserve the right to make literary corrections and to make suggestions to improve brevity, but **the paper must be previously revised for English by the authors**. If English is not the mother tongue of authors, they **must seek** help from one of the following agencies (or other similar official agencies):

www.journalexperts.com
www.sciencedocs.com
www.internationalscienceediting.com
www.writescienceright.com
www.genedits.com

2. Manuscript Preparation

(1) The paper should be divided under the following headings in this order:

Title. Informative of the content of the article (<50 characters + spaces).

Author(s). Initials and Surname, omit professional and official titles. The institute and address where the research was carried out and the current address of each author should be given on the title page.

Abstract. Clearly state the objective of the study, give a concise description of experiment(s), observations, results and conclusions. No references should be cited. **Do not exceed 100 words**.

Key words. Up to six words, in alphabetical order, which describe the document must be given to aid data retrieval and indexing.

Introduction. Review pertinent previous work and cite appropriate references. State the purpose of the investigation.

Materials and Methods. Indicate apparatus, instruments, reagents, etc., giving sufficient detail to allow the work to be repeated.

Results and Conclusions. Results and Conclusions may be presented together or separately. Concisely present results using tables and figures to help justify conclusions (do not present the same information in both forms). Use statistical analysis when appropriate. Unsupported hypotheses should be avoided. Conclusions should point out the significance of the findings and, if possible, relate the new findings to some problem in Food Science and Technology.

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

Units. A list of units particular to the paper may be included.

References. References in the Reference list should be arranged alphabetically (initials of first name, only), and, for the same author, should be arranged consecutively by year, typed double-spaced. Each individual reference should begin flush left (no indentation). Refer to attached examples taken from "Style Guide for Research Papers" by the Institute of Food Technologists (Chicago - I1linois - USA). Literature citations in the text should be referred to by Surname and year in parentheses. If there are more than two authors, give the surname of the first author and add et al. and the year in parentheses. Examples: (SMITH, 2007), (SMITH and JONES, 2008) (SMITH et al., 2008).

(2) Tables should be as few and as simple as possible and include only essential data. Each table must be saved and printed on a separate sheet and have an Arabic number, e.g. Table 4 NOT Tab.

4. Legends must be self-explanatory and on a separate sheet. Use lower-case letters for footnotes in tables and explain below the table in the order in which they appear in the table.

(3) Figures must be prepared and saved separately in **TIF, JPEG, EPS (300 dpi resolution).** They should be prepared so that on 50% reduction, lines, figures and symbols will be clearly legible and not overcrowded. All figures must be given Arabic numbers, e.g. Fig. 3. Legends for figures must be self-explanatory and should be typed on a separate sheet under "Legends to Figures".

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

Common species names should be followed by the Latin binomial (italics) at the first mention. For subsequent use, the generic name should be contracted to a single letter if it is unambiguous.

3. Editorial and Review Policy

Scientific contributions in one of the following forms may be submitted:

Reviews –They can be submitted directly to the Editor-in-Chief or articles can be requested directly by the Editor-in-Chief.

Short Communications, Surveys and Opinions – They do not need to have the formal organization of a research paper; they will receive priority in publication; maximum of five pages allowed.

Papers – The paper must follow the guidelines as specified under the section Manuscript Preparation.

Reviews, Papers, Short Communications and Surveys will be **subjected to critical review by referees.**

(1) Manuscripts will be processed in the order received. The Editor-in-Chief will select papers to enter into the reviewing system based on originality and innovation. A letter will be sent to the authors acknowledging receipt of the manuscript along with a Declaration form stating that it has NOT been previously published, accepted or submitted for publication elsewhere and agreeing to the page charges upon acceptance of the paper. On receipt of the signed Declaration form, the Editor-in-Chief will send the manuscript to a Co-Editor and/or referees for evaluation.

(2) Referees may not be from the same institution as the author. Referees should make their comments and questions in detail and return the paper to the Editor-in-Chief and/or Co-Editor as soon as possible, usually within two weeks. The identity and report of the referees are made know to the Editor-in-Chief, but only the anonymous referee report is sent to the author(s). If all referees recommend acceptance or rejection, the decision stands. If the opinions of the referees tie, the Editor-in-Chief and/or Co-Editors have the freedom to decide upon acceptance or rejection of the paper.

(3) The results of the refereeing process, accompanied by a letter from the Editor-in-Chief or the Co-Editor, will be sent to the author(s). Papers needing revision must be returned to the Co-Editor within the timeframe suggested, otherwise the paper will be considered as withdrawn. A letter announcing acceptance of the manuscript will be sent to the author(s) upon acceptance by the referees.

(4) The authors **will receive galley proofs** of the manuscript along **with the invoice** for the page charges (stated on the first page of each issue) which **must be paid in order to allow for publication**. The proofs will be sent to the corresponding author as a PDF file by e-mail, only. A hard copy will be sent by mail only if the author makes this request when the paper is accepted for publication.

The revised galley proofs must be returned by fax or mail to Chiriotti Editori – 10064 Pinerolo (TO) – Italy –Fax: +39 0121 794480; e-mail: info@chiriottieditori.it

Italian Journal of Food Science would like to thank all the Referees who have contributed to keep up the value of the journal with their important work.

For this reason, all the Referees can download free of charge all the issues of the Italian Journal of Food Science from our website.

REFERENCE EXAMPLES

EXAMPLES of use in a Reference list are given below. The bold-faced parenthetical type of citation above the example is indicated ONLY for information and is NOT to be included in the reference list.

(Anonymous)

Anonymous. 1982. Tomato product invention merits CTRI Award. Food Technol. 36(9): 23.

(Book)

AOAC. 1980. "Official Methods of Analysis" 13th ed. Association of Official Analytical Chemists, Washington, DC.

Weast, R.C. (Ed.). 1981 "Handbook of Chemistry and Physics" 62nd ed. The Chemical Rubber Co. Cleveland, OH.

(Bulletin, circular)

Willets C.O. and Hill, C.H. 1976. Maple syrup producers manual Agric. Handbook No. 134, U.S. Dept. of Agriculture, Washington, DC.

(Chapter of book)

Hood L.F. 1982. Current concepts of starch structure. Ch. 13. In "Food Carbohydrates". D.R. Lineback and G.E. Inglett (Ed.), p. 217. AVI Publishing Co., Westport, CT.

(Journal)

Cardello A.V. and Maller O. 1982. Acceptability of water, selected beverages and foods as a function of serving temperature. J. Food Sci. 47: 1549.

IFT Sensory Evaluation Div. 1981a. Sensory evaluation guide for testing food and beverage products. Food Technol. 35 (11): 50.

IFT Sensory Evaluation Div. 1981b. Guidelines for the prepaation and review of papers reporting sensory evaluation data. Food Technol. 35(4): 16.

(Non-English reference)

Minguez-Mosquera M.I., Franquelo Camacho A, and Fernandez Diez M.J. 1981. Pastas de pimiento. Normalizacion de la medida del color. Grasas y Aceites 33 (1): 1.

(Paper accepted)

Bhowmik S.R. and Hayakawa, K. 1983. Influence of selected thermal processing conditions on steam consumption and on mass average sterilizing values. J. Food Sci. In press.

(Paper presented)

Takeguchi C.A. 1982. Regulatory aspects of food irradiation. Paper No. 8, presented at 42nd Annual Meeting of Inst. of Food Technologists, Las Vegas, NV, June 22-25.

(Patent)

Nezbed R.I. 1974. Amorphous beta lactose for tableting U.S. patent 3,802,911, April 9.

(Secondary source)

Sakata R., Ohso M. and Nagata Y. 1981. Effect of porcine muscle conditions on the color of cooked cured meat. Agric. & Biol. Chem. 45 (9): 2077. (In Food Sci. Technol. Abstr. (1982) 14 (5): 5S877).

Wehrmann K.H. 1961. Apple flavor. Ph. D. thesis. Michigan State Univ., East Lansing. Quoted in Wehrmann, K.H. (1966). "Newer Knowledge of Apple Constitution", p. 141, Academic Press, New York.

(Thesis)

Gejl-Hansen F. 1977. Microstructure and stability of Freeze dried solute containing oil-in-water emulsions Sc. D. Thesis, Massachusetts Inst. of Technology, Cambridge.

(Unpublished data/letter)

Peleg M. 1982. Unpublished data. Dept. of Food Engineering., Univ. of Massachusetts, Amherst.

Bills D.D. 1982. Private communication. USDA-ARS. Eastern Regional Research Center, Philadelphia, PA.

CONTRIBUTORS

Gratitude is expressed to the following entities for contributing to the realization of the Journal by being supporting subscribers for 2012.

ASSOCIATIONS and COMPANIES

Associazione Italiana di Tecnologia Alimentare (A.I.T.A.) - Parma	Fax +39-0521-230507 www.aita-nazionale.it
Società Italiana di Scienze e Tecnologie Alimentari (S.I.S.T.Al) - Perugia	Fax +39-075-5857939 www.sistal.org
Soremartec Italia srl - Alba	Fax +39-0173-313966

CONTRIBUTORS

Gratitude is expressed to the following entities for contributing to the realization of the Journal by being supporting subscribers for 2012.

RESEARCH INSTITUTES

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

Dipartimento di Medicina e Sanità Pubblica Università di Bologna, Bologna Fax +39-051-2094828

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

ISSN 1120-1770 © 2012

CHIRIOTTI EDITORI srl - 10064 Pinerolo - Italy

publishes the technical magazines:



VOLUME XXIV IJFS No. 1, 2012

CONTENTS

PAPERS

SPE-IMS as a New Analysis Technique for Identification and Quantification of Metalaxyl Residue in Cucumber
M. Soleimani, M. Azam, M. Azimi and K. Borhani **3**

Prevalence of Major Pathogens on Sheep Carcasses Slaughtered in Italy
S. Bilei, E.M. Flores Rodas, R. Tolli, P. De Santis, I. Di Domenico, S. Del Frate, P. Palmieri and R. Condoleo **9**

Chemical Composition and Antimicrobial Activities of the Essential Oils from Flowers, Leaves, and Stems of *Wedelia Urticifolia*
L. Zhu, Y.J Tian, Y.C Yin and S.M. Zhu **19**

Effect of Drying Process in Chamber at Controlled Temperature on the Grape Phenolic Compounds
M.T. Frangipane, S. Torresi, D. De Santis and R. Massantini **26**

Physico-Chemical and Electronic Nose Measurements on the Study of Biscuit Baking Kinetics
S. Romani, F. Balestra, A. Angiolini, P. Rocculi and M. Dalla Rosa **32**

Shelf-life of Minimally Processed Blood Oranges as Affected by Modified Atmosphere Packaging
P. Rapisarda, C. Caggia, S.E. Bellomo, P. Pannuzzo, C. Restuccia, N. Timpanaro and C.M. Lanza **41**

Determination of Capsaicinoids from Dried Pepper Fruits by Fast-Gas Chromatography
M. Bononi and F. Tateo **49**

Antioxidant Activity of Sesame Lignan Compounds on Soybean Oil
M. Bahmaei and H. Peyman **55**

SHORT COMMUNICATIONS

The Stability of Frozen Minced African Catfish
J.P. Durães, P.R.C. Oliveira Filho, J.C.C. Balieiro, C.R. Del Carratore and E.M.M. Viegas **61**

Physical, Chemical, Enzymatic and Microbiological Characteristics of Artisanal Rennet Pastes from the Centre of Italy
C. Tripaldi, G. Palocci, S. Bilei, T. Bogdanova, M.F. Scintu and M. Addis **70**

SURVEYS

Dietary Habits of Children Living in the Italian Area of Versilia
D. Fiorini, S. Perseo and R. Ballini **76**

Food Safety Knowledge and Practices of Nursing Students
N. Sanlier, S. Bilici, B. Çelik and E. Memiş **86**

GUIDE FOR AUTHORS **94**

CONTRIBUTORS **97**

PREVIEW ON VOLUME XXIV No. 2, 2012

PAPERS

Effects of Exposure to Gaseous Ozone and Negative Air Ions on Control of Epiphytic Flora and the Development of *Botrytis Cinerea* and *Penicillum Expansum* During Cold Storage of Strawberries and Tomatoes
R. Tuffi, R. Lovino, S. Canese, L.M. Cafiero and F. Vitali

Biochemical and Technological Properties of *Penicillium roqueforti* and *Geotrichum candidum* Strains Isolated from Cabrales, a Spanish Traditional, Blue-Veined, Starter-Free Cheese
C.O. Ferreira, P. Álvarez-Martín, A.B. Flórez, M. Díaz and B. Mayo

Kinetic Characterization of Bacterial Isolates Able to Promote Malolactic Fermentation at Reduced Temperatures
A. Zinnai, F. Venturi, A. Toffanin, M. Agnolucci, M. Nuti and G. Andrich

Volatile Composition of "a La Piedra" Turrón Enriched with Royal Jelly
E. García-García, E. Narbona, P. Jurad, F. Burló, E. Roch and Á.A. Carbonell-Barrachina

Monitoring Bacteriological Quality of Precooked Lasagna
M.A. Marzano and C.M. Balzaretti

Relationship Between the Inner Cellulation of Synthetic Stoppers and the Browning of a White Wine Over Eighteen Months of Storage
M. Lambri, A. Silva and D.M. De Faveri

Comparative Study of the Phenolic Composition of Seeds from Grapes *cv* Cardinal and Alphonse Lavallee During Last Month of Ripening
A. Topalovic, D. Godjevac, N. Perovic and S.Trifunovic

Fast Non-Destructive Determination of Chlorophylls in Apple Skin
A. Venturello, R. Ceccarelli, E. Garrone and F. Geobaldo

Effect of Modified Atmosphere Packaging on Halvas Farsalon Quality During Refrigerated Storage
E. Botsoglou, P. Vareltzi, P. Giannouli and A. Govaris

SHORT COMMUNICATIONS

Quality and Safety of Italian Wine Vinegar
V. Dell'Oro, A. Ciambotti and C. Tsolakis

Effects of Organic Fertilization on Quality and Antioxidant Properties of Hulled Wheats
C. Fares, P. Codianni and V. Menga