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REVIEW

NEW TECHNOLOGIES FOR EXTENDING SHELF LIFE

NUOVE TECNOLOGIE PER PROLUNGARE LA SHELF LIFE

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ABSTRACT

Some of the most important factors that reduce the shelf life of many foods are oxygen, light, spoilage bacteria and inadequate cooling. The strategies followed by Matforsk – Norwegian Food Research Institute in efforts to prevent or limit the damage caused by these factors are 1) to understand the effects of oxygen and light of varying wavelengths on different foods and 2) to develop protection for the food using adequate light and oxygen barrier packaging and protective cultures

RIASSUNTO

Alcuni dei più importanti fattori che riducono la shelf life di molti alimenti sono l'ossigeno, la luce, la contaminazione batterica e la refrigerazione inadeguata. Le strategie seguite dal Matforsk – Norwegian Food Research Institute per prevenire o limitare i danni causati da questi fattori sono 1) comprendere gli effetti dell'ossigeno e della luce di differenti lunghezze d'onda su diversi alimenti e 2) migliorare la protezione degli alimenti utilizzando adeguati confezionamenti che funga-

- Key words: bacteriocin, CO_2-emitter, marinades, oxidation, packaging, shelf life, Time-Temperature Indicator -

and antioxidative marinades. Application of the right packaging gas for different types of food, including the use of an active packaging concept such as a CO_2-emitter, is also crucial for ensuring sensory and microbial shelf life. Fighting spoilage and pathogenic bacteria may also be done by means of protective, bacteriocin-producing bacterial cultures. Inadequate cooling may be detected by means of an intelligent packaging concept such as Time-Temperature Indicators. This paper is a review of seven recent studies conducted at Matforsk that were undertaken in an effort to extend the shelf life of different types of food, including extruded oats, cheese, butter, fresh salmon, smoked salmon, fresh cod, ground beef and cooked poultry meat cuts.

no da barriera per la luce e l'ossigeno, colture microbiche protettive e marinature con funzione antiossidante. L'utilizzazione per il confezionamento di un gas idoneo a ogni determinato alimento e l'utilizzo del concetto di confezionamento attivo, come ad esempio un emettitore di CO_2, costituisce elemento cruciale per garantire la shelf life sensoriale e microbica. Si può anche combattere lo sviluppo di batteri patogeni utilizzando colture batteriche produttrici di batteriocine. Una refrigerazione inadeguata può essere individuata mediante l'impiego di imballaggi intelligenti, ad esempio con indicatori Tempo-Temperatura. Questo lavoro è una rassegna di sette studi recentemente condotti a Matforsk, svolti al fine di prolungare la shelf life di diversi tipi di alimenti, inclusa l'avena estrusa, il formaggio, il burro, il salmone fresco, il salmone affumicato, il merluzzo fresco, il manzo macinato e tagli di carne di pollame cotta.

INTRODUCTION

Ever since man put his foot on Earth, he has faced the challenges of periodical supply and the constant demand for food. Over the centuries, different processing techniques have been developed to secure a constant availability of nutrients; e.g. drying, salting, pickling, smoking, curing and fermentation. Today these processing techniques are regarded by many as merely ways to produce food with desired sensory characteristics, but their original purpose was to increase the shelf life. Developments in food packaging were equally important to safeguard the quality and wholesomeness of the preserved food. The era of simple pottery, baskets and leather bags was followed by the era of the amphora, the wooden barrel and the wooden box. Modern times brought glass jars and bottles, metal cans for retorting, a variety of paper and cardboard packages, and finally, a large variety of different types and combination of plastic materials. In addition, different active and intelligent packaging concepts have been developed to secure or monitor the quality of the packaged food. The scope of this review paper is to present some of the recent studies undertaken at MATFORSK AS – Norwegian Food Research Institute, to prolong, secure or monitor the shelf life and safety of various types of food with emphasis on different packaging techniques.

CO_2-EMITTERS FOR EXTENSION OF SHELF LIFE FOR FRESH, FARMED COD (*GADUS MORHUA* L.)

INTRODUCTION

In modified atmosphere packaging (MAP) of perishable food CO_2 is the most important component in the gas mixture because of its antimicrobial activity. A CO_2-enriched atmosphere is therefore added to achieve a prolonged shelf life and preserve quality. An increased concentration of CO_2 leads to an increased inhibition of bacterial growth (BAGGERAVN *et al.*, 2003; DEVLIEGHERE and DEBEVERE, 2000; DEVLIEGHERE *et al.*, 1998). Processing parameters like gas/product volume ratio (G/P-ratio) and the initial gas concentrations are important factors that determine the final effect of MAP (DEVLIEGHERE *et al.*, 1998).

CO_2-absorption and thereby inhibition of bacterial growth increase with decreasing temperature (GILL and TAN, 1979). Therefore the shelf life of MAP fish is greatly influenced by temperature because CO_2-absorption increases with decreasing temperature. Low storage temperature alone can prolong the shelf life, but in the case of MAP the effect of CO_2 is increased with a temperature as close as possible to 0°C. This is a challenge throughout the whole distribution chain, but is essential in order to achieve the desired shelf life and safety of modified atmosphere packaged seafood.

Trimethylaminoxide (TMAO) forms part of the easily available nitrogen-sources in gadoid fish (OEHLENSCHLAGER, 1992), where it may serve as an osmo-regulator (LOVE, 1988). Lean, trimethylaminoxide-containing fish such as cod should not be stored in the absence of oxygen, because certain spoilage bacteria may convert the TMAO to trimethylamine (TMA), which has an undesirable, rotten smell. An atmosphere containing 60% CO_2 and 40% O_2 has been reported to be the most effective to prevent the production of TMA (DEBEVERE and BOSKOU, 1996) since the availability of oxygen decreases the use of TMAO as a secondary electron acceptor.

The aim of this ongoing research activity is to reduce the headspace and thereby reduce the transport costs of modified-atmosphere packages without compromising quality, safety and shelf life by means of a CO_2-emitter. A CO_2-emitter releases CO_2-gas into the package after sealing to compensate for the gas absorbed by the water and fat of the product. This prevents package collapse and maintains a high CO_2-concentration inside the package (HANSEN *et al.*, 2005; EIE *et al.*, 2003; 2004).

MATERIALS AND METHODS

Farmed cod were harvested, cooled in chilled (2°C) seawater, killed by gill cut, and a blow to the head, transferred to a bleeding tank and gutted immediately after 10-15 min. Half of the fish were filleted pre-rigor and the other half were stored on ice and filleted post-rigor 5 days later. The fish fillet cuts were packaged on the same days as filleting. All the packages were stored at 1.3°C.

The CO_2-emitter was made by mixing dry $NaHCO_3$ and citric acid before placing a humidity absorber inside and adding small amounts of sterile water to each absorber immediately before sealing the package.

High Density Polyethylene trays (127 mL for the G/P 1.6:1 emitter samples and 283 mL for the G/P 4.4:1 MAP samples) were sealed with a Dyno Type 462-VGA tray sealing machine (all from Polimoon as, Kristiansand, Norway) using an EVOH-containing barrier top web (ESB 65 HFP, Wipak, Finland). The 60% CO_2/40% O_2 gas mixture used was supplied by Yara Industrial AS (Oslo, Norway). Vacuum-packed samples were sealed on a Verbomatic C 15-HL vacu-

um-chamber machine (Verbomatic, Bochum, Germany) in 20 μm Polyamide/70 μm Polyethylene vacuum bags (Linvac Plastic Pountivy S.A., Noyal-Puntivy, France).

Headspace CO_2-content (CheckPoint O_2/CO_2 analyser from PBI Dansensor, Ringsted, Denmark), drip loss (weight increase of the humidity absorber), total bacteria count (on Long&Hammer Agar), TMA-content (GC-MS method) and sensory quality (using a trained panel consisting of 10 persons).

RESULTS AND DISCUSSION

The results (average of pre-and post-rigor samples) are shown in Figs. 1-5. Fig. 1 shows that severe drip losses occurred for the three different packaging methods. In the first half of the storage period, the drip loss was much higher for the vacuum-packed samples than for the two MAP samples, probably because vacuum-packaging is a mechanically rough packaging method for high-moisture, delicate foods, such as fish fillets.

Fig. 2 shows that in the traditional MAP samples (MAP G/P 4.4:1) the CO_2-concentration decreased as the CO_2-gas was absorbed by the product. This caused a slight vacuum-effect which increased the O_2-concentration. In the emitter-samples, the CO_2-concentration increased until day 7, more than counterbalancing CO_2 absorption by the cod fillet. Hence, a slight overpressure in the package was observed, which diluted and thereby lowered the O_2-concentration.

Fig. 3 shows more bacterial growth in the vacuum-packed samples due to lack of the bacteria-inhibiting CO_2-gas. No differences in the bacterial growth were observed between the MAP samples and Emitter samples. Figs. 4 and 5 show the combined effect of higher bacterial growth and no available oxygen in the vacuum-packed samples. The TMA concentration was much higher and sensory scores for sour odour were higher than the CO_2-containing packages. As for bacterial growth, no differences were observed between the MAP samples and Emitter samples for TMA content and sensory score.

CONCLUSIONS

- Packaging with reduced headspace and a CO_2-emitter inhibited bacterial growth at least as well as traditional MAP

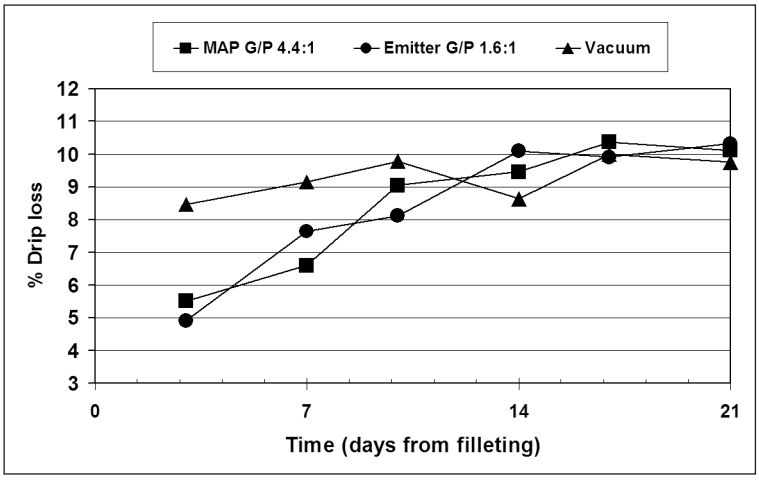


Fig. 1 - Development of drip loss in packages of MAP with G/P-ratio 4.4:1, MAP with a CO_2-emitter and G/P-ratio 1.6:1 and vacuum during storage at 1.3°C (average of pre- and post-rigor filleted farmed cod).

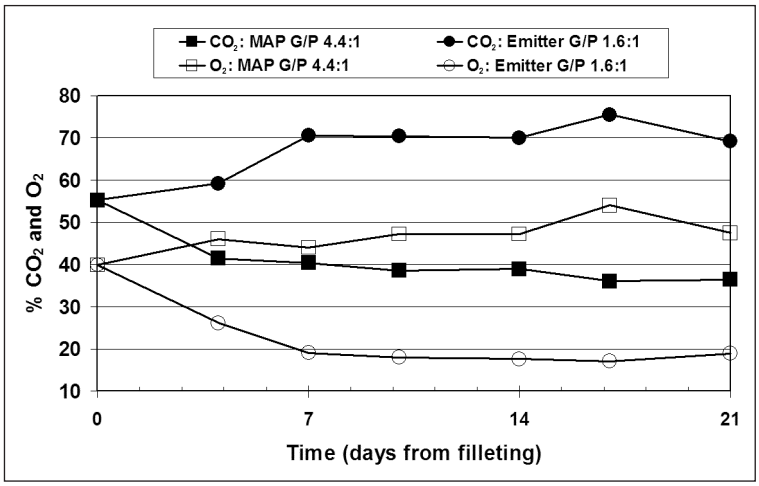


Fig. 2 - Development of CO_2-concentrations and O_2-concentrations in packages of MAP with G/P-ratio 4.4: 1and MAP with a CO_2-emitter and G/P-ratio 1.6:1 during storage at 1.3°C (average of pre- and post-rigor filleted farmed cod).

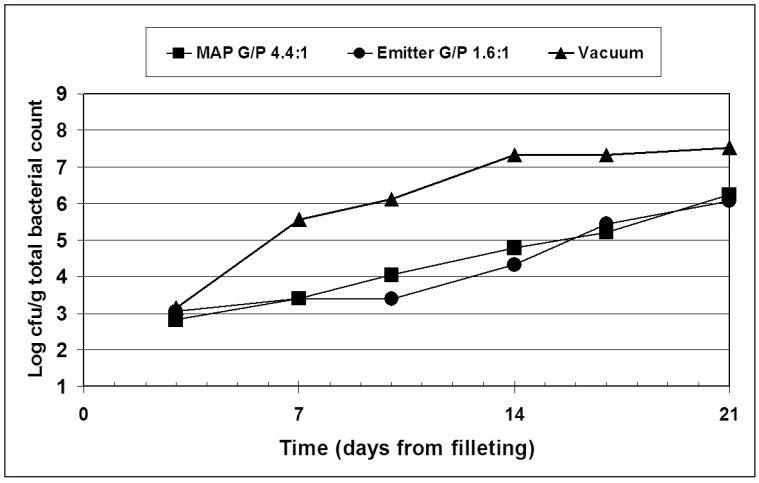


Fig. 3 - Development of total bacterial count on Long & Hammer Agar in packages of MAP with G/P-ratio 4.4:1, MAP with a CO_2-emitter and G/P-ratio 1.6:1 and vacuum during storage at 1.3°C (average of pre- and post-rigor filleted farmed cod).

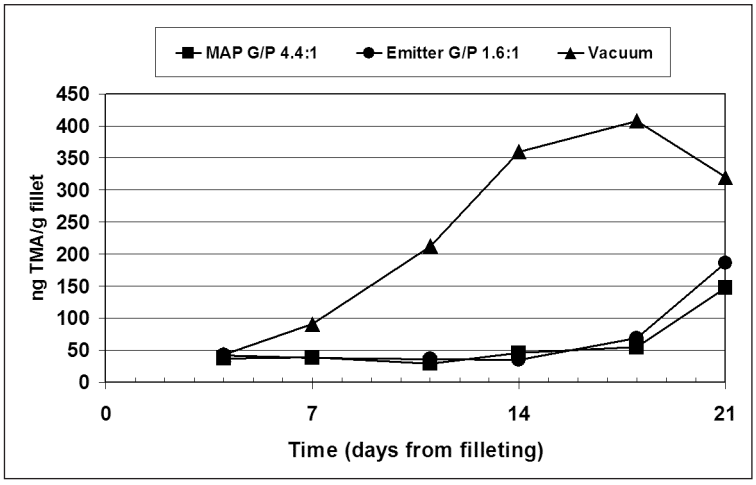


Fig. 4 - Development of TMA (trimethylamine) in packages of MAP with G/P-ratio 4.4:1, MAP with a CO_2-emitter and G/P-ratio 1.6:1 and vacuum during storage at 1.3°C (average of pre- and post-rigor filleted farmed cod).

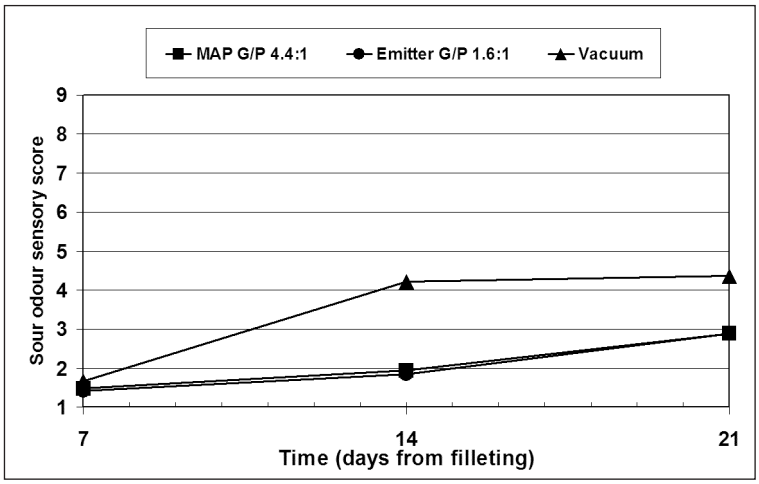


Fig. 5 - Development of sour odour in packages of MAP with G/P-ratio 4.4:1, MAP with a CO_2-emitter and G/P-ratio 1.6:1 and vacuum during storage at 1.3°C (average of pre- and post-rigor filleted farmed cod).

with a larger headspace with respect to inhibition of bacterial growth, prevention of TMA development and development of sour odour.

- Vacuum packaging resulted in greater bacterial growth, higher TMA concentration levels, more drip loss and lower sensory scores than the two modified atmosphere packaging methods.

- A double transport efficiency was achieved with the use of CO_2-emitter compared to traditional MAP-technology.

THE USE OF TIME-TEMPERATURE INDICATOR FOR MAP FRESH MEAT

INTRODUCTION

Time-temperature indicators (TTI) can be defined as simple, inexpensive devices that can show an easily measurable, time-temperature-dependent change that reflects the full or partial temperature history of a food product to which it is attached (TAOUKIS and LABUZA, 1989a). TTI works as a flexible "use by" date where the TTI-indicated shelf life depends on the actual time/temperature history of the product throughout the distribution chain. The TTI adjusts the shelf life indication according to the real distribution temperature, and not the estimated, ideal or regulatory "use by" date. TAOUKIS and LABUZA (1989a, b; 2003) have shown that TTIs can be used as quality monitors for distribution control and stock rotation management instead of, or in conjunction with, "best-before" dates.

The Arrhenius equation states the dependence of a reaction rate as a function of temperature according to:

$$k = A \cdot e^{-(Ea/R \cdot T)},$$

where k= rate of reaction, A= constant, R= universal gas constant, T= Absolute temperature (in °K) and Ea= Arrhenius energy of activation.

The energy of activation (Ea) defines the temperature sensitivity of a given reaction, that is the time/temperature impact needed to provoke a given response, e.g., the total bacterial count needed to reach a certain (unacceptable) level, the sensory properties to become unacceptable and the TTI to change appearance, such as colour. The energy of activation can be found by applying re-

sponse time results from a storage experiment at ≥3 temperatures in an Arrhenius plot. As different food deterioration processes have different temperature sensitivities and different shelf lives at a given temperature, the producer of the TTI must be able to adjust both the energy of activation (Ea) and the response time according to the actual food product.

ROGNERUD (2002) investigated the suitability of a TTI to monitor and communicate the shelf life of two fresh meat products packed in a modified atmosphere.

MATERIALS AND METHODS

The VITSAB® M2-20 TTI (VITSAB AB, Malmö, Sweden) was used to study its suitability for commercially produced and MA-packed shredded pork and ground beef. The VITSAB® M2-20 TTI had an energy of activation of 109 kJ/mol and a response time of 20 days at 2°C, indicated by a colour change from green to yellow. The colour changes of the TTIs at all 3 temperatures were frequently observed visually to determine the time at which the enzyme/substrate/indicator solution inside the indicator changed from green to yellow.

The "use-by" date for the MA-packed shredded pork and ground beef were, respectively, 13 and 14 days at a recommended (and legal) storage temperature of 0°-4°C. The meat products were incubated at 2°, 4° and 8°C (the ideal, legal and abuse temperatures in the distribution).

During the incubation period, the total plate counts on Standard Plate Count Agar (APHA, CM0463, Oxoid Ltd., Hampshire, U.K.) for the products was assessed after 2, 9, 15, 22 and 30 days at 2°C, after 2, 9, 15, 22 and 26 days at 4°C and after 2, 9, 11 and 15 days at 8°C. The bacterial plates were incubated at 20°C for 4 days before counting the colonies. The response times for total plate counts were based on the number of days needed for the total plate count to reach 10^6 cfu/g for the different temperatures and products.

The Arrhenius equation was used to calculate the activation energy for the TTI based on both visual reading of the TTI colour and the time for total plate count to reach 10^6 cfu/g at 2°, 4° and 8°C.

RESULTS AND DISCUSSION

The increase in total plate count at 2°, 4° and 8°C in ground beef and shredded pork is shown in Figs. 6 and 7. Table 1 shows the response times extracted from the curves in Figs. 6 and 7, the corresponding activation energy (Ea) values and the correlation coefficients. Table 1 also includes the response times, Ea-value and correlation coefficient of the VITSAB® M2-20 TTI as specified by the producer, and the values obtained by visual determination of the TTI colour change.

Figs. 6 and 7 and Table 1 show that the temperature had a significant impact on bacterial shelf life and the time needed for the bacterial growth to reach 10^6 cfu/g. They also show that the VITSAB® M2-20 TTI was well suited for the products with respect to activation energy. The values were well within the suggested maximum 10 kcal/mol (42 kJ/mol) discrepancy (TAOUKIS and LABUZA, 1989a). The visual response times at 2° and 8°C fit well with the total plate count response times for both meat products at both temperatures. At 4°C, however, the visual response of the TTI indicated a much longer shelf life (16 days) than the number of days needed to reach 10^6 cfu/g (~13 days). It should be noted that these products were fully edible at 10^6 cfu/g and most probably 3 days later when the TTI changed colour when the bacterial counts were well below 10^7 cfu/g.

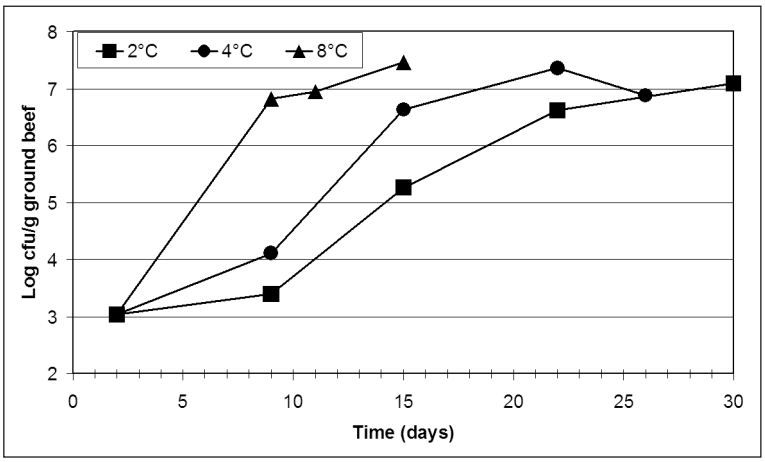


Fig. 6 - Development of total plate count in modified atmosphere packed ground beef at 2°, 4° and 8°C. Data from ROGNERUD (2002).

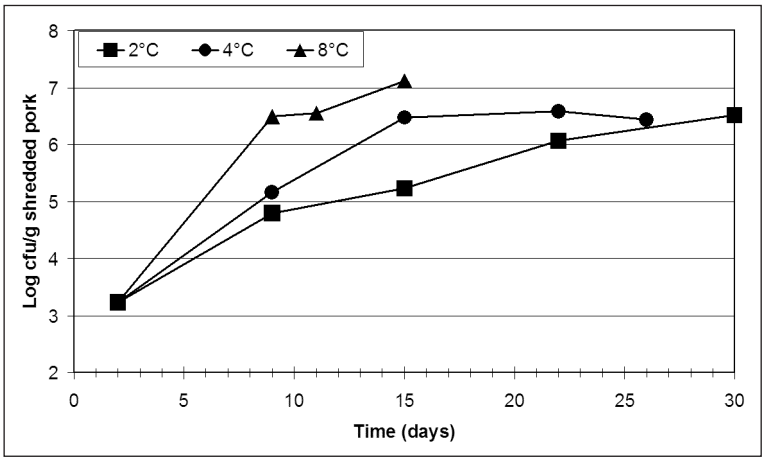


Fig. 7 - Development of total plate count in modified atmosphere packed shredded pork at 2°, 4° and 8°C. Data from ROGNERUD (2002).

Table 1 - Response times (in days) at 2°, 4° and 8°C of TTI (according to manufacturer's specification and as observed visually) and for growth to 10^6 cfu/g of total count bacteria in ground beef and shredded pork and the corresponding, calculated activation energy (Ea) and r^2. Data compiled from ROGNERUD (2002).

	2°C	4°C	8°C	Ea (kJ/mol)	r^2
TTI response according to specification	20.0	14.0	7.2	109.0	1.0000
TTI response, visually observed	22.0	16.0	8.0	108.8	0.9994
Total plate count response, ground beef	22.4	12.8	8.0	106.2	0.9483
Total plate count response, shredded pork	21.5	12.8	8.0	101.7	0.9553

CONCLUSIONS

- The VITSAB® M2-20 TTI followed an Arrhenius behaviour with good linear fit within the tested temperature range.

- The VITSAB® M2-20 TTI was suitable for the tested products with respect to response times. The activation energy (Ea) was within the suggested maximum discrepancy from the total plate count limit of the products.

- TTI has a potential for use with fresh

meat products packed in modified atmospheres.

THE EFFECT OF LIGHT AND OXYGEN ON EXTRUDED OATS

INTRODUCTION

Oxidation of lipids and other food ingredients is one of the major factors limiting the shelf life of microbiologically stable foods, such as dry or frozen products. Lipid oxidation can be limited by reducing the exposure to oxygen and light, which are factors that can be controlled by packaging. The oxygen transmission rate is one of the most important properties that is evaluated when selecting appropriate packaging for a food product.

Increased awareness of the health benefits associated with oats has renewed interest in oat (*Avena sativa* L.) products (MOLTEBERG, 1996). Extruded oats are a common ingredient in many ready-to-eat breakfast cereals. However, since dehydrated extruded oats are susceptible to lipid oxidation (SJÖVALL *et al.*, 1997), the packaging and storage conditions are crucial to ensure a long shelf life for such products.

The Ambient Oxygen Ingress Rate (AOIR) method has been found to be a good alternative or supplement to the isostatic method (Ox-Tran) for measuring the oxygen transmission rate (OTR) of packages. Compared to the Ox-Tran method, AOIR equipment is less expensive, has a greater capacity and the OTR of the packages can be measured under test conditions that are more similar to the temperature and relative humidity conditions used in food storage. Another positive aspect of the AOIR method is that the headspace oxygen concentration in nitrogen flushed packages can be predicted after a given elapsed time (LARSEN *et al.*, 2000, 2002).

The objective of the present work was to study how the oxygen transmission rate of packages, in combination with light and temperature, influences the rancidity of extruded oat pellets (LARSEN *et al.*, 2003).

MATERIALS AND METHODS

Extruded oats were packaged with 100% N_2 on a Tiromat Powerpack 300 thermoforming machine (Bakel, Holland) in films with different oxygen barriers to give packages (120x143x68 mm) with oxygen transmission rates of 0.03-0.09, 0.42-1.59 and 3.93-10.66 mL O_2/pkg·day at 23°-38°C at 50% RH, as determined by the AOIR method (LARSEN *et al.*, 2000).

The packages were stored either in the dark, or in the light (960-1390 Lux, warmwhite 36W/830 fluorescent tubes from Auralight, Oslo, Norway) at 23° and 38°C for 3 months.

The O_2-concentration in the headspace of packages filled with extruded oats was measured after 1, 2 and 3 months of storage by removing 10 mL gas samples with a disposable syringe through self-sealing septa on the packages and injecting the samples into a MOCON Toray oxygen analyzer LC-700F (Modern Controls Inc., Minneapolis, Minnesota, USA).

Paint odour was assessed by descriptive sensory analysis (ISO, 1985) performed by an accredited sensory laboratory employing a panel of 10-11 trained (ISO, 1993) assessors after 1, 2 and 3 months of storage using a 9-point scale. Test samples were compared with reference samples stored either at 23°C in vacuum-packed aluminium foil/PE pouches, or in light at 38°C for 4 weeks in PE pouches. Estimated times for the onset of paint odour (i.e. sensory score for paint odour ≥ 3) were based on linear regression (LARSEN, 2002).

RESULTS AND DISCUSSION

The oat pellets had a high lipid content (10.7% dry solid) with a relatively high proportion of unsaturated fatty acids (79.9% of fat) (LARSEN *et al.*, 2003) which made them susceptible to oxidation. The sensory scores of paint odour after 3 months under the different packaging and storage conditions are shown in Fig. 8. Fig. 9 shows the calculated O_2-concentration in empty N_2-filled packages (based on the equations from the AOIR method (LARSEN *et al.*, 2002) and the actual O_2-concentration in packages filled with extruded oat pellets and stored under the different light and temperature conditions. The arrows in Fig. 9 indicate the estimated onset of paint odour found by linear regression of the sensory results after 1, 2 and 3 months, assuming that rancidity was detected when sensory scores for paint odour were significantly different from the reference sample packed in ALU/PE (i.e. ≥3).

Both Figs. show that light was the design factor that had the greatest effect on the development of paint odour. The

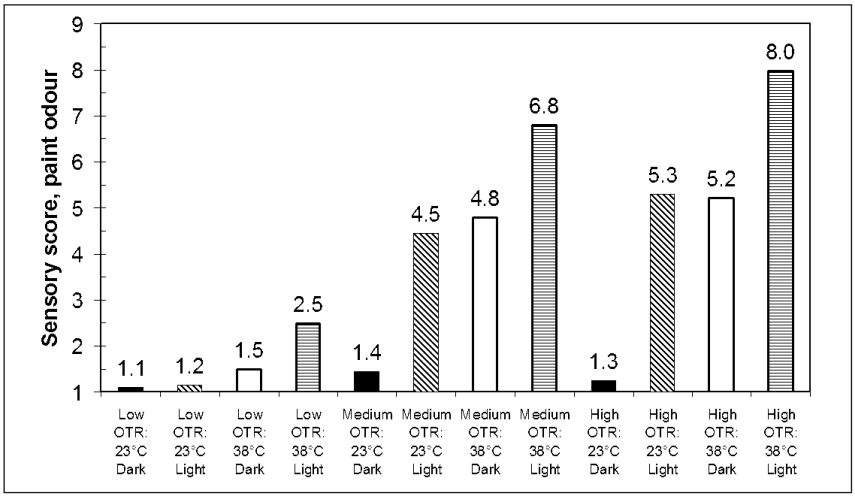


Fig. 8 - Sensory score of paint odour after 3 months of storage in the light or in the dark at 23° or 38°C at 50% RH in packages with low, medium and high OTR. Data from LARSEN *et al.* (2003).

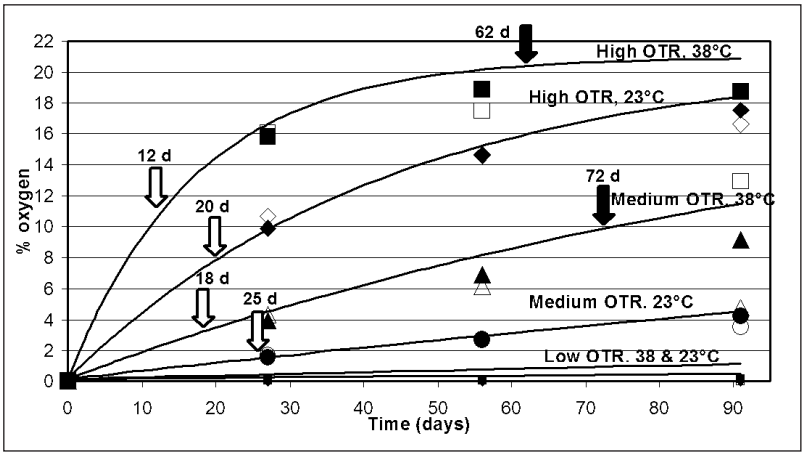


Fig. 9 - Calculated headspace O_2-concentration curves in empty N_2-filled packages and analysed development of O_2 in the same packages, but filled with N_2-packed extruded oats at 23°C/50% RH and at 38°C/50% RH. Arrows indicate estimated time for sensory score ≥3 for paint odour. Open symbols and arrows: illuminated storage. Filled symbols and arrows: Dark storage. Data from LARSEN (2002); LARSEN *et al.* (2003).

effect of light was most predominant when combined with high oxygen levels (i.e. medium and high OTR packages) and high storage temperature. During the 3-month storage period no distinct paint odour developed in packages with low oxygen transmission rates, not even at 38°C with light exposure. Oat pellets packaged in high and medium OTR packages were, in most cases, very rancid (high score of paint odour) after 3 months, except for samples stored at 23°C in the dark. This clearly shows the effect of temperature, oxygen and light on the development of rancidity in extruded oat pellets.

The deviation between the calculated O_2-concentration in empty, N_2-filled packages and the measured O_2-concentration in packages with oat pellets indicates major O_2-consumption due to severe oxidation. This was apparent after 3 months at 23°C and after both 2 and 3 months at 38°C. The deviation was more pronounced in light-exposed samples. These observations are in agreement with those of LABUZA (1971) who distinguished between the induction period of oxidation and the bimolecular period, with the latter having an exponential oxygen uptake. The high oxygen uptake observed after 2 and 3 months in medium and high OTR packages, especially at 38°C in the light-exposed samples, indicates that these samples were in the bimolecular period. There was marked oxidation with an oxygen uptake that exceeded the oxygen transmission into the packages.

Fig. 9 may be regarded as a "shelf life map" where the effect of time, temperature, oxygen and light on shelf life may be considered. These correlations may be used to estimate shelf life for a given packaging material under given temperature and light conditions, or to find the required barrier properties of the packaging material in order to achieve a given shelf life when the temperature and light conditions are known.

In later experiments LARSEN *et al.* (2005) showed that by accelerating the shelf life testing by increasing the temperature from 23° to 38°C, the packaging evaluation test for extruded oats stored in the dark could be performed in one-third the time.

CONCLUSIONS

- Light-exposed oat pellets packed in medium and high OTR packages became highly rancid at 23° and 38°C during a 3-month storage period.
- Oat pellets stored at 38°C in the dark became rancid only in medium and high OTR packages.
- Oat pellets in low OTR packages did not become rancid during the 3-month storage period, not even at 38°C under light exposure.

THE EFFECT OF LIGHT AND OXYGEN ON CREAM CHEESE

INTRODUCTION

A single polymer is often not adequate for a package in most food applications, because it cannot provide all the properties needed to create suitable barrier characteristics (STÖLLMAN *et al.*, 1994). The most common technologies for producing moulded plastic containers for solid or semi-solid foods are injection moulding and sheet thermoforming. Thermoforming is a relatively old and simple process by which a thermoplastic sheet is heated to its softening temperature (above Tg for non-crystalline thermoplastics and near Tg for crystalline ones), usually by means of an infra-red radiant panel heater (GROOVER, 2002; ROBERTSON, 1992). The poor control of thickness distribution is the major limitation of thermoforming. The deeper the draw, the less uniform the wall

thickness and thereby the barrier properties. Irregular thickness can be a serious problem when there is a thin barrier in the composite sheet to be thermoformed and the finished package is intended to be used for perishable foods (PIERGIOVANNI *et al.*, 1996).

Lipid oxidation is a major factor that affects the quality of processed dairy products, especially during long periods of storage (KRISTENSEN and SKIBSTED, 1999). Autoxidation of unsaturated lipids involves the formation of free radicals followed by the formation of hydroperoxides; secondary lipid oxidation products are then formed. The oxidation leads to the formation of various volatile carbonyl compounds, which are responsible for off-flavours. Dairy products are often exposed to light during retail storage and display. Light is known to initiate oxidation processes in foods, which leads to the loss of valuable nutrients and the reduction of sensory quality.

The aim of the present study was to evaluate the effect of different OTR values of packages on the oxidative stability of cream cheese stored in the dark or exposed to light (PETTERSEN *et al.*, 2005).

MATERIALS AND METHODS

Commercial grade, unflavoured cream cheese (28% fat) made from bovine milk and cream, salt and stabiliser was obtained from TINE Meieriet Vest Hardanger (Øystese, Norway).

The cheese was packaged directly from the cheese production line in 195x135x50 mm (1222 mL) thermoformed trays and a 52 μm PET/AlOx/PE lid film (Südpack Verpackungen, Ochsenhausen, Germany) using "soft vacuum" on a Polimoon 500 VG semi-automatic tray-sealing machine (Polimoon, Kristiansand, Norway). Only cheese from the corners of the trays was used for the analyses. The sheets used for thermoforming the trays were all commercially available: 750 μm transparent and 550 μm black A-PET/PE ("Multipet" from Wihury OY Wipak, Nastola Finland) and 750 μm white PS/EVOH/PE ("Ecoform" from Südpack Verpackungen (Ochsenhausen, Germany). The sheets were thermoformed to trays on a Kiefel KL 52 SH thermoforming machine (Paul Kiefel GmbH, Freilassing, Germany) at EKA AS (Hamar, Norway) using plug-assist. The thickness of unconverted sheets and the bottom centre of trays were measured using a 543 Series Digimatic Indicator (Mitutoyo MFG Co. Ltd, Japan).

The OTR of unconverted sheets was analysed on a Mocon Ox-Tran 10/50 (Modern Controls Inc., MN, USA) according to ASTM (1990) at both 23°C 50% RH and at 23°C 100% RH as described in Pettersen *et al.* (2004). The OTR of empty trays was measured according to the AOIR method (LARSEN *et al.*, 2000) at 6°C, 80% RH outside and 100% RH inside. Light transmission of the unconverted packaging material and the bottom of the trays was assessed using a Perkin Elmer Lambda 800 UV/Vis Spectrometer (Perkin Elmer Ltd., Buckinghamshire, UK) with an integrating sphere. Acidulous and sunlight flavours were assessed after 6 months dark/6 months dark+24 h. illuminated storage (5000-6000 Lux, 30 cm from a Coolwhite Delux 840, Auralight) by an 11-person, trained sensory panel according to ISO 8586:1993. Volatile oxidation products were analysed by a dynamic headspace GC-MS method described by MIELNIK *et al.* (2002).

RESULTS AND DISCUSSION

Trade name, supplier, colour, thickness and OTR of packaging materials and trays are presented in Table 2. Light transmission in unconverted sheets and in the bottom of the corresponding trays with 50 mm drawing depths is shown in Fig. 10.

Table 2 - Packaging material trade name and supplier, laminate structure, colour, sheet and tray bottom thickness and OTR of unconverted sheets[1] and formed and sealed trays[2]. Data partly from PETTERSEN *et al.* (2004; 2005).

Trade name	Composition	Colour	Thickness (µm)	Drawing depth (mm)	OTR[c]
Multipet; Wihuri OY	A-PET/PE	Transparent	749	0	2.24[a]/2.43[b]
	A-PET/PE	Transparent	576	50	0.06
Multipet; Wihuri OY	A-PET/PE	Black	550	0	1.15[a]/3.89[b]
	A-PET/PE	Black	372	50	0.08
Ecoform; Südpack	PS/EVOH/PE	White	788	0	1.24[a]/50.37[b]
	PS/EVOH/PE	White	465	50	0.03
PETXL/PE;Südpack	PET/AlOx/PE	Transparent	52	0 (lid film)	3.20[a]/3.03[b]

[1] Measured according to (ASTM, 1990) at [a]23°C 50% RH and at [b]23°C 100% RH: [c]mL O_2/m_2•day•atm;
[2] Measured by AOIR method (LARSEN *et al*., 2000) at 6°C 50% RH outside and 100% RH inside: [c]mL O_2/package•day•atm.

The light transmission is not influenced greatly by converting the transparent or black packages. The transparent trays transmit significant amounts of light from 325 nm and above, which includes the UV-region. The TiO_2 added to the white packaging material effectively blocks the irradiation in the UV region, but the visible and infra-red light is transmitted to a certain degree (up to 18% in the red light region). The light transmission of the white sheet is also affected by converting to 50 mm trays due to the thinning of the material (from 737 µm in an unconverted sheet to 465 µm in the bottom of 50 mm trays). The light transmission is not affected by the conversion of the black sheet because the unconverted sheet and the converted tray effectively block out all the radiation in the measured region (200-890 nm).

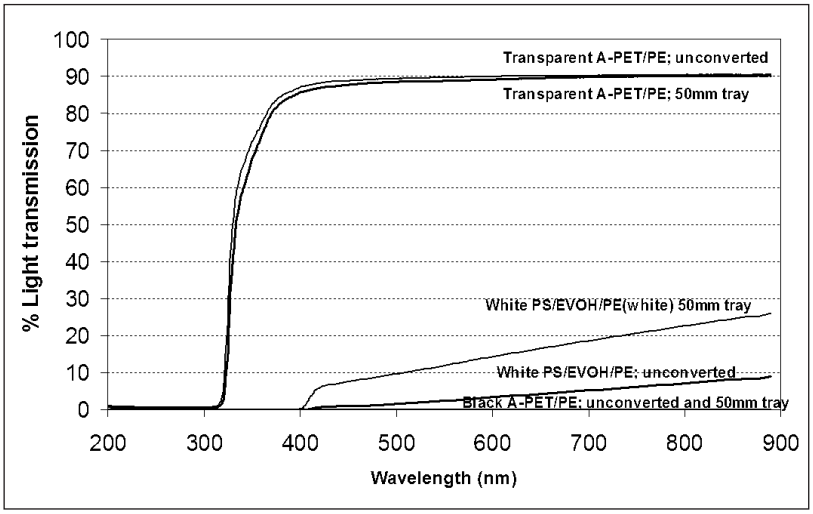


Fig. 10 - Light transmission in unconverted sheets and in the bottom of trays with 50 mm drawing depths. Data from PETTERSEN *et al.* (2005).

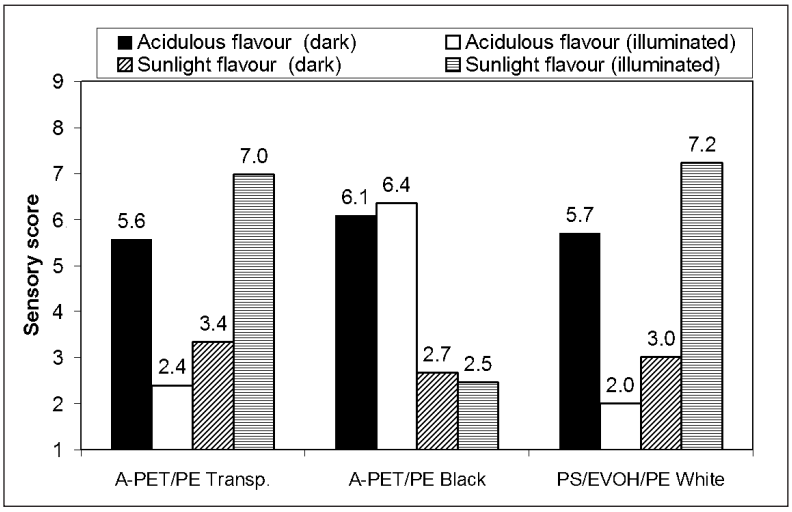


Fig. 11 - Sensory score of acidulous flavour and sunlight flavour in cream cheese stored in the dark for 6 months and thereafter illuminated for 24 hours in transparent A-PET/PE, black A-PET/PE and white PS/EVOH/PE trays with 50 mm drawing depths. All samples were stored at 6°C. Data from PETTERSEN *et al.* (2005).

Fig. 11 shows that the light transmission properties had a statistically significant effect on the sensory score for acidulous and sunlight flavours. Both transparent and white trays had significantly higher acidulous flavour values and significantly lower sunlight flavour values for samples stored in the dark compared with the samples illuminated for 24 hours. No significant differences were observed for the black trays. Differences were also observed between dark and illuminated storage for the white trays. These results demonstrate that light in the visible region (~400-700 nm) is also harmful to dairy products.

Statistically significant differences in the content of 2,3-butadione (diacetyl) and hexanal between samples stored in the dark and those exposed to light were found only in transparent trays (Fig.

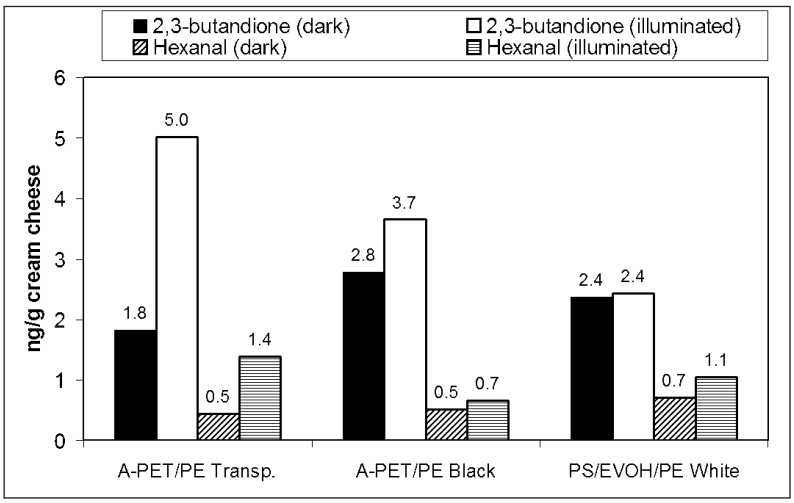


Fig. 12 - Concentrations of 2,3-butadione and hexanal in cream cheese stored in the dark for 6 months and thereafter illuminated for 24 hours in transparent A-PET/PE, black A-PET/PE and white PS/EVOH/PE trays with 50 mm drawing depths. All samples stored at 6°C. Data from PETTERSEN *et al.* (2005).

12). Although 2,3-butadione may contribute positively to the flavour of dairy products (CHRISTENSEN and HØLMER, 1996), this was not enough to counteract the formation of volatiles that had a negative effect on the sensory properties. White packages gave some protection against lighting, but this was not enough to prevent the loss of acidulous flavour and an increase in sunlight flavour.

CONCLUSIONS

- Light has a pronounced negative impact on the quality of cream cheese.
- Dark storage, or packaging in black trays gave the best sensory results and the lowest 2,3-butadione and hexanal content values.
- White trays did not protect the cream cheese from photooxidation which had a negative effect on the sensory properties.

THE EFFECT OF LIGHT AND OXYGEN ON BUTTER

In milk and dairy products, it is generally accepted that riboflavin plays the major role as a photo-sensitizer. Riboflavin absorbs light in the UV region of the spectrum and up to about 500 nm (violet and blue light). Several studies indicate that the 400-500 nm region is the most harmful part of the visible spectral region with regard to photo-oxidation in dairy products (BOSSET, 1994). "Cold" light (rich in the blue and violet parts of the visible spectrum) is more harmful than "warm" light (rich in the yellow, orange and red parts of the visible spectrum). These results are valuable, but not specific in terms of wavelength regions.

WOLD *et al.* (2005) found that in addition to riboflavin, chlorophylls and porphyrins probably act as photosensitisers in cheese. It was shown that the sensory measured photo-oxidation in cheese induced by red light did not differ significantly from that induced by blue light. The most degraded quality was caused by violet light, while green and yellow light (500-600 nm) gave the least adverse effects. Similar results were obtained for milk (HANSEN *et al.*, 1975). These results support the hypothesis that porphyrins and chlorophylls are also active photosensitisers in cheese. This supports the findings of BORLE *et al.* (2001) and BEKBÖLET (1990) who indicated that green and yellow package material can protect milk products to a certain extent because none of these photosensitisers have pronounced light absorption peaks in the green and yellow part of the spectrum (520-610 nm).

The purpose of this study was to evaluate the effect of different photo-sensitizers on the photodegradation of butter as measured by front face fluorescence spectroscopy and sensory analysis performed by a trained sensory panel. Front face fluorescence spectroscopy has proven to be a useful tool to measure light-induced oxidation because it is fast, non-destructive and sensitive (WOLD *et al.*, 2002a, b).

MATERIALS AND METHODS

Commercially produced butter with 1.5% salt (TINE BA, Oslo, Norway) was cut into 10x10x0.4 cm bars and tray-packed (Polimoon 511 VG, Polimoon AS, Kristiansand, Norway) in air with a transparent top-web that was covered with violet, green and red photo studio filters (357 Royal Lavendel, 89 Moss green + 3114 Tough UV filter and 19 Super Fire red + 3114 Tough UV filter, all from Rosco, Stamford, CT, USA) and exposed to the light for 12 h (1.4-

1.3 W/m^2 integrated in the 200-800 nm part of the spectrum) from two HMI 575 W/SE studio lamps (Osram GmbH, Munich, Germany). These metal halide lamps were chosen because of their relatively uniform emission spectrum in the visible and near UV region. Light transmission of the light filters was assessed using a Perkin Elmer Lambda 800 UV/Vis Spectrometer (Perkin Elmer Ltd., Buckinghamshire, UK) with an integrating sphere. Light intensity on the butter surface was measured by a calibrated Apogee SPEC-UV/PAR spectroradiometer (Apogee Instruments Inc., Logan, UT, USA) integrating radiation in the 300-800 nm region of the spectrum.

Oxidised flavour was assessed by a sensory panel consisting of 11 trained assessors according to ISO 8586:1993. Fluorescence emission spectra were measured directly on the light exposed surface of circular (D = 5 cm) samples of butter. The samples were exposed to 380 nm excitation light, and fluorescence emission spectra were recorded in the range 400-750 nm. The excitation light was generated by a 300 W Xenon light source (Oriel 6258, Oriel Corporation, Stratford, CT, USA) and passed through a 10 nm bandwidth interference filter (Oriel 59920). The light was directed onto the samples at an angle of about 45°. The spectra were collected by an imaging spectrograph (Acton SP-150, Acton Research Corp., Acton, MA, USA) connected to a sensitive charge coupled device (CCD-camera) (Roper Scientific NTE/CCD-1340/400-EMB, Roper Scientific, Trenton, NJ, USA). A cut-off filter at 400 nm (Melles Griot 03FCG049, Melles Griot, Rochester, NY, USA) was positioned in front of the spectrograph slit to suppress excitation light reflected from the sample. Exposure time was 1 second for all spectroscopic measurements. Two spectra were collected from each sample, and the average was used for further analysis. The spectrograph and detector were controlled by the software WinSpec Ver. 1.4.3.4 (Roper Scientific, Tucson, Arizona, USA).

RESULTS

Light transmission in coloured filters and UV filter, fluorescence spectra and the results from the sensory analysis of oxidised flavour after 12 h light exposure in air at 4°C are shown in Figs. 13-15.

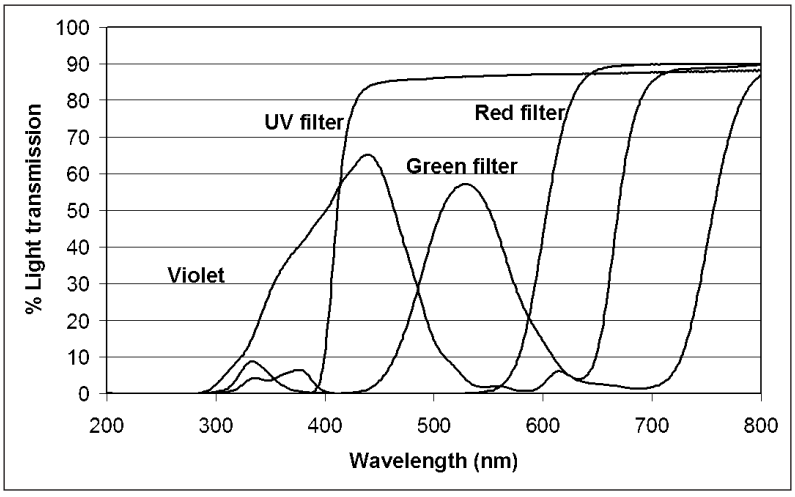


Fig. 13 - Light transmission (percent transmitted light per wavelength) through the coloured filters used in the storage experiment.

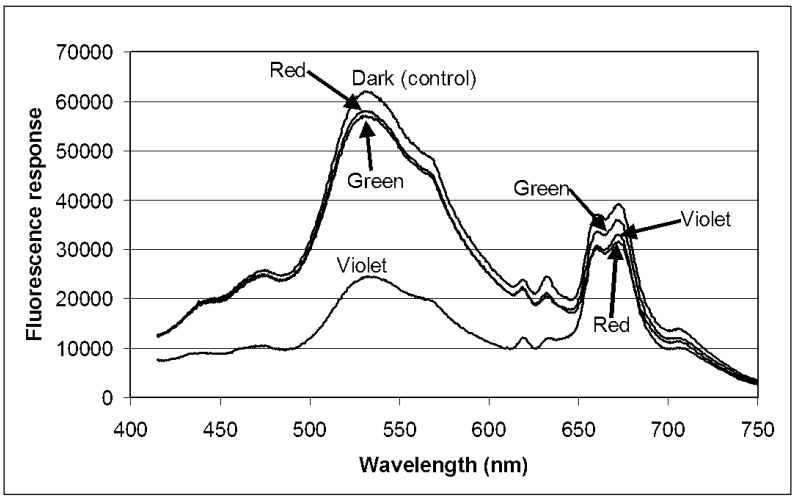


Fig. 14 - Fluorescence spectra from butter stored for 12 hours at 4°C exposed to 1.4-1.3 W/m² (integrated in the 200-800 nm part of the spectrum) + in the dark.

Fig. 13 shows that the lavender (violet) filter allowed a high transmission of the light in the UV-region and in the visible region up to 500 nm. Many biological photoinitiators present in dairy products are known to absorb energy in this region, e.g. porhyrins, chlorophylls and riboflavin. The green and red filters allowed some transmission in the UV-region, but when used together with a UV-filter (as in this investigation), this was filtered away. In the visible region of the spectrum (400-700 nm), the red filter transmits light above 570 nm where both chlorophyll *a* and *b* have additional absorption peaks (at 660 nm and 642 nm, respectively). The green filter transmits most of the light energy between 450 and 625 nm where porhyrines have a number of small absorption peaks.

Fig. 14 shows that the violet light caused severe degradation (i.e. reduced emission peaks) of the different fluorophores in butter: riboflavin (~530 nm), protoporphyrin, (635+705 nm), hematoporphyrin (620 nm), an unidentified compound of chlorophyll nature (661 nm) and chlorophyll *a* (672). The green and red filters (in combination with UV-filter) produced negligible degradation of riboflavin, but the red light caused a greater degradation of the unidentified chlorophyll and chlorophyll *a* than the green light. Photodegradation of the sensitisers usually leads to the formation of free radicals and subsequent oxidation.

The results from the fluorescence spectroscopy were further confirmed by the sensory analysis of oxidised flavour.

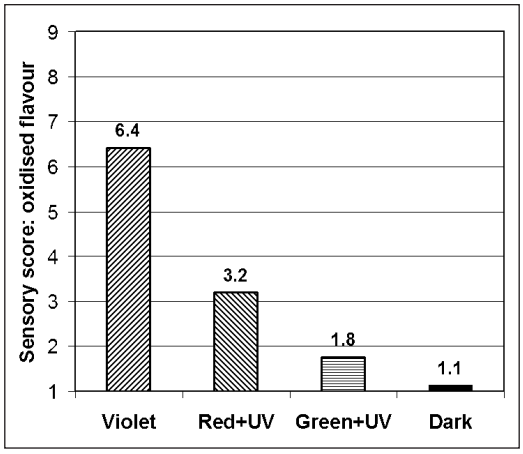


Fig. 15 - Mean sensory scores for oxidised flavour of butter stored for 12 hours at 4°C exposed to 1.4-1.3 W/m² (integrated in the 200-800 nm part of the spectrum) + in the dark.

Violet light induced the highest sensory score of oxidised flavour followed by red light and then green light. This is in agreement with previously reported results on cheese (WOLD *et al.*, 2005). HANSEN *et al.* (1975) evaluated the effects of exposure to different coloured light on the oxidation of milk. They found that, in general, green light had the least adverse effect.

CONCLUSIONS

- Light is harmful to the sensory quality of butter as it triggers photo-oxidation.
- Riboflavin is not the only photosensitizer responsible for photo-oxidation in dairy products.
- Green light is less harmful than violet and red light because the photosensitizers absorb less light in the green area.

THE EFFECT OF MEAT QUALITY AND PACKAGING ON THE COLOUR OF FRESH GROUND BEEF

INTRODUCTION

For fresh raw meat products the main features considered at retail display are a long microbiological shelf life and a stable, bright red colour. To obtain these features, it is crucial to maintain a continuous low temperature chill chain. Most consumers regard a bright red appearance of the product as an indicator of freshness so this is a decisive factor when buying a meat product.

When the product is wrapped in an oxygen-permeable cling film for display in a chill cabinet the microbiological shelf life is short and the colour stability is poor. In Western Europe, the USA and Australia, it is relatively common for the industry to package fresh meat at centralized facilities in modified atmospheres with approximately 70% O_2/30% CO_2. By applying such a high O_2 atmosphere, the time before discolouration and microbiological spoilage occur in the meat is delayed compared to wrapping. The time available for selling the product is often considered too short, even under conditions with high O_2 atmospheres.

Finding a packaging method or gas mixture that prevents microbiological growth on meat while maintaining an attractive bright red colour has been a challenge. Packaging in anaerobic atmospheres with high levels of CO_2 and vacuum packaging increase shelf life by reducing microbiological growth, but without preserving the bright red colour. The Norwegian meat industry was a pioneer in the commercial use of carbon monoxide (CO) starting in 1985 with atmospheres of approximately 0.4% CO, 60% CO_2 and 39.6% nitrogen (N_2). However, in 2004 the use of CO was terminated due to an EU decision not to include CO in the positive list for food additives.

For ground beef, the CO-containing gas was replaced by a 60% CO_2/40% N_2 gas mixture. Under favourable storage and packaging conditions, i.e. low temperature and a low level of residual O_2, this gas mixture will produce a long shelf life (due to the high CO_2-content) and a favourable, dark red deoxy-myoglobin colour of the meat. However, when the residual O_2 in the package is not reduced fast enough, less pleasant grey/brown colours of the meat may be produced due to the formation of met-myoglobin. In order to investigate the influence of different processing and packaging conditions on the colour of ground beef, a study on the effect of salt and the use of fresh vs. frozen meat was undertaken. Results were given as recommendations to the meat industry in Norway. The Norwegian Meat Research Centre, Oslo, gave financial support to this study.

MATERIALS AND METHODS

Beef trimmings (14% fat) were received from the butchery 6 days after slaughter. Half of the meat was vacuum-packed and frozen; the other half was used fresh. The meat was ground twice, first through an 8 mm plate, then through a 4 mm plate, and divided into two subgroups before being packed in trays and plastic bags to produce a total of 4 series:

I fresh, no additions
II fresh, addition of 1% NaCl and 5% water
III frozen (and thawed), no additions
IV frozen (and thawed), addition of 1% NaCl and 5% water.

All ground beef series were packed in trays that were put in EVOH-containing bags that were vacuumed, gas-flushed (55% CO_2/45% N_2) and sealed on a Webomatic C 50-D (Webomatic, Bochum, Germany) chamber machine. All the packed series (approx. 20 packages each) were stored in the dark at 4°C. After 1, 2, 3, 6 and 9 days of storage, CIE a* (redness) values were measured with a Minolta Chroma Meter CR-300 (8 mm opening, D_{65} light source) (Minolta Camera Co., Osaka, Japan). At the same intervals, a visual colour assessment by was made by a 3-person, trained panel using the following scale: 1: fresh red, 2: dark red, 3: slightly grey/brown, 4: some grey/brown and 5: very grey/brown. At the same intervals, and on day 0, the residual O_2 was analysed by means of a Toray PG-100 instrument (Toray Engineering, Osaka, Japan).

RESULTS AND DISCUSSION

The average O_2 headspace values, instrumental colour results and the visual colour scores are shown in Figs. 16-18.

Fresh, unsalted meat reduced the residual O_2 in the headspace much more than the other series (Fig. 16). It seems that the addition of 1% NaCl inhibited this reduction more than freezing, and that the combined effect of freezing and the addition of salt was cumulative. Little can be found in the literature regarding this phenomenon of the reduction of residual O_2 in the headspace. However, the O_2 consumption rate and met-myoglobin reductase activity are both of interest (MANCINI and HUNT, 2005). The effect of high bacterial counts on O_2 consumption was probably neglectible in the first days of this study.

Still, the effect of this reduction in O_2 residual headspace on meat colour is significant. For the fresh, unsalted sample it took less than 3 days for most of the myoglobin to be convert-

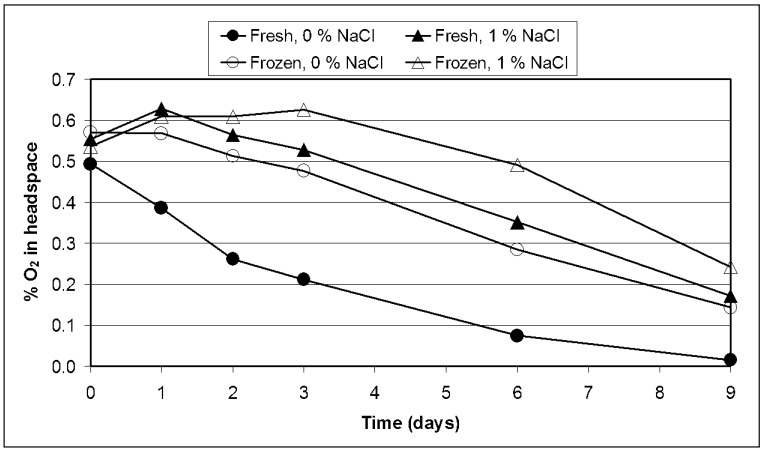


Fig. 16 - Average headspace O_2 concentrations in frozen and fresh ground beef with no salt or 1% salt. All samples were packed with 55% CO_2 and 45% N_2 and stored in the dark at 4°C.

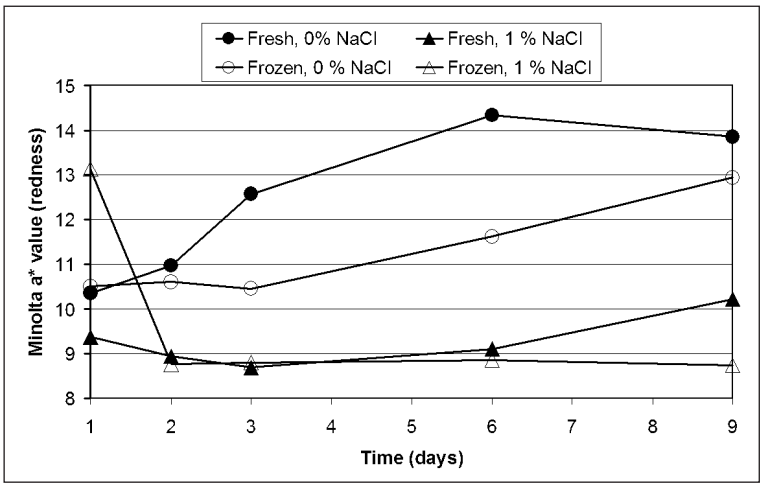
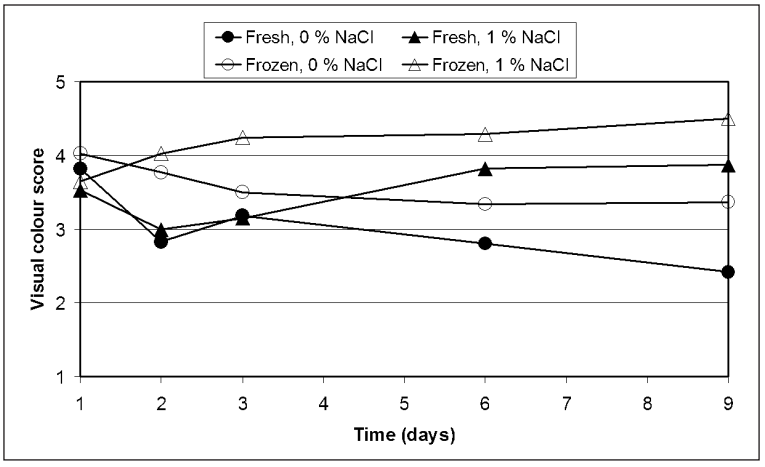


Fig. 17 - Average CIE a* (redness) values in frozen and fresh ground beef with no salt or 1% salt. All samples were packed with 55% CO_2 and 45% N_2 and stored in the dark at 4°C. A favourable red meat colour is achieved with CIE a* values >12.

ed into the favourable dark red deoxymyoglobin, indicated by a CIE a* (redness) value >12 (Fig. 17) and a visual colour score <3 (Fig. 18). For the salted series the residual O_2 headspace was not reduced fast enough, met-myoglobin formed, and an unpleasant brown/grey colour appeared. After one day the frozen sample with 1% NaCl had a high a* (redness) value, probably due to the low ability to reduce residual O_2 headspace. The myoglobin in this sample was still in the oxymyoglobin form. Two days after grinding the CIE a* (redness) value for this sample was as low as for the fresh sample with 1% salt.

After 1, 2 and 3 days both of the fresh series had similar colour scores, but due to the lower ability to reduce residual O_2 headspace, more unfavourable grey/brown met-myoglobin was formed in the salted series, resulting in higher visual colour scores (Fig. 18). In the unsalted series, with a greater ability to reduce the residual O_2 headspace, more favourable, dark red deoxy-myoglobin was formed, resulting in lower visual colour scores.

Fig. 18 - Average visual colour evaluation scores in frozen and fresh ground beef with no salt or 1% salt. All samples were packed with 55% CO_2 and 45% N_2 and stored in the dark at 4°C. The following scale was used: 1: fresh red, 2: dark red, 3; slightly grey/brown, 4: some grey/brown, 5: very grey/brown.



CONCLUSIONS

- Freezing and/or the addition of salt inhibits the breakdown of detrimental, residual headspace O_2 in meat.
- Discolouration of ground beef can be prevented by storage in a CO_2/N_2 mixture with less than 0.1% residual O_2 for more than 2 days.

THE EFFECT OF PROTECTIVE, BACTERIOCIN PRODUCING CULTURES ON SMOKED SALMON

INTRODUCTION

Listeria monocytogenes may cause a serious, sometimes lethal, foodborne illness called listeriosis. Consumption of food infected with *L. monocytogenes* seldom leads to the development of listeriosis in healthy individuals but some groups are especially vulnerable, such as the elderly, pregnant women, unborn babies, newborns and immuno-compromised people (cancer patients, people with HIV/AIDS, those who receive medication to reduce immune reactions and patients with diabetes).

Listeria is found "everywhere" in the environment. Both humans and animals can be healthy carriers of the bacterium which has been found in both human faeces and faeces from many different animals including birds and fish, both wild and domestic. Positive findings of *Listeria* in ready-to-eat seafood (e.g. smoked salmon) are unacceptable in the USA. If found, importation is stopped which causes marked financial losses for the producers. *Listeria* is a tough bacterium and can establish itself in production sites and cold storage rooms where it may attach to surfaces and grow if conditions are favourable. Together with other bacteria, *Listeria* can form a biofilm (slimy layer) on wet, unclean surfaces. Once formed, biofilms are difficult to remove. *Listeria* tolerates high salt concentrations, nitrite and acidity better than many other bacteria. The bacterium grows well in vacuum- and in gas-packaged products, and can grow slowly at refrigeration temperature (4°C). The most effective strategy to avoid contamination with *Listeria* and other pathogenic bacteria in food products is to follow good hygiene practices throughout the entire process from the checks on the raw materials until the food is eaten.

Biopreservation by lactic acid bacteria is a promising strategy to increase the safety and shelf life of food. Since lactic acid bacteria can grow in the product and have an inhibitory effect, they may be used to prevent the growth of *Listeria*. Such a protective culture should inhibit growth of unwanted bacteria, e.g. *Listeria*, and at the same time have little or no effect on the sensory properties of the product. Use of preservatives including protective cultures is to be regarded as an extra safety measure and must never be used to compensate for poor hygiene or temperature-control practices. Use of protective cultures is an innovative method and must be adapted to each product or group of products. The method is still being developed and further research is needed to determine the most suitable cultures and develop application technologies.

KATLA *et al.* (2001) investigated the ability of a sakacin P-producing *Lactobacillus sakei* culture alone or together with purified sakacin P to inhibit *L. monocytogenes* on cold smoked salmon.

MATERIALS AND METHODS

Fresh farmed cold-smoked salmon (*Salmo salar* L.) was obtained from a commercial Norwegian producer. Ten g pieces were aseptically cut from the middle of the loin and vacuum-packed before gamma irradiation (1.5 kGy) to reduce the natural microbial flora. All samples were inoculated with ~$5x10^3$ cfu/g with rifampicin-adapted *Listeria monocy-*

togenes L182 by injecting the surface of the salmon with a syringe through the plastic vacuum bag and a rubber septum. The adaptation to rifampicin was done in order to count this strain in a convenient way. The salmon samples were inoculated with one of the following 0.2 mL preparations:

- Peptone water (control);
- Sakacin P-producing *Lb. sakei* Lb790;
- Non-bacteriocin-producing *Lb. sakei* Lb790;
- Sakacin P-producing *Lb. sakei* Lb790 + 1.1 μg/g purified sakacin P (MØRETRØ, 2000);
- 1.1 μg/g purified sakacin P.

All samples were incubated at 10°C for 28 days with sampling after 0, 3, 7, 14, 21 and 28 days. At sampling, each piece of salmon was homogenised with 50 mL peptone water in a Stomacher Lab-Blender 400 (Seward Medical UAC House, London, UK) for 1 min. The homogenate was analysed for sakacin P according to AXELSSON *et al.* (1998) and serially diluted 10-fold in peptone water and spread on plates to determine the bacterial counts. *L. monocytogenes* was counted after spreading the samples on blood agar plates containing rifampicin (200 μg/mL) and incubated at 37°C. The cell counts of *Lb. sakei* Lb790 strains were determined after plating on MRS containing erythromycin (10 μg/mL) and incubating at 25°C.

RESULTS AND DISCUSSION

Fig. 19 shows that the addition of sakacin P prevented an immediate increase in the growth of *L. monocytogenes*, resulting in initial and lasting counts that were 1-2 log-units less than those of the control. This may indicate that the addition of sakacin P inhibits the initial establishment of this pathogen. When either sakacin P-producing *Lb. sakei* 790 or a non-sakacin-producing strain of *Lb. sakei* 790 was added, no significant growth of *L. monocytogenes* was observed. The lack of any difference between the two *Lb. sakei* strains might be due to the lower pH in the samples to which the non-sakacin-producing *Lb. sakei* 790 was added (data not shown). When 1.1 μg/g sakacin P was added together with a sakacin P-producing strain of *Lb. sakei* 790, a significant reduction in the number of *L. monocytogenes* was observed.

Fig. 20 shows that both the sakacin P-producing and the non-sakacin-producing strains of *Lb. sakei* 790 grew well on

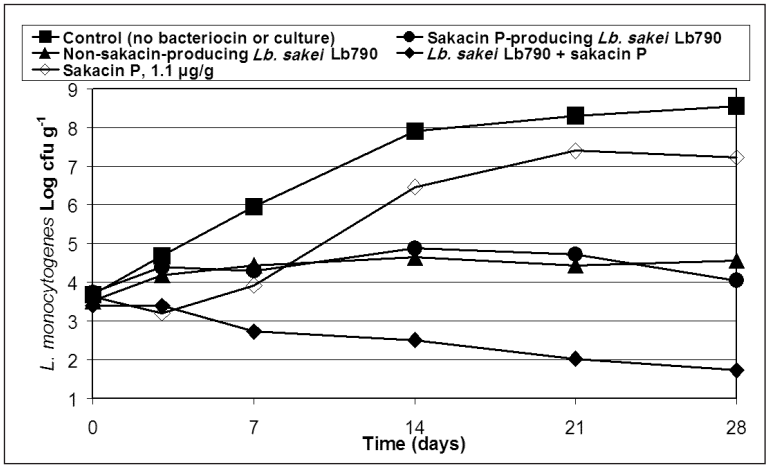


Fig. 19 - Growth of inoculated *Listeria monocytogenes* L182 on gamma-irradiated cold smoked salmon at 10°C with the addition of sakacin P-producing *Lb. sakei* Lb790, non-sakacin-producing *Lb. sakei* Lb790, sakacin P-producing *Lb. sakei* Lb790 and 1.1 μg/g sakacin P, 1.1 μg/g sakacin P or peptone water (control). Data from KATLA *et al.* (2001).

cold smoked salmon, increasing four log-units in 3 days. This probably had an increasingly negative effect on the growth of *L. monocytogenes*.

CONCLUSIONS

- *Lb. sakei* (both Sakacin P-producing and non-producing) inhibits the growth of *L. monocytogenes*.
- Using purified sakacin P together with a sakacin P-producing culture reduced the number of *L. monocytogenes*.
- These effects were observed even with a high initial contamination dosage of the pathogen, and at an "abuse" temperature of 10°C.

THE EFFECT OF ANTIOXIDATIVE MARINADES ON TURKEY THIGHS

INTRODUCTION

Lipid oxidation is one of the major problems in meat and meat products, especially poultry meat products due to the relatively high polyunsaturated fatty acid content, e.g. linoleic and arachidonic acids (IGENE *et al.*, 1981). One way to reduce the sensory deterioration caused by oxidative rancidity is to use the antioxidative power of natural spices, such as rosemary (*Rosmarinus officinalis* L.) and sage (*Salvia officinalis* L.) (MADSEN and BERTELSEN, 1995) as replacements for synthetic antioxidants, such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA).

The objective of this study (MIELNIK *et al.*, 2004) was to investigate the antioxidative effects of marinades made with decoctions of rosemary, sage and thyme on the shelf life of pre-cooked and cold-stored turkey thighs.

MATERIALS AND METHODS

Cuts of turkey thigh meat without skin, obtained from a local poultry meat processing plant the day after slaughter, were soaked overnight in 3 marinades made from decoctions (from Norske Eteriske Oljer, Alvdal, Norway) made of rosemary, sage and thyme with 12% salt, in addition to a 12% saline water control marinade. After wiping with paper towels, the marinated thigh cuts were vacuum-packed in polyamide/polyethylene plastic bags before cooking in water at

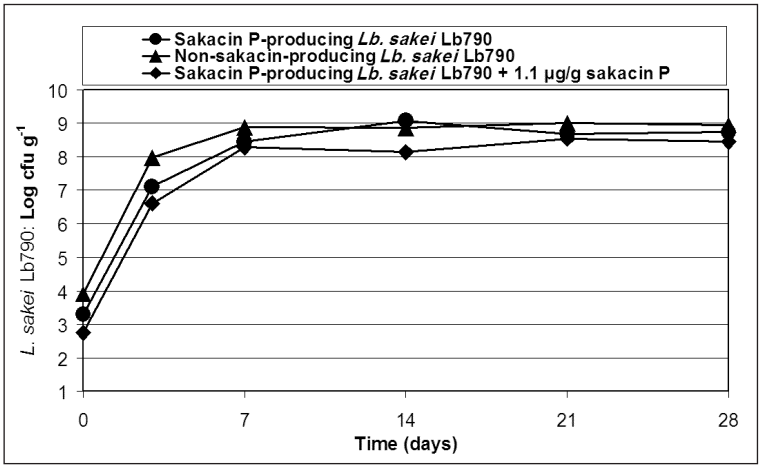


Fig. 20 - Growth of *Lb. sakei* Lb790 strains on gamma-irradiated cold smoked salmon at 10°C inoculated with *Listeria monocytogenes* L182 with the addition of sakacin P-producing *Lb. sakei* Lb790, non-sakacin-producing *Lb. sakei* Lb790 and sakacin P-producing *Lb. sakei* Lb790 and sakacin P. Data from KATLA *et al.* (2001).

85°C for 30 min and cooling for 45 min in running, cold water. The meat cuts were then divided into smaller portions and transferred to plastic trays covered with aluminium foil for storage at 4°C for 5 days.

The antioxidant powers of the different marinades were determined according to BRAND-WILLIAMS *et al.* (1995) with modifications according to MIELNIK *et al.* (2003) and expressed as mg 2,2-diphenyl-1-picrylhydrazyl (DPPH)/mL marinade. The thiobarbituric active reactive substances (TBARS) were determined according to SØRENSEN and JØRGENSEN (1996) and expressed as mg malondialdehyd (MDA)/kg meat. The analysis of hexanal was performed using a semi-quantitative dynamic headspace technique described by MIELNIK *et al.* (2002) and expressed as ng hexanal/g meat x L (L=total volume of purging gas). The turkey thigh cuts were re-heated in sealed vacuum pouches at 70°C for 30 min prior to sensory evaluation by an 11-person, trained sensory panel according to an international standard method (ISO, 1985) using a value of 1.0 for lowest intensity and 9.0 for the highest intensity of rancid flavour. Samples were randomised and served warm in a stainless steel cup covered with a lid.

RESULTS

All three marinades made from the herb decoctions showed good antiradical power (Fig. 21). Rosemary had the highest antioxidative capacity and sage the lowest. This effect is due to the presence of several natural antioxidants in these herbs (MADSEN and BERTELSEN, 1995). This antioxidative capacity results in lower TBARS values (Fig. 22), lower hexanal content (Fig. 23) and lower sensory scores for rancid flavour (Fig. 24) compared to the control samples marinated in saline water only. For rosemary extract there was a good correlation between the high antioxidative activity and the lowest values for TBARS, hexanal content and rancid flavour sensory score.

CONCLUSION

- Herb extracts can potentially be used with poultry meat, to prevent oxidative rancidity, particularly for heat-treated, ready-to-eat products.

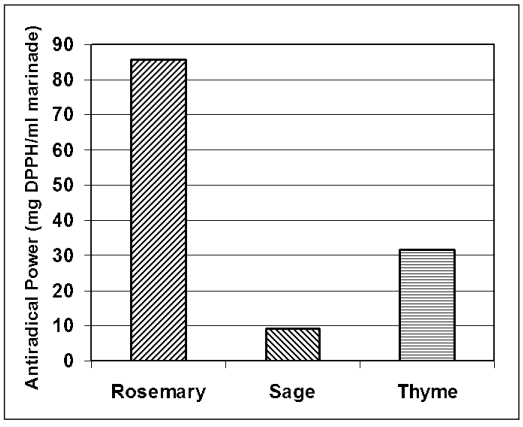


Fig. 21 - Antiradical power of antioxidative marinades made from herb decoctions expressed as mg DPPH/mL marinade. Data extracted from MIELNIK *et al.* (2004).

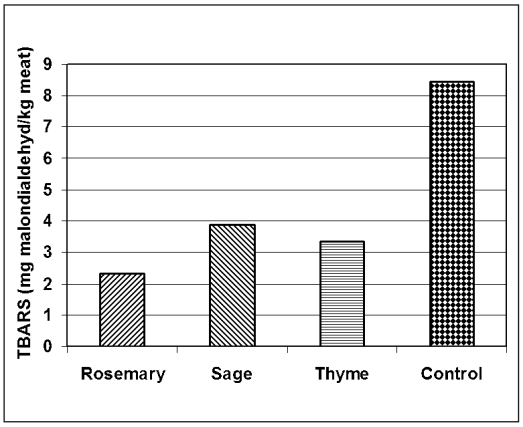


Fig. 22 - TBARS values (mg malondialdehyde/kg meat) in turkey thigh cuts after marinating in herb decoctions, cooking and dark storage for 5 days at 4°C in air. Data extracted from MIELNIK *et al.* (2004).

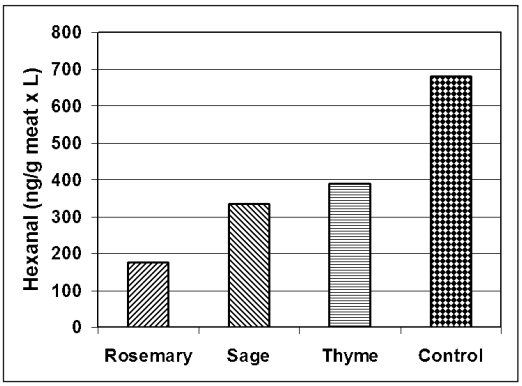


Fig. 23 - Hexanal concentration (ng/g meat x L) in turkey thigh cuts after marinating in herb decoctions, cooking and dark storage for 5 days at 4°C in air.

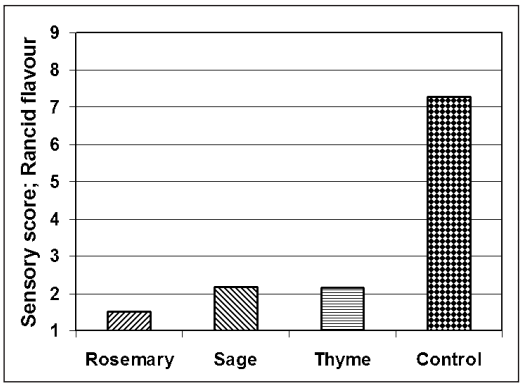


Fig. 24 - Sensory score for rancid flavour in turkey thigh cuts after marinating in herb decoctions, cooking and dark storage for 5 days at 4°C in air.

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PAPER

DETECTION OF *DEKKERA/BRETTANOMYCES BRUXELLENSIS* IN PRESSED SANGIOVESE GRAPES BY REAL TIME PCR

RILEVAMENTO DI *DEKKERA/BRETTANOMYCES BRUXELLENSIS* IN UVE SANGIOVESE PRESSATE MEDIANTE REAL TIME PCR

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ABSTRACT

Dekkera bruxellensis (anamorph *Brettanomyces bruxellensis*) is a controversial yeast that is relevant for the wine industry. Although *Dekkera/Brettanomyces* have been extensively studied, their ecological niche has not been well defined. In this study, a Real Time PCR-based assay was applied to the microbial DNA extracted directly from pressed Sangiovese grapes. Two different DNA extraction procedures were compared in an attempt to obtain a clean and QPCR-amplifiable

RIASSUNTO

Dekkera bruxellensis (forma anamorfa *Brettanomyces bruxellensis*) è un lievito controverso di rilevanza nell'industria enologica. Sebbene questo lievito sia stato oggetto di numerosi studi la nicchia ecologica non è stata ancora ben definita. In questo studio, un metodo basato sulla Real Time PCR era applicato a DNA microbico estratto direttamente da uve Sangiovese pressate. Due differenti procedure di estrazione erano comparate per ottenere un DNA

- Key words: *Dekkera/Brettanomyces bruxellensis*, grapes, pDONR™/Zeo, Real Time PCR, wine yeasts -

DNA from grapes. Plasmidic DNA was used to evaluate both the extraction efficiency and the matrix effect. Such an effect was analysed in order to circumvent the difficulty of using filter-sterilized pressed grapes and to obtain a calibration curve for quantifying *D./B. bruxellensis*. Using specific primers for *D./B. bruxellensis* the number of these yeasts in Sangiovese grapes was in the range of 10^3-10^4 cells mL^{-1}. This evidence supports the hypothesis that *D./B. bruxellensis* belongs to the native Sangiovese grape microflora, and that they can be detected by the method applied. Identifying the environmental reservoir of *D./B. bruxellensis* is of great interest to the wine industry. The method developed in this study, when applied to other vine species, could increase the knowledge about these controversial yeasts.

purificato ed amplificabile mediante QPCR dalle uve. Un DNA plasmidico era usato per valutare sia l'efficienza di estrazione che l'effetto della matrice. Quest'ultimo effetto era analizzato per risolvere la difficoltà di utilizzare uva pigiata sterilizzata per filtrazione e per ottenere una curva di calibrazione per la quantificazione di *D./B. bruxellensis*. Questo approccio ha permesso, impiegando specifici primers per *D./B. bruxellensis* di quantificare questi lieviti in uve Sangiovese pressate in un range di 10^3-10^4 cell mL^{-1}. Questa evidenza è in supporto all'ipotesi che *D./B. bruxellensis* appartiene alla microflora nativa di uva Sangiovese e che il loro rilevamento potrebbe essere una conseguenza del metodo utilizzato. L'identificazione del serbatoio ambientale di *D./B. bruxellensis* è di grande interesse per l'industria enologica. Il metodo sviluppato in questo studio, applicato ad altre specie di vitigni, potrebbe aumentare le conoscenze di questi lieviti controversi, rilevanti nei processi di vinificazione, la cui nicchia ecologica non è ancora stata ben definita.

INTRODUCTION

During the past years, the enological industry has increasingly tried to optimize wine traits, to meet consumer demands that are oriented towards products of constant and high quality. For this reason, there has been an upsurge of interest in the yeasts belonging to *Dekkera/Brettanomyces*, which can produce volatile phenols and acetic acid (compounds that affect wine aroma), and compounds that are noxious to human health, such as biogenic amines.

Indeed, the growth of *Dekkera/Brettanomyces* species in wine, such as *Brettanomyces bruxellensis*, may cause organoleptic modifications that are generally considered negative, due to the production of volatile phenols. 4-Ethyl-phenol and 4-ethyl-guaiacol have been identified as the main compounds responsible for phenolic off-flavour and animal odors, resembling smells of a farm, horse sweat, medicine and animal leather (POLLNITZ *et al.*, 2000).

Recent studies (CARUSO *et al.*, 2002) have shown that *Dekkera/Brettanomyces* are able to produce allergenic com-

pounds such as biogenic amines. These low-molecular-weight organic substances can be metabolically produced and degraded in animals, plants and microorganisms, and are found in a wide variety of food such as fish, meat, cheese, wine, beer and other fermented food (SILLA SANTOS, 1996). The ability of *Dekkera/Brettanomyces* yeasts to produce biogenic amines and volatile compounds seems to be a strain-dependent trait (CHATONNET *et al.*, 1993; FUGELSANG, 1997). Several strains of these yeasts are able to produce up to 10 μg mL^{-1} phenylethylamine from phenylalanine, on an overall concentration of 15 μg mL^{-1} of biogenic amines (CARUSO *et al.*, 2002).

Dekkera/Brettanomyces yeasts exist in two forms: *Brettanomyces*, the asexual nonsporulating form, and *Dekkera*, the sexual sporulating form. These yeasts have been found during alcoholic fermentation of some wines (PEYNAUD and DOMERCQ, 1956; VAN DER WALT and VAN KERKEN, 1960; KUNKEE and AMERINE, 1970), in sparkling wines (CIANI and FERRARO, 1997), during beer fermentation (ANDREWS and GILLILAND, 1952; SHANTHA KUMARA *et al.*, 1993), in some non-alcoholic beverages (KOLFSCHOTEN and YARROW, 1970) and in Sherry wines (IBEAS *et al.*, 1996).

Recently, some strains have been isolated from cheese (COSENTINO *et al.*, 2001; FADDA *et al.*, 2001), during Irish cider fermentation (MORRISEY *et al.*, 2004), from kombucha, a beverage obtained from the fermentation of black and green tea leaves (THEOH *et al.*, 2004), and from tamarind, a traditional Jordanian drink prepared by infusing *Tamarindus indica* dried pulp (NASSEREDDIN and YAMANI, 2005).

In the enological practice, the identification and enumeration of *Dekkera/Brettanomyces* are carried out by using selective/differential microbiological media. However, these methods have a limited efficacy, due to the low growth rate of the yeast and to the low cell density, with respect to other microbial species present in the must-wine. In fact, even if *Dekkera/Brettanomyces* have been extensively studied, their ecological niche has not been well-defined.

According to some authors, this yeast is a contaminating microorganism, whose presence is linked to the lack of hygienic practices in the winery, often connected with the use of wood (BOULTON *et al.*, 1996; FUGELSANG, 1996; CHATONNET *et al.*, 1999); other authors have suggested that this yeast could belong to the indigenous grape microflora (AROJO *et al.*, 1998; PRETORIUS, 2000). Molecular methods such as nested-PCR (IBEAS *et al.*, 1996), fluorescence and *in situ* hybridization (STENDER *et al.*, 2001) and DGGE with specific probes (COCOLIN *et al.*, 2004) were used to determine the presence of these yeasts in wine. The development of specific primers allowed species-specific identification and the *Dekkera/Brettanomyces* isolates from wine were found to belong uniquely to *Dekkera/Brettanomyces bruxellensis* (EGLI and HENICK-KLING, 2001). Recently Real Time or quantitative PCR (QPCR) assays have been developed for the detection and enumeration of *Dekkera/Brettanomyces bruxellensis* in wine (PHISTER and MILLS, 2003; DELAHERCHE *et al.*, 2004).

The presence of this yeast on the surface of the berries of four different grape varieties, *i.e.* Merlot, Chabernet-Franc, Chabernet-Sauvignon an Petit-Verdot, was recently found using an enrichment medium (RENOUF and LONVAUD-FUNEL, 2007).

The aim of this study was to identify *D./B. bruxellensis* in pressed Sangiovese grapes; a microbiological enrichment technique and a quantitative PCR method based on the microbial DNA extracted directly from the matrix are described.

MATERIALS AND METHODS

Samples

Sangiovese grape samples were collected from vineyards located in the Chianti area. Several bunches of grapes were cut randomly and transferred to sterile 50 mL Falcon tubes using gloves, and put in a cooler until laboratory analysis. The berries were then manually pressed in sterile plastic bags.

Enrichment cultures

A modified WL nutrient medium (CAVAZZA *et al.*, 1992) (MWL Oxoid, Milan, Italy) was used to limit the growth of other microorganisms representing most of the indigenous microflora of grapes. One-hundred mg L^{-1} cycloheximide (Sigma-Aldrich, Milan, Italy), 60 mg L^{-1} chloramphenicol (Sigma-Aldrich) and 6% and 9% v/v ethanol final concentrations (Sigma-Aldrich) were added to the WL medium after sterilization. One mL of pressed Sangiovese grapes was inoculated in a flask containing 50 mL of MWL and incubated at 28°C without shaking. Five, ten and nineteen days after inoculation, microbiological analyses were performed using the MWL supplemented with 15 g L^{-1} agar. The plates were incubated at 28°C for up to 10 days. By microscopic inspection, the colonies were identified as yeast cells. Colonies developed on the agar surface were isolated and characterized through molecular methods.

DNA isolation and molecular techniques

Yeast cells were grown overnight in 5 mL malt broth medium (Oxoid). Nucleic acid extraction was performed as described by WHITE *et al.* (1990), with the following modifications: 1.5 mL of fermentation broth was spun down at 10,000 g for 10 min. The cell pellet was resuspended in 0.2 mL of extraction buffer (2.5 mL SDS 10%, 0.5 g of Triton X-100, 0.5 mL NaCl 5 mol L^{-1}, 250 µL Tris 1 mol L^{-1}, 50 µL EDTA 0.5 mol L^{-1}), with 0.3 g glass beads (0.4-0.5 mm in diameter; Sigma-Aldrich) and 0.2 mL phenol/chloroform/isoamyl alcohol (25:24:1 – Fluka, Milan, Italy). The cells were homogenized by vortex and then centrifuged at 10,000 g for 5 min. The aqueous phase was collected and the DNA was precipitated with the same volume of isopropyl alcohol (Sigma-Aldrich) and then centrifuged at 10,000 g for 5 min. The pellet was washed with 70% ethanol and centrifuged at 10,000 g for 5 min, dried and resuspended in 50 mL of TE (TRIS 10 mmol L^{-1}; EDTA 1.0 mmol L^{-1}; pH 7.5). Extracted and purified DNA was analysed by PCR amplification with ITS1-ITS4 primers (WHITE *et al.*, 1990; GRANCHI *et al.*, 1999) and specific primers pB2-ITS4 for *D./B. bruxellensis* (EGLI and HENICK-KLING, 2001). *D./B. bruxellensis* ATCC 10560 and *D./B. bruxellensis* BDF4 (Collection of the Department of Crop Biology, Pisa) were used as reference strains during the characterization of isolates. Reactions were carried out in a 50 µL PCR mixture containing 1x Mg-free Buffer Finnzyme (50 mmol L^{-1} KCl, 10 mmol L^{-1} TrisHCl pH 8.3, 0.1% Triton X-100), 4 mmol L^{-1} $MgCl_2$, 0.2 mmol L^{-1} each dNTPs, 0.4 µmol L^{-1} of each primer, 2.5 U DyNazyme II DNA polymerase (Finnzyme, Celbio, Milan, Italy) and about 80 ng of DNA template. Cycler conditions were: initial denaturation at 94°C for 1 min followed by 30 cycles at 94°C for 1 min, 52°C for 2 min, 72°C for 1 min, with final extension at 72°C for 5 min. Amplification products were analysed by electrophoresis on 1.5% (w v^{-1}) agarose TBE (0.089 mol L^{-1} Tris-borate, 0.089 mol L^{-1} boric acid and 0.0025 mol L^{-1} EDTA) gels. The profiles obtained were captured as TIFF format files by Liscap program for Image Master VDS system (Pharmacia Biotech, Milan, Italy). PCR products were purified with "High pure PCR product purification kit" (Roche,

Monza, Italy) and sequenced with primers ITS1 and ITS4 separately (CRIBI, DNA Sequencing Service, University of Padova, Italy).

Yeast cultures

D./B. bruxellensis ATCC 10560 and *D./B. bruxellensis* BDF4 (Collection of the Department of Crop Biology, Pisa) strains were grown for two days at 30°C in 15 mL Falcon tubes containing 5 mL WLDC medium, i.e. WL nutrient medium supplemented with 100 mg L^{-1} cycloheximide (Sigma-Aldrich, Milan, Italy), 60 mg L^{-1} chloramphenicol (Sigma-Aldrich). The growth was monitored both by cell counting in a Thoma counting chamber and by standard plating procedures on WLDC agar. The plates were incubated at 30°C.

DNA extraction procedures for QPCR

Nucleic acids were extracted from 1 mL of yeast cultures of *D./B. bruxellensis* ATCC 10560 ($4.06 \cdot 10^8$ cells mL^{-1} corresponding to $4 \cdot 10^8$ CFU mL^{-1}) in peptone water and pressed Sangiovese grape samples with MasterPure yeast DNA purification kit (Epicentre Technologies, Tebu-bio, Milan, Italy) (PHISTER and MILLS, 2003) and FastDNA® Spin Kit for Soil (BIO101® Systems, Qbiogene, Resnova, Rome, Italy). Plasmidic DNA (10^8 copies) pDONR™/Zeo (Invitrogen Life Technologies, Milan, Italy) was added to each sample prior to DNA extraction as internal standard. All samples were extracted and finally resuspended in 50 µL according to the manufacturer's instructions (pDONR™/Zeo final concentration of 2.10^6 copies μL^{-1}).

Real Time PCR Assays

Sybr Green Real Time PCR was performed on an iCycler® IQ Multicolor Real Time PCR Detection System (Bio-Rad Laboratories, Milan, Italy) using Master mix iQ™ SybrGreen (Bio-Rad Laboratories). Real Time PCR for pDONR™/Zeo was carried out in a total reaction volume of 50 µL containing 2 µL of extract purified DNA (not diluted and diluted 10- and 100-fold in sterile water), 1x SybrGreen master mix (2x: 100 mmol L^{-1} KCl, 40 mmol L^{-1} Tris-HCl, pH 8.4, 0.4 mmol L^{-1} of each dNTPs, iTaq DNA polymerase 50 U mL^{-1}, 6 mmol L^{-1} $MgCl_2$, SYBR Green I, 20 nmol L^{-1} fluorescein and stabilizers) and 0.3 µmol L^{-1} of each specific primer for plasmidic DNA pDONR™/Zeo, **ZEOF** 5'-TCCCGGAAGTTCGTGGACAC-3' and **ZEOR** 5'-GACGACGTGACCCTGTTCATCA-3' (Beacon Designer 4.0, Premier Biosoft International, Bio-Rad). The reactions were run for 40 cycles, with denaturation at 95°C for 60 sec, annealing at 65°C for 45 sec. An initial 3 min denaturing step at 95°C was used. For pDONR™/Zeo quantitation, a standard curve was created by using 10-fold serial dilution series of plasmidic DNA (10^8 to 10^5 copies μL^{-1}). For *D./B. bruxellensis*, the Real Time PCR was performed in a total reaction volume of 50 µL containing 2 µL of extract purified DNA, 1x SybrGreen master mix (2x: 100 mmol L^{-1} KCl, 40 mmol L^{-1} Tris-HCl, pH 8.4, 0.4 mmol L^{-1} of each dNTPs, iTaq DNA polymerase 50 U mL^{-1}, 6 mmol L^{-1} $MgCl_2$, SYBR Green I, 20 nmol L^{-1} fluorescein and stabilizers) and 0.3 µmol L^{-1} of two different pairs of specific primers for *D./B. bruxellensis, i.e.* **DBRUXF** 5'-GGATGGGTGCACCTGGTTTACAC-3' and **DBRUXR** 5'-GAAGGGCCACATTCACGAACCCCG-3' (PHISTER and MILLS, 2003), and **Brett1** 5'-CGAAGAAGTTGAACGGCCGCATTTG-3' and **Brett2** 5'-TCTTCGATATGCCGTCCAAAAGCTC-3' (DELAHERCHE *et al.*, 2004). Primers DBRUXF and DBRUXR, targeting the D1-D2 subdomain of the large subunit domain of the rRNA gene, were selected by PHISTER and MILLS (2003) to produce a 77 bp fragment specifically from *D./B. bruxellensis*. Primers Brett1 and Brett2 were drawn on

the *rad4* gene by DELAHERCHE *et al.* (2004). The reactions with DBRUXF and DBRUXR were run for 40 cycles, with denaturation at 95°C for 60 sec, annealing at 65°/69°C for 45 sec. An initial 3 min denaturing step at 95°C was used. Whereas the reactions with Brett1 and Brett2 were run for 40 cycles, with denaturation at 95°C for 30 sec, annealing at 69°C for 20 sec and extension at 72°C for 20 sec. An initial 5 min denaturing step at 95°C was used. Each sample was measured in triplicate. For each well, the fluorescent light emission was recorded in real time at 490 nm and data were automatically analysed with iCycler IQ Multicolor Real Time PCR Detection System software version 3.1 (Bio-Rad Laboratories). For *D./B. bruxellensis* quantitation, a standard curve was created by using 10-fold serial dilution series of DNA extracted from the sample 1 mL of yeast culture of *D./B. bruxellensis* strain ATCC 10560 in peptone water ($4.06 \cdot 10^8$ to $4.06 \cdot 10^3$ cell mL^{-1} *i.e.* $1.6 \cdot 10^7$ cell $tube^{-1}$ to $1.6 \cdot 10^2$ cell $tube^{-1}$). Standard curves for quantification of unknown samples and determination of amplification efficiency were generated by plotting the Ct values of QPCR performed on the DNA from dilution series against the log inputs cells per tube.

Yeast identification was performed through molecular techniques such as ITS region amplification and species-specific PCR. The size of the ITS1-5.8S-ITS2 amplicon obtained from the isolated yeast was about 650 bp and no product was obtained by species-specific PCR (Fig. 1), whereas the ITS fragment length of the control strains, *D./B. bruxellensis* ATCC 10560 and *D./B. bruxellensis* BDF4 (Collection of the Department of Crop Biology, Pisa) was 450 bp and species-specific PCR gave a product of 150 bp, according to GRANCHI *et al.* (1999) and EGLI and HENICK-KLING (2001).

For the identification of the isolated yeast, the ITS amplicon was sequenced as described in materials and methods. The sequence was subsequently compared with those available in the NCBI nucleotide sequence database using the basic local alignment search tool (mega BLAST) program on line at the NCBI web page (http://www.ncbi.nlm.nih.gov/BLAST/).

The isolated yeast was referred to the species *Candida ernobii* to which it showed the highest matching sequence identity, *i.e.* a DNA similarity of 91%. By the enrichment technique applied in this study, no colony belonging to *D./B. bruxellensis* appeared.

RESULTS

Yeast isolation and characterization from pressed Sangiovese grapes by microbiological enrichment

After 10 days, the selective enrichment cultures of pressed grapes showed the development of a single yeast colony typology only when 100 mg L^{-1} cycloheximide, 60 mg L^{-1} chloramphenicol and 6% (v/v) ethanol were added.

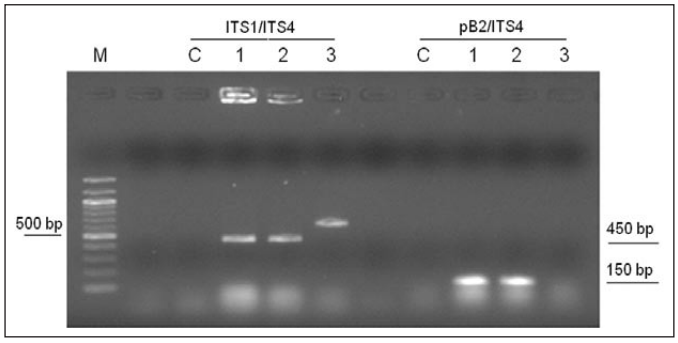


Fig. 1 - ITS region amplification and species-specific PCR performed with primers ITS1/ITS4 and pB2/ITS4. M: molecular weight marker 100 bp (BioLabs, Celbio, Milan, Italy); C: negative control. Lane 1, *D./B. bruxellensis* ATCC 10560; lane 2, *D./B. bruxellensis* BDF4; lane 3, yeast isolated by enrichment cultures.

QPCR assay: evaluation of methods of DNA extraction from pressed grapes

To evaluate the extraction efficiency of the different kits, DONR™/Zeo plasmidic DNA was added, as internal standard, to each analysed sample and a QPCR amplification was carried out using specific primers for this plasmid, designed inside the Zeocin resistance gene and selected to produce a 134 bp fragment.

The melting temperature (T_m) of all analysed samples was 91.0°C, which made it possible to ascertain that no spurious amplification product and/or primer-dimers were generated during the reaction. The linear regression analysis of the amplification plot data gave the following results: slope = -3.703, intercept = 41.692, correlation coefficient = 0.986. The efficiency of the QPCR reaction, calculated on the basis of the slope of the standard curve by the equation "efficiency = [10 $^{(-1/slope)}$] - 1", was 0.862.

Table 1, in which starting quantities (SQ) of plasmid DNA extracted from pressed grape samples are reported, shows that the DNA extracted by MasterPure yeast DNA purification kit (Epicentre Technologies) had QPCR products only when diluted 1:100, corresponding to a mean DNA yield of 46.25%. DNA extracted by Kit FastDNA® Spin Kit for Soil had QPCR amplicons corresponding to a mean DNA yield of 61% even when used undiluted.

The amount of plasmid DNA extracted from peptone water samples was on the same order of magnitude as that of pressed grapes (Table 1, Fig. 2). For this reason, the calibration curve for *D./B. bruxellensis* quantification in pressed Sangiovese grapes by QPCR, was carried out using the DNA extracted from 1 mL of yeast culture of *D./B. bruxellensis* ATCC 10560 in peptone water ($4.06 \cdot 10^8$ to $4.06 \cdot 10^3$ cell mL^{-1} *i.e.* $1.6 \cdot 10^7$ cell tube^{-1} to $1.6 \cdot 10^2$ cell tube^{-1}).

Table 1 - Quantification of DONR™/Zeo plasmidic DNA by real-time PCR in pressed grapes and peptone water samples extracted using different DNA extraction procedures.

Extraction method	Sample		SQ mean±SD* (copies tube^{-1})	Ct mean±SD* (cycles)
MasterPure yeast DNA purification kit	Pressed grapes 1	undiluted	N/A	N/A
	Pressed grapes 1	1:10	N/A	N/A
	Pressed grapes 1	1:100	$1.85 \pm 0.107 \cdot 10^4$ (real value $1.85 \pm 0.107 \cdot 10^6$)	25 ± 0.092
	Peptone water		$2.45 \pm 0.538 \cdot 10^6$	18.08 ± 0.381
Kit FastDNA® Spin Kit for Soil	Pressed grapes 1	undiluted	$2.44 \pm 0.378 \cdot 10^6$	18.05 ± 0.241
	Pressed grapes 2	undiluted	$2.35 \pm 0.457 \cdot 10^6$	18.13 ± 0.334
	Peptone water		$2.69 \pm 0.227 \cdot 10^6$	17.89 ± 0.139

* Starting quantities (SQ) and threshold cycles (Ct) value (mean of three determinations) and ±SD; N/A = no amplicon produced.

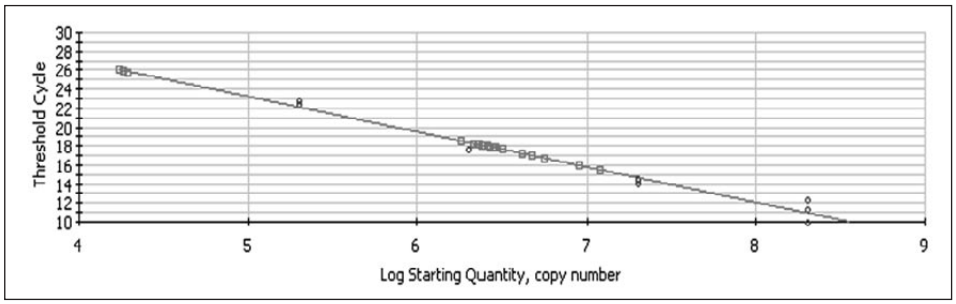


Fig. 2 - Standard curve of 10-fold serial dilution series of pDONR™/Zeo ($2 \cdot 10^8$ to $2 \cdot 10^5$ copies tube^{-1}) and calculated amounts of plasmid DNA extracted from peptone water and pressed grapes samples, plotted as threshold cycle (y-axis) vs target Log copy number (x-axis).

Detection of *Dekkera/Brettanomyces bruxellensis* in pressed Sangiovese grapes by Real Time PCR

DBRUXF/DBRUXR primers specific for *D./B. bruxellensis* were used; they were designed and used by PHISTER and MILLS (2003) to detect the yeast in wine. The melting temperature (T_m) of all analysed samples was 85.0°C. The amplification plot of the experiment generated a slope of -3.821, corresponding to 82.7% efficiency of the PCR assay and to 0.998 correlation coefficient (Figs. 3a,b). Measurements of the starting quantities and threshold cycles of unknown samples, obtained by interpolation from the standard curve, are reported in Table 2. Quantification of the sample extracted from *D./B. bruxellensis* culture in peptone water ($4.06 \cdot 10^8$ cell mL^{-1} *i.e.* $1.6 \cdot 10^7$ cell tube^{-1}) resulted in $3.44 \pm 0.410 \cdot 10^7$ cells tube^{-1} corresponding to $8.72 \pm 1.04 \cdot 10^8$ cells mL^{-1} with a Ct value of 12.38±0.204 cycles (Fig. 3b).

Another specific primer pair of *D./B. bruxellensis*, Brett1/Brett2, drawn on the *rad4* gene by DELAHERCHE *et al.* (2004) and developed for the quantification of this yeast in wine, was used. Since the detection limit for this QPCR assay was 10^4 CFU mL^{-1}, the samples were concentrated 10-fold by precipitation with isopropanol and suspended in a volume that was 10-times smaller (5 μL). Moreover, the amplification protocol was modified to optimize the reaction by increasing primer concentration up to 0.3 μmol L^{-1} and cycle number from 32 to 40. The T_m of all analysed samples was 83.5°C.

The amplification plot of the experiment generated a slope of -3.589, corresponding to a 89.9% efficiency of the PCR assay and a correlation coefficient of 1.0 (Fig. 4). The starting quantities of the analyzed pressed grape samples (1, 2, 3) were $2.21 \cdot 10^5$, $2.32 \cdot 10^5$ and $2.01 \cdot 10^5$ cells mL^{-1} (real value for the samples $2.21 \cdot 10^4$, $2.32 \cdot 10^4$ and $2.01 \cdot 10^4$cells mL^{-1}) with a Ct value of 32.6, 32.5 and 32.8 cycles, respectively.

DISCUSSION

Dekkera/Brettanomyces has been one of the most complex and controversial issues in yeast ecology in recent years. This yeast, a well-known wine spoilage microorganism, is characterized by very slow growth and low cell density, therefore making it difficult to detect and identify in complex matrices.

Although *Dekkera/Brettanomyces* are typically not included in the genera of yeasts found on the grape surface (FLEET

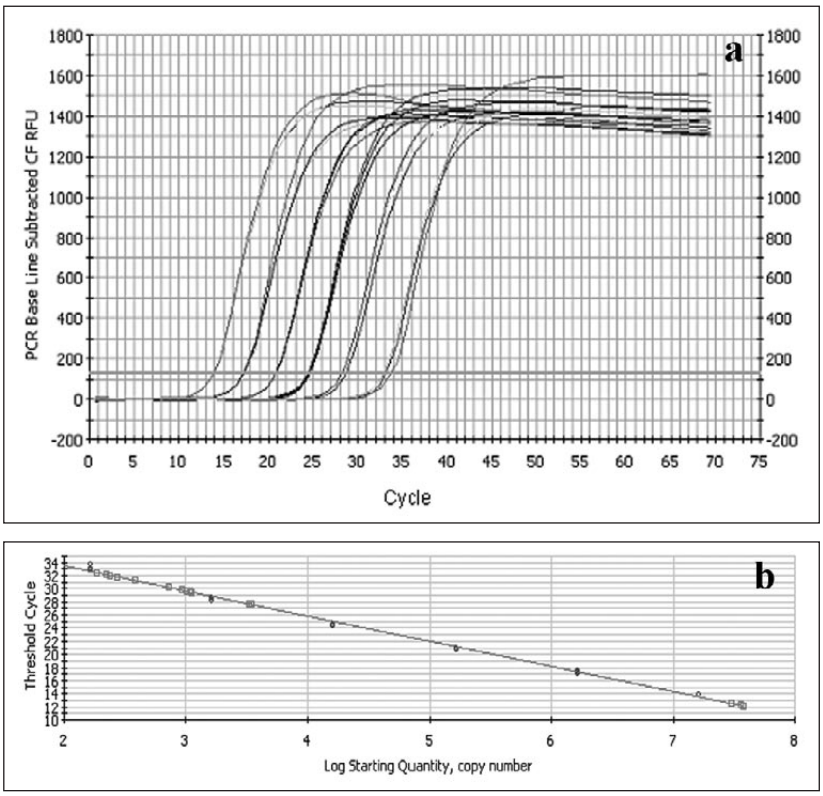


Fig. 3a, b - (a) Amplification plot of 10-fold serial dilution series of *D./B. bruxellensis* ATCC 10560 ($1.6 \cdot 10^7$ to $1.6 \cdot 10^2$ cell tube^{-1} *i.e.* $4.06 \cdot 10^8$ to $4.06 \cdot 10^3$ cell mL^{-1}) in real time detection with the fluorescence dye SYBR Green I after each amplification cycle (x-axis). Relative fluorescence units (RFU) are plotted on the y-axis; (b) Standard curve of serial 10-fold diluted DNA extracted from a culture of *D./B. bruxellensis* ATCC 10560 ($1.6 \cdot 10^7$ to $1.6 \cdot 10^2$ cell tube^{-1}) and calculated amounts of yeast DNA extracted from peptone water and pressed grapes samples, plotted as the threshold cycle on the y-axis, against the target Log copy number per assay (x-axis), relative to DBRUXF/DBRUXR.

and HEARD, 1993), they are commonly found in wineries (FUGELSANG, 1996). They have been repeatedly identified in wine, a difficult environment for most microorganisms due to the low amount of sugars coupled with the presence of ethanol.

In the present study, an enrichment technique and a Real Time PCR-based assay were compared with the aim of identifying *Dekkera/Brettanomyces bruxellensis* in pressed Sangiovese grapes. The enrichment technique did not allow detection of these yeasts. However, it was possible to isolate a yeast and identify it as *Candida ernobii* on the ba-

Table 2 - Quantification of *D./B. bruxellensis* in Sangiovese pressed grapes samples by real-time PCR using DBRUXF/DBRUXR primers.

Pressed grape sample	SQ mean±SD* (cells tube^{-1})	SQ mean±SD* (cells mL^{-1})	Ct mean±SD* (cycles)
1	$1.07 \pm 0.542 \cdot 10^3$	$2.72 \pm 0.138 \cdot 10^4$	29,59 ± 0.084
2	$7.93 \pm 1.21 \cdot 10^2$	$2.01 \pm 0.308 \cdot 10^4$	30,10 ± 0.244
3	$3.39 \pm 0.0718 \cdot 10^3$	$8.61 \pm 0.182 \cdot 10^4$	27.68 ± 0.353
4	$2.96 \pm 0.735 \cdot 10^2$	$7.52 \pm 0.187 \cdot 10^3$	31.75 ± 0.398
5	$2.14 \pm 0.273 \cdot 10^2$	$5.43 \pm 0.693 \cdot 10^3$	32.27 ± 0.218

* Starting quantities (SQ) and threshold cycles (Ct) value (mean of three determinations) and ±SD.

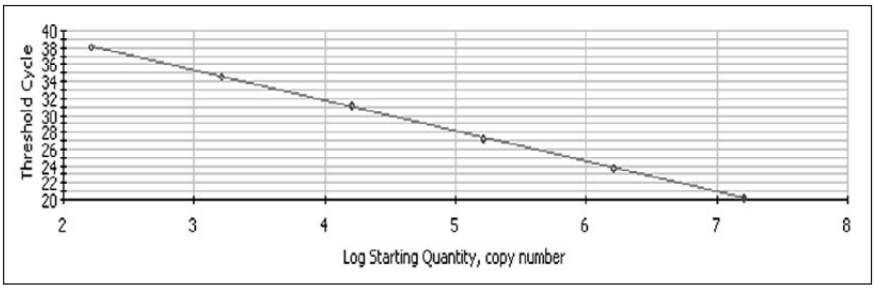


Fig. 4 - Standard curve of 10-fold serial dilution series of *D./B. bruxellensis* ATCC 10560 ($1.6 \cdot 10^7$ to $1.6 \cdot 10^2$ cell $tube^{-1}$) plotted as the threshold cycle on the y-axis, against the target copy number per assay (x-axis) relative to Brett1/Brett2.

sis of ITS analysis. To date, this yeast has not been reported to be associated with the must/wine microflora. KURTZMAN and FELL (2000) described *C. ernobii* as an endosymbiont of the coleopter *Ernobius mollis* (Linnaeus). An in-depth genetic investigation is required to better characterize this yeast, considering that the 91% DNA similarity could be due to the absence of the ITS sequences of the microorganism isolated in this work in the database. Furthermore, considering that this yeast has shown several *Brettanomyces*–like traits, it could be interesting to investigate its ability to produce spoilage substances such as volatile phenols. Recent advances in the field of molecular techniques allow microbial diversity to be assessed without using enrichment and isolation procedures. Furthermore, these methodologies allow viable but nonculturable microorganisms to be detected while bypassing the traditional time-consuming microbiological analyses.

Among these techniques, Real Time PCR assay is used to identify and quantify a target microbial population using genera – and species – specific primers directly on DNA extracted from the native matrix, which, in the case of pressed grapes, is a particularly complex and difficult substrate to analyze. Some substances, like polyphenols, can cause problems during the PCR amplification step. Furthermore, to achieve an absolute quantification of the *Brettanomyces* yeasts in pressed grapes, it would be necessary to inoculate a known quantity of these yeasts in sterile pressed grapes. However it is very difficult to sterilize this matrix without altering its composition.

In this study, a plasmidic DNA was added to each sample in order to evaluate the extraction efficiency of the kits and the effect of the matrix on the extraction. Although DNA extraction yields were comparable, a high-quality and directly amplifiable DNA was obtained by using the FastDNA® Spin Kit for Soil. However, no differences were detected in the concentration of the plasmidic DNA extracted from pressed grapes and cultures in peptone water samples. The sample extracted from *D./B. bruxellensis* yeast cultures in peptone water can therefore be used as a standard for the construction of a calibration curve to quantify this yeast in pressed grape samples. For this purpose, DBRUXF/DBRUXR primers were utilized and the amplification protocol was modified by increasing both the annealing temperature and the primer concentration in order to increase their selectivity.

PHISTER and MILLS (2003) drew these primers on the D1-D2 domain of 26S rDNA of *D. anomala*, *D. bruxellensis*, *B. custersii*, *B. naardenensis*,

B. nanus and *S. cerevisiae* to produce a 77 bp specific product. These primers were tested on various yeasts and bacteria isolated from must/wine and only *D./B. bruxellensis* gave rise to a PCR product.

The results obtained from our QPCR allowed the presence of yeasts belonging to the *Brettanomyces* genus to be detected in pressed Sangiovese grapes samples in a range between 10^3-10^4 cells mL^{-1}. To further verify our results, another primer pair, Brett1/Brett2, was used. By introducing the appropriate changes, as reported in the Results section, it was possible to use these primers and confirm the data already obtained by using DBRUXF/DBRUXR primers.

In conclusion, the approaches developed in this study allow *D./B. bruxellensis* yeasts to be detected in native, even complex, matrices such as pressed grape juice.

On the basis of these preliminary results, we hypothesize that these yeasts might belong to the autochthonous microflora of Sangiovese grapes. Accurate monitoring is now possible which should allow more knowledge to be obtained about the presence and concentrations of *D./B. bruxellensis* in other cultivars and its effect on the quality and flavor of the end products.

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PAPER

INFLUENCE OF HUNTING-SEASON STAGE AND RIPENING CONDITIONS ON DEGRADATION OF SARCOPLASMIC PROTEINS IN VENISON (*CERVUS ELAPHUS*) CHORIZO SAUSAGES

INFLUENZA DELLA FASE DELLA STAGIONE VENATORIA E DELLE CONDIZIONI DI STAGIONATURA SULLA DEGRADAZIONE DELLE PROTEINE SARCOPLASMATICHE NELLE SALSICCE, DI TIPO CHORIZO, DI CARNE DI CERVO (*CERVUS ELAPHUS*)

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ABSTRACT

The influence of hunting-season stage and ripening conditions on the degradation of sarcoplasmic proteins in venison chorizo sausages was studied. Four chorizo batches were prepared, two made with venison hunted at the start of the hunting season and two at the end of the season. Two

RIASSUNTO

È stata studiata l'influenza della fase della stagione venatoria e delle condizioni di stagionatura sulla degradazione delle proteine sarcoplasmatiche negli insaccati tipo "chorizo" di carne di cervo. Sono stati preparati quattro lotti di "chorizo", due preparati con selvaggina cacciata nel periodo iniziale della

- Key words: game meat, hunting season, ripening, sarcoplasmic proteins, SDS-PAGE, venison chorizo sausage -

of them were ripened under controlled conditions and two using a natural system. Sarcoplasmic protein profiles were determined using SDS-PAGE on the raw meat, just after stuffing and at 7, 14 and 21 days of ripening, and after 45 days of vacuum packed storage. The four batches had similar sarcoplasmic protein profiles, showing significant differences in the relative density of some bands. In the early stages of ripening (mainly after 7 days), the 139, 50, 43, 24.5 and 16 kDa fragments disappeared, in contrast, the presence of the 70 and 36 kDa bands gradually increased. Different behaviors were found in some proteins during ripening depending on the ripening conditions and hunting-season stage. Very few sarcoplasmic proteins displayed significant variations during packing. Ripening conditions influenced the relative density of many proteins at the end of ripening.

stagione di caccia e due lotti preparati con selvaggina cacciata alla fine della stessa. Due di questi lotti sono stati stagionati in condizioni controllate e due in condizioni naturali. Sono stati determinati i profili delle proteine sarcoplasmatiche mediante elettroforesi in gel di poliacrilammide (SDS-PAGE) sulla carne cruda, subito dopo l'insaccamento, dopo 7, 14 e 21 giorni di stagionatura e dopo 45 giorni di confezionamento sottovuoto. I quattro lotti avevano simile profilo in proteine sarcoplasmatiche, mostrando differenze significative nelle densità relative di alcune bande. Durante le prime fasi della stagionatura (generalmente dopo 7 giorni) scomparivano i frammenti di 139, 50, 43, 24,5 e 16 kDa, mentre aumentava gradualmente la presenza delle bande di 70 e 36 kDa. Sono riscontrati comportamenti differenti per alcune proteine, durante la stagionatura, in funzione delle condizioni di stagionatura e della fase della stagione venatoria. Solo un numero molto esiguo di proteine sarcoplasmatiche ha mostrato variazioni significative durante il confezionamento. Le condizioni di stoccaggio influenzano un numero rilevante di proteine alla fine della stagionatura.

INTRODUCTION

Consumer interest in game meat has increased over the last years. Game meat is distinguished from that of poultry and domestic animals like cattle, sheep and pigs, because it is generally darker, stronger tasting and sometimes tougher, depending on the type and age of the animal. In addition, game meat has low muscle fat content and a desirable polyunsaturated:saturated fatty acid ratio (HOFFMAN and WIKLUND, 2006).

Hunting is a very important rural activity from an economic standpoint in some Spanish regions such as Castilla-La Mancha. The average total annual invoice is 240 million euros, and hunters take 40,000 big game (red deer, wild boar, roe deer and fallow deer) animals and three million small game (rabbit, partridge and hare) animals annually.

Today there is a very definite tendency towards processed game meat products (sausages, canned meat products and patés, etc.) that are ready to eat and suitable for home consumption (SALGHETTI, 1991). Cured, fermented and dried prod-

ucts from different game species have a restricted presence on the market that depends on the season and culling quotas (PALEARI *et al.*, 2000).

The production of dry sausages is of great importance to the meat industry of the Mediterranean countries. In Spain, cured sausage production amounted to 193,386 tons in 2005 (AICE, 2006). Chorizo is a typical Spanish dry fermented sausage, whose formulation is characterised by the presence of different proportions of paprika.

The ripening of dry sausages involves a set of reactions involving proteins, carbohydrates and lipids. Many different types of end products are formed during the ripening of meat products. Due to proteolytic activity, peptides and amino acids are formed, which may contribute to flavour. Lactic acid and acetic acid are formed from carbohydrates and contribute to the sour flavour. Due to the lipolytic activity, free fatty acids are formed together with flavour components such as aldehydes, ketones and esters (DEMEYER *et al.*, 1995; NAES *et al.*, 1995; FADDA *et al.*, 2001).

The proteins present in skeletal muscle tissues are classified as either myofibrillar or sarcoplasmic proteins. Myofibrillar proteins and collagen account for the hardness of muscle fibres and perform contractile, regulatory and cytoskeletal functions. Sarcoplasmic proteins perform biochemical functions. Curing brings about degradation of the myofibrillar and sarcoplasmic proteins. Several authors (KATO *et al.*, 1994; TOLDRÁ and VERPLAETSE, 1995; JOHANSSON *et al.*, 1996; HIERRO *et al.*, 1999) attribute proteolysis mainly to the action of endogenous enzymes; others (GARCÍA DE FERNANDO and FOX, 1991; FADDA *et al.*, 2002) suggest that lactic acid bacteria play a major role, producing proteolytic enzymes able to break down meat proteins. The relative contribution of endogenous and microbial enzymes, either naturally present in the product or added as a starter culture, is largely governed by the nature of the raw material, the type of product and the processing conditions used (DEMEYER, 1992; MOLLY *et al.*, 1997).

In recent years, several studies have been carried out to characterise the physicochemical composition of red deer meat (STEVENSON *et al.*, 1992; SEMIADI *et al.*, 1993; PEÑA *et al.*, 1993; ZOMBORSZKY *et al.*, 1996), to determine the influence of various feeding regimes on the composition of fatty acids (VOLPELLI *et al.*, 2003) and the sensorial quality (WIKLUND *et al.*, 2001; 2003), to analyse the influence of the slaughtering method on the quality of the meat (WIKLUND *et al.*, 2001) and to study the optimal conditions for packing deer meat (VERGARA *et al.*, 2003) and to determine the effects of processing (smoking and drying) on reindeer meat (SAMPELS, *et al.*, 2004). However, very few studies have been conducted on proteolysis of dry sausages made with deer meat (SORIANO *et al.*, 2006).

The aim of this study was to determine the effect of hunting-season stage and ripening conditions on the changes taking place in the sarcoplasmic proteins extracted from venison chorizo sausages during ripening and following vacuum packing.

MATERIALS AND METHODS

Preparation of chorizo sausages and curing process

Venison chorizo sausages were manufactured in two factories in the Castilla-La Mancha region (central Spain). One factory produced chorizo using a natural drying process, in which the only drying mechanism was the opening and closing of windows. The other company used a ripening system in which chorizo was dried in a room under controlled temperature and relative humidity conditions.

Two batches were obtained from each company: the first made at the start of the 2001-2002 hunting season (November 2001) and the second at the end of the same season (March 2002).

The four batches were coded as follows: batch A: made at the start of the hunting season and ripened under controlled conditions; batch B: made at the end of the hunting season and ripened under controlled conditions; batch C: made at the start of the hunting season and ripened under natural conditions; batch D: made at the end of the hunting season and ripened under natural conditions.

Deer meat from the legs, obtained from hunting, was stored at -18°C until processing. Deer meat and pork back fat (1:1) were ground in a grinder (Mainca, Barcelona, Spain) fitted with a plate containing 12 mm-diameter holes. Ground meat and pork back fat were then kneaded in a mixer (Fuerpla, Valencia, Spain) prior to addition of the remaining ingredients: 90 g/kg of a Ceylamix CH-L (Ceylan, Valencia, Spain) prepared formula containing salt, lactose, dextrose, dextrin, spices, polyphosphates, sodium ascorbate, potassium nitrate (E-252) and paprika, garlic (1.7 g/kg) and sodium nitrite (1 g/kg). The mixture was kneaded for 4 minutes and then stuffed in 30 mm artificial casings using a stuffing machine (Fuerpla, Valencia, Spain). Casings were soaked prior to being immersed in brine for 15-20 minutes.

Batches A and B were ripened in a drying room at a temperature of 10°-12°C and a relative humidity of 76-80% for 3 weeks. Batches C and D were ripened for 3 weeks in a natural drying room in which relative humidity and temperature varied according to outdoor weather conditions. In the natural drying room of batch C, the temperature fluctuated between 3°-14°C and the relative humidity between 41-97%. Batch D was ripened at a temperature of 7°-15°C and a relative humidity of 60-84%.

Three samples from each batch were analyzed at 0 (just after stuffing), 7, 14 and 21 days of ripening. At the end of the ripening period, chorizo sausages were vacuum packed and stored at room temperature. Further analysis was performed 45 days after packing.

Sample preparation of raw meat and sausages

Sausages were deskinned prior to analysis. Raw meat and sausages were ground in a household blender, placed in hermetically-sealed containers and stored at -20°C for future analysis. Samples were analyzed in duplicate.

Physicochemical composition of raw meat

Water activity (a_w) was measured directly using a Decagon CX-2 dew point hygrometer (Decagon Devices Inc., Pullman, WA, USA). pH was determined directly with a Crison 2001 pH meter (Crison Instruments S.A., Barcelona, Spain). The measurement was made three times, changing the insertion place of the electrode in the ground meat. Moisture and fat content were analyzed according to AOAC methods (1990). Protein nitrogen (PN) was determined following the Kjeldahl method, being calculated by multiplying the total nitrogen by 6.25 (AOAC, 1990).

SDS-PAGE

The proteins were extracted following the method described by HUGHES *et al.* (2002). Five grams of sample were homogenized with 35 mL of 0.03 M phosphate buffer pH 7.4 in an Ultra Turrax (Heidolph RZR 2050, Heidolph-Elektro, Schwabach, Germany) for 2 min. The mixture was centrifuged at 10,000 × g for 20 min at 4°C. The supernatant containing the sarcoplasmic proteins was filtered through a glass fibre and 100 μL

of sarcoplasmic extract were dialysed through a minidialysis unit (Amersham Pharmacia Biotech, Uppsala, Sweden) of 7,000 Da in distilled water with magnetic stirring for 20 min.

Total proteins of the sarcoplasmic fraction were determined using the procedure of BRADFORD (1976). The protein concentration of the sarcoplasmic fraction was adjusted to 0.7 mg/mL using the Tris-HCl buffer (pH 8.0) containing 5% (w/v) SDS, 14.5% (v/v) β-mercaptoethanol, 31% (v/v) glycerol, and 0.03% (v/v) bromophenol blue. The samples were heated for 4 min at 100°C prior to electrophoresis.

The proteins were separated by horizontal SDS-PAGE using a Phast System apparatus (Amersham Pharmacia Biotech, Uppsala, Sweden). Porosity gradient polyacrylamide gels, PhastGel 10-15 (Amersham Pharmacia Biotech, Uppsala, Sweden) were used to separate sarcoplasmic proteins. The solid electrode buffer strips were comprised of 0.2 M tricine, 0.2 M Tris, 0.55% (w/v) SDS, pH 8.1. The protein separations were performed at 15°C following the recommendations set out in the instruction manual for the equipment used (AMERSHAM PHARMACIA BIOTECH, 1986a).

Gels were stained using one tablet of Coomassie Blue R-350 dissolved in a water:methanol (4:6) mixture and then diluted to 50% with a 20% (v/v) acetic acid solution. Gels were destained twice with methanol:acetic acid:water (3:1:6). Gel consistency was achieved using 13% (v/v) glycerol. Staining and destaining of the gels was performed according to the recommendations in the PhastSystem manual (AMERSHAM PHARMACIA BIOTECH, 1986b). The gels were air-dried between two sheets of cellophane soaked in 13% (v/v) glycerol for 48-72 h.

Quantification of the bands was carried out by first scanning the gels using the PhotoPhinish program (TDI, Madrid, Spain) and then taking densitometric readings using the 1D-Manager program (TDI, Madrid, Spain).

The molecular masses of the protein bands were calculated from the R_f values by interpolation of the calibration curve constructed using markers of known molecular mass. The markers (Sigma) consisted of the following proteins: myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase b (97 kDa), fructose-6-phosphate kinase (84 kDa), albumin (66 kDa), glutamic dehydrogenase (55 kDa), ovalbumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), trypsin inhibitor (20 kDa), α-lactalbumin (14.2 kDa) and aprotinin (6.5 kDa).

Statistical analysis

Analysis of variance was applied to the data using the SPSS program version 11.0 for Windows 2000 (SPSS, Inc, Chicago, Illinois). The Student-Newman-Keuls test was used to discriminate among means for relative density of the proteins analyzed by SDS-PAGE.

The General Linear Model (GLM) was used to determine the influence of hunting season and ripening conditions on protein profiles. The Least Significant Difference (LSD) test was used to determine differences between batches as a function of these factors.

RESULTS AND DISCUSSION

Physicochemical composition of raw venison

Table 1 presents the means and standard deviations for the physicochemical characteristics and composition of the raw meat used to manufacture the four batches of chorizo sausages. There were no significant differences for pH and water activity (a_w) among the four meats. The protein nitrogen (PN) content was

Table 1 - Means and standard deviations for the physicochemical characteristics and composition of the raw venison used to make the four batches of chorizo.

	A	B	C	D
pH	5.77±0.37	5.48±0.01	5.83±0.11	5.59±0.02
a_w	0.991±0.002	0.988±0.001	0.994±0.003	0.980±0.007
DM (g/100 g sample)	21.97^{a}±0.33	22.87^{a}±0.19	22.31^{a}±0.39	25.36^{b}±1.54
PN (g/100 g DM)	98.22^{a}±0.88	83.27^{b}±3.86	94.09^{c}±1.00	$87.09^{b,c}$±3.12
Fat (g/100 g DM)	1.04^{a}±0.43	8.26^{b}±0.02	2.55^{a}±0.63	11.57^{c}±1.43

DM: dry matter; PN: protein nitrogen.
Different letters within a row indicate significant differences ($P < 0.05$).
A: venison obtained at the start of the hunting season and used for batches ripened under controlled conditions;
B: venison obtained at the end of the hunting season and used for batches ripened under controlled conditions;
C: venison obtained at the start of the hunting season and used for batches ripened under natural conditions;
D: venison obtained at the end of the hunting season and used for batches ripened under natural conditions.

higher in meats obtained at the start of the hunting season (A and C), however fat content was lower in those meats. It could be attributable to the fact that animals at the start of the hunting season (in November) had just been through the rutting season and had obviously consumed significant amounts of their energy reserves, demonstrated both by low fat levels and high pH values (low concentration of muscle glycogen).

SDS-PAGE of raw venison

Table 2 shows mean values and standard deviations for the relative band density (%) of the sarcoplasmic proteins extracted from lean deer meat used to make the four batches studied. Some electrophoretic bands were identified by reference to other authors (McCORMICK *et al.*, 1988; SAVAGE *et al.*, 1990). These authors found the protein pair, phosphorylase *b* and phosphorylase *b* kinase, that migrated together and could not be separated, with an approximate molecular mass of 97 kDa, pyruvate kinase with a molecular mass of about 58 kDa, enolase corresponding to 45-47 kDa, glyceraldehyde-3-phosphate dehydrogenase at 36 kDa and myoglobin at 16 kDa.

The fragment with the highest molecular weight was 139 kDa. It was found at a significantly greater relative density (2.8%) in meat used to make batch B than in the other three batches (1.1-1.9%).

The highest relative density was recorded for myoglobin (16 kDa), which gave values ranging from 13.2-15.0%, with no significant difference among batches.

Differences were found according to the hunting-season stage. The 123.5 kDa fragment and the enolase (47 kDa) were found only in end-of-season meats (B and D), and the 21 kDa fragment was detected only in the start-of-season meats (A and C). The 70 kDa fragment values were higher in meats obtained at the end-of-season (B and D).

Batch D showed significant differences in the relative concentrations of some proteins with respect to the other batches. The concentrations of 50, 47, 24.5 and 21.5 kDa fragments were higher for batch D, however the concentration of the 26.6 and 13 kDa fragments was lower for this batch.

SDS-PAGE of chorizo sausages

Fig. 1 shows the electrophoregram for the sarcoplamic proteins extracted from

Table 2 - Mean values and standard deviations for the relative density (%) of electrophoretic bands corresponding to the sarcoplasmic proteins extracted from venison meat used to make the four batches of chorizo sausages.

Approximate molecular mass (kDa)	A	B	C	D
139	1.57^{a}±0.45	2.83^{b}±0.31	1.89^{a}±0.17	1.10^{a}±0.36
123.5	n.d.	1.94±0.59	n.d.	1.85±0.85
108.5	5.95^{a}±0.71	2.04^{c}±0.36	4.28^{b}±0.59	1.84^{c}±0.48
97 (PHb/PHbK)	1.40±0.21	n.d.	1.51±0.22	1.29±0.21
80	1.24^{a}±0.03	3.01^{c}±0.07	1.98^{b}±0.06	1.58^{a,b}±0.28
70	7.38^{a}±1.41	13.28^{b}±0.81	9.00^{a,b}±0.03	10.66^{a,b}±1.83
65	5.52±2.07	7.30±2.20	4.84±0.20	7.08±1.52
60	2.86±0.59	3.91±0.41	2.34±0.89	2.75±0.75
58 (pyruvate kinase)	1.33±0.17	1.59±0.04	1.70±0.07	1.60±0.42
50	7.79^{a}±1.16	5.33^{a}±0.24	7.19^{a}±0.43	9.96^{b}±0.87
47 (enolase)	n.d.	3.40^{a}±0.55	n.d.	6.39^{b}±0.36
43 (aldolase)	10.19^{a}±2.02	5.03^{b}±0.07	9.10^{a,b}±0.01	7.39^{a,b}±1.17
42	8.27±0.47	4.52±1.86	7.71±0.36	6.46±0.54
38	1.02^{a}±0.03	2.32^{b}±0.24	1.47^{a}±0.15	n.d.
36 (GADPH)	1.23±0.10	1.69±0.28	1.69±0.19	1.49±0.27
31.5	3.17±0.09	3.23±0.05	2.53±0.57	5.28±1.37
31	1.55±0.78	1.04±0.13	1.03±0.07	1.25±0.01
26.6 (TPI)	3.65^{a}±0.59	3.61^{a}±0.14	4.71^{a}±0.18	1.14^{b}±0.37
24.5	2.61^{a}±0.72	3.03^{a}±0.23	2.58^{a}±0.11	4.50^{b}±0.49
24	2.48±0.94	2.40±0.32	2.28±0.44	1.38±0.71
22.5	0.98±0.46	0.95±0.12	1.24±0.31	0.91±0.19
21.5	0.41^{a}±0.16	1.34^{a,b}±0. 38	1.07^{a,b}±0.41	2.12^{b}±0.29
21	1.16±0.07	n.d.	1.18±0.06	n.d.
18	1.41±0.56	1.19±0.04	1.62±0.12	1.46±0.43
16 (myoglobin)	13.95±2.27	13.20±0.03	15.02±0.13	14.18±1.84
13	10.32^{a}±0.84	7.99^{a,b}±1.93	8.12^{a,b}±1.64	4.35^{b}±0.77
12	0.81±0.20	1.78±1.00	1.05±0.16	0.72±0.04
11	1.05±0.18	1.01±0.20	1.65±0.33	1.01±0.60
10.5	0.71±0.53	1.05±0.46	1.22±0.44	0.27±0.06

Different superscripts in each row indicate significant differences ($P < 0.05$).
n.d.: not detected; PHb/PHbK: phosphorylase b/phosphorylase b kinase; GADPH: glyceraldehyde-3-phosphate dehydrogenase; TPI: triosephosphate isomerase.
A: venison obtained at the start of the hunting season and used for batches ripened under controlled conditions;
B: venison obtained at the end of the hunting season and used for batches ripened under controlled conditions;
C: venison obtained at the start of the hunting season and used for batches ripened under natural conditions;
D: venison obtained at the end of the hunting season and used for batches ripened under natural conditions.

venison chorizo sausages from batch A at 0 (just after stuffing), 7, 14 and 21 days of ripening, and after 45 days of being vacuum packed and stored at room temperature.

The four batches presented similar sarcoplasmic protein profiles showing significant differences in the relative density of some bands. In general, the most abundant sarcoplasmic proteins throughout ripening were those corresponding to the molecular mass of 70, 42 and 36 kDa in all batches.

Protein fragments with high molecular weights of 108.5 kDa and 97 kDa, found in venison, were not recorded in any of the batches of chorizo, due to either protein insolubilization or proteolysis during processing. In a study on cured sausage, HUGHES *et al.* (2002) detected a protein

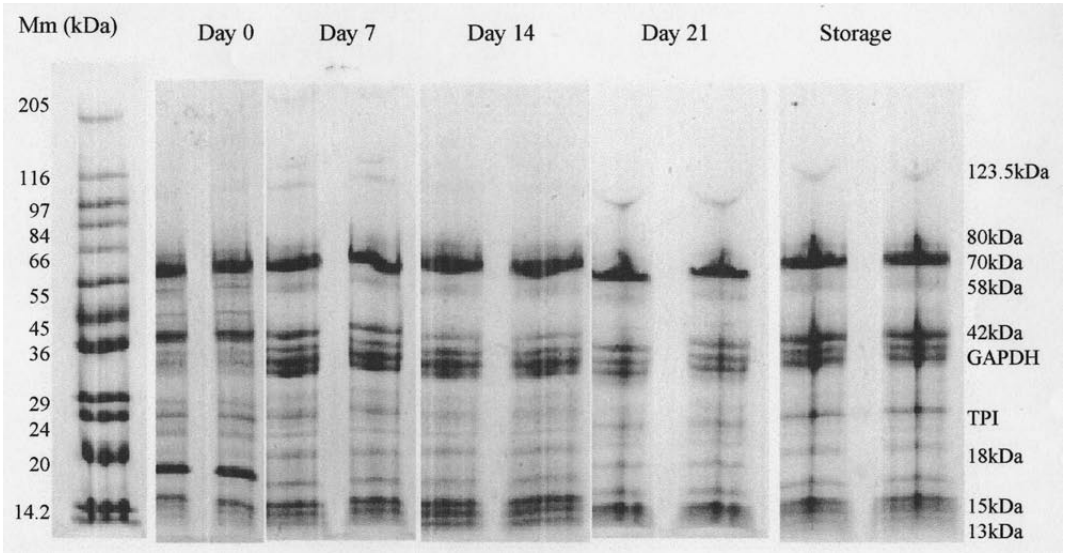


Fig. 1 - Electrophoregram for the sarcoplasmic proteins extracted from a sample (analysed in duplicate) of batch A during ripening and after 45 days of vacuum packed storage. GAPDH: glyceraldehyde-3-phosphate dehydrogenase, TPI: triosephosphate isomerase.

fragment of 97 kDa which disappeared during the first 3 days of ripening.

Tables 3, 4, 5 and 6 show mean values and standard deviations for the relative band density (%) of the sarcoplasmic proteins extracted from the venison chorizo sausages of the four batches studied throughout the ripening process and after 45 days of being vacuum packed at room temperature.

On the whole, three different findings were observed during ripening: electrophoretic bands that ceased to be detectable, bands whose relative concentration underwent changes, and bands that remained constant.

Electrophoretic bands that ceased to be detectable during ripening

The 139, 50, 43, 24.5 and 16 kDa fragments disappeared during the early stages of ripening (mainly after 7 days) in all batches, showing high proteolysis intensity. The 24.5 fragment was not detected in batches B, C and D. The 16 kDa band was not recorded at any time in batch B. JOHANSSON *et al.* (1994) reported a constant fall in the intensity of sarcoplasmic proteins with molecular weights between 20-30 kDa, which almost totally disappeared by the 7th day of ripening of dry sausages. MAURIELLO *et al.* (2002) also reported a drop in the intensity of bands in the 35-50 kDa range during ripening of Italian salami. Disappearance may have been due to proteolysis or to denaturalization induced by salt or acidity. In addition, the water activity values were higher during the first stages of ripening (data not shown), which enhanced the proteolytic enzyme activities.

The protein fragment with the highest molecular weight was 139 kDa, which was initially found at a low relative density in all four batches, but disappeared during the early stages of ripening, except in batch D, where it persisted throughout ripening and storage with no significant variation. In a study on commercial venison sausages, SORIANO *et al.* (2006) noted that the sarcoplasmic protein fragment with the highest molecular weight was 145 kDa, display-

Table 3 - Mean values and standard deviations for the relative density (%) of electrophoretic bands corresponding to the sarcoplasmic proteins extracted from chorizo sausages made at the start of the hunting season and ripened under controlled conditions (batch A), throughout ripening and after 45 days of vacuum packed storage.

Approximate molecular mass (kDa)	Ripening time (days)				Storage
	0	7	14	21	
139	1.39±0.44	0.84±0.52	n.d.	n.d.	n.d.
123.5	1.04±0.52	2.06±1.08	1.09±0.50	2.17±0.80	1.83±0.28
80	4.08±0.37	3.34±1.06	4.73±0.70	3.37±1.21	3.22±0.91
70	15.12^{a}±4.20	19.58^{b}±1.93	24.99^{b}±3.06	21.97^{b}±3.90	23.40^{b}±3.95
58 (pyruvate kinase)	1.53±0.33	1.59±0.88	1.62±0.43	2.20±0.66	2.60±1.53
50	4.36±2.07	n.d.	n.d.	n.d.	n.d.
43 (aldolase)	7.52±1.29	n.d.	n.d.	n.d.	n.d.
42	6.38±0.99	8.73±1.32	6.39±1.91	8.26±3.17	6.55±3.24
38	3.45^{a}±0.92	5.29^{a}±2.91	4.39^{a}±3.39	$7.13^{a,b}$±1.81	9.31^{b}±1.78
36 (GAPDH)	3.88^{a}±1.11	9.47^{b}±0.91	7.81^{b}±3.34	8.96^{b}±1.50	10.07^{b}±3.86
31.5	1.31^{a}±0.48	10.74^{b}±1.28	7.53^{c}±3.53	3.47^{a}±0.29	1.60^{a}±0.35
26.6 (TPI)	3.24±1.21	1.97±1.03	1.95±0.47	2.01±0.54	2.45±1.33
24.5	1.74±0.44	n.d.	n.d.	n.d.	n.d.
24	4.04±1.15	4.08±1.00	4.35±0.84	5.00±0.98	4.46±0.89
22.5	2.07±1.09	1.81±0.92	2.03±1.00	2.15±0.58	2.02±0.61
21.5	2.23±0.80	1.45±0.87	2.20±0.78	2.60±0.70	2.51±1.33
18	1.74^{a}±0.96	3.26^{b}±0.54	3.51^{b}±0.55	3.89^{b}±1.01	4.05^{b}±1.13
16 (myoglobin)	16.93±3.49	n.d.	n.d.	n.d.	n.d.
15	1.03^{a}±0.57	4.30^{b}±0.67	3.20^{b}±0.20	4.69^{b}±1.02	6.51^{c}±1.89
13	6.62^{a}±0.62	$8.15^{a,b}$±1.42	$10.07^{a,b}$±1.66	$9.44^{a,b}$±1.65	11.50^{b}±4.73
12	2.14^{a}±1.32	6.73^{b}±1.37	7.34^{b}±1.40	6.42^{b}±1.10	8.15^{b}±2.27
11	1.91^{a}±0.99	$3.30^{a,b}$±1.31	4.88^{b}±0.69	4.51^{b}±2.11	4.85^{b}±1.85

Different superscripts in each row indicate significant differences ($P < 0.05$).
n.d.: not detected; GADPH: glyceraldehyde-3-phosphate dehydrogenase; TPI: triosephosphate isomerase.

ing a relative density of 2.7%. HUGHES *et al.* (2002) reported a sarcoplasmic protein fragment of 150 kDa which disappeared over the first 3 days of ripening of a semi-dry cured sausage.

The 50 kDa fragment, which displayed high relative-density values in deer meats (5.3-10.0%), declined in chorizos and disappeared after 7 or 14 days of ripening. These results agree with the findings reported by HUGHES *et al.* (2002) for semi-dry fermented sausage.

Initially the high relative-density values of aldolase (43 kDa) were high, then declined until they disappeared. Similar findings were reported by DÍAZ *et al.* (1997) during saucisson ripening.

Myoglobin (16 kDa) bonded to nitric oxide during ripening to form nitrosomyoglobin, which is not soluble in aqueous buffers used to extract sarcoplasmic proteins (MAURIELLO *et al.*, 2002). HUGHES *et al.* (2002) also noted the disappearance of this protein in fermented sausage during the first 3 days of ripening.

Electrophoretic bands whose relative concentration underwent changes during ripening

Increases in relative intensity were recorded for many proteins, mainly with medium and low molecular masses,

Table 4 - Mean values and standard deviations for the relative density (%) of electrophoretic bands corresponding to the sarcoplasmic proteins extracted from chorizo sausages made at the end of the hunting season and ripened under controlled conditions (batch B), throughout ripening and after 45 days of vacuum packed storage.

Approximate molecular mass (kDa)	Ripening time (days)				Storage
	0	7	14	21	
139	0.95±0.49	1.42±0.53	n.d.	n.d.	n.d.
123.5	2.12±0.59	2.15±0.70	1.59±0.89	1.74±0.49	1.63±0.93
80	3.47±1.74	3.69±0.62	4.13±1.88	4.12±2.47	3.63±0.93
70	14.92^{a}±2.43	$16.72^{a,b}$±3.78	$21.80^{b,c}$±4.49	$22.84^{b,c}$±6.01	24.69^{c}±4.89
58 (pyruvate kinase)	1.44±0.51	1.51±0.56	1.89±0.80	2.06±0.91	3.05±2.29
50	1.48±0.20	3.27±0.65	n.d.	n.d.	n.d.
47 (enolase)	3.45^{a}±0.53	2.50^{b}±0.58	$1.82^{b,c}$±0.52	1.02^{c}±0.27	$1.66^{b,c}$±0.21
43 (aldolase)	8.15±1.68	5.55±0.37	n.d.	n.d.	n.d.
42	9.24^{a}±2.08	12.73^{b}±1.52	8.80^{a}±2.99	7.06^{a}±1.08	6.79^{a}±4.06
38	5.60±1.88	7.06±1.44	6.96±1.60	5.31±1.79	7.38±1.71
36 (GAPDH)	4.50^{a}±2.34	$7.23^{a,b}$±1.67	8.38^{b}±1.33	$7.21^{a,b}$±0.38	8.03^{b}±2.42
32	1.90±0.96	1.38±0.70	1.86±0.03	1.61±0.04	1.68±0.53
31.5	1.89±0.67	1.69±0.87	2.57±0.62	2.73±0.51	3.78±2.11
26.6 (TPI)	2.92±0.58	2.58±0.89	1.79±0.77	2.64±1.47	3.37±1.62
24	$5.85^{a,b}$±1.40	3.82^{a}±1.38	7.26^{b}±1.20	4.41^{a}±1.75	3.90^{a}±1.76
22.5	2.26±1.20	2.16±1.08	2.15±0.95	3.15±1.31	2.11±0.53
21.5	2.54±0.72	1.80±1.17	2.43±1.28	3.00±0.87	2.83±0.65
18	3.94^{a}±0.66	2.81^{a}±0.92	3.66^{a}±0.96	5.40^{b}±0.92	3.23^{a}±0.73
15	3.16±1.05	2.80±0.72	3.97±0.79	4.70±1.36	4.68±1.70
13	$7.59^{a,b}$±1.17	6.29^{a}±1.59	$8.27^{a,b,c}$±1.23	10.47^{c}±1.93	$9.41^{b,c}$±2.19
12	7.11±1.54	6.10±1.37	4.58±2.08	7.71±2.89	6.40±3.72
11	1.87^{a}±0.61	1.83^{a}±1.34	$3.73^{a,b}$±2.28	6.06^{b}±1.54	$4.79^{a,b}$±2.13

Different superscripts in each row indicate significant differences ($P < 0.05$).
n.d.: not detected; GADPH: glyceraldehyde-3-phosphate dehydrogenase; TPI: triosephosphate isomerase.

throughout ripening. In the four batches, two fragments, 70 and 36 kDa, increased during ripening. The 70 kDa fragment, which had the greatest relative density, increased after the first week of ripening in batches produced at the start of the hunting season (A and C), and the values were maintained throughout the second and third weeks; by the end of ripening, the overall increases with respect to day 0 were 45% for batch A and 66% for batch C. In batch B, the most marked increase was recorded after the second week of ripening; values were maintained during the third week, by the end of which the increase with respect to day 0 was 53%. In batch D an increase of 23% was obtained after 21 days of ripening. DÍAZ *et al.* (1997) reported a 69 kDa fragment which increased considerably during saucisson ripening, with high concentrations in the final product. On the other hand, glyceraldehyde-3-phosphate dehydrogenase (36 kDa), whose initial relative density in the four batches ranged from 3.9 to 5.1%, increased significantly after the first week of ripening, thereafter displaying no significant variation throughout ripening and storage.

Some different behaviors were observed in some proteins depending on the ripening conditions. This shows the importance of ambient conditions in the

Table 5 - Mean values and standard deviations for the relative density (%) of electrophoretic bands corresponding to the sarcoplasmic proteins extracted from chorizo sausages made at the start of the hunting season and ripened under natural conditions (batch C), throughout ripening and after 45 days of vacuum packed storage.

Approximate molecular mass (kDa)	Ripening time (days)				Storage
	0	7	14	21	
139	1.60±0.52	n.d.	n.d.	n.d.	n.d.
123.5	2.02±1.10	2.51±0.34	2.29±0.86	2.40±0.83	2.46±0.91
80	3.27±0.84	2.76±0.87	3.89±1.93	4.41±1.00	2.80±0.55
70	11.90^{a}±2.22	$18.03^{b,c}$±2.02	15.59^{b}±2.80	19.71^{c}±2.74	$16.39^{b,c}$±1.80
65	6.64±1.49	5.03±2.19	7.82±1.75	5.06±1.38	6.49±1.86
60	2.32±0.89	1.88±0.19	2.01±0.78	2.28±0.64	2.41±0.64
58 (pyruvate kinase)	1.64±0.61	1.72±0.61	2.09±0.54	1.83±0.43	1.86±0.68
50	4.02±0.97	3.28^{a}±0.32	n.d.	n.d.	n.d.
47 (enolase)	2.71±0.99	2.19±0.30	2.59±0.36	3.12±0.72	2.70±0.74
43 (aldolase)	6.20±1.59	n.d.	n.d.	n.d.	n.d.
42	6.88^{a}±1.38	12.99^{b}±1.16	12.51^{b}±1.79	12.54^{b}±2.32	12.79^{b}±1.25
38	4.63±1.63	8.66±3.17	6.64±0.39	6.45±3.34	6.12±2.02
36 (GAPDH)	4.46^{a}±0.95	7.00^{b}±3.97	6.78^{b}±0.99	6.99^{b}±3.20	8.20^{b}±1.41
32	1.64±0.36	2.77±1.19	1.72±0.08	1.74±0.27	2.09±0.54
26.6 (TPI)	4.96^{a}±2.0	$3.07^{a,b}$±0.76	$3.60^{a,b}$±1.51	2.27^{b}±0.30	$3.00^{a,b}$±1.23
24	$5.06^{a,b}$±1.41	$5.29^{a,b}$±1.40	$5.06^{a,b}$±1.11	3.91^{a}±1.00	6.38^{b}±0.73
22.5	4.14^{a}±2.17	4.24^{a}±0.47	$3.56^{a,b}$±0.58	2.33^{b}±0.96	2.08^{b}±0.63
21.5	3.37±0.99	2.68±0.72	2.83±0.39	2.53±0.69	2.61±0.50
18	2.80±0.53	3.48±1.31	3.42±1.57	3.30±0.75	3.25±0.81
16 (myoglobin)	8.65±0.82	n.d.	n.d.	n.d.	n.d.
15	2.00±0.71	3.69±1.12	3.59±1.54	3.25±0.81	3.16±0.82
13	7.07±1.62	7.09±2.59	6.91±2.80	6.90±1.93	7.68±3.20
12	4.61±1.38	5.16±1.83	4.57±1.28	4.43±1.78	3.83±1.71
11	1.26±0.55	0.61±0.95	1.57±2.46	1.76±2.73	1.92±1.31

Different superscripts in each row indicate significant differences ($P < 0.05$).
n.d.: not detected; GADPH: glyceraldehyde-3-phosphate dehydrogenase; TPI: triosephosphate isomerase.

activity of proteolytic enzymes. The relative density of low-molecular-weight fragments, 13 and 11 kDa polypeptides, increased in batches produced under controlled conditions (A and B). In contrast, values did not vary significantly throughout ripening and storage in naturally-ripened batches (C and D). DÍAZ *et al.* (1997) noted a progressive increase in the intensity of 13 and 11 kDa polypeptides during saucisson ripening, and SORIANO *et al.* (2006) reported relative densities between 1.7 and 7.9% in commercial venison chorizo and saucisson. In contrast, enolase (47 kDa) was not detected in batch A and a significant decline was noted in batch B after 7 and 21 days of ripening. However, in naturally-ripened batches (C and D), no significant variation was found in the relative-density values of enolase. On the other hand, in naturally-ripened batches (C and D) the relative density of the 26.6 (triosephosphate isomerase) and 22.5 kDa bands declined significantly during ripening, however these proteins did not display any significant variations in controlled conditions (batches A and B).

Different changes were recorded in certain proteins depending on the hunting-season stage. The 42 kDa increased at the start of ripening in batch C, but

Table 6 - Mean values and standard deviations for the relative density (%) of electrophoretic bands corresponding to the sarcoplasmic proteins extracted from chorizo sausages made at the end of the hunting season and ripened under natural conditions (batch D), throughout ripening and after 45 days of vacuum packed storage.

Approximate molecular mass (kDa)	Ripening time (days)				Storage
	0	7	14	21	
139	1.76±0.29	1.73±0.23	1.59±0.22	1.78±1.35	1.30±0.33
123.5	$1.55^{a,b}$±0.42	3.22^{c}±0.84	3.42^{c}±1.26	$2.65^{b,c}$±0.26	1.29^{a}±0.76
80	3.12±1.02	3.30±0.66	3.51±0.99	3.10±0.33	3.51±0.79
70	11.53^{a}±2.47	$14.77^{a,b}$±3.80	$11.97^{a,b}$±1.33	$14.17^{a,b}$±1.57	16.23^{b}±3.50
65	8.31^{a}±1.02	7.89^{a}±0.25	$6.30^{a,b}$±1.35	4.86^{b}±1.08	5.07^{b}±1.12
58 (pyruvate kinase)	1.61±0.67	1.90±1.03	1.67±0.37	1.94±0.83	2.38±1.18
50	2.29±1.70	n.d.	n.d.	n.d.	n.d.
47 (enolase)	2.49±1.19	2.79±1.19	2.99±0.90	2.94±1.11	2.56±1.15
43 (aldolase)	6.94±1.04	n.d.	n.d.	n.d.	n.d.
42	10.61^{a}±2.55	$14.16^{a,b}$±3.11	15.26^{b}±2.20	$13.33^{a,b}$±2.58	10.51^{a}±1.55
38	4.18^{a}±1.01	$5.83^{a,b}$±0.27	10.29^{c}±2.31	$8.15^{b,c}$±0.89	$8.72^{b,c}$±2.49
36 (GAPDH)	5.14^{a}±1.09	$9.45^{a,b}$±4.62	12.11^{b}±1.29	11.08^{b}±1.69	11.99^{b}±0.83
31.5	1.20±0.29	1.96±1.19	4.04±0.43	1.95±0.57	4.92±3.20
26.6 (TPI)	4.23^{a}±1.45	2.67^{b}±1.23	1.84^{b}±0.57	2.64^{b}±0.33	2.42^{b}±0.86
24	4.25^{a}±0.84	6.43^{b}±0.93	$4.87^{a,b}$±0.62	$5.81^{a,b}$±1.61	$4.59^{a,b}$±1.88
22.5	4.65^{a}±1.45	3.02^{b}±0.65	$1.75^{b,c}$±0.45	$2.16^{b,c}$±1.09	1.53^{c}±0.54
21.5	$2.39^{a,b}$±0.62	2.98^{a}±0.69	1.58^{b}±0.25	$2.39^{a,b}$±0.46	1.64^{b}±0.45
18	2.87^{a}±0.53	3.83^{a}±0.73	3.36^{a}±0.99	3.71^{a}±1.15	5.33^{b}±0.93
16 (myoglobin)	2.05±1.41	n.d.	n.d.	n.d.	n.d.
15	2.80±0.16	4.17±0.48	3.60±1.00	2.85±0.51	3.09±1.23
13	5.68±1.52	6.97±1.53	6.72±1.74	7.15±1.39	7.59±1.42
12	4.18±1.40	4.98±0.50	4.24±0.87	4.65±1.29	5.83±1.59
11	1.85±2.11	4.96±1.50	2.55±1.40	3.31±1.47	4.14±1.57

Different superscripts in each row indicate significant differences ($P < 0.05$).
n.d.: not detected; GADPH: glyceraldehyde 3-phosphate dehydrogenase; TPI: triosephosphate isomerase.

gradually decreased thereafter throughout ripening and storage in batches elaborated at the end of hunting season (B and D). HUGHES *et al.* (2002) noted the appearance of a 42 kDa polypeptide after the first 2 days of ripening of a fermented sausage; afterwards, this fragment remained stable. Initially, the 31.5 kDa protein fragment had low relative density values (1.3%) in batch A, followed by a marked increase during the first week, to a mean 10.7%; values then declined during the second and third weeks, returning to initial levels by the end of ripening; these were maintained throughout storage. In batches B and D, made at the end of the hunting season, the relative-density values did not vary significantly for this protein during ripening and storage; values ranged between 1.2 and 4.9%.

This marked variability in the changes of proteins may be ascribed to the fact that the proteolysis pattern in fermented sausages is governed by a number of variables, including characteristics of the raw material (in this study the most notable difference between raw meat obtained at the start and the end of the hunting season is that animals at the start of the hunting season experienced strong muscle activity and fatigue), the composition

of the product, starter culture and processing conditions, which naturally vary from one product to another.

It should be noted that samples from batch A gave the highest number of proteins whose relative concentration changed during ripening, mainly medium- and low-molecular weight fragments, 70, 36, 18, 15, 13, 12 and 11 kDa, increased after 7 days of ripening. This fact could influence the sensorial characteristics of chorizo sausages, increasing the flavour intensity.

Electrophoretic bands that remained constant during ripening

The relative density of the 80 kDa fragment did not vary significantly throughout ripening and storage in any of the four batches. A similar relative density for this fragment was reported by SORIANO *et al.* (2006) in commercial venison chorizo and saucisson.

Very few sarcoplasmic proteins displayed significant variations during packing. This suggests that variation takes place largely during ripening. The relative density of 70, 24, 18 and 15 kDa fragments increased after storage in batches D, C, D and A, respectively. The relative density of band 123.5 in batch D decreased after storage.

Table 7 shows the results obtained after applying the General Linear Model and the LSD test to determine differences between batches as a function of hunting-season stage and of ripening

Table 7 - Effect of hunting-season stage and ripening conditions on the relative density of the sarcoplamic proteins in the four venison chorizo batches after 21 days of ripening.

Approximate molecular mass (kDa)					*Factor effect*		
	A	*B*	C	D	H	R	H x R
123.5	2.17±0.80	*1.74±0.49*	2.40±0.83	2.65±0.26	*ns*	*ns*	*ns*
80	3.37±1.21	*4.12±2.47*	4.41±1.00	3.10±0.33	*ns*	*ns*	*ns*
70	21.97^{a}±3.90	*22.84^{a}±6.01*	19.71^{a}±2.74	14.17^{b}±1.57	*ns*	*	*
58 (pyruvate kinase)	2.20±0.66	2.06±0.91	*1.83±0.43*	1.94±0.83	*ns*	*ns*	*ns*
42	*8.26^{a}±3.17*	7.06^{a}±1.08	12.54^{b}±2.32	13.33^{b}±2.58	*ns*	*	*ns*
38	7.13±1.81	5.31±1.79	6.45±3.34	8.15±0.89	*ns*	*ns*	*ns*
36 (GAPDH)	$8.96^{a,b}$±1.50	7.21^{a}±0.38	6.49^{a}±3.20	11.08^{b}±1.69	*ns*	*ns*	*
26.6 (TPI)	2.01±0.54	2.64±1.47	2.27±0.30	2.64±0.33	*ns*	*ns*	*ns*
24	$5.00^{a,b}$±0.98	$4.41^{a,b}$±1.75	3.91^{a}±1.00	5.81^{b}±1.61	*ns*	*ns*	*
22.5	2.15±0.58	3.15±1.31	2.33±0.96	2.16±1.09	*ns*	*ns*	*ns*
21.5	2.60±0.70	3.00±0.87	2.53±0.69	2.39±0.46	*ns*	*ns*	*ns*
18	3.89^{a}±1.01	5.40^{b}±0.92	3.30^{a}±0.75	3.71^{a}±1.15	*	*	*ns*
15	4.69^{a}±1.02	4.70^{a}±1.36	3.25^{b}±0.81	2.85^{b}±0.51	*ns*	*	*ns*
13	9.44^{a}±1.65	10.47^{a}±1.93	6.90^{b}±1.93	7.15^{b}±1.39	*ns*	*	*ns*
12	$6.42^{a,b}$±1.10	7.71^{a}±2.89	4.43^{b}±1.78	4.65^{b}±1.29	*ns*	*	*ns*
11	4.51±2.11	6.06±1.54	1.76±2.73	3.31±1.47	*ns*	*ns*	*ns*

Different superscripts in each row indicate significant differences ($P < 0.05$).
* significant differences ($P < 0.05$). ns: not significant differences.
GADPH: glyceraldehyde-3-phosphate dehydrogenase; TPI: triosephosphate isomerase.
A: batch made at the start of the hunting season and ripened under controlled conditions;
B: batch made at the end of the hunting season and ripened under controlled conditions;
C: batch made at the start of the hunting season and ripened under natural conditions;
D: batch made at the end of the hunting season and ripened under natural conditions
H: hunting season;
R: ripening conditions.

conditions on sarcoplasmic protein profiles after 21 days of ripening.

Ripening conditions influenced the relative density of more proteins than did hunting-season stage or the interaction of these two variables. The relative density of the 42 kDa band was significantly lower in batches ripened under controlled conditions. In contrast, the relative density values of low-molecular-weight bands 15, 13 and 12 kDa were higher in these batches. This may be because constant temperature and relative humidity enhance the activity of proteolytic enzymes and thus the generation of low-molecular-weight protein fragments.

The relative density of the 70 kDa band after 21 days of ripening was significantly affected by the ripening process and by the interaction of the two variables; relative density values in batch D were significantly lower than those of the other batches.

Both hunting-season stage and ripening conditions significantly influenced the relative density of glyceraldehyde-3-phosphate dehydrogenase (36 kDa) and the 24 kDa fragment; values were highest in batch D and lowest in batch C. The interaction of the two variables also affected the density of the 18 kDa band, values for batch C were significantly higher than for the other batches.

In conclusion, during the early stages of venison chorizo ripening, mainly after 7 days, proteins with high and medium molecular weight disappeared, showing high proteolysis intensity. Ripening conditions and hunting-season stage influenced the degradation of some sarcoplasmic proteins. Chorizo sausages made with venison from the start of the hunting season and ripened under controlled conditions (batch A) gave the highest number of proteins whose relative concentration changed during ripening, particularly the medium- and low-molecular-weight fragments, 70, 36, 18, 15, 13, 12 and 11 kDa, increased after 7 days of ripening; this fact could influence the sensorial characteristics of chorizo sausages, increasing the flavour intensity. Very few sarcoplasmic proteins displayed significant variations after 45 days of being vacuum packed, so packing is appropriate for conserving the product from a proteolysis point of view.

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PAPER

QUANTITATIVE VARIATIONS OF THE MAIN CAROTENOIDS IN ITALIAN TOMATOES IN RELATION TO GEOGRAPHIC LOCATION, HARVEST TIME, VARIETIES AND RIPENING STAGE

VARIAZIONI QUANTITATIVE DEI PRINCIPALI CAROTENOIDI IN POMODORI DI ORIGINE ITALIANA IN RELAZIONE AL LUOGO DI COLTIVAZIONE, STAGIONE DI RACCOLTA, VARIETÀ E GRADO DI MATURAZIONE

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ABSTRACT

The aim of this study was to evaluate if the carotenoid content of Italian tomatoes is affected by factors such as the geographical area in which the plants are cultivated, harvest time, tomato typology and ripening stage. Three Italian tomato varieties were sampled in different geographical locations throughout Italy in the summer and winter months. Significant differences were found in the lycopene,

RIASSUNTO

L'obiettivo di questo lavoro è stato quello di verificare in pomodori prodotti in Italia se il contenuto dei principali carotenoidi poteva essere influenzato da alcuni fattori, quali la zona geografica in cui le piante erano coltivate, la stagione di raccolta, la varietà di pomodoro e il grado di maturazione. Sono state studiate tre varietà di pomodori italiani e i frutti sono stati campionati in diverse aree geografiche del paese du-

- Key words: carotenoids, harvest time, ripening, tomato, variety -

β-carotene and phytoene contents as a function of tomato variety with the highest carotenoid levels recorded for cherry tomatoes. In relation to the geographic location the only significant differences were lycopene content in salad tomatoes. The harvesting period influenced carotenoid composition, but a definite seasonal trend was not observed. During the summer the lycopene content was higher in summer-harvested tomatoes than in winter-harvested ones, for both cluster and cherry typologies. In relation to the harvesting season, no significant differences were found for the lycopene content in salad tomatoes, while the β-carotene content was significantly different. Ripening stage influenced lycopene and β-carotene levels in all the varieties considered; significant differences in the phytoene values were found in cherry tomatoes. The highest carotenoid levels were recorded in fully ripe cherry tomatoes.

rante i mesi estivi e invernali. In relazione alla varietà, differenze significative sono state riscontrate per i contenuti di licopene, β-carotene e fitoene. I più alti contenuti di carotenoidi sono stati rinvenuti in pomodori appartenenti alla varietà ciliegino. In relazione all'origine geografica delle coltivazioni, si sono riscontrate differenze significative solo confrontando i contenuti di licopene dei pomodori insalatari. Il periodo di raccolta influenzava il contenuto dei composti analizzati, ma non è stato possibile definire un andamento stagionale ben definito. I valori medi di licopene in pomodori delle varietà ciliegino e grappolo determinati nel corso dei mesi estivi erano significativamente più alti di quelli determinati nel corso dei mesi invernali. Per la varietà insalataro differenze significative legate alla stagione di raccolta sono state registrate solo per il contenuto di β-carotene. Il grado di maturazione influenzava in maniera significativa i contenuti di licopene e β-carotene in ciascuna varietà analizzata. Solo nella varietà ciliegino si sono verificate differenze significative anche per il contenuto di fitoene. I maggiori contenuti di carotenoidi sono stati registrati nei pomodori della varietà ciliegino raccolti a completa maturazione.

INTRODUCTION

In recent years, many scientific studies have demonstrated the strong relationship between diet and health. The results of epidemiological studies have shown the association of a high vegetable intake with a reduced risk of many degenerative diseases including some types of cancer (STEINMETZ and POTTER, 1996; RIBOLI and NORAT, 2003), cardiovascular diseases (NESS and POWLES, 1997; JOSHIPURA *et al.*, 2001), etc. The positive correlation between vegetable intake and cancer prevention has been attributed to the presence of antioxidant compounds (MACHLIN, 1995).

During the last decade the physiological and biological functions of carotenoids have been studied. The provitamin A activity of some carotenes has been described in many papers (OLSON, 1989; ROCK, 1997). Other studies have shown that these compounds

have biological functions that can be attributed to their antioxidant activity (BURTON, 1989; PALOZZA and KRINSKY, 1992). Among the carotenoids, lycopene has been considered the most effective for quenching singlet oxygen (DI MASCIO *et al.*, 1989) and for this reason many recent studies have investigated its protective role in human health. Dietary intake of lycopene has been associated with a decreased risk of prostate cancer (GIOVANNUCCI, 1999, 2002) and its function in the prevention of degenerative diseases has been described in numerous papers (RAO and ARGWAL, 1998, 1999).

Tomato is the main dietary source of lycopene and is one of the most frequently consumed vegetables in the Mediterranean area. Recent studies have defined the potential health benefits of a tomato-rich diet. According to many authors the regular consumption of tomatoes is associated with a decreased incidence of certain types of cancer (GIOVANNUCCI, 1999, 2002; RAO and ARGWAL, 1998) and heart diseases (LAVELLI *et al.*, 2000). These beneficial effects on human health have been attributed to the antioxidants, particularly carotenoids and flavonoids (LAVELLI *et al.*, 2000).

It is well known that the qualitative and quantitative composition of tomato is affected by many factors, such as tomato typology, ripening stage, climatic conditions, etc. Differences in composition are due to genotype and several authors have described variations in the carotenoid profile in relation to different cultivars (LEONARDI *et al.*, 2000; ABUSHITA *et al.*, 1997, 2000). Other studies have described the changes that occur during ripening; the biosynthesis of carotenoids is controlled by regulatory mechanisms that allow an increase in carotenoid content (THELANDER *et al.*, 1986; FRASER *et al.*, 1994). In the literature, there have been a few recent reports that carotenoid composition is a function of harvesting period (RAFFO *et al.*, 2006; TOOR *et al.*, 2006) and of the environmental conditions in which the tomato crop is cultivated (DUMAS *et al.*, 2003). These studies have shown that tomato quality is affected by many factors such as temperature and UV radiation (DORAIS *et al.*, 2001); same studies have proven that lycopene synthesis is inhibited by particularly intense solar radiation (ADEGOROYE and JOLLIFFE, 1987).

In Italy, fresh tomatoes and tomato products are the most important source of the dietary intake of lycopene and carotenoids because tomatoes are used in many culinary preparations. In Italy Sicily is the most important tomato-producing area. During the summer, tomato crops are grown throughout the country, because the temperate climate of the Mediterranean area assures optimal conditions for their growth. However, since the microclimates are different in the northern and southern Italian regions it is hypothesized that there could be variations in the lipophilic antioxidant content of tomatoes grown in the different regions. Many tomato varieties cultivated in these regions are harvested and sold at the ripening stage at which they are normally used for culinary purposes.

The aim of this study was to define carotenoid content in the most common tomato cultivars available on the Italian market. The investigation was carried out during different periods of the year and the tomatoes were sampled in different geographical areas in order to determine if there are significant differences in carotenoid content related to harvesting season or to the typical microclimate in which the crops were grown. To compare the carotenoid contents in relation to these variables, three Italian tomato cultivars were analyzed at the ripening stage at which they were sold. However, to complete the investigation, the effect of ripening stage was also evaluated by analyzing some tomatoes harvested at different ripening stages.

MATERIALS AND METHODS

Tomato sampling

Tomatoes were collected in unheated greenhouses located in Sicily, Tuscany and Latium. Plant nutrition and chemical pest and disease control followed commercially acceptable practices. The tomatoes were grown under varying climatic conditions, because the greenhouses were covered with plastic film but temperature and UV radiation were not controlled.

The tomatoes were harvested during May and June 2004; tomatoes from Sicily were also collected during the winter (January-February 2005).

Three different commercial varieties were investigated: a cluster type (cv. Ikram F1), cherry type (cv. Naomi F1) and salad type (cv. Eroe F1).

For each variety grown in the same geographical area and harvested during the same season, ten tomatoes were harvested from ten different representative plants (selection based on their central position in the greenhouse). The tomatoes were combined into one sample that was selected in relation to the ripening stage: salad tomatoes at incomplete ripening (green-orange fruit (G-O)); cluster and cherry tomatoes at full ripening (red fruit (R)). To get a homogeneous group, chromatic coordinates (L*, a*, b*) were determined on the equatorial part of the fruit (MC GUIRE, 1992), using a tristimulus Chroma meter (Minolta, Tokyo, Japan, model Cr 200). Fruit colours was described by the ratio a*/b*.

Reagents and standards

All solvents used were of HPLC grade from BHD (Poole, England). The β-carotene and lycopene standards were produced by Sigma (St. Louis, USA); the lutein standard was purchased from ICN Biochemical Inc. (Ohio, USA). Magnesium oxide and silica gel 60 (0.063-0.200 mm) were from Merck (Darmstadt, Germany).

Sample extraction

To obtain a homogeneous and representative sample for analysis, ten tomatoes were taken at random from each pooled sample and homogenized using an IKA Labortechnik homogenizer, model T25 basic (IKA® WERKE GmbH & Co., Staufen, Germany).

Fresh homogenized tomatoes were weighed and lyophilized for 24 hours (Freeze Dryer Modulyo, Edwards equipped with a Motors BS 5000-11 pump (Edwards High Vacuum. Ltd, Crowley, U.K.). The freeze-dried samples were then weighed and stored at -20°C until analysis.

For carotenoid extraction, a 275 mg sample of homogenized freeze-dried tomatoes (corresponding to 5 g of fresh tomato) was extracted for 2 min using 15 mL of chloroform and then centrifuged at 3,000 rpm for 10 min (ROZZI *et al.*, 2002). This extraction was repeated twice until the pellet became colourless. The organic fractions were collected and evaporated to dryness on a rotavapor. The residue was dissolved in 5 mL of HPLC grade chloroform; 1 mL was filtered through a 0.45 μm Minisart SRP 4 filter (Sartorius, Goettingen, Germany) and injected into the liquid chromatograph. Three replications were carried out for each sample.

HPLC analyses

A Perkin-Elmer LC 410 Series apparatus (Norwalk, CT, USA) equipped with a UV/VIS LC295 Perkin-Elmer detector and a Perkin-Elmer 1022 Plus integrator was used. Elution was carried out using a mixture of methanol:acetonitrile:dichloromethane (50:48:2) at a flow rate of 1.0 mL/min. A reversed-phase LiChrospher 100 RP 18 (5 μm,

125x4.6 mm) Merck column was used and the run time was 35 min (SALEH and TAN, 1991). The injection volume was 10 μL. UV-VIS detector was set at 290 nm for the simultaneous detection of all compounds.

The quantitative analysis of lycopene, β-carotene, lutein and phytoene was based on an external standard method. The quantification of these compounds was carried out using a calibration curve obtained by injecting standards at known and variable concentrations.

Isolation of phytoene standard by open column chromatography (OCC)

To obtain phytoene standard, a previously described OCC method was used (OTLES, 2000). Phytoene was isolated from a carotenoid extract of fresh tomatoes. The organic fraction was evaporated in a rotary evaporator and the residue was dissolved in 2 mL of hexane. OCC was carried out in an open column (15x1.5 cm) packed with MgO: silica gel (1:2) and activated using hexane. Phytoene standard was separated using 100 mL of hexane as the eluting solvent. An aliquot of the isolated fraction was then used to verify the purity by HPLC and UV-VIS spectroscopy. The eluted fraction was evaporated under nitrogen and stored at -20°C until used.

Statistical analysis

A one-way analysis of variance (ANOVA) was performed to independently test the significance of the observed differences (using Stata 9.0, StataCorp LP). Data were analyzed considering tomato variety, geographical locations, harvest time and ripening stage as experimental factors. If the effects were significant ($P \leq 0.05$) the mean values were then compared by Duncan's test to look for grouping.

RESULTS AND DISCUSSION

Carotenoid content determination

Calibration curve parameters (μg/mL - chromatographic peak area ratio) used for carotenoid content determination are described in Table 1.

To verify that the extraction process was reproducible, three portions of different tomato samples (salad, cluster and cherry tomatoes) were collected, lyophilized and analyzed in triplicate for five consecutive days. The obtained CV% values were: 7.5, 3.9, 4.6 and 6.8, respectively, for lutein, lycopene, β-carotene and phytoene.

Effect of tomato variety on carotenoid content

In order to evaluate the effect of variety on the carotenoid content, tomatoes were sampled during the same harvesting period (summer months) and in the same geographical area, Sicily (Table 2). The total carotenoid content was in the range reported in the literature (LEONARDI *et al.*, 2000; ABUSHITA *et al.*, 1996, 2000)

Table 1 - Parameters of lutein, lycopene, β-carotene and phytoene calibration curves.

Compound	Slope	Intercept	R^2	Range (μg/mL)
Lutein	41512	2261	0.9863	0.08 - 20.20
Lycopene	158549	-494	0.9957	8.25 - 200.23
β-carotene	62246	6036	0.9999	2.20 - 20.56
Phytoene	174735	2311	0.9997	0.52 - 60.13

Table 2 - Carotenoid content (mg/100 g of fresh tomato) of different tomato varieties from Sicily (Mean values ± Standard Deviation).

Compound	Tomato cultivar[a]		
	Salad	Cluster	Cherry
Lutein	0.016±0.012	0.020±0.009	0.021±0.012
Lycopene	2.35±0.53 a	8.47±1.17 b	13.27±2.82 c
β-carotene	0.42±0.09 a	0.62±0.22 b	0.71±0.12 b
Phytoene	0.12±0.37 a	0.44±0.28 a	1.99±0.54 b

[a] Different letters, within each row, indicate significant differences according to the Duncan test. No letter is reported if ANOVA results were not significant (P≤0.05) in relation to the tomato cultivar.

Data show that the tomatoes are characterized by relevant differences in lycopene content. In every case, lycopene was the main component of the carotenoid fraction and its mean values ranged from 2.35 to 13.27 mg/100 g of fresh tomatoes. The highest values were detected in cherry-type tomatoes, while the lowest lycopene content as well as the other carotenoids were recorded in salad-type tomatoes. The one-way analysis of variance (ANOVA) showed significant differences for lycopene, β-carotene and phytoene contents. The stage of ripening can explain the quantitative differences that were observed, because the salad tomatoes were analyzed at an incomplete ripening stage, when the carotenoid contents are the lowest (RAFFO *et al.*, 2002; ABUSHITA *et al.*, 1996). According to some authors (LEONARDI *et al.*, 2000) carotenoid content is more affected by ripening stage than by cultivar, the latter determined slight but significant effects, consequently quantitative differences were not observed when different tomato varieties were analyzed at the same ripening stage.

In this study, the lycopene and phytoene contents varied according to varieties for both cluster and cherry tomatoes. Since the cherry and cluster tomatoes were analyzed at the same ripening stage, the differences could be related to tomato typology. As previously described by LEONARDI *et al.* (2000) the highest carotenoid content values were observed in cherry-type tomatoes. Significant differences were not found in lutein content.

Effects of geographical location on carotenoid content

To examine the influence of geographical location on Italian tomatoes, samples were harvested during the summer in three geographically different areas of Italy. Quantitative data on carotenoid composition are presented in Table 3. For each typology investigated, lycopene was the most abundant component ranging from 2.14 mg/100 g (recorded for salad tomatoes grown in Tuscany) to 13.27 mg/100 g (recorded for cherry tomatoes grown in Sicily). The quantitative data obtained for each variety were compared with data obtained for the same variety grown in other geographic locations. Significant differences were found in the lycopene content in salad tomatoes. The carotenoid content in the other varieties was not significantly influenced by geographic location. No significant differences were found in the contents of the other components.

According to some authors (DUMAS *et al.*, 2003; GARCIA and BARRETT, 2006) the lycopene concentration in tomatoes is dependent on the environmental fac-

Table 3 - Carotenoid content (mg/100 g of fresh tomato) in tomatoes grown in Sicily, Tuscany and Latium (Mean values ± Standard Deviation).

Compound	Salad			Cluster			Cherry		
	Sicily	Tuscany	Latium	Sicily	Tuscany	Latium	Sicily	Tuscany	Latium
Lutein	0.016±0.012	0.017±0.011	0.018±0.008	0.020±0.009	0.019±0.008	0.021±0.011	0.021±0.012	0.017±0.010	0.018±0.009
Lycopene	2.35±0.53 ab	2.14±0.68 a	2.80±0.54 b	8.47±1.17	8.53±1.37	8.88±1.42	13.27±2.82	12.44±2.18	13.06±1.94
β-carotene	0.42±0.09	0.39±0.08	0.43±0.09	0.62±0.22	0.71±0.19	0.84±0.23	0.71±0.12	0.83±0.14	0.76±0.14
Phytoene	0.12±0.37	0.11±0.52	0.13±0.48	0.44±0.28	0.52±0.34	0.61±0.26	1.99±0.54	1.85±0.54	1.44±0.50

[a] Different letters, within each row, indicate significant differences according to the Duncan test. No letter is reported if ANOVA results were not significant (P≤0.05) in relation to the geographic locations of the crop.

tors in which the crop is grown. In this study, with the exception of the lycopene content in salad tomatoes, no differences were found in relationship to geographic location. The climatic conditions that occurred in Sicily, Tuscany and Latium in the summer of 2004 were comparable in terms of temperature, UV radiation, etc.

Effects of harvesting seasons on carotenoid content

For this investigation tomatoes were sampled during the summer and winter in unheated greenhouses located in Sicily. During the year, the microclimate of Sicily is subject to seasonal changes in temperature and solar irradiation that could generate variations in lipophilic composition.

As can be seen in Table 4 the carotenoid content was lower during the winter. This is in agreement with studies of RAFFO *et al.* (2006) who reported the highest lutein, lycopene and β-carotene contents in tomatoes sampled during the summer. The seasonal trend of the phytoene contents recovered in our study is not in agreement with these authors because they reported the opposite trend, i.e., a higher content in winter-grown tomatoes.

The quantitative data obtained for each variety analyzed during the summer were compared with data obtained from the same variety grown during the winter. The ANOVA showed that there were significant quantitative differences for the lycopene contents in both cluster- and cherry-type tomatoes. Significant differences in lycopene content were not found for salad-types tomatoes, while the β-carotene contents were significantly different. Based on these results and in agreement with previously published data, no definite seasonal trend of carotenoid content was found in Italian tomatoes (RAFFO *et al.*, 2006; TOOR *et al.*, 2006).

Table 4 - Carotenoid content (mg/100 g of fresh tomato) in tomatoes harvested during the summer and winter (Mean values ± Standard Deviation).

	Salad[a]		Cluster[a]		Cherry[a]	
Compound	winter	summer	winter	summer	winter	summer
Lutein	0.014±0.013	0.016±0.012	0.016±0.009	0.020±0.009	0.019±0.012	0.021±0.012
Lycopene	1.94±0.52	2.35±0.53	6.84±0.89 a	8.47±1.17 b	9.63±2.08 a	13.27±2.82 b
β-carotene	0.21±0.08 a	0.42±0.09 b	0.55±0.12	0.62±0.22	0.64±0.16	0.71±0.12
Phytoene	0.09±0.24	0.12±0.37	0.39±0.52	0.44±0.28	1.83±0.73	1.99±0.54

[a] Different letters, within each row, indicate significant differences according to the Duncan test. No letter is reported if ANOVA results were not significant (P≤0.005) in relation to harvesting season.

Effects of ripening stage on carotenoid content

To complete the study regarding the carotenoid profile in Italian tomatoes, cherry and cluster tomatoes were harvested and analyzed at different ripening stages. Chromatic coordinates were defined and the a*/b* ratio was used as a ripening index (GIOVANNELLI *et al.*, 1999). The mean values were determined for each sample: a*/b* mean values were less than 0.5 in green-orange fruits (G-O), between 0.5 and 1 for orange-red fruits (O-R) and higher than 1 for red fruits (R). Table 5 shows that the carotenoid contents increased during ripening; the highest values were found in fully ripe tomatoes. Lycopene was the most abundant carotenoid compound; at full ripeness its content was six-times greater than unripe tomatoes.

Using ripening stage as an experimental factor, ANOVA showed that the lycopene and β-carotene contents differed significantly in both cluster and cherry tomatoes. These results were confirmed by some studies that reported an increase in the β-carotene and lycopene contents as ripening evolved (RAFFO *et al.*, 2002; ABUSHITA *et al.*, 1997). Significant differences in phytoene content were found in cherry tomatoes. The lutein contents were not significantly influenced by the ripening stage.

Table 5 - Carotenoid content (mg/100 g of fresh tomato) in tomatoes harvested at different ripening stage (Mean values ± Standard Deviation).

	Cluster[a]			Cherry[a]		
Compound	**G-O[b]**	**O-R[b]**	**R[b]**	**G-O[b]**	**O-R[b]**	**R[b]**
Lutein	0.014±0.008	0.016±0.010	0.020±0.009	0.015±0.009	0.020±0.013	0.021±0.012
Lycopene	2.23±0.37 a	4.83±0.96 b	8.47±1.17 c	2.46±0.43 a	8.63±1.09 b	13.27±2.82 c
β-carotene	0.37±0.08 a	0.51±0.11 b	0.62±0.22 b	0.49±0.09 a	0.61±0.13 b	0.71±0.12 b
Phytoene	0.11±0.19	0.35±0.50	0.44±0.28	0.13±0.61 a	1.10±0.52 b	1.99±0.54 c

[a] green-orange fruit (G-O), orange-red fruit (O-R) and red fruit (R).
[b] Different letters, within each row, indicate significant differences according to the Duncan test. No letter is reported if ANOVA results were not significant (P≤0.05) in relation to ripening stage.

CONCLUSIONS

This study provides some interesting new data on the carotenoid content in Italian tomatoes. Our investigation has shown that geographic location, harvesting season, variety and ripening stage are factors that should be taken into consideration when evaluating carotenoid content in Italian tomatoes. The highest carotenoid levels were recorded in fully ripe cherry tomatoes harvested during the summer. All of the various experimental factors considered affected tomato quality in terms of lycopene, β-carotene, phytoene contents but the lutein content was not significantly influenced.

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SHORT COMMUNICATION

IDENTIFICATION OF PHENOLIC ACIDS IN COFFEE (*COFFEA ARABICA* L.) DUST AND ITS ANTIOXIDANT ACTIVITY

IDENTIFICAZIONE DI ACIDI FENOLICI NELLA POLVERE DEL CAFFÈ (*COFFEA ARABICA* L.) E DETERMINAZIONE DELLA LORO ATTIVITÀ ANTIOSSIDANTE

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ABSTRACT

The natural antioxidants present in the by-products of the coffee industry have been focused on because they are related to the reduction of oxidative stress. Furthermore, the phenolic acids present in coffee beans play an important role as oxidative inhibitors. Dust is generated during the process of peeling the coffee bean. The etheric, alcoholic and aqueous extracts of coffee dust were obtained by sequential extraction, and the effect of these solvents (ether, alcohol and water) on the

RIASSUNTO

È stata focalizzata l'attenzione sugli antiossidanti naturali presenti nei sottoprodotti dell'industria del caffè, in quanto correlati alla riduzione dello stress ossidativo. Gli acidi fenolici presenti nelle fave di caffè giocano, inoltre, un ruolo importante quali inibitori dell'ossidazione. La polvere viene prodotta durante il processo di decorticazione delle fave di caffè. Gli estratti eterei, alcolici e acquosi della polvere di caffè sono stati ottenuti per estrazioni successive ed è stato studiato l'effetto di

- Key words: antioxidants, coffee (*Coffea arabica* L.), phenolic acids, residues -

total phenolic content and total antioxidant activity (TAA) was studied. The concentration of phenolic compounds ranged from 288.64 to 424.48 mg and is expressed as gallic acid equivalents (GAE), according to the Folin-Ciocalteau method. The TAA was investigated by the β-carotene/linoleic acid system and the ABTS method, which quantifies the quenching of this radical spectrophotometrically. By using this method, the Trolox Equivalent Antioxidant Capacity values for etheric, alcoholic and aqueous extracts were 735.27, 235.57 and 121.06, respectively. The quantification in free (FPA), esterified (EPA) and insoluble-bound phenolic acids (IPA) were 4,620.94, 2,013.26 and 2,796.56 mg expressed as gallic acid equivalents (GAE), respectively. The identification by gas chromatography showed that salicylic acid was the main component with a concentration higher than 90% in all phenolic acid fractions of the dust. A high correlation between the content of phenolic compounds and the antioxidant index was found. The order of antioxidant activity (TEAC) in different solvents was etheric > alcoholic > aqueous. These data indicate that the coffee residues are an excellent source of natural antioxidants.

questi solventi (etere, alcool ed acqua) sul contenuto fenolico totale e sull'attività antiossidante totale (TAA). La concentrazione dei composti fenolici variava da 288,64 a 424,48 mg, espressa come equivalenti acido gallico (GAE), in accordo con il metodo Folin-Ciocalteau. L'attività antiossidante totale è stata determinata col sistema β-carotene/acido linoleico e il metodo ABTS, che quantifica spettrofotometricamente l'inattivazione di questo radicale. Utilizzando tale metodo, i valori per la capacità antiossidante in equivalenti di Trolox, per gli estratti etereo, alcolico ed acquoso erano rispettivamente pari a 735,27, 235,57 e 121,06. Gli acidi fenolici liberi (FPA), esterificati (EPA) e insolubili in forma polimerica (IPA) erano rispettivamente 4.620,94, 2.013,26 e 2.796,56 mg, espressi come equivalenti di acido gallico (GAE). L'analisi gas-cromatografica mostrava che l'acido salicilico era il componente principale, con concentrazioni superiori al 90% in tutte le frazioni fenoliche della polvere di caffè. È stata riscontrata un'alta correlazione tra il contenuto in composti fenolici e l'indice antiossidante. L'ordine per l'attività antiossidante (TEAC) nei differenti solventi era etere > alcool > acqua. Questi dati indicano come i residui del caffè siano una sorgente eccellente di antiossidanti naturali.

INTRODUCTION

Plant-food processing generates by-products that are rich sources of functional compounds, including phenolic acids (SCHIEBER *et al.*, 2001). The availability of phenolic compounds from agricultural and industrial residues, their extraction and antioxidant activity were objects of a review by MOURE *et al.* (2001). Brazil is the world's largest coffee producer and is becoming a significant player in the specialty coffee industry. It produces around 25% of the world's supply of coffee, eighty percent of which is Arabica. The vast majority of coffees are still processed via the dry method since Brazil is one of the few countries in the world that has the appropriate weather to do so successfully (COFFEERESEARCH, 2005). The objective of the dry method is remove the shell, pulp and seed mu-

cilagens. The bean shell is taken out by rotatory cylinder attrition. In sequence the residues made of dry pulp and mucilagens are removed and represent the dust. Presently, there is no use for the residues of the coffee industry, and the high cost of collecting, transporting and storing them makes their utilization not viable. Dust is generated during the standard process of producing peeled coffee. Every three hundred thousand bags, containing 60 kg of coffee beans per bag, produce, approximately 25 tons of dust per month. This residue has been used as fertilizer and animal feed (SANTA CLARA, 2004).

Phenolic acids, substances that belong to the group of phenolic compounds, are classified into two groups. In the first group are the hydroxy benzoic acids, which are the simplest found in nature. To the second group belong the cinnamic acids; coumarins belong to this group and are lactones of *o*-hydroxycinnamic acids. The phenolic acids are present as three different forms in plants: free, esterified and insoluble-bound (KRYGIER *et al.*, 1982).

Coffee is the major source of chlorogenic acid in the human diet; the daily intake by coffee drinkers ranges from 0.5 to 1 gram, whereas coffee abstainers typically ingest < 100 mg/day (CASTILLO *et al.*, 2002).

The antioxidant properties of coffee beverages and roasted coffee have been widely studied in model systems. However, studies on the free radical scavenging activity of coffee residues are still needed in view of their potential use due to their high content of bioactive compounds (YEN *et al.*, 2005).

Recently, there has been considerable interest in finding natural antioxidants from plant material to replace synthetic ones. Natural antioxidant substances are presumed to be safe since they occur in plant foods; typical compounds that exhibit antioxidant activity include vitamins, carotenoids and phenolic compounds and can be recognized as GRAS substances. In the case of phenolics, their ability to act as antioxidants depends on the redox properties of their phenolic hydroxyl groups that allow them to act as reducing agents, hydrogen-donating antioxidants, and oxygen quenchers (CHANWITHEESUK *et al.*, 2005). However, studies considering the toxicological and physiological aspects of phenolic acids are necessary to assess the dose versus effective response.

Thus, not only does the importance of conducting studies on coffee residues lie on their chemical characterization but also on the possibility of linking the chemical contents with particular functional properties (YEN *et al.*, 2005).

The objectives of this study were to determine total phenolics in the dust of coffee extracted with different solvents, to identify active phenolic acids in the dust fractions (free, esterified and insoluble-bound) by gas chromatography, and to evaluate the total antioxidant activity using the ABTS radical cation decolourization assay. The β-carotene/linoleic acid system was also used to evaluate the antioxidant activity in hydrophilic extracts obtained with alcohol and water.

The ABTS method was chosen for phenolic acid fractions because it is simple, fast, highly reproducible, uses a regular visible-UV spectrophotometer, and is also useful for determining the activity of both hydrophilic and lipophilic species, thus covering a wider range of possible applications (SACCHETTI *et al.*, 2005).

MATERIALS AND METHODS

Materials

The samples of coffee dust were given by the Cooperative of Coffee Producers Cooxupé (Guaxupé - MG), from Brazil. The residue is a result of the dry processing of coffee beans.

Chemicals

Folin and Ciocalteau phenol reagent, ABTS [2,2´-Azino-bis – (3-ethylbenzothiazoneline-6-sulfonic acid)] diammonium salt, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and standards including methyl heptadecanoate, salicylic acid, protocatechuic acid, chlorogenic acid, ellagic acid, cinnamic acid, vanillic acid, *o*-coumaric acid, gentisic acid, quinic acid, *p*-coumaric acid, gallic acid, ferulic acid, caffeic acid and synapic acid were purchased from Sigma-Aldrich Co (St. Louis, MO, USA).

Dry matter

Approximately 10 g of coffee dust were passed through a 60 mesh sieve. The dry matter content of the coffee dust was determined on three samples of 3 g each in a drying oven at 105°C overnight until constant weight; this methodology was used only to determine the dry weight. The dried matter was weighed and the yield was calculated based on the wet weight of the samples. The results were used to express the total phenolic content and the antioxidant activity with respect to dry matter.

Extraction

Etheric, alcoholic and aqueous extracts of coffee dust were obtained in sequential extraction form using 20 g of powder dust, according to MOREIRA and MANCINI-FILHO (2003).

Extraction was applied by shaking flasks with 20 g of sample and 100 mL of ethyl ether for 1 hour at room temperature (25°C±2). The solutions were filtered through filter paper and the volume was made up to 100 mL by adding ethyl ether. The residue in the filter paper was dried at room temperature and in a drying oven at 60°C and used to obtain the other extracts with ethanol and distilled water, following the same procedure used for the first extract. The whole process was completed under dim light to minimize light induced degradation of phenolics, which are generally light sensitive.

Determination of total phenolic compounds

The concentrations of phenolic compounds in the extracts of coffee dust, expressed as gallic acid equivalents (GAEs), were measured according to the method of Folin-Ciocalteau, with some modifications (BUDINI *et al.*, 1980). An aliquot of 100 μL of sample was mixed with 500 μL of Folin and Ciocalteau phenol reagent. After 3 minutes, 1.5 mL of a saturated solution of Na_2CO_3 was added to the mixture and made up to 10 mL by adding distilled water. The reaction was kept in the dark for 2 hours, after which its absorbance was read at 764 nm. A calibration curve was constructed with different concentrations of gallic acid as standard.

Determination of antioxidant activity by ABTS decolourization assay

ABTS [2,2´-Azino-bis – (3-ethylbenzothiazoneline-6-sulfonic acid)] diammonium salt was used as the free radical provider and was generated by reacting this compound (7.4 mM) with potassium persulphate (2.45 mM) overnight (RE *et al.*, 1999). The solution was diluted with ethanol to yield an absorbance of 0.70±0.02 at 734 nm.

After six minutes of incubation in cuvettes containing 980 μL $ABTS^{\bullet+}$ solution and 20 μL of dust extracts, measurements were carried out to quantify the decrease of absorption using a Hewlett-Packard 8425A spectrophotometer (Palo Alto, CA, USA). Ethanol was used as blank. The standard curve was prepared using a similar volume of Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), an analogue of vitamin E. In the presence of color reduction, the percentage inhibition of oxidation was

calculated for each sample relative to a blank absorbance and was plotted as a function of Trolox concentration. The results are expressed as TEAC (Trolox Equivalent Antioxidant Capacity) per gram of organic dust (dry matter).

Determination of antioxidant activity by β-carotene/linoleic acid system

The antioxidant activity of the extracts was based on coupled oxidation of β-carotene and linoleic acid, according to the methodology described by MARCO (1968). This was subsequently expressed as an oxidation inhibition percentage, calculated in relation to the 100% oxidation that occurs in the control (no antioxidant). The antioxidant activities of the BHT and crude alcoholic and aqueous extracts were determined under the same conditions, as a means of comparison.

Attainment of phenolic acid fractions

The free, esterified and insoluble-bound phenolic acid fractions (Fig. 1)

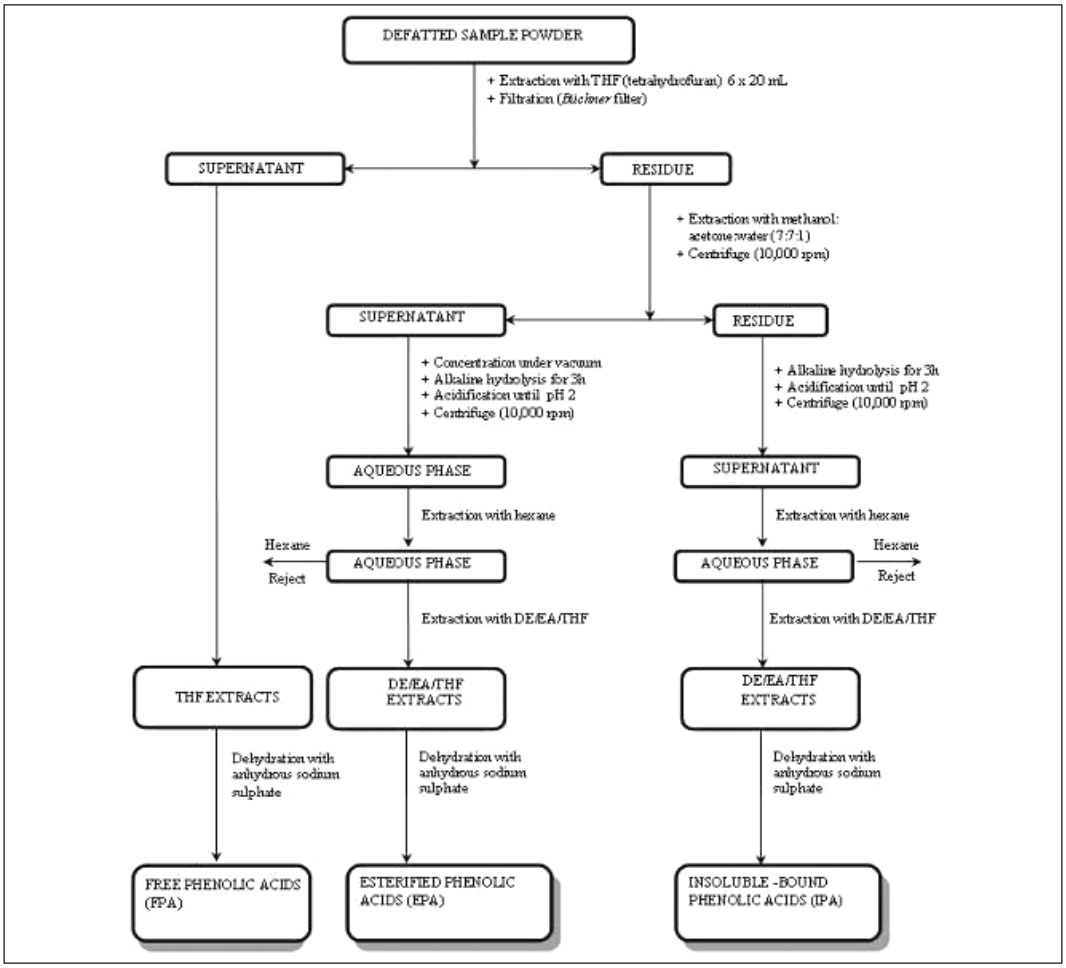


Fig. 1 - Extraction of phenolic acid fractions - free phenolic acids, esterified phenolic acids and insoluble-bound phenolic acids (SOTERO, 2002).

were obtained following the method described by KRYGIER *et al.* (1982), with some modifications (SOTERO, 2002).

Extraction of free phenolic acids

One gram of sample powder was submitted to delipidation with ethyl ether in Soxhlet; this sample was extracted six times with 20 mL tetrahydrofuran and mixed for 5 minutes in a vortex at room temperature (±23°C). The resulting supernatant of the extractions was filtered and dehydrated with anhydrous sodium sulphate. The fraction was concentrated in a rotary vacuum evaporator (Fisatom 802, São Paulo, Brasil), at 40°C and diluted in 5 mL tetrahydrofuran. The extract containing the free phenolic acid fractions was stored under refrigeration and nitrogen atmosphere.

Extraction of esterified phenolic acids

The residue from the extraction of free phenolic acids was used for new processes of extraction. The soluble phenolics of the sample were extracted six times with 20 mL methanol:acetone:water solution (7:7:6). The sample was shaken for 5 minutes and centrifuged. At the end of the extraction, the supernatant was evaporated under vacuum, at 40°C, until aqueous phase. To release the soluble esters, which were esterified with proteins and polypeptides, an equal volume of 4N sodium hydroxide was added (DABROWSKI and SOSULKI, 1984). After three hours of hydrolysis at room temperature (±23°C) in the dark and nitrogen atmosphere, the pH was adjusted to 2 with 6N HCl, followed by centrifugation at 10,000 rpm for 10 minutes. The supernatant was transferred to a separatory funnel and extracted with hexane (1:1), to eliminate free fatty acids and other contaminants, and also to extract the phenolics from the aqueous phase. Twenty mL of diethyl ether:ethyl acetate: tetrahydrofuran solution (1:1:1) was also added six times and under agitation for 5 min. The extracted fraction was filtered and dehydrated with anhydrous sodium sulphate and the solvent was eliminated in a rotary vacuum evaporator at 40°C and made up to 5 mL with tetrahydrofuran. The storage followed the process described in the methodology of DABROWSKI and SOSULSKI (1984).

Extraction of insoluble-bound phenolic acids

The residue from the extraction of soluble phenolics was hydrolyzed with 25 mL of 4N NaOH, for 3 hours, at room temperature (±23°C) in the dark and nitrogen atmosphere. The insoluble-bound phenolic acid extract was acidified until pH 2 using 6N HCl, followed by centrifugation at 10,000 rpm for 10 minutes. From this point, the procedure was the same as that used for the soluble esters filtrate of coffee dust (DABROWSKI and SOSULSKI, 1984).

Identification of phenolic acids by gas chromatography

One-tenth mL of internal standard (methyl heptadecanoate - 1.5 mg/mL) was added to 0.5 mL of each fraction. The sample was derivatized by 0.2 mL N,0-Bis (trimethylsilyl) acetamide (BSA) heated at 60°C for 30 minutes, and after cooling to room temperature, was injected in a Shimadzu CG 17A chromatograph, equipped with a flame ionization detector. The identification of phenolics was based on matching retention times of standards. The chromatographic conditions followed the procedures described by DABROWSKI and SOSULSKI (1984) with modifications made by MOREIRA and MANCINI FILHO (2003).

A DB5 semi polar column (25 m x 0.25 mm, J & W®) was used; operating conditions were as follows: initial temperature of the column 150°C, isother-

mic for 3 minutes; from 150° to 300°C at a rate of 5°C/min; isothermic 300°C for 3 minutes. The temperature of the injection port was 250°C and of detector was 300°C.

A standard solution was prepared diluting the 18 phenolic acids in methanol, and an aliquot of this solution was added to an internal standard (methyl heptadecanoate) previous to injecting them in the chromatograph.

Statistical analysis

All analyses were performed in triplicate. One-way analysis of variance (ANOVA) and Tukey multiple comparisons were carried out to test for any significant differences between the means, by using *Statistica® 6.0*. Differences between means at the 5% ($P < 0.05$) level were considered significant.

RESULTS AND DISCUSSION

Determination of total phenolic content

Data in Table 1 show that the dust extracts contain high levels of total phenolics and that the solvents used for extraction also significantly affected the total phenolic concentration. Ethyl ether was more effective in extracting phenolic compounds from coffee dust than alcohol and water, giving 424.48 mg GAE/100 g dry matter.

In a study concerning the effect of coffee processing on the total phenolic content in organic extracts, SÁNCHEZ-GONZALEZ *et al.* (2005) found values which ranged from 3.9 to 6.91 g/100 g dry matter. In the case of brewed coffee made through various procedures, the values ranged from 1.87 to 3.74 g/100 g dry matter.

Recently, CHANWITHEESUK *et al.* (2005) investigated the antioxidant activity of a large number of plants of Thailand. According to their results, the methanolic extracts of ginger (*Zingiber officinale*) and tamarind (*Tamarindus indica*) gave 60.1 and 121 mg GAE/100 g, respectively. These values are considerably lower than the content of phenolics determined in the dust extracts.

Table 1 shows that the concentration of the phenolic compounds is related to the antioxidant activity measured by both ABTS and β-carotene/linoleic acid. The TAA of coffee dust extracts was affected by the extraction solvents ($P< 0.05$). Their values ranged from 121.02 to 735.27 μM/g dry matter. The order of activity of the dust extracts of coffee was: etheric > alcoholic > aqueous.

As also seen from the results present-

Table 1 - Total phenolics and antioxidant activity of coffee dust extracts.

Extracts	Total phenolics (GAE[1]mg/100g±SD)	ABTS (TEAC[2]μM/g±SD)	β-carotene/linoleic acid inhibition (%)
Aqueous	288.64±7.16[a]	121.06±2.24[a]	40.0
Alcoholic	358.49±8.28[b]	235.57±7.29[b]	84.0
Etheric	424.48±9.10[c]	735.27±7.7[c]	nd

[1] GAE: Gallic Acid Equivalent in mg per 100 mg (dry matter).
[2] TEAC: Trolex Equivalent Antioxidant Capacity in μm per g (dry matter).
[3] % inhibitor of the β-carotene/linoleic acid system.
Values are the means±standard deviation of triplicate determinations.
Different letters in the same column indicate significant differences ($P<0.05$) by Tukey' s test among the samples.
nd: not determined.

ed in Table 2, the dust fractions (FPA, EPA and IPA) had a very high content of phenolics, ranging from 2,013.26 to 4,620.94 mg GAE/100 g dry matter. The free phenolic acid fraction of dust had the highest value, which is twice as high as the content of polyphenols in salvia leaves - 2,260 mg GAE/100 g (MILIAUSKAS *et al.*, 2004). These results show that coffee dust is a good source of phenolic compounds.

Concerning the significant amount of phenolic compounds present in the coffee dust extracts and fractions, it was reasonable to evaluate the antioxidant activity of these residues.

Determination of antioxidant activity by ABTS decolourization assay

The antioxidant capacity of brewed coffee was studied by SÁNCHEZ-GONZÁLES *et al.* (2005). They found the highest antioxidant activity in the Italian method of preparation (117 μM Trolox/g); this value is lower than the results obtained in the present study for the aqueous extract of dust (121.06 μM Trolox/g).

There was a high correlation between the estimated polyphenol content and the radical scavenging activity (r: 0.9585, $P < 0.05$). It would therefore appear that the level of antioxidant activity measured by the *in vitro* testing method is determined mainly by the polyphenol components of the coffee dust extracts (Fig. 2).

As expected, the phenolic acid fractions gave excellent performance as radical scavengers, due to their composition in isolated phenolics. Their values also increased with the total phenolic content, and the best antioxidant was the free phenolic acid fraction, giving 1,743.98 μM Trolox/g (Table 2).

Determination of antioxidant activity by the β-carotene/linoleic acid system

The β-carotene/linoleic acid system is a good method to evaluate the antioxidant activity in polar solvents. The inhibition of oxidation shows that the aqueous extract of coffee dust inhibited 40% of the oxidation. The alcoholic extract of the dust gave 80% inhibition. These results are correlated with the phenolic concentration, 288.64 and 358.49 mg, respectively. It was not possible to measure the antioxidant activity of the etheric extract of the dust with the β-carotene/linoleic acid system.

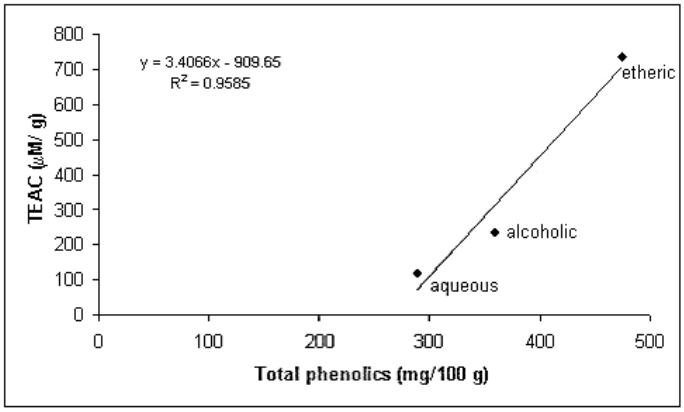


Fig. 2 - Correlation between antioxidant activity and total phenolics of aqueous, alcoholic and etheric extracts of coffee dust.

Table 2 - Total phenolics and antioxidant activity of coffee dust phenolic acid fractions.

Fractions	Total phenolics (GAE[1] mg/100 g±SD)	ABTS (TEAC[2] µM/g±SD)
FPA	4,620.94±8.56[a]	1,743.98±3.33[a]
EPA	2,013.26±10.49[b]	926.68±6.74[b]
IPA Etheric	2,796.56±9.10[c]	926.68±6.74[c]

[1] GAE: Gallic Acid Equivalent in mg per 100 mg (dry matter).
[2] TEAC: Trolox Equivalent Antioxidant Capacity in µm per g (dry matter).
FPA: free phenolic acids; EPA: esterified phenolic acids; IPA: insoluble-bound phenolic acids.
Values are the means±standard deviation of triplicate determinations.
Different letters in the same column indicate significant differences (P<0.05) by Tukey' s test among the samples.

Identification of phenolic acids by gas chromatography

Table 3 reports all of the phenolic acids in the dust fractions identified by comparison with the retention times of the standards. Salicylic acid was predominant in all the fractions, giving values over 90%.

Caffeic acid has been reported to be the most abundant phenolic compound in coffee brew, and it is endowed with strong antioxidant activity *in vitro* and *in vivo* (NATELLA *et al.*, 2002). All dust fractions had caffeic acid, with concentrations (0.12; 1.77 and 1.04 for FPA, EPA and IPA, respectively) less than only the salicylic and gentisic acids.

SROKA and CISOWSKI (2003) investigated the anti radical activity of some phenolic acids with the DPPH• method. The results showed that the strongest antioxidant scavenging of DPPH• radical activity was exhibited by gallic acid, with three hydroxyl groups bonded to the aromatic ring in an *ortho* position in relation to each other. Phenolic acids with two hydroxyl groups bonded to the aromatic ring in the *ortho* position, caffeic and protocatechuic acids, showed strong antioxidant and anti-radical activ-

Table 3 - Phenolic compound composition (%) in the coffee dust fractions.

Phenolic Acids	FPA	EPA	IPA
Salicylic	98.82±0.45	94.41±0.46	95.48±1.55
Protocatequinic	0.36±0.16	0.19±0.00	_
Chlorogenic acid	0.35±0.00	0.24±0.01	_
Ellagic	0.77±0.00	0.46±0.12	_
Cinnamic	0.09±0.00	_	_
Vanillic	0.07±0.00	_	_
o-Coumaric	0.21±0.00	_	_
Gentisic	0.26±0.00	0.87±0.02	2.52±0.14
Quinic	_	_	0.21±0.01
p-Coumaric acid	0.07±0.00	_	0.18±0.02
Gallic	_	_	0.21±0.02
Ferulic acid	0.13±0.00	0.77±0.03	0.21±0.01
Caffeic acid	0.12±0.00	1.77±0.09	1.04±0.06
Synaptic	0.13±0.00	0.5±0.02	1.02±0.05

FPA: free phenolic acids; EPA: esterified phenolic acids; IPA: insoluble-bound phenolic acids; respectively.
Values are the means±standard deviation of triplicate determinations.
_ trace levels.

ity. Compounds with one hydroxyl group such as salicylic acid exhibited the lowest anti-radical activity. The results obtained showed that the anti-radical activity of phenolic acids correlated positively with the number of hydroxyl groups bonded to the aromatic ring. Concerning the data presented in this study, it becomes clear that, despite the high concentration of salicylic acid in all fractions, it was not responsible for the antioxidant activity.

Other phenolic acids present in low concentrations in the coffee dust fractions can be determinant for the antioxidant activity. The phenolic and cinnamic acids present in soy grains, defatted soy flour and soy protein isolate were investigated by SOARES (2002). Four phenolic acids presented significant antioxidant activity - chlorogenic acid (higher content and activity), caffeic acid, *p*-coumaric acid and ferullic acid.

Some studies have shown that bioactive compounds such as chlorogenic acid, caffein, caffeic acid, nicotinic acid, trigonelline, and 5-(hydroxymethyl) furfuraldehyde are present in coffee (TAMURA and SHIBAMOTO, 1991; MARTIN *et al.*, 1998; KY *et al.*, 2001).

In the present study, most of the phenolic acids were detected in free form in coffee dust. It is also interesting to note that gallic and quinic acids, which were present in the insoluble-bound form, were present at trace levels in the free and esterified forms in the dust fractions.

The esterified phenolic acid (EPA) fraction gave the highest content in caffeic acid (1.77%), as can be seen in Table 3. However, its antioxidant activity was lower than the other fractions (926.68 μM Trolox/g). These findings suggest that an individual evaluation of the antioxidant activity of each compound would be interesting in order to ensure their real contribution to the total radical scavenging capacity of coffee dust.

CONCLUSIONS

The results obtained demonstrate that the etheric extract of coffee dust gave a significant antioxidant activity, which increased with the total phenolic content of the extracts and fractions. Among the phenolic acids identified in the dust fractions, salicylic acid was predominant, although its presence does not seem to be responsible for the antioxidant capacity of this residue. Besides knowledge of potential antioxidant activity of the food phenolic compounds, the study of their action *in vivo* is extremely important.

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SHORT COMMUNICATION

STUDY OF PHENOLIC COMPOUNDS IN ALEATICO GRAPES DRIED IN A FORCED AIR TUNNEL

STUDIO DEI COMPOSTI POLIFENOLICI IN UVE ALEATICO APPASSITE IN UN TUNNEL AD ARIA FORZATA

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ABSTRACT

Grape drying is a technology used in the production of dessert wines. In this study the phenolic compounds were determined in Aleatico grapes dried in a forced air tunnel. In this system temperature and relative humidity are accurately controlled. The results showed that there was no reduction in the polyphenol content. So this technology opens a new prospect for producing a dessert wine from Aleatico grapes without altering the phe-

RIASSUNTO

L'appassimento delle uve è una tecnica che viene utilizzata per la produzione dei vini da dessert. Il presente lavoro ha studiato i composti polifenolici in uve di Aleatico sottoposte ad una tecnica di disidratazione artificiale, che permette il controllo accurato di tutti i parametri ambientali e in particolare della temperatura e dell'umidità relativa. I risultati ottenuti sono piuttosto interessanti in quanto non si è assistito al calo previsto della concentrazione poli-

- Key words: anthocyanins, drying system, grapes, HPLC, polyphenols -

nolic compounds which are extremely important for organoleptic characteristics.

fenolica. Pertanto, con l'ausilio di questa tecnica, si aspira alla produzione di un vino Aleatico che realizzi un connubio tra tradizione ed innovazione, cercando di conservare più inalterata possibile la sua componente polifenolica che influenza in modo determinante le caratteristiche organolettiche.

INTRODUCTION

The Aleatico grape variety produces a small cluster of vermilion grape, which has a transparent skin and is very rich in bloom, giving rise to its opaque black appearance. This grape is well acclimated to the hills surrounding Bolsena Lake in the region of Lazio, Italy, where "Aleatico di Gradoli" wine, in liqueur and stock typology is produced. The wine received the Controlled Denomination of Origin (DOC), with D.P.R. in 1972. "Aleatico di Gradoli" DOC is a typical product obtained from an autochthonous variety. Current market trends are directed towards more characteristic wines.

Grape drying is a technology used in the production of dessert wines. Dried grapes have a higher sugar concentration which gives a wine that is richer in "flavour", extractive substances and glycerine, conferring body and aromatic qualities to the wines (FERRARINI, 1982). Natural sun-drying is still the most common method used to produce dessert wines. In this system ripe grapes are exposed to the sun on racks. It is usually used in the sunny southern regions. Considerable loss may occur during natural sun-drying due to various factors, such as rodents, birds, insects, rain and microorganisms. The quality of the dried product may also be significantly lower. In the sunless drying method, grapes are placed in pans or on racks in ventilated rooms. This method is usually used in the colder northern regions (DI STEFANO *et al.*, 1995).

An alternative to the traditional techniques which could help overcome some of these problems is the use of a dehydration tunnel. This is an innovative drying system based on an air speed of 1 m/sec which allows the temperature and relative humidity (R.H.) to be controlled (BELLINCONTRO *et al.*, 2004). Dehydration time and intensity affect the qualitative characteristics of grapes (TONUTTI and MENCARELLI, 2005) such as sugars, tartaric and malic acids (CANCELLIER and MICHELET, 1994). Little information has been reported on the effects of drying on the secondary metabolic compounds, such as polyphenols and terpenes.

DI STEFANO *et al.* (1995) reported a decrease in polyphenol concentration in the skins and juice caused by oxidative phenomena due to high temperature (Di STEFANO *et al.*, 1997; 2001; DI STEFANO and MAGGIOROTTO, 1995; BORSA and DI STEFANO, 2000). Temperature influences the enzymatic systems and therefore it can compromise, preservation of aromatic and phenolic compounds during dehydration. Recently a new technology for grape drying has been used (BELLINCONTRO *et al.*, 2002; FRANGIPANE *et al.*, 2005) This system permits grapes to be dehydrated as it maintains lower and more constant temperatures than those regularly used.

This study was undertaken to deter-

mine if the wine produced from Aleatico grapes subjected to drying maintained its varietal composition. Hence, the fate of phenolic compounds during the grape drying process was investigated.

MATERIALS AND METHODS

The tunnel for artificial dehydration was manufactured by Europanel SpA (Laives, Bozen, Italy) (patent MI2001A 002198). The pilot plant used has a capacity of 300 kg. The small tunnel is insulated and has a double roof where the evaporative coil, condenser and fans are located. The rest of the cooling plant and the control panel display are outside. During the dehydration tests the thermohygrometric atmospheric conditions were monitored with a HYGROclip model probe (Rotronic AG, Bassersdorf, Switzerland) connected to HYGROwin software to record the data. The air speed was measured by means of a Terman hot wire anemometer (LSI SpA, Milan, Italy).

Over-ripe Aleatico grapes were carefully picked and immediately placed in perforated plastic boxes (wall height 20 cm) to reach a weight of 6 kg (±500 g). The boxes were immediately placed inside the tunnel, with an air speed of 1 m/sec and the temperature and relative humidity (40%) were monitored continuously. Initially the temperature was held at 15°C and then progressively increased to 23°C in order to facilitate the dehydration process; subsequently the temperature was reduced (17°C) in order to slow down the respiration process, as weight decrease was still under 10%. This is considered the first critical point in the drying process (TONUTTI and MENCARELLI, 2005). The temperature was then increased to 30°C in order to reduce grape drying time. The experiment was carried out for thirteen days; every day clusters were weighed and samples taken for chemical analysis. The method of Di Stefano and Cravero (1991) was followed for sample extraction. Total anthocyanins and total polyphenols were determined on the skin extracts (Di Stefano *et al.*, 1989); polyphenols were also determined in the juice. The single anthocyanins of the skins were characterized by HPLC, according to the method of Ritchey and Waterhouse (1999). After filtration on Millipore filters (0.22 μm diameter) the samples were directly injected in a Dionex chromatograph (loop 20 μm) with a photodiode detector. A C18 column, 300 mm x 3.9Ø (Waters), kept at 22°C was used as stationary phase, with a mobile phase flow rate of 0.5 mL/min. Solvent A = 50 mM 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. Two wavelengths were monitored: 280 nm for phenolic acids and 520 nm for anthocyanins. Peak identification was carried out by comparison to standards as well as by comparison with standards previously characterized on the HPLC system by both relative elution times and spectral matching.

All analyses were done in triplicate and analytical results were submitted to statistical analysis. 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 dehydration process was slow, probably due to the concentration of bloom on the Aleatico berries, so on October 3, the temperature was increased to 30°C for two days. This caused a 26% weight loss (Table 1). At the same time the sugar content increased due to rise in temperature. Anthocyanins and total polyphenols increased when weight loss

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

Sampling date	Temperature (°C)	Sugars (°Brix)	Increase in sugars (%)	Weight loss (%)
24/09/2004	15.00	23.82	0.00	0.00
27/09/2004	16.80	25.30	6.21	3.21
29/09/2004	23.00	26.10	9.57	6.84
01/10/2004	16.80	27.14	13.94	9.03
04/10/2004	30.00	30.66	28.72	21.18
06/10/2004	30.00	31.86	33.75	26.48

was approximately 7% (Table 2). This increase could be due to phenolic induction due to water stress as confirmed by other studies (BELLINCONTRO *et al.*, 2004; Tonutti and Mencarelli, 2005).

Subsequent dehydration and final thermal shock induced a significant oxidative stress which brought anthocyanins and total polyphenols to their initial values (FRANK *et al.*, 2005). The concentrations are expressed as mg/1000 berries in order to compare the treatment to the number of berries and not to the weight (PAPUCCI *et al.*, 2000).

In Table 3 the evolution of some phenolic acids in juice and skins is reported. The results show that gallic and caffeic acids are present in the juice; generally they are mainly found in the skins. Trans-caftaric acid values in the juice remained almost constant until approximately midway through the drying proc-

Table 2 - Mean values of anthocyanins and total polyphenols in skins and juice of grapes during the drying process.

Sampling date	Anthocyanins (mg malvin/1000 berries)	Total Polyphenols (mg gallic ac. /1000 berries)	
	skin	skin	juice
24/09/2004	1,085±*54*	2,706±*104*	178±*8.90*
27/09/2004	1,215±*61*	2,800±*140*	178±*8.70*
29/09/2004	1,285±*64*	3,070±*153*	178±*8.80*
01/10/2004	1,160±*58*	2,942±*147*	169±*8.45*
04/10/2004	1,135±*57*	2,751±*137*	145±*7.25*
06/10/2004	1,036±*52*	2,615±*138*	142±*7.10*

Table 3 - Evolution of phenolic acids (mg/L) in skins and juice of grapes during the drying process.

	24/09/2004		29/09/2004		06/10/2004	
Phenolic acids	juice	skin	juice	skin	juice	skin
Trans-caftaric acid	19.58±*0.979*	17.34±*0.868*	20.81±*1.041*	30.13±*1.505*	63.47±*3.173*	16.52±*0.826*
Syringic acid	0.08±*0.004*	0.70±*0.035*	0.12±*0.005*	0.86±*0.043*	0.05±*0.003*	0.5±*0.025*
Gallic acid	3.02±*0.151*	-	2.98±*0.148*	-	0.50±*0.012*	-
Caffeic acid	0.30±*0.015*	-	0.19±*0.010*	-	0.66±*0.033*	-

ess, then its concentration tripled at the end of the process. Trans-caftaric acid in the skins assumed a slightly increasing trend until approximately midway through the drying, and then it came back to the initial values. Syringic and caffeic acids however did not change in either the juice or the skins. Finally the amount of gallic acid in the juice decreased during the drying process. For anthocyanins determined by HPLC (Fig. 1) the same trend of polyphenols was ob-

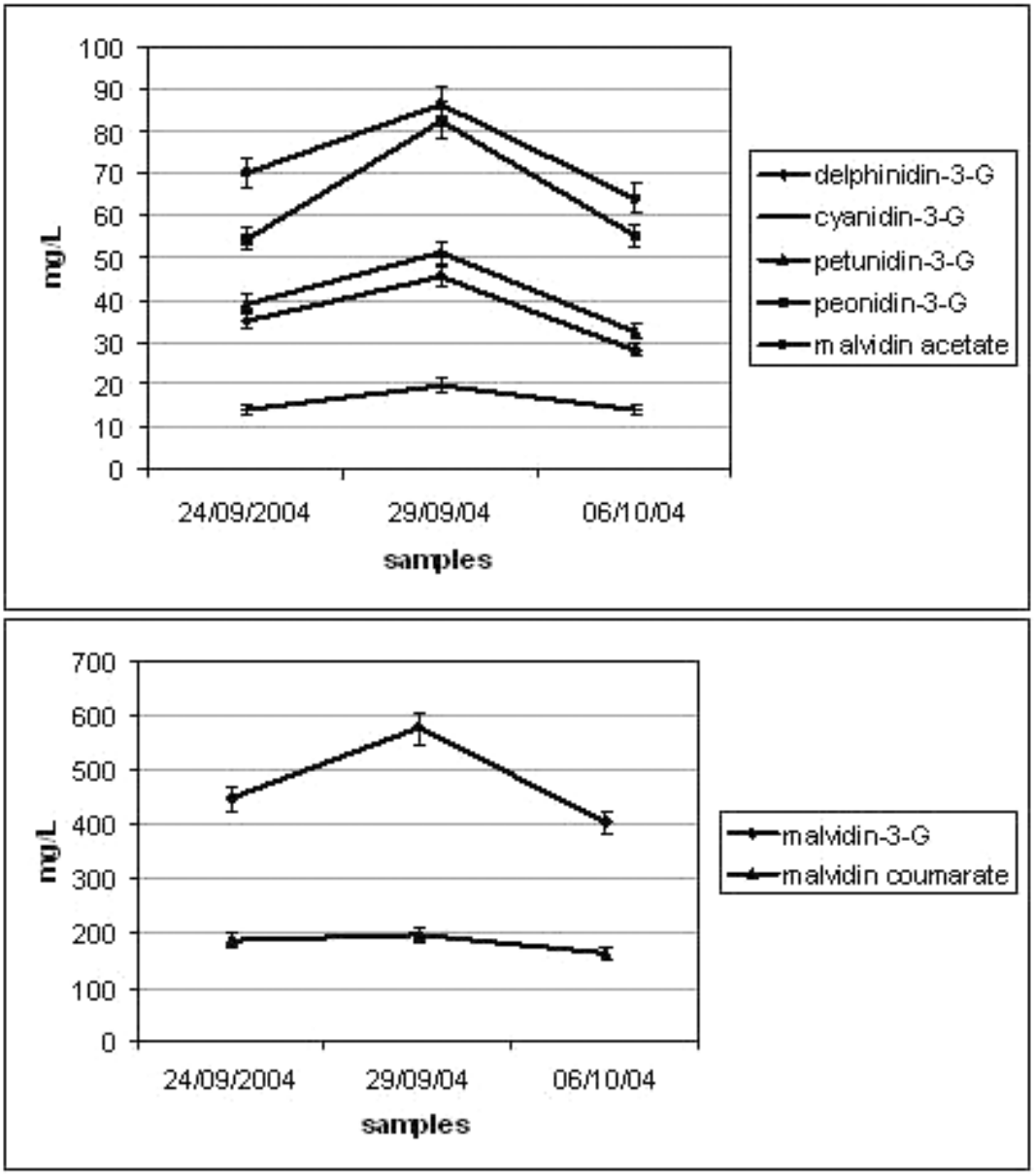


Fig. 1 - Evolution of skin anthocyanins during the drying process.

served. The percentage of each anthocyanin however was unchanged.

In conclusion, the study carried out on the Aleatico grapes dried with a new technology showed that in a short time the sugar concentration can increase to approximately 34%. Furthermore the phenolic concentration remained unchanged. Moreover oxidation that can take place with the traditional drying techniques is minimized. So drying grapes in a forced air tunnel can be a valid alternative to traditional drying methods and the grapes maintain an anti-oxidant power that will be able to guarantee the production of wine with good organoleptic characteristics.

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SHORT COMMUNICATION

DEVELOPMENT OF MEATBALLS FROM FINFISH, PRAWNS AND MOLLUSKS: QUALITY AND SENSORY ANALYSIS

ANALISI QUALITATIVA E SENSORIALE DI POLPETTE DI PESCE, GAMBERI E GASTROPODI

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ABSTRACT

Meatballs, a value-added product made from fish (*Scombfroidfs tol)*, prawn (*Penaeus semisulcatus*) and a gastropod (*Chicoreus ramosus*) were evaluated for their quality during storage. The protein content of the meatballs was higher than the raw meat. The FFA, TMA-N and TVB-N values of the meatballs increased slightly during storage, but the increase was within acceptable limits. The total plate count of bacteria varied in meatballs, but no pathogenic bacteria were found.

RIASSUNTO

È stata valutata la qualità durante lo stoccaggio di prodotti alimentari quali: polpette di pesce (*Scombfroidfs tol)*, gamberi (*Penaeus semisulcatus*) e gastropodi (*Chicoreus ramosus*). Il contenuto proteico delle polpette di carne era più alto rispetto a quello della carne cruda. I valori di acidi grassi liberi, di trimetilammina e di Azoto Basico Volatile Totale delle polpette aumentava leggermente durante lo stoccaggio, restando comunque entro limiti accettabili. Nelle polpette la conta totale di bat-

- Key words: meatballs, physico–chemical properties, sensory analysis -

The taste, texture and appearance and long shelf-life under frozen storage were rated good.

teri risultava aumentare, ma non sono stati ritrovati batteri patogeni. Il gusto, la consistenza, l'aspetto e la lunga conservazione per congelamento, sono risultati ottimali.

INTRODUCTION

Human consumption of seafood chiefly consists of finfish, followed by crustaceans and mollusks. Seafood is an excellent source of animal protein, vitamins and minerals. Fish represents about 14% of all animal protein and about 5% of the total protein consumed on a global basis (SOMVANSHI, 1998). It is estimated that 60% of the people in developing countries obtain 40-100% of animal protein from fish (CLUCAS and WARD, 1996).

The dietary habits of people are changing rapidly due to improved socio-economic status, the availability of new food resources and of enriched, prepared foods for customer convenience. Present market trends indicate an increased demand for ready-to-serve or ready-to-cook convenience products made from a variety of fish and shell fish (JAYACHANDRAN *et al.*, 1988). Value-added means any additional process that changes the nature and presentation of the product and that adds sale value. Rapid industrialization and urbanization provide ample incentive for the development of such products in India. However, ready-to-eat products of reasonable shelf-life are not common. Systematic studies on their nutritional quality and storage characteristics are required before popularizing such products (NURAL HUDA *et al.*, 2001). In the present study meatballs were developed using the meat of finfish, crustaceans and mollusks and their quality and shelf-life were assessed.

MATERIALS AND METHODS

Meatballs were prepared using three different sea foods including needle scaled queen fish (*Scombfroidfs tol),* tiger prawn (*Penaeus semisulcatus)* and a gastropod (*Chicoreus ramosus*). One kilogram of finfish and prawns were bought at the marine landing and taken to the laboratory in an icebox. They were cleaned and the edible portions were separated out. The edible parts were washed in potable water, minced in a Waring blender and used to make meatballs. For the gastropods, the shell was broken, the soft body removed and the edible parts, foot and adductor muscle were cut and separated. The edible meat, washed meticulously two to three times in potable water, was cut into small pieces and dried in a mechanical drier for 48 h at 50°-60°C and ground into a powder using a pulverizer.

Preparation of meatballs

Three different seafood meatballs were prepared using the minced meat from the finfish and prawns and the dried, powdered gastropod meat. The ingredients are given in Table 1. The onions were cleaned, chopped into small pieces and fried in oil until golden brown. The sesame seeds were fried in a pan over low heat until they burst; they were then removed and cooled. The minced finfish and prawn meat, and the dried meat powder were thoroughly mixed with corn flour, curry powder, pepper powder, fried onions and sesame seeds. Finally

Table 1 - Ingredients used for making meatballs.

Ingredients	Quantity
Minced meat of prawn or finfish or dried and powdered gastropod meat	500 g
Corn flour	175 g
Sesame seeds	16 g
Big onions	n=5
Curry powder	3.5 g
Pepper powder	3.5 g
Ascorbic acid	0.166 g
Sodium benzoate	0.166 g
Salt	9 g

the preservatives, ascorbic acid and sodium benzoate, were added and mixed. The mixture was then seasoned to taste with salt and mixed thoroughly. This mixture was then made into a dough by adding a little water and then shaped into small balls of uniform size (about 2-3 cm) by rolling sufficient amounts of the dough between one's palms.

Proximate composition, packaging and storage

The proximate composition such as carbohydrate, lipid and protein were determined. The carbohydrate content was estimated by the method of DUBOIS *et al.* (1956) and the lipid content was determined by the method of BLIGH and DYER (1959). The amount of protein present was estimated following the Biuret method (RAYMONT *et al.,* 1964).

The meatballs were wrapped with aluminum foil, packed in laminated Low Density Polyethylene (LDPE) pouches and sealed with an electric sealing machine model No. PSA 15/OB (India) and stored at -18°C in a deep freezer until used.

Shelf-life

The shelf-life was determined by analyzing the thaw and drip loss, moisture content, pH, total volatile base nitrogen (TVB-N), tri-methyl amine nitrogen (TMA-N) and free fatty acid (FFA) content.

The thaw and drip loss was estimated by thawing the meatballs in the polythene bags. The drip loss in the bags was measured using a graduated cylinder and values are expressed in milliliters. The moisture content was determined by drying the meatballs in a mechanical drier for 2 days at 50°-60°C till a constant weight was reached. For pH, 10 g of the sample was homogenized with 50 mL of distilled water and the pH was measured using a digital pH meter. The total volatile base nitrogen was estimated by the Conway micro-diffusion method. Tri-methyl amine-Nitrogen (TMA-N) was estimated using the Conway micro diffusion method of BEATTY and GIBBONS (1937). Free fatty acids were estimated by the method of KE *et al.* (1976). Microbiological analysis was done by checking the total plate count and total fungal count (APHA, 1992). Pathogens such as *E. coli, Vibrio* and *Salmonella* were enumerated by the pour plate method of the U.S. Food and Drug Administration (U.S. FDA, 1998).

Sensory evaluation

The frozen meatballs were thawed and fried in refined sunflower oil to a uniform brown colour. Sensory evaluation was done by judging the colour, texture, appearance, odour and taste. The fried meatballs were tested by a taste panel of 6 to 8 members and the overall acceptability was determined using a hedonic scale of 1 to 9 (AMERINE *et al.*, 1965) with a rating of 9 (excellent), 6 (good) and below 4 (unacceptable). The analysis was carried out weekly over a 7-week period and the changes in quality were noted.

Statistical analysis

Analysis of variance (ANOVA) was used to analyze the data to determine if there were any differences in the biochemical, microbiological and organoleptic characteristics during storage. Results are the means of three replications.

RESULTS AND DISCUSSION

The percentages of protein, carbohydrates and lipids in the meat of the three species are shown in Table 2. The nutritive value of the raw finfish meat was 24.2% protein, 6.3% carbohydrate and 6.3% lipid. The raw prawn meat contained 20.5% protein, 4.9% carbohydrate and 1.3% lipids. The gastropod meat had 14.4% protein, 5.2% carbohydrate and 1.3% lipid. The nutritive value of the meatballs was also analyzed. The finfish meatballs had 24.6% protein, 7.1% carbohydrate and 7.4% lipid, while the prawn meatballs had 20.7% protein, 5.4% carbohydrate and 7.4% lipid, and the gastropod meatballs had 14.5% protein, 5.6% carbohydrate and 5.6% lipid. The finfish had the highest protein level followed by prawns and gastropods. The nutritive value was slightly higher in the meatballs than in the raw meat which may be due to the ingredients added to make the meatballs. The protein value of finfish meatballs (24.6%) was higher than the shark fish balls (17 to 19%) of Indonesia (FAWZYA,1992) and bream fish balls (11.09-11.25%) of Thailand (YAMPRAYOON *et al.*, 1991).

During storage, the percentage moisture content increase was significant ($P<0.01$) in finfish and gastropod meatballs (Tables 3 and 4), but it was not significant in prawn meatballs (Table 5). The thaw and drip loss of finfish and prawn also increased gradually and significantly ($P<0.01$) with increased storage time. The duration and temperature of frozen storage are the most important factors that influence drip loss. Moisture loss was accompanied by greater drip loss during thawing of frozen crabs (GEORGE, 1973). Similar results have been obtained with frozen storage of other sea foods (GEORGE, 1974; RAJESWARI and HAMEED, 1998; GEORGE *et al.*, 1990).

The pH of the meatballs decreased in all three species during 49 days of frozen storage (Tables 3, 4, 5). The pH of muscles has been reported to be one of the most important factors in determining the drip loss of thawed meat (LAWRIE, 1974) and the reduction in pH is due to an increase in drip loss percentage (HAARD, 1971).

Over the 49 days of storage, the TVB-N values increased steadily for all three species as did the TMA-N values. These values were very low, indicating a high level of fish freshness and were within acceptable limits. The changes in all the parameters were significantly different ($P<0.01$) between the storage days. TVB-N and TMA-N are the most important indices of fish freshness and indicate the ammonia and other basic volatile nitrogenous compounds associated with seafood spoilage. The low levels may have been due to the addition of the preservative sodium benzoate and also the use of aluminium foil and air tight packaging which minimized moisture and gas permeation to some extent during frozen storage.

The free fatty acid levels (% oleic acid) of all three meatball types increased with storage (Tables 3, 4, 5). This may have been due to the anti-oxidative effect of ascorbic acid added during the preparation of the meatballs.

The changes in total plate counts during frozen storage of the three types

Table 2 - Percentage (%) of protein, carbohydrate and lipid in raw meat and meatballs.

Species	*Scombfroidfs tol* (Finfish)		*Penaeus semisulcatus* (Tiger prawn)		*Chicoreus ramosus* (Gastropod)	
	Raw meat	Meatballs	Raw meat	Meatballs	Raw meat	Meatballs
Protein	24.2	24.6	20.5	20.7	14.4	14.5
Carbohydrate	6.3	7.1	4.9	5.4	5.2	5.6
Lipid	6.3	7.4	1.32	7.45	1.3	5.6

Table 3 - Physicochemical characteristics of finfish, *S. tol*, meatballs.

Parameters	Storage (days)								F -value
	0	7	14	21	28	35	42	49	
pH	7.38±0.26	7.16±0.12	6.99±0.30	6.84±0.10	6.74±0.20	6.65±0.10	6.54±0.12	6.38±0.19	2.657NS
FFA (% oleic acid)	0.64±0.01	1.64±0.007	2.65±0.01	3.66±0.01	4.65±0.01	5.66±0.01	6.66±0.01	7.67±0.01	3.257*
TMA-N (mg/100 g)	2.5±0.4	2.58±0.52	2.73±0.21	2.81±0.22	2.87±0.62	3.52±0.48	3.55±0.40	3.59±0.30	3.487*
TVB-N (mg/100 g)	2.14±0.19	2.47±0.48	2.5±0.40	2.55±0.13	2.65±0.43	2.80±0.21	2.91±0.21	3.46±0.47	3.803*
Moisture (%)	58.37±0.02	59.12±0.44	59.42±0.45	59.52±0.55	59.6±0.73	60.6±0.66	61.78±0.73	63.01±0.80	8.155**
TPC (x10^2 CFU/g)	24.33±2.08	36±2.64	56.3±1.52	65±3.6	75±3	85.33±1.15	95.66±2.08	103.33±2.51	391.15**
Thaw drip (mL)	0.028±0.003	0.036±0.002	0.045±0.00	0.059±0.004	0.082±0.004	0.17±0.026	0.28±0.036	0.35±0.002	183.2**

Mean±SD; n=3; * - P<0.05; ** - P<0.01; NS - Not significant.
FFA = Free Fatty Acid; TMA-N = Tri-methyl amine-Nitrogen; TVB-N = Total volatile base nitrogen; TPC = Total Plate Count.

Table 4 - Physicochemical characteristics of gastropod, *C. ramosus*, meatballs.

Parameters	Storage (days)								F -value
	0	7	14	21	28	35	42	49	
pH	7.56±0.27	7.45±0.27	7.44±0.10	7.4±0.36	6.76±0.19	6.64±0.27	6.54±0.41	6.28±0.33	8.8743**
FFA (% oleic acid)	0.391±0.01	0.42±0.01	0.44±0.01	0.46±0.007	0.475±0.01	0.497±0.01	0.51±0.01	0.52±0.008	0.0676NS
TMA-N (mg/100 g)	2.48±0.365	2.53±0.35	2.62±0.46	3.02±0.10	3.04±0.10	3.14±0.145	3.15±0.063	3.44±0.44	3.963*
TVB-N (mg/100 g)	1.81±0.171	2.41±0.22	2.41±0.22	2.497±0.397	2.57±0.41	2.64±0.25	2.72±0.209	2.8±0.13	4.157**
Moisture (%)	61.47±0.367	62.46±0.45	62.53±0.48	62.68±0.252	62.87±0.66	63.39±0.35	63.57±0.37	64.47±0.40	12.833**
TPC (x10^2 CFU/g)	21±1	33±1	46.3±1	65.33±0.57	69±1	77.66±1.52	88.66±1.52	100.33±1.52	1421.053**
Thaw drip (mL)	0.050±0.04	0.054±0.06	0.061±0.038	0.134±0.029	0.264±0.029	0.334±0.071	0.54±0.05	0.644±0.04	2.657NS

Mean±SD; n=3; * - P<0.05; ** - P<0.01; NS - Not significant.
FFA = Free Fatty Acid; TMA-N = Tri-methyl amine–Nitrogen; TVB-N = Total volatile base nitrogen; TPC = Total Plate Counts.

Table 5 - Physicochemical characteristics of tiger prawn, *P. semisulcatus*, meatballs.

Parameters	Storage (days)								F -value
	0	7	14	21	28	35	42	49	
pH	7.91±0.93	7.34±0.49	6.76±0.56	6.67±0.52	6.63±0.487	6.61±0.31	6.49±0.13	6.32±0.27	2.657NS
FFA (% oleic acid)	0.164±0.01	0.17±0.01	0.181±0.07	0.186±0.01	0.192±0.008	0.205±0.01	0.224±0.011	0.238±0.01	0.1383NS
TMA-N (mg/100 g)	2.34±0.52	2.60±0.37	2.63±0.42	2.65±0.44	2.75±0.22	2.98±0.26	3.36±0.48	3.56±0.371	2.787*
TVB-N (mg/100 g)	1.79±0.3	2.11±0.13	2.35±0.19	2.4±0.26	2.47±0.19	2.56±0.355	2.63±0.255	2.73±0.28	4.231 **
Moisture (%)	39.42±0.389	39.53±0.939	39.56±0.52	40.88±0.69	42.16±.251	42.65±0.340	42.66±0.438	43.62±0.455	2.657NS
TPC (x10^2 CFU/g)	24.31±1.52	42±1	52±1	61±1	70±1	73±1	87±1	95±5	1399.628**
Thaw drip (mL)	0.058±0.01	0.064±0.01	0.076±0.008	0.092±0.008	0.166±0.01	0.173±0.016	0.280±0.016	0.605±0.003	151.414**

Mean±SD; n=3; * - P<0.05; ** - P<0.01; NS - Not significant.
FFA = Free Fatty Acid; TMA-N = Tri-methyl amine-Nitrogen; TVB-N = Total volatile base nitrogen; TPC = Total Plate Counts.

Table 6 - Organoleptic characteristics of finfish, *S. tol*, meatballs.

Parameters	Storage (days)								F -value
	0	7	14	21	28	35	42	49	
Appearance	8.47±0.057	8.63±0.153	8.47±0.153	8.53±0.153	8.17±0.153	8±0.2	7.97±0.153	7.87±0.153	0.01802*
Colour	8.5±0.1	8.17±0.153	8.5±0.1	8.2±0.2	8.43±0.153	8.27±0.153	8.2±0.2	7.9±0.115	0.018056*
Odour	8.43±0.153	8.17±0.153	8.17±0.153	8.23±0.153	8.03±0.153	7.93±0.115	7.83±0.208	7.83±0.153	0.016962*
Taste	8.67±0.153	8.5±0.1	8.6±0.2	8.5±0.2	8.43±0.153	8.2±0.2	8±0.2	8.03±0.153	0.018622*
Texture	8.57±0.153	8.63±0.153	8.53±0.251	8.5±0.2	8.4±0.2	8.27±0.25	8.2±0.2	8.17±0.153	0.018873*
Flavor	8.5±0.2	8.4±0.153	8.4±0.208	8.3±0.3	8.2±0.153	8.2±0.153	8±0.153	7.9±0.251	0.01789*
Overall acceptability	8.77±0.153	8.63±0.153	8.5±0.2	8.47±0.153	8.1±0.1	8.2±0.2	8.17±0.153	8±0.2	0.018556*

Mean±SD; n=3; * - P<0.05.

Table 7 - Organoleptic characteristics of tiger prawn, *P. semisulcatus*, meatballs.

Parameters	Storage (days)								F -value
	0	7	14	21	28	35	42	49	
Appearance	8.67±0.153	8.57±0.153	8.63±0.153	8.53±0.153	8.4±0.1	8.2±0.2	7.97±0.153	8.03±0.153	0.018676*
Colour	8.5±0.1	8.8±0.1	8.6±0.2	8.7±0.1	8.3±0.2	8.2±0.2	8.07±0.252	8±0.1	0.018804*
Odour	8.43±0.153	8.17±0.153	8.23±0.153	8.17±0.153	8±0.2	8±0.1	7.9±0.1	7.83±0.153	0.017038*
Taste	8.6±0.1	8.33±0.153	8.27±0.153	8.4±0.2	8.2±0.1	8±0.2	8±0.1	7.97±0.057	0.017766*
Texture	8.7±0.1	8.8±0.1	8.77±0.153	8.7±0.1	8.6±0.2	8.47±0.153	8.4±0.2	8.3±0.1	0.020018*
Flavour	8.7±0.2	8.5±0.1	8.73±0.153	8.57±0.153	8.3±0.1	8.4±0.1	8.07±0.153	8.2±0.2	0.019027*
Overall acceptability	8.87±0.058	8.77±0.153	8.67±0.153	8.6±0.1	8.1±0.1	8.5±0.2	8.23±0.208	8.27±0.208	0.019443*
Mean±SD; n=3; * - P<0.05.									

Table 8 - Organoleptic characteristics of gastropod, *C. ramosus*, meatballs.

Parameters	Storage (days)								F -value
	0	7	14	21	28	35	42	49	
Appearance	8.66±0.153	8.56±0.13	8.563±0.15	8.5±0.153	8.6±0.1	8.5±0.2	7.76±0.152	8.70±0.154	0.018867*
Colour	8.4±0.1	8.8±0.1	8.7±0.2	8.9±0.1	8.1±0.2	8.9±0.2	8.06±0.252	8.1±0.1	0.018804*
Odour	8.43±0.153	8.17±0.153	8.23±0.153	8.17±0.153	8±0.2	8±0.1	7.9±0.1	7.83±0.153	0.017148*
Taste	8.9±0.1	8.06±0.153	8.29±0.153	8.2±0.2	8.1±0.1	8.1±0.2	8.2±0.1	7.94±0.057	0.016776*
Texture	8.9±0.1	8.6±0.1	8.87±0.153	8.4±0.1	8.8±0.2	8.50±0.153	8.42±0.2	8.5\3±0.1	0.070018*
Flavor	8.9±0.2	8.7±0.1	8.83±0.153	8.59±0.153	8.2±0.1	8.01±0.1	8.09±0.153	8.5±0.2	0.019427*
Overall acceptability	8.9±0.058	8.82±0.155	8.46±0.143	8.3±0.1	8.4±0.2	8.2±0.2	8.41±0.209	8.60±0.218	0.019544*
Mean±SD; n=3; * - P<0.05.									

of meat are given in Tables 3, 4 and 5. Pathogenic bacteria, such as *E. coli, Vibrio* sp. and *Salmonella* sp., were absent in all the samples. The increase in total plate count (TPC) (CFU/g) was significant ($P<0.01$) in all three meatball types. The sanitation standard (3×10^6 CFU/g) for frozen food was used throughout the storage period to determine the plate count limits. The counts were found to be well within limits of acceptability.

Organoleptic ratings of the product during frozen storage (Tables 6, 7, 8) gradually decreased for all the characteristics, including overall acceptability and the differences were significant ($P<0.01$). The scores were always well above the acceptability level of five.

CONCLUSION

The meatballs developed using finfish, prawn and gastropod meat had good protein and carbohydrate contents with low fat. The FFA, TMA-N and TVB-N values of the meatballs increased slightly with storage, but the increase was within acceptable limits. The texture and colour of all the meatballs under frozen storage were good till the end of storage. Thus this type of product could be used as a new ready-to-cook product.

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PROTEINS AND AMINO ACIDS IN TABLE OLIVES: RELATIONSHIP TO PROCESSING AND COMMERCIAL PRESENTATION

PROTEINE E AMINOACIDI NELLE OLIVE DI TAVOLA: IL RAPPORTO CON LA LAVORAZIONE E LA FORMA COMMERCIALE

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ABSTRACT

A survey of the protein levels and amino acid composition of a variety of commercial presentations of table olives was carried out. The three main processing types (Spanish-style, directly-brined, and ripe olives) were considered. A highly significant correlation was found between the protein content calculated as the sum of the amino acids and protein content calculated from nitrogen. All presentations showed an excellent balance of total essential amino acids when compared with the FAO/

RIASSUNTO

È stata effettuata un'indagine dei livelli proteici e della composizione in aminoacidi di diverse varietà di olive da tavola commerciali. Sono stati considerati i tre processi principali (sistema Sivigliano, salamoia diretta ed olive mature). È stata osservata una correlazione altamente significativa fra il contenuto proteico calcolato come somma degli aminoacidi ed il contenuto proteico calcolato in aminoacidi essenziali totali in confronto al pattern aminoacidico della proteina modello di riferimento secon-

- Key words: amino acids, discriminant analysis, principal component analysis, protein quality, table olives -

WHO/UNU reference protein pattern; lysine was the limiting amino acid in most presentations. Data analysis by Principal Components Analysis (PCA) did not differentiate among the processing types or cultivars, but Discriminant Analysis (DA) was able to discriminate among the processing types.

do FAO/WHO/UNU; la lisina è risultata essere l'aminoacido limitante nella maggior parte delle forme commerciali. L'analisi di dati utilizzando l'analisi delle componenti principali (PCA) non ha consentito la differenziazione fra le differenti lavorazioni o fra le cultivars, ma l'analisi discriminante (DA) è stata in grado di discriminare fra le differenti lavorazioni.

INTRODUCTION

The world production of table olives reached 1,456,000 tonnes in 2001/2002 (IOOC, 2003). Table olives constitute an important part of the Mediterranean diet. Their industrial processing is restricted to only a few types or styles, each of which can be found in various commercial presentations (that is, in a great variety of products offered to the consumer). The most common are green olives (Spanish- or Sevillian-style); directly-brined olives (turning colour or naturally black); and ripe olives (Californian-style). A detailed description can be found elsewhere (GARRIDO *et al.*, 1997). The number of cultivars used for table olives is limited, and some of them can be processed in various styles. In turn, each cultivar within the same style may have several commercial presentations. The commercial presentations of green olives are numerous, and include the use of diverse stuffing materials, which may produce variations in the composition of their respective final products. In some commercial presentations of directly-brined olives, the olives are broken or cut along their longitudinal diameter and/or seasoned with natural products or their flavours. Commercial presentations of ripe olives are limited to plain (whole), pitted, and sliced olives, and, sometimes, olive paste. Therefore, in order to create an up-to-date nutrient database of table olives, it is necessary to include the widest possible range of commercial presentations.

Although the protein content of vegetables, including olives, is low, knowledge of the amino acid composition is important, particularly in the dietary treatment of patients with amino acid and protein metabolism disorders (BREMER *et al.*, 1996). It has been reported (ZAMORA *et al.*, 2001) that proteins from the olive fruit mesocarp constitute 1.3-1.8% of the dry weight of the olive fruit, and cultivar and fruit ripening do not produce important changes in mesocarp protein content or composition. Fermented (Spanish-style) green table olives have been reported (MONTAÑO *et al.*, 2005) to have a high content of total essential amino acids (EAA) when compared with the FAO/WHO/UNU (1985) reference protein pattern for adults. To date, no accurate calculation of protein quality has been done for the other two abovementioned processing types. Moreover, the diversity of commercial presentations can produce different amino acid profiles, which may, in turn, affect the protein quality.

Amino acid profiles have been widely used to differentiate or classify various foods, such as vinegar (DEL SIGNORE *et*

al., 2000), wine (ARVANITOYANNIS *et al.*, 1999), almonds (SERON *et al.*, 1998) and honey (COTTE *et al.*, 2004) using chemometric techniques like Principal Component Analysis (PCA) and Discriminant Analysis (DA).

The main objective of this work was to supply protein content and amino acid profiles for commercial presentations of the three main processing types of table olives. The results may be used by the industry for requirements of nutritional labelling or by nutritionists to estimate amino acid intakes in diets that include table olives. A further objective of this study was to determine if the amino acid composition together with chemometric methods could be used to classify table olives according to processing type and olive cultivar.

MATERIALS AND METHODS

Samples

The study was carried out on sixty-six composite samples of different commercial presentations of table olives. Each composite sample was made up of 3 to 8 units (depending on the size of the units, cans, jars, or plastic pouches) from 1-5 processing companies, and with different packing dates, depending on their availability on market shelves. Brine was separated by a procedure similar to that established in the International Standards for Table Olives Applicable in International Trade (IOOC, 1980) to determine olive net weight. Fruit containers were emptied onto a standard no. 8 circular sieve of 20 cm diameter. The olives were evenly distributed over the sieve, which was inclined at an angle of 17-20° to facilitate drainage. The product was allowed to drain for 20 minutes. Drained olives of the different commercial presentations were pitted (if appropriate). Average time from packing was about three months. Producers kindly supplied those not available in the local markets. The samples of Spanish-style green olives, directly-brined olives, and ripe olives that were analysed are reported in Tables 1, 2, and 3, respectively.

Chemical analyses

To estimate water content, aliquots of homogenised freeze-dried samples were weighed before and after freeze-drying. Fat and nitrogen contents were determined using the Soxhlet technique and Kjeldahl method, respectively, as described previously (FERNANDEZ *et al.*, 1985).

For the determination of the amino acids, except for tryptophan, freeze-dried and defatted samples (100 mg) were subjected to acid hydrolysis with 5 mL of 6 M HCl under nitrogen atmosphere for 24 h at 110°C. Each hydrolysate was washed in a 50 mL volumetric flask and made up to mark with Milli-Q water. An aliquot was spiked with DL-norleucine (Sigma, St Louis, MO, USA) as internal standard, and dried under vacuum on a rotary evaporator. The dried mass was washed with Milli-Q water, evaporated to dryness, and 1 mL of 20 mM HCl was added to the residue. The amino acids were subjected to HPLC analysis after derivatisation with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC), following the method described in the Waters AccQ.Tag chemistry package (MILLIPORE, 1993). Because of the conversion of asparagine and glutamine into aspartic acid and glutamic acid, respectively, during hydrolysis, the data for asparagine plus aspartic acid and glutamine plus glutamic acid are reported as Asx and Glx, respectively. Variation coefficients of the amino acid determination, including hydrolysis, were from 1.8 to 5.1%.

Cysteine and methionine were analysed as cysteic acid or methionine sulphone, respectively, after oxidation of the sample with performic acid and hydrolysis with 6 M HCl, according to the

Table 1 - Samples of Spanish-style green table olives analysed according to cultivars and commercial presentations.

Olive cultivar	Commercial presentation	Code
Gordal	Plain	AV-GH
	Seasoned	AV-GALP
	Pitted	AV-GP
	Stuffed with formulated red pepper strips	AV-GPP
	Stuffed with natural red pepper	AV-GPN
	Stuffed with almond	AV-GA
	"Salads" (a mixture of olives and formulated red pepper strips)	AV-GS
	Stuffed with cucumber	AV-GV
	Stuffed with onion	AV-GC
	Stuffed with garlic	AV-GAJ
	Stuffed with jalapeño pepper	AV-GJP
Manzanilla	Plain	AV-MH
	Plain anchovy- flavoured	AV-MAS
	Plain seasoned	AV-MALE
	Seasoned ("gazpachas")	AV-MGA
	Pitted	AV-MP
	Pitted "salads"	AV-MSL
	Slices	AV-MR
	Slices "salads"	AV-MRS
	Stuffed with formulated red pepper strips	AV-MPP
	Stuffed with formulated anchovy strips	AV-MAN
	"Alcaparrado" (with capers)	AV-MAP
	Stuffed with natural red pepper	AV-MPN
	Stuffed with almond	AV-MA
	Stuffed with formulated tuna strips	AV-MAT
	Stuffed with onions	AV-MC
	Stuffed with red pepper and almond	AV-MPAL
	Stuffed with capers	AV-MALC
	Stuffed with garlic	AV-MAJ
	Stuffed with hazelnut	AV-MAV
	Stuffed with "piquillo" pepper	AV-MPQ
	Stuffed with natural hot pepper	AV-MVG
	Stuffed with formulated hot pepper strips	AV-MPPP
	Stuffed with ham	AV-MJ
	Stuffed with formulated and marinated anchovy strips	AV-MBV
	Stuffed with lemon	AV-ML
	Stuffed with formulated orange strips	AV-MN
	Stuffed with formulated jalapeño strips	AV-MJP
	Stuffed with formulated garlic strips	AV-MAJP
	Stuffed with cheese	AV-MQ
Carrasqueña	Pitted	AV-CP
	Pitted "salads"	AV-CS
	"Alcaparrado" (with capers)	AV-CAP
Hojiblanca	Plain	AV-HH
	Pitted	AV-HP
	Slices	AV-HR
	Stuffed with formulated red pepper strips	AV-HPP

Table 2 - Samples of directly-brined table olives analysed according to cultivars and commercial presentations.

Olive cultivar	Commercial presentation	Code
Gordal	Broken seasoned turning colour	AS-GMALP
Manzanilla	Turning colour in brine	AS-MM
	Turning colour seasoned	AS-MMA
	Green in brine (ecologic)	AS-ME
Hojiblanca	Seasoned	AS-HA
Arbequina	Seasoned	AS-SA
Aloreña	Seasoned prepared from fresh olives	AS-AC
	Seasoned prepared from stored olives	AS-AA
Verdial	Green broken seasoned	AS-VALP

Table 3 - Samples of ripe olives (by alkaline oxidation) analysed according to cultivars and commercial presentations.

Olive cultivar	Commercial presentation	Code
Gordal	Plain	AN-GE
Manzanilla	Pitted	AN-MP
Carrasqueña	Plain	AN-CE
	Pitted	AN-CP
Hojiblanca	Plain	AN-HE
	Pitted	AN-HP
	Slices	AN-HR
Cacereña	Plain	AN-CCE
	Pitted	AN-CCP
	Slices	AN-CCR

method described by MOORE (1963). Cysteic acid and methionine sulphone were derivatised with AQC and separated as mentioned above. Method precision for cysteine and methionine was 3.4 and 5.1%, respectively.

Tryptophan was analysed after alkaline hydrolysis with 4.2 M NaOH according to the method described by NIELSEN and HURRELL (1985). Chromatographic conditions were similar to those used by DELHAYE and LANDRY (1986), except that a μ-Bondapak C18 (10 μm, 3.9x300 mm) column (Waters), maintained at 40°C, and fluorimetric detection (excitation at 285 nm, emission at 345 nm) were used. Method precision was 1.4%. The chemical score of the limiting amino acid was determined on the basis of the FAO/WHO/UNU (1985) recommendations for adults. All amino acid analyses were performed in duplicate.

Statistical analysis

All the computations for basic and multivariate statistics were carried out using Statistica version 6.0 software package (STATSOFT, 2001). Principal component analysis (PCA) with varimax rotation was performed on the data matrix to classify data according to their amino acid profile, without making prior assumptions on processing types or cultivars. To decide on the number of principal components (PCs), the Kaiser criterion was followed and only factors with eigenvalues greater than 1 were retained. A discriminant analysis (DA) model was built following the backward stepwise analysis option. The values of probability to include or to remove were fixed at 0.05 and 0.1, respectively, and the number of steps was set at 100. The minimum tolerance was fixed at 0.01 and no variable was forced to enter in any model. The prior probabilities were established proportional to the number of samples in each group. A leaving-one-out cross validation procedure was performed to assess the performance of the classifi-

cation rule. The scores of the table olive samples based on processing types were plotted on the canonical axes (discriminate coordinates) to visualize group discrimination.

RESULTS AND DISCUSSION

Proteins in commercial table olives

Moisture, fat, and protein (calculated from N) contents of the commercial presentations analysed are reported in Table 4. Mean contents of moisture and fat for presentations of Spanish-style olives did not significantly ($p<0.05$) differ from those of ripe olives. Presentations of directly-brined olives had lower moisture and higher fat contents. The protein content averaged 1.2% in both Spanish-style and directly-brined olives, whereas the mean content (0.9%) of ripe olives was significantly ($p<0.05$) lower. Apparently, the numerous lye treatments and water washes in the processing of ripe olives result in greater loss of nitrogenous compounds when compared with that of Spanish-style olives. The range for protein content expressed as g/100 g edible portion within presentations of directly-brined olives or ripe olives was relatively small (0.9-1.4 and 0.6-1.1, respectively), but within presentations of Spanish-style olives, the protein content varied widely (0.7-3.7), as expected due to the many types of stuffing materials or ingredients. The protein content calculated from nitrogen was significantly higher than that calculated as the sum of the individual amino acids, as indicated by the highly significant ($p<0.001$) slope of the regression (Fig. 1):

$$\text{Protein from N} = 1.265 \times (\text{protein from amino acids}) + 0.098 \qquad R = 0.963$$

The intercept 0.098 was also significant ($p<0.05$).

For individual processing types, the following equations were obtained (the intercept did not significantly differ from 0 in any case):

Spanish-style olive

$$\text{Protein from N} = 1.292 \times (\text{protein from amino acids}) + 0.04 \qquad R = 0.978$$

Directly-brined olives

$$\text{Protein from N} = 2.070 \times (\text{protein from amino acids}) - 0.33 \qquad R = 0.881$$

Ripe olives

$$\text{Protein from N} = 0.822 \times (\text{protein from amino acids}) + 0.36 \qquad R = 0.710$$

The slopes were significantly ($p<0.05$) higher than 1 in both Spanish-style and

Table 4 - Moisture, fat, and protein levels (g/100 g edible portion) of commercial presentations of table olives.

	Spanish-style olives (n=47)			Directly-brined olives (n=9)			Ripe olives (n=10)		
	Water	Fat	Protein[a]	Water	Fat	Protein	Water	Fat	Protein
Mean	75.7	15.1	1.2	68.7	21.8	1.2	77.1	16.0	0.9
CV (%)	4.7	21.3	48.1	6.7	17.2	14.8	5.0	22.4	17.4
Min	65.2	8.7	0.7	58.5	18.7	0.9	71.4	9.2	0.6
Max	83.4	23.2	3.7	73.0	30.7	1.4	85.2	22.0	1.1

[a] Calculated as N x 6.25; CV = coefficient of variation.

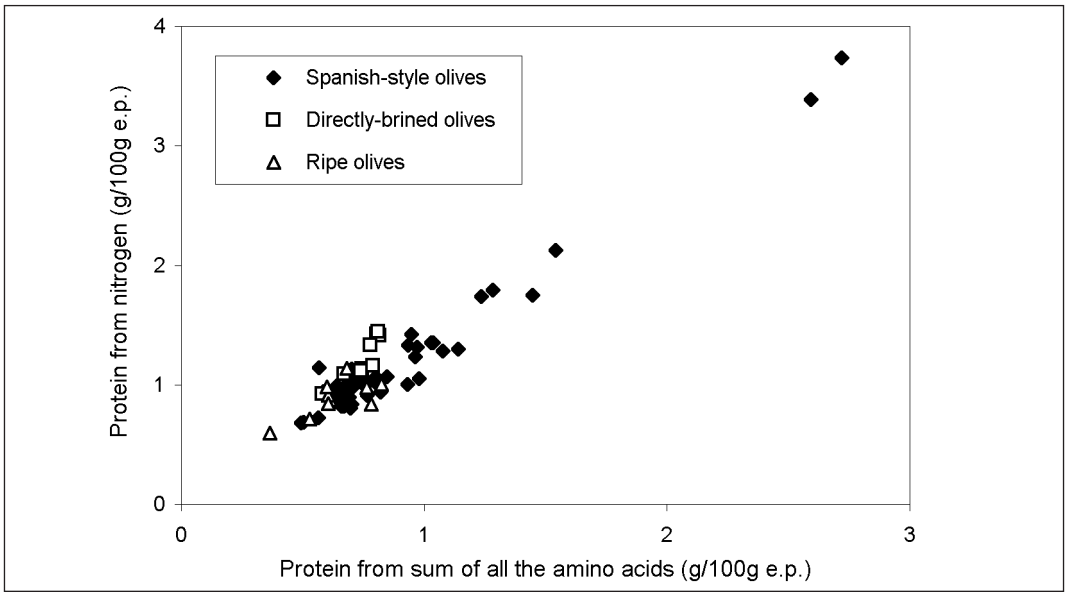


Fig. 1 - Relationship between protein content calculated as the sum of the amino acids and protein content calculated from nitrogen for commercial presentations of table olives (n=66).

directly-brined olives, but did not differ from 1 in ripe olives.

The highest protein contents were found in presentations using almonds as stuffing material (AV-MA, 3.7 g/100 g edible portion; AV-GA, 3.4 g/100 g edible portion; AV-MPAL, 2.1 g/100 g edible portion), followed by that with hazelnut (AV-MAV, 1.8 g/100 g e.p.) and those with stuffing materials of animal origin (AV-MJ, 1.8 g/100 g e.p.; AV-MAT, 1.7 g/100 g e.p.). These results are not surprising, because almond contains a high percentage of protein (21.26 g/100 g e.p., USDA 2004), similar to the protein content of foods traditionally used as protein source, such as meat and fish.

Individual amino acid content in commercial table olives

The results of the amino acid analyses are shown in Table 5. In Spanish-style green olives, the number of commercial presentations of the Manzanilla cultivar (29) was considerably larger than those of the Gordal (11), Hojiblanca (4) and Carrasqueña (3) cultivars. The major amino acids were Glx, Asx, and leucine, as previously reported in plain Spanish-style green olives (FERNANDEZ *et al.*, 1985; MONTAÑO *et al.*, 2005). The range for the Glx/Asx ratio was 0.9-5.2. Since this ratio is quite close to the value of 1.0 in table olives (VEGA *et al.*, 1981; BREMER *et al.*, 1996; MONTAÑO *et al.*, 2005; USDA, 2004), higher values could indicate that monosodium glutamate (MSG) had been added. This compound is a flavour enhancer that is frequently added to table olives to give an "anchovy flavour" (REJANO and SANCHEZ, 1996). In presentations where MSG was declared on the label, the Glx/Asx ratio ranged from 1.4 (in presentation AV-GH) to 5.2 (in AV-MJ). In the remaining presentations (those without label + those not declaring MSG on the label) the Glx/Asx ratio was <1.2, with the exception of presentations AV-GA (2.1), AV-MAV (1.6), AV-MPAL (1.7), and AV-MALC (2.3). According to the USDA (2004), al-

Table 5 - Levels of individual amino acids in presentations of table olives according to processing types.

	Spanish-style olives (n=47)			Directly-brined olives (n=9)			Ripe olives (n=10)		
Amino acid[a]	Mean	CV(%)	Range	Mean	CV(%)	Range	Mean	CV(%)	Range
Asx[b]	98	49	60-299	89	11	70-97	76	19	46-95
Serine	45	39	29-120	44	12	34-54	37	21	21-48
Glx[c]	188	81	62-651	93	10	75-103	79	18	50-99
Glycine	45	50	29-144	41	11	32-45	37	23	21-50
Histidine	23	48	14-69	20	11	16-23	15	34	8-25
Arginine	59	79	29-267	51	15	40-67	38	26	20-56
Threonine	42	30	26-94	41	9	33-46	36	22	20-48
Alanine	47	41	29-128	47	13	35-54	39	18	24-47
Proline	45	42	28-128	44	18	31-52	36	19	21-46
Tyrosine	36	37	22-93	33	14	25-38	30	32	14-48
Valine	52	39	32-136	51	11	39-56	43	19	25-53
Lysine	15	80	4-53	6	79	1-13	2	82	1-6
Isoleucine	48	39	30-127	45	10	35-49	39	19	23-48
Leucine	74	42	46-209	70	10	54-76	60	19	36-75
Phenylalanine	48	50	29-156	43	12	33-50	40	30	20-64
Tryptophan	17	26	11-30	18	17	13-23	13	26	7-17
Cysteine	6	60	2-23	7	19	5-9	4	31	3-6
Methionine	11	32	5-23	9	7	8-10	8	28	5-12
Total	898	49	493-2746	752	10	584-817	631	21	364-816
Glx/Asx	2	63	0.9-5.2	1.1	5	1.0-1.1	1.0	3	1.0-1.1
Total EAA[d]	51	9	41-57	54	2	52-56	56	2	54-58
Chemical score[e]	101	31	48-160	60	77	12-122	21	61	9-54

[a] mg/100 g edible portion; [b] Aspartic acid plus asparagine; [c] Glutamic acid plus glutamine; [d] Essential amino acid expressed as g/16 g N, corrected to 100% nitrogen recovery; [e] Using the FAO pattern for adults (FAO/WHO/UNU, 1985) as reference and lysine or methionine + cysteine as the first limiting amino acid.

monds and hazelnuts have Glx/Asx ratios of about 2, which could explain the relatively high values for samples AV-GA, AV-MAV, and AV-MPAL in the absence of added MSG. Capers have been reported (VEGA and RAMOS, 1987) to have similar amounts of Glx and Asx. Therefore, the relatively high value of Glx/Asx ratio in presentation AV-MALC (supplied without label) indicates that MSG had probably been added.

Values for total essential amino acids (EAA) ranged from 41 g/16 g N in presentation AV-MAS to 57 g/16 g N in presentations AV-GJP and AV-CAP, which are higher than the FAO/WHO/UNU (1985) reference protein pattern value for adults (12.7 g/16 g N) or a two to five year-old child (33.9 g/16 g N). As a result, the amino acid profile of all presentations within this processing type showed a good balance of total EAA. Based on the FAO pattern for adults, lysine was the first limiting amino acid in most presentations. However, in 14 of the 47 presentations the most limiting amino acids were the sulphur-containing amino acids cysteine and methionine. In presentations based on olives alone, the protein chemical score was low for the Manzanilla and Hojiblanca cultivars (e.g. AV-MH, 56%; AV-HR, 49%), but greater than 100% for presentations AV-GH and AV-CP of the Gordal and Carrasqueña cultivars, respectively. The chemical scores previ-

ously reported in green table olives referred only to the Manzanilla cultivar, and ranged from 45 to 60% (MONTAÑO *et al.*, 2005). In olives with stuffing materials, the highest scores corresponded to presentations AV-MALC (155%) and AV-GALP (160%). Considering all the presentations, the average chemical score was 101%.

In directly-brined olives, with the exception of the limiting amino acid (lysine), the amounts of each amino acid varied relatively little between presentations (Table 5). MSG is usually not added to this type of processing, which is reflected in the Glx/Asx ratio values that were quite close to 1. Total EAA content averaged 54 g/16 g N, which was slightly higher ($p<0.05$) than the mean value for commercial Spanish-style presentations (51 g/16 g N). Based on the chemical score, the presentations AS-GMALP, AS-VALP, and AS-MMA showed the best protein quality, while the worst was for presentation AS-ME. On average, protein quality, indicated by the mean value of the chemical score in this type of processing, was lower than in the case of Spanish-style olives.

For the presentations of ripe olives studied, the mean values of each amino acid, expressed as mg/100 g e.p., were slightly lower than those for directly-brined olives, but the amino acid profiles were quite similar. Total EAA content averaged 56 g/16 g N (Table 5), which was slightly higher ($p<0.05$) than the mean value for directly-brined olives. However, the lysine content was the lowest in the ripe olives (Fig. 2). This is not surprising, because this style of olive requires several lye treatments and water washes, and a sterilisation treatment, which favour the possibility of lysine loss due to lysinoalanine formation or Maillard reaction (MEADE *et al.*, 2005). The chemical score was low, averaging 21%, and, thus, the protein quality was the poorest of all the processing types.

Chemometric study of the amino acid composition of table olives

The data were successfully checked, using the Kolmogrov-Smirnov-Lilliefors test, for normal distribution. The multivariate analysis of variance (data not shown) resulted in significant differences among processing styles and

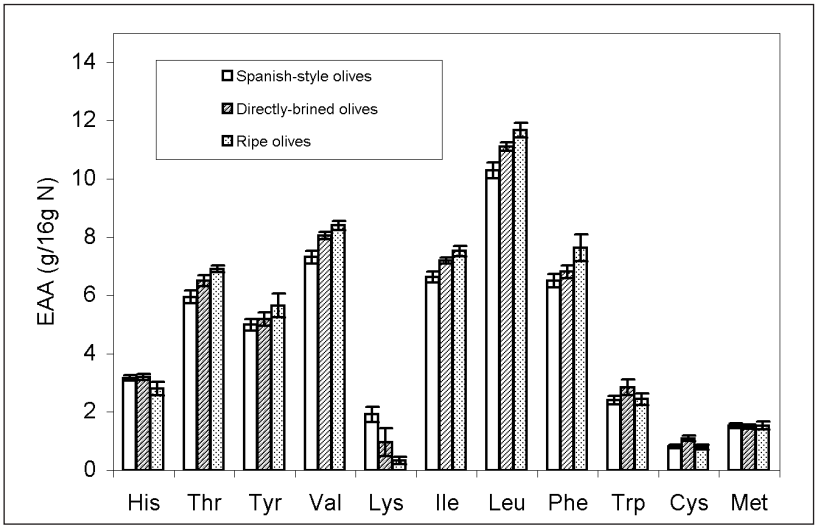


Fig. 2 - Essential amino acid (EAA) profile of commercial presentations of table olives from the three processing types. Data represent mean values of 47, 9, and 10 samples of Spanish-style olives, directly-brined olives, and ripe olives, respectively. Error bars were obtained at 95% confidence intervals.

cultivars. The data showed appropriate characteristics to be subjected to the chemometric study. PCA was applied without making any assumption of sample processing styles. Basically, the extraction of the principal components (PCs) amounts to a variance maximizing (varimax) rotation of the original variables. A new set of only two PCs was generated. Their eigenvalues were 12.85 and 1.62 and they accounted for a cumulative variance of 90.45%. The plot of the variable loadings on the PCs plane is shown in Fig. 3. The graph permits a visualization of the relative position of the variables and their respective correlations. Two groups were evident, one formed by cysteine and methionine and other by the rest of the amino acids. Correlations within groups (cosine of the angle of their respective vectors) were very strong but fairly low between them. Most of the amino acids were related to PC1, with high loads, while the group composed of cysteine and methionine was closest to PC2. A similar distribution (not shown) with cysteine and methionine that was clearly separated from the other amino acids was also found when data from a different product like pickled garlic (previously published by MONTAÑO *et al.*, 2004) were analysed by PCA. This appears to be a reflection of the different procedure (sample preparation including an oxidation step) used to analyse cysteine and methionine in comparison to that used for the other amino acids.

The plot (not shown) of the sample scores in the plane of the two PCs did not lead to a classification of the samples according to processing styles or cultivars. Consequently, predictive DA was applied. The back stepwise procedure retained that only the following amino acids significantly contributed to discrimination: histidine, Asx, valine, isoleucine, phenylalanine, alanine, tyrosine, cysteine, tryptophan, and lysine, in decreasing order of F to-remove values. The canonical analysis of the data deduced two orthogonal functions (number of groups -1), identified as Factor 1 and Factor 2, the classification abilities of which were observed by plotting the case scores on the plane of these functions (Fig. 4). It was evident that they were able to discriminate among the three different processing styles. In this plot, ripe olives were related to low Factor 1 values and high Factor 2 values. Directly-brined olives (with relatively wide dispersion) had Factor 1 values that were similar to those of ripe olives, but lower Factor 2 values. Green olives were characterized by values around 0 for both factors and also showed marked dispersion. The contribution of each var-

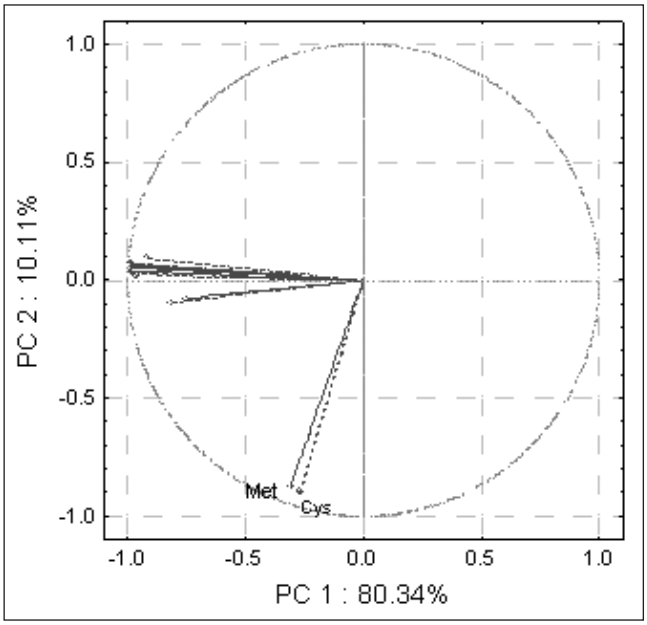


Fig. 3 - Projection of loadings of the variables (amino acids) in the first two principal components.

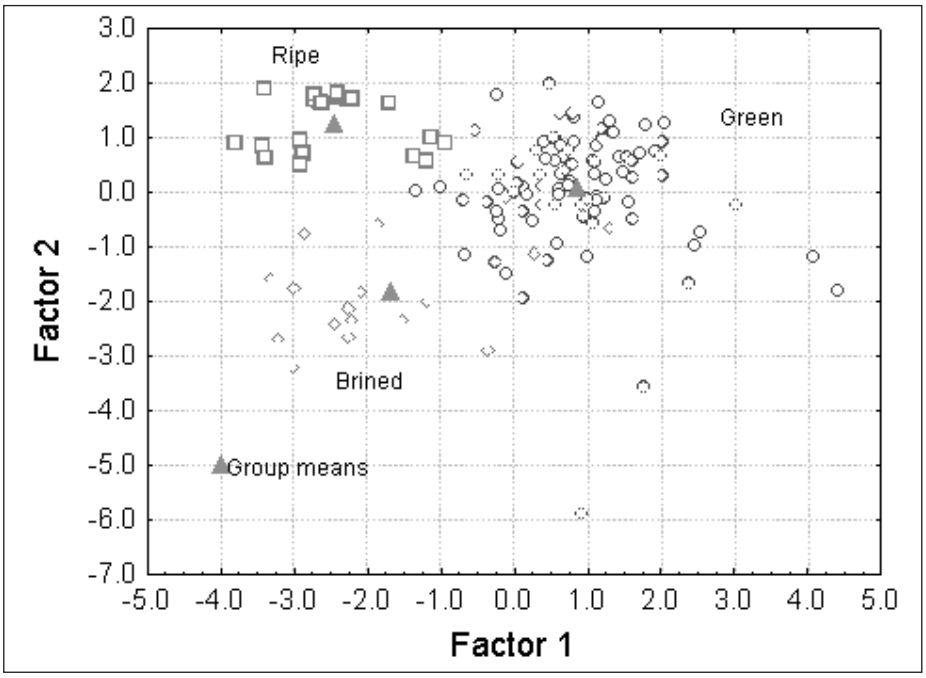


Fig. 4 - Plot of sample scores and group means, according to processing styles, as a function of the two canonical discriminant functions.

iable (amino acid) within each factor was assessed by their standardized (by within group variances) coefficients. Those with the highest contributions in Factor 1 were: valine (-14.9), isoleucine (12.8), phenylalanine (-5.4), Asx (5.0), histidine (4.7), and tyrosine (2.7); those with the highest values in Factor 2 were alanine (-5.5), isoleucine (5.9), histidine (-3.0), valine (-2.1), Asx (2.1), tyrosine (1.8), and phenylalanine (1.6). The classification functions correctly assigned 85, 78, and 99% of the ripe, directly-brined and green olive samples, respectively, with 94% correct answers overall. The cross-validation resulted in similar rates: 85, 72, 98, and 92%, respectively. These achievements showed that the algorithm developed could be used for the classification of future samples.

When the predictive DA was used to discriminate among cultivars, the results were less conclusive and only 63% of the samples were correctly assigned. These results may indicate that the amino acid content in most cultivars are fairly similar. The apparent increase in correct answers in the classification according processing styles with respect to cultivars may be due to some effects of the processing treatments (e.g. lye treatments, washings, etc.) or to differences between the raw materials.

CONCLUSIONS

An accurate calculation of protein content and protein quality was made based on the corresponding amino acid composition for the three main types of commercial table olives. The Glx/Asx ratio was used as an indicator of the presence or absence of MSG in table olives. On average, the total EAA content for each type of processing was high when compared with the

FAO reference protein, but the chemical score was low in both directly-brined (60%) and ripe (21%) olives. As a result of more commercial presentations with a diversity of stuffing materials which increased the lysine content notably, the chemical score for the Spanish-style product averaged 101%. Data analysis by PCA did not differentiate among processing types or cultivars. However, DA was successfully able to discriminate among processing types. This indicates that the amino acid content may be affected by the processing treatments or the maturity level of the raw material.

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SURVEY ON THE PRESENCE OF ENTEROVIRULENT *ESCHERICHIA COLI*, INCLUDING *E. COLI* O157, IN CHEESE MANUFACTURED IN THE ITALIAN ALPINE REGION

INDAGINE SULLA PRESENZA DI *ESCHERICHIA COLI* ENTEROVIRULENTI, INCLUSO *E. COLI* O157, IN FORMAGGI PRODOTTI NEL COMPRENSORIO ALPINO ITALIANO

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ABSTRACT

The aim of the present study was to investigate the prevalence of enterovirulent *Escherichia coli* strains in cheese manufactured in the Italian Alpine Region. Cheese samples obtained from raw and pasteurised milk were collected from cheese-making plants. Enumeration of *E. coli* and the presence of verocytotoxigenic (VTEC), enteropatho-

RIASSUNTO

La presente ricerca è stata eseguita con lo scopo di osservare la prevalenza di ceppi enterovirulenti di *Escherichia coli* in formaggi prodotti sul territorio alpino italiano. Dai caseifici alpini sono stati raccolti campioni di formaggi prodotti sia da latte crudo che pastorizzato. Per ogni campione si sono determinate le cariche di *E. coli* totali e la presenza di

- Key words: cheese, pathogenic, *E. coli*, PCR -

genic (EPEC) and enterotoxic (ETEC) *E. coli* were carried out. A multiplex-PCR for the amplification of the sequences of VT1, VT2, *eae, ehxA*, ST, LT genes was used to identify pathogenic *E. coli* strains. The presence of *E. coli* O157 was investigated using an immunomagnetic separation technique. *E. coli* O157 was not recovered in cheese made from raw or pasteurised milk. The multiplex PCR approach detected at least one virulence determinant in only ten cheese samples, in four of them, a verotoxin-producing gene (VT2 and/or VT1) was detected and in the remaining six only the attaching and effacing lesion genes (*eae*) were found. No ETEC strains were detected. The statistical analyses of the results demonstrated significant associations between the use of raw milk in cheese production and the presence of detectable virulence genes in cheese. Moreover, high *E. coli* counts were also associated with the use of raw milk in fresh and ripened cheese production. The results revealed the survival and recovery of pathogenic strains even after a 60-day ripening period, highlighting the importance of the hygienic and sanitary requirements for milk used in the production of both fresh and ripened cheese.

ceppi verocitotossici (VTEC), enteropatogeni (EPEC) ed enterotossici (ETEC). I ceppi patogeni sono stati identificati mediante l'utilizzo di una PCR multipla per l'amplificazione di frammenti dei geni VT1, VT2, *eae*, *ehxA,* ST ed LT. La ricerca di *E. coli* O157 è stata eseguita utilizzando la tecnica della separazione immunomagnetica. I risultati hanno permesso di evidenziare l'assenza di *E. coli* O157, mentre in 10 formaggi la PCR ha messo in evidenza la presenza di geni di patogenicità: in 4 campioni sono stati evidenziati i geni codificanti per la produzione di verotossina (VT1 e/o VT2), nei rimanenti si è evidenziato solo il gene responsabile delle attaching and effacing lesions (*eae*), mentre non si sono evidenziati ceppi ETEC. L'analisi statistica dei risultati ha permesso di evidenziare che la presenza di geni di virulenza è associata a formaggi prodotti da latte crudo. Inoltre è stata osservata un'associazione tra alte cariche di *E. coli* e prodotti a latte crudo (freschi e stagionati). I risultati della presente ricerca hanno evidenziato la sopravvivenza di ceppi patogeni anche in formaggi stagionati per periodi superiori ai 60 giorni, sottolineando l'importanza delle condizioni igienico-sanitarie del latte utilizzato per la produzione sia di formaggi freschi che stagionati.

INTRODUCTION

Escherichia coli, is physiologically present in the intestines of humans and warm-blooded animals. Although usually harmless, it was defined as a foodborne pathogen in 1971, when nearly 400 people became ill after eating contaminated cheese (MARIER *et al.*, 1973). At present, at least five virulence groups of *E. coli* are recognized with respect to disease syndromes, characteristics, serological grouping and effects on certain cell cultures (JAY *et al.*, 2005). Verotoxin-producing *E. coli* (VTEC) are often associated with the onset of foodborne diseases. These strains may produce two kinds of verotoxins (VT1 and VT2). More than 200 VT-producing *E. coli* serotypes have been isolated, but only a few have caused severe human diseases; the O157 serotype has been responsible for most of these infections (CAP-

RIOLI *et al.*, 2005; MALDONADO *et al.*, 2005). Two virulence factors may affect the pathogenicity of VTEC: the *eae* and *ehxA* genes. The former is responsible for the production of attaching and effacing lesions (AE lesions) in the intestinal mucosa; the latter encodes for the haemolysin called EHEC enterohaemolysin (*ehxA*) (BEUTIN *et al.*, 2004).

While consumption of undercooked beef meat has long been considered the main cause of this infection, *E. coli* O157 and VTEC strains have often been detected in the environment (MALDONADO *et al.*, 2005). In addition, raw milk, as well as cheese and yogurt, have also been associated with outbreaks and sporadic cases of illnesses due to VTEC contamination (GILLEPSIE *et al.*, 2003; LIPTAKOVA *et al.*, 2004; HUSSEIN and SAKUMA, 2005).

E. coli O157 are currently the most common VTEC in many regions of the world (ARMSTRONG *et al.*, 1996). However, other serotypes, such as O26, O103, O111, O118 and O145 have also been recovered from infected patients and are considered a serious threat to public health (CAPRIOLI *et al.*, 1997; ALLERBERGER *et al.*, 2003).

Some non-VTEC strains, named enterotopathogenic *E. coli* (EPEC) and enterotoxigenic *E. coli* (ETEC), can induce sporadic diarrhoea. The former group causes AE lesions in the human small intestine causing diarrhoea, but does not generally produce enterotoxins (CORTÉS *et al.*, 2005). The latter group is a major cause of sporadic diarrhoeic diseases in humans, affecting mainly children in developing countries (NATARO and KAPER, 1998). ETEC strains have also been detected in Europe, in particular Switzerland, in 20% of the raw milk and dairy products (ALLMANN *et al.*, 1995).

At present, conventional microbiological diagnostics in food only include the determination of the *E. coli* count per gram without requiring any further characterization of the isolated strains; the analyses on serogroups and detection of pathogenic determinants are only carried out when foodborne outbreaks occur. Therefore, information about the presence of potentially pathogenic *E. coli* in dairy products is lacking, particularly regarding traditional cheeses produced in specific geographic areas.

The aim of this study was to investigate the occurrence of all potentially enterovirulent *E. coli* (VTEC including *E. coli* O157, EPEC, ETEC strains) in raw and pasteurised milk cheeses manufactured in the Italian Alpine Region, which is known for its high standards in dairy production. The investigation was carried out by coupling the traditional immunoseparation technique used to detect O157 with the newer multiplex PCR technique used to detect other pathogenic *E. coli* strains.

MATERIALS AND METHODS

Sampling

Two-hundred and three soft and ripened cheeses were collected over a 17-month period from 50 cheese-making plants in the Italian Alpine Region. The samples included 143 (70.4%) raw milk cheeses and 60 (29.6%) pasteurised milk cheeses. Soft cheeses (less than 10-days maturation) and ripened cheeses (more than 10-days maturation) were the most frequent cheese types. All of the ripened and most of the soft cheeses analysed were obtained by enzymatic coagulation. Table 1 reports the sample distribution according to ripening time (days), cheese texture, heat treatment and dairy species of origin of the milk used in cheese production. After collection, samples were refrigerated and transported to the laboratory where the samples were stored at 4°C and then processed the following day.

Enumeration of *E. coli*

The total *E. coli* count was done according to the UNI method (2002). Serial dilutions of peptone water were prepared and 1 mL of each dilution was poured onto Tryptone Bile Glucuronide Medium (TBX, Oxoid Italiana, Garbagnate Milanese, Milan, Italy). Plates were incubated at 44°C for 18-24 h and *E. coli* were counted on plates with typical blue/green colonies.

Detection of pathogenic *E. coli* strains

For each sample, 25 g of product were added to 225 mL of modified Tryptic Soy Broth (Oxoid Italiana, Garbagnate Milanese, Milan, Italy) supplemented with novobiocin (20 μg/mL, Oxoid Italiana, Garbagnate Milanese, Italy) and incubated at 41.5°C for 6 h. In compliance with the ISO (2001) procedure, two 50-μL aliquots of broth were streaked onto one plate of Sorbitol MacConkey agar (SMAC, Oxoid Italiana, Garbagnate Milanese, Milan, Italy), and one plate of Sorbitol MacConkey agar supplemented with Cefixime tellurite (CT-SMAC, Oxoid Italiana, Garbagnate Milanese, Italy), in order to increase the selectivity of the medium for *E. coli* detection. Both media were incubated at 37°C for 18-24 h.

If present, five sorbitol-negative and five sorbitol-positive colonies from each plate were inoculated into tryptone water for 24 h at 37°C. These suspensions were sub-cultured onto Nutrient agar (Oxoid Italiana, Garbagnate Milanese, Milan, Italy), emulsified in 5 mL of sterile saline solution, inoculated onto API 20E strips (BioMérieux, Rome, Italy), and incubated at 37°C for 24 h. The same suspensions were also processed to prepare DNA templates for multiplex PCR targeting *E. coli* virulence factors, following the published protocol (BOTTERO *et al.*, 2004) for DNA extraction and amplification. However, in order to effectively identify pathogenic *E. coli* strains, further investigations were performed when multiplex-PCR detected at least one virulence factor when tryptone water-suspended colonies were tested. These were sub-cultured on TBX and at least five isolates per plate were analysed singularly with the same multiplex-PCR method and serotyping.

Isolation of *E. coli* O157

All samples were examined for the presence of *E. coli* O157 following the ISO (2001) procedure and using an immunomagnetic separation (IMS) technique. Each sample underwent pre-enrichment in modified Tryptic Soy Broth (Oxoid Italiana, Garbagnate Milanese, Milan, It-

Table 1 - Cheeses sampled according to days of ripening (A); number of raw and pasteurized milk cheeses according to cheese texture and species of milk origin (B).

Ripening period (days)	Sampled cheeses
<10	74
10-30	45
31-60	47
61-90	28
>90	9

A

	Texture		Species of origin of milk				
Cheeses	Soft	Ripened	Cow	Goat	Cow/Goat	Cow/Sheep	Cow/Goat/Sheep
Raw milk	50	93	115	11	11	4	2
Pasteurised milk	29	31	56	1	2	0	1

B

aly) supplemented with novobiocin (20 μg/mL, Oxoid Italiana, Garbagnate Milanese, Milan, Italy) as described above. Immunomagetic separation was performed according to the manufacturer's instructions (Dynal, Oslo, Norway); however, the IMS was repeated after an additional 24 h of incubation.

In compliance with the ISO (2001) procedure, two 50-μL aliquots of the IMS-enriched broth were analyzed as described previously. Suspected *E. coli* O157 colonies were tested for indole production using Kovacs reagent (Merck & Co, Milan, Italy).

Serotyping

Serotyping was performed by means of slide agglutination with an *E. coli* O157 Latex test kit (Oxoid Italiana, Garbagnate Milanese, Milan, Italy) on API 20 E identified *E. coli* isolates, which revealed at least one virulence gene when tested with multiplex PCR. The results were confirmed by tube agglutination of heat-treated cultures (Denka Seiken Co., Ltd., Tokyo, Japan).

Data analysis

In the present study three associations were evaluated with the heat treatment of milk used in the cheese-making process: the first was the total *E. coli* count, the second the presence of virulence genes in colonies isolated from cheese samples and the third the presence of the VT gene in isolated colonies. In order to analyse the data, microbiological and PCR results were coded as binary variables; for *E. coli* count, a value of 1 was attributed to samples where the *E. coli* count was greater than 10^3 cfu/g, and 0 was assigned if the count was less. To evaluate the presence of a virulence gene, a value of 1 was given when at least one isolated colony from the cheese samples showed the presence of at least one virulence factor amplification product with multiplex PCR. Similarly, when the association between milk heat treatment and the presence of the VT gene was evaluated a value of 1 was given to all the samples that had at least one isolated colony with VT1 and/or VT2. The heat treatment of milk used in the cheese-making process was also coded as 1 and 0 for raw and pasteurised milk, respectively. For the evaluation of the association between heat treatment of milk and *E. coli* count, a total of 200 cheese samples were analysed. Since cheese type is associated with both *E. coli* count in cheese and the heat treatment of the milk used, samples were divided into fresh (n=83) and ripened (n=117) cheeses and a stratified analysis was performed in order to check for the confounding effect of cheese type. On investigating the other two associations, 195 cheese samples were analysed, dividing them into fresh (n=78) and ripened (n=117) cheeses. In all the contingency tables the Fisher Exact Probability test (F.E.P.) and the Mantel-Haenszel χ^2 were used to assess the significance of the results. Mantel-Haenszel Odds Ratio (O.R.) values and 95% Confidence intervals (95% C.I.) were evaluated as measures of association.

RESULTS

Enumeration of *E. coli* and pathogenic *E. coli* identification

The *E. coli* counts are reported in Table 2. In the raw milk cheese samples less than 10 cfu/g of *E. coli* were present in 70 cheeses (49% of the samples). Among these samples, 21 (30%) and 49 (70%) were soft and ripened cheeses, respectively. In contrast, 55 (92%) pasteurised milk cheeses had *E. coli* counts less than 10 cfu/g.

Presumptive *E. coli* colonies on SMAC and CT-SMAC were recovered in 123 (61%) and 93 (46%) cheese samples, respectively. When subsequently tested

Table 2 - *E. coli* distribution in cheeses related to heat treatment of the milk used for cheese production.

	E. coli counts (cfu/g)					
Cheeses	< 10	10-10^2	10^2-10^3	10^3-10^4	10^4-10^5	>10^5
Raw milk	70	14	19	15	16	9
Pasteurised milk	55	2	2	1	0	0

with API 20 E, these colonies were identified as *E. coli*. When multiplex PCR was performed on tryptone water from isolated SMAC/CT-SMAC colonies, the presence of at least one virulence gene was detected in 16 (8%) cheeses. In particular, when PCR was performed on SMAC colonies, 6 soft (3%) and 8 ripened (4%) cheeses showed amplification products of at least one virulence factor. Pathogenicity determinants were detected when PCR was performed on tryptone water from isolated CT-SMAC colonies, in 5 (2.5%) soft and 4 (2%) ripened cheeses. Frequencies of virulence determinants in the different kinds of cheeses are reported in Table 3. In particular, the VT2 gene was detected in 3 (2%) of the positive SMAC samples, whereas the VT1 and VT2 genes, respectively, were detected in 2 (2%) and 3 (3%) of the positive CT-SMAC samples. Both genes were detected in only one positive CT-SMAC sample. The *eae* was the most commonly amplified gene, whereas the *ehxA* gene was only detected in 3 isolates in association with VT2 or VT1 genes, detected in three and two isolates, respectively. Tryptone water inoculated with PCR positive samples were sub-cultured on TBX, and both multiplex PCR and serotyping were performed on 5 isolated colonies. Virulence determinants were only detected in 10 samples. Table 4 shows the presence of virulence determinants in these 10 cheese samples: 9 raw milk, ripened cheeses, and one pas-

Table 3 - Number of cheeses with isolated *E. coli* colonies on SMAC (A) or CT-SMAC (B) and with multiplex PCR positive results, according to cheese texture and heat treatment of the milk. The percentage of positive results calculated over the total sample size for each type is reported in parentheses.

	Cheeses	n	SMAC (%)	*ehxA* (%)	*eae* (%)	ST (%)	LT (%)	VT1 (%)	VT2 (%)
Soft	Raw milk	52	28 (53)	3 (6)	3 (6)	------	------	------	------
	Pasteurised milk	31	12 (39)	------	1 (3)	------	------	------	------
Ripened	Raw milk	91	63 (69)	3 (4)	4 (6)	------	------	------	3 (4)
	Pasteurised milk	29	12 (41)	------	------	------	------	------	------
	Total	203	123 (61)	6 (3)	8 (4)	------	------	------	3 (1.5)

A

	Cheeses	n	CT-SMAC (%)	*ehxA* (%)	*eae* (%)	ST (%)	LT (%)	VT1 (%)	VT2 (%)
Soft	Raw milk	52	27 (52)	5 (10)	2 (4)	—	—	1 (2)	1 (2)
	Pasteurised milk	31	7 (23)	—	—	—	—	—	—
Ripened	Raw milk	91	47 (52)	2 (2)	2 (2)	—	—	1 (1)	2 (2)
	Pasteurised milk	29	12 (41)	—	—	—	—	—	—
	Total	203	93 (46)	7 (3.5)	4 (2)	—	—	2 (1)	3 (1.5)

B

Table 4 - Cheeses with at least one *E. coli* colony isolated from SMAC or CT-SMAC that showed positive reactions to one or more virulence gene targeted in the Multiplex PCR. The total *E. coli* count is also reported for each sample.

	E. coli count	Multiplex PCR				
Cheese	cfu/g	*ehxA*	*eae*	VT1	VT2	*uidA*
1	<10	+	-	-	+	+
2	<10	-	-	-	+	+
3	<10	+	+	+	+	+
4	230	+	+	+	-	+
5	1,100	-	+	-	-	+
6	<10	-	+	-	-	+
7	<10	-	+	-	-	+
8	160	-	+	-	-	+
9	43,000	-	+	-	-	+
10	<10	-	+	-	-	+

teurised, fresh milk cheese. Four of these isolates were characterized as VTEC, and none as ETEC, since no LT or ST genes were amplified.

Isolation of *E. coli* O157

When samples were tested with the ISO immunoconcentration technique (2001) the presence of *E. coli* O157 was not detected.

Serotyping

When serotyping was performed on ten cheese samples from which isolates with pathogenic determinants were recovered, three isolates, O26, O113, and O146, were identified, the rest were not able to be typed. Interestingly, only the attaching and effacing lesion encoding gene was detected in the O26 isolate, whereas in the O113 strain, isolated from a 70-day ripened cheese, both VT2 and *eae* genes were detected. In the O146 strain, isolated from a mixed raw cow-sheep milk cheese, all four virulence genes (*eae, ehxA*, VT1, VT2) were detected.

Measure of association

The use of raw milk for cheese production was significantly associated with *E. coli* counts in cheeses when the stratified analysis was performed (Mantel-Haenszel $\chi^2 = 17.79$; $p<0.001$). In particular, cheeses produced from raw milk were more associated with an *E. coli* count greater than 10^3 cfu/g than cheeses made from pasteurised milk (Mantel-Haenszel O.R.= 29.74; 95% C.I.: 3.63-531.53).

The association between raw milk cheeses and the presence of at least one virulence gene in isolated *E. coli* colonies was also statistically significant (Mantel-Haenszel $\chi^2 = 3.95$ $p<0.05$), indicating that cheeses made from raw milk are more associated with positive PCR results than the ones produced with pasteurised milk (Mantel-Haenszel O.R.=2.31 95% C.I.: 1.11-5.05). In contrast, the presence of the VT gene was not associated with the use of raw milk for cheese production (F.E.P. >0.05).

DISCUSSION

The aim of the present study was to monitor the presence of pathogenic *E. coli* strains and related virulence determinants (*eae*, *ehxA*, VT1, VT2, ST, LT) in raw- and pasteurised-milk cheese produced in the Italian Alpine Region, an area that has high dairy production standards. Conventional techniques for the differential detection of *E. coli* virulent strains in dairy products are not applicable due to the absence of known phenotypic traits related to strain discrimination. Therefore, in the last few years, biomolecular approaches have been extensively explored as strain differentiation tools as an alternative to conventional phenotypic methods.

Genes encoding for virulence determinants, such as ST, LT (ALLMANN *et al.*, 1995), VT1, VT2, *ehxA*, and *eae* (PIERARD *et al.*, 1997; BOUVET *et al.*, 2001;

CHAPMAN *et al.*, 2001) have been used for PCR detection of VTEC, including *E. coli* O157, EPEC, and ETEC. The usefulness of these PCR assays has long been debated. In fact, even if PCR assays can be applied for *E. coli* pathogenic strain detection in human faecal samples, the applicability of these techniques on environmental samples is severely limited by the widespread presence of virulence genes in *E. coli* strains that are only rarely associated with human diseases.

However, despite these limitations, PCR might allow strains to be identified that are not usually detected by conventional techniques. Some VTEC strains belonging to serogroups O26, O103, O111, O145 and O157 isolated from human diseases such as HC and HUS (CAPRIOLI *et al.*, 1997; BEUTIN *et al.*, 2004), were not detected by traditional techniques and were investigated only after the occurrence of food poisoning outbreaks (BEUTIN *et al.*, 2004). In this context, the use of PCR for monitoring *E. coli* virulence factors is important for identifying potentially dangerous foods as part of the HACCP plans, in order to detect and avoid the spread of pathogenic *E. coli,* before food poisoning outbreaks occur.

The results of this research revealed that in 98.3% of the pasteurised-milk cheeses and in 72% of the raw-milk cheeses the *E. coli* counts were less than 10^3 cfu/g, the maximum level allowed (Commission Regulation No 2073/2005) in cheeses made from heat-treated milk. The significantly low *E. coli* count in the Italian Alpine cheese sampled in this investigation, is undoubtedly related to the strict hygiene procedures used in milk and cheese production. However, the analysis of the association between raw milk cheeses with *E. coli* counts greater than 10^3 cfu, and positive multiplex PCR isolated colonies showed a statistically significant association. These results indicate the importance of milk quality and hygiene prior to processing, for both fresh and ripened cheese production.

In the present investigation, the presence of pathogenic *E. coli* in cheeses was evaluated by combining the traditional ISO (2001) procedure for *E. coli* O157 detection, with the detection of *E. coli* virulence determinants through multiplex PCR after pre-enrichment of cheese samples. This approach could detect EPEC, ETEC and VTEC by PCR amplification of virulence determinants, revealing the presence of potentially pathogenic *E. coli* strains (besides O157) in cheeses which might not be detected by standard identification procedures. However, to effectively identify pathogenic *E. coli* strains, other investigations (e.g. serotyping) were performed when multiplex-PCR detected at least one virulence factor when tryptone water-suspended colonies were tested. Using this approach pathogenic *E. coli* were isolated in only 10 cheese samples out of 16 which presented multiplex PCR amplification products. None were serotyped as O157, three were identified as O26, O113, and O146. Four of the isolates were VTEC, but none were ETEC. These findings suggest that by combining the two methods pathogenic *E. coli* isolates, which are likely to pass undetected when only traditional methods are used, can be detected. In particular, the pathogenic *E. coli* levels are usually lower than those of non pathogenic *E. coli* and, when plated, the colonies share the same morphology, thus making it difficult to isolate the pathogenic strains. Hence, from a practical point of view, the presence of such small concentrations of pathogens might be important for the monitoring process needed in HACCP plans in order to control the spread of these strains. In fact, low levels of pathogenic *E. coli* can also be a serious hazard for consumers' health. The infectious dose of strains containing VT2, that is well associated with human disease (HUSSEIN and SAKUMA, 2005), is quite low. TILDEN *et al.* (1996) suggested that the infectious dose evaluated in an *E. coli* O157:H7 outbreak from dry fermented salami consumption

was less than 50 bacteria, and STRACHAN *et al.* (2001) estimated that an infectious dose could be as low as 4-24 ingested organisms.

Enterovirulent *E. coli* contamination of milk and dairy products (HUSSEIN and SAKUMA, 2005) has been investigated in a limited number of studies. Detection of VTEC strains in raw milk, milk filters and cheeses was reported in Belgium, Canada, Germany, the UK and the U.S.A., but only a few isolates were identified as belonging to serogroups known to cause human illness. CONEDERA *et al.* (2004) analysed 2948 Italian dairy products and *E. coli* O157:H7 was detected in only one sample. Similarly, no O157 VTEC were isolated when 1011 raw cow milk samples were examined in the Netherlands (HEUVELINK *et al.*, 1998).

Likewise, these research results showed a very low prevalence of pathogenic *E. coli* strains in cheeses: no O157 strains and no ETEC were detected. The virulence genes (VT1, VT2, *eae,* and *ehxA*) were detected in only 1 of the 10 isolates presenting virulence determinants. The source of this isolate was a 60-day-old raw-milk cheese, that had an *E. coli* count of less than 10 cfu/g. Similarly, considering multiplex PCR results, most of the genes coding for toxin production were detected in isolates from raw-milk ripened cheeses, which were also characterized by low levels of total *E. coli* contamination. The absence of any correlation between the *E. coli* count and the presence of *E. coli* O157, as well as other VTEC and ETEC strains, has already been observed (CHAPMAN *et al.*, 2001; CONEDERA *et al.*, 2004). These findings suggest that VTEC and ETEC strains could be more resistant than other *E. coli* strains to stress conditions (fermentation and maturation of cheeses) as already observed for *E. coli* O157 (KASRAZADEH and GENIGEORGIS, 1995; REITSMA and HENNING, 1996).

The association between the presence of virulence genes and the heat treatment of milk used in the cheese-making process was also evaluated, in particular the use of raw milk was associated with the presence of virulence genes for both fresh and ripened cheese, indicating that the ripening process alone (even if 60 days) does not guarantee the absence of pathogenic *E. coli* strains. Hence, contrary to what was claimed by QUINTO and CEPEDA (1997), both soft and ripened cheeses should be considered as possible vehicles of infection.

The results of this investigation demonstrate the presence of *E. coli* virulence determinants in raw milk cheese regardless of the ripening process. *E. coli* virulence testing is therefore needed particularly in all raw milk products or other dairy products processed at temperatures below 45°-48°C. This testing is currently not included in routine diagnostic activities. Therefore, to address consumer protection issues there must be a careful control of all the cheese production phases, starting from milking procedures, as well as more detailed information on product labels that indicates the heat treatment of the milk used in production.

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THE CHEMICAL PHYSICS OF FOOD

Edited by Professor Peter Belton, School of Chemical Sciences and Pharmacy, University of East Anglia, UK
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BAKED PRODUCTS
Science, Technology and Practice

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9 The opportunities for new product development

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Whole Grains and Health presents a science-based discussion of whole grains and their expanding role in health and disease. An international collection of authors presents current perspectives on grains, the many opportunities for further research into whole grains and the remarkable growth potential for product development.

3RD INTERNATIONAL WORKSHOP ON "BIOSENSORS FOR FOOD SAFETY AND ENVIRONMENTAL MONITORING" FEZ - Morocco 18th to 20th October, 2007

The workshop will feature all aspects of the highly interdisciplinary area of biosensors with emphasize on the application of biosensors on food safety and environmental monitoring. It will cover the following topics:

- Enzyme-based biosensors;
- Nucleic acid sensors;
- Immunosensors;
- Whole cell-based biosensors;
- Nanotechnology and biosensors;
- Validation of biosensors in real environmental and food samples;
- Commercial developments and markets.

Invited speakers

Kevin W. Plaxco, University of California, Santa Barbara (U.S.A.): "Folding-based biosensors".

Alexander Kuhn, University of Bordeaux 1 (France): "Multiscale tailored electrode surfaces in bioelectroanalysis".

Otto S. Wolfbeis, University of Regensburg, Institute of Analytical Chemistry (Germany): "Advancements in Fluorescence Optical Biosensors, and Possible Applications in Food Safety and Environmental Monitoring".

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Important deadlines

June 1st, 2007: Second announcement

September 17th, 2007: Submission of the abstracts

September 24th, 2007: Information to authors about acceptance of abstracts

September 29th, 2007: Early registration with reduced fee

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SELECTIVE ENUMERATION OF BIFIDOBACTERIA IN MILK AND MILK PRODUCTS

Brussels, 4 April 2007 - IDF has just released a draft standard method on bifidobacterial enumeration describing how the method was developed and assessed for its performance based on selectivity, reproducibility and repeatabil-

ity. This method can be used as a roadmap for developing a microbiological standard method.

Bifidobacteria play an important role as probiotic microorganisms in fermented dairy products. Depending on the individual properties of the strains as well as on their density in the product and the survival conditions in the human intestinal tract, several beneficial effects have been demonstrated for certain bifidobacteria in numerous studies. In this context, selective viable count and bacterial viability have been identified as key criteria for determining the quality of probiotic products.

As far as bifidobacterial enumeration is considered, several methodologies have been proposed during the last two decades, however, with limited applicability due to laborious procedures and/or lacking selectivity. According to experience, the accompanying microflora often negatively influences the discrimination among the different groups of bacteria contained in a product. On this account, an IDF group of experts has elaborated a procedure for bifidobacterial enumeration, which is easy to perform and of high selectivity.

"Selective Enumeration of Bifidobacteria in Dairy Products: Development of a Standard Method" is the new Bulletin of IDF n. 411/2007. It can be ordered in electronic form via the IDF website at http: www.fil-idf.org

IDF GUIDING PRINCIPLES FOR TRACEABILITY/PRODUCT TRACING

Brussels, 5 April 2007 - IDF has just released thirteen guiding principles as a consensus view of the worldwide dairy sector on how to apply traceability/product tracing in the dairy production chain.

The dairy sector has taken the viewpoint that traceability/product tracing as a tool is not only relevant in the context of food inspection and certification system but may also be applied for industry-driven business reasons and is mainly concerned with food safety aspects.

Over the past years, a number of countries have introduced requirements for traceability/product tracing in national regulations as an effective means to contribute to both product safety and consumer confidence. The FAO/WHO Joint Food Standards Programme (Codex Alimentarius) has recently adopted Codex Principles for Traceability/Product Tracing as a Tool within a Food Inspection and Certification System.

IDF Guiding Principles for Traceability/Product Tracing, a new IDF publication is available free of charge from the IDF Internet homepage: http://www.fil-idf.org/WebsiteDocuments/412-2007.pdf

EVIDENCE-BASED BENEFICIAL HEALTH EFFECTS OF PRO-AND PREBIOTICS

Scientific reviews published in the supplement to the Journal of Nutrition

Brussels, 2 March 2007 - The evidence for claims on beneficial effects of functional foods is frequently called into question. In a collection of independent scientific articles published in a supplement to the Journal of Nutrition, leading nutrition scientists review the evidence on the effects and on the underlying mechanisms of pro-and prebiotics.

These scientific studies strengthen the positive influence of probiotics in maintaining and improving health and quality of life including preventive and therapeutic application possibilities on infectious

or antibiotic-associated diarrhea, influence on the intestinal flora, and the reduction of pathogenic mircoorganisms, metabolites, and enzymes as well as immunostimulating properties and the reduction of allergenic and atopic complaints. Recent investigation has also revealed beneficial effects on infectious intestinal diseases and irritable bowel syndrome.

The prevention or alleviation of different infectious diseases including common cold, other respiratory infections and also allergy has also been demonstrated. Very recently, there have been new findings that enable to understand how probiotics may strengthen immune defence and reduce allergic reactions.

Effects of probiotics and prebiotics, Supplement to the Journal of Nutrition, March 2007, Volume 137, Number 3S-II. http://jn.nutrition.org

HEALTH-ENHANCING MILK COMPONENTS: TECHNOLOGICAL ADVANCES AND HEALTH BENEFITS

Brussels, 25 April 2007 - This IDF publication provides insights into the latest and emerging scientific knowledge about the characteristics of major and minor bioactive component groups occurring naturally in milk, focusing on components with positive health attributes. Novel technologies for extraction of bioactive components from milk and product innovations based on these components are also presented.

The rapid scientific progress in the knowledge of milk bioactives has put a lot of pressure on the development of appropriate technologies for recovery, fractionation and application of such components in active form. To this effect, membrane separation processes have been developed and applied successfully to industrial scale.

Beneficial health effects of many milk or colostrum-derived bioactive components have been demonstrated in animal model studies and clinical trials in humans. Examples of documented health benefits delivered through such value-added products are also given in this IDF Bulletin. "Health-enhancing Milk Components: Technological Advances and Health Benefits", Extended abstracts of the conference held at the IDF World Dairy Congress in Shanghai, China, October 2006, is the new Bulletin of IDF n. 413/2007. It can be ordered in electronic form via the IDF website at http: www.fil-idf.org

GUIDE FOR AUTHORS

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The paper must also be submitted by e-mail or on a digital support (cd-rom or floppy disk). Indicate which word processor was used to generate the file and save the file also in format "Text only", DCA-RTF or ASCII, if you do not have programs for Macintosh; **graphics, pictures and diagrams must be saved at 300 dpi in TIF, JPEG, EPS or PICT formats** (not included in MsWord documents).

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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, article number or thesis fulfillment may be included.

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parentheses (only the initials in capital letters). If there are more than two authors, mention the first author and add *et al.*

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