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PAPER

PROPERTIES OF KASHAR CHEESE COATED WITH CASEIN AS A CARRIER OF NATAMYCIN

CARATTERISTICHE DEL FORMAGGIO KASHAR RICOPERTO CON CASEINA COME CARRIER DI NATAMICINA

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ABSTRACT

The effects of casein-coating on some properties of Kashar cheese and its effectiveness in carrying natamycin to prevent mould growth were studied. Five cheese groups were prepared: no-coating (A), vacuum-wrapped (B), coated with casein (C), coated with casein containing natamycin (D) and dipped in natamycin solution (E). While samples A and C had significant mould growth after only one week, no visible mould growth was detected on the surface of sample D for about one month. Sample

RIASSUNTO

È stato studiato l'effetto del rivestimento superficiale con caseina su alcune proprietà del formaggio Kashar e la sua efficacia, quale carrier di natamicina, nel prevenire lo sviluppo di muffa. Sono stati preparati cinque gruppi di formaggi: non trattati superficialmente (A), confezionati sotto vuoto (B), rivestiti con caseina (C), rivestiti con caseina contenente natamicina (D) e immersi in una soluzione di natamicina (E). Mentre i campioni A e C presentavano uno sviluppo di muffa significati-

- Key words: casein, coating, kashar cheese. natamycin -

E showed mould growth after just three weeks of ripening probably due to the presence of non-treated areas on the cheese surface. Sample B had no visible mould growth throughout the ripening period. Sensory evaluation and electrophoretic analyses showed no significant differences among cheese samples. These results show that casein-coating with natamycin can suppress mould growth for about one month without any adverse effects to cheese quality.

vo dopo una sola settimana, sulla superficie del campione D non era stato riscontrato nessun segno visibile di sviluppo di muffa per un periodo di circa un mese. Il campione E presentava uno sviluppo di muffa solo dopo tre settimane di stagionatura, probabilmente dovuto alla presenza sulla superficie del formaggio di aree non trattate. Il campione B non presentava alcun segno visibile di sviluppo di muffa durante l'intera stagionatura. Valutazioni sensoriali ed analisi elettroforetiche non mostravano differenze significative fra i diversi campioni di formaggio. Tali risultati dimostrano che il rivestimento superficiale caseinico con natamicina può evitare lo sviluppo di muffe per circa un mese senza alcun effetto negativo sulla qualità del formaggio.

INTRODUCTION

Milk and milk products are basic components of the human diet and among milk products, cheese holds an important place. Kashar (Kaşar) cheese is a traditional semi-hard Turkish cheese. According to Turkish standards, it is classified as either fresh or matured depending on the length of ripening time (TSI, 1999).

The composition of a cheese along with the environmental conditions during cheese-ripening often foster extensive mould development on the cheese surface, which considerably reduces quality. Mould growth is responsible for substantial economic losses in cheese-making and may even constitute a health risk due to the production of mycotoxins (KIERMEIER and ZEIER, 1975; IDF, 1977; BASILICO *et al.*, 2001). Coating the cheese with an anti-mould agent could be an effective way to protect cheese from mould growth during ripening. Vacuum-packaging can retard mould growth to a certain extent but it may adversely affect flavour development (STIPKOVA *et al.*, 1976; PENELSKI, 1979; NUNEZ *et al.*, 1986). Potassium sorbate and natamycin are currently used to prevent surface spoilage on cheddar cheese (FRANSSEN *et al.*, 2004). Natamycin destroys moulds at a concentration considerably lower than other known fungicides. It is generally applied to the cheese surface by dipping in or spraying with an aqueous dispersion containing 200-300 ppm of natamycin. The heavy molecular weight of natamycin (666 g mol^{-1}) and its conjugated double-bond structure makes it extremely insoluble in water and most food-grade solvents. The inability to apply natamycin in true solution results in untreated areas on the cheese surface where moulds may grow (MOLL, 1966; SHAHANI *et al.*, 1985). This problem could be overcome by using an edible protein coating as a carrier but the antifungal properties of natamycin could be reduced due

to the interaction of natamycin with the coating materials.

An edible film is generally defined as a thin layer of edible material formed on a food as a coating or placed on or between food components (KESTER and FENNEMA, 1986; GUILBERT, 1986; KROCHTA and DE MULDER-JOHNSTON, 1997). Edible films and coatings are typically derived from lipids, proteins, carbohydrates or various combinations of the three (KESTER and FENNEMA, 1986; BRANDENBURG *et al.*, 1993; YILDIRIM and HETTIARACHCHY, 1998). Edible films and coatings can be a carrier of food ingredients and additives. In some recent studies protein films were used as carriers of antimicrobials (OZDEMIR and FLOROS, 2003; FRANSSEN *et al.*, 2004). A slow release is desirable in this type of application in order to maintain a critical surface concentration of the preservative. The shelf-life of many products, including cheese, can be extended by using edible protein coatings to maintain a minimum level of antimicrobial compound on the surface of the product.

To the best of our knowledge, there has been no work reported on the effect of natamycin on Kashar cheese quality nor on the use of a casein-coating as a carrier of natamycin to retard mould growth on the cheese surface. Therefore, the purpose of this study was to evaluate the effectiveness of using a casein-coating containing natamycin to prevent mould growth, and to investigate its effects on some properties of Kashar cheese.

MATERIALS AND METHODS

Materials

Whole cows' milk was obtained from a commercial dairy. *Streptococcus salivarus* subsp. *thermophilus* E was used as the starter culture (Wiesby GmbH & Co. KG, Germany). Casein containing 85.3% (w/w) protein was prepared from skim milk by acid precipitation (GÜRSEL, 2001). The analytical grade reagents used were purchased from Merck (Darmstadt, Germany) and Sigma Chemical Co. (St. Louis, MO, USA).

Methods

Cheese production

Cheese production was carried out in a commercial dairy plant. The fat content of the milk was standardized to 2.9% (w/w) by using a separator for cheese production. One hundred fifty kg of milk were used for each replicate. The milk was pasteurized at 72°C for 40 seconds and then cooled to 33°C. The starter culture (10 mL kg^{-1}) and $CaCl_2$ (200 mg kg^{-1}) were added. When the pH of the milk reached to 6.30-6.40, rennet diluted in water (1:10) was added. After 1 h the curd was cut into 1 cm cubes and allowed to settle for 15 min. The curd was cooked by increasing the temperature from 33°C to 40°C over 35 min with an increase of 1°C every 5 min; the curd was gently agitated. After cooking, the curd was pressed for 2 h to drain out 105-110 kg of whey. The pressed cheese curd was fermented until the pH reached 5.10-5.20. The fermented curd was then cut into slices about 0.5 cm thick, and then machine-stretched in 8% (w/w) brine at 85°C. The stretched curd was put into cylindrical plastic molds that were open at both ends and turned after 30 min to form a flat surface. The fresh cheese was cooled at room temperature and the molds were removed. After that, the fresh cheese was allowed to develop its yellow colour for 48 h at 18-20°C. Each Kashar cheese wheel was approximately 250 g. The cheese wheels were divided into five groups (8 wheels per group) and the following treatments were applied: (A) no coating (control), (B) vacuum-wrapping with composite plastic film (Cryovac, W.R. Grace, Lausanne, Switzerland) with an oxygen permeability of $3.3x10^{-9}$ m^3 m^{-2} h^{-1} kPa^{-1} (at 23°C and 75% relative humidity) and a water va-

pour permeability of $2.5x10^{-6}$ kg m^{-2} h^{-1} kPa^{-1} (at 38 °C and 90% relative humidity) using Webomatic type: D463 (Webomatic Vacuum Packaging Systems, Bochum, Germany), (C) coating with casein solution, (D) coating with casein solution containing natamycin and (E) dipping in natamycin solution. The cheeses were then stored for 90 days at 6°C and a relative humidity of 85%. Kashar cheese samples were analyzed after 0, 30, 60 and 90 days of storage.

Coating solutions

The coating solutions were prepared by modifying the procedures of CHICK and HERNANDEZ (2002) and MEZGHENI *et al.* (1998) by dispersing acid casein (7.5%, w/w) in deionized water, adjusting the pH to 10.0 with 2 N or 0.1 N NaOH and heating at 75°C for 20 min. After cooling to 25°C, the pH was adjusted to 7.0 and then 2.5% (w/w) glycerol, 0.0125% (w/w) $CaCl_2$ and 0.07% (w/w) natamycin were added to the solution. The natamycin concentration was chosen as 0.07% to approximate the suggested concentration of 0.05% (JAY, 1998). Cheese wheels (group D) were coated by dipping them into this solution for 60 seconds. Group C was coated with the same solution but without natamycin and group E was coated by dipping the cheese wheels in a 0.07% (w/w) natamycin solution for 60 seconds. To provide uniform coating, one of the surfaces of the cheese wheels was dipped into the coating solution for 60 seconds and then left to dry for 2 h. Then, the other surface was dipped in the same way and allowed to dry.

Chemical analysis

The total dry matter content was determined by the gravimetric method (IDF, 1982), the salt content by the Mohr method (TSI, 1995) and the fat content by the Van-Gulik method (TSI, 1978). The pH was measured on the cheese slurry prepared from 10 g cheese and 6 mL distilled water (BSI, 1976).

Determination of nitrogen fractions

To determine the nitrogen fractions, a sodium citrate-cheese extract was prepared as described by GRIPON *et al.* (1975). An aliquot was taken from this extract to determine the total nitrogen (TN) which was estimated by the Kjeldahl method (IDF, 1993). The soluble nitrogen fraction (SN) was determined after precipitating out the insoluble nitrogen fraction (caseins) in the sodium citrate-cheese extract at pH 4.40. To indicate how much of the TN became soluble during storage, the ripening index (RI) was calculated by dividing SN by TN according to HALKMAN *et al.* (1994).

Urea-polyacrylamide gel electrophoresis (urea-page)

Polyacrylamide gel electrophoresis of cheese samples was carried out according to the method of ANDREWS (1983) with some modifications. The assays were carried out in a vertical vat (E90693, Consort, Turnhout, Belgium) using a Consort E815 power supply. The slab gels consisted of a 4% (w/w) stacking gel and a 12% (w/w) resolving gel. Runs were performed at 20 mA until the end of the stacking gel, followed by a current of 30 mA. Gels were stained with commassie brillant blue R250 (Sigma Chemical Co., St. Louis, MO, USA).

Mould growth

Mould growth on the surface of the cheese was evaluated visually. The cheese samples were inspected for the appearance of mould. The first appearance of mould marked the end of treatment effectiveness.

Sensory analysis

Cheese samples, aged for 90 days, were scored by 8 trained panellists, 3 women and 5 men whose mean age was 32. The cheese samples were presented in such a manner that the panellists could not identify the treatment. The attributes of characteristic odour, surface

appearance, cross-sectional appearance, texture and taste were evaluated. The intensity of each attribute was rated on a scale from 1 to 5. The maximum total score was 25, with odour contributing 5, surface appearance 5, cross-sectional appearance 5, texture 5, and taste 5. The cheese samples were equilibrated at 20°C for an hour before being scored. The samples were presented to panellists as 15-20 g pieces of cheese; water and bread were provided for them to clean their palates between samples.

Statistical analysis

The data obtained from three replications (the cheeses were produced and coated for each replicate and all the physico-chemical analyses were performed twice for each replicate) were analyzed to determine the effect of coating type and storage time using the general linear model procedure of SAS (1995) to determine differences between treatment means. Pairwise comparison of all treatment means was performed using the Least Significant Difference (LSD) procedure with a significance level of $p < 0.05$.

RESULTS AND DISCUSSION

Physico-chemical characteristics

Changes in the mean pH values of the cheeses during the 90 days of ripening are given in Fig. 1. The pH of the samples increased slightly until the 30th day of ripening followed by a slight decrease. The increase in pH could be attributed to peptide bond cleavage and the formation of new groups, resulting in an increased buffering capacity of the cheese. An increase in pH during storage was noted by METZGER *et al.* (2000), GUO *et al.* (1997) and GUINEE *et al.* (2002) in Mozzarella cheese. There were no significant differences in pH between samples A, C, D, and E at the end of the ripening period ($p > 0.05$). However, sample

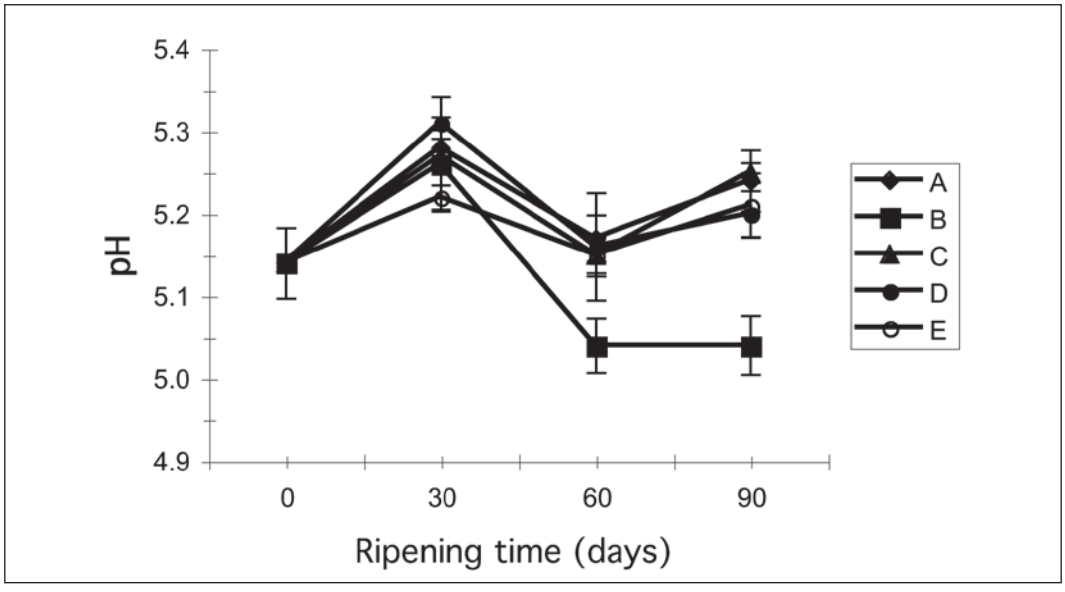

Fig. 1 - Changes in the pH values of Kashar cheese during ripening. (A) No coating (control), (B) vacuum-wrapped with plastic film, (C) coated with casein solution, (D) coated with casein solution containing natamycin, and (E) dipped in natamycin solution. Error bars represent standard deviations.

B was significantly different ($p < 0.05$). This may be related to the higher water and lower salt in moisture contents of sample B.

Due to the drying of the cheeses, the dry matter content of samples A, C, D, and E increased until the 60th day of storage ($p < 0.05$) after which it did not change significantly ($p > 0.05$) (Fig. 2). By the end of the ripening period, samples A, C, D, and E had attained the required total dry matter content of at least 60% (w/w). This indicates that the casein-coating did not restrict moisture loss. The total dry matter content of sample B was nearly constant ($p > 0.05$) throughout the ripening period due to the fact that vacuum-wrapping with plastic film restricted moisture loss. The total dry matter percentages were comparable to those published by previous authors on Kashar cheese (HALKMAN *et al.* 1994; ATASEVER *et al.* 2003).

Similar to the total dry matter content, the fat content in all the cheeses except for sample B increased with increasing ripening time ($p < 0.05$). The lowest fat content was observed in sample B (27.3%, w/w) and by the end of the ripening period it was significantly different from samples A (31.3%, w/w), C (30.4%, w/w), D (30.9%, w/w) and E (30.4%, w/w) ($p<0.05$).

Salt in moisture concentration is one of the major factors in the ripening process, which in the end determines the final quality of the product. High salt levels are related to a delay in the ripening process, while low levels of salt lead to excessive proteolysis, which results in bitterness and other undesirable attributes. The salt in moisture content of all the samples increased with increasing ripening time except for sample B (Fig. 3), which had the lowest value and was significantly different from the other

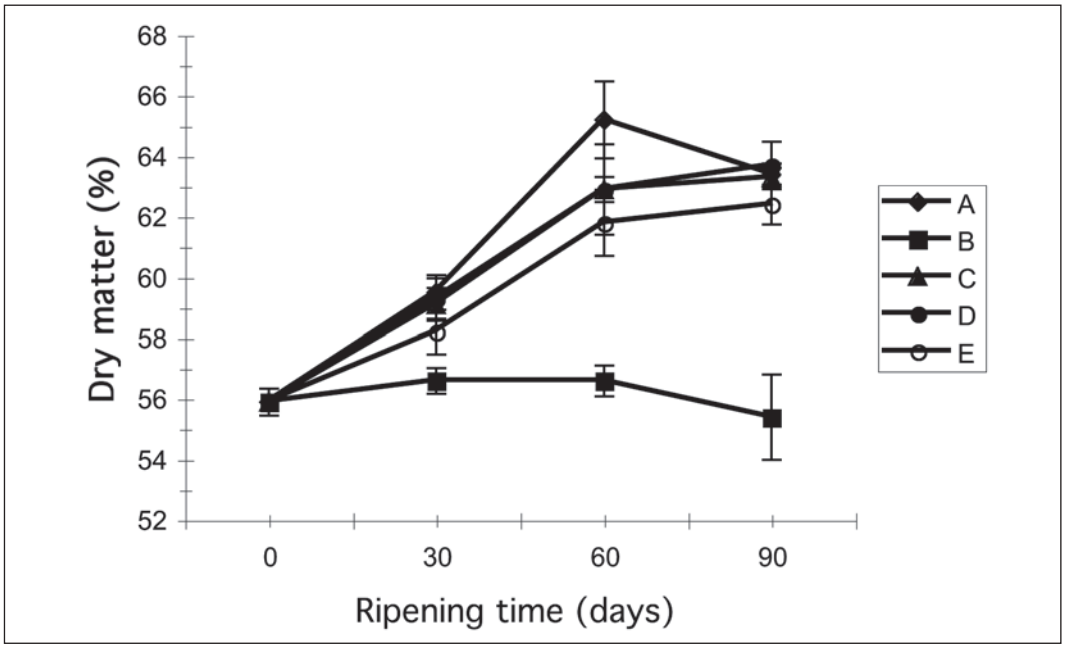

Fig. 2 - Changes in the dry matter content of Kashar cheese during ripening. (A) No coating (control), (B) vacuum-wrapped with plastic film, (C) coated with casein solution, (D) coated with casein solution containing natamycin, and (E) dipped in natamycin solution. Error bars represent standard deviations.

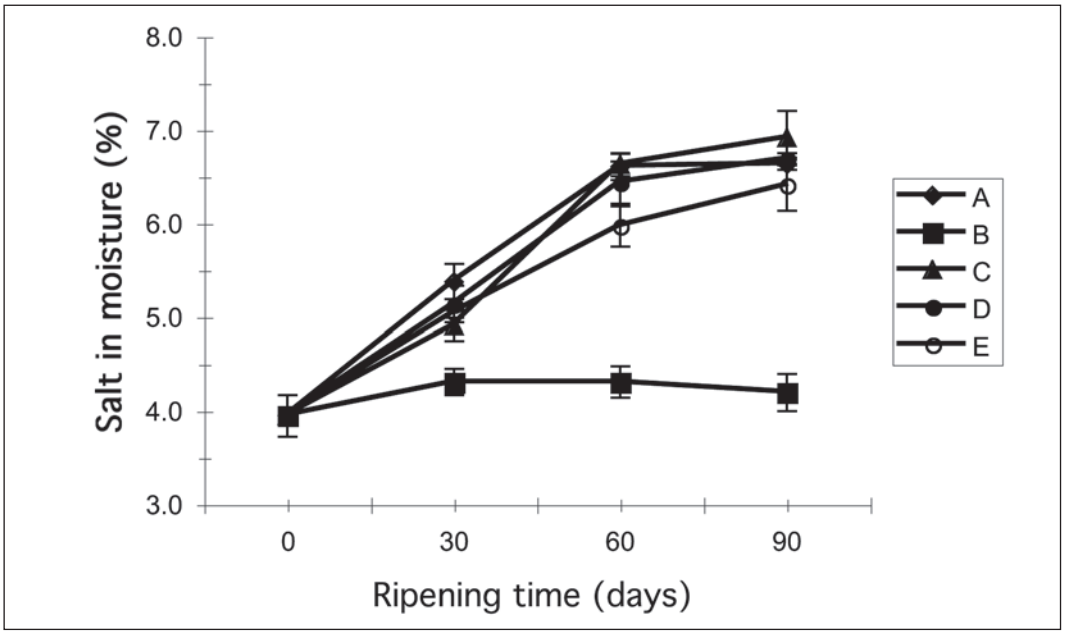

Fig. 3 -. Changes in the salt in moisture content of Kashar cheese during ripening. (A) No coating (control), (B) vacuum-wrapped with plastic film, (C) coated with casein solution, (D) coated with casein solution containing natamycin, and (E) dipped in natamycin solution. Error bars represent standard deviations.

samples at the end of ripening ($p < 0.05$). This showed that there was no relationship between the salt in moisture content of the samples and casein-coating (with or without natamycin).

The total nitrogen (TN) content in the cheeses increased with increasing ripening time (Fig. 4); the values were similar to those reported by HALKMAN *et al.* (1994) and ATAMER *et al.* (1997). Samples A, C, D, and E showed a significant increase in TN content with time ($p < 0.05$) due to the drying of the cheeses. There were no statistical differences in the TN content of sample B throughout the ripening period ($p > 0.05$) since vacuum-wrapping with plastic film restricted moisture loss. Casein-coating with or without natamycin (samples C and D) did not affect the TN content of cheeses compared with the control (A) during the 90-day ripening period ($p > 0.05$).

Level of proteolysis

In order to determine if coating types affected the level of proteolysis, soluble nitrogen (SN) was determined and electrophoretic analyses were carried out. The formation of SN compounds during cheese ripening is an index of the rate and extent of proteolysis, in that, it is an indicator of casein hydrolysis brought about by the action of rennet, starter culture enzymes and milk proteases present at the beginning of ripening. During ripening, proteases hydrolyze paracasein to polypeptides and peptidases break down these polypeptides to smaller peptides and free amino acids (KOSIKOWSKI and MISTRY, 1997). These compounds are soluble in water. The highest SN value was obtained for the casein-coated sample C at the end of ripening (Fig. 5), but there were no significant differences among the SN values of the cheeses ($p > 0.05$). The SN con-

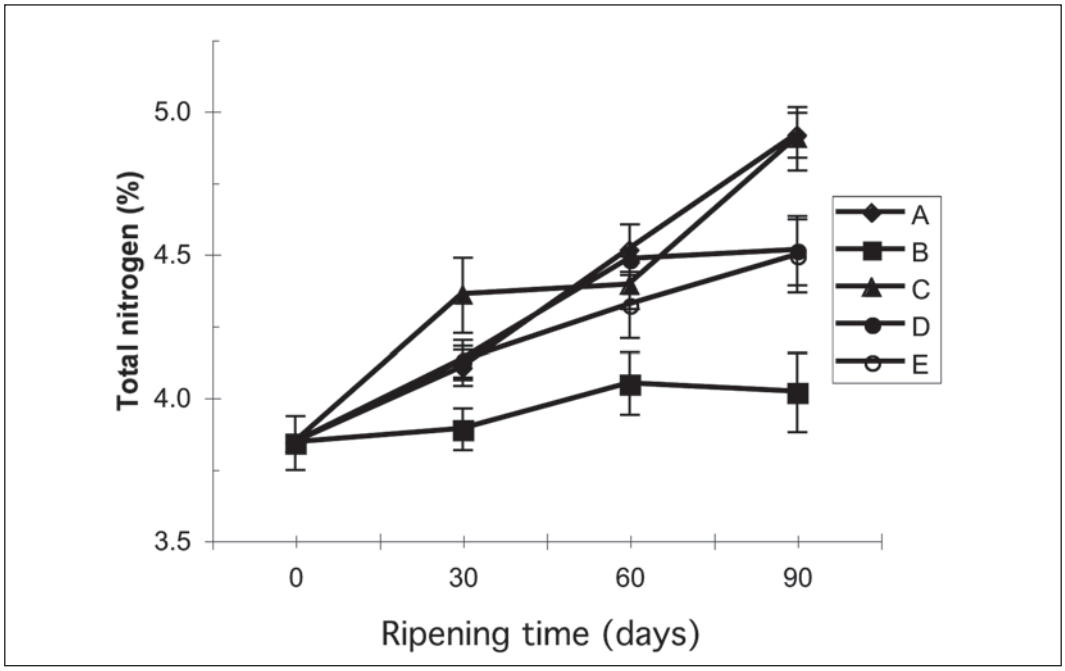

Fig. 4 - Changes in the total nitrogen content of Kashar cheese during ripening. (A) No coating (control), (B) vacuum-wrapped with plastic film, (C) coated with casein solution, (D) coated with casein solution containing natamycin, and (E) dipped in natamycin solution. Error bars represent standard deviations.

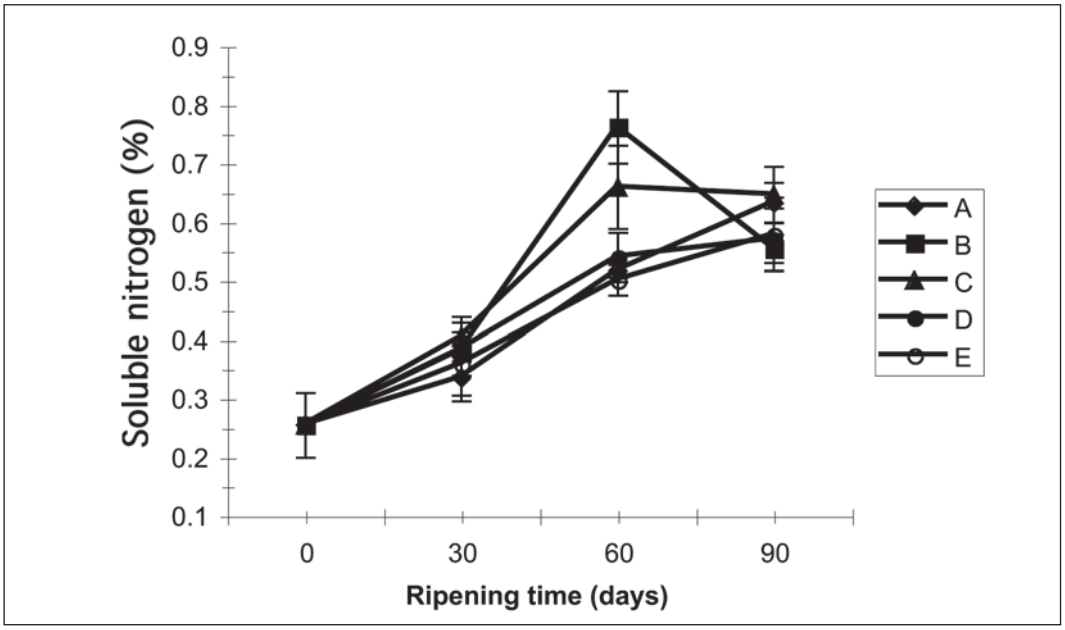

Fig. 5 - Changes in the soluble nitrogen content of Kashar cheese during ripening. (A) No coating (control), (B) vacuum-wrapped with plastic film, (C) coated with casein solution, (D) coated with casein solution containing natamycin, and (E) dipped in natamycin solution. Error bars represent standard deviations.

tent in the various cheese samples was similar to those reported by HALKMAN *et al.* (1994) and ATAMER *et al.* (1997) for Kashar cheese.

The ripening index (RI) of the samples increased steadily until day 60 of ripening (Fig. 6). The highest RI was obtained for sample B, although there were no significant differences between the cheeses at the end of ripening ($p > 0.05$). This was probably due to the high water and low salt in moisture content of sample B, which resulted in enhanced starter activities. KELLY *et al.* (1996) found that the RI in cheddar-type cheese increased with decreasing NaCl concentration.

The urea-PAGE electrophoretogram (Fig. 7) for the cheeses (replicate 1) shows that α_{s1}-1-casein, a breakdown product of α_{s1}-casein, γ-casein, fragments of C-terminal residues of β-caseins and other peptides were formed after 90 days of ripening. By the end of ripening, the quantities of α_{s1}-1-casein, γ-casein and other peptides were quite similar in all the cheeses. There were no significant differences among the samples with respect to the breakdown products. According to these results, including the SN/TN values, after 90 days of ripening there were no significant differences in casein hydrolysis among the various cheeses. This indicates that the casein-coating with or without natamycin did not adversely affect casein hydrolysis.

Mould growth

Visual evaluation of cheeses confirmed that the control (A) and casein-coated cheeses (C) had extensive mould growth with almost the entire surface covered with mould colonies after only one week of ripening. The cheeses coated with natamycin (E) showed mould growth after only three weeks of ripening. This was probably due to the presence of non-treated areas on the cheese surface. The inability to apply natamycin in true solution leaves void or non-treated areas on the cheese surface which may allow

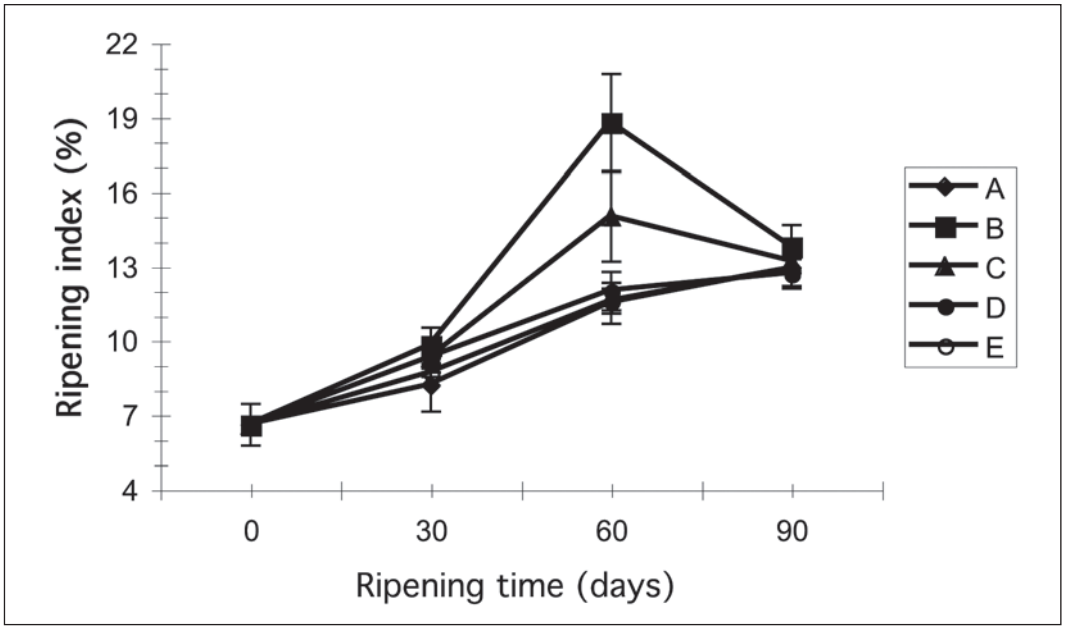

Fig. 6 - Changes in the ripening indexes of Kashar cheese during ripening. (A) No coating (control), (B) vacuum-wrapped with plastic film, (C) coated with casein solution, (D) coated with casein solution containing natamycin, and (E) dipped in natamycin solution. Error bars represent standard deviations.

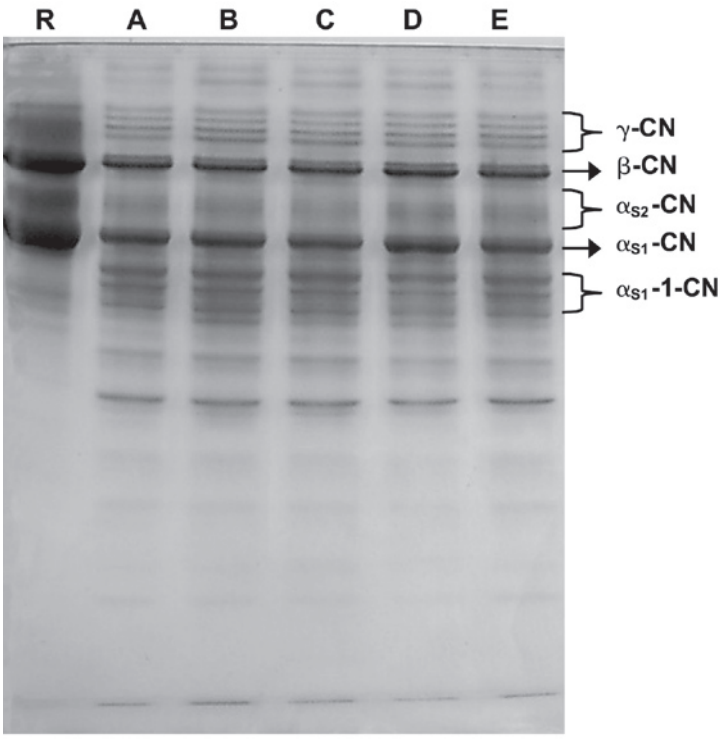

Fig. 7 - Urea-page electrophoretogram of Kashar cheese during ripening. Lane R: reference bovine milk casein, lane A: no coating (control), lane B: vacuum-wrapped with plastic film, lane C: coated with casein solution, lane D: coated with casein solution containing natamycin, and lane E: dipped in natamycin solution.

moulds to grow (MOLL, 1966; SHAHANI *et al.*, 1985). While casein-coating (with natamycin) (D) retarded visible mould growth for about one month, vacuum-wrapping (B) suppressed visible mould growth throughout the ripening period. The results showed that casein may have prevented the formation of non-treated areas, and did not significantly reduce the effectiveness of the natamycin. In order to retard visible mould growth for longer than one month, the natamycin concentration and/or the number of coatings should be increased.

Sensory evaluation

The results of the sensory evaluation of the cheeses after 90 days of ripening are given in Fig. 8. There were no statistical differences among the cheeses with respect to odour, surface appearance, cross-sectional appearance, texture, taste and total scores ($p > 0.05$). The casein-coated cheese (C) received the highest surface appearance score (3.8 out of 5), and the panellists did not perceive any significant differences among the cheeses. Sample D had the highest texture score (4.2), while vacuum-wrapped cheese (B) had the lowest (2.9). Sample B had the lowest odour and taste scores (4.0). The control (A) and casein-coated cheeses (C and D) received the same total score, 20.6 as opposed to 18.6 for the vacuum-wrapped cheese (B), and 20.0 for the natamycin-coated cheese (E). These results showed that casein-coating did not adversely affect any of the sensorial properties of the cheese.

The results of this study show that the total dry matter, fat, salt, and total nitrogen content values of cheese D were similar to those of cheeses A, C and E, but were higher than those of cheese B after 90 days of ripening. No visible mould growth was detected on the surface of cheese D for about one month. There were no significant differences in the sensory evaluation among the cheeses. Electrophoretic analysis and the SN/TN values showed that casein hydrolysis was similar in all the cheese samples after 90 days of ripening. All the results indicate that the use of casein-coating with natamycin can suppress mould growth for about one month without any

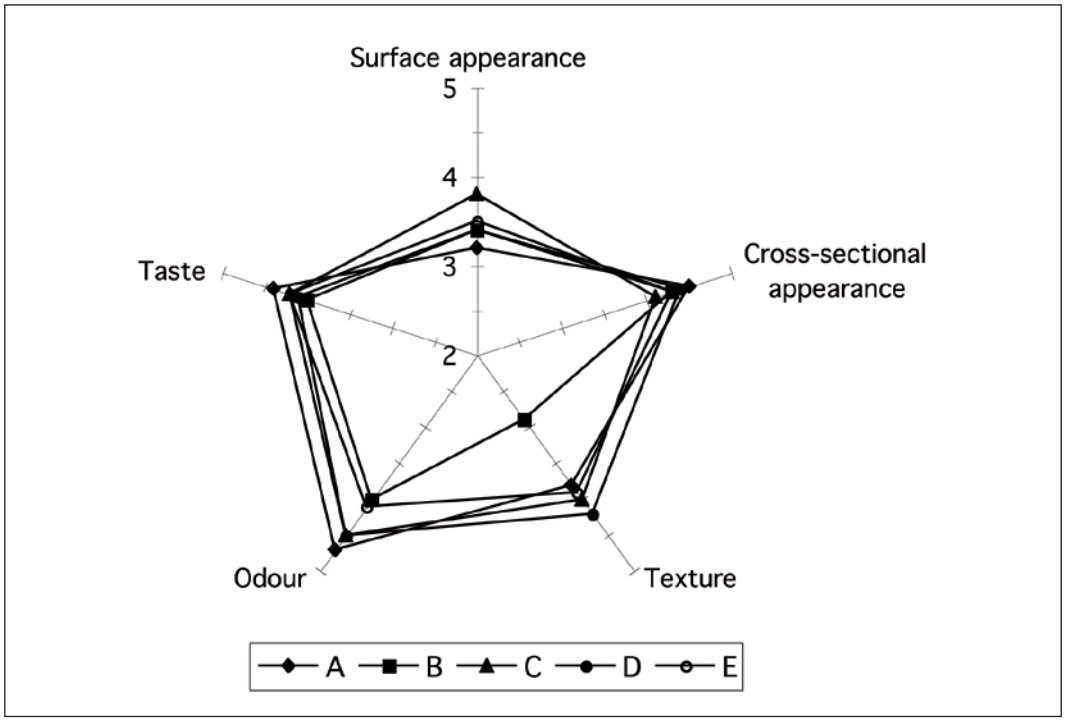

Fig. 8 - Results of sensory evaluation on Kashar cheese after 90 days of ripening. (A) No coating (control), (B) vacuum-wrapped with plastic film, (C) coated with casein solution, (D) coated with casein solution containing natamycin, and (E) dipped in natamycin solution.

adverse effects to cheese quality. In order to prevent visible mould growth for longer than one month, further research is needed to determine the most suitable natamycin concentration and coating thickness.

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PAPER

EFFECT OF HEAT TREATMENT AND STARTER CULTURE ON PROTEOLYSIS AND LIPOLYSIS OF KULEK CHEESE DURING RIPENING

EFFETTO DEL TRATTAMENTO TERMICO E DELLE COLTURE STARTER SULLA PROTEOLISI E SULLA LIPOLISI DEL FORMAGGIO KULEK DURANTE LA STAGIONATURA

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ABSTRACT

The effect of heat treatment and starter culture on proteolysis and lipolysis of Kulek cheese (raw milk cheese without starter culture, raw milk cheese with starter culture, and heat-treated milk cheese with starter culture) during ripening was examined. Heat-treated milk cheese with starter had lower pH, and higher titratable acidity and salt than the raw milk cheeses. Water-soluble and soluble nitrogen in 12% trichloroacetic acid levels were the highest in the cheeses made from heat-treat-

RIASSUNTO

È stato valutato l'effetto del trattamento termico e delle colture starter sulla proteolisi e sulla lipolisi del formaggio Kulek (formaggio da latte crudo senza colture starter, formaggio da latte crudo con colture starter e formaggio ottenuto da latte trattato termicamente con colture starter) durante la stagionatura. Il formaggio ottenuto da latte trattato termicamente con aggiunta di colture starter presentava un pH inferiore, una maggiore acidità titolabile ed un maggiore contenu-

- Keywords: cheese, heat treatment, lipolysis, proteolysis, starter culture -

ed milk with starters. Conversely, soluble nitrogen in 5% phosphotungstic acid level was the highest in the cheeses made from raw milk with starters. Cheese made from heat-treated milk with starter had the lowest residual α_s- and β-casein content and the highest γ-caseins and peptides. Heat treatment increased lipolysis significantly. Starter culture addition to the raw milk cheeses did not change the lipolysis.

to in sali rispetto ai formaggi ottenuti da latte crudo. I livelli di azoto solubile in acqua ed in una soluzione al 12% di acido tricloroacetico risultavano maggiori nei formaggi ottenuti da latte trattato termicamente con aggiunta di starters. Diversamente, il livello di azoto solubile in una soluzione al 5% di acido fosfotungstico risultava maggiore nei formaggi prodotti da latte crudo con starters. I formaggi prodotti da latte trattato termicamente con starters avevano il contenuto minore di α_s- and β-caseina e il maggiore in γ-caseine e peptidi. I trattamenti termici aumentavano significativamente la lipolisi. L'aggiunta di colture starter ai formaggi prodotti da latte crudo non influenzava la lipolisi.

INTRODUCTION

Kulek cheese, a ripened acid-curd cheese made with the addition of rennet, is one of the most important cheese varieties in Turkey, primarily in the Black Sea Region. It is produced in a way similar to acid curd-uncreamed Cottage cheese, but it has more compact curd structure because it is consumed after a ripening period of 3 months (DERVISOGLU and YAZICI, 2001). The traditional manufacturing process and some properties of this cheese were described by YAZICI *et al.* (1998). A standardized method for production of Kulek cheese without starters was reported by DERVISOGLU and YAZICI (2001).

Traditionally, Kulek cheese ripens for 1-3 months in goat skin, wood or clay containers (YAZICI *et al.*, 1998). Therefore, organoleptical and rheological characteristics of Kulek cheese vary according to the area and the season of manufacture. DERVISOGLU and YAZICI (2001) improved the microbiological, biochemical and sensory properties of Kulek cheese by controlling the manufacturing conditions (4°-7°C and 85-90% relative humidity). Cheese ripening may be accomplished by (1) rennet or rennet substitute; (2) indigenous milk enzymes; (3) starter proteinases and peptidases; (4) enzymes from secondary starters, and (5) non-starter bacteria (FOX *et al.*, 1993). Cheese starter cultures are used among others to modify cheese organoleptic characteristics. However, the textural and sensory characteristics of cheese made from pasteurized milk with commercial starter cultures may not be similar to their traditional counterparts made without starter culture. The use of an appropriate commercial starter culture may result in a market product with standard characteristics.

The effect of milk pasteurization temperature (ALBENZIO *et al.*, 2001; BEUIVER *et al.*, 1997; GRAPPIN and BEUVIER, 1997; MENDIA *et al.*, 2000;

O'REILLY *et al.*, 2001, 2002; PSONI *et al.*, 2005; RYNNE *et al.*, 2004; SOMERS and KELLY, 2002; XANTHOPOULOS *et al.*, 2000) and increasing pasteurization temperature (EL-KOUSSY *et al.*, 1997; GREEN, 1990; GUINEE *et al.*, 1998; KELLY, 1999; LEBEUF *et al.*, 1998; MARSHALL, 1986) on different properties of cheese including proteolysis and the use of starter culture have been reported in the literature. Heat treatment, 72°-75°C for 15-30 s, may modify several properties of milk such as elimination of milk microorganisms, activation-inactivation of indigeneous milk pro-enzymes and enzymes, denaturation of serum proteins and modification of the rennetability of milk and activity of starter acid bacteria (GRAPPIN and BEUVIER, 1997). LAU *et al.* (1991) reported that cheese made from pasteurized milk had lower soluble nitrogen and free amino acid contents and higher concentrations of amino acids such as asparagines, serine and proline than cheese made from raw milk. Although pasteurization temperatures higher than 72°C have usually not been used in cheese making because of their negative effects on curd formation and syneresis, the positive effect on cheese yield due to increased moisture content and more effective recovery of whey proteins has stimulated researchers to study the effect of high heat treatment on cheese making (RYNNE *et al.*, 2004). It has been found that increasing pasteurization temperature significantly increased the levels of whey protein denaturation, moisture and non-expressible serum; it influenced the degradation profiles of α_{s1}- and β-caseins, and did not significantly affect the levels of pH 4.6-soluble nitrogen (RYNNE *et al.*, 2004).

This study was undertaken to determine the effect of a commercial starter culture on proteolysis during the ripening of Kulek cheese produced from raw and heat-treated milk.

MATERIALS AND METHODS

Materials

Whole cow milk was used to manufacture cheese at a cheese factory (Uzunoglu Food Company, Samyo, Samsun-Turkey). Animal rennet was obtained from Mayasan Company (Istanbul, Turkey), $CaCl_2$ from Solvay (Rosignano, Italy), and a commercial cheese culture (FRC-65- mixture of *Lactococcus lactis* subsp. *lactis, Lactococcus lactis* subsp. *cremoris, Streptococcus thermophilus* and *Lactobacillus delbruckii* subsp. *bulgaricus)* were used (Peyma Chr. Hansen's Laboratory, Istanbul, Turkey). The chemicals used in this study were Sodium Dodecyl Sulphate (J.T. Baker, The Netherlands), TEMED, Tris-base (hydroxymethylaminomethane), 2 β-mercaptoethanol, ammonium persulfate, acrylamide, N' N'-Bis-methyleneacrylamide, Commassie blue R-250, α- and β-casein standards (Sigma Aldrich Chem., Steinheim, Germany), glycerol, bromophenol blue, glycine (Merck, Darmstadt, Germany), trichloroacetic acid (TCA; Carlo Erba, Rodano, Italy) and phosphotungstic acid (PTA; Merck, Darmstadt, Germany).

Cheesemaking

The procedure outlined in Fig. 1 was followed to prepare three types of cheese: (A) raw milk cheese without starter culture; (B) raw milk cheese with starter culture, and (C) heat-treated milk cheese with starter culture. The whole procedure was repeated three times.

Chemical analysis

Dry matter, salt content and titratable acidity (TA) were determined according to BRADLEY *et al.* (1992). pH was measured using a pH meter (inoLab, Weilheim, Germany) according to the Official Methods of the AOAC (1992). Lipolysis

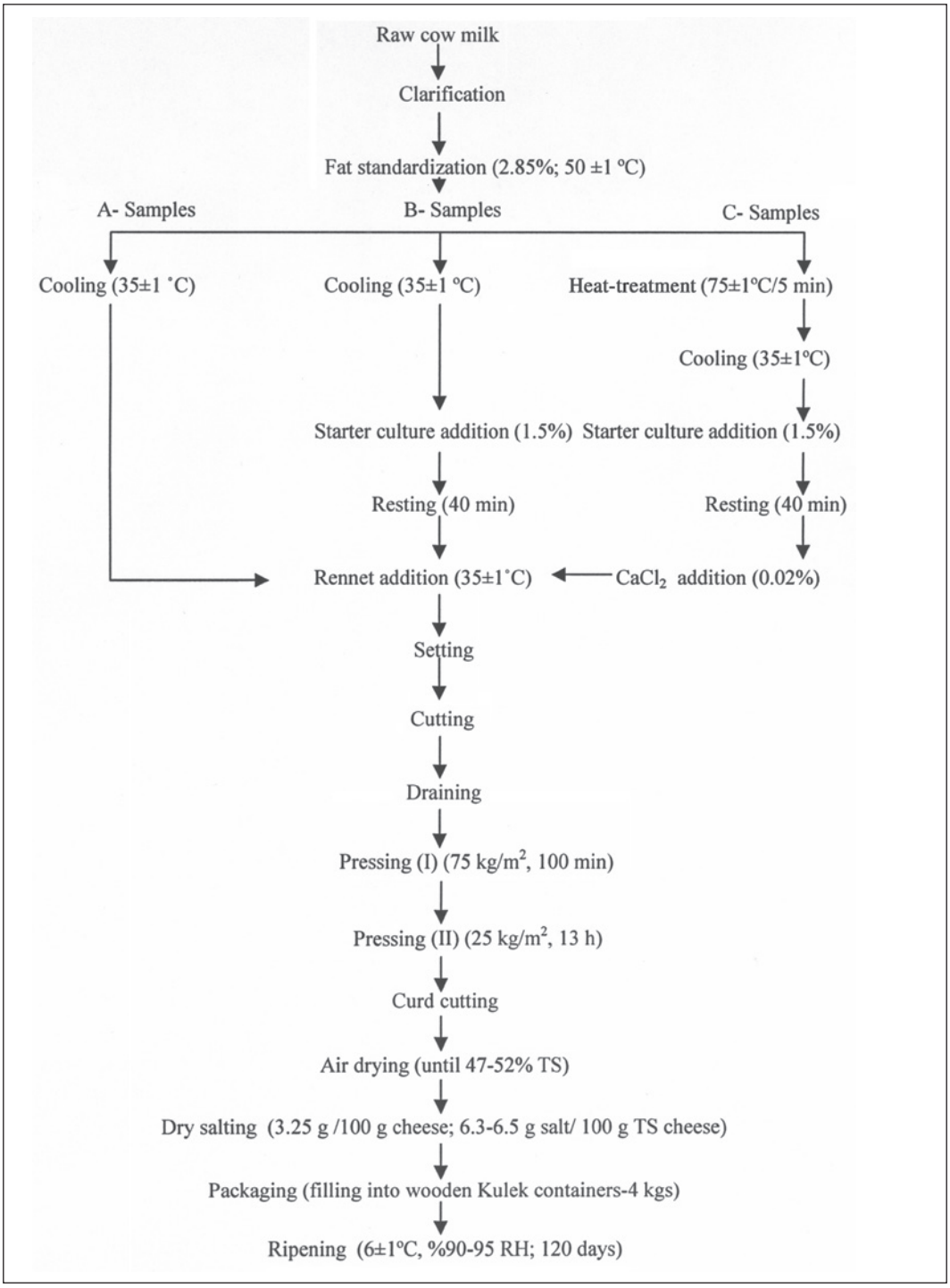

Fig. 1 - Outline of Kulek cheese production.

as Acid Degree Value (ADV) was measured according to the method of SALJI and KROGER (1981) and BRADLEY *et al.* (1992).

Nitrogen fraction analysis

The total nitrogen (TN) and nitrogen fractions were determined by the Kjeldahl method (BRADLEY *et al.*, 1992). Water-soluble cheese extracts were prepared according to the procedure described by BUTIKOFER *et al.* (1993). Soluble nitrogen in 12% trichloroacetic acid (12% TCAN) was determined as described by KATSIARI *et al.* (2001). Soluble nitrogen in 5% phosphotungstic acid (5% PTAN) was determined as described by FREITAS *et al.* (1997). All WSN, 12% TCA and 5% PTA values are expressed as the percentage of the total nitrogen content of the cheese.

Preparation of cheese proteins and electrophoresis

The procedure used by YAZICI and DERVISOGLU (2003) was followed for preparing the cheese samples and SDS-PAGE gels as well as for the electrophoresis running conditions, and for evaluating the electrophoretograms. The only modification was that the final protein concentration in the sample preparation was 0.3% instead of 0.25%.

Statistical analysis

Statistical analysis of data was performed by one-way ANOVA using Minitab statistical software (MINITAB, 1996). Linear regression analysis was also done to correlate the casein fractions with each other and with pH, TA, salt in dry matter, acid degree value and nitrogen fractions (KITCHELL and SAW, 1975). Significant ($P<0.05$) differences between means were identified using the Duncan multiple range test (O'MAHONY, 1986).

RESULTS AND DISCUSSION

The average composition of the cheese made from heat-treated milk with starter culture at the first day of ripening was 48.9% TS, 3.3% total nitrogen, 6.4 % salt in dry matter, 39.5% fat in dry matter and 4.4% ash (data not shown). The initial pH values of raw milk cheese were significantly higher than heat-treated milk cheese ($P<0.05$). The pH values of the B- and C-samples did not significantly change throughout the ripening period, while in A-samples the pH decreased slightly at the end of the ripening period. TA values of samples increased significantly during ripening. Heat and starter culture treated cheese had higher TA values than the samples made from raw milk with or without starters ($P<0.05$). The ripening time did not significantly affect the salt content of the samples, but the salt content of the C-samples was significantly higher than A- and B-samples ($P<0.05$).

The levels of WSN/TN, 12% TCAN/TN and 5% PTAN/TN increased significantly throughout the ripening time (Table 1). The water-soluble fraction includes whey proteins, high-, medium- and low-molecular-weight peptides and free amino acids (MACEDO and MALCATA, 1997). In general, the highest WSN/TN levels were found in the cheese made from the pasteurized milk with starter. Moreover, significant ($P<0.05$) differences between means at 1, 30 and 60 days of ripening were recorded. It also shows that starter culture decreased significantly the levels of WSN of raw milk cheeses at 30 and 60 days of ripening. This may have been due to elimination of the bacteria of the native lactic-acid flora in the raw milk (ELISKASES-LECHNER *et al.*, 1997). No difference was observed in WSN/TN contents between raw and pasteurized cheeses by BEUVIER *et al.* (1997) in Swiss-type cheese and by MCSWEENEY *et al.* (1993) in Cheddar cheese. In contrast, raw milk cheeses had higher WSN/

Table 1 - Some biochemical properties of Kulek cheeses during ripening*.

Variable	Cheese**	Ripening time (d) 1	30	60	90	120
WSN/TN	A	7.43±0.13$^{a\,E}$	12.39±0.53$^{b\,D}$	14.21±0.6$^{a\,C}$	16.07±0.13$^{a\,B}$	17.2±0.15$^{a\,A}$
	B	7.47±0.05$^{a\,D}$	11.45±0.1$^{c\,C}$	13.19±0.23$^{b\,B}$	16±0.24$^{a\,A}$	16.3±0.38$^{a\,A}$
	C	6.93±0.07$^{b\,D}$	13.62±0.42$^{a\,C}$	14.6±0.12$^{a\,C}$	15.68±0.3$^{a\,B}$	17.1±1.16$^{a\,A}$
12 % TCAN/TN	A	3.04±0.25$^{b\,E}$	7.25±0.26$^{a\,D}$	8.47±0.31$^{b\,C}$	10.3±0.15$^{b\,B}$	11.52±0.67$^{b\,A}$
	B	3.32±0.08$^{b\,E}$	7.21±0.24$^{a\,D}$	7.99±0.18$^{c\,C}$	10.83±0.22$^{b\,B}$	13.18±0.14$^{a\,A}$
	C	4.25±0.04$^{a\,E}$	7.54±0.05$^{a\,D}$	9.49±0.04$^{a\,C}$	11.65±0.4$^{a\,B}$	12.41±0.35$^{a\,A}$
5% PTAN/TN	A	1.33±0.13$^{a\,D}$	1.66±0.15$^{a\,C}$	2.07±0.12$^{b\,B}$	2.95±0.15$^{b\,A}$	2.99±0.2$^{b\,A}$
	B	1.37±0.09$^{a\,C}$	1.66±0.1$^{a\,C}$	2.7±0.04$^{a\,B}$	3.54±0.24$^{a\,A}$	3.66±0.33$^{a\,A}$
	C	1.33±0.06$^{a\,C}$	1.56±0.14$^{a\,C}$	2.08±0.23$^{b\,B}$	2.83±0.15$^{b\,A}$	3.12±0.39$^{ab\,A}$

*A-E: Means with same letters in a row within the same category are not significant at P>0.05.
a-c: Means with same letters in a column within the same category are not significant at P>0.05.
** A: Cheese made from raw milk without starter culture.
B: Cheese made from raw milk with starter culture.
C: Cheese made from heat-treated milk with starter culture.

TN content in St Paulin (BEUVIER, 1990) and in Canestrato Pugliese cheese (ALBENZIO *et al.*, 2001) than in pasteurized cheeses.

In general, 12% TCAN/TN values were the highest (P<0.05) in C cheeses during ripening. These results are in contrast with the data reported for cheeses made from pasteurized milk by others (GAYA *et al.*, 1990; LAU *et al.*, 1991; BEUVIER *et al.*, 1997), possibly due to the manufacturing process and the microbial characteristics of fresh raw and heat-treated milk cheeses. Kulek cheese is traditionally air-dried after pressing and cutting the curd on a polyethylene-cloth in a layer of 4-5 cm and occasionally total solids reach the level of 47-52%. It was observed that cheeses made from heat-treated milk needed considerably longer time to reach this level than raw milk cheeses. The longer drying time may contribute to the differentiation of the microflora of the cheeses. Another reason might be the extent of the heat treatment which was much higher in our study than the reported pasteurization temperatures. In general, the levels of 12% TCA increased significantly throughout the ripening period (P<0.05).

In general, the values of 5% PTAN/TN in the samples made from raw milk with starters were higher than A and C cheeses and the differences were significant (P<0.05) at 60, 90 and 120 days of ripening. In general heat-treated milk cheeses with starter had similar 5% PTAN/TN levels as raw milk cheeses. GRAPPIN and BEUVIER (1997) reported that 5% PTAN/TN contents were always lower in pasteurized milk cheese than in raw milk cheese, but according to ROSENBERG *et al.* (1995), there was no difference between the two. In a similar study, MENDIA *et al.* (2000) observed that raw milk cheese with starter had the highest total free amino acid content which is consistent with the our findings.

Fig. 2 shows the electrophoretograms for the casein fractions of α_s-, β-, and γ and other peptides of Kulek cheeses during storage. The degradation of α_s- and β-casein and the accumulation of γ-caseins and other peptides increased with increasing storage time, especially after 60 days of storage. In order to quantify

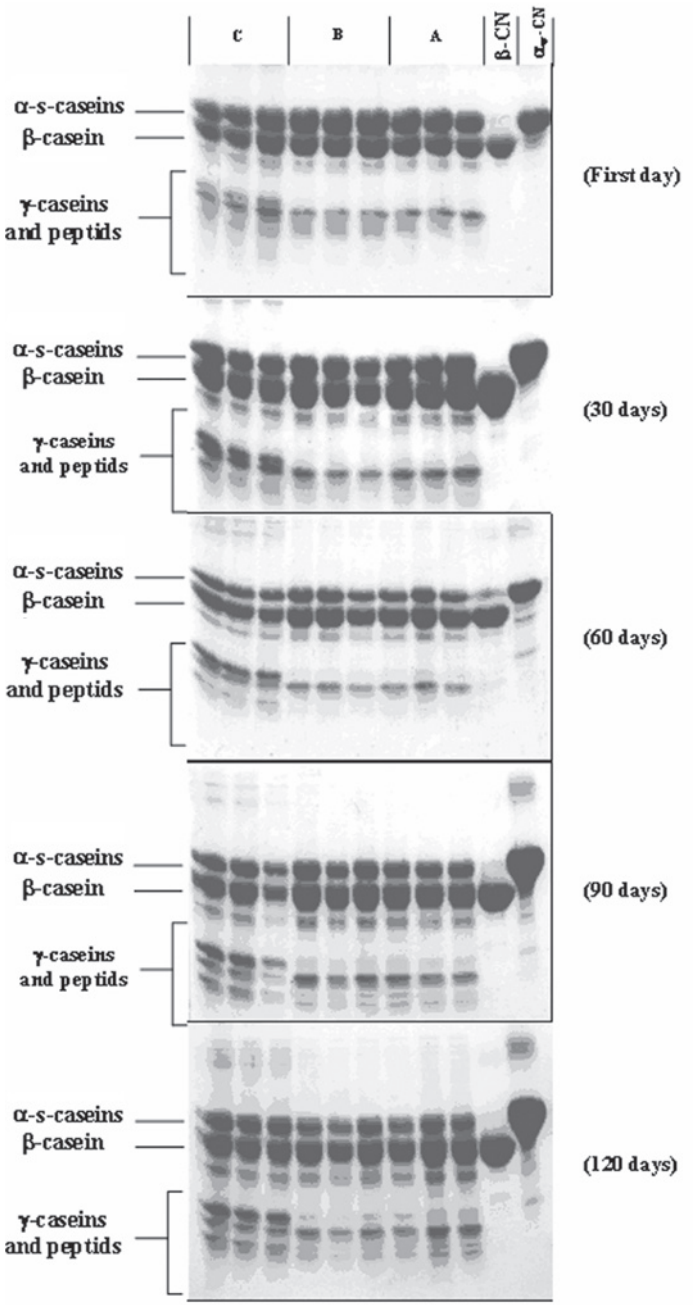

Fig. 2 - SDS-PAGE profiles of Kulek cheeses during ripening*.
A: Cheese made from raw milk without starter culture.
B: Cheese made from raw milk with starter culture.
C: Cheese made from heat-treated milk with starter culture.
α-s-CN and β-CN: standards.

proteolysis in Kulek cheese, densitometric evaluation was done after SDS-PAGE and the mean relative proportions of the casein fractions of three trials are presented in Table 2. Cheese made from raw milk had significantly higher residual α_s-casein content than cheeses made from heat-treated milk ($P<0.05$) until the 60th day of ripening. Although hydrolysis of α_s-casein during ripening was approximately 52.5% for both A- and B- samples, in C-samples it was considerably lower (41%). This indicated that starter culture addition to raw milk did not have a great effect on the hydrolysis of α_s-casein during ripening. However, it should be noted that the level of residual α_s-casein of C-samples was the lowest at the first day of ripening although all of the cheeses were made from the same milk with the same procedure. The ratio of casein hydrolysis was significantly higher compared to the values found in Kulek cheese made from pasteurized milk without starter culture addition (DERVISOGLU and YAZICI, 2001). Our results showing that heat-treated milk cheese had lower levels of α_s-casein are consistent with the findings of GAYA *et al.* (1990) and BEUVIER (1990). The lower level of residual α_s-casein in these cheeses was attributed to the greater retention of rennet (GRAPPIN and BEUVIER, 1997).

β-casein did not change significantly in A- and B-cheeses throughout ripening but it decreased significantly in C-cheeses at the end of the ripening period. This may be explained by the activity of indigenous proteolytic enzyme, plasmin, for which β-casein is the most susceptible subsrate (FOX *et al.*, 1993). Pasteurization increases plasmin activity in milk by inactivation of plasmin inhibitors or by increasing the rate of activation of plasminogen (GRUFFERTY and FOX, 1988). The lower level ($P<0.05$) of β-casein in heat-treated milk cheese can be attributed to the activation of plasminogen and/or the increase in plasmin activity following heat treatment.

γ-caseins and peptides increased significantly during ripening. Since γ–caseins are degradation products of β-casein (MAYER, 1996, 1997; HASSAN and EL-DEEB, 1988; MARSHALL *et al.*, 1988), changes of γ-caseins during ripening cor-

Table 2 - Relative contents of casein fractions of Kulek cheeses during ripening (% of total scanning density)*.

Casein fraction	Cheese**	Ripening time (d)				
		1	30	60	90	120
α-s-casein	A	39.4±1.6a A	28.8±1.65a B	28.4±2.73a B	22.47±1.87a C	18.7±0.49a D
	B	41.6±0.88a A	27.4±1.59a B	28.1±1.35a B	21.7±0.47a C	19.8±1.53a C
	C	31.7±1.96b A	24±1.51b B	24±0.77b B	19.7±2.72a C	18.7±2.06a C
β-casein	A	38.1±2.54a B	40.4±0.55a AB	42.1±1.7a A	41.7±1.86a AB	41.6±2.1a AB
	B	37.5±1.04a B	41±1.73a A	41.5±0.98a A	40.4±1.57a A	39.5±1.92a AB
	C	35±1.36a A	34.2±0.96b A	33.8±1.74b A	31.2±0.9b B	29.9±0.35b B
γ-caseins and peptides	A	22.5±1.6b A	30.7±1.38b B	29.6±2.73b B	35.9±2.9b A	39.7±2.51b A
	B	21±0.2b D	31.5±1.55b C	30.4±0.45b C	37.8±1.84b B	40.7±2.36b A
	C	33.3±1.44a C	41.8±2.4a B	42.1±1.67a B	49.1±3.43a A	51.3±2.36a A

A-D: Means with same letters in a row within the same category are not significant at $P>0.05$.
a-b: Means with same letters in a column within the same category are not significant at $P>0.05$.
** A: Cheese made from raw milk without starter culture.
B: Cheese made from raw milk with starter culture.
C: Cheese made from heat-treated milk with starter culture.

Table 3 - Acid degree values (mg KOH/g fat) of Kulek cheeses during ripening*.

Cheese**	Ripening time (d)				
	1	30	60	90	120
A	0.52±0.11a D	0.91±0.14b C	1.14±0.06b B	1.33±0.16b B	1.63±0.08b A
B	0.36±0.08a D	0.57±0.21b CD	0.74±0.21b BC	0.91±0.18c B	1.37±0.07b A
C	0.57±0.08c C	2.1±0.25b B	2.43±0.34a B	4.42±0.15a A	4.79±0.5a A

*A-C: Means with same letters in a row within the same category are not significant at P>0.05.
a-c: Means with same letters in a column within the same category are not significant at P>0.05.
** A: Cheese made from raw milk without starter culture.
B: Cheese made from raw milk with starter culture.
C: Cheese made from heat-treated milk with starter culture.

related with the degradation of β-casein in the C-samples.

The degree of lipolysis measured as acid degree value (ADV) increased significantly during ripening (Table 3). The cheese made from heat-treated milk with starter had significantly higher ADV values compared with the raw milk cheeses. It has been reported that pasteurized milk cheese always has a lower level of free fatty acids than raw milk cheese (GRAPPIN and BEUVIER, 1997). Therefore, these results are not in agreement with the literature. This may be related to the manufacturing process of Kulek cheese and possible higher microbial load of curd from heat-treated milk may be higher after air drying than that of raw milk cheeses A and B because B cheese took a longer time to dry although no microbial counts are reported in this study.

There was a positive and significant correlation between α_s- and β-casein fractions because both fractions break down during ripening. The increase in the γ-caseins and peptides is due to the degradation of α_s- and β-casein fractions. There was a highly significant and negative correlation between the acid degree value and the α_s- and β-casein fractions ($P<0.05$). This indicates that lipolysis and proteolysis proceeded at the same time throughout ripening.

CONCLUSIONS

Heat treatment of milk prior to Kulek cheese manufacture influences proteolysis and lipolysis during cheese ripening. Heat treatment and addition of starter contributed to higher titratable acidity, WSN/TN, 12% TCAN/TN, lipolysis, and β-casein hydrolysis and lower pH and residual α_s-casein compared to raw milk cheese samples during ripening. Starter culture addition to the raw milk did not have any significant effect on the casein degradation in the cheese compared to raw milk cheeses without starter culture addition, but affected pH and lipolysis.

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PAPER

FREE FATTY ACIDS IN "PROVOLA DEI NEBRODI", A HISTORICAL SICILIAN CHEESE

GLI ACIDI GRASSI LIBERI DELLA "PROVOLA DEI NEBRODI", FORMAGGIO STORICO SICILIANO

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ABSTRACT

The FFA content of Provola dei Nebrodi during ripening and in different cheesemaking seasons was investigated. Twenty-eight components from C_4 to C_{20} were quantified and myristic, palmitic, stearic and oleic acids were the major FFA in all the samples analysed. The level of FFA after 90 days of ripening ranged from 651.52 to 840.45 mg 100 g^{-1} of cheese, depending on the cheesemaking season. There were significant differences ($P \leq 0.05$) between the average content of even-car-

RIASSUNTO

Gli Autori riportano il contenuto di acidi grassi liberi (FFA) della Provola dei Nebrodi, formaggio tipico Siciliano a pasta filata, in campioni a diversa maturazione durante un intero anno di produzione. Sono stati identificati acidi grassi da C_4 a C_{20}; tra questi, gli acidi miristico, palmitico, stearico ed oleico erano i più rappresentati. Il livello di FFA dopo 90 giorni di maturazione era compreso tra 651,52 e 840,45 mg 100 g^{-1} di formaggio; l'analisi statistica dei dati ottenuti ha evidenziato differenze significa-

- Key words: cheese ripening, free fatty acids, "Pasta filata" Sicilian cheese, Production season, Provola dei Nebrodi -

bon number fatty acids during ripening and within the production seasons. The high content of FFA and the significant differences observed are discussed and correlated with the artisanal cheese production technique.

tive ($P \leq 0,05$) per gli acidi grassi a numero pari di atomi di carbonio relative alla maturazione ed al periodo di produzione. Il contenuto elevato di FFA e le differenze riscontrate vengono discusse e correlate con la tecnica artigianale di produzione.

INTRODUCTION

Free fatty acids (FFA) composition is an important marker for cheese characterisation. FFA strongly contribute to cheese flavour which is one of the most important quality criteria of fresh and aged cheese. Since consumer acceptability of cheese depends mainly on its sensory qualities, the flavour is determinant.

Short chain fatty acids (SCFA), butyric (rancid, sour, repellent), caproic and caprylic acids (cheesy, rancid, sweat-like) are the most significant examples of cheese flavour notes (AKIN, *et al.*, 2003; HA and LINDSAY, 1991; URBACH, 1997; NAJERA *et al.*, 1994). Fatty acids with more than 12 carbon atoms have less influence on flavour, even though they are present in a fair amount in cheese (SABLE and COTTENCEAU, 1999). Medium chain free fatty acids (MCFA), especially branched ones, are responsible for mutton fat flavour, while long chain (LCFA) are perceived as oily, waxy and soapy (ASHURST, 1999). FFA also act as precursors of many aromatic compounds such as alcohols, aldehydes, methylketones, lactones, esters and thioesters (McSWEENEY and SOUSA, 2000).

Free fatty acids are released mainly from milk lipids through the lipolytic activity of lipases. Lipolysis in cheese is well documented and it is known that lipases may originate from milk, indigenous or added micro-organisms (STEAD, 1986), rennet and, if utilised, exogenous lipases. Furthermore short chain fatty acids can also be produced by nonenzymatic oxidation of long and/or unsaturated fatty acids, especially of linoleic and linolenic acids (MACEDO and MALCATA, 1996). Protein and carbohydrate metabolism by bacteria are other possible sources for free fatty acids in ripened cheese: branched chain fatty acids derive from reductive and oxidative deamination of amino acids (CONTARINI *et al.*, 1989); acetic and propionic acids and butyric acids, to some extent, arise from microbial fermentation of lactate (McSWEENEY and SOUSA, 2000).

Several authors have studied the FFA composition of different types of cheese and significant changes during ripening have been observed (WOO *et al.*, 1984a; FARKYE and FOX 1990; MACEDO and MALCATA, 1996; POVEDA *et al.*, 1999; PAVIA *et al.*, 2000; MALLATOU *et al.*, 2003; ADDIS *et al.*, 2005; MOATSOU *et al.*, 2004; GEORGOLA *et al.*, 2005; VIRTO *et al.*, 2003). Due to its increase during ripening, the FFA content has been suggested as a ripening index, even if it has been reported to be less useful than proteolytic and glycolytic indicators (FARKEY and FOX, 1990; WOO and LINDSAY, 1984b; WOO *et al.* 1984a). In contrast, there are fewer reports of changes during time of the year (MACEDO and MALCATA, 1996; CHAVARRI *et al.*, 1999; COLLOMB *et al.*, 2003).

"Provola dei Nebrodi" is classified as a historical and typical Sicilian dairy product; it is artisanally produced in several areas in the Nebrodi Mountains, near Messina, Sicily, Italy. It is a "pasta fila-

ta" cheese derived from full-fat raw cow milk with the addition of farmhouse rennet paste. Salting takes place in saturated brine. Well known for its size (4-5 kg), it has a peculiar oblong, pear-like shape, with a short neck and a small round top; it can be eaten fresh (one month) or aged (3-4 months) since it is the only "Provola" subjected to ripening.

As part of a larger research project, which aims at the chemical characterisation of typical Sicilian cheeses (VERZERA *et al.*, 2004; ZIINO *et al.*, 2005), the FFA composition of "Provola dei Nebrodi", and its dependence on ripening and on the cheesemaking season were investigated. Samples produced in winter, spring, summer, and autumn were analysed immediately after cheesemaking and during ripening. Statistical analysis gave interesting information on the similarities and differences in the FFA composition during the ripening time and throughout different cheesemaking seasons.

MATERIALS AND METHODS

Cheesemaking

The cows grazed on wet pasture from April to June (spring) and from October to December (autumn), and on dry pasture from July to September (summer), and with hay and concentrated feed from January to March (winter).

A flowchart for the production of "Provola dei Nebrodi" is reported in Fig. 1. "Provola dei Nebrodi" was manufactured from evening and morning milk; the evening milk was refrigerated (4 °C) overnight and mixed with the morning milk of the following day. Milk was coagulated with kid or lamb rennet paste without addition of starter culture. The native microflora of the raw milk provided the acid-producing bacteria for cheesemaking. The rennet was prepared by the same cheesemaker in the following way: the abomasus of suckling lamb or kid

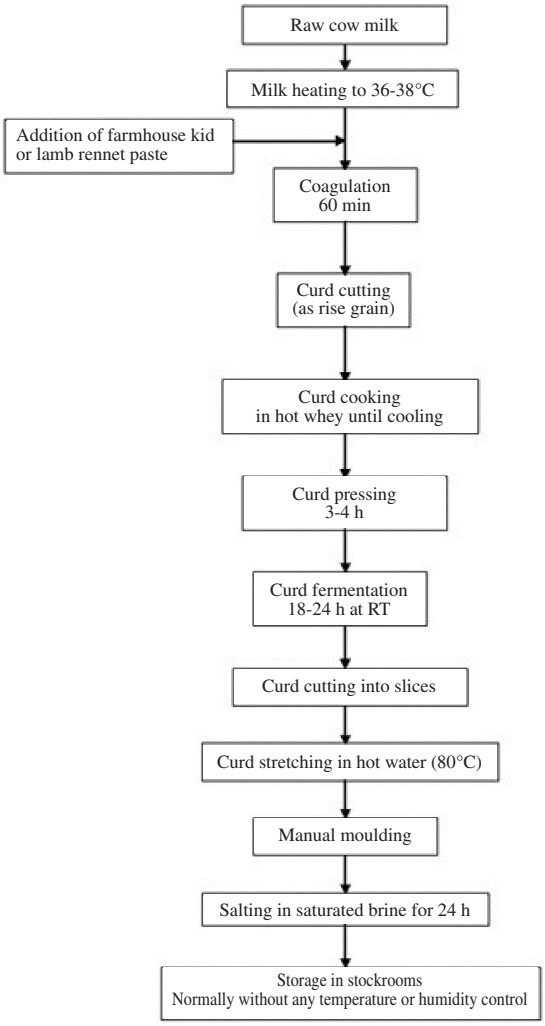

Fig. 1 - Flowchart of "Provola dei Nebrodi" production.

was salted, dried until 13-15% of water content remained and then ground up with the addition of salt in the ratio 1:5 w/w salt/ rennet paste. The amount of rennet used for the milk coagulation was in the range of 20-40 g 100 L^{-1} of milk. After coagulation, the curd was broken to the size of a rice grain, and the whey drained. The curd was then covered with the hot whey (70-80°C) for about 3 h until it cooled to room temperature. It was then removed from the vat and left to

drain and ripen (18-24 h depending on room temperature).

After maturation, the curd was cut into thick slices (ca. 1 cm), stretched in hot water (80°C) and manually moulded. Finally, it was salted in saturated brine (24 h, RT).

The cheese was ripened in farmhouse stockrooms, tied with a cord across a wooden rafter, usually without any temperature or humidity control.

Sampling

The cheese samples analysed were provided by three producers. Sampling was carried out in the months of January, April, July and October in the year 2003. For each producer, in each month four successive cheese productions were followed, within the same week. For each production one sample was analysed immediately, whereas three samples were stored away and analysed after 7, 30 and 90 days of ripening. The ripening took place in each producer's farmhouse stockrooms. Each cheese, prior to the analyses, was divided in two portions and each portion was analysed in duplicate.

Compositional analysis

Samples of cheese were analysed for moisture, protein and fat content according to the International Dairy Federation (IDF) methods. The moisture content was determined gravimetrically after oven drying at 102°C (IDF, 1982), protein content by the Kjeldahl method (IDF, 1993) and fat content gravimetrically (IDF, 2004). The pH was measured with an electrode for solids connected to an Inolab pH level 1 standard pH meter (WTW GmbH, Weilheim, Germany).

Free fatty acid analysis

Free fatty acids were extracted according to the method described by CONTARINI *et al.* (1989): 20 g of cheese were homogenised with anhydrous Na_2SO_4, suspended in ethanol acidified to a pH < 2 and extracted with three aliquots of diethyl ether. The organic extracts were combined and potassium hydroxide (0.1 N) was added to make the solution basic. The FFA were then extracted with distilled water; the aqueous solution was dehydrated at 105 °C, the FFA methylated with sulphuric acid (96%)/methyl alcohol 3:1 v/v and analysed by gas chromatography.

A Shimadzu 17A gas chromatograph with a flame ionisation detector (FID) (Shimadzu Italia s.r.l., Milano, Italy) was used to analyse the FFA. Injector temperature: 200 °C; injection mode: splitless; column: RTX-225 (Chrompack Italia s.r.l., Milano, Italy), 30 m, 0.32 mm i.d., 0.25 μm film thickness; oven temperature: 50 °C held for 2 min, then increased to 100 °C at a rate of 10 °C min^{-1} and to 200 °C at a rate of 3 °C min^{-1}. Carrier gas: helium at constant pressure of 10 psi; detector temperature: 210 °C; integrator: Hewlett Packard HP3394.

FFA were identified by comparing the retention time of each known standard with that of each unknown. The quantitative determination was carried out using internal standards, $C_{7:0}$ and $C_{13:0}$ (Sigma-Aldrich, Milan, Italy), which were absent in the sample analysed or present as traces.

Statistical analysis

The SPSS 11.5 (2002) software package (SPSS Inc., Chicago, IL, USA) was used for statistical treatment of the data. One way analysis of variance (ANOVA) was applied to the data to determine the presence of significant differences (Duncan test, significant level $P \leq 0.05$) in the FFA content during the ripening time and throughout the cheesemaking seasons.

RESULTS AND CONCLUSION

The average values for moisture, protein and fat contents, and pH of all the samples analysed, grouped according to the ripening time and the production season, are shown in Table 1. Average values for fat and protein were between 26.35-27.95 and 31.66-33.02 g 100 g^{-1} of cheese at 90 days of ripening; fat content values were similar to average values of Provolone and, Caciocavallo Pugliese and Silano cheeses, but protein contents were higher than the average values of the same cheeses, as reported by BATTISTOTI and CORRADINI (1993) and GOBBETTI *et al.* (2002). Moisture content decreased during ripening and reached the final values of 32.73-36.37 g 100 g^{-1} of cheese after 90 days of ripening, as expected for a semi-hard cheese. pH values varied from 5.05 to 5.38 in all the samples analysed, and they are similar to those of other types of "pasta filata" cheeses (GOBBETTI *et al.*, 2002).

Table 2 shows the average concentrations (mg 100 g^{-1} cheese) of individual FFA for all the analysed samples grouped according to the ripening time and the production season. Saturated, monounsaturated and polyunsaturated fatty acids from C_4 to C_{20} were quantified and the long chain fatty acids constituted most of the fat fraction, followed by medium and short chain. Myristic, palmitic, stearic and oleic acids were the major FFA in all the samples analysed, which is typical of bovine milk fat (ALAIS, 1984).

Table 1 - Chemical composition (g 100 g^{-1} cheese) of "Provola dei Nebrodi" throughout ripening in different production seasons.

Production season	Ripening time/days	Moisture g 100 g^{-1} $\bar{X}^d$	pH $\bar{X}$	Fat g 100 g^{-1} $\bar{X}$	Protein g 100 g^{-1} $\bar{X}$
spring	0	42.92 a	5.21 A	21.97 cB	28.08 bB
	7	41.31 ab	5.38 A	23.90 cb	28.67 b
	30	38.76 b	5.09	24.17 bAB	29.12 b
	90	32.73 cB	5.15	26.35 a	33.02 a
summer	0	43.61 a	5.21 A	21.94 bB	28.64 bAB
	7	40.33 b	5.32 AB	22.76 b.	28.80 b
	30	39.64 b	5.35	21.04 bB	29.64 b
	90	33.24 cB	5.22	26.58 a	31.94 a
autumn	0	40.80 a	5.08 B	24.80 bA	29.94 abA
	7	40.67 a	5.05 C	23.99 b	28.99 b
	30	37.49 b	5.15	26.38 aA	28.87 b
	90	34.01 cAB	5.30	27.95 a	31.66 a
winter	0	42.43 a	5.14 B	22.36 bB	27.41 bB
	7	42.05 a	5.10 CB	23.26 b	30.01 b
	30	40.44 a	5.10	24.29 ab AB	28.94 b
	90	36.37 bA	5.24	27.27 a	32.03 a

[d] mean value: 12 cheeses (4 cheeses/ producer), for each cheese two samples, each sample in duplicate.
Means with different letters (a-c) in the same column are significantly different from each other for the ripening time ($P \leq 0.05$). Means with different capital letters (A-C) in the same column are significantly different from each other for the production season ($P \leq 0.05$) at the same ripening time. Letters are not reported where significant differences did not result ($P > 0.05$).

Table 2 - Free Fatty Acid concentration (mg 100 g^{-1} cheese) in "Provola dei Nebrodi" throughout ripening in different production seasons.

		Free Fatty Acids[d]										
Production Season	Ripening time/days	$C_{4:0}$	$C_{5:0}$	$C_{6:0}$	$C_{8:0}$	$C_{9:0}$	$C_{10:0}$	Total SCFA	$C_{11:0}$	$C_{12:0}$	$iC_{14:0}$	$C_{14:0}$
Spring	0	7.94 cA	0.10	12.52 bB	8.73 bB	0.15	21.29 dB	50.74	0.27	19.85 bB	0.58 b	44.58 bC
	7	12.18 bAB	0.11	15.04 bB	11.20 bA	0.21	26.42 cA	65.16	0.27	18.07 bB	0.55 b	43.44 bC
	30	14.95 aB	0.09	17.03 aB	14.68 aB	0.20	30.67 bAB	77.62	0.25	23.74 bC	0.62 bB	58.62 bC
	90	16.23 aB	0.11	17.14 aB	15.83 aB	0.32	40.85 aB	90.48	0.45	41.48 aAB	0.82 aB	74.10 aB
Summer	0	8.41 cA	0.10	15.17 bA	11.74 bA	0.14	26.87 dA	62.43	0.30	22.33 bAB	0.73 b	54.57 bB
	7	14.05 bA	0.14	17.36 bA	11.65 bA	0.19	31.80 cA	75.19	0.27	20.41 bB	0.66 b	54.37 bB
	30	20.23 aA	0.08	20.85 aA	16.13 aA	0.31	39.94 bA	97.54	0.47	39.58 abA	0.78 bB	78.20 abAB
	90	24.14 aA	0.15	23.15 aA	18.53 aA	0.31	45.17 aA	111.45	0.50	39.35 aAB	1.07 aB	87.14 aB
Autumn	0	5.66 bB	0.09	9.52 bB	7.80 bB	0.12	21.63 cB	44.80	0.25	24.73 bA	0.86 b	67.43 bA
	7	9.92 bB	0.05	12.76 bB	8.61 bB	0.17	22.02 cB	53.54	0.26	25.54 bA	0.71 b	52.22 bB
	30	18.95 aAB	0.11	14.82 aB	12.53 aB	0.18	27.31 bB	73.89	0.33	27.63 abB	1.05 bA	85.40 abA
	90	21.89 aB	0.14	16.60 aB	12.39 aB	0.19	30.10 aC	81.32	0.37	37.33 aB	1.28 aA	107.76 aA
Winter	0	9.21 bA	0.14	11.96 bB	9.11 bB	0.21	21.74 cB	52.37	0.37	22.13 bAB	0.51 b	58.21 bB
	7	11.78 bAB	0.12	14.05 bB	9.77 bAB	0.29	22.12 cB	58.12	0.37	24.42 bAB	0.49 b	62.82 bA
	30	13.65 aB	0.17	16.03 aB	12.08 aB	0.28	27.63 bB	69.85	0.52	27.91 bB	0.58 bB	65.44 bBC
	90	15.66 aB	0.15	16.11 aB	12.42 aB	0.46	36.36 aB	81.16	0.77	44.22 aA	0.62 aB	86.02 aB

Table 2 (continued).

Free Fatty Acids[d]													
Production Season	Ripening time/days	$iC_{15:0}$	$aiC_{15:0}$	$C_{15:0}$	$C_{14:1}$	$iC_{16:0}$	$C_{16:0}$	$C_{16:1w7}$	Total MCFA	$iC_{17:0}$	$aiC_{17:0}$	$C_{17:0}$	$C_{17:1}$
Spring	0	1.22 B	2.45	4.96 B	0.51 b	1.19 b	115.02 bC	1.70 cB	192.31	0.40	0.79 B	1.69 B	0.37
	7	1.11	2.27 B	4.57 B	0.51 bB	1.00 bB	102.40 bC	1.50 cB	175.69	0.33 B	1.30 B	1.94	0.28
	30	1.42 B	2.79 B	5.67 B	0.52 bB	1.58 bB	125.60 bC	1.80 bB	222.59	0.58 B	1.49 B	2.42 B	0.77
	90	1.90 B	3.48 B	7.43 B	3.54 aA	1.83 aB	186.92 aC	3.97 aB	325.92	0.69 B	1.81 B	2.84 C	1.26
Summer	0	1.48 AB	2.77	6.28 A	0.38 b	1.54 b	153.60 bAB	2.06 bAB	246.04	0.35	1.46 AB	2.42 AB	0.37
	7	1.33	2.54 B	5.46 AB	0.62 bB	1.38 bB	142.57 bB	2.72 bAB	232.33	0.59 B	1.50 B	2.12	0.35
	30	1.72 AB	3.14 B	6.12 B	0.92 bA	1.76 bB	161.35 bB	3.34 bAB	297.37	0.58 B	1.83 B	2.51 B	0.76
	90	2.31 AB	4.39 AB	9.25 AB	1.68 aB	2.52 aAB	261.25 aB	4.65 aAB	446.10	1.59 B	2.47 B	3.66 B	0.94
Autumn	0	1.73 A	2.64	4.97 B	0.52 b	1.50 b	154.15 cAB	1.69 bB	260.46	0.27	2.40 A	2.98 A	0.28
	7	1.41	3.44 A	6.30 A	0.83 bA	1.96 bA	180.22 cA	1.99 bB	274.89	1.21 A	2.18 A	2.26	0.49
	30	2.31 A	4.58 A	8.48 A	0.60 bB	2.61 bA	238.36 abA	2.91 bAB	374.27	1.46 A	2.67 A	3.97 A	0.73
	90	3.11 A	6.17 A	11.21 A	1.64 aB	3.35 aA	295.35 aA	4.24 aAB	471.81	1.61 A	3.87 A	5.13 A	0.81
Winter	0	1.20 B	2.78	5.44 AB	0.71 b	1.21 b	182.99 bA	2.50 bA	278.06	0.69	1.12 B	2.81 A	0.56
	7	1.09	2.51 B	5.16 B	0.98 bA	1.56 bAB	160.75 bAB	3.23 bA	263.37	0.43 B	1.79 B	2.63	0.64
	30	1.41 B	3.47 B	7.96 A	0.90 bA	1.54 bB	180.08 bB	3.19 bA	292.99	0.77 B	2.57 A	3.78 A	0.69
	90	1.49 B	3.26 B	6.90 B	2.11 aAB	1.97 aB	234.55 aB	5.63 aA	387.53	0.54 B	2.06 B	3.19 B	0.93

Table 2 (continued).

Free Fatty Acids[d]										
Production Season	Ripening time/days	$C_{18:0}$	$C_{19:0}$	$C_{18:1w9}$	$C_{18:1w7}$	$C_{20:1}$	$C_{18:2w6}$	$C_{18:3w3}$	Total LCFA	Total FFA
Spring	0	49.63 bB	0.31	70.73 cC	4.27 bB	0.37	4.83 bB	0.66 b	134.06	377.11
	7	40.06 bC	0.25	80.59 bB	4.30 bB	0.29 B	3.30 bB	0.98 b	133.62	374.47
	30	53.10 bB	0.31	98.27 abB	4.87 bB	0.25 B	13.81 aA	2.18 aA	178.05	478.26
	90	75.09 aB	0.53	128.60 aB	5.37 aC	0.84 B	12.12 aAB	2.98 aB	231.13	647.52
Summer	0	49.19 bB	0.18	82.71 cB	5.91 bB	0.69	5.35 bB	0.77 b	149.40	457.87
	7	47.86 bB	0.28	84.95 bB	6.04 bAB	0.59 B	6.55 bAB	0.80 b	151.63	459.15
	30	50.40 bB	0.42	108.98 abB	6.27 bAB	0.55 B	7.91 aB	1.30 aB	181.51	576.42
	90	75.44 aB	0.52	146.66 aAB	11.89 aAB	0.82 B	11.78 aAB	1.47 aC	257.25	814.80
Autumn	0	53.73 cA	0.24	80.12 bB	5.33 bB	0.26	4.54 bB	0.46 b	150.61	455.87
	7	63.31 cA	0.18	93.96 bA	4.65 bB	0.74 A	5.75 bAB	0.58 b	175.32	503.74
	30	75.58 abA	0.47	141.09 aA	5.25 bB	1.12 A	6.28 bB	0.80 bC	239.41	687.57
	90	96.97 aA	0.48	149.05 aAB	9.53 aB	1.05 A	10.42 aB	1.05 aC	279.96	833.10
Winter	0	51.15 bB	0.15	111.04 bA	8.01 bA	0.45	8.25 bA	0.50 b	184.73	515.16
	7	58.59 bA	0.28	100.02 bA	8.84 bA	1.02 A	9.42 bA	0.63 b	184.29	505.78
	30	59.49 bB	0.32	121.03 abAB	9.78 bA	1.06 A	9.84 abA	0.71 abC	210.03	572.87
	90	62.61 aB	0.36	164.90 aA	14.88 aA	1.15 A	14.30 aA	3.27 aA	248.19	716.89

[d] mean value: 12 cheeses (4 cheeses/producer), for each cheese two samples, each sample in duplicate.
Means with different letters (a-c) in the same column are significantly different from each other for ripening time ($P \leq 0.05$). Means with different capital letters (A-C) in the same column are significantly different from each other for the production season ($P \leq 0.05$) at the same ripening time. Letters are not reported where there were no significant differences ($P > 0.05$).

Analysis of variance of the data obtained showed statistically significant differences ($P \leq 0.05$) during the ripening time and within the cheesemaking seasons. During ripening, since the P-value was less than 0.05, there were statistically significant differences between the mean of all fatty acids from 0 to 90 days (95% confidence level), except for the odd-carbon-number fatty acids and eicosenoic acid ($P > 0.05$). Using homogeneous groups (Duncan's test), for each free fatty acid ($P < 0.05$), the multiple-range test determined statistically significant differences as a result of ripening and production season, which are summarised as follows: 1) between 0 and 7 days no statistically significant differences resulted for all free fatty acids considered, except for butanoic, decanoic acids and for oleic acid in summer and spring; 2) there were significant differences between 7 and 30 days for even-carbon number fatty acids from C_4 to C_{10} and between 30 and 90 days from C_{10} to $C_{18:3}$; 3) in ripened samples even-carbon number fatty acids from C_4 to C_{10} had a significantly higher content in summer, all saturated even-carbon number fatty acids from C_{14} to C_{18} in autumn, while those unsaturated in winter; 4) the amount of myristic, palmitic and stearic acids were significantly lower in spring.

The total content of free fatty acids was higher than that reported by WOO *et al.* (1984 b) for other Italian cheese varieties. The significant increase of all the even carbon number fatty acids observed during the ripening period has also been reported for a great majority of cheeses (NAJERA *et al.*, 1994; CONTARINI and TOPPINO, 1995; MACEDO and MALCATA, 1996; SOUSA and MALCATA, 1997; FREITAS and MALCATA, 1998; MALLATOU *et al.* 2003; AKIN *et al.* 2003; GEORGALA *et al.*, 2005).

The increase of the FFA content during ripening is a consequence of the progressive lipolytic process that involves the hydrolysis of glycerides. The lipolytic system, responsible for lipolysis in Provola dei Nebrodi, at first hydrolyses the external position sn-3 of triglycerides, producing butyric and caproic acids, then during ripening hydrolyses the other external position sn-1, producing oleic, stearic and palmitic acids.

Although Provola dei Nebrodi manufacture involved the utilisation of rennet paste contained the lipase, pregastric esterase (PGE), apparently the PGE action is not evident. In fact PGE is highly specific for SCFA at the sn-3 position of mono, di, and triglycerides of milk fat.

The lipolytic action in Provola dei Nebrodi seems to be due to other less selective lipolytic enzymes, such as microbial enzymes. These non specific enzymes can release indiscriminately short chain fatty acids from the sn-3 position, long chain fatty acids from the sn-1 position and medium chain fatty acids from the sn-2 position by conversion in sn-1 or sn-3 isomers (SOMERHARJU *et al.*, 1978; NILSSON-EHLE *et al.*, 1973).

The main differences in the SCFA composition were between 7 and 30 days in all the production seasons considered as previously reported by VERZERA *et al.* (2004), and the highest amount of each SCFA were observed in summer. Probably the higher temperature of the summer season forces the hydrolysis of triglycerides, moreover, in these samples lactic acid bacteria, enterococci and propionic acid bacteria reached their maximum value (8.68-8.96, 5.41-5.75, 4.18-4.70 log CFU g^{-1} cheese, respectively) in the second week of ripening (unpublished data). Lactic acid bacteria are weakly lipolytic (STADHOUDYERS and VERINGA, 1973), but enterococci, which are part of the dominant flora in raw milk cheese (CENTENO *et al.*, 1996), have been used on numerous occasions to accelerate cheese maturation due to their proteolytic and lipolytic activity (CENTENO *et al.*, 1999) and propionic acid bacteria are well known for

their high lipolytic activity, 10-100 times more than lactic acid bacteria (CHAMBA and PERREARD, 2002). In this light, the microbial population could contribute to the lipolysis of "Provola dei Nebrodi". Regarding the concentration of butanoic, decanoic and oleic acids it was noted that they continuously increased throughout the ripening period. For these fatty acids, in analysing white pickled cheese from raw cow milk, AKIN *et al.* (2003) observed a similar behaviour in cheese samples made with the addition of pregastric lamb lipase.

The period of lactation, animal feeding and the local weather are the major factors responsible for the differences observed among the seasonal cheeses. The major content of FFA in ripened cheese samples was observed in autumn and the lowest in spring (Fig. 2) which was also the case for the main free fatty acids: myristic, palmitic and stearic. In autumn, the end of lactation determined a significantly higher fat content of cheese samples produced in this season (Table 1) as previously reported by MACEDO and MALCATA (1996). This leads to a higher amount of FFA since lipolysis is expected to occur faster if the substrate concentration is increased (IRVINE *et al.*, 1948). In spring, cows which have been stalled throughout the winter are moved to the pasture and this sudden change causes a significantly lower fat content in the cheese samples (ALAIS, 1984). In winter the significantly higher amount of unsaturated fatty acids mainly oleic, linoleic and linolenic was probably due to the concentrated feed containing oil seeds.

In conclusion, "Provola dei Nebrodi" cheese is characterised by a high amount of FFA (up to 840.45 mg 100 g^{-1} cheese) which was higher than the values reported for other types of cheese (MALLATOU *et al.* 2003; POVEDA *et al.* 1999; PARTIDARIO, 1999; FREITAS and MALCATA, 1998) except for blue and smear cheeses and long ripened "pasta filata" cheeses (DE LA FUENTE *et al.*, 1999; WOO *et al.*, 1984a; BATTISTOTI and CORRADINI,

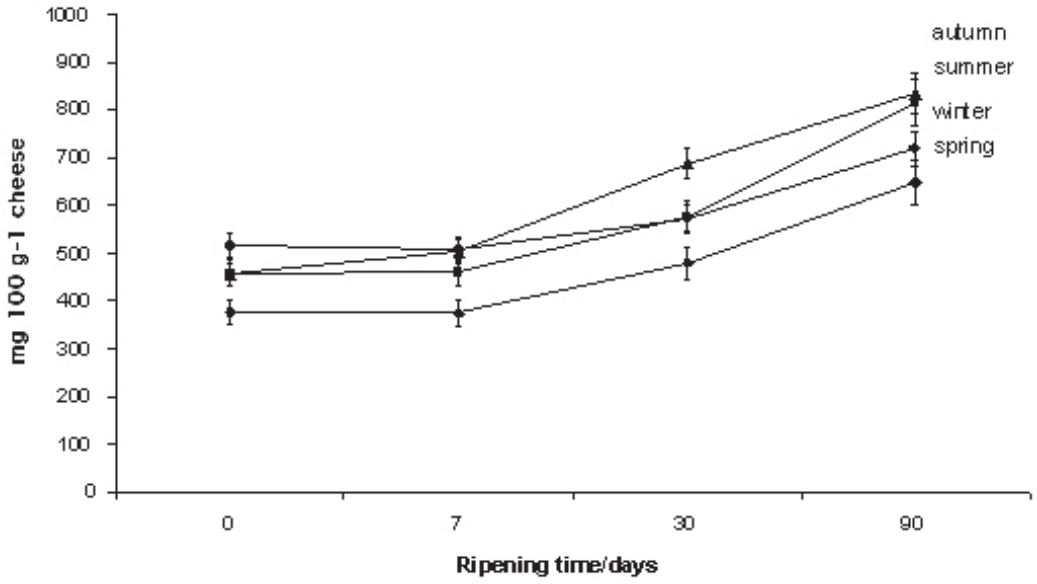

Fig. 2 - Total free fatty acid content (mg 100 $g^{-1}$of cheese) throughout ripening in the different production seasons.

1993). The statistically significant differences observed for samples from different seasons with equal ripening is coherent with the artisanal nature of Provola dei Nebrodi production, since the cheese makers do not standardise the milk. From the data obtained, the FFA composition could become a powerful tool to establish a protocol for the characterization of this product.

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PAPER

EFFECT OF A POST-PROCESSING PHASE ON THE VOLATILE FLAVOUR COMPOUNDS OF ASÌNO CHEESE

RUOLO DELLA FASE DI STAGIONATURA SUI COMPONENTI VOLATILI RESPONSABILI DEL FLAVOUR DEL FORMAGGIO ASÌNO

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ABSTRACT

Asìno is a traditional Italian cheese which, after a short ripening period in the warehouse, is soaked in a special brine, called *"salmuerie"*, which consists of whey, salt, milk and milk cream. The effect of immersing this cheese in this unusual brine on the characteristics of this product and, particularly, on the profiles of the volatile compounds and the short-chain free volatile fatty acids was investigated. The study demonstrated that fatty acids prevail in the aromatic fraction and

RIASSUNTO

Il formaggio Asìno è un prodotto tradizionale italiano, ottenuto a partire da forme che, dopo un breve periodo di stagionatura in magazzino, vengono immerse in una particolare salina, chiamata *"salmuerie"*, costituita da siero, sale, latte e panna. Scopo di questo lavoro è stato quello di valutare l'influenza esercitata dal periodo di affinamento del prodotto in questa peculiare salina sulle caratteristiche distintive di questo formaggio, con particolare attenzione all'evoluzione dei profili dei componen-

- Key words: cheese characterization, flavour profile, free volatile fatty acids, HS-SPME, migration process, volatile compounds -

that these compounds migrate from the *salmuerie* into the cheese as a result of the concentration differential existing between the two systems.

ti volatili e degli acidi grassi a corta catena. Lo studio condotto ha dimostrato che nella frazione aromatica prevale la classe degli acidi grassi e che tali composti migrano progressivamente dalla *salmuerie* all'interno del formaggio immerso in essa in seguito a un processo diffusivo determinato dal gradiente di concentrazione inizialmente esistente tra i due sistemi.

INTRODUCTION

In northeastern Italy a particular, traditional semi-hard cheese, called Asìno, is manufactured. It is usually made from raw or heated and partially skimmed cow's milk which is inoculated with natural milk culture or selected starters. The milk is coagulated at 34°-36°C using calf rennet and after cutting the gel into nut-sized pieces, the curd is cooked at 40°-44°C. After removal from the vat, the cheese is salted in a brine (16-18% w/w of sodium chloride) and then ripened for 7 days in a warehouse at a temperature of 13°-15°C and a relative humidity of 75-85%. After this time, the cheese is immersed in a special brine, called "*salmuerie*", which is kept in wooden vats in rooms at 13°-15°C and 75-85% relative humidity. The brine consists of about 4% w/w of sodium chloride, with the addition of milk, milk cream and whey. The *salmuerie* is never completely renewed, but it is frequently oxygenated by mixing and periodically adjusted by adding the above-mentioned components, according to the experience and judgement of the cheese-maker. Ripening for about 100 days is required for the characteristic flavour to develop. The cheese has a cylindrical shape, 5-15 cm high and 30-40 cm in diameter, and is characterized by an intense aroma and flavour, with a marked pungent note.

The aim of this study was to describe this traditional product and define the relevance of this specific maturation process on its characteristics. The main composition parameters, the proteolysis index and the fatty acid content were determined during the maturation which takes place with the cheese immersed in the brine.

Moreover, since one of the foremost criteria determining consumer preference is flavour, it is very important to define which specific volatile compounds are mainly responsible for the particular sensory characteristics of the Asìno cheese. Thus, particular attention was also paid to the analysis of the volatile fraction and the effect of immersing it in the in *salmuerie*.

Several studies have been carried out on the volatile compounds of different types of cheese (MOIO *et al.*, 1993; KUBÍCKOVÁ and GROSCH, 1998; JOU and HARPER, 1998; DIRINCK and DE WINNE, 1999; DI CAGNO *et al.*, 2003; PINHO *et al.*, 2004). Because of its reduced sample preparation time, high sensitivity and limited risks of producing artefacts, the characterization and determination of the volatile compounds is increasingly carried out by the headspace solid-phase microextraction method coupled with gas chromatography (CHIN *et al.*, 1996; PILLONEL *et al.*, 2002; FRANK *et al.*, 2004; PINHO *et al.*, 2004). In this study, a fib-

er assembly coated with divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) was chosen because previous studies had proved that this is the most appropriate type of fiber for analyzing the volatile fraction of the cheese matrix with regard to sensitivity and repeatability (PÉRÈS *et al.*, 2001; LECANU *et al.*, 2002; BELLESIA *et al.*, 2003).

MATERIALS AND METHODS

A) *Salmuerie* characterization

Over a period of 6 months, random samplings of the *salmuerie* were performed and a total of 14 different brine samples were taken from different wooden vats for analysis. In order to obtain a representative and uniform sample, the brine was always thoroughly mixed before 500 mL was removed.

Samples were analysed for pH, acidity and moisture (D.M. n. 88, 1986). Protein content was determined by the Kjeldahl method (AOAC, 1980), NaCl by FIL-IDF 7A (1972) and fat by FIL-IDF 5A (1972).

B) Cheese characterization during immersion in the *salmuerie*

Three different and successive cheesemaking processes were performed. All the trials were organized according to the following scheme. At each cheesemaking trial 3,500 kg of milk were used and a total of 30 cheeses were produced from the vat. The cheese was then ripened in a traditional warehouse for 7 days at a temperature of 13°-15°C and a relative humidity of 75-85%. After this time, they was divided into two groups: 15 cheeses were soaked for 100 days in the *salmuerie* (experimental samples), while the other 15 cheeses were ripened in the warehouse (control samples). From the start to the end of the soaking phase (at 0, 15, 42, 53, 67, 83 and 100 days), samples were taken from the two groups of cheeses and from the *salmuerie* to be analysed for moisture (D.M. n. 88, 1986), NaCl (FIL-IDF 7A, 1972), fat (FIL-IDF 5A, 1972), free volatile fatty acids (INNOCENTE *et al.*, 2000) and volatile compounds (POLENTARUTTI *et al.*, 2001). Total and water-soluble nitrogen analysis was performed on the cheese samples alone to evaluate the proteolysis index (INNOCENTE, 1997).

To obtain a representative sample of cheese, at each sampling time, a whole segment was cut from the centre to the edge of the cheese, the rind (2 cm) was removed and the sample (about 500 g) was finely grated. As mentioned above, the brine was always thoroughly stirred in the wooden vats before sampling.

C) Analytical determinations

Analysis of volatile compounds

Volatile compounds were determined by the headspace solid-phase microextraction (HS-SPME) method coupled with gas chromatography (GC).

Ten grams of sample were placed in a 50 mL vial, closed with a teflon septum and an aluminium seal, and then placed in a water bath at 40°C for 30 min. A fiber assembly coated with divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) 50 and 30 μm, 2 cm long (Supelco, Bellefonte, PA, USA) was exposed for 20 min in the headspace of the sample kept in the water bath at 40°C (CHIN *et al.*, 1996, modified according to PÉRÈS *et al.*, 2001).

The volatile compounds of the SPME extracts were separated using a Model 3400 Varian gas chromatograph (Walnut Creek, CA, USA) equipped with a flame ionization detector (FID, set at 250°C). Volatile compounds were thermally-desorbed from the SPME fiber in the injector port (250°C) in a splitless mode and the split valve was opened 3 min after injection. The carrier gas was helium with a column flow rate of 1 mL/min. Separation was performed on ECONO-CAP Ec-

Wax column, 30 m x 0.25 mm i.d., 0.25 μm film thickness (Alltech, State College, PA, USA). The oven temperature was held at 50°C for 5 min, then raised to 230°C (10°C/min, held 10 min) before reaching a final temperature of 250°C (10°C/min, held 10 min) (POLENTARUTTI *et al.*, 2001). The absolute peak area of each compound was used for comparing and interpreting the data.

Identification of the compounds was achieved using a Model 3400 Varian gas chromatograph coupled with a model Ion Trap Detector (ITD) Varian Saturn mass spectrometer. Volatile compounds were thermally-desorbed from the SPME fiber in the injector port (250°C) in a split mode (split ratio 1: 30). Helium carrier gas flow rate was 2 mL/min; a mixture of C_8-C_{30} *n*-alkanes was used to set the column flow to match the retention times with the column of the gas chromatograph equipped with FID. Separation was performed on an ECONO-CAP Ec-Wax column, 30 m x 0.32 mm i.d., 0.25 μm film thickness. The oven temperature was set as previously described. The ITD mass spectrometer conditions were as follows: the temperatures of the manifold and the transfer line were 170° and 250°C, respectively, electron impact mass spectra were recorded at 70 eV, the ionization current was 10 μA and the scan rate was 1.5 scans/s. Compounds were identified by comparing their mass spectrum with those of the mass spectrum library Wiley 5. Moreover, Kovats' retention indexes were determined and compared with retention indexes of the compounds available in the literature.

Analysis of short-chain free volatile fatty acids

The free volatile fatty acids (FVFAs) were evaluated by the method set up by INNOCENTE *et al.* (2000). Thirty-five milliliters of water and 3 mL of internal standard solution (crotonic acid) were added to 10 g of sample. This mixture was homogenized with a Politron (Kinematica, Lucerne, Switzerland) for 2 min and then centrifuged (Beckman, mod. Avanti™ J-25, Palo Alto, CA, USA) at 5000 rpm for 20 min at 15°C to obtain an aqueous extract that was acidified to pH 3-4 to free volatile fatty acids present as salts. The FVFAs were then extracted from 1 mL of the acidified aqueous solution using 2 mL of diethyl ether. High-resolution gas chromatography was performed using a Varian instrument (model 3700, Varian Instruments, Sunnyvale, CA, USA) equipped with a split injector, a flame ionization detector (FID) and a Hewlett-Packard integrator (model 3396 A, Hewlett-Packard, Palo Alto, CA, USA). A Nukol™ fused-silica wide-bore column (15 m x 0.53 mm i.d.) with a film thickness of 0.5 μm (Supelco, Bellefonte, PA, USA) was used. The carrier gas was helium with a flow rate of 25 mL/min, and the split ratio was 1:1. The oven temperature was maintained at 80°C for 5 min and then increased to 180°C at a rate of 8°C/min. Injector and detector temperatures were 180° and 200°C, respectively.

D) Statistical analysis

Three different cheesemaking processes were monitored and each time all the analyses were carried out three times on the samples. Thus, the data shown are the averages of the nine values obtained. Student's T-test was used to compare two means, while one-way analysis of variance (F-test) and Tukey's test were used for multiple comparison. In both cases, the differences between the means were considered statistically significant for P values ≤ 0.05. All statistical analyses were conducted using the software Statistical Discovery JMP 3.0 for Windows.

RESULTS AND DISCUSSION

The chemical composition of the *salmuerie* was performed. The mean values of the pH, acidity, moisture, total pro-

Table 1 - Mean values and relative standard deviations of chemical and compositional parameters measured in the *salmuerie* samples during a 6-month period.

Number of *salmuerie* samples analysed	pH		Acidity Lactic acid g/100 g		Moisture g/100 g		Fat g/100 g		NaCl g/100 g		Proteins g/100 g	
	X	R.S.D.	X	R.S.D.	X	R.S.D.	X	R.S.D.	X	R.S.D.	X	R.S.D.
1	4.87a	0.41	1.60a	2.50	72.18a	0.21	13.59a	1.84	4.16a	0.96	6.48a	1.08
2	4.89a	0.41	1.60a	1.25	72.25a	0.08	13.45a	2.38	4.12a	1.94	6.41a	0.78
3	4.90a	0.41	1.63a	0.61	72.42a	0.08	13.53a	2.59	4.24a	0.94	6.49a	0.92
4	4.90a	0.41	1.60a	1.88	72.32a	0.14	13.78a	1.16	4.21a	1.90	6.35a	1.57
5	4.89a	0.20	1.64a	1.83	72.38a	0.08	14.12a	2.62	4.16a	1.44	6.33a	1.58
6	4.88a	0.20	1.61a	0.62	72.34a	0.05	13.75a	1.16	4.22a	1.66	6.45a	1.86
7	4.88a	0.20	1.63a	1.23	72.25a	0.08	14.09a	1.63	4.14a	1.69	6.33a	1.58
8	4.89a	0.41	1.62a	0.62	72.29a	0.01	13.52a	2.29	4.12a	0.97	6.33a	2.21
9	4.88a	0.20	1.65a	1.82	72.22a	0.08	13.72a	1.31	4.21a	1.19	6.35a	1.89
10	4.90a	0.41	1.63a	1.23	72.34a	0.14	13.59a	1.91	4.23a	1.42	6.46a	2.17
11	4.87a	0.21	1.66a	1.81	72.26a	0.17	13.46a	1.56	4.19a	1.43	6.45a	1.55
12	4.88a	0.41	1.64a	0.61	72.32a	0.04	13.54a	1.85	4.21a	1.19	6.46a	2.01
13	4.89a	0.20	1.65a	1.21	72.27a	0.08	14.71a	0.73	4.16a	0.48	6.64a	1.51
14	4.87a	0.21	1.66a	0.60	72.35a	0.14	14.22a	2.46	4.21a	0.71	6.55a	0.92

X = mean value of three repetitions; R.S.D. = relative standard deviation (%).
Mean values within the same column followed by the same letters do not differ significantly at P≤0.05 according to Tukey's test.

Table 2 - Mean values, expressed as μg/g of *salmuerie*, and relative standard deviations of the short-chain free volatile fatty acid content measured in the salmuerie samples during a 6-month period.

Number of *salmuerie* samples analysed	Acetic acid		Propionic acid		Butyric acid		Caproic acid	
	X	R.S.D.	X	R.S.D.	X	R.S.D.	X	R.S.D.
1	3595a	3.25	597a	1.34	454a	1.32	306a	3.27
2	3769a	2.92	603a	1.33	468a	1.71	323a	2.79
3	3653a	3.67	598a	1.84	471a	0.64	312a	2.24
4	4004a	3.27	614a	2.12	478a	1.67	317a	3.79
5	3594a	3.34	601a	2.33	466a	2.15	305a	2.95
6	4011a	3.84	620a	1.94	452a	1.99	298a	1.68
7	3726a	2.55	598a	1.84	478a	2.93	322a	3.11
8	3664a	5.65	596a	0.84	451a	3.55	301a	3.65
9	4027a	4.62	613a	1.47	467a	3.21	320a	3.13
10	4006a	4.57	623a	2.89	454a	4.19	317a	1.58
11	3646a	4.25	594a	1.85	452a	1.11	307a	2.61
12	3583a	5.08	604a	0.83	466a	0.86	303a	1.65
13	3663a	3.41	601a	1.33	448a	1.34	307a	0.98
14	4014a	3.69	591a	1.35	461a	1.52	323a	2.48

X = mean value of three repetitions; R.S.D. = relative standard deviation (%).
Mean values within the same column followed by the same letters do not differ significantly at P≤0.05 according to Tukey's test.

teins, salt, fat and free volatile fatty acids from 14 different brine samples are reported in Tables 1 and 2. There were no significant changes in the composition of the brine taken from different wooden vats during the 6-month period (Tukey's test, P≤0.05). The high presence of short-chain fatty acids in the *salmuerie* is probably the result of the lipolytic activity of the enzymes produced by the microflora. A previous study showed that the brine is the natural habitat for *Micrococcaceae* (INNOCENTE *et al.*, 2002); nevertheless, a more exhaustive characterization of the microflora of the brine and the Asìno cheese during the cheesemaking process is under investigation.

In order to study the effect of the soaking phase in the *salmuerie* on the characteristics of Asìno, the chemical composition and the volatile compounds of the cheese samples (both the Asìno cheese immersed in *salmuerie* and the control cheese ripened in the warehouse) and of the *salmuerie* liquid itself were determined during the immersion period.

The data in Table 3 show that no important changes were measured in the moisture and fat contents of the Asìno samples. On the other hand, the salt content increased rapidly in the Asìno cheese during the first two months of immersion, while it remained almost steady in the brine; after this time an equilibrium condition was reached between these two systems. The increase in proteolysis measured in the Asìno cheese immersed in *salmuerie* and in the control cheese ripened in the warehouse were very similar (Fig. 1). So, it is clear that the *salmuerie* soaking phase does not

Table 3 - Mean values and relative standard deviations of chemical and compositional parameters measured in the *salmuerie*, in the Asìno cheese soaked in *salmuerie* and in the control cheese ripened in the warehouse during 100 days of immersion.

	Moisture						Fat						NaCl					
	Asìno cheese in salmuerie g/100 g		*Salmuerie* g/100 g		Control cheese in warehouse g/100 g		Asìno cheese in salmuerie g/100 g c.d.m.		*Salmuerie* g/100 g		Control cheese in warehouse g/100 g c.d.m.		Asìno cheese in salmuerie g/100 g c.d.m.		*Salmuerie* g/100 g		Control cheese in warehouse g/100 g c.d.m.	
Days	X.	R.S.D.	X.	R.S.D.	X.	R.S.D.	X.	R.S.D.	X.	R.S.D.	X.	R.S.D.	X.	R.S.D.	X.	R.S.D.	X.	R.S.D.
0	45.70a,b	0.26	73.91a,b	0.31	45.70a	0.26	49.76b	0.73	13.02b	1.03	49.76a	0.73	2.44e	1.31	3.98a	1.89	2.44a	1.31
15	46.07a	0.32	74.21a	0.16	-	-	50.93a	0.25	13.35a,b	0.53	-	-	2.93d	1.79	4.00a	1.77	-	-
42	46.06a	0.36	73.31b,c	0.31	44.00b	0.25	50.13a,b	0.20	13.64a	1.62	49.41a	0.89	3.40c	2.75	3.99a	0.71	2.51a	0.61
53	45.51b	0.15	73.29b,c	0.79	-	-	49.82b	0.17	13.19b	0.83	-	-	3.74b	0.75	4.04a	0.99	-	-
67	45.78a,b	0.26	72.84c	0.27	43.15c	0.76	51.08a	1.73	13.32a,b	1.58	49.57a	1.04	4.08a	0.45	4.04a	2.42	2.43a	1.23
83	45.68a,b	0.71	73.25b,c	0.23	-	-	50.65a,b	0.42	13.09b	0.69	-	-	4.09a	1.82	4.02a	1.14	-	-
100	45.58b	0.90	72.79c	0.36	41.82d	0.79	49.66b	0.45	13.30a,b	1.20	49.72a	0.67	4.03a	1.39	4.01a	1.25	2.42a	1.03

X = mean value of nine repetitions (three for each cheesemaking process); R.S.D. = relative standard deviation (%).
c.d.m. = cheese dry matter.
Mean values within the same column followed by the same letters do not differ significantly at P≤0.05 according to Tukey's test.

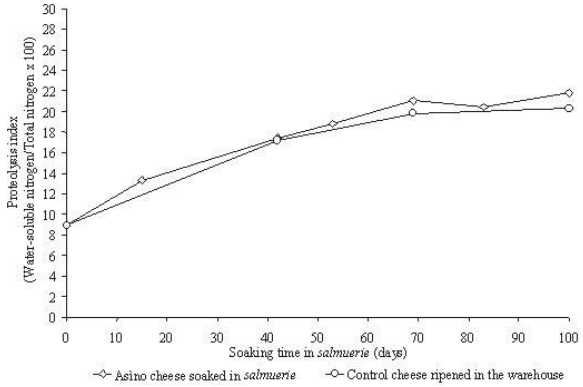

Fig. 1 - Proteolysis increase, expressed as the percentage ratio of water-soluble nitrogen to total nitrogen, during 100 days of immersion. Each value is the mean of nine repetitions (three for each cheesemaking process).

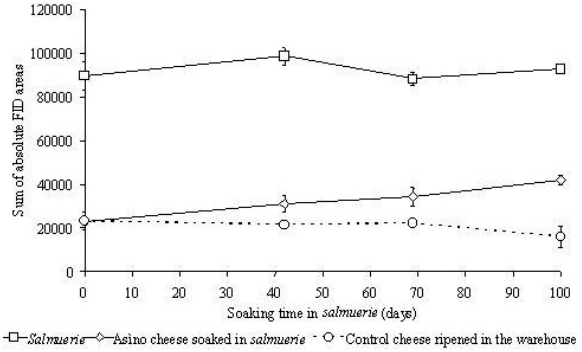

Fig. 2 - Total volatile profile, expressed as the sum of the averages of the absolute areas measured in the headspace of *salmuerie*, of Asìno cheese soaked in *salmuerie* and the control cheese ripened in the warehouse during 100 days of immersion. Each value is the mean of nine repetitions (three for each cheesemaking process).

influence the regular behaviour of the proteolytic ripening of the cheese.

The total volatile fraction profiles, expressed as averages of the sum of the absolute areas of the peaks measured in the headspace of the cheese samples and *salmuerie* during immersion, are shown in Fig. 2. The total profile of the *salmuerie* is very high and almost steady. As regards the cheeses, in the Asìno sample soaked in *salmuerie* the aromatic fraction gradually increased (Tukey's test, $P \leq 0.05$), while in the warehouse-ripened control cheese the profile

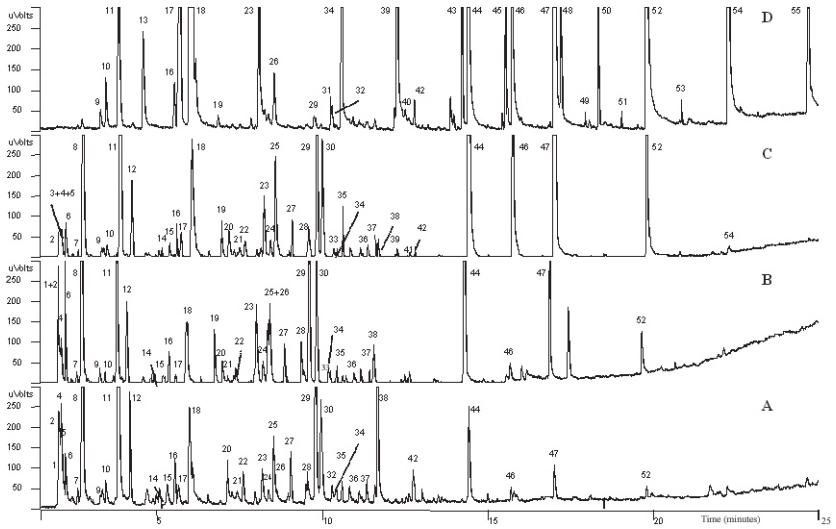

Fig. 3 - GC-FID chromatograms, volatile compound extraction was performed using DVB/CAR/PDMS fiber and chromatographic separation was carried out by a ECONO-CAP Ec-Wax column. Identification numbers correspond to those reported in Table 4. A: Cheese at the beginning of the *salmuerie* soaking phase; B: Control cheese after 100 days of ripening in the warehouse; C: Asìno cheese after 100 days of the *salmuerie* soaking phase; D: *Salmuerie*.

was always significantly lower (Student's T-test, P≤0.05). Therefore, it is possible that immersion in the special brine has a direct effect on the volatile fraction of the cheese.

Fig. 3 shows some examples of chromatographic profiles obtained by the SPME method of the headspace of the cheese samples at the start of the soaking phase (A) and after 100 days (B, C) and in the headspace of the *salmuerie* (D). The identification numbers of the peaks correspond to the compounds listed in Table 4. The compounds were

Table 4 - Volatile compounds in the headspace of the cheese and *salmuerie* samples by GC-MS analysis during 100 days of immersion.

Peak identification number	Compound	Kovats' retention index		Identification method
		I Obs.	I Ref.	
	Hydrocarbons			
12	3-methylpentane	980		MS
24	p-xylene	1146	1140[a]	MS, RI
25	m-xylene	1151	1147[a]	MS, RI
28	o-xylene	1192	1191[a]	MS, RI
37	1,2,3-trimethylbenzene	1290		MS
	Terpenes			
16	α-pinene	1013	1039[a], 1036[f]	MS, RI
22	β-pinene	1104	1120[a,f]	MS, RI
27	β-mircene	1167	1156[a], 1168[f]	MS, RI
29	limonene	1198	1190[b], 1206[a,f]	MS, RI
35	γ-terpinene	1251	1251[a,f], 1247[f]	MS, RI
36	p-cimene	1284	1272[a,f], 1275[f]	MS, RI
	Aldehydes			
20	hexanal	1074	1074[b], 1084[a]	MS, RI
33	2-hexenal	1235	1216[b]	MS, RI
	Ketones			
8	2-propanone	842	810[a], 814[c], 820[b]	MS, RI
10	2-butanone	907	907[a]	MS, RI
38	3-hydroxy-2-butanone	1293	1250[c]	MS, RI
	Alcohols			
11	ethanol	970	924[d], 932[b]	MS, RI
17	2-butanol	1018	975[a]	MS, RI
18	1-propanol	1051	1002[a], 1030[b]	MS, RI
26	1-butanol	1154	1113[a]	MS, RI
30	1-pentanol	1208	1213[a]	MS, RI
41	3-pentanol	1349	1335[a], 1337[b]	MS, RI
42	1-hexanol	1357	1357[b], 1359[d]	MS, RI
	Esters			
9	ethyl acetate	904	872[a], 893[c], 892[b]	MS, RI
13	propyl acetate	994	962[a]	MS, RI
19	n-butyl acetate	1069	1059[a]	MS, RI
23	propyl butanoate	1122	1110[a]	MS, RI
31	butyl butanoate	1238	1207[a]	MS, RI
32	propyl pentanoate	1240	1200[a]	MS, RI
34	ethyl hexanoate	1248	1223[a], 1234[e]; 1238[d]	MS, RI
39	pentyl hexanoate	1338		MS
40	ethyl heptanoate	1347	1321[a]	MS, RI
43	ethyl octanoate	1446	1423[a]	MS, RI

Table 4 (continued).

Peak identification number	Compound	Kovats' retention index		Identification method
		I Obs.	I Ref.	
45	propyl octanoate	1518	1498[a]	MS, RI
48	ethyl decanoate	1665	1624[a]	MS, RI
55	ethyl hexadecanoate	2274		MS
	Fatty acids			
44	acetic acid	1457	1448[b], 1451[e]	MS, RI
46	propanoic acid	1533	1525[c]	MS, RI
47	butanoic acid	1659	1588[c],1626[b]	MS, RI
51	pentanoic acid	1752	1667[b]	MS, RI
52	hexanoic acid	1833	1841[b], 1852[d]	MS, RI
53	heptanoic acid	1978	1904[c], 1962[b]	MS, RI
54	octanoic acid	2079	2008[c]	MS, RI
50	not identified	1741		

I Obs.: observed Kovats' retention index.
I Ref.: reference Kovats' retention index, reported by different authors:
a: JENNINGS and SHIMABOTO, 1980
b: VICHI *et al.*, 2003;
c: LECANU *et al.*, 2002;
d: LOPEZ *et al.*, 1999;
e: BAEK and CADWALLADER, 1999;
f: DAVIES, 1990.
Identification method:
MS: tentatively identified by Wiley 5 mass spectra library search;
RI: tentatively identified by comparison of elution order according to different authors (a, b, c, d, e, f).

identified by matching their mass spectra with the reference mass spectra of the Wiley 5 library and by comparing their Kovats' retention indexes with those reported in the literature.

In the headspace of the samples a total of 43 compounds were identified, grouped according to the following chemical classes: hydrocarbons (5), terpenes (6), aldehydes (2), ketones (3), alcohols (7), fatty acids (7) and esters (13).

Tables 5 and 6 show the mean values, standard deviations and relative standard deviations of the sum of the absolute areas of the volatile compounds (grouped according to chemical class), which were measured at the start and the end of the soaking phase in the cheeses and in the *salmuerie* samples, respectively. The data reported in these tables show that alcohols, fatty acids and esters were the most significant contributors to the volatile profile of the samples.

More specifically, as regards the alcohols, the profile of the *salmuerie* was high and remained almost steady during the 100 days of immersion (Table 6), while both cheeses had lower values and no significant differences were measured between them (Table 5). It is quite possible that oxygenation of the brine caused by periodically stirring it induced oxidation of the fatty acids, which in turn led to the production of aldehydes and methylketones that are rapidly transformed into alcohols or acids by the enzymatic systems present (MARILLEY and CASEY, 2004). As in all high-fat foods, lipids present in cheese can undergo oxidative degradation; however because of the negative oxidation-reduction potential of cheese, oxi-

dation of cheese lipids is probably limited (McSWEENEY and SOUSA, 2000) and this could explain the very low values measured in the headspace of the cheese samples. The main alcohol identified in the aromatic fraction was eth-

Table 5 - Mean values, standard deviations and relative standard deviations of the sum of the absolute areas of the peaks of volatile compounds, grouped according to chemical classes, measured in the cheese samples by GC-FID analysis during 100 days of immersion.

	Asìno Cheese at the start of soaking in *salmuerie* Sum of the absolute areas			Asìno cheese after 100 days of soaking in *salmuerie* Sum of the absolute areas			Control cheese after 100 days of ripening in warehouse Sum of the absolute areas		
	X	S.D.	R.S.D.	X	S.D.	R.S.D.	X	S.D.	R.S.D.
Hydrocarbons	1504a	83	6	1642a	192	12	1140a	296	26
Terpenes	3187a	78	2	2982a,b	465	16	2131b	540	25
Aldehydes	619a	6	1	451b	78	17	267c	49	18
Ketones	7663a	2379	31	3074b	101	3	3007b	581	19
Alcohols	5984a	720	12	4294a	869	20	3817a	771	20
Fatty Acids	1049b	18	2	27525a	2208	8	1863b	111	6
Esters	431a	15	3	1093a	340	31	1101a	243	22

X = mean value of nine repetitions (three for each cheesemaking process); S.D.= standard deviation; R.S.D.= relative standard deviation (%).
Mean values within the same row followed by different letters differ significantly at P≤0.05 according to Tukey's test.

Table 6 - Mean values, standard deviations and relative standard deviations of the sum of the absolute areas of the peaks of volatile compounds, grouped according to chemical class, measured in the *salmuerie* samples by GC-FID analysis during 100 days of immersion.

	Salmuerie at the start of the soaking phase Sum of the absolute areas			*Salmuerie* after 100 days of the soaking phase Sum of the absolute areas		
	X	S.D.	R.S.D.	X	S.D.	R.S.D.
Hydrocarbons	traces	-	-	traces	-	-
Terpenes	324a	21	6	259a	33	13
Aldehydes	traces	-	-	traces	-	-
Ketones	415a	24	6	375a	86	23
Alcohols	12060a	1063	9	9798a	1249	13
Fatty Acids	49593a	3520	7	55230a	3569	6
Esters	27113a	2749	10	27244a	1688	6

X = mean value of nine repetitions (three for each cheesemaking process); S.D. = standard deviation; R.S.D.= relative standard deviation (%).
traces =sum of absolute areas < 100.
- = not calculated.
Mean values within the same row followed by the same letters do not differ significantly at P≤0.05 according to Student's T-test.

anol, which probably derives from lactose metabolism.

As concerns the fatty acids, the *salmuerie* samples showed very high amounts that remained almost steady with time (Table 6). On the other hand, in the Asìno cheese they started at a very low value and then dramatically increased during soaking, while in the control sample there were no significant changes (Table 5). As shown in the chromatograms of Fig. 3, only acetic acid, propanoic acid, butanoic acid and hexanoic acid (peaks 44, 46, 47 and 52) were present in the cheese after soaking (C), while the *salmuerie* (D) also showed the significant presence of octanoic acid (peak 54).

Finally, as regards the ester compounds, the *salmuerie* samples had higher total area values (Table 6) and, as shown in chromatogram D in Fig. 3, medium-chain esters prevail (peaks 39, 43, 45, 48 and 55). It is well-known that esters derive from reactions occurring between fatty acids and alcohols (MOLIMARD and SPINNLER, 1996). The *salmuerie* were found to be the richest in both classes that are involved in these reactions and this could explain the very high intensity measured for the ester compounds. Concerning the cheese samples, both the Asìno and the control cheese had similar profiles (Table 5) in which short-chain esters prevail (peaks 9, 19, 23, 34 in chromatograms C and B in Fig. 3). The low signal intensity of these compounds in cheese is probably due to the scarcity of alcohols in the Asìno samples; nevertheless, it should not be excluded that the greater formation of ester compounds in the *salmuerie* with respect to the cheese could be explained by differences in the microbial enzymatic systems existing between the two systems which in turn lead to a different influence on the occurrence of esterification reactions.

In conclusion, the analysis of the volatile compounds proved that the *salmuerie* soaking phase plays an important role in differentiating the profile of the Asìno cheese. In particular, it was demonstrated that in the volatile fraction of this cheese the fatty acids class constituted by short-chain compounds (C_2, C_3, C_4 and C_6) prevails. These compounds were not measured at such high intensities in the control samples, while the *salmuerie* had the highest value. Therefore, it might be surmised that the high amount of the fatty acids measured in the Asìno cheese does not derive from internal lipolytic processes, but rather is due to the migration of these compounds from the *salmuerie*. Octanoic acid (C_8), which gave a very high signal intensity in the *salmuerie* but not in the cheese, is probably unable to migrate into the cheese because of its lower water solubility.

To test this hypothesis, the acetic, propionic, butyric and caproic acid profiles were quantitatively evaluated at different times of the soaking phase by the method set up by INNOCENTE *et al.* (2000). Their trends are shown in Figs. 4, 5, 6 and 7, respectively. From the profiles of Fig. 4, it is clear that while the acetic acid content is high in the *salmuerie* and remains almost steady for the duration of the soaking, in the Asìno sample it starts at very low values and then rises and reaches an equilibrium with that of the brine. On the other hand, in the control cheese ripened in the warehouse the increase in acetic acid is very small and never reaches the high values of the sample soaked in *salmuerie*. Similar considerations can also be made for the other short-chain fatty acids (Figs. 5, 6, and 7); however, these compounds take a longer time to reach equilibrium with the *salmuerie*, probably because of their lower content. All these profiles prove that the short-chain fatty acids in Asìno cheese do not derive from internal lipolysis, but migrate from the *salmuerie*, in which they are present in large amounts, into the cheese, where initially they are scarce.

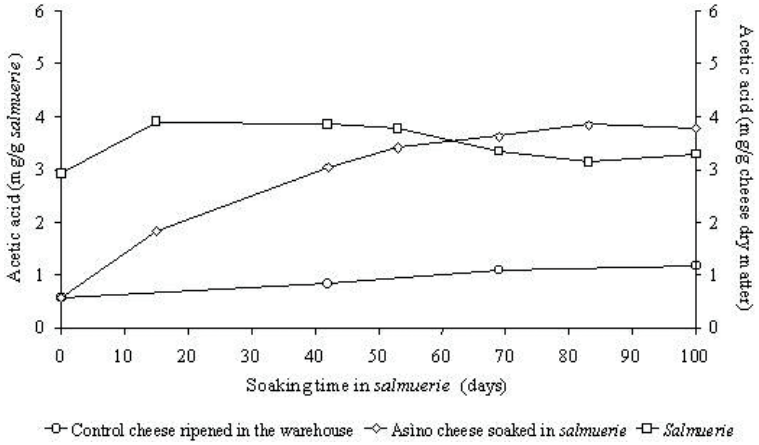

Fig. 4 - Trend of acetic acid content, expressed as mg/g cheese dry matter and as mg/g *salmuerie*, during 100 days of immersion. Each value is the mean of nine repetitions (three for each cheesemaking process).

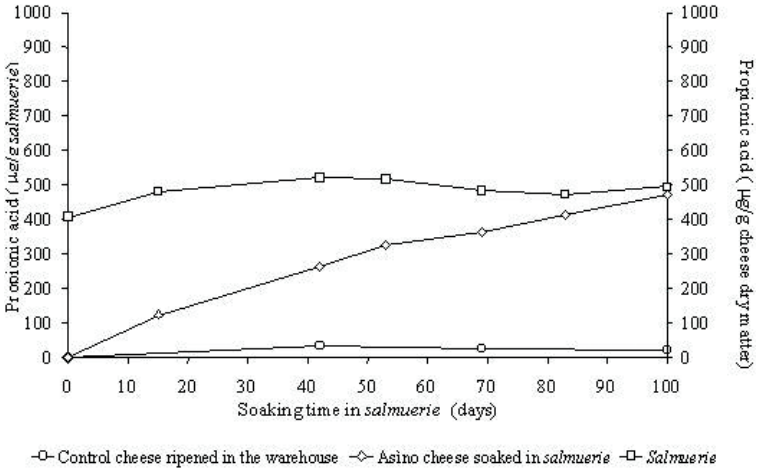

Fig. 5 - Trend of propionic acid content, expressed as µg/g cheese dry matter and as µg/g *salmuerie*, during 100 days of immersion. Each value is the mean of nine repetitions (three for each cheesemaking process).

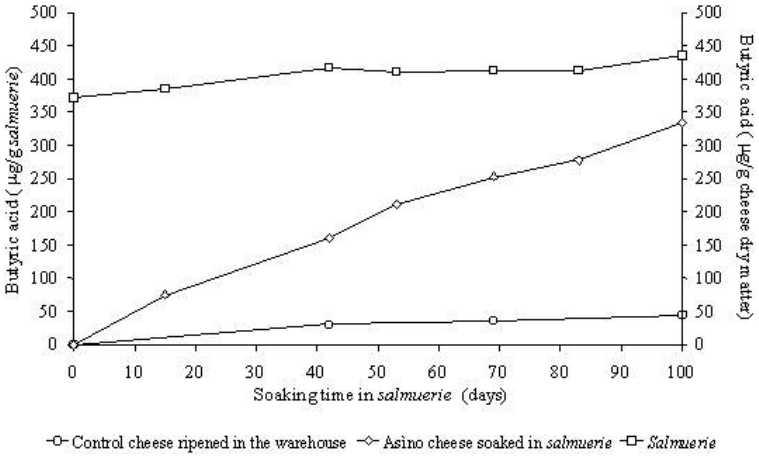

Fig. 6 - Trend of butyric acid content, expressed as µg/g cheese dry matter and as µg/g *salmuerie*, during 100 days of immersion. Each value is the mean of nine repetitions (three for each cheesemaking process).

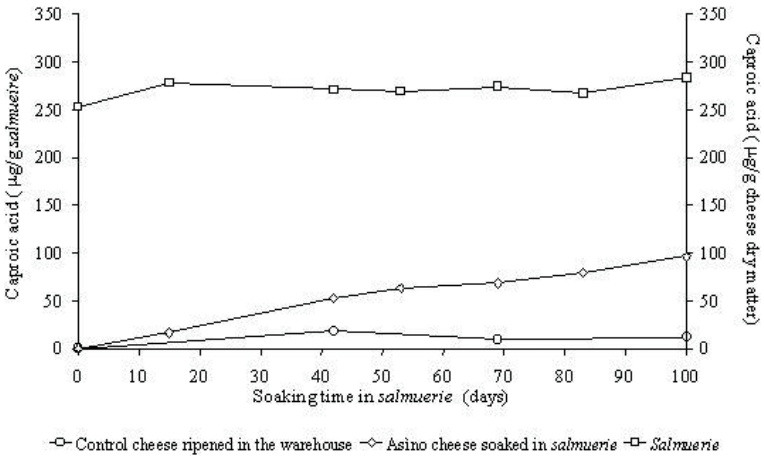

Fig. 7 - Trend of caproic acid content, expressed as μg/g cheese dry matter and as μg/g *salmuerie*, during 100 days of immersion. Each value is the mean of nine repetitions (three for each cheesemaking process).

Previous studies have demonstrated that the sensory profile of the Asìno cheese differs from the warehouse-ripened control sample (INNOCENTE *et al.*, 2003); it is characterized by a very intense aroma and flavour, in which the pungent descriptor prevails (BIASUTTI, 2004). It is well-known that short-chain fatty acids directly contribute to the flavour of a number of cheeses and impart a marked taste and a slightly pungent sensation (MOLIMARD and SPINNLER, 1996; MCSWEENEY and SOUSA, 2000; MARILLEY and CASEY, 2004). It is reasonable to suggest that the peculiar flavour characteristics of the Asìno cheese are related to the high amounts of these compounds measured in the product.

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PAPER

PHENOLIC COMPOSITION OF RED GRAPES GROWN IN SOUTHERN ITALY

COMPOSIZIONE FENOLICA DI UVE ROSSE DEL SUD ITALIA

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ABSTRACT

The phenolic composition of red grapes native to Southern Italy (Aglianico, Carignano, Frappato, Gaglioppo, Negro Amaro, Nero d'Avola, Primitivo, Tintilia, and Uva di Troia) and an "international" grape (Cabernet Sauvignon) introduced into the Apulia region were investigated. Results showed that these cultivars could be divided into two groups on the basis of both their anthocyanin content and the presence of ortho-hydroxylated groups. Further differences regarded the ratio between fla-

RIASSUNTO

Lo studio ha riguardato la composizione fenolica di uve rosse native del Sud Italia (Aglianico, Carignano, Frappato, Gaglioppo, Negro Amaro, Nero d'Avola, Primitivo, Tintilia e Uva di Troia) e di un'uva internazionalmente diffusa (Cabernet Sauvignon) introdotta in Puglia. I risultati ottenuti hanno evidenziato che queste uve possono essere divise in due gruppi sulla base del contenuto in antociani e della presenza di gruppi –OH in posizione orto. Ulteriori differenze sono state evidenziate in re-

- Key words: anthocyanins, phenolic composition, proanthocyanidins, red grapes, wine-making -

vans reacting with vanillin and proanthocyanidins. The anthocyanin profile of the skin of Negro Amaro, Primitivo and Uva di Troia grapes was found to be a specific characteristic of the grape variety which was affected only slightly by the place of growing. The different phenolic composition of the cultivars determines a different aptitude to wine production. The Cabernet Sauvignon grapes, due to their high concentration in polyphenolic substances, could be added to the native grape varieties in order to produce wines with a more complex aroma.

lazione al rapporto tra i flavani che reagiscono con la vanillina e le proantocianidine. Inoltre, per le uve Negro Amaro, Primitivo ed Uva di Troia, il profilo antocianico delle bucce è risultato strettamente correlato alla cultivar e poco influenzato dal luogo di allevamento. La differente composizione fenolica determina differenti attitudini alla vinificazione per le varie uve. Le uve Cabernet Sauvignon, grazie al loro elevato contenuto in sostanze polifenoliche, possono essere efficacemente aggiunte con le uve originarie dell'Italia meridionale allo scopo di ottenere vini con aromi più complessi.

INTRODUCTION

Since the different chemical compounds present in grapes have considerable effect on wine quality, knowledge of the chemical composition of the grape is critical for the processing of certain types of wine. In addition to sugar concentration, pH and total acidity, the most important parameter to take into account is the polyphenolic content (in particular, anthocyanins, tannins and proanthocyanidins) that affects both the sensory characteristics of the wine (KOSIR *et al.*, 2004) and consumer health (LEIGHTON *et al.*, 1998; PECHÁNOVA *et al.*, 2002; SOLEAS *et al.*, 2002). These nutraceutical compounds are represented by natural antioxidants and have been found to be able to prevent cancer, cardiovascular diseases and dermatitis (YILMAZ and TOLEDO, 2004) and to inhibit LDL oxidation (FRANKEL *et al.*, 1993). The parts of the berry richest in phenols are skins and seeds (Amerin and Joslyn, 1987) which, in the traditional process, are discarded. Pulp is poor in phenolic compounds and as a consequence, red wines (obtained by maceration in the presence of the skins) are richer than the white ones. The phenolic composition of the seed is qualitatively and quantitatively different from that of the skins. The former are rich in flavonoids such as monomers, dimers, trimers and polymers of the flavanols (Haslam, 1980) and phenolic acids (gallic and ellagic) (YILMAZ and TOLEDO, 2004). In addition to these compounds, the skins of the red berries contain anthocyanins responsible for the red-purple colour. Skins and seeds also contain rutin, quercetin and trans-resveratrol (the cis-form is found in the wine but not in the grape) (PALOMINO *et al.*, 2000).

Grapes with red-coloured berries are characterized by the presence of anthocyanins. The anthocyanin profile may be used as a chemotaxonomic marker for classifying red grape cultivars (REVILLA *et al.*, 2001). Since the concentration of the different anthocyanins is strictly dependent on the genetic code (BAKKER and TIMBERLAKE, 1985; SCIENZA *et al.*, 1985; ROGGERO *et al.*, 1986; CRAVERO and DI STEFANO, 1992), it could be considered as an index of cultivar biodiversity together with the content of oth-

er phenolic compounds such as the hydroxycinnamyl tartaric acids (ROMEYER *et al.*, 1985; DI STEFANO and MORUNO, 1986; TAMBORRA *et al.*, 2003), the catechins and the proanthocyanidins (KONTEX, 1986; LEE and YAWORSKI, 1987; CRAVERO and DI STEFANO, 1990; KOVAC *et al.*, 1990).

The purpose of this study was to characterize the polyphenolic fraction of some red grape varieties native to southern Italy, in order to assess the influence of this fraction on the wine-making potential of these varieties. Comparison was made with the "international" Cabernet Sauvignon grape grown in the same climatic and soil conditions.

MATERIALS AND METHODS

The study was focused on the following grapes typical of Southern of Italy (the native regions are reported in brackets):

- Aglianico (Basilicata);
- Tintilia (Molise);
- Gaglioppo (Calabria);
- Negro Amaro, Primitivo and Uva di Troia (Apulia);
- Carignano (Sardinia);
- Frappato and Nero d'Avola (Sicily).

In order to investigate the variability of the anthocyanin content as a function of the growing environment, each of the grapes from Apulia was grown in three different locations in the region: Negro Amaro at Sandonaci, Mesagne and Leverano; Primitivo at Santeramo, Sava and Manduria and Uva di Troia at Barletta, Canosa and San Severo.

Grapes of the Cabernet Sauvignon cultivar, grown in the areas around Cerignola (Province of Foggia, in the Apulia Region) were also included in this study. The reason for this choice is that Cabernet Sauvignon is considered an enhancing grape and it is often added to the native grapes to improve the quality of the wine.

Sampling

Grape sampling and extraction of the phenolic substances were carried out according to DI STEFANO and CRAVERO (1991). Three hundred and fifty berries were considered for each type of grape and then, 3 samples consisting of 10 berries were withdrawn from them (3 replicates).

Analyses

Berries were separated into skins, seeds and pulp. The pulp was pressed and the must obtained was analysed for sugar, pH and total acidity according to the EEC 2676 standard procedure (1990). Tartaric and malic acids were analysed by high performance liquid chromatography (HPLC) according to the method proposed by CANE (1990).

The seeds of 10 berries were separated from the pulp by means of a lancet whereas the skins were carefully scraped from the pulp. Successively, seeds and skins were separately put into 100 mL beakers containing 25 mL of a tartaric buffer solution at pH 3.20. The buffer solution was previously prepared by dissolving 5 g tartaric acid, 22 mL of 1N NaOH, 2 g sodium metabisulfite and 125 mL 95% ethanol in distilled water up to a final volume of 1 litre in a calibrated flask. The final pH was carefully controlled. Seeds and skins were kept in the buffer solution for 12 h (in the dark, at room temperature) and then filtered. Skin extracts were analysed for total flavonoids, total anthocyanins, proanthocyanidins and flavans reacting with vanillin according to DI STEFANO *et al.* (1989), whereas the anthocyanin profile was determined by HPLC-DAD (DI STEFANO and CRAVERO, 1991). In addition to the analysis of the seed extracts for total flavonoids, proanthocyanidins and flavans reacting with vanillin, the flavan composition was also determined by HPLC-DAD according to UMMARINO *et al.* (2001).

Statistical analysis

For each determination, average and standard deviation were calculated on three replicates. Principal Component Analysis was applied to the anthocyanin content in order to evaluate the differences among samples using Winstat, ver. 6.0 (Statsoft, Tulsa, USA) software.

RESULTS AND DISCUSSION

All the grapes were harvested at a good stage of ripeness, showing a total sugar content greater than 210 g/L, pH values between 3.16 and 4.15, total acidity ranging from 4.50 to 9.70 g/L, tartaric acid from 3.33 to 9.48 g/L and malic acid from 0.73 to 3.98 g/L (Table 1).

Table 2 reports the percentage of anthocyanins in the skin extracts, whereas Tables 3 and 4 report the phenolic concentrations of skin and seed extracts, respectively.

In comparing the anthocyanin profiles (Table 2), it was possible to divide the cultivars into two groups. The first group included Aglianico, Cabernet Sauvignon, Carignano, Nero d'Avola, Primitivo, Tintilia and Uva di Troia. The skins of these varieties had an anthocyanin profile characterized by a predominance of malvidin-3-glucoside and by percentages of anthocyanins containing ortho-hydroxylated groups (delphinidin-3-glucoside, cyanidin-3-glucoside and petudinin-3-glucoside) less than 26%. This low amount indicates a good wine-making potential as these compounds react with oxygen, giving rise to compounds called chinons which form stable structures with heavy metals that precipitate and weaken the wine colour (USSEGLIO-TOMASSET, 1985).

The skin extracts also had a high content of proanthocyanidins (P), flavans reacting with vanillin (responsible for the sensorial characteristics of astringency and bitter taste) (FRV), and values of the FRV/P ratio ranging from 0.15 for Carignano to 0.83 for the Uva di Troia (Table 3). The relevant amount of anthocyanins different from those containing ortho-hydroxylated groups increases the probability of their interaction with flavans and ethanol, leading to a stable red pigmentation of the wine. These particular characteristics make the grapes suitable for vinification, including frequent aeration and brief or medium-length maceration, so as to induce the extraction of the skin polyphenols only. The grapes of these varieties can be used to produce several types of wine: rosé, nouveau, young wines and wine for aging, obviously according to the degree of ripeness of the grape and the length of time of maceration of the skins and seeds in the must. The seed extracts showed a

Table 1 - Chemical characteristics of grapes at vintage.

Cultivar	Sugars (g/L)	pH	Total acidity (g/L)	Tartaric acid (g/L)	Malic acid (g/L)
Aglianico	216±4	3.29±0.02	6.97±0.08	5.77±0.21	0.85±0.11
Cabernet Sauvignon	236±3	3.65±0.02	5.77±0.07	5.44±0.18	3.85±0.24
Carignano	212±5	4.15±0.02	4.55±0.24	3.82±0.12	0.90±0.08
Frappato	273±7	3.16±0.02	9.70±0.20	9.48±0.32	1.00±0.21
Gaglioppo	242±5	3.87±0.03	4.50±0.18	3.33±0.14	1.60±0.23
Negro Amaro	238±3	3.38±0.03	7.88±0.09	6.73±0.25	2.58±0.18
Nero d'Avola	216±5	3.39±0.03	8.77±0.15	6.90±0.41	3.98±0.35
Primitivo	258±5	3.38±0.01	6.50±0.09	6.00±0.23	1.47±0.13
Tintilia	230±4	3.45±0.02	5.70±0.10	5.30±0.17	1.60±0.22
Uva di Troia	210±4	3.61±0.03	5.10±0.17	4.62±0.31	0.73±0.13

Table 2 - Percentage (%) of the various anthocyanin forms in the grape skin extracts.

Cultivar	Delphinidin-3-glucoside	Cyanidin-3-glucoside	Petunidin-3-glucoside	Peonidin-3-glucoside	Malvidin-3-glucoside	Acetylate forms	p-Coumarate forms
Aglianico	4.8±0.1	0.5±0.0	5.6±0.1	2.7±0.1	54.4±0.8	7.7±0.2	24.3±0.4
Cabernet Sauvignon	9.6±0.2	1.9±0.1	5.7±0.2	6.7±0.2	47.9±0.6	19.4±0.4	8.8±0.2
Carignano	n.d.	n.d.	15.1±0.4	3.7±0.2	36.1±0.4	8.8±0.2	36.3±0.7
Nero d'Avola	10.2±0.2	2.4±0.0	9.7±0.2	3.8±0.1	44.7±0.4	18.3±0.4	10.9±0.3
Primitivo	4.4±0.1	0.9±0.0	6.2±0.2	3.9±0.1	49.0±0.7	6.1±0.1	29.5±0.7
Tintilia	7.9±0.2	5.1±0.2	12.9±0.3	7.3±0.2	66.8±1.0	n.d.	n.d.
Uva di Troia	5.0±0.2	0.8±0.0	4.6±0.1	3.2±0.1	28.2±0.3	24.2±0.5	34.0±0.6
Frappato	13.0±0.3	12.0±0.3	14.0±0.3	21.9±0.4	37.0±0.5	n.d.	2.1±0.1
Gaglioppo	5.6±0.1	36.5±0.6	5.3±0.2	37.2±0.6	15.4±0.3	n.d.	n.d.
Negro Amaro	15.3±0.3	3.8±0.1	15.2±0.4	3.6±0.1	35.3±0.5	4.8±0.1	22.0±0.5

n.d.: not detectable (<0.1%).

Table 3 - Phenolic concentration of skin extracts (mg/kg).

Cultivar	Total Flavonoids	Total Anthocyanins	Proanthocyanidins (P)	Flavans Reagent with Vanillin (FRV)	FRV/P
Aglianico	2940±43	700±12	1205±30	548±24	0.45±0.03
Cabernet Sauvignon	2113±32	845±18	3354±39	1120±28	0.33±0.04
Carignano	2503±35	418±7	2040±38	307±24	0.15±0.01
Nero d'Avola	2080±24	809±15	3064±87	959±33	0.31±0.02
Primitivo	2560±28	712±6	1199±26	773±27	0.64±0.04
Tintilia	4674±64	1439±32	1454±39	307±12	0.21±0.01
Uva di Troia	2887±31	714±14	1509±35	1258±58	0.83±0.02
Frappato	2266±18	627±11	2059±64	740±17	0.36±0.02
Gaglioppo	2543±28	467±27	1831±27	654±16	0.36±0.01
Negro Amaro	2916±15	1088±14	2893±67	803±25	0.28±0.03

modest amount of phenolic compounds, with the exception of Nero d'Avola and Cabernet Sauvignon and a FRV/P ratio generally higher than that of the skins (from 0.59 for Carignano to 0.97 for Cabernet Sauvignon) (Table 4). These high FRV/P values could lead to a greater reactivity of the flavans due to the low degree of tannin polymerisation.

The other group of grapes is represented by Gaglioppo, Frappato and Negro Amaro. These cultivars had an anthocyanin profile characterized by percentages of malvidin-3-glucoside less than 37% and percentages of anthocyanins containing ortho-hydroxylated groups greater than 34% (Table 2). The considerable reactivity of these anthocyanins could be the cause of the strong colour changes observed in wines made from Gaglioppo and Negro Amaro grapes (LOVINO *et al.*, 2000; 2001). In fact, the colour of these wines becomes orange-red the first summer after wine-making. The polyphenolic content of the skins derived from these grapes (Table 3) was good and they had a low FRV/P ratio indicating that tannins should not be too reactive. Instead, the seed extracts of these grapes had a high FRV/P ratio, indicating the presence of more reactive tannins (Table 4).

The polyphenolic profile of the grapes belonging to the second group suggests

Table 4 - Phenolic concentration of the seed extracts (mg/kg).

Cultivar	Total Flavonoids	Proanthocyanidins (P)	Flavans Reagent with Vanillin (FRV)	FRV/P
Aglianico	878±23	511±18	480±26	0.94±0.02
Cabernet Sauvignon	3987±65	4022±75	3885±81	0.97±0.05
Carignano	781±32	977±38	580±24	0.59±0.01
Nero d'Avola	2973±78	3048±67	2877±72	0.94±0.04
Primitivo	931±12	870±31	835±34	0.96±0.02
Tintilia	977±38	1082±43	929±28	0.86±0.01
Uva di Troia	182±21	600±24	432±14	0.72±0.01
Frappato	2280±55	2141±58	1806±43	0.84±0.03
Gaglioppo	751±18	650±14	617±31	0.95±0.01
Negro Amaro	1848±11	1967±42	1701±37	0.86±0.03

that a wine-making process should be chosen that is able to extract the greatest amount of anthocyanins and polyphenols from the skins and the lowest amount of tannins from the seeds. In fact, since tannins promote the formation of stable red pigment, they would be useful in the early stages of vinification due to their high reactivity but, on the other hand, a high tannin content could affect the gustative characteristics in a negative way. Furthermore, the wine-making techniques should include the use of small containers in the early stages of production and aeration or oxygenation only after the alcohol is produced. In this way, the increased quantity of flavans produced should enhance the formation of stable red pigments (flavanols-anthocyanins-acetaldehyde) (DALLAS *et al.*, 1996). Another wine-making technique suitable to process these grapes is the staggered extraction of the anthocyanins and the separate fermentation of the solid parts (DI STEFANO and BOSSO, 2002) which would enable the reactions between anthocyanins and flavans and allow the formation of more stable red pigments to increase. In fact, this technique ensures a greater production of acetaldehyde that has a primary role in the formation of these pigments. On the other hand, it is necessary to avoid the polymerisation of monomeric/oligomeric flavans and monomeric anthocyanins, since these reactions cause a decrease in the anthocyanin content and undesirable precipitation phenomena.

Cabernet Sauvignon grapes could be advantageously blended with all the considered grapes in order to add methoxypyrazines (ALLEN *et al.*, 1989) that are molecules giving a greatly appreciated flavour to wines, making them suitable for a wide group of consumers. Furthermore, the addition of Cabernet Sauvignon grapes could improve the wines produced from the grapes of the second group by increasing the content of malvidin-3-glucoside and thus contributing to the formation of more stable red pigments.

The anthocyanin skin profiles relative to the three Apulian grapes (Table 5) were submitted to Principal Component Analysis and the results are reported in Fig. 1. From the data reported in Table 5 and Fig. 1, it is evident that cultivar significantly affected the anthocyanin profile, whereas the influence of the growing place was less important. These results are similar to those obtained by other authors (SAINT-CRICQ *et al,* 1999; GLORIES, 2001; Revilla *et al,.* 2001; GONZÁLEZ-NEVES *et*

Table 5 - Percentage (%) of the anthocyanins in the grape skin extracts from the three Apulian varieties grown in different areas.

Cultivars	Growing Areas	Delphinidin-3-glucoside	Cyanidin-3-glucoside	Petunidin-3-glucoside	Peonidin-3-glucoside	Malvidin-3-glucoside	Acetylate forms	p-Coumarate forms
Negro Amaro	Sandonaci	15.3±0.4	3.8±0.2	15.0±0.3	3.6±0.1	35.5±0.4	4.8±0.2	22.0±0.7
	Mesagne	10.7±0.8	10.7±0.9	12.0±0.5	11.0±0.8	34.5±0.3	4.6±0.3	16.5±0.5
	Leverano	17.0±0.5	3.3±0.0	17.0±0.3	2.8±0.1	34.7±0.5	4.7±0.2	20.5±0.5
Primitivo	Santeramo	4.7±0.1	1.4±0.1	6.4±0.2	4.0±0.1	44.8±0.8	7.4±0.2	31.3±0.8
	Sava	5.9±0.1	2.3±0.2	8.0±0.1	11.3±0.7	48.0±0.5	6.0±0.1	18.5±0.7
	Manduria	4.4±0.0	1.2±0.0	6.2±0.0	4.3±0.2	49.0±0.7	5.4±0.3	29.5±0.7
Uva di Troia	Barletta	5.0±0.2	0.8±0.0	4.7±0.1	3.2±0.2	28.1±0.3	24.2±0.4	34.0±0.8
	Canosa	5.2±0.1	1.1±0.1	5.0±0.3	3.2±0.1	31.2±0.3	22.7±0.3	31.6±0.9
	San Severo	4.2±0.1	0.9±0.0	4.6±0.0	3.0±0.1	28.4±0.4	22.9±0.5	36.0±0.5

al., 2004) who found that the anthocyanic profiles of the grapes are typical of each cultivar even if the amount of the different anthocyanins can change as a consequence of the environmental and pedoclimatic conditions and management of the vineyard. Fig. 1 shows that it is possible to classify the three varieties as a function of specific anthocyanins: Uva di Troia grapes are distinguishable for their content of acetylated and p-coumarate forms; Primitivo for their content of malvinidin-3-glucoside and peonidin-3-glucoside; Negro Amaro for their content in cyanidin-3-glucoside, petunidin-3-glucoside, delphi-

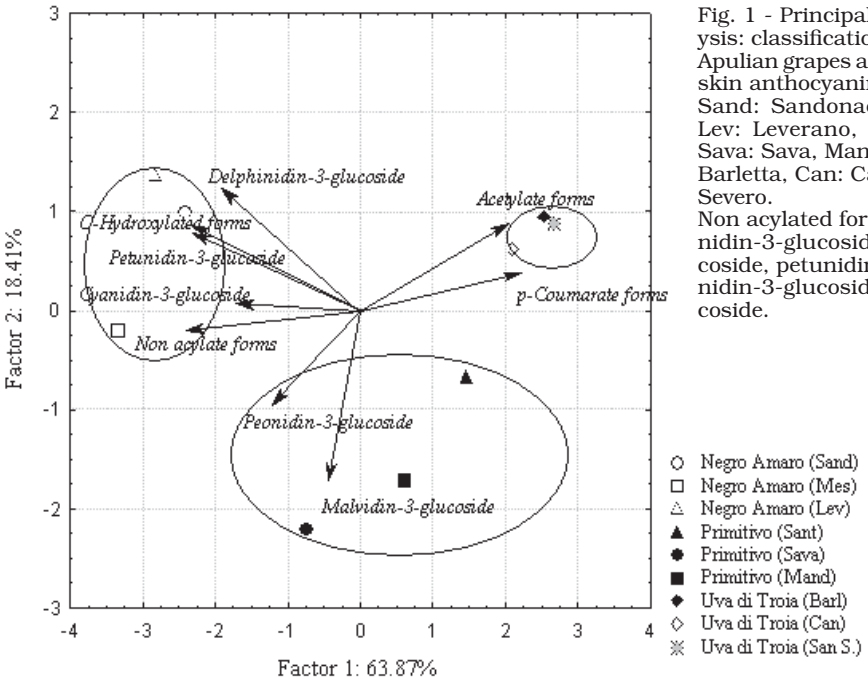

Fig. 1 - Principal Component Analysis: classification map of the three Apulian grapes as a function of their skin anthocyanin profile.
Sand: Sandonaci, Mes: Mesagne, Lev: Leverano, Sant: Santeramo, Sava: Sava, Mand: Manduria, Barl: Barletta, Can: Canosa, San S.: San Severo.
Non acylated forms: sum of delphinidin-3-glucoside, cyanidin-3-glucoside, petunidin-3-glucoside, peonidin-3-glucoside, malvidin-3-glucoside.

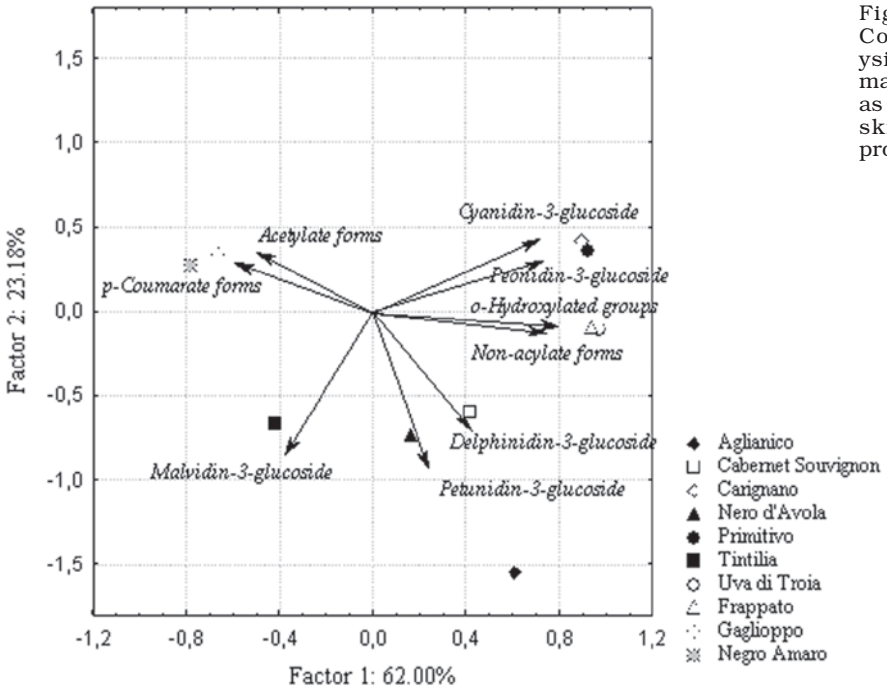

Fig. 2 - Principal Component Analysis: classification map of the grapes as a function of their skin anthocyanin profile.

nidin-3-glucoside, non acylated forms and o-hydroxylated forms. The first factor described over 63% of the explained variance. These results are confirmed by the application of Principal Component Analysis to the ten varieties considered (Fig. 2). It is worth noting that grapes native to the same region had different anthocyanin profiles and that the quantitative-qualitative anthocyanin composition is specific to each variety. In this case the first factor described 62% of the explained variance.

Finally, as shown in Fig. 3, the seed flavans are mainly composed of (+)-catechin, (-)-catechin and catechin-gallate followed by the procyanidins B2 and B3.

CONCLUSIONS

The grape varieties considered showed marked differences in their phenolic composition, thus suggesting dividing them into two different groups on the basis of their content of anthocyanins having ortho-hydroxylated groups. The first group was characterised by a low amount of these compounds, whereas the second had a higher percentage of them. Concerning Negro Amaro, Primitivo and Uva di Troia grapes, the anthocyanin profile was only slightly affected by the growing place, whereas the cultivar showed the greatest influence.

Different harvesting time and wine-making processes have to be carefully chosen for each variety of grape. There are very few grape varieties that, if used alone in a wine-making process, are able to produce a high-quality, harmonious wine. Indeed, many of the best-known wines are obtained by using a mixture of grapes or by blending wines produced from different cultivars. Cabernet Sauvignon grapes grown in Puglia could be blended with native cultivars to produce wines with a higher content of stable red

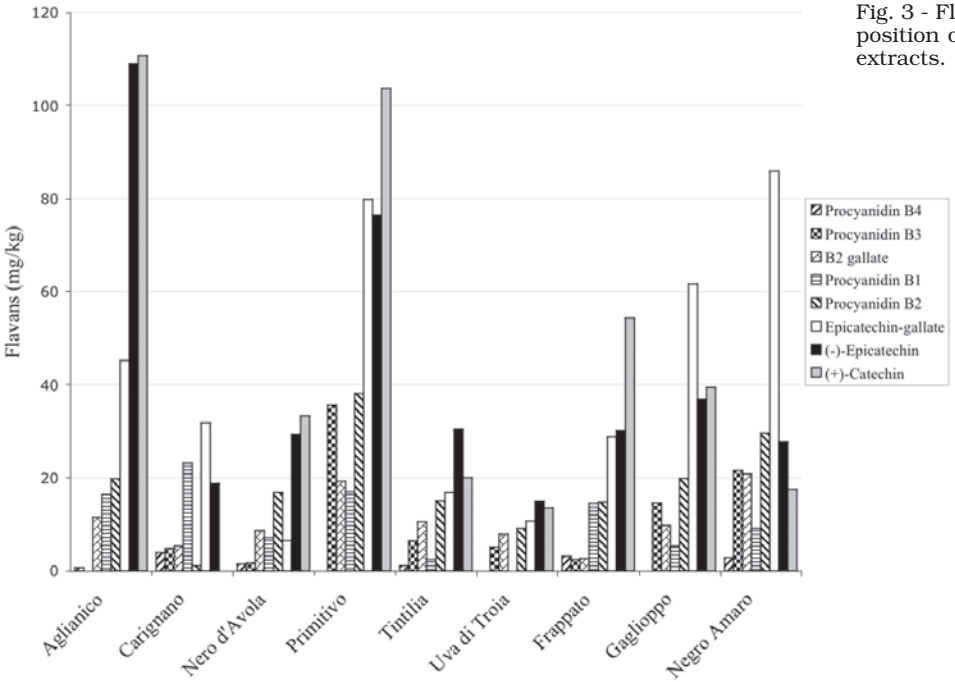

Fig. 3 - Flavan composition of the seed extracts.

pigments and characterised by a more complex flavour.

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PAPER

ANTIOXIDANT ACTIVITY OF MALVASIA ISTRIANA GRAPE JUICE AND WINE

ATTIVITÀ ANTIOSSIDANTE IN SUCCO D'UVA E VINO DI MALVASIA ISTRIANA

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ABSTRACT

The phenolic composition, including hydroxybenzoic acids, hydroxycinnamic acids and flavan-3-ols, was identified and quantified in grape juice and wine produced from cv. Malvasia istriana (*Vitis vinifera* L.) grafted on two rootstocks, Kober 5BB and 420A, by using high-performance liquid chromatography (HPLC) coupled with diode array detection. Protocatechuic acid, *p*-coumaric acid, (+)-catechin and (-)-epicatechin were the major phenols determined in the samples. The antioxidant

RIASSUNTO

È stata determinata, tramite cromatografia ad alta prestazione (HPLC) accoppiata ad un rivelatore a serie di diodi (DAD), la composizione qualitativa e quantitativa in composti fenolici, inclusi gli acidi idrossibenzoici, idrossicinnamici e i flavan-3-oli, di succo d'uva e vino ottenuti da cv. Malvasia istriana (*Vitis vinifera* L.), innestata su portinnesti Kober 5BB e 420A. I principali componenti fenolici nei campioni in esame sono risultati: l'acido protocatechico, l'acido p-cumarico, la (+)catechina e la (-)epi-

- Key words: antioxidant activity, BCB, DPPH, Malvasia istriana, polyphenols -

activity of grape juice and wine was measured by the β-carotene bleaching (BCB) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging methods. Good correlation was observed between the total phenol content of grape juice and wine and the antioxidant activity measured by the BCB and DPPH methods as well as between the results of both methods.

catechina. L'attività antiossidante del succo d'uva e del vino è stata determinata con il metodo della decolorazione del β-carotene (BCB) e con il metodo del DPPH (2.2 difenil-1-picril-idrazil) radical scavenging. È stata osservata una buona correlazione tra il contenuto di fenoli totali del succo d'uva e l'attività antiossidante misurata con i due metodi del BCB e del DPPH e tra tra i risultati ottenuti dai due metodi.

INTRODUCTION

The phenolic compounds of grapes and wines are very important, as they contribute to sensory characteristics, particularly color, astringency and bitterness (ROBICHAUD and NOBLE, 1990) and they influence the oxidation reactions (Oszmianski, 1989), interactions with proteins (HO *et al.*, 1980) and the ageing potential of wines (HASLAM, 1980). The phenolic composition varies due to a wide range of factors, including the degree of grape ripeness, variety, season, region, growing conditions and processing practices (JACKSON, 1993).

In contrast to red winemaking, white grape processing technology is aimed at limiting the duration of extraction of phenolic compounds. White wine contains significantly lower amounts of total polyphenols compared with red wines, mainly hydroxycinnamic acids, hydroxybenzoic acids and flavan-3-ols (MAKRIS *et al.*, 2003). The total phenol content of white wines, vinified with minimal skin contact, falls in the range between 100 and 250 mg/L. Approximately 30 mg/L of this amount is accounted for by flavonoid phenols, mainly flavan-3-ol (-)-epicatechin and (+)-catechin and its dimmers, oligomers and polymers (FISHER and NOBLE, 1994; NOBLE, 1990). Phenolic acids in grape berries are concentrated primarily in the skin and grape pulp (RAMOS, 1999).

The antioxidant activity of phenolic acids and their esters depends on the number of hydroxyl groups in the molecule strengthened by steric hindrance (RICE–EVANS, 1996). Hydroxycinnamic acids (HCA) (C_6-C_3) are derivatives of cinnamic acid. Caffeic acid has been demonstrated to be a strong antioxidant against LDL oxidation, with the following order of potency: caffeic acid>ferulic acid>*p*-coumaric acid. Conjugation with tartaric acid apparently enhances the antioxidant potential of all three acids (MEYER, 1998a). Hydroxybenzoic acids (HBA) have a general structure of a C_6-C_1 benzoic ring derived directly from benzoic acid and they differ according to hydroxylation and methoxylation of the aromatic ring. During fermentation and ageing of wine, various reactions take place in which HCA and HBA acids change their form and content (SOMERS *et al.*, 1987).

There is increasing interest in antioxidants, particularly in those intended to prevent the presumed deleterious effects of free radicals in the human body, and to prevent the deterioration of fats and other constituents of foodstuffs. In both cases, there is a preference for anti-

oxidants from natural rather than synthetic sources (ABDALLA and ROOZEN, 1999). There is therefore a parallel increase in the use of methods for estimating the efficiency of such substances as antioxidants (SANCHEZ-MORENO, 2002; SCHWARZ *et al.*, 2001; BADERSCHNEIDER *et al.* 1999*;* FOGLIANO *et al.* 1999; SANCHEZ-MORENO *et al.*, 1999a).

There are only a few publications related to phenolic compounds determined in must and wine of grape varieties grown in Croatia, and most of them deal with red wines (PILJAC *et al.*, 2005; KATALINIĆ *et al.*, 2004; BUDIĆ-LETO *et al.*, 2003). Almost no attention has been given to the non-colored phenolic acids of native Croatian white grape varieties. Therefore, this study was focused on determining the concentration of seven phenolic acids and (+)-catechin, (-)-epicatechin and (-)-epicatechin gallate of the native grape variety Malvasia istriana (*Vitis vinifera* L.) from the Istrian peninsula. Individual phenolic compounds were identified and quantified by HPLC with UV-DAD detection. In addition to the phenolic composition, the antioxidant activity of Malvasia istriana grape juice and wine was determined with respect to different rootstocks and different bud loads per vine on both rootstocks. For a more reliable assessment, the free radical scavenging capacity of Malvasia istriana grape juice and wine was measured by two different methods: DPPH (2,2-diphenyl-1-picrylhydrazyl) and the BCB (β-carotene bleaching).

MATERIALS AND METHODS

Samples

Monovarietal Malvasia istriana wine was made from grapes grown on two rootstocks, Kober 5BB and 420 A. The grapes were harvested at the technological state of ripeness in September 2003. The experiment was carried out in a completely randomized manner by choosing 5 well-balanced vines with three repetitions for bud load (20, 30 and 40 buds per vine) on both rootstocks.

The wines were produced in the wine cellar *Minivinifikacija* of the Institute for agriculture and tourism in Poreć. Grape juice samples were collected immediately after pressing. The wine-making procedure consisted of the following steps: after pressing, the must was placed in stainless steel tanks and 20 g/hL of potassium-metabisulphite was added followed by sedimentation at 12°C for 48 hours. Pure must was decanted. A culture of multiplied selected *Saccharomyces cerevisiae,* under the commercial name VIN13 (Anchor Bio-technologies, Cape Town, South Africa), was added to the quantity of 20 g/hL and the yeast nutrient Zimovit (Ever, Pramaggiore, Italy) was also added to the quantity of 20 g/hL. The fermentation temperature was kept at 14°C. After fermentation (sugar content below 2.5 g/L), the wine was decanted. The stainless steel tanks were protected by inert gas (nitrogen). The wine was kept under these conditions and regularly controlled for levels of free SO_2. Corrections were made if necessary. The wine was decanted again. In the period between March and May 2004, wine clarification was carried out with 100 g/hL bentonite (Fortbenton-Ever, Pramaggiore, Italy). After that, the wine was filtered through a filter (Strassburger, Westhofen, Germany) followed by microfiltration (∅ 0.65 μm). Before bottling, the wine was sulfurated with SO_2, so that the amount of free SO_2 was 20 mg/L. The wines were bottled and stored at the temperature of 16°C, corresponding to the temperature of the wine cellar.

Chemicals

Vanillic, syringic and *p*-coumaric acids were obtained from Fluka (Fluka Chemie, Buchs, Switzerland). Ferulic

acid, gallic acid, protocatechuic acid, (+)-catechin, (-)-epicatechin and (-)-epicatechin gallate were obtained from Sigma (Sigma-Aldrich Chemie, Steinheim, Germany). Caffeic acid and HPLC grade methanol were obtained from Merck (Darmstadt, Germany). Formic acid and Folin-Ciocalteu reagent were of analytical grade and were supplied by Kemika (Zagreb, Croatia). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and linoleic acid were obtained from Aldrich (Sigma-Aldrich Chemie, Steinheim, Germany). DPPH (2,2-diphenyl-1-picrylhydrazyl) and Tween 40 were purchased from Fluka (Sigma-Aldrich Chemie, Steinheim, Germany). β-carotene was obtained from Sigma (Sigma-Aldrich Chemie, Steinheim, Germany).

Total phenol content

The total phenol concentration in selected samples was determined spectrophotometrically according to the Folin-Ciocalteu colorimetric method (SINGLETON and ROSSI, 1965). The measurements were calibrated against gallic acid standards and the results are expressed as mg/L of gallic acid equivalents (GAE). Data are averages of three measurements.

Antioxidant activity determined with the DPPH radical scavenging method

The samples were analyzed according to the technique reported by BRAND-WILLIAMS *et al.* (1995). Twenty microliters of Malvasia istriana wine was added to 0.094 mM 2-diphenyl-1-picrylhydrazyl (DPPH) in methanol and made up to 1 mL. The free radical scavenging activity using the free radical DPPH reaction was evaluated by measuring the absorbance at 515 nm after 60 min of reaction at 20°C in a water bath. The reaction was carried out in closed eppendorf tubes shaken at 20°C. The results are expressed as mmol/L Trolox equivalents, a vitamin E analogue (YAMAGUCHI *et al.*, 1998). All measurements were performed in triplicate.

Antioxidant activity determined with β-carotene bleaching method

The antioxidant activity with the BCB method was measured using the procedure of VON GADOW *et al.* (1997). Two hundred microliters of undiluted Malvasia istriana wine was added to the reaction mixtures. Readings of all samples were taken immediately (t=0) and at 15 min intervals for 2h (t=120 min) on a UV-Vis Unicam, Helios β spectrophotometer at 470 nm. The vials were placed in a water bath at 50°C between measurements.

The antioxidant activity coefficient (AAC) was calculated from the data according to the formula (MALLET *et al.*, 1994):

$$AAC = [(A_{A(120)} - A_{C(120)}) / / (A_{C(0)} - A_{C(120)})] \times 1000$$

where $A_{A(120)}$ is the absorbance of the anti oxidant at t = 120 min, $A_{C(120)}$ is the absorbance of the control at t = 120 min and $A_{C(0)}$ is the absorbance of the control at t = 0 min. All determinations were performed in triplicate.

HPLC-DAD analysis

The wine samples were filtered through a 0.45 μm filter (Nylon Membranes, Supelco, Bellefonte, PA, USA) before HPLC analysis. Twenty microliters of each sample was injected for HPLC analysis using a Varian Pro Star Solvent Delivery System 230 (Varian, Walnut Creek, CA, USA) and a Varian Pro Star 330 Photodiode Array detector (Varian, Walnut Creek, CA, USA) using a Pinnacle II C-18 reversed-phase column (Restek, Bellefonte, PA, USA) (250x4.6 mm, 5 μm i.d.). The solvents consisted of water, 3% formic

acid (solvent A) and HPLC grade methanol (solvent B) at a flow rate of 1 mL/min. The elution was performed with a gradient starting at 2% B to reach 32% B at 20 min, 40% B at 30 min and 95% B at 40 min, and became isocratic for 5 min. Chromatograms were recorded at 278 nm.

Detection was performed with a Photodiode Array Detector by scanning between 200-400 nm, with a resolution of 1.2 nm. Phenolic compounds were identified by comparing the retention times and spectral data with those of authentic standards. Quantitative determinations were performed using standard curves of determined hydroxycinnamic acids, hydroxybenzoic acids and flavan-3-ols. The data acquisition and treatment were conducted using the Star Chromatography Workstation Version 5 software. All analyses were repeated three times, and the results are expressed as mean values in milligrams per liter of wine ± SD.

Statistical analysis

Significant differences between the samples were calculated by analysis of variance (ANOVA) using a significance level of $p<0.05$ and Duncan's multiple range test. Results were processed by the computer programme Statistica 6.0 (StatSoft Inc., Tulsa, OK, USA).

RESULTS AND CONCLUSIONS

Table 1 summarizes the content of the hydroxycinnamic acids identified in the grape juice and wine samples. These acids are well known in grapes as secondary metabolites of phenylalanine (MACHEIX *et al.*, 2000). The following hydroxycinnamic acids were identified: caffeic acid, *p*-coumaric acid and ferulic acid. The content of caffeic and *p*-coumaric acids in the grape juice samples was on the average 0.49±0.04 and 0.71±0.06 mg/L, respectively, and in the wine samples on average 2.18±0.10 and 2.17±0.07 mg/L. The highest contents of caffeic and *p*-coumaric acids were determined in wine samples 2 and 5. The average content of ferulic acid in grape juice and wine amounted to 0.29± 0.03 and 1.48±0.08 mg/L, respectively. Significantly higher values of hydroxycinnamic acid content ($p<0.05$) were observed for the wine samples. Free hydroxycinnamic acids in wine probably originate from the hydrolysis of hydroxycinnamic tartaric esters during fermentation (CHEYNIER *et al.*, 1986).

Table 1 - Content of hydroxycinnamic acids identified in the grape juices and wines.

Sample*	Caffeic acid		*p*-Coumaric acid		Ferulic acid		Total hydroxycinammics	
	grape juice	wine	grape juice	wine	grape juice	wine	grape juice	wine
420A (20)	0.42±0.04^{d}	1.97±0.21^{a}	0.66±0.09^{c}	2.16±0.19^{a}	0.24±0.06^{a}	1.40±0.21^{c}	1.32±0.11^{c}	5.53±0.35^{d}
420A (30)	0.53±0.09^{ab}	2.24±0.33^{c}	0.75±0.12^{ab}	2.25±0.20^{a}	0.25±0.02^{a}	1.55±0.09^{ab}	1.53±0.15^{ab}	6.04±0.40^{bc}
420A (40)	0.46±0.13^{ab}	1.74±0.15^{f}	0.64±0.16^{dc}	1.94±0.09^{a}	0.26±0.09^{a}	1.32±0.25^{d}	1.36±0.22^{c}	5.04±0.31^{f}
Kober 5BB (20)	0.51±0.09^{ab}	2.33±0.16^{b}	0.72±0.17^{bc}	2.16±0.11^{a}	0.32±0.10^{a}	1.45±0.25^{bc}	1.55±0.22^{ab}	5.94±0.32^{c}
Kober 5BB (30)	0.55±0.08^{a}	2.65±0.31^{a}	0.79±0.13^{a}	2.30±0.14^{a}	0.34±0.12^{a}	1.63±0.16^{a}	1.68±0.19^{a}	6.58±0.38^{a}
Kober 5Bb (40)	0.49±0.11^{b}	2.14±0.18^{d}	0.71±0.22^{bc}	2.19±0.28^{a}	0.31±0.03^{a}	1.50±0.09^{b}	1.51±0.25^{b}	5.83±0.34^{c}
Mean value	0.49±0.04	2.18±0.10	0.71±0.06	2.17±0.07	0.29±0.03	1.48±0.08	1.49±0.07	5.83±0.15

* Rootstock and bud load per vine in parentheses.
Each value is expressed as mean value in mg/L sample ± standard deviation (n=3). Values within each column followed by the different superscript letter are significantly different ($p<0.05$).

Hydroxybenzoic acids represent the class of phenolic compounds present at the lowest concentrations in the grape juice (1.0-1.10%) and wine samples (0.68-0.76%) (Table 2). Protocatechuic acid was the major hydroxybenzoic acid, followed by gallic acid, syringic acid and vanillic acid. It is also evident that the content of all the identified hydroxybenzoic acids was higher in the grape juice and wine samples produced from grapes grown on the Kober rootstock. The bud load of 30 buds per vine on this rootstock yielded the highest value. In comparing the content of individual hydroxybenzoic acids in grape juices and wine, it was observed that the content of gallic, syringic and protocatechuic acids was lower in wine, whereas the content of vanillic acid showed a slight increase. One of the ways of biosynthesis of vanillic acid is through β-oxidation of ferulic acid, thus, the increased level of vanillic acid in wine may be attributed to the transformation of ferulic to vanillic acid (LAZARO *et al.*, 1990). There were significant differences between the hydroxycinnamic acid content of grape juice and wine ($p<0.05$). In comparison with the previously reported data of non-colored phenolics in some white wines (MAKRIS *et al.*, 2003; RAMOS *et al.*, 1999), Malvasia istriana wine is characterized by relatively low HBA and HCA contents.

The most important group of phenolic compounds causing bitterness in red and white wines is flavan-3-ols. These phenols are extracted from the skins, stems and seeds of grapes in the course of vinification. (+)-Catechin, (-)-epicatechin and (-)-epicatechin gallate were identified from the group of flavan-3-ols (Table 3). The (+)-catechin content varied from 6.13±0.29 to 7.58±0.17 mg/L (mean 7.13±0.09 mg/L) in grape juices, and from 5.84±0.36 to 6.15±0.27 mg/L (mean 6.03±0.12 mg/L) in the wine samples. The content of (-)-epicatechin ranged from 3.22±0.11 to 3.61±0.10 mg/L (mean 3.52±0.04 mg/L) in grape

Table 2 - Content of hydroxybenzoic acids identified in the grape juices and wines.

Sample*	Gallic acid		Protocatechuic acid		Vanillic acid		Syringic acid		Total hydroxybenzoics	
	grape juice	wine	grape juice	wine	grape juice	wine	grape juice	wine	grape juice	wine
420A (20)	0.99±0.22^{cd}	0.34±0.05^{c}	1.37±0.13^{b}	0.98±0.18^{c}	0.17±0.05^{a}	0.26±0.08^{c}	0.57±0.09^{d}	0.37±0.07^{d}	3.10±0.28^{c}	1.95±0.21^{c}
420A (30)	1.04±0.28^{c}	0.39±0.08^{b}	1.31±0.19^{b}	0.93±0.09^{c}	0.23±0.12^{a}	0.36±0.11^{b}	0.62±0.11^{d}	0.41±0.09^{c}	3.20±0.38^{c}	2.09±0.19^{c}
420A (40)	0.93±0.29^{d}	0.33±0.11^{c}	1.26±0.31^{b}	0.92±0.11^{c}	0.13±0.09^{a}	0.19±0.02^{c}	0.41±0.08^{e}	0.17±0.12^{f}	2.73±0.32^{d}	1.61±0.20^{d}
Kober 5BB (20)	1.15±0.19^{b}	0.38±0.06^{ab}	1.76±0.41^{a}	1.22±0.16^{a}	0.20±0.07^{a}	0.29±0.12^{c}	0.82±0.15^{b}	0.43±0.14^{c}	3.93±0.48^{ab}	2.32±0.25^{b}
Kober 5BB (30)	1.31±0.17^{a}	0.59±0.12^{a}	1.80±0.21^{a}	1.18±0.21^{ab}	0.23±0.05^{a}	0.41±0.04^{b}	0.91±0.23^{a}	0.76±0.06^{a}	4.25±0.36^{a}	2.94±0.25^{a}
Kober 5BB (40)	1.09±0.21^{bc}	0.42±0.05^{ab}	1.67±0.09^{a}	1.14±0.06^{b}	0.21±0.06^{a}	0.48±0.10^{a}	0.74±0.11^{c}	0.54±0.09^{b}	3.71±0.26^{b}	2.58±0.16^{b}
Mean value	1.09±0.09	0.41±0.04	1.53±0.10	1.06±0.06	0.20±0.03	0.33±0.04	0.68±0.07	0.45±0.10	3.49±0.15	2.25±0.10

* Rootstock and bud load per vine in parentheses.
Each value is expressed as mean values in mg/L sample ± standard deviation (n=3). Values within each column followed by the different superscript letter are significantly different ($p<0.05$).

Table 3 - Content of flavan-3-ols identified in the grape juices and wines.

Samples*	(+)-Catechin		(-)-Epicatechin		(-)-Epicatechin gallate		Total flavan-3-ols	
	grape juice	wine	grape juice	wine	grape juice	wine	grape juice	wine
420A (20)	6.13±0.29^{e}	6.09±0.34^{a}	3.69±0.04^{a}	2.24±0.08^{c}	0.59±0.11^{b}	0.37±0.05^{c}	10.41±0.31^{b}	8.70±0.35^{c}
420A (30)	7.46±0.33^{a}	5.98±0.42^{a}	3.34±0.15^{c}	2.14±0.09^{d}	0.43±0.14^{d}	0.31±0.08^{cd}	11.23±0.39^{c}	8.43±0.42^{d}
420A (40)	7.12±0.21^{d}	5.84±0.36^{a}	3.22±0.11^{d}	2.03±0.12^{e}	0.40±0.09^{d}	0.22±0.04^{e}	10.74±0.25^{d}	8.09±0.38^{e}
Kober 5BB (20)	7.58±0.17^{a}	6.15±0.27^{a}	3.69±0.05^{a}	2.68±0.14^{a}	0.59±0.13^{b}	0.46±0.11^{b}	11.86±0.22^{a}	9.29±0.32^{b}
Kober 5BB (30)	7.21±0.08^{bc}	6.09±0.11^{a}	3.61±0.10^{a}	2.72±0.05^{a}	0.69±0.16^{a}	0.59±0.15^{a}	11.51±0.20^{bc}	9.40±0.19^{a}
Kober 5BB (40)	7.26±0.10^{b}	6.01±0.22^{a}	3.56±0.06^{b}	2.35±0.07^{a}	0.54±0.14^{c}	0.29±0.09^{de}	11.36±0.18^{b}	8.65±0.25^{c}
Mean value	7.13±0.09	6.03±0.12	3.52±0.04	2.36±0.04	0.54±0.05	0.37±0.04	11.19±0.12	8.76±0.13

* Rootstock and bud load per vine in parentheses.
Each value is expressed as mean values in mg/L sample ± standard deviation (n=3). Values within each column followed by the different superscript letter are significantly different ($p<0.05$).

juices and from 2.03±0.12 to 2.72±0.05 mg/L (mean 2.36±0.04 mg/L) in wines. The content of (-)-epicatechin gallate was higher in grape juices and the calculated mean was 0.54±0.05 mg/L of (-)-epicatechin gallate, whereas in wines the mean content of this compound was 0.37±0.04 mg/L.

The identified flavan-3-ols represent 4.24 and 3.51% of the total phenolics in grape juice and wine, respectively. This is less than in most white wines in which flavonoids account for approximately 20% of the phenolic content (BADERSCHNEIDER and WINTERHALTER, 2001). In red wines, flavonoids account for more than 85% of the phenolics.

Analysis of variance and Duncan´s multiple range test (Tables 1, 2, 3) showed significant differences ($p<0.05$) among results of grape juice samples and also of wine samples.

The total phenol content and the antioxidant activity of Malvasia istriana grape juices are given in Table 4. The values, determined according to the Folin-Ciocalteu method, varied from 234±2.71 to 312±2.51 mg/L GAE (mean 269±0.92 mg/L GAE). Malvasia istriana wine contained lower amounts of total phenols, ranging from 219±0.91 to 288±1.35 mg/L GAE (mean 252±0.72 mg/L GAE) (Table 5). The highest mean total phenol values were found in grape juices and wine

Table 4 - Total phenol content and antioxidant activity of the grape juice samples.

Juice samples*	Total phenols (mg/LGAE)	DPPH (mmol/L Trolox)	BCB (AAC)
420A (20)	234±2.71	0.435±0.03	307.15±2.26
420A (30)	258±1.83	0.463±0.09	351.88±1.32
420A (40)	230±3.45	0.450±0.05	295.01±2.33
Kober 5BB (20)	301±0.52	0.487±0.11	384.22±4.52
Kober 5BB (30)	312±2.51	0.504±0.05	406.30±4.10
Kober 5BB (40)	279±1.29	0.471±0.22	369.13±1.22
Mean value	269±0.92	0.468±0.01	352.28±1.19

* Rootstock and bud load per vine in parentheses.
Values are expressed as mean ± standard deviation of three separate measurements.

Table 5 - Total phenol content and antioxidant activity of the wine samples.

Wine samples*	Total phenols (mg/LGAE)	DPPH (mmol/L Trolox)	BCB (AAC)
420A (20)	223±1.12	0.814±0.51	368.06±5.18
420A (30)	240±1.62	0.867±0.21	406.31±6.21
420A (40)	219±0.91	0.801±0.64	346.28±5.22
Kober 5BB (20)	272±2.65	0.908±0.41	437.89±2.34
Kober 5BB (30)	288±1.35	0.925±0.36	459.55±2.87
Kober 5BB (40)	265±2.24	0.882±0.72	418.35±3.33
Mean value	252±0.72	0.866±0.21	406.07±1.81

* Rootstock and bud load per vine in parentheses.
Values are expressed as mean ± standard deviation of three separate measurements.

samples produced from grapes grown on the Kober rootstock, especially for the bud load of 30 buds per vine (Tables 4 and 5). In comparing the total phenol content and antioxidant activity of the samples, it can be observed that the content of total phenols in wine is lower than in grape juice, whereas the values of the antioxidant activity of wine measured by the DPPH and β-carotene bleaching methods are higher than in grape juice. These results point to a good correlation between the total phenol content of grape juices and their antioxidant activity measured by the DPPH method (r = 0.9626). An even better correlation (r = 0.9743) was observed for the wine samples. Also, good correlation was observed between the total phenol content of grape juice and wine samples and their antioxidant activities determined by the β-carotene bleaching method (r_{juice} = 0.9822 and r_{wine} = 0.9713) as well as between the results of both methods used (r_{juice} = 0.9418 and r_{wine} = 0.9952).

Determination of the radical scavenging ability of edible plant products and wines using discoloration of 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) has been widely used due to its stability, simplicity and reproducibility. The reaction is based on reduction of the DPPH radical in a reaction with a phenolic compound that donates a hydrogen atom, which leads to the loss of violet color (MOLYNEUX, 2004; DE BEER, 2003). The DPPH reacts directly with the phenolic compound, while β-carotene bleaching coincides with the primary oxidation of linoleic acid. Malonaldehyde forms from the decomposition of hydroxyperoxides during secondary oxidation reactions of linoleic acid. These differences could lead to variations in the measurement of antioxidant activity. Because the methods used to measure the antioxidant activity are extremely dependent on the reaction conditions and the substrates or products, all methods do not yield the same values for the activity (FUKUMOTO and MAZZA, 2000). FRANKEL (1993) and WARNER (1997), reviewing limitations of antioxidant activity assays, suggested that antioxidant activity be measured using more than one method, by detecting the primary and secondary oxidation products, and using tests that detect specific substrates or products.

When the antioxidant activity of the samples determined by the DPPH method was compared with the total content of hydroxycinnamic and hydroxybenzoic acids and flavan-3-ols, good correlation was observed. In grape juices, these correlation coefficients decreased in the order: hydroxycinnamic acid (r = 0.9581) > hydroxybenzoic acid (r = 0.9043) > flavan-3-ols (r = 0.8885). In wine samples, the correlation coefficients followed the same order with the values of r = 0.9115,

r = 0.9000 and r = 0.8344. In comparing the antioxidant activity of grape juices determined by the BCB method with the total content of hydroxycinnamic acids, hydroxybenzoic acids and flavan-3-ols, the highest correlation coefficient was observed between the antioxidant activity values and hydroxycinnamic acid (r = 0.9670) followed by hydroxybenzoic acid (r = 0.9530) and flavan-3-ols (r = 0.8894). In wine samples, the correlation coefficients followed the same order with the values of r = 0.9318, r = 0.9178 and r = 0.8605. Some authors have observed that certain hydroxycinnamates are less effective antioxidants compared with flavonoids (CUVELIER *et al.*, 2000), but other investigations have indicated that caffeic acid and its derivatives may be potent inhibitors of LDL oxidation (MEYER *et al.*, 1998a,b) in vitro. Furthermore, caffeic acid exhibited a stronger antioxidant effect compared to both (+)-catechin and (-)-epicatechin, according to the β-carotene and DPPH methods (FUKUMOTO and MAZZA, 2000). Similar studies dealing with the antioxidant activity of individual phenolic compounds showed that the activity of phenolic acids according to the DPPH method decreases in the order caffeic acid > ferulic acid > *p*-coumaric acid (MAKRIS *et al.*, 2003; SANCHEZ-MORENO *et al.*, 1999b). It can be concluded that cinnamic acid derivatives are better antioxidants than their benzoic acid counterparts (ferullic acid > vanillic acid; caffeic acid > protocatechuic acid). This observation is accounted for by the CH=CHCOOH group, which participates in stabilizing the radicals of cinnamic acid derivatives by resonance (CUVELIER *et al.*, 1992).

Generally, the content of phenolic compounds with similar structures followed similar trends in all methods. For the benzoic and cinnamic acid derivatives, flavonols and anthocyanidins, an increase in the number of hydroxyl groups led to higher antioxidant activity. The compounds with three hydroxyl groups on the phenyl ring of phenolic acids exhibited high antioxidant activity. The loss of one hydroxyl group caused a slight decrease in the activity, whereas the loss of two hydroxyl groups significantly decreased the activity (FUKUMOTO and MAZZA, 2000).

It is interesting to point out that the antioxidant activity of grape juices and wine is not a property of a single phytochemical group of compounds, but is widely distributed among the phenolic constituents (FRANKEL *et al.*, 1995, SIMONETTI *et al.*, 1997). Therefore, it is important to bear in mind that the polyphenols identified in this paper represent a part of the total polyphenols found in grape juice and wine, indicating that the other non-identified compounds contribute in a significant manner to the antioxidant activity of the samples studied.

The study presented herein provides new insights into the polyphenolic composition of Malvasia istriana grape juice and wine and highlights some interesting points about the correlation between the major classes of white wine polyphenols and their antioxidant properties.

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PAPER

FATTY ACIDS, CHOLESTEROL OXIDES AND CHOLESTEROL IN BRAZILIAN PROCESSED CHICKEN PRODUCTS

ACIDI GRASSI, OSSIDI DI COLESTEROLO E COLESTEROLO IN PRODOTTI BRASILIANI INDUSTRIALI A BASE DI POLLO

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ABSTRACT

Total lipids extracted from 7 types of processed chicken products were analyzed for total fat, fatty acid composition, cholesterol oxides and cholesterol. The Chester® chicken frankfurters had the highest levels of cholesterol (64 mg/100 g) and the lowest total lipid content (8 g/100 g). The Chester® chicken hamburger showed the lowest cholesterol level (31 mg/100 g) and the chicken mortadella had the highest total lipid content (19 g/100 g). All samples showed C18:3n-3 varying from 0.3

RIASSUNTO

Gli estratti lipidici totali ottenuti da 7 differenti tipologie di prodotti industriali a base di pollo sono stati analizzati per quanto riguarda il tenore in lipidi totali, ossidi di colesterolo e la composizione in acidi grassi. L'hamburger di pollo Chester® presentava i livelli più alti di colesterolo (64 mg/100 g) e il più basso contenuto di lipidi totali (8 g/100 g). L'hamburger di pollo Chester® presentava il più basso valore di colesterolo (31 mg/100 g) e la mortadella di pollo presentava il più alto contenuto di lipidi to-

- Key words: chicken products, cholesterol, cholesterol oxide, fatty acid -

g/100 g in chicken frankfurters to 0.1 g/100 g in the other samples analysed. No cholesterol oxides were found in the samples probably due to the presence of the antioxidant sodium erythorbate in the processed chicken products. In general, the processed chicken products had higher total lipid, saturated and monounsaturated fatty acid contents than the Chester® chicken products, however the cholesterol and the polyunsaturated fatty acid contents were similar for both products.

tali (19 g/100 g). Tutti i campioni presentavano un contenuto in C18:3n-3 compreso fra 0,3 g/100 g, nei würstel di pollo Chester®, e 0,1 g/100 g negli altri campioni analizzati. Non sono stati rilevati ossidi di colesterolo nei campioni analizzati, probabilmente per la presenza dell'antiossidante eritorbato di sodio nei prodotti a base di pollo. In generale i prodotti industriali a base di pollo presentavano un maggior tenore di lipidi totali, acidi grassi saturi e monoinsaturi rispetto ai prodotti di pollo Chester®, tuttavia il tenore in colesterolo ed acidi grassi polinsaturi era simile per entrambi i prodotti.

INTRODUCTION

The production of domestic poultry has increased over the last 20 years. New species have been genetically developed, such as quick growing species with well developed carcasses, especially the breast and thigh. Perdigão Industrial, a Brazilian company that sells chicken meat and derivatives, developed a species of chicken called Chester®, which has 70% of the meat concentrated in the breast and thighs (PEREIRA *et al.*, 2000). In 2005, Brazilian broiler meat exports were projected to increase by 10% fuelled by competitive pricing, market promotion efforts, favourable exchange rates and avian flu related import bans on major competitors (USDA, 2005). In the first part of 2004, broiler meat exports were up 22% at 1 million tons. This increase was largely attributed, among other things, to Brazil's marketing strategy to increase value-added poultry meat such as high-end cuts and processed broilers. The vast majority of Brazil's exports are found in the top import-markets and have a strong domestic and foreign demand.

The AMERICAN HEART ASSOCIATION (2005) recommends that for individuals with normal blood cholesterol levels the daily ingestion of lipids should not exceed 30% of the total calorie intake, saturated fat should not exceed 10% of the total calorie intake and cholesterol intake should be less than 300 mg/day. However, for individuals with cardiovascular disease, cholesterol intake should be less than 200 mg/day. Furthermore, cholesterol can be oxidized under harsh conditions forming various cholesterol oxides which could contribute to the initiation of the formation of arteriosclerotic deposits (KUMAR and SINGHAL, 1991) and other undesirable biological effects such as mutagenicity, cytotoxicity and carcinogenicity (GUARDIOLA *et al.*, 1996). *Trans* fatty acids are of greater concern than saturated ones, since, in addition to increasing the level of low-density lipoproteins, they decrease the level of high density lipoproteins (LAMBERTSON, 1992). On the other hand, the polyunsaturated fatty acids (PUFA) are considered beneficial to health and there is evidence that C18:3n-3 independently reduces the risk of heart disease (RENAUD

and LANZMANN-PETITHORY, 2001). However, the beneficial effects of PUFA depend on the ratio of omega 6 (n-6) to omega 3 (n-3) fatty acids. The ideal ratio in the daily diet has still not been established, however, recent reports indicate an optimal n-6/n-3 balance between 2:1 and 3:1 (SIMOPOULOS *et al.*, 2000). According to BENGMARK (1998), the n-6/n-3 ratio had been about 2:1 in the human diet since the paleolithic period and nowadays it is about 1:1 in the Eskimo diet and is as high as 50:1 in most industrialized societies.

With the aim of providing data on these constituents which are important in human health, an integrated study on the fatty acid composition, cholesterol oxides, cholesterol and total lipids of processed chicken products sold in Brazil was carried out.

MATERIALS AND METHODS

Materials

Three types of chicken meat products (frankfurter, sausage and mortadella) and four types of Chester® ckicken meat products (mortadella, frankfurter, hamburger and sausage) were analysed. Table 1 shows the basic composition of these products. The frankfurters, sausages and mortadella should be stored under refrigeration conditions (up to +8°C). The shelf life for frankfurters is up to 60 days and up to 90 days for the sausages and mortadella, while the hamburger should be kept frozen (-12° to -18°C) with a shelf life of up to 120 days.

One brand of each of these products was examined, with the exception of the chicken frankfurters, for which two brands were examined. For each brand, three batches with different expiration dates were examined. Each batch consisted of three units. The samples were examined immediately after being acquired from supermarkets in Campinas, São Paulo, Brazil. The whole sample was ground up and then blended in a multiprocessor until a homogeneous mass was obtained. Fifty-gram samples of these homogenates were analysed in duplicate.

Methods

The lipids were extracted as described by FOLCH *et al.* (1957). Aliquots of the same extracts were taken for the determination of the total lipids, fatty acid composition, cholesterol oxides and cholesterol.

Determination of the fatty acid composition by GC

To determine the fatty acid composition, 0.1 g of the extracted lipid was converted to methyl esters by reaction with a solution of ammonium chloride and sulphuric acid in methanol (HARTMAN and LAGO, 1973). Fatty acid methyl esters were separated on a gas chromatograph (model 4000A HRCG KONIK, Barcelona, Spain) equipped with a split injector (75:1), fused silica capillary column (50 m x 0.25 mm i.d., 0.20 μm film thickness of cyanosilicone) (CP-SIL 88, Chrompack, Middelburg, The Netherlands), flame ionisation detector and workstation (Borwin, Fontaine, France). The GC oven temperature programme began at 180°C for 2 min and then ramped to 225° at 5°C per min, the injector temperature was set at 270°C and the detector temperature at 300°C. Hydrogen was used as carrier gas at a flow rate of 0.5 mL/min and nitrogen as make-up gas at 30 mL/min. The fatty acids were identified by comparing the retention times of the sample with those of the standards and by spiking. A total of 37 saturated, monounsaturated and polyunsaturated fatty acid standards (37 FAME Mix 47885-U, Sulpeco™, Bellefonte, PA, USA) were used to veri-

Table 1 - Basic composition of the processed meat products.

Products	Ingredients
Chicken	
Frankfurter	Chicken meat, mechanically deboned chicken meat, chicken fat, soy protein, salt, starch (max. 2%), natural condiments, monosodium glutamate, sodium nitrite and nitrate and sodium erythorbate.
Sausage	Chicken meat, soy protein, salt, natural condiments, sodium nitrite, monosodium glutamate and sodium erythorbate.
Mortadella	Chicken meat, mechanically deboned chicken meat, chicken giblets, chicken skin, starch (max. 5%), soy protein, natural condiments and aromas, monosodium glutamate, glucose, salt, sodium nitrite and nitrate, sodium erythorbate.
Chester® chicken	
Frankfurter	Chester® meat, mechanically deboned Chester® meat, vegetable protein, glucose, salt, starch (max. 2%), natural condiments, sodium nitrate and nitrite and sodium erythorbate.
Sausage	Chester® breast, bacon, salt, starch, spices, sodium nitrite and nitrate and sodium erythorbate.
Mortadella	Chester® meat, mechanically deboned Chester® meat, starch (max. 5%), vegetable protein, glucose, salt, natural aromas, sodium nitrate and nitrite and sodium erythorbate.
Hamburger	Chester® meat, vegetable protein, monosodium glutamate, spices and sodium erythorbate.

fy the identity and the accuracy of the method. Quantification was calculated as area percentages.

Analysis of cholesterol and cholesterol oxides by HPLC

Aliquots of the lipid extracts were dried in a rotary evaporator, cold saponified and the non-saponifiable material was extracted with ethyl ether (SANDER *et al.*, 1989; BAGGIO and BRAGAGNOLO, 2004; BAGGIO *et al.*, 2005).

A Shimadzu liquid chromatograph (Kyoto, Japan) was used; it was equipped with a quaternary solvent delivery system (LC-10ATVP), a rheodyne injector with a 20 μL loop, photodiode array (SPD-M10AVP) and refractive index (RID-10A) detectors, a column heater (CTO-10ASVP) and software (CLASS – LC 10). Operating conditions were as follows: the mobile phase was *n*-hexane/isopropanol (96:4) at a flow rate of 1.0 mL/min, column Nova Pak CN HP, 300x3.9 mm column, 4 μm (Waters, Milford, MA, USA) set at 32°C and preceded by a Hypersil BDS CN 7.5x4.6 mm, 5 μm guard column. Absorption spectra were taken between 200 and 400 nm and the chromatograms were taken at 210 nm.

7α-hydroxycholesterol was obtained from Steraloids Inc. (Wilton, CT, USA). Cholesterol, cholesta-3,5-dien-7-one, 20α-hydroxycholesterol, 25-hydroxycholesterol, 7-ketocholesterol, 5,6α and 5,6β-epoxycholesterol and 7β-hydroxycholesterol were purchased from Sigma Chemical Company (St. Louis, MO, USA). HPLC grade *n*-hexane and isopropanol were obtained from Mscience (Darmstadt, Germany) and all other solvents were analytical grade from Merck (Darmstadt, Germany). The HPLC solvents were filtered through a 0.22 μm membrane filter Millipore (Bedford, MA, USA) under vacuum and degassing by ultrasound prior to use.

Quantification was done by external standardization, with a concentration range from 0.5 to 2.22 mg/mL for cholesterol and from 0.5 to 64.0 μg/mL for the cholesterol oxides. Cholesterol and 5,6α e 5,6β-epoxycholesterol were quantified using a refractive index detector, since in this case, cholesterol is better sepa-

rated and 5,6α e 5,6β-epoxycholesterols because they contain no π electron and do not absorb ultraviolet light. The other cholesterol oxides were quantified using the photodiode array detector. The detection limits were 0.14 μg/g of the sample to 20α-hydroxycholesterol and 25-hydroxycholesterol, 0.12 μg/g of the sample to 5,6α and 5,6β-epoxycholestrol and 7α and 7β-hydroxycholesterol and 0.09 μg/g of the sample to 7-ketocholesterol and cholesta-3,5-dien-7-one, calculated according to KEITH *et al.* (1983).

Identification and confirmation of cholesterol and its oxides were performed by comparing the retention times of the samples with those of the standards, co-chromatography, the characteristics of their absorption spectra and mass spectra. The mass spectras were obtained with a gas-chromatograph-mass spectrometer (Hewlett-Packard 6890 GC and 593 MS, Palo Alto, CA, USA) operated under electron ionization conditions with an electron energy of 70 eV (BAGGIO *et al.,* 2002).

Statistical analysis

The results were submitted to analysis of variance (ANOVA). Tukey's test was used to compare the means at a 5% significance level.

RESULTS AND DISCUSSION

Total lipids and fatty acid composition

New epidemiological studies conclude that the type of fat supplied in the diet is an important health factor. It has been proven that the substitution of saturated fat by unsaturated fat is more effective in decreasing the risk of heart disease than only reducing total fat intake. In addition, the influence of the different types of saturated fatty acids on the cholesterol levels and risk of heart disease has been reported (HU *et al.*, 2001).

The total lipid content (Table 2) varied from 8.4 g/100 g in the Chester® chicken frankfurters to 19.1 g/100 g in the mortadella. There was no significant difference ($p>0.05$) between the values found for total lipids in brand 2 of the chicken frankfurters and the Chester® chicken sausage. Significant differences were found among the other processed chicken products, probably due to their different compositions. On the other hand, the low standard deviation values found among the batches for all the brands indicated standardization on the part of the industry, offering consumers products with a uniform lipid content.

The lipid content in the chicken sau-

Table 2 - Cholesterol (mg/100 g) and total lipid (g/100 g) content of the processed chicken products.

Meat Products	Cholesterol M±SD*	Total lipids M±SD*
Chicken frankfurter (brand 1)	39.6±2.6 c	10.5±0.7 de
Chicken frankfurter (brand 2)	61.9±3.5 a	13.7±0.5 c
Chester® chicken frankfurter	64.1±4.2 a	8.4±0.8 f
Chicken sausage	45.0±1.8 bc	11.4±0.7 d
Chester® chicken sausage	40.4±1.3 c	13.5±0.1 c
Chicken mortadella	46.3±3.3 bc	19.1±0.3 a
Chester® chicken mortadella	51.9±7.0 b	17.4±0.2 b
Chester® chicken hamburger	31.0±1.3 d	9.7±0.9 e

*Means and standard deviations of three batches analyzed in duplicate for each brand. Values in the same column with the same letters are not significantly different at the 5% level.

sage was similar to that found by PEREIRA *et al.* (2000) (11.4 g/100 g). However, they found lower values in the Chester® chicken sausages (9.5 g/100 g). The USDA (2003) table reports a higher total lipid content (19.5 g/100 g) in chicken frankfurters.

Few studies on processed chicken products were found in the literature, so the results of the present work were compared with those of other meat products. NOVELLI *et al.* (1998) found higher total lipid contents in pork mortadella (34.5 g/100 g) and in Milan-type salami (36.0 g/100 g), as did MCCANCE and WIDDOWSON (1994) (25.0 g/100 g) and PIIRONEN *et al.* (2002) (16.0-35.8 g/100 g) in frankfurters. However, similar values were found in beef hamburgers (13.8 g/100 g) by RODRIGUEZ-ESTRADA *et al.* (1997) and in turkey hamburgers (12.0 g/100 g) and turkey frankfurters (9.8 g/100 g) by BAGGIO *et al.* (2005). Lower values were found in beef meatballs (6.6 g/100 g) by LARKESON *et al.* (2000) and in processed turkey meat (1.1-5.3 g/100 g) by BAGGIO *et al.* (2005).

Tables 3 and 4 report the fatty acid content (% area) found in the processed chicken meat products. The main fatty acids found were: C18:1n-9, C16:0, C18:2n-6, C18:0, C16:1n-7 and C18:3n-3. PEREIRA *et al.* (2000) found the same main fatty acids in the chicken and

Table 3 - Fatty acid composition (% area) of the chicken frankfurters and hamburger.

Fatty Acids	Chicken Frankfurter 1* M±SD***	Chicken Frankfurter 2** M±SD***	Chester® chicken Frankfurter M±SD***	Chester® chicken Hamburger M±SD***
C14:0	0.8±0.1 b	0.6±0.0 c	0.6±0.0 c	0.8±0.0 b
C15:0	0.4±0.1 a	0.3±0.1 b	0.4±0.0 a	0.4±0.0 a
C16:0	26.3±1.9 a	22.8±0.4 b	23.5±0.1 b	24.9±0.1 b
C17:0	0.3±0.1 b	0.5±0.1 a	0.3±0.1 b	0.2±0.0 b
C18:0	6.5±0.4 b	6.0±0.0 b	6.3±0.2 b	6.1±0.1 b
C20:0	0.2±0.0 a	tr	tr	0.1±0.0 b
C22:0	tr	tr	tr	0.2±0.0 a
C24:0	tr	0.1±0.0 a	0.2±0.0 a	0.1±0.0 a
C16:1n-7	5.7±0.4 a	4.4±0.2 b	4.7±0.2 b	5.7±0.3 a
C17:1n-7	0.3±0.1 b	0.3±0.0 b	0.4±0.1 b	0.1±0.0 c
C18:1n-9*t*	0.3±0.0 a	0.1±0.0 b	0.1±0.0 b	0.2±0.1 ab
C18:1n-9	36.9±0.6 c	35.2±1.4 c	35.9±0.4 c	38.2±0.1 b
C20:1n-11	0.2±0.0 c	0.3±0.0 c	0.3±0.0 c	0.3±0.0 c
C18:2n-6	20.5±1.6 bc	26.6±2.0 a	24.6±0.3 a	21.0±0.1 bc
C18:3n-6	0.2±0.0 a	0.2±0.0 a	0.2±0.0 a	0.2±0.0 a
C18:3n-3	1.0±0.1 c	2.0±0.2 a	1.5±0.0 b	1.1±0.0 c
C20:2n-6	0.1±0.0 b	0.2±0.0 b	0.2±0.0 b	0.1±0.0 b
C20:4n-6	0.5±0.1 b	0.6±0.0 b	0.9±0.0 a	0.5±0.0 b
Saturated	35 a	30 c	32 b	33 ab
Monounsaturated	43 b	40 c	41 c	44 a
Polyunsaturated	22 de	30 a	27 b	23 d
Total n-3	1.0	2.0	1.5	1.1
Total n-6	21.4	27.5	25.8	21.8
PUFA/SFA	0.6	1.0	0.8	0.7
n-6/n-3	21.4	13.7	17.2	19.8
Total lipids (g/100 g)	10.5	13.7	8.4	9.7

*Brand 1; **Brand 2; ***Means and standard deviations of three batches analyzed in duplicate for each brand; tr = traces (<0.1); PUFA/SFA = Polyunsaturated/Saturated; values in the same line in Tables 3 and 4 with the same letter are not significantly different at the 5% level.

Table 4 - Fatty acid composition (% area) of the chicken mortadella and chicken sausage.

Fatty Acids	Chicken Mortadella M±SD*	Chester® chicken Mortadella M±SD*	Chicken Sausage M±SD*	Chester® chicken Sausage M±SD*
C14:0	0.8±0.0 b	0.6±0.0 c	0.7±0.1 bc	1.1±0.1 a
C15:0	0.2±0.1 c	0.2±0.1 c	0.4±0.0 a	0.4±0.0 a
C16:0	24.1±0.7 b	24.1±0.3 b	24.8±2.6 b	23.8±0.8 b
C17:0	0.3±0.0 b	0.2±0.1 b	0.1±0.0 c	0.5±0.0 a
C18:0	7.0±0.2 b	5.9±0.1 b	6.6±0.2 b	8.6±0.4 a
C20:0	0.1±0.0 b	tr	0.1±0.0 b	0.1±0.0 b
C22:0	0.1±0.0 a	tr	0.2±0.0 a	0.2±0.0 a
C24:0	0.1±0.0 a	0.1±0.0 a	0.1±0.0 a	0.2±0.0 a
C16:1n-7	4.6±0.1 b	5.2±0.2 ab	5.1±0.3 ab	3.8±0.2 c
C17:1n-7	0.3±0.1 b	0.3±0.0 b	0.2±0.1 b	0.5±0.0 a
C18:1n-9*t*	0.2±0.0 ab	0.1±0.0 b	0.2±0.1 ab	0.3±0.0 a
C18:1n-9	39.3±0.9 a	37.1±0.4 c	36.8±0.6 c	39.8±0.4 a
C20:1n-11	0.4±0.1 b	0.2±0.1 c	0.2±0.1 c	0.6±0.1 a
C18:2n-6	20.7±0.3 bc	23.7±0.4 a	22.3±1.0 a	18.2±0.7 c
C18:3n-6	0.2±0.0 a	0.2±0.0 a	0.2±0.0 a	0.1±0.0 b
C18:3n-3	1.1±0.2 c	1.3±0.0 c	1.2±0.1 c	0.9±0.1 d
C20:2n-6	0.2±0.0 b	0.2±0.0 b	0.1±0.0 b	0.5±0.0 a
C20:4n-6	0.4±0.0 b	0.5±0.0 b	0.7±0.1 b	0.6±0.1 b
Saturated	33 ab	31 c	33 ab	35 a
Monounsaturated	44 a	43 b	42 bc	45 a
Polyunsaturated	23 d	26 bc	25 c	20 e
Total n-3	1.0	1.3	1.2	0.9
Total n-6	21.5	24.6	23.3	19.3
PUFA/SFA	0.7	0.8	0.8	0.6
n-6/n-3	21.5	18.9	19.4	21.4
Total lipid (g/100 g)	19.1	17.4	11.4	13.5

*Mean and standard deviation of three batches analyzed in duplicate; tr = traces (<0.1); PUFA/SFA = Polyunsaturated/Saturated; values in the same line in Tables 3 and 4 with the same letter are not significantly different at the 5% level.

Chester® chicken sausages. Higher values were recorded for the fatty acids C18:1n-9, C18:0 and C16:1n-7 in the chicken sausage, with lower values for C18:2n-6. Higher levels for C18:3n-3 were recorded in both chicken and Chester® chicken sausage.

The fatty acid composition, when expressed in g/100 g of sample, is directly related to the total lipid content. Consequently, meat products processed with a higher total lipid content will have proportionally higher fatty acid content. Thus the samples of chicken and Chester® chicken mortadella had the highest values for the main fatty acids found. The saturated fatty acid content varied from approximately 2.7 g/100 g in the Chester® chicken frankfurters to 6.3 g/100 g in the chicken mortadella. The C16:0 fatty acid was found in greatest concentration, followed by C18:0. According to KRIS-ETHERTON and YU (1997), among the saturated fatty acids, C14:0 and C12:0 are presumably those that exert the greatest influence in raising cholesterol levels. In the samples analysed, C12:0 was not detected and C14:0 contributed about 0.1 g to the total saturated fatty acid fraction.

The lowest concentration of monounsaturated fatty acids was found in the Chester® chicken frankfurters (3.4 g/100 g) and the highest concentration was in the chicken mortadella (8.4 g/100 g). Similar values were found in the Chester® chicken hamburgers (4.3 g/100 g), brand 1 of the chicken frankfurters (4.5 g/100 g) and

chicken sausage (4.8 g/100 g). The fatty acid that was found in the greatest amount was C18:1n-9, followed by C16:1n-7. The intake of monounsaturated fatty acids has been inversely associated with the risk of cardiovascular disease; the association is weaker for polyunsaturated fatty acids (PUFA) (HU *et al.*, 1997).

The total PUFA content was the lowest in the Chester® chicken hamburger (2.2 g/100 g) and the highest in the Chester® chicken mortadella (4.5 g/100 g). Similar contents were found in the Chester® chicken hamburgers and frankfurters and the brand 1 of the chicken frankfurters, and in the chicken and Chester® chicken mortadella. The fatty acid C18:2n-6 was found in the greatest amounts. The polyunsaturated and saturated fatty acid ratio was the highest in brand 2 of the chicken frankfurters (1.0) and the lowest in brand 1 of the chicken frankfurters, and Chester® chicken sausages (0.6).

The only n-3 PUFA found in the samples analysed was C18:3n-3. The levels were 0.3 g/100 g in brand 2 of the chicken frankfurters, 0.2 g/100 g in the chicken and Chester® chicken mortadella and 0.1 g/100 g in the other samples analysed. The n-3 PUFA are considered beneficial to health and there is evidence that C18:3n-3 independently reduces the risk of heart disease (RENAUD and LANZMANN-PETITHORY, 2001). This amount cannot be considered nonsignificant, taking into account that, for example, salmon paté contains 0.75 g/100 g (ECHARTE *et al.*, 2004). The n-6/n-3 ratio was high in all samples, varying from 13.7 in brand 2 of the chicken frankfurter to 21.5 in the chicken mortadella.

The only *trans* fatty acid isomer detected in the samples of processed chicken products analysed was C18:1n-9, varying from 0.008 g/100 g in the Chester® chicken frankfurter to 0.04 g/100 g in the chicken mortadella and Chester® chicken sausage. This amount can be considered very low when compared to a medium portion of french fries containing 5-6 g/100 g (KATAN, 2000). ARO *et al.* (1998) reported that the total *trans* fatty acid content in chicken meat samples from fourteen European countries varied from 0.2 to 1.7%, which is higher than those found in the present study (0.1 to 0.3%). According to SALES *et al.* (1996), the amount of *trans* fatty acids found in the meat of non-ruminant animals, such as poultry, is usually low and is related to the presence of *trans* fatty acids in the feed.

Cholesterol and cholesterol oxides

Table 2 reports the cholesterol levels found in the chicken samples analysed. There were no significant differences ($p>0.05$) in the cholesterol content between brand 1 of the chicken frankfurter and the Chester® chicken sausage, between brand 2 of the chicken frankfurter and the Chester® chicken frankfurter or between the chicken sausage and the chicken mortadella. There was a significant difference ($p<0.05$) between the two brands of chicken frankfurters. The highest cholesterol content was found in the Chester® chicken frankfurter and the lowest content was in the Chester® chicken hamburger. The greatest variation in cholesterol content within a batch was found in the Chester® chicken mortadella and frankfurter samples, with standard deviation values of 7.0 and 4.2, respectively. The variation in cholesterol content between batches could be related to the composition of the raw materials, such as the meat and other meat products used.

PEREIRA *et al.* (2000) found cholesterol contents of 44 mg/100 g in chicken and Chester® chicken sausages; these values were close to those found in the present study. The USDA (2003) table shows a higher cholesterol content (101 mg/100 g) for chicken frankfurters.

Since few studies about processed chicken products were found, the results of the present study were compared with those of other meat products. KING *et al.*

(1998) obtained higher values for cholesterol in turkey ham (116 mg/100 g) and turkey pastrami (118 mg/100 g). NOVELLI *et al.* (1998) found higher values in Milan salami (78-102 mg/100 g) and in pork mortadella (87-138 mg/100 g) and similar results for both samples (42-67 mg/100 g). RODRIGUEZ-ESTRADA *et al.* (1997) also found higher values in beef hamburger (83 mg/hamburger). However, similar values were shown for beef hamburgers and meatballs (46 and 39 mg/100 g, respectively) by LARKESON *et al.* (2000), for processed turkey products (32-43 mg/100 g) by BAGGIO *et al.* (2005) and for hot-dog frankfurters (36-75 mg/100 g) by PIIRONEN *et al.* (2002).

None of the cholesterol oxides analysed (cholesta-4,6-dien-3-one, 20α-hydroxycholesterol, 25-hydroxycholesterol, 5,6α-epoxycholesterol, 5,6β-epoxycholesterol, 7α-hydroxycholesterol, 7β-hydroxycholesterol and 7-ketocholesterol) were found in the samples. The absence of cholesterol oxides in the processed meat products could be related to the addition of the antioxidant sodium erythorbate in the formulations, as reported in Table 1. According to TAI *et al.* (1999), some antioxidants have been found to reduce the formation of cholesterol oxidation products. Similar results were also found in beef hamburger, beef jerky and liver sausage by PARK and ADDIS (1985). In addition, 7-ketocholesterol was only detected at very low levels in cheese after 1 year of storage at 37°C (KRISTENSEN *et al.*, 2001).

Cholesterol oxides are related to a variety of in vitro and in vivo biological effects, which could contribute to the development of disease and cholesterol metabolism (GUARDIOLA *et al.*, 1996; MOREL and LIN, 1996). Cholesterol oxides are absorbed within a few hours after consuming the food containing them (LINSEISEN and WOLFRAM, 1998). Thus diets containing cholesterol oxides could contribute to the development of arteriosclerosis, while diets free of such components could have an important role in preventing and treating arteriosclerosis. Considering that the processed chicken meat products analysed in this study had no cholesterol oxides, their consumption apparently does not represent a health risk.

CONCLUSIONS

The processed chicken meat products had total lipid contents above 5 g/100 g, so they cannot be classified as low-fat containing food (FOOD ADVISORY COMMITTEE, 1990). On the other hand, the cholesterol content varied from 31 to 64 mg/100 g, well below the maximum recommended level of 300 mg/day (AMERICAN HEART ASSOCIATION, 2005) and no cholesterol oxides were found. In addition, the amount of *trans* fatty acids in the samples was low and the levels of C18:3n-3 varied between 0.3 and 0.1 g/100 g. In contrast, the polyunsaturated/saturated fatty acid ratio varied from 0.6 to 1.0, which exceeds the recommended value of 0.45 for the overall diet, according to the BRITISH DEPARTMENT OF HEALTH (1994). Finally, the n-6/n-3 ratio varied from 13.7 to 21.4, which is also above the optimal ratio of 2:1 and 3:1 (SIMOPOULOS *et al.*, 2000). These deficiencies could be compensated by other components of the diet.

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PAPER

NUTRITIONAL AND TECHNOLOGICAL REASONS FOR EVALUATING THE ANTIOXIDANT CAPACITY OF VEGETABLE PRODUCTS

RAGIONI NUTRIZIONALI E TECNOLOGICHE PER LA VALUTAZIONE DELLA CAPACITÀ ANTIOSSIDANTE DI PRODOTTI VEGETALI

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ABSTRACT

Oxygen Radical Absorbance Capacity (ORAC), phenolic and flavonoid determinations were performed on "ready-to-eat" fresh or cooked vegetables, sprouts, frozen vegetables and fresh and dehydrated fruit. The peel and pulp of fresh fruit was compared as well. In the minimally processed vegetables, the reduction of phenolic and ORAC values ranged from 10 to 40% in comparison with the fresh vegetables; a reduction of 80% was found in frozen vegetables, depending on the texture of the vege-

RIASSUNTO

Questo lavoro presenta l'applicazione del metodo ORAC, ovvero della capacità di assorbimento dei radicali perossilici, da parte di estratti vegetali e frutti. La determinazione del parametro ORAC, della concentrazione di polifenoli e flavonoidi è stata fatta su vegetali freschi o cotti pronti per l'uso, su germogli di vari semi, vegetali congelati, frutti disidratati e frutti freschi. Sui frutti freschi è stato fatto un confronto fra la buccia e la polpa. I valori ORAC dei vegetali trasformati e pronti per l'uso, mostrava-

- Key words: antioxidant capacity, dehydrated fruits, flavonols, frozen vegetables, phenols, "ready-to-eat" vegetables, sprouts -

table and the consequent blanching time. Some sprouts were shown to possess a high ORAC and can be considered useful ingredients to increase the antioxidant capacity of salads. Dry fruit showed a 25-55% decrease in ORAC when compared with fresh fruit. In two cultivars of apple and pear the ORAC value of the peel was 6-9 fold higher than that of the pulp. The results are discussed from the standpoint of nutritional value and quality of the processing technology used.

no un decremento variabile tra il 10 e il 40% rispetto ai vegetali freschi; nei vegetali surgelati il decremento massimo rilevato è stato dell'80% a seconda della durezza del vegetale e del corrispondente tempo di blanching. I frutti disidratati mostravano un decremento di ORAC rispetto ai freschi variabile dal 25 al 55%. In due cultivar di mele e pere, l'ORAC delle buccia era 6-9 volte più elevato del valore della polpa. I risultati sono discussi dal punto di vista del valore nutrizionale e della qualità della tecnologia di trasformazione.

INTRODUCTION

Phenolic compounds are nutritional phytochemicals contained in fruit and vegetables. They are divided into several sub-groups: phenolic acids, flavonoids, coumarins, stilbenes, lignins and condensed tannins (ANTONOLOVICH *et al.*, 2000). All of these express antioxidant activity since they are able to scavenge free radicals or bind pro-oxidant metal ions by means of their OH groups (RICE-EVANS *et al.*, 1997). Phenolic compounds contribute to organoleptic qualities of food and exhibit various beneficial "*in vivo*" physiological properties, including vasodilatatory (HANASAKI *et al.*, 1994), anti-inflammatory (READ, 1995), anti-bacterial, anti-viral (HANASAKI *et al.*, 1994) and anti-proliferative (YANG *et al.*, 2001) properties. They are considered chemo-preventive agents (WILLETT and TRICHOPOULOS, 1996), which should be preserved as much as possible in both fresh and processed vegetables (GIL *et al.*, 1998; NINFALI and BACCHIOCCA, 2004) to promote consumer health (GARIBALLA and SINCLAIR, 1998). One of the most frequently used parameters in the evaluation of the protective effect of vegetables is the antioxidant capacity that is measured by several methods (PRIOR and CAO, 1999). Among them, the Oxygen Radical Absorbance Capacity (ORAC) assay is one of the most reproducible and reliable (OU *et al.* 2002; HUANG *et al.*, 2005).

The guidelines for chronic disease prevention recommend a nutrition regime based on the consumption of fresh fruit and vegetables (AMES, 1998). However, due to lack of time for meal preparation, many people do not have the opportunity to eat fresh vegetables every day and therefore include processed vegetables in their diet. In order to reconcile the need to save time and eat fresh vegetables, people resort to the alternative possibility of "ready-to-eat" vegetables. These are minimally processed fresh vegetables that are cleaned and washed and the non-edible parts are removed after which they are submitted to modified atmosphere packaging (MAP). When necessary, they are cooked by grilling or steaming before packaging and then vacuum-packed (MASSANTINI and SALCINI, 2003). The organoleptic characteristics and phenolic concentrations of these vegetables strictly depend on their processing history, type of storage and MAP (WATADA and QI, 1999). The two

latter aspects seem to be relevant in determining the bioavailability of the phenolic compounds in humans (ROLLE and CHRISM, 1987; SERAFINI *et al.*, 2002).

Another category of "ready-to-eat" vegetable products is sprouts. They have been used for centuries in many dishes in the Orient but only recently have they become popular in Western countries. Sprouts have a soft and slightly crispy texture and an attractive fragrance. From a nutritional point of view they are a good source of amino acids, minerals, fibre and phenolic compounds (LORENZ, 1980). For each type of sprout, there is a pattern in the increase of phenolic content as seedling days progress (KIM *et al.*, 2004), but the absolute content of phenolics and ORAC of sprouts has never been studied in detail.

Frozen foods are cheaper than "ready-to-eat" vegetables but they are subjected to blanching during processing with the consequent loss of phenolics when compared to fresh vegetables (NINFALI and BACCHIOCCA, 2003).

Dehydrated fruit is used instead of fresh fruit both in homemade desserts or fruit salads and in the catering sector. They are easy to use and have satisfying organoleptic properties, however their antioxidant capacity before and after industrial processing has seldom been investigated. To our knowledge, only one paper (WU *et al.*, 2004) has reported the ORAC values of lipophilic and hydrophilic components in nuts, figs, prunes and raisins and compared them with the same parameters of fresh fruit.

The antioxidant capacity may be associated with the potential protective health effect of the vegetable products and can be a relevant parameter to evaluate the mildness of processing, the nutritional quality and health properties of these foods. It could also be used to direct the food choices of a large number of people who eat out every day. The present paper reports on the use of the ORAC assay to compare fresh and processed vegetables and fruits.

MATERIALS AND METHODS

Chemicals

2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH) was purchased from Tebu-Bio (Milan, Italy); 6-Hydroxy-2,5,7,8-tetramethyl-2-carboxylic acid (Trolox), Fluorescein sodium salt, Folin-Ciocalteu phenol reagent and all other reagents were purchased from Sigma Aldrich (Milan, Italy).

Vegetables and fruit

Major brands of "ready-to-eat" fresh vegetables were bought from March to May 2005 in the marketplace. Considering that the average shelf-life was 8±1 days, they were used at day 5 from the date of packaging. Sprouts were obtained from the market and used 4 days after the production date, taking into consideration that the average shelf-life is 7±1 days. "Ready-to-eat" cooked vegetables were obtained from a local producer, who guarantees 6 months of shelf-life when maintained refrigerated at 2°-6°C; vegetables were used 3 months from the date of packing. Frozen vegetables of a major brand were purchased from a local market: aubergines and carrots were in thin slices and broccoli was flowered. Dehydrated fruit of a major brand was organically grown; the shelf life was 9-12 months and it was used 6 months from the date of packaging. Since they contained an average of 40% residual water, complete dehydration to 5% residual water was carried out by mean of a ventilated oven and a lyophilizer (Edwards). Fresh products were obtained from farmers and used for the experiments within 2 days from harvest. For industrially processed products the same cultivars and the same stage of growth were

used; information was obtained by consulting the consumer services of the producer brands.

Extract preparation

Fresh vegetables and fruit were cleaned, chopped and homogenized in a food processor; frozen and dehydrated products were ground directly in a food processor. Ground material was weighed and suspended (1:5 w/v) in 40:20 v/v acetone/5% perchloric acid (PCA), shaken 30 min at 4°C, then centrifuged 10 min at 3,000 g (NINFALI and BACCHIOCCA, 2003). The extraction was repeated twice and the supernatants were collected and used for the phenolic and flavonoid assays. For the ORAC assay, the extracts were diluted as needed with 0.075 M phosphate buffer pH 7.0.

Assays

Phenolic compounds were assayed according to the Folin-Ciocalteu method (SINGLETON *et al.*, 1999). Total phenolic content is expressed as mg/g caffeic acid equivalents. Flavonoids were determined by the method of EBERHARDT *et al.* (2000).

The ORAC assay was carried out using a Fluostar Optima plate reader (PBI International, Milan) equipped with a temperature-controlled incubation chamber and an injection pump. Incubator temperature was set at 37°C. The reaction mixture for the assay was the following: 200 μL of 0.096 μM fluorescein sodium salt in 0.075 M Na-phosphate buffer, pH 7.0, 20 μL sample or Trolox. A calibration curve was made each time with the standard Trolox (100, 50, 25, 12.5 and 6.25 μM). The blank was 0.075 M Na-phosphate buffer, pH 7.0. The reaction was initiated with 40 μL of 0.33 M AAPH and fluorescence was read at 485 nm ex. and 520 em. until zero. ORAC values are expressed as μmol Trolox Equivalents (TE)/g of fresh or dry weight.

Statistics

To establish reproducibility of the analytical method, sample preparation of each vegetable product was repeated four times on independent material. Each assay was carried out in duplicate. Differences among means were evaluated with ANOVA, using the Origin 2.8 (Microsoft Software) statistics program.

RESULTS AND CONCLUSIONS

Table 1 compares phenolics, flavonoids and ORAC values of fresh and "ready-to-eat" vegetables. "Ready-to-eat" carrot and red chicory had phenolic values quite similar to the fresh vegetables, while rocket and valerian had significantly lower values in the "ready-to-eat" form. The same pattern was found for flavonoids. ORAC values of carrots, rocket and valerian showed a significant loss of antioxidant capacity in the "ready-to-eat" form as compared with the fresh form, while flavonoids and ORAC in red chicory did not differ significantly. This means that red chicory has the best adaptability to processing and storage under MAP. The high ORAC value of red chicory, as compared to other vegetables, is likely due to its content of anthocyanins, which have a notably high antioxidant capacity (ARNOUS *et al.*, 2002) and possible bioavailability (CAO *et al.*, 1998).

Rocket and valerian need better processing conditions to maintain phenol levels closer to those of fresh products. Carrot probably loses antioxidants due to slicing which requires time and activates phenol oxidases thus favouring phenolic oxidation. The low ORAC value of carrot is due to the low activity of β-carotene in the scavenging of peroxyl radicals used in the assay. This low value does not mean a lesser importance of this vegetable in the diet. In fact, β-carotene exploits its maximal activity towards singlet oxygen (CARLSSON *et al.*,

Table 1 - Total phenols, flavonoids and ORAC of fresh and "ready-to-eat" vegetables.

	Total phenols (mg/g fresh w.)		Flavonoids (mg/g fresh w.)		ORAC (μmolTE/g fresh w.)	
	Fresh	Ready-to-eat	Fresh	Ready-to-eat	Fresh	Ready-to-eat
Carrot	0.35±0.02	0.30±0.02	0.13±0.02	0.12±0.02	6.96±0.7	4.99±0.5*
Red Chicory	1.29±0.21	1.37±0.36	0.89±0.12	0.94±0.15	35.37±3.1	37.84±2.9
Rocket	1.36±0.12	0.61±0.03*	0.46±0.02	0.11±0.01*	23.27±2.1	10.12±0.9*
Valerian	1.40±0.12	1.08±0.74*	1.05±0.12	0.44±0.02*	22.57±2.0	18.20±1.3*

Values are reported on fresh weight basis and are the mean ± S.D. of four independent determinations. * significantly different from fresh samples by ANOVA ($p<0.05$).

1976), a radical species generated by UV light and therefore this vegetable must be maintained in the diet to protect against a wider spectrum of radical species.

Table 2 shows the phenolic, flavonoid and ORAC values of four minimally processed cooked vegetables. Values are expressed on edible weight basis; the loss of weight during grilling or steaming was in the order of 10% and was disregarded. There was a 25-30% loss of ORAC units in all processed vegetables in comparison with the fresh products, while the loss of phenolic content was less pronounced. In the case of cooked vegetables the ORAC assay is able to detect the loss of antioxidant capacity due to processing better than the phenolic assay.

Table 3 shows parameters of sprouts from different seeds. Regarding leek and radish, the absolute values of phenolics and ORAC in the sprouts was only slightly lower than those of the mature vegetable previously studied (NINFALI *et al.*, 2005). In the case of Sango sprouts, a Japanese radish recently introduced into Western countries, there is no known data on the adult vegetable, however in comparison with the other more common sprouts, the phenolic and ORAC values are notably higher. This indicates that, beyond the importance of sprouts for their high digestibility and tenderness, they are also an important ingredient for preparing salads with high antioxidant capacity. In fact, a simple salad

Table 2 - Total phenols, flavonoids and ORAC of fresh and minimally processed cooked vegetables.

Vegetable	Processing	Phenolics (mg/g edible weight)	Flavonoids (mg/g edible weight)	ORAC (μmolTE/g fresh w.)
Yellow pepper	Fresh	1.54±0.07	0.18±0.01	9.50±0.89
	Grilled	1.45±009	0.11±0.01 *	6.94±0.81 *
Cauliflower florets	Fresh	0.62±0.09	0.32±0.02	9.25±0.97
	Steamed	0.54±0.06	0.10±0.01 *	6.20±0.58 *
Carrot slices	Fresh	0.32± 0.03	0.12±0.02	2.71±0.51
	Steamed	0.18±0.02 *	0.06 ±0.01 *	2.63±0.18
Aubergine slices	Fresh	0.26±0.02	0.09±0.01	3.81±0.15
	Steamed	0.19±0.02 *	0.04±0.01 *	2.45±0.26 *

Values are the mean ± S.D. of four independent determinations. * Significantly different from the corresponding fresh vegetable by ANOVA ($p<0.05$).

Table 3 - Total phenols, flavonoids and ORAC of sprouts of different seeds.

Sprouts	Total phenols (mg/g fresh weight)	Total Flavonoids (mg/g fresh weight)	ORAC (μmolTE/g fresh weight)
Alfalfa	0.47±0.03	0.087±0.006	15.10±2
Leek	0.29±0.02	0.11±0.001	8.21±07
Radish	0.89±0.07	0.36±0.004	21.84±2
Sango	1.5±0.9	0.72±0.006	35.78±3
Soy	0.42±0.02	0.12±0.001	9.62±0.9

Sprouts of the indicated seeds were obtained in plastic containers as ready-to-be-consumed.
Values are the mean ± S.D. of four independent determinations. * Significantly different from the corresponding fresh vegetable by ANOVA ($p<0.05$).

with lettuce and tomatoes has an ORAC value of 7.0 μmol TE/g fresh weigh (NINFALI *et al.*, 2005); the ORAC value of one gram of Sango sprouts is seven-fold higher, so that it is possible to double the ORAC units of 100 g of lettuce-tomato salad with the addition of 20 g of Sango sprouts. Therefore it is important to continue to search for sprouts, which can be mixed with fresh vegetables to increase the nutritional value due to the antioxidant metabolites contained in them.

Table 4 compares fresh and frozen vegetables and shows that frozen vegetables have phenolic, flavonoid and ORAC values invariably lower than fresh ones. These differences are more evident in beet green and broccoli, probably as a consequence of their texture that requires a long blanching time, generally 40-60 sec at 98°C, before freezing. Spinach is harvested very young and tender and requires a very brief blanching time, 10-20 sec at 98°C, as well as aubergine that are sliced and blanched quickly before freezing. An additional factor can be the leakage of juice from the vegetable during processing. This factor makes the ORAC decrease attributable to both degradation and loss of antioxidant components. It is worth noting that the degree of the ORAC decay is more pronounced than that expressed by flavonoids and phenolics thus indicating that the ORAC values are very sensitive markers of phenolic "damage" induced during processing.

Table 5 shows the phenolic and ORAC values of fresh and dry fruit. Results are expressed as "as consumed"

Table 4 - Phenolic, flavonoids and ORAC values of fresh and frozen vegetables.

	Total phenols (mg/g edible weight)		Flavonoids (mg/g edible weight)		ORAC (μmolTE/g edible weight)	
	Fresh	Frozen	Fresh	Frozen	Fresh	Frozen
Aubergine	0.32±0.02	0.23±0.02*	0.09±0.02	0.07±0.02	3.44±0.7	0.37±0.5*
Beet green	0.97±0.07	0.44±0.05*	0.47±0.12	0.22±0.08*	26.24±2.1	11.68±1.1*
Broccoli	1.09±0.10	0.60±0.06*	0.61±0.13	0.12±0.01*	35.29±3.1	4.96±0.9*
Spinach	1.01±0.11	0.81±0.07*	0.32±0.10	0.28±0.10	27.32±2.3	16.87±1.3*

Values are reported on fresh weight basis and are the mean ± S.D. of four independent determinations. * significantly different from fresh samples by ANOVA ($p<0.05$).

and "after complete dehydration". Complete dehydration eliminates the difference in water content left after processing, and the ORAC and phenol values allow the fruits brought down to 5% residual water to be compared. Complete dehydration was carried out by using a ventilated oven followed by lyophilization, a mild process that does not significantly change the phenol and ORAC values. The value "as consumed" refers to the conditions of about 40 and 90% residual water in dehydrated and fresh fruits, respectively. The results show that ORAC of heat-dehydrated products decreased 25-55% in apple, apricot, peach, prune and pear. In the white raisin, the decrease was about 10%. The loss of phenolics during processing can be considered moderate in dehydrated fruit; they are consumed in a quantity which is one half that of fresh fruit but they contain twice the amount of phenolics, therefore, they furnish the same amount of antioxidants as a fresh fruit portion.

An additional aspect to be taken into account in comparing the antioxidant capacity of fruit is the presence or absence of the peel. Some fruit, such as apples and pears, are commonly commercially dehydrated without the peel. The contribution of the peel is minimal in fresh fruit due to the low weight of the peel compared to the total mass, but when the fruit is dehydrated the loss of weight makes the peel antioxidant contribution significant.

Table 6 shows the comparison between the peel and pulp of two cultivars of fresh pears and apples. The results confirm that there is a major difference (from 6 to 9 fold) in the phenolic, flavonoid and ORAC values of peel and

Table 5 - Total phenols and ORAC of fresh and dehydrated fruits.

Fruit	Total phenols (mg/g)		ORAC (μmolTE/g)	
	a as consumed	b after complete dehydration	a as consumed	b after complete dehydration
Apple f.	1.48±0.2	9.25±0.96	21.7±2.4	135.6±13.7
Apple d.	2.87±0.3	4.50±0.42*	66.81±7.8*	101.91±10.4*
Apricot f.	0.58±0.06	4.46±0.53	10.58±1.1	81.42±8.7
Apricot d.	2.20±0.2	3.45±0.38*	32.34±3.4*	51.30±5.3*
Peach f.	1.04±0.1	9.50±0.99	15.93±1.6	144.51±13.8
Peach d.	2.51±0.2	3.80±0.43*	42.22±4.6*	63.9±7.2*
Prune f.	1.46±0.1	9.10±0.94	22.12±2.7	138.11±12.9
Prune d.	3.76±0.4	4.51±0.41*	68.40±7.4*	101.42±10.4*
White raisin f.	1.45±0.1	8.01±0.8	9.30±0.92	51.2±5.1
White raisin d.	3.30±0.3	5.1±0.51*	41.88±4.6*	65.4±6*
Pear f.	1.49±0.1	7.45±0.67	29.41±3.8	147.05±12.5
Pear d.	6.02±0.5	7.16±0.63	94.96±8.4*	113.04±10.3*

Fresh (f.) and dehydrated (d.) fruit in column a were assayed as obtained from the local market and the values refer to g fresh or partially dehydrated fruit (average residual water 40±5% of the fresh). In column b, fruit was assayed after complete dehydration performed as reported under methods and values refer to dry weight basis (average residual water 5%).
All values are the means ± S.D. of 4 independent determinations. * Significantly different from the fresh fruit by ANOVA with $p \leq 0.05$.

Table 6 - Total phenols, flavonoids and ORAC of different cultivars of apple and pear.

Fruit	Cultivar		Total phenols (mg/g fresh)	Flavonoids (mg/g fresh)	ORAC (μmolTE/g fresh)
Apple	Golden Delicious	peel	2.26±0.19	2.01±0.19	40.02±3.19
		pulp	0.38±0.01*	0.42±0.03*	7.07±6.57*
	Stark	peel	4.21±0.32	3.09±0.28	69.65±5.32 **
		pulp	0.49±0.05*	0.42±0.02*	10.21±8.68*
Pear	Abate	peel	4.14±0.40	3.58±0.31	83.55±7.21
		pulp	0.70±0.05*	0.58±0.04*	9.21±7.56*
	Conference	peel	2.67±0.31	1.31±0.12	56.97±4.20**
		pulp	0.27±0.02*	0.16±0.01*	11.67±2.06*

Values are reported on fresh weight basis. * The pulp is significantly different from the peel of the same fruit and ** the peel is significantly different from the peel of the other cultivar by ANOVA with $p \leq 0.05$.

pulp of the same fruit. Likewise there is a significant difference in the content of antioxidants between the two cultivars of both fruits. These results confirm previous studies on apples (ESCARPA and GONZÀLEZ, 1998) and add information regarding two pears cultivars, undescribed until now. The results on the content of antioxidants in the different parts of the fruit suggest consuming organically cultivated fruit dehydrated with the peel after careful washing in order to ingest a considerable part of the phenolics.

In conclusion, these results show the applicability of the ORAC method to evaluate the antioxidant capacity of some commercial vegetable products in order to follow quality loss during processing. The data are limited to a small number of products of four major brands and are based only on the cultivar and the stage of growth of the vegetables. Since other factors, including agronomic conditions, could influence the phenol content the results cannot be generalized. However, they focus on the applicability of the ORAC assay as a useful tool both for producers wishing to improve the industrial process and consumers desiring higher quality products. When it is not possible to consume fresh vegetables and fruit, "ready-to-eat" products can represent a valid alternative. Though the antioxidant content is lower compared to the fresh product, they still maintain a significant antioxidant content. The correlation ascertained between vegetable consumption and the reduction in the incidence of chronic diseases and the increased consumption of processed vegetables rather than fresh, make processing technologies preserving the phenolic content and antioxidant capacity of vegetables and fruits important factors in safeguarding public health. To obtain chemoprevention through the diet, epidemiologists agree that it is not necessary to eat enormous amounts of fruit and vegetables nor any particular potent vegetable but, on the contrary, to systematically eat a variety of fruit and vegetables every day which have maintained a satisfactory content of vitamins and phenolics intact.

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MICROBIOLOGICAL MONITORING IN THE PUBLIC CATERING SECTOR

MONITORAGGIO MICROBIOLOGICO DI ALIMENTI PRODOTTI IN MENSE

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ABSTRACT

The hygienic-sanitary condition of products in some catering services in Salerno (Italy) were evaluated. The sampling done between July 2003 and July 2004 included mixed salad, sandwiches, ice cream and pastry products. No pathogens were detected in the food tested. In terms of total coliforms non compliance of the products was in the following order: Mixed salads (87%)>Pastry (86%)>Ice cream (67%)>sandwiches (30%), while for total plate count only 28% of the mixed

RIASSUNTO

Questo lavoro ha avuto come scopo la valutazione della qualità microbiologica di alimenti prodotti in mense nella provincia di Salerno. I campionamenti sono stati eseguiti da luglio 2003 a luglio 2004 e hanno riguardato insalate miste, panini farciti, gelati e prodotti di pasticceria. Per i parametri indicatori di contaminazione ambientale (coliformi totali) i prodotti sono risultati non conformi in media del 67%. Ciò dovuto alla inadeguata preparazione del personale riguardo alle vigenti norme igieniche e

- Key words: catering, food microbiology, microbiological contamination, pathogens -

salad samples did not comply with the regulations in force. It is concluded that more detailed education of the staff and more controls should be promoted for effective food quality control.

agli insufficienti controlli delle Autorità Sanitarie competenti. Educare maggiormente il personale addetto ed eseguire un numero maggiore di controlli degli esercizi sono le strategie da intraprendere.

INTRODUCTION

The world of public catering has significantly changed through the years and is progressively developing due to many factors including different type of organization, types of clients and food. The clients have higher expectations than previously and pay more and more attention to the quality of products and services (NATIONAL INSTITUTE OF NUTRITION, 1997). There are more than 65,000 food catering organizations present in Italy, which include restaurants, self-service and fast-food establishments and more than 30,000 hotels with restaurants (GUIDA *et al.*, 2003). Many operations such as insufficient cooking or thawing of frozen food make an ideal growth medium for microorganisms responsible for toxinfections (BERTINI *et al.*, 2004) and many complications due to bacterial, viral and parasitic infections transmitted by food have been reported (MOSSEL *et al.*, 1999). Each year, an estimated 76,000,000 people experience a foodborne infection in the United States. Five foodborne pathogens – *E. coli* O157:H7, *Salmonella*, *Campylobacter*, *Listeria* and *Toxoplasma* – together cause an estimated 3.5 million cases of foodborne illness (TAUXE, 2002). Among the many pathogens, *Salmonella* spp. is the most frequently reported cause of foodborne diseases in the world (PANISIELLO *et al.*, 2000; BEMRAH *et al.*, 2003), including Italy (SCUDERI *et al.*, 1996) and France (LECLERC *et al.*, 2002). It has been reported that *Salmonella* spp. caused more than one-quarter of the outbreaks of foodborne disease associated with passenger ships (ROONEY *et al.*, 2004). Various pathogens have also been recorded in relation to foodborne outbreaks in England and Wales (O'BRIEN *et al.*, 2002). *E. coli* O157:H7 has been associated with many foodborne outbreaks found in a great variety foodstuff (ground beef, USA; ham, Turkey, cheese sandwiches, Ontario, Canada; yoghurt, UK, milk, Scotland, etc.) (McCLURE, 2000). It has been demonstrated that the edible portion of carrots and, to a lesser extent, onions have been contaminated when grown in soil containing contaminated manure or irrigated with contaminated water (ISLAM *et al.*, 2005). For these reasons the training of food establishment personnel to control the hygienic and sanitary conditions of food with respect to the law has become mandatory (ZICARI, 2003).

In this study, some rapidly consumed food (sandwiches, sweets, ice-cream, mixed-salad) prepared in the public catering sector in the Province of Salerno (Southern Italy) were monitored for pathogenic contamination with regard to the EU 2073/2005 criteria (G.U., 2005). Total plate count, total coliforms, fecal coliforms, *Staphylococcus aureus*, *Escherichia coli* O157:H7 and *Salmonella* bacteria were determined according to present Italian and international laws. All of these bacteria had been previously monitored in different food (except pastry) and equipment in mass catering establishments (LEGNANI *et al.*, 2004) and

many of the products were found to be contaminated. *S. aureus* was also detected in some food refrigeration equipment (EVANS *et al.*, 2004).

MATERIAL AND METHODS

Sampling

This study was carried out between July 2003 and July 2004 on a total 197 samples of sandwiches (105), ice cream (42), mixed salads (15) and pastry (35) randomly collected from two public catering companies located in Salerno (Southern Italy). The samples were put into sterile plastic ice bags and transported to the laboratory within 1 hour.

For total plate count and enumeration of total coliforms, fecal coliforms, *Escherichia coli* O157:H7 and *Staphylococcus aureus* an aliquot of 10 g of each food sample was homogenized in a Stomacher for 30 seconds with the addition of 90 mL of 1% peptone water. For the detection of *Salmonella* spp., 25 g of sample was homogenized in 225 mL of the respective enrichment broth (LEGNANI *et al.*, 2004).

Microbiological parameters and incubation medium and conditions are given in Table 1. The methods followed for the determination of microorganisms are given in Table 2.

The colonies were directly counted on the plates using a colony-counter (Model Colony Counter 560 Suntecs, Milano, Italy) equipped with a magnifying lens at 5X (GUIDA *et al.*, 2004).

The results are expressed as colony forming units (CFU)/g of sample. All data refer to average values.

The number of microorganisms per millilitre of sample was obtained with the statistical formula of Poisson :

$$N = N = \frac{\sum C}{V\,(n_1 + 0.1n_2)d} \quad (1)$$

Table 1 - Microbiological identification techniques used.

Microbiological parameters	Medium	Incubation	Identification procedures
Total plate count	Plate count agar (Oxoid, # CM325B)	30°C for 48 h	Enumeration of colony forming units
Total coliforms	Violet Red Bile Agar (Oxoid, # CM107B)	32°C for 24h	Enumeration of colony forming units (lactose fermenting)
Fecal coliforms	Violet Red Bile Agar (Oxoid, # CM107B)	44°C for 48 h	Enumeration of colony forming units
Staphylococcus aureus	Baird parker selective agar (Oxoid, # CM275B)	36°C for 48 h	API 20 Staph. Biochemical tests (Biomerieux, # 20500)
Salmonella spp.	Pre-enrichment in buffered peptone water (Oxoid, # CM509B), Isolation in Hektoen enteric agar (Oxoid, # CM419B)	Pre-enrichment: 37°C for 24 h Isolation: 37°C for 24 h	API 20E Biochemical tests (Biomerieux, # 20100)
Escherichia coli 0157:H7.	Enrichment in Lauril Sulphate Broth (Oxoid, # CM451B), Isolation in Tryptone water (Oxoid, # CM087B) and Indol reagent	Enrichment at 37°C for 24 h, Isolation at 44°C for 24 h	MPN technique based on the frequency of dilutions

Table 2 - Microbiological reference standards for various foods.

Foods		Standard value		Reference
		n= 5 m(cfu/g)	c =2 M (cfu/g)	
Sandwiches	Total plate count at 32°C	10^5	10^6	EMILIA ROMANIA REGION (1992)
	Total coliforms	10^2	10^3	LOMBARDIA REGIONAL GENERAL DIRECTION OF HEALTH (2001)
Mixed salad	Total plate count at 30°C	5.10^5	5.10^6	OFFICIAL JOURNAL OF FRANCE (1993)
Pastry (sweets)	Total plate count at 32°C	10^5	10^6	TIECCO (2000)
	Total coliforms	10^2	10^3	
Ice cream	Total plate count at 32°C	10^5	5.10^5	DPR/54 (1997)
	Total coliforms	10	10^2	

c: number of units sampled in which the presence of the microorganism considered can be allowed;
n: number of samples;
m: limit within which the result is acceptable;
M: limit above which the result is not acceptable.

where $\sum C$ = sum of the colonies in the considered plates, V = volume of the inoculation in mL sown in each plate, n_1 = number of plates considered for the first dilution, n_2 = number of plates considered for the second dilution, d = dilution factor corresponding to the first dilution.

The reliability of the results was calculated with the "mean of Poisson" using formula 2:

$$\frac{C_1 - C_2}{\sqrt{C_1 + C_2}} \leq 20 \qquad (2)$$

where C_1 represents the sum of the counts of the first dilution tested and C_2 is the sum of the counts of the second dilution tested.

RESULTS AND CONCLUSIONS

None of the samples analysed resulted positive for *Salmonella* spp., *Staphylococcus aureus*, *Escherichia coli* O157: H7 and fecal coliforms.

Varying ranges of total plate count and total coliform bacteria, indicators of lack of hygiene in the production environment, were detected in the different types of food. Fig. 1a illustrates the variation of both bacteria in different ice cream products. There was no correlation between bacteria and ice cream type. The highest level of total plate count bacteria was in cacao-based ice cream, while total coliforms were the highest in cream-based ice cream which requires considerable handling and contains mainly milk.

As shown in Fig. 1b, both bacteria varied widely in mixed salad ranging from 20 to 500 CFU/g. There was no correlation between the two types of bacteria. There was also no correlation between the two types of bacteria and variations due to product type for the pastry products (Fig. 1c). The pastries made with milk (cream, deliziosa, bignè) and requiring more handling, displayed higher levels of the bacteria, whereas the products cooked for a longer time had lower levels. The sandwiches that were mostly cooked products showed the same

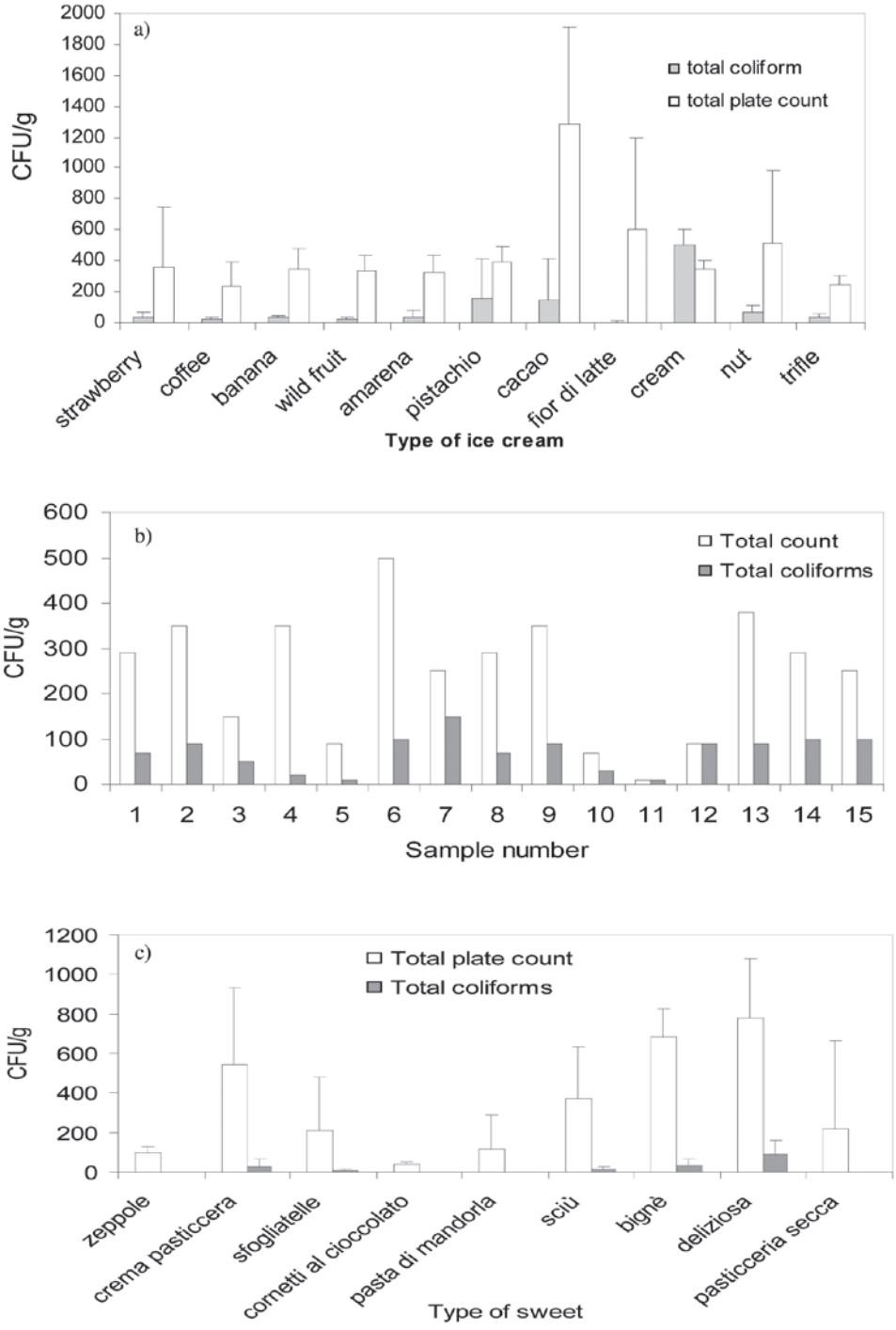

Fig. 1 - Variations of total plate count and total coliforms bacteria in (a) ice cream products (b), mixed salads (c) and pastry products.

tendency (data not shown). These results are in accordance with the observations of LEGNANI *et al.* (2004), who reported that 83.9% of the first and second courses consisting of 220 samples were in compliance with the legal limit of total count bacteria at 32°C (85.2% of 220 samples were in compliance with the legal limit for coliforms).

When the present data were evaluated for compliance to present laws (Table 2), the results of the total plate count of the sandwiches complied 100%, while for total coliforms, 30% of the samples did not comply. In the case of mixed salads, 28% of the samples were unacceptable for total plate count, while 87% were unacceptable for total coliforms. The work by LEGNANI *et al.* (2004) also showed a similar trend for raw vegetables (58.3% of 43 samples were within the limit, 33.4% were acceptable for total count at 32°C). Meanwhile, DROSINOS *et al.* (2005) reported that cheese used in filling salads should be considered in checking for contamination of food.

Regarding the pastry, 100% of the samples complied for total plate count bacterial load, whereas 86% were unacceptable for total coliforms. Of all the ice cream products, only total coliforms did not comply with the limits (67% unacceptable).

The results showed that the identification techniques to monitor these microbiological parameters were well chosen (EVANS *et al.*, 2004; LEGNANI *et al.*, 2004), however, further study should be considered to provide effective hygienic control according to the HACCP system (SUN and OCKERMAN, 2005).

The results of this study demonstrate that the hygienic quality of the food tested can be considered satisfactory in terms of pathogens (Fecal coliforms, *Salmonella* spp., *Staphylococcus aureus*, *Escherichia coli* O157:H7) but not for total plate count (mixed salad, 28% unacceptable) and total coliform bacteria (all foods) according to present laws.

In terms of total coliforms non compliance of the products can be ordered in the following sequence: Mixed salads (87%)>Pastry (86%)>Ice cream (67%)>sandwiches (30%). These results indicate significant environmental contamination and the need for strict hygienic control in production places. Meanwhile, good training of the staff involved in hygienic control in these public food producing and consuming places is essential because the public catering sector is the easiest way to control food quality before consumption (DROSINOS *et al.*, 2005).

Besides the necessity of establishing major and more severe sanitary and hygienic regulations, specifying the application procedures of self-control and better hygienic practices has become obligatory. Although the current HACCP system helps to control food from the source, a continuous improvement of qualitative standards of the equipment and finished products and regular analytical control of equipment is recommended. Furthermore, as stated by DROSINOS *et al.* (2005), close attention should be given to milk products.

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TRACE ELEMENTS IN BOVINE MILK FROM DAIRY FARMS IN SICILY

ELEMENTI IN TRACCE NEL LATTE BOVINO PROVENIENTE DA VARI ALLEVAMENTI DELLA SICILIA

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ABSTRACT

The levels of trace elements in 36 milk samples were determined in dairy farms in Sicily. The determination of As, Cd, Cu, Pb, Se and Zn were performed using an atomic absorbtion spectrophotometer. Among the toxic elements, the highest concentrations were those of Pb (range 0.4-170.1 μg/kg) of which 22 samples were above the Maximum Residue Level of 20 μg/kg. The lowest levels were those of Cd (range <0.01-20.1 μg/kg), while intermediate values were those of As (range

RIASSUNTO

Lo scopo di questo studio è stato quello di determinare i livelli di elementi in tracce in 36 campioni di latte proveniente da vari allevamenti della Sicilia. La determinazione di As, Cd, Cu, Pb, Se e Zn è stata effettuata mediante spettrofotometria di AA. Tra gli elementi tossici, le concentrazioni più elevate sono risultate quelle del Pb (range 0,4-170,1 μg/kg), e di cui 22 campioni con livelli superiori ai Limiti Massimi Residuali (LMRs = 20 μg/kg). Le concentrazioni più basse sono state quelle

- Key words: bovine milk, heavy metals, MRL, Sicily -

<0.2-188.7 μg/kg). Regarding essential elements, the highest concentrations were those of Zn (range 3.7-9.6 mg/kg), followed by Cu (range 9.1-229.9 μg/kg) and Se (range 6.4-41.5 μg/kg).

del Cd (range <0,01-20,1 μg/kg) mentre l'As (range <0,2-188,7 μg/kg) mostrava valori intermedi. Per quanto riguarda gli elementi essenziali, le concentrazioni più elevate sono state quelle dello Zn (range 3,7-9,6 mg/kg), seguite da Cu (range 9,1-229,9 μg/kg) e Se (range 6,4-41,5 μg/kg).

INTRODUCTION

Rapid urbanisation, technological progress, industrial development and increased traffic have resulted in worldwide environmental pollution. Among the various pollutants metal contamination is threatening human health via the food chain. Moreover, agricultural activities, e.g. the use of fertilizers can also be important sources of environmental contamination (CASARETT and DOULL, 2000).

Milk, recognised as the most complete food in the human diet, can contain numerous xenobiotic substances (pesticides, disinfectants, drugs, metals and various environmental contaminants), which constitute a technological risk factor for dairy products, for their commercial image and above all for the health of the consumer. Today, the consumer is more demanding than in the past and expects "healthy" milk, rich in nutrients, with high biological value. Determination of the residual concentrations of metals in milk could be an important "direct indicator" of the quality of milk and/or of products derived from it, as well as an "indirect indicator" of the degree of pollution of the environment where the milk was produced.

Particularly high levels of trace elements (Cd, Pb and Zn) in milk have been reported in the international liter-

Table 1 - Ranges of trace elements in milk samples from international references.

	Concentration (μg/kg w.w.)	References				
		WOJCIECHOWSKA-MAZUREK *et al.*, 1995	TRIPATHI *et al.*, 1999	ALAIS, 2000	SIMSEK *et al.*, 2000	MARTINO *et al.*, 2001
				Toxic Elements		
As	<0.15-188.71	nd	nd	40-100	0.2-50	nd
Cd	<0.01-20.11	2-10	0.07	20-30		0.47
Pb	0.39-170.13	25-47	1.70	50-1,000	18-49	1.8
				Essential Elements		
Cu	9.07-229.87	120-280	43.2	200-300	390-960	60
Se	6.38-41.48	nd	nd	11-27	nd	12.8
Zn	3,690-8,570	24,000-39,000	3,177	3,000-6,000	3,770-5,010	3,800

nd = not determined.

Table 2 - Ranges of trace elements in milk samples from Italian references.

	Concentration (µg/kg w.w.)	References								
		BALDINI et al., 1990	CONI et al., 1994	CONI et al., 1996	CAMPILLO et al., 1998	CERUTTI, 1999	IMPARATO et al., 1999	TIECCO, 2000	LICATA et al., 2004	
Toxic Elements										
As	<0.15-188.71	nd	nd	nd	nd	ndnd	<2.6	<0.15-684		
Cd	<0.01-20.11	0.2-1.9	0.066-0.180	0.128-0.183	nd	nd-20	3.4	nd		<0.01-22.8
Pb	0.39-170.13	4-50	0.092-0.160	0.049-0.109	nd	nd-1500	46.3	120-15,800		<0.10-9.92
Essential Elements										
Cu	9.07-229.87	40-630	0.873-12.1	0.453-0.784	462	50-200	nd	<2,000		<0.136-737.58
Se	6.38-41.48	nd	nd	nd	nd	10-30	nd	<110		<1.060-110.58
Zn	3,690-8,570	3,300-7,300	21.6-41.5	16.8-26.0	28,000	300-6,000	nd	4,000-5,000		<24.73-4961

nd = not determined.

ature (Table 1). There have also been several studies of metal contamination in milk from some Italian regions (Table 2).

In a previous investigation (LICATA *et al.*, 2004) the concentration of trace elements (As, Cd, Cr, Cu, Pb, Se and Zn) was evaluated in samples of bovine milk from various dairy farms in Calabria. Regarding toxic elements, the highest values were those of As and Pb, while the lowest concentrations were those of Cd (Table 2). However, these concentrations do not pose toxicological risks to the consumer. The essential elements Zn and Se showed the highest concentrations followed by Cu. The trace element concentrations found in milk samples from dairy farms are similar to those reported by other authors from various Italian regions (Table 2).

At present, there are no data concerning the concentrations of trace elements in milk from Sicily. The area of Messina, due to its particular location, is subject to intense road traffic and industrial development: oil-refinery of Milazzo, graphite electric power station of Giammoro and various urban waste disposal sites that are periodically closed. The aim of this investigation was to determine the levels of trace elements in milk samples from various dairy farms from the area of Messina (Sicily).

MATERIALS AND METHODS

Thirty-six samples of bovine milk were taken from cows (after carefully washing the udder with alcohol and water to avoid possible organic contamination of the milk samples) on dairy farms from Messina in March 2004, placed in PET containers and frozen at -20°C until analysis. The cows were kept in free stalls and were of varying breed, age and weight; dur-

ing the winter the cows were fed on fodder, while during the other seasons they were fed on grass.

After homogenization and agitation with a Vortex, about 2 g of each milk sample were mixed with 2 mL of HNO_3 and 0.5 mL of H_2O_2 (GHIDINI *et al.*, 2002) and mineralized in a microwave oven (model MDS-2100, CEM Corporation, Matthews, NC, USA; power 950±50 W at 100%) in Teflon PFA reactors, equipped with a pressure regulation system (by means of a vessel acting as a sensor). Analytical blanks were prepared using reagents without samples. After mineralization, the samples and the blank solution were brought to a volume of 10 mL with ultrapure water. To avoid extraneous contamination, the glassware was treated with a dilute solution of 10% HNO_3. The concentrations of trace elements were determined by three methods of atomic absorption spectroscopy:

1) Analysis by graphite furnace atomic absorption spectrometry for Cd, Cu, Pb and Se was carried out using an AA Varian model 220/Zeeman Spectrophotometer equipped with a single-element hollow cathode lamp for each element, and a Varian PSD autosampler, (Varian Australia Pty Ltd, Mulgrave, Victoria, Australia) using Zeeman-effect background correction (ALEGRIA *et al.*, 1994; ALEIXO *et al.*, 2000; LICATA *et al.*, 2004). For these investigations, graphite tubes ("Varian, Partition tubes coated GTA" type) were used.

2) For determination of As, a VGA 77 adapted to a Varian AA-475 spectrophotometer was used. Arsenic was determined by the hot vapor generation technique. For the acid channel, 10 M HCl was used and for the reducing channel, 0.6% $NaBH_4$ in 0.5% NaOH was used; the flow was 1.0 mL/min. The As was treated with 1.0% $NaBH_4$ in order to reduce all the As^V to As^{III}.

3) Zn was determined using the air/acetylene flame method on a Varian AA-475 spectrophotometer.

Matrix modifiers: 1% magnesium nitrate, 0.1% monobasic ammonium phosphate, 0.5% palladium chloride and 0.056% ascorbic acid were provided by Sigma (Italy).

Other reagents and chemical mixtures: 30% hydrogen peroxide, sodium borohydride and sodium hydroxide were provided by Sigma (Italy), ultrapure water, 75% nitric acid and 37% hydrochloric acid for analysis of trace metals were provided by J.T. Backer (Mallinckrodt Backer, Milan, Italy).

The experimental conditions used for each element are reported in Table 3. The value obtained for each metal is the mean value of five determinations.

Good laboratory practice (GLP) was applied throughout and blanks were also analyzed. For all elements the blank concentrations were lower than the respective detection limits. Good laboratory practice guidelines were applied in all phases of the study: resources (organization, personnel, facilities, equipment), rules: protocols, (Standard Operating Procedures), characterization (test items, test systems), documentation (raw data, final report, archives) and quality assurance (independent observers). The quality of the analytical data was checked by analysing certified reference material (Non-Fat Milk Powder of National Bureau of Standards –1549, Gaithersburg, MD) and comparing the results with certified values. Accuracy and precision of the analysis are expressed as percentage error (e) and relative standard deviation (s_r), respectively (Table 4).

In order to determine the detection limit of an element, two solutions were prepared: the concentration of the first solution was close to the detection limit and, the concentration of the second solution was double the detection limit concentration. The test was repeated twelve times (Table 4) (LICATA *et al.*, 2004). The detection limits were in accordance with the following formula:

Table 3 - Working conditions adopted for the determination of As, Cd, Cr, Cu, Pb, Se and Zn.

GFAAS	MATRIX MODIFIER	WAVELENGTH (nm)	SLIT (nm)	STAGE	TEMP. (°C)	TIME (sec)	GAS FLOW (L/min)
Cadmium	Magnesium nitrate 1%; monobasic ammonium phosphate 10%	228.8	0.5	1	120	20	1.0
				2	250	10	1.0
				3	300	10	1.0
				4	300	3	0. 0H*
				5	1,500	3	0. 0H*
Copper	Palladium chloride 0.5%; ascorbic acid 0.056%	324.8	0.5	1	120	20	1.0
				2	250	10	1.0
				3	500	10	1.0
				4	500	3	0. 0H*
				5	2,300	3	0. 0H*
Lead	Palladium chloride 0.5%; ascorbic acid 0.056%	283.3	0.5	1	120	20	1.0
				2	250	10	1.0
				3	300	10	1.0
				4	300	3	0. 0H*
				5	1,800	3	0. 0H*
Selenium	Palladium chloride 0.5%; ascorbic acid 0.056%	196.0	1.0	1	120	20	1.0
				2	250	10	1.0
				3	600	10	1.0
				4	600	3	0. 0H*
				5	2,200	3	0. 0H*

FAAS	WAVELENGTH (nm)	SLIT (nm)	FLAME COMPOSITION
Zinc	213.9	1.0	Air/Acetylene

HG-AAS	WAVELENGTH (nm)	SLIT (nm)	FLAME COMPOSITION	REDUCTANT CHANNEL	ACID CHANNEL
Arsenic	193.7	0.5	Air/Acetylene	0.6% $NaBH_4$; 0.5% NaOH	10 M HCl

*0. 0H: High sensitivity mode.

Table 4 - Accuracy and precision of the analytical procedure (Standard Reference Material: Non-Fat Milk Powder NBS-1549) and detection limits. Number of determinations, 5.

Element	Certified concentration[a] (Mean values ± SD)	Observed concentration[b] (Mean values)	e (%)	s_r (%)	Absolute detection limit (3 SD) ng/mL
Cd	0.0005±0.0002	0.00051	+2.0	21.3	0.01
Cu	0.7±0.1	0.68	-2.8	2.9	0.1
Pb	0.019±0.003	0.020	+5.2	5.0	0.6
Se	0.11±0.1	0.109	-0.9	4.1	1.1
As	—	—	—	—	0.2
Zn	46.1±2.2	47.03	+2.1	1.5	24.7

[a] of Standard Reference Material expressed in µg/g;
[b] of Standard Reference Material expressed in µg/g;
e (%): accuracy data, expressed as relative error;
s_r (%): precision of five independent determinations, expressed as relative standard deviation;
Absolute detection limit in ng/mL = 3xSD(control)xConc.(stand.)/average absorbance of standard;
SD (control) = Standard deviation of control;
Conc. (stand.) = Lowest concentration of standard used (ng/mL);
Average absorbance of standard = Abs signal at lowest concentrations.

$$\text{Detection limit in ng/mL} = \frac{\text{3xStandard Deviation (control)x Lower Concentration (standard)}}{\text{average absorbance of standard signal at the lower concentration used}}$$

The percentage of non detected, geometric mean (g.m.), median and range values for each metal are reported in Tables 5 and 6.

RESULTS AND DISCUSSION

Table 5 shows the toxic element concentrations (As, Cd, and Pb) in the milk samples. The highest concentrations were those of Pb (G.M. 21.8±35.2, median 25.8 and range 0.4-170.1 µg/kg). In particular 22 samples had concentrations (21.0-170.1 µg/kg) above the MRL (20 µg/kg). These Pb levels represent a potential risk for the health of the consumer. The Pb concentrations found in the milk samples were similar to those reported in national and international literature (BALDINI *et al.*, 1990; WOJCIECHOWSKA-MAZUREK *et al.*, 1995; ALAIS, 2000; TIECCO, 2000). The Pb concentrations were higher than those found in samples from dairy farms in Calabria. The higher Pb concentrations found in milk in this investigation may be due to various factors: transhumance along roads and/or motorways, fodder contamination, climatic factors, such as wind and the use of pesticides, urban waste disposal sites, industrial activity, etc. In particular, the farms are located in areas near an oil refinery and a graphite electric power station in Giammoro (Messina).

The levels of Cd were low (G.M. 8.8±4.5, median 12.2 and range <0.01-20.1 µg/kg), with 44.4% of the samples being non detectable (Table 5). The As concentrations were intermediate (G.M. 14.8±45.2, median 15.7 and range <0.2-188.7 µg/kg), with 5.6% of the samples being non detectable. These concentrations are similar to those reported in the literature (CERUTTI, 1999; ALAIS, 2000). The As concentrations in all milk sam-

ples do not represent a toxicological risk for the consumer. However, comparing these data with data from Calabria, albeit at concentrations presenting no toxicological risk, As concentrations found in milk samples from areas of Messina were higher and Cd concentrations were lower than in milk samples from Calabria.

Table 6 shows essential element levels (Cu, Se, and Zn). The highest concen-

Table 5 - Toxic elements (As, Cd and Pb) in whole raw bovine milk (μg/kg w.w.) from various dairy farms in Sicily.

SAMPLE	As	Cd	Pb
1	10.4	12.2	22.9
2	5.1	11.4	10.3
3	7.8	9.7	0.4
4	3.9	8.6	9.3
5	<0.2	9.5	2.5
6	160.7	9.1	2.4
7	7.1	12.1	14.7
8	2.7	12.3	16.4
9	4.4	13.8	21.0
10	<0.2	12.3	2.4
11	188.7	13.7	11.6
12	1.7	20.1	49.4
13	36.5	14.1	11.2
14	4.2	12.3	170.1
15	139.6	13.3	35.6
16	3.4	16.1	16.8
17	2.8	14.4	30.0
18	44.3	1.5	17.3
19	82.6	<0.01	57.3
20	10.1	<0.01	28.9
21	10.7	<0.01	96.5
22	16.2	<0.01	99.3
23	17.7	<0.01	30.6
24	19.1	<0.01	64.6
25	27.3	<0.01	89.5
26	16.0	<0.01	62.9
27	28.5	<0.01	55.8
28	31.2	<0.01	9.6
29	31.4	<0.01	33.4
30	19.9	<0.01	32.3
31	15.2	1.1	25.3
32	17.6	<0.01	32.0
33	18.6	<0.01	18.4
34	15.3	<0.01	26.2
35	8.5	<0.01	25.2
36	12.0	1.1	75.5
% of non detected samples	5.6	44.4	
GM	14.8	8.8	21.8
S.D.	45.2	4.5	35.2
Median	15.7	12.2	25.8
Range	<0.2-188.7	<0.01-20.1	0.4-170.1

Table 6. Essential elements (Cu and Se (μg/kg w.w.) and Zn (mg/kg w.w.)) in whole raw bovine milk from various dairy farms in Sicily.

SAMPLE	Cu	Se	Zn
1	76.6	13.4	3.9
2	80.2	21.8	4.3
3	229.9	41.5	6.9
4	131.4	28.4	4.8
5	48.6	20.8	4.2
6	20.5	21.2	3.7
7	35.3	17.4	4.2
8	166.3	13.7	6.0
9	203.0	23.7	9.2
10	75.1	24.3	6.4
11	43.2	23.5	6.0
12	30.1	13.8	6.3
13	41.8	34.0	7.0
14	30.5	24.1	6.4
15	16.4	16.6	4.0
16	18.2	27.4	6.1
17	78.8	29.9	7.0
18	18.8	24.0	6.0
19	31.6	7.0	5.4
20	9.1	24.8	6.2
21	103.9	21.4	7.2
22	92.5	17.1	6.1
23	74.3	12.5	6.6
24	135.3	19.4	9.6
25	79.3	14.4	5.8
26	75.7	22.2	8.6
27	68.8	11.1	5.6
28	86.7	20.7	5.4
29	33.7	22.3	6.2
30	48.8	17.2	6.7
31	63.1	17.3	4.3
32	98.2	6.4	6.2
33	60.4	14.4	7.1
34	14.1	11.0	4.2
35	33.0	11.0	5.0
36	42.1	13.2	6.3
% of non detected samples			
GM	53.3	18.1	5.8
S.D.	51.6	7.4	1.4
Median	61.7	20.1	6.1
Range	9.1-229.9	6.4-41.5	3.7-9.6

trations were those of Zn (G.M. 5.8±1.4, median 6.1 and range 3.7-9.6 mg/kg), while the lowest levels were those of Se (G.M. 18.1±7.4, median 20.1 and range 6.4-41.5 μg/kg); intermediate values were those of Cu (G.M. 53.3±51.6, median 61.7 and range 9.1-229.9 μg/kg). The concentrations of these elements were similar to those reported in the literature (WOJCIECHOWSKA-MAZUREK *et al.*, 1995; CERUTTI, 1999; ALAIS, 2000; SIMSEK *et al.*, 2000). The concentrations of Cu and Se were lower than in milk collected in Calabria, although the Cu levels were similar. Zn concentrations were higher than those in milk samples from Calabria (LICATA *et al.*, 2004).

Accuracy and precision of the analysis were good in all cases, confirming the quality of the data (Table 4).

In conclusion, in milk samples from various dairy farms from areas of Messina the Pb concentrations were above the MRL, while for the other toxic and essential elements there were no significant data because there are no specific MRLs in milk fixed by EC Regulation n. 2001/466 for these metals. For greater food safety it would be advisable to establish MRLs for the various metals, not only for milk and other dairy products, but for all food.

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FINAL PROGRAM

International Association for Food Protection
Calgary, Alberta, Canada
August 13-16, 2006

The meeting has earned recognition as the leading food safety conference. Online registration and program information is available on the Association Web site at: www.foodprotection.org.

The meeting kicks off Sunday evening, August 13, with the Ivan Parkin Lecture followed by the Opening Reception held in the Exhibit Hall. This year's honored lecturer is Dr. Arthur Liang from the Centers for Disease Control and Prevention. Monday morning starts three days of diversified sessions with over 500 presentations including 24 half-day symposia, three round-table sessions, 80 technical and 350 poster presentations. A sample of symposia topics includes: Symposium on *Salmonella*: The Saga Continues; How Risk Managers Decide on Risk from Different National Perspectives; A New Crack at Egg Safety: From the Hen House to Your House; Disaster Preparedness and Response; Making Food Safer: How Outbreaks Can Influence Change; and many others. The Third Annual Silliker Lecture will be presented on Wednesday afternoon, featuring Dr. William Sperber, Cargill. For additional educational opportunities, IAFP 2006 will present three pre-meeting workshops on August 11 and 12.

Attendance is expected to exceed 1,600 of the top industry, academic and government food safety professionals. This broad mix of attendees includes professionals in quality control, processing operations, regulatory inspections, consulting groups, risk assessment, research and development, microbiological research, plant management, technical services, and HACCP management. The meeting will also showcase more than 90 companies demonstrating the latest products and technologies in the Exhibit Hall.

Three Workshops at IAFP 2006

The International Association for Food Protection announces three workshops to be held in conjunction with IAFP 2006 at the Telus Convention Centre in Calgary, Alberta, Canada.

Workshop I - Saturday, August 12, 8:00 a.m. - 5:00 p.m. - Developing and Improving Your Food Microbiology Laboratory

This workshop will present ways to operate a food microbiology laboratory more effectively and efficiently. Participants will learn in a friendly and interactive environment, the critical elements of a food microbiology testing laboratory. Also, laboratory layout as it applies to efficiency and data quality will be addressed. Workshop participants will learn how to build technical competence through training and the three pillars of quality. Analysis of variables to be considered when determining whether to build or up grade an internal microbiology laboratory including a review of experiences and challenges with in-house testing will be presented. The workshop will include time for a round-table dis-

cussion and a binder of information to reinforce the practical experience gained during the workshop for future use.

Workshop II - Saturday, August 12, 8:00 a.m. - 5:00 p.m. - Methods, Methods Everywhere but Which is Right for Me? Selection and Verification of Methods

Selecting the analytical tools for microbiological analysis that best meets your needs is a critical task. This workshop will teach you about selecting a microbiological method that is "fit for purpose". Experience a first-time release and the demonstration of an AOAC "online" learning center to understand the various international approaches to method validation schemes. Speakers will address practical considerations in method selection both for corporate and single manufacturing site labs; the concept of uncertainty of measurement as a key component of method verification; and the Canadian experience in expectations of accrediting authorities for methods verification.

Workshop Ill - Friday and Saturday, August 11-12, 8:00 a.m. - 5:00 p.m. - Global Food Standards: Food Safety Auditing

In today's global food market it is vital that there are food safety standards in place that can be used by companies in determining a supplier base for their foodstuffs. To this end there has been an increase in the development and evolution of Global Food Safety Standards. The recently launched ISO 22000 Standard is the latest in the range of standards. Currently, the most widely used is the British Retail Consortium (BRC) Global Standard Food. This is used by approved Certification Bodies as the standard to audit against in ensuring a consistent, safe food supply.

The Standard covers a wide range of topics including, HACCP, Quality Management Systems, Factory Environment Standards, Product Control, Process Control and Personnel. One of the problems with auditing is ensuring consistency between auditors. This workshop will cover all aspects of both the Standard and auditing techniques to guarantee consistency. This course is certified by the British Retail Consortium and is recognized as the required Internal Auditor training for any company seeking certification.

Detailed descriptions and registration information regarding these workshops may be obtained by visiting our Web site at www.foodprotection.org.

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5th International Symposium on the Challenge to Sheep and Goats Milk Sectors 18-20 April 2007 Alghero/Sardinia, Italy

The challenge to the dairy sheep and goat sectors lies in the improvement of their technical and economic efficiency, whilst maintaining the characteristics that confer an excellent image of tradition and high quality on their products. The main objective of this Symposium is to provide the economic stakeholders and scientists with a global revision of the situation of the sector and its evolution and better understand the present demands of the international markets particularly within the context of quality, safety and regulations.

Who should attend?

Scientists and other professionals involved in the sheep and goats dairy sec-

tors (milk producers, dairy industry, trade associations, academia, research institutes, government).

The Symposium will be a platform for exchange of knowledge and information among international experts in the field.

Visit the event's website regularly to be kept up-to-date!

Contact: Dr. Antonio Pirisi or Dr Giovanni Piredda, Istituto Zootecnico e Caseario per la Sardegna, Regione Bonassai, 07040 Olmedo, Italy, Tel.: +39 079 387277/387272 or +33 079 389444 Fax: +39 079389450, e-mail: info@sheepgoatsmilk.fil-idf-pr.com, webside and first announcement are available at: http://sheepgoatsmilk.fil-idf-pr.com

3rd International Symposium on milk genomics and human health Brussels, Belgium September 19-21, 2006

International experts in nutrition, genomics, bioinformatics and milk will gather at the Hilton Brussels to address the status of milk-specific genomic research. The event is being organized by the California Dairy Research Foundation and the International Dairy Federation.

The symposium will feature three sessions over the two-and-a-half day period focusing on such topics as the regulation of lactation genes, milk peptides sequence and targets, and updates from the International Milk Genomics Consortium. Special sessions for Consortium members also will be held during the event. A complete speaker program will be announced in mid-April.

The Consortium's goals include leveraging existing resources for the assembly of genetic instructions for milk molecules, linking the scientific community for a better understanding of the biological values of milk, creating tools for an interactive, Web data exchange, coordination of pre-competitive research to develop baseline data, and providing a foundation for the development of exclusive/competitive research. Information about the Milk Genomics Consortium can be obtained by emailing info@imgconsortium.org.

Symposium registration is $425 (approximately €355) through July 12, 2006 and $475 (approximately €395) thereafter and includes all program materials and daytime meals as well as a Tuesday evening reception. Members of the International Milk Genomics Consortium can register at the rate of $400 (approximately €335) through July 12, 2006 and $450 (approximately €375) thereafter. Registration fees will be collected in US dollars. Student rates also are available. For registration information, visit http://milkgenomics.fil-idf-pr.com or register online at www.acteva.com/go/cdrf.

For further information, please contact: Marylene Tucci, IDF Communications and Public Affairs, Tel.: +32 2 706 86 44, Fax: +32 2 733 04 13, e-mail: MTucci@fil-idf.org

International Conference Bubbles in Food: Novelty, Health and Luxury Low Wood Hotel & Conference Centre on Lake Windermere Cumbria, UK September 11th-13th, 2006

We have had a fantastic response to our earlier call for papers for this conference, with over 70 abstracts submitted from all over the world and covering a wide variety of aerated foods. This has allowed us to put together an excellent programme, the details of which are now available at http://www.umanitoba.ca/bubblesinfood2/schedule.shtml, along with links to abstracts.

Highlights include sessions on novel processing of aerated foods, new methods for detection and quantification of bubbles in food products including ultrasonics, image analysis and X-ray tomography, sensory and textural aspects of aerated foods, dairy foams, baked products, mathematical modelling, bubble stability, ingredient effects, along with an opening address from Professor Graeme Jameson from the University of Newcastle in Australia, to encourage us in our "Thinking outside the bubble".

The aim of the conference is to bring together scientists and engineers from academia and industry working to understand and exploit bubbly and porous systems in order to create new and improved food products. The refereed proceedings of the conference will be published as a fully-edited book by AACC International.

The website at http://www.umanitoba.ca/bubblesinfood2 gives full details of the conference arrangements, including the registration form.

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IFIS dictionary of food science and technology

Hardback July 2005
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The International Food Information Service (IFIS) has been producing quality comprehensive information for the world's food science, food technology and nutrition community since its foundation in 1968 and through its production of FSTA – Food Science and Technology Abstracts®, has earned a worldwide reputation for excellence.

Distilled from the extensive data held and maintained by IFIS, the Dictionary is easy to use and has been rigorously edited and cross-referenced. This landmark publication features:

- Over 7800 entries;
- Reflects current usage in the scientific literature;
- Includes local names, synonyms and Latin names, as appropriate;
- Extensive cross-referencing;
- Scientific editing from the team at IFIS.

This dictionary is an invaluable resource for all food scientists, food technologists, chemists and nutritionists in universities, research establishments, food and pharmaceutical companies. Libraries in all establishments where these subjects are studied and taught should ensure that they have copies on their shelves.

IFIS is a not-for-profit organisation providing international products and services, commissioning research and providing education in information science for the international food science, food technology and human nutrition community. Further information can be found at the IJIS website, www.foodsciencecentral.com.

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The Dictionary is available online at: www.blackwellpublishing.com or by contacting: Blackwell Publishing Ltd., 9600 Garsington Road, Oxford OX4 2DQ, UK, Tel: +44(0)1865 776868, Fax: +44(0)1865 714591, Vikki.Beale@oxon.blackwellpublishing.com

Dietary Supplements and Functional Foods

Geoffrey P. Webb, University of East London, School of Health & Bioscience, UK

256 pages, paperback, Publishing january 2006
ISBN: 1405119098
Price: £ 39.50
Publisher: Blackwell Publishing

The study of nutritional supplements has become increasingly important within orthodox establishments throughout the world, and as the market for these products continues to grow, so does the need for comprehensive scientifically sound information about these products, their properties and potential health effects. Dr. Geoffrey P. Webb, in this excit-

ing and most useful new book, not only looks at the accepted uses of dietary supplements, such as the use of fish oils in the prevention of heart disease and arthritis, but also explores the wider picture, identifying common themes and principles or particular categories of supplements.

Dietary Supplements and Functional Foods provides an excellent introductory text on this fascinating subject. An invaluable source of reference for students and professionals in nutrition, dietetics, nutritional therapy, food science and technology and other health professions including nursing, pharmacy and pharmacology. Personnel within food and pharmaceutical companies involved with supplement and functional food development and all libraries in institutions where this subject is studied and taught will find this book an important addition to their shelves.

- Written with a strategic overview approach applied to each chapter.

- Evidence based assessment of supplements and their contribution to the prevention and treatment of disease.

- Detailed discussion on individual supplements and functional foods including vitamins, minerals, antioxidants and probiotics.

GUIDE FOR AUTHORS

ITALIAN JOURNAL OF FOOD SCIENCE - IJFS

1. Manuscript Preparation

(1) Manuscripts must be typed, double-spaced and **two** copies submitted along **with** the computer disk. There should be liberal margins on top, bottom and sides (2.5 cm). English is the official language. Authors who are not fluent in written English must seek help from a person fluent in scientific English. The Assistant Editor reserves the right to make literary corrections and to make suggestions to improve brevity, but **the paper must be revised by a native English speaker** before submission.

Pages and lines on all pages, including those pages for "References" and figure legends, **must be electronically numbered in the left margin**, beginning with number one at the top of the page.

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).

(2) Every paper should be divided under the following headings in this order:

Title. Informative of the content of the article (<50 characters + spaces). Author(s). Initials and Surname, omit professional and official titles. The Institute and address where the research was carried out and the current address of each author should be given as a footnote on the title page.

Abstract. Clearly state the objective of the study, give a concise description of experiment(s), observations, results and conclusions. No references should be cited. DO NOT EXCEED 100 WORDS. An abstract and title in Italian (corresponding to the English) must also be included.

Keywords. Up to six words, in alphabetical order, which describe the document must be given to aid data retrieval and indexing.

Introduction. Review pertinent previous work and cite appropriate references. State the purpose of the investigation.

Materials and Methods. Indicate apparatus, instruments, reagents, etc., giving sufficient detail to allow the work to be repeated.

Results and Conclusions. Results and Conclusions may be presented together or separately. Concisely present results using tables and figures to help justify conclusions (do not present the same information in both forms). Use statistical analysis when appropriate. Unsupported hypotheses should be avoided. Conclusions should point out the significance of the findings and, if possible, relate the new findings to some problem in Food Science and Technology.

Acknowledgments. Acknowledgments of assistance are appropriate provided they are not related to analyses, or other services performed for a fee. Financial support, thanks for assistance, article number or thesis fulfillment may be included.

Units. A list of units particular to the paper may be included.

References. References should be arranged alphabetically, and for the same author should be arranged consecutively by year, typed double-spaced. Each individual citation should begin flush left (no indentation). Refer to attached examples taken from "Style Guide for Research Papers" by the Institute of Food Technologists (Chicago - Illinois - USA). Literature citations in the text should be referred to by name and year in

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

(3) Tables should be as few and as simple as possible and include only essential data. Each table must be saved and printed on a separate sheet, and have an Arabic number, e.g. Table 4 NOT Tab. 4. Legends must be self-explanatory and on a separate sheet. Use lower-case letters for footnotes in tables and explain below the table in the order in which they appear in the table.

(4) Figures must be drawn and saved by separate in TIF, JPEG, EPS or PICT formats (300 dpi resolution). They should be drawn so that on 50% reduction, lines, figures and symbols will be clearly legible and not overcrowded. A photocopy of how the figure should appear must be included. Photographs must be unmounted, glossy prints or slides. All figures must be given Arabic numbers, e.g. Fig. 3, in the text and in the final copy only on the back where the title of the paper, the senior author's surname and the top of the illustration must also be marked; for reviewing procedures, do not include this information in the first submitted copies. Legends for figures must be self-explanatory and should be typed on a separate sheet under "Legends to Figures".

(5) Standard Usage, Abbreviations and Units. The Concise Oxford and Webster's English Dictionaries are the references for spelling and hyphenation. Statistics and measurements should always be given in figures, e.g. 10 min, except when the number begins a sentence. When the number does not refer to a unit of measurement it is spelled out unless it is 100 or greater. Abbreviations should be used sparingly, only when long or unwieldy names occur frequently, and never in the title; they should be given at the first mention of the name. International Standard abbreviations should generally be used except where they conflict with current practice or are confusing. For example, 3 mm rather than $3x10^{-3}$ m. Abbreviations should be defined the first time that they are used in the text and they should be used consistently thereafter. Temperatures should be expressed in the Celsius (centigrade) scale. Chemical formulae and solutions must specify the form used, e.g. anhydrous or hydrated, and the concentration must be in clearly defined units. Common species names should be followed by the Latin binomial (italics) at the first mention. For subsequent use, the generic name should be contracted to a single letter if it is unambiguous.

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Opinions and Reviews - Papers may be sent directly to the Editor-in-Chief who will decide upon publication or articles will be requested directly from the authors by the Editor-in-Chief.

Short Communications and Surveys - They do not need to have the formal organization of a research paper; they will receive priority in publication;

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Short Communications, Surveys and Papers will be subjected to critical review by the referees. Upon receiving papers from authors, the Advisory Board with the Editor-in-Chief will select papers in relationship to innovation and originality and send copies to the referees. A letter stating that the paper has been accepted for refereeing will be sent to the authors. Papers needing revision will be returned to the author, and the author must return the revised manuscript to the Editor-in-Chief, otherwise the paper will be considered as withdrawn. Papers not suitable for publication will be returned to the author with a statement of reasons for rejection.

3. Editorial Policy

Referees may not be from the same institution as the author. Referees should make their comments and questions in detail and return the paper to the Editor-in-Chief as

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The proofs will be sent to the corresponding author as a PDF file by e-mail only. A hard copy will be sent by mail only if the author makes this reguest when the paper is accepted for publication.
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EXAMPLES of use in a Reference list are given below. The bold-faced parenthetical type of citation above the example is indicated ONLY for information and is NOT to be included in the reference list.

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