ISSN 1120-1770

ITALIAN JOURNAL OF FOOD SCIENCE

Rivista italiana di scienza degli alimenti

IJFS

Volume XV
Number 4
2003

Spedizione in ab. post. comma 26 - art. 2 - legge 549/95 n. 4/2003 - Torino

CHIRIOTTI EDITORI

ITALIAN JOURNAL OF FOOD SCIENCE

(RIVISTA ITALIANA DI SCIENZA DEGLI ALIMENTI)

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Publisher:
Alberto Chiriotti
Chiriotti Editori s.p.a., Viale Rimembranza 60, I-10064 Pinerolo, Italy
Tel. +39 0121 393127 - Telefax +39 0121 794480
E-mail: info@chiriottieditori.it - URL: www.chiriottieditori.it

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

Impact Factor: 0.639 published in the 2002 Journal of Citation Reports, Institute for Scientific Information

Subscription Rate: 2004: Volume XVI

Ordinary	€	120.00
Supporting	€	500.00

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

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REVIEW

FUROSINE AND OTHER HEAT-TREATMENT INDICATORS FOR DETECTING FRAUD IN MILK AND MILK PRODUCTS

FUROSINA E ALTRI INDICATORI DI DANNO TERMICO
PER IL RICONOSCIMENTO DI ALCUNE FRODI IN LATTE E DERIVATI

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ABSTRACT

Besides having wide application as criteria for heat-classification of several milk products, heat-treatment indicators can help discover frauds. When the level of 8.6 mg furosine/100 g protein is exceeded in peroxidase-positive pasteurized milk, without doubt, either reconstituted milk powder (RMP) or high-temperature treated milk has been added. The ratio between the levels of furosine and lactulose allows the presence of RMP to be detected in UHT milk or in-bottle sterilized milk. Different lev-

RIASSUNTO

Gli indicatori di trattamento termico, oltre ad avere applicazione per classificare molti prodotti lattieri sulla base del carico termico, possono aiutare a riconoscere frodi. Quando nel latte pastorizzato perossidasi-positivo il valore di furosina supera 8,6 mg/100g proteine, è dimostrata la presenza di latte in polvere ricostituito (RMP) o di latte trattato ad alta temperatura. Il rapporto furosina/lattulosio permette di riconoscere la presenza di RMP nel latte UHT e nel latte sterilizzato in bottiglia. Dif-

- Key words: dairy products; furosine; heat-treatment; lactulose; lysinoalanine; recomposed milk -

els of furosine in skim milk and in the fat globule membrane from heat-treated whole milk demonstrate that the milk was recomposed from skim milk and cream heated under different conditions. The unnatural amino acid lysinoalanine is a suitable indicator of the use of caseinates in cheesemaking. The advanced Maillard compound galactosyl-β-pyranone allows directly and indirectly heated UHT milk to be distinguished.

ferenti valori di furosina delle proteine del latte magro e di quelle sulla membrana del globulo di grasso, determinati nel latte intero trattato termicamente, dimostrano l'avvenuta ricomposizione di latte magro con crema trattati in condizioni termiche diverse. L'amminoacido innaturale lisinoalanina è utile indicatore per riconoscere l'utilizzo di caseinati in caseificazione. Il galattosil-β-piranone, derivante dalla reazione di Maillard avanzata, permette di distinguere il latte UHT diretto da quello indiretto.

INTRODUCTION

Several indicators have been proposed for evaluating the severity of heat-treatment of milk and milk products. In a comprehensive review PELLEGRINO *et al.* (1995a) reported that heat-treatment indicators originate from two types of chemical reactions:

i) degradation, denaturation and inactivation of heat-labile components, e.g. whey proteins or enzymes;

ii) formation of new molecules which are not present, or only at trace levels, in the unprocessed product, e.g. lactulose or molecules originating from the Maillard Reaction (MR).

An excellent review with a similar approach has been published by CLAEYS *et al.* (2002). Even the most recent literature (CLAWIN-RÄDECKER *et al.*, 2000; MORALES *et al.*, 2000; SHARMA *et al.*, 2000; DATTA *et al.*, 2002; BLOCK de *et al.*, 2003) refers to the same heat-treatment indicators which are presently applied on a world-wide scale due to the availability of robust and easy-to-use analytical methods.

Among these parameters, furosine is sometimes used as an index for assessing quality or, more specifically, the genuineness of both milk and dairy products. Indeed, this molecule describes the extent of the early Maillard reaction (FINOT and MAURON, 1972) and therefore its level is very sensitive to the entire heat-damage a milk product has undergone, including that occurring during storage. For this reason, the furosine index *per se* often does not allow different types of heat-treated milk (e.g. pasteurized milk from high-temperature pasteurized milk) to be distinguished. However, the level of furosine can give extra information when it is combined with another index which describes the actual heat-treatment used in processing.

Some examples of this original approach are briefly presented in this review, while details concerning experimental design, type of samples considered and statistical parameters for each specific application are described in the related original papers. It should be mentioned that some authors (SHIN *et al.*, 2000; VILLAMIEL *et al.*, 2000; OHTA *et al.*, 2002) have recently adopted the furosine index *itself* as a parameter for assessing genuineness of milk and dairy products in a more pragmatic way which, however, can have poor sensitivity.

Pasteurized milk

Furosine can be determined by a direct and sensitive ion-pair reversed phase HPLC method (RESMINI at al., 1990; ISO/DIS 18329/IDF 193, 2003) which allows the routine determination of this molecule in all types of food and its quantification at picomole levels without interference in raw milk, as well. The furosine level is rather constant in raw bulk milk, ranging from 3.8 to 4.8 mg/100 g protein (RESMINI *et al.*, 1992a), and it is always less than 7 mg/100 g protein in peroxidase-positive pasteurized milk, while it increases with pasteurization temperature, up to conditions leading to peroxidase-negative pasteurized milk (Fig. 1). It is clear that furosine is not a suitable parameter for discriminating between peroxidase-positive and peroxidase-negative pasteurized milk. However, the furosine index represents a useful parameter for evaluating the genuineness of both raw milk and peroxidase-positive pasteurized milk (M.D., 2000). The pasteurization process specified by the EU Directives 92/46 and 92/47 must lead to milk still peroxidase-positive. Data obtained on commercial samples of pasteurized milk collected in 9 European countries and analyzed by 12 different laboratories confirm that the furosine value is always less than 8.6 mg/100 g protein ($P<0.001$) in peroxidase-positive pas-

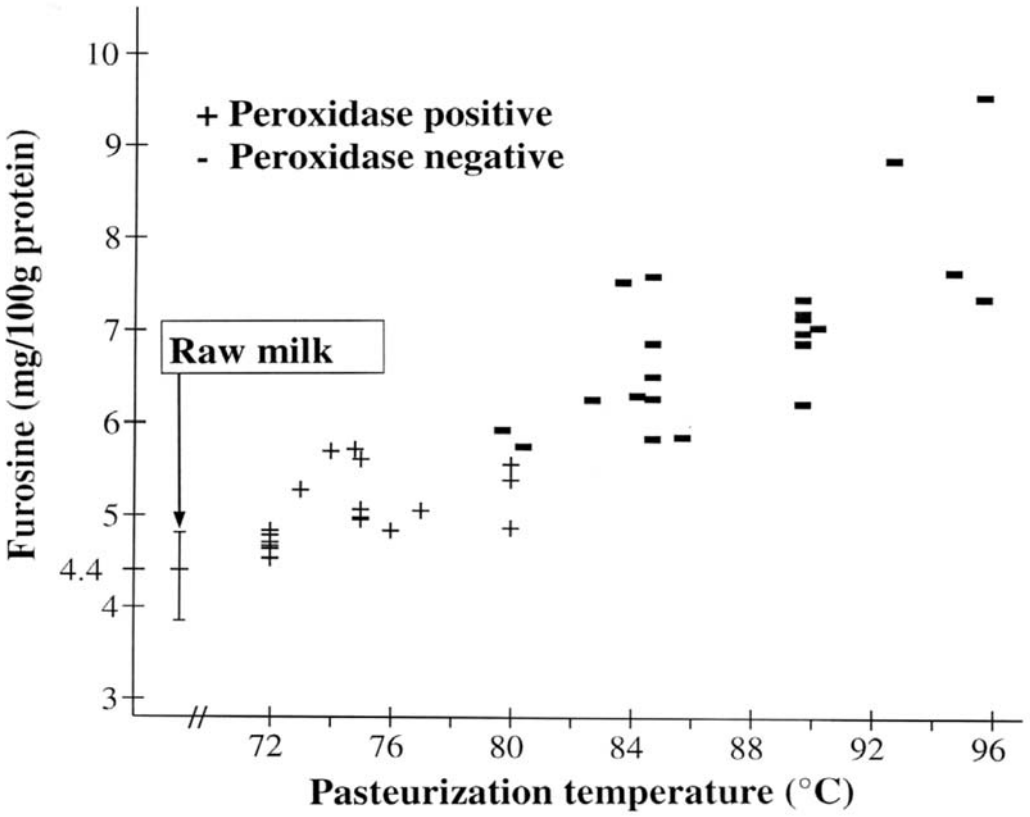


Fig. 1 - Furosine level in milk samples pasteurized at 9 industrial plants under 20 different processing conditions. Pasteurization temperature from 72° to 96°C; holding time from 15 to 38 sec.

teurized milk (Fig. 2) (PELLEGRINO *et al.*, 1996a). These data perfectly agree with those of other authors (CLAWIN-RÄDECKER and SCHLIMME, 1995; VAN RENTERGHEM and BLOCK de, 1996).

As the MR is highly enhanced by heat processing conducted at low levels of water activity (a_w=0.6-0.7), milk powder and other dried milk products, even when processed under mild heating conditions, have furosine levels 5-10 times higher than those found in fluid low-heat treated products. The furosine value of milk powders can range from 70 to over 500 mg/100 g protein, depending on the heat class and keeping conditions (RESMINI and PELLEGRINO, 1994). Values higher than 8.6 mg/100 g protein in peroxidase-positive pasteurized milk prove the presence of reconstituted milk powder (RMP) (Fig. 3) (RESMINI *et al.*, 1992b) or liquid milk heated under more severe conditions.

Sterilized milk

As the level of furosine dramatically rises when milk is processed under sterilization conditions, this parameter itself is not useful for assessing the manipulation of UHT milk (e.g. addition of RMP). A close relationship between the content

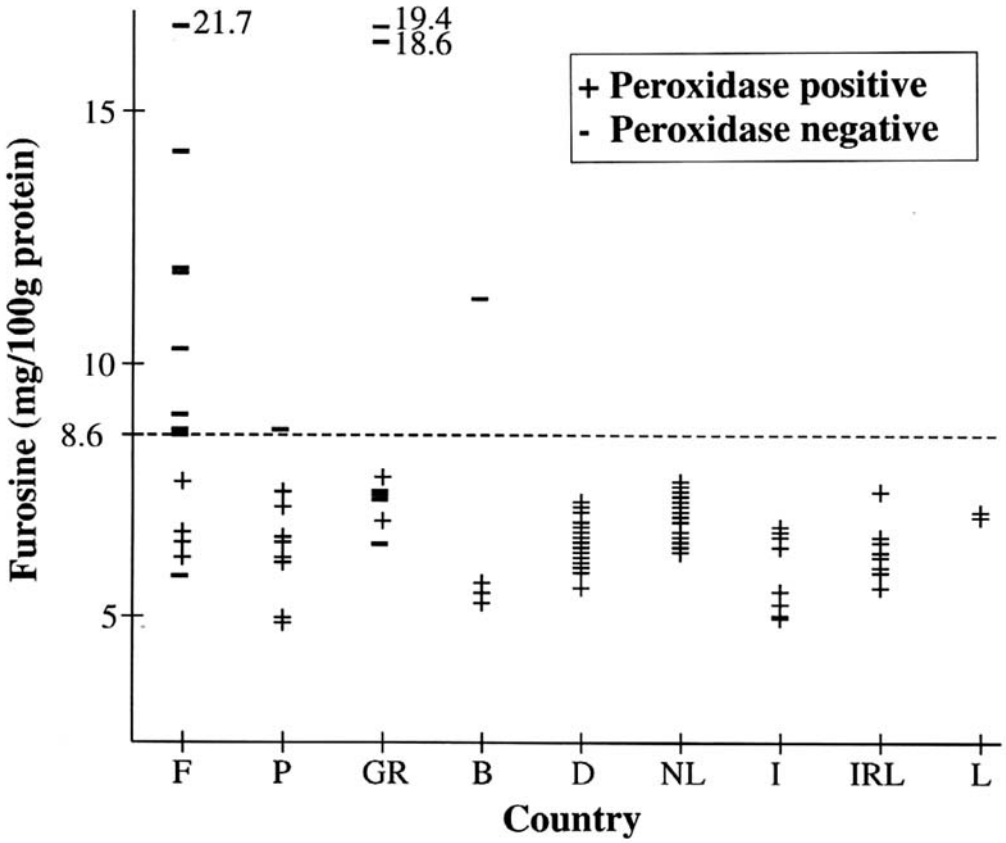


Fig. 2 - Furosine level in 104 commercial samples of pasteurized milk produced within the European Union.

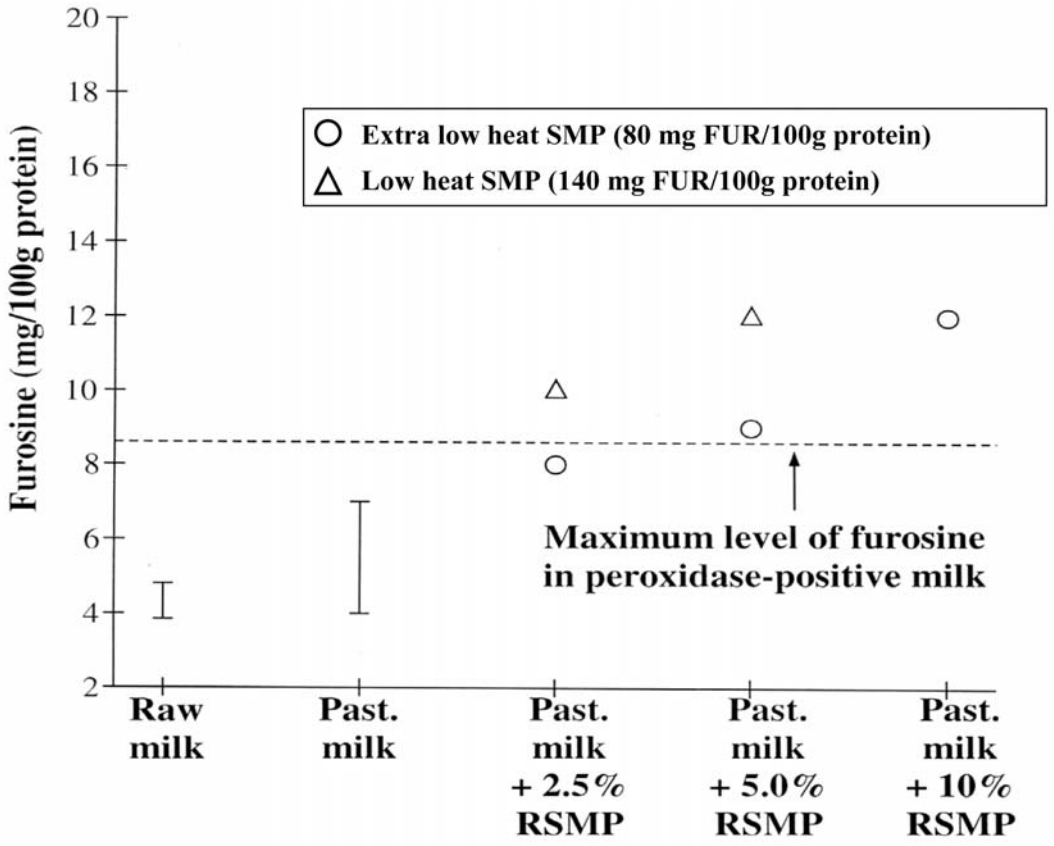


Fig. 3 - Furosine level in raw milk and in peroxidase-positive pasteurized milk containing different amounts of reconstituted skim milk powder (RSMP).

of lactulose, describing the extent of heat-induced lactose isomerization, and the level of furosine has been found in freshly prepared UHT milk and in-bottle sterilized milk samples of known origin (Fig. 4) (PELLEGRINO *et al.*, 1995b). This relationship is not respected in two cases: (i) when RSMP is present; (ii) when the pH value of raw milk is adjusted before the heating process to improve thermal stability (Fig. 5). Therefore, the indicator furosine in combination with lactulose is useful for evaluating the quality of high-heat treated drinking milk, provided that the milk has been stored no longer than 2 weeks.

When heating is severe and prolonged, ε-lactulosyl-lysine is converted into advanced glycosylation end products (AGEs) through the advanced MR. A pathway which brings about the formation of galactosyl-β-pyranone (GAP) *via* the intermediate diketone 1-deoxyosone was proposed by KRAMHÖLLER *et al.* (1993). This AGE, which occurs under free form and can be directly determined by HPLC, has been recently proposed for evaluating the severity of milk sterilization (PELLEGRINO *et al.*, 2001). Furthermore, it is suitable for both distinguishing between directly and indirectly heated UHT milk (Fig. 6) and for pointing out overprocessing. Neither furosine nor other

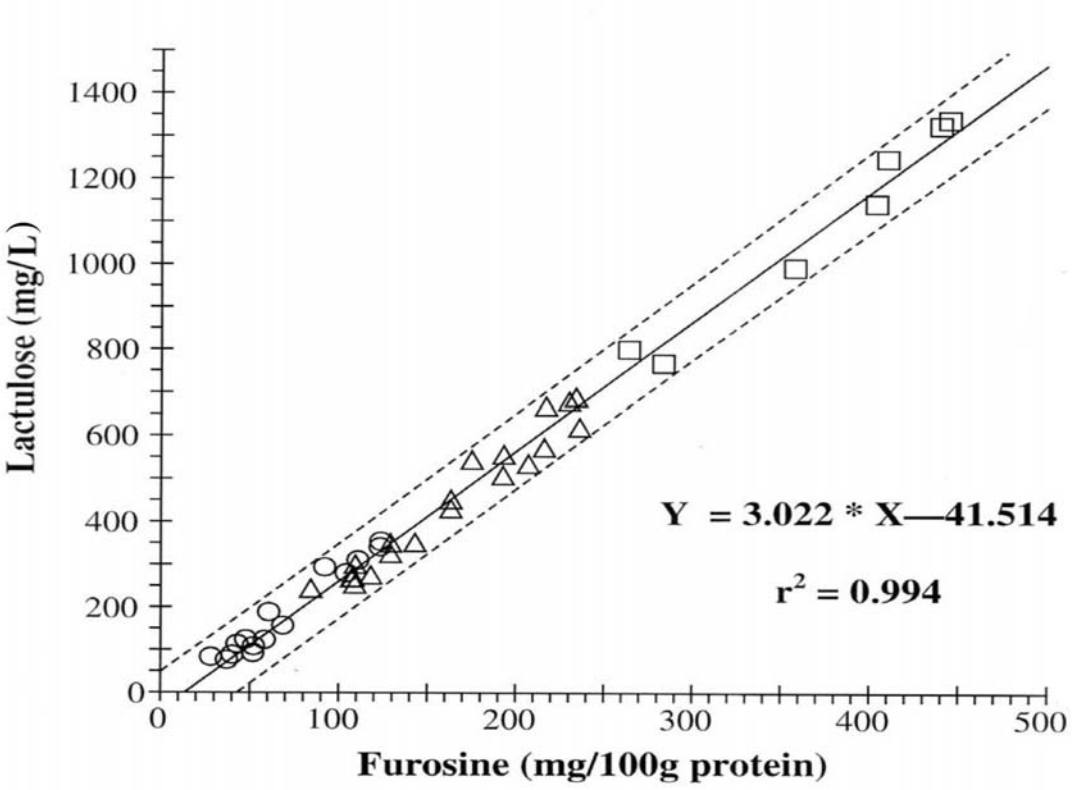


Fig. 4 - Correlation between lactulose and furosine in freshly prepared samples of directly (○, n=17) or indirectly (Δ, n=22) heated UHT milk and in-bottle sterilized milk (□, n=7).
(---) = confidence limits at ±2σ.

chemical indicators proposed to date allow a clear distinction between the two classes of UHT milk (DATTA *et al.*, 2002).

Recomposed milk

The presence in drinking milk of components which were separately submitted to different heat treatments can be detected by measuring furosine according to an original approach (CATTANEO *et al.*, 2002). When milk is heated, regardless of the heating conditions, all milk protein fractions will show the same level of furosine. On the contrary, when skim milk and cream are separately heat treated and then reblended, the level of furosine of these two components will be different depending on the severity of the individual heat treatments they have undergone. Therefore, a possible way to recognize when whole milk has been obtained by mixing such components is to separate them again in order to measure the respective levels of furosine. Upon heating, casein micelles bind irreversibly to the milk fat globule membrane (MFGM). As a consequence, heat-treated cream contains a large amount of structures, like that shown in Fig. 7, where both MFGM and the layer of casein micelles bound to it are "labelled" by the same level of furosine. If the cream is then blended with skim milk these structures can be easily separated by centrifugation and submitted to furosine determination after a suitable sample preparation pro-

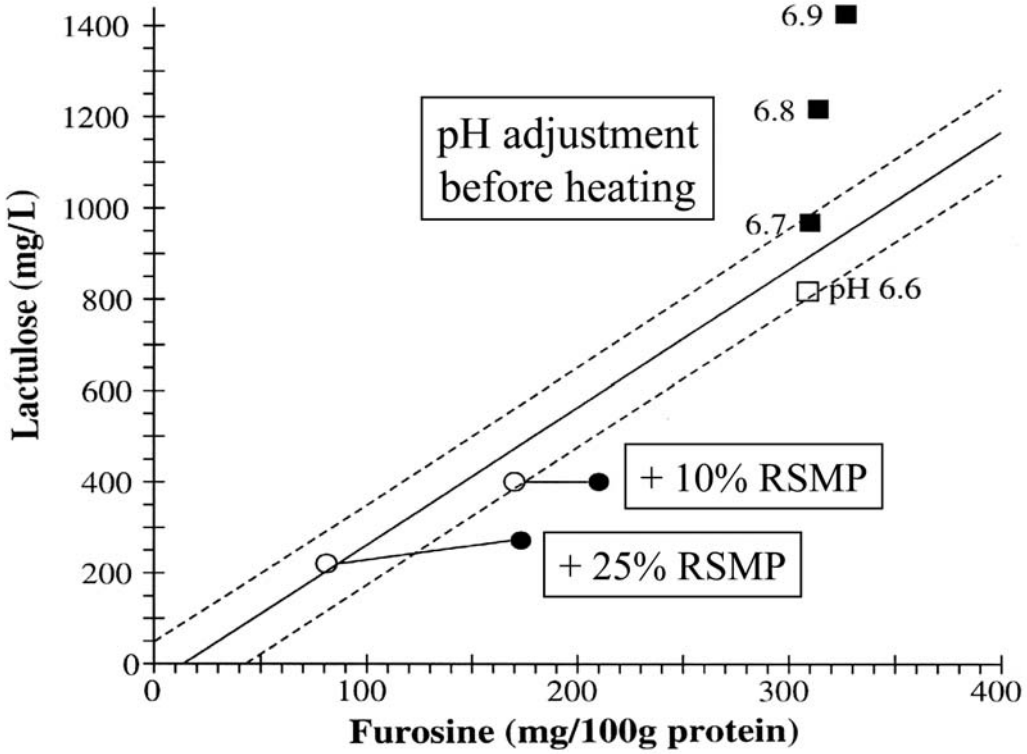


Fig. 5 - Formation of lactulose and furosine in manipulated sterilized milk: direct UHT milk before (○) or after (●) the addition of reconstituted skim milk powder (RSMP); milk autoclaved (110°C/10 min) at natural pH (□) or after pH adjustment to different values (■). Correlation line as in Fig. 4.

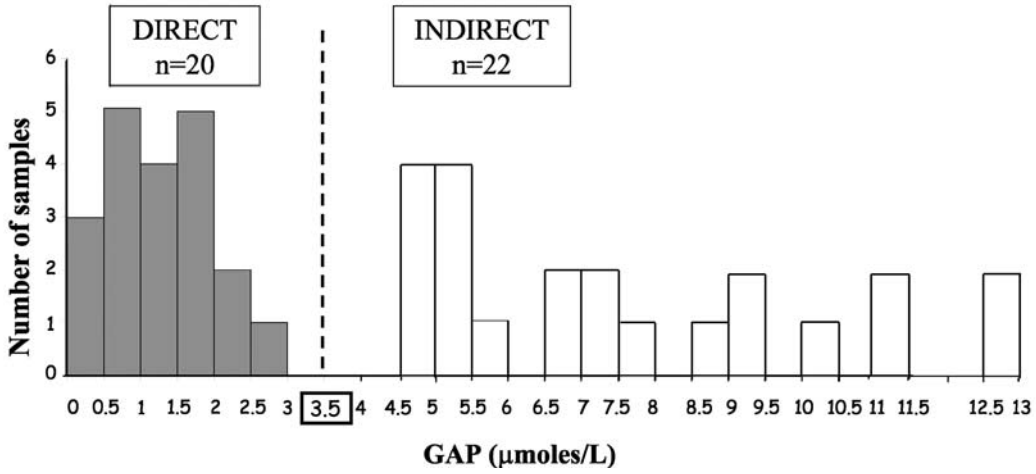


Fig. 6 - Levels of galactosyl-β-pyranone (GAP) in directly or indirectly heated UHT milk.

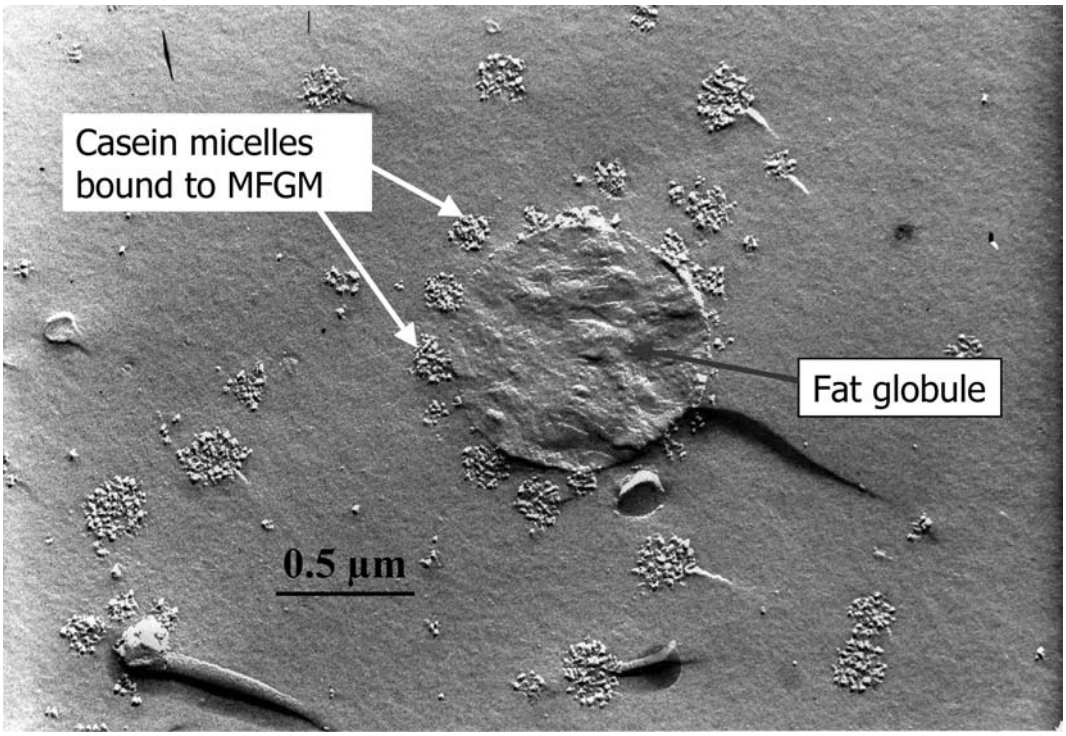


Fig. 7 - Transmission electron microscopy (freeze-fracturing preparation) of casein micelles in heated whole milk; arrows indicate casein micelles bound to the milk fat globule membrane.

cedure. If the level of furosine differs from that of the corresponding skim milk by more than 10% of the arithmetic mean of the two values, this proves that the cream and skim milk heated differently have been recomposed.

Data obtained following this approach are reported as an example of its feasibility. Table 1 shows that both whole milk and the corresponding skim milk and cream have the same level of furosine, regardless of the type and severity of heat treatment. When skim milk and cream are heated separately (Table 2, samples 1, 3 and 4), the furosine level of MFGM is different from that of skim milk and the difference is still detectable after a severe heat treatment of the recomposed milk (sample 2). The presence of RMP in the recomposed whole milk can be identified on the basis of this approach before (sample 5) and after heating (sample 6).

Application of this approach to control samples of microfiltered drinking milk allows the different processing steps to be pointed out (Table 3).

Table 1 - Furosine level in 3 fractions of different types of drinking milk.

	Furosine (mg/100 g protein)		
Sample	Whole Milk	Skim Milk	MFGM [(a)]
Raw milk	5.3	5.5	5.3
Pasteurized milk	6.4	6.0	6.3
UHT milk	84	82	85

[(a)] MFGM = milk fat globule membrane.

Table 2 - Furosine level in 3 fractions of milk samples recomposed with components heated separately under different conditions.

N.	Sample	Furosine (mg/100 g protein)		
		Whole Milk	Skim Milk	MFGM
1	Pasteurized skim milk with the addition of 20% UHT cream	44.3	15.2	161
2	Same as 1, heated at 95°C/30 min	258	235	268
3	Raw skim milk with the addition of 20% cream heated at 70°C/30 min	6.4	5.8	8.8
4	Skim milk heated at 70°C/30 min with the addition of 20% raw cream	13.9	13.0	9.0
5	Reconstituted SMP with the addition of pasteurized cream	273	260	9.3
6	Same as 5, heated at 95°C/30 min	310	291	148

Table 3 - Furosine level in 3 fractions of commercial microfiltered (MF) drinking milk.

N.	Sample	Furosine (mg/100 g protein)		
		Whole Milk	Skim Milk	MFGM
1	MF raw milk from F (a)	6.0	4.5	9.0
2	MF pasteurized milk from UK [b]	13.0	10.5	24.1
3	MF pasteurized milk from GE [b]	8.6	7.9	11.6
4	MF pasteurized milk from GE [c]	5.5	6.0	6.6

[a] Cream was UHT-treated separately and added to MF raw milk; [b] APV process: cream was UHT-treated separately and added to MF pasteurized milk; [c] Raw cream was added to MF raw milk and the recomposed milk was pasteurized.

Pasta-filata cheeses

The evaluation of furosine may also be used for the quality control of fresh cheese. The raw material for pasta-filata cheeses produced under GMP can not be submitted to severe heating without the curd losing its ability to be stretched and moulded. RESMINI *et al.* (1992a) reported that controlled industrial cheesemaking of Mozzarella, including cheesemilk pasteurization, coagulation, curd draining and acidification, final stretching and moulding with hot water did not produce values of furosine higher than 8 mg/100 g protein (Fig. 8). This value was exceeded in the finished cheese when amounts higher than 3% RMP were added to pasteurized cheesemilk. Furosine values found in several commercial samples of Mozzarella cheese on the EU market ranged from 4 to 38 mg/100 g protein. Use of dried dairy ingredients which were not declared on the label was suspected in more than 50% of these samples. Presently, legal threshold values of furosine have been fixed at 10 mg/100 g protein for certified Mozzarella produced in the EU (EU Regulation n. 2527/98) and at 12 mg/100 g protein for Mozzarella and other fresh pasta-filata cheeses produced in Italy (M.D., 2000) (Fig. 8).

The MR takes place when dairy products containing significant amounts of lactose are heated. Lacking a reducing sugar, the MR is largely suppressed by the competitive reaction of the lysine residues with dehydroalanine, arising from the β-elimination reactions on milk heteropro-

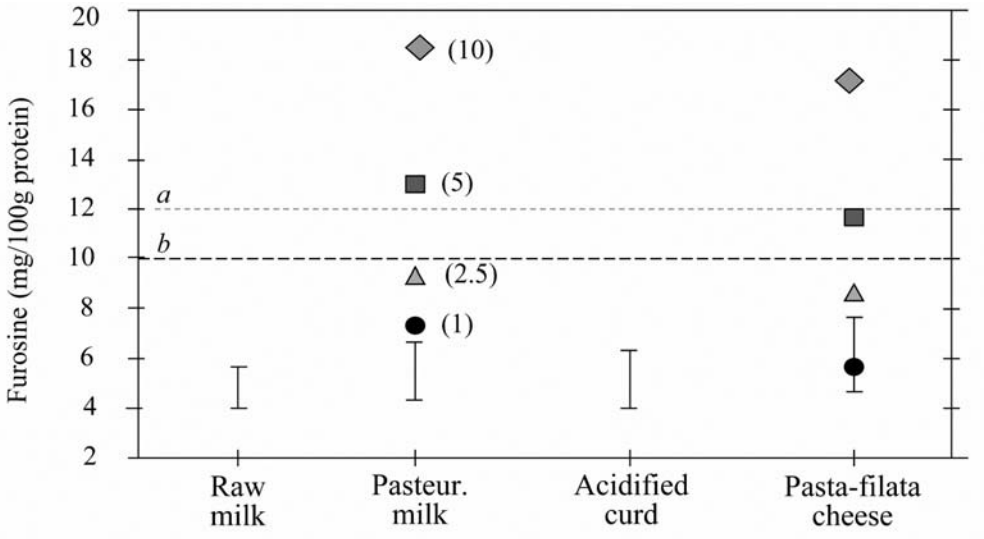


Fig. 8 - Furosine level in Mozzarella cheese produced under controlled conditions. I = range of values in genuine samples from 8 industrial cheesemakings from 7 industrial plants; ( ) = % reconstituted milk powder; a = upper limit for Mozzarella and other fresh pasta-filata cheeses in Italy; b = upper limit for Mozzarella cheese with certificate of specific character in the EU.

teins. This reaction mechanism leads to the formation of lysinoalanine (LAL) as one of the main products (Fig. 9) (CUQ and CHEFTEL, 1985). Because casein and caseinates are industrially dried at neutral or alkaline condi-

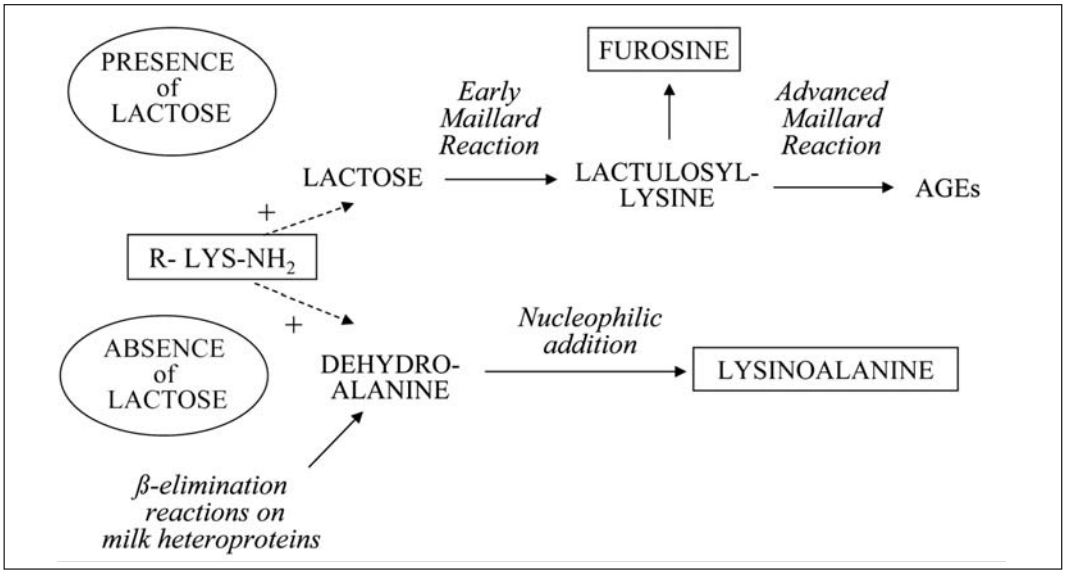


Fig. 9 - Heat-induced competitive reactions of lysine residues in milk protein in the presence or absence of lactose.

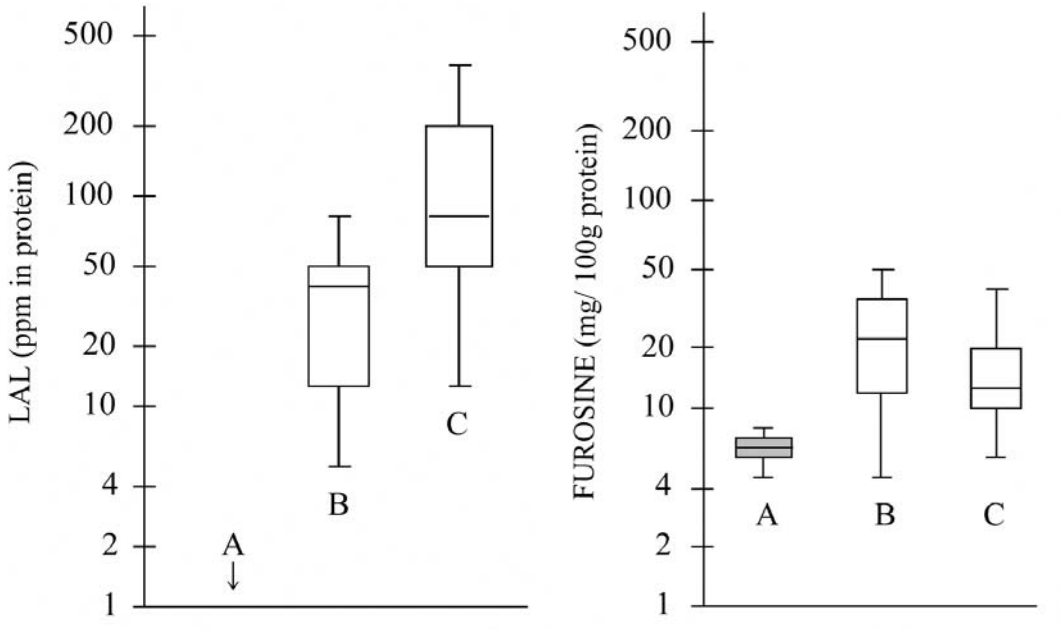


Fig. 10 - Levels of lysinoalanine (LAL) and furosine in commercial samples of natural Mozzarella cheese (A, n=11), imitation Mozzarella cheese (B, n=42), and casein and caseinates (C, n=11).

tions and after removal of most of the lactose, these ingredients may contain high levels of LAL and low levels of furosine because of the suppression of the MR. Therefore, LAL represents a useful marker for indicating the presence of casein and caseinates in natural cheeses, mainly in fresh pasta-filata cheeses which can be easily distinguished from imitation products (PELLEGRINO *et al.*, 1996b).

The capability of both LAL and furosine indices to distinguish natural Mozzarella from the substitutes is shown in Fig. 10. This distinction was always achieved by the LAL index (left), whereas furosine values of natural Mozzarella cheese are low and close to those of commercial casein and caseinates. Thus only the LAL index allows a clear-cut distinction ($P<0.001$) between natural Mozzarella cheese and imitation products.

CONCLUSION

The main results obtained by our group over the last decade on heat treatment indicators and related applications in the quality control of milk and dairy products have been reviewed. These studies have brought about the specification of new parameters for describing the quality of food and they have been incorporated in both Italian and European legislation. A key step in this work was the careful study of reliable analytical methods which have been adopted as international standards by organizations like ISO and IDF. This recognized transfer of basic knowledge to the practice of food quality control demonstrates that the study of the modifications induced by food processing can bring about unforeseen applications.

A form of this paper was presented at Congrilait 2002, the 26th IDF World Dairy Congress, 24-27 September 2002, Paris (France)

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Paper received February 13, 2003 Accepted July 30, 2003

PAPER

QUALITY OF MILK AND CACIOCAVALLO CHEESE FROM FARMS REARING PODOLICA AND ITALIAN FRIESIAN COWS

QUALITÀ DEL LATTE E DEL CACIOCAVALLO OTTENUTO DA ALLEVAMENTI DI VACCHE PODOLICHE E FRISONE ITALIANE

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ABSTRACT

Protein and fat content and total and free fatty acid and amino acid compositions were determined in Caciocavallo cheese obtained with milk from 4 farms rearing Podolica (n=2) and Italian Friesian (n=2) cow breeds in Puglia. Podolica milk had a higher content of protein, fat and somatic cells than Friesian milk. Higher values of $C_{6:0}$-$C_{14:0}$ total fatty acids and of stearic and linolenic acids, and lower percentages of palmitic, palmitoleic, oleic and linoleic total fatty acids were found in Podolica

RIASSUNTO

Sono stati confrontati il contenuto in proteine ed in grasso, la composizione aminoacidica e quella in acidi grassi totali e liberi del latte e del Caciocavallo Silano ottenuto da vacche delle razze Podolica e Frisona Italiana allevate in Puglia. Nel latte prodotto dalle vacche Podoliche è stato rilevato un contenuto più elevato in proteine ed in grasso rispetto a quello delle vacche Frisone, nonché un più alto contenuto in cellule somatiche. Nei formaggi ottenuti dal latte Podolico è stata rilevata una

- Key words: amino acids, Caciocavallo Silano Cheese, cow breeds, fatty acids, milk quality -

cheese. More acetic acid and medium- and long-chain free fatty acids, except for linoleic acid, were found in Podolica cheese. Podolica cheese had higher concentrations of alanine, methionine, proline and aspartic acid, while Friesian cheese had higher levels of phenylalanine, isoleucine, leucine and glycine. The results of this study show that cow breed and the cattle production system associated with the breeds, such as herd management, feeding regimen, milking techniques and milk collection, can greatly affect milk and cheese composition.

più alta percentuale degli acidi grassi da $C_{6:0}$ a $C_{14:0}$ e degli acidi stearico e linolenico, nonché una più bassa incidenza di acido palmitico, palmitoleico, oleico e linoleico rispetto a quelli ottenuti dal latte di vacche Frisone. Maggiori concentrazioni di acido acetico e di tutti gli acidi grassi liberi a media e lunga catena, con la sola eccezione dell'acido linoleico, sono stati rilevati nei caciocavalli Podolici. Questi ultimi hanno anche evidenziato maggiori concentrazioni di alanina, metionina, prolina e acido aspartico, mentre i caciocavalli ottenuti dal latte di vacche Frisone hanno presentato una maggiore incidenza di fenilalanina, isoleucina leucina e glicina. I nostri risultati evidenziano che la razza bovina ed il sistema di allevamento possono fortemente condizionare la qualità del latte e del formaggio da esso ottenuto.

INTRODUCTION

There is a growing need to certify production processes and production materials in order to assure high quality and well-defined organoleptic characteristics of the product. The use of different methods and/or raw materials can lead to a loss of specificity or, in the worse case, to fraudulent actions and food hoaxes.

Caciocavallo Silano is one of the 400 cheese varieties presently produced in Italy. The definition "Silano" refers to the production area of the cheese (BATTISTOTTI and CORRADINI, 1993). About 1,100-1,300 tons of Caciocavallo Silano cheese are produced annually using the "pasta filata" procedure. This cheese received the Italian legal designation of "DOC" (Controlled Denomination of Origin) on May 10, 1993 and the European "PDO" (Protected Designation of Origin) in June 1996. Caciocavallo Silano cheese must have the following requisites: a) it has to be produced in the southern Italian regions of Puglia, Campania, Molise, Basilicata or Calabria; b) it has to be obtained from the milk of cows raised in the five regions mentioned in point a); c) it has to be produced using a well-defined process.

While these rules are important for typifying the geographical origin of Caciocavallo Silano cheese, they are very general and at the end of the process, there may be marked differences in the organoleptic characteristics and physical-chemical properties of the cheese produced.

Many parameters (not mentioned in DOC and PDO definitions) can influence cheese quality: differences in rennet type (SALVADORI DEL PRATO, 1998), feeding systems (SUCCI and CROVETTO, 1996), and, above all, the breed of the cow (GRAVERT, 1987; CHIANESE *et al.*, 1988). About 100,000 Podolica cattle are reared in the southern regions of Italy and about 22,000 head of Podolica cattle are regis-

tered in the herd-book, of which 60% are cows. Milk yield is generally less than 10 L/day/cow, aside from the milk suckled by the calf. The lactation period may vary from 4 to 7 months, due to forage availability (MONTEMURRO, 1996). The casein polymorphism of Podolica milk has been found to be well suited for cheese-making (FERRARA *et al.*, 1986).

The aim of this work was to demonstrate that Caciocavallo cheese produced from milk of Podolica and Italian Friesian cows differs in some chemical properties that are of primary importance for the organoleptic characteristics of cheese. The degree of difference is such that it is possible to distinguish the milk source. This may indicate the need for a more complete legal definition of Caciocavallo Silano cheese.

MATERIALS AND METHODS

Herd management and milk processing

Four farms situated in the province of Foggia (Puglia), were involved in the experiment. On two farms, the herds consisted of more than 100 Italian Friesian cows (about 140 days of lactation). The cows were kept in free stalls provided with cubicles on straw litter. They were fed diets of barley silage, alfalfa hay and a pelletted concentrate (forage : concentrate = 0.6 : 0.4), which were given as total mixed rations twice daily. The cows on these farms were milked twice daily using pipeline milking machines. The average milk production was about 29 L/day/cow.

On the other two farms the herds consisted of about 70 Podolica cows (about 80 days of lactation). They were fed on pasture in a typical Mediterranean brush land during the day and housed in open cowsheds at night. The cows on these farms were hand milked once a day. The average milk yield was 6 L/day/cow.

Friesian cheese was obtained from an industrial process following the standard procedure (SALVADORI DEL PRATO, 1998). The same procedure was adopted for manufacturing Podolica cheese. Cheese from Friesian milk was manufactured in an industrial dairy plant using stainless steel machines, whereas Podolica cheese was manufactured on a traditional dairy farm using wooden and copper tools. In addition, the curd was mechanically and manually stretched in the production of Friesian and Podolica cheese, respectively. Full fat raw milk was used for all the cheeses. No starters were used. Milk was stored for 12 to 18 hours in refrigerated tanks before cheese-making. Cheeses had a mean weight of 2.2 kg; about 20 and 22 litres of Podolica and Friesian milk, respectively, were used to produce the experimental cheeses. The cheeses were ripened at 12±1°C ambient temperature and 80±2% relative humidity in controlled rooms.

Milk sampling and analysis

Forty-eight bulk milk samples (each in triplicate) were collected from the four herds over a four-month period (April to July of 1999) at monthly intervals (three samples per month for each breed). The milk samples were taken from the collecting tanks present on the farms on three consecutive days during four sampling sessions. Milk samples were collected in 200 mL sterile plastic containers, which were taken immediately to the laboratory in a refrigerated tank at 4°C. The following measurements were made on the milk: pH, total protein, fat and lactose contents using an infra-red spectrophotometer (Milko Scan 133B; Foss Electric, DK-3400 Hillerød, Denmark) according to the International Dairy Federation (IDF, 1990) standard; somatic cell count (SCC) using a Fossomatic 90 cell counter (Foss Electric, Denmark) (IDF, 1995).

Cheese sampling and analysis

The bulk milk from which the milk samples were taken for analysis was used to produce the Podolica and Friesian cheeses. The cheeses were sampled after 140±5 days of ripening. For each cheese, three different sampling depths (surface, middle and core) were tested in order to study the influence of this parameter on cheese composition. The surface sample was obtained by cutting a 3 mm-deep slice after removing a thin (<1 mm) superficial layer. Each chemical analysis was done in triplicate and the mean value for each parameter was used for data processing.

Moisture, NaCl and pH were determined as described by the International Dairy Federation (IDF, 1970; 1988; 1989). Total protein content, pH 4.6 water soluble N and fat content were determined using the micro-Kjeldhal method and the Soxhlet method, respectively, as previously described (ALBENZIO *et al.,* 2001).

Total fatty acid (TFA) determination

Fat recovery from cheese was accomplished by microwave extraction using a CEM model MDS 2100 microwave extractor (Italian CEM Corporation, Cologno al Serio – Bergamo, Italy). One gram of ground cheese was put into the extraction vessel, together with 50 mL of diethyl ether and subjected to microwave extraction. The operating extraction parameters were: temperature, 103°C; duration of "temperature at constant pressure (80 psi)": 30 min; duration of the extraction procedure: 35 min (AVILA *et al.,* 1996).

The ether extract was filtered and the solvent was removed from the fat by distillation under vacuum using a VV2000 Rotary Evaporator (Heidolph, Schwabach, Germany). The derivatization of the fatty acids was accomplished by methylation using a methanol / sulfuric acid / zinc chloride (100 : 10 : 1 w / w / w) mixture for 9 h at 90°C. The total fatty acid composition as FAMEs (fatty acid methyl esters) was determined using a Fisons model GC 8160 gas chromatograph (Italian Fisons, Milan, Italy) equipped with a split injector. A RTX 2330 fused silica column (60 m x 0.25 mm i.d., 0.20 µm film thickness; Restek Corporation, Bellafonte, PA, USA) and a flame ionization detector (FID) connected to Chromcard Thermoquest acquisition software were used.

The carrier gas was helium at a linear gas velocity of 35 cm / sec. Operating temperatures: injector 250°C, detector 280°C. Sample injection volume: 2 µL. Oven temperature program: 60°C for 1 min, raised to 250°C at increments of 8°C / min and held for 10 min. A standard mixture of fatty acid methyl esters was used for the calibration in order to obtain the correction factors.

Free fatty acid (FFA) determination

Free fatty acids were extracted according to the DE JONG and BADINGS (1990) procedure. The purification of FFA was performed by Solid Phase Exctraction using Bond Elut aminopropyl columns. The neutral lipids were eluted from the column with a 4 mL chloroform / propanol mixture (2:1 v / v) and then eluted with 4 mL of diethyl ether containing 2% formic acid.

Analyses were performed using a Fisons model GC 8160 gas chromatograph (Fisons Italia, Milan) equipped with a column injector. Secondary cooling was used for 1 min. Stabilwax-DA fused silica column (30 m x 0.53 mm i.d, 0.5 µm film thickness; Phenomenex – Torrance CA, USA) and a flame ionization detector (FID) connected to a Fisons DP 700 integrator were used.

The carrier gas was helium at a linear gas velocity of 25 cm / sec. Operating temperature of the detector: 280°C. Sample injection: 0.5 µL. Oven temperature

program: 65°C for 1 min, raised to 240°C at increments of 10°C / min and held for 35 min.

All solvents were analytical grade and were obtained from J.T. Baker (Phillipsburg, NJ, USA). Bond Elut aminopropyl columns (500 mg, 2.8 mL) were obtained from Lab Service Analytica (Bologna, Italy). FFA pure standards for chromatographic analysis were from Larodan Fine Chemicals (Malmöe, Sweden).

Total amino acid (TAA) determination

Total and individual free amino acids were analysed on freeze-dried water-soluble extracts of the cheeses by means of RP-HPLC analysis. A standard amino acid mixture (Beckman, High Wycombe, UK) was used to calibrate the column and norleucine (Sigma Aldrich-Italia, Milan) was added to all samples before being injected as an internal standard. Each freeze-dried sample (2.5-5.0 mg) was dissolved in sample buffer (0.2 M sodium citrate, pH 2.2), filtered through Whatman 0.22 m filters and 50 L of filtrate loaded onto the column. Amino acid determination was preceded by acid or base hydrolysis and a precolumn derivatization with FMOC-Cl.

Statistical analysis

Milk data and cheese protein and fat contents were subjected to analysis of variance, using the GLM procedure of the SAS (1990) statistical software; the model used for all variables was Y = breed + replication (breed) + time of sampling + breed x time of sampling + error, where Y was the milk and cheese data of each breed. The statistical significance of the variations due to cow breed, time of sampling, cheese sample depth and their interactions on the amino acid and total and fatty acid contents in cheeses, were also tested using the GLM model, having replication within cheeses as the error term. For all parameters, model effects were declared significant at $P<0.05$, unless otherwise stated. Since the effects of the interactions and of the categorical factors, other than milk source, were significant in very few cases for amino acids and fatty acids, only the effects on these parameters due to the different cow breed are reported in the tables and discussed. Results are presented as the least squares means of milk and cheese in each breed and variability of the data is expressed as the SE of the mean response over the whole experimental period.

TAA, TFA and FFA data analysis were submitted to principal component analysis (PCA) (MASSART *et al.,* 1988) in order to investigate more thoroughly the possible relationships between milk source and fatty acid and amino acid profiles in the cheeses. The data set included 48 objects (cheese samples) and 16 variables for total fatty acids, 15 variables for free fatty acids and 18 variables for amino acids. The objects were classified according to cow breed, sampling time and sample depth.

RESULTS AND DISCUSSION

Milk quality

The breed significantly affected the composition of the milk (Table 1). Podolica cows had higher protein and fat contents in their milk ($P<0.01$) than Friesian cows. Significant effects of time of sampling and of breed x time of sampling ($P<0.01$) were also found. Differences between breeds throughout the trial period were probably the results of genetic effects (FORMIGONI and PIVA, 1996) and of the smaller and markedly uneven volume of milk yielded by the Podolica cows (AULDIST *et al.,* 1996). This suggests that the scarcity of forage and the adverse effects of climatic extremes on both pasture quality and the metabolic and endocrine status of cows may

Table 1 - Protein, fat and lactose contents and somatic cell counts (SCC) in the milk, and protein and fat contents in cheeses from Podolica and Italian Friesian cows. Values are least squares means ± SE.

Item	Cow breed									SE	Effects, P		
	Podolica				Friesian								
	April	May	June	July	Mean	April	May	June	July	Mean		Cow breed	Time of sampling	Cow breed x Time of sampling
Milk														
Protein content, %	3.56	3.73	3.86	3.57	3.68	3.20	3.15	3.11	3.14	3.15	0.003	<0.01	<0.01	<0.01
Fat content, %	3.85	3.78	4.24	4.41	4.07	3.14	3.18	3.53	3.39	3.31	0.014	<0.01	<0.01	<0.01
Fat to protein ratio	1.08	1.01	1.10	1.23	1.11	0.98	1.01	1.13	1.08	1.05	0.03	NS	<0.001	NS
Lactose content, %	5.13	5.19	5.10	5.02	5.11	5.12	5.03	5.09	5.08	5.08	0.039	NS	NS	NS
Somatic cell count (log_{10} of somatic cells/mL)	5.51	5.44	5.39	5.86	5.55	5.20	5.41	5.25	5.18	5.26	0.05	<0.001	<0.01	NS
Cheese														
Protein content, % of total solids	44.57	42.71	43.92	42.32	43.38	42.44	43.74	43.33	42.65	43.04	0.256	NS	<0.05	<0.005
pH 4.6 Soluble N/Total N, %	22.11	18.81	18.89	18.55	19.59	13.75	13.35	11.26	12.64	12.75	1.35	<0.001	<0.001	<0.01
Fat content, % of total solids	41.67	43.38	43.81	45.06	43.38	42.16	41.37	40.26	42.57	41.59	0.178	<0.001	<0.01	<0.01

strongly hamper the production of Podolica milk for cheese manufacturing (KAUFMANN and HAGEMEISTER, 1987; KEFFORD *et al.*, 1995; OWEN, 1987). It is also known that the stage of lactation affects the milk fat content, because the concentrations of the milk constituents increase as the lactation period progresses and the milk yield gradually decreases. However, given that the Friesian cows were more advanced in their lactation period than the Podolica cows, the effect of lactation stage should be excluded in explaining the higher milk fat content in the Podolica than in the Friesian milk.

In contrast, the milk lactose content was similar in both breeds and remained substantially constant throughout the experiment. This could be expected given that, regardless of production levels, the lactose concentration in milk tended to remain unchanged, since lactose is the main osmotically active component in milk (SEVI *et al.*, 2000).

Both the small volume of milk yielded and possibly the poorly controlled sanitation conditions of the animals and of milking procedures may account for the higher somatic cell count ($P<0.001$) in Podolica milk. Somatic cell count in Podolica milk exceeded 400,000 cells/mL, which is the maximum accepted level in the European Union (EU directive 46/92) for raw milk destined for cheese production.

Cheese quality

Cheeses from farms rearing Podolica cows had a significantly higher fat content ($P<0.001$) than Friesian cheeses. The protein content did not change with cow breed, but the pH 4.6 soluble nitrogen was significantly higher in the Podolica than in the Friesian cheeses (Table 1). Differences in cheese fat content may be ascribed in part to the higher fat content and fat to protein ratio in the Podolica milk, and in part to changes in the recovery of fat in cheese as a conse-

quence of different stretching methods (manual for Podolica cheese and mechanical for Friesian cheese). In addition, a higher casein to protein ratio cannot be excluded in explaining the higher fat content in the Podolica cheese. Indeed, casein is primarily incorporated into the curd during cheese-making and fat is entrapped within the network created by the casein micelles (DALGLEISH, 1993).

The higher content of soluble nitrogen found in the Podolica cheese suggests a higher level of proteolysis during ripening, which may be ascribed to a more intense activity of microbial and endogenous enzymes (CORSETTI *et al.,* 2001), that is probably a consequence of breed and process differences. In particular, manual stretching and wooden tools were used for Podolica cheeses, while mechanical stretching and stainless steel tools were used for Friesian cheeses. DI MATTEO *et al.* (1982) and FOX *et al.* (1996) have reported that kneading and stretching methods may affect the rennet and the plasmin activity, thus contributing to proteolysis in cheeses. Also the higher milk SCC was probably responsible of the enhanced proteolysis in Podolica cheese via the increase in plasmin activity (AULDIST *et al.,* 1996).

Significant effects of time of sampling and of cow breed x time of sampling were found for both parameters, which may be ascribed to the fact that Podolica cheese quality changed during the study period.

No differences were found in cheese pH and dry matter and NaCl contents of the cheese. The mean values of pH, moisture, and of NaCl content were 5.33, 32.68%, and 5.6% in the Friesian cheeses and 5.21, 29.89%, and 7.1% in the Podolica cheeses, respectively.

The results of the total amino acid composition analysis of the cheese samples are shown in Table 2. A higher total amino acid content was found in Podolica cheeses ($P<0.05$), which would be expected given that there was more intense proteolysis in Caciocavallo cheeses from the Podolica cows. In addition, higher concentrations of threonine and valine ($P<0.05$) and of alanine, aspartic acid, methionine, proline, serine and tyrosine ($P<0.01$) were observed in Podolica cheeses, whereas glycine and lysine ($P<0.05$), and phenylalanine, isoleucine and leucine were more abundant ($P<0.01$) in Friesian cheeses. Such differences may be due to differences between the proteolytic activity induced by native microflora and in the processing of the Podolica and Friesian cheeses. However, differences in the amino acid composition of the raw milk cannot be excluded. In particular, the increased alanine content in the Podolica cheeses, together with a reduction in leucine and isoleucine content, may be ascribed to a greater uptake of amino acids by the muscle tissue (HARPER, 1983). A greater availability of essential amino acids may account for the higher proline content found in Podolica cheeses, given that this amino acid may be synthesized in the mammary tissue using excess essential amino acids as precursors (ANNISON, 1983).

From a nutritional point of view, a notable aspect of Podolica cheeses is represented by their high concentrations of valine, methionine and threonine. There is evidence that these amino acids are involved in the synthesis of leukocytes and immunoglobulins (VOET and VOET, 1990; DEFA *et al.,* 1999).

It is of interest that valine, threonine, proline and alanine, the main sources of sweet and bitter tastes in "pasta filata" cheeses (RESMINI *et al.,* 1988), were more abundant in the Podolica caciocavallo cheeses.

The results of ANOVA obtained using the GLM procedure on the data sets relevant to TFA and FFA analysis in the Podolica and Friesian cheeses are reported in Tables 3 and 4, respectively. Cow breed effect was highly significant ($P<0.01$) on the content of all the fatty

Table 2 - Amino acid content (mg/g and % of total amino acid content) in Caciocavallo cheeses manufactured from Podolica and Italian Friesian cow milk. Values are least squares means of the averages of the three different sampling depths ± SE.

Item	Cow breed		SE	Effects, P
	Podolica	Friesian		Cow breed
Alanine	0.152	0.133	0.002	<0.01
	4.7%	*4.2%*		
Arginine	0.119	0.114	0.003	NS
	3.7%	*3.6%*		
Aspartic acid	0.165	0.138	0.003	<0.01
	5.1%	*4.4%*		
Cystine	0.246	0.243	0.007	NS
	7.6%	*7.7%*		
Glycine	0.077	0.083	0.001	<0.05
	2.4%	*2,6%*		
Glutamic acid	0.449	0.426	0.007	NS
	13.9%	*13.4%*		
Histidine	0.286	0.261	0.009	NS
	8.9%	*8.2%*		
Isoleucine	0.108	0.147	0.004	<0.01
	3.3%	*4.6%*		
Leucine	0.237	0.301	0.007	<0.01
	7.3%	*9.5%*		
Lysine	0.206	0.252	0.01	<0.05
	6.4%	*8.0%*		
Methionine	0.086	0.064	0.017	<0.01
	2.7%	*2.0%*		
Phenylalanine	0.126	0.150	0.004	<0.01
	3.9%	*4.7%*		
Proline	0.328	0.293	0.004	<0.01
	10.2%	*9.2%*		
Serine	0.112	0.096	0.003	<0.01
	3.5%	*3.0%*		
Threonine	0.093	0.082	0.003	<0.05
	2.9%	*2.6%*		
Tryptophan	0.095	0.093	0.001	NS
	2.9%	*2.9%*		
Tyrosine	0.162	0.140	0.005	<0.01
	5.0%	*4.4%*		
Valine	0.181	0.152	0.008	<0.05
	5.6%	*4.8%*		
Total amino acid content	3.228	3.168		

acids of the TFA profile, as well as on the levels of the acetic acid and the free fatty acids from $C_{14:0}$ to $C_{18:3}$, while this effect was not significant for the short-chain saturated free fatty acids from $C_{3:0}$ to $C_{12:0}$.

Compared to the Friesian cheeses, Podolica cheeses (Table 3) had lower contents of palmitic, oleic and linoleic acids (-11, -7 and -12%, respectively); 7 to 21% higher contents of $C_{4:0}$ to $C_{14:0}$ fatty acids and 21 to 290% higher levels of stearic

Table 3 - Total fatty acid composition (g/kg methyl esters and % of total fatty acids) in Caciocavallo cheeses manufactured from Podolica and Italian Friesian cow milk. Values are least squares means of the averages of the three different sampling depths ± SE.

Item	Cow breed		SE	Effects, P
	Podolica	Friesian		Cow breed
4:0	27.3	24.7	0.8	<0.01
	2.7%	*2.5%*		
6:0	20.8	17,2	0.4	<0.01
	2.1%	*1.7%*		
8:0	13.6	11.2	0.2	<0.01
	1.4%	*1.1%*		
10:0	30.2	27.1	0.5	<0.01
	3.0%	*2.7%*		
12:0	34.4	31.9	0.5	<0.01
	3.5%	*3.2%%*		
14:0	117	104	1.3	<0.01
	11.8%	*10.4%*		
14:1	15.6	14.6	0.2	<0.01
	1.6%	*1.5%*		
15:0	13.4	12.6	0.2	<0.01
	1.3%	*1.3%*		
16:0	290	327	2.9	<0.01
	29.1%	*32.8%*		
16:1	20.4	24.4	0.4	<0.01
	2.0%	*2.4%*		
17:0	9.2	7.4	0.2	<0.01
	0.9%	*0.7%*		
18:0	116	95.7	1.8	<0.01
	11.7%	*9.6%*		
18:1	249	267	2.4	<0.01
	25.0%	*26.8%*		
18:2	24.3	27.6	0.5	<0.01
	2.4%	*2.8%*		
18:3	11.9	4.1	0.6	<0.01
	1.2%	*0.4%*		
20:0	2.2	1.5	0.1	<0.01
	0.2%	*0.2%*		
Total fatty acid content	995.3	998		

and linolenic acids. As for the TFA analyses, the total amount of free fatty acids (Table 4) was 71% higher in the cheese manufactured from Podolica milk. In particular, acetic acid and all the free fatty acids from $C_{14:0}$ to $C_{18:3}$ were more abundant in Podolica cheeses, except for linoleic acid, which was higher in Friesian cheeses.

Podolica cheeses had a higher proportion of total short-chain and medium-chain fatty acids and fewer long-chain

Table 4 - Free organic and fatty acids (mg/kg) and % of total free fatty acids in Caciocavallo cheeses manufactured from Podolica and Italian Friesian cow milk. Values are least squares means of the averages of the three different sampling depths ± SE.

Item	Cow breed		SE	Effects, P
	Podolica	Friesian		Cow breed
2:0	154	55.3	10.3	<0.01
	10.3%	*6.7%*		
3:0	9.4	6.4	1.6	NS
	0.6%	*0.8%*		
4:0	139	116	8	NS
	9.3%	*14.0%*		
6:0	48.6	58.1	2.3	NS
	3.2%	*7.0%*		
8:0	18.2	17.5	1.5	NS
	1.2%	*2.1%*		
10:0	46.7	49.7	3	NS
	3.1%	*6.0%*		
12:0	37.5	36.8	3.2	NS
	2.5%	*4.4%*		
14:0	56.4	40.3	1.7	<0.01
	3.8%	*4.9%*		
14:1	33.2	27.6	1.4	<0.01
	2.2%	*3.3%*		
16:0	536	210	26.1	<0.01
	35.7%	*25.3%*		
16:1	34.3	24.7	1	<0.01
	2.3%	*3.0%*		
18:0	5.4	2.0	0.3	<0.01
	0.4%	*0.2%*		
18:1	316	126	15.9	<0.01
	21.1%	*15.2%*		
18:2	42.9	54.4	2.5	<0.01
	2.9%	*6.5%*		
18:3	22.5	6.1	2.8	<0.01
	1.5%	*0.7%*		
Total free fatty acid content	1500.08	830.9		

fatty acids. The fact is probably related to the higher intake of fiber-rich forage by Podolica cows. In fact, acetic and butyric acids originating from ruminal fermentation are the major carbon sources for *de novo* short-chain fatty acid synthesis in the mammary gland (GRUMMER, 1991). It is known that the amount of volatile fatty acids increases and, in particular, the flow of acetic and butyric acids from the rumen increases with the dietary forage to concentrate ratio at the expense of propionic acid, which leads to an increase of long-chain body fatty acids and, consequently, to a reduction of *de-novo* milk fat synthesis in the mammary gland (KAUFMANN and HAGEMEISTER, 1987).

From a nutritional point of view, there was a lower percentage of total palmitic

and higher percentage of total linolenic acid in Podolica compared to Friesian cheeses. This is of interest in light of evidence suggesting that the consumption of ω-3 fatty acids may reduce the risk of atherosclerosis and, conversely, that palmitic acid has a hypercholesterolemic effect on human plasma (GRUMMER, 1991).

The higher amount of FFA found in Podolica cheeses may be attributed to the autochthonous microflora, and primarily to lactic acid bacteria and moulds, which are regarded as the main sources of lipolytic enzymes in ripening cheeses (GOBBETTI *et al.*, 1999; CORBO *et al.* 2001). It is likely that hand milking, pasture feeding, and manual stretching increased the variety and enhanced the growth of the microbial species in the cheeses.

Higher concentrations of FFA, especially of short-chain fatty acids, contribute to giving the typical "piccante" flavor to long-ripened "pasta filata" cheeses (FOX and GUINEE, 1987; BATTISTOTTI and CORRADINI, 1993; CORSETTI *et al.*, 2001). Furthermore, amino acids may play a main role in giving a cheese its typical taste (FOX and GUINEE, 1987; BATTISTOTTI and CORRADINI, 1993).

In order to get a general perspective of the effects of cow breed, time of sampling and sample depth on TAA, TFA and FFA compositions of the examined cheeses, the data sets were subjected to PCA. Figures 1, 2 and 3 report the results for the first two principal components, which account for 53.0, 61.3 and 55.2 of total variance, respectively.

Figures 1-3 show the bicentrate plots of the scores of the cheese samples and of the loads of the measured amino acids, total fatty acids and free fatty acids,

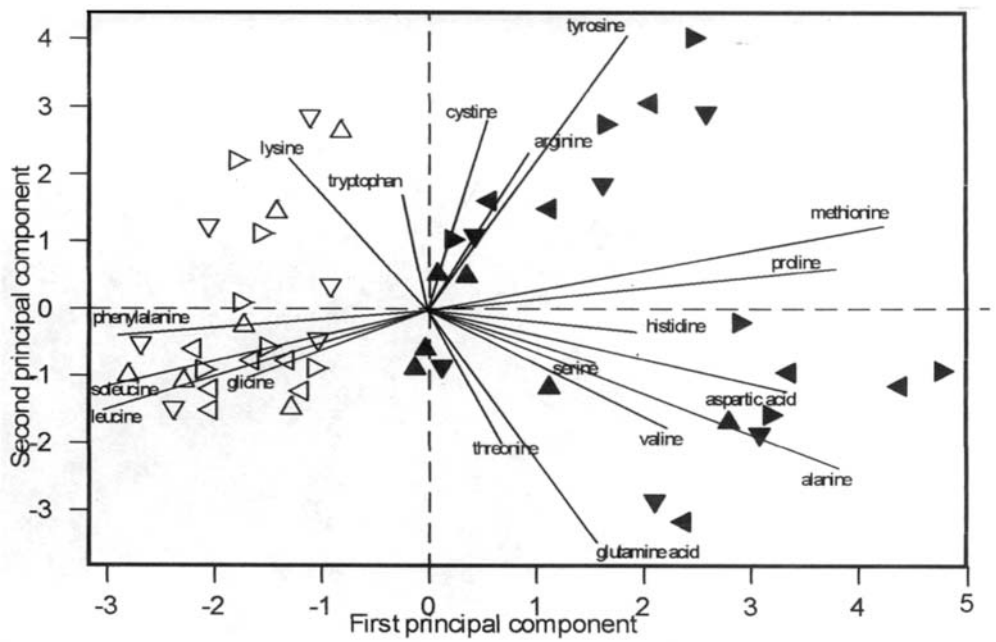


Fig. 1 - Principal component analysis of the amino acid composition of 48 cheese samples: 24 each (2 farms x 4 sampling sessions x 3 cheese sampling depths) from Podolica (▲,▶,▼,◀) and Italian Friesian (△,▷,▽,◁) cows, respectively. The first two principal components display 53.0% of total variance.

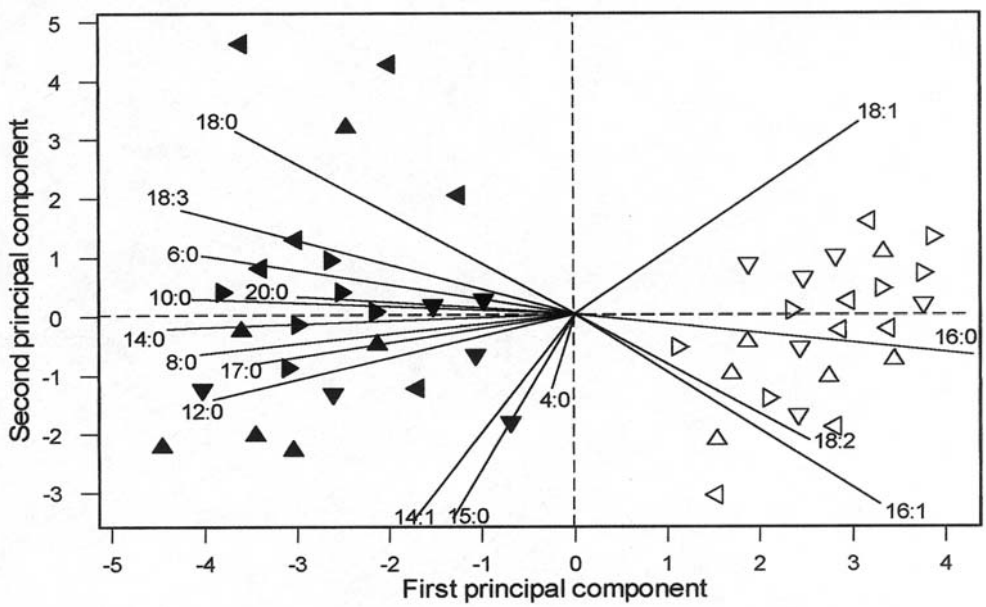


Fig. 2 - Principal component analysis of the total fatty acid composition of 48 cheese samples: 24 each (2 farms x 4 sampling sessions x 3 cheese sampling depths) from Podolica (▲,▶,▼,◀) and Italian Friesian (△,▷,▽,◁) cows, respectively. The first two principal components display 61.3% of total variance.

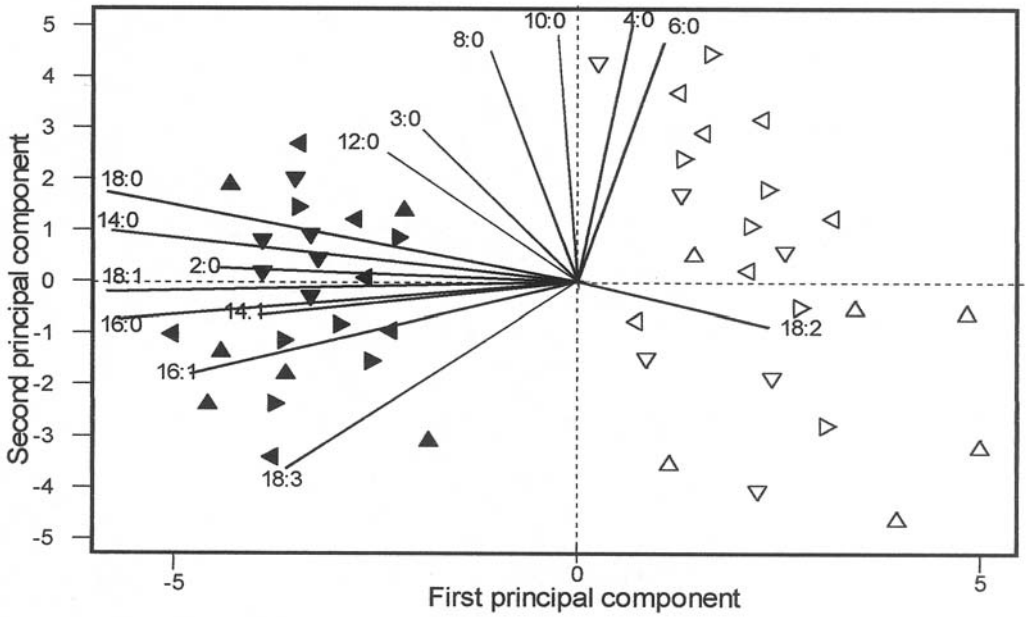


Fig. 3 - Principal component analysis of the free organic and fatty acid composition of 48 cheese samples: 24 each (2 farms x 4 sampling sessions x 3 cheese sampling depths) from Podolica (▲,▶,▼,◀) and Italian Friesian (△,▷,▽,◁) cows, respectively. The first two principal components display 55.2% of total variance.

respectively. These representations of the samples showed two well-separated groups, each comprised of the cheese samples obtained from Podolica and Friesian cow milk, respectively. This separation means that cow breed affected the examined chemical characteristics of the cheeses to such an extent that it was possible to discriminate between cheeses from Podolica cows and those from Friesian cows.

The plot of the loadings of the measured variables onto the first two principal components, emphasized the role of each variable in separating the groups.

For the amino acid analysis (Fig. 1), the first principal component (describing 40.5% of total variance) is represented principally by phenylalanine, isoleucine, leucine and glycine, the amino acids which were present in higher amounts in Fresian cheese, and alanine, methionine, proline, and aspartic acid, which characterized the Podolica cheese. The second principal component is composed predominantly of tyrosine and cystine, and by glutamic acid and threonine. These amino acids do not actually separate the groups, but they spread out the cheese samples on the second principal component, especially the Podolica cheese samples.

For the TFA analysis (Fig. 2), the fatty acids with higher weights on the first component (describing 48.6 % of the total variance) were $C_{16:0}$, $C_{16:1}$, $C_{18:1}$ and $C_{18:2}$, which strongly characterized the Friesian group, while all the other saturated fatty acids from $C_{6:0}$ to $C_{20:0}$ plus $C_{18:3}$ typified the Podolica cheese group.

The score plot of the FFA (Fig. 3) shows that, with respect to Friesian cheese samples, Podolica cheese samples were characterised by more long-chain fatty acids, $C_{14:0}$, $C_{16:0}$, $C_{18:0}$ and $C_{18:1}$, and fewer $C_{18:2}$. The short-chain fatty acids $C_{4:0}$, $C_{6:0}$, $C_{8:0}$ and $C_{10:0}$ were not influenced by the cow breed.

The score plots in Figures 1-3 do not show any separation of the cheese samples related to the different time of sampling or cheese depth sampling, confirming the assumption that only the breed and/or the cattle production system influenced the amino acid and fatty acid compositions of the examined cheeses.

In conclusion, cow breed and/or the differences in herd management and feeding system strongly affected milk and Caciocavallo cheese composition. Indeed, the amino acid and fatty acid profiles, which play a main role in giving a cheese its nutritional properties and typical taste, changed with milk source so that a sharp characterization of Podolica and Friesian Caciocavallo cheeses was possible when amino acid and fatty acid data were subjected to principal component analysis. The effect of milk origin was not investigated in the present trial. However, it should be considered more carefully in the legal definition of Caciocavallo cheese because herbage quality, which largely depends on the climatic and soil characteristics of different production areas, may greatly affect the cheese quality, especially when manufactured from the milk of pasture-grazing animals.

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Revised paper received March 21, 2003 Accepted June 20, 2003

PAPER

STABILITY AND RECOVERY OF MAIZE DNA DURING FOOD PROCESSING

STABILITÀ E RESA DEL DNA DI MAIS DURANTE
IL PROCESSAMENTO TECNOLOGICO DI ALIMENTI

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ABSTRACT

The identification and quantification of food ingredients based on DNA detection depends strictly on DNA stability during the food preparation treatment and on the efficiency of DNA recovery. Investigations into DNA stability carried out on polenta by real time PCR revealed that after 65 min of processing the amplifiable DNA decreased to 40% of the amount detectable before processing. Four commercial kits for DNA extraction were compared with the hexadecyltrimethyl ammoni-

RIASSUNTO

L'identificazione e la quantificazione di ingredienti alimentari effettuata mediante analisi del DNA dipende in particolar modo dalla stabilità del DNA durante i trattamenti per la preparazione dell'alimento e dall'efficienza della resa del DNA estratto. Indagini sulla stabilità del DNA condotte in polenta mediante real time PCR rivelavano che dopo 65 min di cottura il DNA amplificabile diminuiva fino al 40% della quantità presente prima della cottura. Quattro kit commerciali di estrazione di DNA

- Key words: DNA extraction methods,
DNA stability, maize foodstuffs, processing treatment, real time PCR -

umbromide (CTAB)-based method for DNA extraction from different maize foodstuffs: flour, canned maize, corn chip snacks, cheese corn puff snacks, chocolate corn flakes and an infant formula having a maize flour ingredient. The methods were evaluated for recovered DNA quality and yield by agarose gel electrophoresis, PCR and real time PCR targeting of the maize zein gene. No detectable DNA could be extracted from the chocolate corn flakes, whereas highly degraded DNA was extracted from all the other materials tested except flour which yielded high quality DNA. Among the methods tested, CTAB, the Wizard method and Wizard Magnetic DNA Purification System kits gave the highest DNA yields. The overall data indicate that quantitative detection of ingredients in processed food products may not reflect the original amount of DNA due to DNA degradation and low yield of DNA recovery.

sono stati comparati con il metodo CTAB, basato sull'utilizzo dell'esadeciltrimetilammoniobromuro, su differenti alimenti derivati da mais: farina, mais in scatola, snack di mais, snack soffiati al formaggio, corn flakes al cioccolato e un omogeneizzato per bambini contenente mais come ingrediente. I metodi sono stati valutati per la qualità e la resa del DNA estratto mediante elettroforesi in gel di agarosio, PCR e real time PCR. La PCR aveva come bersaglio il gene della zeina. Da corn flakes al cioccolato non era estratto DNA, mentre DNA altamente degradato era estratto da tutti gli altri alimenti saggiati. Tra i metodi saggiati, CTAB, Wizard e il kit Wizard Magnetic DNA Purification System davano le rese più elevate di DNA. I dati nel loro insieme indicano che l'analisi quantitativa di ingredienti in prodotti alimentari cotti può non riflettere l'originale quantità presente, ciò dovuto alla degradazione del DNA e alla bassa resa.

INTRODUCTION

The labelling of genetically modified foodstuffs is mandatory in the European Community according to the Regulation 49/2000/EEC, setting a 1% (w/w) threshold as the maximum limit for the accidental presence of genetically modified organism (GMO) material in each ingredient. The methodology used to fulfill this labelling regulation is based largely on the use of the polymerase chain reaction (PCR) technique. In recent years many analytical methods based on PCR have been developed to detect qualitatively and/or quantitatively the presence of a modified sequence of nucleic acid in food matrices (ANKLAM *et al.*, 2002).

Prerequisites for PCR feasibility are the quantity, quality and purity of the isolated DNA. These depend on various factors like the type of foodstuff and, as most food products are processed before reaching the market, the degree of treatment the sample has undergone during such processing. Finally, another key point is the DNA extraction method which influences the final yield of the target DNA for the PCR.

During processing, physical and chemical processes like heat, pressure and pH changes can lead to DNA degradation (fragmentation) and damage (hydrolysis, depurination and enzymatic degradation). During processing, the degree of DNA degradation in a given ingredient affects the amount of residual DNA available for quantitation by PCR in the final food product, influencing the estimation of the amount of the original ingredient. This can pose prob-

lems for correct quantitation of an ingredient in foodstuffs for which ingredient limits are foreseen, as, for example, in products labelled with the GMO-free label.

Another critical point for ingredient identification by PCR analysis is DNA recovery, for which suitable methods must be used. Several studies dealing with DNA isolation methods from raw and processed materials have been published [ZIMMERMAN *et al.*, 1998; MEYER and JACCAUD, 1997; HURST *et al.*, 1999; PAULI *et al.*, 2000; TENGEL *et al.* 2000; 2001; LIPP *et al.*, 2001; HAHNEN *et al.*, 2002], but only a few studies have dealt with the direct comparison of existing DNA extraction procedures (ZIMMERMAN *et al.*, 1998; TENGEL *et al.* 2000). Further information is needed for a better insight into the best extraction methods, in terms of DNA yield and quality, for use in the analysis of different food matrices. Currently, methods using nucleic acid binding resins and the hexadecyltrimethylammoniumbromide (CTAB)-based method are preferred for GMO detection. In fact, as reported by ZIMMERMANN *et al.* (1998), such methods, even though they often give very poor yields with respect to those obtained with simpler and faster methods (e.g. chelex and rose-based methods), isolate high quality DNA suitable for PCR amplification.

The aim of this study was to evaluate how food processing and DNA extraction methods could affect, respectively, the stability and final recovery of maize DNA from processed food products. The influence of thermal treatment on maize DNA stability was investigated using real time PCR to determine the amount of the residual amplifiable target DNA during polenta processing. Five DNA extraction methods, namely the CTAB-method, and four commercially available kits were tested and compared with respect to the quality and yield of DNA isolated from different foodstuffs derived from maize.

MATERIALS AND METHODS

Food samples

The samples used, maize flour, canned maize, corn chip snacks, cheese corn puff snacks, chocolate corn flakes and infant formula, represent different degrees of processing and contain different amounts of maize; they were purchased at a local supermarket. The polenta (corn meal mush) was prepared as already described RIZZI *et al.*, 2001.

Isolation and characterisation of genomic DNA

The foodstuffs were ground to a fine powder with a mortar and pestle and aliquots were stored at –20°C until DNA extraction. For the isolation of the DNA the CTAB-based method (LIPP *et al.*, 1999) and four commercially available kits were tested: the Wizard (based on the Wizard DNA Purification Resin, WP, LIPP *et al.*, 1999), Wizard Magnetic Purification System (WM, Promega, Milan, Italy), GenElute Plant Genomic kit (GE, Sigma, Milan, Italy) and QIAamp DNA Stool Mini kit (QI, Quiagen, Milan, Italy). The procedures were performed according to the manufacturer's guidelines. The starting material was 200 mg for WP, CTAB and QI methods, 1 g for WM and 100 mg for GE. The DNA was eluted or resuspended in different volumes of final buffer, following the manufacturer's guidelines: WP and CTAB, 50 µL; WM and GE, 100 µL; QI, 200 µL. Five and ten µL of DNA solutions were used for PCR and real-time PCR experiments respectively. For each extraction method the DNA in the solution used for the PCR experiments (5 µL) corresponded to the following amounts of original sample: WP and CTAB, 20 mg; GE and QI, 5 mg; WM, 50 mg.

The polenta and maize flour samples were extracted with the Wizard method; starting material was 60 mg for maize

flour and 300 mg for polenta (polenta was prepared with a flour:water ratio of 1:4.16).

The quality and efficiency of the DNA extraction was evaluated by agarose gel electrophoresis (1.5% agarose) using lambda DNA digested with *Hind*III/*Eco*RI (Boehringer Mannheim, Milan, Italy) as the molecular weight marker. The gels of polenta were photographed under UV light by the Gel Doc 2000 image system (Biorad, Milan, Italy) and band intensities were determined by comparison with lambda marker (Sigma, Milan, Italy) using image processing software (Diversity Database). Bands were quantified using the linear regression equation, determined by the software on the basis of the marker bands in the gel: Conc = - a Vol + b, where Conc is the concentration based on the standard marked in the image and Vol, Volume, is the sum of the intensities of the pixels within the volume band boundary pixel area. The concentration of DNA in solution was determined by UV absorption at 260 nm on a SmartSpec™ 3000 spectrophotometer (Biorad, Milan, Italy) with microvolume cuvettes; its purity was checked on the basis of 260/280 nm absorption ratio.

Heat-treated DNA solutions were obtained by heating aliquots of a maize flour DNA solution (40 ng/µL) at 99.9°C in a Gene Amp 2400 thermocycler (Applied Biosystems, Milan, Italy) for 1, 10, 20, 35, 50, 65 min.

DNA target sequences and primer oligonucleotides

Primer sequences used in the detection of the maize specific zein gene were taken from STUDER *et al.* (1997). Primer pair Zein 1/2 amplifies a 485 bp amplification product and the nested primer sets Zein 3/4 generates a 277 bp fragment of the zein gene.

For the real time PCR the PCR system amplifying a 72 bp fragment of the endogenous *10-kDa zein* gene was used. The Taqman probe (5'-FAM-catcactggcatcgtctgaagcgg-TAMRA-3') was labelled with the fluorescent reporter dye 6-carboxy fluoresceine (FAM) on the 5' end and with the quencher dye 6-carboxytetramethylrhodamine (TAMRA) on the 3' end. The primers used were the following: Zea F (5'-tcatgttaggcgtcatcatctgt-3'; GenBank Accession X07535, position 203-222) and Zea R (5'-tgcagcaactgttggcctta-3'; GenBank Accession X07535X, position 252-274X). All the primers and the probe were synthesised by MWG Biotech (Ebersberg, Germany).

Polymerase chain reaction

Nested amplifications of the maize specific zein sequence were carried out in a final volume of 50 µL in 0.2 mL tubes on a GeneAmp 2400 thermocycler. End concentrations of PCR components were as follows: 1 PCR buffer (Amersham Pharmacia Biotech, Milan, Italy), 2.5 mM $MgCl_2$, 200 M of each dNTP (Amersham Pharmacia Biotech), 0.5 µM of each primer (Amersham Pharmacia Biotech), 1 unit of Taq DNA polymerase (Amersham Pharmacia Biotech). PCR were performed using 5 µL of the isolated DNA. The thermal conditions for the reactions consisted in an initial denaturation at 95°C for 5 min, followed by 25 cycles of amplification performed for the first PCR (Zein 1/2) and 30 cycles with the nested primer set (Zein 3/4). Two microliters from the first reaction were used as the template for the second PCR. Cycles of amplification were performed with denaturation at 95°C for 40 s, primer annealing at 60°C for 40 s and primer extension at 72°C for 3 min.

PCR products containing 0.2 volume of loading buffer (40% saccharose, 0.05% bromophenol blue, 0.1 mM EDTA, pH 8) were separated on a 2% agarose gel and electrophoresed in 0.5 TBE buffer (45 mM Tris-borate, 1 mM EDTA) at 7 V/cm for 1h. After staining with ethidium bro-

mide (1 g/mL), the gel images were recorded under UV light by the Gel Doc 2000 image system. As size reference a 100 bp ladder (Amersham Pharmacia Biotech) was used.

Real time polymerase chain reaction

Calibration curves were generated by diluting stock DNA from 100% transgenic maize, measured by U.V. absorption, to the following concentrations: 30, 10, 3.3, 1.1 and 0.37 ng/μL. Ten microliters were included in the amplification reaction, giving ranges from 300 to 3.7 ng of endogenous and transgenic DNA. Real time PCR assays were performed with the i-Cycler (Biorad, Milan, Italy) in a 96-well plate. Each assay contained, in a total volume of 50 μL, 3 μM of the sense and anti-sense primer and 2 μM of the probe. The "Platinum Quantitative PCR Supermix-UDG" (Life Technologies, Milan, Italy) was used for preparation of the PCR assay. Ten microliters of the DNA extracted from foodstuffs were added to the master mix. For the study of DNA stability, 10 μL of a 1/10 dilution of the extracted DNA from polenta and 10 μL of the heat-treated DNA solutions were used as template for the PCR reaction.

The PCR program consisted of an initial decontamination step of 10 min at 50°C to activate the uracil-N-glycosylase (UNG), followed by 3 min at 95°C to activate the DNA polymerase. Finally a two-step protocol was used for amplification with 50 cycles: denaturation at 95°C for 15 sec and annealing and elongation at 60°C for 60 sec. At the end of the cycling, Cycle threshold (Ct) values were calculated by the software according to the manufacturer's instructions. In order to calculate the μg of DNA present in the sample, the Ct value was compared with the calibration curves. The genomic equivalents were calculated considering that one haploid maize genome corresponds to 2.725 pg (KAY and VAN DEN EEDE, 2001).

RESULTS AND DISCUSSION

In order to study DNA stability during food processing a simple food, polenta, was used as the model. The influence of thermal treatment with respect to processing time was investigated. The results of DNA quantification by U.V. absorption revealed that the samples taken at the different processing times of polenta showed no more than 13% difference in the amount of DNA extracted with respect to that of the not heat-treated sample (flour). The amount of amplifiable target DNA was determined by real time PCR in samples of polenta withdrawn at different processing times. Data obtained from two independent experiments showed similar curves (Fig. 1A). The fraction of amplifiable DNA decreased in the course of the heat treatment, as expected from the decrease in high molecular weight DNA (hmw) and, hence, of intact sequences available for PCR (Fig. 1B and 2). The amount of amplifiable DNA in polenta was compared with the amount of amplifiable DNA in heat-treated DNA samples and with the fraction of hmw DNA in polenta (Fig. 1B), measured by agarose gel electrophoresis (Fig. 2). In Fig. 1B the data are expressed as percentages with respect to the initial untreated sample. The DNA concentration in polenta and in solution, measured by real time PCR, showed the same decrease during processing, dropping to 40% of the DNA concentration before the thermal treatment. This indicates that in the case of polenta the matrix did not significantly influence the thermal stability of amplifiable DNA. The fraction of hmw DNA decreased much faster with respect to processing time, and at 65 min there was only 10% of the initial value with respect to the 40% of the amplifiable DNA still present. The DNA stability of heat-treated samples has already been investigated using a quantitative competitive PCR system (HUPFER *et al.*, 2000) and, in this study,

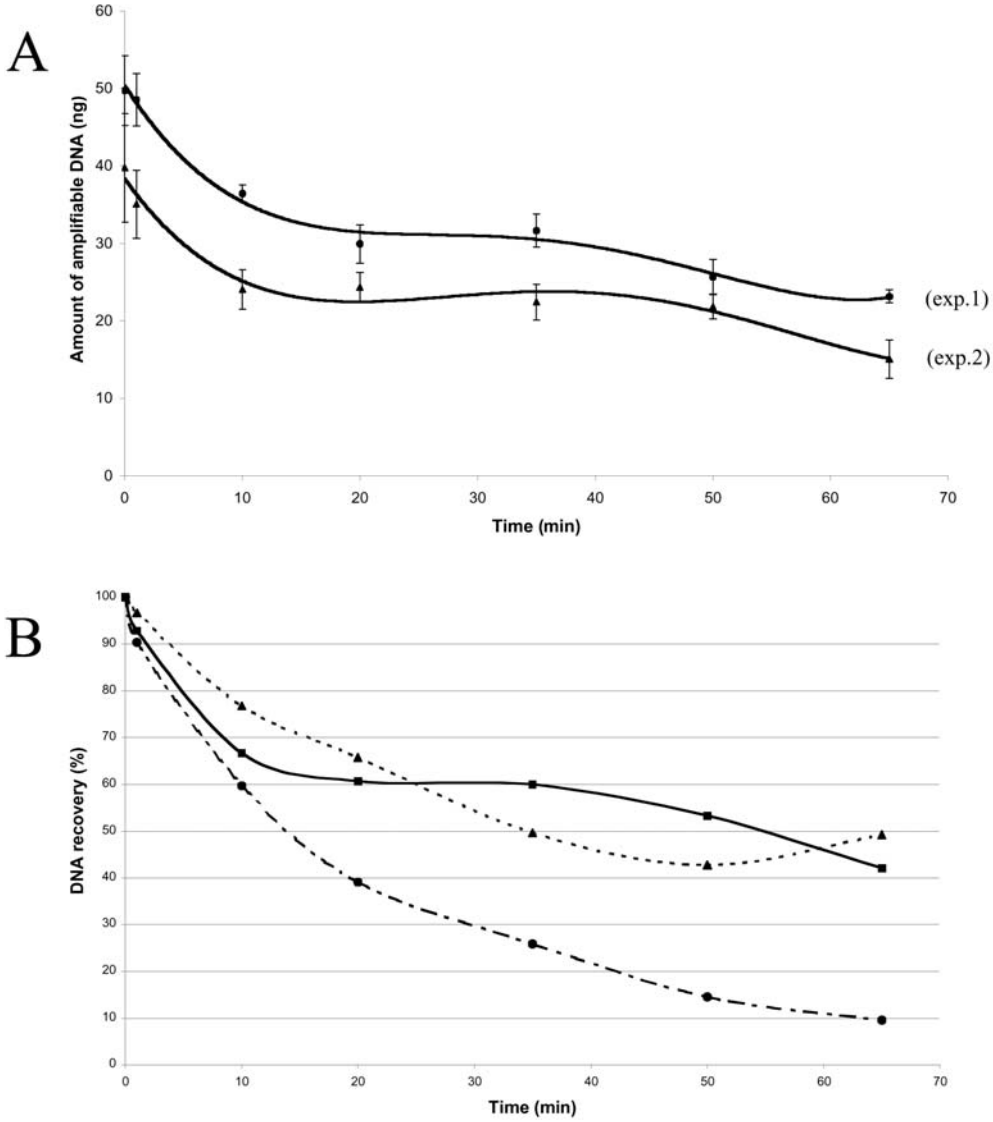


Fig. 1 - (A) Amount of amplifiable target DNA determined by real time PCR at various times of processing. Data presented are from two independent experiments (exp.1, exp.2) of polenta preparations; real time PCR was performed in triplicate; bars represent standard deviation.
(B) Recovery of amplifiable DNA from polenta (–■–), and from heat-treated DNA solution (--▲--) measured by real time PCR, and of high molecular weight DNA extracted from polenta (–● -) measured by agarose gel electrophoresis at different times of processing. Values are expressed as percentage with respect to the untreated sample. Data are the mean of two experiments.

after 60 min of thermal treatment the final recovery of amplifiable DNA was in the range of 2-5%. The difference between this and the present results can be explained by the higher sensitivity of real time PCR which is based on the amplification of very short DNA fragments: the competitive PCR system set

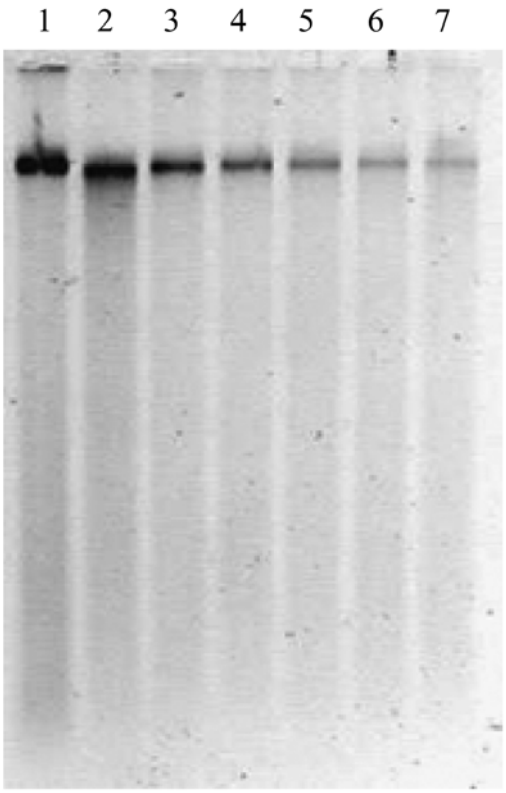


Fig. 2 - Agarose gel electrophoresis of polenta DNA at different times of processing. Samples are the same as exp. 1 in Fig. 1(A). Ten microliters of DNA solution, corresponding to 30 mg of starting material, were loaded in each lane. Lane 1, untreated sample; lanes 2-7, polenta at 0, 1, 10, 20, 35, 50, 65 min of processing.

up by HUPFER *et al.* (2000) targeted a 226 bp fragment, while the real time PCR system used in this study targeted a 72 bp fragment.

Error could occur when absolute target gene quantification is performed on a particular food because of the possible different amplification efficiency of the food DNA with respect to the reference material (flour DNA or plasmid DNA) used as standard. However GMO-quantification by real time PCR, determining the ratio of GM-maize DNA to endogenous maize DNA, eliminates this error. This normalisation is possible because the degradation of GM-specific sequences and endogenous maize sequences is the same (HUPFER *et al.*, 2000). However, quantification can be uncertain when there are only small absolute amplifiable sequence amounts in the final sample. In fact, the practical limit of quantification, which takes into account the distribution probability of the copies in the template DNA solution, is about a few tens of copies (BERDAL and HOLST-JENSEN, 2001). Consequently, the way the food is processed appears to be very important. In polenta there should be no problem in determining the GMO content, as only a simple boiling process is carried out and, as we have shown here, a consistent amount of extracted DNA is still available for amplification after processing; instead in complex products, because of the DNA-degrading action of some manufacturing processes, quantification of the ingredients can be more difficult and uncertain, or, indeed, even impossible.

Another critical point which may negatively affect the PCR detection of an ingredient in a food product is the quantity and quality of the template DNA, as these depend on the type of DNA extraction method used. Five DNA extraction methods were compared for their efficiency in extracting DNA from six food products. The general characteristics and the principle of DNA purification on which the different extraction methods are based are summarised in Table 1. All the kits, except the CTAB-based method, base DNA purification on the binding of DNA to specific resins.

DNA obtained from the different methods was checked for integrity by agarose gel electrophoresis (Fig. 3). As expected, high-molecular weight DNA was observed only in maize flour samples. In the case of canned maize grain and corn chip snacks, the extracted DNA was highly degraded, running below 500 bp. The DNA extracted from the other proc-

Table 1 - General characteristics and principle of DNA purification of the extraction methods used in this study.

Method	Principle	Characteristics	Reference
CTAB	Selective precipitation of nucleic acids	Widely used, cheap, official method of German Test method	ZIMMERMANN *et al.* 1998; PAULI *et al.* 2000
WP Wizard method	Selective binding of nucleic acids to specific resin	Widely used, official method of Swiss Food Manual	ZIMMERMANN *et al.* 1998; PAULI *et al.* 2000
WM Wizard Magnetic DNA Purification System	Selective capture of nucleic acids by a specific magnet	Tested protocols for raw and processed material	
GE GenElute Plant Genomic kit	Selective binding of nucleic acids to a specific resin	Protocols for different matrices	
QI QIAamp DNA Stool Mini kit	Selective binding of nucleic acids to a specific resin	Suitable for samples with high concentration of inhibitors	TENGEL *et al.* 2000; TENGEL *et al.* 2001

essed samples was not detectable on gel with any of the extraction methods used. Drastic technological processing, like the long term heating in the case of infant formula and the combination of pressure/heat for the corn flakes, probably led to significant DNA degradation. Judging from the agarose gel electrophoresis, the different methods, ranked in terms of DNA yield, was the following:

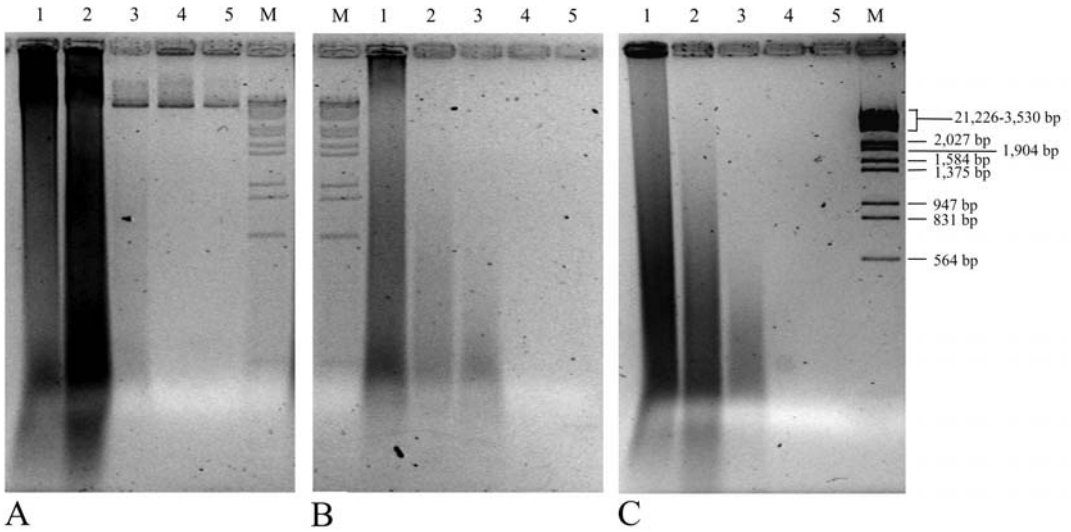


Fig. 3 - Gel electrophoresis of Bt-maize DNA extracted from flour (A), canned maize (B) and corn chip snacks (C). Each lane was loaded with 30 µL of DNA solution, corresponding to different amounts of starting materials: WP and CTAB, 120 mg; GE and QI, 30 mg; WM, 300 mg. Lanes M, molecular size marker (lambda DNA *Eco*RI/*Hind*III); lane 1, WP; lane 2, CTAB; lane 3, WM; lane 4, GE; lane 5, QI.

WP > CTAB > WM (Fig. 3). To confirm the amount of extractable DNA and to check its purity, DNA was evaluated by U.V. absorption at 260 nm (Table 2). Agarose electrophoresis showed that CTAB and WP gave the highest DNA yields. The greatest amount of extractable DNA was observed in maize flour and in low processed foods such as canned maize and corn chip snacks. Readings for the other processed samples proved unreliable due to the low concentration or to the presence of impurities which affected the test. For example, samples of chocolate corn flakes yielded a very low A_{260}:A_{280} absorption ratio (<1.5), suggesting the presence of a significant amount of contaminants, whereas with corn chip snacks and infant formula, the A_{260}:A_{280} absorption ratio was between 1.6 and 1.8 (SAMBROOK *et al.*, 1989). High DNA yield did not always correspond to positive signals of amplificability (Table 2), suggesting the poor reliability of U.V. measurement in the DNA solutions from complex, processed foods.

The extracted DNA was tested for suitability in PCR amplification. At first the nested Zein 1/2-3/4 PCR system, targeting the endogenous, single copy,

Table 2 - Quantification of DNA released by different extraction methods as determined by U.V. adsorption at 260 nm and by real time PCR determined only for corn chip snacks, chocolate corn flakes and infant formula. Amplifiability of DNA was evaluated with the zein PCR nested system.

Food		CTAB	WP	WM	GE	QI
Flour	Yield by UV[a] (µg/100 mg)	19.6±2.7	9.3±0.7	1.8±0.2	0.8±0.1	4.3±0.7
	Yield by RT-PCR[b] (µg/100 mg)	N.D.	N.D.	N.D.	N.D.	N.D.
	Amplifiability[c]	8/8	8/8	8/8	8/8	8/8
Canned maize	Yield by UV[a] (µg/100 mg)	8.0±0.3	3.5±0.6	0.5±0.07	0.6±0.2	0.6±0.1
	Yield by RT-PCR[b] (µg/100 mg)	N.D.	N.D.	N.D.	N.D.	N.D.
	Amplifiability[c]	8/8	8/8	8/8	1/8	2/8
Cheese corn puff snacks	Yield by UVa (µg/100 mg)	0.2±0.01	1.3±0.2	0.4±0.02	0.8±0.2	0.3±0.08
	Yield by RT-PCRb (µg/100 mg)	N.D.	N.D.	N.D.	N.D.	N.D.
	Amplifiability[c]	8/8	8/8	1/8	6/8	5/8
Corn chip snacks	Yield by UV[a] (µg/100 mg)	4.3±1.1	4.1±0.6	0.2±0.06	0.9±0.1	0.3±0.08
	Yield by RT-PCR[b] (µg/100 mg)	0.6±0.05	0.5±0.14	0.2±0.005	<0.01	0.02±0.01
	Amplifiability[c]	8/8	8/8	8/8	0/8	3/8
Chocolate corn flakes	Yield by UV[a] (µg/100 mg)	0.6±0.2	2.4±0.3	0.7±0.2	0.5±0.08	0.1±0.04
	Yield by RT-PCR[b] (µg/100 mg)	<0.01	N.D.	N.D.	<0.01	<0.01
	Amplifiability[c]	1/8	0/8	1/8	0/8	1/8
Infant formula	Yield by UV[a] (µg/100 mg)	0.4±0.06	1.0±0.1	0.1±0.07	0.7±0.1	0.1±0.03
	Yield by RT-PCR[b] (µg/100 mg)	<0.01	<0.01	<0.01	<0.01	<0.01
	Amplifiability[c]	3/8	0/8	0/8	0/8	0/8

[a] Each value is the mean (± standard deviation) of eight samples; [b] Each value is the mean (± standard deviation) of four samples; [c] Number of positive samples out of eight samples each separately extracted; n.d.: not detected; N.D. not determined.
The $A_{260/280}$ ratio ranged for flour ranged from 1.8-2, for canned maize and cheese corn puff snacks 1.6-1.9, for corn chip snacks and infant formula 1.6-1.8 and for chocolate corn flakes 1.3-1.5.

zein gene of maize, was used. Fig. 4 shows some amplification examples obtained with DNA extracted from corn chip snacks and Table 2 summarizes the results. All the methods tested on flour samples gave the expected bands (277 bp). The CTAB, WP and WM methods yielded amplified products in almost all the reactions tested on DNA from canned maize, corn chip snacks and cheese corn puff snacks, while only WM was partially successful with samples of cheese corn puff snacks. Probably the food matrix interfered with the process of DNA isolation carried out through magnetic capture, as indicated by the negative result of the control that was performed by adding external maize DNA to some of the food samples, such as corn puff snacks (data not shown). With all the methods tested very few samples from chocolate corn flakes gave amplified fragments. Although cocoa is a well known inhibitor of Taq polymerase (GASH *et al.*, 1997), PCR products were generated in the control reactions with added maize DNA (data not shown). For cocoa-containing foods we used the optimised protocol developed for WM; successful DNA extraction from cocoa-containing products with the QI method has already been reported (TENGEL *et al.*, 2000). These results suggested that the low PCR yield was due to a low amount of DNA coupled with poor DNA quality, rather than to the presence of PCR inhibitors in the DNA solution. Among the infant formula samples tested only those obtained with the CTAB method yielded the expected PCR products in three of the eight samples. Failure to obtain amplification was possibly due to the low amount of maize-DNA in the food product tested (<5% of total ingredients). The low amount of ingredient coupled with the possible partial degradation of maize DNA due to processing did not permit the clear detection of the maize ingredient. In this case, as also suggested by other authors (ANKLAM *et al.*, 2002; HURST *et al.*, 1999), the use of large volumes could

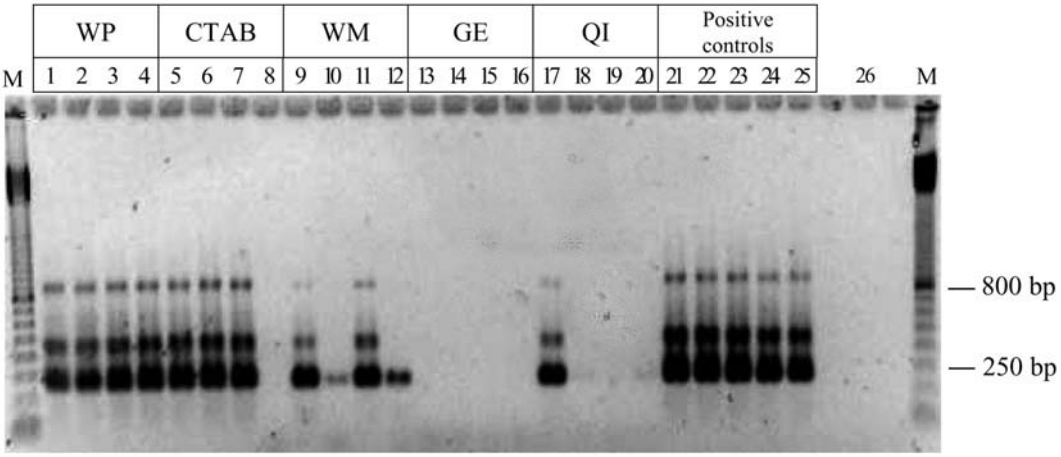


Fig. 4 - Agarose gel electrophoresis of PCR products obtained with nested primer set Zein 1/2-3/4 DNA extracted from corn chip snacks with the different extraction methods. Four PCR products from DNA extracted from four independent samples are shown for each method. Lanes M, 100 bp ladder; lanes 1-4, WP; lanes 5-8, CTAB; lanes 9-12, WM; lanes 13-16, GE; lanes 17-20, QI; lanes 21-25, control reactions performed adding external maize-DNA to samples extracted with WP, CTAB, WM, GE, QI methods respectively; lane 26, negative control (reaction added with water).

Table 3 - Concentration of DNA and number of theoretical copies of haploid maize genome measured by real time PCR in corn chip snacks, chocolate corn flakes and infant formula treated with different DNA extraction methods.

Food		ng/reaction[a]	N. copies[b]	R^2, a, b[c]
Corn chip snacks	CTAB	263	97,400	
	WP	227	84,000	R^2 = 0.996
	WM	21	7,770	a = 3.436
	GE	0.02	7	b = 31.045
	QI	2.58	900	
Chocolate corn flakes	CTAB	0.015	5	
	WP	n.d.	n.d.	R^2 = 0.996
	WM	n.d.	n.d.	a = 3.376
	GE	0.006	2	b = 31.075
	QI	0.003	1	
Infant formula	CTAB	2.7	1,000	
	WP	0.019	7	R^2 = 0.998
	WM	0.264	97	a = 3.372
	GE	0.003	1	b = 31.116
	QI	0.043	15	

[a] Each PCR reaction was assayed with 10 µL of the isolated DNA; each value is the mean of four samples; [b] Number of the theoretical copies of haploid maize genome considering that one haploid genome equivalent corresponds to 0.0027 ng; [c] R^2, a and b are values of the regression equation Threshold Cycle (Ct) = a log (ng of DNA) + b; n.d.: not detected.

allow the extraction of enough DNA for PCR detection.

Real time PCR experiments were performed with DNA isolated from corn chip snacks, chocolate corn flakes and infant formula in order to gain further information on the quality and yield of the extracted DNA. The real time PCR system permits successful PCR also with highly degraded DNA, like that in many processed foods, because the PCR target consists of very short DNA fragments which may be more reliably amplified (BERDAL and HOLST-JENSEN, 2001). The real time PCR data are summarized in Tables 2 and 3. The CTAB and WP methods show similar amounts of DNA quantified by real time PCR in corn chip snacks, while the other methods yielded much less DNA. With the chocolate corn flakes and infant formula very low yields of amplifiable DNA were obtained, suggesting that the DNA was extensively fragmented with small amounts of available targeting sequences. In these cases, when operating with low copy numbers of DNA, the distribution probability of the template must be taken into account for absolute quantification (BERDAL and HOLST-JENSEN, 2001). These experiments indicate that for quantitative purposes the choice of the DNA extraction method is a key point for reliable determination.

CONCLUSIONS

The results obtained on DNA stability in polenta showed that heat treatment severely affects DNA integrity, however real time PCR revealed that a relatively high amount of amplifiable DNA is still present in the final processed product.

From among the methods tested, CTAB, WP and WM gave the best results with regard to quality and quantity of the DNA isolated from the various maize foodstuffs, and suggest that DNA detection in some processed foods requires more than one technique. The overall data showed that both processing treatments in food preparation and DNA extraction strongly influence the quantitative detection of maize DNA, contributing to potential biases in the quantitative estimation of ingredients in processed foods.

ACKNOWLEDGEMENTS

This work was financially supported by ANPA (Agenzia Nazionale per la Protezione dell'Ambiente), contract "Sviluppo di metodologie per il monitoraggio di vegetali transgenici e derivati e della diffusione dei transgeni nell'ambiente". The authors thank Biorad for kindly providing the sequences of primers used in the real time analysis.

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Paper received January 9, 2003 Accepted May 16, 2003

PAPER

MEDIUM POLARITY LIPIDS FROM FRIED OILS PROMOTE LDL OXIDATION *IN VITRO*

I LIPIDI DI MEDIA POLARITÀ DA OLI DI FRITTURA PROMUOVONO L'OSSIDAZIONE *IN VITRO* DELLE LDL

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ABSTRACT

Some frying by-products of intermediate to total polarity and normal triglycerides, called medium polarity materials (MPMs), were examined for their biological effect on low density lipoprotein (LDL) *in vitro*. LDL was oxidized by MPM at different concentrations to oxidative levels comparable to those of copper sulphate (5 μM). The effect on LDL was expressed as % mean oxidation (% MO). The tested MPMs present in the absorbed oil from batches of restaurant-supplied French-fried potatoes

RIASSUNTO

Alcuni prodotti secondari di frittura formati da intermedi a polarità media e da trigliceridi normali, chiamati materiali di media polarità (MPM), sono stati esaminati per la loro attività biologica *in vitro* sulle lipoproteine di densità bassa (LDL). La LDL è stata ossidata dai MPM in concentrazioni diverse, fino a livelli di ossidazione comparabili con quelli del solfato di rame (5 μM). La loro azione sulla LDL è stata espressa come % di ossidazione media (%MO). I MPS presenti nell'olio assor-

- Key words: fried oils, lipids, *trans*,*trans*-2,4-decadienal, low density lipoprotein, low density lipoprotein oxidation -

(150 g), gave 97.6-105.5% MO. The MPMs in 1, 2, 3 and 8 successive domestic deep-frying operations in sunflower oil gave 58.1, 64.0, 64.9 and 67.6% MO, respectively. The oxidative effect of MPM was inhibited (approx. 30%) by Vitamin E (20 μM). An IC_{50}=0.016 AU*s was calculated for the oxidative effect of Vitamin E on native LDL.

bito dalle patate fritte (150 g) fornite da ristoranti hanno mostrato una ossidazione media compresa tra 97,6 e 105,5%. I MPS formati nel corso di diverse fritture (1,2,3 e 8) in olio di girasole hanno prodotto ossidazioni medie pari a 58,1; 64,0; 64,9; e 67,6%. L'effetto ossidante dei MPM veniva inibito (circa 30%) dalla vitamina E (20 μM). È stato calcolato un valore di IC_{50}=0.016 AU*s per il suo effetto sulla ossidazione della LDL nativa.

INTRODUCTION

Frying is a widespread cooking process used for foods of either plant or animal origin. Foods are fried to develop the desirable characteristic flavors imparted by the frying oil or fat (STIER, 2000). During heating, cooking oils and fats deteriorate due to various chemical processes including oxidation, polymerization, isomerization, cyclization and hydrolysis (GERTZ, 2000). Oil deterioration increases with the number of successive frying operations (ANDRIKOPOULOS *et al.*, 2002c). Frying causes the formation of several by-products derived mainly from the oxidative alteration of triglycerides (TGs). These by-products make up a group called total polar materials (TPMs) which can be separated from the non-polar materials, consisting mainly of normal oil TGs, by glass column absorption chromatography (GRUHR *et al.*, 1981).

Because cooking oils and fats are absorbed by foods, TPMs become constituents in the diet (ANDRIKOPOULOS *et al.*, 1989; 2002b; DOBARGANES *et al.*, 2000). The health aspects of thermoxidized oils and fats were recently reviewed by BILLEK (2000). The toxic classes of compounds determined were (a) cyclic fatty acid (FA) monomers isolated as non-urea adducted substances and (b) oxidatively modified aldehydic TGs that remain in the oil as non-volatile cleavage products of the TG alkoxy radicals produced from normal TGs during frying. Thermal stressing of polyunsaturated fatty acid (PUFA) cooking oils generates high levels of aldehydic products (predominantly *trans*-2-alkenals, *trans,trans*-alka-2,4-dienals and n-alkanals) (GROOTVELD *et al.*, 1998). These species arise from the fragmentation of conjugated hydroperoxy-diene precursors which have been proven to be cytotoxic in experimental animals (WITZ, 1989).

ANDRIKOPOULOS *et al.* (2002a) reported the isolation of some frying by-products produced during domestic deep-frying of French-fried potatoes in edible vegetable oils using reversed-phase high performance liquid chromatography (RP-HPLC). Elution of these products in both TPM and non-polar material fractions by glass column absorption chromatography in almost equal amounts led us to characterize them as "medium polarity materials" (MPM). A correlation among MPM, TPMs and polymerized TGs (PTGs) has also been reported for both domestic (ANDRIKOPOULOS *et al.*, 2002a) and commercial (ANDRIKOPOULOS *et al.*, 2003) frying.

Low density lipoprotein (LDL) is the

carrier of cholesterol in the blood. When it adheres to endothelial tissue cells, LDL contributes to cholesterol deposition, forming foaming cells that are responsible for the formation of atheromatic plaque which increases the risk of cardiovascular heart disease (CHD) (GOLDSTEIN and BROWN, 1997). LDL in its oxidized form strongly promotes this phenomenon (STEINBRECHER *et al.*, 1989; 1990). Epoxid-FAs have been reported to have a non-health function during LDL synthesis (RIEMERSMA *et al.*, 1994; RIEMERSMA, 1996). LDL, isolated from animals fed with oxidized lipids, has been found to be more susceptible to oxidation (SUTHERLAND *et al.*, 1999). Oxidized lipids in the diet could play a significant role in lipoprotein oxidation. Oxidized FAs have been found to accelerate fatty streak formation in cholesterol-fed rabbits (STAPRANS *et al.*, 1996). In rats, the levels of oxidized lipids in chylomicrons and in serum VLDL and LDL fractions were correlated with the quantity of oxidized lipids in the diet by STAPRANS *et al.*, 1993a; 1993b. These results indicate that oxidized lipids in the diet are absorbed by the small intestine and are transported in chylomicrons by the blood where they contribute to the total body pool of oxidized lipids.

To the best of our knowledge, the effect of the MPM fraction on the oxidized modification of native LDL has never been reported. Given the obvious importance of the dietary intake of frying byproducts and the role of oxidized LDL in atherosclerosis, the role of MPMs in oxidizing LDL *in vitro* in a dose-dependent manner was investigated.

MATERIALS AND METHODS

Materials

Adequate quantities of MPM were extracted from fried oils and purified by RP-HPLC. Two series of fried potato samples, one in sunflower oil and a second in vegetable shortening oil, were obtained after one to eight successive, domestic deep-frying sessions (ANDRIKOPOULOS *et al.*, 2002b). Two other series of samples fried in highly unsaturated vegetable oil and cooking oils and fats from a previously performed survey of 63 restaurants (ANDRIKOPOULOS *et al.*, 2003) were also used. The fried samples were kept at -20°C until use.

Sterilized LDL was lyophilized from 1 mL of LDL solution in 0.15 M NaCl and 0.01% EDTA at pH 7.4, with 6.3 mg protein per 400 mg LDL. Vitamin E (95%) (Sigma, St. Louis, Missouri) was prepared from synthetic phytol, as indicated by the manufacturer. Phosphate buffer saline (PBS) tablets were purchased from Sigma. Standard malonaldehyde and *trans*,*trans*-2,4-decadienal were purchased from Aldrich (Steinheim, Germany) and thiobarbituric acid was purchased from Serva (Heidelberg, Germany).

HPLC-grade solvents were obtained from LabScan (Dublin, Ireland) and all other reagents and chemicals of analytical grade were purchased from Merck.

Lipid extraction

The fried oil samples were grouped together and each group was diluted in hexane/isopropanol (4:1, v/v) to obtain 20% working solutions. MPMs were isolated from the fried oil groups according to the method proposed by ANDRIKOPOULOS *et al.* (2002a). In brief, the oil solution was extracted with methanol/water (6:4 v/v). The methanolic-aqueous extract was removed and the oil solution was further extracted with acetonitrile. Because rotary evaporation resulted in the partial loss of some semi-volatile compounds of the MPM fraction (e.g. *trans*,*trans*-2,4-decadienal), the acetonitrile extracts were

left overnight in a 37°C water bath to evaporate the solvents. The MPMs retained in the residue were redisolved in 2 mL of acetonitrile to form a stock solution which was stored at -20°C. Appropriate volumes from the stock solution were redissolved in acetonitrile to form working solutions of known MPM content before the biological experiments. The MPM content of the working solutions was quantified by RP-HPLC analysis.

RP-HPLC and GC/MS analyses

The RP-HPLC analysis of MPMs obtained from the fried oils was performed according to the method proposed by ANDRIKOPOULOS *et al.* (2002a). In brief, using a quaternary solvent system consisting of water (acidified with phosphoric acid to pH 3), methanol, acetonitrile and isopropanol, gradient elution on a Nucleosil 120-5C18 column (Macherey-Nagel, Düren, Germany) and UV (280 nm) detection was carried out. An Agilent Technologies (former Hewlett Packard, HP, Avondale, PA) HPLC model HP 1050 combined with an autosampler and diode array detector was used. Peak areas were calculated utilizing a HP ChemStation data system. The same method was also applied for the standard *trans*,*trans*-2,4-decadienal analysis.

The MPM fractions derived from RP-HPLC analysis were extracted by applying the lipid extraction method proposed by BLIGH and DYER (1959). They were then subjected to GC/MS analysis in chloroform solutions. A Hewlett Packard 6890 gas chromatograph (Waldbronn, Germany) coupled with a 5972 quadropole mass spectrometric detector (MSD) with a cross-linked 5% diphenyl-95% dimethyl siloxane capillary column (30 m x 0.2 mm I.D., 0.25 μm film thickness, HP-5MS) was used. Helium was used as a carrier gas at a flow rate of 0.6 mL/min. Injection port and transfer line temperatures were maintained at 250°C. A 1 mL sample volume was injected in the split mode (1:20). The initial oven temperature was set at 70°C for 5 min, then increased 15°C/min to 250°C where it was held for 5 min. The elution time for standard *trans*,*trans*-2,4-decadienal was 11.45 min for the main peak and 11.20 min for the 10% pre-eluted compound appearing the same mass spectra as 2,4 decadienal and characterized as the isomer of *trans*,*trans*-2,4-decadienal. MSD was run in full scan mode (mass range 30-600amu) with electron impact ionization (70Ev). A standard solution of *trans*,*trans*-2,4-decadienal was prepared with 150 μg/mL of chloroform.

LDL oxidation tests

The method of LDL oxidation by copper ions (LDLox) proposed by BALLA *et al.* (1991) was applied with slight modifications. In brief, 1.0 mL of LDL suspended in PBS was mixed with 1.0 mL $CuSO_4$ (10 μM) as oxidizing agent. It was then incubated at 37°C for 1h, 3h, and 6h and the resulting thiobarbituric acid reactant substances (TBARS), mainly malonaldehyde, were measured spectrophotometrically at 532 nm with a UV-VIS spectrophotometer (Kontron, Milano, Italy), after reaction with 2 mL of alkaline TBA solution. TBARS were evaluated using a standard curve of standard malonaldehyde at different concentrations versus absorption at 532 nm. An LDL-blank test was also performed in the absence of copper ions. Several concentrations of MPM were tested as above as oxidizing agents instead of copper ions. The residue was redissolved in 10 μL of ethanol before the addition of the other test reagents. This ethanolic volume had no effect on the test results, which was in agreement with GORDON and WEN (1992). All experiments were carried out four times.

RESULTS

Total MPMs fractionated by RP-HPLC analysis were expressed as absorbance units multiplied by seconds (AU*s), corresponding to the area of HPLC eluting peaks under the experimental conditions used. The effect of the MPM fraction on LDL was assayed by the TBA assay after different incubation times of several quantities of MPM with native LDL. Incubation time never exceeded six hours, because TBARS tended to stabilize even before the six-hour incubation was completed (data not shown). For the *in vitro* LDL oxidative modification, 5 μM $CuSO_4$ were always used (VISIOLI and GALLI, 1994). To exclude interference from phosphoric acid present in the HPLC eluents in the biological experiment, native LDL was tested. Phosphoric acid present in concentrations equal to those in the HPLC eluents was found to be inactive (data not shown). An LDL blank test was done to estimate LDL autoxidation. The results were expressed as % mean oxidation (%MO), considering LDLox (5 μM $CuSO_4$ induced oxidation) as 100% MO.

The oxidative effect of $CuSO_4$ and 4.05 AU*s MPMs is given in Table 1 for all three incubation times and is expressed as %MO. The influence of 20 μM of vitamin E on the LDLox test and MPM-induced oxidation is also given. GORDON and WEN (1992) previously identified 20 μM of vitamin E as the optimal concentration for the maximum inhibitory effect. The influence of 4.05 AU*s MPM against the *in vitro* LDL oxidation, together with the inhibitory activity of vitamin E (20 μM), is presented in Fig. 1. As shown, 4.05 AU*s of the MPM fraction exhibited practically the same oxidation on native LDL as the 100% exhibited by adding copper ions as oxidizing agent. Vitamin E was a stronger antioxidant on LDLox, limiting MO to 56.0%, while it induced 30% inhibition on MPM oxidation, reducing MO from 97.6 to 68.6%.

In Table 2 a dose-dependent effect of the MPM fraction on LDL oxidation can be observed which is associated with AU*s simulation of RP-HPLC analysis and the deterioration of oils used to cook a batch of French-Fried potatoes, either at home or in restaurants. Low, mean or maximum deterioration is defined as the % TPM content in the oil. Thus, MPMs corresponding to a batch of potatoes french-fried in oils undergoing mean (4.05-5.9 AU*s, 14.0-20.0%TPM) deterioration exhibited 97.6-99.2%MO, while

Table 1 - Effect of MPM and Vit E (TBARS, μM) on LDL expressed as % mean oxidation (%MO).

Nr (i)	Tests (n=4)	Incubation time (hours)			
		1 h	3 h	6 h	%MO
-	LDLox (5 μM $CuSO_4$-induced oxidation)	1.412	2.294	2.694	100.0
1	MPM-induced oxidation	1.406	2.130	2.378	97.6
2	Vit. E vs. MPM-induced oxidation	1.244	1.412	1.301	68.6
3	Vit. E vs. $CuSO_4$-induced oxidation	1.092	1.174	0.921	56.0
-	LDL blank	0.000	0.100	0.223	4.5

S.D. (standard deviation) ranged from 0.0006 to 0.0032.
LDLox and LDL blank tests as in the "Materials and Methods" section.
MPM: medium polarity materials (4.05 AU*s). TBARS: thiobarbituric acid reactant substances. Vit. E: solution of standard vitamin E (20 μm).
% MO = $\{\{[\sum(O_t)]_i/3\} \times 100$, where $O_t = \{[TBARSi / (LDLox - LDL\ blank)]_t\} \times 100$; e.g. for test 1: % MO={{[1.406 / (1.412 – 0.000)] + [2.130 / (2.294 – 0.100)] + [2.377 / (2.694 – 0.223)]} /3} x 100 = 97.6.

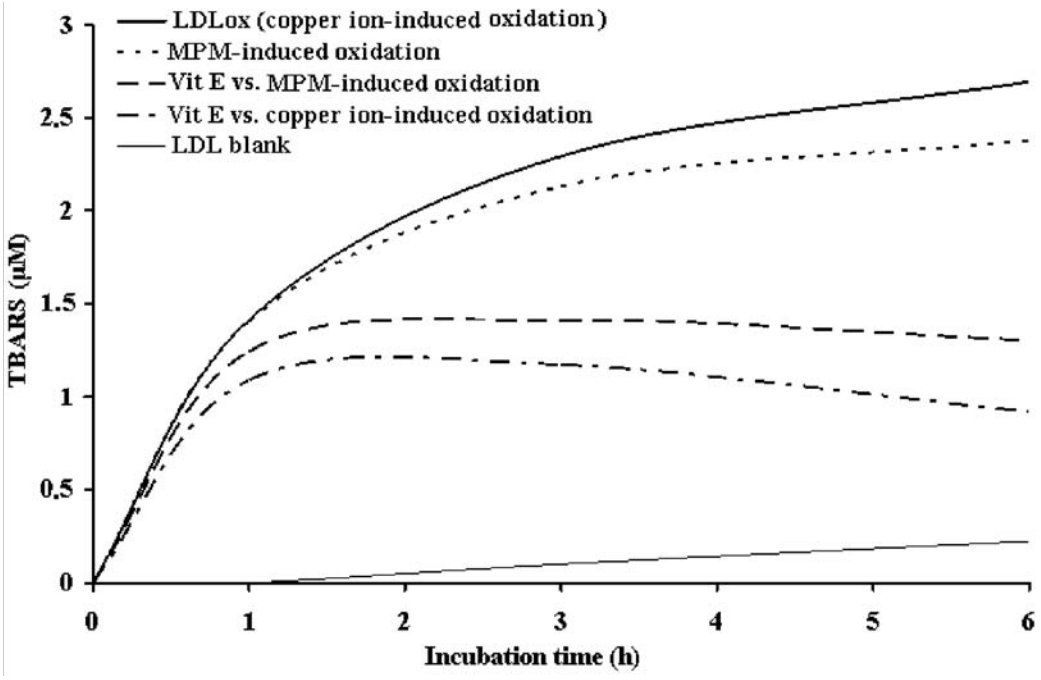


Fig. 1 - Oxidative effect of 5 μM $CuSO_4$ (LDLox) and medium polarity materials (MPM, 4.05 AU*s) on native LDL, *in vitro*. Inhibitory effect of vitamin E (Vit E, 20 μM). LDLox and LDL blanks.

Table 2 - Comparison of the oxidative effect on LDL of different MPM concentrations and 100% $CuSO_4$-induced oxidation (5 μM).

Corresponding to the oil (15 g) absorbed from a batch (150 g) of French-Fried potatoes in:	MPM in RP-HPLC (AU*s)	MPM per test (AU*s)	MPM-induced oxidation (%MO)
Cooking oils and fats of maximum deterioration from restaurants (40.0% TPM)[a]	20.5	15.4	105.5
Cooking oils and fats of mean deterioration from restaurants (20.0% TPM)[a]	7.86	5.9	99.2
Highly unsaturated vegetable oils of mean deterioration from restaurants (14.0% TPM)[a]	5.4	4.05	97.6
Vegetable shortening oil after 8 successive domestic fryings (9.0% TPM, MUFA/PUFA:0.70)[b]	0.7	0.525	89.3
Sunflower oil after 8 successive domestic fryings (9.0% TPM, MUFA/PUFA:0.56)[b]	0.6	0.45	67.6
Sunflower oil after 3 successive domestic fryings (8.0% TPM)[b]	0.2	0.15	64.9
Sunflower oil after 2 successive domestic frying session (7.0% TPM)[b]	0.1	0.075	64.0
Sunflower oil after 1 domestic frying (6.5% TPM)[b]	0.05	0.0375	58.1

%MO as in Table 1; TPM: total polar materials; MUFA/PUFA: Monounsaturated fatty acids/Polyunsaturated fatty acids; [a] ANDRIKOPOULOS *et al.*, 2003; [b] ANDRIKOPOULOS *et al.*, 2002b.

those corresponding to a batch of potatoes french-fried in oils undergoing maximum (15.4 AU*s, 40.0%TPM) deterioration exhibited 105.5%MO, exceeding the 100.0%MO induced by $CuSO_4$. MO induced by 0.0375-0.525 AU*s MPMs that correspond to one-to-eight successive domestic French-fryings in sunflower oil, undergoing low (6.5-9.0%TPM) deterioration, ranged from 58.1 to 67.6%. In Fig. 2 a logarithmic dose-response curve is given in order to evaluate the IC_{50} for MPM. Using the equation given, IC_{50}=0.016 AU*s.

DISCUSSION

Oxidative stress, implicated in human disease by a growing number of facts, results from metabolic reactions that use oxygen. It has been defined as a disturbance in the equilibrium between the prooxidant and antioxidant systems in intact molecules and cells. The definition of oxidative stress implies that molecules and cells have intact prooxidant and antioxidant systems that continuously generate and detoxify oxidants during normal aerobic metabolism. When additional oxidative events occur, the pro-oxidant systems overbalance the anti-oxidant systems, potentially producing oxidative damage to lipids, proteins, carbohydrates, and nucleic acids and ultimately leading to cell death in severe oxidative stress (HARRISON *et al.*, 2003). LDL particles are particularly susceptible to oxidative stress (Belcher *et al.*, 1999). The circulating lipoproteins in an undisturbed native form do not appear to be significantly irritating or toxic; but oxidized LDL is the main offender (PIETZSCH and JULIUS, 2001). If oxidative damage to LDL is sufficient to damage the protein moiety, macrophages take up oxidized LDL in an unregulated manner via scavenger receptors, resulting in the formation of large numbers of lipid-laden macrophages that accumulate to form a fatty streak which leads to

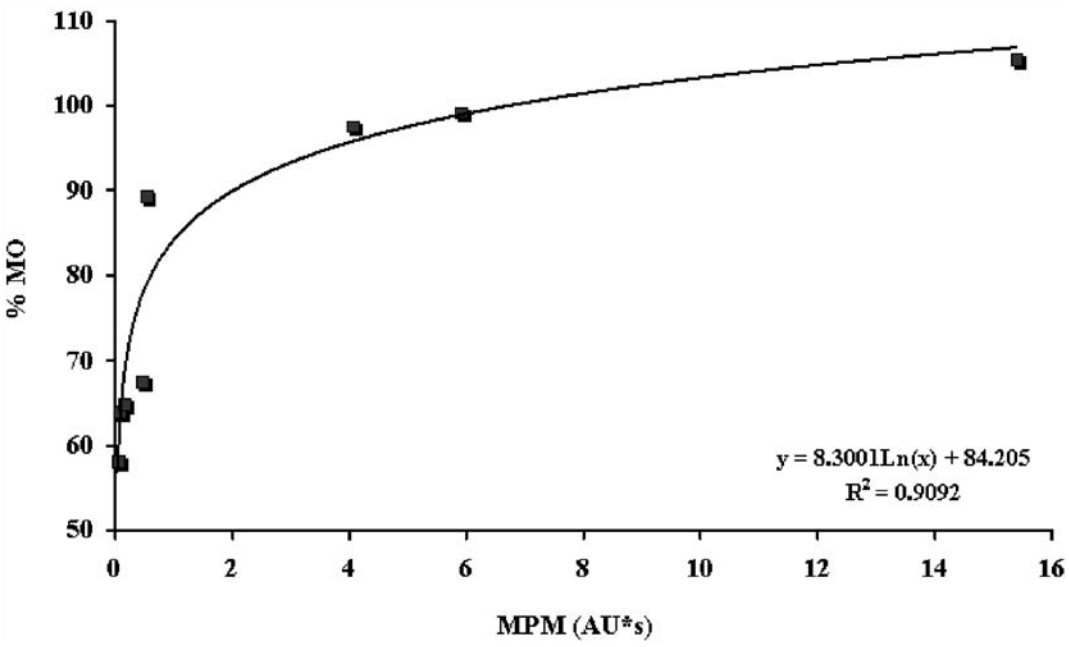


Fig. 2 - Oxidative effect of different quantities of MPM on native LDL. Calculation of IC_{50}.

the onset of atherosclerosis (MINAMI *et al.*, 2001; NICHOLSON *et al.*, 2001).

Oxidized lipids taken up via thermoxidized oils and foods have been implicated in atherosclerosis, as their intake is correlated to enhanced oxidized lipoproteins in plasma (STAPRANS *et al.*, 1993a; 1993b). The *in vitro* effect of oxidized lipids of medium polarity on LDL is presented for the first time in the present report.

These findings suggest that the MPM fraction obtained from oils, either from domestic frying or from frying in restaurants, is highly oxidative as indicated from the IC_{50} value of 0.016 AU*s. Consumption of French-fries produced after the first domestic frying process caused less LDLox risk than those produced after the five subsequent frying sessions. These findings indicate that MPMs, even in low concentration, drastically influence LDL. There are some differences in the level of influence between MPMs produced during commercial frying and those produced during domestic frying. Household fryers have an oil capacity of up to four batches of potatoes per frying, which allows up to eight successive frying operations before the oil reaches the fryer minimum indication. Therefore, in the normal domestic frying process the maximum number of frying sessions is usually eight. A plateau is observed at 4.05 AU*s, above which increasing the MPM concentrations does not markedly affect % MO (Table 2, Fig. 2), indicating the upper limit of the oxidative effect. It is possible that the LDL concentration imposes a limit, or that polymerization of the polar compounds released after enhancing successive frying operations results in a decrease in the reactive intermediates. Consuming French–fries in restaurants cooked in oils under mean or maximum deterioration causes similar LDL oxidation risk. Almost twice as much LDL oxidation ocuured in commercial French-fries compared to first frying in a domestic frier. The MPM effect is correlated to the quantity of oil and its deterioration (Table 2). Concerning vegetable shortening and sunflower oils after eight successive domestic fryings, although these underwent exactly the same deterioration (9.0% TPM, ANDRIKOPOULOS *et al.*, 2002b), the derived MPM fraction of vegetable shortening was higher than that of sunflower (Table 2). This is due to a high PUFA content which generates MPM, as shown by the MUFA/PUFA ratios found, 0.70 and 0.56, respectively (ANDRIKOPOULOS *et al.*, 2002b). The above results are also supported by the lower content of linoleic acid found in fresh and fried vegetable shortening oil (53.5 and 52.5%, respectively, ANDRIKOPOULOS *et al.*, 2002c).

GC/MS of MPMs fractionated by RP-HPLC analysis indicated that *trans, trans*-2,4 decadienal was the main constituent (data not shown). This undesirable frying by-product has also been found in previous studies (WARNER *et al.*, 2001). Thus, standard *trans, trans*-2,4-decadienal was tested on native LDL modification. The %MO values obtained indicated that this aldehyde was the biologically active constituent, because at concentrations applied as those in 0.45, 4.05 and 15.4 AU*s of MPMs, the %MOs were 41.6, 57.0 and 72.7, respectively. Thus, the aldehydes were responsible for 61.5, 58.4 and 68.9% of the oxidative effect, respectively (compared to 67.6, 97.6 and 105.5%MO, Table 2).

The vitamin E that remained in the fried oil was almost equal to that which was absorbed by the food (DOBARGANES *et al.*, 2000) and this might have contributed to LDL protection. Notable quantities of vitamin E remain unaffected (30-50%) in fried oils, used in either commercial (ANDRIKOPOULOS *et al.*, 2003) or domestic friers (ANDRIKOPOULOS *et al.*, 2002). These quantities were equal to those in fried foods. Nevertheless, the contribution to LDL protection

was considered to be relatively low given the above defined experimental conditions, since the amount of vitamin E added to the LDL-MPM mixture (5.5 g / 0.84 mg LDL-ox) was 20-times higher than that which could possibly be transferred to blood (0.75 g / 0.9 mg LDL-ox) after consuming a batch of French-fried potatoes (i.e. with 200 mg/kg vitamin E in the absorbed oil). Perhaps the oxidative effect of MPMs obtained from a batch of French-fries could be significantly offset by vitamin E obtained simultaneously from a salad oil and the absorbed frying oil.

Bearing in mind that the human LDL level is 2 mg/mL blood, the effect of the tested quantities of MPMs on 0.84 mg LDL in the cuvette may correspond well to their real transfer to LDL particles in humans. Thus, the findings of this *in vitro* study give strong evidence of the undesirable *in vivo* properties and effects that MPMs exert on human health.

ACKNOWLEDGEMENT

The laboratory assistance of Mrs. Margarita Christea is appreciated.

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Revised paper received May 19, 2003 Accepted June 30, 2003

PAPER

THE ROLE OF BUFFERS ON THE KINETICS OF L–ASCORBIC ACID OXIDATION CATALYZED BY COPPER (II)

IL RUOLO DI SOLUZIONI TAMPONE NELLA CINETICA DELL'OSSIDAZIONE DELL'ACIDO L-ASCORBICO IN PRESENZA DI RAME CU(II) COME CATALIZZATORE

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ABSTRACT

The chemical role of copper chelates on ascorbic acid oxidation was examined. The kinetics of uncatalyzed and Cu(II) ion- catalyzed oxidation of ascorbic acid in aerated and buffered (e.g., acetate, citrate, tartrate and phthalate) solutions were studied at 20°C. The rate of the oxidation reaction in the presence of cupric ion as the catalyst, in the pH range investigated (pH 3.2-6.5), was found to be a first - order reaction with respect to ascorbic acid. The effects of pH and cupric ion concentra-

RIASSUNTO

È stato esaminato il ruolo chimico dei chelati di rame nell'ossidazione dell'acido ascorbico insieme alla cinetica di ossidazione dell'acido ascorbico con 1'ossigeno molecolare sia senza catalizzatore che in presenza di ioni Cu(II) come catalizzatore, in presenza di diverse soluzioni tampone (per esempio: acetato, citrato, tartrato, ftalato), alla temperatura di 20°C. All'interno di valori di pH compresi tra 3,2 e 6,5, la velocità della reazione di ossidazione in presenza di catalizzatore - ioni Cu si è

- Key words: *A*scorbic acid, buffer organic acids, copper (II) -catalyzed oxidation, kinetics -

tion on the reaction rate suggest a mechanism that may involve the formation of a transition complex between monohydrogenascorbate ion and Cu(II) – buffer chelate. The differences in the reaction rates in the various buffers are due to the different complexing abilities of Cu(II) with the various buffer salts. Coordinatively unsaturated Cu(II) - buffer complexes which are comparatively less stable than the higher chelates were more active in the Cu(II) - induced oxidation of ascorbic acid.

rivelata essere di primo stadio rispetto all'acido ascorbico. Gli effetti del pH e della concentrazione di ioni Cu sulla velocità di reazione hanno suggerito un meccanismo che potrebbe interessare la formazione del complesso di transizione fra ione di monoidrogenascorbato ed il tampone del chelato di Cu(II). La differenza di velocità delle reazioni in presenza di varie soluzioni tampone dipendente è dipendente dalla capacità del Cu(II) di formare diversi complessi con i sali delle soluzioni tampone. Si può affermare che le soluzioni tampone di complessi insaturi di Cu(II), meno stabili degli altri chelati, sono più attive nell'ossidazione indotta del Cu(II) dell'acido ascorbico.

INTRODUCTION

Vitamin C (ascorbic acid) is widely used in soft drinks as a water-soluble antioxidant and to restore its losses in fruit juices, particularly citrus juices due to manufacturing. Its stability in these products varies widely according to the composition and oxygen content of the solution (OTTAWAY, 1993). Traces of transition metal ions like Cu also act as catalysts in the oxidation of ascorbic acid. Because of its biochemical importance in the food and pharmaceutical industries, the Cu(II)-catalyzed oxidation of ascorbic acid by molecular oxygen has been extensively studied (WEISSBERGER and LU VALLE, 1944; KHAN and MARTELL, 1967a,b; OGATA *et al.,* 1968; DAVIDSON and GRIEGER-BLOCK, 1977; BIRCH and PEPPER, 1983; IMER *et al.,* 1989). Recently, it has been found that the catalytic efficiency of Cu(II) complexes depends on the nature of the ligands and on the coordination geometry of the metal ion (MARTELL, 1982; DAVIES, 1992). The complexation of Cu(II) by certain ligands such as †EDTA, CDTA, NTA, citrate, bis–histidine and glutathione modifies its catalytic activity (KHAN and MARTELL, 1967b; SCHWERTNEROVA *et al.,* 1976; MİKOVA *et al.,* 1981; ŞAHBAZ and SOMER, 1993; SCARPA *et al.,* 1996; OHTA *et al.,* 2000). There is little information in the literature about the catalytic effects of buffered organic acid - Cu(II) chelates in the oxidation of ascorbic acid.

Citric and tartaric acids are the most common acids in fruits and acetic acid is an important metabolic intermediate that occurs naturally in body fluids and plant juices. These acids merit special attention due to their presence in numerous natural products and the allowance of their use in food for various technical purposes.

Since buffers interact with Cu(II) to varying degrees and form complexes that are a complicating factor, the aim of this paper is to propose an original approach to explain the complex role that is played by the buffer in the copper- catalyzed oxidation of ascorbic acid.

†*Abbreviations:* CDTA, Cyclohexanediaminetetracetic acid; EDTA, Ethylenediaminetetracetic acid; NTA, Nitrilotriacetic acid.

MATERIALS AND METHODS

Reagents

All chemicals used in the experiments were analytical grade and were supplied by E. Merck (AG, Darmstadt, Germany). L-ascorbic acid which is mentioned as ascorbic acid in the text was used without further purification. 2,6-dichlorophenol-indophenol, metaphosphoric/acetic acid (3%/8%), buffer solutions (pH 3.2-6.5) were used and the catalyst was cupric nitrate. Acetate buffer (0.5 M), citrate buffer (0.5 M), ortho-phthalate and tartrate buffers (0.2 M) were prepared according to BIRCH and PEPPER (1983), ŞAHBAZ and SOMER (1993) and WEAST (1982), respectively. Deionized distilled water was used to prepare all of the solutions.

Method

The AOAC Official Method 967.21 (final action) (AOAC, 1998) was used for the ascorbic acid assay. The principle of this method is a titration with 2,6-dichlorophenol-indophenol (DCPIP) as the indicator with a precision of ±1%. Ascorbic acid reacts with DCPIP, changing the colour from blue to colourless. The quantity of DCPIP used gives a direct measure of the amount of ascorbic acid present.

The pH measurements were made using a Metrohm 632 Digital – pH meter (CH-9100 Herisau, Switzerland) with a combined pH electrode. The ionic strength of the reaction medium was maintained at I = 0.5 M in order to keep the activity coefficient of the species constant during the kinetic process. To compensate for the changes in organic buffer ionizations over the pH range studied, the solution was adjusted by adding appropriate amounts of sodium or potassium nitrate salts. All the experiments were carried out at 20°C by means of a thermostated system (±0.5°C) which contained an immersion circulator (Thermomix 1419 B. Braun model, AG Melsungen, W. Germany).

Sample preparation and kinetic measurements

A stock cupric nitrate solution was prepared with a Cu^{2+} concentration of 1 mg mL^{-1}. For daily use, a solution with a Cu^{2+} concentration of 15.7 x 10^{-5} M was used; 1 mL of stock solution was diluted in 100 mL reaction mixture. Sample solutions containing KNO_3, 0.075 M buffer and Cu^{2+} in the concentration range from 3.15 x 10^{-5} to 31.5 x 10^{-5} M were prepared in a 100 mL volumetric flask and then placed in a 250-mL Erlenmeyer flask in contact with a water bath thermostated at 20°C. During this time a stream of air (flow rate = 60 L h^{-1}) was passed through the Erlenmeyer flask and the solution was saturated with oxygen. The air was presaturated with water vapor by passing through a wash-bottle. Since the rate of reaction is slow compared to the rate of dissolution of oxygen, the reacting solution was considered to be saturated with oxygen at all times. Ascorbic acid (100 mg) was added immediately to each sample and the reaction was timed. After the addition of ascorbic acid, the pH of the sample solutions was measured. Two milliliter aliquots were taken periodically for titration and 5 mL of metaphosphoric/acetic acid was added as a stabilizer to prevent further oxidation of ascorbic acid. The residual ascorbic acid concentration was determined according to the AOAC (1998) methods. All tests were done in duplicate and the results are presented as means.

RESULTS AND DISCUSSION

Theoretical consideration

The pKa values of ascorbic acid are 4.1 and 11.79 (WEAST, 1982). The undissociated (H_2A) and monoionic (HA-) forms of ascorbic acid are mainly present in the solutions in the pH range 3.2 to 6.5. Cupric nitrate was used as the catalyst because among the inorganic anions, copper (II) forms the least stable complex with nitrate. Sulphate and chloride form more stable complexes with copper, though all of them are relatively weak.

The oxidation of ascorbic acid in the presence of an excess of oxygen (saturation) was assumed to follow a first order reaction (KHAN and MARTELL, 1967 a,b; OGATA *et al.,* 1968). The reaction rate can be expressed as

$$d [H_2A] / dt = k [H_2A]$$

where $[H_2A]$ is the concentration of ascorbic acid, t is the time and k is the first-order rate constant which can be calculated from a plot of Log $[H_2A]$ *versus* time. It gives a straight line with a slope - k / 2.303.

Spontaneous and copper (II)- ion - catalyzed oxidation

In order to determine the spontaneous and Cu(II) ion-catalyzed oxidation of ascorbic acid (5.67×10^{-3} M) solutions with copper levels of 0, 3.15×10^{-5}, 7.87×10^{-5}, 15.7×10^{-5} and 31.5×10^{-5} M were studied at 20°C. The reaction was observed for 60 min over the pH range 3.2-6.5 in various buffer solutions. During the first hour the uncatalyzed (no copper present) oxidation of ascorbic acid occurred at a ratio of only 1.5%. The copper - catalyzed oxidation of ascorbic acid with respect to time was evaluated with a compensation for the uncatalyzed oxidation. The first-order rate constants were calculated from the slopes of the straight lines by linear regression analyses. The rate increased as the copper concentration increased at a certain pH in all the buffers except tartrate. Kinetic data for the oxidation of ascorbic acid in citrate buffer are presented in Fig. 1.

Effect of pH on the reaction rate of ascorbic acid

The reaction rate increased as the pH increased to about 5.5 in acetate buffer (Fig. 2). However, it increased with pH to about 4.5 and decreased to pH 6.0 in citrate buffer for all the Cu(II) concentrations (Fig. 3). In phthalate buffer, the reaction rate decreased with increasing pH for the higher concentrations to about 4.5 and increased at pH 6.0 for all of the Cu(II) concentrations (Fig. 4). In tartrate buffer, the reaction rate increased with pH to about 5.5 (Fig. 5).

Effect of Cu(II) concentration on the reaction rate of ascorbic acid

The difference between the first order rate constants in the presence and in the absence of Cu(II) ion was taken as the

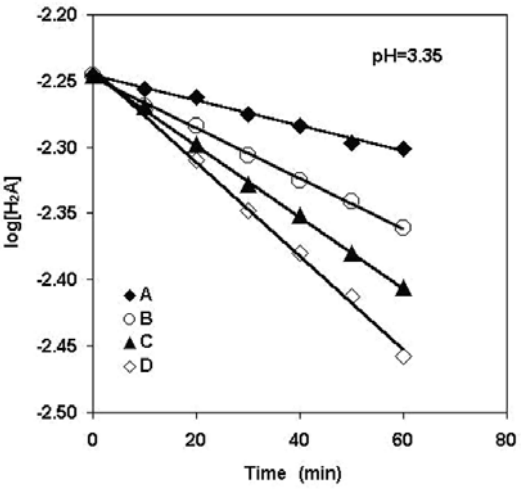


Fig. 1 - Kinetic data for oxidation of ascorbic acid in citrate buffer at different Cu(II) concentrations [(A: 3.15, B: 7.87, C: 15.7, D: 31.5).10^{-5} M].

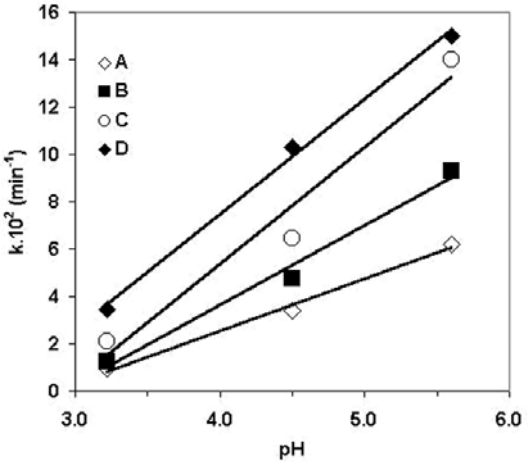


Fig. 2 - Dependence of the rate constant, k, on the pH values in acetate buffer, for the oxidation of ascorbic acid, Cu(II) concentrations [(A: 3.15, B: 7.87, C: 15.7, D: 31.5).10^{-5} M].

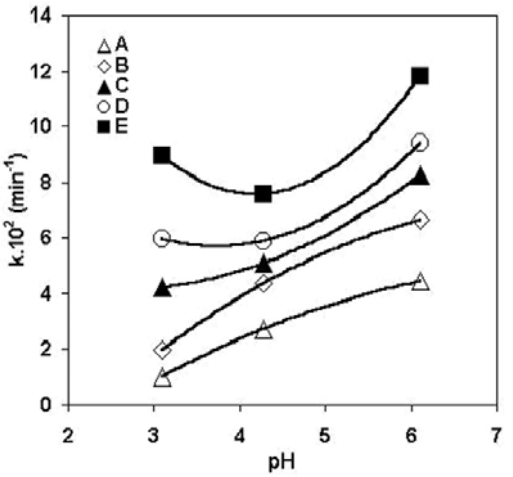


Fig. 4 - Dependence of the rate constant, k, on the pH values in phthalate buffer, for the oxidation of ascorbic acid, Cu(II) concentrations [(A: 3.15, B: 7.87, C: 15.7, D: 23.5, E: 31.5).10^{-5} M].

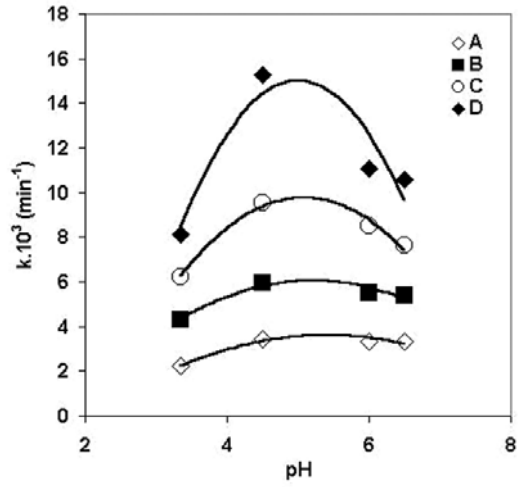


Fig. 3 - Dependence of the rate constant, k, on the pH values in citrate buffer, for the oxidation of ascorbic acid, Cu(II) concentrations [(A: 3.15, B: 7.87, C: 15.7, D: 31.5).10^{-5} M].

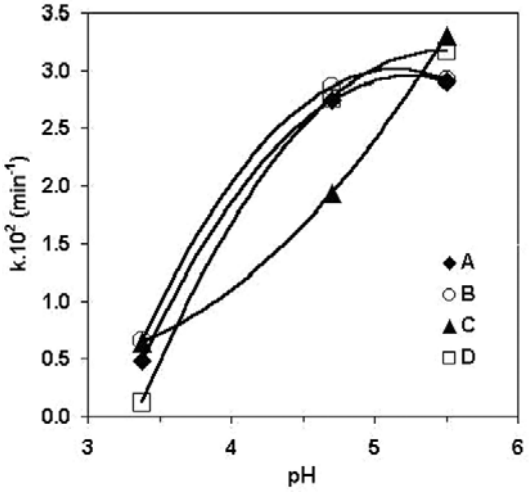


Fig. 5 - Dependence of the rate constant, k, on the pH values in tartrate buffer for the oxidation of ascorbic acid, Cu(II) concentrations [(A: 3.15, B: 7.87, C: 15.7, D: 31.5).10^{-5} M].

rate characteristics. The variations of the rate constant (k_d), which is equal to K[Cu(II)] with respect to the total Cu(II) concentration at various pH values, in acetate buffer are shown in Fig. 6. The specific rate constant could not be calculated for tartrate buffer because a similar relation was not observed. The specific rate constants (K) given in Table 1 were calculated by the linear regression analysis of the plot of k_d versus copper (II) concentration.

The specific rate constants (K) of the Cu(II) ion –catalyzed oxidation of ascor-

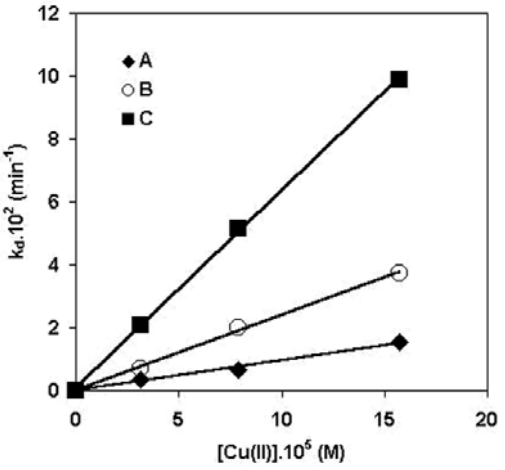


Fig. 6 - Catalytic effect of acetate buffer on the oxidation of ascorbic acid in the presence of Cu(II) ion, k_d = Difference between the first-order rate constants in the presence and in the absence of copper, pH values (A: 3.2, B: 4.5, C: 5.6).

bic acid in citrate buffer were lower than those in phthalate and acetate buffer in the same pH range (Table 1).

The effects of pH, cupric ion concentration and the kind of buffer on the rate of the oxidation reaction of ascorbic acid are as follows:

1) The rate increased as the concentration of Cu(II) increased,

2) The rate increased as pH increased to about 5.5 in acetate and tartrate buffers and the rate also increased with pH to about 4.5 and decreased at pH 6.5 in citrate buffer, the rate increased with pH to about 6.0 in phthalate buffer but at lower copper concentrations ($\leq 23.5 \times 10^{-5}$ M).

In the acetate-buffered solutions over the pH range 3.2-6.0, the specific rate constants (K) increased with pH. The pKa of acetic acid is 4.76 at 25°C (Weast, 1982), and the cumulative stability constants (Log β_n) of Cu – acetate complexes are 2.16 and 3.20 for the mono- and bis- complexes, respectively (INCZÉDY, 1976). The highest reaction rate with Cu(II) - acetate was achieved at pH 5.5 (Fig. 2). However, in the citrate-buffered solutions in the same pH range, K changed with pH, reaching a maximum at pH 4.5. This result is attributed to the differing catalytic effects of the Cu(II)-citric acid chelates formed at varying pH values (WARNER and WEBER, 1953). The main species are $CuHCi^-$ at pH 3.5; $CuHCi^-$ and $CuCi^{2-}$ at pH 4.5 and $CuCi^{2-}$ at pH 6.0. As the pH increases, more stable complexes form and the binding between Cu(II) and citrate anions becomes stronger. Finally, $CuCi^{2-}$ is the most stable chelate, and practically all cupric ions are bound to this complex at pH 6.0. The pKa values of citric acid are $pKa_1 = 3.13$, $pKa_2 = 4.76$ and $pKa_3 = 6.40$. The stability constant (Log β_1) of the mono–complex with HCi^{2-} is 4.35 and of the mono-complex with Ci^{3-} is 14.2. Thus, it is apparent that a partially stable and protonated mono-complex with HCi^{2-} is catalytically more favorable than a very stable and deprotonated copper (II) complex with Ci^{3-} (Fig. 3).

As seen in Table 1, for the phthalate buffered-solutions in the same pH range, the specific rate constants decreased with pH to about 4.5 and then increased at pH 6.0. The pKa values for orthophthalic acid are $pKa_1 = 2.95$ and $pKa_2 =$

Table 1- Specific rate constants (K) of Cu(II) ion – catalyzed oxidation of ascorbic acid in various buffers.

Buffer	pH	$K \pm S_K$ (M^{-1} min^{-1})	r^2
Acetate	3.22	95.04 ± 6.22	0.9915
	4.50	240.7 ± 8.83	0.9973
	5.60	628.2 ± 10.8	0.9994
Citrate	3.35	31.06 ± 2.93	0.9825
	4.50	48.56 ± 0.994	0.9992
	6.00	40.64 ± 1.19	0.9983
	6.50	33.76 ± 2.36	0.9918
Phthalate	3.10	247.4 ± 7.41	0.9977
	4.27	142.9 ± 6.51	0.9850
	6.19	233.6 ± 8.82	0.9750

S_K = Standard deviations of K values, r^2 = regression coefficient.

5.41 and the stability constants (Log β_n) of the corresponding Cu(II) complexes are Log β_1 = 3.46 and Log β_2 = 4.83. It seems that mono- and bis- complexes of phthalic acid with Cu(II) are both catalytically active to different extents (Fig. 4).

The pKa values of tartaric acid are pKa_1 = 3.2 and pKa_2 = 4.8 and the stability constants of copper complexes with n = 1,2,3,4 are (Log β_n): 3.2, 5.11, 4.78 and 6.51, respectively. On the other hand, a hydrolyzed complex with tartrate i.e., $[Cu(OH)_2 tartr.]^{2-}$, had a much higher stability constant of Log K_f = 19.4. As observed with other organic acid buffers, a partly protonated mono- or bis- complex of tartaric acid with Cu(II) should have more catalytic activity than the higher, more stable complexes (Fig. 5).

The corresponding concentrations of free Cu(II) may only be important in the Cu – acetate system where the ratio of free Cu(II) to total Cu(II) is about 1 / 20 for the total Cu(II) ion and buffer concentration range studied. In all the other systems the free Cu(II) concentration was negligible in a solution containing an excess (more than 100 - fold) of a stable complex - forming ligand like citrate.

The use of buffers which interact to various degrees with cupric ions to form complexes has been a complicating factor in many of the previous reports on copper- catalyzed oxidation reactions of ascorbic acid (MILLER and JOSLYN, 1949). Therefore the Cu(II) – buffer chelates formed as a function of pH and their relative stabilities must be taken into account. Two mechanisms have been considered for the Cu(II) chelate-catalyzed oxidation of ascorbic acid: (1) a dissociation mechanism, which may be due to the free Cu(II) ion in equilibrium with the chelate compound or (2) the direct participation of Cu(II) - buffer chelates in an electron transfer process (MARTELL, 1982). If the first hypothetical mechanism prevails, the observed reaction rate should only be a function of Cu(II) dissociated from the Cu – buffer ligand complex, and should not be dependent on the nature of the Cu – chelate involved. Actually, for all of the organic acid buffers studied, the second mechanism, i.e., the direct participation of the Cu(II) - chelate species in the oxidation of ascorbic acid, can be followed as a proposed mechanism in the study by ŞAHBAZ and SOMER (1993).

$$H_2A \leftrightarrow HA^- + H^+ \quad (1)$$

$$HA^- + Cu(II) - L \leftrightarrow HA - Cu(II) - L \quad (2)$$

$$HA - Cu(II) - L \xrightarrow{slow} \bullet AH + Cu(I) - L \text{ [or } Cu(I) + L] \quad (3)$$

$$\bullet AH + O_2 \rightarrow A + HO_2\bullet \quad (4)$$

$$2\,Cu(I) - L + O_2 + 2\,H^+ \rightarrow 2\,Cu(II) - L + H_2O_2 \quad (5)$$

$$2HO_2\bullet \rightarrow H_2O_2 + O_2 \quad (6)$$

where ligand,L, stands for the organic acids used in the buffers.

According to this mechanism the copper chelate with the buffer ligand may form a saturated ternary complex with an ascorbic acid anion (HA^-) which may be converted to the Cu(I) - chelating buffer complex and radical product of ascorbic acid (• AH) in a slow reaction. Following the slow reaction, the lower valence form of the copper complex is rapidly reoxidized in the solution by molecular oxygen and regenerates the Cu(II) to form H_2O_2 with the hypothetical reaction order (YAMAZAKI *et al.*,1960; MCALPINE *et al.* 1972).

It is proposed that copper (I) forms in a series of reactions in a chelated form which is presumably oxidized into the corresponding copper (II) chelate and H_2O_2. In other words, free copper (I) ions may not interact with H_2O_2 in a Fenton - type reaction (DEUTSCH, 1998; LAWRENCE and YUAN, 1996).

CONCLUSION

The chemical role of buffer components on ascorbic acid stability in Cu(II) solutions, under various pH conditions, is of importance for practical applications

in food processing, storage and formulation. The spontaneous and copper (II)-ion catalyzed oxidation of ascorbic acid was studied by varying copper concentrations and pH values in different aerated buffer solutions. The specific first - order rate constants were calculated.

In accordance with the above criterium, since the citrate buffer forms the strongest complexes, it is the least active buffer in copper - induced ascorbic acid oxidation. Between pH 3.1-3.3, phthalate buffer is the most active (i.e.the highest K constant). Because phthalic acid loses a proton around this pH range, it may form an unsaturated complex with copper. Between pH 4.2-6.2, acetate is the most active buffer because starting from pKa = 4.8, acetic acid loses a proton and may form an unsaturated complex with copper. Furthermore, acetate is a monodentate ligand which can not form saturated complexes with copper, so the unsaturated copper to acetate complex binds to ascorbic acid to form an intermediate for oxidation. At pH 4.5, ascorbic acid in phthalate - buffered solutions tended to be protected due to the copper complexing power. Thus the coordinatively unsaturated Cu(II)- buffer complexes which are comparatively less stable than the higher chelates proved to be more active in the Cu(II) - induced oxidation of ascorbic acid.

It is obvious from the data that ascorbic acid stability is dependent on the type of buffer under various pH conditions. Citric acid, commonly present as a stabilizer in fruit juices and beverages, prevented ascorbic acid oxidation. The specific rate constant (K) obtained at pH 6.50 is one of the lowest values in Table 1, showing that the relatively more stable chelate with citrate does not enhance electron transfer.

ACKNOWLEDGEMENT

This research was supported by the Yildiz Technical University Scientific Projects Coordination Department, Istanbul, Turkey. Project Number: 92-B-01-02-01.

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Revised paper received May 5, 2003 Accepted June 27, 2003

PAPER

PHYSICO-CHEMICAL PROPERTIES OF ALMOND SEED PROTEIN CONCENTRATE

PROPRIETÀ FISICO-CHIMICHE DI CONCENTRATI PROTEICI DI SEMI DI MANDORLE

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ABSTRACT

The proximate analysis of almond seed and the physico-chemical characteristics of almond seed oil were found to be within the range of conventional oil seeds, except for a high protein content of 30.13%. The maximum extraction of solubilized and precipitated almond seed protein was found in the defatted sample at a 1:10 flour-solvent ratio using 0.03M NaOH and an extraction time of 20 minutes. Minimum and maximum nitrogen solubility index values were observed around pH 4 and 10,

RIASSUNTO

La composizione dei semi di mandorle e le caratteristiche fisico-chimiche dell'olio estratto sono risultate comprese all'interno dei valori convenzionali degli oli di semi, con l'eccezione dell'elevato contenuto in proteine (30,13%). L'estrazione massima di proteine di semi di mandorle solubilizzate o precipitate è stata rilevata nei campioni preventivamente sgrassati uitlizzando come soluzione estraente NaOH 0,03M per 20 minuti. Gli indici minimi e massimi di solubilità dell'azoto sono stati

- Key words: Almond seed, physico-chemical properties, protein concentrate -

respectively. The protein concentrate had 4.25% moisture, 89.95% protein, 3.15% carbohydrate, 2.15% ash and no trace of fat. Water absorption, oil absorption, foaming, emulsion capacities and least gelation percentages were higher in the protein concentrate (220, 165, 69, 310 and 25%, respectively) than in the seed flour (198, 110, 38, 220 and 14, respectively). Almond seed was found to be a good source of calcium, potassium, magnesium, iron and phosphorous.

osservati rispettivamente a pH 4 e pH 10. Il concentrato proteico ha mostrato un contenuto di umidità pari al 4,25%, di proteine pari all'89,95%, di zuccheri pari a 3,15%, di ceneri pari a 2,15% ed una assenza totale di grassi. L'assorbimento di acqua e di olio, la capacità schiumogena ed emulsionante e le percentuali di gelatinizzazione minime si sono dimostrate più elevate nel concentrato proteico (rispettivamente 220, 165, 69, 310 e 25%) che nella farina (rispettivamente 198, 110, 38, 220 14). Il seme di mandorla si è dimostrato una buona fonte di calcio, potassio, magnesio, ferro e fosforo.

INTRODUCTION

Increase in the world population and a decrease of available high quality proteins have prompted the need for new sources of food proteins. In most developing countries, diets are mainly from cereals with animal proteins used as supplements (OBATOLU and COLE, 2000). The high cost of animal protein has advanced the need for alternative sources of protein, especially from plant sources. In this regard, various kinds of economical, protein-rich plant mixtures are used in various, areas of Africa to improve the nutrition of children (MOSHA and SVANBERG, 1990).

The search for new, economical raw materials for seeds has become one of the most important lines of research in animal nutrition. The idea is to develop feeds that will avoid excessive dependence on a single ingredient or on a few ingredients.

Potato protein concentrate has been reported to be a good replacement for soy protein or powdered milk in feeds designed for pig fattening (URBANCZYCK *et al.*, 1983) and as a partial substitute for milk (CHARLET-LERY *et al.*, 1985) and broiler chicken feeds (HELDER and VERSTEEGH, 1977).

The purpose of this study was to characterize the physico-chemical, functional and nutritional properties of almond seed flour, protein and oil. It is believed that this information will enhance the potential of almond seed as an ingredient in fortification of foods for both human beings and animals.

MATERIALS AND METHODS

Materials

Sun-dried soft-shelled sweet type almond fruits were hand-picked from the ground under the trees at Asero Abeokuta, Ogun State, Nigeria. The dried fruits were manually dehulled and shelled carefully using a carpenter's hammer. The seeds were oven-dried at 110°C for 24 hours, ground and packed in a clean polyethene sack. The proximate analysis, functional properties and mineral composition of the seed flour were carried out before oil and protein were extracted from the seed flour.

Proximate analysis

The proximate analysis of the oven-dried almond seed flour such as moisture, ash, fat and crude protein were carried out according to methods described by AOAC (1990). All analyses were done in triplicate. Mineral composition was determined after wet ashing using an atomic absorption spectrophotometer.

Analysis of oil

The oil was extracted from the dried almond seed flour by the Soxhlet extraction method using n-hexane as solvent. The oil was analysed for free fatty acids, peroxide number, iodine value, saponification value, density and refractive index at 40°C according to AOAC (1990).

Functional properties

The least gelation concentration, water and oil absorption and oil and foaming capacities were determined using the method of XU and DIOSADY (1994), while the emulsion capacity was determined by the method of IGE *et al.* (1984).

Protein solubility

The solubility of the protein over the pH range of 2 to 12 was measured according to the method of OSHODI and EKPERIGIN (1989).

Effect of flour: solvent ratio on protein extraction

The flour sample was dispersed in 0.03M NaOH, Na_2SO_3 and $Ca(OH)_2$, at flour: solvent ratios of 1:50, 1:10 and 1:20, respectively. The suspensions were then blended for 20 min in a Warring blender and centrifuged at 3,000 rpm for 10 min.

Aliquots from the peptized liquor were taken for protein determination using the Kjeldahl method (AOAC, 1990) and results are reported as percentage solubilized protein based on the total protein content of the different flour samples. Protein in the extraction liquor was precipitated at pH 4 by drop-wise addition of 0.1M HCl. The precipitates were removed by centrifuging and the difference between the amount of protein originally present in the dispersion and that left after removal of the precipitate (i.e. in the whey) gave a measure of the amount of protein isolated. The protein isolated is reported as the percentage of the total solubilized protein.

Effect of extractant concentration on protein solubility

Almond flours were extracted with 0.01, 0.02, 0.03 and 0.04M NaOH, Na_2SO_3 and $Ca(OH)_2$ in a Warring blender for 10 min at a flour:solvent ratio of 1:10. Solubilized and precipitated protein was determined.

Effect of extraction time on protein solubility

The flour sample was dispersed in a 0.03M Na0H, Na_2SO_3 and $Ca(OH)_2$ solution at a flour solvent ratio of 1:10. Each suspension was blended in a Warring blender for 10, 20, 30 and 60 minutes. Solubilized and precipitated protein was determined.

Effect of defatting on protein solubility

Oil in the flour sample was extracted with hexane by the Soxhlet method at room temperature and the solvent was removed from the flakes in air. The solvent-free flakes were ground to a fine powder and used as defatted flour.

The defatted seed flour was suspended in 0.03M NaOH, Na_2SO_3 and $Ca(OH)_2$ respectively, at a flour:solvent ratio 1:10 and blended for 20 min in a Warring

blender. Solubilized and precipitated protein was determined.

Preparation of protein concentrate

The protein concentrates were prepared by modifying the method of IGE *et al.*, (1984). Defatted flour (250g) was mixed in distilled water at room temperature at a flour:solvent ratio of 1:10 and the slurry was stirred for one hour. The pH of the slurry was then adjusted to 10 by dropwise addition of 0.03M NaOH. It was then centrifuged at 8,000 rpm for 30 min. The protein in the supernatant was precipitated by drop-wise addition of 0.1M HCl at pH 4.0 and centrifuged at 8,000 rpm for 30 min. The curd obtained was then sun-dried.

RESULTS AND DISCUSSION

Proximate composition of seed flour

The proximate composition of the almond seed flour is presented in Table 1. The sample had a low moisture content (5.88%) making it not susceptible to attack by microorganisms. The value is in agreement with those reported by IGE *et al.* (1984) for *C. vulgaris* (8.23%), by PADILLA et al. (1996) for soybean (8%) and by PRINYAWIWATKUL et al. (1993) for peanut (7.27-8.67%). But, JEREMIAH (1992) reported a higher value of 20.50% for almond fruit (which enclosed the seed). The ash content (6.66%) was within the general range for legumes. The low ash content makes the seeds suitable for human consumption and most especially for animal feed.

The high oil content of 47.70% is comparable to oil seeds such as palm oil fruit and palm kernel 47-56% (HARTLEY, 1967). The high contents of crude protein (30.13%) and carbohydrate (9.63%) suggest that both protein and carbohydrate can be extracted from almond seed for both human and animal feed.

Table 1 - Proximate analysis (%) of almond seed flour.

Moisture	5.88 ± 0.07
Fat	47.70 ± 0.31
Crude Protein*	30.13 ± 0.12
Carbohydrate (by difference)	9.63
Ash	6.66 ± 0.09

* conversion factor 6.25
determinations were done in triplicate

Physico-chemical analysis of oil

The physico-chemical parameters of almond seed oil are given in Table 2. The refractive index (1.46) and density (0.92) of almond seed oil fall within the recommended standard for oils (EGAN, 1981). These values are comparable to those of palm oil that has a refractive index of 1.44 and density of 0.89 (HARTLEY, 1967).

The saponification value of 184 is similar to that of palm oil (199) and groundnut oil (192) (EGAN, 1981). This value implies that almond oil can be used for the production of soap.

The iodine value of 96.83 is comparable to that of groundnut oil (98.0) (EGAN, 1981). This indicates that almond seed oil has a nutritional value, unsaturated fatty acid content and oxidative stability similar to groundnut oil. The rancidity of the crude almond seed oil was measured in term of its peroxide number which was found to be 4.0. This value is very close to that of crude palm oil (3.80) (EGAN, 1981). This implies that crude almond oil and palm oil would have the same shelf-life, probably due to the presence of similar natural antioxidants.

The probability of crude almond seed oil undergoing rancidity is very low due to the lower amount of free fatty acid (0.42%) (Table 2). Crude palm oil has a higher amount of free fatty acid (4.0%) (ASHRI, 1989) than that of crude almond seed oil, but soybean oil and maize oil

Table 2 - Physico-chemical characteristics of crude almond seed oil.

Refractive Index at 40°C	1.46 ± 0.01
Density (at 40°C/ water at 30°cg/cm^3	0.92 ± 0.10
Saponification value (mg KOH/g)	184 ± 1.23
Iodine value (mg of KOH of oil)	96.83 ± 2.01
Peroxide number (meq. O_2/kg)	4.00 ± 1.53
Free Fatty Acid (% Oleic)	0.42 ± 0.02
Acid Value (mg OH/g)	0.83 ± 0.03
Determinations were done in triplicate.	

have similar low free fatty acids, 0.7% and 0.03%, respectively (SALUNKHE *et al.*, 1992).

Protein solubility

Almond seed protein had the lowest solubility at pH 4 (16%) and a maximum at pH 10 (98%) (Table 3). The low protein solubility at pH 4 suggests that the protein recovery process in some way altered the native structure of the almond seed protein. This may be explained by the alkaline (pH 10) conditions used in the recovery process. At this high pH, the repulsive forces within the protein molecules are large and cause the molecules to open up and unfold. Similar results were found by GOMEZ-JUAREZ *et al.* (1999) for globin protein (pH 4 and pH 12 for minimum and maximum solubility, respectively).

Table 3 - Protein solubility of almond seed with respect to pH.

pH	%
2	20
4	16
6	50
8	70
10	98
12	96
14	94

Protein extractability

The effect of the flour-solvent ratio on the solubilization and precipitation of proteins from almond seed flour is shown in Table 4. The highest amount of protein was solubilized and precipitated at the 1:10 ratio for all the solvents used. The solvent concentration of 0.03M was the best concentration and sodium hydroxide was the best solvent (Table 5). The best extraction time was 20 min for all the solvents (Table 6).

The defatted flour sample gave the highest yield of solubilized and precipitated protein (Table 7). In summary, the maximum solubilization and precipitation of almond seed protein was achieved with the defatted sample at a flour-solvent ratio of 1:10 using 0.03M Na0H and an extraction time of 20 minutes.

Proximate composition of seed protein concentrate

The proximate composition of almond seed protein concentrate is shown in Table 8. The protein concentrate had 4.25% moisture content, no trace of fat, 89.95% protein, 3.15% carbohydrate and 2.15% ash content. The protein concentrate had lower moisture, carbohydrate, ash and fat but higher protein content when compared with almond seed flour (Table 1).

Functional properties

Functional properties of almond seed flour and protein concentrate are given in Table 9. Water and oil absorption capacity of the protein concentrate was higher than the seed flour (Table 9). The water absorption capacity of the almond seed flour (198%) and protein concentrate (220%) were lower than what has been reported for soya protein (461%) (XU and DIOSADY, 1994) and soybean meal (301.3%) (MACZK *et al.*, 1985). But water absorption capacities of melon seed

Table 4 - Effect of flour-solvent ratio on the solubilization and precipitation of proteins from almond seed flour.

Solvent	Flour-Solvent Ratio	Protein solubilized (%)	Whey proteins (%)	Protein precipitated (%)
0.03M NaOH	1:50	70.32	23.61	76.39
	1:10	79.41	10.39	89.61
	1:20	76.83	16.15	83.85
0.03 M Na_2SO_3	1:50	71.78	22.20	77.80
	1:10	76.43	15.72	84.28
	1.20	72.62	20.50	79.50
0.03M Ca $(OH)_2$	1:50	69.19	29.17	70.83
	1:10	75.56	19.87	80.13
	1:20	71.38	21.81	78.19

Table 5 - Effect of extractant concentration on the solubilization and precipitation of proteins from almond seed flour.

Solvent	Concentration	Protein solubilized (%)	Whey proteins (%)	Protein precipitated (%)
NaOH	0.01M	66.54	29.75	70.25
	0.02M	70.54	14.10	75.90
	0.03M	79.41	10.39	89.61
	0.04M	75.80	17.16	82.84
Na_2SO_3	0.01M	67.29	29.85	70.15
	0.02M	70.14	21.65	78.35
	0.03M	76.43	15.72	84.28
	0.04M	71.81	19.78	80.22
$Ca(OH)_2$	0.01M	64.80	37.30	68.70
	0.02M	68.21	29.90	70.10
	0.03M	75.56	19.87	80.13
	0.04M	72.90	21.48	78.52

flour (120%), pumpkin seed flour (70%) and gourd seed flour (100%) (OLAOFE *et al.,* 1994) were lower than almond seed flour and protein concentrate. The higher water and oil retention capacities of the protein concentrate of almond seed may be due to an increase in the number of hydrophobic groups on the proteins.

The foaming and emulsion capacity of the protein concentrate was higher than that of the seed flour. This implies that the protein concentrate of almond seed can be used to promote oil-in-water emulsions.

The protein concentrate (25%) had a higher least gelation percentage with respect to seed flour (14%) which may be due to the presence of oil in the seed flour (Tables 1 and 8). BURGESS and KELLY (1979) reported that the presence of lip-

Table 6 - Effect of extraction time on the solubilization and precipitation of protein from almond seed flour.

Solvent	Time (minutes)	Protein solubilized (%)	Whey proteins (%)	Protein precipitated (%)
0.03M NaOH	10	69.87	29.16	70.84
	20	79.20	10.45	89.55
	30	75.35	19.58	80.42
	60	73.40	22.69	77.31
0.03M Na_2SO_3	10	71.45	19.89	80.11
	20	76.40	15.69	84.31
	30	70.62	22.76	77.24
	60	68.52	29.77	70.23
0.03M $Ca(OH)_2$	10	71.63	29.55	70.45
	20	75.47	19.80	80.20
	30	72.82	22.78	77.22
	60	70.14	30.10	69.90

Table 7 - Effect of flour treatment on the solubilization and precipitation of protein from almond seed flour.

Solvent	Treatment	Protein solubilized (%)	Whey proteins (%)	Protein precipitated (%)
0.03M NaOH	Undefatted	79.41	10.39	89.61
	Defatted	85.67	7.78	92.22
0.03M Na_2SO_3	Undefatted	76.43	15.72	84.23
	Defatted	80.83	12.45	87.55
0.03M $Ca(OH)_2$	Undefatted	75.56	19.87	80.13
	Defatted	79.94	17.88	82.12

id may reduce the ability of protein to gel due to competition of the lipid components for the hydrogen bonding sites involved.

Mineral composition

The mineral composition of the almond seed flour is presented in Table 10. The almond seed contained appreciable amounts of potassium, calcium, phosphorus, iron, magnesium and copper. The bio-availability of these minerals is important as it may be influenced by their interaction with other food constituents. Almond seed has been shown to be richer in sodium, potassium, phosphorus and magnesium than common legume seeds such as soya beans (TEMPLE *et al.,* 1991). Africanyan bean (ENE-

Table 8 - Proximate composition (%) of almond seed protein concentrate.

Moisture	4.25 ± 0.04
Fat	ND
Crude Protein*	89.95 ± 0.20
Carbohydrate (by difference)	3.15 ± 0.02
Ash	2.15 ± 0.04

* conversion factor 6.25
determinations were done in triplicate.

Table 9 - Functional properties of almond seed flour and protein concentrate.

	Seed flour	Protein concentrate
Water Absorption Capacity (%)	198	220
Oil Absorption Capacity (%)	110	165
Foaming Capacity (%)	38	69
Emulsion Capacity (mL oil/g)	220	310
Least gelation (%)	14	25

Determinations were done in triplicate.

Table 10 - Mineral composition of the almond seed flour based on dry weight of sample.

Mineral	mg/100 g
Ca	176
K	476
Mg	104
Na	3.80
Fe	1.80
Cu	1.60
P	378
Zn	ND
Pb	1.50
Cd	ND
Cr	ND
Co	1.60
Mn	3.90

Determinations were done in triplicate.

OBONG and CARNOVALE, 1992) and melon seed (BADIFU and OGUNSUN 1991). But all these legume seeds contained more calcium, iron and zinc. However, caution should be exerted in the consumption of almond seed because of the presence of some heavy metals (toxicological metals) such as lead (1.50 mg/100 g), cobalt (1.60 mg/100 g) and manganese (3.90 mg/100 g).

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Revised paper received December 16, 2002 Accepted February 12, 2003

PAPER

MONITORING OF PYRETHROID RESIDUES IN SARDINIAN HONEY BY SOLID PHASE EXTRACTION AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

MONITORAGGIO DI RESIDUI DI PIRETROIDI IN MIELE SARDO CON ESTRAZIONE IN FASE SOLIDA E CROMATOGRAFIA LIQUIDA AD ALTA PRESTAZIONE

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ABSTRACT

A rapid, economical and simplified multi-residue method, involving an extraction step and further HPLC analysis is described for the determination of fenpropathrin, cyfluthrin, deltamethrin and permethrin residues in honey. The method was tested on 74 honey samples, produced in Sardinia during 2000-2001. Pyrethroids were isolated from the matrix by means of solid-phase extraction (SPE) with octadecylsilane tandem-florisil cartridges. Recoveries of the pyrethroids at four dif-

RIASSUNTO

Viene descritto un metodo multiresiduo rapido, semplice ed economico che consiste in una estrazione e successiva analisi in HPLC per la determinazione di residui di fenpropathrin, cyfluthrin, deltamethrin e permethrin nel miele. Il metodo è stato applicato per l'analisi di 74 campioni di miele, prodotti in Sardegna durante gli anni 2000-2001. I residui furono isolati dalla matrice mediante estrazione in fase solida (SPE) utilizzando un tandem di cartucce di octadecylsilane e florisil. Il

- Key words: honey; HPLC analysis, pyrethroids; SPE -

ferent concentrations ranged from 93.6 to 99.2%. The estimated limits of detection ranged from 0.2 to 1.6 μg kg^{-1} and limits of quantification ranged from 0.6 to 5.2 μg kg^{-1}. The stability of the four pyrethroids and their isomers was investigated on a multifloreal honey sample. The pesticides had a different degree of persistence in this matrix after 10 months. Traces of pyrethroid pesticides were detected in only four samples and their concentrations were lower than the limit of quantification. No residues were found in the other samples.

range dei recuperi dei piretroidi a quattro diverse concentrazioni fu dal 93,6 al 99,2%. Il limite pratico di determinazione del metodo fu compreso tra 0,2 a 1,6 μg kg^{-1} e i limiti di quantificazione erano compresi tra 0,6 e 5,2 μg kg^{-1}. La stabilità dei quattro piretroidi e dei loro isomeri è stata valutata su un campione di miele multifloreale. I peiretroidi mostravano un differente grado di persistenza in questa matrice dopo 10 mesi. Tracce di insetticidi piretroidi furono trovate solo in quattro campioni, in concentrazione al disotto del limite quantificabile. Negli altri campioni non furono trovati residui.

INTRODUCTION

Bluetongue (BT) is an insect-spread disease of wild and domestic ruminants (mainly sheep, goats and cows). Insects of the *Culicoides* genus are the main virus vectors of BT in Mediterranean countries (SULIS *et al.*, 2000).

In Sardinia (Italy) large quantities of pyrethroids were used to eliminate these pests in 2000 and 2001. Synthetic pyrethroids are exstensively used in agricultural production and veterinary use because of their good efficacy against a wide spectrum of pests. In particular, they have a selective toxicity towards insects and are effective at low doses. Furthermore they have low environmental persistence and are quite stable upon exposure to air and light (TOMLIN, 1994). In a formulation they may be present as a mixture of two isomers or as a mixture of several stereo- and optical isomers (BLAB and WEILER, 1990).

Several methods have been published for the multi-residue determination of pyrethroids in agricultural products (CHEN and WANG, 1996; DI MUCCIO *et al.*, 1997; PANG *et al.*, 1994, 1995a,b). ZUCCARI BISSACOT and VASSILIEFF (1997) determined them in biological matrices such as cow milk and blood and ARREBOLA *et al.* (1999) detemined them in human urine. DI MUCCIO *et al.* (1999), LENTZA RIZOS *et al.* (2001) and RAMESH and BALASUBRAMANIAN (1998) determined phyrethroids in fatty materials of plants and animals and CHEN and WANG (1996) and LEE *et al.* (2002) detemined them in environmental matrices such as water and soil. They have been found in apiarian products, honey and beeswax, mainly as a consequence of acaricide treatments, because pyrethroid acaricides, such as fluvalinate, acrinathrin and flumethrin are used worldwide to control the honeybee parasite *Varroa jacobsoni* (BERNAL *et al.*, 2000; COSSU *et al.*, 2001; FERNÁNDEZ MUIÑO and LOZANO, 1993; FRISON *et al.*, 1999; KORTA *et al.*, 2001).

Acaricide residues in honey are usually extracted by treating the sample with an organic solvent or in solid phase by passage through an octadecylsilane cartridge (BERNAL *et al.*, 2000; MARTEL and ZEGGANE, 2002; IMPARATO *et al.*, 2000) after dilution of the honey sample with water or by supercritical fluid extraction (SFE) (ATIENZA *et al.*, 1993) or solid phase

micro-extraction (SPME) (JIMÉNEZ *et al.*, 1998a). The extract can be subjected to a clean-up step using an octadecylsilane (LODESANI *et al.*, 1992; LEE *et al.*, 2002; van der HOFF, 1996) or florisil column (FERNÁNDEZ MUIÑO *et al.*, 1995; GARZIA *et al.*, 1996; JIMENEZ *et al.*, 1998b), liquid-liquid partitioning (LENTZA RIZOS *et al.*, 2001; JIMENEZ *et al.*, 1996) or thin layer chromatography (CHEN and WANG, 1996).

Depending on the nature and number of the pesticides to be determined, the extract is commonly analyzed by gas chromatography (GC) (ARREBOLA *et al.*, 1999; BERNAL *et al.*, 2000) or high–performance liquid chromatography (HPLC) (ATIENZA *et al.*, 1993; COSSU, *et al.* 2001; GOMIS *et al.*, 1996; JIMENEZ *et al.*, 2000; KORTA *et al.*, 2001; MARTEL and ZEGGANE, 2002).

In the years 2000-2001, environmental contamination could have taken place in Sardinia because of the widespread use of pyrethroids to control *Culicoides imicola*. Fenpropathrin, cyfluthrin, deltamethrin and permethrin were the formulations mainly used.

European norms allow the use of flumethrin and fluvalinate as apiarian acaricides without a maximum allowable limit in the honey. These norms however do not take into account the environmental contamination of the pyrethroids in honey, so their allowable level in this food could be the generic maximum limit of 10 $\mu g\ kg^{-1}$, according to Italian Regulations (O.M., 1985).

Some studies have demonstrated the stability of pyrethroids in acidic media such as honey (KORTA et. al., 2001; TSIGOURI *et al.*, 1997; TACCHEO and DE PAOLI, 1994), which is frequently used as an indicator of environmental contamination. Therefore, in this study a simple multiresidual method was evaluated to determine fenpropathrin, cyfluthrin, deltamethrin and permethrin in Sardinian honeys. Their degradation in this matrix was monitored in order to evaluate if the intensive use of these pyrethroids contaminated the honey and if some factors such as the honeybee mortality owing to intensive treatments, or the periods of disinfestations (September-October) of *Culicoides* or the environmental persistence of pyrethroid pesticides, could affect honey contamination. Only a few reports have been found on multiresidues of pyrethroids in honey (IMPARATO *et al.*, 2000; JIMÉNEZ *et al.*, 1998a,b). In these methods, GC-ECD and GC-MS analyses were used after solid phase extraction (SPE) or solid phase micro-extraction (SPME).

One problem with the use of gas chromatography is that pyrethroid pesticides decompose readily when heated, so they were quantified by determining their degradation products by GC-MS (FITCH *et al.* 1988; VALVERDE *et al.*, 2001). The degradation resulting from the thermal lability of the analyte can be overcome by using high-performance liquid chromatography (usually with UV detection), although the sensitivity achieved by HPLC is somewhat lower than that with GC (ATIENZA *et al.*, 1993; VALVERDE *et al.*, 2001; ZUCCARI BISSACOT and VASSILIEFF, 1997).

In the present work, a modified method (COSSU *et al.*, 2001) is proposed. The procedure involves loading of a honey sample on an octadecylsilane packed cartridge which is subsequently eluted with methanol and then purified on a florisil cartridge. An HPLC-UV procedure was used to analyse the pyrethroids. The method was verified on 74 unrefined (without heating and/or filtration) honey samples, produced in different areas of Sardinia in 2000-2001.

MATERIALS AND METHODS

Reagents and apparatus

The reference pyrethroid standards (fenpropathrin, cyfluthrin, deltamethrin and permethrin) were obtained from Dr.

Ehrenstorfer (Augsburg, Germany). The certified purity was 91-99%.

Residue analysis-grade methanol, acetonitrile and acetone were supplied by Riedel-deHaen (Milan, Italy). The solvents used for chromatography, were ultra pure-water prepared with the MilliQ R4 system Millipore (Milan, Italy) and acetonitrile Chromasolv used for gradient elution in HPLC, obtained from Riedel-deHaen (Milan, Italy). Membrane filters (0.45 μm pore size) from Millipore were used to filter of the mobile phase and 0.45 μm nylon filter units were used for the samples (Alltech, Milan, Italy).

ODS Sep-Pak cartridges of 500 mg (Alltech) and dried florisil (60–100 mesh) cartridges of 350 mg obtained from Carlo Erba (Milan, Italy) were used to purify the samples using an Alltech vacuum manifold.

A rotary evaporator equipped with a thermostated water bath and a vacuum system was supplied by Buchi (Plawil, Switzerland).

Preparation of standards and samples

For each pyrethroid insecticide, a stock standard solution (500 μg L^{-1}) was prepared by dissolving 50.0 mg of the pyrethroid in acetonitrile and diluting it to 100 mL in a volumetric balloon; the standard solutions were stored in a refrigerator. Working standard solutions were prepared by diluting the stock solutions to obtain final concentrations ranging from 25 to 500 μg L^{-1}. The solutions could be stored at 5°C for one week.

Monofloral and multifloral honey samples (74) produced in different areas of Sardinia (Fig. 1) during 2000 and 2001 were analyzed. The samples, purchased from beekeepers and local markets, were stored at 5°C.

Solid-phase extraction of samples

Five grams (prec. 0.001 g) of honey were mixed with 10 mL of a water/acetone (1:1) mixture and shaked for 5 min. Simultaneously, an ODS cartridge was conditioned by eluting, 10 mL of water, using gentle suction with vacuum. The sample was then eluted at a rate of about 0.5 mL/min and washed with 6 mL of water. The cartridge was dried with nitrogen for about 15 min. Finally, the cartridge was eluted by gravity with 2 mL of methanol after soaking for 5 min. The sample was evaporated to dryness in a rotary evaporator at 40°C under vacuum and then reconstituted with 0.5 mL of acetonitrile and purified in a florisil cartridge (350 mg), treated with 6 mL of acetonitrile. After elution and washing with acetonitrile (2x0.5 mL), the sample was evaporated to dryness and the residue was dissolved in 500 μL of acetonitrile and filtered through a 0.45 μm pore-size nylon filter.

The residue extracts, stored at –10 °C, were stable for 3 days.

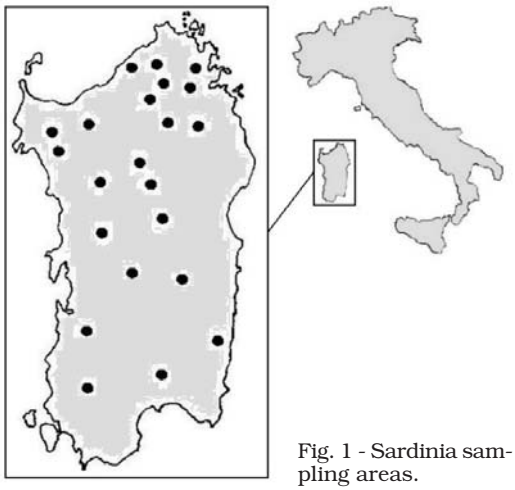


Fig. 1 - Sardinia sampling areas.

HPLC apparatus and analysis

The following instruments were used: Hewlett–Packard 1050 series quaternary pump, variable-wavelength UV–Vis spectrophotometer detector and auto sam-

pler. The peak areas determined with a 3390 integrator were used for quantification (Hewlett–Packard Waldbronn, Germany).

The chromatographic separation was performed using an ODS Adsorbosil 5 μm column, 150 mm, 3.2 mm I.D. stationary phase, preceded by a Nucleosil RP-18 5 μm guard column, (Alltech, Milan). Twenty microliters of extracted samples or calibration standards were directly injected onto the column and eluted with a gradient made of acetonitrile (A) and water (B). Zero-time conditions were A–B (55/45). After 20 min, the gradient was adjusted to A–B (80:20) until termination of the run at 30 min., with a constant flow rate of 1 mL/min at room temperature. The system was equilibrated for 15 min using the starting conditions before injecting the next sample. Detection was carried out by monitoring the absorbance signals at 210 nm (0.1 AUFS) which was the best wavelength for the simultaneous determination of the four pyrethroids. At the end of each working day, the column was washed with the zero-time solvent mixture. Mobile phases were filtered with a 0.45 μm pore size nylon membrane filter and degassed before use.

Validation

The validation procedure, parameters and acceptance criteria were based on USP 23 (1995) guidelines and recommendations in the literature (QUATTROCCHI *et al.*, 1992; PRIETO *et al.*, 2003). The method was validated regarding accuracy and precision, concentration range, linearity, limit of detection (LOD) and limit of quantification (LOQ). Determination of limits of detection (LOD) and quantification (LOQ) were performed according to the following equations: $LOD = (Y_{bl} + 3\ S.D._{bl})/b$ and $LOQ = (Y_{bl} + 10\ S.D._{bl})/b$, where Y_{bl} is the blank response calculated as the intercept of the linear regression curve, $S.D._{bl}$ is the standard deviation of the blank response calculated as the intercept of the curve and b is the slope of the calibration curve. The LOD is defined as the lowest analyte concentration in a sample that can be detected, but not necessarily quantified, by the method of analysis. The LOQ is defined as the lowest analyte concentration in a sample that can be determined under the stated operating conditions of the method. Repeatability of the method was studied by preparing six separate samples, each of which was analysed once. Intermediate precision was studied by preparing the samples on six separate days. The short-time repeatability of the detector was confirmed by calculating the pooled standard deviation of the peak areas.

To verify matrix interference with standard peak resolution, matrix-matched calibration standards were prepared by adding the pyrethroids to blank extracts. Sensitivity and linearity of the analyte detector response were examined by injecting either matrix-matched standards at five concentrations in the range 25.0 to 500.0 μg L^{-1} or corresponding standard solutions at the same concentrations in order to evaluate the possible differences related to the matrix interferences in the detector response. The accuracy and precision of the method were assessed by determining five replicate recoveries at each of the four fortification levels ranging from 5.0 to 50.0 μg kg^{-1}.

Stability of pyrethroids in honey

The stability of the four pyrethroids was investigated on a multifloreal honey sample spiked with 50 μg kg^{-1} of each insecticide put in closed glass containers and kept at room temperature without direct exposure to sunlight. Three portions of honey were collected at regular intervals, extracted and injected into the HPLC system in triplicate to obtain an average value.

RESULTS AND DISCUSSION

Calibration graphs for each compound were constructed by plotting concentration against peak area. Good linearities were achieved in the range 25.0-500.0 μg L^{-1} with correlation coefficients between 0.999 and 0.998. Under the optimum conditions, estimated limits of detection ranged from 0.2 to 1.6 μg kg^{-1} and limits of quantification ranged from 0.6 to 5.2 μg kg^{-1} for all four pyrethroids in the extracts, obtained from a honey sample of 5.0 g and a final volume of 0.5 mL (Table 1).

The recovery of the four pyrethroids was checked by adding 0.5 mL of each acaricide standard solution at different concentrations (from 5.0 to 50.0 μg kg^{-1}) to untreated samples of honey. The pesticides were extracted, cleaned up and analysed by HPLC–UV under the optimal conditions as described in materials and methods. Five repetitions were carried out for each fortification level. Blank samples from the same honey without fortification were treated, analysed and compared with spiked honey samples. A good recovery yield was obtained for each compound tested (Table 2) with a range from 93.6 to 99.2%. In detail, the averages were: fenpropathrin (98.2%), cyfluthrin 1 (93.8%), cyfluthrin 2 (97.5%), deltamethrin (97.3%), permethrin 1 (98.5%) and permethrin 2 (97.6%) and the relative standard deviations (RSDs) varied from 0.6 to 7.2%.

To evaluate repeatability, a honey sample spiked with a mixture of four pyrethroids at a concentration of 20 μg kg^{-1} was extracted six times; for each average amount found, the relative standard deviation (RSD) ranged from 1.2 to 2.8%.

Relatively short retention times ranging from 16.2 to 20.8 min. for the detection of the four pyrethroids and their isomers (Fig. 2), were obtained using the selected chromatographic conditions.

Quantification was carried out using the calibration curves of pyrethroid standards in acetonitrile because the efficiency of the modified procedure of extraction and clean-up (C_{18} tandem-florisil) was satisfactory. There were slight differences between the conventional calibration and the calibration with matrix-matched standards; in fact there were no interfering peaks on the chromatogram, at the same retention time interval as the pyrethroid standard solutions. Comparison between Figs. 3 and 4 demonstrates the importance of C_{18}-florisil clean up regarding the improvement in chromatographic peak resolution.

On the contrary, the use of only C18 cartridges did not sufficiently reduce the

Table 1 - Retention times (Rt), retention factor (K), range of concentrations, coefficients of linearity (r^2), limits of detection (LOD) and limits quantification (LOQ) of the four pyrethroids.

	isomers (medio)	Rt μg/L	K[a] μg/L	Range*[,b] μg/L	r^2	LOD[c]	LOQ[c]
Fenpropathrin	1	16.2	20.6	25.0 – 500.0	0.999	1.6	5.2
Cyfluthrin	1	16.8	21.4	"	0.999	0.4	1.3
Cyfluthrin	2	17.3	22.1		0.998	0.8	2.8
Deltamethrin	1	18.4	23.5	"	0.999	0.2	0.6
Permethrin	1	19.4	24.9	"	0.999	0.4	1.4
Permethrin	2	20.8	26.7		0.998	1.5	5.1

* expressed as of sum of the isomers; [a] K=(Rt-To)/To, (To=Retention time of solvent front); [b] expressed in micrograms/liter; [c] expressed in micrograms/kg of honey

Table 2 - Recoveries of four pyrethroids and their isomers in a honey sample at different fortification levels using an SPE RP-C_{18} tandem florisil column (n=5).

Compound	Added µg/kg	Found ± SD[a] µg/kg	Average recovery ± RSD[b]
Fenpropathrin	5.0	4.90 ± 0.2	98.01 ± 4.7
Fenpropathrin	10.0	9.88 ± 0.5	98.81 ± 5.7
Fenpropathrin	20.0	19.70 ± 1.1	98.50 ± 5.7
Fenpropathrin	50.0	48.83 ± 1.3	97.66 ± 2.6
Cyfluthrin 1	2.92	2.75 ± 0.2	94.18 ± 5.7
Cyfluthrin 1	5.84	5.46 ± 0.2	93.57 ± 3.9
Cyfluthrin 1	11.68	10.94 ± 0.8	93.68 ± 7.2
Cyfluthrin 1	29.20	27.34 ± 1.0	93.63 ± 3.7
Cyfluthrin 2	2.08	2.03 ± 0.1	97.58 ± 6.0
Cyfluthrin 2	4.16	4.07 ± 0.2	97.79 ± 4.2
Cyfluthrin 2	8.32	8.12 ± 0.2	97.55 ± 2.2
Cyfluthrin 2	20.80	20.23 ± 0.1	97.25 ± 0.7
Deltamethrin	5.0	4.82 ± 0.2	96.49 ± 4.9
Deltamethrin	10.0	9.77 ± 0.2	97.68 ± 1.9
Deltamethrin	20.0	19.73 ± 0.4	97.63 ± 1.8
Deltamethrin	50.0	48.72 ± 0.3	97.44 ± 0.6
Permethrin 1	1.96	1.94 ± 0.1	99.20 ± 3.8
Permethrin 1	3.92	3.84 ± 0.3	97.85 ± 7.4
Permethrin 1	7.84	7.75 ± 0.3	98.86 ± 3.3
Permethrin 1	19.60	19.24 ± 0.8	98.17 ± 4.1
Permethrin 2	3.04	2.99 ± 0.1	98.25 ± 1.6
Permethrin 2	6.08	5.95 ± 0.2	97.79 ± 3.8
Permethrin 2	12.16	11.81 ± 0.2	97.14 ± 2.0
Permethrin 2	30.40	29.58 ± 0.6	97.29 ± 2.1

[a] Standard deviation; [b] Relative standard deviation

influence of the matrix on the chromatogram. In this case, a matrix-matched standard calibration may be required to reduce the influence of the matrix in the quantitative analysis.

Fig. 5 shows the degradation (evaluated over 10 months) of four pyrethroids and their isomers in a honey sample spiked with 50 µg kg^{-1} of each insecticide. The degradation percentages were determined with respect to the measured initial concentration. Data showing the decrease of residues in a spiked honey sample are reported in Table 3. It is clear that the degradation of residues in a honey

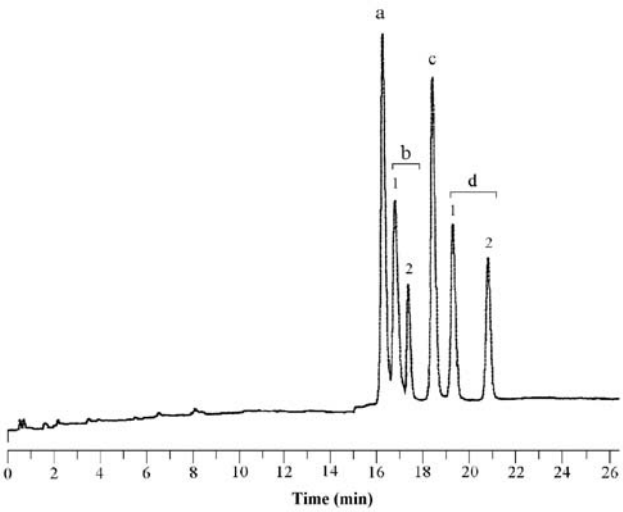


Fig. 2 - HPLC chromatogram of standards of the four pyrethroids and their isomers: (a) fenpropathrin, (b, 1-2) cyfluthrin, (c) deltamethrin and (d, 1-2) permethrin.

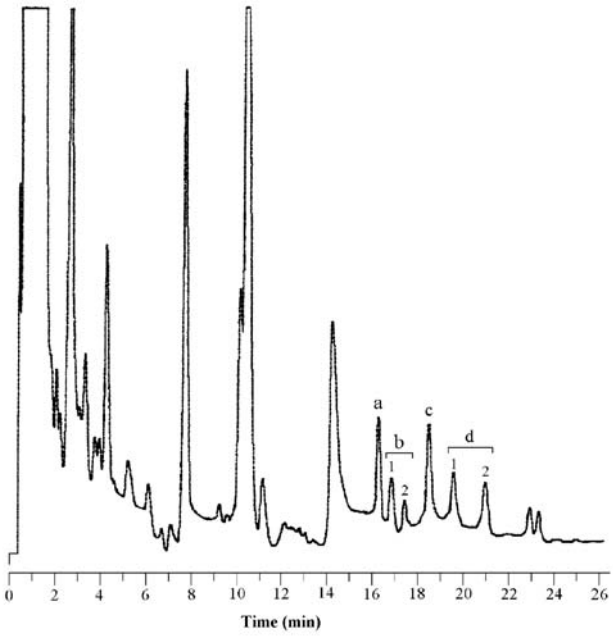


Fig. 3 - HPLC chromatogram of the four pyrethroids and their isomers in a spiked honey sample (20 µg/kg) obtained after the SPE octadecylsilane cartridge extraction procedure.

sample after ten months was the greatest for permethrin followed by deltamethrin, while that of cyfluthrin and fenpropathrin was less. Very little information is available on the persistence of pyrethroids in honey. KORTA *et al.* (2001), KUBIK *et al.* (1996), TACCHEO and DE PAOLI (1994) and TSIGOURI *et al.* (1997) reported a similar stability and persistence for fluvalinate and flumethrin (used in beehives as acaricides) in honey over nine months.

The column performance was checked periodically during the analyses of the 74 samples by injecting the pyrethroid standard solutions in the HPLC, either in organic solvent or in a blank extract at the same concentration. The resolution of the pyrethroid peaks and the LOD and LOQ values did not change during the sample analyses.

The results of the analysis of 74 honey samples collected during 2000-2001 from beehives from different areas of Sardinia (Italy) showed that fenpropathrin and deltamethrin were not present in amounts above the limit of determination. Four samples contained traces of cyfluthrin and permethrin below their limits of quantification and always below the maximum allowable limit (10 µg kg^{-1}), established by Italian Regulations (O.M., 1985).

The results demonstrate that the intensive treatments with pyrethroid insecticides used to combat *Culicoides imicola* in Sardinia did not contaminate the honey samples. These re-

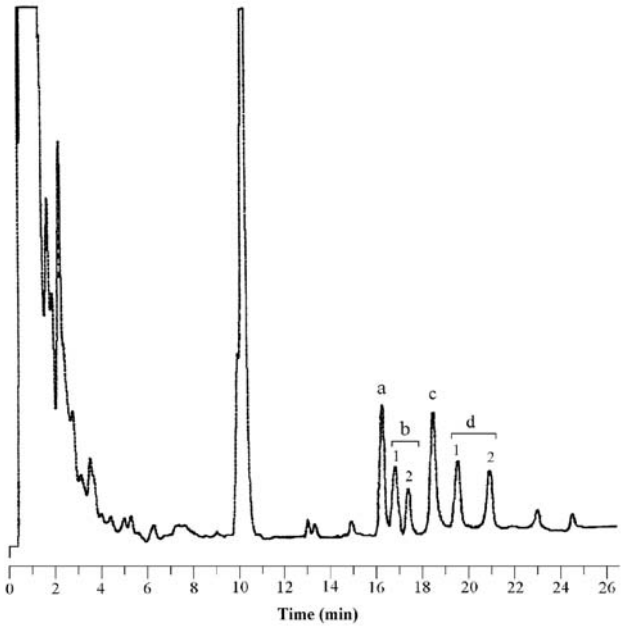


Fig. 4 - HPLC chromatogram of the four pyrethroids and their isomers in a spiked honey sample (20 µg/kg) obtained after the SPE octadecylsilane tandem-florisil cartridge extraction procedure.

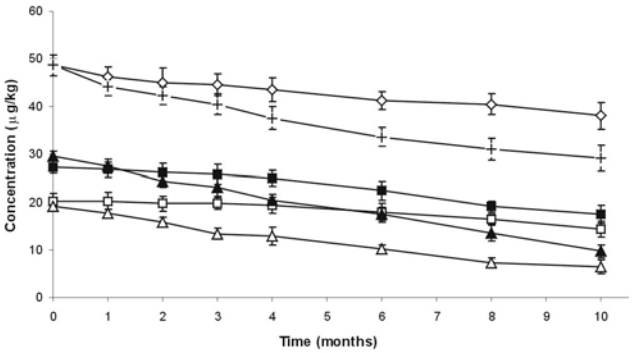


Fig. 5 - Degradation of the four pyrethroids and their isomers in a honey sample spiked with 50 μg/kg of each insecticide evaluated (n=3) over a 10 month period; (◊) fenpropathrin, (□) cyfluthrin 1, (■) cyfluthrin 2, (+) deltamethrin, (△) permethrin 1, (▲) permethrin 2.

Table 3 - Percentage decrease in residues in a honey sample, spiked with 50 μg/kg of each insecticide, after 10 months (n=3).

	loss% ± RSD [b]
Fenpropathrin	21.9 ± 3.4
Cyfluthrin [a]	34.2 ± 0.7
Deltamethrin	40.1 ± 2.3
Permethrin [a]	66.9 ± 2.4

[a] - sum of isomers

[b] - relative standard deviation

sults could have been influenced by a high honeybee mortality owing to treatments of fields (as honey-keepers claim); disinfestation treatments for insects of the *Culicoides* genus carried out in September-October while the honeybees were resting and low environmental persistence of pyrethroid pesticides.

The method is rapid, accurate and inexpensive and can also be used to evaluate honey quality after acaricide treatment. The moderate persistence of pyrethroids in honey is in accord with other studies about pyrethroid stability in acidic media and indirectly confirms that the samples examined were not contaminated by these insecticides.

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Paper received January 23, 2003 Received March 27, 2003

PAPER

QUALITY OF FILM WRAPPED "MIYAGAWA" SATSUMAS STORED AT 20°-22°C

CAMBIAMENTI QUALITATIVI IN FRUTTI DI SATSUMA "MIYAGAWA" CONSERVATI A 20°-22°C

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ABSTRACT

"Miyagawa" satsumas treated or not treated with imazalil and left unwrapped or wrapped with two different plastic films were stored at 20°-22°C for 30 days. Transpiration in unwrapped fruit caused a rapid decline in freshness and a loss of commercial value, while no important changes occurred in pH, titratable acidity, total soluble solids and vitamin C content. The film (MR) that was more permeable to gases and water vapour in combination with imazalil had a positive effect on

RIASSUNTO

Frutti di Satsuma "Miyagawa" trattati o non trattati con imazalil e confezionati in contenitori di polistirolo con due film plastici con differenti caratteristiche di permeabilità o non confezionati, sono stati conservati a 20°-22°C per 30 giorni e ispezionati ogni 10 giorni. A causa dell'eccessiva traspirazione i frutti non confezionati hanno perso rapidamente la loro freschezza iniziale ed il loro valore commerciale anche se non sono state rilevate sostanziali variazioni della componente chimico-nutrizio-

- Key words: Citrus fruit, decay, passive atmosphere, postharvest physiology, quality evolution, storage -

the chemical and sensory evolution of the fruit with minimal losses due to decay. Use of the other film (MY) was detrimental to fruit quality and caused a higher incidence of decay.

nale (pH, acidità titolabile, solidi solubili totali, vitamina C). Il film (MR), il più permeabile ai gas ed al vapore acqueo, in combinazione con l'imazalil ha avuto un effetto positivo sulle caratteristiche chimiche e sensoriali mentre le perdite per marciumi erano molto contenute. L'uso dell'altro film (MY) ha alterato profondamente le caratteristiche qualitative dei frutti e causato una più elevata incidenza dei marciumi.

INTRODUCTION

"Miyagawa" is a very early ripening satsuma which can be harvested in Italy at the very beginning of October. While the fruit does not have the same flavour and taste as the more preferred cultivars of mandarins and clementines, its precocity makes it very interesting from a commercial point of view. Its commercial life lasts from the beginning of October to mid-November when the earlier selections of clementines start to ripen (D'AQUINO *et al.*, 1996a). Due to their short commercial life, after harvest satsumas are sent directly to market without refrigeration. As in other loose-skinned mandarins (HARDENBURG *et al.*, 1986), the thin layer of peel, which adheres loosely to the segments, shrinks very rapidly after harvest. Consequently, the freshness of the fruit only lasts a few days, especially when the environmental vapour pressure deficit is high during transportation and retailing. In addition, physiological changes associated with water loss can reduce the permeability of the peel to gases (PIGA *et al.*, 1998) causing anaerobic respiration of the flesh with negative consequences on nutritional and sensory quality.

Since the major cause of loss of quality in "Miyagawa" satsumas, as in other citrus fruit is weight loss (BEN-YEHOSHUA, 1969), a possible way to prolong shelf-life could be to maintain a high level of humidity in the environment during transportation and retailing. Film wrapping seems to be the cheapest and most suitable way to reduce transpiration and maintain fruit freshness. Wrapping offers the advantage of creating a humid environment regardless of temperature and vapour pressure differences in the various environments encountered along the distribution chain which are generally unpredictable and difficult to control.

However, if the film characteristics do not meet the requirements of the commodity, anaerobic respiration may be stimulated, resulting in an increased rate of breakdown of organic acids and sugars (KADER, 1986; HARDENBURG, 1971). The vapour transmission rate of the film is also important because, if the in-package atmosphere is saturated, the risk of decay is enhanced. In addition, if condensation forms inside the package, it will greatly impair gas diffusion and make it difficult to see the product.

The objective of this study was to evaluate the possibility of lengthening the shelf-life of "Miyagawa" satsumas at room temperature (20-22°C) by testing two plastic films with different physical properties and to study the quality and physiological changes that occurred in the fruit over a 30-day storage period.

MATERIALS AND METHODS

Plant material

"Miyagawa" satsumas were harvested the first week of October 2001 from the experimental station of Oristano (west-central Sardinia). Defect-free and uniformly sized (95-105 g) fruits (n=2280) were selected for storage and 150 fruits were used for the destructive analysis carried out at the time of harvest.

Fruit treatments and storage conditions

About 40 kg of fruit were put in a 120-L tank, which was then filled with tap water. Two technicians simultaneously washed each fruit with a sponge and placed them in boxes. The washing operation for each lot of fruit took 4-5 minutes. The water was changed for each batch of fruit. The fruits were allowed to dry and were then subjected to fungicidal treatments. For fungicidal treatment half of the satsumas were dipped in a 500 mL/L solution of imazalil at 20°C for 2 minutes, while the other half were dipped in water at the same temperature for the same length of time. Both the imazalil-treated and non-treated fruits were then placed in polystyrene trays (8 per tray) and the trays of each group were divided into 3 lots. The first lot of trays of each group (imazalil-treated and imazalil non-treated fruit) was wrapped with a 15 µm thick polyolefinic heat shrinkable film called MR (water transmission rate = 23 g/24 h m^2 at 38°C and 100% delta relative humidity (RH); CO_2 permeance = 26500 cm^3/24 h m^2 bar at 23°C and 0% RH; O_2 = permeance 9500 cm^3/24 h m^2 bar at 23°C and 0% RH). The second lot of trays for each group was wrapped with a 20 µm thick polyolefinic film, called MY film (water transmission rate = 8 g/24 h m^2 at 38°C and 100% delta RH; CO_2 permeance = 6400 cm^3/24 h m^2 bar at 23°C and 0% RH; O_2 = permeance 2500 cm^3/24 h m^2 bar at 23°C and 0% RH). The third lot was left unwrapped. Both films were provided by Cryovac (Milan, Italy). Trays were sealed using a Minipack FM/75 wrapping machine (Costruzioni Meccaniche Torre, Dalmine, Italy). The fruit was then stored at 20-22°C and 60-65% RH for 30 days and inspected at 10-day intervals.

Respiration, endogenous and in-package gas composition

Ten fruits per treatment were individually put into 1-litre jars which were then sealed with rubber serum stoppers for 2 h. Wrapped fruits were placed in the jars 6 hours after the film had been removed. After shaking the jars 10 times, the headspace atmosphere inside the jars was sampled. A 30 mL syringe was used to withdraw a 20-mL sample which was then injected into a CO_2/O_2 analyser (Servomex, Crowborough, England). The respiration rate was expressed as mL of CO_2/kg·h. Respiration measurements of the unwrapped fruit were carried out after three days of storage and then every 3 or 4 days until the end of the trial.

The in-package CO_2 and O_2 levels were first measured 2 days after the fruit was film wrapped and then at 10-day intervals. Ten packages were analysed per treatment and the same CO_2/O_2 analyser was used for all trials.

To collect samples of the endogenous atmosphere, the calyx of the fruit was removed and a 2-mL syringe was inserted through the button scar. After immersing the fruit in water, a 2-mL sample was withdrawn. The procedure of removing the button and inserting the needle through the scar took 4-5 seconds. Endogenous CO_2 and O_2 values were determined by gas chromatography, as previously reported by D'AQUINO *et al.* (1996b).

Juice analysis

Chemical analysis of the juice was carried out as previously reported by D'AQUINO *et al.* (1998a), and included pH, titratable acidity (TA) expressed as grams of citric acid in 100 mL of juice), total soluble solids (TSS) expressed as degrees Brix and vitamin C (mg ascorbic acid/100 g of juice). The ratio TSS/TA was used as a maturity index.

Weight loss, decay, deformation, firmness, overall appearance and acceptability

Weight loss was measured on fifty fruits per treatment. At each inspection time, the fruit was checked for decay, which was expressed as percentage of rotten fruit, and overall appearance, based on a subjective scale of 1-9, where 9 indicated fruit as fresh as at the time of harvest; 5, the fruit at the limit of saleability and 1, very old fruit. The percentage of saleable fruit was determined as the difference between the total number of fruits and the sum of the rotten fruit plus the fruit rated less than 5. A test panel composed of 7 untrained laboratory technicians conducted a sensory analysis. Ten fruits per treatment were peeled, divided into segments and placed on a dish; each panelist tasted different segments from each dish before giving a judgement on crispness, sourness, sweetness, presence of off-flavour and overall acceptability. Each parameter was evaluated on the basis of a subjective scale ranging from 1 (the lowest score) to 9 (the highest score), according to the judgement for each single attribute.

Firmness was measured by puncturing the peel on two opposite sites of the cheeks with a 2-mm needle using an Effegi penetrometer (TR Turoni & C., Forlì, Italy); the same fruits (10 per treatment) were then used to determine deformation (D'AQUINO, *et al.*, 1998a), which is the difference in the transversal diameter of the fruit measured before and after applying a 1 kg weight for 5 seconds.

Statistical analysis

The experiment was arranged according to a 4x3x2 completely randomised factorial design, where the four levels of the first factor (storage time) were the number of inspections, including the first one carried out at harvest, or after 2 days for in-package atmosphere analysis; the three levels of the second factor (wrapping) were the two films used and the unwrapped treatment; the two levels of the third factor (fungicide treatment) were the fruit treated with imazalil and those that were only dipped in water. All data were subjected to analysis of variance and the separation of the means was carried out using the Duncan multiple range test. The Statgraphics software (plus 5 version, Manugistics, Rockville, Maryland, U.S.A.) was used.

RESULTS AND DISCUSSION

Respiration, endogenous and in-package gas composition

In unwrapped fruit respiration decreased markedly from the time of harvest to the end of the first three days, 19.6 and 11 mL CO_2/kg.h, respectively. This was then followed by a slower, progressive reduction reaching a final value of about 5 mL CO_2/kg.h at 30 days. There were no statistically significant differences between imazalil-treated fruit and imazalil non-treated fruit (Fig. 1a). The respiration rate of the wrapped fruit, six hours after the film had been removed, also decreased during storage, but at a slower rate than in the unwrapped fruit (Fig. 1b). The respiration decreased more in fruit wrapped in MY film than in that wrapped in MR film,

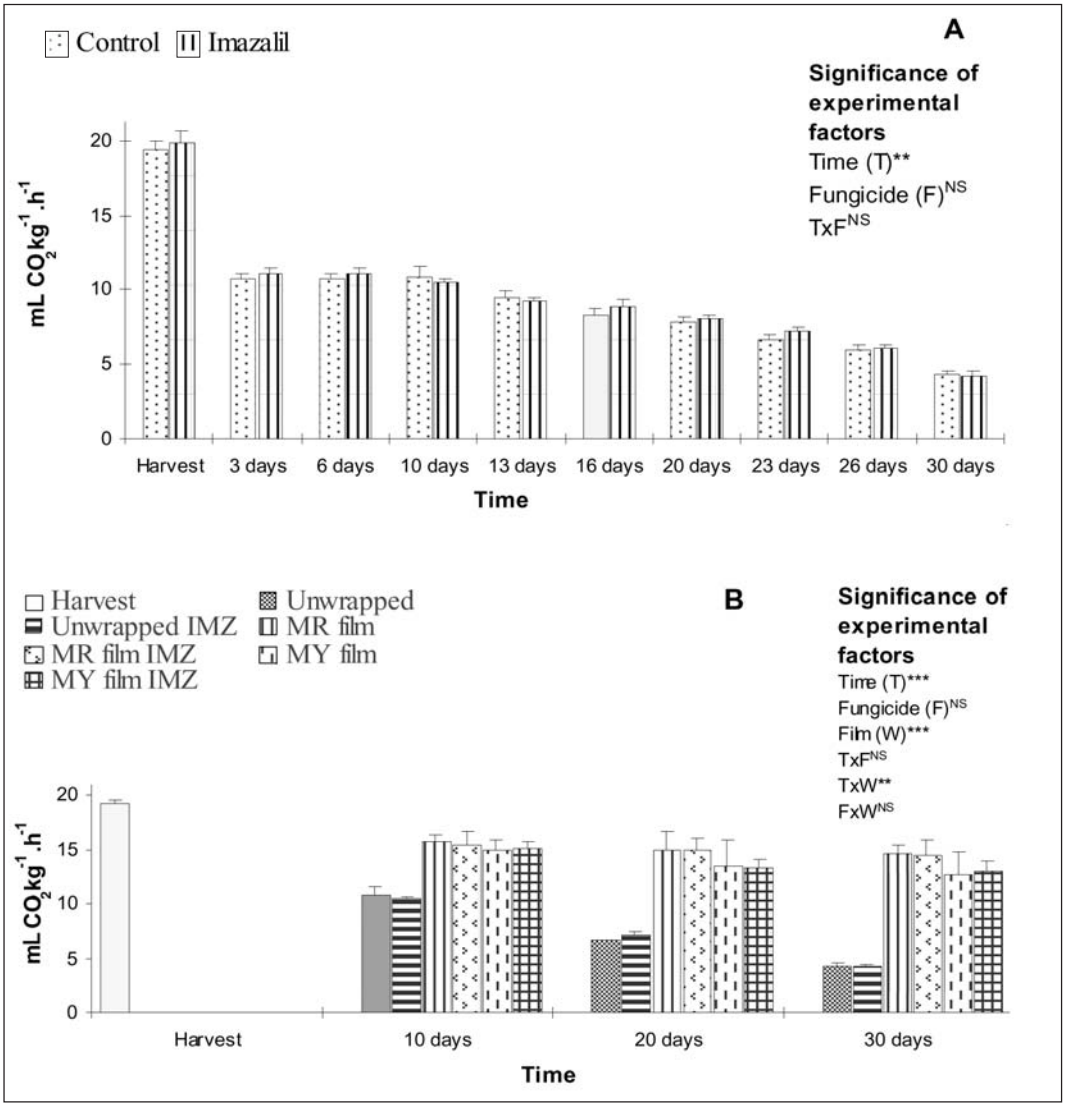


Fig. 1 - Respiration rate of unwrapped fruit (A) and unwrapped or wrapped fruit six hours after removing the film (B). Vertical bars represent the standard error (n=10).

and the differences between the two treatments increased during storage (Fig. 1b).

Endogenous CO_2 increased continuously in unwrapped fruit, exceeding 4.5 kPa after 30 days of storage (Fig. 2a). An opposite trend was observed in wrapped fruit, with a slight, progressive reduction, with significantly lower values for the MY-wrapped fruit than the MR-wrapped fruit (Fig.2a). Endogenous O_2 partial pressure, which was about 20 kPa at harvest, underwent slight modifications. While there were no statistically significant differences in wrapped fruits, there was a general and progressive decrease

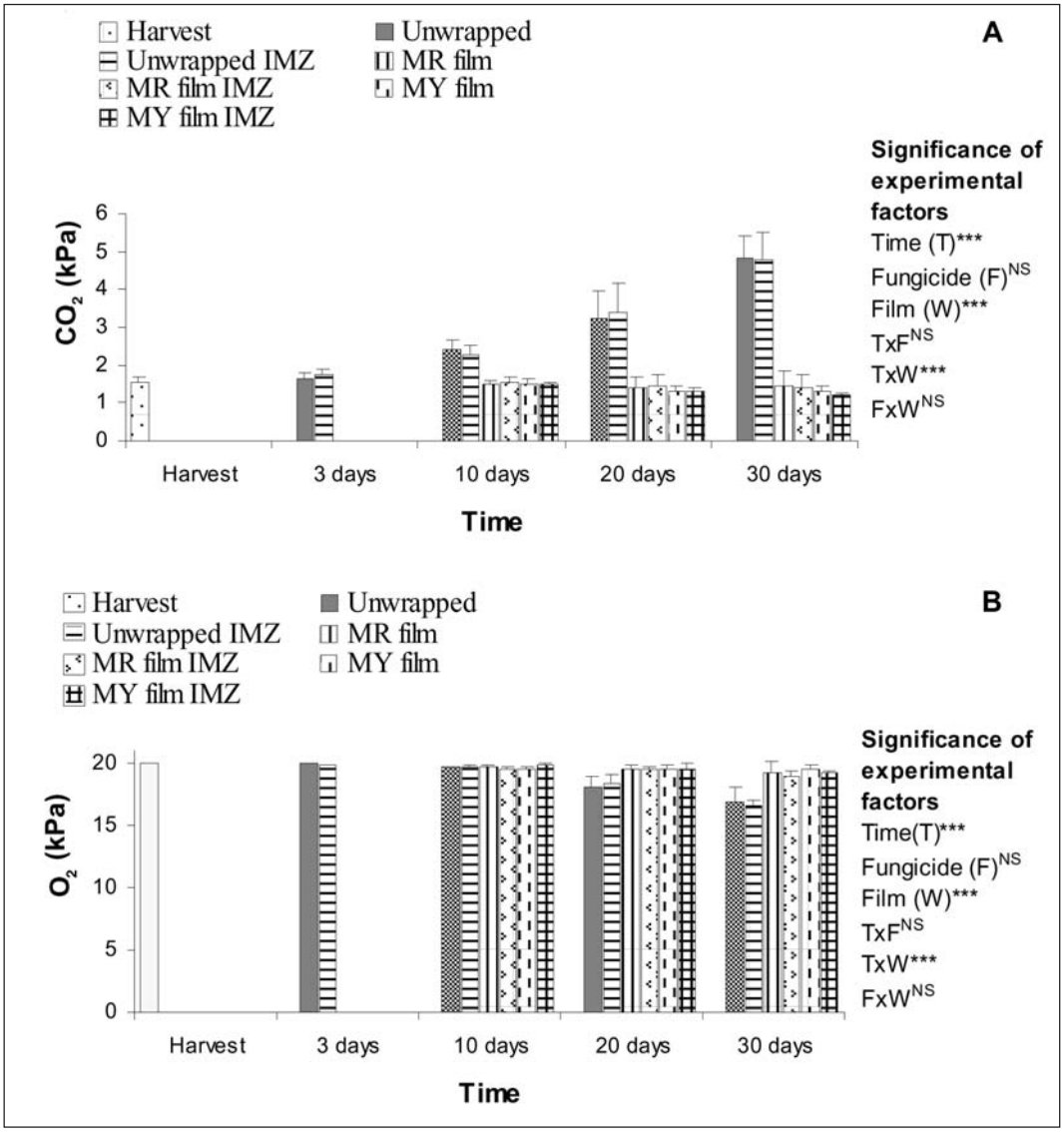


Fig. 2 - Endogenous CO_2 (A) and O_2 (B) in unwrapped or wrapped Miyagawa satsumas 6 hours after removing the film and stored at 20°-22°C and 60-65% RH for 30 days. Vertical bars represent the standard error (n=10).

in the unwrapped fruit, which was statistically significant at both 20 and 30 days of storage (Fig. 2b). No statistically significant differences were observed with respect to the fungicidal treatment (Figs 2a, b).

In-package CO_2 levels were significantly higher when fruit was wrapped with MY film compared with fruit wrapped with MR film (Fig. 3a). The CO_2 levels decreased slightly with both films during storage (Fig. 3a). Conversely, in-package O_2 partial pressure was higher with MR film than with MY film, but the val-

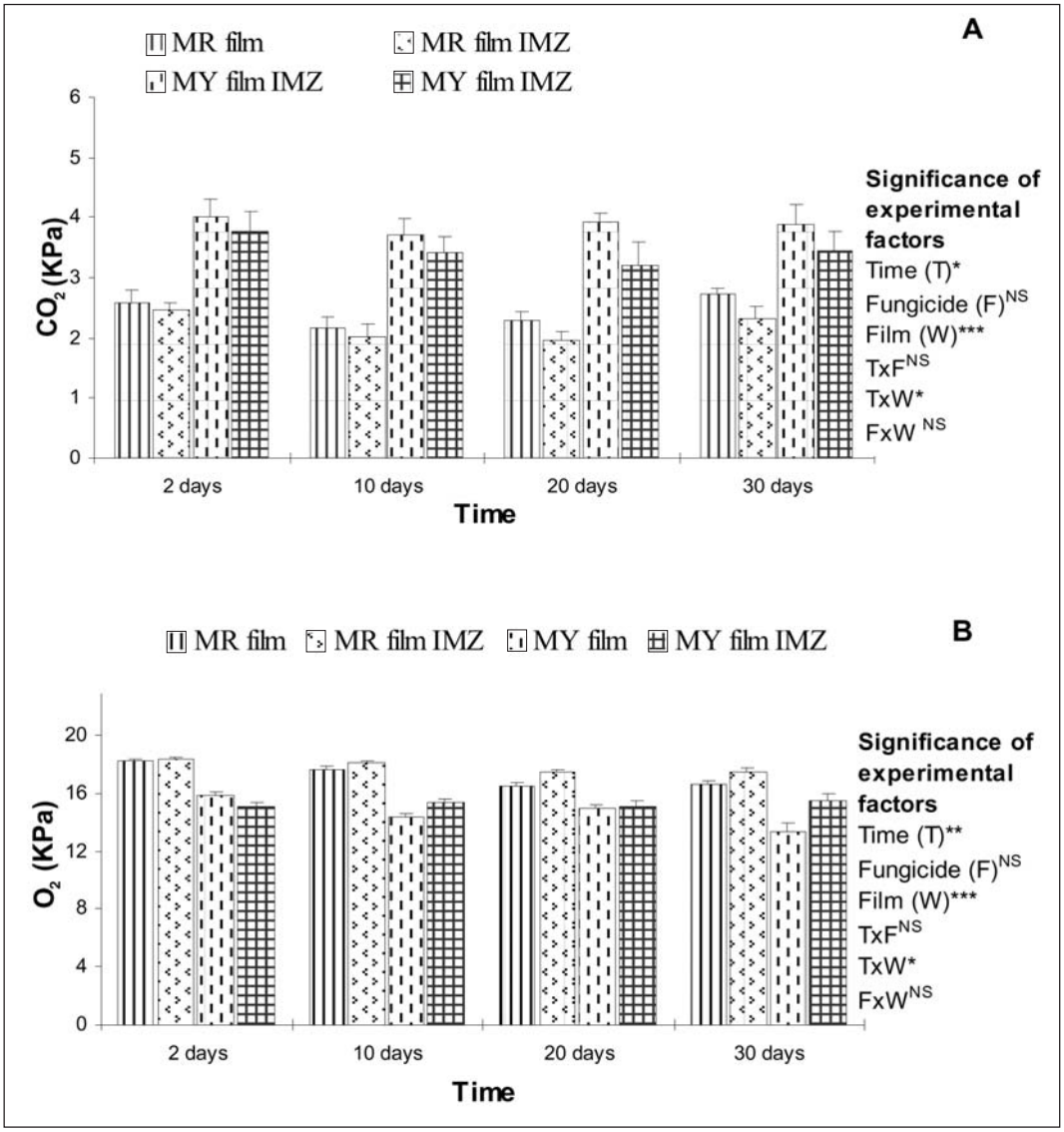


Fig. 3 - In-package CO_2 (A) and in-package O_2 (B) sealed with MR or MY film during storage. Vertical bars represent the standard error (n=10).

ues varied little over storage time (Fig. 3b). The data refer to decay-free packages only. In packages containing rotten fruit, whether treated with imazalil or not and regardless of the kind of film used, the CO_2 partial pressure values increased dramatically, varying from 11 to 20 kPa, depending on the number of rotten fruits, while O_2 partial pressure values dropped as low as 1kPa (data not shown).

The respiration rate of unwrapped fruit decreased more during the first three days of storage than during the remain-

ing 27 days (Fig. 1a). The values measured at harvest were probably amplified by a series of stresses, including microwounds, bruises and pressure damage, which temporarily affected the fruit during the harvesting operation. EAKS (1970) and VINES *et al.* (1968) also reported a transient increase in respiration activity in citrus fruit due to mechanical or temperature stresses.

The progressive decline in the respiration rate during the remaining 27 days of storage was partially due to a reduction in respiratory substrates, as well as to the physiological consequences of transpiration. Water loss due to transpiration increases resistance to gas diffusion in the rind and alters the partial pressure gradient between the inner tissues and the environment (DAVIS *et al.*, 1967; BEN-YEHOSHUA, 1969; PIGA *et al.*, 1998; D'AQUINO *et al.*, 1998b). These changes cause the endogenous CO_2 partial pressure to increase while the endogenous O_2 partial pressure decreases. High levels of CO_2 and reduced O_2 concentrations may lower respiratory activity (SMOCK, 1979), if the O_2 concentration does not decrease to the extinction point, below which a shift from aerobic to anaerobic metabolism occurs (KADER, 1987). If the reduced respiration is the result of a depletion of respirable substrate, then the endogenous CO_2 should also decrease. However, endogenous CO_2 increased during storage (Fig. 2a), while the O_2 concentration decreased (Fig. 2b). A lower respiratory rate has been reported in fruit of different citrus species when held at low humidity (64% RH, 20°C) compared with fruits kept at high humidity (92% RH, 20°C) which was due to a greater rate of weight loss due to transpiration (MURATA AND YAMAWAKI, 1989). Transpiration was negligible in the wrapped fruit and no important changes occurred in rind permeability to gases. Wrapped fruit was exposed to an atmosphere with a CO_2 partial pressure ranging from 2.5 kPa to 4 kPa, while O_2 ranged from 17 kPa to 14 kPa, respectively, in MR and MY packages. Although the endogenous atmosphere was not measured when fruit was still wrapped, it is obvious that endogenous CO_2 partial pressure must have been higher than in-package CO_2 partial pressure, and the endogenous O_2 level would have been lower than in-package O_2. This means that aerobic respiration in wrapped fruit could have taken place simultaneously with anaerobic respiration. Similar results were obtained by D'AQUINO *et al.* (1998b; 1999) with other citrus cultivars and the relationship between transpiration and endogenous gas composition, respiration rate and resistance to gas diffusion of the peel has been well documented in other experiments (BEN-YEHOSHUA, 1969; BEN-YEHOSHUA *et al.*, 1979; BEN-YEHOSHUA *et al.*, 1985; EAKS 1991; D'AQUINO *et al.*, 1997; PIGA *et al.*, 1998).

Juice analysis

Table 1 shows a significant increase in pH and a concomitant decrease in titratable acidity occurred during storage in all treatments. There were significant differences in pH and titratable acidity values at each inspection time between wrapped and unwrapped fruit: pH was always lower in unwrapped fruit, while titratable acidity was always higher in unwrapped fruit than in wrapped fruit, especially when MY film was used. TSS were very stable in wrapped fruit, but increased significantly in unwrapped fruit (Table 1). The SST/TA ratio rose in all treatments, particularly in fruit wrapped with MY film due to the concomitant increase of TSS and decrease in titratable acidity. Surprisingly, the Vitamin C levels increased during the first 20 days of storage in all treatments and then decreased slightly. Final values were similar to harvest values in wrapped fruit but were still significantly higher in unwrapped fruit.

Table 1 - Effect of film wrapping on chemical parameters of "Miyagawa" satsumas after 10, 20 or 30 days of storage at 20°-22°C and 60-65% RH.

STORAGE	pH	TA (% citric acid)	TSS (°Brix)	Maturity Index (TSS/TA)	Vitamin C (mg/100g)
Harvest	3.36	1.09	9.2	8.4	35.36
10 Days					
Unwrapped	3.65a	1.02b	9.3a	9.1a	39.41 b
MR film	3.76b	1.01b	9.2a	9.1a	39.24 b
MY film	3.78b	0.96a	9.3a	9.7b	37.68 a
20 Days					
Unwrapped	3.71a	0.96c	9.6b	10.0a	41.87 c
MR film	3.80b	0.90b	9.3a	10.3a	39.30 b
MY film	3.81b	0.84a	9.2a	11.0b	38.50 a
30 Days					
Unwrapped	3.81a	0.91c	9.7b	10.7a	38.87 b
MR film	3.93b	0.85b	9.3a	10.9a	35.56 ab
MY film	3.95b	0.76a	9.3a	12.2b	35.03 a

For each storage time, the means in a column followed by different letters are statistically significant at P≤ 0.05. Means were separated by the Duncan's multiple range test.

Imazalil had no influence on the chemical parameters (data not shown).

In other studies with citrus fruit, the pH values always increased, while titratable acidity and TSS generally decreased, with TSS decreasing at a slower rate than acidity (SAMSON, 1986) or slightly increasing before decreasing (D'AQUINO *et al.* 1997, 1999). The postharvest behaviour of TSS can be explained by the breakdown and subsequent solubilization of polysaccharides in the juice which come from the cell walls of the juice vesicles or from the segment membranes (BURNS, 1990; Echeverria and Valich, 1989) and from a possible concentration effect in unwrapped fruit. The same mechanism may explain the vitamin C level which increased during storage. In fact, segment membranes and juice vesicles contain higher levels of ascorbic acid than juice (Kefford, 1959). Increasing vitamin C concentrations in citrus fruit juices have also been reported by SCHIRRA (1992) and MULAS *et al.*(1993). On the other hand, the negligible transpiration of wrapped fruit together with a higher rate of degradation due to the possible presence of anaerobic metabolism may explain the consistently lower concentration of vitamin C in wrapped fruit with respect to unwrapped fruit, especially in MY packages which had higher anaerobic conditions than the MR packages.

Sensory evaluation

During storage, unwrapped fruit became progressively sweeter, while no appreciable changes in sweetness occurred in MR-wrapped fruit, but a significant decrease in sweetness occurred in MY-wrapped fruit (Table 2). Sourness decreased dramatically in wrapped fruit, especially in the fruit wrapped in MY film, while there was only a slight reduction in sourness in the unwrapped fruit (Table 2). The panelists did not

Table 2 - Effect of film wrapping on sensory parameters[1] of "Miyagawa" satsumas after 10, 20 or 30 days of storage at 20°C and 65% RH.

STORAGE	Crispness	Sourness	Sweetness	Off-flavour	Acceptability
Harvest	9	7	6.8	1	8
10 Days					
Unwrapped	6.8 a	6.6 b	7.3 c	1 a	8.4 c
MR film	9 b	5.5 ab	6.6 b	1 a	7.8 b
MY film	9 b	4.1 a	6.1 a	2.6 b	6.2 a
20 Days					
Unwrapped	5.2 a	6.2 c	7.6 c	3.2 b	6.8 b
MR film	8.6 b	4.5 b	6.8 b	2.1 a	7.6 c
MY film	8.5 b	3.3 a	5.5 a	5.8 c	4.1 a
30 Days					
Unwrapped	2.9 a	5.6 c	8.2 c	3.6 b	5.7 b
MR film	8.5 b	4.3 b	6.4 b	2.4 a	6.5 c
MY film	8.7 b	2.8 a	5.2 a	7.5 c	2.6 a

[1] Sensory quality was evaluated by a test panel made up of 7 untrained laboratory technicians who gave a judgment for each parameter on the basis of a subjective scale ranging from 9 to 1, according to the intensity of the attributes. For each storage time means in a column followed by different letters are statistically significant at $P \leq 0.05$. Means were separated by the Duncan's multiple range test.

detect any changes in crispness in wrapped fruit during storage, whereas it changed dramatically in the unwrapped fruit after the first inspection. At the end of the storage time the fruits were flaccid. There was no alteration in flavour after 10 days of storage in the unwrapped fruit or in fruit wrapped in MR film. From 20 days on, a slight build-up of off-flavour occurred in the MR-wrapped fruit, while off-flavours were slightly stronger in the unwrapped fruit; but the presence of off-flavours in both treatments did not compromise the acceptability (Table 2). The build-up of off-flavour in MY-wrapped fruit, however, was detrimental. The panelists detected the presence of strange odours during the first inspection and, after 20 days of storage, the flavour was so altered that the fruit was inedible. Regarding overall acceptability, after 10 days of storage unwrapped fruit was judged better than wrapped fruit, while between the two films, MR-wrapped fruit was preferred to MY-wrapped fruit (Table 2). After 20 days, the panelists did not detect appreciable changes in MR-wrapped fruit with respect to the first inspection. The unwrapped fruit was judged as still acceptable, but the scores decreased from 8.4 to 6.8, while MY-wrapped fruit was judged non-edible due to the presence of off-flavours. MR-wrapped fruit maintained a fairly good acceptability even after 30 days of storage, while unwrapped fruit, although showing evident signs of ageing and segment flaccidity, was still considered edible.

A moderate water loss due to transpiration can increase both the sugar and acid concentrations in the juice with a positive effect on taste. However, if transpiration is severe, fruit loses its turgidity with negative consequences on acceptability. The permeability of the thin-layered "Miyagawa" satsuma rind, can

be reduced dramatically by marked dehydratation. This can increase off-flavours of the inner tissue of the flesh which negatively affect flavour and taste. The two kinds of films gave different results; MR-wrapped fruit had a slight decrease in sweetness and sourness during storage with no alteration in the sensory features, while fruit wrapped with MY film underwent a marked reduction in sweetness, sourness and accumulated volatile gases which altered the taste and flavour.

Decay, deformation, weight loss, firmness, overall appearance and acceptability

Deformation increased sharply, peaking at 5 mm and was associated with a dramatic loss of turgidity in unwrapped fruit during the first 20 days of storage, followed by a slight decrease with final values slightly higher than 4 mm (Fig. 4a). There was little deformation in wrapped fruit, but it progressively increased with values always significantly lower than those of the unwrapped fruit (Fig. 4a). The evolution of firmness showed opposite trends in the unwrapped and wrapped fruit, with the former increasing during the entire storage period and the latter decreasing (Fig. 4b). Deformation and firmness were not affected by imazalil (Figs. 4a, b).

Water loss had a detrimental effect on the unwrapped fruit (Fig. 5); after 30 days of storage the weight had decreased 19% with respect to harvest time. In contrast, wrapping almost completely inhibited transpiration and at the end of storage, weight loss was less than 2% in both film-wrapped fruits. A close relationship was noted between weight loss and visual appearance. During the first 20 days of storage, wrapped fruit maintained its aesthetical aspect with only a few signs of ageing, such as stem-end pedicel (button) browning or button detachment, appearing in some fruits at the end of storage; no other visible alterations were noted. In contrast, the peel of unwrapped fruit showed evident symptoms of senescence such as shrivelling and necrosis of the button and the area surrounding the stem after the 10^{th} day of storage. At the second inspection, the peel was marked by diffuse brown blotches and either the rind or the flesh of the fruit was softer. After 30 days of storage the peel became hard and leathery, while the flesh remained soft. About 90% of the unwrapped fruit was graded higher than 5 after 10 days of storage, but this dropped to 2.5% after 20 days and none were judged as marketable after 30 days. Wrapped fruit was never rated less than 7.9 and even after 30 days of storage the wrapped fruit was still totally marketable. These results confirm the beneficial effect of wrapping to reduce ageing, improve appearance and lengthen the storage life of "Miyagawa" satsumas and concur with the results from other studies on citrus fruit (BEN-YEHOSHUA, 1985; BEN-YEHOSHUA *et al.*, 1979; D'AQUINO *et al.*, 1996a; 1998a; 2001a; 2001b; MILLER and MCDONALD, 1989).

The incidence of decay was negligible in unwrapped fruit but was very high in wrapped fruit. After 30 days of storage, 30% of the fruit was rotten (28.9 and 33.3%, respectively, for MR- and MY-film) (Table 3). The factors which may have promoted decay in the wrapped fruit were: a) a water-saturated environment; b) the presence of condensation; c) a non-optimal storage temperature; and d) a possibly toxic action caused by high CO_2 levels. Since factors "a", "b" and "d" occurred at higher levels in MY-packages than in MR-packages, they could account for the higher incidence of decay in packages wrapped with MY film.

Imazalil prevented the development of decay especially in wrapped fruit, where the conditions for microbial growth were

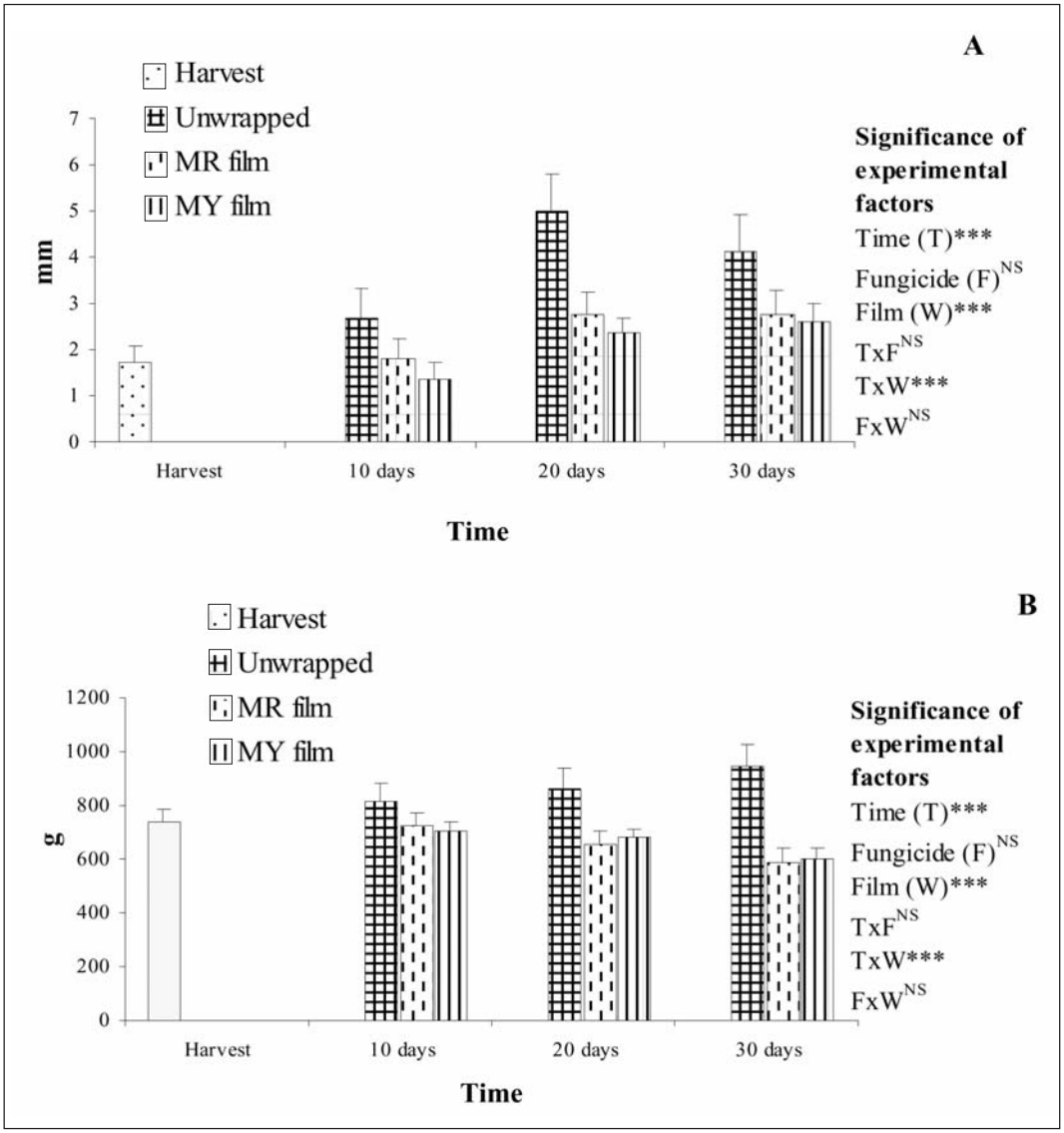


Fig. 4 - Changes in deformation (A), i. e. the difference in the transversal diameter of the fruit measured before and after applying a 1-kg weight for 5 seconds and firmness (B), i. e. the resistance to a 5 mm penetration of a 2-mm needle applied by an Effegi penetrometer. Vertical bars represent the standard error (n=10)

more favourable. Most of the losses were caused by *Penicillium digitatum* (green mold); less than 1% of the decay was due to other fungi (*Penicillium italicum*, botrytis and anthracnose) (data not shown). Fruit decay was not time-dependent in unwrapped fruit, while in wrapped fruit, the number of rotten fruit increased with storage time. The rapid desiccation of the peel in unwrapped fruit probably created unfavourable conditions for microbial development.

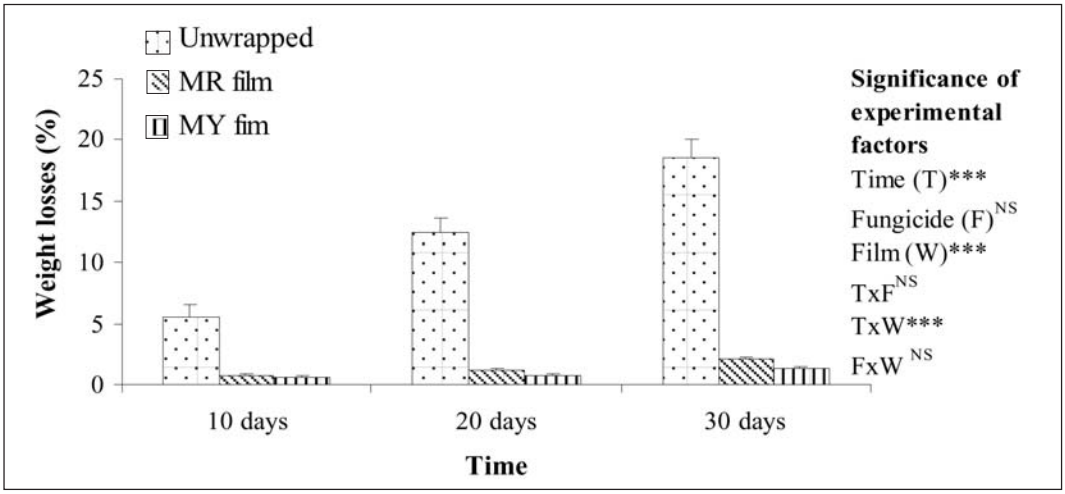


Fig. 5 - Weight loss of "Miyagawa" satsumas stored at 20-22°C and 60-65% RH during storage. Vertical bars represent the standard error (n=50).

Table 3 - Effect of film wrapping and fungicide treatment on appearance, incidence of decay and percentage of saleable fruit of "Miyagawa" satsumas after 10, 20 or 30 days of storage at 20°-22°C and 60-65% RH.

STORAGE	Appearance[1] (index number)	Fruit rated more than 5 (%)	Rotten fruit (%)	Saleable fruit [2] (%)
Harvest	9	100	-	
10 Days				
Unwrapped	5.8a	86	2.7	83.7
Unwrapped -IMZ	6.1	92	0	92
MR film	9b	100	12.2	87.8
MR film-IMZ	9	100	2.2	97.8
MY film	9b	100	13.3	86.7
MY film-IMZ	9	100	0	100
20 Days				
Unwrapped	4.2a	2	5.5	1.9
Unwrapped -IMZ	4.4	3	1.1	3
MR film	8.8b	100	12.2	87.8
MR film-IMZ	8.7	100	4.4	95.6
MY film	8.5b	100	17.7	82.3
MY film-IMZ	8.6	100	5.6	94.4
30 Days				
Unwrapped	2.5a	0	5.6	0
Unwrapped -IMZ	2.4	0	3.3	0
MR film	8.1b	100	28.9	71.1
MR film-IMZ	8.0	100	6.7	93.3
MY film	8.3	100	33.3	66.7
MY film-IMZ	7.9	100	7.8	92.2

[1]Visual appearance was evaluated according to a subjective scale, where 9 indicated that the fruit was as fresh as at harvest, 5 the fruit was at the limit of commerciability and values less than 5 indicated aged and unmarketable fruit.
[2]Saleable fruit were determined by subtracting decayed fruit and fruit rated less than 5 from the total number of fruits.
For each storage time, the means in a column followed by different letters are statistically significant at $P \leq 0.05$. Means were separated by the Duncan's multiple range test.

CONCLUSION

Easy-peeling citrus fruit lose water very rapidly due to transpiration if they are not properly handled and become old and unmarketable in a few days. Film wrapping is a good way to reduce ageing and lengthen the shelf-life of satsumas and mandarins, especially when fruit is kept at room temperature. However, the barrier created by the film can considerably reduce the availability of O_2 to the fruit, while increasing the level of CO_2. In contrast to other fruits, which benefit from a moist, CO_2-saturated environment and low O_2 concentrations, citrus fruit only benefits from high humidity. This is particularly true when fruit is stored at room temperature, because the respiration rate is many times faster than in fruit stored at low temperatures and the O_2 requirements are considerably higher. Poor permeability to gases may shift aerobic metabolism toward anaerobic metabolism, resulting in a rapid depletion of respirable substrates, an increase in off-flavour and odours and a weakening of the tissue which will make it more prone to decay, particularly with the elevated in-package humidity. In our study the high permeability to gases of the MR film created better conditions than the MY film. The rate of alteration of visual appearance, which is the only parameter used by consumers at the time of purchasing, was not indicative of the taste alteration in MY fruit which became inedible even when the aesthetical appearance was still good. Imazalil prevented decay in the citrus fruit, even at very low concentrations.

In conclusion, a suitable plastic film, such as MR, can be used to prolong the shelf-life of very perishable citrus fruit, such as "Miyagawa" satsumas for 30 days, even at room temperature, provided that the fruit is treated with an effective fungicide.

ACKNOWLEDGEMENTS

Research supported by MiPAF. Progetto: "Ricerche e Sperimentazioni nel settore dell'Agrumicoltura Italiana". Paper n° 46.

The authors wish to thank Mr. Monaldi (Cryovac, Milan, Italy) for providing the films used.

Authors contributed equally to this study.

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Paper received January 13, 2003 Accepted June 26, 2003

PAPER

EFFECT OF LONG-TERM CONTROLLED ATMOSPHERE STORAGE ON THE SENSORY QUALITY OF TOMATOES

EFFETTO DELLA CONSERVAZIONE
IN ATMOSFERA CONTROLLATA A LUNGO TERMINE
SULLE CARATTERISTICHE QUALITATIVE DEL POMODORO

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ABSTRACT

Tomato (*Lycopersicon esculentum* Mill.) fruit were harvested at the mature green and pink stages of maturity and held for 60 days at 15°C (mature green fruit) or 13°C (pink fruit) under three different controlled atmosphere (CA) conditions: 3.2% CO_2, 6.4% CO_2 or 9.1% CO_2, combined in all cases with 5.5% O_2. Mature green fruit were then kept for additional 10 days at 20°C to simulate shelf life conditions. Different sensory tests were conducted which included analytical tests (tomato flavour

RIASSUNTO

I frutti di pomodoro (*Lycopersicum esculentum* Mill.) sono stati raccolti allo stadio di maturazione verde e rosa e mantenuti per 60 giorni a 15°C (frutti verdi) o 13°C (frutti rosa) a tre differenti condizioni di atmosfera controllata (CA): 3,2% di CO_2, 6,4% di CO_2 o 9,1% di CO_2, in unione, in ogni caso, con 5,5% di O_2. I frutti a maturazione verde sono stati poi mantenuti per ulteriori 10 giorni a 20°C per simulare le condizioni normali di vita di scaffale. Sono stati condotti diversi test sensoriali,

- Key words: acidity, controlled atmosphere, flavour,
Lycopersicon esculentum, sweetness, sourness, tomato -

and two flavour descriptors, sweetness and sourness) and affective tests (acceptability). Objective measures (total soluble solids, titratable acidity and TSS/titratable acidity ratio) were also obtained. Control tomatoes showed significantly higher flavour scores, and were sweeter and more acceptable than those exposed to long-term CA storage. Although there were no significant differences in flavour, sweetness and acceptability scores among mature green and pink fruit stored at different gaseous combinations, the acceptability levels were slightly higher in tomatoes stored in 9.1% CO_2. The sourness score for tomatoes treated with 9.1% CO_2 was significantly lower than that for tomatoes treated with other CO_2 concentrations. Although no significant differences were found among the three CA storage treatments, TSS values and TSS/acidity ratios in fruit treated with 9.1% CO_2 plus 5.5% O_2 suggested that this combination displayed a better performance for long-term storage (slightly lower ripening rate, and slightly higher tomato flavour and consumer acceptability).

inclusi test analitici (aroma del pomodoro e due descrittori di aroma, dolcezza ed amarezza) e test affettivi (accettabilità). Sono inoltre state effettuate alcune misurazioni oggettive (solidi totali solubili, acidità titolabile e rapporto TSS/acidità titolabile). I pomodori di controllo mostravano valori aromatici significativamente più elevati ed erano più dolci e più accettati di quelli sottoposti a conservazione in atmosfera controllata a lungo termine. Anche se non sono risultate differenze significative in aroma, dolcezza ed accettabilità tra i frutti verdi e quelli rosa conservati a diverse combinazioni gassose, i livelli di accettabilità erano debolmente superiori nei pomodori conservati sotto CO_2 al 9,1%. La valutazione dell'amaro dei pomodori trattati con CO_2 al 9,1% è stata significativamente più bassa di quella dei pomodori trattati alle altre concentrazioni di CO_2. Anche se non sono state rilevate differenze significative nei tre trattamenti di conservazione, l'analisi dei valori del rapporto TSS/acidità nei frutti trattati con CO_2 al 9,1% ed O_2 al 5,5% ha suggerito che questa combinazione sarebbe leggermente preferibile (minor tasso di maturazione e maggior aroma ed accettabilità da parte del consumatore).

INTRODUCTION

Firmness and colour are the two primary characteristics regarding the purchase quality of fresh tomatoes (YANG and CHINNAN, 1988). Sensory properties also play an important role in the selection of tomatoes. Tomatoes that do not have an acceptable flavour, appearance, colour and consistency are rejected by consumers. KADER *et al.* (1978) has stated that appearance and flavour are the main quality factors for fresh tomatoes. To achieve acceptable colour and flavour development, tomatoes should be left to fully ripen on the vine. However, "field-ripened" fruit must be marketed without delay (HOBSON, 1989). Problems arise when tomatoes are transported to other regions or where a longer shelf life is required. Tomato quality continues to change during ripening after harvest, resulting in loss of quality and reduced shelf life (GEESON *et al.*, 1985).

Delaying ripening and extending the shelf life of tomatoes could be achieved by using controlled atmosphere (CA)

storage. CA usually means removal of O_2 or ethylene and addition of CO_2 resulting in a composition around the commodity different from that of air (KADER, 1992). In general, reduced O_2 and increased CO_2 concentrations in the storage atmosphere decrease the respiration rate of fruit and vegetables (GEESON, 1984).

There is little information in the literature on the sensory evaluation of tomatoes after long-term CA storage. SALUNKHE and WU (1973) found that lycopene synthesis was completely inhibited for 60 days in tomatoes stored at 1% O_2, 99% N_2, and 12.78°C, and for 40 days when tomatoes were stored at 3% O_2 and 97% N_2. YANG *et al.* (1987) showed that sealed-packaged tomatoes displayed lower accumulation of lycopene during ripening but no delay in ripening was observed beyond the pink stage of maturity. YANG and CHINNAN (1988) found high off-flavour scores in tomatoes treated with 5 to 15% CO_2 and 5% O_2 during 45 days but relatively low off-flavour scores were found after 30 days of storage.

In this study, analytical tests (tomato flavour and two flavour descriptors, sweetness and sourness), affective tests (acceptability), and objective measurements (total soluble solids, titratable acidity and TSS/titratable acidity ratio) were conducted in order to evaluate the effect of long-term controlled atmospheres on tomato fruit quality.

MATERIALS AND METHODS

Plant materials

Tomatoes (*Lycopersicon esculentum* cv 'Criterium') were obtained from a greenhouse at the Silsoe Research Institute in Beds, England. Freshly harvested tomatoes at either the pink or mature green stage of maturity were used. At the pink stage, more than 30% but no more than 60% of the surface, in the aggregate, shows pinkish, red or reddish yellow colour. At the mature green stage, fruit are entirely light to dark green, but mature and capable of ripening (GRIERSON and KADER, 1986). Control tomatoes were harvested at the pink stage 60 days later from the same plants and then kept for 10 days at 13°C. Thus, the stage of ripeness was similar in control and CA stored tomatoes.

Controlled atmosphere (CA) storage

The Minolta colour a*/b* values of tomatoes submitted to CA storage were 0.58-0.69. On the basis of skin colour, tomatoes were at a very similar stage of ripeness (BATU and THOMPSON, 1994). Fruit treated with CA were exposed to the desired gas mixture in a closed system. Six tomatoes were placed in each of 12 non-sealed plastic boxes (18 cm length, 9 cm depth and 11 cm width) which were placed in air-tight 50 litre polyethylene containers (Model C217, Mailbox International Ltd, Cheshire, UK), giving 72 tomatoes per container. The containers were connected to tubes of a gas distributor (Mercury, UK. serial No.ss13306). The gas distributor was connected to a computer controlled gas blender (Signal Instrument Co. Ltd. Surrey, UK., 850 series) which was connected to compressed oxygen, carbon dioxide and nitrogen cylinders. Controlled atmosphere combinations of O_2, CO_2 and N_2 were made according to the gas concentration needed in each controlled atmosphere container. After sealing the containers, the O_2 concentration was rapidly reduced by flushing with a mixture of the desired concentration of gases. The appropriate atmospheres were obtained within a maximum of 24 hours after harvest. The gas output from the gas blender and the controlled atmosphere storage containers were monitored for oxygen and carbon dioxide levels twice a day during the first week of the experiments, then twice a week, using

an Oxystat 2 Fruit Store Analyser fitted with an Infra Red Gas Analyser and a type 770 Paramagnetic Oxygen Analyser (David Bishop, Sussex, UK).

During the experiments, mature green tomatoes were stored at 15°C and 76±2% RH, while pink tomatoes were stored at 13°C and 76±2% RH. At equilibrium, CO_2 concentrations were 3.2 (2.9-3.3) %, 6.4 (6.2-6.5) % and 9.1 (8.8-9.3) % plus 5.5 (5.3-6.0) % O_2 in all cases. After 70 days (60 days in CA storage plus 10 days in air for mature green tomatoes) and 60 days (for pink tomatoes) of storage, titratable acidity and total soluble solids were measured and sensory evaluation was carried out.

Color, total soluble solids and titratable acidity evaluation

Skin colour was measured at three points on the equatorial region of the fruits using a Minolta chromameter (model CR 200, Osaka, Japan) The instrument was calibrated against a standard white colour plate (Y=93.9, x=0.313, y=0.321) (ANONYMOUS, 1992). Total soluble solid content was determined with a model PR1 Atago digital refractometer (Atago Co. Ltd, Tokyo, Japan). Acidity was determined by titrating juice to pH 8.1 with 0.3125 N NaOH using a model 3020 Jenway digital pH meter (Leeds, UK).

Sensory evaluation

Tomatoes were harvested at either the pink or the mature green stage, and stored in different controlled atmosphere environments for 60 days. Then, mature green tomatoes were transferred to air (20°C, 63 ± 2% RH) for 10 days. Control tomatoes were harvested at the pink stage 10 days before the taste panel assessment and kept at 13°C in order to reach an acceptable red colour. At the time of assessment all fruit were fully red. Ten semi-trained judges were selected from a pool of PhD students from different countries. They were trained to use the score sheet. A scoring test was used and the results were statistically analysed (LARMOND, 1987). Three tomatoes from each treatment were sliced into 4 pieces. Each panelist rated one diced sample from each treatment. Samples were ranked by using scales for four attributes: fresh tomato flavour, sweetness, sourness and acceptability levels. Each sample was ranked from 1 to 5. *Flavour*: 1: poor, 2: weak, 3: moderate, 4: good enough, 5: strong tomato flavour; *Sweetness*: 1: not sweet, 2: slightly sweet, 3: moderate, 4: good enough, 5: very sweet; *Sourness*: 1: not sour, 2: slightly sour, 3: moderate, 4: sour, 5: very sour; *Acceptability*: 1: not acceptable, 2: less acceptable, 3: acceptable (moderate), 4: more acceptable, 5: perfect.

Total soluble solids (TSS)

Six replicate samples of 12 fruits per treatment were homogenised in a laboratory blender at high speed for 1 min. The homogenates were filtered and the TSS content (°Brix) of the resulting clear juice samples was determined by placing 2-3 drops of the undiluted juice in a model PR1 bench top Atago digital refractometer (Atago Co. Ltd., Tokyo, Japan). Total soluble solids content is expressed as previously described (KOPELIOVITCH *et al.*, 1982).

Titratable acidity

Two 10-mL aliquots of extracted tomato juice were titrated to pH 8.1 with 0.1 N NaOH using a model 3020 Jenway digital pH meter (Leeds, UK) and the results were converted to percent citric acid.

Statistical analysis

The experiments were arranged in a completely randomised factorial design. Ten samples were obtained from each treatment to analyse TSS and titratable acidity of the mature green and pink to-

matoes. Significant differences between treatments were detected using least significant differences (LSD), at a significance level 0.05. Genstat statistical package was used for data analysis using the general linear model (STEAL and TORRIE, 1987).

RESULTS

Control tomatoes had the highest scores for tomato flavour, sweetness and consumer acceptability (P=0.05) (Fig. 1). There were no significant differences (P=0.05) in flavour, sweetness or acceptability scores among the three CA storage treatments for tomatoes harvested at the mature green or pink stages of maturity (Figs. 1, 2). Sweetness scores for tomatoes harvested at the pink stage and stored in 6.4% CO_2 (Fig. 2) were significantly (P=0.05) higher than those for fruit stored in 3.2% CO_2. Sourness scores of the tomatoes harvested at the pink stage and stored in 9.1 % CO_2 were significantly lower than the others (Fig. 2). Tomatoes harvested at the pink stage of maturity and stored in 3.2% CO_2 had the lowest scores for tomato flavour, sweetness, and acceptability, although their sourness was similar to that of fruit stored in 6.4% CO_2 and the control fruit. Tomatoes harvested at the pink stage and stored in 9.1% CO_2 had medium sweetness and the highest tomato flavour and consumer acceptability, while their sourness was significantly lower than that of the other treatments.

The consumer acceptability score of the mature green tomatoes kept in 9.1% CO_2 was slightly higher than the others. On the other hand, there were no significant differences in all four sensorial scores among the three CA storage treatments (Fig. 1). The sourness score of the mature green tomatoes kept in 6.4% CO_2 was slightly higher than the other treatments. This might be due to the fact that these fruits had small amounts of decay and some off-flavours produced by the decay could have been interpreted as sourness.

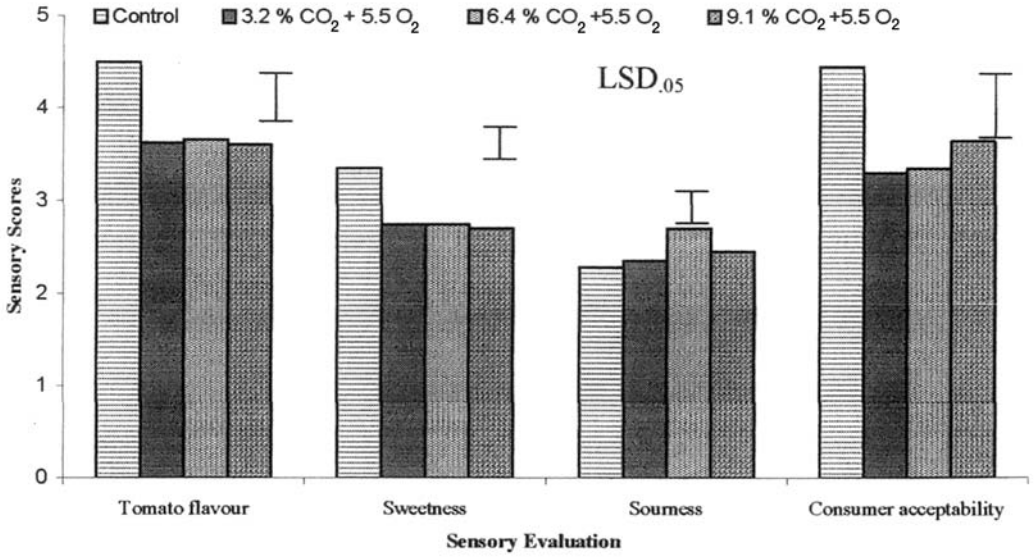


Fig. 1 - Means of sensory scores of tomatoes harvested at the mature green stage of maturity and kept in CA storage for 60 days at 15°C and 82 (±2) % RH plus 10 days in air at 20°C and 63 (±2) % RH.

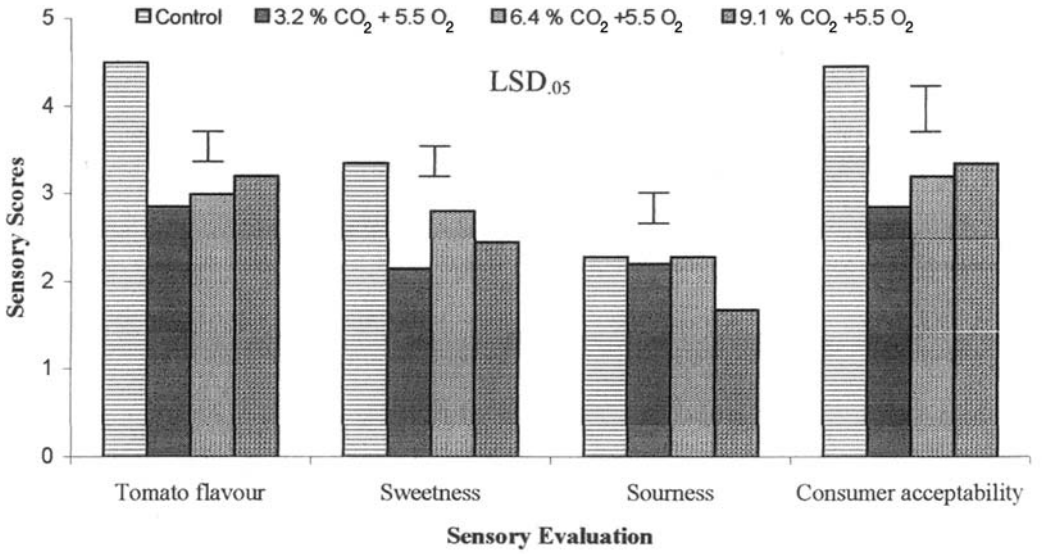


Fig. 2 - Means of sensory scores of tomatoes harvested at the pink stage of maturity and kept in CA storage for 60 days at 13°C and 82 (±2) % RH.

Control fruits had the highest TSS and titratable acidity scores and the lowest TSS over titratable acidity ratio (Fig. 3). TSS values generally increased with increasing CO_2 levels but there was not a significant variation in TSS values among the treatments. Both mature green and pink tomatoes stored in 9.1% CO_2 had slightly higher TSS at the end of 70 days of storage in comparison to fruit stored in 3.2% and 6.4%.

There were no differences in the titratable acidity of tomatoes either at the mature green or pink stage of maturity which corresponds to the results of the taste panel.

Results of the correlation analysis between the sensory analysis and the objective measurements for CA stored tomatoes are summarised in Table 1. Regression analyses showed that there was a positive correlation between tomato flavour and TSS and acidity for both the mature green and pink tomatoes. Sweetness was positively correlated with TSS, while it was negatively correlated with titratable acidity. Sourness was negatively correlated with TSS, but positively correlated with titratable acidity. Similar correlations were shown by MALUNDO *et al.* (1995).

DISCUSSION

In this study the effect of CA storage on the eating quality of long-term CA stored tomatoes was evaluated. It is quite difficult to obtain information on the eating quality of fruit stored in CA because control fruit stored in air has a considerably shorter storage life. In all experiments, fruit from the same plants were utilized, so as to minimize variations due to different sources. Control fruit was stored following commercial practices. KADER *et al.* (1978) showed that vine ripened tomatoes have better flavour. Our results confirmed that control fruit obtained higher scores than CA stored fruit. CA storage considerably extended the shelf life of tomatoes held at 13°C or 20°C, irrespective of their initial stage of maturity. This effect has

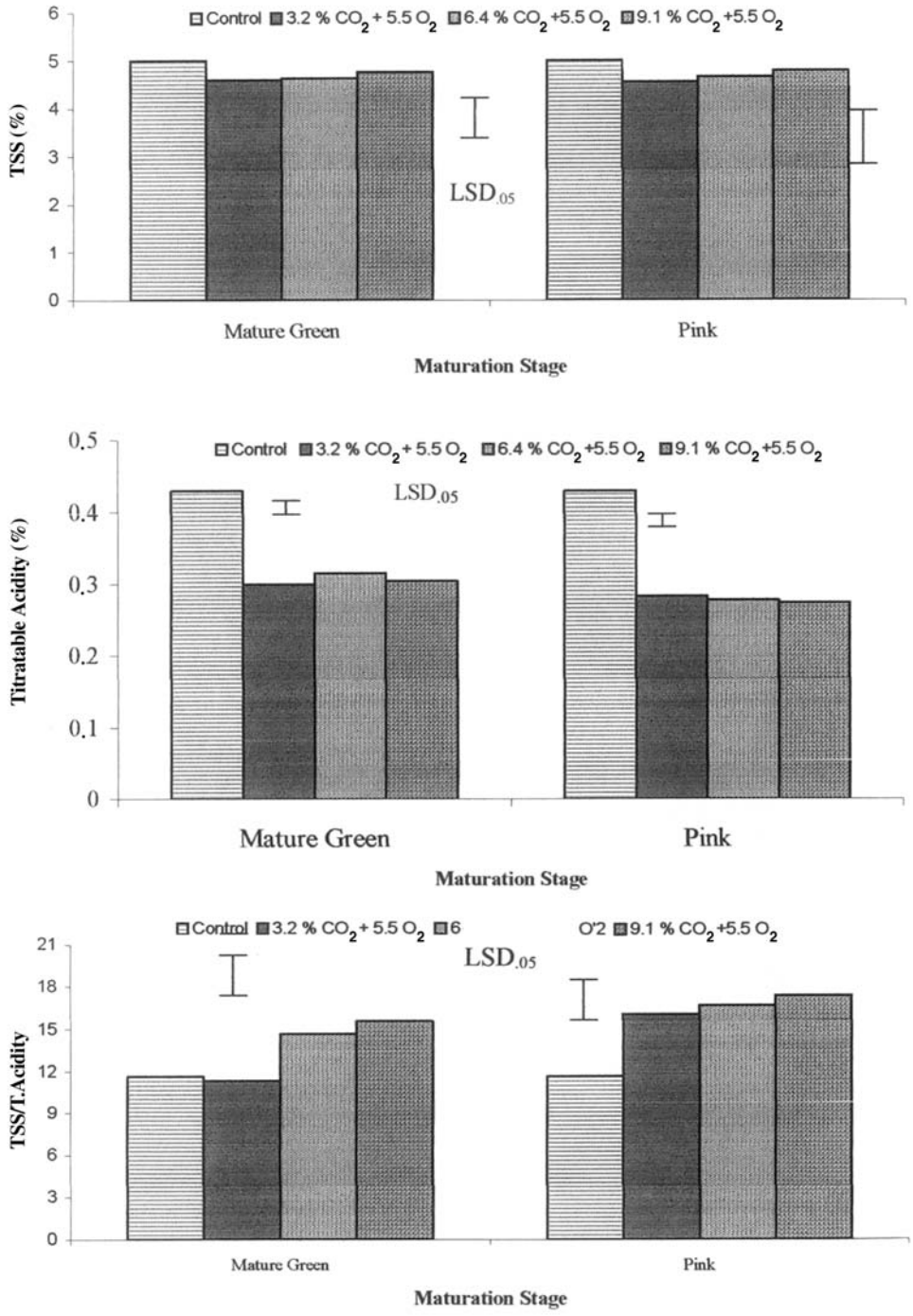


Fig. 3 - Means of TSS, titratable acidity (TA) and TSS/T. Acidity of tomatoes harvested at the mature green stage (stored in CA for 60 days at 15°C plus 10 days at 20°C) and at the pink stage (stored in CA for 60 days at 13°C plus 10 days in air at 20°C).

Table 1 - Correlation coefficients (r) for sensory scores and objective measurements of tomatoes harvested at the mature green and pink stages of maturity and kept in CA storage (3.2%, 6.4% and 9.1% CO_2 plus 5.5% O_2 in all cases) (n=40).

Mature Green	Tomato Flavour	Sweetness	Sourness	Acceptability
TSS	0.96*	0.95*	-0.25	0.87
Titratable acidity	0.99**	-0.99**	0.49	0.92
Pink	**Tomato Flavour**	**Sweetness**	**Sourness**	**Acceptability**
TSS	0.96*	0.94*	-0.24	0.88
Titratable acidity	0.99**	-0.99**	0.46	0.91

*, ** correlations significant of the 5% and 1% level, respectively.

been shown previously (MORRIS and KADER, 1974; DENNIS *et al.*, 1979; GRIERSON and KADER, 1986; BATU, 2003).

Sugar and acidity are the parameters that panelists perceive most quickly. Long-term storage of tomatoes always results in poorer sensory characteristics in comparison to those stored short term. The flavour of the tomato fruit depends on the complex interaction of sugars, organic acids and more specialized flavour compounds, including a wide range of volatiles that are lost during long-term storage. Hence it is particularly important to maintain acid levels in stored fruit so as to avoid a bland and uninteresting impression when the fruit is consumed. It has been shown that tomato fruit flavour reaches the most acceptable level when acidity and sugars are high (KADER *et al.*, 1977; GRIERSON and KADER, 1986; HOBSON and GRIERSON, 1993). High sugar and high acidity are associated with high flavour intensity (STEVENS *et al.*, 1977). High acidity and low sugar content give tart fruit, while high sugar content and low acidity give bland tasting fruit (GRIERSON and KADER, 1986).

STEVENS (1986), KADER *et al.* (1978) and RESURRECCION and SHEWFELT (1985) have shown that variations in sugar and acidity and in the sugar/acidity ratio are important to tomato flavour. It has also been reported that sugar and acidity are not the only contributors to sweetness and sourness. Tomato flavour intensity is also affected by the organic volatile compounds produced by the fruit (STEVENS *et al.*, 1977). These were not measured in this study, but may have contributed to the flavour and acceptability scores of the taste panels.

High CO_2 and low O_2 maintain better acidity levels than low CO_2 and high O_2. KADER (1980; 1987) reported that high CO_2 concentrations delay fruit ripening. Thus, acidity and total sugar levels could be higher in less ripe fruit than in ripe or over ripe fruit. Acidity and sugar contents decrease with ripening, especially after the pink stage of maturity (GRIERSON and KADER, 1986; HO and HEWITT, 1986). Although SALTVEIT (1989) indicated that levels higher than 3% CO_2 for green tomatoes and 5% CO_2 for red tomatoes may cause CO_2 injuries, no injury was observed in this work with the application of either 6.4% or 9.1% CO_2 for 60 days of CA storage.

In conclusion, the results of this preliminary study on the sensory quality of tomato after long-term storage in CA indicate that the quality was still acceptable after CA storage. The use of 9.1% CO_2 combined with 5.5% O_2 were the best conditions for storage of pink tomatoes for 60 days and for mature green tomatoes for 70 days, resulting in a lower rip-

ening rate with slightly higher tomato flavour and consumer acceptability.

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Revised paper received February 5, 2003 Accepted April 24, 2003

SHORT COMMUNICATION

QUALITY CONTROL OF A TYPICAL "PASTA FILATA" CHEESE BY FT-IR SPECTROSCOPY

CLASSIFICAZIONE DI FORMAGGI A PASTA FILATA MEDIANTE SPETTROSCOPIA FT-IR

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ABSTRACT

FT-IR spectroscopy was used to analyse 112 samples of "Provola" cheese, coated with biodegradable wax or paraffin, at 90 and 120 days of shelf-life, in order to verify the discriminating power of this spectroscopic technique. The influence of the two different kinds of wax was also studied. Spectral data were processed using Mean Centre algorithm and transformed into second derivative using the Savitzky-Golay algorithm. Principal Component Analysis was applied to optimise the infor-

RIASSUNTO

Campioni 112 di formaggio "Provola", rivestiti con paraffina e con cera biodegradabile, sono stati analizzati mediante spettroscopia FT-IR a 90 e 120 giorni di shelf-life con l'obiettivo di verificare le potenzialità discriminanti della tecnica. È stata inoltre studiata la possibilità di classificare i campioni anche in base al tipo di rivestimento impiegato. I dati spettrali sono stati elaborati applicando l'algoritmo "Mean Centre", e successivo calcolo della derivata seconda delle assorbanze utiliz-

- Key words: chemometrics, FT-IR spectroscopy, "pasta filata" cheese, shelf-life -

mation given by the spectral data sets. FT-IR spectroscopy was able to classify samples on the basis of shelf-life and to detect the differences in the lipolytic trend due to the nature of the wax used.

zando l'algoritmo "Savitzky-Golay". L'applicazione della statistica multivariata come analisi esplorativa, utilizzando l'Analisi delle Componenti Principali (PCA), ha permesso di verificare come la spettroscopia FT-IR sia stata in grado di classificare i campioni in funzione del periodo di shelf-life considerato, rilevando differenze di andamento di degradazione delle componenti grasse per lo più attribuibili al tipo di rivestimento impiegato.

INTRODUCTION

"Pasta filata" cheese, such as Provola and Provolone, has particular process characteristics which makes it special and original (SALVADORI DEL PRATO and MESSINA, 1990). This type of cheese is different from other cheese because critical parameters have been found by analysing the technological steps used in its production. In particular, the use of raw milk and the curd "stretching" process contribute to conferring typical characteristics of structure and aroma (BOTTAZZI and BATTISTOTTI, 1973). Furthermore, coagulation is carried out at 37-38°C using lamb and kid paste rennet, rich in lipolytic enzymes. For this reason, during ripening, the cheese fat undergoes a hydrolytic process which could contribute to giving it its piquant taste. The action of these enzymes leads to the presence of short chain free fatty acids, like butyric acid, which are the precursors of other aroma compounds such as alcohols, esters, methylketones and lactones. These free fatty acids are responsible for the piquant taste of the cheese and the piquant intensity can be correlated to the total amount of free fatty acids. Provolone cheese is the classical result of the application of this technology. Another type of "pasta filata" cheese includes small size Provola cheese, which is characterised by a short ripening time (10 days) and a sweet and refined taste. The development of a piquant taste is not desirable for this type of cheese.

After ripening, this cheese is normally sealed completely using a thick wax layer to protect the product. This prevents drying out and damage during storage and transportation, thus maintaining the original taste during shelf-life. The wax usually used is paraffin, which is chemically inert and non-biodegradable under room conditions. The most recent alternative is based on the use of biodegradable polymers such as polyhydroxy-alkanoates (PHAs), produced by microbiological fermentation processes using renewable resources e.g. fatty acids (EU-PROJECT, 2001).

During the shelf-life of this "pasta filata" cheese, lipolysis continues to occur. However, consumers do not accept such a strong development of flavour. Aroma standardisation is one of the most important issues in the production of short-ripened "pasta filata" cheese. Therefore, chromatographic techniques, such as GC and GC-MS, are used to check the characteristics of the final product, even during shelf-life.

In the last decade, Fourier Transform InfraRed (FT-IR) spectroscopy has been used to classify other kinds of cheese during ripening. DUFOUR *et al.* (2000)

discriminated sixteen semi-hard cheeses according to ripening times on the basis of the shift of the methylene bands in the infrared region. CHEN *et al.* (1998) applied FT-IR spectroscopy to study Cheddar cheese of different fat levels and corresponding changes in the IR region during ripening. The advantages of applying FT-IR spectroscopy in the dairy field are greater when coupled with multivariate analysis such as Principal Component Analysis (PCA). Some studies have been performed using FT-IR spectroscopy together with statistical techniques to classify dairy products like butter and vegetable margarine (DUPUY *et al.*, 1996), cream (DUFOUR and RIAUBLANC, 1997) and milk fat globule membrane (DUFOUR *et al.*, 1999).

The aim of this work was to evaluate whether FT-IR spectroscopy could be applied to classify small size "Provola" cheese, coated with biodegradable wax or paraffin, during its shelf-life. The influence of the two different kinds of wax on the lipolytic trend was also studied.

MATERIALS AND METHODS

Small size Provola cheese samples were produced at Latteria Soresinese (Soresina, Lodi, Italy), a medium-sized industrial dairy plant. One hundred and twelve samples, produced on the same day, were taken from the same batch and ripened for 10 days. Samples were divided into two sets (56 samples each) on the basis of the coating, paraffin and biodegradable wax. Twenty-eight samples from each set were analysed during shelf-life at 90 days and the others at 120 days.

Analyses were carried out at room temperature. Samples were cut into small pieces (72 x 11 x 1 mm) from the cheese core, and cheese films were placed on the surface of the FT-IR cell.

Spectra were collected using an FT-IR/420 spectrometer (Jasco Corporation, Tokyo, Japan) coupled with attenuated total reflectance (ATR). The ATR cell was made of horizontal ZnSe crystal (IRE), with 12 internal reflections, having an incident angle of 45°. The spectra were recorded as %T in the range 4,000-600 cm^{-1} with a resolution of 4 cm^{-1} using 16 scans/sample using Jasco Spectra Manager Software. The background was recorded with an empty ATR cell and it was repeated for each measurement. Both the interferometer and the sample-detector compartments of the instrument were purged with dry air to reduce interfering water vapor and CO_2.

Data were processed by GRAMS/32 AI Galactic Software (Galactic Industries, Salem, NH, USA) by using the Mean Centre algorithm which calculates the average spectrum of all spectra and then subtracts the results from each spectrum. This technique was designed to show the differences among spectra. Preprocessed data were transformed into second derivative using the Savitzky-Golay algorithm (five points).

Explorative analysis was applied to the full set of data using Principal Component Analysis to optimise the information given by the spectral data sets without any hypothesis or any external information concerning the samples studied (JOLLIFFE, 1986).

Chemometric results were confirmed by sensory analysis. The sensory evaluation was carried out through a triangle test (LARMOND, 1977) made by a panel of 15 half-trained panelists.

RESULTS AND DISCUSSION

In Fig. 1, the mean FT-IR spectra of "pasta filata" cheese coated with paraffin and biodegradable waxes are shown. Apparently, only the water content was modified during shelf-life independent of the type of wax covering the cheese, as seen in the 3,300-3,400 cm^{-1} spectral range.

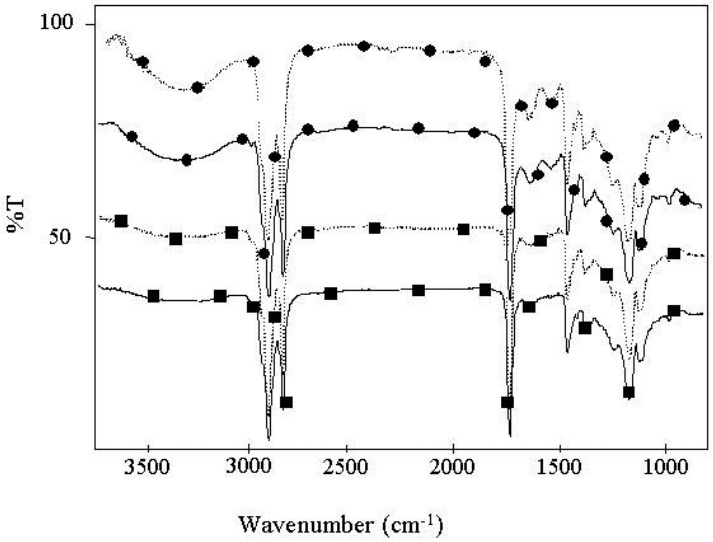


Fig. 1 - Examples of the original FT-IR spectra of small size "Pasta filata" cheese taken at 90 (•) and 120 (■) days of shelf-life, and coated with paraffin (...) and biodegradable wax (-). Spectra were shifted to enhance differences.

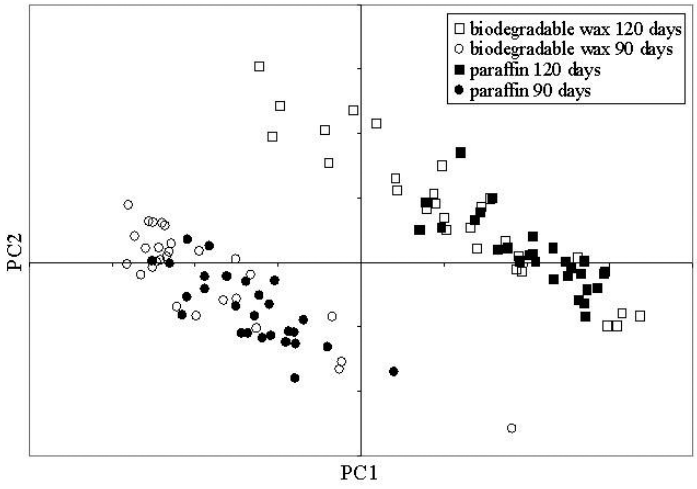


Fig. 2 - PCA similarity map defined by the first (PC1) and second (PC2) Principal Component for FT-IR spectral data collected between 1,100 and 1,035 cm^{-1}.

Principal Component Analysis was applied to the whole set of FT-IR data. Results obtained using the whole IR range did not show satisfactory discrimination among samples (data not shown). For this reason, PCA was applied to selected IR regions: 1,100-1,035 and 1,720-1,690 cm^{-1}.

The PCA similarity map, obtained on the 1,100-1,035 cm^{-1} IR region, in which C-OH (1,040 cm^{-1}) and C-O-C (1,080 cm^{-1}) stretching of sugars (LEFIER *et al.*, 1996; AMIEL *et al.*, 2000) absorb, and defined by the Principal Components 1 and 2, is shown in Fig. 2. A clear separation between the two sets of cheese with regard to shelf-life, 90 and 120 days, can be seen along the PC1 (explaining 86% of the total variance). Although the PC2 (explaining 6% of the total variance) did not lead to a total discrimination between cheese covered with paraffin and biodegradable waxes, there was a general trend. The variation in the carbohydrate absorption may explain the differences among the samples having different shelf-lives.

Fig. 3 shows the PCA similarity map obtained on the 1,720 and 1,690 cm^{-1} IR region, which is the group frequency zone. This spectral region is related to the C=O stretching of carboxylic acids. These functional groups are typical of organic molecules such as lactic acid and free fatty acids (SILVERSTON *et al.*, 1991) derived from fat and sugar hydrolysis. A good discrimination was obtained between the two groups of cheese with regard not only to shelf-life, but also to the

type of coating used. It is interesting to observe how the scores relating to cheese covered with paraffin were better separated than those covered with biodegradable wax. The first two principal components took into account 99% of the total variance, mainly along the PC1. The separation between samples coated with different wax, and stored for 90 days, was obtained along the PC2.

Furthermore, some differences were detected in the lipolytic trend between the two sets of cheeses stored for 120 days, which is probably connected to the nature and the adhesion of the wax to the cheese surface. Weight loss values confirmed the influence of the type of coating on the final characteristics of the products, detectable by PCA analysis, as reported in Fig. 4.

There was a higher percentage of weight loss in samples coated with the biodegradable wax than those coated with paraffin. The lipolytic trend during shelf-life was influenced by such weight loss. This was confirmed by the sensory results that assigned a sweet taste to the cheese coated with paraffin at 90 days of shelf-life, development of a light piquant taste to the cheese coated with paraffin at 120 days, and a piquant taste to the cheese coated with biodegradable wax at 120 days of shelf-life. The longer the shelf-life period, the stronger the development of piquant taste.

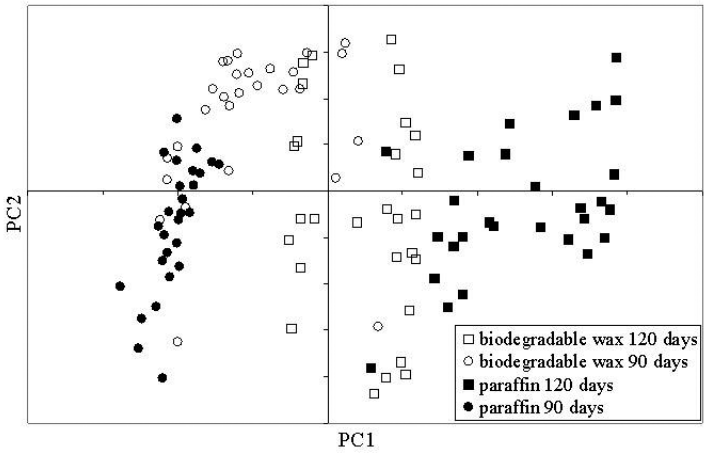


Fig. 3 - PCA similarity map defined by the first (PC1) and second (PC2) Principal Component for FT-IR spectral data between 1,720 and 1,690 cm^{-1}.

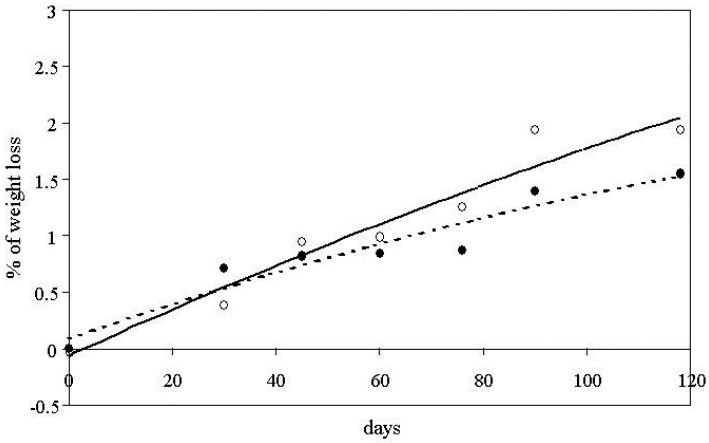


Fig. 4 - Weight loss during cheese shelf-life: cheese coated with paraffin (-●-), cheese covered with biodegradable wax (-○-).

CONCLUSIONS

The results show that FT-IR spectroscopy could be used in combination with multivariate statistical analysis to classify small size "Provola" cheese samples on the basis of both the days of shelf-life and the type of coating.

The lipolytic activity is an important factor of variance in short-ripening "pasta filata" cheese, and differences in this activity were detected by FT-IR. Further

investigations are required to support IR data with classical methods which could confirm these preliminary observations.

The advantages of this technique, coupled with chemometric analysis, are: i) fast collection and processing of data; ii) suitability to classify cheese in real-time on the basis of its shelf-life; iii) ease of use; iv) no chemical treatments required during the sample preparation step; v) no waste.

ACKNOWLEDGEMENTS

This work was partially supported by the EU-PROJECT. 2001, BES2-2659.

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Paper received February 12, 2003 Accepted June 20, 2003

SHORT COMMUNICATION

INFLUENCE OF DIET AND AGE OF KIDS ON ENZYMATIC ACTIVITIES OF KID RENNET PASTES

INFLUENZA DELLA DIETA E DELL'ETÀ DEI CAPRETTI SULLE ATTIVITÀ ENZIMATICHE DEL CAGLIO IN PASTA

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ABSTRACT

The influence of diet and age of kid goats was determined on the total clotting activity, content of milk clotting enzymes (chymosin and pepsin) and lipolytic and esterase activities of home-made rennet paste from three different groups of kids. Both chymosin and lipolytic activities were higher in rennet pastes obtained from kids that only suckled milk than in rennets from kids that suckled and ate mixed feed. In the groups that suckled and ate mixed feed, both activities decreased in the older kids. Overall the results show that diet appears to influence the enzymatic pattern present in rennet paste more than age.

RIASSUNTO

L'influenza della dieta e dell'età dei capretti è stata valutata sul contenuto in chimosina, pepsina e l'attività lipolitica nei cagli in pasta ottenuti da tre differenti gruppi di capretti. L'attività della chimosina e quella lipolitica erano più alte nei capretti alimentati con solo latte, rispetto agli altri due gruppi che avevano avuto un'alimentazione mista. A parità di alimentazione, le due attività diminuivano con l'età. I risultati ottenuti indicano che la dieta ha una maggiore influenza rispetto all'età sul contenuto enzimatico del caglio in pasta di capretto.

- Key words: chymosin, esterase, lipase, pepsin, rennet paste -

INTRODUCTION

Rennet pastes are used traditionally in Italian cheese manufacturing and are prepared from the abomasum of calves, lambs or kids slaughtered after suckling. The enzymatic content of rennet pastes is very heterogeneous, and includes proteolytic, lipolytic and milk clotting enzymes. These enzymes take part both in milk clotting and cheese ripening. The fourth stomach (or abomasum) of ruminants secretes, as zymogens, three milk-clotting aspartic proteases called: chymosin (EC 3.4.23.4), pepsin A (EC 3.4.23.1), and gastricsin (EC 3.4.23.3). Chymosin and pepsin represent 97-100% of the total clotting activity. The lipases present in rennet paste are commonly referred to as pre-gastric esterase (of oral origin) and gastric lipase. Pre-gastric esterases are secreted by the lingual serous glands in the oral cavities of young animals (NELSON *et al.*, 1977) and exhibit high specificity for short chain fatty acids, especially butanoic acid. Gastric lipases are present in some rennet paste preparations but not in commercial rennet. Gastric lipases hydrolyze milk fat very slowly, but may have a role in cheese flavour development (RICHARDSON *et al.*, 1971).

The rennet paste from kids is used in cheese-making of some typical Italian cheeses such as Provolone or Pecorino Romano (RAMPILLI and BARZAGHI, 1995). The characteristic flavour of Italian-type cheeses has been attributed primarily to short chain fatty acids. These fatty acids are produced mainly by lipolytic enzymes contained in rennet paste (LEE and RYAN, 1980). The enzyme contents of rennet paste have been shown to be strictly dependent on age (SPONCET *et al.*, 1984), diet and the physiological state of the animal at slaughter (PIREDDA and ADDIS, 1998). The aim of this study was to investigate the influence of diet and age of kids on the rennet paste characteristics, and in particular on the content of coagulant, proteolytic and lipolytic activity, as they could be of interest for the production of some Italian cheeses.

MATERIALS AND METHODS

Rennets

Three different kid rennet pastes prepared at the "Istituto Sperimentale per la Zootecnia", Bella, Potenza, from 30 kids (race: syrian derivate) were analyzed. Rennet MF35 was obtained from the 1st group of kids, slaughtered at the age of 30-35 days. The kids in this group suckled milk but were also allowed to graze and eat hay and commercial mixed feed. Rennet MK35 was obtained from the 2nd group. Kids of this group only suckled milk and were slaughtered at the age of 30-35 days. Rennet MF25 was obtained from the 3rd group, slaughtered at the age of 25 days. Kids in this group suckled milk but were also allowed to graze and eat hay and commercial mixed feed. Each rennet paste was prepared from kids slaughtered after suckling milk. Samples were prepared as described in (CALANDRELLI *et al.*, 1997).

Determination of total clotting activity

For the determination of total milk clotting activity, 3x10 g of each rennet paste (prepared as described above) were blended in 50 mL acetate buffer, stirred for 30 min at room temperature (20°C) and filtered through sterile gauze. The filtrate was tested for total clotting activity according to the FIL-IDF 157:1992 standard (FIL-IDF, 1992). For each sample, the total clotting activity was determined in triplicate. Enzymatic activity is expressed in IMCU/g of rennet (International milk clotting units/g rennet).

Determination of pepsin and chymosin content

The pepsin and chymosin content in the rennet pastes was determined according to the FIL-IDF 110A:1987 standard (FIL-IDF, 1987). The sample used was prepared by blending 3x10 g of rennet in 50 ml deionised water for 30 min at room temperature. The sample was filtered through sterile gauze and dialyzed against 25 mM piperazine – HCl buffer, pH 5.3 overnight at 4°C. After centrifugation at 49,000 x g at 4°C for 1 h, 1 mL of supernatant was analyzed according to the FIL-IDF 110A:1987 standard (FIL-IDF, 1987). For each sample the analyses were done in triplicate.

Determination of esterase activity

Esterase activity was determined using the colorimetric method with p-nitrophenylbutyrate (Sigma) as the substrate (RUA *et al.*, 1993). The sample was prepared by blending 3x10 g of rennet in 50 mL 100 mM sodium phosphate, pH 7.0, for 30 min at room temperature. Then the sample was centrifuged at 31,000 x g at 4°C for 30 min and the supernatant was filtered through a 0.45 µm filter. Esterase activity was determined at 400 nm using a standard curve obtained by using *p*-nitrophenol (Sigma). Enzymatic activity is expressed in EU/g rennet. One unit is the amount of enzyme that liberates 1 µmol of *p*-nitrophenol/min under the above conditions. For each sample the analyses were done in triplicate.

Determination of lipolytic activity

Lipolytic activity was determined by titration of acids liberated from tributyrin. The substrate for the assay was prepared by vigorously stirring 5 mL of tributyrin (Sigma) in an ultraturrax with 5 g of arabic gum for 2 min. It was then diluted with deionised water to a final volume of 250 mL. The pH was adjusted to 6.0 with 0.01 N NaOH.

For the assay, 3x10 mL of assay mixture and 2 mL of rennet extract (obtained by blending 10 g of rennet in 50 mL deionised water for 30 min at room temperature, and filtering through a sterile gauze) were pipetted into a test tube and incubated in a 37°C shaker water bath for 30 min. The reaction was stopped by placing the tube in ice and adding 10 mL of cold absolute ethanol (LEE *et al.*, 1980). Then the acids liberated from the assay mixture were titrated with 0.01 N NaOH to pH 7.0 with a pH-meter. For each sample the analyses were done in triplicate. Lipolytic activity is expressed as µeq of NaOH / min/g rennet.

Lipolytic activity was also determined using the gas chromatographic method by evaluating the total and individual free fatty acids (FFA) produced from UHT cream (24% fat, Granarolo, Italy).

Rennet extract (1.25 mL) was added to 25 g of UHT cream. The mixture was incubated at 37°C for 5 days on an orbital stirrer (AROS 150) at 150 rpm. The reaction was stopped by freezing the reaction mixture at -20°C. A blank was prepared in the same conditions as the sample except that the reaction mixture was immediately frozen at -20°C. Extraction and analysis of FFA was carried out according to LENCIONI *et al.*(1998) using 0.5 g of reaction mixture. For each sample the extraction of FFA was repeated. All analyses were done in triplicate. Lipolytic activity is expressed as mg of FFA / 100 g cream.

Statistical analysis

All assays were carried out on three samples for each group of rennet pastes. All analyses were done in triplicate. Results are presented as means ± SD. Significant differences between all parameter values were evaluated by one-way analysis of variance (ANOVA) followed by

comparison with the LSD test. Differences were considered significant at $P<0.05$.

RESULTS

Total clotting activity and pepsin and chymosin content

Table 1 shows the milk clotting activity and the content of pepsin and chymosin. Total clotting activity was higher in the rennet obtained from kids that only suckled milk and were slaughtered at the age of 30-35 days, (group MK35) than in rennets from kids that suckled milk and ate mixed feed, regardless of age (groups MF35 and MF25). The difference in total clotting activity between rennets obtained from kids slaughtered at different ages but with a similar diet (groups MF35 and MF25), was less than that between rennets obtained from kids slaughtered at the same age but with different diets (rennets MF35 and MK35). The chymosin content was higher in rennet from MK35 than in rennets from MF35 and MF25. However, the pepsin content was higher in rennets MF35 and MF25 than in MK35.

Lipolytic and esterase activity

Table 2 shows the lipolytic and esterase activities of the kid rennet pastes. The lipolytic activity, related to the pregastric lipase, was higher in rennet MK35 than in MF35 and MF25. The esterase activity was significantly lower in MF35 than in MK35 and MF25.

Fig. 1 shows the lipolytic activity determined by gas chromatography of the total free fatty acids released by hydrolysis of milk cream (FFA mg/100g cream). There were significant differences between the three rennets. In particular rennet MK35 showed higher lipolytic activity than MF35 and MF25 rennets. Acylic profiles obtained by gas chromatography (Fig. 2) also confirmed a higher lipolytic activity for short chain fatty acids. The MK35 rennet showed a higher propensity than the other rennets to release short chain fatty acids (C4:0 and C6:0). It also showed a lower percentage of C10:0 and C18:1 fatty acids. No significant differences were found between the C8:0, C12:0 and C16:0 fatty acids. All the others were significantly different. The MF35 rennet released a lower

Table 1 - Milk clotting activity and enzymatic content of the 3 rennets.

Rennet		MF35	MK35	MF25
Total clotting activity ($LSD_{95\%}$=0.88)	IMCU/g	$63.2^{a}\pm0.9$	$106.4^{b}\pm1.0$	$87.5^{c}\pm0.9$
Chymosin content ($LSD_{95\%}$=0.59)	%	$75.0^{a}\pm0.6$	$88.5^{b}\pm0.6$	$81.5^{c}\pm0.6$
Pepsin content ($LSD_{95\%}$=0.32)	%	$25.0^{a}\pm0.4$	$11.5^{b}\pm0.3$	$18.5^{c}\pm0.3$

Mean values followed by different letters (a, b, c) indicate significant differences between groups (one-way ANOVA; $p<0.05$).
IMCU: International milk clotting units.
MF35: rennet obtained from kids that suckled milk, and ate hay and commercial mixed feed and were slaughtered at 30-35 days.
MK35: rennet obtained from kids that suckled milk only and were slaughtered at 30-35 days.
MF25: rennet obtained from kids that suckled milk and ate hay and commercial mixed feed and were slaughtered at 25 days.

Table 2 - Lipolytic and esterase activities of the 3 rennets.

Rennet		MF35	MK35	MF25
Lipolytic activity ($LSD_{95\%}$=0.15)	µeq /min*g	3.62^{a}±0.18	6.37^{b}±0.15	5.81^{b}±0.14
Esterase activity ($LSD_{95\%}$=0.01)	EU/g	0.54^{a}±0.01	0.72^{b}±0.01	0.64^{c}±0.01
Mean values followed by different letters (a, b, c) indicate significant differences between the groups, p>0.05).				

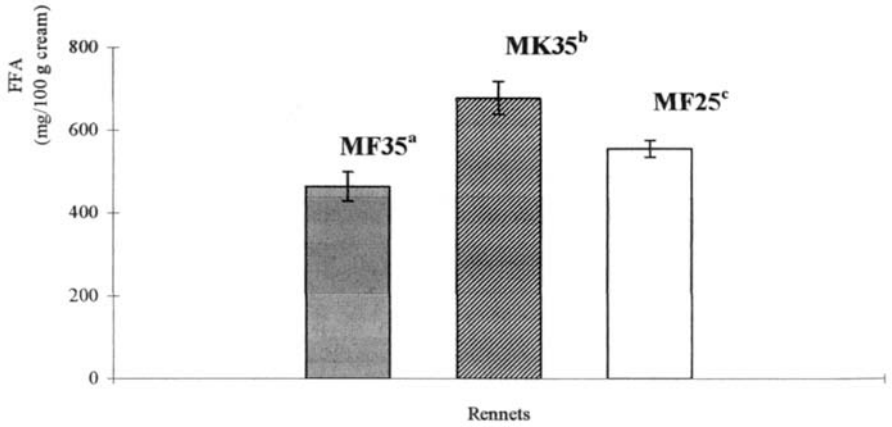


Fig. 1 - Lipolytic activity determined by gas chromatographic analysis of free fatty acids obtained from cream.
Abbreviations MF35, MK35 and MF25 followed by different letters (a, b, c) indicate significant differences between the groups $LSD_{95\%}$ = 31.8; p<0.05).

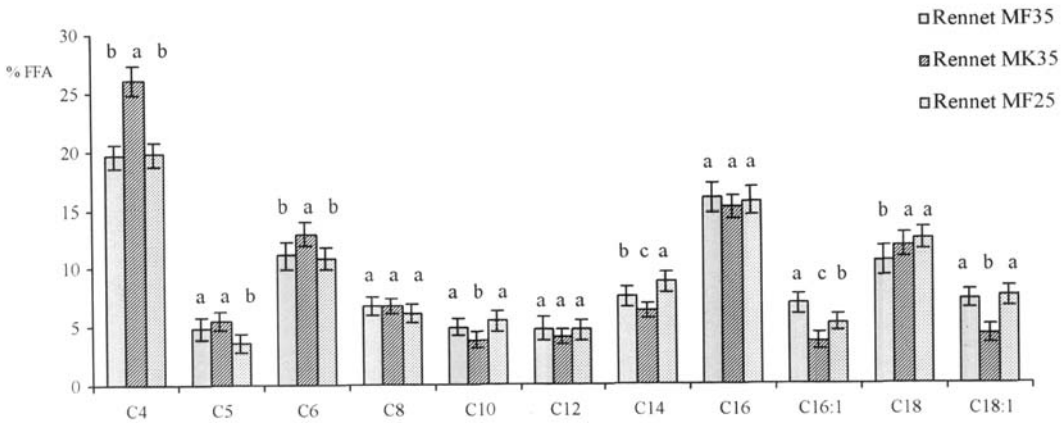


Fig. 2 - Acylic profiles obtained by gas chromatographic analyses of free fatty acids released from cream.
For each group, columns followed by different letters represent significant differences (p<0.05).

amount of C18:0 in comparison to the other rennets, whereas the release of C14:0 and C16:1 were different for all rennets.

DISCUSSION

The aim of this study was to investigate the influence of diet and age of kids on the enzymatic characteristics of home-made rennet pastes, as they could be of interest for the production of some Italian cheeses. Results show that diet influenced the enzymatic pattern present in rennet paste more than age. Kids whose diet was based on milk only gave rennets with higher lipolytic activity, due to pregastric lipase, whereas kids whose diet was based on mixed feed gave rennets with lower lipolytic activity. This may be useful for the preparation of home-made rennets with high lipolytic activity, which are required for the production of cheeses with typical characteristics such as the Italian cheeses, Romano and Provolone. In fact, it is known that in these Italian cheeses the characteristic spicy tastes can be primarily ascribed to the presence of short chain fatty acids produced mainly by the pre-gastric esterases (FOX and GUINEE, 1987). Although short chain fatty acids can also be released by other enzymes, such as the autochthonous milk lipase LPL (HA and LINDSAY, 1990), or can derive from carbohydrates or some amino acids (NAKAE and ELLIOT, 1965a,b), the lipolytic activity of pregastric esterases remains dominant. Hence, the observed effect of diet on these enzymes is relevant.

Studies have been carried out mainly on calves and lambs, but there has been no report on kid rennet paste. GUILLOTEAU *et al.* (1985) reported the influence of diet on the enzyme pattern of the abomasum and pancreas of calves of a similar age using a completely different approach and studying the effects of weaning and feeding a liquid supplement. Weaning induced a decrease in chymosin and an increase in pepsin, whereas the liquid supplement lowered the enzyme content in general, with the exception of chymosin. Similar results were found by MARTIN and CORRE (1984).

With regard to milk-clotting activity and chymosin content, the present paper clearly shows that both are reduced with age. Reduction of the milk-clotting activity and chymosin content of the abomasum and an increase in pepsin content have also been observed in older lambs (IRIGOYEN *et al.*, 2001), but the coagulating activity did not decrease when the lamb rennet paste was stored in the cold for 1 year (BUSTAMANTE *et al.*, 2000).

In conclusion, the present study indicates how feeding of kids with milk alone may be useful for the preparation of home-made kid-rennet pastes to be used for the preparation of cheeses with spicy tastes due to their higher lipolytic activity.

ACKNOWLEDGEMENTS

The paper was corrected by Richard Lusardi, a lecturer in English language at the University of Bari.

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Revised paper received March 31, 2003 Accepted July 15, 2003

SHORT COMMUNICATION

PRELIMINARY STUDY ON ACRYLAMIDE IN BABY FOODS ON THE ITALIAN MARKET

SUL CONTENUTO DI ACRILAMMIDE IN PRODOTTI ALIMENTARI ITALIANI PER L'ALIMENTAZIONE DEI BAMBINI

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ABSTRACT

The acrylamide content in some baby foods available on the Italian market was measured using a previously developed GC/MS method. The levels were found to range from <50 to 403 µg/kg in baby biscuits and from 126 to 385 µg/kg in oven-baked confectionery products. Less than 148 µg/kg were found in snack foods with chocolate. In homogenised meat, the acrylamide content was less than 50 µg/kg. The data obtained for most of the products

RIASSUNTO

La quantità di acrilammide in alimenti per bambini distribuiti sul mercato italiano è stata determinata usando un metodo GC/MS precedentemente sviluppato. I livelli risultavano compresi fra <50 e 403 µg/kg nei biscotti, tra 126 e 385 µg/kg nelle merendine. Negli snack al cioccolato considerati è risultata inferiore a 148 µg/kg. In alcuni campioni di omogeneizzati alla carne analizzati il contenuto di acrilammide è sempre risultato inferiore a 50 µg/kg.

- Key words: acrylamide, baby foods, confectionery products, GC/MS, snacks -

tested were not significantly different from those reported by other institutes for the same category of products, but provide preliminary data on the levels of acrylamide in some baby foods sold on the Italian market.

I dati ottenuti per la maggior parte dei prodotti esaminati non risultano di ordine di grandezza differente da quelli riportati da altri Istituti per prodotti della stessa categoria, ma tuttavia costituiscono dati preliminari sui livelli di acrilammide in alcuni prodotti "baby foods" reperiti sul mercato italiano.

INTRODUCTION

In studies carried out by TÖRNQVIST *et al.* (1998) at the University of Stockholm, acrylamide was shown to produce adducts with Hb and emerged as a substance associated with neurotoxic, genotoxic and cancerogenic risk. On the basis of the observed background Hb adduct levels (BERGMARK *et al.*, 1997; HAGMAR *et al.*, 2001), it could be estimated that a background, general exposure to acrylamide could pose a potential cancer risk. The average Hb adduct levels of acrylamide recorded in Swedish adults without known exposure stimulated further research on the acrylamide content in fried foods (TAREKE *et al.*, 2000). Because it was assumed that acrylamide is produced at high temperatures during cooking, the acrylamide content produced in the process of heating various foods for human consumption was subsequently determined (TAREKE *et al.*, 2002).

In July 2002, the Scientific Committee on Food of the European Commission expressed its opinion on new findings regarding the presence of acrylamide in food, and recommended that levels of acrylamide should be as low as reasonably achievable (SCF, 2002). Steps were taken nationally and internationally to embark on multidisciplinary research, including the analysis of acrylamide levels in food and assessments based on long term dietary exposure.

At present, several analytical methods are available for determining acrylamide in foods. The U.S. Department of Health and Human Services (FDA, 2002a) has published exploratory data suggesting a method of analysis and the Swiss Federal Office of Public Health (SFOPH, 2002b) has also published findings concerning Swiss food, suggesting a different method. Some methods are based on the GC/MS or GC-MS/MS technique (CASTLE, 1993; SFOPH, 2002a; TAREKE *et al.*, 2002; GERTZ and KLOSTERMANN, 2002), while others use LC-MS/MS analysis (SFOPH, 2002a; TAREKE *et al.*, 2002; FDA, 2002b; ROSEN and HELLENÄS, 2002).

Realising the need to devise new, simplified methods, TATEO and BONONI (2003) developed an EI-GC/MS method (MID mode) for underivatized acrylamide determination. In the present paper, this method was applied to various baby foods and other well-known products available on the Italian market, to obtain results on the levels of acrylamide in these foods. The aim was not to compare statistically data obtained for various brands but to determine the acrylamide content in random samples of three packages belonging to the same lot for each type of product. This type of sampling is sufficiently representative of the random choice made by the consumer and at the same time enables a more immediate evaluation to be made, useful for preliminary screening.

MATERIALS AND METHODS

Twenty-nine food samples were obtained from local supermarkets and were subjected to the following extraction procedure. Fifty grams of material were taken from each of three packages of the same lot, mixed, and finely crushed; extraction was carried out on 10 g aliquots, using the operating conditions described by TATEO and BONONI (2003). After defatting, the 10 g aliquot was extracted with 50 mL of methanol (VWR International, Milano, Italy, 99.8%) and 30 mL of the extract were recovered and concentrated to 2 mL. This extract (1 µL) was directly injected into a GC/MS QP 2010 (Shimadzu; Milan, Italy) in EI mode. The column was a Supelcowax (30 m x 0.25 mm i.d., 0.25 µm film thickness; Supelco; Milan, Italy) and the temperature program was: isothermal for 1 min at 60°C, with an increase of 10°C/min to 240°C.

The methanol extracts were analysed in the Multiple Ion Detection mode (MID) to establish the identity of acrylamide at a retention time of 15.09±0.04 min. As suggested by TATEO and BONONI (2003), the molecular ion $[C_3H_5NO]^{\bullet+}$ (m/z = 71) and product ions $[C_3H_3O]^+$ (m/z = 55) and $[C_2H_3]^+$ (m/z = 27) were monitored. Acrylamide standard (99%) was obtained from Sigma-Aldrich, Milan, Italy.

RESULTS AND CONCLUSIONS

The calibration curves produced by measuring the area counts of the m/z 71 peak for standard solutions of acrylamide in methanol are reported in Figs. 1 and 2. The area count values refer to 1 µL (autosampler) of standard solution injected in the splitless mode (1 min). Fig. 3A shows the trace of the m/z 71 ion produced in the SIM mode for the quantitative determination of a baby biscuit containing 293 µg/kg of acrylamide and Fig. 3B shows the traces of m/z 71, 55, 27 ions produced in the MID mode for confirming the identity of the peak.

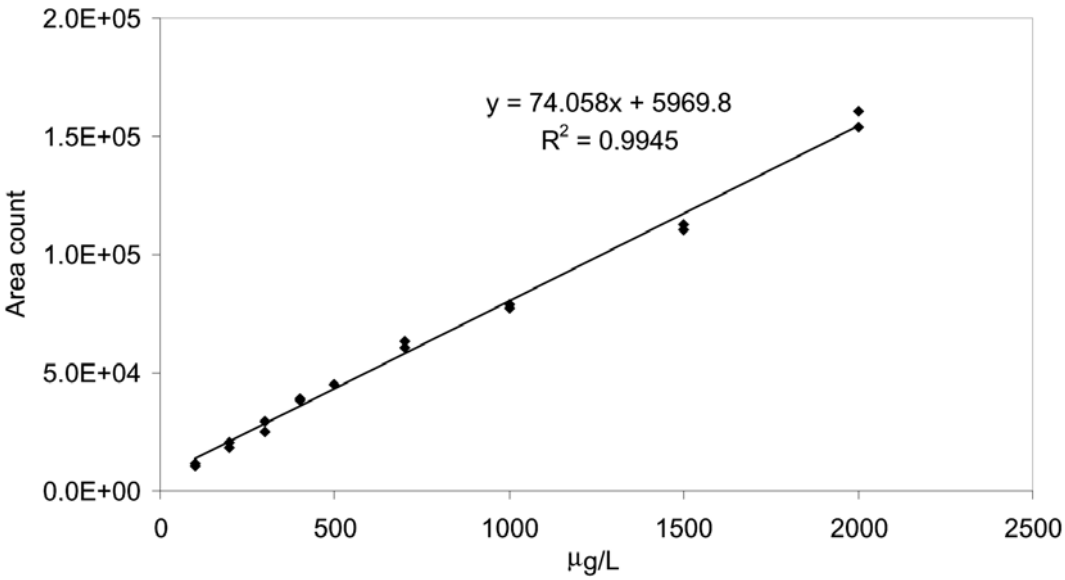


Fig. 1 - Calibration curve for acrylamide in the 100-2,000 mg/L range, GC/MS detection.

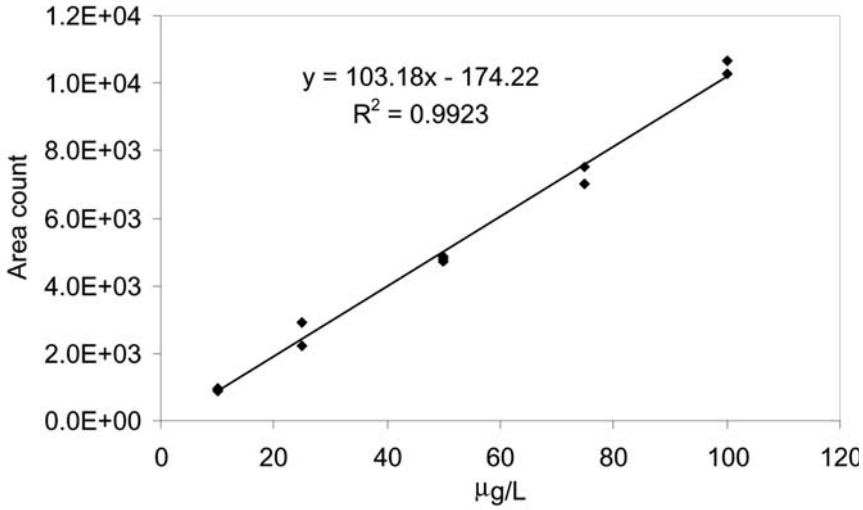


Fig. 2 - Calibration curve for acrylamide in the 10-100 mg/L range, GC/MS detection.

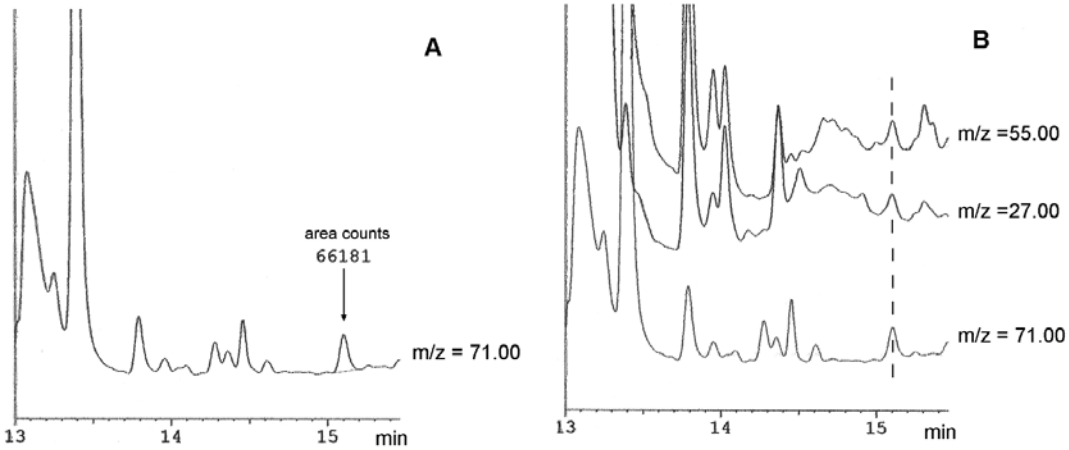


Fig. 3 - Trace of the m/z 71 ion (A = SIM mode) for identification and quantitative determination of acrylamide in a biscuit (293 mg/kg) and traces of m/z 71 55, 27 ions (B = MID mode).

The calibration curve in Fig. 1 was linear (R^2 = 0.9945) in the 100-2,000 µg/L range. Average recoveries were between 78% and 84% (acrylamide-spiked between 100 and 500 µg/kg for biscuits, oven-baked confectionery products, and snacks with chocolate) and the CV was between 8.0% and 2.8%. For extracts with area counts lower than 10,000, the level of acrylamide found in food samples was less than 50 µg/kg. As Fig. 2 shows, the calibration curve was linear (R^2 = 0.9923) between 10 and 100 µg/L, and the average recovery was 65% for products containing less than 80 µg/kg acrylamide. Recovery was evaluated by spiking unbaked flour with 40 µg/kg of acrylamide (Fig. 4).

The acrylamide content found in various foods by several organizations is reported in Table 1. Since the highest value reported for bread, the most com-

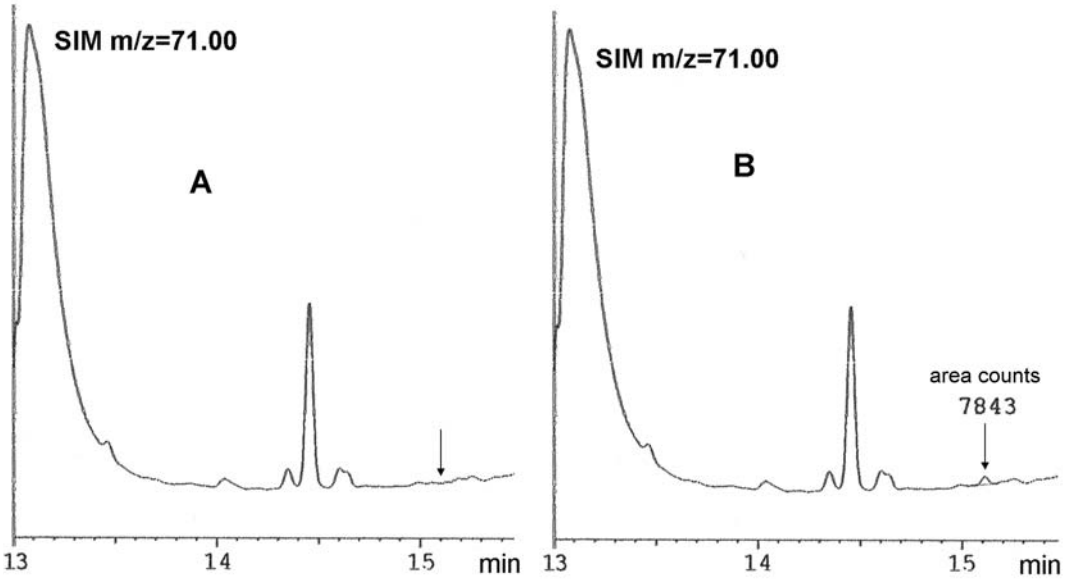


Fig. 4 - Recovery evaluated by spiking unbaked flour with 40 mg/kg of acrylamide (A= unbaked flour, B = spiked unbaked flour).

Table 1 - Acrylamide concentration (μg/kg) in various foods reported by different sources*.

	SNFA April 2002	FDA September 2002	SFOPH October 2002	TAREKE *et al.* (2002) University of Stockholm
Potato crisps	322-2,287	117-1,096	190-2,000	1,300-3,897
French fries	301-1,104	21-1,036	85-250	314-732
Biscuits	<30-652	36-199	<50-2,000	—
Crackers	534	26-504	<50-560	—
Crispbread	<30-1874	—	—	—
Breakfast cereals	<30-1340	47-266	<50-240	—
Corn snack foods	120-180	—	—	—
Bakery products	30-162	ND – 364	<20-510	—
Bread **	<30-45	ND-36	<50	13-49
Baby Foods	—	ND – 130	<20-120	—

*SNFA = Swedish National Food Administration;
FDA = Food and Drug Administration;
SFOPH = Swiss Federal Office of Public Health;
**Bread = Samples classified as common and not special bread.

Table 2 - Acrylamide levels (μg/kg) in 29 food samples identified with the lot number reported on the original packaging. Each value is the average of three determinations. Values were corrected for recovery as described in the text.

Product		Lot n.	Acrylamide
Biscuits	Dieterba organic biscuits	225453	293
	Esselunga organic biscuits	L1863	309
	Gerber biscuits	2542	228
	Nipiol 6 cereals biscuits	223512	252
	Mellin biscuits	LT171	214
	Milupa biscuits	LNOFEB	<50
	Milupa 6 cereals biscuits	1372	403
	Plasmon “after first months” biscuits	2277E3	206
	Plasmon cocoa biscuits	218053	242
	Plasmon “first months” biscuits	224923	331
Homogenised meat	Plasmon veal homogenised	2296C 06038 A007	<50
	Mellin veal homogenised	L T321 VT 021	<50
Oven baked	Barilla cocoa “Crostatina”	7012	147
confectionery products	Barilla cocoa “Tegolino”	6882C	234
	Barilla cocoa “Trancino”	6962C	222
	Barilla milk “Flauti”	7092	228
	Barilla plumcake	6942B	180
	Buondì snack	4L2787	142
	Casalini cocoa roll	2872A	153
	Carrefour cocoa “Crostatina”	2724	166
	Carrefour plumcake	2690	132
	Kinder Ferrero “brioss”	309LB1	252
	Nuova Forneria cocoa roll	2788	385
	Parmalat plumcake	282B	126
Snack foods	Nestlè choco snack “Kit Kat”	A3	128
	Masterfoods choco snack “Bounty”	N3B6	148
	Masterfoods choco snack “Twix”	G 2B2	78
	Masterfoods choco snack “Mars”	F2C	107
	Ferrero nut cream “Nutella”	——-	<50

These exploratory data cover a limited number of food categories, products and brands and do not allow absolute comparisons. They only refer to the samples analysed and they are not statistically representative of the lot.

mon product in the diet, was 49 μg/kg (Table 1), we do not consider values less than 50 μg/kg as having a safety risk. Table 2 reports the results of the acrylamide content found in the 29 food items considered in this study. All data from extracts with area counts less than 10,000 are reported as <50 μg/kg. Baby food biscuits were in the range found by other organisations (Table 1), but for other baby foods there is insufficient data for comparison.

ACKNOWLEDGMENT

Thanks are due to A. Rosa for the analytical collaboration.

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Revised paper received June 19, 2003 Accepted July 31, 2003

HETEROCYCLIC AMINES AND CARCINOGENESIS

FFE 585/03/SME69

New research indicates that many plant foods may be able to protect humans against the detrimental effects of heterocyclic amines (HA). This is one of the many new results from a European research project previously introduced by Flair-Flow (see one-pager number FFE 439/01/SME19 at http://www.flair-flow.com/industry-docs/ffe43901.html).

Heterocyclic amines in foods have been on the spotlight for many years. They develop in some heated foods at part per billion levels. Several epidemiological studies published in recent years show a correlation between the intake of fried meat and the development of cancer in the large intestine, breast and prostate, whilst other studies show no such relationships.

The objective of this project is to increase our understanding of the impact of heterocyclic amines on the development of cancers, in particular developing robust analytical methods for more than 20 known HA, assessing the exposure and identifying biological markers as well as dietary protectants against the harmful effects of the heterocyclic amines.

One part of the project focuses on dietary protectants against the toxic effect of HA. Researchers found out using a biological model system that several extracts from plant foods strongly reduced the detrimental effect of HA. Among the most effective of these protectants were extracts from green tea, red wine, blueberries, blackberries, red grapes, kiwi, watermelon, parsley and spinach.

Other important results include:

- Development of a validated analytical method (LC-MS)
- Improved knowledge of the exposure of HA. Estimated intake is between 0 and 15 microgram per day

F-FE PROJECT LEADER

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- Better understanding of the conditions for HA development by heat processing methods
- New knowledge of a considerable influence of the animal genotype (pigs) on HA development in heat treated meat

Project No: QLK1-1999-01197 (H C AMINES) http: //www.cis.tugraz.at/ilct/hca/hca,html

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NATURAL ANTIMICROBIALS DO THEY WORK?

FFE 592/03/SME70

Several natural antimicrobials may contribute to extending food shelf-life and improving its safety by reducing the presence of pathogens such as *Listeria*, Salmonella or *Staphylococcus*, according to research work funded by the EU. Protection of food products such as fruit juices, pork sausages, beer, beef or salmon fillets may be provided through different combinations of chitosan extracted from shellfish, bacteriocins produced by bacteria and components from essential oils.

The scientists developed mathematical models for growth of *Listeria monocytogenes* and *Penicillium chrysogenum* in a model food, using natural preservatives, pH, water activity and temperature as variables.

They mainly emphasised the synergistic effects of the antimicrobials, but also included other components such as salt, hydrogen peroxide, sulphite, organic acids and EDTA. New bacteriocins from two lactic acid bacteria were found to protect beer and beverages, and a combination of chitosan, carvacrol and hydrogen peroxide showed to be efficient in surface cleaning of stainless steel, reducing counts of *Listeria*, Salmonella, *Staphylococcus* and *Saccharomyces*. Finally, a novel combination of chitosan and carnocin allowed a reduced content of sulphite addition to chilled pork sausages, improved shelf life and a reduced risk of *Listeria innocua.* Other synergistic effects between the preservatives found in this study were:
- Chitosan + benzoate, in juices and culture media
- Chitosan + sulphite, in comminuted pork and sausages
- Bacteriocins for the prevention of spoilage in alcoholic beverages
- Bacteriocin combinations for chilled dairy products
- Oregano essential oil + EDTA, in salads
- Carvacrol + cymene, in culture media
- Carvacrol + soy sauce against *Bacillus cereus* in rice

Project No: FAIR-CT96-1066

Project Co-ordinator: Prof. Sibel Roller, Head of Human Sciences, Faculty of Health and Human Sciences, Thames Valley University, 32-38 Uxbridge Road, London W5 2BS, UK; Tel. +44 20 82805108; Fax +44 20 82805289; E-mail: Sibel.Roller@tvu.ac.uk

OLIVE OIL AND OXIDATÍVE DARNAGE

FE 597103/HP72

Human volunteers from all over Europe are helping researchers to find out about the beneficial effects of olive oil on health Specifically, the EUROLIVE project will evaluate the impact of different types of olive oil and their antioxidant phenolic compounds on oxidative stress and DNA damage.

The Mediterranean diet, which usually contains olive oil, has been associated with reduced risk of diseases such as cancer, cardiovascular and neurodegenerative diseases. The benefit of consuming olive oil has been linked to its high content of monounsaturated fatty acids (MUFA) and antioxidants such as phenolic compounds, vitamin E and beta- carotene.

180 healthy volunteers in Northern, Central and Southern Europe will take a daily 'dose' of 25 mL of either refined (without phenolic compounds), common (with low phenolic content) or extra virgin (with high phenolic content) olive oil per day; 25 mL is close to the amount used daily in Mediterranean countries,

The objectives of the study are:

- To compare the impact of these oils on oxidative damage and LDL fatty acid composition
- To investigate the bioavailability of olive oil phenolic compounds in humans
- To determine the relationship between olive oil ingestion and the binding of phenolic compounds to LDL
- To explore differences in lipid peroxidation and fatty acid composition of LDL among healthy men from different European areas
- To determine whether addition or substitution of MUFA in the diets of participants from Central and Northern Europe results in a more favourable plasma LDL fatty acid composition after 3 months of consuming the olive oil.

So far, the project investigators have prepared the olive oils to be used for the study, a pilot study on the bioavailability of the olive oil phenolic compounds has been conducted, and a method has been developed to measure the plasma concentrations of the olive oil compounds tyrosol and hydroxytyrosol. The project website will be updated with results as they become available.

Project Reference: QLKI-2001-00287 (EUROLIVE) www.kepka.org/eurolive

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PREPARING THE WAY TO SCIENTIFICALLY VALIDATED HEALTH CLAIMS

FFE 601/02/SME73

A process for the assessment of scientific support for claims on food is under preparation within a European Concerted Action, called PASSCLAIM, in which a large number of health scientists, organisations, and industries are taking part.

The action has the following objectives:

- To produce a generic tool with principles for assessing the scientific support for health-related claims for foods and food components
- To evaluate critically the existing schemes in the EU and worldwide that assess the scientific substantiation of claims
- To select common criteria for how markers should be identified, validated and used in well designated studies to explore the links between diet and health.

PASSCLAIM builds on the results from an ended concerted action called FUFOSE, coordinated as the present one by ILSI Europe, which developed the science base for functional foods and defined two health related claims, an "enhanced function claim" and a "reduced risk of disease claim" (Brit. J. Nutr. 1999, 81, Suppl. 1).

The first year of the action resulted in reports within 4 theme groups: 1) diet-related cardiovascular diseases, 2) bone health and osteoporosis, 3) physical performance and fitness, and 4) review of existing codes. Based on these reports, the participants worked out at a workshop a draft set of interim criteria for the substantiation of health claims on foods and food ingredients. Those results were published in March 2003 in the European Journal of Nutrition and will be further discussed in a Flair-Flow synthetic report on functional foods to be out soon. During its second year, the project is focusing on insulin sensitivity and risk of diabetes, diet-related cancers, mental state and performance and gut health and immunity.

At the same time, a new directive is expected to regulate the approval of these claims and will be relevant to the functional foods industry. The EC draft proposal, "Regulation of the European Parliament and of the Council on Nutrition, Functional and Health Claims made on Foods" (SANCO/1832/2002), operates on nutritional, functional and health claims. While most countries have adopted the nutrition claims following the Codex Alimentarius recommendations, functional and health claims have been more controversial, particularly regarding the influence of foods on disease.

Project No: QLKI-2000-00086(PASSCLAIM) http://europe-ilsi.orp/passclaim/

Project Co-ordinator: Dr. Laura Contor, International Life Sciences Institute - ILSI Europe, Avenue E. Mounier 83 bte 6, BE-1200 Brussels, BELGIUM, Tel. +32 2 7710014; Fax +32 2 7620044; E-mail: dpannemans@ilsieurope.be

OCHRATOXIN A AND DNA DAMAGE

FFE 605/03/HP74

A study is now underway to investigate how, and why, ochratoxin A exposure can result in DNA damage and tumour induction.

Ochratoxin A is a mycotoxin, which can contaminate a number of foods, such as grapes, cereals, animal products, and even coffee and beer. Human exposure is therefore inevitable, and ochratoxin A has been detected in human blood samples, but generally at low levels in healthy people. Low levels of ochratoxin A in foods are not considered to pose a risk to health at all, however, at high levels it is considered to be a potential carcinogen: animal studies have shown that a type of ochratoxin A can cause tumours in the kidney and other organs. In humans, exposure to high levels of ochratoxin A has been linked with chronic renal disease and an increased incidence of urinary tract tumours.

The present project forms part of a EU-funded cluster of projects on mycotoxins, and will obtain crucial information on the dose-response curve for ochratoxin A-induced toxic effects. It will also improve understanding of the mechanisms resulting in DNA damage and its contributions to tumour induction by ochratoxin A. This, in turn, will serve as a basis for establishing scientifically sound maximum permitted levels for ochratoxin A within the EU.

Most of the experimental work is currently underway, but, so far, experimental models have been selected and established. Rat studies have shown that exposure to ochratoxin A in feed, for a period of 6 months, resulted in slight, but consistent, modification in terms of genetic damage in some tissues and organs. The induction of primary DNA lesions has also been observed in liver and bone marrow cells, but at this stage it is not possible to say whether these lesions are induced by oxidative stress or generated by adducts to DNA of ochratoxin A metabolites.

Project Reference: QLKI-2001-01614 (OTA RISK ASSESSMENT)
http://www.uni-wuerzburg.de/toxikologie/EU-OTA/OchratoxinA.html

Project contact: Prof Dr. Wolfgang Dekant, Department of Toxicology, University of Würzburg, Versbacher Straße 9; 97078 Würzburg, GERMANY; Tel. +499312013449; Fax +499312013865; e-mail: dekant@toxi.uni-wuerzburg.de; URL: http://www.uni-wuerzburg.de/toxikologie/Englisch/1stPage.html

DIETARY FAT AND ALZHEIMER'S DISEASE

FFE 616/03/CG76

Diet, and specifically the type and amount of lipids (fats) consumed, may have a role in the development of Alzheimer's disease. An EU-funded research project called "LIPIDIET" with researchers from seven countries, is focusing on the connection between dietary fat and this disease.

In recent years it has been discovered that cholesterol decreases the formation of amyloid, which has a link with Alzheimer's disease. Cholesterol levels can be

influenced by diet, but there is still a lack of knowledge about the relationship between cholesterol, diet and the disease.

The work in the project is divided into three main parts:

- The substances that could reduce amyloid production are screened in laboratory conditions
- These substances are then added to animal food and their function analysed in laboratory animals.
- A third step is to investigate the diet in humans and the relationship between dietary habits and the disease in a selected group of people suffering from it.

Later, the project partners hope to find out more about prevention, delay or slow down of Alzheimer's disease through dietary components.

Alzheimer's disease is a fatal disease affecting higher brain function. During the average lifetime, approximately one third of the EC population develop Alzheimer's disease, making it the major neural disorder in Europe. The disease usually becomes apparent after the sixties, but it can also start earlier. Alzheimer's disease is clearly recognised as a disease and not a process of normal ageing.

The disease starts with small difficulties to remember things or to perform certain tasks. Later, patients do not even remember their closest relatives and are completely dependent on full-time assistance by others. At the moment, there is no cure for it. The cause of the disease is not fully understood. In most cases the disease is not inheritable but may depend on several environmental and genetic factors.

Project Reference: QLKI -2002-00172 (LIPIDIET) http://www.lipidiet.org/

Project contact: Dr. Tobias Hartmann, Center for Molecular Biology Heidelberg (ZMBH), University of Heidelberg, Im Neuenheimer Feld 282, D-69120 Heidelberg, GERMANY, Phone +49-6221-54-6844; Fax +49-6221-54-5891; E-mail: tobias.hartmann@zmbh.uni-heidelberg.de

IAFP 2004 Call for Abstracts

The International Association for Food Protection is now accepting abstracts for IAFP 2004 to be held in Phoenix, Arizona, August 8-11, 2004. The deadline for submissions is January 5, 2004. IAFP accepts abstracts online or via E-mail. Call for Abstracts (Submission Form, General Information and Instructions), Speaker Reference Guide, Policy for Commercialism and information on the Developing Scientist Awards Competition are available at our Web site, www.foodprotection.org.

Poster or oral presentation formats are available. Abstracts are limited to 250 words and must report the results of original research pertinent to the subject matter. Papers may also report subject matter of an educational and/or nontechnical nature. Abstracts submitted for IAFP 2004 will be evaluated for acceptance by the Program Committee. Information in the abstract data must not have been previously published in a copyrighted journal.

Questions regarding abstract submission should be directed to Bev Brannen, IAFP Public Relations, at bbrannen@foodprotection.org or call 515.276.3344; 800.369.6337.

International Food Conference Thinking Beyond Tomorrow a safe and nutritious food chain for the consumer

Background

The conference is being organised, with the support of the Research directorate-General of the European commission, as part of a major scientific programme to mark the Irish Presidency of the European Union in 2004. The event is being organised by Teagasc-The Irish Agriculture and Food Development authority, University College Dublin, University College Cork and the Department of Agriculture and Food, with the support of Superquinn Ltd, and the Carbery Group.

Scope

The conference will address critical issues along the total food chain, focusing on key new technologies from "fork-to-farm", while at the same time aiming to reassure the consumer of the quality and safety of food.

It will feature the following five main sessions, dealing with key aspects of food quality, safety and consumer concerns along the food chain, specifically in the context of the application of innovative technologies:

- a nutritious food chain for the consumer
- key innovative technologies throughout the food chain
- food safety and the food chain
- future food chains and the "omic" revolution
- Policy, science and the consumer – an open forum.

The conference will provide a unique and innovative forum in which leading world specialists will present the latest research findings and engage in debate with other industry stakeholders. A significant outcome will be to inform stakeholders of opportunities and challenges facing the European food industry as it seeks to adopt new technologies while ensuring a safe and nutritious food supply.

Poster presentation

Abstracts for poster presentations on

the conference themes are invited. Please go to the "Abstract Submission" page of the conference website for further information on the submission process.

Deadline for submission is 31st March 2004.

Further information

Further information concerning the conference i.e., registration instructions, registration form, accommodation booking, etc., will be available on the conference website. You are cordially invited to visit the website – www.foodchain2004.com – and encouraged to register your e-mail address in order to be informed of all future developments.

Contact:
Conference Secretariat
Thinking Beyond Tomorrow
TEAGASC
The National Food Centre
Ashtown, Dublin 15 – Ireland
e-mail: info@foodchain2004.com
Tel. +353 1 8059500
Fax +353 1 8059550

IDF/FAO/OIE INTERNATIONAL SYMPOSIUM DAIRY HYGIENE AND SAFETY 'A Farm to Table Approach for Emerging and Developed Dairy Countries' Cape Town, South Africa 2-5 March 2004

The main objective of this symposium is to promote the production of safe and suitable dairy products. The application of a risk-based approach and the principles of HACCP and Good Agricultural Practices throughout the food chain will be emphasized. Particular consideration will be given to the needs of countries with developing dairy industries.

The provisional programme comprises six main sessions with 40 presentations by leading experts. Areas that will be targeted include animal health, milking hygiene, animal feeding and water, and the environment. Reference will be made to the IDF/FAO Guide on Good Dairy Farming Practices that should be finalized late this year. Also featured in the programme are the latest developments and new insights into the management of microbiological and chemical hazards at the dairy processing level. Visits to dairy farms and dairy factories in or around Cape Town will form an integral part of the symposium to highlight in practice the principles to be discussed at the main sessions. The symposium will end up with a session to exchange experiences and outline challenges in achieving dairy hygiene and safety in developing countries.

The events website http://www.fil-idf.org/ubisi2004/ provides a portal enabling access to a broad range of information and registration details.

A reduced registration fee will facilitate participation of experts from developing countries.

For more information, please contact:
Caroline Brooks, IDF Events Manager, +32 2 706 86 45
CBrooks@fil-idf.org
Marylène Tucci, IDF Communications and Public Affairs Assistant, +32 2 706 86 44
MTucci@fil-idf.org

4th IDF Symposium CHEESE: RIPENING, CHARACTERIZATION AND TECHNOLOGY Prague, Czech Republic 21-25 March 2004

The symposium will explore challenges and opportunities in the fields of cheese ripening, microbiology, characterization, flavour formation and technology.

The program will cover the latest scientific findings about important microbial and enzymatic activities during cheese ripening, control of flavour formation and chemical analysis, sensory studies, texture, structure and functionality of cheese

and the influence of technology on cheese ripening not forgetting characterization of cheese and the economic aspects.

In twelve sessions the Symposium will deal in detail with a wide range of scientific and technological aspects of cheese and cheesemaking. The object is exchange of information, ideas and experience between people in research and people in practical cheese making from many countries.

Full Symposium and registration details are available online at http://www.conference.cz/IDF/

For more information, please contact:
Caroline Brooks, IDF Events Manager, +32 2 706 86 45
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First Announcement
8th International Symposium on
KINETICS IN ANALYTICAL CHEMISTRY
Rome, Italy (Villa Mondragone)
8-10 July 2004

The scientific programme will include plenary lectures, oral presentations and poster sessions to cover the following topics:

Analytical chemistry applications based on the kinetics of:
- Electrochemical reactions
- Spectrometric systems
- Antigen-Antibody interaction
- Redox reactions
- Enzyme systems.

DEADLINE SCHEDULE:
15 January: 2nd Announcement
15 March: Abstract submission

Further details, including registration form, scientific programme, travel and accommodation, will be sent with the second announcement and will be available soon on the KAC-2004 web site (www.uniroma2.it/dipartim/BEAT/KAC2004.

Communications should be directed to:
Francesca Dominici
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Via della Ricerca Scientifica, 00133 Rome, Italy
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PRESENTATION

We are honoured to announce to all IJFS readers the founding of the first Italian Society of Food Science and Technology (SISTAL) which has become a supporting subscriber of IJFS.



SOCIETA' ITALIANA DI SCIENZE E TECNOLOGIE ALIMENTARI
(ITALIAN SOCIETY OF FOOD SCIENCE AND TECHNOLOGY)

The Società Italiana di Scienze e Tecnologie Alimentari (SISTAL) was founded in January 2003. It includes Italian university teaching staff, who perform teaching and research activities within the field of Food Science and Technology.

SISTAL is a non-profit association and is aimed at contributing to scientific advances in the field of preservation and processing of foods for human feeding.

In particular, it intends to contribute to improvement and innovation of food quality and safety throughout the entire food production chain by both developing production methods and analytical techniques and managing production systems for product quality to meet requirements of the market and, in general, of the Community.

According to its Statute, the main aims of SISTAL are:

- to promote study, research, study boards and diffusion of the results;
- to organise and support congresses, round tables, etc.;
- to publish and support journals, newsletters, monographs, etc.;
- to administer foundations and funds intended to promote research, to award scientific prizes, to invite distinguished foreign scientists and to offer recognition to outstanding personalities for their particular merits in any field specific to SISTAL.

The Society supports both private and public bodies as a link between the production sphere, research field and consumers. As a result, it intends to represent a source for addressing and spurring both national and international research in the food sector.

The Society's Governing Bodies are the following:

Governing Council
Prof. Paolo Fantozzi, President
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Prof. Margherita Rossi, Vice-President
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University of Naples

Prof. Vincenzo Gerbi, Treasurer
University of Turin

Prof. Gianni Lercker, Council member
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Prof. Bruno Zanoni, Council member
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College of Arbiters

Prof. Aureliano Amati, University of Bologna
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Prof. Carlo Pompei, University of Milan

College of Auditors

Prof. Ennio La Notte, University of Foggia
Prof. Biagio Mincione, University of Calabria
Prof. Giovanni Spagna, University of Catania
Prof. Fernando Tateo (Deputy), University of Milan

SISTAL
SOCIETÀ **I**TALIANA DI **S**CIENZE E **T**ECNOLOGIE **AL**IMENTARI

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BOOKS

NEW FOOD SAFETY: FROM THE HACCP SYSTEM TO THE TRACEABILITY SYSTEM FOR AGRICULTURAL FOOD CHAIN AND AGROFOOD INDUSTRIES

The most important mandatory and voluntary rules

By G. Manzone
224 pages (in Italian)
ISBN:
Price € 25,00
Publisher: M&M Associati srl - Via P. Metastasio 55 - 95127 Catania - Italy - Tel. +39 095 371717 - Fax +39 095 7224656 - e-mail: info@mmconsul.it

The most important points of Regulation (EC) no. 178/2002 (of the European Parliament and of the Council of 28 January 2002) are reviewed. It extends the field of application of food safety legislation to the level of primary production, including feed (where that feed is intended for food-producing animals), and establishes at all stages of production, processing and distribution the traceability of food, feed, food-producing animals, and any other substance intended to be, or expected to be, incorporated into a food or feed from 1 January 2005.

Moreover, the book analyses the certification standards of the HACCP method (UNI 10854), those of the traceability system (of food products) for the agricultural food chain (UNI 10939) and those of the traceability system (of food products) for the agrofood industries (UNI 11020).

It also analyses the operating procedure in the HACCP system in the light of the most recent developments of Community and Italian norms. The main objective is to better explain the responsibilities to each operator as to the safety of food products.

IDF Publications

New Applications of Mid-infra-red Spectrometry for the Analysis of Milk and Milk Products

H. van den Bijgaart, J.M. Van Crombrugge, C. Baumgartner, A. Landgraf, J. Buermeyer and A.B. Bendtsen

Mid-infra-red spectrometry has long been used successfully for the compositional analysis of milk and its fat, protein and lactose components. This paper looks at new applications of the technology to cover analysis of urea, freezing point, pH, specific sugars, lactic acid, casein, free fatty acids and citric acid.

32 pp - English only

Proceedings of IDF Symposium on Advancement in Analytical Techniques, Holstebro, 21 May 2003

J. Tønder, E. Wolthers, J.C. Jennings, A.J. Whetsell, N.R. Nicholas, B.M. Sweeney, M.B. Klaften, S.B. Kays, G.F. Hartnell, R.P. Lirette & K.C. Glenn, C.

Sidi, D.K. Pedersen, P. Gowik, B. Juul, M. Tomaska and G. Suhren

In today's climate of technological advances in food production, it is important to consumers and companies alike that reliable methods of compositional analysis exist. This paper looks at some of the technologies available to provide confidence in biotechnological developments and at the public and private control measures in operation.

28 pp - English only

Total: 60 pp
Bulletin N° 383/2003 - 60 Euro

WORLD DAIRY SITUATION 2003

Results of annual survey presented by the chair of the Standing Committee "Dairy Policies and Economics" at the IDF World Dairy Summit. September 2003.

The 2003 edition of the World Dairy Situation report includes the customary collation of figures and comments from IDF National Committees and other sources where such Committees do not exist. Contributions from the ZMP which, on behalf of IDF, continued its networking with other institutions working on the economy of the world dairy sector have significantly added to the publication.

Milk production, industrial milk processing, trade in dairy products, development of demand and consumption and prices for 2002 are presented, along with some dairy product information and forecasts for 2003. Country reports provided by experts from member states, describing developments and sometimes subjective prognosis, are presented. The WDS Forum section comprises four papers: OECD and EU projections on agricultural markets. EU enlargement and dairy markets. EU dairy reform decisions and beyond butter, cheese and powder: non-traditional dairy products: facts, implications and challenges.

69 pp - English only
Bulletin N° 384/2003 - 70 Euro

FERMENTED MILK SYMPOSIUM PROCEEDINGS (Kolding, Denmark, June 2002)

Latest scientific information and technological status on aroma and texture of fermented milk, with a focus on cultures, processing and product evaluation. Use of fermentation technology for compounds that can be used as natural food preservatives, natural nutraceutical products such as bioactive peptides or as natural flavour ingredients, or as a source of specific enzymes or enzyme cocktails.

Sections devoted to - Cultures, traditional and novel, and their properties:

- Processing techniques and monitoring; effects on the product
- Product evaluation in relation to physical (rheological, etc) and nutritional properties and sensory testing.

404 pp. ± A5 format - in English only
S.I. N° 0301 - 99 Euro - 2003 ISBN 92 9098 036 2

RUMINANT MAMMARY GLAND IMMUNITY VARIOUS AUTHORS

This paper summarizes the current knowledge of bovine mammary gland im-

munity, describes the major factors affecting the immune system and shows potential approaches currently available to modulate immune defences of the udder. Its point of depart is the IDF's amended classification of the immunological components of the mammary gland. It is the combination of the position papers presented at the IDF Seminar on Mammary Gland Immune Defences in Stresa on 11-14 June 2000 together with the contributions of the participants.

128 pp. ± A5 format - in English only
S.I. N° 0302 - 39 Euro - 2003 - ISBN 92 9098 037-0

FIL-IDF - Building Diamant, Boulevard Auguste Reyers 80, B-1030 Brussels, Belgium - Tel. +32 2 7339888 - Fax +32 2 7330413 - E-mail: info@fil-idf.org - Web Site: http://www.fil-idf.org

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CONTRIBUTORS

Gratitude is expressed to the following entities for contributing to the realization of the Journal by being supporting subscribers for 2003.

Si ringraziano i seguenti Enti, Ditte ed Istituti per aver voluto contribuire fattivamente alla realizzazione della Rivista, sottoscrivendo un abbonamento sostenitore per il 2003.

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ITALIAN JOURNAL OF FOOD SCIENCE
Rivista Italiana di Scienza degli Alimenti
DIRETTORE RESPONSABILE: Giovanni Chiriotti
AUTORIZZAZIONE: n. 3/89 in data 31/1/1989
del Tribunale di Perugia
Proprietà dell'Università di Perugia
TIPOGRAFIA Giuseppini - Pinerolo
Una copia € 6.00

ISSN 1120-1770 © 2003

CHIRIOTTI EDITORI spa - 10064 Pinerolo - Italy

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