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PAPER

ELECTROCHEMICAL METHOD FOR THE EVALUATION OF THE NUMBER OF EGGS IN EGG PASTA

DETERMINAZIONE DEL NUMERO DI UOVA NELLA PASTA ALL'UOVO MEDIANTE UN METODO ELETTROCHIMICO

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ABSTRACT

A specific and rapid method to determine the number of eggs in egg pasta based on the determination of ovalbumin thiol groups is described. Pasta water extracts were analysed by a Flow Injection Analysis (FIA) system with an electrochemical detector operating in amperometry with a tungsten sensor working at -0.2 V vs SCE. There was a good linear correlation between the SH content obtained using the proposed electrochemical method and the number of eggs in egg pasta. Moreover

RIASSUNTO

Viene descritta una procedura specifica basata sulla determinazione dei gruppi tiolici dell'ovoalbumina per la valutazione del numero di uova nelle paste all'uovo. Gli estratti acquosi della pasta vengono analizzati con un sensore di tungsteno operante in amperometria ad un potenziale fisso di -0,2 V vs SCE in un sistema a flusso tipo FIA con rivelazione elettrochimica. I risultati riportati mostrano una buona correlazione di tipo lineare tra il numero di uova contenute nelle paste all'uovo

- Key words: electrochemical sensor, Flow Injection Analysis (FIA), ovalbumin, thiols -

to confirm the results of the electrochemical method, the ovalbumin content of egg pasta was also evaluated by electrophoretic SDS-Page analysis. Because of the speed of analysis, the electrochemical method could be useful for the rapid determination of the number of eggs in egg pasta.

e la concentrazione di SH valutata con il metodo proposto. Inoltre, al fine di confermare i risultati del metodo, l'ovoalbumina contenuta nelle paste all'uovo è stata valutata anche con l'analisi elettroforetica SDS-Page. Data la velocità dell'analisi il metodo elettrochimico è utile per la rapida determinazione del numero di uova contenute nelle paste all'uovo.

INTRODUCTION

The term "egg pasta" means a pasta product containing a minimum of 4 whole eggs per kilogram, corresponding to 200 g of egg per kilogram of durum wheat semolina. Italian legislation (DPR, 2001) has established that egg pasta must contain a minimum 0.145 g of sterols per 100 g of dried matter. This limit was set considering that sterols are present in egg yolk at a relatively constant concentration (1.45-1.50 g per 100 g of yolk) (POWRIE and NAKAI, 1986). However, modern hen feeding practices have caused a decrease in the sterol content in eggs to values lower than 1.45 g per 100 g of egg yolk. For this reason, egg pasta makers are now forced to use a larger number of eggs or add egg yolk in order to meet legal requirements.

The official method of analysis for the determination of the number of eggs in pasta is based on the evaluation of the sterol content (AOAC, 1990). Another method reported in the literature is based on the HPLC determination of cholesterol as an indicator of egg solids (HURST *et al.*, 1985).

Considering that the information about the egg content in egg pasta is important from both the production and the regulatory points of view, a new method based on the determination of ovalbumin thiol groups has been developed. Ovalbumin (M.W. 45000 D) represents more than 50% (w/w) of the albumen proteins and it is the only egg white protein containing free SH groups (FOTHERGILL and FOTHERGILL, 1970; NISBET *et al.*, 1981).

In a previous paper, COSIO *et al.* (2000) set up an electrochemical method to determine the thiol content in whey proteins to evaluate milk heat treatment. Likewise, in the present work a procedure to evaluate the SH content of egg pasta is described. It involves operating with a tungsten electrode at -0.2V (SCE) in a flow injection system with a carrier solution containing mercury ions.

MATERIALS AND METHODS

Apparatus and reagents

The flow-injection system consisted of a Jasco 880 PU HPLC pump, a Rheodyne model 7010 sample injection valve and EG&G PAR Model 400 electrochemical detector equipped with a tungsten wire working electrode (0.3 mm diameter and 20 mm length), 99.95% purity (Good Fellow, Cambridge), a reference saturated Calomel electrode (SCE) and a platinum auxiliary electrode. Current outputs were displayed on a strip chart recorder (REA 112, Radiometer, Denmark).

All solutions were prepared from rea-

gent grade chemicals. L-cysteine, mercury chloride, sodium chloride and acetic acid were purchased from Merck (Darmstadt Germany). Ovalbumin was from Sigma Chemical Co (St. Louis, MO, USA).

Samples

Ten commercial samples of egg pasta containing 4 or 6 eggs (16.7 or 23.1% egg/kg product) were analyzed. Seven samples of pasta containing from 0 to 6 eggs, produced by the Institute of Cerealicoltura in Rome, were also tested. The pasta samples were dried at low temperature (LT) (50°C x 18 h). Moreover ten pasta samples, without egg, produced by the same institute using different cultivars of durum wheat flour were also analysed. All samples were dried at low temperature (as egg pasta) and at high temperature (73°C x 11 h).

Sample preparation

All samples were ground using a laboratory mill and passed through a 0.188 mm sieve. One gram of sample was weighed in a centrifuge tube, dispersed in 10 mL of distilled water, mechanically stirred for 30 min at room temperature and centrifuged for 20 min (10,000 rpm; 15°C). The supernatant was filtered through a Whatman n°4 filter, diluted 1:1 with the carrier solution and then injected in the flow system.

Electrochemical procedure

Amperometric flow injection measurements of thiol compounds were performed using a potential of -0.2V versus SCE and a carrier solution composed of 0.1 mol L^{-1} acetic acid containing 0.04 mol L^{-1} NaCl and 37 μmol L^{-1} $HgCl_2$. A flow rate of 1.0 mL min^{-1} and an injection volume of 20 μL were used. All aqueous standard solutions were prepared fresh each day.

Determination of the "water soluble" proteins

The "water soluble" proteins (albumins plus globulins) were determined by the Biuret method (GORNALL *et al.*, 1949). This method is based on a colorimetric reaction. The extent of the coloration of a solution is a function of peptide linkages and subsequently of the protein concentration. Water extracts (0.5 mL) of samples were added to 2 mL of the Biuret reagent and then incubated at room temperature for 30 min. Absorbance at 540 nm was immediately recorded using a DU 650 Beckmann spectrophotometer (Instruments Inc., Fullerton, CA, USA).

Electrophoresis SDS-PAGE

Sodium dodecyl sulfate-polyacrylamide gel was prepared using 15 mL of polyacrylamide (29.1% polyacrylamide, 0.9% N,N-methylenebisacrylamide from Biorad-Hercules, CA, USA), 3.3 mL of ammonium persulphate 1.5% from Fluka, 30 mL of phosphate buffer (pH 7.1, 0.2 M containing 0.2% sodium dodecyl sulfate from Sigma), 70 μL of TEMED (NNN'N'-tetramethylene diamine) from Merck and 11.63 mL of distilled water. After pre-running the gel at 200 V (current 150 mA) for 30 min, 10 μL of 0.6 mg mL^{-1} protein solution containing bovine serum albumin, carbonic anhydrase, lysozyme and ovalbumin (from Sigma), all together with water extracts of the pasta samples, were loaded and subjected to electrophoresis at about 20 mA for 10 min and then 200 mA for 3 h. The gel was stained with 1% Comassie Brilliant blue R-250 from Merck in methanol/TCA/sulfosalicylic/water (30%/10%/5%/55%) and destained with ethanol/acetic acid/water (30%/10%/60%) (WRINGLEY *et al.*, 1982). Electrophoretic gel images were obtained by digitalization using a plane scanner (HP Scanjet 6100C) connected to a Pentium PC platform equipped with Adobe Photoshop software version 5.5.

The image was processed with CREAM software (Cross Radio-Immuno Electrophoresis Analysis Manager).

RESULTS AND DISCUSSION

Optimisation of the experimental conditions

The electrochemical method is based on the measurement of the excess amount of mercury (II) ions in the carrier solution after reactions with organic thiols (RSH) to form $(RS)_2Hg$ complexes. Complexation diminishes the Hg^{2+} activity in the solution and thus reduces the cathodic current measured by the electrochemical detector. In order to optimize the working conditions for the samples studied, injections of standard solutions of $HgCl_2$ in 0.1 mol L^{-1} acetic acid at a potential range from -0.1V to -0.25V (SCE) were performed. Optimal responses were obtained by operating with 37 µmol L^{-1} of $HgCl_2$ at a potential of -0.2V and these conditions were used in all subsequent experiments. The effect of NaCl concentration on the $HgCl_2$ response was evaluated by adding different concentrations of NaCl (10-80 mmol L^{-1}) to the carrier solution. Chloride ions have the function of increasing mercury solubility and also of improving carrier solution conductivity. The best signal/background ratio was obtained with 40 mmol L^{-1} of NaCl. This concentration was therefore added to the carrier solution in all subsequent experiments.

Using these optimized conditions, amperometric responses of increasing concentrations of cysteine and ovalbumin solutions were recorded. Fig. 1 shows that the detector responded rapidly to the dynamic changes of cysteine and ovalbumin concentration, allowing about 40 determinations per hour. In particular cysteine was used as reference standard in the determination of the SH content in the pasta samples. The calibration plot

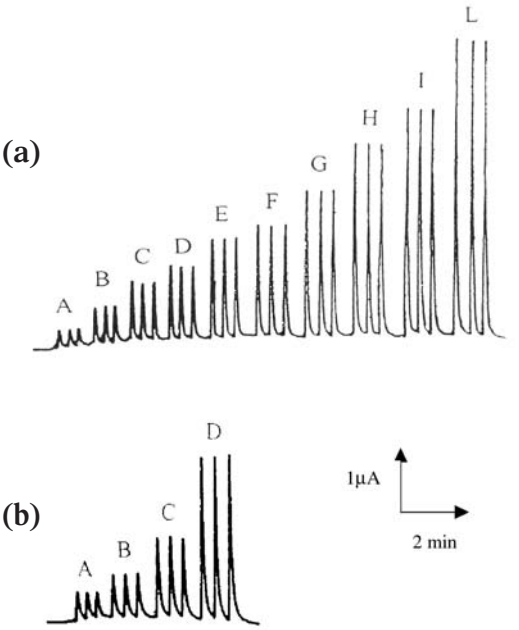


Fig. 1 - Flow injection responses for increasing concentrations of cysteine (a) and ovalbumin (b). Cysteine concentrations: A: 0.5 µg mL^{-1}; B: 1 µg mL^{-1}; C: 1.5 µg mL^{-1}; D: 2 µg mL^{-1}; E: 2.5 µg mL^{-1}; F: 3 µg mL^{-1}; G: 4 µg mL^{-1}; H: 5 µg mL^{-1}; I: 6 µg mL^{-1}; L: 8 µg mL^{-1}.
Ovalbumin concentrations: A: 62.5 µg mL^{-1}; B: 125 µg mL^{-1}; C: 250 µg mL^{-1}; D: 500 µg mL^{-1}.
Operating conditions: Working potential -0.2V (SCE); flow rate 1 mL min^{-1}; carrier solution: acetic acid 0.1 mol L^{-1}, 37 µmol $L^{-1}HgCl_2$ and 40 mmol $L^{-1}NaCl$; injection volume 20 µL.

was highly linear over the 0.1-8 µg mL^{-1} concentration range (r^2=0.9998), the slope and the intercept were 0.497 µA mL/µg and 0.025 µA, respectively. The relative standard deviation (RSD) at the concentration level of 2.5 µg mL^{-1} was 1.5% (n=20) and the estimated detection limit calculated using the linear regression technique from MILLER and MILLER (1993) was 0.12 µg mL^{-1}.

Optimisation of the extraction procedure

Since the methodology developed is based on the determination of ovalbumin thiol groups, it was necessary to find an

extraction procedure that could minimize the extraction of thiols present in wheat proteins.

The best results were obtained by using water as extractant since only albumins of durum wheat (7-10% of total durum wheat proteins) and ovalbumin are water soluble. Typical amperometric responses of pasta and egg pasta water extracts are shown in Fig. 2. The amperometric signal of pasta with durum wheat semolina was about two times lower than that of pasta with 2 eggs and about four times lower than that of pasta with 6 eggs. The height of the peak of pasta with durum wheat semolina is related to the presence of thiol groups in durum wheat extracted with water and depends greatly on the drying temperatures. It is well know that the use of a high or a low temperature drying profile in pasta can have a great influence on the extraction of albumins; this is clearly show in Table 1, where results ob-

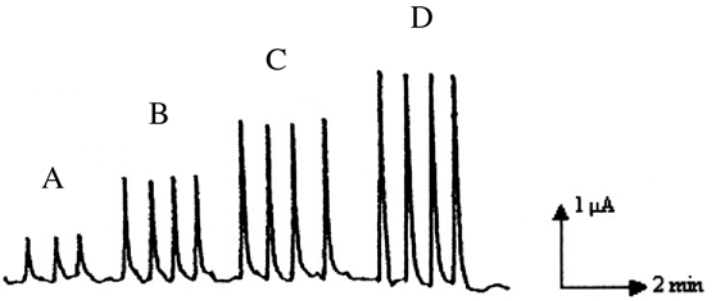


Fig. 2 - Flow injection responses of pasta with durum wheat semolina (A), egg pasta with 2 eggs (B), egg pasta with 4 eggs (C), egg pasta with 6 eggs (D). Operating conditions as in Fig. 1.

Table 1 - Content of "water soluble" proteins (N_{sol}) and SH groups in the pasta samples produced from different cultivars of durum wheat flour without egg.

Pasta samples	Drying*	N_{sol} g/100 g dried matter [a]	SH µM cysteine eq/100 g of dried matter [a]
	LT	1.23±0.12	40.3±3.2
1	HT	1.18±0.11	31.5±2.5
	LT	1.25±0.14	42.6±3.3
2	HT	1.11±0.10	28.6±1.9
	LT	1.25±0.14	39.6±3.0
3	HT	1.15±0.13	26.3±2.0
	LT	1.26±0.13	38.0±2.9
4	HT	1.05±0.11	22.0±1.4
	LT	1.31±0.15	46.2±3.3
5	HT	1.12±0.10	29.9±2.8
	LT	1.21±0.13	40.0±2.9
6	HT	1.11±0.11	30.2±2.2
	LT	1.32±0.14	38.0±2.8
7	HT	1.26±0.12	22.0±2.1
	LT	1.28±0.13	42.5±2.9
8	HT	1.15±0.10	22.0±1.8
	LT	1.49±0.15	43.8±3.4
9	HT	1.18±0.09	22.8±1.4
	LT	1.55±0.15	46.8±2.9
10	HT	1.01±0.09	25.2±1.6

[a] (mean S.D., n=3); * LT= Low temperature; HT= High temperature

tained from the analysis of pasta samples of known origin are reported. Samples of pasta dried at low temperature (LT) had SH values in the range of 38-46.8 while those obtained at high temperature (HT) were in the range of 22-31.5. This is in agreement with some studies reported in the literature (ALARY and KOBREHEL, 1987; PAGANI *et al.*, 1986; DEXTER *et al.*, 1981) stating that pasta dried at high temperature has a higher degree of protein insolubilisation with respect to low temperature dried pasta.

Table 2 - Content of SH groups in the egg pasta samples produced with an increasing number of eggs.

Experimental egg pasta samples (number of eggs)	SH concentration (µmol cysteine eq/100 g of dried matter) [a]
0	44.9±3.2
1	85.5±2.8
2	109.0±3.8
3	133.3±4.2
4	162.7±6.2
6	203.7±5.7

[a] (mean S.D., n=3).

Application to egg pasta samples

To verify if the electrochemical method was suitable to determine the number of eggs in egg pasta, pasta samples containing from 0 to 6 eggs, produced from the same durum wheat semolina and dried at low temperature (50°C), were analyzed. The results are reported in Table 2. The SH content is expressed as µmol of cysteine equivalent per 100 g of dried matter. As expected the SH content increased in relation to the number of eggs and the increase per egg was about 30-40 µmol of cysteine equivalent per 100 g of dried matter. The pasta sample made with 4 eggs had a SH content of about 160 µmol of cysteine equivalent per 100 g of dried matter. As shown in Fig. 3 there was a good linear correlation between the SH content obtained by the proposed electrochemical method and the number of eggs (r=0.994).

The SH content determined in commercial pasta samples produced with 4 or 6 eggs is reported in Table 3. There was no great variability among samples produced with the same number of eggs. This is related to the low drying temperature used in order to avoid denaturation of proteins or enzymatic browning that could cause the egg pasta to darken (NAMIKI, 1988; WALSH, 1970).

To confirm the results of the electrochemical method, the ovalbumin content of egg pasta water extracts was evaluated by electrophoretic SDS-PAGE analysis. A calibration curve of ovalbumin standard solutions was developed

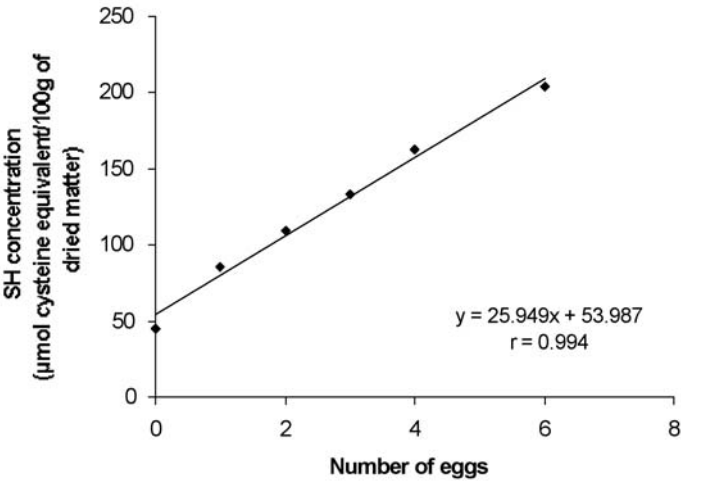


Fig. 3 - Linear correlation plot between the number of eggs in the egg pasta samples and the SH concentration determined using the proposed method.

(y = 12057x - 123.57, r^2=0.999) and the correlation between the SH content and the ovalbumin concentration of experimental and commercial egg pasta samples was evaluated (Fig 4). The good linear correlation (r=0.981) confirms that the proposed electrochemical method based on the measurement of the ovalbumin SH groups is useful for determining the number of eggs in egg pasta. Free cysteine that could come from egg yolk did not seem to affect the accuracy of the method.

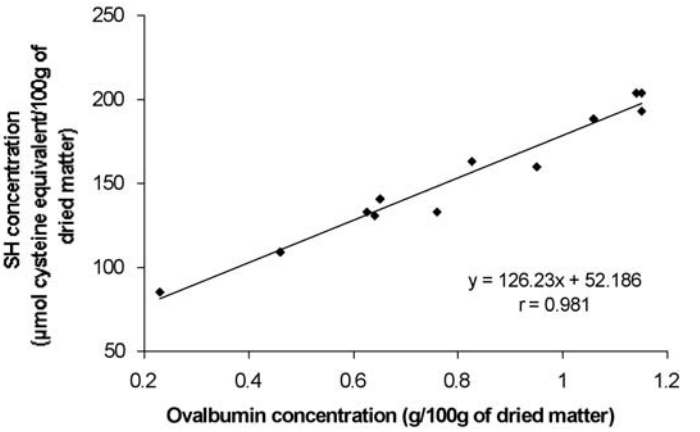


Fig. 4 - Linear correlation plot between the ovalbumin concentration of the experimental and commercial egg pasta samples and SH concentration determined using the proposed method.

CONCLUSION

The proposed electrochemical method has been successfully employed for the direct, rapid and reliable monitoring of the number of eggs in egg pasta. This new method offers an alternative to the difficult and time-consuming AOAC method based on the determination of the sterol content. Furthermore the proposed method based on the determination of the SH groups of ovalbumin, gives results that are not related to hen feeding and, because of the speed of the analysis, it is useful for on-line or routine determination of the number of eggs in egg pasta.

Table 3 - Content of SH groups in commercial egg pasta samples with 4 eggs (samples 1 to 6) or 6 eggs (samples 7 to 10).

Commercial egg pasta samples	SH concentration (mol cysteine eq/100 g of dried matter) [a]
1	140.4±5.5
2	132.9±6.5
3	141.2±5.2
4	130.4±4.5
5	134.8±6.0
6	136.9±3.9
7	160.0±5.8
8	193.0±7.2
9	188.2±8.2
10	204.2±9.9

[a] (mean S.D., n=3).

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PAPER

CHEMOMETRIC DETERMINATION OF THE GEOGRAPHICAL ORIGIN OF COW MILK USING ICP-OES DATA AND ISOTOPIC RATIOS: A PRELIMINARY STUDY

DETERMINAZIONE CHEMOMETRICA DELL'ORIGINE GEOGRAFICA DEL LATTE VACCINO ATTRAVERSO ICP-OES E DATI ISOTOPICI: STUDIO PRELIMINARE

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ABSTRACT

The geographical origin of twenty cow milk samples from two different geographical areas of the Apulia region in southern Italy was determined using Inductively Coupled Plasma Emission Spectroscopy (ICP-OES) for the metals

RIASSUNTO

La differenziazione dell'origine geografica di venti campioni di latte vaccino provenienti da due differenti aree di produzione della Puglia, nel sud Italia, è stata investigata attraverso spettroscopia atomica ad emissione di plasma

- Key words: heavy metals, multivariate statistical analysis, PDO -

(Ba, Mn, Zn, Al, Fe and Cu), and isotope ratio mass spectrometry (IRMS). The phosphorous concentration was determined by colorimetry. The data were processed using the following multivariate statistical methods: Principal Components Analysis (PCA), Hierarchical Clustering (HCA) and Linear Discriminant Analysis (LDA). The samples were divided into two groups according to their origin and were classified by discriminant analysis which gave 100% success. This method could be extended to classify dairy products like mozzarella and cheese in order to verify and safeguard Protected Designation of Origin (PDO) products.

induttivamente accoppiato (ICP-OES), per la determinazione dei metalli e spettrometria di massa dei rapporti isotopici (IRMS). Il contenuto di fosforo totale è stato anche misurato per via colorimetrica. I dati ottenuti sono stati processati attraverso l'analisi statistica multivariata: Analisi delle Componenti Principali (PCA), Analisi di Clustering Gerarchico (HCA) ed Analisi Discriminante (DA). I campioni sono stati divisi in due gruppi in base all'origine e sono stati classificati mediante l'analisi discriminante ottenendo il 100% di capacità di classificazione. Uno sviluppo di questo studio potrebbe consistere nell'estensione del metodo a prodotti lattiero-caseari come formaggio e mozzarella contribuendo alla salvaguardia dei prodotti recanti il marchio di Denominazione di Origine Protetta.

INTRODUCTION

The growing interest of consumers in genuine and traditional products has increased the need for methods to control the quality of the foods produced within the European Community as well as those imported from other countries.

The EU regulation 2081/92 (E.U., 1992) protects consumers through a system of effective and impartial controls by which Protected Designation of Origin (PDO) products can be distinguished from non PDO products. At the same time, this regulation meets producers' requirements. That is, it is in the interest of the producers that the controls to be as objective and clear as possible so as to protect the authenticity of the products that are to be promoted on the market.

A greater appreciation of qualified national products, together with a guaranteed reference for consumers, has been developed in the field of dairy products. The PDO trademark has been assigned to numerous Italian cheeses (like Parmigiano Reggiano, Mozzarella di Bufala Campana, etc.) and many cheese producers in Italy are actively seeking PDO classification. In order to receive this designation, the raw materials must be produced and processed in the specified region from which the product gets its specific name. For this reason, authentication of the geographical origin of a product is becoming a major challenge. MANCA *et al.* (2001) recently identified some compositional parameters that allow Italian cheese samples to be distinguished according to their region of origin. In order to determine the authenticity of typical dairy products, the geographical origin of the milk must be determined. Studies on the geographical origin of milk samples have been conducted by means of Isotope Ratio Mass Spectrometry (IRMS) (KORNEXL *et al.*, 1997; ROSSMANN *et al.*, 1998).

The question of the geographical identification of food products often involves small areas of production. Wines, for instance, are often produced in restricted areas defined as DOC (Denomination of Controlled Origin) and there are often several DOC areas within a given region that are not far from one another.

Metal composition has been widely used to assign the geographical origin of various matrices such as wine, honey and other food products (BRESCIA *et al.*, 2002a; REBOLO *et al.*, 2000; PARAMAS *et al.*, 2000). Isotopic and trace elements measurements have provided very interesting results in the case of wine (MARTIN *et al.*, 1999).

The objective of this study was to determine whether the concentrations of selected metals (Ba, Mn, Zn, Al, Fe and Cu) and phosphorous, and the isotopic ratios of carbon and nitrogen ($^{13}C/^{12}C$ and $^{15}N/^{14}N$) could be used to identify the specific area of origin of a milk sample within the Apulia region of Italy. The isotopic and trace element measurements were combined and statistically evaluated.

MATERIALS AND METHODS

Milk samples

Twenty samples of cow milk were supplied by some farms in Apulia, a region in Southern Italy. Ten samples came from five farms [Troia (T), Castelluccio (C), Volturara Appula (V), Foggia (F) and Lucera (L)] in the province of Foggia, in Northern Apulia and ten samples came from four farms [Alberobello (A), Noci (N) and Martina Franca (M1 and M2)] situated in a characteristic area in the centre of Apulia, called "Murgia". For this preliminary study, cows in the 0-3 month lactation period were selected in order to eliminate the variability in milk composition associated with the lactation period. All samples were collected in polypropylene bottles and stored in a freezer at -80°C until analysis.

Phosphorus determination

Phosphorous content was determined by adding 0.3 g of ammonium persulphate and 2 mL of 30% sulphuric acid to 0.2 g of milk. This mixture was mineralised for 2 h at 100°C, then neutralised with 30% NaOH, filtered and diluted to 100 mL with deionised water. Afterwards, 0.05 g of ascorbic acid and 10 mL of an acid mixture consisting of 0.13 g/L of potassium antimony tartrate, 5.6 g/L of ammonium molybdate and 70.0 mL/L of concentrated sulphuric acid were added to 20 mL of the sample. After 30 min, the absorbance at λ=800 nm was measured with a LP1W photometer (DrLange, Düsseldorf, Germany).

Metal determinations

The concentrations of Ba, Fe, Mn, Al, Cu and Zn were determined using a Liberty 110 (Varian Inc., Palo Alto, USA) inductively coupled plasma optical emission spectrophotometer (ICP-OES), equipped with an ultra-sound nebulizer and a VGA 77P hydride generator. Quantitative determinations were carried out using the external standard method. Other heavy metals (Co, Ni, Pb and Cd) were determined. However, in all cases the levels were less than the detection limits (1.5, 0.4, 0.08 and 0.4 mg/kg, respectively).

The samples were subjected to a mineralising process: 0.5 g of freeze-dried milk was dissolved in 6 mL of HNO_3 and 1 mL of H_2O_2 and put into a 1200 MEGA microwave digestion unit (Milestone, Bergamo, Italy) with a well defined temperature program. The operating conditions for measurements are reported in Table 1.

Hydrochloric and nitric acids were ul-

Table 1 - Operating conditions for ICP-OES measurements.

	Ba	Fe	Mn	Al	Zn	Cu
λ (nm)	455.4	259.9	257.6	396.1	213.9	324.7
Viewing Height (mm)	10	14	12	15	10	6
Search Window (nm)	0.080	0.040	0.040	0.080	0.027	0.040
Amplification Signal (V)	800	800	800	650	800	600
RF Power (kW)	1.2	1.2	1.2	1.0	1.2	1.2
Plasma (L/min)	18.0	18.0	18.0	15.0	18.0	18.0
Auxiliary (L/min)	1.50	1.50	1.50	1.50	1.50	1.50
Pump Speed (rpm)	15	15	15	15	15	15

tra-pure grade (J.T. Baker, Deventer, The Netherlands); deionized water was obtained using a Milli-Q system (Millipore, Billerica, USA).

Isotopic ratio determinations

The isotopic ratios, $^{13}C/^{12}C$ and $^{15}N/^{14}N$, were determined using a Carlo Erba 1110 elemental analyser (Carlo Erba, Milano, Italy) connected on line to a Finnigan MAT delta S mass spectrometer (Thermo Finnigan, San Jose, USA) according to previously described methodologies (BREAS *et al.*, 1994; BRESCIA *et al.*, 2002b). Isotopic contents ($^{13}C/^{12}C$ and $^{15}N/^{14}N$) are expressed as isotopic deviations, δ, defined as:

$$\delta(‰) = \frac{R_s - R_{Ref}}{R_{Ref}} \cdot 1{,}000$$

Where R_s is the isotopic ratio measured for the sample and R_{Ref} is the isotopic ratio of the following international standards: Pee Dee Belemnite (PDB) for carbon and atmospheric N_2 for nitrogen.

Multivariate analysis

Multivariate statistical techniques were used to evaluate the possibility of differentiating the milk samples according to their geographical origin. Multivariate analyses were performed using Statistica software (StatSoft Inc., Tulsa, OK, USA). The chemometric approaches used were: Principal Component Analysis (PCA), Hierarchical Clustering Analysis (HCA) and Linear Discriminant Analysis (LDA).

PCA calculates orthogonal linear combinations of the auto-scaled variables by using a correlation matrix based on the maximum variance criterion. Such linear combinations are called principal component scores and the coefficients of the linear combinations are called loadings (MASSART *et al.*, 1988). The numerical loading value for each variable of a given principal component shows how much the variable has in common with that component.

HCA is used to identify groupings among the samples on the basis of multivariate measurements. In the hierarchical methods, objects are grouped on the basis of inter-object distances in high-dimensional space and defined by an agglomerative algorithm. In this work, the Manhattan distance was computed and a Ward linkage procedure was applied (FRANK and TODESCHINI, 1994). The results obtained were confirmed by hierarchical cluster analysis in which a complete linkage procedure was applied on the Euclidean distances. PCA and HCA are both unsupervised statistical methods that give complementary information about the similarities and groupings of the samples considered. If a trend exists, it is of interest to evaluate the possibility of classi-

fying the samples. LDA obtains discriminant functions calculated to maximise the distances between groups defined *a priori*. The purpose is to calculate class models and boundaries, giving a rule of classification based on a set of known objects (training set). This rule can be applied to define the classification of unknown objects (test set).

RESULTS AND DISCUSSIONS

The values of the parameters determined in the cow milk samples are summarised in Table 2. The marked variance of the values for some metals and phosphorous in both groups reflects the wide variation in the composition of the milk samples.

Table 2 - Mean values and standard deviations of the parameters determined for the milk samples from the two groups. All metals are in ppm, isotopic ratios are in δ‰, and P is in mg/100 g.

Variables	Murgia		Foggia	
	mean	SD	mean	SD
Ba	0.49	0.15	0.69	0.12
Mn	0.11	0.04	0.09	0.04
Fe	1.18	0.45	1.35	0.53
Zn	16.19	7.14	17.48	5.99
Al	10.89	8.81	6.51	1.69
Cu	0.32	0.23	0.31	0.10
$\delta^{15}N$	3.15	0.30	4.15	0.59
$\delta^{13}C$	-24.22	1.28	-21.09	0.78
P	85.57	25.94	76.81	8.89

PCA was applied on a data set consisting of twenty samples. Five Principal Components (PC) were extracted covering 84% of the variance. Since a five-dimensional display is difficult to present, the first two PCs were used for the score plot shown in Fig. 1.

A separation between milk samples according to their origin was found on PC1. All samples from Foggia except one had positive scores on the PC1. Milk samples from Murgia were characterised by negative scores on the same principal component. Both groups of samples showed a separation on PC2. This behaviour may be partly due to the different farms from which the samples came. For this reason samples were coded on the score plot according to the farm. Except for those samples that came from the same farm in Martina Franca (M1), that are grouped together, this effect is not clear and a trend cannot be determined.

From the loadings of the variables on the first and second principal components (Fig. 2), barium, $\delta^{15}N$ and $\delta^{13}C$ were the dominant variables in the first principal component, representing 24.78% of the total variance; while zinc dominated in the second principal component, representing 19.25% of the total variability. HCA was carried out on the first five principal components. The dendrogram in Fig. 3 shows the clustering of milk samples on the basis of their geographical origin, confirming the results of PCA. The compo-

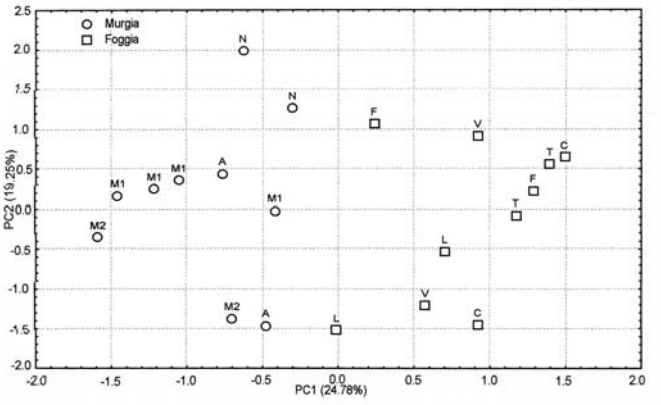


Fig. 1 - Scatter plot of the scores on the first two principal components PC1 and PC2 obtained from metals (Ba, Mn, Zn, Al, Fe and Cu), phosphorous and isotopic ratios ($^{13}C/^{12}C$ and $^{15}N/^{14}N$). Sample labels indicate the farm of origin.

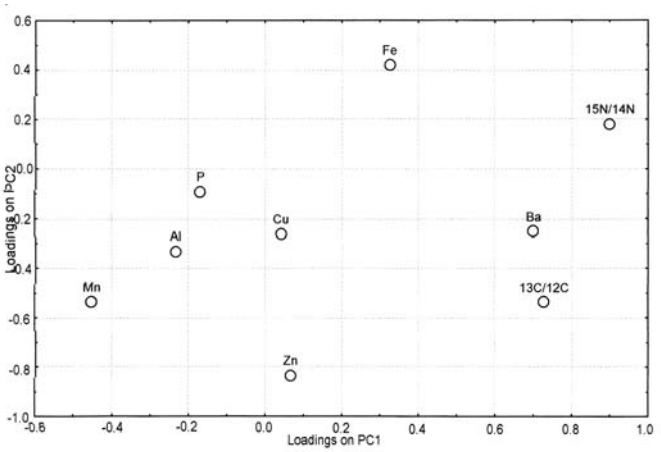


Fig. 2 - Loadings of the variables on the first two principal components obtained from metals (Ba, Mn, Zn, Al, Fe and Cu), phosphorous and isotopic ratios ($^{13}C/^{12}C$ and $^{15}N/^{14}N$).

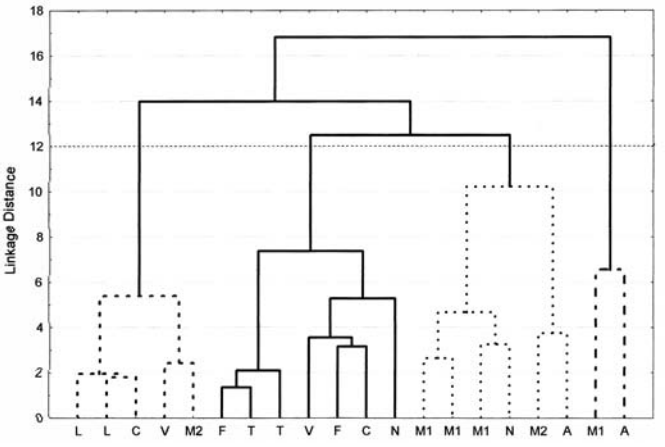


Fig. 3 - Dendrogram of milk samples obtained using the first five Principal Components. Murgia (A, N, M1, M2); Foggia (T, C, V, F, L).

sition of the clusters indicates that the parameters used gave information useful for classifying the samples. At a similarity level of 12, there were four clusters, which can be identified as follows: the first two clusters contained 10 samples from Foggia and 2 from Murgia; the third and fourth clusters included milks only from Murgia.

Discriminant analysis was then applied in order to classify the milk samples into groups. In order to use this classification method appropriately, the ratio between the number of variables and the number of samples should be ≤1:3 (DEFERNEZ and KEMSLEY, 1997). For this reason LDA was performed on the first five PCs. Samples were randomly split into a training set, formed by 66% of the samples (7 milk samples from Murgia and 7 from Foggia), in order to develop a discriminant model and a validation set, formed by the remaining 33% of the samples (3 from Murgia and 3 from Foggia), on which the model could be tested. This division provided a sufficient number of samples in the training set as well as a representative number of milk samples in the test set. In order to obtain a good estimate of the stability of the model, this validation test was repeated three times and each time a different subset was selected for the validation set. In all cases the prediction ability of the model for the training set was 100% and the same model applied to the validation set gave 100% success. The training and test milk samples on the canonical function obtained with LDA are shown in Fig. 4.

CONCLUSIONS

This preliminary study has demonstrated that multivariate analyses applied to a chemical data set are capable of extracting information that is useful for classifying cow milk samples accord-

ing to their geographical origin. The most discriminating variables were the isotopic ratios ($^{15}N/^{14}N$ and $^{13}C/^{12}C$) and Ba concentration. This confirms the fact that isotopic data carry important information about the geographical origin of food products and its discriminating potential can be enhanced by including metal concentrations.

The results obtained can be considered a good starting point for extending this study to include a broader base of milk samples from cows at different stages of lactation and from various production areas as well as samples taken at different times of the year. Other types of milk (buffalo, goat and sheep milk) will also be considered. Further investigations should also be conducted on processed dairy products like cheeses. This could be a very important application of the procedure since the differentiation of geographical origin is essential for the PDO classification of these products. Finally, this type of investigation could be used to determine misrepresentations in cheeses and other milk products which should discourage potential fraud and increase consumer confidence in PDO products.

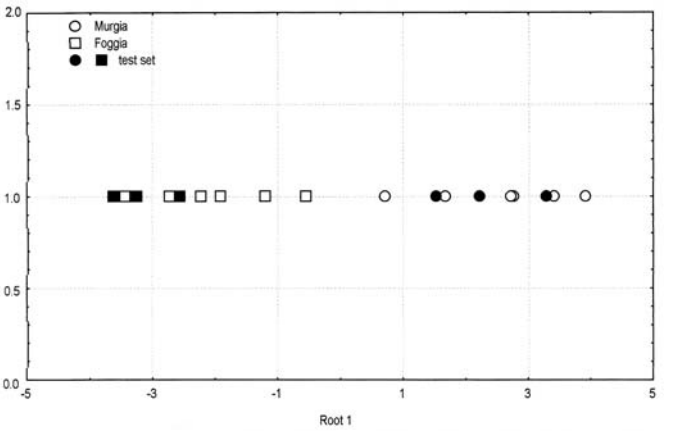


Fig. 4 - Plot of milk samples on the canonical function obtained using the first five PCs to differentiate samples according to geographical origin. The assignment of test set samples is also represented.

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PAPER

A METHOD FOR ANTHOCYANIN EXTRACTION FROM FRESH GRAPE SKIN

UN METODO PER L'ESTRAZIONE DELLE ANTOCIANINE DA BUCCE FRESCHE D'UVA

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ABSTRACT

Tartaric and citric acid solutions were used to extract anthocyanins from fresh grape skins, in a non continuous process. The type of solvent and concentration were significant factors in the extraction operation, and tartaric acid was more efficient than citric acid in the extraction yield. Comparative trials were conducted using sulphur dioxide and acidified ethanol as solvents. The extraction yields using the optimal tartaric acid solution differed slightly from those obtained with acidified ethanol

RIASSUNTO

Sono state impiegate soluzioni di acido tartarico e citrico per l'estrazione, in discontinuo, di antocianine da bucce fresche d'uva. Natura e concentrazione del solvente costituivano i fattori significativi per la riuscita del sistema, con una maggiore efficienza dell'acido tartarico rispetto al citrico quanto a rese d'estrazione. Prove comparate impiegavano come solventi anidride solforosa ed etanolo acidificato. Con le soluzioni di acido tartarico i valori delle rese d'estrazione differivano leggermente ri-

- Key words: tartaric and citric acids; storage temperatures and atmospheric conditions -

but were higher than those obtained with sulphur dioxide. Total amounts of polyphenols, proanthocyanidins and flavans were determined in the extracts obtained from the tartaric acid solutions. Solvent concentration was not a significant factor for flavan extraction yield but was highly significant for the extraction of the other compounds. The stability of the anthocyanins was evaluated at four different storage temperatures and atmospheric conditions. The combined effects of low pH of the extract (2.4), low temperature (2.0°C) and modified atmosphere (N_2) provided a long shelf-life. Based on the results, a 0.75% tartaric acid solution is recommended for anthocyanin extraction from fresh grape skin, and could substitute the widely used sulphur dioxide.

spetto a quelli ottenuti impiegando etanolo acidificato, in ogni caso più elevati di quelli ottenuti con anidride solforosa. Negli estratti ottenuti dalle soluzioni di acido tartarico sono stati analizzati, polifenoli totali, proantocianidine e flavani. La concentrazione del solvente non costituiva un fattore significativo per l'estrazione dei flavani, mentre risultava altamente significativo ai fini dell'estrazione di altri composti. La stabilità delle antocianine è stata valutata secondo quattro diverse condizioni di temperatura e d'atmosfera. Gli effetti combinati di basso pH dell'estratto (2,4), bassa temperatura (2,0°C) ed atmosfera modificata (N_2) davano luogo ad un prodotto con un'elevata shelf-life. In base ai risultati, è raccomandata per l'estrazione di antocianine da bucce fresche d'uva una soluzione di acido tartarico allo 0,75% che potrebbe sostituire l'anidride solforosa largamente utilizzata.

INTRODUCTION

Judging of food quality beyond its microbiological and nutritional characteristics is greatly influenced by sensorial properties such as colour, taste, flavour and texture. Depending on the food, these factors play different roles in the valuation of overall quality. Colour, however, is one of the most important properties of foods and beverages and is a basis for their identification and acceptability (CLIFFORD, 1996; HARDY, 2000).

Normally, food colour is due to naturally occurring pigments, but synthetic or artificial colorants are often added in order to confer the desired colour to the final product. Because there is increasing consumer demand for natural foods, industrially practical and economic production of natural food colorants is a desirable goal for food ingredients industries. However, availability of natural pigment sources, extraction process requirements and stability of colorants and fitness of use must be taken into account in the production of such ingredients.

Flavonoids are receiving increasing interest on the part of consumers and food manufactures for several reasons (KÜHNAU, 1976). Epidemiological studies have suggested associations between the consumption of polyphenol-rich foods or beverages and prevention of diseases (STEINMETZ and POTTER, 1996; YANG *et al.*, 2001). Anthocyanins, one of the main classes of flavonoids, appear to contribute significantly to their powerful antioxidative properties (VINSON, 1998; LEPIDOT *et al.*, 1999). They are an important source of the naturally occurring colorants of red fruits such as cherries, plums, strawberries, raspberries, blackberries, grapes, red currants and black currants

(JACKSON *et al.*, 1978; FRANKEL *et al.*, 1995; LEPIDOT *et al.*, 1999). Restrictions in the use of synthetic colorants in foods have led to the interest in the potential use of anthocyanins as a food colorant in beverages, syrups, fruit juices, jellies, jams, ice-creams, pastries candies and yoghurts, as well as in tooth paste, pharmaceuticals, cosmetics and similar products (CLYDESDALE and FRANCIS, 1976; DURANTE, 1995).

Pigments present in the skins of red grapes are industrially extracted using extraction processes together with sulphur dioxide. The production of this pigment is a way not only to recover a winery by-product but also to obtain a highly marketable, valued ingredient. The commercial method of producing this complex of pigments may vary slightly from one manufacturer to another, depending on the characteristics of the pomace available and the market demand. Carpenè and Comboni started to produce it in 1879 using ethanol as solvent (DIECI, 1967; GAROGLIO, 1980). It was soon realized, however, that its recovery could be improved considerably with the use of a mild solution of sulphur dioxide in water.

Attempts have been made to replace the sulphur dioxide extraction process due to health risks that this compound can cause, even in low concentrations. The effects of methanol, ethanol and water, containing or not containing acetic and chloric acids, on the degree and rate of extraction were investigated by Metiver, as reported by HANG, (1988). CALVI and FRANCIS (1978) described a process of recovering anthocyanins from grape juice pomace with 0.01% citric acid in ethanol. The same method was used by MAIN *et al.* (1978), but to produce a spray-dried colorant.

The present study was conducted to develop a method to extract anthocyanins from fresh grape skins using tartaric and citric acid solutions as solvents, to evaluate if this could be a valid alternative for the use of sulphur dioxide. The optimal solvent concentration and the yield and rate of extraction were investigated, and compared with sulphur dioxide and acidified ethanol. Stability of the extracts in four different storage conditions was evaluated.

MATERIALS AND METHODS

Grape harvesting and skin removal

Vitis vinifera grapes var. Ancellotta were purchased directly from producers in eight locations around Reggio Emilia (Italy). Grapes were harvested at the industrial maturation state. All 8 vineyards were located in the plains (20 m a.s.l.) on homogeneous land, with similar climatic conditions (temperature and relative humidity ranged from 15° to 30°C and from 40 to 60%, respectively, under normal levels of rainfall). The fresh grapes were stored at 2.0°C in nitrogen atmosphere for 24 h. Grapes were sampled from 5 randomly selected clusters of each vineyard. Berries were snipped from the top, middle and bottom of each cluster, and from the inner and outer surface. To facilitate skin and pulp tissue separation the whole berries were put into an ethylene-glycol bath kept at a constant temperature of -35°C for 4 min; they were then immediately put in a water bath at 18°C for 30 sec. After the berries were removed from the water bath, they were thoroughly wiped with absorbent paper. This procedure allowed only the pulp to remain frozen, and increased the ease of skin removal which was done by squeezing the grapes between the thumb and the forefinger.

Anthocyanin extraction

A non-continuous extraction process was employed throughout. Ten-gram samples of fresh skin (wet weight) were soaked in 25 mL of an aqueous tartaric or citric acid solution at the desired concentration (between 0.25 and 2.00%).

They were soaked with mild stirring at 25°C for almost 20 min, which is the time needed to reach mass transfer equilibrium, as described by DURANTE (1995). Immediately the extract was transferred to a dark glass bottle, in which nitrogen was being pumped simultaneously (a nitrogen flush was performed throughout the entire bottle, before and during filling), and the bottles were then stored at 2.0°C. Subsequently, a new 25 mL aliquot of solvent was added to the skins (moisture content 22.26%; peel thickness 185 μm), and the extraction process described above was repeated. This procedure was repeated until the extract formed was clear and a constant anthocyanin concentration was achieved. The same procedure was used for an aqueous 0.20% sulphur dioxide solution as extracting solvent. A comparative extraction was carried out using a hydroalcoholic-chloride solution (70:30) as extracting solvent in order to determine total extractable anthocyanins (GLORIES, 1978). The values of pH in all the extractions varied with the concentration of citric and tartaric acid in comparison to the pH of the control (pH 1.60 at 25°C), the hydroalcoholic-chloride solution. Samples (5.0 g) of fresh skins (wet weight) were soaked in 50 mL of 70% ethanol containing 1% HCl (v/v) and then slightly stirred at 25°C for 30 min. After each extraction, the extract was put in a dark glass bottle, in which nitrogen was being pumped simultaneously, and then stored at 2.0°C. After that, a new 50 mL aliquot of solvent was added to the skins and the extraction operation was carried out as above. This procedure was repeated 25 times until the solvent was completely clear, confirmed by an absorbance <0.05. Difference tests were performed at different liquid/solid ratios, but the ratio of 5.0 g of fresh skins soaked in 1.25 L of solvent was the most advantageous.

All extractions were made in triplicate and the results are reported as the arithmetic mean. The t-Test was used to estimate 95% confidence intervals of means of all determinations.

Evaluation of extract stability

Four extraction runs were carried out simultaneously using a 0.75% tartaric acid solution as the extracting solvent (highest extraction yield) following the method described above. The first extracts of each run were put in dark glass bottles and stored in four different conditions: in air at 2.0° and 25°C and in nitrogen atmosphere at 2.0° and 25°C. The headspaces of the bottles were the same as the external storage atmosphere. The three extracts from each run were added to the bottles and after all the extracts were mixed and homogenized, a "zero time" sample was taken for each run. Sampling was done at 30-day intervals for 240 days and the samples were immediately analysed. The stability was evaluated as colour intensity, which is related to colour quantity, according to POMPEI *et al.* (1983). The analyses were performed without any preparation of the samples by reading their absorbances at 420 and 520 nm on a Cary 1E spectrophotometer (Varian, Mulgrave-Victoria, Australia). Colour intensity was estimated as the sum of both absorbance values ($E_{420} + E_{520}$). The first sum was taken as 100% of colour intensity and extract stability was evaluated as decrease in colour intensity as a function of time.

The extracts from tartaric acid solutions were analysed for total anthocyanins, total polyphenols, flavans and proanthocyanidins. With slight modification, total anthocyanin analysis was performed using the spectrophotometric technique described by DI STEFANO *et al.* (1989). According to RIGO *et al.* (2000), the sample was diluted 20 to 40 times with the hydroalcoholic-chloride solution (70:30) cited above and absorbances were read at the wavelength of maximum absorption in the visible range (536-540 nm) on a Cary 1E spec-

trophotometer (Varian, Mulgrave-Victoria, Australia). The total anthocyanins were directly quantified on the basis of the maximum absorbance in the visible range (536-540 nm) against a blank (hydroalcoholic-chloride solution) The amount of the extracted pigment was calculated in milligrams/liter by assuming an average absorbance of the mixture of anthocyanins (average MW=500) extracted from Cabernet Sauvignon grapes to equal 18,800 M^{-1} cm^{-1} (GLORIES 1984; DI STEFANO *et al.*, 1989).

Total polyphenol analysis was performed using the Folin-Ciocalteau reagent and the phenolic compounds redox reaction method, in alkaline medium, as described by SINGLETON and ROSSI (1965); results are reported as gallic acid. Flavan determinations were made using the strong acidic vanillin reaction method described by MARGHERI and FALCIERI (1972); results are reported as catechins. Proanthocyanidins were estimated by their transformation into anthocyanins in hot acidic media, according to the method described by MARGHERI and FALCIERI (1972); results are reported as cyanidin chloride. The solvents and reagents not expressly specified had a high degree of purity (RPE) and were supplied by Carlo Erba (Rodano, Italy). All analyses were done in triplicate and the results are reported as the arithmetic mean. The t-Test was used to estimate 95% confidence intervals of the means of all determinations.

RESULTS AND DISCUSSION

Anthocyanin extraction

Tartaric and citric acids were effective in extracting anthocyanins from fresh grape skins. The pigment extracted as a function of solvent concentration is shown in Fig. 1. Both curves had simi-

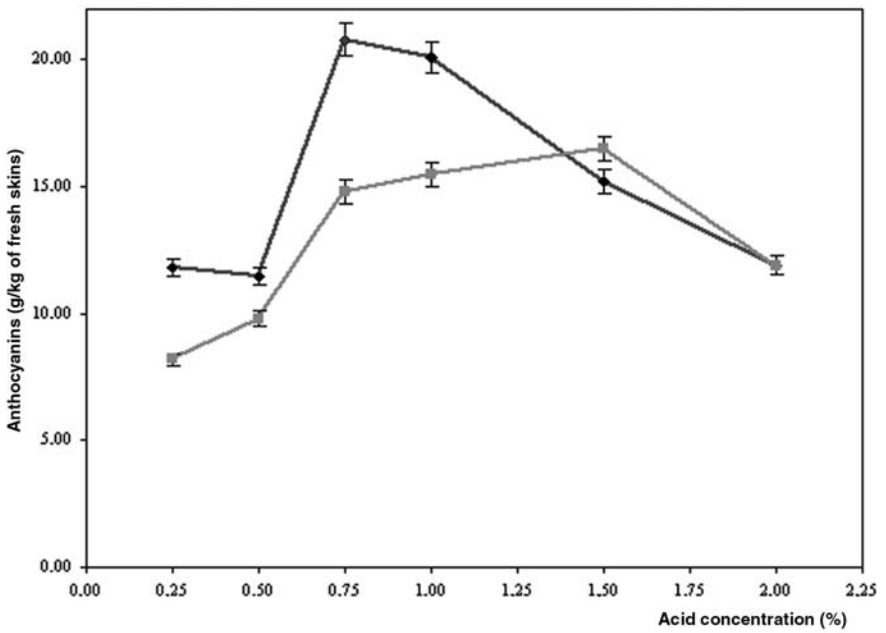


Fig. 1 - Anthocyanins extracted using tartaric and citric acids as solvents. (◆) Tartaric acid, 20 extractions for 0.75, 1.0 and 1.5% concentrations, 24 extractions for all other concentrations; (■) citric acid, 25 extractions for all concentrations. Data are the means of five replicates; $P<0.05$.

lar trends with a higher extraction yield and rate at the lower concentrations of tartaric acid. The nature of the acids affected the extraction yield and rate significantly ($p \geq 0.05$). This difference could be due to the larger molecular size of citric with respect to tartaric acid, making it more difficult for citric acid to diffuse into intra-lamellar regions of the skin tissue, where the pigments are present. The extraction processes are based on the stability of anthocyanins in polar solvents. Anthocyanins are found in nature as cations bound to organic acid anions.

A decrease in pH from 2.90 to 2.30 (pH interval of the tartaric acid solutions used) increased extraction of the anthocyanins, while an increase in pH decreased the dissociated form of tartaric acid; equilibrium was reached at 0.75% concentration. The optimal concentration of tartaric acid could be explained by a hypotonic condition formed in low concentration solutions, which causes water absorption by skin cells. As the acid concentration increases, the cells swell continuously up to a maximum state and then break, liberating the pigments which were in their vacuoles. Instead, at high concentrations, a hypertonic situation develops, in which the cells lose water and harden, diminishing membrane permeability and protecting them from rupturing. At this point, anthocyanin molecules remain in the vacuoles and mass transfer becomes difficult.

Extraction using different solvents is shown in Fig. 2. Sulphur dioxide is widely used today in anthocyanin production and the concentration used in this study is the most commonly used in industrial production (MAZZA *et al.*, 1999; MAZZA and MINIATI, 1993). Hydroalcoholic-chloride solutions are commonly used for analytical purposes, in the isolation of anthocyanins from fruits and vegetables, as they are capable of extracting all pigments contained in the plant materials

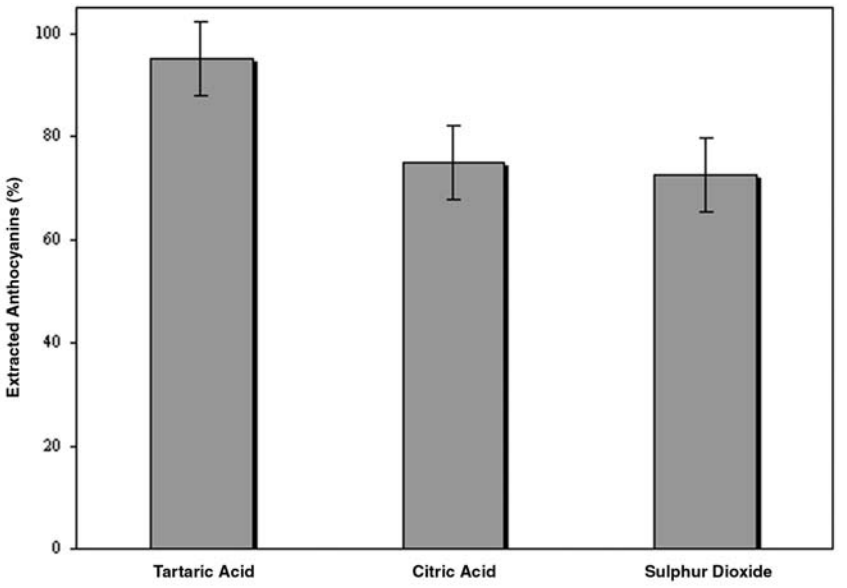


Fig. 2 - Anthocyanin extraction yield using different solvents. 0.20% sulphur dioxide, 15 extractions; 0.75% tartaric acid, 20 extractions; 2.0% citric acid, 25 extractions. Data are the means of five replicates; error bars are ± S.D.; $P<0.05$.

(DI STEFANO *et al.*, 1989; GLORIES, 1978; LAMIRANKA, 1989; SAN JOSÉ and DI STEFANO, 1990; GLORIES, 1984; DI STEFANO *et al.*, 1989), but it is more expensive that tartaric or citric acids. As the best extraction yield (21.91 g anthocyanins/kg fresh skin) was obtained with this solvent, it was taken as the reference (100% of extractable anthocyanins) for the other trials. Tartaric acid was more efficient than sulphur dioxide ($p \geq 0.05$) but there was no significant difference between tartaric acid and the hydroalcoholic-chloride solution ($p \leq 0.05$). Sulphur dioxide augments membrane permeability (CAPPELLI and VANNUCCHI, 2000) and binds itself to anthocyanins, but high concentrations are necessary when fresh skins instead of spent pomace are used.

Besides anthocyanins, the extraction of other phenolic compounds with tartaric acid were investigated and the results are presented in Fig. 3. With respect to anthocyanins, there were three different levels of extraction rate and yield (Fig. 3a). Low and high concentrations (0.25, 0.50 and 2.0%) gave the same profile ($p<0.05$) with respect to the total amount extracted, but the extraction rate was higher with the 2.0% solution. After 9 extractions, 97% of the total pigment was extracted, whilst with other two solutions (0.25 and 0.50%), 16 extractions were necessary to reach 97%. Instead, with the concentrations of 0.75 and 1.0%, it was possible to extract more pigment more rapidly. Three extractions yielded more pigment than the

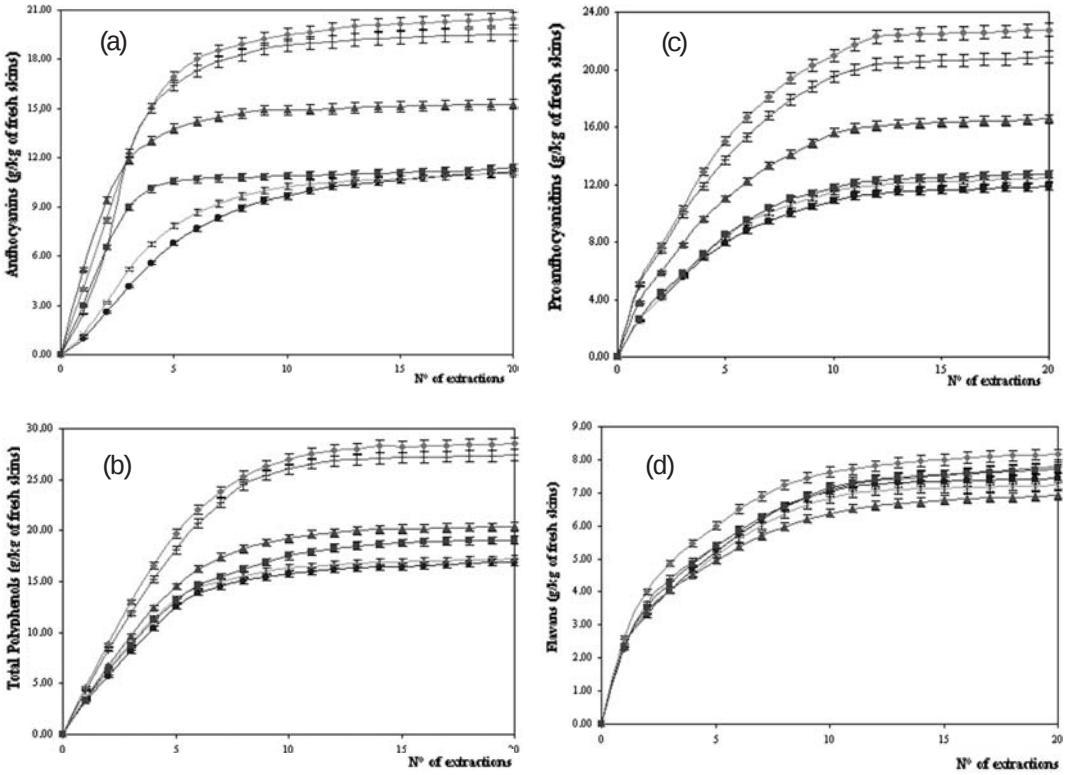


Fig. 3 - Pigment extracted with tartaric acid. (a) anthocyanins; (b) total polyphenols reactive to the Folin-Ciocalteau; (c) proanthocyanidins; (d) flavans; (●) 0.25%; (x) 0.50%; (◆) 0.75%; (+) 1.0%, (▲) 1.50%; (■) 2.0%, cumulative values. Data are the means of five replicates; error bars are ± S.D.; $P<0.05$.

total obtained with all other solutions, and ten extractions were necessary to achieve 95% of the final anthocyanin concentration. The 1.5% solution gave an intermediate profile, for both extraction yield and rate. The differences in the amount extracted and the rate of extraction can be explained by the theory of hypotonic and hypertonic conditions discussed above. The extraction profile of total polyphenols (Fig. 3b) and proanthocyanidins (Fig. 3c) were quite similar to that of anthocyanins. The final amounts obtained for each analyses were proportional to those of anthocyanins attained in the corresponding analyses. In addition, the rates of extraction were the same as for anthocyanins. In the total polyphenol extraction, the curve of the 1.5% solution also gave an intermediate profile but was closer to the first group of curves (0.25, 0.5 and 2.0%). The high amounts of polyphenols obtained could be due to the low pH of the solutions (from 2.0 to 3.0), which enhances polyphenol extraction (POMPEI, 1983), but the hypertonic state formed by high concentrations of tartaric acid could have hindered extraction, suggesting that pH plays a secondary role in the efficacy of the process. A combination of pH and solvent concentration indicate the 0.75% solution to be the best solvent. Flavan extractions are shown in Fig. 3d. Based on the results, the extraction performance gave a somewhat different profile with respect to the other compounds. Solvent concentration did not affect extraction yield and rate significantly ($p<0.05$). As total amounts of polyphenols attained were high, the results indicate that anthocyanins and proanthocyanidin are the dominant extracted constituents and therefore extraction with tartaric acid is preferential to these compounds but not to flavans. All extractions were carried out at a constant low temperature (25°C) in order to minimize pigment degradation by heat.

The colour intensity decreased in the

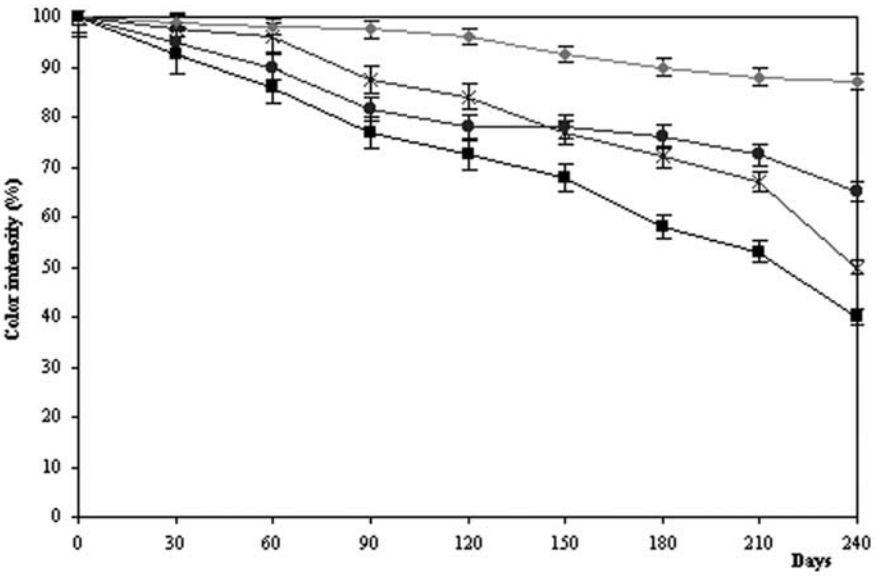


Fig. 4 - Anthocyanin stability in different storage conditions (2° and 25°C) and atmosphere (air and nitrogen) at constant pH (2.4); (◆) N_2 at 2°C; (●) air at 2°C; (*) N_2 at 25°C; (■) air at 25°C. Data are the means of five replicates; error bars are ± S.D.; $P<0.05$.

different storage conditions as shown in Fig. 4. Therefore, the combined effects of low pH of the extract (2.4), low temperature, protection against light, high concentration of phenols and polyphenols and modified atmosphere resulted in a long and stable product.

CONCLUSIONS

The use of tartaric acid as solvent in anthocyanin extraction has been shown to be a simple and inexpensive methodology, which provides a high extraction yield and a stable extract using mild storage conditions. Besides, tartaric acid solutions extract anthocyanins and proanthocyanidins more easily than flavans. Because, tartaric acid can be easily removed from the extract, it could be suitable to substitute the commonly used sulphur dioxide used industrially.

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PAPER

PHENOLIC COMPOUNDS IN RED-BERRY SKINS OF UVA DI TROIA AND BOMBINO NERO GRAPES (*VITIS VINIFERA* L.)

COMPOSTI FENOLICI IN BUCCE DI UVE A BACCA NERA DELLE VARIETÀ UVA DI TROIA E BOMBINO NERO (*VITIS VINIFERA* L.)

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ABSTRACT

The amounts of anthocyanins, flavonol glycosides and hydroxycinnamoyl tartaric acids in the skins of two red grape varieties (*Vitis vinifera* L.) from southern Italy, Bombino nero and Uva di Troia, were determined. Arbor and trellis training systems in the different growing areas were also compared. Skin extracts were analyzed by a direct HPLC-DAD method for the simultaneous detection of the selected flavonoids and phenolic acids. Although the phenolic profiles were similar in the two

RIASSUNTO

Il contenuto e la composizione in antociani, flavonoli glicosilati e acidi idrossicinnamil tartarici, sono stati determinati sulle bucce di Uva di Troia e Bombino nero (*Vitis vinifera* L.), varietà a bacca rossa tipiche del sud Italia. Le uve, oggetto di sperimentazione, sono state campionate in differenti aree di produzione ed in vigneti con sistemi di allevamento diversi. Un metodo diretto HPLC-DAD è stato impiegato per l'analisi simultanea dei differenti costituenti fenolici presenti negli

- Key words: anthocyanins, flavonol glycosides, red-berry varieties, tartaric esters of hydroxycinnamic acids (HCTA) -

varieties, the absolute content of acylated forms of malvidin and peonidin-3-(6-O-p-coumaroyl-glucoside) was higher in Uva di Troia. This particular acylated anthocyanin content could be useful to promote the varietal character of the respective rosé and young red wines. Hydroxycinnamoyl tartaric acids were significantly affected by the area of production and training system.

estratti delle bucce. Il profilo fenolico delle bucce delle due varietà è risultato simile, tuttavia il contenuto delle forme acilate della malvidina e della peonidina-3-*p*-cumarilglucoside nell'Uva di Troia era significativamente più elevato rispetto al Bombino nero. Questa peculiare dotazione in antocianine acilate potrebbe costituire un carattere varietale distintivo anche per i relativi vini rossi e rosati. L'area di produzione e il sistema di allevamento hanno, invece, significativamente influenzato la dotazione di acidi idrossicinnamil tartarici in entrambe le varietà.

INTRODUCTION

The phenolic composition of a wine depends mainly on the phenolic content of the grapes from which the wine is made, juice extraction and winemaking techniques, and on the numerous reactions that occur during wine aging. The initial phenolic content of the berries is of great taxonomic importance in viticulture for classifying grape cultivars (MACHEIX *et al.*, 1990; BOSS *et al.*, 1996). Moreover, the synthesis and concentration of phenols in red grapes depend on a number of environmental factors, particularly fruit ripeness, climatic conditions, soil features and crop load (JACKSON and LOMBARD, 1993; REYNOLDS *et al.*, 1996; ROUBELAKIS-ANGELAKIS and KLIEVER, 1986).

In making rosè and young or aged red wines, the phenolic compounds in the grape skin, particularly anthocyanins, have a relevant role in the choice of berry crushing and pressing techniques and vatting time (GAO *et al.*, 1997; GOMEZ-PLAZA *et al.*, 2000). Moreover, in recent studies specific health-related properties, especially with respect to heart disease, have been ascribed to particular flavonoids from grape skin: anthocyanins (WANG *et al.*, 1997) and flavonols (HERTOG *et al.*, 1997).

A number of studies have demonstrated the key role of the phenolic compounds in grapes in the characterization and classification of widespread international grape varieties (MAZZA *et al.*, 1999); however, few data (ROMANI *et al.*, 1996) are available about typical Italian grapes used for wines destined to a niche connoisseur market.

The aim of this study was to investigate two autochthonous red grape varieties (*Vitis vinifera* L.) from Apulia (TAMBORRA *et al.*, 2000), a key wine-producing region in southern Italy, in order to improve the winemaking techniques, and to promote the varietal character of the respective young red wines. To achieve this purpose the qualitative and quantitative anthocyanin, flavonol glycoside and hydroxycinnamoyl tartaric acid (HCTA) profiles of berry skins of Bombino nero and Uva di Troia varieties, used for the production of Controlled Denomination of Origin Castel del Monte wines, were investigated in rela-

tion to various production areas and training systems.

MATERIALS AND METHODS

Plant material

Grape samples of Bombino nero cv. and Uva di Troia cv. were harvested at technological ripeness in 5 and 8 vineyards, respectively. The vineyards were located in the areas delimited for the region of Controlled Denomination of Origin Castel del Monte, and arbor and trellis training systems were used. In each vineyard, 300 berries were collected from 30 bunches produced by vines with similar vegetative and productive characteristics (age, amount of buds). Berries were picked, cutting and leaving part of the peduncle, from different zones of the bunch. A random selection of 3 replicates of 10 berry sub samples were weighed before phenolic extraction.

Extraction of phenolic compounds

Phenolic extraction was carried out according to DI STEFANO and CRAVERO (1991). The berry skins were manually removed from the pulp, dried with filter paper, and then immersed in an HCl/water/ethanol solution (1/ 30/ 70) for 20 hours (in the dark and at room temperature) to keep the extracts.

Chemicals and reference compounds

The reagents (Carlo Erba, Milan, Italy) were analytical or HPLC grade, as required. Malvidin 3-glucoside chloride and rutin were purchased from Extrasynthèse (Genay, France); caffeic acid, and quercitin dihydrate were from Fluka (Buchs, Switzerland).

HPLC-DAD analysis

For the phenolic fractionation, in order to save solvents and to reduce time and the number of analyses, the extract was diluted 5 times with water, filtered (0.2 µm) and analyzed by direct HPLC-DAD separation. Therefore, the compound identification and quantification were carried out with minimal pre-treatment of the sample (LAMUELA-RAVENTOS and WATERHOUSE, 1994; TAMBORRA and MINAFRA, 1998; RITCHEY and WATERHOUSE, 1999).

Detection and quantification of phenolic compounds were carried out by HPLC using an HP 1090 L apparatus (MILFORD, MA, USA) with a photodiode array detector and an injection valve (20 µL loop). The stationary phase was a Symmetry C_{18} column, 4.6x100 mm, 3.5 µm particle size from Waters (MILFORD, MA, USA). The flow rate was 0.5 mL min^{-1} and the solvents used for the separation were: Solvent A = 50 m*M* dihydrogen ammonium phosphate adjusted to pH 2.6 with orthophosphoric acid; Solvent B = 20% A with 80% acetonitrile; and Solvent C = 0.2 *M* orthophosphoric acid adjusted with ammonia to pH 1.5. Table 1 gives the solvent gradient conditions.

The eluates were detected at 25°C, at 280 nm for all phenols, at 313 nm for hydroxycinnamic derivatives, at 365 nm for flavonols and at 520 nm for anthocyanins. In addition, UV-visible absorp-

Table 1 - Solvent gradient conditions with linear gradient.

Time (min)	Solvent A (%)	Solvent B (%)	Solvent C (%)
0	100	0	0
5	100	0	0
15	96	4	0
25	92	8	0
25.01	0	8	92
45	0	20	80
50	0	30	70
55	0	40	60
60	0	80	20
65	100	0	0

tion spectra (260-600 nm) of each peak were obtained. The identification was performed by chromatographic separation by HPLC and by comparing spectra with authentic standards, when available, and with data from the literature.

Each single anthocyanin, hydroxycinnamoyl tartaric acid (HCTA) and flavonol was quantitatively determined (mg/kg of berry) from the areas of the chromatographic peaks, using calibration curves prepared from standards of malvidin 3-monoglucoside, caffeic acid and quercitin, respectively. The phenol concentration in each sample is the mean of three replicates.

Statistics

A three-way Analysis of Variance (variety * production area * training system), fixed effects and interactions, was performed on each chemical parameter to get an indication of the variation in the material and level of error of the data.

In addition, Principal Component Analysis (PCA) of the correlation matrices was carried out on all variables simultaneously in order to summarise the information on differences between samples. Each variable was standardised at unit variance prior to analysis.

The analyses were performed using SPSS (1991), for univariate analyses, and UNSCRUMBLER Software version 7.01 (CAMO ASA, Trondheim, Norway) for PCA.

RESULTS AND DISCUSSION

The HPLC-chromatograms of Bombino nero berry skin extracts are reported in Fig. 1.

Results of the three-way Analyses of Variance (Table 2) showed significant differences between the varieties for their content in cinnamoyl tartaric ac-

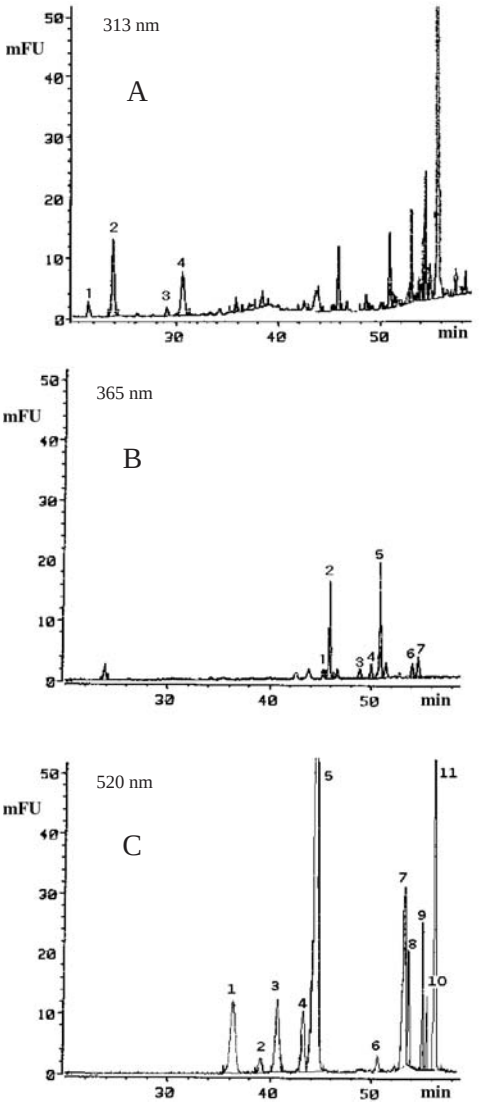


Fig. 1 - HPLC-chromatograms of Bombino nero berry skin extract, recorded at 313 nm (A) peaks: 1=*cis*-caftaric acid, 2=*trans*-caftaric acid, 3=*cis*-coutaric acid, 4=*trans*-coutaric acid; at 365 nm (B) peaks: 1=myricetin-3-O-glucuronide, 2=myricetin-3-O-glucoside, 3=rutin, 4=quercitin-3-O-glucuronide, 5=quercitin-3-O-glucoside, 6=kaempferol-3-O-glucuronide, 7=kaempferol-3-O-glucoside; and at 520 nm (C) peaks: 1=delphinidin-3-O-glucoside, 2=cyanidin-3-O-glucoside, 3=petunidin-3-O-glucoside, 4=peonidin-3-O-glucoside, 5=malvidin-3-O-glucoside, 6=peonidin-3-O-acetylglucoside, 7=malvidin-3-O-acetylglucoside; 8=malvidin-3-O-caffeoylglucoside, 9=petunidin-3-(6-O-*p*-coumaroyl-glucoside), 10=peonidin-3-(6-O-*p*-coumaroyl-glucoside), 11=malvidin-3-(6-O-*p*-coumaroyl-glucoside)

ids, acylated forms of malvidin and peonidin-3-(6-O-p-coumaroyl-glucoside), thus confirming the marked genetic basis of the synthesis of these compounds present in the grapes (CALÒ et al, 1994) and wines (MATTIVI *et al.*, 1995). There were also significant differences at the 95% confidence level for area of production (*trans*-caftaric, *cis*-coutaric and *trans*-coutaric acids), and training system (*trans*-caftaric and *trans*-coutaric acids, at 95% and *cis*-coutaric acid at 99% confidence, respectively). These results indicate that, besides being determined by genetic factors (DI STEFANO and MAGGIOROTTO, 1995), the skin content of hydroxycinnamate is significantly affected by the area of production and training system.

Phenol profiles of the two varieties for the different areas of production and training systems are listed in Tables 3 and 4.

Among the phenolic acids in the berry skin, the most abundant non-flavonoid phenol was *trans*-caftaric acid (62% of total HCTA). BOURSIQUOT (1987) found the percentage of this acid to vary from 36% in the skins of Carig-

Table 2 - Analysis of Variance. F-values for effects (variety, production area, training system and interactions) on phenolic compounds in red-berry skins of Uva di Troia and Bombino nero grapes (*Vitis vinifera* L.).

Variables	F-Variety	F-Production Area	F-Training System	F-Variety x Area	F-Variety x System	F-Area x System
cis-caftaric acid	82.8 ***	2.5	0.0	2.8	0.1	2.8
trans-caftaric acid	33.9 **	7.1 *	11.0 *	5.0	6.3	1.1
cis-coutaric acid	93.6 ***	19.0 *	33.7 **	24.6 **	15.6 *	1.2
trans-coutaric acid	37.3 **	12.7 *	9.6 *	13.7 *	4.4	0.9
delphinidin-3-O-glucoside	0.3	3.7	0.0	0.5	0.5	0.1
cyanidin-3-O-glucoside	0.1	2.9	0.0	0.4	1.6	0.3
petunidin-3-O-glucoside	0.3	4.6	0.0	0.6	0.0	0.1
peonidin-3-O-glucoside	2.1	4.3	2.4	1.5	0.9	3.0
malvidin-3-O-glucoside	0.1	2.8	0.1	0.0	0.4	0.8
delphinidin-3-O-acetylglucoside	1.9	0.2	0.9	0.5	2.4	0.6
peonidin-3-O-acetylglucoside	1.1	0.0	1.0	0.5	1.5	0.2
malvidin-3-O-acetylglucoside	13.2 *	0.0	1.8	0.4	6.4	0.7
malvidin-3-O-caffeoylglucoside	15.9 *	1.3	0.9	0.6	8.5 *	0.8
delphinidin-3-(6-O-*p*-coumaroyl-glucoside)	4.3	0.4	2.0	0.0	1.7	0.4
petunidin-3-(6-O-*p*-coumaroyl-glucoside)	2.1	0.1	0.7	0.4	3.4	0.0
peonidin-3-(6-O-*p*-coumaroyl-glucoside)	15.2 *	0.1	6.1	0.0	13.6 *	2.7
malvidin-3-(6-O-*p*-coumaroyl-glucoside)	11.1 *	0.0	4.5	0.2	9.2 *	1.2
myricetin-3-O-glucuronide	0.1	0.1	0.2	0.9	0.7	1.4
myricetin-3-O-glucoside	1.6	1.2	0.7	0.3	1.1	1.3
rutin	0.5	0.9	0.0	1.4	2.3	1.6
quercitin-3-O- glucuronide	0.9	1.1	0.1	1.6	3.7	2.6
quercitin-3-O- glucoside	0.9	5.6	1.8	4.7	1.0	2.9
kaempferol-3-O-glucuronide	0.0	0.0	0.1	1.7	0.7	1.2
kaempferol-3-O-glucoside	0.0	0.2	0.1	0.4	0.0	1.2

* P<0.05; ** P<0.01; *** P<0.001.

Table 3 - Phenolic concentration (mg/kg) of berry skins from Bombino nero grapes.

Training system	Arbor		Trellis				
Production Area	Ruvo di Puglia Calendano	Andria S. Domenico	Ruvo di Puglia Calendano	Andria S. Domenico	Corato Bosco	Average (n=5)	SD (n=5)
Phenolic Compounds							
cis-caftaric acid	0.75±0.1	0.75±0.1	0.99±0.1	0.42±0.0	0.85±0.1	0.8	0.2
trans-caftaric acid	25.3±2.1	20.1±1.9	31.9±2.5	14.2±1.5	20.4±2.8	22.4	6.6
cis-coutaric acid	2.8±0.3	1.9±0.2	3.3±0.3	1.6±0.2	2.8±0.5	2.5	0.7
trans-coutaric acid	12.8±1.5	8±1.1	15.6±1.1	5.9±1.4	8.5±1.3	10.2	3.9
delphinidin-3-O-glucoside	125.5±15.2	66.4±4.2	90.7±8.2	107.3±10.2	127.9±18.6	103.6	25.7
cyanidin-3-O-glucoside	63.3±4.9	1.7±0.2	9.4±1.2	15.5±1.8	9.8±1.1	19.9	24.7
petunidin-3-O-glucoside	107.4±9.8	62.2±4.2	79.4±5.1	88.5±5.4	116.5±11.2	90.8	21.7
peonidin-3-O-glucoside	172.7±21.3	32.6±2.8	42.3±3.2	54.5±5.3	46.6±3.9	69.7	58.1
malvidin-3-O-glucoside	620.9±36.7	419.8±28.9	563.9±31.8	544.6±28.4	663.5±37.5	562.5	92.6
Total non-acylated anthocyanins	1,089.8	582.7	785.7	810.4	964.3	846.6	192.1
delphinidin-3-O-acetylglucoside	8.5±1.3	6.9±0.9	7.8±1.1	14.5±1.3	11.3±1.2	9.8	3.1
peonidin-3-O-acetylglucoside	8.9±1.1	9.6±2.1	7.8±1.2	15.1±1.4	7.7±1.0	9.8	3.1
malvidin-3-O-acetylglucoside	80.8±5.7	77.3±5.4	88.5±6.2	151.1±19.5	101.5±10.1	99.8	30.1
malvidin-3-O-caffeoylglucoside	25.2±2.9	17.3±1.8	27.9±2.5	37.7±3.1	33.3±2.9	28.3	7.8
delphinidin-3-(6-O-*p*-coumaroyl-glucoside)	0.5±0.1	4.4±0.7	2.9±0.4	2.9±0.4	0.5±0.0	2.2	1.7
petunidin-3-(6-O-*p*-coumaroyl-glucoside)	4.7±0.8	14.2±1.5	21.3±1.8	20.3±2.4	18.6±1.9	15.8	6.8
peonidin-3-(6-O-*p*-coumaroyl-glucoside)	28.1±2.3	24.3±2.1	31.1±2.6	36.9±2.8	31.8±3.1	30.4	4.7
malvidin-3-(6-O-*p*-coumaroyl-glucoside)	285.5±21.6	259.6±18.4	324.5±26.1	338.5±30.1	271.5±19.8	295.9	34.1
Total acylated anthocyanins	441.8	413.6	511.8	617.0	476.2	492.1	79.0
myricetin-3-O-glucuronide	1.95±0.2	0.36±0.0	0.6±0.1	1.31±0.7	1.84±0.3	1.2	0.7
myricetin-3-O-glucoside	11.06±1.0	3.91±0.3	10.32±1.2	12.34±1.5	14.29±1.6	10.4	3.9
rutin	3.14±0.4	0.36±0.0	0.61±0.2	1.25±0.8	1.68±0.5	1.4	1.1
quercitin-3-O- glucuronide	2.25±0.3	0.73±0.1	2.01±0.3	2.41±0.4	2.78±0.4	2.0	0.8
quercitin-3-O- glucoside	26.06±2.1	5.03±0.6	18.11±1.6	13.85±1.7	19.54±1.1	16.5	7.8
kaempferol-3-O-glucuronide	3.43±0.6	0.36±0.1	0.61±0.2	1.57±0.6	2.43±0.8	1.7	1.3
kaempferol-3-O-glucoside	4.13±0.8	1.38±0.2	1.82±0.4	2.62±0.7	3.01±1.0	2.6	1.1

Each value is the mean ± SD of three replicates of 10 berry sub samples.

Table 4 - Phenolic concentration (mg/kg) of berry skins from Uva di Troia grapes.

Training system	Arbor						Trellis			
Production area	Minervino Murge	Ruvo di Puglia II Piano	Ruvo di Puglia Calendano	Ruvo di Puglia Capocasale	Andria Rivera	Andria S. Domenico	Ruvo di Puglia	Andria S. Domenico	Average (n=8)	SD (n=8)
Phenolic Compounds										
cis-caftaric acid	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.1	0
trans-caftaric acid	6.1±0.8	6.9±0.9	6.9±0.8	10.3±1.6	5.1±0.9	6.7±0.8	17.9±1.6	17.5±1.5	9.7	5.2
cis-coutaric acid	0.9±0.1	0.9±0.2	0.76±0.1	0.91±0.2	0.74±0.1	0.79±0.1	1.87±0.2	1.9±0.2	1.1	0.5
trans-coutaric acid	3.6±0.5	3.5±0.5	3.4±0.4	5.28±0.8	2.89±0.3	3.81±0.6	7.81±0.9	7.8±0.8	4.8	2.0
delphinidin-3-O-glucoside	120.6±12.6	86.2±6.4	187.7±29.1	143.3±22.4	69.6±7.2	93.7±10.2	149.1±25.6	69.2±7.9	114.9	42.6
cyanidin-3-O-glucoside	28.1±2.8	16.6±2.0	29.4±2.3	17.4±1.9	9.3±1.5	16.6±2.1	42.8±3.5	9.8±1.8	21.3	11.4
petunidin-3-O-glucoside	97.4±9.3	81.5±8.9	151±19.7	117.3±11.5	64.3±7.1	81.8±8.4	127.6±15.4	61.4±7.0	97.8	31.8
peonidin-3-O-glucoside	85.7±8.2	50.8±5.1	94.3±9.8	75.8±8.2	32.2±2.8	52.7±5.9	51.3±5.6	32.3±2.6	59.4	23.4
malvidin-3-O-glucoside	557.2±34.6	480.9±30.1	798.2±59.8	766.9±63.4	434.1±29.7	564.8±39.7	550.6±38.1	491.1±29.8	580.5	132.6
Total non-acylated anthocyanins	889.0	716.0	1260.6	1120.7	609.5	809.6	921.4	663.8	873.8	225.5
delphinidin-3-O-acetylglucoside	18.8±2.9	15.3±2.3	26.4±4.5	15.7±2.1	10.4±1.4	19±3.5	9.8±1.2	8.7±1.4	15.5	5.9
peonidin-3-O-acetylglucoside	19.9±3.5	15.8±2.9	29.9±3.1	14.3±2.2	11.8±1.1	20.4±2.5	10.1±1.6	8.7±1.5	16.4	6.9
malvidin-3-O-acetylglucoside	216.8±38.2	201.3±30.1	257.5±41.3	201.3±29.4	167.9±27.6	243.7±38.5	130.6±17.3	134.8±19.2	194.2	46.8
malvidin-3-O-caffeoylglucoside	56.4±6.9	50.7±7.3	62.6±7.5	47.5±7.0	42.6±5.9	49.5±6.2	38.2±4.8	31.4±4.2	47.4	9.9
delphinidin-3-(6-O-*p*-coumaroyl-glucoside)	18.3±2.5	15.1±2.2	9.6±2.0	7.8±1.5	7.3±1.3	12.8±2.0	0.5±0.0	7.8±1.1	9.9	5.5
petunidin-3-(6-O-*p*-coumaroyl-glucoside)	26.3±4.0	18.7±2.3	18.6±2.8	27.6±3.8	14.9±2.6	22.8±5.2	15.3±2.9	20.4±4.8	20.6	4.7
peonidin-3-(6-O-*p*-coumaroyl-glucoside)	54.1±5.9	52.1±5.0	59.3±8.3	56.3±7.2	42.8±6.2	58.1±7.4	26.9±4.8	36.6±5.1	48.3	11.7
malvidin-3-(6-O-*p*-coumaroyl-glucoside)	468.4±35.8	470.9±39.2	525.4±46.3	527.3±49.5	409.4±38.9	560.6±55.2	272.6±44.2	365.9±50.1	450.1	96.3
Total acylated anthocyanins	879.0	839.9	989.3	897.8	707.1	986.9	504.0	614.3	802.3	176.8
myricetin-3-O-glucuronide	1.75±0.2	0.48±0.0	1.21±0.1	0.62±0.0	0.66±0.1	1.09±0.3	1.24±0.3	1.2±0.2	1.0	0.4
myricetin-3-O-glucoside	15.99±2.0	9.31±1.1	14.01±1.9	13.22±2.3	8.82±1.5	12.65±2.3	11.81±2.0	10.77±1.9	12.1	2.4
rutin	1.17±0.2	0.48±0.3	0.78±0.2	1.21±0.2	0.48±0.3	1.08±0.2	1.64±0.4	1.32±0.8	1.0	0.4
quercitin-3-O-glucuronide	2.56±0.2	2.01±0.2	2.28±0.1	2.47±0.1	1.88±0.1	2.43±0.2	1.81±0.1	1.86±0.1	2.16	0.3
quercitin-3-O-glucoside	13.24±2.3	11.11±2.6	10.11±2.5	17.04±2.1	5.49±1.3	12.35±2.9	16.41±3.1	16.92±3.7	12.8	4.0
kaempferol-3-O-glucuronide	1.94±0.2	1.25±0.1	1.45±0.1	1.21±0.1	0.98±0.0	2.64±0.2	1.75±0.1	1.86±0.2	1.6	0.5
kaempferol-3-O-glucoside	2.75±0.3	1.58±0.2	5.05±1.1	1.81±0.4	3.49±0.8	1.09±0.1	1.92±0.4	2.82±0.5	2.6	1.3

Each value is the mean ± SD of three replicates of 10 berry sub samples.

nane berries to 83% in the skins of Alfohonse Lavallée. Although the *trans*-caftaric:*trans*-coutaric ratio was about 2:1 in both varieties, the average content of HCTA in Bombino nero (Table 3) was double that of Uva di Troia (Table 4). DI BENEDETTO and TAMBORRA (1992) and TAMBORRA *et al.* (1994) also found a ratio higher than 1 in Primitivo berries, whereas the ratio was lower than 1 in Aleatico, Malvasia di Lecce, Malvasia di Brindisi, Negroamaro and Gaglioppo di Cirò. It should be noted that, as these compounds are involved in the browning reaction of must and wine, they can affect their sensorial properties (MACHEIX *et al.*, 1991; VRHOVSEK, 1998).

Delphinidin, cyanidin, petunidin, peonidin, and malvidin 3-monoglucosides are the only anthocyanins found in Pinot noir (RIBÉREAU-GAYON, 1982), while these anthocyanins, along with various forms acylated by acetic, coumaric and caffeic acids, are found in Cabernet Sauvignon (NAGEL and WULF, 1979; TAMBORRA and DI BENEDETTO, 1991), Merlot and the Syrah varieties (MAZZA, 1995). Acylated derivatives were also found in Bombino nero and Uva di Troia berries, and a total of 13 anthocyanins were quantified by HPLC analysis. The anthocyanin composition of both varieties, which was similar to Cabernet Sauvignon, mainly consisted of malvidin 3-monoglucoside (on average, 66.4% of the non-acylated forms) and a rather high relative amount of acetate derivatives. With regard to the acylated forms, the malvidin derivatives, 3-acetylglucoside (about 20%), 3-*p*-coumarylglucoside (about 60%) and 3-caffeoylglucoside (about 6%), were the principle ones found in both varieties. The acylated forms (the greatest quantity in Uva di Troia berries) contributed the most to distinguishing the two varieties. In fact, the average concentration of the acylated anthocyanins in Uva di Troia (802 mg/kg) was about twice as much as that in Bombino nero (492 mg/kg), with non-acylated/acylated anthocyanin ratios of about 1:1 and 2:1, respectively.

The flavonol varietal profile of grapes (PIGELLA *et al.*, 1998; TAMBORRA and MINAFRA, 1998) and wines (MCDONALD *et al.*, 1998; VUORINEN *et al.*, 2000) has been demonstrated to be useful for cultivar characterization and classification of wines on the basis of their geographical origin.

In Uva di Troia and Bombino nero, quercitin-3-O-glucoside and myricetin-3-O-glucoside were the most represented flavonols with non-significant differences due to variety. Moreover, the average quercitin (3-glucuronide plus 3-glucoside forms): myricetin (3-glucuronide plus 3-glucoside forms) ratio was slightly higher than 1 in both varieties.

Principal Component analysis was performed on all variables simultaneously to classify the samples (Figs. 2 and 3). A sample classification map was obtained in relation to acylated anthocyanin (negative loadings) and cinnamoyl tartaric acid (positive loadings) contents on the first dimension (Fig. 2) that describes most of the differences (41% explained variance). From the sample distribution plot (Fig. 3), Uva di Troia samples scored in the opposite position to Bombino nero (all positively scored on the abscissa). These varietal differences could be attributed to different biosynthetic pathways of anthocyanin and cinnamate esters (MACHEIX *et al.*, 1990).

Non-acylated anthocyanins and glycosidic flavonols (positive loading on the second dimension) did not contribute to product discrimination by variety, as confirmed by results from ANOVA.

There were few differences between samples of the two varieties trained using the trellis system (located in a central position on the plot). Note that the

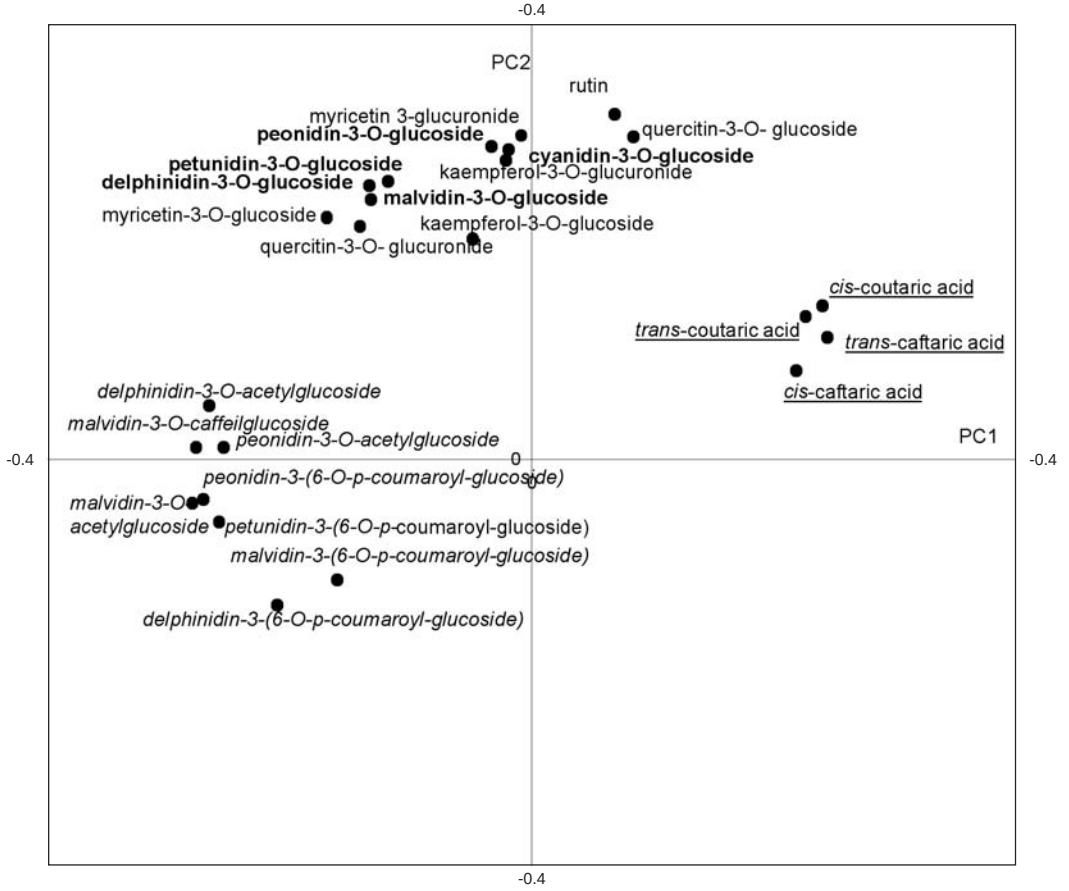


Fig. 2 - PCA - Loadings of variables for factors 1 and 2.

Variety identification: Uva di Troia (in bold); Bombino nero (block capitals) Training system identification: trellis (underlined); arbor (italic).

amount of HCTA in Uva di Troia was higher in the grapes grown using the trellis system.

Area of production gave no particular groupings, probably due to a limited variability of soil and climatic conditions in the growing regions.

CONCLUSIONS

The study has demonstrated that red-berry skins of the two varieties, Uva di Troia and Bombino nero, had an analogous composition in anthocyanins, flavonol glycosides and hydroxycinnamoyl tartaric acids. However, the absolute content of cinnamoyl tartaric acids, acylated forms of malvidin and peonidin-3-(6-O-p-coumaroyl-glucoside), significantly different in the two varieties, highlight a genetic basis for the synthesis of these compounds in the grapes.

The particular content of acylated anthocyanins of the two varieties could be useful to promote the varietal character of the respective young red wines.

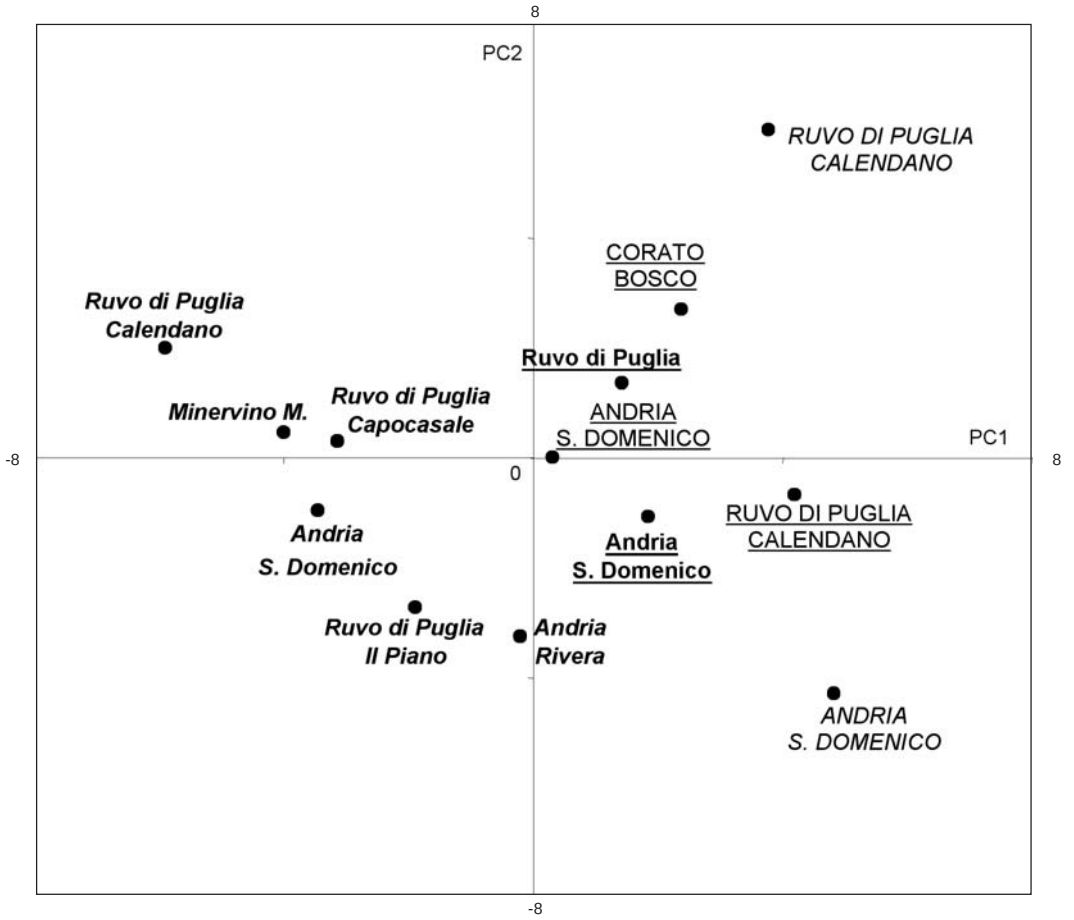


Fig. 3 - PCA - Scores of samples for factors 1 and 2.

Variety identification: Uva di Troia (in bold); Bombino nero (block capitals) Training system identification: trellis (underlined); arbor (italic).

Furthermore, HCTA were significantly affected by the area of production and training systems in both varieties.

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PAPER

EFFECT OF SODIUM LACTATE AND POTASSIUM SORBATE ON QUALITY CHARACTERISTICS AND SHELF-LIFE OF MEDITERRANEAN MUSSEL (*MYTILUS GALLOPROVINCIALIS*) MEAT DURING CHILLED STORAGE IN POUCHES WITH WATER

EFFETTO DEL LATTATO DI SODIO E SORBATO DEL POTASSIO SULLE CARATTERISTICHE QUALITATIVE E LA VITA DI SCAFFALE DELLA CARNE DI VONGOLE MEDITERRANEE (*MYTILUS GALLOPROVINCIALIS*) REFRIGERATA CON ACQUA IN SACCHE DI PLASTICA

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ABSTRACT

The quality characteristics and shelf-life of Mediterranean mussel (*Mytilus galloprovincialis*) meat (shucked mussels), stored at 5±2°C in plastic pouch-

RIASSUNTO

Sono state studiate le caratteristiche qualitative e la vita di scaffale della carne di vongole mediterranee (*Mytilus galloprovincialis*) conservata in acqua a

- Key words: Mediterranean mussel, *Mytilus galloprovincialis*, potassium sorbate, sodium lactate -

es with water, were studied. The control was 150 g of mussel meat with 150 mL of water. Sodium lactate (1% and 2%) and potassium sorbate (0.35%) were added to 150 g of mussel meat in 150 mL of water. Potassium sorbate significantly ($p<0.05$) retarded the growth of mesophilic aerobic bacteria and psychrotrophic bacteria, and especially the growth of lactic acid bacteria. In mussel meat with potassium sorbate, the pH was slightly reduced and the total volatile basic nitrogen (TVBN) and thiobarbituric acid (TBA) values were significantly ($p<0.05$) lower than all other treatments. Based on sensory attributes the shelf-life of the product with potassium sorbate was increased from 6 to 8 days. The potassium sorbate content of the mussel meat was initially less than 0.185%, and after the 5th day, less than 0.15%. The shelf-life of the control was 6 days. Sodium lactate at both levels had no effect ($p>0.05$) on microbial counts, TVBN and TBA and shelf-life, but the pH was higher ($p<0.05$) than the control at the end of the storage period and the mussels were saltier.

5±2°C in sacche di plastica. Al controllo (150 g di carne di vongole in 150 mL di H_2O) sono stati aggiunti, rispettivamente 1 e 2% di lattato di sodio e sorbato di potassio (0,35%). Il sorbato di potassio ha ritardato in modo significativo ($p<0.05$) la crescita di batteri aerobi mesofilici e psicrotropici ed in particolare la crescita di batteri lattici. Nei campioni con sorbato, il pH si è debolmente ridotto e i valori di TVBN e TBA sono stati significativamente ($p<0,05$) più bassi di quelli degli altri trattamenti. Sulla base degli attributi sensoriali, la vita di scaffale delle vongole aggiunte di sorbato di potassio è aumentata da 6 ad 8 gg. Il contenuto in sorbato di potassio è stato inizialmente inferiore allo 0,185% e, dopo il quinto giorno, allo 0,15%. La vita di scaffale del controllo è stata di 6 giorni. Il lattato di sodio, ad ambedue i livelli di concentrazione, non ha mostrato effetti ($p>0,05$) sulla conta microbica totale, e i valori di TVBN e TBA e sulla vita di scaffale. Il valore del pH, invece, è stato significativamente ($p<0,05$) più elevato del controllo alla fine del periodo di conservazione le vongole sono apparse più salate.

INTRODUCTION

The Mediterranean mussel (*Mytilus galloprovincialis*) contains about 79-83% moisture, 11-13% protein of high biological value, 2.0-2.3% carbohydrates, 1.5-1.8% fat, 2.5% ash and 0.35% non-protein nitrogen compounds (METAXATOU, 1998), and a high iron content, about 110 ppm (KARAKOLTSIDIS *et al.*, 1995). It is considered a low-fat and low-cholesterol food (KING *et al.*, 1990; HOLLAND *et al.*, 1994) and gives about 87 kcal/100 g (HOLLAND *et al.*, 1994). More than 40.8% of its fatty acids are polyunsaturated (MILETIC *et al.*, 1991), with eicosapentaenoic (C20:5, n-3) and docosahexaenoic (C22:6, n-3) acids being the prevalent ones (KARAKOLTSIDIS *et al.*, 1995; ORBAN *et al.*, 2002). Mussel meat has a high n-3/n-6 fatty acid ratio with positive effects on human health. Raising the level of n-3 fatty acids in the blood contributes to low incidence of cardiovascular diseases and mortality from atherosclerosis and heart disease, reduced inflammation associated with rheumatic arthritis and psoriasis and low blood pressure and viscosity (KINSELLA, 1986; ACKMAN, 1995).

In Greece, more than 25000 ton of mussels are produced annually, the greatest part of which is exported to other countries. In Greece they are retail mar-

keted in plastic pouches which contain water and either live mussels in the shells or shucked mussels. The product which is sealed in pouches, placed in boxes and covered with crushed ice for chilling and transport, has a shelf-life of 4 to 6 days.

Sodium lactate is currently considered as a GRAS (Generally Recognized As Safe) substance and is used as an emulsifier, flavour enhancer, humectant and pH control agent at levels not exceeding current good manufacturing practice (DUXBURY, 1988; BREWER *et al.*, 1991). It has also been shown to retard bacterial growth and to increase the shelf life of beef and pork meat and meat products (BREWER *et al.*, 1991; MACA *et al.*, 1997; BLOUKAS *et al.*, 1997; LIN and LIN, 2002), turkey (MAAS *et al.*, 1989) and salmon (PERLOY *et al.*, 1994). Sodium lactate has been reported to delay growth and toxin production by *Clostridium botulinum* in cooked fish products and to control growth of *Listeria monocytogenes* in meat and poultry frankfurters (SOFOS, 1995) and fish products (PERLOY *et al.*, 1994; NYKANEN *et al.*, 2000) and growth of *Escherichia coli* O157:H7 in commercial beef patty formulations (BYRNE *et al.*, 2002). WILLIAMS *et al.* (1995) used 2% sodium lactate to preserve fresh catfish fillets and found that it affects its shelf-life and consumer acceptance under simulated retail conditions.

Sorbic acid and its salts, known as sorbates, are regarded as GRAS substances and were approved for use in foods in 1950. Traditionally, they serve as broad spectrum inhibitors of yeasts and moulds and are useful to inhibit aerobic, catalase positive bacteria and pathogenic microorganisms, such as salmonellae, *Staphylococcus aureus* and *Clostridium botulinum* (SOFOS, 1989). They have been used throughout the food industry to extend the shelf-life of many foods, including bakery items, cereals, butter, cheese and meat products (ROBACH and SOFOS, 1982; LIEWEN and MARTH, 1985). Potassium sorbate has been used for the preservation of many products (FEY and REGENSTEIN, 1982; FLETCHER *et al.*, 1988; SAWAYA *et al.*, 1993; KARABULUT *et al.*, 2001; ANDERS *et al.*, 2002), to control the growth of *Listeria monocytogenes* on cooked Grawfish (*Procambarus clarkii*) tail meat (DORSA *et al.*, 1993), and as an additive in edible films and wrapping materials (BALDWIN *et al.*, 1996; OZDEMIR and FLOROS, 2001).

KASTANIDOU-MANOUSOU *et al.* (1982) studied the keeping quality of Mediterranean mussel meat stored at refrigeration temperatures (3°-4°C), but the shucked mussels were not kept in pouches with water. There have been no reports in the literature on the effects of sodium lactate and potassium sorbate on the quality characteristics and shelf-life of Mediterranean mussel meat.

The objective of this study was to investigate the effect of the levels of sodium lactate (1% and 2%) and potassium sorbate (0.35%) on the quality characteristics of Mediterranean mussel (*Mytilus galloprovincialis*) meat and their effect on shelf-life under chilled storage (5°±2°C) in plastic pouches with water.

MATERIALS AND METHODS

Experimental design

Fresh (live) mussels were harvested from approved shellfishing waters in an area near Thessaloniki. Within 1 hour the mussels were taken to the laboratory in plastic boxes covered with crushed ice, and were immediately shucked by forcing a knife between the two valves of the mollusk shell, cutting appropriately the adductor muscle from each valve and thus freeing the meat. The shucked mussels were carefully washed with potable tap water and separated into samples of 150 g, containing about 14-16 pieces of shucked mussels, each.

Four treatments were carried out on 150 g samples of shucked mussels, putting them into plastic pouches as follows:

1. C: Control treatment; the mussels were mixed with 150 mL of water.
2. SL 1%: The mussels were mixed with a 150 mL solution containing 1% sodium lactate.
3. SL 2%: The mussels were mixed with a 150 mL solution containing 2% sodium lactate.
4. PS 0.35%: The mussels were mixed with a 150 mL solution containing 0.35% potassium sorbate.

A 60% pure commercial sodium lactate solution (PURAC Bioquimica S.A., Montmelo, Spain) was used for treatments 2 and 3, and pure solid potassium sorbate E202 (Merck) was used for treatment 4.

Twelve pouches with shucked mussels were prepared for each treatment. All pouches were carefully closed with a heat (conductance) sealer and kept in chilled storage at 5°±2°C. Two pouches per treatment were taken on the 0, 3, 5, 7 and 10th day of storage for analyses. The experiment was repeated three times in three different months from April to September.

Physicochemical analysis

pH was measured in a homogenate prepared by blending 20 g of mussel meat with 80 mL of distilled water for 30 s. Readings were taken with a WTW, model pH 521 digital pH meter and a WTW, type E 56 combination electrode (WTW-Wissensehaftlich-Technische Werkstaetten GmbH, Weilheim, Germany). Total volatile basic nitrogen was determined according to the method described by BOTTA *et al.* (1984) on 10 g of mussel meat applying direct distillation with MgO, using the micro-Kjeldahl distillation apparatus and titrating with 0.1N H_2SO_4. Results are expressed as mg N/100g. The thiobarbituric acid (TBA) value was determined according to the method described by BOTSOGLOU *et al.* (1994) on 2 g of mussel meat using 5% aqueous TCA (8 mL) and 0.8% BHT in hexane (5 mL). The sample was Ultra-Turraxed for 30 s and centrifuged at 3000 g for 3 min. The bottom layer was made to 10 mL with 5% TCA and a 2.5 mL aliquot was taken and mixed with 1.5 mL 0.8% aqueous TBA. Following incubation for 30 min at 70°C, the sample was cooled and submitted to third-derivative spectrophotometry against a blank reaction mixture. The results are expressed as mg of malonaldehyde/kg. The potassium sorbate content was determined according to the AOAC (1990) procedure. Duplicate samples from each treatment were analyzed for all physicochemical parameters.

Microbiological analysis

A 20 g sample of mussel meat was homogenized in a stomacher Lab-blender 400 (Seward Medical, London, UK) with 180 mL sterile 0.1% peptone water for 2 min. Appropriate dilutions of samples were prepared in sterile 0.1% peptone water blank and plated in duplicate on different growth media. The following media and incubation conditions were used: (1) Plate Count Agar (Oxoid) at 30°C for 72 h, for total aerobic mesophilic count; (2) Plate Count Agar (Oxoid) at 7°C for 10 days, for psychrotrophic bacterial count; (3) deMan Rogosa Sarpe (MRS) Agar (Oxoid) at 25°C for 5 days and anaerobic incubation (BBL "GasPack" System with H_2/CO_2 cartridges), for lactic acid bacterial count. Results are expressed as $\log_{10}$ numbers of colony forming units/gram (cfu/g).

Sensory shelf-life

An experienced ten-member panel was used to evaluate mussel meat during storage. At each sampling time stored mussels were compared with fresh ones

just after being put in a pouch and mixed with water (reference sample). They were evaluated for appearance and odour when the pouches were opened, and odour and taste after cooking. Mussel meat with acceptable odour was washed with tap water, put in boiling water for 5 min, cooled under running tap water and served to the panelists in a random manner together with cooked fresh mussel meat for comparison and evaluation of odour and taste. Tap water was also provided to the panelists to cleanse the palate between samples. The sensory evaluation scales used for evaluating the odour during opening of pouches and the odour and taste of cooked mussel meat are given in Table 1. The time in days for the mussels to be considered at the limit of acceptability was defined as the shelf-life of shucked mussel meat under the specified storage conditions. In terms of panel scores given, this corresponded to the score of 3, both for odour during opening of the pouches and odour and taste of the cooked mussel meat.

Table 1 - Sensory evaluation scales used to evaluate the mussel meat.

Odour during opening of pouches

5 = odour of fresh shucked mussels
4 = slightly off-odour, but still pleasant
3 = moderately off-odour, but acceptable
2 = slightly sour, not acceptable
1 = strongly sour or putrid, highly objectionable

Odour and taste of cooked mussel meat

5 = excellent
4 = good
3 = acceptable
2 = fair
1 = unacceptable

Statistical analysis

Data were analyzed by a split-plot design in a completely randomized system, with treatment as a whole plot and storage time and treatment by storage time

Table 2 - Analysis of variance on the effect of sodium lactate (1 and 2%) and potassium sorbate (0.35%) on the microbiological, physicochemical and sensory attributes of Mediterranean mussel (*Mytilus galloprovincialis*) meat in pouches with water during chilled storage (5±2°C) (F-value for independent variables and interactions).

Parameter	Source of variance		
	Treatment[a] (A)	Storage time (B)	Interaction (AxB)
Microbiological			
Total aerobic mesophilic count	493*	385.35***	3.06**
Psychrotrophic bacteria	2.97NS	283.48***	3.48**
Lactic acid bacteria	40.90***	278.59***	6.09***
Physicochemical			
pH value	25.30***	511.40***	36.24***
Total volatile basic nitrogen (TVBN)	4.89*	245.76***	5.21***
Thiobarbituric acid value (TBA)	2.67NS	122.18***	6.71***
Sensory attributes			
Off-odour during opening of pouches	2.24NS	550.82***	5.29***
Odour and taste of cooked mussels	19.94***	20.69***	8.63***

*Significant at p<0.05; **significant at p<0.01; ***significant at p<0.001; NS, not significant;
[a] C: Control; Sodium lactate 1%; Sodium lactate 2%; Potassium sorbate 0.35%.

as the sub-plot. Means were compared by using the $LSD_{0.05}$ test. Data analyses were performed using the MSTAT (1985) program (Version 3.0, Michigan State University, East Lansing, Ml, USA). Simple correlations were determined between selected response variables.

RESULTS AND DISCUSSION

Sodium lactate and potassium sorbate slightly affected ($p<0.05$) the total aerobic mesophilic count, had no effect on the psychrotrophic bacterial count, but significantly affected ($p<0.001$) the lactic acid bacterial count (Table 2). Storage time and the interaction of treatment by storage time significantly affected ($p<0.001$) all microbial counts.

Total aerobic mesophilic count, psychrotrophic bacterial count and lactic acid bacterial counts of treatments with sodium lactate did not differ ($p>0.05$) from the control (Fig. 1). WILLIAMS *et al.* (1995) also reported that 1% and 2% sodium lactate had no effect on phychrotrophic bacteria during chilled storage of fish fillets. However, they found that 2% sodium lactate affected the total aerobic mesophilic count. LEMAY *et al.* (2002) found that sodium lactate had no effect on aerobic mesophilic counts, while it delayed the growth of *Brochothrix thermosphacta* and lactic acid bacteria in a cooked and acidified chicken model. They suggest that the spectrum of activity of sodium lactate is probably narrow or specific to a certain group of organisms, and that with time, other microorganisms take over. Two main mechanisms have been proposed for the bacteriostatic mode of action of lactate: a) passage across the cell membrane of the undissociated acid, which is dissociated within the cell to reduce the intracellular pH, and b) acting as a humectant by lowering the water activity (SHELEF, 1994; LEMAY *et al.*, 2002). The treatment with potassium sorbate had lower ($p<0.05$) total aerobic mesophilic counts, phychrotrophic bacterial counts and lactic acid bacterial counts than all other treatments throughout storage. Potassium sorbate significantly ($p<0.05$) inhibited the growth of lactic acid bacteria even at day "0". At the end of the storage period (10^{th} day) the treatment with potassium sorbate had about a 1-2 $\log_{10}$ cfu/g lower phychrotrophic bacterial count and a 3.0-3.5 $\log_{10}$ cfu/g lower lactic acid bacterial count than the other treatments.

Sodium lactate, potassium sorbate and storage time, as well as the interaction of treatment by storage time, significantly affected ($p<0.001$) the pH of the mussel meat (Table 2). The initial pH of the mussel meat was about 6.5 (Fig. 2a). KASTANIDOU-MANOUSOU *et al.* (1982) reported similar pH values. The treatment with potassium sorbate caused the least reduction in pH during the storage period, while the control treatment caused the largest. At the 10^{th} day of storage the pH of the control dropped to 5.0, that with sodium lactate to about 5.6 and that with potassium sorbate to 6.0. A high negative correlation ($r = -0.95$, $p<0.05$) was found between the pH and all microbial counts. No difference in pH was found between the treatments with 1% and 2% sodium lactate throughout the storage period. Similar results were reported by WILLIAMS *et al.* (1995) during storage of fish fillets.

Total volatile basic nitrogen (TVBN) was slightly ($p<0.05$) affected by treatment, while the storage time and the interaction of treatment by storage time had a significant ($p<0.001$) effect on it (Table 2). After the 3^{rd} day of storage the mussel meat with potassium sorbate had significantly ($p<0.05$) lower TVBN than other treatments (Fig 2c). A high positive correlation was found between TVBN and total aerobic mesophilic count ($r = -0.95$, $p<0.05$) and TVBN and psychrotrophic bacterial count ($r = -0.96$, $p<0.05$).

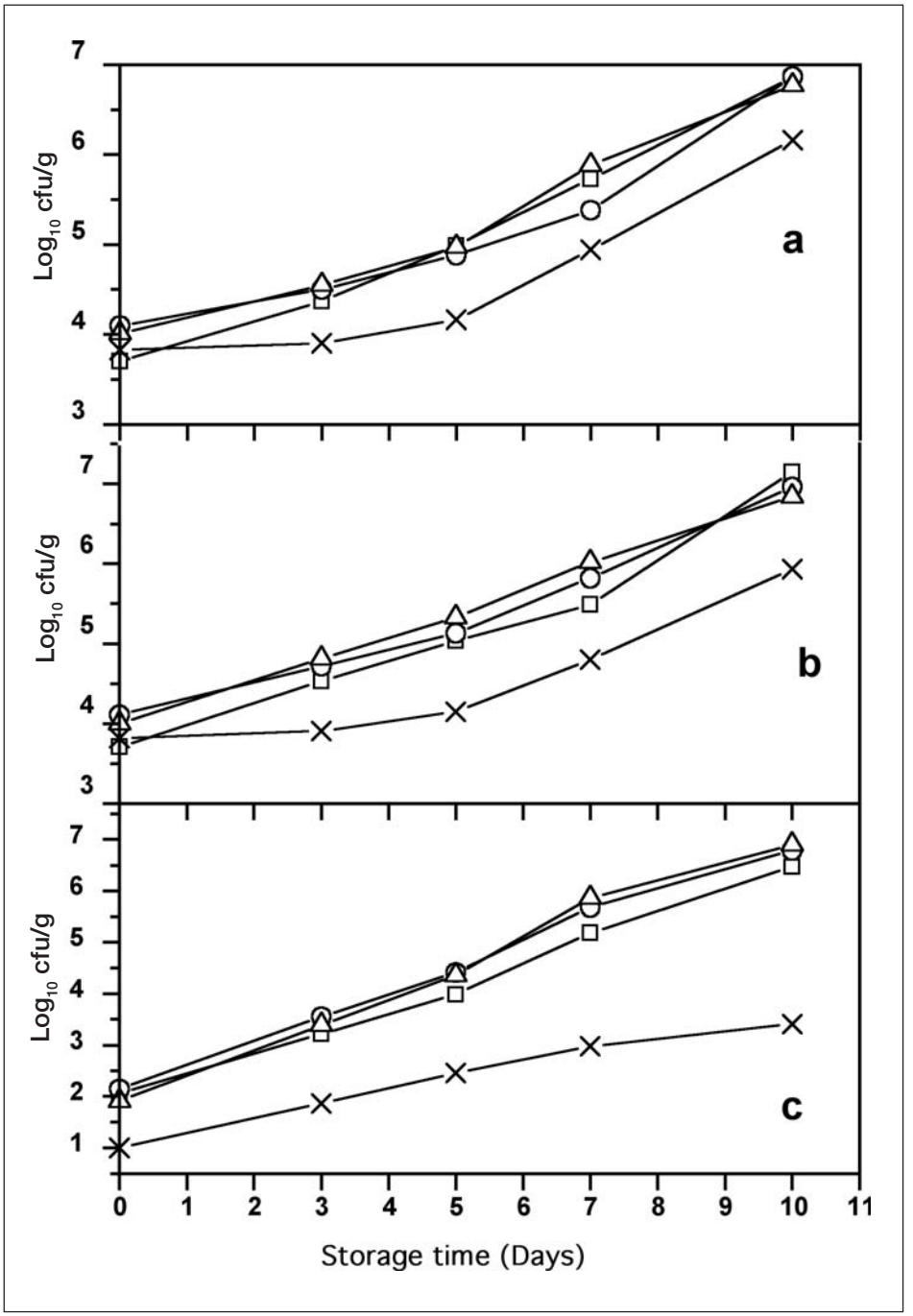


Fig. 1 - Effect of sodium lactate and potassium sorbate on (a) Total aerobic mesophilic count, (b) Psychrotrophic bacterial count, and (c) Lactic acid bacterial count of Mediterranean mussel meat during chilled storage in pouches with water. □: Control; ○: Sodium lactate 1%; Δ: Sodium lactate 2%; X: Potassium sorbate 0.35%.

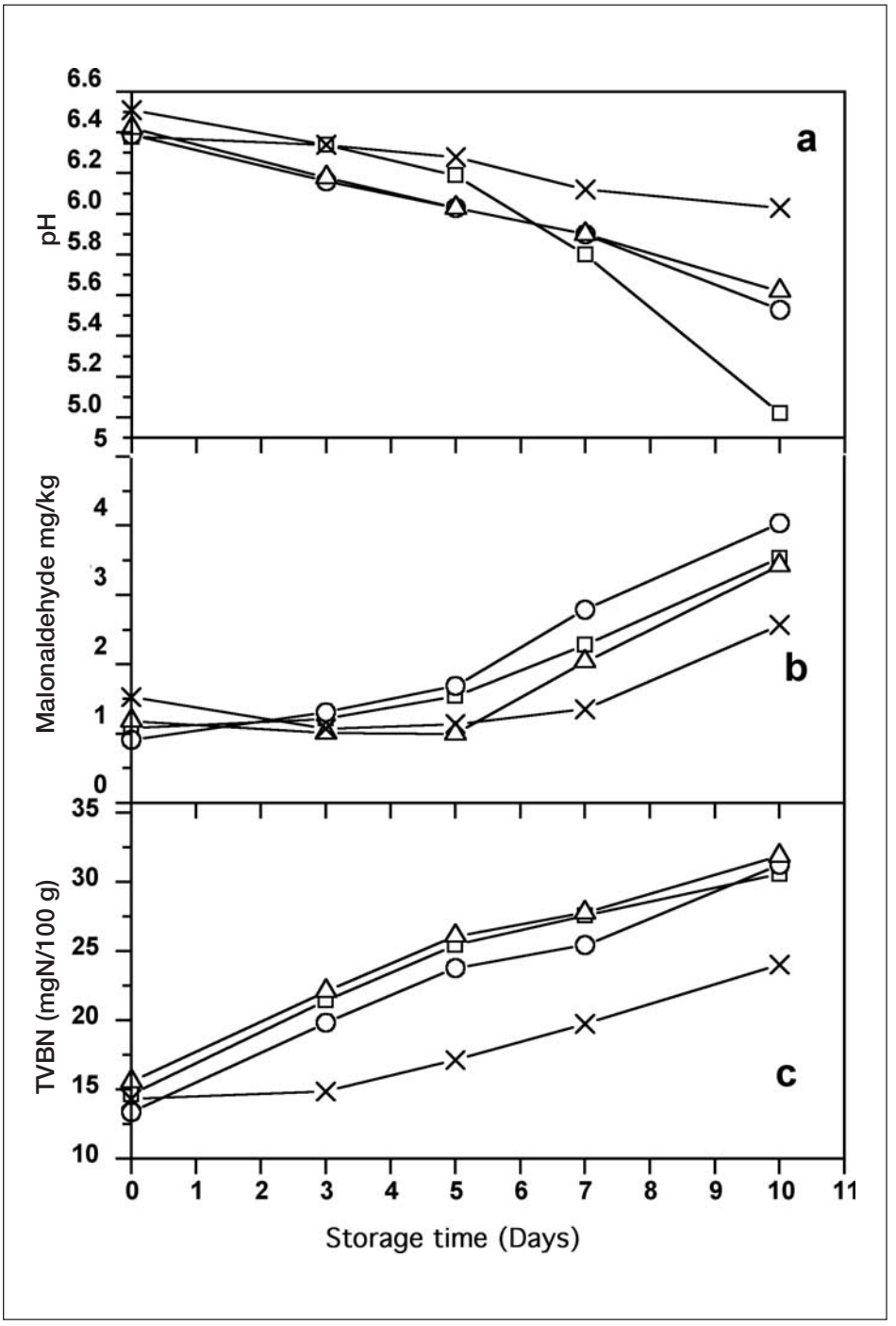


Fig. 2 - Effect of sodium lactate and potassium sorbate on (a) pH, (b) Thiobarbituric Acid (TBA), and (c) Total Volatile Basic Nitrogen (TVBN) of Mediterranean mussel meat during chilled storage in pouches with water. □: Control; ○: Sodium lactate 1%; Δ: Sodium lactate 2%; X: Potassium sorbate 0.35%.

Sodium lactate and potassium sorbate had no effect ($p>0.05$) on the thiobarbituric acid (TBA) value, while storage time and the interaction of treatment by storage time significantly ($p<0.05$) affected it (Table 2). The TBA value continuously increased in the control and 1% sodium lactate treatment (Fig. 2b). It remained constant for 5 days in the treatment with 2% sodium lactate and for 7 days in the treatment with potassium sorbate and after that time it increased rapidly. The treatment with 1% sodium lactate had a higher ($p<0.05$) TBA value than the control and 2% sodium lactate. Similar results and similar TBA values were reported by WILLIAMS *et al.* (1995) for fish filets. The antioxidant activity of sodium lactate is attributed in part to the Fe^{3+}-lactate complexation or lactyl radical reduction of Fe^{3+} to Fe^{2+} and subsequent chelation of Fe^{2+} by the carboxylate species (NNANNA *et al.* 1994). The treatment with potassium sorbate had the lowest ($p<0.05$) TBA value on the 7th and 10th days of storage.

In the treatment with 0.35% potassium sorbate, the potassium sorbate which had been absorbed by the mussel meat was determined. It was found that up to the 3rd day of storage the potassium sorbate content of the mussel meat was 0.16-0.18% and decreased to 0.14-0.15% between the 5th and 10th days of storage. MUNZNER *et al.* (1990) investigated the possible genotoxic action of potassium sorbate. In all the *in vitro* and *in vivo* tests they found no signs of genotoxicity. However, BINSTOK *et al.* (1998) showed that sorbates can react with nitrites to form mutagenic reaction products and they suggested that the combined use of nitrites and sorbates should be avoided.

Sodium lactate and potassium sorbate had no effect ($p>0.05$) on odours during opening of the pouches, while storage time and the interaction of treatment by storage time significantly ($p<0.05$) affected it (Table 2). The odour scores during opening of the pouches continuously decreased with storage time for all treatments. Close to the 6th day of storage the control and the treatments with sodium lactate had odour scores at the limit of acceptability. On the contrary, the treatment with potassium sorbate had an acceptable odour score up to the 8th day of storage (Fig. 3a). Sodium lactate, potassium sorbate, storage time and the interaction of treatment by storage time significantly ($p<0.05$) affected the odour and taste of cooked mussel meat (Table 2). The cooked mussel meat that was treated with potassium sorbate had significantly higher ($p<0.05$) scores for odour and taste throughout storage than all other treatments. The mussel meat of this treatment had an acceptable odour and taste score up to the 8th day of storage. All other treatments gave cooked meat with an unacceptable odour and taste after the 6th day of storage (Fig. 3b).

Based on these sensory attributes, the shelf-life of Mediterranean mussel (*Mytilus galloprovincialis*) meat (shucked mussels), stored at 5±2°C in plastic pouches with water, was determined to be 6 days for the control treatment and the treatments with sodium lactate. However, the shelf-life in the treatment with potassium sorbate was increased to 8 days. Sodium lactate (1% and 2%) had no effect ($p>0.05$) on the shelf-life of the product compared to the control. The panelists noticed that mussel meat with sodium lactate was saltier, even on the first day of storage, and became more pronounced during storage. This is probably due to the addition of sodium ions (Na^+) from sodium lactate. WILLIAMS *et al.* (1995) also reported that fish filets with sodium lactate had a more pronounced salty taste compared to the control and that this taste was confirmed by 60% of the panelists.

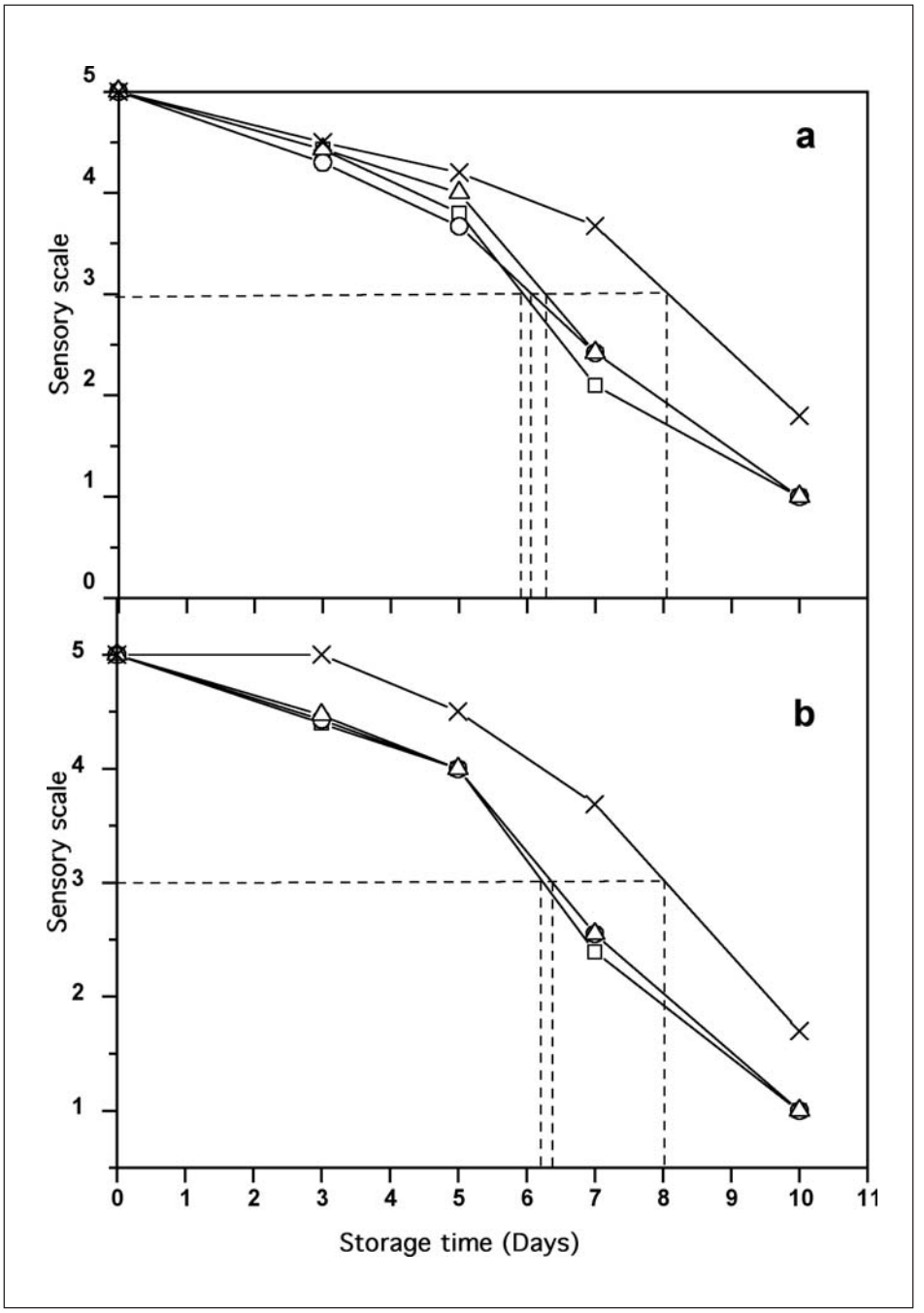


Fig 3 - Effect of sodium lactate and potassium sorbate on (a) Odour during opening of pouches, and (b) Odour and taste of cooked Mediterranean mussel meat during chilled storage in pouches with water. □: Control; ○: Sodium lactate 1%; Δ: Sodium lactate 2%; X: Potassium sorbate 0.35%. Horizontal dotted lines represent the limit of acceptability of mussel meat for each sensory attribute and vertical lines the shelf-life of mussel meat for each treatment.

CONCLUSION

Potassium sorbate (0.35%) can be used to increase the shelf-life of Mediterranean mussel (*Mytilus galloprovincialis*) meat (shucked mussels), stored at 5±2°C in plastic pouches with water, up to 8 days. Such an improvement in shelf life can have an important economic impact by reducing losses attributed to spoilage and by allowing the product to reach distant and new markets. On the contrary, sodium lactate at 1 or 2% had no effect on the shelf-life of the product and increased its saltiness.

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PAPER

EFFECT OF SMOKING ON QUALITY CHARACTERISTICS AND SHELF-LIFE OF MEDITERRANEAN MUSSEL *(MYTILUS GALLOPROVINCIALIS)* MEAT UNDER VACUUM IN CHILLED STORAGE

EFFETTO DELL'AFFUMICATURA SULLE CARATTERISTICHE QUALITATIVE E SULLA VITA DI SCAFFALE DELLA CARNE DI VONGOLE MEDITERRANEE (*MYTILUS GALLOPROVINCIALIS*) CONSERVATE SOTTOVUOTO E REFRIGERATE

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ABSTRACT

A smoking procedure was developed for Mediterranean mussel meat (*Mytilus galloprovincialis*). Fresh mussels were washed in running water and steamed at 80°C (air temperature) for 10 min, the shells were removed and the meat

RIASSUNTO

È stata sviluppata la seguente tecnica di affumicatura per la carne di vongole del mediterraneo: le vongole fresche erano lavate in acqua corrente e bollite a 80°C per 10 min, Dopo aver rimosso le conchiglie, la carne era po-

- Key words: Mediterranean mussel, mussel meat, *Mytilus galloprovincialis*, smoking -

was put in a brine with 4% salt for 15 min, drained for 5 min, dried in a smokehouse at 60°-65°C for 13 min, smoked at air temperature 65°-80°C for 17 min to obtain a $F_{70°C}$ value of 40.2 min, vacuum packed in pouches and cold stored at 2°-3°C. The season (spring and autumn) of harvesting had no effect ($p>0.05$) on the physicochemical and microbiological parameters of the smoked mussels. Steaming and smoking affected ($p<0.05$) the chemical composition of the mussel meat, reduced the TBA value, the total plate count and lactic acid bacteria count and increased the TVBN, but had no effect ($p>0.05$) on the pH value. During storage of mussel meat for 90 days at 2°-3°C the total plate count increased by 0.8 $\log_{10}$ cfu/g and the lactic acid bacteria count by 0.4 $\log_{10}$ cfu/g, the pH decreased by 0.2 units, the TBA value increased by 0.2 mg of malonaldehyde/kg and the TVBN increased from 18 to 29 mg N/100 g. Based on sensory attributes the shelf-life of Mediterranean mussel meat under the specified smoking procedure and storage conditions can be extended to 70 days.

sta in salamoia salina al 4% per 15 min, scolata per 5 min ed essiccata a 60°-65°C per 13 min ed affumicata a temperatura ambiente per 17 min, ottenendo un valore di $F_{70°C}$ pari a 40,2 min. Finalmente la carne era confezionata sottovuoto e conservata a 2°-3°C. La stagione di raccolta delle vongole (autunno e primavera) non ha mostrato alcun effetto ($p>0,05$) sulle caratteristiche fisico-chimiche e microbiologiche della carne. La cottura e l'affumicamento ne hanno invece influenzato ($p<0,05$) la composizione chimica, riducendo, da un lato, il valore TBA, la conta microbica totale e la conta dei batteri lattici ed aumentando, dall'altro, il valore TVBN. Nessun effetto ($p>0,05$) si è osservato sul pH. Dopo conservazione della carne di vongole per 90 gg a 2°-3°C, la conta microbica totale è incrementata di 0,8 $\log_{10}$ cfu/g, la conta di batteri lattici di 0,4 $\log_{10}$ cfu/g, mentre il pH è diminuito di 0,2 unità. Il valore di TBA è aumentato di 0,2 mg di aldeide malonica/kg. Il valore di TVBN è aumentato da 18 a 29 mg N/100 g. Sulla base delle valutazioni sensoriali la vita di scaffale della carne di vongole del mediterraneo, conservata nelle condizioni descritte, può essere estesa a 70 gg.

INTRODUCTION

Mediterranean mussel meat (*Mytilus galloprovincialis)* contains proteins with a high biological value and lipids with a high content of polyunsaturated fatty acids with more than 20 C atoms (MILETIC *et al.*, 1991). Eicosapentaenoic (C20:5, n-3) and docosahexaenoic (C22:6, n-3) are the prevalent fatty acids among the polyunsaturated fatty acids (KARAKOLTSIDIS *et al.*, 1995; FERNANDES-REIRIZ *et al.*, 1996; ORBAN *et al.*, 2002; FREITES *et al.*, 2002). They also have a low level of n-6 fatty acids, a high ratio of n-3/n-6 fatty acids (ORBAN *et al.*, 2002) and a high iron content, about 110 ppm, (KARAKOLTSIDIS *et al.*, 1995), and are also considered to be a low-fat and low-cholesterol food (HOLLAND *et al.*, 1994; ORBAN *et al.*, 2002). The meat of *Mytilus galloprovincialis* is also appreciated by consumers for its organoleptic properties which remain even after processing (ORBAN *et al.*, 2002).

More than 25,000 tons of Mediterra-

nean mussel are produced annually in Greece, the largest part of which is exported to other countries, usually at a low price. On the Greek retail market mussels are mainly sold in plastic pouches with water, which contains either live mussels or shucked mussels. Sealed pouches containing mussels, placed in boxes and covered with crushed ice for chilling and transport, have a shelf-life of about 4 to 6 days (VASAKOU *et al.*, 2003). Frozen mussels are also produced in a limited quantity.

Smoking, as well as salting and drying, are used as preservation techniques of fish and other sea foods that have remained virtually unaltered from prehistory to the present day. Today the preservation of fish and other seafood products is still the prime objective of smoking in most parts of the world. Smoked seafood products are regarded as delicatessen food items, to be consumed on special occasions, or presented as tempting alternatives in our diet (HORNER, 1992).

KASTANIDOU-MANOUSSOU *et al.* (1982) studied the keeping quality of Mediterranean mussel meat stored at refrigerator temperatures of 3°-4°C and found that its shelf-life was limited to 4 days. VASAKOU *et al.* (2003) studied the effect of sodium lactate and potassium sorbate on the quality characteristics of Mediterranean mussel meat. They found that the shelf-life of shucked mussels under chilled storage (5°±2°C) in plastic pouches with water could be extended to 8 days by the addition of 0.35% potassium sorbate. However, no research has been conducted on the effect of smoking on the quality characteristics and shelf-life of Mediterranean mussel meat.

The objective of this study was a) to standardize a smoking procedure for Mediterranean mussel meat and b) to investigate the quality characteristics and the shelf-life of the smoked product under chilled storage (2°-3°C).

MATERIALS AND METHODS

Experimental design

Fresh (live) mussels were harvested from approved shellfishing waters in an area near Thessaloniki. The length of the mussels was about 5-6 cm and their total weight was about 35 g. The mussels were transported within 1 hour to the laboratory in isothermal plastic boxes and immediately separated into samples of about 300 g. These samples were used in a series of preliminary experiments to determine the optimum conditions for processing and smoking of mussels as described below.

The fresh (live) mussels, about 12 kg, were washed in running tap water and the byssus thread removed. In order to open the shells and free the mussel meat, cleaned mussels were steamed in an oven with live steam at an air temperature of about 80°C for 10 min. The mussel meat was placed in a 4% sodium chloride brine at room temperature for 15 min in order to improve its taste and consistency. The ratio of mussel meat to brine was 1:2. The brined mussel meat was placed in a canister, drained for 5 min in a refrigerator and after that placed on racks in a special smokehouse, which was heated by electricity. The mussel meat was initially dried at an air temperature of 60°-65°C for 13 min. After drying and when the air temperature was 65°C, the mussel meat was hot smoked for 17 min with smoke which was produced in a smoke generator by burning wood shavings under forced air flow. The smokehouse was switched-off when the air temperature reached 80°C. A thermocouple (Ellab cmc 821, Denmark) was used to monitor the core temperature of the mussel meat. The $F_{70°C}$ value, based on the destruction of D-steptotococcus (REICHERT and THUEMEL, 1986) was calculated from the core temperature data during hot smoking of the mussel meat. The smoked mussel meat was left

in ambient conditions to cool at room temperature and weighed in order to calculate the processing yield. It was then separated into samples of about 75 g, which were vacuum packed (-1 bar vacuum level) in film pouches (Inplast, Oberaudorf, Germany) with a Weromatic vacuum packaging machine (Werner Bonk, Bochum, Germany) and stored in the dark at 2°-3°C for 90 days. The film pouches were made of polyethylene / polyamide (80/80 μm thickness) with an oxygen transmission rate of 77 cm^3/m^2/24h/1atm (23°C, 0%RH) (KOTZEKIDOU and BLOUKAS, 1996).

Samples were taken for analysis from fresh (live) mussels and mussels after steaming and smoking, as well as from smoked mussel meat after 5, 15, 30, 45, 60 and 90 days in chilled storage.

Physicochemical analyses

Moisture, protein, fat (ether-extractable) and sodium chloride content were determined according to standard AOAC (1990) procedures. pH was measured in a homogenate prepared by blending 20 g of mussel meat with 80 mL of distilled water for 30 s. Readings were taken with a WTW, model pH 521, digital pH meter and a WTW, type E 56, combination electrode (WTW-Wissensehaftlich-Techiniche Werkstaetten GmbH, Weilheim, Germany). Total volatile basic nitrogen (TVBN) was determined according to the method described by BOTTA (1995) and the results are expressed as mg N/100g. Thiobarbituric acid (TBA) value was determined according to the method described by RAHARJO and SOFOS (1993) and the results are expressed as mg of malonaldehyde/kg.

Microbiological analysis

A 20 g sample of mussel meat was homogenized in a stomacher Lab-blender 400 (Seward Medical, London, UK) with 180 mL sterile 0.1% peptone water for 2 min. Appropriate dilutions of samples were prepared in sterile 0.1% peptone water blank and plated in duplicate on different growth media. The following media and incubation conditions were used: (1) Plate Count Agar (Oxoid) at 30°C for 72h, for total plate count (total aerobic mesophilic count); (2) deMan Rogosa Sarpe (MRS) agar (Oxoid) at 25°C for 5 days under anaerobic conditions (BBL "Gas-Pack" System with H_2/CO_2 cartridges), for lactic acid bacteria count. Results are expressed as $\log_{10}$ numbers of colony forming units/gram (cfu/g).

Sensory shelf-life

An experienced five-member panel was used to evaluate smoked mussel meat during processing and storage. A preparatory session was held prior to testing so that each panel member could thoroughly discuss and clarify each attribute to be evaluated. Testing was initiated after the panelists had agreed on the specifications.

The panelists were offered a package of smoked mussel meat but they did not know its storage time. They were asked to evaluate the appearance and colour of the smoked mussel meat in the package according to the following scale: 5 = excellent appearance, light colour similar to that of fresh mussel meat; 4 = very good appearance, slightly dark colour; 3 = good appearance, fairly dark but acceptable colour; 2 = not good appearance, dark colour rather unacceptable; 1 = completely unacceptable appearance, very dark colour completely unacceptable. The package was subsequently opened and the mussel meat was served to the panelists. Panelists were asked to evaluate the following attributes using the respective scale: consistency: 5 = soft, 4 = slightly dry, 3 = dry but acceptable, 2 = rather dry, 1 = too dry and brittle; odour and taste: 5 = excellent, 4 = good, 3 = acceptable, 2 = fair, 1 = unacceptable, smoke intensity: 7 = very intensive-

ly smoked and unacceptable, 6 = intensively smoked and unacceptable 5 = slightly smoked, 4 = acceptable smoke intensity, 3 = slightly unsmoked, 2 = rather unsmoked and unacceptable, 1 = completely unsmoked and unacceptable.

The time in days determined by the panelists to consider the quality to be at the limit of acceptability was defined as the shelf-life (BLOUKAS *et al.*, 1997) of mussel meat under the specified smoking and storage conditions. In terms of scores given, this was when scores of 3 and 5 were given by the panelists for smoke intensity, and a score of 3 for appearance and colour, consistency and odour and taste, or when the score exceeded the above limits, as measured by interpolation, between the scores given at two successive storage times.

Statistical analysis

Four replications were carried out for chemical composition and sodium chloride content of the fresh (live) mussels harvested in April and May 1997. The smoking of mussel meat and its preservation under vacuum in chilled storage (2°-3°C) was repeated 10 times. Six replications were made for samples harvested from March to May and four for samples harvested from September to October 1997. Data collected for physicochemical and microbiological parameters of fresh (live) mussels were analyzed by one-way analysis of variance in order to test for significant differences between the two harvesting seasons (spring and autumn) and processing of the mussels. Data collected from fresh mussels and mussel meat after steaming and smoking were also analyzed by one-way analysis of variance in order to test for significant processing fluctuations for each parameter considered. In both cases means were compared using the $LSD_{0.05}$ test. Data collected during the chilled storage of smoked mussel meat were analyzed by a two factor factorial analysis in a completely randomized design. The two factors were: (1) the season (spring and autumn) of processing the mussels and (2) the storage time. Means were compared using the Duncan test. Data analysis was performed with the software SPSS program (Version 11 for Windows).

RESULTS AND DISCUSSION

The chemical composition of fresh (live) mussels as well as their physicochemical and microbiological parameters are given in Table 1.

Fresh mussels, harvested in April and May, had a moisture content of 77.38%±2.83, with a very low coefficient of variation (CV%). MILETIC *et al.* (1991) reported the same moisture content (77.92%) for *Mytilus galloprovincialis*. However, KARAKOLTSIDIS *et al.* (1995) found that mussels harvested during the same season had a moisture content of 87%±0.1. The protein content of the fresh mussel meat was 9.27%±1.16 or about 41%, expressed as % of dry matter. KARAKOLTSIDIS *et al.* (1995) reported a protein content of 8%±0.2 for Mediterranean mussel and MILETIC *et al.* (1991) reported 42.06-44.27% on dry matter. The fat content of fresh mussels was 1.51%±0.65 and was close to that reported by KARAKOLTSIDIS *et al.* (1995). Expressed on the dry matter basis, the fat content was 6.67%. MILETIC *et al.* (1991) found that *Mytilus galloprovincialis* had 9.61% fat content on dry matter. There was a high (43%) coefficient of variation for fat content, indicating that the fat content of fresh mussels is quite variable. The sodium chloride content of fresh mussels was 1.42%±0.26, with a coefficient of variation (CV%) 18.30%, and the water phase salt content (brine) was 1.80%±0.17. A significant seasonal fluctuation has been reported for the chemical composition of mussels (KARAKOLTSIDIS *et al.*, 1995; FERNANDES-REIRIZ *et*

Table 1 - Effect of steaming and smoking on the physicochemical and microbiological parameters of Mediterranean mussel meat (*Mytilus galloprovincialis*).

	Fresh mussels			Steamed mussels			Smoked mussels		
Parameters	Mean	SD	CV%	Mean	SD	CV%	Mean	SD	CV%
Processing yield (%)							9.5	2.10	4.6
Moisture (%)	77.38^{a}	2.83	3.65	71.10^{b}	1.55	2.18	62.60^{c}	3.19	5.11
Protein (%)	9.27^{c}	1.16	12.51	11.96^{b}	0.77	6.49	15.59^{a}	1.63	10.46
Fat (%)	1.51^{c}	0.65	43.00	2.01b^{c}	0.71	35.35	3.29^{a}	1.31	39.82
Sodium chloride %	1.42	0.26	18.30	1.26	0.22	17.82	1.62	0.31	19.14
Brine (%)*	1.80	0.17	13.8	1.74	0.12	11.02	2.52	0.23	15.6
pH value	6.47	0.10	1.54				6.22	0.16	2.57
TVBN (mg N/100g)	12.84^{b}	1.06	8.25				18.35^{a}	0.78	4.25
TBA value**	1.18^{a}	0.61	51.16				0.32^{b}	0.14	43.75
Total plate count***	3.14^{a}	0.57	18.16				1.94^{b}	0.55	35.03
Lactic acid bacteria	2.58^{a}	0.26	10.0				1.90^{b}	0.27	14.67

* Brine or water phase salt content (%) was calculated using the formula: [(NaCl%)/(NaCl% + H_2O%)] x 100,
**Expressed as mg of malonaldehyde/kg, ***Expressed as $\log_{10}$ cfu/g,
SD=standard deriation, CV%= coefficient of variation,
$^{a\text{-}c}$ Means within the same row with different superscript letters are significantly different ($p<0.05$)

al., 1996; ORBAN *et al.*, 2002), reflecting the stage of gonadal development as well as the availability of food.

The effect of steaming and smoking on processing yield, composition and physicochemical and microbiological parameters of mussel meat is given in Table 1. The average processing yield of smoked mussel meat during the spring was 9.5%±1.12. This low processing yield is attributed to the small meat portion of fresh (live) mussels, ranging from 25 to 35 g/100 g of fresh weight, and to their high moisture content, which accounts for high water losses during processing. The meat content of mussels shows seasonal variations (OKUMUS and STIRLING, 1998; ORBAN *et al.*, 2002), which affects the processing yield of smoked mussel meat.

Steaming and smoking of mussels under the specified conditions reduced ($p<0.05$) the moisture content and increased ($p<0.05$) the protein and fat content, as a result of water removal. The moisture content of smoked mussel meat was 62.60%±3.19, the protein content 15.59%±1.63 and the fat content 3.29%±1.31. The sodium chloride content decreased to 1.26%±0.22 during steaming and increased to 1.62%±0.31 after brining and smoking, but these changes were not significant ($p>0.05$). However, brining and smoking significantly increased ($p<0.05$) the water phase salt content (brine %) from 1.74%±1.12 to 2.52%±0.23.

The season of harvesting had no effect ($p>0.05$) on the microbiological and physicochemical parameters of fresh (live) mussels (Table 2).

The total plate count and lactic acid bacteria count of fresh (live) mussels was low, 3.14±0.57 and 2.58±0.26 $\log_{10}$ cfu/g respectively (Table 1). VASAKOU *et al.* (2003) reported a total plate count of 3.7 $\log_{10}$ cfu/g and a lactic acid bacteria count of 3.22 $\log_{10}$ cfu/g for fresh mussels harvested in different shellfishing areas.

The growth retarding and lethal effect of smoking on spoilage and pathogenic microflora depends on the content of salt in the water phase of the product, the

Table 2 - Analysis of variance on the effect of season and storage time on the physicochemical and microbiological parameters of fresh (live) mussels and smoked musel meat (F-values of indepentent variables and interaction).

Parameter	Source of variance			
	Fresh mussels	Smoked mussel meat		
	Aa	A	B[a]	AxB
Total plate count	0.35ns	0.29ns	4.37**	0.67ns
Lactic acid bacteria	1.02ns	0.06ns	1.50ns	0.18ns
pH	4.50ns	0.83ns	0.511ns	0.97ns
TVBN	0.01ns	3.64ns	5.98***	0.31ns
TBA	0.30ns	3.54ns	1.00ns	0.22ns

*p<0.05; **p<0.01; ***p<0.001; ns=not significant.
[a] A=season; B=storage time.

temperature, humidity and density of the smoke, the duration of smoking, the concentration of active components in the smoke and the time and temperature of heating. A very intensive heat process was applied during hot smoking of mussel meat equal to $F_{70°C}$ = 40.2 min (Table 3), which is significantly higher than the $F_{70°C}$ =14 min value required for a 5D destruction of D-streptococcus (REICHERT and THUEMEL, 1986).

As result of the intensive heat applied, smoking significantly reduced the total plate count and the lactic acid bacteria count ($p<0.05$) of the mussel meat (Table 1). During subsequent storage of the smoked mussel meat under the specified conditions, the total plate count continuously increased from 1.94 to 2.77 $\log_{10}$ cfu/g (Fig. 1a). Storage time significantly ($p<0.05$) affected the total plate count, while the season of harvesting and processing, as well as the interaction of season and storage time had no effect (Table 2). The lactic acid bacteria count of smoked mussel meat also increased during chilled storage from 1.90 to 2.28 $\log_{10}$ cfu/g (Fig. 1b), but neither the season of processing nor the storage time had a significant effect on it (Table 2). The very small increase in total plate

Table 3 - Temperature change during smoking of mussel meat and calculated $F_{70°C}$ value.

Time (min)	Smokehouse air temperature (°C)	Mussel meat core temperature (°C)	Lethality Rate* (min)
2	23	16.8	
8	54	30.1	
16	63	54.2	0.010
17	64	57.6	0.057
18	65	60.7	0.120
19	68	63.5	0.226
20	70	65.5	0.357
21	72	67.5	0.565
22	74	69.1	0.812
23	76	70.6	1.151
24	77	71.9	1.551
25	78	73.1	2.046
26	79	74.0	2.512
27	80	74.9	3.097
28	80	75.8	3.817
29	79	76.6	4.599
30	78	77.2	5.275
31	77	77.8	6.106
32	65	78.2	6.692
33	54	76.4	4.599
34	40	60.0	0.100
		Total	40.256

* For *D. streptococcus* (REICHERT and THUEMEL, 1986).

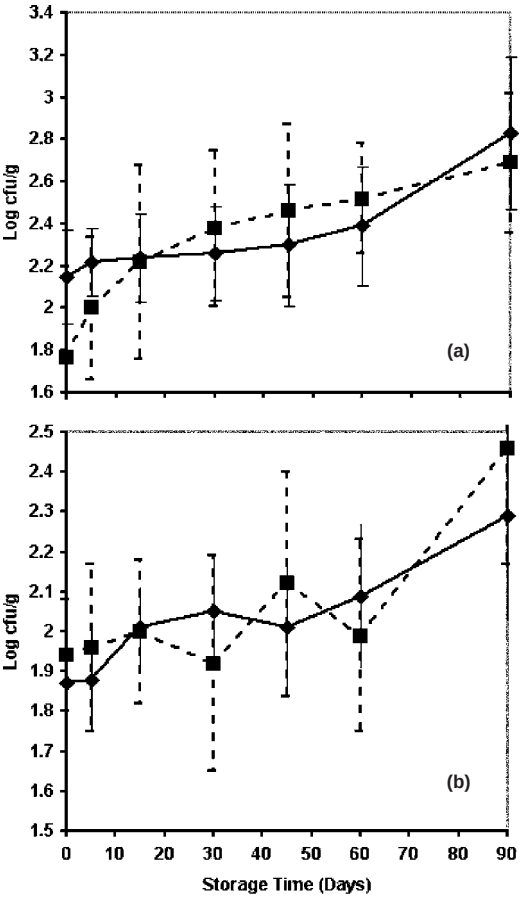


Fig. 1 - Effect of smoking on (a) total plate count and (b) lactic acid bacteria count of Mediterranean mussel meat under vacuum in chilled storage (2°-3°C). ◆ spring; ■ autumn.

count and lactic acid bacteria count during chilled storage, 0.83 and 0.38 $\log_{10}$ cfu/g, respectively, is due to the very low initial microbial population, as well as to the combined action of brining and smoking of the mussel meat, packaging under vacuum and storage at low temperatures (2°-3°C). Salting reduces the water activity of the product, while during smoking, the surface of the product is dried, providing a physical barrier to the passage of microorganisms and a hostile environment for aerobic microbial proliferation, while antimicrobial substances, such as phenols and formaldehyde, are transferred to the product inhibiting microbial growth (HORNER, 1992).

Among the pathogenic bacteria, *Listeria monocytogenes* is able to survive, and in many cases to increase in numbers, on vacuum-packed smoked seafood products under conditions of chilled storage (HARTEMINK and GEORGSSON, 1991; RORVIK *et al.*, 1991; PETERSON *et al.*, 1993; EMBAREK, 1994; THAM *et al.*, 2000). NG and SEATH (1995) isolated *L. monocytogenes* from smoked mussels and a small outbreak of listeriosis was reported by BRETT *et al.* (1998) to be associated with smoked mussels. BREMER and OSBORNE (1995) found that the thermal-death time of *L. monocytogenes* in green shell mussels (*Perna canaliculus*) prepared for hot smoking was $D_{60°C}$ = 5.49 min and z = 4.25°C. The $D_{60°C}$ = 5.49 min value for *L. monocytogenes*, which is equivalent to $D_{65.5°C}$ = 0.27 min, is much lower than the $D_{65.5°C}$ = 0.5-1.0 min of mesophilic non-spore-bearing spoilage bacteria (HELDMAN and HARTEL, 1997), in which D-streptococcus are included. Therefore, the heat process applied to the mussel meat during smoking, $F_{70°C}$ = 40.2 min (Table 3), is adequate for the safety of the product from *L. monocytogenes*. Other pathogenic bacteria associated with smoked seafood products are the non-proteolytic *Clostridium botulinum* type E, *Staphylococcus aureus* and *Salmonella* species (KOLODZIESKA *et al.*, 2002). All these pathogenic bacteria are also less heat resistant than D-streptococcus and therefore the heat process applied during smoking of the mussel meat is adequate for the safety of the product.

The pH of fresh mussels was 6.47±0.10 with a very low coefficient of variation (1.54%). Similar pH values for fresh mussels have been reported by VASAKOU *et al.* (2003) and KASTANIDOU–MANOUSOU *et al.* (1982).

Smoking slightly reduced the pH value of mussels to 6.22±0.16, but the difference was not significant (Table 1). During chilled storage of smoked mussel meat under vacuum, the pH was slightly reduced by about 0.2 units (Fig. 2a). This small change in pH during chilled storage of smoked mussel meat is probably due to the low initial level of lactic acid bacteria as well as to the combined action of all the above-mentioned factors which inhibit their growth. Values of pH 6.0-6.3 have also been reported for vacuum-packed smoked salmon (CIVERA *et al.*, 1995).

The total volatile basic nitrogen (TVBN) of fresh mussels was 12.84±1.06 mg N/100g. Similar values (14.67 mg N/100g) were reported by VASAKOU *et al.* (2003). According to CONNEL (1990), some TVBN, about 20 mg N/100g, is present even in very fresh fish. Smoking significantly increased ($p<0.05$) the TVBN to 18.35±0.78 mg N/100g (Table 1). Storage time had a significant effect ($p<0.001$) on TVBN levels (Table 2). TVBN remained almost constant the first 45 days of chilled storage, after which it continuously increased ($p<0.05$) to 29.43 mg N/100g (Fig. 2b). However, this value is lower than the upper limit of 35-40 mg N/100g beyond which round, whole chilled fish can be considered too spoiled for most uses, and much lower than the upper limit of 100-200 mg N/100g for a variety of salted and dried fish (CONNEL, 1990). CIVERA *et al.* (1995) suggested 45 mg N/100g as the acceptable limit of TVBN for cold smoked fish products and CANTONI *et al.* (1993) recommended 40 mg N/100g as the maximum TVBN content for smoked salmon. A low correlation ($r = 0.47$, $p<0.01$) was found between TVBN and total plate count.

The thiobarbituric acid (TBA) value of fresh mussels, expressed as mg of malonaldehyde/kg, was 1.18±0.61 with a high (51.16%) coefficient of variation.

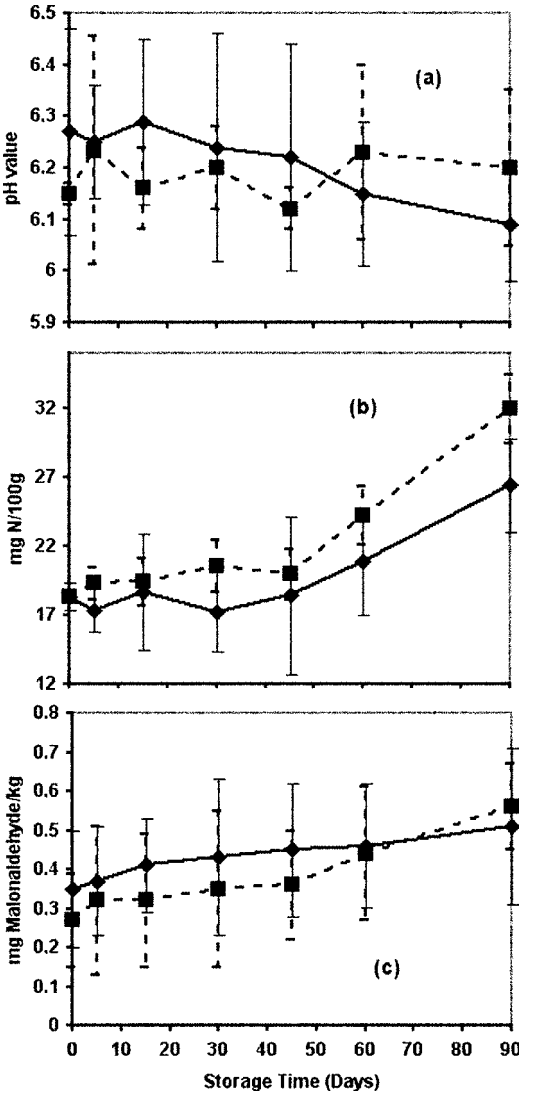


Fig. 2 - Effect of smoking on (a) pH value, (b) total volatile basic nitrogen (TVBN) and (c) TBA value of Mediterranean mussel meat under vacuum in chilled storage (2°-3°C) ◆ spring; ■ autumn.

VASAKOU *et al.* (2003) found a TBA value for fresh mussels of 1.08 mg of malonaldehyde/kg. According to CONNEL (1990) the TBA values should be lower than 1-1.5 mg of malonaldehyde/kg for fresh fish. Smoking significantly reduced ($p<0.05$) the TBA value of mussel meat to 0.32±0.14 mg of malonaldehyde

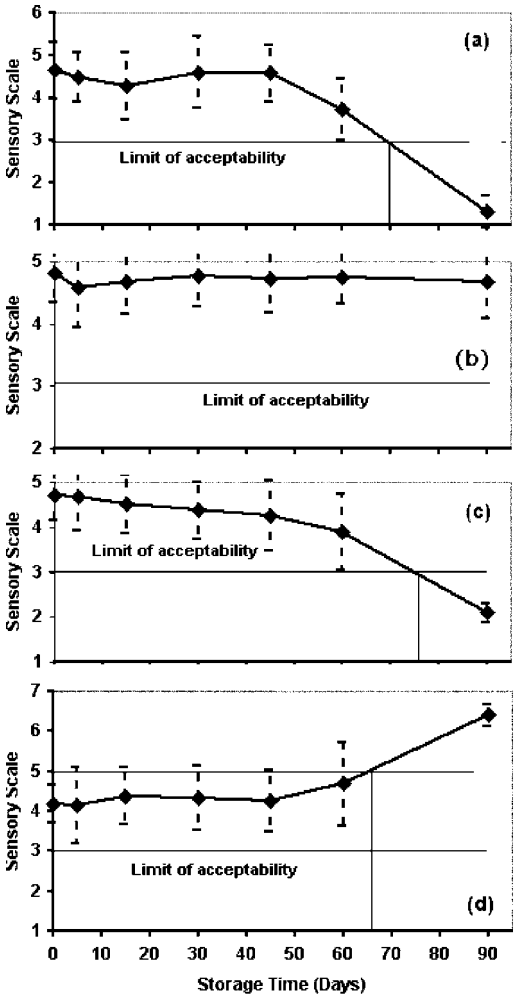


Fig. 3 - Effect of smoking on (a) appearance and colour, (b) consistency, (c) odour and taste and (d) smoke intensity of Mediterranean mussel meat under vacuum in chilled storage (2°-3°C).

/kg (Table 1). This reduction may be due to the fact that volatile compounds which are responsible for the TBA value are lost during the steaming and smoking of mussels. During chilled storage of the smoked mussel meat for 90 days under the specified conditions the TBA value remained almost constant, since it increased by only about 0.2 mg of malonaldehyde/kg (Fig. 2c). This is probably due to the antioxidant action of the smoke which contains phenolic antioxidant compounds that delay the autoxidation and rancidity of highly unsaturated fatty acids (HORNER, 1992).

During storage of the smoked mussel meat for 90 days under the specified conditions, the consistency of the smoked mussel meat remained constant (Fig. 3b). All other sensory attributes also remained constant for 45 days. However, between the 68th and 74th day the colour of the meat changed from light to dark (Fig. 3a), the odour and taste decreased (Fig. 3c) and the intensity of smoke increased and exceeded the limit of acceptability (Fig. 3d). Based on these sensory attributes the shelf-life of Mediterranean smoked mussel meat, in chilled storage (2°-3°C) under vacuum, was restricted to about 70 days. These results are in agreement with those of CIVERA *et al.* (1995) who found that the effective shelf-life of vacuum-packed smoked salmon is nearly 40-50 days and it can be increased to 80 days if the fish product is kept at temperatures of 2°-3°C.

CONCLUSION

Smoking can be used for the processing of Mediterranean mussel meat and the production of a delicatessen food. The shelf-life of smoked Mediterranean mussel meat under vacuum in chilled storage (2°-3°C) can be extended to about 70 days.

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PAPER

SHELF LIFE EXTENSION OF DURUM WHEAT BREAD

ESTENSIONE DELLA SHELF LIFE DI PANE DI SEMOLA DI GRANO DURO

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ABSTRACT

The aim of this study was to investigate packaging methods to prolong the shelf life of durum wheat bread packaged in a bag type package without refrigeration beyond three days (the current limit for this bread). Two different gas compositions (40%CO_2/60%N_2 and 80%CO_2/20%N_2) and an oxygen scavenger were tested in conjunction with two high barrier multilayer films, giving six different packaging combinations. The shelf life tests were carried out at 30°C, monitoring the crumb elas-

RIASSUNTO

Nel presente lavoro è presentato un studio sull'effetto sia dell'atmosfera modificata sia di assorbitori d'ossigeno sulla shelf life di pane ottenuto da semola di grano duro. A tale scopo sono state utilizzate due diverse composizioni di gas (40%CO_2/60%N_2 e 80%CO_2/20%N_2) e un assorbitore d'ossigeno. I test per valutare la shelf life sono stati eseguiti a 30°C, ogni campione veniva visionato giornalmente per rilevare l'eventuale presenza di muffe. Inoltre, sono state eseguite prove di compres-

- Key words: crumb firmness, modified atmosphere packaging, oxygen scavenger, shelf life -

tic modulus and evaluating the samples for visible signs of mold. The results showed that it is possible to prolong the shelf life of durum wheat bread from three days to about 18 days by using modified atmosphere packaging with high carbon dioxide concentration or by means of oxygen scavengers.

sione allo scopo di determinare il grado di avanzamento del fenomeno di raffermimento della mollica. I risultati ottenuti mostrano che è possibile prolungare la shelf life del pane preso in esame dagli attuali tre giorni a 18 giorni utilizzando sia atmosfera protettiva con elevata concentrazione di CO_2 sia gli oxygen scavenger.

INTRODUCTION

Generally the shelf life of bread is limited by several deterioration processes including microbiological spoilage, the loss of water and staling. Staling refers to a broad set of sensory and chemical changes that affect the crispiness of the crust, the textural properties of the crumb and the aroma. Enzymatic treatment of bread has proven to be very effective in delaying crumb firming, and the use of antimicrobial agents is effective in inhibiting mold growth.

Several regions of southern Italy have started research programs aimed at valorizing typical products, i.e., products manufactured in a limited geographical area by using traditional technologies and raw materials grown in the same area. One of these products is bread made from two durum wheat cultivars (75% Simeto and 25% Duilio) and fermented using sourdough. This durum wheat bread has great potential for export since in the local area consumer demand is satisfied. It is generally characterized by a soft crust, and a crumb with low porosity and high water content. This type of bread is usually packed in a bag-type package made of either micro-perforated or continuous film. Because of the short shelf life, this durum wheat bread is currently sold only at local markets. In order for this bread to be exported, the shelf life should be extended beyond the present time of three days.

Preliminary studies have shown that (for this bread) spoilage takes place after a few days of packaging. It is caused either by the presence of visible signs of mold or by crumb attribute modification. The former spoilage phenomenon is the main cause of unacceptability when the bread is packaged using continuous film, while the latter phenomena, accompanied by a rapid loss of aroma, occurs when it is packed using micro-perforated film.

Generally, it is difficult and expensive to delay mold growth and the crumb firming process without modifying the bread composition and technology. In fact, crumb textural properties are affected by several factors such as: temperature, crumb water content, the degree of starch retrogradation and the extent of interaction between starch and lipids (PONTE *et al.*, 1993). Microbial spoilage caused by the growth of aerobic microorganisms such as mold, depends on the temperature, concentration of oxygen in the package head space and on the extent of bread contamination prior to packaging.

According to the data in literature, it is possible to delay both microbial growth and the crumb firming process by packaging under carbon dioxide in conjunction with high barrier films (SMITH *et al.*, 1983; AVITAL *et al.*, 1990; KNORR and TOMLINS, 1985; CENCIC *et*

al., 1996). It has been reported that modified atmosphere packaging using CO_2 and N_2 has been successfully applied to bakery products to prolong their shelf life (AVITAL *et al.*, 1990; KNORR and TOMLINS, 1985). In some cases, modified atmosphere packaging fails to prolong the shelf life of food items, especially porous products, where some oxygen usually remains inside the package. Gas flushing and analogous physical methods are rarely used to maintain the oxygen level below the threshold value because they are not economical (BERENZON and SAGUY, 1998). To overcome this problem, oxygen scavengers have been successfully used to reduce the oxygen partial pressure in the package headspace (BERENZON and SAGUY, 1998).

The purpose of this research was to investigate ways of extending the shelf life of durum wheat bread beyond three days without refrigeration by acting only on the packaging technology without modifying the bread composition and technology used to make it.

MATERIALS AND METHODS

Materials

Commercially available sliced durum wheat bread was supplied by Valle del Dittaino (Cooperativa Agricola Valle del Dittaino, Enna, Italy), and it was used as received. Two commercially available barrier multilayer films were used. Film 1, composed of PE(40 μm)/EVOH(5 μm)//PE(40 μm)//PET(12 μm), was supplied by Di Mauro (Di Mauro Officine Grafiche, Cava dei Tirreni, Italy); film 2, composed of PP(75 μm)/Nylon6,6 (15 μm), was supplied by Cryovac (Cryovac Sealed Air Corp., Passirana di Rho, Italy). As reported by HERNANDEZ (1994), the water and oxygen permeability of water sensitive films such as polyamides and EVOH depends on the water activity inside and outside the package. In these cases, the water and oxygen permeability coefficient cannot be determined by a single measurement. A more accurate analysis of the permeation process is necessary to correctly determine the barrier properties of the film in actual working conditions. A complete characterization of the water and oxygen transport properties of the multilayered films used goes beyond the aim of this paper and will therefore not be considered here. A rough estimate was made of the water and oxygen permeability at 30°C based on the data reported in the literature (BAKER, 1986; BRANDRUP and IMMERGURT, 1975): film1/O_2 = $1 \cdot 10^{-10}$ $cm^3(STP) \cdot cm \cdot cm^{-2} \cdot s^{-1} \cdot atm^{-1}$; film1/$H_2O$ = $9 \cdot 10^{-7}$ $cm^3(STP) \cdot cm \cdot cm^{-2} \cdot s^{-1} \cdot atm^{-1}$; film2/$O_2$ = $4 \cdot 10^{-9}$ $cm^3(STP) \cdot cm \cdot cm^{-2} \cdot s^{-1} \cdot atm^{-1}$; film2/$H_2O$ = $6 \cdot 10^{-7}$ $cm^3(STP) \cdot cm \cdot cm^{-2} \cdot s^{-1} \cdot atm^{-1}$.

The oxygen scavenger used was Ageless FX100 produced by Mitsubishi Gas Chemical Company (Tokyo, Japan). It is sold in the form of a powder contained in a little sachet.

Six different packaging combinations were investigated by combining the described packaging materials; for the sake of simplicity they will be referred to as: A1 = Film 1 + 40% CO_2/60% N_2; B1 = Film 1 + 80% CO_2/20% N_2; C1 = Film 1 + oxygen scavenger; R1 = Film 1 + air; A2 = Film 2 + 40% CO_2/60% N_2; B2 = Film 2 + 80% CO_2/20% N_2; C2 = Film 2 + oxygen scavenger; R2 = Film 2 + air. R1 and R2 were used as controls.

Packaging procedure

Modified atmosphere packaging: 1.08 cm thick bread slices were packed individually in 35x30 cm pouches using a model MG 400 Vuotomac (Vuotomac, Alessandria, Italy). The pouches were evacuated, gas flushed with a previously prepared gas mixture (40% CO_2/60%N_2 and 80%CO_2/20%N_2) and then heat-sealed. The time taken to evacuate and flush the pouches with the gas mixture was the same for all the packages.

This ensured that the amount of oxygen entrapped in the porous structure of the bread did not change from one pouch to another.

Oxygen scavenger: 1.08 cm thick bread slices were packed in 35x30 cm pouches. After introducing the oxygen scavenger into the pouch, it was heat sealed using a model 421 Magulta sealer (Gaudius Saldatrici, Milan, Italy).

Reference sample: 1.08 cm thick bread slices were packed in 35x30 cm pouches using both types of film and then heat sealed using a model 421 Magulta sealer (Gaudius Saldatrici, Milan, Italy).

Mechanical tests

Uniaxial compression tests were carried out using a model 4301 Universal Testing Machine (Instron Ltd Italian Branch, Milan, Italy, maximum load allowed 100 N) at a crosshead speed of 10 mm/min. Cylindrically shaped samples, with a diameter of 2.93 cm and a height of 1.08 cm, were punched out of the bread slice immediately after removing the slice from the pouches, and then compressed according to the above procedure.

Evaluation of the crumb water content

Immediately after removing the slice from the pouches, part of the crumb was separated from the slice; it was weighed and then placed in an oven at 110°C. The weight of the crumb was monitored till it reached a constant value. The crumb water content was evaluated by means of the following relationship:

$$\frac{P_{in.} - P_{fin.}}{P_{in.}}$$

where: $P_{in.}$ is the initial weight of the crumb, $P_{fin.}$ is the final steady weight attained in the oven.

Microbiological spoilage

For each type of packaging combination tested, three bread slices were packaged separately according to the above procedures and analyzed daily for visible signs of mold. The bread was considered unacceptable if one of the three slices showed visible signs of mold. A conventional pour plate microbial count was not carried out for two reasons: firstly, during the homogenization phase, the mycelia always broke up, giving rise to a very large number of colony forming units (cfu) (BOURGEOIS *et al.*, 1988); secondly, since the bread slice was not uniformly contaminated, estimation of the average microbial count would not be representative of the local microbial population. In fact, the method used in the present study to determine microbial spoilage of the bread tested is very close to the consumer's perception of bread quality.

RESULTS AND DISCUSSION

As reported above, bread can be rejected by the consumer either on the basis of a change in the textural properties of the crumb or because of the presence of visible signs of mold. These two aspects will be discussed separately.

Microbial shelf life

For the purpose of this study microbial shelf life is defined as the time elapsed from the time the bread is packaged to the time the packaged slice shows visible signs of mold. The first column in Table 1 reports the values of the microbial shelf life obtained for the different packaging combinations tested. For samples C1 and C2, a value is not reported since the bread slices stored in these packages did not show any visible signs of mold

Table 1 - Microbial shelf life, textural shelf life and shelf life of the samples.

Sample	Microbial Shelf Life days	Textural Shelf Life days	Shelf Life days
A1 Film 1 + 40% CO_2/60% N_2	12	14.6 [12.0-17.7]*	12
B1 Film 1 + 80% CO_2/20% N_2	22	19.5 [17.2-22.6]*	19.5 [17.2-22.6]*
C1 Film 1 + oxygen scavenger	> 38	16.6 [14.6-18.7]*	16.6 [14.6-18.7]*
R1 Film 1 + air (control)	3	-	3
A2 Film 2 + 40% CO_2/60% N_2	12	18.0 [15.8-21.0]*	12
B2 Film 2 + 80% CO_2/20% N_2	22	18.9 [16.5-21.4]*	18.9 [16.5-21.4]*
C2 Film 2 + oxygen scavenger	> 38	19.8 [17.5-22.3]*	19.8 [17.5-22.3]*
R2 Film 2 + air (control)	3	-	3

* The values reported in the brackets are the lower and upper limit of the confidence interval (95%).

during the entire time of observation (i.e., 38 days). The microbial shelf life of the two reference packages (three days) was considerably shorter than that of the other packages. The results show that the oxygen scavenger is an effective solution for reducing the growth of aerobic spoilage microorganisms such as mold. In modified atmosphere packaging the microbial growth rate diminished because of the bacteriostatic effect of carbon dioxide coupled with a decrease in oxygen from the package head space. In fact, the microbial shelf life of bags containing a high concentration of CO_2 had a longer shelf life. In the case of the oxygen scavenger, the active compound put into in the package reacts continuously with the oxygen present inside the package, reducing its concentration, and consequently the microbial growth rate. These results suggest that oxygen scavengers are more effective in prolonging the microbial shelf life probably because they eliminate the oxygen that remains trapped inside the porous structure of the food product during packaging.

Data reported in Table 1 show that the oxygen barrier properties of the two multilayer films used did not influence the microbial shelf life of the bread packaged in modified atmosphere, suggesting that the oxygen trapped in the porous structure of the bread is quite important for microbial spoilage of the packed food product.

Textural shelf life

Textural shelf life is defined as the time elapsed from the time the bread is packaged to the time it is rejected by the consumer on the basis of changes in the textural properties of the crumb. As reported in the literature, the textural properties of the crumb are frequently evaluated through compression tests (BAKER *et al.*, 1986; 1987; BAKER and PONTE, 1987; PIAZZA and MASI, 1995). The resulting stress-strain curves depend on the porous structure of the crumb (or, more simply, on the relative density of the crumb, defined as the ratio between the crumb density and the density of its solid matrix) as well as on the elastic modulus of its solid matrix (HILYARD, 1982). However, during storage, the relative density of the crumb does not change substantially, while the elastic modulus of its solid matrix increases due to several phenomena including the loss of water, the retrogradation of starch and increase in the extent of the interactions between gluten and starch (PONTE *et al.*, 1993). In view of the above scenario of the firming process, decay in crumb quality during storage can, in principle, be followed by monitoring the elastic modulus of its solid matrix. However, since the relative density of the crumb does not change during storage, the elastic modulus of the solid matrix of the crumb is directly proportional to the elastic modulus of the crumb (HILYARD, 1982). For the above reason, in this study increase in crumb firmness was followed by monitoring its elastic modulus during storage.

The elastic modulus of the crumb can be evaluated from the initial slope of the compressive stress-strain curve. However, since the surfaces on which the compressive load is applied are not perfectly parallel (as they should be), estimation of the elastic modulus of the crumb based on such a technique is generally affected by an error that is quite significant. For this reason, a mathematical model capable of describing the entire compressive stress-strain curve is proposed. Considering both the complexity of the deformation mechanisms and their dependence on the porous structure of the crumb, a suitable mathematical model can be obtained by factoring the compressive behavior of the crumb in two parts: an elastic modulus and some function of the strain (HILYARD, 1982):

$$\sigma(\varepsilon) = E_C \cdot \varepsilon \cdot \xi(\varepsilon) \qquad (1)$$

where: ε is the engineering strain, i.e. the absolute deformation divided by the initial height of the sample, σ is the engineering stress, E_C is the elastic modulus of the crumb and $\xi(\varepsilon)$ is a function that reflects the influence of the buckling of the cell elements of the crumb and their subsequent compaction on the shape of the compressive stress-strain curve. To be representative of the above phenomena, the function $\xi(\varepsilon)$ has to fulfill the following requirements:

$$\xi(0) = 0, \left[\frac{d\xi(\varepsilon)}{d\varepsilon}\right]_{\varepsilon=0} = 0.$$

In the present investigation the following empirical relationship for $\xi(\varepsilon)$ is proposed:

$$\xi(\varepsilon) = \{-K_1 \cdot \varepsilon^{1.1} \cdot \exp(\varepsilon \cdot K_2) + \exp[(K_3 \cdot \varepsilon)^{K4}]\} \qquad (2)$$

where: the values of K_i are constant and have to be regarded as fitting parameters. Substituting equation (2) in equation (1) the following equation is obtained:

$$\sigma(\varepsilon) = E_C \cdot \varepsilon \cdot \{-K_1 \cdot \varepsilon^{1.1} \cdot \exp(\varepsilon \cdot K_2) + \exp[(K_3 \cdot \varepsilon)^{K4}]\} \qquad (3)$$

Fig. 1 shows the compressive stress-strain curve relative to sample C1 along with the most suitable fit of the data obtained by means of equation (1). It is evident that the ability of the proposed model to fit the experimental data is quite satisfactory.

Figs. 2 and 3 show the crumb elastic modulus of the packaged bread slices ($\bar{E}_C$) plotted as a function of storage time for all the packaging combinations, while Figs. 4 and 5 show the crumb water content of the packaged slice plotted as a function of storage time. Each of the values of $\bar{E}_C$ reported in Figs. 2 and 3 were obtained by taking the average of the values of the elastic modulus of four cylinders punched out of a single slice of bread. For each cylinder the elastic modulus was obtained by fitting the compressive stress-strain curve by means of equation (1). The error bars appearing in Figs. 2 and 3 are the standard errors of the mean (MANDEL, 1964) characterizing each value of $\bar{E}_C$. The data relative to the two reference samples are not reported in the figures because of their short microbial shelf life, three days, for which it was useless to measure the $\bar{E}_C$, and consequently the crumb water content, for periods of time longer than three days. As shown in these figures, for all the samples studied there was an increase in the storage time of the crumb elastic modulus with a decrease in the crumb water content.

As is the case of white bread (GIOVANELLI and PAGLIARINI, 1996), the unacceptability threshold of the elastic modulus of the crumb, $\bar{E}_C^{Threshold}$, was set equal to three times the value of the elastic modulus of the fresh crumb, $\bar{E}_C^{Fresh}$ (i.e., $\bar{E}_C^{Threshold} = 3 \cdot \bar{E}_C^{Fresh}$). To determine the textural shelf life of the packaged bread, it was assumed that the data reported in Figs. 2 and 3 can be described by means of the following expression:

$$\bar{E}_C = A_1 + A_2 \cdot t \qquad (4)$$

where A_1 and A_2 values are constant and are regarded as the fitting parameters. It is worth noting that A_1 is equal to

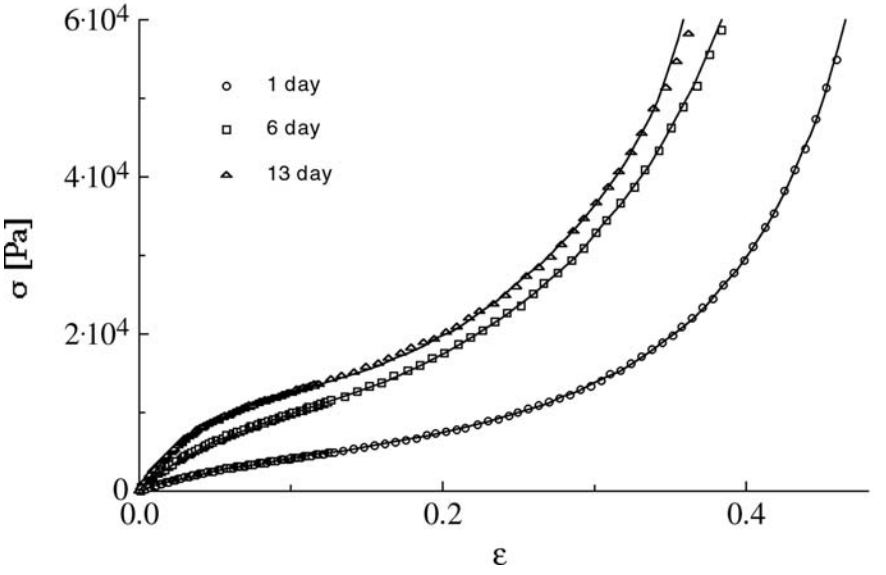


Fig. 1 - Compressive stress plotted as a function of strain for sample C1.

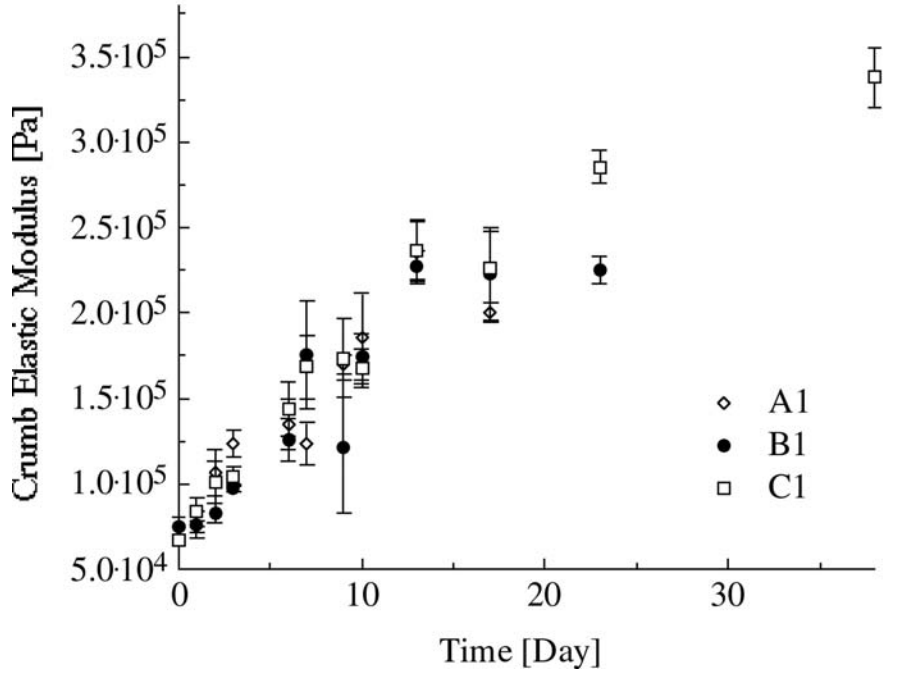


Fig. 2 - EC plotted as a function of storage time of samples packaged with film 1.

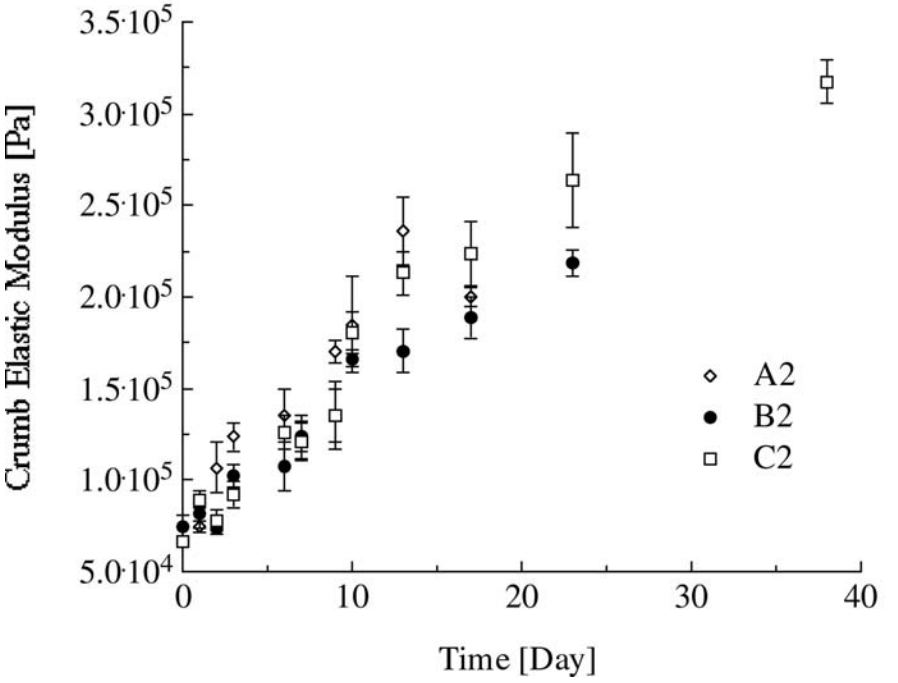


Fig. 3 - EC plotted as a function of storage time of samples packaged with film 2.

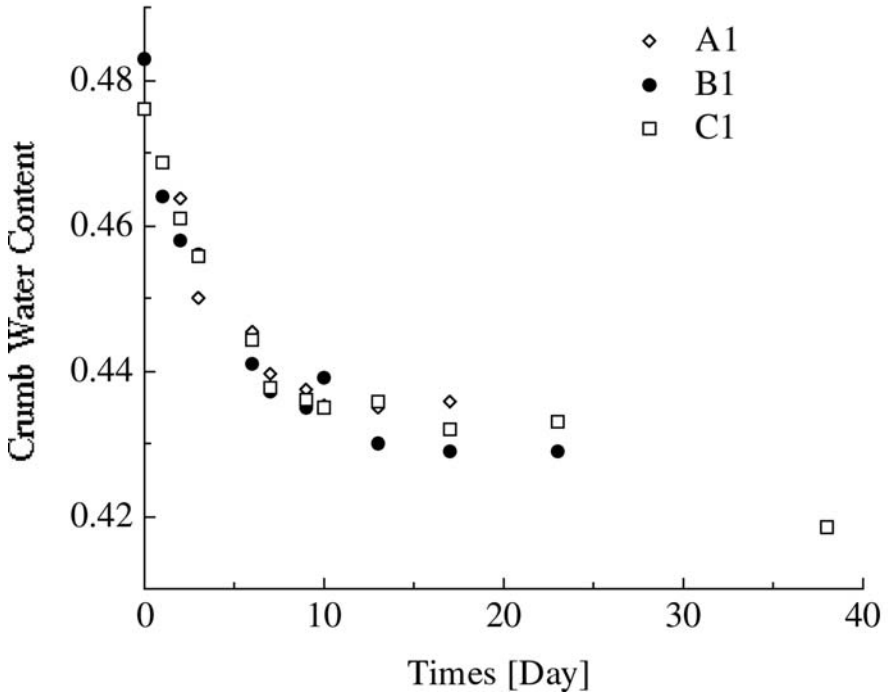


Fig. 4 - Mass fraction of water present in the crumb versus storage time of samples packaged with film 1.

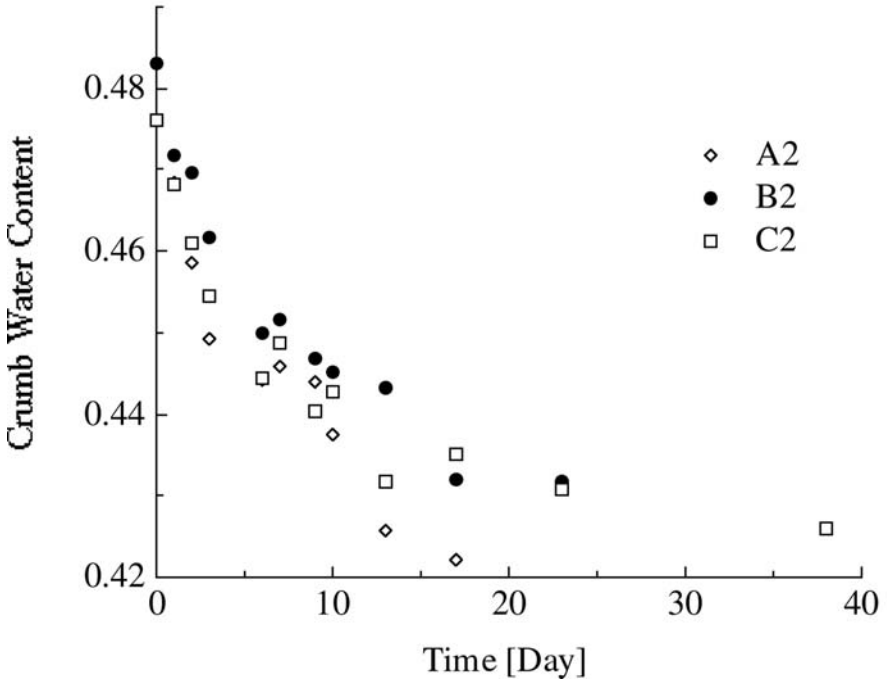


Fig. 5 - Mass fraction of water present in the crumb versus storage time of samples packaged with film 2.

$\bar{E}_C^{Fresh}$. Equation (4) can be rearranged as follows:

$$\bar{E}_C = A_1 \cdot \left(1 + \frac{2}{t_{S.L.}} \cdot t\right) \qquad (5)$$

where $t_{S.L.}$ is the textural shelf life of the packaged bread slice. Equation (5) was used to fit the data reported in Figs. 2 and 3; the fitting was done by taking into account the error that characterized each data point. The results obtained are reported in Table 1; the confidence intervals (95%) were evaluated on the basis of 500 converging interactions. Unfortunately, due to the width of the confidence intervals it was not possible to take into consideration the differences in the textural shelf life of the packaging combinations studied. The average value of the textural shelf life, which was evaluated by averaging the values obtained for the six packaging combinations tested, is equal to 17.9 days.

CONCLUSIONS

This investigation shows that it is possible to prolong the shelf life of the durum wheat bread investigated from 3 to 18 days acting only on the packaging technology. Depending on the packaging combination tested, unacceptability of the product was caused either by the presence of visible signs of mold or by a change in the textural properties of the crumb. In particular, the oxygen scavenger was much more effective than the modified atmosphere packaging with regard to suppression of molds, but an extension of the shelf life beyond 18 days was limited by the textural deterioration of the bread. On the other hand, in the case of modified atmosphere packaging, shelf life was limited by microbial growth in low carbon dioxide concentration and the firming process in high carbon dioxide concentration.

The results point out that using either modified atmosphere packaging or oxygen scavengers, it is possible to prolong the shelf life to about 18 days. Further extension of the shelf life is limited by textural changes. The choice between these two packaging technologies can be made only on an economical basis.

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PAPER

PROTEOLYSIS AND PRODUCTION OF BIOGENIC AMINES IN TOMA PIEMONTESE PDO CHEESE DURING RIPENING

PROTEOLISI E SVILUPPO DI AMMINE BIOGENE NEL CORSO DELLA MATURAZIONE DEL FORMAGGIO TOMA PIEMONTESE DOP

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ABSTRACT

Proteolysis of Toma Piemontese PDO cheese (large/long–ripened cheese and small/short-ripened cheese types) during ripening was studied by monitoring changes in nitrogenous compounds (protein content, SDS-PAGE, pH 4.6 soluble-nitrogen and ripening index). Changes in the concentration of four biogenic amines and their precursor amino acids were also determined, showing the presence of histamine and tyramine (up to a total concentration of 145 mg kg^{-1}) in long-ripened cheese.

RIASSUNTO

La proteolisi del formaggio Toma Piemontese DOP (tipologia "grande a lunga maturazione" e tipologia "piccola a breve maturazione") è stata studiata, durante la maturazione, analizzando la variazione della frazione azotata (contenuto in proteine totali, N solubile a pH 4.6, indice di maturazione) nonché della frazione proteica per SDS-PAGE. Sono state determinate anche alcune ammine biogene ed i loro aminoacidi precursori, evidenziando la presenza di istamina e tiramina (valore massimo complessivo

- Key words: biogenic amines, cheese, proteolysis, SDS-PAGE, Toma Piemontese -

The total concentration of biogenic amines in the commercial samples analyzed did not exceed 219 mg kg^{-1}. In comparison to short-ripened Toma, long-ripened Toma had a higher level of proteolysis (ripening index up to 20.8% after 64 days ripening) with the characteristic disappearance of the α_{s1}-casein band in the electrophoresis profiles. The increase in proteolysis during ripening was confirmed by PCA, showing the possibility of using this technique to assess the age of unknown cheese samples. The main variables contributing to the first principal component were ripening index, % soluble N, phenylalanine and α_{s1}-casein.

pari a 145 mg kg^{-1}) nella tipologia a lunga maturazione. Il contenuto totale in ammine biogene dei campioni commerciali analizzati non ha superato i 219 mg kg^{-1}. Lo stesso tipo di formaggio ha inoltre presentato il maggior grado di proteolisi (IM 20.8% a 64 giorni di maturazione), con la caratteristica scomparsa della banda relativa all'α_{s1}-caseina dai profili elettroforetici. L'uso della PCA ha confermato l'aumento della proteolisi durante la maturazione, indicando la possibilità futura di datare campioni di formaggio di età sconosciuta. Le principali variabili che incidono sulla componente principale sono: indice di maturazione, % di N solubile, fenilalanina e α_{s1}-caseina.

INTRODUCTION

Proteolysis in cheese is a fundamental process which, together with glycolysis and lipolysis, contributes to the texture, aroma and taste of ripened cheeses. Proteolysis may be regarded as the most important biochemical event in the ripening of many cheese varieties (FOX *et al.*, 1994).

Toma Piemontese is a typical PDO Italian cheese (Italian law DPCM, 10/05/1993, CE regulation 1263/96), widely consumed in northern regions of Italy. The production of Toma Piemontese in 2001 was about 1,233 t (official data obtained from Associazione Regionale Produttori Latte Piemonte).

Toma Piemontese is a semi-hard cheese (coagulation temperature: 32°-35°C), made from cows' milk (raw or pasteurized milk, acidity: 3.6°SH/50). There are two different types of this cheese: the first, called "Toma Piemontese PDO" (40% minimum fat as dry matter), is produced from whole milk; the second, called "Toma Piemontese semigrasso" (20% minimum fat as dry matter), is produced from partially skimmed cows' milk. Each type of Toma cheese is currently produced in two different sizes: small cheese (1.5-2 kg, 15 days minimum ripening time) and large cheese (6-8 kg, 60 days minimum ripening time). About 70% of the total production is the large-type, of which 30% is "Toma semigrasso". The long-ripened Toma is currently sold and consumed within 30 days after the required 60 days of ripening.

Only a few scientific papers have reported data on the chemical composition of Toma Piemontese cheese and are previous to the PDO certification (DELFORNO, 1966; 1977; BATTAGLINI and FORTINA, 1992). However, since 1995 the Agricultural Department of the Piedmont Region has coordinated interdisciplinary research on this topic (SOSTER, 1998). The preliminary results showed the existence of at least three different types of Toma Piemontese cheese: "classic", "cheese factory" and "Biellese" which differ for some chemical parameters (dry matter, fat, protein, DL lactic acid). The use of sensory analysis (based on the use of 20 descriptor parameters) and clus-

ter analysis has partially confirmed the differences among these three Toma cheeses (AMBROSOLI *et al.*, 1998).

As reported in many studies on different cheeses (MAYER, 1996; DEWETTINCK *et al.*, 1997; MOONEY *et al.*, 1998), the evolution of primary proteolysis can be readily monitored using SDS-PAGE and/or urea-PAGE. The formation and accumulation of biogenic amines in cheese (as well as in other fermented/seasoned foods) is a well-studied subject (BRINK *et al.*, 1990; HALASZ *et al.*, 1994; VALE and GLORIA, 1998). The formation of some biogenic amines (histamine, tyramine, tryptamine and 2-phenylethylamine) and their related precursor amino acids have been studied in other Italian cheeses, for example, Gorgonzola PDO cheese (MARTELLI *et al.*, 1993).

This paper describes proteolysis in Toma Piemontese PDO during ripening. Variations in the total and soluble nitrogen fractions, as well as some parameters related to the proximate composition, were monitored. The final goal was to study the changes in the nitrogenous fraction during ripening in order to obtain some ripening markers, useful for assessing the age of this cheese. The same parameters were also determined on some commercial samples of unknown age, purchased directly from retail shops.

MATERIALS AND METHODS

Cheese sampling

Samples of Toma Piemontese cheese were manufactured from whole milk on a commercial scale in an industrial plant, according to the standard protocol reported in PDO regulations. These samples were kindly supplied from "Consorzio di Tutela del Toma Piemontese". In one trial consisting of "small" type cheese (3 different whole cheeses of about 1.5 kg) samples were taken at 4, 11 and 19 days of ripening. "Large" type cheeses (from 5 different whole cheeses of about 7 kg) were sampled at 4, 19, 29, 39, 49 and 64 days of ripening. This trial was done with cheese produced in the winter and another trial with cheese produced in spring.

A total representative sample of 100 g was obtained by grating and homogenising the pieces collected from each whole cheese; each final sample was then analysed, avoiding any loss of moisture.

All commercial samples were purchased at retail shops and supermarkets located throughout the Piedmont Region.

Compositional analysis

Cheese moisture, ash, fat and total acidity were determined according to the Italian Official Methods for cheese analysis (Official Italian Journal, 1986).

Total and pH 4.6-soluble nitrogen values were determined by the Kjeldahl method (AOAC, 1990) using the Kjeltec system (Tecator, Sweden); protein was calculated using the conversion factor, 6.38.

Total fat was determined using the Soxhlet apparatus with dichloromethane for 14 hours.

Biogenic amines (tyramine, histamine, tryptamine and 2-phenylethylamine) and their precursor amino acids were determined using an ion-pair HPLC method previously optimised for Gorgonzola PDO cheese (ARLORIO *et al.*, 1998). Each sample (10 g) was extracted with 50 mL of trichloroacetic acid (5%, w/v). All chemical reagents (reagent grade) were purchased from Fluka Chemie AG (Buchs, Switzerland).

SDS-PAGE electrophoresis

SDS-PAGE was performed according to LAEMMLI (1970) using a Mini Protean II Dual Slab Cell (Bio-Rad Laboratories

S.r.l., Segrate, Italy) on a urea-soluble fraction of cheese (obtained using a 6 M urea solution, pH 8.5). Fluor-S MultiImager and Quantity One Software (Bio-Rad) were used to analyse all gels. Data collected are expressed as relative percent for each lane and were used for PCA statistical analysis.

Statistical methods

All results are reported as the mean of three (or more) independent determinations ± standard deviation. One-way Anova test was conducted on long-ripened cheese, using Microsoft Excel Software. Principal Component Analysis (PCA), performed employing Mathematica Software, was used to analyse all data collected from the study.

RESULTS AND DISCUSSION

The values for the principal parameters (fat, protein, moisture) of well-ripened cheeses (19 days and 64 days, respectively, for two different types of Toma) were in accord with the minimum values prescribed by the PDO rules (CE Rules 1263, 1/7/96) (Tables 1, 2). A slight increase in the total nitrogen content was evident for both types of Toma Piemontese (Tables 1, 2); this is normal for cheese due the loss of moisture during ripening (MADKOR *et al.*, 1987; HAASNOOT *et al.*, 1989; FOX *et al.*, 1996; GOBBETTI *et al.*, 1997). The pH 4.6 soluble nitrogen fraction, as well as the ripening index (R.I.; ratio of soluble to total nitrogen), showed a constant increase. The R.I. of the short-ripened Toma increased from 8.1 to 14.4% in 19 days; the R.I. of long-ripened Toma increased from 9.8 to 20.8% in 64 days. Although not comparable, both increases in R.I. are important parameters for PCA.

Differences in the degree of ripening observed in the winter and spring long-ripened cheeses may be related to the influence of environmental parameters (quality of the original milk, microbial quality and count, climatic conditions) on the cheesemaking processes. These winter and spring-produced cheeses did not exhibit significant differences using Anova statistical tests, although the spring series showed a higher level of some proteolysis parameters. The data reported in Table 2 are the average of spring and winter produced long-ripened cheeses.

The total acidity of the two types of Toma cheese had an opposite trend (Tables 1, 2). It decreased during the ripening of short-ripened Toma which could be partially related to the increase in proteolysis and the release of basic compounds. In contrast, it increased during ripening in long-ripened Toma. The degree of proteolysis at 19 days was similar for both cheese types; therefore, the reason why acidity increased in large Toma may be the higher moisture content, with higher whey and lactose retention in the cheese. These conditions could promote greater lactic acid production.

Tyrosine, histidine, phenylalanine and tryptophan showed no marked changes in the short-ripened Toma (data not shown). Long-ripened Toma cheese had a significant increase in these amino acids during ripening (Table 3). This may be a useful marker, as reported for other cheeses (BÜTIKOFER and FUCHS, 1997; INNOCENTE, 1997). Phenylalanine, for example, had a constant and significant increase during ripening, which has also been reported in other Italian cheeses analysed using the same approach (Gorgonzola PDO, Raschera PDO, Bra PDO, etc., unpublished data) or in some Spanish cheeses (GARDE *et al.*, 2002). Apparently, decarboxylation of phenylalanine did not occur, in contrast to tyrosine and histidine (CHRISTENSEN *et al.*, 1999). JOOSTEN (1987) did not identify phenylalanine-decarboxylating bacterial strains; the formation of 2-phenylethyl-

Table 1 - Some parameters (%) of Toma Piemontese PDO (small, whole short-ripened cheese type) during ripening (mean±S.D.).

Days	Moisture	Total fat	Ash	Protein	Soluble nitrogen	R.I.[a]	Total acidity[b]
4	43.6±0.4	28.9±0.2	2.77±0.04	20.3±0.3	0.26±0.02	8.1±0.39	3.62±0.01
11	43.1±0.2	29.2±0.1	3.13±0.08	21.1±0.1	0.31±0.02	9.3±0.45	1.73±0.19
19	42.2±0.1	29.5±0.2	3.68±0.23	21.5±0.3	0.49±0.03	14.4±0.82	1.06±0.28
Significance	**	*	***	**	***	***	***

[a] R.I.: Ripening index (ratio between soluble and total nitrogen x 100). [b] expressed in meq/100g of cheese. Significance of time of ripening: ANOVA test, *** p<0.001, ** p<0.01, * p<0.05. n=3 samples for each day.

Table 2 - Some parameters (%) of Toma Piemontese PDO (large, whole long-ripening cheese type) during ripening (mean±S.D.).

Days	Moisture	Total fat	Ash	Protein	Soluble nitrogen	R.I. [a]	Total acidity[b]
4	46.0±0.3	28.2±0.5	2.02±0.43	20.9±0.7	0.32±0.07	9.8±2.0	1.94±0.15
19	45.0±0.4	28.2±0.7	2.95±0.03	21.1±0.6	0.52±0.04	15.6±1.1	2.39±0.10
29	44.7±0.5	28.6±0.2	3.14±0.18	21.0±0.3	0.62±0.13	18.7±3.8	2.45±0.07
39	44.1±0.4	29.3±0.2	3.40±0.05	21.3±0.3	0.69±0.16	20.5±4.7	2.53±0.12
49	43.8±0.6	29.4±0.3	3.58±0.17	21.5±0.4	0.67±0.13	19.8±3.8	2.68±0.13
64	43.0±0.9	29.4±0.3	3.71±0.21	21.9±0.2	0.71±0.05	20.8±1.5	2.74±0.31
Significance	***	***	***	**	***	***	***

[a] R.I.: Ripening index (ratio between soluble and total nitrogen x 100). [b] expressed in meq/100g of cheese. Significance of time of ripening: ANOVA test, *** p<0.001, ** p<0.01, * p<0.05. Data are the average of winter and spring produced cheeses. n=6 samples for each day.

Table 3 - The concentration (mg kg^{-1}) of biogenic amines and related precursor amino acids in Toma Piemontese PDO (large whole, long-ripening cheese type) during ripening (mean±S.D).

Days	Tyrosine	Histidine	Phenylalanine	Tryptophan	Tyramine	Histamine[a]
4	85±39	24± 1.4	98±40	8.8± 3.0	Tr	Nd
19	170±23	56±29	295±27	18 ± 1.6	10± 1	12±1
29	141±23	98±49	333±35	16 ± 3.7	26± 4	14±3
39	180±25	108±31	439±42	23 ± 5.5	34± 8.8	15±1
49	188±25	93±56	504±60	28.7± 9.0	60±14	22±9
64	254±23	253±94	709±36	57.3±21	108±46	37±9
Significance	***	***	***	***	***	**

[a] Present only in spring trial (n=3). Significance of time of ripening: ANOVA test, *** p<0.001, ** p<0.01, * p<0.05. Tr: traces, Nd: not detected. Data are the average of winter and spring produced cheeses. n=6 samples for each day.

amine could be related to the action of other enzymes, for example tyrosine-decarboxylase, that has shown a slight activity towards phenylalanine, structurally-related to tyrosine.

Biogenic amines were not found in short-ripened Toma samples, presumably because of the short time of ripening (19 days). In long-ripened Toma cheese, tyramine ranged from 10 to 108 mg kg^{-1};

the presence of histamine was detected only in the long-ripened spring Toma, ranging from 12 to 37 mg kg^{-1}. The increase in the concentration of each amine during ripening was significant (respectively $p<0.001$ for tyramine and $p<0.01$ for histamine). 2-Phenylethylamine and tryptamine were absent in all samples.

General hygienic conditions during industrial/artisanal cheesemaking and decarboxylation by some starter and non-starter bacterial strains are two fundamental parameters directly related to the accumulation of biogenic amines (VOIGT and EITENMILLER, 1977; JOOSTEN and STADHOUDERS, 1987; HALASZ *et al.*, 1994). For this reason, tyramine is a potential marker for the control of hygienic conditions during cheese production. The formation of biogenic amines in cheese is enhanced by temperature (mainly if >18°C), pH (>5) and low salt content as demonstrated by ANTILA *et al.* (1984) and JOOSTEN (1987). The ripening and storage temperatures are parameters which influence the formation of biogenic amines in cheese (MARTELLI *et al.*, 1993).

The maximum total concentration of biogenic amines in the samples of long-ripened Toma Piemontese at the end of the ripening period (64 days, about 145 mg kg^{-1} as the sum of histamine and tyramine) does not present any toxicological risk. A total content of 500 mg kg^{-1} of biogenic amines is considered a critical concentration able to cause the "cheese reaction". About 200 mg kg^{-1} of biogenic amines represents a risk for the health of the consumer only in the case of simultaneous consumption of Mono Amino Oxidase-inhibitor (i-MAO) drugs (enzymes involved in the catabolism of amines) as well as in the case of continuous excessive consumption of cheese by sensitive patients (SMITH, 1980; BRINK *et al.*, 1990).

The SDS-PAGE profile of the total proteins obtained for short-ripened Toma showed a slight decrease of the α_{s1}-casein band (about 30 kDa) (Fig. 1, lane 3). The SDS-PAGE pattern of the pH 4.6-soluble nitrogen fraction did not provide a useful fingerprint of proteolysis (data not shown). The degree of proteolysis in short-ripened Toma Piemontese was limited, in agreement with the other chemical indexes (Table 1). The SDS-PAGE data for long-ripened Toma Piemontese cheese showed a sharp decrease of the α-casein band (about 30 kDa); the β-casein band (molecular weight 28 kDa) was observed in all samples (Fig. 1b).

Even in long-ripened Toma, the pH 4.6-soluble nitrogen fraction analyzed by SDS-PAGE did not give any significant results (data not shown). An analysis of this soluble fraction by RP-HPLC is surely preferable, as previously suggested (BICAN and SPAHNI, 1993; SINGH *et al.*, 1993; 1995).

Some commercial samples of Toma (unknown age, large type) were then analysed for free amino acids, biogenic amines, total nitrogen and pH 4.6-soluble nitrogen, R.I., SDS-PAGE. They showed a different degree of proteolysis (Table 4); the R.I. ranged from 19.5 to 35.1%, indicating a large variability of proteolysis. The data for most samples were quite close, as also confirmed by PCA analysis (Fig. 2). Most commercial samples had R.I. values higher than the R.I. of the 64 day-ripened samples (Table 2). On the contrary, free amino acid values of the commercial samples showed more variability (Table 5).

Commercial samples showed the presence of tyramine (from 31 to 178 mg kg^{-1}) and histamine (from traces to 67 mg kg^{-1}). 2-Phenylethylamine and tryptamine were absent in all commercial samples, as well as in the control samples analyzed.

The total concentration of biogenic amines in the commercial samples did not exceed 219 mg kg^{-1} (Table 5); this value, however, was higher than the values obtained for the 64 day-ripened control samples (Table 3).

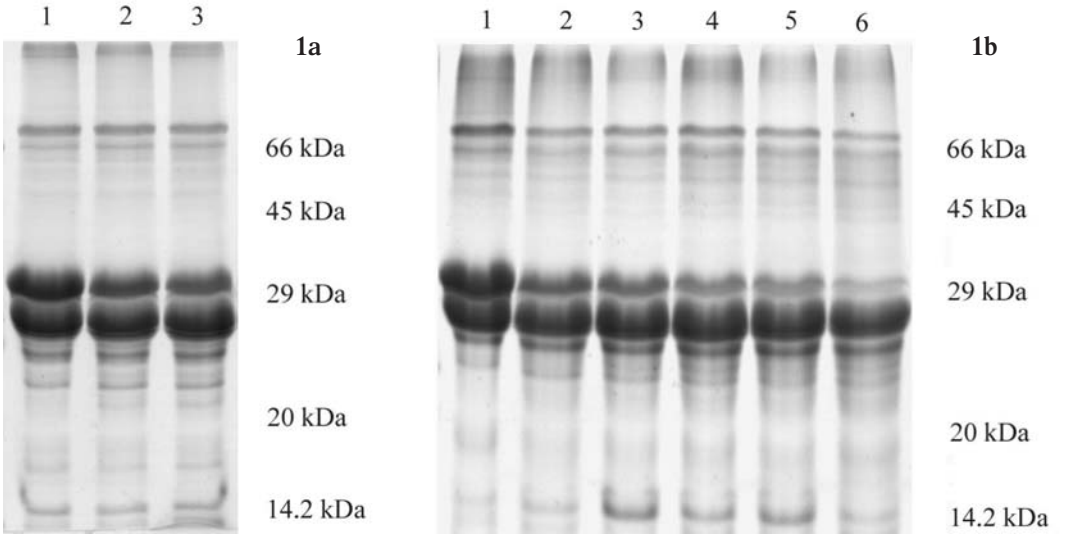


Fig. 1a - SDS-PAGE of Toma Piemontese PDO cheese (small cheese, short-ripened type) during ripening. Lane 1: 4 days; lane 2: 11 days; lane 3: 19 days. 1b - SDS-PAGE of Toma Piemontese PDO cheese (large cheese, long-ripened type) during ripening. Lane 1: 4 days; lane 2: 19 days; lane 3: 29 days; lane 4: 39 days; lane 5: 49 days; lane 6: 64 days.

Table 4 - Values (%) of chemical parameters related to the proteolysis in some commercial samples of Toma Piemontese PDO cheese. R.I.: ripening index.

Samples	Protein	Soluble (pH 4.6) nitrogen	R. I.
1	21.8±0.2	0.69±0.04	20.2
2	22.2±0.3	1.22±0.02	35.1
3	25.2±1.5	0.94±0.06	23.8
4	24.3±0.3	0.99±0.06	26.1
5	23.1±0.6	0.96±0.01	26.5
6	24.4±0.6	1.12±0.03	29.2
7	23.8±0.6	0.73±0.02	19.5
8	24.4±0.6	1.19±0.04	31.2
9	28.0±0.5	1.08±0.02	24.6

SDS-PAGE showed a different degree of proteolysis of the commercial samples, in agreement with the R.I. data (Fig. 3, Table 4). Different R.I. values could be related mainly to the residual coagulant used in cheesemaking, but also to the action of the bacterial flora (starter strains and typical non-lactic bacterial flora). Storage conditions of the cheese (refrigeration temperature, size of the cheese portions) and maintenance of refrigeration in retail shops or supermarkets are other significant parameters that influence the proteolysis of commercial samples.

All data obtained for the reference cheese samples and the commercial samples were submitted to PCA analysis. The first principal component (score: 52.6%), for both types of Toma

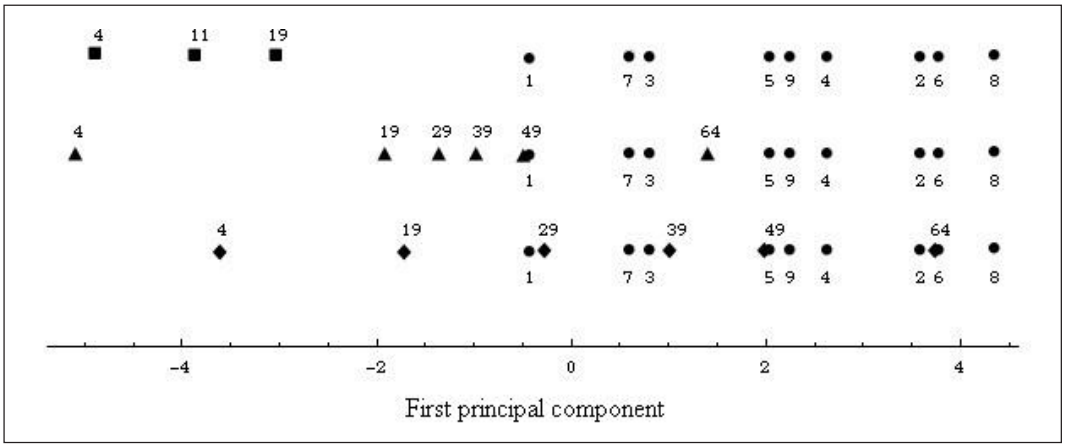


Fig. 2 - Linear representation of the first principal component (PCA analysis using all parameters, see the text for explanation) showing ripening trends of different types of Toma. ■: small type, short-ripened reference Toma; ▲: large type, long-ripened Toma winter-produced; ◆: large type, long-ripened Toma spring-produced; ●: commercial samples. Numbers above all symbols represent the age of the cheese; numbers below symbols (1-9) represent numbers of commercial samples (Tables 4-5).

Table 5 - Concentration (mg kg^{-1}) of biogenic amines and related precursor amino acids in some commercial samples of Toma Piemontese PDO cheese. Tr: traces; Nd: not detected.

Commercial sample n.:	Tyrosine	Histidine	Phenylalanine	Tryptophan	Tyramine	Histamine
1	81±0.8	83±8	278±12	Tr	39±1	Nd
2	196±2	26±2	823±25	87±4	178±7	Nd
3	Tr	97±34	312±119	19± 5	95±28	40±12
4	107±4	503±12	459±77	54±8	58±9	67±3
5	50±2	241±18	471± 93	25±1	79±6	29± 1
6	358±18	677±10	965±14	57± 20	Nd	Nd
7	19±8	Tr	508± 21	12±4	146±29	Nd
8	90±7	Tr	673±16	75±6	165±1	54±2
9	341±50	355±50	708±35	37±3	31±2	27±6

(short-ripened and long-ripened reference series) increased with the age of the sample and can thus be used as a ripening index. The main variables contributing to the first principal component were ripening index (correlation coefficient: 0.3334), % soluble N (correlation coefficient: 0.3306), phenylalanine (correlation coefficient: 0.3231) and α_{s1}-casein (correlation coefficient: 0.3211).

The commercial samples were located in lines created using the three reference samples (Fig. 2). Firstly, the two series of long-ripened Toma (winter and spring) showed different ripening trends, confirming different proteolysis for spring and winter Toma. Considering the three reference series, the commercial samples were correctly clustered as "long-ripened" Toma. Using this approach, it is obvious that some commercial samples were aged for less than the minimum ripening time required by the CE regulation for long-ripened Toma (60 days). Apparently, the use of PCA could be usefully employed to assess the age of unknown samples

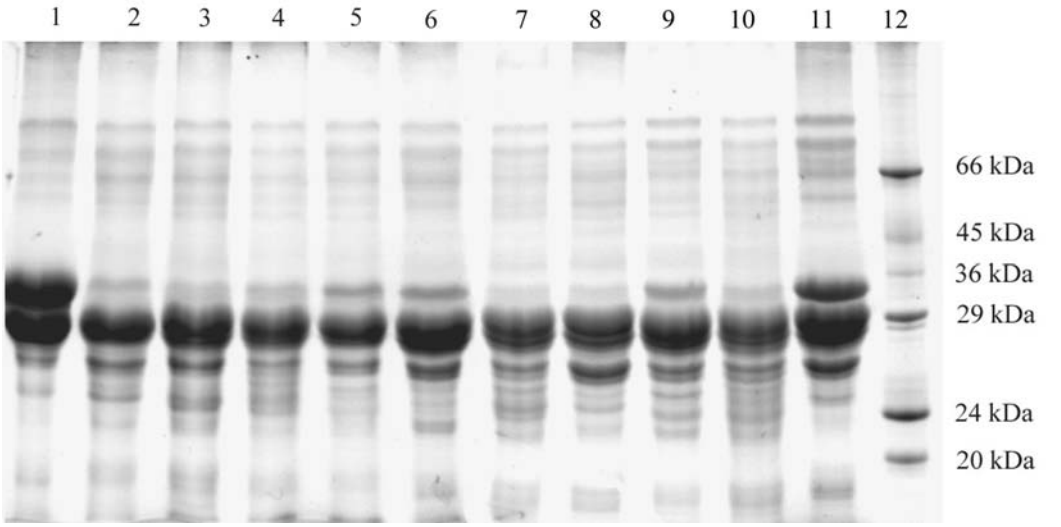


Fig. 3 - SDS-PAGE profiles of some commercial samples of Toma Piemontese PDO cheese. Lane 1: 4 days long-ripened type; lane 2: 64 days long-ripened type; lane 3-11 commercial samples n. 1-9; lane 12 molecular weight marker.

of Toma. It is suggested that more reference samples be analyzed to standardize and validate a PCA-based method for assessing cheese age. This technique could be a useful tool for "Consorzio di Tutela" to monitor the correct ripening-time before sale. In conclusion, the assessment of proteolysis (particularly SDS-PAGE, free amino acids and biogenic amines, R.I.) confirmed different ripening trends for the two types of Toma (large long-ripened and small short-ripened cheeses) as well as for the season of cheesemaking. In conclusion, PCA can be used to classify and assess the age of unknown commercial samples.

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PAPER

DIP TREATMENTS FOR FRESH ROMAINE LETTUCE

TRATTAMENTI PER IMMERSIONE
DI LATTUGA FRESCA ROMANA

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ABSTRACT

The effects of dip treatments with chlorine, citric acid, ascorbic acid and $CaCl_2$ on the storage life of Romaine lettuce leaves were investigated. While the presence of chlorine delayed degradation related to the activity of the native and exogenous microflora, the other compounds did not improve the storage life of the product when used alone. A combination of citric and ascorbic acid had a beneficial effect on the overall visual quality (color, texture, brightness). The ratio of ascorbic acid con-

RIASSUNTO

È stato studiato l'effetto del trattamento con cloro, acido citrico, acido ascorbico e $CaCl_2$ sulla vita di scaffale della lattuga Romana. Mentre la presenza di cloro è fondamentale per ritardare la degradazione collegata all'attività della flora nativa ed esogena, gli altri additivi, quando applicati da soli, non hanno migliorato la vita di scaffale del prodotto. La combinazione di acido citrico e acido ascorbico ha avuto un effetto vantaggioso sulle caratteristiche organolettiche (colore, tessitura e lucen-

- Key words: disinfection, leafy vegetable quality, sensory attributes, storage -

tents in samples treated and not treated with ascorbic acid as additive was about 4.2:1 immediately after the dip treatment but dropped to about 2.1:1 after 10 days of storage.

tezza). Il rapporto tra il contenuto in acido ascorbico nei campioni trattati e non trattati con questi composti, è stato 4.2:1 immediatamente dopo il trattamento, ma è scesa a 2.1:1 dopo 10 giorni di conservazione.

INTRODUCTION

Lettuce is a very perishable product, extremely sensitive to abuse such as temperature or mishandling. One of the most common processing-induced disorders is discoloration. Techniques that reduce surface and edge browning include low storage temperatures (BOLIN and HUXSOLL, 1991), modified or controlled atmospheres with low oxygen (2%) and/or relatively high carbon dioxide levels (7%) (KE and SALTVEIT, 1989a; MATEOS *et al.*, 1993a,b; HEIMDAL *et al.* 1995) and chemical additives (McEVILY *et al.*, 1992).

Several chemical compounds are reported to have inhibitory effects on enzymatic browning of various commodities. SAPERS *et al.* (1990) reduced darkening of "Russet Burbank" and "Katahdin" potatoes by using a dip treatment with ascorbic acid, $CaCl_2$ and citric acid. $CaCl_2$ dips have been reported to delay ripening of pears and bananas (WILLS and TIRMAZI, 1982, POOVAIAH, 1986), retard flesh softening (BANGERTH *et al.*, 1972) and retain the vitamin C content of apple fruit (DRAKE and SPAYD, 1983). Dip treatments with ascorbic acid and citric acid alone have been shown to retard enzymatic browning in freshly prepared vegetables (BAUERNFEIND and PINKERT, 1970; ANONYMOUS, 1983). However, PONTING *et al.* (1972) found that ascorbic acid or $CaCl_2$ alone did not prevent discoloration of refrigerated apple slices, but when used in a combination treatment they were very effective. Calcium used alone or combined with other substances such as ascorbic acid or sulfur dioxide has been shown to maintain firmness of pear and strawberry slices (ROSEN and KADER, 1989), and carrot sticks (BRUEMMER, 1987).

Several authors have studied the quality of head and shredded iceberg lettuce as affected by processing conditions. Although iceberg is the predominant lettuce used for prepared salad, other types of lettuce are now used in salad mixes. Information on the postharvest behavior of these other types of lettuce is limited, especially with regard to their physiology and quality as a minimally processed product (LOPEZ-GALVEZ *et al.*, 1996). Lettuce quality is reduced and its shelf life shortened by tissue browning. Some of the most common post-harvest browning disorders of iceberg lettuce tissue are russet spotting (RS), brown stain (BS) (SALTVEIT, 1997), leaf surface browning (LSB), leaf edge browning (LEB) (LÓPEZ-GÁLVEZ *et al.*, 1996) and stem browning (SB).

BOLIN *et al.* (1977) stated that refrigeration temperature is used to extend the shelf life of shredded lettuce. Lettuce stored at 5° or 10°C had a statistically shorter shelf life (10 days) than samples held at 2°C (26 days) (BOLIN et al., 1977).

BOLIN and HUXSOLL (1991) found that dipping solutions of $CaCl_2$, citric acid or $ZnCl_2$ were ineffective in retaining the quality of salad-cut lettuce, but dipping in 0.5% ascorbic acid increased the shelf life by about 10%. KRAHN (1977) reported that sodium bisulfite and $CaCl_2$ were of little or no value in extending the shelf life of cut head lettuce. IZUMI and WATA-

DA (1995) reported that the addition of chorine to a $CaCl_2$ dip enhanced the desirable effect of calcium on zucchini squash.

Taking into account that fresh horticultural crops differ in morphological structure, in composition and in general physiology, it is clear that requirements and recommendations for maximum post-harvest shelf life will differ. KE and SALTVEIT (1989b) and COUTURE *et al.* (1993) reported differences in the browning activities of various cultivars of lettuce when shredded and kept in different storage conditions.

The effectiveness of chemical dip treatments to increase post-harvest storage life is affected by pH, temperature, water activity, initial microbial load and storage atmosphere. Moreover, when different additives are used together they may cause either synergistic, additive or antagonistic effects (WILEY, 1997).

The purpose of the present work was to determine the response of whole Romaine lettuce leaves to various chemical treatments in order to extend its shelf life.

MATERIALS AND METHODS

Sample preparation

Heads of Romaine lettuce (*Lactuca sativa*, type Cos, variety Logifolia) were harvested at optimal maturity when they had reached a marketable size (approximately 20-24 leaves per head). They were immediately transported to the laboratory and analyzed by a sensory panel. Outer leaves were discarded and only photosynthetic leaves (green leaves) were included in the samples. Lettuce leaves were dipped in the various baths for 4 minutes at room temperature (ca. 20°C) at a weight ratio 1:10. The dipping baths were: 1) unchlorinated water (tap water with the following cation compositions: calcium 20 mg/L, magnesium 10 mg/L, sodium 250 mg/L, potassium 15 mg/L, total iron 0.05 mg/L, manganese 0.05 mg/L, and a water pH of 6.8-7.0); 2) chlorinated water (Control). The source of chlorine was liquid sodium hypochlorite (NaOCl) and to achieve the best balance of activity and stability of the hypochlorous acid, the pH of the water was maintained between 6.5 and 7.5; 3) chlorine plus ascorbic acid (Chl-AA); 4) chlorine plus citric acid (Chl-CI); 5) chlorine plus $CaCl_2$ (Chl-CA); 6) chlorine plus ascorbic acid plus citric acid (Chl-AA-CI); 7) chlorine plus ascorbic acid plus $CaCl_2$ (Chl-AA-CA); 8) chlorine plus citric acid plus $CaCl_2$ (Chl-CI-CA); 9) chlorine plus ascorbic acid plus citric acid plus $CaCl_2$ (Chl-AA-CI-CA). The chlorine concentration was 0.1% sodium hypochlorite, the ascorbic acid concentration was 0.5%, the citric acid concentration was 0.1% and the $CaCl_2$ concentration was 1% in the baths that included each of these compounds. Lettuce leaves were removed from the baths without rinsing and were centrifuged for 30 s at 200 rpm in a salad spinner to eliminate excess solution adhering to the tissue surface. Leaves were piled up in 100 g stacks, placed in open plastic containers and covered with a 15 m polyvinyl chloride film, with an O_2 permeability of 620-2,248 $cm^3.m^{-2}.day^{-1}$ and a CO_2 permeability of 4,263-8,138 $cm^3.m^{-2}.day^{-1}$ (1 atm-22°-25°C). Each container of 100 g stacks corresponded to a duplicate of independent lots. These containers were placed in holding boxes at a relative humidity (RH) of 97-99% and a temperature of 4±1°C for 10 days. Beakers with saturated K_2SO_4 solutions were placed in the boxes to maintain RH ca. 98%.

Microbiological studies

Lettuce leaves (25 g) were macerated in PO_4H_2K buffer solution ($1.68x10^{-4}$ g/L adjusted to pH = 7.2 with 1 mol/L NaOH), with a homogenizer (Stomacher 400 Circulator Homogenizer, London,

UK). The enumeration and differentiation of microorganisms were performed using the following culture media and culture conditions: mesophilic aerobic bacteria on PCA (Plate Count Agar) incubated at 35°C for 48 hours (ICMSF, 1983; MOSSEL and MORENO GARCÍA, 1985); psychrotrophic bacteria on PCA incubated at 5°C for 3-4 days (ICMSF, 1983); molds and yeasts in YGC (yeast-glucose-chloramphenicol) medium and incubated at 25°C for 5 days (ICMSF, 1983). Microbial counts were performed in duplicate on three independent lots, therefore count values are the mean of six determinations.

Determination of reduced ascorbic acid

Ascorbic acid content was determined by the titrimetric assay described by PELLETIER (1985). Ground lettuce (20 g) was torn manually by hand and added to 100 mL 6% metaphosphoric acid and homogenized for 3 min, it was made up to 250 mL with 3% metaphosphoric acid and filtered through Whatman # 42 filter paper. Aliquots (5 mL each) of the filtrate were titrated with 2,6-dichloroindophenol. Ascorbic acid concentrations (mg/100 g) are reported on a wet basis. All assays were performed in duplicate on three independent lots, therefore each value represents the mean of six determinations.

Calcium content

The calcium concentration of samples treated with $CaCl_2$ was calculated using the method described by ROSEN and KADER (1989). Samples were filtered using Whatman N. 12 filter paper and analyzed on a Varian AA-375 Series Atomic Absortion Spectrophotometer (Varian Techtron Pty. Ltd, Springuale, Australia) and recorded on a dry-weight basis. All assays were performed in duplicate on three independent lots and each value represents the mean of six determinations.

Sensory evaluation

Six judges, aged 30-45 years (4 females and 2 males, all members of the UNMDP Food Engineering Research Group), with sensory evaluation experience with leafy vegetables, were trained in the quality evaluation of Romaine lettuce. At each sampling time (0, 2, 4, 6 and 10 days of storage), lettuce leaves were removed from the plastic containers 20 min prior to evaluation to reach room temperature. Lettuce leaves from three independent lots were subjected to sensory evaluation in duplicate. The coded (3 digit) samples were presented one at a time in random order to the judges who sat at around a table and made independent evaluations. Samples from each treatment were evaluated for percentage and severity of injury and subsequent decay. Evaluated indexes were overall visual quality (OVQ: color, texture and brightness) (KADER *et al.*, 1973), leaf surface browning (LSB) (LÓPEZ-GÁLVEZ *et al.*, 1996), stem browning (SB) and russet spotting (RS). OVQ was rated on a scale from 5 to 1, where 5 = excellent and 1 = unusable. An OVQ rating of 3 was considered the limit of saleability. LSB, SB and RS were scored on a scale from 1 to 5, where 1 = lack of defects and 5 = severe defects. A score of 3 was considered the limit for saleability. For each of these defects, an index was calculated by multiplying the scores by the percentage of pieces affected.

Statistical analysis

ANOVA was used to establish the levels of significance of the differences among the mean values from different assays. Regression analysis was used to draw least square lines representing the degradation of sensory attributes over

time. Differences among slopes were tested as indicated by VOLK (1958).

RESULTS AND DISCUSSION

Sensory attributes

Scores for leaf superficial browning, stem browning and overall visual quality of Romaine lettuce samples dipped in chlorinated water over a 10-day storage period at 4ºC and 98% RH are presented in Fig. 1. The regression equations for the different quality parameters of the samples from all the dip treatments are reported in Table 1. Lower slopes represent lower rates of degradation. In comparing samples dipped in unchlorinated and chlorinated water, there were higher rates of degradation for OVQ indexes ($p<0.01$) for the unchlorinated samples, but there were no differences in the slopes for the LSB and SB indexes. This was due to the fact that, in the unchlorinated samples, the native and exogenous flora were still active and could have caused necrosis and other deleterious effects (Fig. 2A). On the other hand, the fact that chlorine did not affect the development of LSB and SB indicates that they reflect mostly enzymatic phenomena and are not directly affected by the presence of chlorine. The activity of phenylalanine ammonia lyase is involved in the phenylpropanoid pathway in phenolic metabolism, leading to lettuce browning (PEISER *et al.*, 1998).

The addition of ascorbic acid, citric acid or $CaCl_2$ to the chlorine dipping solution did not make any improvements ($P<0.10$) in the sensory indexes as compared to lettuce treated with chlorinated water (control) (Table 1).

Samples treated with ascorbic acid (Chl-AA) had higher slopes for OVQ, LSB and SB ($P<0.10$) than with chlorine alone. BOLIN and HUXSOLL (1991) reported that 0.5% ascorbic acid increased the shelf life of iceberg lettuce by 10%. A possible explanation could be a different response of the varieties (Iceberg and Romaine) and the different storage conditions used.

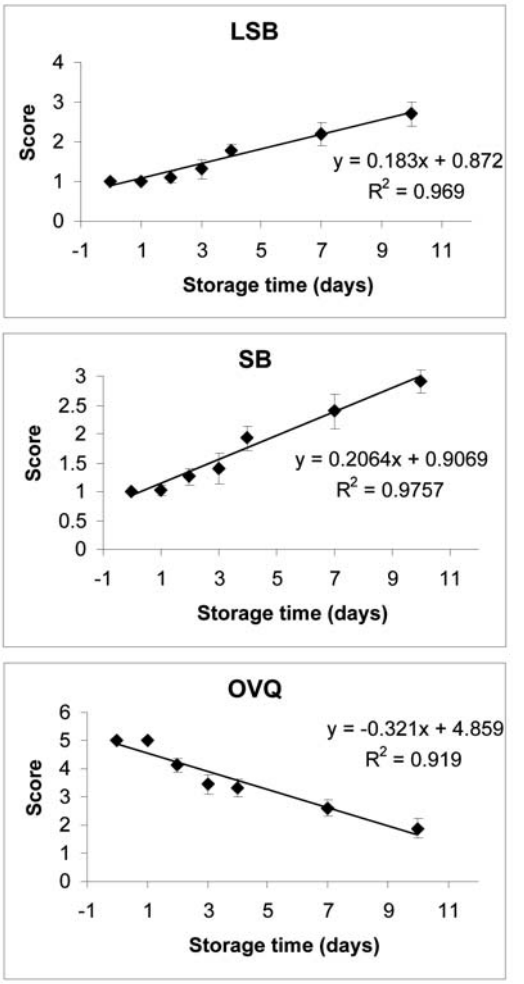


Fig. 1 - Evolution of LSB, SB and OVQ in lettuce leaves dipped in water with 100 ppm of chlorine (Chlorinated water) during 10 days of storage at 4°C. Means of six determinations. Vertical lines represent SD. Some SD bars are masked by the graph symbol.

Samples treated with chlorine and citric acid (Chl-CI) had a faster decline in OVQ ($P<0.10$), than with chlorine alone. This is contradictory to the reports that citric acid can benefit the storage life of

Table 1 - Regression equations for changes in the quality parameters (indexes) over 10 days of storage time at 4±1°C.

DIP treatments	Quality parameters		
	OVQ	LSB	SB
Unchlorinated water	y=-0.461x + 4.808 b	y=0.249x + 0.700 n.s.	y=0.280x + 0.825 n.s.
Chlorinated water (Chl)	y=-0.321x + 4.859	y=0.183x + 0.870	y= 0.206x + 0.898
Chl + ascorbic acid	y=-0.491x + 4.925 b	y=0.293x + 0.792 b	y=0.358x + 0.776 b
Chl + citric acid	y=-0.441x + 4.601 b	y=0.235x + 0.878 n.s.	y=0.217x + 0.927 n.s.
Chl + $CaCl_2$	y=-0.326x + 4.509 n.s.	y=0.328x + 0.737 c	y=0.195x + 1.018 n.s.
Chl + ascorbic acid + citric acid	y=-0.232x + 4.995 a	y=0.170x + 1.003 n.s.	y=0.280x + 0.834 n.s.
Chl + ascorbic acid + $CaCl_2$	y=-0.414x + 4.881 b	y=0.160x + 0.887 n.s.	y=0.310x + 0.822 n.s.
Chl + citric acid + $CaCl_2$	y=-0.512x + 5.028 b	y=0.241x + 0.739 n.s.	y=0.142x + 0.988 n.s.
Chl + ascorbic acid + citric acid + $CaCl_2$	y=-0.262x + 4.431 a	y=0.412x + 0.898 c	y=0.326x + 0.844 n.s.

a Slope significantly lower than slope of dip treatment with chlorinated water (Chl) at P<0.10.
b Slope significantly higher than slope of dip treatment with chlorinated water (Chl) at P<0.10.
c Slope significantly higher than slope of dip treatment with chlorinated water (Chl) at P<0.05.
n.s. No significant differences.
OVQ = overall visual quality, LSB = leaf superficial browning, SB = stem browning.

lettuce by lowering the pH (BOLIN *et al.*, 1977) or by preventing enzymatic browning by chelation of Cu in polyphenol oxidase (WILEY, 1997).

Samples treated with chlorine and $CaCl_2$ (Chl-CA) had a higher slope for LSB ($p<0.05$), with respect to chlorine alone. KRAHN (1977) also reported that the use of $CaCl_2$ did not produce any benefits in extending the shelf life of cut head lettuce. Also, the storage life of shredded lettuce (BOLIN *et al.*, 1977) was not extended by calcium treatment. The lack of response of lettuce to calcium compared to other products such as apples, strawberries, pears and carrots, may be due to differences in its form, the concentration of the solution and storage temperature (IZUMI and WATADA, 1994). The concentration of the remaining $CaCl_2$ on the lettuce was determined. After treatment, the concentration was 4 times higher (1,344 μg/g) than in non treated samples (330 μg/g). This concentration might be too high and produce negative effects on Romaine lettuce. SAMS *et al.* (1993), studying the effectiveness of post-harvest $CaCl_2$ treatments to maintain firmness and reduce decay of whole apples, found that excessive calcium could result in fruit injury.

The use of solutions containing a combination of two additives with chlorinated water produced mixed results. The simultaneous addition of citric and ascorbic acid (Chl-AA-CI) produced a lower slope for the OVQ index ($p<0.10$) compared with lettuce treated with chlorinated water (control), without significantly affecting indexes that show enzymatic

degradation such as LSB and SB. This could be due both to the lowering of the pH and to citric acid strengthening the action of ascorbic acid (WILEY, 1997).

The combination of ascorbic acid and $CaCl_2$ (Chl-AA-CA) and the combination of citric acid and $CaCl_2$ (Chl-CI-CA) produced greater declines in the OVQ index ($p<0.10$).

The simultaneous addition of ascorbic and citric acids and $CaCl_2$ (Chl-AA-CI-CA) gave beneficial effects on the OVQ index, but deleterious effects on the LSB index. Probably the presence of a high concentration of remaining calcium on the lettuce leaves after the $CaCl_2$ treatment, produced this negative effect.

Only the simultaneous addition of ascorbic and citric acids to chlorinated water (Chl-AA-CI) gave an improvement over chlorinated water alone (control).

Russet spotting (RS) is a major physiological disorder of harvested Iceberg lettuce. It is induced by exposure to ethylene and is more severe at a temperature of 5°C (RITENOUR *et al.*, 1995). These authors indicated that when harvested Iceberg lettuce was exposed to 10 ppm of ethylene at 5°C, symptoms appeared after approximately 3 days of storage. In all the samples, the development of russet spotting showed no significant increase up to 6 days of storage. By day 10 of storage, the indexes increased slowly regardless of the bath treatment, reaching final values of 1.6 to 2.0.

Microbiological counts

A fundamental problem in extending the shelf life of minimally processed fruits and vegetables is microbial proliferation which must be retarded. Growth of disease-causing microorganisms is a food safety concern, especially with higher pH vegetables (in contrast to lower pH fruits). Visible growth and off-odors caused by microorganisms are aesthetically unacceptable. The ecological niche caused by the composition of the food will determine the kind of microorganisms that will grow.

PONCE *et al.* (2002) reported large differences in the initial microbial counts among different lots of fresh leaves of Swiss chard obtained by conventional methods that could not be entirely explained by the variability of the assessment methods. Numerous factors, such as ambient conditions during harvest, amount of soil accompanying the product, time elapsed between last irrigation and harvest or post-harvest handling, could be responsible for these differences. Since some of these factors can not be controlled, initial counts would be necessary whenever the effect of post-harvest processes on the micro flora of this type of product is evaluated.

Fig. 2 shows the trend of a) mesophilic bacteria, b) psychrotrophic bacteria and c) yeast and mold populations on lettuce leaves dipped in the various baths. Initial counts for mesophilic aerobic microorganisms were in the range of $1.3x10^5$ to $2.5x10^6$ CFU/g for all sample treatments. The initial counts corresponding to control samples (chlorinated water) were $7\text{-}8x10^5$ CFU/g, the highest counts corresponded to samples with unchlorinated water (tap water) with $2\text{-}3x10^6$ CFU/g and the lowest counts corresponded to samples with chlorine plus ascorbic acid plus citric acid (Chl-AA-CI) with $1\text{-}2x10^5$ CFU/g (Fig 2A). These samples maintained the lowest counts up to 2 days of storage. Chlorine treatment reduced ($P<0.01$) the initial population of natural contaminants. Hypochlorite dips are commonly used in postharvest practices for sanitizing fruits and vegetables, particularly in the fresh cut industry (BEHRSING *et al.*, 2000). Chlorine acts on the microbial flora of vegetables (WILEY, 1997; WATADA, 1997). Microbial activity has been shown to be associated with the development of necrosis during storage of lettuce leaves (NGUYEN-THE and PRUNIER, 1989; JACQUES and MORRIS, 1994). By 10 days no differenc-

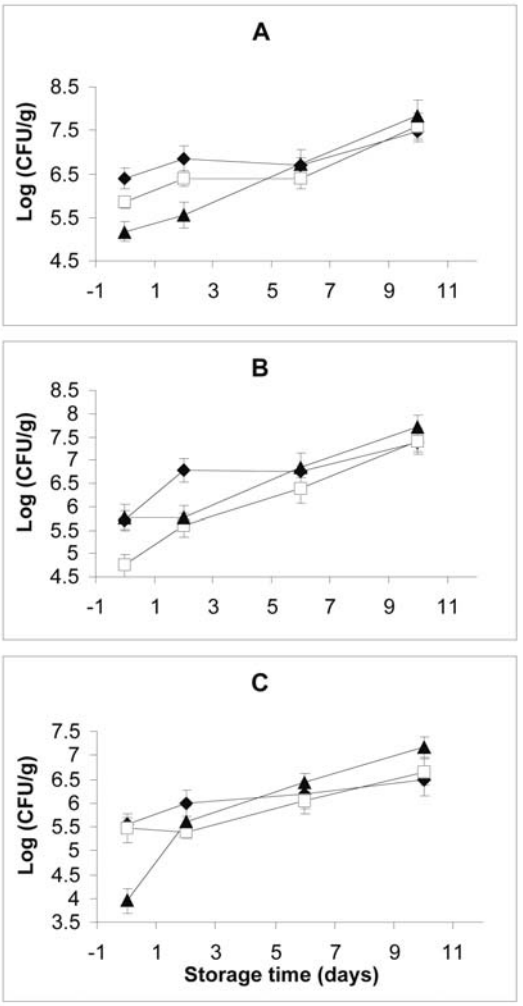


Fig. 2 - Mean microbial Log_{10} counts of lettuce leaves during storage: Mesophilic bacteria (A), Psycrotrophic bacteria (B) and Yeasts and molds (C). Values correspond to a mean of duplicates, obtained from three independent lots, therefore values are the means of six determinations. Vertical lines represent SD. Some SD bars are masked by the graph symbol.
◆ - Unchlorinated water treatment, □ - Chlorinated water treatment, ▲ - Chl-AA-CI.

es ($P<0.01$) in the populations of mesophilic aerobic microorganisms were noted on lettuce regardless of type of bath (1.5-8x10^7 CFU/g). For minimally processed vegetables, French legislation allows a maximum of 5x10^7 CFU/g at the stage of consumption (BARRIGA *et al.*, 1991), hence the microbiological quality could be considered satisfactory during 10 days of storage.

The levels and trend of psychrotrophic microorganisms were similar to those of mesophilic aerobic microorganisms (Fig. 2B). The population increased from an initial level of 0.25-6x10^5 to 2-6x10^7 CFU/g. In general, the numbers of psychrotrophic microorganisms were acceptable. With the various chemical treatments investigated, no differences in the initial counts ($P<0.01$) were found. The lowest initial counts corresponded to the sample treated with chlorinated water.

The lowest initial counts for yeasts and molds (3-9x10^3 CFU/g) were for lettuce dipped in chlorine plus ascorbic acid (Chl-AA), chlorine plus ascorbic acid plus citric acid (Chl-AA-CI), chlorine plus ascorbic acid plus calcium (Chl-AA-CA), chlorine plus citric acid plus calcium (Chl-CI-CA) and chlorine plus ascorbic acid plus citric acid plus calcium (Chl-AA-CI-CA). This could probably be due to the low pH of these baths (pH 4-5). By day 10 of storage no differences ($p<0.01$) were found among dip treatments (Fig. 2C). BEUCHAT and GOLDEN (1989) studied antimicrobials that occur naturally in foods, and suggested that some organic acids can exert fungicidal and fungistatic action which is related directly to lowering the pH of the substrate.

The ability of both mesophilic and psychrotrophic bacteria and yeast and molds to multiply at the low temperatures assayed indicates their potential ability to cause lettuce spoilage.

Ascorbic acid contents

The initial ascorbic acid content of fresh leaf samples was 8.3 mg/100g of fresh weight. The endogenous ascorbic acid concentrations in the fresh lettuce used in this study were higher than those reported by ALBRECHT (1993) for Ro-

maine lettuce. The ascorbic acid concentrations of samples dipped in treatments free of ascorbic acid were not significantly different ($P<0.05$). On the other hand samples treated with ascorbic acid had about a 4 times higher initial ascorbic acid content without significant differences among them. Fig. 3A presents the mean values obtained for the ascorbic acid concentration of samples treated without ascorbic acid (treatments with unchlorinated water, chlorinated water, Chl-CI, Chl-CA and Chl-CI-CA) and Fig. 3B presents the mean values obtained for the ascorbic acid concentration of samples treated with ascorbic acid (treatments with Chl-AA, Chl-AA-CI, Chl-AA-CA and Chl-AA-CI-CA). The difference between the means of both groups during storage was always significant ($p<0.01$).

The rate of ascorbic acid loss was lower in samples without ascorbic acid treatments than in samples with ascorbic acid treatments. For example, by day 4, samples without ascorbic acid had lost 50% of their initial ascorbic acid content, while samples with ascorbic acid had lost 77%. This difference could be attributed to the ascorbic acid being on the surface and more exposed to oxidation.

Nevertheless, in spite of the higher rates of ascorbic acid degradation in ascorbic acid-treated samples, their absolute concentrations remained higher than in the other samples during storage.

CONCLUSIONS

The effect of dip treatments with chlorine, $CaCl_2$, citric acid and ascorbic acid on the quality of Romaine lettuce leaves during storage was investigated. Chlorine may be required to inhibit the growth of bacteria responsible for necrosis. Neither citric acid nor ascorbic acid

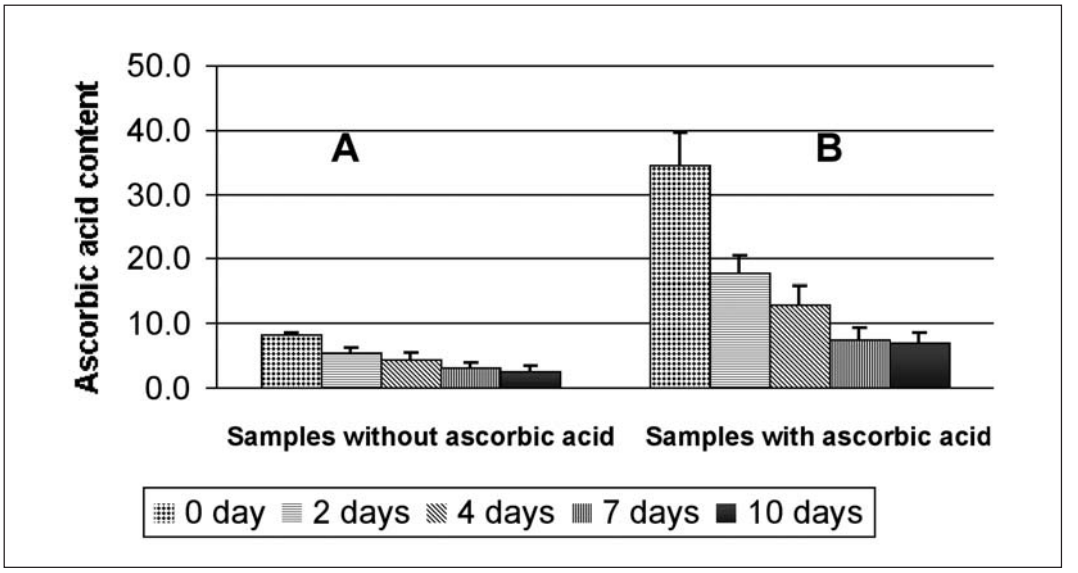


Fig. 3 - Ascorbic acid content (mg per 100g of fresh vegetable) in samples treated (A) without ascorbic acid (Chlorinated water, Unchlorinated water, Chl-CI, Chl-CA and Chl-CI-CA) and (B) samples treated with ascorbic acid (Chl-AA, Chl-AA-CI, Chl-AA-CA, Chl-AA-CI-CA) during 10 days of storage at 4°C. Means of six determinations. Vertical lines represent SD.

in chlorinated solutions resulted in improvements in LSB, SB, OVQ indexes, which is in contrast with published results on Iceberg lettuce. However, the combination of both acids resulted in a slower decline of the overall visual quality of the Romaine lettuce leaves. The addition of calcium chloride did not result in improvements in the visual quality and could be responsible for faster leaf surface browning. Samples treated with ascorbic acid maintained higher contents of ascorbic acid throughout storage, although the rate of ascorbic acid loss was higher than in samples not dipped in ascorbic acid.

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PAPER

EFFECTS OF A WHEAT GERM-ENRICHED DIET ON SKELETAL MUSCLE REGENERATION

EFFETTI DI UNA DIETA ARRICCHITA IN GERME DI GRANO SULLA RIGENERAZIONE DEL MUSCOLO SCHELETRICO

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ABSTRACT

One of the common ingredients used in dietary supplements is wheat germ and its derivatives. In this study the effect of a wheat germ-enriched diet (WGED) on tissue repair was investigated in a model of skeletal muscle damage induced by the anesthetic bupivacaine. Rats were fed normal feedstuff or a WGED; injections of bupivacaine caused skeletal muscle necrosis followed by tissue regeneration. Morphometric analyses were performed on the muscles in order to quantify cell size

RIASSUNTO

Uno dei costituenti più utilizzati negli integratori è il germe di grano ed i suoi estratti. In questo studio abbiamo voluto saggiare gli effetti di una dieta arricchita in germe di grano (WGED) sulla rigenerazione muscolare in un ben noto modello sperimentale di danno indotto da un anestetico locale di uso comune (la bupivacaina). Muscoli di ratti alimentati con normale mangime o con WGED sono stati iniettati con bupivacaina che causa necrosi seguita da rigenerazione del tessuto. Sono state

- Key words: lectins, muscle regeneration, myofibers, wheat germ -

and nuclei migration toward the cell periphery because these are indices of muscle regeneration. Statistical analysis shows that myoblast maturation, determined by nuclei migration to a peripheral position, was significantly delayed in rats fed WGED compared to rats fed the control diet. No statistically significant differences were observed in the myocellular areas. This study provides evidence that dietary wheat germ can interfere with muscle regeneration.

eseguite quindi analisi morfometriche considerando quali indici di rigenerazione le dimensioni delle cellule rigeneranti e la migrazione dei nuclei alla periferia. L'analisi statistica dei dati mostra che la maturazione dei mioblasti, valutata misurando la migrazione dei nuclei, era significativamente ritardata nel tempo nei ratti alimentati con WGED rispetto ai controlli. Differenze statisticamente non significative sono state invece osservate per quanto concerne le dimensioni cellulari. Si può quindi ipotizzare che il germe di grano, assunto per via orale, abbia effetti sulla rigenerazione muscolare.

INTRODUCTION

Increasing importance is being given to natural foods and nutritional supplements. For example, nutritional supplements are currently being promoted for athletes due to their supposed beneficial effects on muscle growth and repair (MAUGHAN, 1999). Similar supplements are also used in the paediatric field. Although there is some experimental evidence to support the use of some of these products, the claims made for others are not based on controlled experiments.

Many dietary supplements contain wheat germ derivatives that may include, among other proteins, wheat germ agglutinin (WGA). WGA is a lectin, a member of the class of carbohydrate-binding proteins whose effects on several physiological functions have been addressed in numerous studies (MANAGE *et al.*, 1972; MITCHELL and CLARKE, 1979; LIVINGSTONE and PURVIS, 1980; NOAH *et al.*, 1980; PUSZTAI, 1991; PUSZTAI *et al.* 1993; VAN DAMME *et al.*, 1998). Lectins, including WGA, bind glycoconjugates expressed at the cell surface level including those of the gastrointestinal tract, where they may cause functional disorders (nausea, vomiting, diarrhoea) and anatomical alterations (hyperplasia and hypertrophy) (VAN DAMME *et al.*, 1998; PUSZTAI *et al.*, 1993). Some lectins are remarkably toxic upon ingestion as is the case for lectins from *Ricinus communis* (PUSZTAI, 1991) and *Phaseolus vulgaris* (MANAGE *et al.*, 1972). Direct cytotoxic activity has also been demonstrated for WGA *in vitro* (VAN DAMME *et al.*, 1998), although this lectin is structurally and functionally unrelated to both *R. communis* and *P. vulgaris* lectins. Due to their capacity to cross the gastrointestinal barrier in a biologically active form, lectins can also have systemic effects, such as hypersensitivity reactions (MITCHELL and CLARKE, 1979; PUSZTAI, 1991), increased lipogenesis and pancreatic hypertrophy (LIVINGSTONE and PURVIS, 1980). In particular, WGA has been shown to interfere with several functions of the immune system in both *in vitro* and *in vivo* assays (GREENE *et al.*, 1985; TORIBIO *et al.*, 1985; MURAILLE *et al.*, 1999). It is important to note that, along with other lectins, WGA has the ability to promote the secretion of pro-inflammatory cytokines (HAAS *et al.*, 1999). However, little is known about the effects

of these proteins on the possible accumulation resulting from their long-term intake and on their possible interactions with other dietary components when given in amounts comparable to those present in a common diet. These effects would be shown more clearly in *in vivo* assays with complex mixtures that are similar to natural foodstuffs.

Bupivacaine is a well-studied drug that causes extensive skeletal muscle necrosis followed by rapid muscle regeneration with established kinetics and histologic characteristics (JIRMANOVA and THESLEFF, 1972; CARRARO *et al.*, 1983; MUSSINI *et al.*, 1987). An injection of bupivacaine causes sarcolemmal lysis of most of the fibers with extensive dilation of the sarcoplasmic reticulum accompanied by overcontraction and overstreching of the myofilaments (BRADLEY, 1979). Most fibers are totally disrupted, leaving empty basal lamina bags (BRADLEY, 1979). These effects can be observed 1 h after injection when sector lesions appear; they are similar to those which occur in Duchenne muscular dystrophy as reported by MOKRY and ENGEL (1975) and CRAWFORD *et al.* (2000). Two days after injection, phagocytes begin to infiltrate the muscle and satellite cells become prominent below the basement membrane (BRADLEY, 1979). By day 4, regeneration of the muscle is prominent even occurring side-by-side with necrotic fibers (BRADLEY, 1979). On day 6, fiber regeneration is extensive but fibers are still smaller and rounder than normal, noninjured ones. The nuclei occupy a central position in the fiber. In the rat model, myoblasts progressively increase their cytoplasm and finally show peripheral migration of their nuclei (BRADLEY, 1979).

The above model of tissue damage by necrosis/inflammation and of tissue repair was used to test the possible interactions of a wheat germ-enriched diet (WGED) with a complex series of pathophysiological pathways. The myocellular areas and the fraction of central myonuclei were selected as parameters correlated with muscle regeneration. WGED delayed tissue repair and this delay appeared to be related to a specific alteration of the molecular mechanism(s) that drives the localisation of the myonuclei in muscle fibers.

MATERIALS AND METHODS

Animals, diet composition and feeding procedures

Three-month-old Wistar rats, reared at the Università di Padova, were used. They were fed with normal feedstuff (NF) for rodents (Harlan Italy, Udine, Italy) containing 18% protein or with the wheat germ-enriched diet (WGED). The WGED was obtained by adding 1:1 (w:w) wheat germ to NF (see Table 1 for diet composition). The WGED was continuously administered during the experiment starting 4 days before muscle injury. Animals were housed individually. The experiments were carried out according to current Italian laws (D.L. 116, 1992) in agreement with the directive 86/609/CEE on animal experimentation.

WGA determination in wheat germ and in the dietary formulations

The WGA content in the normal rat feedstuff, wheat germ and WGED was determined by ELISA assays as described by VINCENZI *et al.* (2002). Briefly, samples were solubilised (1:10 w/v) with phosphate-buffered saline (PBS) and centrifuged for 15 min at 10,000 g. Samples were loaded onto Ovalbumin-coated microtiter plates. Bound WGA was detected using specific antibodies and its concentration in unknown samples was determined by comparing OD values with those obtained in parallel for standard dose-response assays carried out with purified WGA (Sigma-Aldrich, Milano, Italy).

Table 1 - The average composition of normal feedstuff (NF) and wheat germ-enriched diet (WGED) (main components only).

Component	[1]NF	[2]WGED	Ratio (WGED/NF)
Protein %	18.90	22.80	1.20
Fat %	5.70	7.45	1.30
Carbohydrate %	57.30	51.00	0.89
Na %	0.23	0.11	0.47
K %	0.68	0.81	1.19
Ca %	1.01	0.53	0.52
Mg %	0.20	0.23	1.15
Fe mg/kg	226.00	155.50	0.68
Cl %	0.40	0.24	0.60
P %	0.65	0.85	1.30
Cu mg/kg	15.21	12.10	0.79
Zn mg/kg	77.00	123.50	1.60
Vitamin A mg/kg	4.65	2.32	0.49
Vitamin B1 mg/kg	16.50	14.25	0.86
Vitamin B2 mg/kg	14.90	11.05	0.74
Vitamin B6 mg/kg	18.50	25.75	1.39
Vitamin B12 mg/kg	0.08	0.04	0.50
Biotin mg/kg	0.30	0.27	0.90
Vitamin C mg/kg	0.00	0.00	—
Vitamin E mg/kg	101.00	160.00	1.58
Energy value MJ/kg	13.70	14.40	1.05
[3]WGA mg/100 g	<0.03	20	>600

[1] Composition of NF diet was taken from Harlan Teklad (18% Protein Rodent Diet, see www.teklad.com/global/data/2018.htm).
[2] Composition of WGED was calculated by taking the average compositions of wheat germ (McCANCE and WIDDOWSON, 1997) and of NF and by considering the 1:1 mixture.
[3] Determination of WGA was from our own experiments (see the text for details).

Tissue damage/regeneration procedures

Six control NF-fed and six WGED-fed rats were anaesthetised with 0.1 mg/g ketamine chloridrate. Soleus muscles from the right hind limbs were identified, raised and then a strip of parafilm was passed below the muscle belly. The muscles were injected with 200 µL of 0.5% bupivacaine in 0.9% NaCl and soaked in the same solution. After 5 min, the muscles were replaced in the normal anatomic position and the skin was sutured. Fifteen days after surgery, the animals were sacrificed under anaesthesia and the muscles were explanted and immediately frozen in liquid nitrogen at resting length. The muscles were stored at -80°C until used for the morphologic and morphometric analyses.

Morphologic, morphometric and statistical analyses

The 12 frozen muscles were sectioned at the mid-belly using a cryostat. Three 10 µm thick sections were put on glass slides and stained with hematoxylin and eosin (H&E) using conventional techniques. Images were obtained using an Olympus IX70 microscope (Olympus Italia, Milano, Ita-

ly) connected to a 2-CDD camera (model KYF58, JVC Professional Products, Milano, Italy). The Image-Pro Plus software (Media Cybernetics, Silver Spring, USA) was used for the morphometric analysis.

Six control NF-fed and six WGED-fed rats were used in these assays. The morphometric measurements of the myocellular areas were performed at 20X magnification (in a range of 3 to 6 fields for each muscle), while the determination of the myonuclei position was performed at 40X (in a range of 4 to 8 images). In both analyses microscopic fields were randomly selected.

Myocellular area determinations

Tissue preparations from one control and one WGED-fed rat were excluded from the analysis because of an incorrect injection procedure which was revealed by histologic inspection. On the average 624 cells per rat were analysed for the control NF-fed group and 1,004 cells per rat for the WGED-fed group for a total of 3,120 and of 5,020 cells analysed, respectively. Data from each rat were collected and the mean±SD areas calculated. The mean areas were then compared using the Student *t*-test.

Determination of myonuclei position

Tissue preparations from one control and one WGED-fed rat were excluded from the analysis as mentioned above. On the average 435 cells per rat were analysed for the control NF-fed group and 810 cells per rat for the WGED-fed group for a total of 2,175 and 4,050 cells analysed, respectively. The fraction of cells with nuclei clearly in a central position were calculated for each rat and the data were subjected to analysis of variance using the *F*-test.

RESULTS

WGA determination in wheat germ, in NF diet and in WGED

LIS and SHARON (1981) reported a 30±20 mg /100 g WGA/WG ratio in food-store available products. Due to a high variability in the concentrations of WGA lectin in the wheat germ used in our assays and in the NF (largely based on raw cereal derivatives) these concentrations should be directly determined. Using a sensitive and specific ELISA method (VINCENZI *et al.*, 2002), the WGA concentration in the wheat germ was found to be 40±1 mg /100 g whereas in NF it was found to be below the ELISA sensitivity threshold (30 ng/mL corresponding to 0.03 mg/100 g). Western Blot analysis of the NF revealed the presence of a faint WGA band (data not shown). The concentration of WGA in WGED (1:1 wheat germ:NF) was found to be 20±0.2 mg /100 g as expected. The WGA content constituted the main difference in the composition of NF and WGED feedstuffs, whereas no significant differences were observed for the other components (Table 1).

Effects of wheat germ on muscle damage/regeneration responses

No differences in the dietary intake (approximately 20 g/day) or of common physiological parameters (e.g. modification of body weight, fur status) were observed during the course of the experiments carried out with rats on NF and WGED. The histological profiles at day 15 of the regeneration kinetics are shown in Fig. 1. In both control NF-fed and WGED-fed groups, the rat skeletal muscles still exhibited a great variability in myofiber diameter with a certain proportion of the nuclei in the central position. These morphometric parameters are correlated with an incomplete recovery of

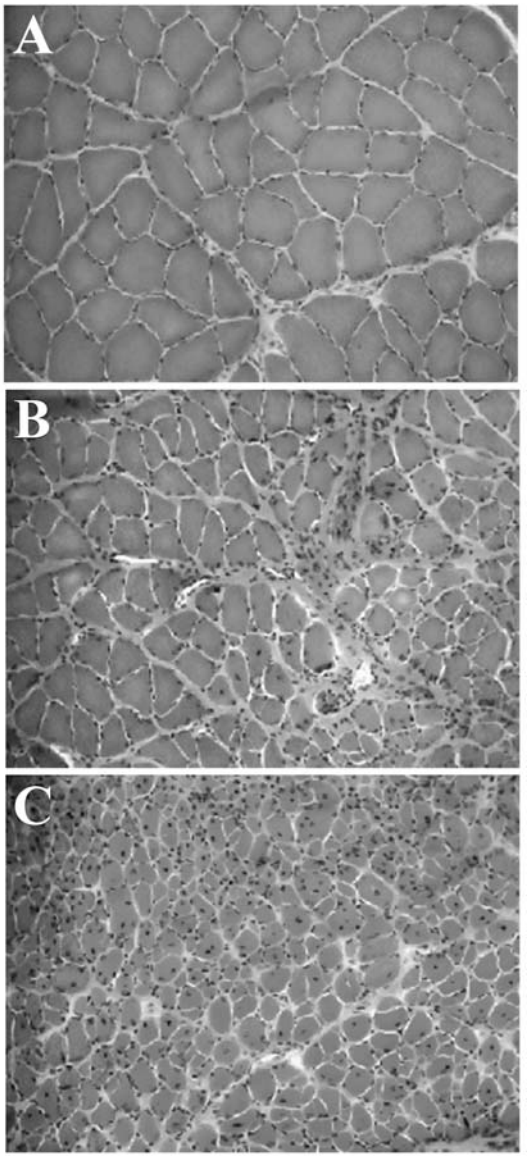


Fig. 1 - Transversal mid-belly section of normal rat soleus muscle (Panel A), of recovering soleus muscle after bupivacaine-induced injury (day 15) in control NF-fed rats (Panel B) or in WGED-fed rats (Panel C). H&E stain at 20X magnification.

the muscles (panels B and C). A section of tissue from a rat muscle not subjected to tissue injury is also shown for comparison purposes (panel A). The regenerating muscles in panels B and C show an incompletely restored fascicle architecture with sheaths of fibrotic tissue enclosing groups of fibers.

In comparison to normal tissue (panel A), the mean myofiber diameters were 23.3±16.6% and 21.3±12.8% in muscles from the control NF-fed and from the WGED-fed rats, respectively. The mean myocellular areas±SD measured in tissue preparations from each rat in the two analysed groups are shown in Fig. 2. Great variability characterised this parameter even within each group of rats. Overall, the areas in the NF-fed groups appear slightly greater than in the WGED-fed group as expected for a higher degree of myofiber recovery. No significant difference was found between the control NF-fed and WGED-fed rats (*t*-test, $p>0.05$).

The determination of the myonuclei position in the control NF-fed and in the WGED-fed rats is shown in Table 2. A larger fraction of the myonuclei occupied a central position in WGED-fed rats compared to tissue preparations from the NF-fed rats. The differences were statistically significant (*F*-test, $p<0.01$).

DISCUSSION

Knowledge that bioactive molecules that enter the body via the alimentary tract can influence a number of pathophysiological responses is not new (KILPATRICK, 1999; MAUGHAN, 1999). For example, some dietary plant lectins have been shown to modulate the activity of the immune system (TORIBIO *et al.*, 1985; HAAS *et al.*, 1999; KILPATRICK, 1999). Bupivacaine-induced skeletal muscle injury is a well established model of muscle necrosis and regeneration that includes a phase of active inflammation (JIRMANOVA and THESLEFF, 1972; CARRARO *et al.*, 1983; MUSSINI *et al.*, 1987). It is a way to analyse the possible interactions between dietary signals and animal patho-physiological pathways with-

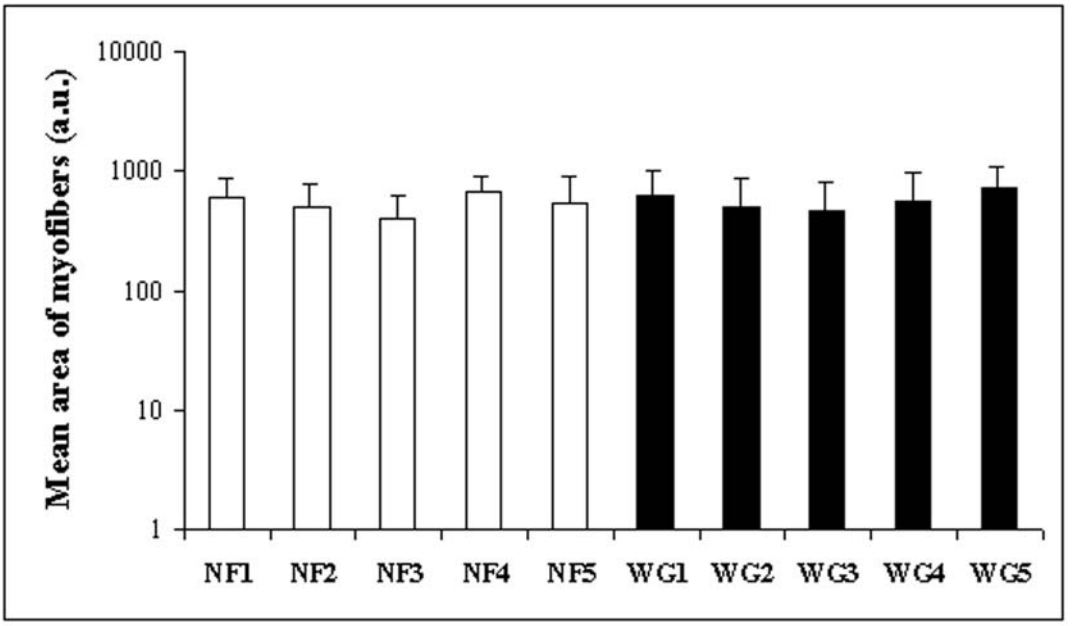


Fig. 2 - Measures of mean±SD myocellular areas in tissue sections of skeletal muscles from control NF-fed (white bars) and WGED-fed (black bars) rats.

Table 2 - Determination of myonuclei position in recovering skeletal muscles of control NF-fed and WGED-fed rats.

Animals	WGED-fed rats			NF-fed rats		
	Total cells	Cells with central nuclei	[1] Ratio	Total cells	Cells with central nuclei	Ratio
1	748	215	0.287	323	73	0.226
2	633	278	0.439	438	67	0.153
3	967	321	0.322	388	56	0.144
4	791	299	0.378	583	82	0.141
5	911	303	0.333	443	110	0.248
Total	4,050	1,416		2,175	388	
Mean	810	283.2	0.352	435	77.6	0.182
SD	132.6	41.07	0.058	95.82	20.42	0.05

[1] Differences in the ratio values between the two treatment groups were evaluated using the *F*-test. F^{**}=23.92 ($F_{0.01}$=11.26).

in a complex framework of regulatory cells and signals, including immune cells, cytokine production which regulates the extent of inflammation and a number of signals involved in the regeneration of myofibers. Included in the latter are growth factors (basic fibroblast growth factors), insulin-like growth factors I and II and transforming factor (BORNEMANN and ANDERSON, 2000).

Consequently, the general nutritional condition of the organism is important for muscle recovery, as well as any other factor affecting local circulation (e.g. infections or the presence of a foreign body leading to local proliferation of connective tissue) (FIELD, 1960).

A diet is a complex mixture of molecules that can potentially act at different levels in the body. Among the dietary molecules, plant lectins attract attention because they can interfere with several physiological pathways in the animal (LIVINGSTONE and PURVIS, 1980; KILPATRICK, 1999). In particular, WGA can reduce the digestibility of dietary proteins and induce anatomical modifications of the gut that alter animal growth (PUSZTAI, 1991; PUSZTAI *et al.*, 1993). WGA can alter mammalian metabolism at the endocrine level by mimicking the effects of insulin on adipocytes, thereby stimulating the synthesis of triglycerides and the transport and oxidation of glucose in fat cells (LIVINGSTONE and PURVIS, 1980). A WG-enriched diet, thus containing WGA, would therefore be expected to interfere with skeletal muscle regeneration.

It should be stressed that studies that investigate the role of dietary signals (e.g. lectins) on some functional responses usually analyse the effects of artificially-introduced high doses of purified molecules in experimental animals (PUSZTAI *et al.*, 1993). The effective role of dietary molecules under physiological conditions would therefore not be included in such studies. Moreover, the possible interactions of the candidate molecule with other components of the diet and/or the cumulative effects resulting from the continuous intake of low doses of these compounds would not be explored. For these reasons the present work used complex diet formulations, the NF diet, that had undetectable levels of WGA, and the WGED, that contained WGA at a concentration of 20 mg/100 g. The potential effect of WGA, as well as the other components of the diet, on muscle regeneration were therefore studied under nutritional conditions that resembled natural ones (e.g., the diet of a wild rat living in a mill). In fact, we failed to observe the typical systemic effects of WGA in WGED-fed rats that have been reported in other studies that used much higher doses of WGA (PUSZTAI *et al.*, 1993).

A significant difference in the regeneration of skeletal muscle myofibers was observed between control NF-fed and WGED-fed rats with respect to the localisation of myonuclei, whereas no differences were observed for the measured myocellular areas. The position of myonuclei is a parameter correlated with the later phases of muscle regeneration. Hence, the observed differences do not appear to be related to a delay in the general maturation of myofibers but rather to some specific alteration of the mechanisms by which nuclei migrate to the periphery at a later time. Unfortunately, little is known about the nuclear movement at the molecular level. The modified regeneration induced by WG may be of help in future studies aimed at clarifying these mechanisms in more detailed time-course assays.

In conclusion, this study provides evidence that dietary wheat germ can interfere selectively with complex patho-physiological pathways under standard non-limiting nutritional conditions. Dietary wheat germ altered skeletal muscle regeneration. This fact should be considered when claims are made about the beneficial effects of natural foods and, above all, of nutritional supplements. Many supplements are promoted as having beneficial effects on muscle growth and regeneration and are therefore recommended for athletes (MAUGHAN, 1999). Similar supplements are largely used in the paediatric area and in the diet of recovering patients. Since food supplements are not subjected to regulation (MAUGHAN, 1999), as are drugs, and since their use is frequently self-determined by the consumer, the results of the present

work suggest that these products should be evaluated more critically.

ACKNOWLEDGEMENT

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SHORT COMMUNICATION

GAS CHROMATOGRAPHIC DETERMINATION OF AZOXYSTROBIN, DINOCAP, FENARIMOL, PENCONAZOLE AND QUINOXYFEN DURING WINE MAKING

DETERMINAZIONE DI FUNGICIDI
IN PRODOTTI VITIVINICOLI MEDIANTE HRGC-ECD

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ABSTRACT

A quick gas chromatographic method is described to determine residues of azoxystrobin, dinocap, fenarimol, penconazole and quinoxyfen in grapes, must, marc and wine. The samples were extracted with hexane-ethyl acetate and directly analyzed using a high resolution gas chromatograph fitted with an electron-capture detection system (ECD). No clean-up was necessary because there were no interferences in the chromatogram. Recoveries from untreated samples spiked with known

RIASSUNTO

In questo lavoro viene descritto un metodo rapido per la determinazione simultanea di azoxystrobin, dinocap, fenarimol, penconazolo e quinoxyfen in uve, mosti, vinacce e vini mediante HRGC-ECD. I campioni, estratti con esano:acetato di etile sono analizzati direttamente, senza nessuna purificazione. I valori di recupero, determinati a tre diversi livelli di fortificazione in triplo, oscillano per l'azoxystrobin da 80,4% (vinacce) a 100,7% (uve); per il dinocap da 81,8% (vinacce) a 110,4%

- Key words: fungicides, grapes, marc, must, wine -

amounts of the pesticides (0.01, 0.1 and 1.0 mg/kg) ranged from 80.4% (marc) to 100.7% (grapes) for azoxystrobin; from 81.8% (marc) to 110.4% (wine) for dinocap; from 82.8% (grapes) to 98.2% (wine) for fenarimol; from 80.9% (marc) to 97.3% (wine) for penconazole; from 87.0% (wine) to 95.8% (wine) for quinoxyfen. The limits of quantitation were 0.010 mg/kg for dinocap and 0.005 mg/kg for the other fungicides; the detection limits were $6x10^{-4}$ ng for dinocap and $3x10^{-4}$ ng for the others.

(vini); per il fenarimol da 82,8% (uve) a 98,2% (vino); per il penconazolo da 80,9% (vinacce) a 97,3% (vino); per il quinoxyfen da 87,0% (vino) ta 95,8% (vino). I limiti di quantificazione sono pari a 0,010 mg/kg per il dinocap e 0,005 mg/kg per gli altri fungicidi; i limiti di rivelabilità sono pari a $6x10^{-4}$ ng per il dinocap and $3x10^{-4}$ ng per gli altri prodotti.

INTRODUCTION

The grapevine is subject to attack by numerous parasites. The most frequent diseases caused by fungi are downy mildew, powdery mildew and gray mold. The pesticides commonly used to control powdery mildew (*Uncinula necator*) belong to the chemical classes of acylanilines, triazols and dicarboximides. Among these, dinocap, fenarimol and penconazole are widely used. In the 1990s azoxystrobin was used and, more recently, quinoxyfen (belonging to the family of the quinolines) is used as a fungicide.

Numerous methods aimed at isolating and extracting fungicides in grapes, must and wine appear in the literature, but often the extraction procedure is complex and expensive. SCARPONI *et al.* (1992) used an opportune solvent and a separatory funnel to extract the wine sample; OLIVA *et al.* (1999) used a micro on-line extraction method and NAVARRO *et al.* (1999) homogenized the wine sample with solvents and then filtered it through celite and a NaCl support. Because no data have been reported on the determination of dinocap residues in vines or wine, this study was designed to standardize a quick analytical method for their extraction in the wine products with a single and sensitive procedure.

MATERIALS AND METHODS

Field trials

The trial was carried out on grapes from three Italian vineyards: one located in Tuscany (Franci of Magliano farm), one in Campania (Vadiaperti of Montefrondone farm) and one in Sicily (Giuffrida of S. Venerina farm). Wines from Tuscany were produced from 25-year-old plants, cultivated on a calcareous soil (100 m elevation); vines were grafted with the Sangiovese variety (Morellino clone). Wines from Campania were produced from 25-year-old plants, cultivated on a clay soil (700 m elevation); vines were grafted with the Fiano d'Avellino variety. Sicilian wines were produced from 15 to 20-year-old plants, cultivated on a volcanic soil (300 m elevation); vines were grafted with the Inzolia and Carricante varieties in a ratio of 1:1.

Pesticides were used at the doses recommended by the manufacturer and were sprayed with a Fox manual sprayer (Fox Motori, Poviglio – Re, Italy). Each fungicide treatment was applied every 7-12 days during blossoming and maturation (from April to August). The last treatment was carried out, on average, 28 days before the grape harvest. The fungicide treatments were: 1,000 mg/kg

of azoxystrobin, 400 mg/kg of dinocap, 300 mg/kg of fenarimol, 300 mg/kg of penconazole and 350 mg/kg of quinoxyfen. Only dinocap was applied 3 times, because, it is usually used to start the defence strategy. A random-block design was used with three replications for each test, and each block contained 60 plants.

Wine making process

The grapes were placed in crates, refrigerated at 4°C and transported quickly to the Genna Cellar (Marsala – TP) where vinification took place.

White Vinification. Newly – cropped *Vitis vinifera* from Sicily and Campania were crushed and destemmed; they were then soft pressed using a pneumatic press. The first must obtained was treated with SO_2 (30 g/hL), pectolytic enzymes (1.5 g/hL) and vitamin C (5 g/hL) to favour clarification before fermentation; the temperature was maintained at 8°C for 24 hours. Clear must was spiked with 20 g/hL of thiamin and ammonium phosphate as fermentation coadjuvant and 30 g/hL of selected yeasts were added for fermentation which was kept at 15°C. To remove the lees, after fermentation the wine was decanted into a tank and spiked with SO_2 (5 g/hL). After 10 days, the wine was decanted again and treated with SO_2 (5 g/hL). Subsequently the wine was filtered twice through 1 µm and 0.45 µm cardboard filters, spiked with SO_2 (2-3 g/hL) and bottled in dark bottles at 4°C for the duration of the experiment. Each sample from Sicily and Campania were separately vinified following the protocol mentioned above.

Red vinification. Newly – cropped *Vitis vinifera* from Tuscany were crushed and destemmed and then spiked with SO_2 (5 g/hL) and selected yeasts were added (30 g/hL) and let to ferment for 10 days at 28°C, effecting three fullings a day. The wine was drawn from the vat and the marc was pressed with a hydraulic press. After 4 weeks the lees were removed and the wine was spiked with SO_2 (2-3 g/hL). Subsequently the wine was spiked again with SO_2 (2-3 g/hL) and bottled in dark bottles and kept at 4°C for the duration of the experiment.

Reagents

Analytical standards of the pesticides were purchased from Dr. Ehrenstorfer (Angsburg, Germany) (azoxystrobin, dinocap, penconazole, and bromophos-methyl) and from Dow Agrosciences (Rotterdam, The Netherlands) (fenarimol and quinoxyfen). Bromophos-methyl was used as the internal standard.

N-hexane (Carlo Erba, Milano, Italy) and ethyl-acetate (Baker, Deventer, The Netherlands) were used for pesticide residue determination. N-hexane was used to solubilize the pesticide standards and the solutions were stored at +4°C in a refrigerator.

Apparatus and chromatography

An HRGC 5300 Mega Series gas chromatograph (Carlo Erba Instruments) was used to determine the five fungicides. It was fitted with an electron capture detection (ECD) system and split-splitless injector, connected to an HP 3394 A reporting integrator (Hewlett Packard, Avanlade, PA, USA).

An SPB-5 fused – silica column (30 m x 0.25 mm i.d.) was employed, with 5% diphenyl 95% dimethyl siloxane as liquid phase (film thickness 0.25 µm) (Supelco, Bellefonte, PA, USA). The injector and detector operated at 250° and 280°C, respectively.

The sample (1 µL) was injected in the split mode (1:3), and the oven temperature was programmed as follows: 200°C (1 min) raised to 270°C (5°C/min), held for 20 min. Helium was the carrier gas and nitrogen the make-up gas both at 150 KPa.

Extraction procedure

Ten grams of the sample (grapes, must, marc and wine) were collected in a 100 mL Erlenmeyer flask with a teflon-lined screw-cap. Twenty milliliters of hexane-ethyl acetate (9:1, v/v) were added and the mixture was sonicated (Transsonic T420, Elma) for 30 min. The supernatant (2 mL) was evaporated under nitrogen flow; 0.2 mL of the internal standard solution (bromophos-methyl 1.00 mg/kg) was added, and the mixture was directly analyzed by gas chromatography. No clean-up was necessary because there was no interference in the chromatogram.

Recovery trials

Previously, a blank assay was used to check for the absence of residues in the grapes, must, marc and wine. Untreated samples of grapes, must, marc and wine from the three regions were spiked with 0.01, 0.1 and 1.0 mg/kg of the fungicides. The samples were allowed to equilibrate for 60 min prior to extraction, and were processed according to the procedure described earlier. The recovery assays were replicated 3 times. Since the extraction procedure of the active ingredients was not influenced by the place of origin of the samples, and the amount recovered did not depend on the cultivar, soil or climate, the recovery percentage was calculated from the data of the three regions.

RESULTS AND DISCUSSION

Azoxystrobin, fenarimol, penconazole, and quinoxyfen were confirmed using the retention times of the standard solutions, ranging from 0.05 to 1 mg/kg. Dinocap was identified using the retention times of the six isomers.

The active ingredients were quantitatively determined by the internal standard method; the calibration curves were obtained by plotting peak heights versus concentrations. Good linearity was achieved in the range 0.05 to 1 mg/kg; the correlation coefficients were 0.9986 for azoxystrobin, 0.9977 for dinocap, 0.9980 for fenarimol, 0.9987 for penconazole, and 0.9980 for quinoxyfen.

The results of the recoveries reported in Table 1 show that the method is simple, fast and repeatable. Natural components of skins, grapes, etc. did not interfere with the analyses. The recovery values ranged from 80.4% (marc) to 100.7% (grapes) for azoxystrobin; from 81.8% (marc) to 110.4% (wine) for dinocap; from 82.8% (grapes) to 98.2% (wine) for fenarimol; from 80.9% (marc) to 97.3% (wine) for penconazole; from 87.0% (wine) to 95.8% (wine) for quinoxyfen. These values show that the extraction method described is appropriate for the determination of residues of these compounds.

The quantitation limit (QL) for azoxystrobin, fenarimol, penconazole and quinoxyfen was 0.005 mg/kg and 0.010 mg/kg for dinocap. The determination of the QL was based on the standard deviation of the response and the slope of the calibration curve (PHARMEUROPA, 1999). The detection limits (setting a signal to noise ratio of 2) (PHARMEUROPA, 1999) were $3x10^{-4}$ ng for azoxystrobin, fenarimol, penconazole and quinoxyfen and $6x10^{-4}$ ng for dinocap. The results are reported in Table 2.

The azoxystrobin residues in grapes ranged from 0.005 mg/kg (Campania) to 0.077 mg/kg (Sicily); in marc from 0.016 mg/kg (Campania) to 0.078 mg/kg (Sicily); in must from 0.014 mg/kg (Campania) to 0.034 mg/kg (Sicily); in wines from 0.005 mg/kg (Campania) to 0.021 mg/kg (Sicily). The residue levels in the wines were nearly 75% lower than in the marcs, and nearly 51% lower with respect to the must. CABRAS *et al.* (1998) reported that azoxystrobin residues are preferably adsorbed on the skins.

In the grapes treated with dinocap, all

Table 1 - Recovery of azoxystrobin, dinocap, fenarimol, penconazole and quinoxyfen (% ±s.d.) from grapes, marc, must and wine.

	Fortification level (mg/kg)	azoxystrobin	dinocap	fenarimol	penconazole	quinoxyfen
grapes	1	100.7±4.9	89.4±3.7	87.9±2.8	88.7±2.8	95.1±3.6
	0.1	87.6±3.2	90.5±3.8	92.8±2.5	91.4±3.9	93.3±3.9
	0.01	82.9±2.1	94.1±4.8	82.8±2.6	85.5±2.3	87.2±2.8
marc	1	80.4±2.2	89.8±3.0	91.6±3.7	88.7±3.1	87.2±2.9
	0.1	84.4±3.6	93.8±3.9	92.6±3.7	90.9±3.9	87.8±2.3
	0.01	87.3±2.8	81.8±2.9	92.7±4.1	80.9±2.5	93.3± 3.7
must	1	93.8±4.0	101.3±4.7	90.3±3.6	89.8±3.9	87.6±2.4
	0.1	94.7±4.5	85.3±2.8	88.6±4.2	89.9±3.9	88.8±3.3
	0.01	87.6±2.5	87.7±2.9	88.2±3.6	86.4±3.0	87.2±2.2
wine	1	86.6±3.5	110.4±5.0	98.2±5.4	97.3±4.5	93.4±4.3
	0.1	92.5±3.3	96.5±4.4	88.9±3.5	93.9±3.8	87.0±3.0
	0.01	90.2±4.1	89.5±2.6	90.6±3.7	94.7±3.9	95.8±4.7

Table 2 - Fungicide residues (mg/kg ±s.d. x 10^{-3}) in grapes, marc, must and wine. Each value is the mean of three determinations.

	azoxystrobin	dinocap	fenarimol	penconazole	quinoxyfen
grapes s	0.077±9	≤0.010	0.046±6	0.065±6	≤0.005
marc s	0.078±12	0.023±3	0.282±17	0.093±9	0.030±3
must s	0.034±4	≤0.010	0.051±5	0.039±3	0.006±1
wine s	0.021±6	≤0.010	0.013±2	0.010±1	≤0.005
grapes c	0.005±1	≤0.010	0.011±2	0.042±4	0.007±1
marc c	0.016±3	≤0.010	0.051±6	0.104±5	0.098±8
must c	0.014±2	≤0.010	0.043±5	0.032±2	0.006±1
wine c	0.005±1	≤0.010	0.006±2	0.008±2	≤0.005
grapes t	0.038±5	≤0.010	0.146±2	0.030±3	≤0.005
marc t	0.076±9	0.024±3	0.293±15	0.109±7	0.028±3
must t	0.024±3	≤0.010	0.165±13	0.050±5	≤0.005
wine t	0.012±2	≤0.010	0.070±6	0.012±1	≤0.005

s Sicily; c Campania; t Tuscany.

the values were below the QL; in Sicilian marc there were 0.023 mg/kg of dinocap and in Tuscan marc 0.024 mg/kg; in must and wine all the values were below the QL.

Fenarimol residues in grapes ranged from 0.011 mg/kg (Campania) to 0.146 mg/kg (Tuscany); in marc from 0.051 mg/kg (Campania) to 0.293 mg/kg (Campania); in must from 0.043 mg/kg (Campania) to 0.165 mg/kg (Tuscany); in wine from 0.006 mg/kg (Campania) to 0.070 mg/kg (Tuscany). The decrease in pesticide residues in wine and marc was very high (almost 86%). These values are comparable to results of other studies (NAVARRO *et al.*, 1999).

Penconazole residues in grapes ranged from 0.030 mg/kg (Tuscany) to 0.065 mg/kg (Sicily); in marc from 0.093 mg/kg (Sicily) to 0.109 mg/kg (Tuscany); in must from 0.032 mg/kg (Campania) to 0.050 mg/kg (Tuscany); in wines from 0.008 mg/kg (Campania) to 0.012 mg/kg (Tuscany). There was a 75% decrease

in penconazole residues in wine with respect to the must. NAVARRO *et al.* (1999) reported that a 85% reduction of penconazole residue is usually observed from must to wine.

Quinoxyfen residues in grapes ranged from 0.005 to 0.007 mg/kg (Campania); in marc from 0.028 mg/kg (Tuscany) to 0.098 mg/kg (Campania); in must from 0.005 to 0.006 mg/kg (Campania); all the values were below the QL in wine.

The residues of quinoxyfen tend to accumulate completely in the solid fraction, leaving no residues in the wine (CABRAS *et al.*, 2000; DUGO *et al.*, 2001).

As reported in Table 2, quinoxyfen decreased more rapidly with respect to fenarimol and penconazole. The levels observed after the same time in the same samples were much lower than the fenarimol and penconazole residues. These data showed that this compound could also be used to produce wine free from detectable amount of residues.

In conclusion, the residues found in all wines were below the limits that the Italian law (D.M. 19 May 2000) has established for grapes (2 mg/kg for azoxystrobin, 1 mg/kg for dinocap, 0.3 mg/kg for fenarimol, 0.1 mg/kg for penconazole and 0.5 mg/kg for quinoxyfen) and wines (0.5 mg/kg for azoxystrobin and 0.01 mg/kg for quinoxyfen).

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SHORT COMMUNICATION

EFFECT OF SALT DOSE ON THE QUALITY OF DRY SMOKED BEEF

EFFETTO DI DIVERSE CONCENTRAZIONI DI SALE SULLA QUALITÀ DELLA CARNE BOVINA ESSICCATA

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ABSTRACT

Longissimus dorsi muscles from beef carcasses were dry-salted at doses of 0, 25, 50, 75 and 100 g salt/kg meat. Salted samples were kept in a cooler at 2°C for 5 days after which time one treatment group of the cured meat was pre-dried at 35°C for 2 h, smoked at 88°C for 6 h and then dried at 95°C to a constant weight for about 52 h. Proximate analysis showed that the moisture and protein contents of the samples decreased with increased salt content of the meat, while the ash content

RIASSUNTO

Le carni di *Longissimus dorsi*, provenienti da carcasse bovine, sono state salate a secco con dosi pari a 0, 25, 50 e 100 g di sale/kg di carne. I campioni salati sono stati mantenuti a 2°C per 5 giorni per poi essere pre-essiccati a 35°C per 2 ore, affumicati a 88°C per 6 ore e finalmente essiccati a 95°C fino a peso costante per circa 52 ore. L'analisi prossimata ha mostrato che l'umidità ed il contenuto in proteine dei campioni diminuiva con l'aumentare del contenuto salino, mentre il contenuto in ceneri

- Key words: beef, dry, salted, smoked -

increased. The pH of the fresh meat increased with increasing salt. In general, the addition of salt significantly (P<0.05) increased the degree of oxidation, while smoking produced antioxidant activity. Iron and phosphorus decreased with increasing salt content. With salting, smoking and drying of the meat the microbial loads generally decreased but the halophilic microorganisms increased.

aumentava. Il pH della carne fresca aumentava all'aumentare del sale. In generale, l'aggiunta di sale aumentava significativamente (p<0.05) il grado di ossidazione, mentre l'affumicamento favoriva l'attività antiossidante. Il contenuto in ferro e fosforo diminuiva all'aumentare del contenuto in sale. Con la salatura, l'affumicamento e l'essiccamento della carne, la carica microbica totale tendeva a diminuire, permettendo, però un aumento della concentrazione dei microrganismi alofili.

INTRODUCTION

At high ambient temperatures, meat spoils within a few hours after the onset of rigor mortis due to its high moisture and protein contents. In tropical African countries, and particularly in Cameroon, the major meat producing areas are far from large consumer centres. The principal constraints for the distribution of meat and meat parts are the lack of distribution and refrigeration facilities. In rural areas, the conservation of meat by relatively unsophisticated techniques is of paramount importance. Among the many preservation methods, drying and smoking were two of the earliest and most effective methods developed in rural areas where refrigeration was not available (MUSONGUE *et al.*, 1994; DZUDIE and OKUBANJO, 1992; OCKERMAN *et al.*, 2000).

Another method of preserving meat is by curing it with salt followed by drying and / or smoking (ALONGE and AGBA, 1981; ZAPATA *et al.*, 1990). In temperate developed countries, a wet-curing process has been developed which involves the use of a solution of salt, sodium nitrate and / or nitrite. In addition, light smoking is often used to impart a desirable flavour to the product. However, in order to limit undesirable microbiological activity, a low ambient temperature is necessary while the salt solution is penetrating into the meat. The dependence on the availability of refrigeration facilities has prevented widespread application of this technique in developing countries. Though salted, smoked and dried beef has been accepted for many years in Cameroon, little is known regarding the chemical, nutritional and microbial quality of such products.

With the aim of increasing and diversifying the supply of locally-produced and processed meat in urban areas, the present study was carried out to determine the effects of curing, smoking and drying on the physico-chemical and microbial properties of beef.

MATERIALS AND METHODS

Materials

Beef filets (musculature surrounding the dorsal part of the spinal column, mainly *longissimus dorsi*) were obtained from a local retail butcher. All visible fat and connective tissue were trimmed from the filets. Each filet was then divided into portions of approximately 400 g each. For each replication, ten portions were obtained and randomly assigned to two treatment groups of five portions each.

Salting

In each treatment group, dry curing salt was applied uniformly to the 5 portions at 0, 25, 50, 75 and 100 g per kg meat, respectively, which was applied at two-day intervals. The total curing time was five days at 2°C. Cuts were weighed before and after curing to determine weight loss.

After curing, portions of meat from one treatment group were packed in cellulose film and stored at 2°C for further analysis. Portions from the other group were smoked and dried. The smoke-dried meats were packed in cellulose film and stored at room temperature (ca 25°C) until required for analysis.

Smoking and drying

The smoking and drying processes were carried out in a Matindex air-conditioned smokehouse (model 74560 Lamurax, Halmaln, Germany) with a matindex smoke generator (model 80, Lamurax, Halmaln, Gremany). The meat portions were placed on wire racks such that no portion touched another during the smoking process. They were pre-dried at 35°C for 2 h (HR=90%), smoked at 90°C for 6 h (HR=80%) and dried at 95°C for 52 h (HR=60%). The meat portions were weighed at 2 h intervals during each processing step to determine weight loss. During processing, shelf temperatures were monitored using copper thermocouples with a digital temperature recorder (Didalab-model 918, Gesvrine, France).

Analytical techniques

All analyses were carried out on samples that were ground twice through a 4 mm plate (Moulinex Model 133, Alençon, France). Proximate analysis, mineral composition, pH and thiobarbituric acid (TBA) measurements were carried out on fresh and smoke-dried meat, while the microbiological analysis were carried out on smoke-dried samples. The moisture content was determined by oven drying, the protein content by using the Kjedahl nitrogen estimation, the fat content by soxhlet extraction with petroleum ether and ash content by incineration at 525°C as described in the AOAC methods (1980). Sodium chloride as chloride was determined by dissolving the ash in boiling water and titrating against nitrate (AOAC, 1980).

To measure pH, 10g of ground meat sample was homogenized in 100 mL of distilled deionised water. The pH was determined using a Minisi pH meter (Model 8000, Nantes, France). Thiobarbituric acid (TBA) reactive substances were measured as described by WITTE et al.(1970). The results are expressed as mg malonaldehyde / kg of meat.

Samples for mineral analysis were prepared according to AOAC (1980) procedures, and the minerals (except phosphorus) were assayed spectroscopically using an atomic absorption spectrophotometer (Model 1100, Perkin-Elmer, Norwalk, USA)). Phosphorus was determined following the colorimetric molybdenum (Mo)-blue method (AOAC, 1980).

Microbiological analysis

To count the viable aerobic microorganisms and fungal growth, 30 g of ground samples from each treatment group were homogenised in a sterile lab blender stomacher (Model 400, London, UK) in 90 mL of sterile peptone (0.1 g/ 100 mL) water. Subsequent dilutions were also made in peptone water. Difco standard plate count agar was used to count the viable, aerobic microorganisms. These plates were incubated at 32°C for 48 h. To enumerate fungal growth, 10% tartaric acid was used. The plates were incubated at 25°C for 120 h. Sulfite reducing bacteria were determined by transferring 10 mL of initial homogenate to a 20 mL test tube, which was heated to 80°C for 10 min before plating anaerobically on trypticase sulfite

(TSN) agar containing neomycin and incubated at 37°C for 24 h.

For Salmonella, 25 g of minced sample were blended with 75 mL of sterile peptone water in a stomacher bag. A 5 mL aliquot of the homogenate was incubated in 10 mL selenite cystine broth at 37°C for 18 h, after which 1 mL was spread on the surface of Salmonella agar which was incubated at 37°C for 24 h. Colonies were counted after incubation.

Statistical analysis

The experiments were performed in triplicate and the means ± standard deviation of three values are reported. Data were subjected to one way analysis of variance, and Ducan's multiple range test (STEEL and TORRIE, 1980) was used to determine the significant differences among means.

RESULTS AND DISCUSSION

Fig. 1 shows weight changes during pre-drying, smoking and drying of samples. During the first 12 h of processing, all the samples dried at the same rate, after which, the unsalted meat dried faster than the salted meat. As the dose of salt in the meat increased, the salt served as a great obstacle to the diffusion of water from within the muscle to the surface of the meat by acting as a barrier against the establishment of an appropriate osmotic gradient. At the end of the drying period, the unsalted meat had lost more weight than the salted cuts. DZUDIE and OKUBANJO (1992) reported that at the end of the ageing period (27°C for 4 weeks), cured rabbit cuts lost less weight than uncured ones. However, KEMP *et al.* (1986) observed that injected hams absorbed salt faster but lost more weight than non-injected hams. The discrepancy in the published data could be attributed to the differences in the curing methods and drying temperatures.

The means for the proximate analysis are shown in Table 1. The moisture content of the fresh meat significantly ($P<0.05$) decreased as the salt concentration in the meat increased. The highest percent weight loss recorded for the salted samples over the unsalted ones during curing could account for the observed results. During smoking and drying of the meat the salt formed a dried layer on the surface of the meat, reducing the rate of diffusion of water from within the muscle cells to the surface. Consequently, the moisture content of the dried meat increased with the amount of salt applied to the meat.

A slight decrease in the protein content of the meat was observed as the salt dose in the meat increased. During osmotic withdrawal of water from the interior of the meat cuts, part of the salt-soluble protein is lost as drip.

The lipid content of the meat was not affected by the salt treatments. Increase in the lipid content of the dried meat over the fresh meat was due to the dehydration effect of smoking and drying. The ash content in the two types of meat significantly ($p<0.05$) increased as the amount of salt applied to the surface increased. This was expected and can be attributed to the increasing salt concentrations in the different samples. OWEN *et al.* (1986) found that the mean ash content of processed cured chicken was significantly higher than that of fresh uncured chicken.

The pH of the fresh meat significantly ($p<0.05$) increased as the amount of salt added to the meat increased (Fig. 2). The same trend was observed with the smoke-dried samples, except at 50 g added salt/kg meat, the pH of the samples started to decrease. The increase in the pH of the fresh and salted meat can be attributed to the effect of salting on muscle proteins. BABJI *et al.* (1982) found that the pH of salted turkey breast muscles was higher than that of unsalted samples. In the present study, the variation observed in the pH changes of the sam-

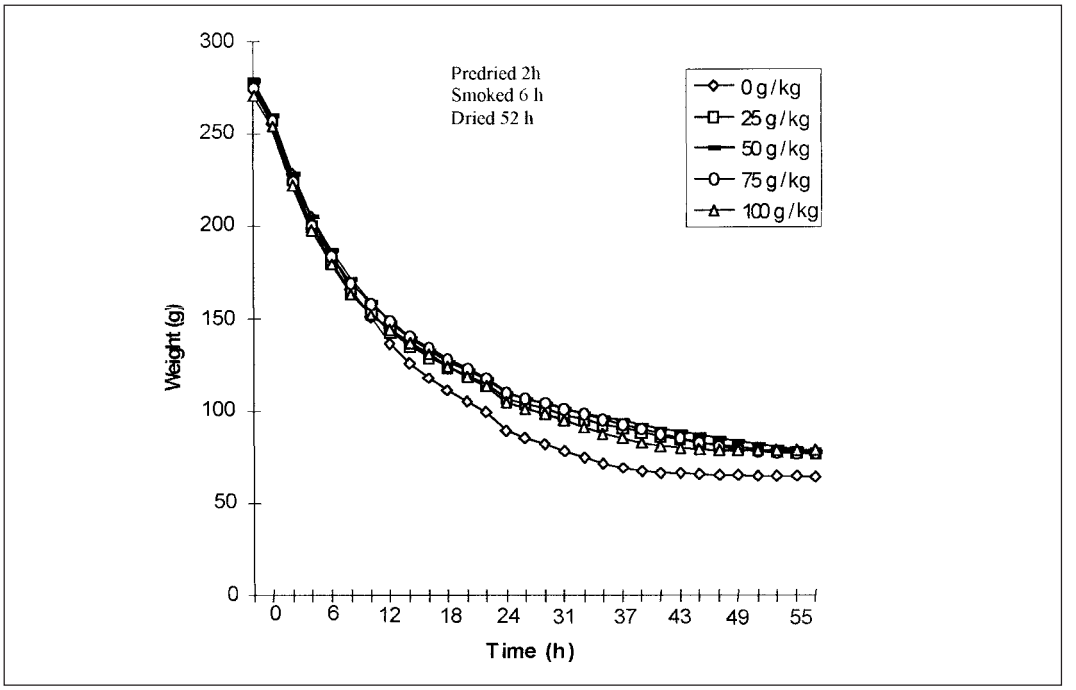


Fig. 1 - Weight changes during pre-drying, smoking and curing of meat with different concentrations of salt.

Table 1 - Proximate composition* of fresh and smoke-dried meat subjected to salt treatments.

		Salt treatment (g/kg)				
	Meat	0	25	50	75	100
Moisture	Fresh	76.95±0.57^{a}	74.24±0.47^{b}	73.63±0.6±1^{b}	71.68±0.50^{c}	69.68±0.93^{d}
	Smoke-dried	7.61±0.25^{d}	12.85±0.15^{c}	12.88±0.14^{c}	13.80±0.22^{b}	15.45±0.39^{a}
Proteins	Fresh	19.10±0.95^{a}	18.372±2.21^{b}	18.30±0.89^{b}	17.52±0.43^{c}	16.62±0.45^{c}
	Smoke-dried	65.27±1.20^{a}	56.12±0.10^{b}	54.11±0.10^{b}	52.45±1.46^{b}	45.26±0.24^{c}
Lipids	Fresh	4.02±0.09^{a}	4.26±0.99^{a}	4.43±0.79^{a}	4.50±0.17^{a}	5.09±0.26^{a}
	Smoke-dried	7.73±0.63^{a}	8.19±0.39^{a}	7.83±0.67^{b}	8.50±0.05^{b}	8.63±0.14^{c}
Ash	Fresh	1.14±0.01^{d}	3.12±0.06^{c}	5.27±0.42^{b}	5.45±0.03^{b}	7.90±0.01^{a}
	Smoke-dried	7.80±0.17^{d}	12.37±0.22^{c}	18.24±1.32^{b}	20.99±0.46^{b}	22.43±0.93^{a}

* values expressed as % standard deviation;
^{abcd} values in the same row with different superscripts are significantly different ($p<0.05$).

ples during smoking and drying of the meat can be attributed to the change in the ionic strength surrounding the muscle proteins. DZUDIE and OKUBANJO (1992) reported that the pH of smoke-dried rabbit meat was related to the salt

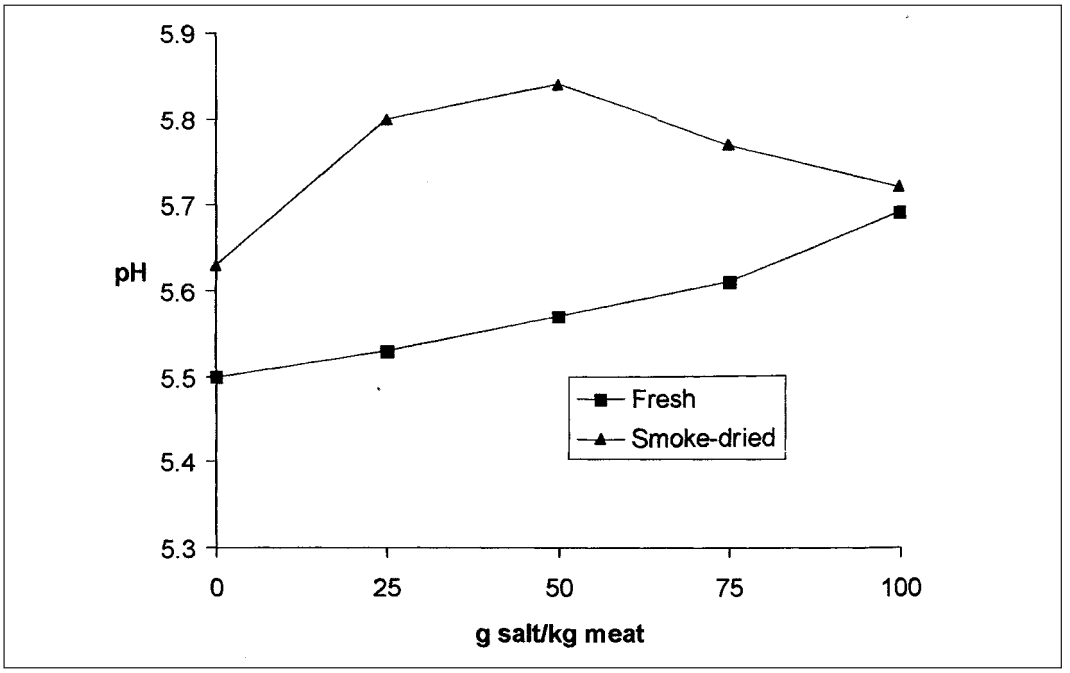


Fig. 2 - Effect of salt doses on the pH of fresh and smoke-dried meat.

content of the meat. In the present study, the pH values of the smoke-dried meat were higher than those of the fresh and salted samples. In contrast, VISHWANATH *et al.* (1998) found that the pH of smoked mud eel fish was more acidic than that of the fresh fish. The discrepancies between the results of these tests could be due to differences in processing methods because the above-mentioned investigators did not treat their samples with salt as in the present study.

The stability of lipids in the fresh and smoke-dried meat cured at different salt levels was evaluated by the TBA procedure (Fig. 3). Overall, the results indicated that each salt level had a pro-oxidant effect on the lipids in the fresh samples. These results confirm those of WHEELER *et al.* (1990) who found that TBA values in restructured beef steaks were significantly ($P < 0.05$) higher in salted samples than in unsalted ones.

In a second study, concerned with the effect of smoke and the level of salt on the stability of lipids in smoke-dried beef, the antioxidant activity of the smoke was evident. The TBA values of the smoke-dried meat were significantly lower ($P < 0.05$) than those of the non-smoked counterparts. The preservative effect of wood smoke on food is the result of partial surface dehydration and deposition of anti-oxidant compounds from the smoke on the surface on the meat. As observed in the present study, there was first an apparent decrease followed by an increase in the TBA values of the smoke-dried meat as the level salt added to the meat increased. On the other hand, CUPPETT *et al.* (1989) found that adding salt significantly ($P<0.05$) increased the TBA values of smoked great lakes white fish. Obviously TBA values are not good indices of the quality for these dried meats. ZAPATA *et al.* (1990) reported that malonaldehyde is not an end product of the oxidative changes in meat products.

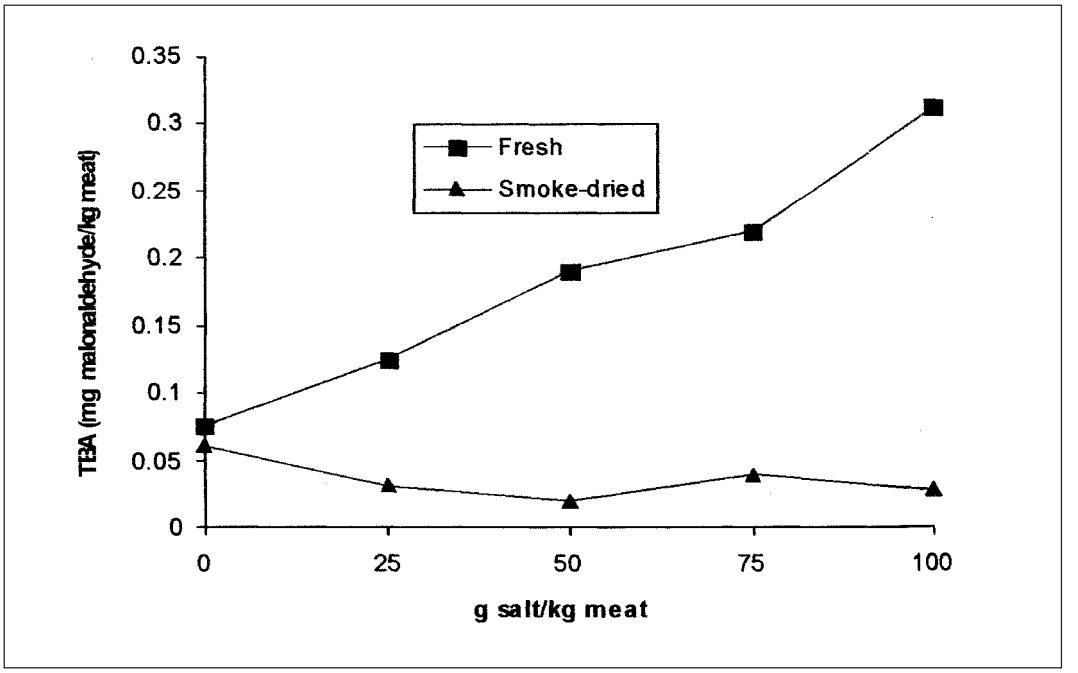


Fig. 3 - Effect of salt doses on TBA of fresh and smoke-dried meat.

Table 2 - Mineral content and microbiological count* of fresh and smoke-dried meat.

		Salt treatment (g/kg)				
	Meat	0	25	50	75	100
NaCl (%)	Fresh	ND	4.01±0.18^{d}	5.47±0.17^{c}	5.77±0.19^{b}	8.80±0.12^{a}
	Smoke-dried	ND	5.69±0.16^{d}	11.82±0.57^{c}	13.03±0.61^{b}	14.600.14^{a}
Calcium	Fresh	17.06±2.14^{b}	18.0±41.87^{c}	17.54±1.97^{c}	20.48±1.12^{b}	26.821.79^{a}
(mg/100g)	Smoke-dried	29.63±1.17^{d}	33.9±11.82^{c}	51.31±4.30^{b}	78.92±2.01^{a}	78.581.86^{a}
Phosphorus	Fresh	446.57±119.78^{a}	253.22±74.29^{b}	236.60±16.11^{b}	229.00±38.16^{b}	180.71±38.81^{c}
mg/100g)	Smoke-dried	1114.81±173.11^{a}	748.98±62.96^{b}	691.06±82.22^{b}	484.14±62.33^{c}	431.89±37.12^{c}
Magnesium	Fresh	22.28±11.56^{c}	59.75±18.1^{b}	49.03±12.51^{b}	67.73±18.75^{a}	73.28±11.73^{a}
(mg/100g)	Smoke-dried	292.66±20.69^{c}	314.04±13.12^{c}	324.24±16.74^{d}	341.54±12.16^{a}	348.64±10.77^{a}
Iron	Fresh	0.94±0.15	0.56±0.10	0.21±0.17	0.25±0.16	0.28±0.10
(mg/100g)	Smoke-dried	2.96±0.10^{a}	1.97±0.31^{a}	0.55±0.16^{c}	0.55±0.18^{c}	0.56±0.11^{c}
Aerobic mesophilic (CFU/g)	Smoke-dried	$1.03.10^{3}$	$2.33.10^{3}$	$1.80.10^{3}$	$1.43.10^{3}$	$1.05.10^{3}$
Fungal counts (CFU/g)	Smoke-dried	$5.1.10^{3}$	$6.2.10^{3}$	$7.0.10^{3}$	$9.8.10^{3}$	$9.0.10^{3}$

* values standard deviation; ^{abcd} values in the same row with different superscripts are significantly different ($p<0.05$); ND: Not determined.

Regardless of the dose of salt added to the meat, the mineral content of the smoked-dried meats was higher than that of the fresh-salted samples (Table 2). MBOFUNG (1993) found that the mineral content of Kilishi was higher than that of fresh beef. Sodium chloride, calcium and magnesium values of fresh, as well as smoked-dried meat, increased with the dose of salt added. This increase is reflected in the increased ash content. A general decrease in phosphorus and iron values could be attributed to the loss of these minerals through drip during dry curing. Because this product is a typical food in very restricted areas, no specific data have been found in the literature about its mineral composition. Table 2 shows that aerobic plate counts decreased as the dose of salt added to the meat increased. These results were expected due to the effect of salt on microbial growth. An inverse trend was observed with fungal counts. OTENG-GYANG (1984) reported that halophilic microorganisms constituted the principal microorganisms of salted and smoked meat products. Salmonella and sulfite-reducing bacteria were not present in any of the samples analysed

Data from this study show that the greatest weight loss was recorded for the unsalted meat. Salting of the meat both increased the pH and TBA values of the meat. The smoke-dried meat underwent less oxidation than the fresh samples. The high salt content of the meat should not be a problem because it could be used for stewing or cooking without using any additional salt or after desalting it with water.

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SHORT COMMUNICATION

INFLUENCE OF HYPOCHLORITE CONCENTRATION, pH AND TEMPERATURE ON INACTIVATION OF *LISTERIA MONOCYTOGENES*

INFLUENZA DELLA CONCENTRAZIONE DI IPOCLORITO, DEL pH E DELLA TEMPERATURA SULL'INATTIVAZIONE DI *LISTERIA MONOCYTOGENES*

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ABSTRACT

The effect of hypochlorite on *Listeria monocytogenes* at varying concentrations, pH, temperature, and type of solution (with or without peptone in phosphate buffer) was studied. *L. monocytogenes* was reduced by about 3.55, 4.17, 4.56 and 5.59 log units, respectively, after 30 sec of exposure to 5, 10, 50 and 100 mg L^{-1} hypochlorite in phosphate buffer (pH 6.5) at 30°C. There was an increase in the rate of disinfection ($p<0.05$) with an increase in temperature from 20° to 40°C. Hypochlorite had

RIASSUNTO

È stato studiato l'effetto di diverse concentrazioni di ipoclorito sull'inattivazione di *Listeria monocytogenes* al variare del pH, della temperatura e di diversi tipi di soluzione (con e senza peptone in tampone fosfato). La concentrazione iniziale di *L. monocytogenes* si è ridotta di 3,55, 4,17, 4,56 e 5,59 unità logaritmiche dopo esposizione per 30 sec a 5, 10, 50 e 100 mg L^{-1} di ipoclorito in tampone fosfato (pH 6,5) a 30°C. Si è notato un incremento del grado di inattivazione ($p<0,05$) con incrementi di

- Key words: antimicrobial effect, hypochlorite, *Listeria monocytogenes* -

a greater antimicrobial effect on *L. monocytogenes* at pH 4.5 than at 5.5, 6.5 and 8.5. Addition of 0.1% peptone provided a marked neutralization of the chlorine in the phosphate buffer. Survival curves showed two distinct phases in the destruction of *L. monocytogenes*. At all hypochlorite concentrations, the first phase (about 30 sec) was characterized by a higher rate of inactivation, shown by a steeper inactivation slope, which was followed a more gradual slope.

temperatura tra 20° e 40°C. È stato osservato un maggior effetto antimicrobico dell'ipoclorito su *L. monocytogenes* a pH 4,5 rispetto a pH di 5,5, 6,5 e 8,5. L'aggiunta di peptone allo 0,1% ha portato ad una marcata neutralizzazione dell'ipoclorito nel tampone fosfato. Le curve di sopravvivenza hanno mostrato due distinte fasi di inattivazione *di L. monocytogenes*. A tutte le concentrazioni di ipoclorito, la prima fase (di ca. 30 sec) è caratterizzata da un maggior grado di inattivazione, evidenziato da una maggior pendenza della curva di inattivazione, seguita poi da una pendenza più dolce nella seconda fase.

INTRODUCTION

Food, such as milk, milk products, meat, frankfurters, poultry and seafood, are usually preserved by refrigeration. Refrigeration temperatures, however, offer an appropriate environment for multiplication of *Listeria monocytogenes* (SWAMINATHAN, 2001). *L. monocytogenes* is a small Gram-positive bacterium which can be found in food equipment, soil and water (KIM *et al.*, 2000; SWAMINATHAN, 2001). This organism has been implicated in several outbreaks of food and water-borne diseases (ERKMEN, 2000; KIM *et al.*, 2000; RAY, 1996; SWAMINATHAN, 2001). Listeriosis can be transmitted through consumption of *Listeria* contaminated foods or from a wide range of infected animals (ADAMS and MOSS, 1995; RAY, 1996). *L. monocytogenes* is ubiquitous, moderately heat resistant and psychrotrophic and can grow under refrigeration in the absence of oxygen (ADAMS and MOSS, 1995; FOEGEDING and LEASOR, 1990; RAY, 1996; SWAMINATHAN, 2001).

Sanitizing practices are necessary to improve the microbiological safety of food products (FAVIER *et al.*, 2001; RAY, 1996). Chlorine is widely used as a disinfectant in the food industry and for the treatment of drinking water in community distribution systems and reservoirs (ODLAUG, 1981; HAN *et al.*, 2002). Chlorine compounds are effective against bacteria, yeasts, molds, spores and viruses and require short to moderate contact time. Chlorine is readily available in several forms (liquid chlorine, hypochlorites, inorganic and organic chloramines, and chlorine dioxide).

Unlike some of the other disinfection methods, several simple test kits exist to measure the availability of chlorine (residual chlorine). The antimicrobial (germicidal) action of the chlorine compounds is due to the oxidizing effect of chlorine on the –SH group in many enzymes and structural proteins. In addition, damage to membranes, destruction of protein synthesis, reactions with nucleic acids, and interference with metabolism have been suggested (RAY, 1996). Its efficacy against a broad spectrum of microorganisms is well documented (TRUEMAN, 1971; ODLAUG, 1981).

The overall objective of this study was to evaluate the behavior of *L. monocytogenes* against different hypochlorite

concentrations at different pHs, temperatures and types of solutions.

MATERIALS AND METHODS

Bacterial strain

L. monocytogenes type 4a KUEN 136 was obtained from the Microorganisms Collection Center, Faculty of Medicine, University of Istanbul (Istanbul, Turkey). The culture was maintained on brain heart infusion agar (BHIA; Difco, Detroit) slants and stored at 4°C. The cultures were subcultured twice by inoculating them into 10 mL of brain heart infusion broth (BHIB; Difco, Detroit) and incubating at 35°C for 18 h in a bench-type water bath shaker ST-402 (NÜVE; Sanayi Malzemeleri Imalat ve Ticaret A.S., Istanbul Turkey) with shaking (120 strokes per min). After centrifugation supernatant fluids were discarded and cell pellets were washed twice with a 0.1 M phosphate buffer (pH 6.5) solution. They were then suspended in phosphate buffer solution to yield about $2.2x10^8$ colony forming unit (cfu) mL^{-1} and used in the experiments. Cell suspensions were prepared immediately before each treatment.

Preparation of test solutions

All glassware was thoroughly cleaned and rinsed with water free of residual chlorine before use. A stock hypochlorite solution was prepared from household bleach (sodium hypochlorite with 92.66 g L^{-1} of available chlorine) with phosphate buffer to provide 5, 10, 50, and 100 mg L^{-1} hypochlorite concentrations when mixed with the inoculum. A sodium thiosulphate solution (0.1 mol L^{-1}) was used to neutralize the antimicrobial activity of hypochlorite. All solutions were prepared fresh.

Available chlorine concentration in the original and prepared solutions was determined by the standard idiometric titration method (ANONYMOUS, 1985).

Procedure

The system was designed to determine rates of bacterial inactivation but not intended to simulate natural environments. Thus, hypochlorite concentrations employed here may not be directly comparable with those reported elsewhere.

Hypochlorite solutions (5, 10, 50, and 100 mg L^{-1}) were prepared from household bleach and phosphate buffer at pH 4,5, 5,5, 6,5 and 8,5; in sterile 100 mL Erlenmeyer flasks, 49 mL of each was mixed with 1 mL of bacterial suspension (final concentration about $2.2x10^8$ cfu mL^{-1}) and placed in a water bath at 20, 30 and 40°C. Ten mg L^{-1} of hypochlorite solution in phosphate buffer containing 0.1% peptone was also prepared at pH 4.5. The pH of the phosphate buffer was prepared by mixing 0.1 M K_2HPO_4 and 0.1 M KH_2PO_4 solutions.

After exposure of *L. monocytogenes*, 1 mL of 0.1 mol L^{-1} of sodium thiosulfate was added to neutralize the hypochlorite in solution. Each sample from the initial inoculum and after hypochlorite treatment was serially diluted with 0.1% peptone (Difco, Detroit) solution. Initial counts and the surviving *L. monocytogenes* were determined by spread plating of 0.5 mL of diluted (or non diluted) samples on duplicate plates of BHIA. The plates were incubated at 35°C for 48 h, after which all the characteristic visible colonies on BHIA were counted (ERKMEN, 2000). Each experiment was carried out in duplicate and repeated two times on separate days. The average number of colonies from the duplicate plates was then recorded for each sample.

Analysis of variance was performed on data obtained at different rates of *L. monocytogenes* inactivation of hypochlorite treatment using the computer program, Statgraphics 2.0 (Stsc., Inc., Rockville, MD).

RESULTS AND DISCUSSION

Effect of hypochlorite concentrations

The antimicrobial effect of various hypochlorite concentrations on *L. monocytogenes* in phosphate buffer at pH 6.5 and at 30°C is shown in Fig. 1. After 30 sec exposure to 5, 10, 50, and 100 mg L^{-1} hypochlorite, initially inoculated *L monocytogenes* (about 8.34 log units) dropped by 3.35, 4.17, 4.56 and 5.59 log units, respectively. The sterilization effects (i.e., no survival cells in plate of 1 mL of treated sample) of 5, 10, 50 and 100 mg L^{-1} hypochlorite at pH 6.5 at 30°C on *L. monocytogenes* were observed after 210, 150, 120 and 100 sec, respectively. Inactivation increased with increasing hypohlorite concentration from 5 to 100 mg L^{-1}. The antimicrobial effect of hypochlorides has been previously observed on other microorganisms; about 8 mg L^{-1} chlorine was effective in killing viable *Vibrio cholerae* in broth culture (SOUSA *et al.*, 2001). KIM *et al.* (2000) reported that application of 13 mg L^{-1} of chlorine water (pH 3.90) achieved more than 7.0 log reductions on *L. monocytogenes* in 30 sec and complete elimination in one min. ZHAO *et al.* (2001) reported that chlorine water is even more effective (0.25 mg L^{-1} of free chlorine) and achieved more than 7.0 log reductions on *E. coli* 0157:H7 in one min.

Effect of pH

The effect of 10 mg L^{-1} hypochlorite in phosphate buffer at various pHs at 30°C is shown in Fig. 2. Decreasing the pH of hypochlorite from 8.5 to 4.5 increased the disinfection of *L. monocytogenes* due to the synergistic effect of chlorine and the acidic environment. Hypochlorite showed maximum activity in acid solutions. All initially inoculated (about 8.34 log units) *L. monocytogenes* were completely inactivated after 80 sec exposure to 10 mg L^{-1} hypochlorite at pH 4.5, while it was reduced about 3.59, 5.94 and 7.59 log units were at pH 8.5, 6.5 and 4.5, respectively. The least antimicrobial effect of hypochlorite was observed at alkaline pH (8.5). BLOOMFIELD and MILES (1979) suggested that the bactericidal action of chlorine was produced by hypochlorous acid (HOCl). It probably enters the cell and reacts with the –SH group of proteins. HOCl is stable at acid pH and thus more effective; at alkaline pH, it dissociates to H^+ and OCl^- (hypochlorite ions), which reduces its bactericidal effectiveness. It is also less effective on microorganisms in the presence of organic matter. There were significant ($P<0.05$) differences among the various pH treatments in the number of *L. monocytogenes*. This suggests the importance of the H^+ ion concentration besides HOCl on the survival of *L. monocytogenes*.

Effect of temperature

Effects of 10 mg L^{-1} hypochlorite in phosphate buffer (pH 6.5) at 20, 30 and 40°C is shown in Fig. 3. Reduction in the number of *L. monocytogenes* after 30 sec was about 3.81, 4.17 and 5.32 log units at 20°, 30° and 40°C, respectively. The hypochlorite was found to be more effective ($P<0.05$) at high temperature. The inactivation of *L. monocytogenes* was significantly different ($P<0.05$) at the various temperatures, indicating that the sensitivity of *L. monocytogenes* to temperature changes was not the same.

Effect of composition of solution

The effect of 10 mg L^{-1} hypochlorite in phosphate buffer (pH 6.5) containing 0.1% peptone at 30°C is shown in Fig. 4. A relatively low peptone concentration (0.1%) caused a large decrease in bactericidal activity of hypochlorite against *L. monocytogenes*. All of inoculated *L. monocytogenes* (8.34 log units) were inactivated after 35 min with 10 mg L^{-1} hypochlo-

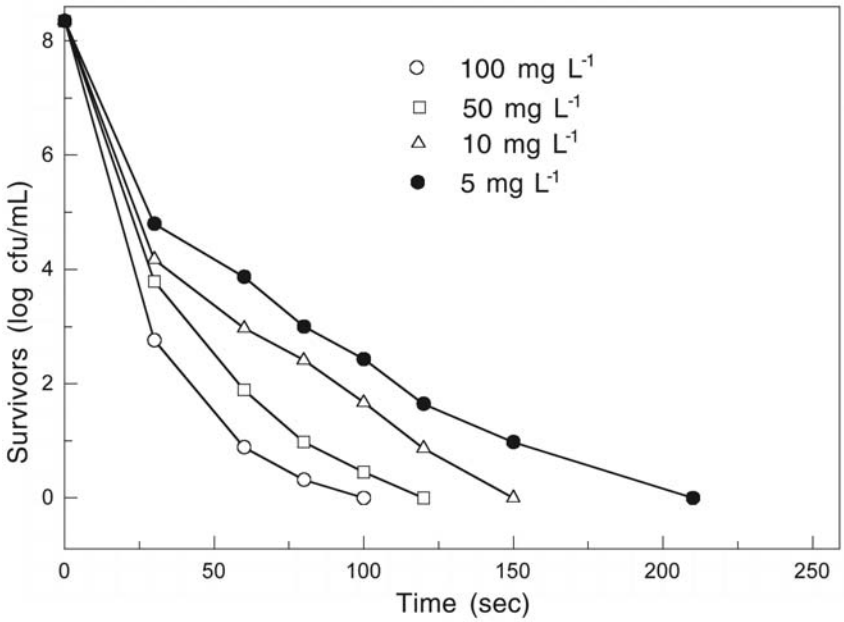


Fig. 1 - Effect of 5, 10, 50, and 100 mg L^{-1} hypochlorite on *L. monocytogenes* in phosphate buffer at pH 6.5 at 30°C.

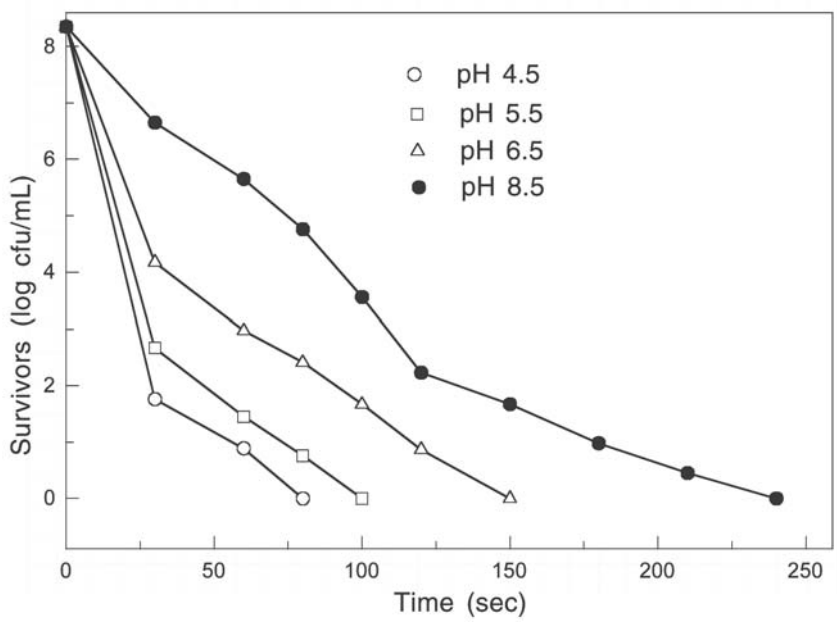


Fig. 2 - Effect of 10 mg L^{-1} hypochlorite at pH 4.5, 5.5, 6.5, and 8.5 on *L. monocytogenes* at 30°C.

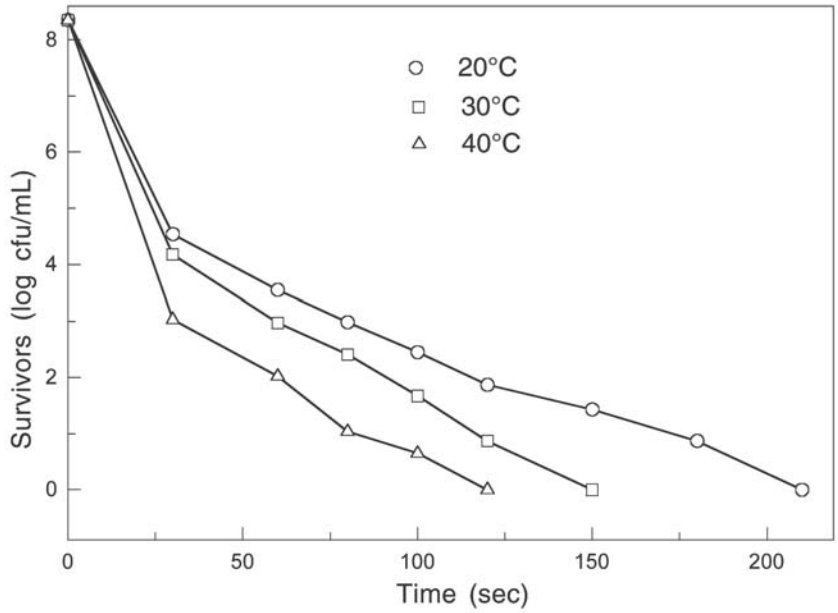


Fig. 3 - Effect of 10 mg L^{-1} hypochlorite at 20°, 30° and 40°C on *L. monocytogenes* in phosphate buffer at pH 6.5.

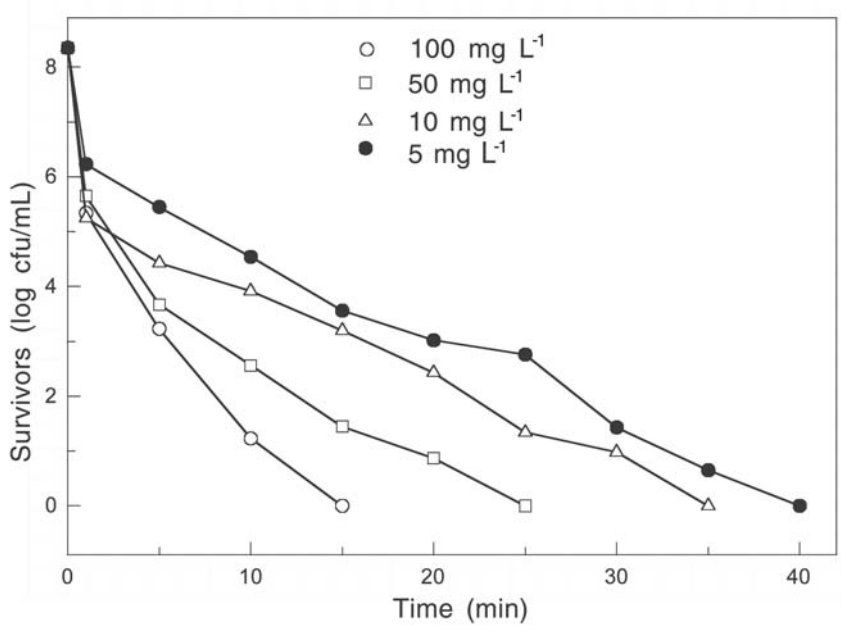


Fig. 4 - Effect of 5, 10, 50, and 100 mg L^{-1} hypochlorite in phosphate buffer containing 0.1% peptone on *L. monocytogenes* at pH 6.5 at 30°C.

rite in phosphate buffer containing 0.1% peptone at pH 4.5 at 30°C, while *L. monocytogenes* was completely inactivated after 80 sec in chlorinated phosphate buffer without peptone (Fig. 2). This suggests that the composition of the solution protected *L. monocytogenes* in the treatment with hypochlorite. Chlorination and oxidation reactions between the amino groups and chlorine are most likely responsible for this antimicrobial effect (RAY, 1996). Peptone containing nitrogenous compounds such as proteins should be thoroughly removed before using hypochlorite as a disinfectant (EL-KEST and MARTH, 1988; PAZ *et al.*, 1993). Washing salad leaves with 0.01% hypochlorite decreased mesophilic aerobic bacteria by 2.5 logs after 5 min of contact (PIERNAS and GUIRAUD, 1997). *Yersinia enterocolitica* inoculated onto eggshell surface, was exposed to 100 mg L^{-1} hypochlorite, which resulted in a reduction of about 1.28 log units (FAVIER *et al.*, 2001).

Survival curves (Figs. 1-4) revealed two different rates of *L. monocytogenes* inactivation for each concentration of hypochlorite. This suggests a complex mechanism in the inactivation of bacteria with hypochlorite. At any hypochlorite concentration, the earlier phase (about 30 sec) was characterized by a higher inactivation, which continued slowly thereafter with a straight-line inactivation. This suggests that the diffusivity of hypochlorite into cells occurs in a very short time (within 30 sec) and cells could then be stressed or sensitized and inactivated by hypochlorite. TRUEMAN (1971) indicated that the hypochlorite antimicrobial activity correlates with the concentration of undissociated hypochlorite (HOCl) molecules. An increased microbicidal HOCl effect may be due to its diffusion through microbial walls (PAZ *et al.*, 1993) followed by oxidation of cellular components (PIERNAS and GUIRAUD, 1997).

Hypohlorite concentration, pH, temperature, physical and chemical properties of suspending solution and exposure time were effective factors in the inactivation of *L. monocytogenes*. There were significant differences ($P<0.05$) among inactivation rates of *L. monocytogenes* with respect to hypochlorite concentrations at constant temperature. The higher hypochlorite concentrations and longer treatment times were more effective in reducing the bacterial numbers. Particulate matter associated with *L. monocytogenes* can decrease the bactericidal activity of hypochlorite. These considerations are particularly important in determining the efficiency of chlorination used for disinfection of equipment and water. Food is usually not contaminated with large numbers of *L. monocytogenes* (8.34 log units). Therefore hypochlorite can be considered as an effective antimicrobial agent in the disinfection of *L. monocytogenes* in terms of food production equipment. This study indicates that hypochlorite appears to adequately control this pathogen.

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SURVEY

PESTICIDE RESIDUES IN EPRW´98 MELONS

RESIDUI DEI PESTICIDI NEI MELONI DELL´EPRW´98

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ABSTRACT

The objective of this study was to compare the results of pesticide residue analysis carried out by different official laboratories in Europe, particularly for recently introduced pesticides. At the closing session of EPRW´98 (2nd European Pesticide Residue Workshop), 200 delegates from 35 countries each received two melons from a homogenous lot. Delegates working in pesticide residue control laboratories were invited to analyze the melons and send the results to the EPRW´98 Sec-

RIASSUNTO

L'esperimento è stato progettato per valutare quali fossero i risultati di laboratori ufficiali di diverse nazioni per valutare residui di pesticidi, soprattutto per quelli di recente introduzione. Nell'ultima sessione dell'EPRW'98 (2nd European Pesticide Residue Workshop), 200 delegati di 35 Paesi hanno ricevuto una scatola contenente due meloni. I delegati dei laboratori di controllo dei residui di pesticidi hanno spedito i risultati al Segretariato dell'EPRW'98. Sui meloni sono stati effettuati trattamenti

- Key words: melons, pesticide monitoring, residues -

retariat. Melons had been treated with 16 pesticides, including some relatively new active ingredients. No residues of the pesticides used were determined in the EPRW´98 melons by the seven participating laboratories. Surprisingly, low levels of two pesticides not used during the production of the melons (endosulfan and procymidone) were found by a number of laboratories. Some hypothesis about the causes of these findings are proposed.

con 16 pesticidi, alcuni dei quali "relativamente nuovi". Nessun residuo degli insetticidi usati è stato trovato nei meloni dai sette laboratori che partecipavano all'esperimento. Sorprendentemente, bassi livelli di due insetticidi, non usati durante la produzione dei meloni (endosulfan e procimidone), sono stati trovati da alcuni laboratori. Su questo aspetto sono state avanzate alcune ipotesi.

INTRODUCTION

One of the major tasks of the food control agencies in most of the developed countries is monitoring for pesticide residues in foods. The main objective of the pesticide residue monitoring programs is to enforce compliance with the legislated national (or international) maximun residue limits (MRLs) (ANDERSEN and POULSEN, 2001; EU COMMISSION, 2001; MAPA, 2001). Monitoring data are also used to assess the dietary exposure of pesticide residues from food (ANDERSSON *et al.*, 2001; CAMONI *et al.*, 2001; MAFF/PSD/HSE, 2000; WHO, 1997). These monitoring programmes are usually executed on an annual basis and encompass the sampling and analysis of a pre-determined number of samples of different foods, mainly fruits and vegetables. In general, samples included in the monitoring programs are "surveillance" samples (there is no prior knowledge about the possible presence of pesticide residues). This is the reason for which the analytical methods routinely used in almost all the food control laboratories to determine pesticide residues in foods are multiresidue methods (CAIRNS and SHERMA, 1992), i.e., methods capable of detecting a large number of pesticides in one procedure.

According to the laboratory manuals published by different agencies (MPHWS, 1996; NFA, 1998; FDA, 1994) and recent reviews on this general area of analysis (SCHENZLER and THIER, 2001; LEHOTAY, 1998), most pesticide residue analyses are performed using multiresidue methods involving ethyl acetate, acetone or acetonitrile extraction; liquid-liquid partitioning, gel permeation chromatography (GPC) or solid phase extraction (SPE) clean-up; and gas chromatography (GC) or liquid chromatography (LC) determination. Without doubt, capillary GC with different detection systems, including mass spectrometry (MS), is by far the predominant method of analysis in multiresidue determination. However, LC combined with UV, diode-array, or fluorescence detection is also used in many pesticide residue laboratories as a complementary technique to determine many of the new pesticides developed during the last decade. Also, some important laboratories are just now starting to use various LC-MS multiresidue methods in routine analyses for most of the non-GC-amenable pesticides (VALVERDE, 2000).

The total number of pesticides covered by the multiresidue methodologies used in the food control laboratories

may be very different depending on the analytical capabilities of each laboratory. The number can vary from 40 to 300. This is one of the reasons (but not the only one) for which the results of the monitoring programs of different countries, or of different regions in a country, are not totally comparable. However, it is interesting to note that the percentages of fruit and vegetable samples found to contain pesticide residues above the MRLs in the different national monitoring programs are very similar, and usually vary from 2 to 4% (EU COMMISSION, 2001).

One general and important limitation of all the pesticide residue monitoring programs is that the introduction of a new pesticide in the routine multiresidue analytical methodology of the control laboratories is usually carried out several years after the pesticide has been introduced on the market. For this reason, it is not unusual that residues of some pesticides widely used by the growers in different countries are not found for several years in the respective monitoring programs.

The objective of this paper is to report the results obtained from a unique experiment carried out during the celebration of the "2nd European Pesticide Residue Workshop -EPRW´98" (Almería, Spain, 24-27 May, 1998), which was named "EPRW´98 Melons Survey". At the end of the closing session of EPRW´98, about 200 delegates from 35 countries from all over the world received two melons, cultivar "Galia" and cultivar "Cantaloupe". The melons were produced in two different plastic greenhouses in Almería (Spain) under rigorous supervision by the organisers. Delegates working in pesticide residue laboratories were invited by the organisers to take the melons home and analyse them in their laboratories. They were then asked to send the pesticide residue levels found in each melon to the EPRW´98 Secretariat.

This experiment was initially planned to evaluate the variability of the results obtained by different pesticide residue control laboratories in the analysis of individual pieces of melons from a "homogeneous lot" (same plantation, same pesticide treatments, and same harvest date) after having been handled and stored under different and unknown conditions before analysis. It is important to note that the experiment was not planned to be an interlaboratory test, but a "survey" on the pesticide residues which, from a "formal" point of view, consumers of different countries (or regions) would ingest by eating the "same" melons.

The EPRW´98 melons were produced using the typical horticultural practices of the Almería plastic-greenhouse plantations, which include the use of many pesticides recently introduced on the European market. Therefore, the experiment was also planned to evaluate the real capability of the current pesticide residue monitoring programmes to control "relatively new" pesticides. Almería is a Spanish province that has the highest concentration of plastic greenhouses in the world. More than 3 million metric tons of fruit and vegetables are produced yearly, and about half of the total production is exported to different European countries. For this reason, Almería is one of the places in Europe where many new pesticides, mainly insecticides, acaricides and fungicides, start to be marketed.

The results of this experiment were presented as an "informal poster" at the 3rd European Pesticide Residue Workshop (VALVERDE *et al.*, 2000). However, the interest generated by this presentation, and the importance of some of the results obtained, led to this publication.

MATERIALS AND METHODS

"Galia" and "Cantaloupe" melons were cultivated in two different commercial greenhouses of 5,000 m^2 surface area each (CampoNix S.L., Nijar-Almería,

Spain), using state-of-the-art good agricultural practices. Melons were planted on the 25th (Galia) and 27th (Cantaloupe) of February, 1998 at a planting density of 1 plant/m^2 in both cases. From each plantation, about 1% of the total production (400 melons; 700-800 g/each) were collected on the 26th of May, 1998 to be packed and distributed as "EPRW´98 Melons". Pesticide treatments given to the melons (Tables 1 and 2) were rigorously controlled and recorded by the organisers throughout the production process, but they were unknown to the participants in the survey. The pre-harvest intervals for all the pesticides were respected since they were, in all the cases, less than 15 days. For those compounds applied in the last treatment (on the 6th or 7th of May), the pre-harvest intervals were 3 days, except for methomyl (7 days) and carbendazim (15 days) (DE LIÑAN, 1997).

Melons were packed in specially designed boxes on the 27th of May, 1998, after receiving a routine industrial brushing. Each EPRW´98 box contained one "Galia" melon and one "Cantaloupe" melon, with written instructions for the participants. In total, 200 boxes of EPRW´98 melons were distributed to the EPRW´98 delegates on the 27th of May, 1998. According to the enclosed instructions, delegates working in pesticide residue laboratories, and interested in participating in the survey were to take the melons to their laboratories, analyse them, and send the pesticide residue levels found in each melon to the EPRW´98 Secretariat. The two melons were to be analysed separately, and the analyses were to be done on the whole, fresh melons, using the analytical methodologies routinely used by their laboratories in their monitoring programmes.

RESULTS AND DISCUSSION

Seven laboratories analysed the melons according to the instructions given and sent the results to the organisers. Table 3 shows the names and addresses of the participating laboratories. The total number and type of pesticides covered by the analyses carried out in each laboratory are given in Table 4, and the results obtained are reported in Table 5. In Tables 4 and 5 the names of the laboratories are not specified as they were identified with a randomly assigned number. After receiving the results from the seven laboratories, melons were also analysed by the organisers at the University of Almeria (Ualm) by the supercritical fluid extraction method developed by VALVERDE *et al.* (1996) and GC determination of 43 GC-amenable pesticides, including endosulfans and procymidone. Results obtained in these analyses are also included in Table 5.

Results in Table 5 indicate that no residues of the pesticides used in the two plantations of melons were found by the participating laboratories. Out of the seventeen different pesticides used, eleven could not be determined because they were not included in the scope of the analytical methods routinely applied by all the laboratories at the time of making the analyses. These eleven pesticides are: abamectin, azadirachtin, *Bacillus thuringiensis*, copper oxychloride, cymoxanil, imidacloprid, kasugamycin, kresoxim-methyl, pyrifenox, pyriproxyfen, and tetraconazol. Except for copper oxychloride (an inorganic fungicide not included in the pesticide residue monitoring programmes) all these pesticides can be considered as "new pesticides" recently introduced on the European market. Regarding the other six pesticides used on the melons, only chlorothalonil was analysed by all the laboratories (including the University of Almeria), whereas methomyl, carbendazim, thiophanate-methyl, mancozeb and maneb were only analysed by five (four for methomyl) of the seven laboratories participating in the experiment. For these six pesticides, it could be assumed that the residue levels in the melons were below the

Table 1 - Pesticide treatments given to the "Galia" melons.

Date of Treatment	Days before harvest	Active ingredients	Type of pesticide
March 29, 1998	58	Abamectin	Insecticide/Acaricide
		Imidacloprid	Insecticide
		Thiophanate-methyl	Fungicide
		Mancozeb	Fungicide
		Cymoxanil	Fungicide
April 9, 1998	47	Copper oxychloride	Fungicide
		Kasugamycin	Bactericide/Fungicide
		Azadirachtin	Insecticide
May 6, 1998	20	Chlorothalonil	Fungicide
		Carbendazim	Fungicide
		Imidacloprid	Insecticide
		Kresoxim-methyl	Fungicide
		Methomyl	Insecticide

Table 2 - Pesticide treatments given to the "Cantaloupe" melons.

Date of Treatment	Days before harvest	Active ingredients	Type of pesticide
March 25, 1998	62	Pyrifenox	Fungicide
		Thiophanate-methyl	Fungicide
		Mancozeb	Fungicide
		Cymoxanil	Fungicide
April 18, 1998	38	Abamectin	Insecticide/Acaricide
		Methomyl	Insecticide
		Maneb	Fungicide
April 28, 1998	28	Cymoxanil	Fungicide
		Mancozeb	Fungicide
		Tetraconazol	Fungicide
		Pyriproxyfen	Insecticide
		B. Thuringiensis	Insecticide
May 7, 1998	19	Abamectin	Insecticide/Acaricide
		Pyriproxyfen	Insecticide

Table 3 - Names and addresses of the laboratories that participated in the EPRW´98 Melons Experiment.

Kantonales Laboratorium, Sanitätsdepartement des Kantons Basel-Stadt: Kannenfeldstrasse 2, Postfach, 4012 Basel, Switzerland.
Gestione Laboratori di Analisi, Progetto Natura: Via Giovanni Marradi 41, 59100 Prato, Italy.
Laboratoire Interregional de la Represión des Fraudes: 369 Rue Jules Guesde, 59651 Villeneuve D´Ascq, France.
Swedish National Food Administration-AnalyCen Nordic AB: Livsmedelsverket, Box 622, 75126 Uppsala, Sweden.
Laboratorio Agrario, Conselleria Agricultura-Valencia: Pintor Goya 8, 46100 Burjassot, Valencia, Spain.
Laboratorio SOIVRE-Almería, Ministerio Economía y Hacienda: General Tamayo 11, 04004 Almería, Spain.
Laboratorio Sanidad Vegetal-Almería, Consejería Agricultura-Andalucía: Hermanos Machado 4, 3ª planta, 04004 Almería, Spain.

Table 4 - Type and number of pesticides covered by the analyses carried out by the participating laboratories (Numbers were randomly assigned to the various laboratories).

	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7
GG-Multiresidue Method (Organophosphorous, Organochlorine, Pyrethroids, others)	62	65	18	48	123	50	198
HPLC Methods (Benzimidazols, Carbamates, Benzoyl/Phenyl-ureas, others)	11	20	-	-	25	4	49
Dithiocarbamate Method (as CS_2)	Yes	Yes	-	-	Yes	Yes	Yes

"–" indicates that these methods were not used.

Table 5 - Pesticide residues levels (in mg/kg) determined in the EPRW´98 Melons.

	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Ualm
"Galia" Melon								
Endosulfan	0.08	-	-	0.04	-	-	0.11	-
"Cantaloupe" Melon								
Endosulfan	0.03	-	0.04	0.04	-	0.01	0.03	0.01
Procymidone	0.01	-	0.03	-	-	0.02	-	0.02

"–" indicates below limit of determination.

respective analytical limits of determination, which were 0.01 mg/kg for almost all the pesticide/laboratory combinations.

Surprisingly, residues of two pesticides not used during the production of the melons were determined in the EPRW´98 melons by a number of laboratories. These pesticides are endosulfan and procymidone, which were included in the scope of the analytical methods used by all the laboratories. Specifically, residues of endosulfan were determined by three laboratories in the "Galia" melons (0.04–0.11 mg/kg) and by five laboratories in the "Cantaloupe" melons (0.01-0.04 mg/kg), whereas residues of procymidone were determined by only three laboratories in the "Cantaloupe" melons (0.01-0.03 mg/kg). Residues of endosulfan (0.01 mg/kg) and procymidone (0.02 mg/kg) were also found in the "Cantaloupe" melon in the analyses carried out at the University of Almeria. The only explanation to justify these results is that earlier crops of eggplants in the two greenhouses used to produce the EPRW´98 melons, received two treatments with endosulfan in August, 1997 and October, 1997, and one treatment with procymidone in December-1997. After removing the eggplants and cleaning the greenhouses (at end of January, 1998), some residues of these two pesticides remained in the greenhouses as contaminants, mainly in the soil and/or on the plastic cover. The presence of residues of both pesticides in the melons could have originated from the natural process of water condensing on the inside plastic cover of the greenhouse (which takes place every night throughout the production cycle) and the subsequent dripping of the water containing the pesticides on the melons. In the case of procymidone, which is a systemic fungicide, the contamination of the melons could also have originated from absorption from the soil through the roots of the melon plants and translocation to the fruit (TOMLIN, 1994).

The results obtained for endosulfan are especially important, since this pesticide has been recently prohibited in Europe for use on almost all vegetable crops (cucumber, eggplant, lettuce, green beans, etc.). However, it is still authorised to be used on melon, pepper and tomato (EUROPEAN COMMUNITIES, 2000). According to the results obtained in this experiment, some routine (and "good") agricultural practices in greenhouse cropping, such as making crop rotation without taking into account if the earlier crop was treated with endosulfan, should be questioned in some cases. For example, the melon-cucumber rotation, which is a very usual crop rotation in the Almería greenhouses, should be avoided if the melon crop had been treated with endosulfan.

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FLAIR-FLOW EUROPE



RISK ASSESSMENT IN FOOD AND DIET

FFE 575/03/SME65

Consumers are greatly concerned by the risk of chemicals in food, in particular of pollutants, chemical additives, agrochemicals and veterinary drugs. The EU introduced its new and improved food safety policy a few years ago through the EC White Paper on Food Safety (12 January 2000 Comm (1999) 719 Final), where a reduced load of risky chemicals and an improved risk assessment procedure were announced, together with the set up of the European Food Safety Authority.

Further, the World Trade Organization (WTO) is also dealing with risk assessment, and in the UN, the Codex Alimentarius has developed standards, guidelines and recommendations in order to protect human health from food borne hazards.

However, the area of risk assessment is complex. Scientific progress has been made but a lack of consensus and of European harmonization exists. For these reasons, a European Concerted Action has now focused this field.

A risk assessment comprises: 1) hazard identification, 2) hazard characterisation, 3) exposure assessment, and 4) risk characterisation. The Concerted Action follows this structure, setting up individual theme groups of experts, critically examining and developing each area.

The project explored means of improving the principles applied to, and scientific basis of, risk assessment with respect to food additives and contaminants, micronutrients and nutritional supplements, macronutrients and whole foods.

The first report from this Action has now been published in Food and Chemical Toxicology 40 (2002), 137-428, (Pergamon, ISSN 0278-6915), and the final report on risk characterisation is expected at the beginning of 2003.

Project No: QLK1-1999-00156 (FOSIE) http://www.ilsi.org/misc/fosie/

Project Co-ordinator: Dr. Juliane Kleiner, ILSI Europe, 83, Avenue E. Mounier, B-1200 Brussels, Belgium, Tel. +32 (0)2 7710014, Fax +32 (0)2 7620044, E-mail: jkleiner@ilsieurope.be

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DECODING THE CAUSES OF EATING DISORDERS

FFE 578/03/HP65

Obesity is considered by many health professionals to be an eating disorder, and there is additional concern that inappropriate and unnecessary attention to body weight could trigger diseases such as *Anorexia nervosa* and *Bulimia nervosa*.

European scientists have embarked on a project aimed at furthering the understanding of the aetiology of these eating disorders. The environment, genome and brains of individuals from hundred of families are all being investigated in a very complex study in order to identify and measure psychosocial variables and traits which are risk factors for these diseases.

Researchers are investigating:

- Personal, environmental and developmental risk factors, by studying pairs of sisters, one of whom has a disorder.
- The role of dieting in triggering eating disorders, by evaluating obese adolescents in 1000 twin families.
- Candidate genes to define genetic risk factors, by studying 600 families and 600 cases and controls with the three disorders.
- Neuronal responses to food in the brain, by using scanning techniques (magnetic resonance imaging, PET).

Males and females from across Europe will be studied to account for gender and cultural differences. The researchers hope to use the results to improve the level of healthy eating in the population by reducing the risk factors for eating disorders and obesity; to educate experts and the general public about the reasons these disorders develop, thus removing stigma and discrimination, and introduce new pharmacological treatments.

Project Reference: QLK1-1999-00916 (FACTORS IN HEALTHY EATING) www.eatingresearch.com

Project contact: Prof Janet Treasure and Dr David A Collier, The Institute of Psychiatry; Division of Psychiatry, Eating Disorders Unit, PO Box 053; De Crespigny Park, Denmark Hill, London SE5 8AF, UK, Tel. +44 20 78480180/0631, Fax +44 20 78480560/0051, E-mail: j.treasure@iop.kcl.ac.uk; d.collier@iop.kcl.ac.uk

DETECTING GLUTEN IN FOODS: A RELIEVE FOR COELIAC PATIENTS

FFE 580/03/CG64

An easy-to-use tool for the detection of gluten in foods will be developed in an EU-funded project coordinated by Prof. Ludvig Sollid, which will help people with coeliac disease to avoid problem foods.

An every day torment for coeliac disease patients is having to scrutinise the ingredients of all foods they are going to eat. **Gluten**, which causes the symptoms of the disease, is common in foodstuffs, even if the product itself is not a cereal product: due to its increasing use as a food additive its presence in many products is not immediately obvious.

Coeliac disease affects approximately one million Europeans. The intolerance to gluten results causes a damage to the small intestine mucosa which is reversible with avoidance of dietary gluten. The damaged mucosa disturbs the absorption of water and nutrients. This can cause malnutrition, which can be severe, especially for small children and adolescents. Coeliac disease is different from allergy: some people with wheat allergies are not gluten intolerant and can eat rye and barley. According to present knowledge, oat is allowed for coeliac patients, under supervision of a physician.

The symptoms of coeliac disease can vary with each individual and they do not always involve the digestive system. They can range from no symptoms at all, to severe ones, such as gas, bloating, diarrhoea, abdominal pain, irritability, muscle cramps or fatigue. Because of the broad range of symptoms that coeliac disease presents, it can be difficult to diagnose.

Scientists in this project will use new knowledge on the development of reliable and easy to handle test systems for the detection of gluten in food products. You can find more information of the project from their web page http://immunology.no/cd/eu/

Project No: QLK1-2000-00657 (GLUTEN EPITOPES IN CD)

Project Co-ordinator: Prof Dr Ludvig Sollid, University of Oslo, Institute of Immunology, Rikshospitalet, 0027 Oslo, Norway, Tel. +47-23073811, Fax +47-2307 3822/3510, E-mail: l.m.sollid@labmed.uio.no

CHEESE PACKED TO LAST LONGER

FFE 581/03/CG65

The shelf-life of cheeses may triple from the present 2-3 months up to 9 months, with the help of a new concept in food packaging. This improvement does not require more food additives in the cheese itself, but in the packaging material.

Participants in a project named Biopack are developing a new approach to the use of oxygen scavengers, and other preservatives, as active, protective agents in a new biobased packaging material. In the first instance, this new active packaging will be targeted at improving the quality and safety of cheese, but the project is also expected to improve the shelf-life after opening the package at home, reducing the growth of moulds and development of rancid taste.

The importance of foods being properly packaged is seldom noticed in everyday life. However, an excellent product can be totally wasted if the packaging of it is not appropriate. Packages are not only an important communication channel to the consumers, they also protect food from microbiological spoilage and mechanical damage.

The new technology developed, and knowledge obtained from this project, will also be applicable in other packaging concepts, to extend the shelf-life of a broad variety of food products from meat to fruits and vegetables, and improve their quality for the consumer.

Further information is available at the project web site http://www.biopack.org/

Project No: QLK5-2000-00799 (BIOPACK)
Project Co-ordinator: Prof Grete Bertelsen, Centre for Advanced Food Studies (LMC-KVL), The Royal Veterinary and Agricultural University, Rolighedsvej 30, DK-1958 Frederiksberg C, Denmark, Tel. +45 3528 3212, Fax +45 3528 3190, E-mail: grb@kvl.dk

BRAIN FOOD AND FATS

FFE 586/03/HP67

Work has just started to investigate the role of lipids in preventing age-related brain neurodegeneration and Alzheimer's disease (AD). AD is the most common neurodegenerative disorder amongst EU citizens, affecting about one third of the population, so treatment and prevention are key challenges.

The cause of the AD is not yet fully understood, although certain key processes involved in its development have been established over the last 20 years. In most cases, the disease depends on several environmental and genetic factors, but in about 5% of cases the disease is genetic, resulting from mutations in 3 different genes. A protein called Amyloid Precursor Protein (APP) is central to the disease causing process. The protein occurs normally in most cells, and sections of the protein (known as A-beta) may be 'cut out' by protease enzymes. The A-beta section accumulates over time in the brain and forms a dense amyloid cluster, or plaque. All people produce these plaques to some extent, but not all develop AD. It appears that a specific form of A-beta called A-beta 42 specifically increases the likelihood of developing AD at an earlier age. Efforts to treat or prevent AD have been directed towards decreasing production of A-beta 42 or helping the brain to clear it.

Recently, research on AD has found that cholesterol increases the activity of the protease enzymes responsible for A-beta production. Treatment of patients with AD using cholesterol-lowering drugs (statins) has shown promising results, with decreased production of A-beta 42. Cholesterol levels can also be influenced by diet.

The Lipidiet project began in 2002 and it is expected to end in 2005. It will develop and evaluate the role of dietary lipids (fats) and related food additives in the prevention and treatment of AD. Three main strands of investigation will be carried out:

1) Cellular and molecular studies on the **role** of lipids in brain neurodegeneration

2) Potential lipid-related **treatments** for brain neurodegeneration and AD will be formulated and assessed using *in vivo* models

3) Modified lipid **diets** to slow down, and eventually prevent, AD in humans will be studied *in vivo*.

Project Reference: QLK1-2002-00172 (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, Tel. +49 6221 546844, Fax +49 6221 545891, E-mail: tobias.hartmann@zmbh.uni-heidelberg.de

MAKING MOVES ON HEART DISEASE PREVENTION

FFE 587/03/HP68

Does moving to a different country and changing your diet alter your risk of developing cardiovascular disease (CVD)? Researchers across Europe are trying to find out the answer to this question using populations in Italy, Belgium and the United Kingdom.

Cardiovascular diseases are the major cause of death in Europeans, but risk is not evenly distributed across Europe. Compared to the Italian population, the rate of CVD is three times higher in the UK and twice as high in Belgium. Ecological studies showed that a low rate of CVD in Mediterranean populations was associated with, for example, low consumption of saturated fatty acids (such as those found in butter, cream, pies and pastries) and consumption of red wine with meals. Unfortunately in the last 20 years, there has been a tendency for Italians and other southern Europeans to adopt more northern European food habits, and this has been reflected in an increase in the prevalence of obesity. Paradoxically, populations in Northern Europe, with higher prevalence of CVD, are moving towards a more Mediterranean-style diet, for example by using more olive oil. Therefore the impact of diet on the CVD risk profile amongst European populations needs to be re-evaluated. Clearly, genetic risk factors may be implicated too, and genes also interact with environmental factors.

The *Immidiet* project will evaluate the present dietary habits of three European communities (UK, Belgium and Italy) at different risk of myocardial infarction (MI). The impact of migration on risk factors for MI will be evaluated from Italian migration to Belgium – the integration of the two populations will be considered as a model of gene-environment interaction. Data are now being collected, including measurements of diet composition, biomarkers of food intake (fatty acids, selenium and zinc), anthropometric variables, and many others.

It is anticipated that the study will facilitate prevention campaigns in Belgium, Italy and the UK specifically by involving general practitioners (GPs) through whom primary prevention can be implemented.

Project Reference: QLK1-2001-00100 (IMMIDIET) http://www.negrisud.it/immidiet/

Project contact: Dr Licia Iacoviello, “Angela Valenti” Laboratory of Genetic and Environmental Risk Factors for Thrombotic Disease, Department of Vascular Medicine and Pharmacology, Consorzio Mario Negri Sud, Via Nazionale, 66030 Santa Maria Imbaro (Ch) Italy, Tel. +39 0872 570298/302, Fax +39 0872 570299, E-mail: immidiet@cmns.mnegri.it

Announcement

10th SEMINAR OF THE EUROPEAN NUTRITION LEADERSHIP PROGRAMME
Luxembourg, 17-25 March 2004

The aim of the programme is to assist in the development of future leaders in the field of human nutrition in Europe. Emphasis is given to understanding the qualities and skills of leaders, team building, communication of nutrition information in a broader context, and to understanding the role of nutrition science in society. The participants of the 2003 Seminar aptly expressed the vision of the seminars as follows: "To provide a European platform where young scientists meet in harmony, realise their potential and leave as leaders". Seminars have been held annually since 1994, except in 1999. The programme is organized by a group of leading European nutritionists in close collaboration with and support from the European Commission and the European food industry.

The programme is designed for final year PhD students and postdoctoral fellows in human nutritional science in Europe. Preference will be given to candidates under the age of 35 years. From the applications received, thirty candidates will be selected by an international selection committee to attend the seminar.

The fee for attending the seminar is € 1400. This includes full board and lodging from the evening of 17 March to the morning of 25 March 2004 at the seminar hotel. Travel and personal costs such as drinks and phone calls are not included.

The ENLP organization may provide grants of € 600 towards the seminar fee and a small number of travel grants up to € 500. Those interested in applying for such grants, should send, together with the application form, a letter including a budget justifying the application for a grant towards the seminar fee and cost of travel.

Deadline for applications: 15 November 2003

Further information and application forms can be obtained from:

Mrs Lous Duym-Brookman - Division of Human Nutrition, Wageningen University, P.O. Box 8129, 6700 EV Wageningen, the Netherlands - Tel. +31 317 483054, Fax +31 317 483342 - Email: lous.duym@wur.nl Internet: www.enlp.info

Final Announcement

IDF WORLD DAIRY SUMMIT
7-12 September 2003
Bruges, Belgium

100 years of collaboration and networking between the dairy communities of so many countries all over the world is really an extraordinary achievement. IDF and Belgium are very happy to invite you to join them in celebrating IDF's first one hundred years of existence, in Bruges from September 7-12. This will be 100 years to the day of the First International Dairy Congress, held in Brussels, which saw the creation of IDF. Of course IDF has seen many changes in the course of its first century. Now we have brought together modern communication technology and traditional

networking, always a major factor in IDF's effectiveness for the dairy sector.

Conferences will address:

1) The Dairy (R)evolution – 100 Years of Change

2) Advances in Fractionation and Separation: Processes for Novel Dairy Applications

3) Quality Management at Farm Level

4) Effects of Pro- & Prebiotics on Health Maintenance

5) Energy Consumption & Life Cycle Assessment towards Sustainability

6) 100 Years with Liners and Pulsators in Machine Milking.

Further information is available at www.idf2003.com or

IDF Secretariat - Attn. Mrs. Caroline Brooks - Boulevard Auguste Reyers - 80 Diamant Building – B-1030 Brussels, Belgium - Tel. +32 2 733 98 88, Fax +32 2 733 0413 - e-mail: Cbrooks@fil-idf.org

Confectionery Manufacturing Expo 27 to 29 April 2004 Wiesbaden, Germany

Confectionery Manufacturing Expo is a biennial event that takes place every other year. It is organised by Simply Events, a leading international trade shows organiser and publisher. It is the only pan-European major exhibition exclusively featuring suppliers to the confectionery industry.

A seminar programme is currently the subject of discussion and negotiation with the industry and will be announced later in the year. Themes include:

- an innovations theatre
- seminars on how East European companies can compete beyond their traditional marketplaces
- workshops designed to provide ingredient buyers with the tools to successfully forecast market supply and demand.

For further information contact:

Gillian Pattison, Show press office - Tel: +44 (0)20 8542 9090 - E-mail: press@simply-events.com - www.cm-expo.com

REPORT

Ethical Corporation Magazine "Does the Structure of Europe's Food Supply Chain Work For or Against Our Environmental Objectives?"

The report reviews Europe-wide food production, processing and retail networks, focusing on the effects of large-scale consolidation, food clustering and agricultural intensification on markets and the environment.

Data from over 320 accredited pan-European sources has been collated to illustrate the report's key thrust – the shift from a food supply chain to a food demand chain. The report provides industry stakeholders with a clear explanation of the trends that are shaping the future of retail, processing, production, policy, environmental issues and institutional investment in the food system.

Risk exposure is becoming increasingly concerning for companies involved in large-scale diversification throughout the food supply chain – the report outlines the threats and opportunities presented by vertical integration from seed to supermarket for enterprises contemplating such a move.

The report also evaluates the strengths and weaknesses of Europe's retailers, processors and growers, as well as trends in branding and consumer spending. A detailed analysis of the environmental impact of agricultural intensification and biodiversity issues is also provided.

This groundbreaking report will be released to coincide with the announcement of the speaker roster for the "European Food Supply Chains 2003 Conference" to be held in Brussels on November 5-6, 2003. The event will be Europe's first-ever independent forum to address the concerns of retailers, processors, growers and environmentalists about the risks to the food supply chain in the EU and beyond.

To receive your **free** pre-release copy of the report, please contact Kathryn Hingston on +44 (0) 20 73757201 / kathryn@ethicalcorp.com.

For information on speaking at, or attending the conference, contact John Bodenham on +44 (0) 20 73757500 / john@firstconf.com.

BOOKS

IDF Publications

HEALTH EFFECTS OF PROBIOTICS AND CULTURE-CONTAINING DAIRY PRODUCTS IN HUMANS

A.C. Ouwehand, B. Bianchi Salvadori, R. Fondén, G. Mogensen, S. Salminen, R. Sellars

This scientific summary reviews the latest research related to the wide array of health benefits attributed to probiotics and culture-containing dairy products.

17 pp - English only

DETECTION OF FERMENTATION-PRODUCED CHYMOSIN (FPC) IN RENNET

J.-C. Collin, C. Repelius, M.K. Harboe

This study deals with the use of enzymes in cheese-making. It describes the methodology for determining the presence of certain milk-clotting enzymes in cheese.

5 pp - English only

LABORATORIES IN TRANSITION

S. Coates, R. López-Fandiño, G. Wilson, H. van den Bijgaart, S. Holroyd

A symposium session organized during the IDF/ISO/AOAC International Analytical Week 2002 (Toledo, Spain, April 2002) dealt with the accreditation of laboratories, notably the validation of methods, the new ISO Standard on accreditation and the application of modern information technology to the handling and communication of analytical data.

32 pp - English only

Total: 57 pp

Bulletin N° 380/2003-60 Euro

REVIEW OF PRACTICES FOR CLEANING AND SANITATION OF MILKING MACHINES

D.J. Reinemann, G.M.V.H. Wolters, P. Billon, O. Lind, M.D. Rasmussen

This paper gives an overview of cleaning and sanitation practices for milking machines with an update of recent research. Moreover, the paper provides a very practical section for trouble-shooting cleaning problems and guidance on strategic sampling.

18 pp - English only

MEASURING VACUUM IN MILKING MACHINES

M.D. Rasmussen, D.J. Reinemann, G.A. Mein

The technical condition of milking equipment may have a direct or indirect influence on udder health and milk quality. This document explains how measurements carried out in the milking unit can be interpreted and why faults are

likely to affect the outcome of the milking.

14 pp - English only
Total: 32 pp

Bulletin N°381/2003-60 Euro

GUIDE FOR DAIRY MANAGERS ON WASTAGE PREVENTION IN DAIRY PLANTS

Increasingly stringent environmental requirements demand a new revised approach to dairy wastage management. This guide covers process description, types of waste, sources of loss, and waste reduction practices together with advice on minimizing environmental impact and cost. Technical aspects and systematic wastage management make up an overall environmental management system. Methods of measuring flow, sampling and methods of analysis are also covered.

19 pp - English only

Bulletin N°382/2003-60 Euro

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GUIDE FOR AUTHORS

ITALIAN JOURNAL OF FOOD SCIENCE - IJFS

1. Manuscript Preparation

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

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

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

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

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

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

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