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EDITOR'S LETTER

LETTER FROM THE EDITOR

Following the announcement made on page 609 of n. 4, volume 15 in 2003, regarding the founding of the first Italian Society of Food Science and Technology (SISTAL), I am very pleased to share with you that, during the Society's annual meeting held in Perugia on February 13, 2004, the decision was made to select IJFS as the Official Journal of the Society.

During our first 15 years, we have definitely received several important recognitions: the support of the Italian National Research Council, selection as the Official Journal of SISTAL, awarding of the Impact Factor from the Institute for Scientific Information (I.S.I.) and coverage by many of the world's most renowned Abstract Services.

On behalf of the IJFS Advisory Board, I wish to thank all of the referees and authors who have contributed to making these achievements possible.

Paolo Fantozzi

PAPER

TENSILE PROPERTIES, WATER VAPOUR PERMEABILITIES AND SOLUBILITIES OF STARCH-METHYLCELLULOSE-BASED EDIBLE FILMS

PROPRIETÀ ELONGAZIONALI, PERMEABILITÀ AL VAPOR D'ACQUA E SOLUBILITÀ DI FILM EDIBILI A BASE DI AMIDO-METILCELLULOSA

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ABSTRACT

The objective of this investigation was to develop a starch-methylcellulose edible film by evaluating and optimising the combined effects of plasticiser (glycerol) content and blending levels of methylcellulose (MC) with starch on film properties using response surface methodology. The influence of two independent variables (plasticiser and MC content) on the water solubility, water vapour permeability (WVP) and mechanical properties of edible films was investigated. The formulation with an

RIASSUNTO

L'obiettivo della ricerca è stato quello di sviluppare un film edibile a base di amido-metilcellulosa valutando ed ottimizzando gli effetti combinati della quantità di plasticizzante (glicerolo) e del grado di miscelazione della metilcellulosa (MC) con l'amido sulle proprietà del film utilizzando la metodologia della superficie di risposta. Lo studio ha investigato l'influenza delle due variabili indipendenti (quantità di plasticizzante e di MC) sulla solubilità in acqua, sulla permeabilità al vapor d'ac-

- Key words: edible films, formulation optimisation, functional properties, plasticiser, response surface methodology -

MC to starch ratio (w/w, dry basis) of 31:69 and a glycerol content of 20% was determined as optimal for edible film production and for providing acceptable functional properties of the film during use.

qua (WVP) e sulle proprietà meccaniche del film edibile. La formulazione con un rapporto tra MC e amido di 31:69 (p/p, sostanza secca) e un contenuto di glicerolo del 20% è stata identificata come ottimale per la produzione di basi filmogene e per ottenere film con proprietà funzionali accettabili.

INTRODUCTION

In recent years, edible films and coatings have received increasing attention from researchers and industry as a potential food packaging material. In view of their beneficial impact on the environment, natural polymers have been studied extensively for the development of edible packaging. Materials that can be used to make edible films include polysaccharides (NISPEROS-CARRIEDO, 1994), proteins (GENNADIOS *et al.*, 1994a) and lipids (HERNANDEZ, 1994), or combinations of these. Additives such as plasticisers and emulsifiers may also be used (DEBEAUFORT and VOILLEY, 1995; 1997).

Composite films make it possible to combine the advantages associated with different components. Lipids can create effective barriers to water vapour, whereas hydrocolloids provide structural support, as well as limiting oxygen and carbon dioxide transfer (KESTER and FENNEMA, 1986; GONTARD *et al.*, 1996; BALDWIN *et al.*, 1997).

The functions of edible films and coatings are: to reduce water vapour, oxygen, lipid and flavour transfer in multicomponent foods, and between food and the surrounding atmosphere; to serve as a carrier of food ingredients and additives; or to provide mechanical protection. The functional properties of edible films depend on parameters such as formulation, film-forming technology, solvent characteristics and additives (GONTARD *et al.*, 1992; 1993).

Cellulose and its derivatives, such as ethers and esters, together with starch, are the most important raw materials for the preparation of films. The usefulness of cellulose as a material for edible films may be extended by chemical modification. One such modified version is methylcellulose (MC), a water-soluble ether with good film-forming properties. Glycerol and polyethylene glycols have been shown to be the most effective plasticisers for MC (DONHOWE and FENNEMA, 1993; PARK *et al.*, 1993). Plasticisers are necessary to reduce the brittleness of polysaccharide or protein-based edible films but their use entails a decrease in the barrier properties of edible packaging (PSOMIADOU *et al.*, 1996; DEBEAUFORT and VOILLEY, 1997; SHAW *et al.*, 2002).

Cellulose-based films are efficient oxygen and aroma barriers but ineffective against water vapour because they are hydrophilic (PARK *et al.*, 1993; DEBEAUFORT and VOILLEY, 1994). Water vapour barrier properties of cellulose-based films have been reported to be improved by the addition of lipids (KAMPER and FENNEMA, 1985; KESTER and FENNEMA, 1989; KOELSCH and LABUZA, 1992). Furthermore, NELSON and FENNEMA (1991) developed methylcellulose films to prevent lipid migration in confectionery products.

Starch is a natural polymer which can

be readily cast into films. It has poor physical properties, but these can be improved by blending starch with cellulose derivatives and proteins (ARVANITOYANNIS *et al.*, 1996, 1998; PSOMIADOU *et al.*, 1996). The interest in starch and other polymers of agricultural origin stems from their inherent biodegradability, availability and low cost (GUILBERT *et al.*, 1997).

The aims of this investigation were to develop a starch-methylcellulose edible film and to use response surface methodology to evaluate and optimise the combined effect of plasticiser content and starch-methylcellulose blending levels on film properties.

MATERIALS AND METHODS

Experimental design

A two-factor, five-level, central composite design was chosen to study the effects of MC and glycerol content on the edible film properties of the film-forming dispersion. The blends of MC and starch (0:100; 9:91; 31:69; 53:47; 62:38 w/w) and glycerol content (13, 15, 20, 25 and 27% on dry matter as starch plus MC plus glycerol) were established after preliminary tests. The five levels of each variable were coded as -1.41, -1, 0, +1 and +1.41. Table 1 presents the experimental design selected, which consists of nine design points. The central point was replicated three times, and the interior points twice, for a total of 19 samples. The sequence of experiments was fully randomised to avoid systematic errors.

Preparation of film-forming dispersions

Edible film-forming dispersions were obtained by dispersion and solubilisation of MC (high substitution, 27-32% methoxyl content, BDH, Poole, UK) in 75 mL of a distilled water-ethyl alcohol mixture (2/1, v/v) at 75°C for 10 min, and dispersion and gelatinisation of corn starch (27% amylose, S-4126, Sigma, St. Louis, MO, USA) in 75 mL of water at 95°C for 30 min. Gelatinised starch was homogenised at 4000 rpm for 1 min (Polytron PT 3000, Kinematica AG, Lucerne, Switzerland). Subsequently, glycerol (Baker, Deventer, Holland) was added to the MC and the solution was homogenised at 6000 rpm

Table 1 - Experimental levels of independent variables used in the central composite design.

Design point	Coded values		Actual values	
	X_1	X_2	MC[a] (% d.b.)	glycerol[b] (% d.b.)
1	- 1	- 1	9.04	15
2	- 1	+1	9.04	25
3	+1	- 1	53.14	15
4	+1	+1	53.14	25
5	- 1.41	0	0	20
6	+1.41	0	62.18	20
7	0	- 1.41	31.09	13
8	0	+1.41	31.09	27
9	0	0	31.09	20

[a] MC = (MC ·100) / (MC + starch); [b] glycerol = (glycerol ·100) / (MC + starch + glycerol).

for 1 min, then kept for 10 min in the same conditions. The components were mixed with a magnetic stirrer (800 rpm). Variations in MC and glycerol content are illustrated in Table 1. Dry matter was maintained at a constant weight of 5.8 g per 150 mL of water-ethyl alcohol mixture. MC and starch preparations were mixed together, homogenised at 6,000 rpm for 90 sec, and maintained at 75°C for 10 min under stirring before being spread onto glass plates (25x50x1 cm) with a thin layer chromatography spreader. Films were dried at 25°C, at a relative humidity of about 40%, for 15 hours. Film thickness was 30±3 μm (Elcometer 345 digital micrometer, Elcometer Instruments, Mancaster, UK). Before measurements, films were maintained at a constant relative humidity of 53% and a temperature of 25°C for ten days, using a saturated magnesium nitrate solution (Fluka, Buchs, Switzerland).

Water vapour permeability (WVP)

Water vapour transfer rate (WVTR) at a constant difference of relative humidity (RH), and at 25°C, was measured gravimetrically, using a modification of ASTM standard method E 96-80 (ASTM, 1990). The test film was sealed to a glass permeation cell containing distilled water, and the cell was placed in a climatic room maintained at 22% RH, with a saturated salt solution of potassium acetate (Sigma, St. Louis, MO, USA). The climatic room was equipped with fans to eliminate stagnant air above the test cell. Cell weight was recorded periodically by a computer to evaluate stationary-state water vapour transfer. WVTR ($g\ m^{-2}s^{-1}$) and WVP ($g\ m^{-1}s^{-1}Pa^{-1}$) were calculated using the following equations:

$$WVTR = \frac{\Delta m}{A \cdot \Delta t} \qquad (1)$$

$$WVP = \frac{WVTR \cdot x}{p_1 - p_2} \qquad (2)$$

where Δm is the weight loss of the permeation cell, with $4.9.10^{-4}\ m^2$ exposed area (A), during time (Δt), x is the film thickness and $p_1 - p_2$ is the real vapour partial pressure difference (Pa) across the film. The real vapour partial pressure at the film inner surface (p_1) was corrected for the stagnant air gap inside the test cell, according to GENNADIOS *et al.* (1994b). After this correction, the difference in relative humidity varied between 22-75% and 22-77%.

Water solubility

The solubility percentage (S, %) expresses the percentage of film dry matter solubilised after immersion for 24 hours in water at 25°C, according to GONTARD *et al.* (1992). The test was performed under slow agitation every 30 min. Film solubility was calculated from the following equation:

$$\%S = \left(\frac{wt_{initial} - wt_{final}}{wt_{initial}} \right) \qquad (3)$$

where $wt_{initial}$ and wt_{final} are the initial and final dry film weights, respectively.

Mechanical properties

Tensile strength (TS, MPa) and percentage elongation (E, %) at breakpoint were measured uniaxially by stretching the specimen (10x2.5 cm) in one direction at 50 mm/min using an Instron Universal Testing Instrument (Model 4301, High, Wycombe, UK). The films were analysed in a climatic room at 53% RH and 25°C. Initial grip separation was set at 4 cm. TS was calculated by dividing the maximum load by the cross-sectional area of the film, and E was expressed as a percentage of change in the

original specimen length between grips (4 cm), according to the ASTM standard method D 882-88 (ASTM, 1989).

Moisture content

Moisture content was determined by drying at 105°C for 15 hours in an air convection oven and weighing the dried samples (Salvis Thermocenter, Rotkreuz, Switzerland).

Statistical analysis of data

Data are reported as the mean of twelve measurements from three different preparations for the formulation corresponding to the central point of the experimental design, and eight measurements from two different preparations for the other samples.

Data were analysed by multiple regression using a second-order polynomial model (Statistica software, version 5, 1997) containing linear, quadratic and interaction terms for the two independent variables. The response surfaces were generated from the following equation:

$$Y = b_0 + b_1 \cdot X_1 + b_2 \cdot X_2 + b_{12} \cdot X_1 \cdot X_2 + b_{11} \cdot X_1^2 + b_{22} \cdot X_2^2 \quad (4)$$

where Y represents the response; X_1 and X_2 are the encoded values of MC and glycerol, respectively; b_0 is a constant; b_1 and b_2 are linear coefficients; b_{12} is the interaction coefficient; and b_{11} and b_{22} are quadratic coefficients.

RESULTS AND DISCUSSION

Film preparation

A film-forming dispersion was obtained with starch and MC in a water / ethanol solvent. Although MC is a water-soluble hydrocolloid, a solvent blend was used to facilitate MC solubilisation, and to reduce drying time, because of the low boiling point of ethanol. A relatively low concentration of ethanol was chosen, in line with the reports of DONHOWE and FENNEMA (1993). Ethanol concentration in excess of 25% did not completely dissolve MC and was consequently detrimental for the mechanical properties and appearance of the resulting edible films. Furthermore, high ethanol concentrations could cause precipitation of the starch in film-forming dispersions.

Several starch films were prepared using dry-process approaches, such as thermoplastic extrusion, which are based on the thermoplastic properties of polymers when plasticised and heated above their glass-transition temperature under low water-content conditions (WARBURTON *et al.*, 1993; ARVANITOYANNIS *et al.*, 1996; PSOMIADOU *et al.*, 1996; ARVANITOYANNIS and BILIADERIS, 1998). The disadvantage of extruded films is that they cannot be used to cover irregular surfaces. For this reason, the present study adopted a wet-process mechanism, based on a film-forming dispersion in which polymers are first dispersed or solubilised into a liquid phase, and then dried. The wet process was preferred because it permits the application of coatings in liquid form directly onto food products by dipping, brushing or spraying.

Functional properties

Table 2 illustrates the experimental design formulations and the corresponding properties of the various starch / MC edible films. Solubility and mechanical properties differed considerably, depending on formulation, but WVP showed less variation from sample to sample. Water solubility ranged from 17 to 72% as MC increased from 0 to 62%. Water resistance is an important characteristic of edible films in applications where there is high water activity, such as food pro-

Table 2 - Solubility, water vapour permeability (WVP), and mechanical properties of different formulations of glycerol-plasticised, starch-MC-based films.

Trial number	MC[a] (% d.b.)	Glycerol[b] (% d.b.)	Solubility (% d.b.)	WVP (10^{-11}g ·m^{-1} ·s^{-1} ·Pa^{-1})	TS (MPa)	E (%)
1	9.04	15	15.24±1.53	26.82±2.65	22.64±0.40	10.68±0.98
2	9.04	25	18.64±1.39	29.80±1.58	10.98±0.54	31.26±0.36
3	53.14	15	56.78±5.88	26.20±2.04	32.32±3.01	20.30±0.14
4	53.14	25	63.38±5.17	27.38±2.25	26.54±2.46	39.48±0.32
5	0	20	16.94±0.74	28.36±3.75	20.17±0.98	12.55±0.36
6	62.18	20	72.05±7.49	22.58±2.28	31.60±3.39	30.50±3.50
7	31.09	13	34.08±3.42	26.20±2.13	37.39±0.08	13.20±0.93
8	31.09	27	35.61±3.76	28.69±4.38	14.19±0.26	33.55±1.22
9	31.09	20	32.33±2.86	27.66±2.93	18.30±0.15	18.20±1.30

[a] MC = (MC ·100) / (MC + starch); [b] glycerol = (glycerol ·100) / (MC + starch +glycerol); Mean ± standard deviation.

tection, or when the film comes into contact with water during food processing. An application of a starch-based film in osmotic dehydration of fruit was reported by CAMIRAND *et al.* (1992). In contrast, high solubility is an advantage for applications such as edible candy wrap films, which should dissolve quickly in the mouth.

WVP changed from 22.58±2.28 (·10^{-11}· ·g·m^{-1}·s^{-1}·Pa^{-1}) to 29.80 ± 1.58 (·10^{-11}·g·m^{-1}· ·s^{-1} ·Pa^{-1}) in different samples (Table 2). These results fall within the range for other edible films. WVP values of 5.2-15.1 (·10^{-11}·g·m^{-1}·s^{-1}·Pa^{-1}) have been reported for starch-cellulose and starch-sodium, caseinate-based, edible films at 5% water content (PSOMIADOU *et al.*, 1996; ARVANITOYANNIS and BILIADERIS, 1998). Synthetic films, such as cellophane and LDPE, gave WVP values of 8.4 and 0.02 (·10^{-11}·g·m^{-1}·s^{-1}·Pa^{-1}), respectively (SHELLHAMMER and KROCHTA, 1997). The poor water vapour barrier properties of all the samples tested are due primarily to the hydrophilic nature of starch and MC. Several approaches have been reported for improving the water vapour barrier properties of cellulose and starch-based films by adding lipids (KAMPER and FENNEMA, 1985; GREENER and FENNEMA, 1989; RICO-PEÑA and TORRES, 1990; GARCÍA *et al.*, 2000).

Mechanical properties showed substantial variations, depending on film formulation. Tensile strength ranged from 10.98 MPa to 37.39 MPa and elongation varied from 10.68 to 39.48% (Table 2). Similar values were reported for methylcellulose-based films by DEBEAUFORT and VOILLEY (1997). TS values are in the same range as for low-density polyethylene film (8.3-27.6 MPa) (PARK *et al.*, 1993; ARVANITOYANNIS *et al.*, 1998). High values of TS and E are required to maintain the integrity of edible packaging during application and food handling. In general, increasing MC content in the starch-MC blend had a beneficial effect, resulting in improvement in the tensile strength and elongation of edible films, according to PSOMIADOU *et al.* (1996). This is probably due to the presence of a greater number of interacting molecular chains participating in the formation of the network structure (PERESSINI *et al.*, 2003). WOLFF *et al.* (1951) and PARK *et al.* (1993) reported improvements in film mechanical properties when the molecular weight of biopolymers was increased. In contrast, branched polymer chains were detrimental (WOLFF *et al.*, 1951; ZOBEL, 1988). The chemical het-

erogeneity of starch, deriving from the presence of both amylose and amylopectin, influences its film-forming properties. Linear amylose molecules have the ability to form strong, flexible films, whereas branched amylopectin-based films are weak and brittle (WOLFF *et al.*, 1951; ZOBEL, 1988). Corn starch used in this work contained 73% amylopectin, which was probably one of the main factors responsible for the poor mechanical properties of the formulation prepared with starch only (n. 5). The linear molecules of MC probably have better orientation and packing ability than the branched molecules of amylopectin, resulting in an improvement in mechanical properties as linear molecule content increases.

TS decreased and E increased substantially as glycerol content increased, in agreement with previous studies (WOLFF *et al.*, 1951; PARK *et al.*, 1993; PSOMIADOU *et al.*, 1996). Glycerol displayed a pronounced plasticising action. When glycerol is incorporated into the polysaccharide network, there is competition for hydrogen bonds between polymer-polymer chains and polymer-plasticiser, partly reducing direct interactions of starch or MC chains (TURHAN *et al.*, 2001). The result is a decrease in intermolecular forces along polymer chains, which increases film flexibility. Samples with low glycerol content (13-15%) exhibited brittle behaviour similar to that of synthetic polymers in the glassy state. Higher plasticiser content induced changes in structure, from glassy to rubbery, because the glass transition temperature (Tg) fell below 25°C, as demonstrated by the shape of the tensile stress-strain curves (Fig. 1) and in agreement with results in the literature (PSOMIADOU *et al.*, 1996). Samples with 13-15% glycerol levels had moisture contents of

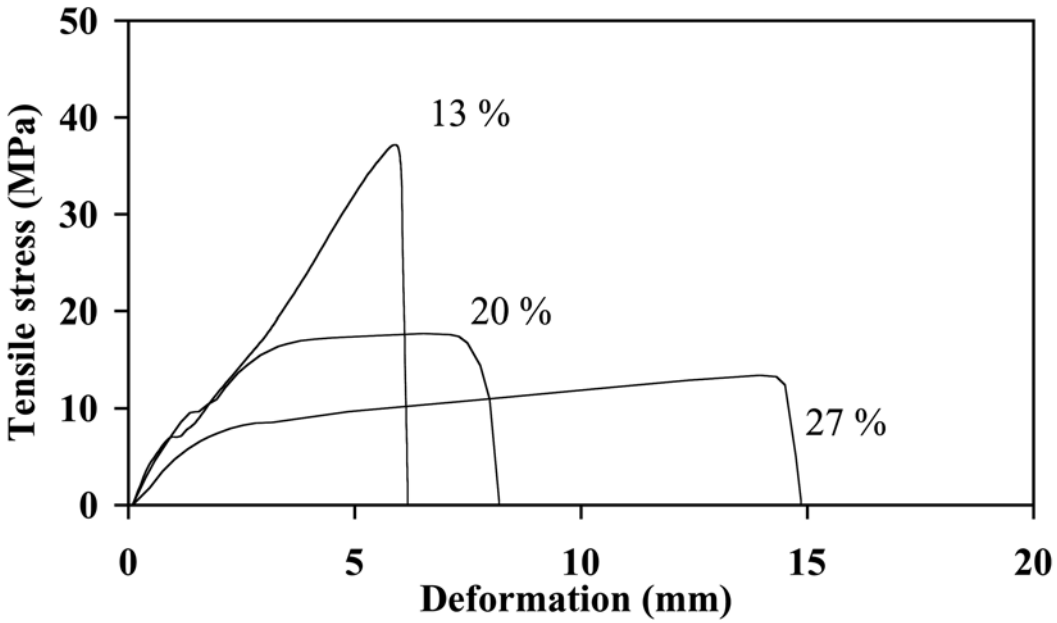


Fig. 1 - Tensile stress-strain curves of starch-MC-based films at 31% MC with different glycerol contents (53% RH; 25°C).

10.30-11.62%, which increased to 15.87-17.33% for formulations with 20-27% glycerol content because of the hygroscopic properties of glycerol. This suggests that water may also have contributed to the plasticisation of the starch-MC-based films.

Mechanical properties depend on biopolymers and the selection and concentration of plasticisers (PARK *et al.*, 1993).

Response surface analysis

Table 3 shows coefficients and analysis of variance of the second-order models, fitted with the responses from the nine combinations used in the central composite design. Models fitted for solubility and mechanical properties were highly significant ($P < 0.0001$), with satisfactory coefficients of determination (R^2 = 0.90-0.98), whereas the model developed for WVP was less predictive because of the low level of R^2 (Table 3). MC had a quadratic positive effect on solubility but glycerol had no significant effect. Both independent variables had a significant effect on mechanical properties. The model for TS showed a positive second-order effect for both glycerol and MC but a significant negative main effect was found for glycerol. Second-order terms for MC and glycerol and the linear term for glycerol had significant effects on elongation.

The relationship between independent variables and dependent responses can be better understood by examining the series of two-dimensional contour plots generated for each of the fitted models (Figs. 2-4). Solubility increased as methylcellulose levels rose, and was insensitive to changes in glycerol content (Fig. 2). The area of optimised response (low solubility) was defined by low levels of methylcellulose.

The contour plot for tensile strength (TS) showed that TS decreased in the direction of high glycerol and low MC content (Fig. 3). Elongation (E) increased as glycerol and MC levels rose (Fig. 4). The influence of glycerol on TS and E was greater than the influence of MC. The best way to improve the mechanical

Table 3 - Regression coefficients and analysis of variance of second-order models for solubility, tensile strength, and elongation of glycerol-plasticised, starch-MC-based films.

Coefficient	Solubility (% d.m.)	WVP (10^{-11} g m^{-1} s^{-1} Pa^{-1})	TS (MPa)	E (%)
Constant				
b_0	28.997	25.040	100.003	33.497
Linear				
b_1	0.018	0.122	-0.358	-0.001
b_2	-1.454	-0.025	-6.688 ***	-3.637 **
Interaction				
b_{12}	0.008	-0.004	0.009	-0.003
Quadratic				
b_{11}	0.011***	-0.002	0.006 **	0.005 **
b_{22}	0.037	0.009	0.127 **	0.136 ***
R^2	0.985	0.370	0.855	0.930
p-level	<0.000001	<0.054	<0.00001	<0.000001

* Significant at 10%, ** significant at 5%, *** significant at 1%; b_1 = methylcellulose; b_2 = glycerol.

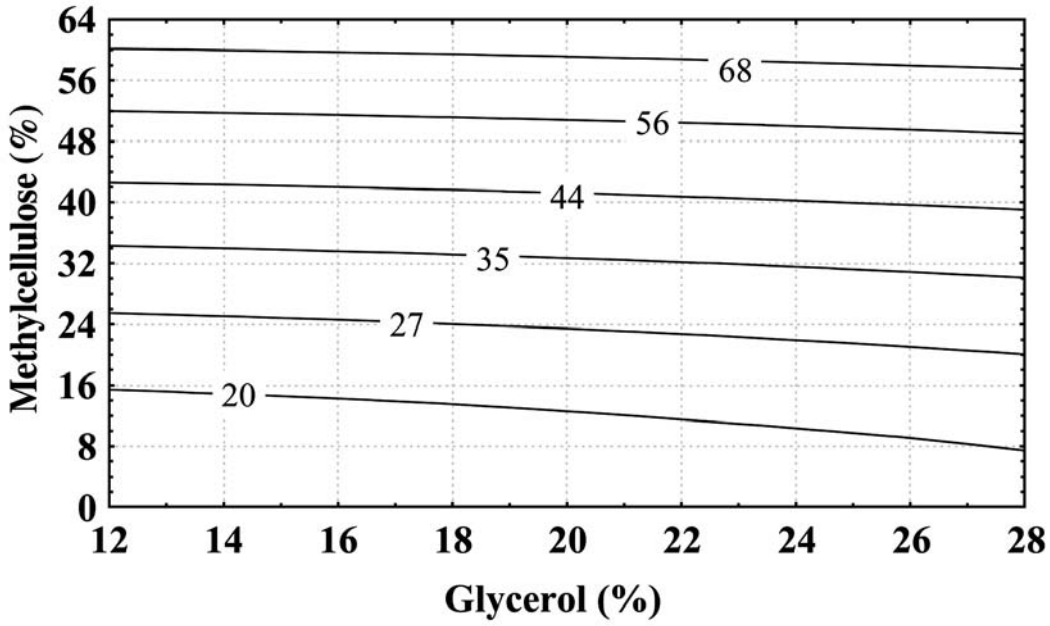


Fig. 2 - Contour plot of the effect of MC and glycerol on solubility of starch-MC-based films.

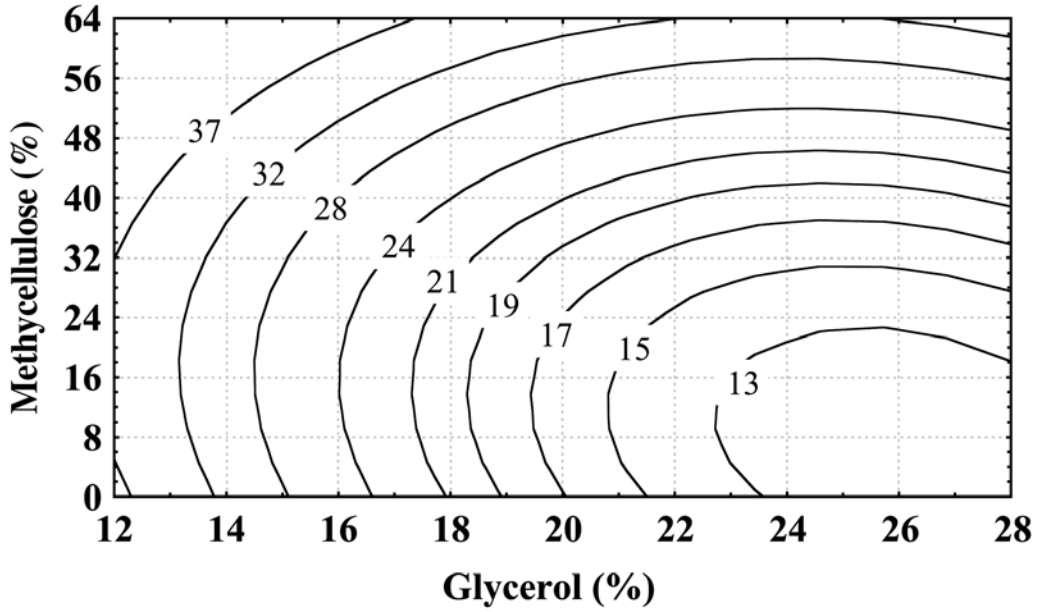


Fig. 3 - Contour plot of the effect of MC and glycerol on tensile strength of starch-MC-based films.

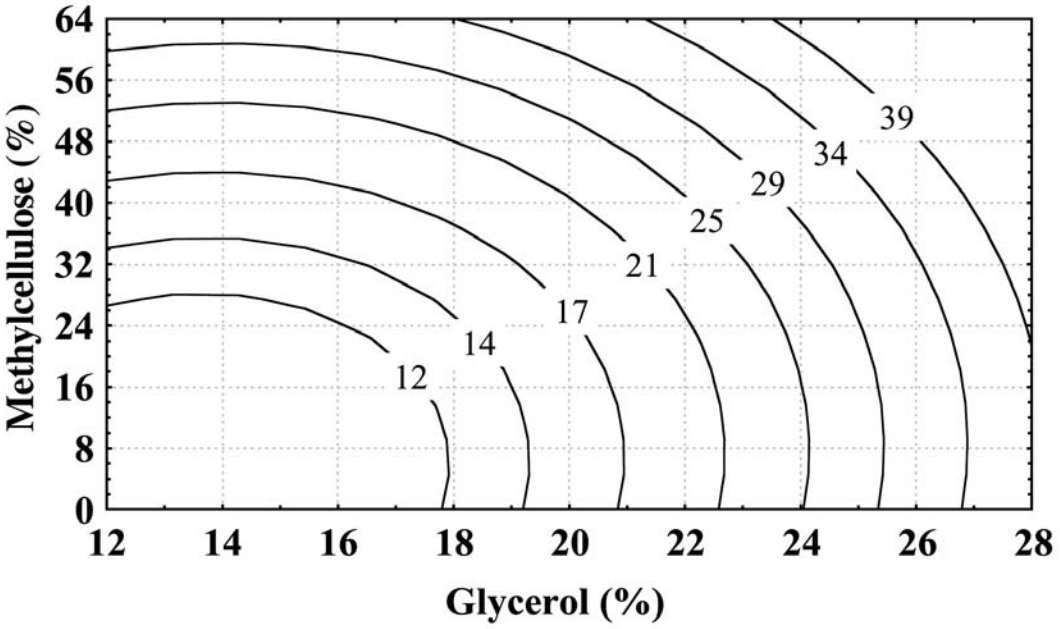


Fig. 4 - Contour plot of the effects of MC and glycerol on elongation of starch-MC-based films.

properties was to find a compromise between high TS and E values, since the areas of optimised response for the two parameters are in conflict.

Figure 5 was obtained by superimposing the previous three contour plots (Figs. 2-4). The crosshatched area shows MC and glycerol contents that produce films with satisfactory characteristics. The shaded area fell within the central region of the experimental design. From these results for solubility and mechanical properties, MC levels should fall between 25-34%, whereas glycerol should exceed 19% and be lower than 21.5% (Fig. 5).

PERESSINI *et al.* (2003) reported that formulations containing 31% MC showed rheological properties of the film-forming dispersion that ensure optimal performance for the production of laminated films. Experimental formulations with an MC content in excess of 31% led to the formation of very viscous dispersions associated with levelling problems during spreading. In addition, high viscosity is undesirable during the preparation of liquid films because it is more difficult to disperse the ingredients and eliminate visible air bubbles, which are responsible for discontinuities in solid film. Samples with an MC content lower than 31% were subject to low viscosity-related gravity effects. The best compromise of levelling and sagging behaviours was achieved for systems with an MC content of around 31%. Taking the above considerations into account, the formulation corresponding to the central point of the experimental design was identified as optimum for edible film production. Levels of 31% MC and 20% glycerol assure both satisfactory rheological properties for the liquid film and acceptable functional properties for the film during use. The addition of soybean oil (20% d.b.) to the optimised formulation has been reported to improve the water vapour barrier properties of starch-MC based edible film (BRAVIN *et al.*, 2002).

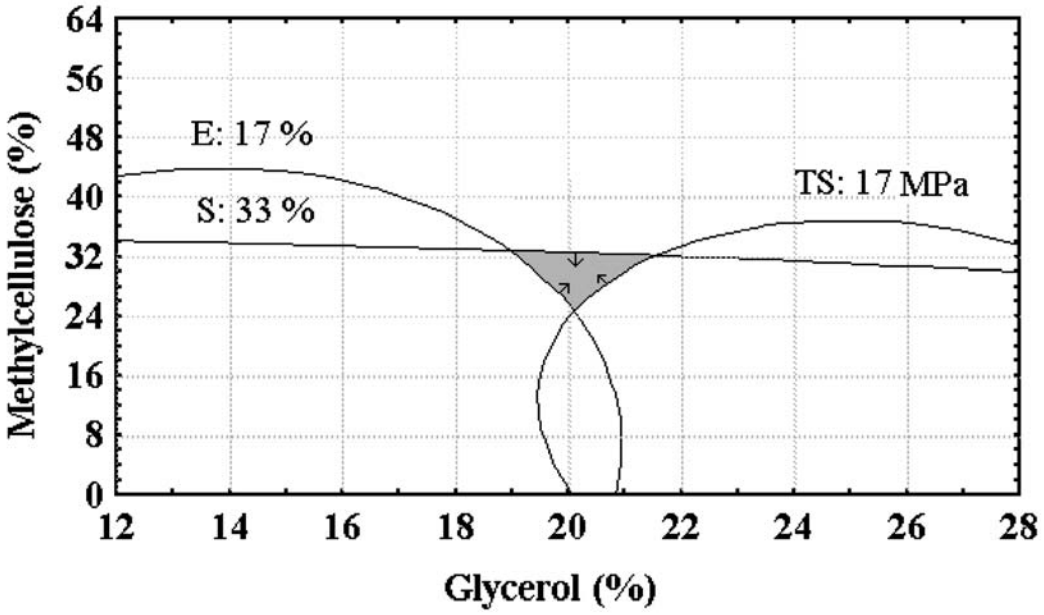


Fig. 5 - Acceptable area obtained by overlapping contour plots of functional properties of starch-MC-based films as a function of MC and glycerol content.

The application of edible film to a low a_w-type food model, such as crackers, whose regular, constant geometry enables uniform coating and control over exchange surfaces, confirmed the potential of edible film to become an integral part of the food and reduce water vapour transmission in a high-a_w environment (BRAVIN *et al.*, 2002).

CONCLUSIONS

Central composite design and response surface methodology were used successfully to estimate the effect of MC-starch ratio and glycerol content on the functional properties of edible film, and to determine the optimal formulation. Levels of 31% for MC and 20% for glycerol were identified as optimal for edible film production because these proportions assured both satisfactory rheological properties in the liquid film and acceptable functional properties in the film.

ACKNOWLEDGEMENTS

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PAPER

EVALUATION OF THE *IN VITRO* ANTIOXIDANT PROPERTIES OF BROCCOLI EXTRACTS (*BRASSICA OLERACEA* L.)

VALUTAZIONE *IN VITRO* DELLE PROPRIETÀ ANTIOSSIDANTI DI ESTRATTI DI BROCCOLI (*BRASSICA OLERACEA* L.)

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ABSTRACT

Water and ethanol extracts of broccoli (*Brassica oleracea* L.) florets were studied for the following *in vitro* antioxidant properties: total antioxidant activity, reducing power, free radical scavenging, superoxide anion radical scavenging, hydrogen peroxide scavenging, and metal chelating activities. Both extracts exhibited strong total antioxidant activity. The concentrations of 20, 40, and 60 μg/mL of the water and ethanol extracts of broccoli showed 74, 83, 91 and 87, 94 and 99% inhibi-

RIASSUNTO

Su estratti acquosi ed alcolici di fiori di broccoli sono state valutate, *in vitro*, le seguenti proprietà antiossidanti: Potere antiossidante totale, potere riducente, attività scavenger di radicali liberi, attività scavenger di radicale anionico superossido, attività scavenger di perossido di idrogeno ed attività di chelazione di metalli. Ambedue gli estratti hanno dimostrato un forte potere antiossidante totale. Gli estratti sia acquosi che alcolici di broccoli, alla concentrazione di 20, 40 e 60 μ/mL hanno

- Keywords: Antioxidant activity; *Brassica oleracea* L., broccoli florets, phenolic content -

tion of peroxidation of linoleic acid emulsion, respectively. Controls at a concentration of 60 μg/mL of butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and α-tocopherol exhibited 97, 99, and 61% inhibition of peroxidation of linoleic acid emulsion, respectively. Both extracts of broccoli florets displayed effective reducing power, free radical scavenging, superoxide anion radical scavenging, hydrogen peroxide scavenging, and metal chelating activities at 20, 40, and 60 μg/mL. Total phenolic compounds in both broccoli floret extracts were determined as gallic acid equivalents and found to show no correlation with the antioxidant activities of the extracts.

dato luogo ad una riduzione della perossidazione di una emulsione di acido linoleico pari, rispettivamente, al 74, 83, 91% e 87, 94 e 99%. I controlli, ad una concentrazione di 60 μ/mL, di BHA, BHT ed alfa-tocoferolo, hanno dato luogo ad una riduzione della stessa emulsione pari a 97,99 e 61%. Ambedue gli estratti di fiori di broccoli hanno mostrato effettivi potere riducente, attività scavenger di radicali liberi, attività scavenger di radicale anionico superossido, attività scavenger di perossido di idrogeno ed attività di chelazione di metalli a concentrazioni pari a 20, 40 e 60 μ/mL. Il contenuto in polifenoli totali dei fiori di broccoli è stato determinato come equivalente di acido gallico. La loro concentrazione non si è dimostrata in correlazione con l'attività antiossidante degli estratti.

INTRODUCTION

Fatty acids are susceptible to attack by highly reactive oxygen species (ROS) such as OH·. Hence any reaction or process which forms ROS could potentially stimulate lipid oxidation. Hydrogen abstraction is easier in unsaturated fatty acids than in their saturated counterparts, thus making them more susceptible to ROS attack. Oxygen and ROS are among the major sources of primary catalysts that initiate oxidation *in vivo* and *in vitro*. The electronic structure of oxygen has two unpaired electrons at energy levels of p antibonding, in the triplet state ($^3\sum_g$) (KORYCKA-DAHL and RICHARDSON, 1978). The reaction of oxygen with ground state molecules of singled multiplicity such as polyunsaturated fatty acids (PUFA) is spin-forbidden. However, this barrier does not apply to reactions, which involve single electrons, hydrogen atoms, and molecules containing unpaired electrons, such as transition metal complexes and free radicals. Therefore, the triplet state oxygen can react with other molecules to yield ROS such as hydrogen peroxide (H_2O_2), superoxide ($O_2\cdot^-$), and the hydroxyl radical (OH·) (BORG, 1993; HALLIWELL and GUTTERIDGE, 1985; KANNER *et al.*, 1987).

Exogenous compounds from food and endogenous metabolic processes in the human body or in the food system might produce highly reactive free radicals, which in high enough concentrations are capable of oxidizing biomolecules, leading to oxidative stress, cell death and tissue damage. Oxidative damage plays a significant pathological role in human diseases. Excessive generation of ROS induced by various stimuli can overwhelm the compensatory antioxidant capacity of the body leading to a variety of pathophysiological processes including inflammation, diabetes, genotoxicity and cancer (GÜLÇIN *et al.*, 2002a; KOUR-

OUNAKIS *et al.*, 1999). The body is typically protected from free radical damage by enzymes such as superoxide dismutase and catalase or antioxidant compounds such as ascorbic acid, tocopherol and gluthatione (NIKI *et al.*, 1994).

Normal aerobic respiration in mitochondria, stimulated polymorphonuclear leukocytes, macrophages and peroxizomes are the main endogenous sources of ROS in the body. Exogenous sources of ROS include tobacco smoke, ionising radiation, certain pollutants, organic solvents and pesticides. ROS are continuously produced during normal physiological processes and are removed by antioxidant mechanisms. There is a balance between the generation of ROS and the antioxidant system in the body. The imbalance between ROS and antioxidant defence systems leads to oxidative modification in the cellular membrane or intracellular molecules (BÜYÜKOKUROĞLU *et al.*, 2001). Antioxidants can interfere with the oxidation process by reacting with free radicals, chelating catalytic metals, and also by acting as oxygen scavengers (SANCHEZ-MORENO *et al.*, 1999; SHAHIDI and WANASUNDARA, 1992; MAU *et al.*, 2002).

Antioxidant supplements or food containing antioxidants may be used to protect the human body from oxidative damage (HALLIWELL and GUTTERIDGE, 1984; MAU *et al.*, 2001; GÜLÇIN *et al.*, 2002b). The most common antioxidants used as food additives are BHA, BHT, propyl gallate (PG), and tert-butylhydroquinone (TBHQ) (SHERWIN, 1990). However, these compounds are suspected of being responsible for liver damage and carcinogenesis in laboratory animals (GRICE 1986, WICHI, 1988). Therefore, the development and utilization of more effective antioxidants of natural origin is desirable (GÜLÇIN *et al.*, 2002a, OKTAY *et al.*, 2003).

Fruit and vegetables contain several classes of compounds that can potentially contribute to antioxidant activity, including vitamins, phenolics, sulphur-containing compounds and glucosinolates (CAO *et al.*, 1996). Broccoli is an important vegetable that has recently become more popular due to its high nutritional value. Some metabolic changes in broccoli flower buds during short-term storage of broccoli were investigated by LEJA *et al.* (2001). Polyphenolic compounds with antioxidant activities are relatively abundant in broccoli (PLUM *et al.*, 1997). In particular, broccoli is a significant source of several classes of biologically active dietary components such as flavonol glycosides and sulphur containing compounds such as glucosinolates (PRICE *et al.*, 1997; FENWICK and HEANY, 1983), hydroxycinnamic acids (PRICE *et al.*, 1997), carotenoids and ascorbate (KURILICH *et al.*, 1999). The glucosinolates are secondary metabolites derived from protein and non-protein amino acids (MANSON *et al.*, 1997; SMITH *et al.*, 1998). Therefore, glucosinolate regulation and final concentrations in tissues of cruciferous crops may be important for the human diet and health (RANGKADILOK *et al.*, 2002). PLUMB *et al.* (1996) examined the free radical scavenging properties of purified glucosinolates from cruciferous vegetables. Hydroxycinnamic acids, another phytochemical class of compounds in broccoli, are attracting interest as a group with protective activity against degenerative diseases (PRICE *et al.*, 1997).

KURILICH *et al.* (2002) determined the antioxidant capacity of hydrophilic and lipophilic extracts in eight broccoli genotypes using the oxygen radical absorbance capacity assay. They indicated that the antioxidant capacity of hydrophilic extracts is higher than that of lipophilic extracts. CAO *et al.* (1996) measured the antioxidant activity of broccoli flowers using the automated oxygen radical absorbance capacity assay. However, there are no reports about the *in vitro* antioxidant properties of water or ethanol extracts of broccoli floret tissue using the

thiocyanate method, reducing power, free radical scavenging, superoxide anion radical scavenging, hydrogen peroxide scavenging, and metal chelating antioxidant activity assays.

Although numerous procedures are available for evaluating the antioxidant activity of pure compounds, most of these procedures are not suitable for determining the antioxidant activity of plant extracts. Various solvents have been used for extraction (PLUMB *et al.*, 1997; AZUMA *et al.*, 1999; LEJA *et al.*, 2001), however, from a toxicological point of view, water and ethanol, as solvents, are safer than acetone, methanol and other organic solvents. Therefore, the aim of the present study was to investigate the antioxidant activity of water and ethanol extracts from broccoli florets using several antioxidant assays including total antioxidant activity, reducing power, free radical scavenging, superoxide anion radical scavenging, hydrogen peroxide scavenging, and metal chelating activity. Total phenolic content (as gallic acid equivalents) of aqueous and ethanolic extracts of broccoli florets was also determined.

MATERIALS AND METHODS

Chemicals

Ferrous chloride, polyoxyethylene-sorbitan monolaurate (Tween-20), α-tocopherol, 1,1-diphenyl-2-picryl-hydrazyl (DPPH·), 3-(2-pyridyl)-5,6-bis (4-phenyl-sulfonic acid)-1,2,4-triazine (Ferrozine), nicotinamide adenine dinucleotide (NADH), butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and trichloracetic acid (TCA) were purchased from Sigma (Sigma-Aldrich GmbH, Sternheim, Germany). Ammonium thiocyanate was purchased from Merck (Merck, D-6100 Darmstad, Germany). All other chemicals used were of analytical grade and were obtained from Sigma (Sigma-Aldrich GmbH, Sternheim, Germany). BHA, BHT and α-tocopherol were dissolved in ethanol.

Plant material and extraction

Fresh broccoli cannot be found during all four seasons in Turkey. It is commonly used in Turkish folk medicine to treat prostatitis and urinary tract infections, hence, it is also available in the dried form on the market. In the present study dried broccoli was obtained from a local market at Erzurum, Turkey. The entire broccoli heads were cut into small pieces, left to dry on a bench under ambient temperature for over three days after which it was ground in a blender. The extraction procedures were then performed according to GÜLCIN *et al.* (2004a; 2004c). For water extraction, 20 g of dried broccoli floret tissue were ground into a fine powder in a mill and then mixed with 400 mL of boiling water using a magnetic stirrer for fifteen minutes. Then the extract was filtered through Whatman No.1 filter paper. The filtrates were frozen and lyophilised at 5 μm Hg pressure at -50°C (Labconco Corp., Freezone 1L, Kansas City, MO, USA). For ethanol extraction, 25 g of dried broccoli floret tissue were ground into a fine powder in a mill and then extracted five times with 100 mL ethanol. Extraction continued until the solvent became colourless (total solvent volume was 500 mL). The extracts were filtered (Whatman No.1 paper) and the ethanol was removed using a rotary evaporator (RE 100 Bibby, Stone Staffordshire, U.K.) at 50°C to obtain the dry extract. Each broccoli floret extract was put in a plastic bottle and stored at -20°C until used.

Total antioxidant activity

The total antioxidant activity of extracts of broccoli florets was determined using the thiocyanate method described

by MITSUDA et al (1996). Ten milligrams of the lyophilised water extract of broccoli floret tissue were dissolved in 10 mL water. Ten milligrams of the ethanol extract of the broccoli floret tissue were dissolved in 10 mL ethanol. Preparation of standards was performed under the same conditions. Then different concentrations (20, 40, and 60 μg/mL) or standard samples were prepared separately in 2.5 mL of potassium phosphate buffer (0.04 M, pH 7.0). These solutions were added to 2.5 mL of linoleic acid emulsions in potassium phosphate buffer (0.04 M, pH 7.0). Five millilitres of linoleic acid emulsion contained 17.5 μg Tween-20, 15.5 μL linoleic acid and 0.04 M potassium phosphate buffer (pH 7.0). Five millilitres of the control included 2.5 mL linoleic acid emulsion and 2.5 mL potassium phosphate buffer (0.04 M, pH 7.0). The mixed solution was incubated at 37°C in a glass flask in the dark. After stirring the mixture for 3 min, 100 μL $FeCl_2$ and 100 μL thiocyanate were added and the peroxide value was determined by reading the absorbance at 500 nm in a spectrophotometer (8500 II, Bio-Crom Gmb, Zurich, Switzerland). During linoleic acid oxidation, peroxides form and oxidize Fe^{+2} to Fe^{+3}. Fe^{+3} ions form a complex with SCN^- and this complex has a maximum absorbance at 500 nm. Therefore high absorbance indicates high linoleic acid oxidation. Control solutions without the addition of extracts of broccoli florets were used as blanks to measure background absorbance. The percentage inhibition of lipid peroxidation was calculated using the following equation:

$$\% \text{ Inhibition} = [(A_o - A_1)/A_o] \times 100$$

where A_o was the absorbance of the control reaction and A_1 was the absorbance in the p resence of the extracts of broccoli floret tissue (DUH *et al.*, 1999; GÜLÇIN *et al.*, 2004d).

Reducing power

The reducing power of extracts of broccoli florets was determined by investigating the Fe^{3+} - Fe^{2+} transformation according to the method of OYAIZU (1986). The various concentrations of aqueous or ethanolic extracts of broccoli floret tissue (20, 40, 60 μg/mL) in 1 mL of distilled water were mixed with phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (2.5 mL, 1%). The mixture was incubated at 50°C for 20 min. An aliquot (2.5 mL) of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged for 10 min at 1,000 g (MSE Mistral 2000, U.K.) The upper layer of solution (2.5 mL) was mixed with distilled water (2.5 mL) and $FeCl_3$ (0.5 mL, 0.1%), and the absorbance was measured at 700 nm in a spectrophotometer (8500 II, Bio-Crom Gmb, Zurich, Switzerland). Higher absorbance of the reaction mixture indicated greater reducing power.

Free radical scavenging activity (DPPH assay)

The free radical scavenging activity of water and ethanol extracts of broccoli florets was measured by 1,1-diphenyl-2-picryl-hydrazil (DPPH·) using the method of BLOIS (1958). Briefly, a 0.1 mM solution of DPPH· in ethanol was prepared and 1 mL of this solution was added to 3 mL of broccoli floret extract solutions. The mixture was shaken vigorously and allowed to stand at room temperature for 30 min. Then the absorbance was measured at 517 nm in a spectrophotometer (8500 II, Bio-Crom Gmb, Zurich, Switzerland). Lower absorbance of the reaction mixture indicated higher free radical scavenging activity. The DPPH· concentration (mM) in the reaction medium was calculated from the following calibration curve, determined by linear regression (R^2: 0.9769):

$$\text{Absorbance} = 104.09 \times [\text{DPPH}\cdot].$$

The DPPH radical concentration was calculated using the following equation:

$$\text{DPPH}\cdot \text{ Scavenging Effect (\%)} = = 100 - [(A_o - A_1/A_o) \times 100],$$

where A_o was the absorbance of the control reaction and A_1 was the absorbance in the presence of the broccoli floret extracts (GÜLÇIN *et al.*, 2003).

Superoxide anion scavenging activity

Measurement of the superoxide anion scavenging activity of extracts of broccoli florets was based on the method described by LIU *et al.* (1997) with slight modifications (OKTAY *et al.*, 2003; GÜLÇIN *et al.*, 2004b). Superoxide radicals are generated in PMS-NADH systems by oxidation of NADH and assayed by the reduction of nitroblue tetrazolium (NBT). Superoxide radicals were generated by mixing 3 mL of Tris-HCl buffer (16 mM, pH 8.0) containing 1 mL of NBT (50 μM) solution, 1 mL NADH (78 μM) solution and water or ethanol extracts of broccoli florets (from 20 to 60 μg/mL). The reaction was initiated by adding 1 mL of phenazine methosulphate (PMS) solution (10 μM) to the mixture. The reaction mixture was incubated at 25°C for 5 min and the absorbance at 560 nm (Spectrophotometer 8500 II, Bio-Crom Gmb, Zurich, Switzerland) was measured against blank samples. L-Ascorbic acid was used as a standard. Decreased absorbance of the reaction mixture indicated increased superoxide anion scavenging activity. The percentage inhibition of superoxide anion generation was calculated using the following formula:

$$\text{\% Inhibition} = [(A_o - A_1)/A_o] \times 100,$$

where A_o was the absorbance of the control (L-Ascorbic acid), and A_1 was the absorbance of extracts of broccoli floret and standards (YE *et al.*, 2000).

Metal chelating activity

The chelating of ferrous ions by water and ethanol extracts and standards was estimated by the method of DINIS *et al.* (1994). Briefly, extracts (20-60 μg/mL) were added to a solution of 2 mM $FeCl_2$ (0.05 mL). The reaction was initiated by the addition of 5 mM ferrozine (0.2 mL) and the mixture was shaken vigorously and left to stand at room temperature for ten minutes. After the mixture had reached equilibrium, the absorbance of the solution was then measured spectrophotometrically at 562 nm (8500 II, Bio-Crom Gmb, Zurich, Switzerland). Percent inhibition was calculated using the following formula.

$$\text{\% Inhibition} = [(A_o - A_1)/A_o] \times 100$$

where A_o was the absorbance of the control ($FeCl_2$ and ferrozine absent), and A_1 was the absorbance of the broccoli floret extracts and standards.

Scavenging of hydrogen peroxide

The ability of water and ethanol extracts of broccoli florets to scavenge hydrogen peroxide was determined according to the method of RUCH *et al.* (1989). A solution of hydrogen peroxide (40 mM) was prepared in phosphate buffer (pH 7.4). Hydrogen peroxide concentration was determined spectrophotometrically at 230 nm (8500 II, Bio-Crom Gmb, Zurich, Switzerland). Aqueous and ethanolic extracts (20-60 μg/mL) in distilled water and ethanol were added to a hydrogen peroxide solution (0.6 mL, 40 mM). Absorbance of hydrogen peroxide at 230 nm was determined after ten minutes against a blank solution of phosphate buffer without hydrogen peroxide. The percentage of scavenged hydrogen

peroxide of broccoli floret extracts and standard compounds was calculated by the following formula:

$$\% \text{ Scavenged } [H_2O_2] = [(A_o - A_1)/A_o] \times 100$$

where A_o was the absorbance of the control, and A_1 was the absorbance in the presence of the broccoli floret extracts and standards. BHA, BHT and α-tocopherol were used as standards.

Total phenolic content

The concentration of the total soluble phenolic compounds in water and ethanol extracts of broccoli florets was determined with the Folin-Ciocalteu reagent (SLINKARD and SINGLETON, 1977). Gallic acid was used a standard phenolic compound. Briefly, 1 mL of water and ethanol extracts of broccoli floret solutions (each containing 1,000 μg extract) were separately pipetted into a volumetric flask and diluted with distilled water (46 mL). One millilitre of Folin-Ciocalteu reagent was added and the contents of the flask were mixed thoroughly. After 3 min, 3 mL of Na_2CO_3 (2%) was added and the mixture was allowed to stand for 2 hours with intermittent shaking. The absorbance was measured at 760 nm in a spectrophotometer (8500 II, Bio-Crom Gmb, Zurich, Switzerland). The concentration of total phenolic compounds in the broccoli floret extracts was determined as μg of gallic acid equivalents using an equation that was obtained from the standard gallic acid graph (OKTAY *et al.*, 2003):

Absorbance = 0.0053 × Total Phenols [Gallic Acid Equivalent (μg)] - 0.0059.

Statistical analysis

Results of the total antioxidant activity are the average of duplicate analyses of each extract. All other results are expressed as the mean ± S. D. of three replicate measurements. Analysis of variance was performed by ANOVA procedures using SPSS 9.0 software (SINGLETON *et al.*, 1999). Significant differences between means were determined by Duncan's Multiple Range tests. *P* values < 0.05 were regarded as significant and *p* values < 0.01 as highly significant.

RESULTS AND DISCUSSION

Numerous antioxidant methods and modifications have been proposed to evaluate antioxidant activity and to explain how antioxidants function. Of these, the total antioxidant activity, reducing power, DPPH assay, metal chelation and quenching assays for active oxygen species such as H_2O_2, $O_2\cdot^-$, and OH· are often used for evaluating the antioxidant activities of extracts (DUH *et al.*, 1999; AMAROWICZ *et al.*; 2000, CHANG *et al.*, 2002). The water and ethanol extraction yields (20 g dried broccoli) were 1.20 g and 1.54 g, respectively.

Total antioxidant activity

The total antioxidant activity of broccoli floret extracts was determined by the thiocyanate method. Both extracts of broccoli florets exhibited effective antioxidant activity at all concentrations. The effects of various concentrations of water and ethanol extracts of broccoli florets (from 20 to 60 μg/mL) on peroxidation of linoleic acid emulsion are shown in Table 1. The antioxidant activity of both extracts of broccoli florets increased with increasing concentration. The percent inhibition of peroxidation in the linoleic acid system by the water and ethanolic extracts of broccoli were 74, 83, and 91%, and 87, 94 and 99%, respectively. The different concentrations of water and ethanol extracts (20, 40 and 60 μg/mL) of broccoli florets had higher antioxidant activities than that of α-to-

Table 1 - Inhibition of lipid peroxidation of linoleic acid emulsion, reducing power and free radical scavenging activity by DPPH radicals of various concentrations of water extracts of broccoli (WEB), ethanol extracts of broccoli (EEB), BHA, BHT and α-tocopherol.

	Inhibition of lipid peroxidation (%)			Reducing power			DPPH radical scavenging activity (%)		
	20μg/mL	40μg/mL	60μg/mL	20μg/mL	40μg/mL	60μg/mL	20μg/mL	40μg/mL	60μg/mL
BHA	88	91	97	1.120	1.916	2.217	47	56	62
BHT	89	94	99	0.573	1.106	1.486	49	56	60
α-tocopherol	51	54	61	0.298	0.623	0.988	25	30	31
WEB*	74	83	91	0.437	0.767	1.007	10	28	53
EEB†	87	94	99	0.469	0.900	1.224	31	44	59

*:1.20 g dried water extract was found in 20 g dried floret tissue; †: 1.54 g dried water extract was found in 20 g dried floret tissue.

copherol. On the other hand, the percent inhibition of the 60 μg/mL concentration of BHA and BHT was found to be 97 and 99%, respectively. AZUMA *et al.* (1999) evaluated the antioxidant activity of plant extracts in linoleic acid emulsion and phospholipid bilayers and found the ethanolic extracts to have a higher antioxidant activity than the aqueous extract.

Reducing power

The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity (MEIR *et al.*, 1995). The antioxidant activity of putative antioxidants has been attributed to various mechanisms, among which are prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, reducing power and radical scavenging (DIPLOCK, 1997).

Table 1 shows the reducing power of water and ethanol extracts of broccoli florets compared to BHA, BHT and α-tocopherol. As seen with total antioxidant activity, the reducing power of both extracts of broccoli florets increased with increasing concentration. All concentrations of water and ethanol extracts showed higher activities than the controls and these differences were highly significant ($p<0.01$). The ethanol extract exhibited higher reducing power than the water extract ($p<0.05$). Both extracts of broccoli florets had higher reducing power than α-tocopherol, but lower than BHA and BHT. The reducing power of water and ethanol extracts of broccoli florets and standard compounds followed the order: BHA > BHT > ethanol extract > water extract > α-tocopherol.

Free radical scavenging activity (DPPH)

Scavenging activity using the stable DPPH radical is a popular method for evaluating antioxidant activities. The effect of antioxidants on DPPH radical scavenging is thought to be due to their hydrogen donating ability. DPPH· accepts an electron or hydrogen radical to become a stable diamagnetic molecule (SOARES *et al.*, 1997). The reduction capability of DPPH radicals was determined by the decrease in its absorbance at 517 nm. The maximum absorption of a stable DPPH radical in ethanol is at 517 nm. The decrease in absorbance of DPPH radicals is due to the reaction between antioxidant molecules and the radical.

It is visually noticeable as a discoloration from purple to yellow. Hence, DPPH· can be used as a substrate to evaluate antioxidant activity (DUH *et al.*, 1999; CHANG *et al.*, 2002).

As seen in Table 1, there is a significant (P<0.01) decrease in the concentration of the DPPH radical due to the scavenging ability of both extracts and standards. At 60 μg/mL, water and ethanol extracts of broccoli florets, BHA, and BHT showed similar DPPH scavenging activity, while that of α-tocopherol was significantly lower (p<0.01). The scavenging effect of water and ethanol extracts and standards on the DPPH radical decreased in the order of BHA = BHT = ethanol extract of broccoli floret = water extract of broccoli floret > α-tocopherol and were 62, 60, 59, 53 and 31% at a concentration of 60 μg/mL, respectively. These results indicated that both broccoli extracts had a noticeable effect on scavenging of the DPPH free radical. Free radical scavenging activity also increased with increasing concentration.

It has been reported that oxidative stress, which occurs when free radical formation exceeds the body's ability to protect itself, forms the biological basis of chronic conditions such as arteriosclerosis (FATIMAH *et al.*, 1998). Based on the data obtained from this study, both extracts act as free radical inhibitors or scavengers, as well as primary antioxidants that react with free radicals and may limit free radical damage occurring in the human body.

Superoxide anion scavenging activity

In the PMS-NADH-NBT system, superoxide anions, derived from dissolved oxygen by the PMS-NADH coupling reaction, reduce NBT. The decrease in absorbance at 560 nm indicates the consumption of the superoxide anion in the reaction mixture. Table 2 shows the percent inhibition of superoxide radical generation by 60 μg/mL of the water and ethanol extracts of broccoli florets and compares them with same concentration of BHA, BHT, and α-tocopherol. Both extracts of broccoli florets exhibited higher superoxide radical scavenging activity than BHT and α-tocopherol (p<0.01). Both aqueous and ethanolic extracts had superoxide radical scavenging activity similar to BHA. The percent inhibition of superoxide generation by 60 μg/mL of water and ethanol extracts was 73 and 76%, respectively, which was comparable or greater than

Table 2 - Percent of inhibition of superoxide radical generation, metal chelation activity and hydrogen peroxide scavenging activity of various concentrations of water extracts of broccoli (WEB), ethanol extracts of broccoli (EEB), BHA, BHT and α-tocopherol.

	Inhibition of superoxide radical generation (%)	Metal chelation activity (%)			Hydrogen peroxide scavenging activity (%)		
	60 μg/mL	20μg/mL	40μg/mL	60μg/mL	20μg/mL	40μg/mL	60μg/mL
BHA	77	19	47	59	12	17	23
BHT	34	56	60	71	3	12	20
α-tocopherol	26	6	44	56	7	20	28
WEB*	73	65	77	84	34	57	70
EEB**	76	70	81	89	39	61	81

*: 1.20 g dried water extract was found in 20 g dried floret tissue; †: 1.54 g dried water extract was found in 20 g dried floret tissue.

the same concentration of BHA, BHT, and α-tocopherol (77, 34 and 26%), respectively. Superoxide radical scavenging activity followed the order: BHA = ethanol extract of broccoli floret = water extract of broccoli floret > BHT > α-tocopherol.

Metal chelating activity

The chelation of ferrous ions by both extracts was estimated by the method of DINIS *et al.* (1994). Ferrozine can quantitatively form complexes with Fe^{2+}. In the presence of chelating agents, the complex formation is disrupted, resulting in reduction of the red colour of the complex. Measurement of colour reduction therefore allows estimation of the chelating activity of the coexisting chelator (YAMAGUCHI *et al.*, 2000). In this assay both broccoli floret extracts and standard antioxidant compounds interfered with the formation of ferrous ions and the ferrozine complex, suggesting that they have chelating activity and can compete for the capture of the ferrous ion without ferrozine.

Iron can stimulate lipid peroxidation by the Fenton reaction, and also accelerate peroxidation by decomposing lipid hydroperoxides into peroxyl and alkoxyl radicals that can themselves abstract hydrogen and perpetuate the chain reaction of lipid peroxidation (CHANG *et al.*, 2002, HALLIWELL, 1991).

$$H_2O_2 + Fe^{+2} \longrightarrow Fe^{+3} + OH^- + OH\cdot$$
(Fenton Reaction)

As shown in Table 2, the formation of the Fe^{2+}-ferrozine complex is not complete in the presence of water and ethanol extracts of broccoli florets, indicating that both extracts of broccoli floret tissue chelate iron. The absorbance of the Fe^{2+}-ferrozine complex linearly decreased in a dose dependent manner with increasing extract concentrations (from 20 to 60 μg/mL). The difference between both extracts and the control was statistically significant ($p<0.01$). The percent metal chelating capacity of 60 μg/mL concentration of water and ethanol extracts of broccoli florets was found to be 84 and 89%, respectively. The percent metal chelating capacity of α-tocopherol, BHA, and BHT at 60 μg/mL was 56, 59, and 71%, respectively. The metal scavenging effect of the extracts and standards decreased in the order of ethanol extract of broccoli floret = water extract of broccoli floret > BHT > BHA = α-tocopherol.

Metal chelating capacity is significant since it reduces the concentration of the catalysing transition metal in lipid peroxidation (DUH *et al.*, 1999). It has been reported that chelating agents which form σ-bonds with a metal are effective as secondary antioxidants because they reduce the redox potential thereby stabilizing the oxidized form of the metal ion (GORDON, 1990). The data reported in Table 2 show that both extracts of broccoli floret tissue demonstrate a marked capacity for iron binding, suggesting that their action as peroxidation protectors may be related to their iron binding capacity.

Scavenging of hydrogen peroxide

The scavenging ability of water and ethanol extracts of broccoli florets on hydrogen peroxide is shown in Table 2 and compared with BHA, BHT and α-tocopherol as standards. Extracts of broccoli florets were capable of scavenging hydrogen peroxide in a concentration–dependent manner. Water and ethanol extracts of broccoli florets exhibited 70 and 81% scavenging activity on hydrogen peroxide, respectively. BHA, BHT, and α-tocopherol at 60 μg/mL showed 23, 20, and 28% hydrogen peroxide scavenging activity, respectively. These results showed that both extracts of broccoli florets had effective hydrogen peroxide scavenging activity which was statisti-

cally higher than the controls ($p<0.01$). The hydrogen peroxide scavenging effect at 60 μg/mL of both extracts of broccoli florets and standards decreased in the order of ethanol extract > water extract > α-tocopherol = BHA = BHT. Hydrogen peroxide itself is not very reactive, but it can sometimes be toxic to the cell by giving rise to hydroxyl radicals. H_2O_2 can cross membranes and may slowly oxidize a number of biomolecules and compounds. H_2O_2 is formed in vivo by superoxide dismutates and also by many oxidase enzymes. H_2O_2 at micromolar levels is poorly reactive. However, higher levels of H_2O_2 can attack some energy-producing systems. H_2O_2 inactivates the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase. In addition, H_2O_2 also forms OH· in the presence of metal ions and oxygen facilitates this reaction. Thus, removing excessive H_2O_2 is very important for living organisms and for protecting food systems (HALLIWELl 1991; GÜLÇIN *et al.*, 2004b).

Scavenging of H_2O_2 by both extracts may be attributed to their phenolic content. Phenolics can donate electrons to H_2O_2, thus neutralizing it to water (HALLIWELL and GUTTERIDGE, 1985). H_2O_2 scavenging capacity may be attributed to the structural features of the active phytochemicals, which determine their electron donating abilities (WETTASINGHE and SHAHIDI, 2000).

Determination of total phenolic compounds

Phenolic compounds may contribute directly to antioxidative action (DUH *et al.*, 1999). It has been suggested that polyphenolic compounds have an inhibitory effect on mutagenesis and carcinogenesis in humans, when up to 1.0 g is ingested daily from a diet rich in fruits and vegetables (TANAKA *et al.*, 1998). In addition, it has been reported that phenolic compounds are associated with antioxidant activity and play an important role in stabilizing lipid peroxidation (YEN *et al.*, 1993).

Phenols are very important plant constituents because of their radical scavenging ability due to their hydroxyl groups (HATANO *et al.*, 1989). According to recent reports, a highly positive relationship between total phenols and antioxidant activity has been found in many plant species (VINSON *et al.*, 1998; VELIOGLU *et al.*, 1998; GÜLÇIN *et al.*, 2004c; OKTAY *et al.*, 2003). In 1mg of the water and ethanol extracts of broccoli, 25.3 and 19.6 μg gallic acid equivalents of phenols were detected, respectively. The total phenolic content is lower than previous reports. LEJA *et al.* (2001) reported a considerable increase in the total phenol level during short-term storage of broccoli at room temperature. The difference between the phenolic contents of the water and ethanol extracts of broccoli was not significant. According to these results, there does not appear to be a relationship between total phenols and total antioxidant activity.

CONCLUSION

The results of this study indicate that both aqueous and ethanolic extracts of broccoli florets have powerful antioxidant activity against various free radical inducing systems *in vitro.*

Broccoli florets can potentially be used as an easily accessible source of natural antioxidants and may be used as a possible food supplement or in the pharmaceutical industry. The various antioxidant mechanisms of extracts of broccoli florets may be attributed to strong hydrogen donating capacity, metal chelation ability and their effectiveness as scavengers of hydrogen peroxide, superoxide and other free radicals.

It has been suggested that a higher consumption of fruit and vegetables with high phytochemicals can inhibit, prevent or retard chronic diseases. The results

of this study imply that dietary phytochemicals from broccoli may supply substantial antioxidants which may provide health-promoting effects to consumers. The compounds responsible for the antioxidative activity of both extracts of broccoli florets are not well known. Therefore, it is suggested that further work could be performed on the isolation and identification of the antioxidant compounds in broccoli.

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PAPER

EFFECTS OF BLANCHING ON FIRMNESS OF SLICED POTATOES

EFFETTI DI TRATTAMENTI DI BLANCHING
SULLA CONSISTENZA DI PATATE IN FETTE

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ABSTRACT

Response surface methodology was used to investigate variables affecting the firmness of blanched potato slices such as type of blanching, time of treatment, NaCl or $CaCl_2$ concentrations and lactic acid concentration. Results showed that the mathematical models were able to provide a good estimate of the effects of individual and interactive factors on the retention of firmness of processed potato. The best results were obtained with microwave blanching of slices dipped in calcium chloride-con-

RIASSUNTO

Allo scopo di studiare l'influenza sulla consistenza di patate in fette sottoposte a scottatura di variabili quali la modalità di scottatura, il tempo di trattamento, la concentrazione di cloruro di sodio o di cloruro di calcio e la concentrazione di acido lattico sulla consistenza di fette di patate, è stata adottata la metodologia delle superfici di isorisposta. I risultati hanno dimostrato la capacità dei modelli matematici ottenuti di fornire una buona stima degli effetti delle variabili, considerate indi-

- Key words: calcium chloride, microwave, pre-heating, rheological property, starch gelatinization, RSM -

taining solution. In addition to firmness analysis, weight loss, moisture and starch crystallinity levels were used to determine blanching treatment effectiveness.

vidualmente ed in interazione con le altre, sulla consistenza delle patate in fette dopo l'operazione di scottatura. In particolare, i migliori risultati in termini di ritenzione della consistenza, sono stati ottenuti con la scottatura in soluzioni contenenti cloruro di calcio. Oltre alla determinazione della consistenza, la valutazione della variazione di sostanza secca ed acqua e il grado di cristallinità dell'amido sono stati usati come indici dell'efficacia dei trattamenti di scottatura studiati.

INTRODUCTION

Blanching as a pre-treatment in the production of canned, frozen and dehydrated vegetables is performed for several reasons: inactivation of enzymes such as polyphenoloxidases, responsible for browning (MAPSON *et al.*, 1963; Collins and McCarty, 1969) and peroxidase, which leads to the development of off-flavours (PINSENT, 1962). It also decreases the reducing sugar content on the surface of the product, preventing changes in colour due to caramelization and Maillard reactions (SMITH, 1975). Blanching can be performed by exposing vegetables to hot water (the most common method), steam (KIDMOSE and MARTENS, 1999) or microwaving (CHEN *et al.*, 1971; RAMASWAMY and VAN DE VOORT, 1990; PONNE *et al.*, 1991) for several seconds or minutes. A certain cooking effect connected to a blanching operation can be quite acceptable since most vegetables have to be cooked prior to consumption. Blanching in boiling water has some disadvantages such as loss of soluble solids, high water use and, consequently, a large volume of water to drain away (SEVERINI *et al.*, 2001). Heat treatment also causes changes in textural characteristics (probably because of swelling of gelatinized starch), loss of cell turgor and activation/inhibition of pectic enzymes. All these factors can cause a decrease in firmness (MALTINI *et al.*, 1993).

To ameliorate firmness retention, calcium chloride can be added to the blanching solution (BOUWKAMP, 1985). Calcium and other divalent metal ions are known to increase firmness retention due to cross-linking with pectic substances (LEE and HOWARD, 1999), according to the "egg box" model proposed by VAN BUREN (1979). In this way, the rate of cleavage of pectic substances by β-elimination during heat processing and that of extraction by hot water are decreased. Furthermore, it has been found that the addition of a vacuum-infiltrated base followed by tissue neutralization reduces softening (WALTER *et al.*, 1992), because in this way demethylation of cell wall pectins by endogenous pectin methylesterase enzymes happens.

Low temperature blanching (performed at 62°C) has been found to increase firmness retention (ANDERSON *et al.*, 1994; FUCHIGAMI *et al.*, 1995; HOWARD *et al.*, 1997; TRUONG *et al.*, 1998; VAN DIJK *et al.*, 2002). BARTOLOME and HOFF (1972) postulated a mechanism for the firming effect of precooking

on potatoes performed at moderate temperatures. According to them, low temperature blanching causes the disruption of cytoplasmic membranes, allowing the passage of electrolytes (potassium ions, in particular) from the cytoplasm to the middle lamella. Here, pectinesterase is bound to the negatively charged pectins in the cell wall, but the presence of electrolytes causes its release. The mobilized and activated pectinesterase hydrolyzes the methyl ester linkages in the pectin molecules. Finally, the de-esterifed pectins form calcium bridges between free carboxyl groups.

DI CESARE *et al.* (1997) compared microwave blanching of apple cubes with or without water to traditional blanching in boiling water. They found that the best firmness was obtained by microwave blanching at 80°C without water. With reference to textural changes in carrots during blanching, GLASSCOCK *et al.* (1982) found higher shear values for carrots following microwave blanching than following water blanching. KIDMOSE and MARTENS (1999) found the same tendency even if the difference among the methods was not significant. Furthermore, a significantly higher content of dry matter was obtained by microwave blanching than by steam and water blanching.

With acidifying blanching two objectives can be achieved: reduction of the total microbial load and decrease in the pH to 4.5, which is the upper limit recommended by the Food and Drug Administration (FDA, 2002). This allows sensorial acceptability to be maintained and the safety and the microbial stability of the packed product to be met by using only a mild heat treatment.

In this paper, Response Surface Methodology (RSM) was applied to investigate the way in which variables such as types of blanching (by dipping in boiling solutions or by microwaves), time of treatment, addition of sodium or calcium chloride and lactic acid, affect the firmness of blanched potato slices. Three factor-five level, second order central composite designs were developed to analyze and model the considered variables. In addition to firmness analysis, weight loss, moisture, and starch crystallinity level were used as indices to measure the effectiveness of the blanching treatments.

MATERIALS AND METHODS

Raw materials

Trials were performed on potato tubers (*Solanum tuberosum* var. *Primura*) purchased at a local market. This Italian variety, characterized by an early ripening time, is the one most commonly used in the food industry due to its regular shape and good resistance to cooking of its large tubers. Tubers were stored at room temperature and in the dark.

After grading, tubers were washed under running water, wiped with blotting paper, hand-peeled and cut into round slices (4 cm diameter, 1 cm thickness).

Blanching treatments

Blanching was performed by:

- microwaves (Sfornatutto, mod. Combi&Function Convection, De Longhi, Milan, Italy) at 850 watt upon immersion in either a sodium chloride-lactic acid solution or a calcium chloride-lactic acid solution;
- dipping in either a boiling sodium chloride-lactic acid solution or a boiling calcium chloride-lactic acid solution.

For each trial, about 100 g of potato slices were blanched. Salts and acid concentrations and time of treatment were in accordance with the combinations of the two Central Composite Designs described below. The product:solution ratio was 1:4 (w/w). After blanching, samples were cooled in

tap water, drained and wiped with blotting paper.

Unblanched potato slices were used as controls.

Experimental design

The five levels chosen for each of the three independent variables were as follows: time of treatment (t) from 2 to 6 min, lactic acid concentration (LA%) from 0.5 to 2.5% and sodium chloride concentration (NaCl%) from 1.0 to 5.0%. Alternatively, time of treatment and lactic acid concentration were the same as listed above, whereas calcium chloride ($CaCl_2$%) was used instead of sodium chloride, with concentrations ranging from 0.1 to 0.5%. Concentrations were always expressed as %w/w. Lactic acid was used to avoid an acidic taste of the samples, because of its very low organoleptic impact; furthermore, it has a good acidifying power. Sodium and calcium chloride are generally used in blanching of vegetables. In particular, calcium was chosen because it is well known to increase firmness retention.

The effects of the individual and interactive variables were investigated by modulating their levels according to a three factor-five level Central Composite Design (BOX *et al.*, 1978). The application of a Central Composite Design allowed the number of possible combinations to be reduced to a manageable size because it uses only a fraction of the total number of factor combinations for experimentation. The 17 combinations obtained for each of the two Central Composite Designs are reported in Table 1.

Table 1 - Combinations of the three factor-five level Central Composite Designs.

Samples	t (min)	LA%	NaCl% or $CaCl_2$%	
1	5	1.0	4.0	0.4
2	3	1.0	4.0	0.4
3	3	1.0	2.0	0.2
4	5	1.0	2.0	0.2
5	3	2.0	4.0	0.4
6	5	2.0	4.0	0.4
7	5	2.0	2.0	0.2
8	3	2.0	2.0	0.2
9	4	1.5	3.0	0.3
10	4	1.5	3.0	0.3
11	4	2.5	3.0	0.3
12	4	0.5	3.0	0.3
13	4	1.5	5.0	0.5
14	4	1.5	1.0	0.1
15	6	1.5	3.0	0.3
16	2	1.5	3.0	0.3
17	4	1.5	3.0	0.3

Analyses

On raw and blanched samples, the following analyses were carried out:

- Weight loss (g): weighing samples before and after treatments.

- Moisture (%): placing a carefully weighed sample in a static oven (ISCO s.r.l, Milan, Italy) at 105°C until it reached a constant weight (AOAC, 1984).

The data are reported as the average of at least three replications.

- Firmness: using a model 4301 Universal Testing Machine (Instron International LTD, High Wycombe, UK), equipped with a standard plunger (ϕ 3 mm) at a crosshead speed of 200 mm/min, compression force was taken as the maximum peak of the recorded force expressed as Newtons (N) (MALTINI *et al.*, 1993). For each treatment, at least ten determinations were performed (coefficient of variation less than 8%).

- Starch crystallinity degree: samples were previously freeze-dried, gently ground and vacuum stored until analysis. Crystallinity level was determined with an X-ray diffractometer (Philips AXR, software PW 1710). Wide angle X-ray diffraction patterns were obtained with nickel-filtered CuKα radiation. For each 2q value, determination of the intensity was repeated six times; hence, each diffraction pattern consisted of six different sets of data. To determine the

degree of starch crystallinity, the six sets of data were simultaneously fitted with a series of Gaussian functions.

Statistical analysis

The data was submitted to statistical analysis using Winstat ver. 5.1 (Statsoft, Tulsa, USA) software.

The mathematical modeling was carried out in two steps. The first involved a stepwise regression to identify the relevant variables; the second consisted in multiple regression (Standard Least Square Fitting) to fit a second order mathematical model, according to the following polynomial equation:

$$y = B_0 + \sum B_{i\chi_i} + \sum B_{ii\chi_{ii}}{}^2 + \sum B_{ij\chi_i\chi_j}$$

where y is the dependent variable (firmness of the blanched samples), B0 is a constant value, χ_i and χ_j are the independent variables in coded values and B_i, B_{ii}, B_{ij} are the regression coefficients of the model. This model allowed the effects of the linear (χ_i), quadratic (χ_i^2) and combined ($\chi_i\chi_j$) terms of the independent variables (t, LA% and NaCl% or $CaCl_2$%) to be assessed on the dependent variable.

In order to describe the individual and interactive effects of the independent variables on the positive outcome of the blanching treatments (in terms of firmness retention), iso-response surfaces were developed, considering one of the independent variables constant at the central level of the Central Composite Design.

RESULTS AND DISCUSSION

During blanching, in addition to heat transfer, mass transfer (of solids and water) occurs. The dry matter balance of blanched potato slices consisted in the release of starch (mainly) into water and in a gain in soluble solids (lactic acid and sodium or calcium chloride). The water balance consisted in a water gain/loss.

Data (Tables 2-5) show that samples lost weight as a consequence of all the

Table 2 - Mass and water transfer balance of potato slices blanched in boiling NaCl-LA solutions.

Samples	Dry matter (g)	H_2O (g)	Weight loss (g/100 g)*	Dry matter gain/loss (g/100 g)	H_2O gain/loss (g/100 g)
1	17.73	82.27	-5.09	+0.23	-5.31
2	16.89	83.11	-3.78	-0.35	-3.43
3	17.56	82.44	-3.68	-1.61	-2.07
4	18.79	81.21	-3.60	-0.41	-3.19
5	18.82	81.18	-3.58	-0.37	-3.21
6	18.63	81.37	- 3.42	-0.53	-2.89
7	17.90	82.10	-4.43	-1.41	-3.02
8	16.21	83.79	-3.19	-2.83	-0.36
9	14.15	85.85	-3.33	-4.84	+1.51
10	17.92	82.08	-3.44	-0.51	-2.93
11	17.25	82.75	-4.14	-1.27	-2.86
12	14.31	85.69	-2.78	-3.90	-1.18
13	18.39	81.61	-3.05	+0.02	-3.07
14	17.07	82.93	-2.15	-1.11	-1.04
15	17.41	82.59	-3.26	-0.97	-2.29
16	14.02	85.98	-2.27	-4.11	+1.84
17	16.46	83.54	-2.69	-1.79	-0.90

* expressed as the difference between weight after blanching and weight before blanching.

Table 3 - Mass and water transfer balance of potato slices blanched in boiling $CaCl_2$-LA solutions.

Samples	Dry matter (g)	H_2O (g)	Weight loss (g/100 g)*	Dry matter gain/loss (g/100 g)	H_2O gain/loss (g/100 g)
1	12.04	87.96	-1.21	-4.37	+3.16
2	14.99	85.01	-0.62	-1.37	+0.75
3	14.62	85.38	-0.72	-2.46	+1.74
4	15.48	84.52	-0.38	-1.56	+1.18
5	15.40	84.60	-0.61	-1.67	+1.06
6	15.44	84.56	-2.17	-1.88	-0.29
7	14.84	85.16	-3.26	-2.62	-0.64
8	17.78	82.22	-2.14	+0.42	-2.56
9	15.07	84.93	-1.95	-2.20	+0.25
10	14.81	85.19	-1.67	-6.82	+5.15
11	16.06	83.94	-2.47	-5.72	+3.25
12	15.25	84.75	-2.31	-6.48	+4.17
13	15.78	84.22	-1.54	-5.84	+4.30
14	12.44	87.56	-2.01	-4.08	+2.07
15	13.15	86.85	-2.92	-3.50	+0.58
16	13.35	86.65	-2.08	-3.20	+1.18
17	15.49	84.51	-1.75	-1.05	-0.70

* expressed as the difference between weight after blanching and weight before blanching.

Table 4 - Mass and water transfer balance of potato slices blanched by microwaves upon immersion in NaCl-LA solutions.

Samples	Dry matter (g)	H_2O (g)	Weight loss (g/100 g)*	Dry matter gain/loss (g/100 g)	H_2O gain/loss (g/100 g)
1	14.80	85.20	-2.80	-0.89	-1.91
2	16.51	83.49	-2.33	+0.85	-3.18
3	15.26	84.74	-2.39	-0.38	-2.01
4	16.12	83.88	-2.65	+0.42	-3.07
5	16.25	83.75	-2.08	+0.64	-2.72
6	15.96	84.04	-2.56	+0.28	-2.84
7	13.06	86.94	-2.17	-2.49	+0.32
8	14.70	85.30	-2.46	-0.93	-1.53
9	14.14	85.86	-2.26	-1.45	-0.81
10	13.18	86.82	-2.67	-2.44	-0.23
11	16.24	83.76	-2.80	-0.43	-2.37
12	16.95	83.05	-2.48	+0.31	-2.79
13	16.43	83.57	-2.39	-0.18	-2.21
14	13.00	87.00	-1.76	-3.45	+1.69
15	14.38	85.62	-2.85	-2.25	-0.60
16	15.27	84.73	-1.85	-1.23	-0.62
17	13.90	86.10	-2.48	-1.71	-0.77

* expressed as the difference between weight after blanching and weight before blanching.

Table 5 - Mass and water transfer balance of potato slices blanched by microwaves upon immersion in $CaCl_2$-LA solutions.

Samples	Dry matter (g)	H_2O (g)	Weight loss (g/100 g)*	Dry matter gain/loss (g/100 g)	H_2O gain/loss (g/100 g)
1	17.42	82.58	-2.23	-4.35	+2.12
2	14.23	85.77	-1.16	-7.31	+6.15
3	16.09	83.91	-1.51	-2.43	+0.92
4	15.62	84.38	-1.44	-2.88	+1.44
5	16.52	83.48	-1.68	-2.04	+0.36
6	12.46	87.54	-1.29	-5.98	+4.69
7	16.69	83.31	-1.93	-2.56	+0.63
8	16.46	83.54	-1.91	-2.78	+0.87
9	15.23	84.77	-1.57	-3.94	+2.37
10	16.13	83.87	-1.95	-3.11	+1.16
11	15.41	84.59	-1.31	-3.72	+2.41
12	18.19	81.81	-1.33	-0.98	-0.35
13	17.59	82.41	-1.33	-1.57	+0.24
14	16.95	83.05	-1.04	-2.16	+1.12
15	11.60	88.40	-2.79	-7.65	+4.86
16	13.22	86.78	-0.98	-5.84	+4.86
17	16.81	83.19	-1.43	-2.36	+0.93

* expressed as the difference between weight after blanching and weight before blanching.

blanching treatments. The weight loss was principally due to the water loss for samples blanched in the NaCl-containing solution (Tables 2 and 4), whereas it was strongly due to the dry matter loss for samples blanched in the $CaCl_2$-containing solutions (Tables 3 and 5). In particular, blanching in the boiling NaCl-LA solution produced a decrease in both dry matter and water content (Table 2). Samples blanched in the boiling $CaCl_2$-LA solutions showed a decrease in dry matter and an increase in water content (Table 3). The differences between these two kinds of treatments were significant: p=0.0346 for Δdry matter and <0.00001 for Δwater content). The same occurred in samples blanched by microwaves and, in this case p was less than 0.00001 for both Δdry matter and Δwater content (Tables 4 and 5).

The partial dehydration caused by sodium chloride may be due to the high osmotic pressure of the solutions containing this solute which ranged from 1.0 to 5.0%. The water gain in the samples dipped in the $CaCl_2$-containing solutions was due to the fact that calcium causes an increase in Tg (glassy transition temperature) and, as a consequence, there is a greater water demand for starch gelatinization (NURUL and AZEMI, 1994); furthermore, the calcium chloride concentrations were lower (0.1-0.5%).

With respect to dipping in the NaCl-containing solutions, the difference in mass transfer between traditional and microwave blanching was negligible (p≥0.2049). The same occurred for dipping in the $CaCl_2$ solutions (p≥0.3935). Probably, the different way of heat transfer did not affect mass transfer if the microwave treatment was carried out upon immersion in saline-acid solutions.

The crystallinity level of a food matrix containing starch can be considered as an indirect index of heat-induced starch gelatinization. Fig. 1 reports the X-ray

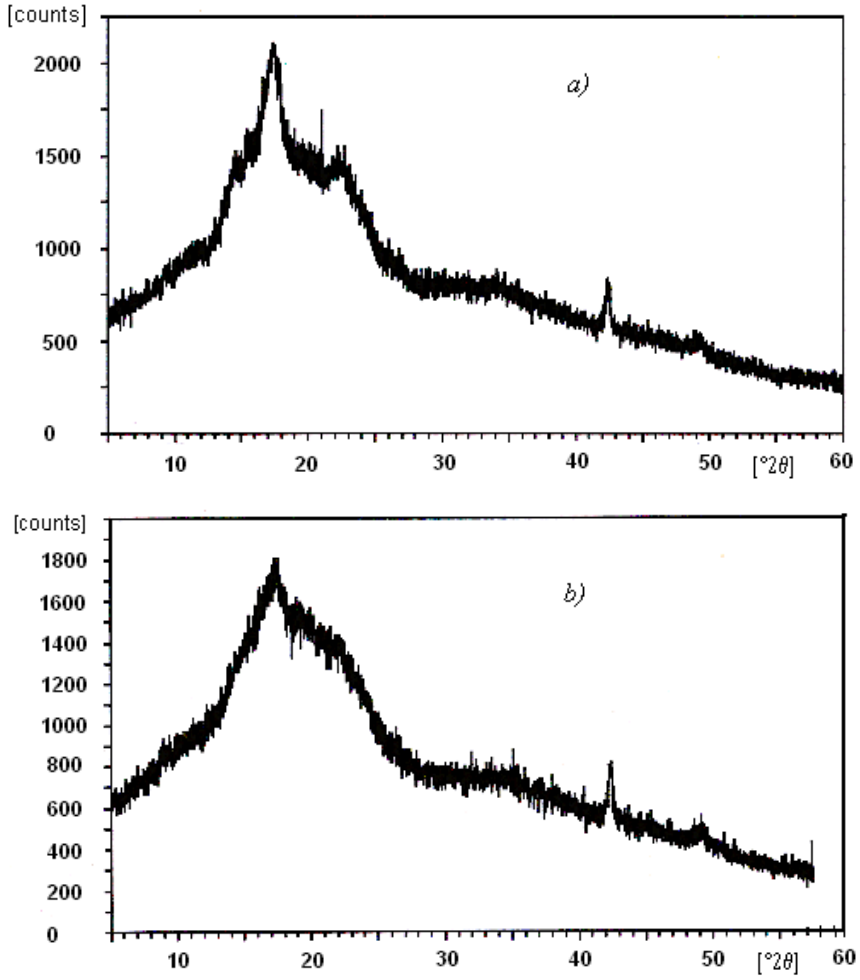


Fig. 1 - X-ray diffraction patterns of a) unblanched potato slices (ungelatinized) and b) potato slices blanched for 6 min in a boiling 0.3% $CaCl_2$-1.5% LA solution (completely gelatinized).

diffraction patterns of *a)* unblanched potato slices (ungelatinized starch) and *b)* samples blanched for 6 min in a boiling 0.3% $CaCl_2$-1.5% LA solution (thoroughly gelatinized starch). The difference between the two profiles consisted in the absence of the second peak (°2θ) from the diffractogram of the gelatinized sample. Interpretation of the crystallinity data of the potato matrix is difficult because of the already very low crystallinity level of the unblanched sample (5.38%). In any case, with respect to potato slices dipped in the boiling 0.3% $CaCl_2$-1.5% LA solution (Table 6), it is very evident that traditional blanching caused a significantly (p=0.0028) greater decrease in the crystallinity level than the microwave treatment: this is in accordance with PIAZZA *et al.* (1994) who found that the higher temperature reached in traditional blanching increased the endothermic effect of amylopectin crystal fusion. Furthermore, the

Table 6 - Crystallinity degree of starch in potato slices: comparison between blanching in a boiling solution and blanching by microwaves upon immersion in the same solution (crystallinity degree of raw material=5.38%).

Time of treatment (min)	Starch crystallinity degree %	
	Blanching in boiling 0.3% $CaCl_2$-1.5% LA solution	Blanching by microwaves in 0.3% $CaCl_2$-1.5% LA solution
2	3.72	4.62
3	2.74	4.98
4	3.38	3.81
5	3.65	3.70
6	3.91	4.52

crystallinity level dramatically decreased within the first 2 min of treatment and then was almost constant up to 6 min. This was due to the low crystallinity level of the raw potatoes: a brief heat treatment was already able to induce full gelatinization.

Table 7 reports the firmness values of all the blanched samples. The firmness of raw potato slices was equal to 10.24 N, hence it is clear that each blanching treatment caused a dramatic decrease in firmness. With respect to traditional blanching, samples dipped in the $CaCl_2$-containing solutions were significantly (p=0.0457) firmer than slices dipped in the NaCl-containing solutions. The same was found for samples blanched by mi-

Table 7 - Firmness, measured immediately after treatment, of potato slices blanched according to the Central Composite Designs.

Samples	Firmness (N)			
	Blanching in boiling solutions		Blanching by microwaves upon immersion	
	NaCl-LA	$CaCl_2$-LA	NaCl-LA	$CaCl_2$-LA
1	4.363	5.304	5.637	6.323
2	6.176	3.794	5.284	6.559
3	5.343	4.510	6.618	5.588
4	3.951	4.823	5.294	6.137
5	4.970	4.520	5.069	6.618
6	4.696	5.176	6.049	5.520
7	3.833	6.029	5.882	6.284
8	4.470	5.500	5.470	6.774
9	5.000	5.725	5.912	5.225
10	5.294	4.892	6.412	5.520
11	3.559	4.784	5.882	6.098
12	3.323	5.451	5.843	7.118
13	4.637	5.598	6.627	6.549
14	3.696	5.284	6.010	6.833
15	4.323	4.294	6.510	4.539
16	4.010	4.412	7.255	8.431
17	4.431	4.176	6.294	5.490
Mean values	4.475	4.957	6.003	6.212

crowaves, even if the difference was not significant (p=0.4193). This effect was likely due to the presence of calcium which caused a greater retention of firmness thanks to the formation of calcium pectate (LEE and HOWARD, 1999; VAN BUREN, 1979). Samples treated by microwaves were significantly firmer than the traditionally blanched ones (p<0.00001). This is in accordance with PIAZZA *et al.* (1994) and data concerning both mass transfer and crystallinity level.

The general equations that refer to the four different kinds of blanching and that were obtained by applying the stepwise backward regression and multiple regression to the firmness experimental data, are the following:

- Blanching in boiling NaCl-LA solution

$$\text{Firmness} = 9.65214-1.94473*(t)-1.48167*(LA\%)^2+1.05309*(t)*(LA\%)$$
$$r=0.908,\ p<0.00002;$$

- Blanching in boiling $CaCl_2$-LA solution

$$\text{Firmness} = 10.0596-1.1801*(t)-18.6815*(CaC_{l2}\%)+4.3608*(t)*(CaCl_2\%)$$
$$r=0.904,\ p<0.00002;$$

- Blanching by microwaves upon immersion in NaCl-LA solution

$$\text{Firmness} = 10.28858-1.39671*(t)-0.82138*(NaCl\%)-$$
$$-0.64612*(LA\%)^2+0.20776*(t)*(NaCl\%)+0.47917*(t)*(LA\%)$$
$$r=0.923,\ p<0.00013;$$

- Blanching by microwaves upon immersion in $CaCl_2$-LA solution

$$\text{Firmness} = 10.33327 - 1.72482*(t)+0.14774*(t)^2+$$
$$+20.44243*(CaCl_2\%)^2+0.76912*(LA\%)^2-8.16448*(CaCl_2\%)*(LA\%)$$
$$r=0.915,\ p<0.00021.$$

The independent variables compared in these equations were significant. Together with these data, Figs. 2-5 show the tri-dimensional plots described by the partial equations which were calculated from the equations reported above, considering the value of one of the three independent variables constant at the central level of the Central Composite Design (time=4, LA=1.5%, NaCl=3.0% or $CaCl_2$=0.3%).

With reference to blanching in the boiling NaCl-LA solutions, lactic acid concentration and time of treatment were the factors mainly affecting sample firmness (Fig. 2); the negative value related to the coefficients of these variables means that they affected the retention of firmness in a negative way. The highest firmness values were obtained at the lowest times of treatment and acid concentrations. Whereas the negative correlation between firmness and time of blanching is obvious, the negative coefficient with respect to the acid concentration could be due to the higher pectin solubility in the acid medium which determined a leaching phenomenon. The concentration of NaCl does not appear in the general equation; it had no effect on firmness probably because it does not form cross-linking with pectins.

Time of treatment and $CaCl_2$ concentration were the variables affecting the retention of firmness as a consequence of blanching in the boiling $CaCl_2$-LA solution; the contribution of lactic acid was negligible if compared to the great effect of the addition of calcium. The effect of calcium chloride on firmness retention was evident already at low concentra-

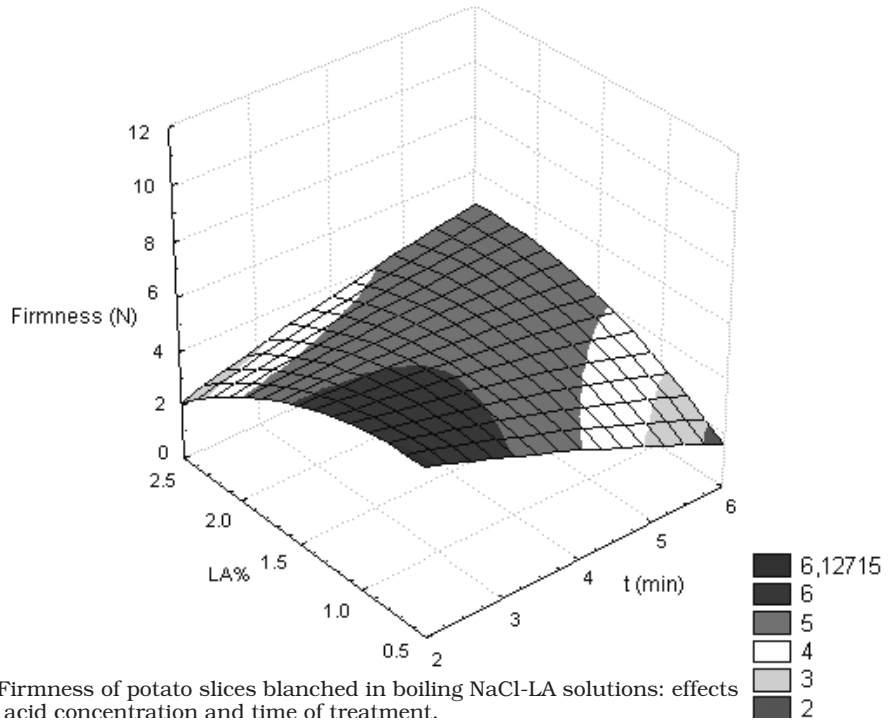


Fig. 2 - Firmness of potato slices blanched in boiling NaCl-LA solutions: effects of lactic acid concentration and time of treatment.

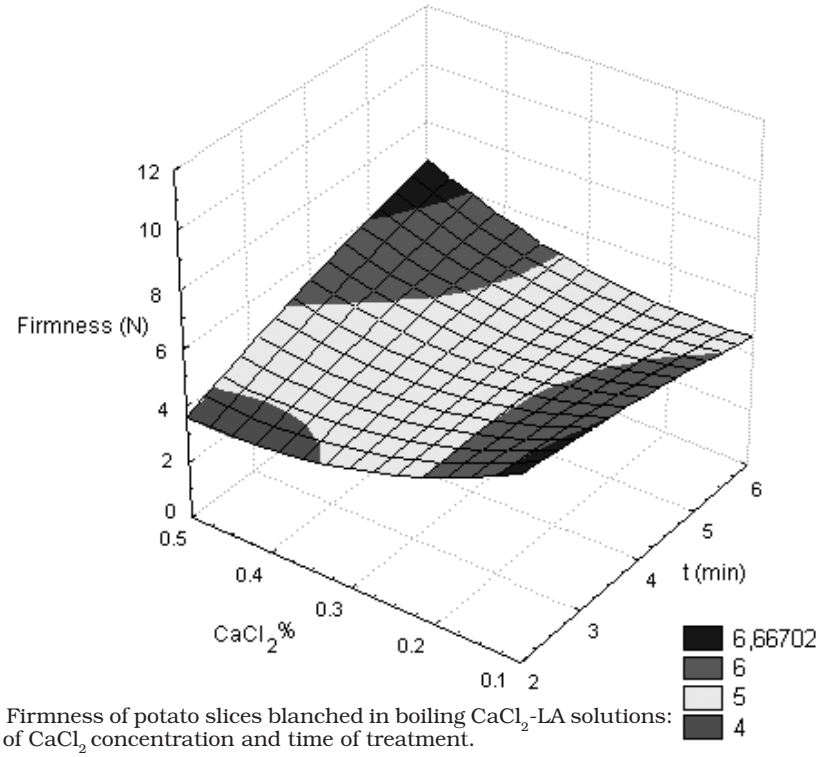


Fig. 3 - Firmness of potato slices blanched in boiling $CaCl_2$-LA solutions: effects of $CaCl_2$ concentration and time of treatment.

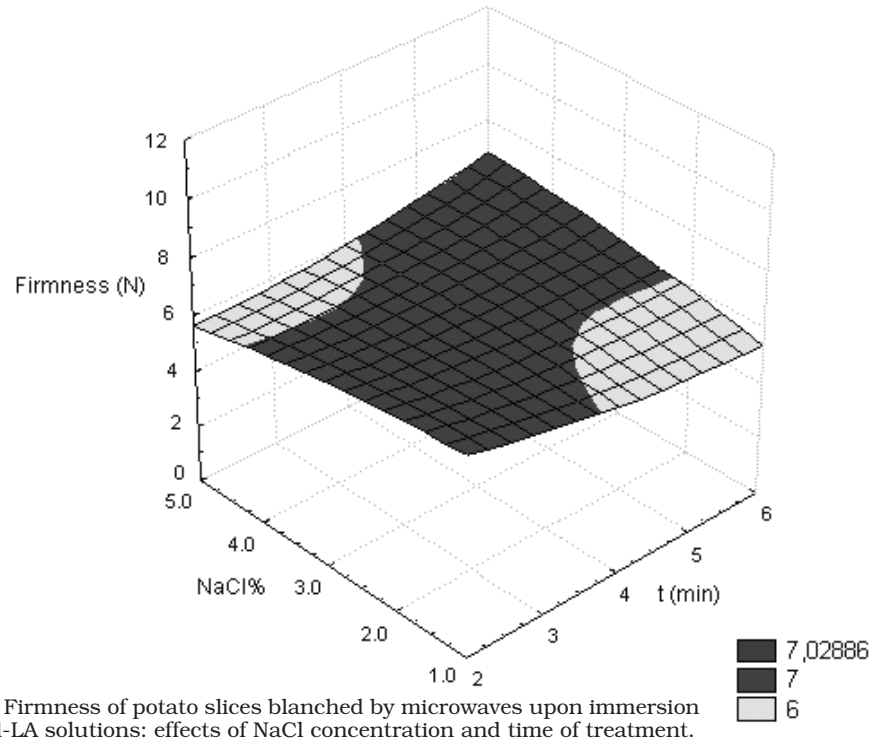


Fig. 4 - Firmness of potato slices blanched by microwaves upon immersion in NaCl-LA solutions: effects of NaCl concentration and time of treatment.

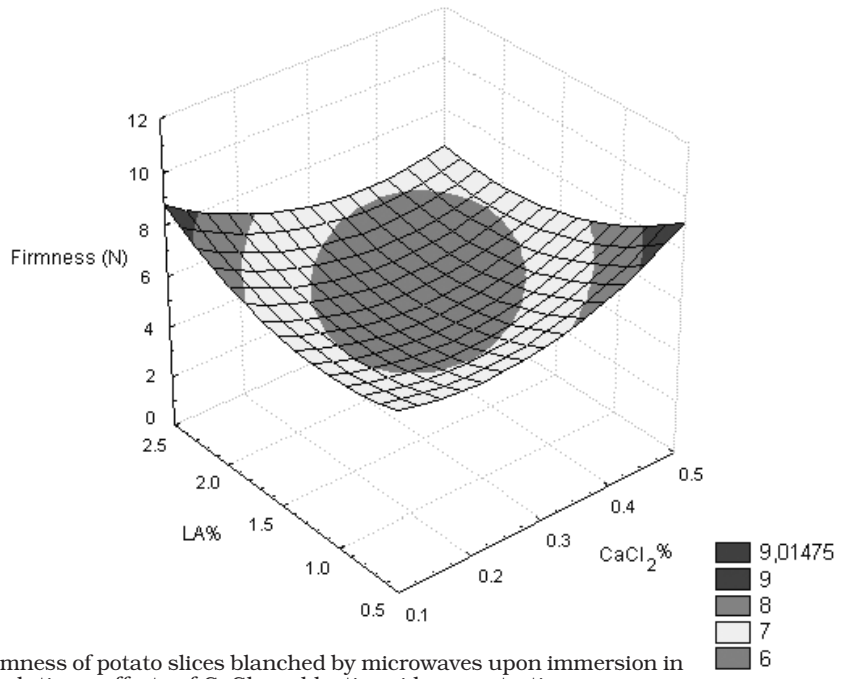


Fig. 5 - Firmness of potato slices blanched by microwaves upon immersion in $CaCl_2$-LA solutions: effects of $CaCl_2$ and lactic acid concentrations.

tions for brief times of blanching; extending the time of treatment, a higher $CaCl_2$ concentration was necessary (Fig. 3).

Considering the microwave blanching of potato slices dipped in the NaCl-LA solutions, NaCl concentration seemed to have little influence due to the small values of its coefficients. Rather, the effect of lactic acid on firmness was already evident at low concentrations for brief times of blanching; extending the time of treatment, a higher concentration was necessary (data not shown). At low concentrations, NaCl allowed a certain retention of firmness for only short times of blanching, otherwise it was necessary to increase the concentration; in the other cases, firmness was lower (Fig. 4). For all NaCl concentrations, firmness retention was good at intermediate lactic acid concentrations (data not shown).

With reference to microwave blanching in the $CaCl_2$-LA solution, firmness decreased as time of treatment increased (Fig. 5). At a given time of treatment, it is sufficient that either the lactic acid or calcium chloride concentration is higher to allow good retention of firmness. At an intermediate concentration, samples showed the lowest firmness, resulting in negative interactions.

CONCLUSIONS

These trials showed that Response Surface Methodology is a suitable method to specify the best operating conditions which should be applied to carry out a blanching treatment able to ensure good firmness retention in potato slices.

Results show that the addition of sodium chloride to the blanching solution caused a partial dehydration of potato slices with an evident loss in cell turgor, whereas the presence of calcium chloride had a strong preserving effect thanks to the high water gain. Traditional blanching induced greater gelatinization than microwave blanching. The best retention of firmness was obtained with microwave blanching of potato slices dipped in a calcium-chloride containing solution. The addition of lactic acid caused the pH to decrease below 4.5 and inhibit the potential growth of naturally occurring pathogens which could negatively affect firmness.

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PAPER

MICROBIOLOGICAL, COMPOSITIONAL AND BIOCHEMICAL CHARACTERISATION OF PDO CANESTRATO PUGLIESE CHEESE

CARATTERIZZAZIONE MICROBIOLOGICA, BIOCHIMICA DEL CANESTRATO PUGLIESE PDO

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ABSTRACT

Microbiological, compositional and biochemical characterisation of the Protected Designation of Origin (PDO) Canestrato Pugliese cheese made from raw ewes' milk is reported. Very few differences were found between two batches

RIASSUNTO

Il formaggio Canestrato Pugliese PDO è stato caratterizzato da un punto di vista microbiologico, fisico-chimico e biochimico durante 150 giorni di maturazione. Le analisi condotte su due lavorazioni di formaggio hanno

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

of cheese analysed during 150 d of ripening. Fully ripened cheese contained a total of log 7.7 cfu g^{-1} mesophilic bacteria, log 4.6 cfu g^{-1} presumptive enterococci and log 4.0 cfu g^{-1} presumptive staphylococci. Coliforms were detectable in fresh but not in fully ripened cheeses. Streptococci decreased, while lactobacilli increased during ripening. *Lactobacillus delbrueckii*, *L. fermentum*, *L. helveticus*, *Streptococcus thermophilus*, *Lactococcus lactis* subsp. *lactis*, *L. plantarum*, *L. casei* and *L. reuteri* were identified during ripening; the thermophilic strains were probably derived from the natural starter used, the others were probably components of the adventitious microflora. Urea-PAGE electrophoresis of the water-insoluble fraction and the Reverse Phase-HPLC of the ethanol-soluble sub-fractions were good indices of cheese-ripening time. As shown by multivariate statistical analysis, samples after 90 to 150 d of ripening were differentiated from the others. Fully ripened cheese contained ca. 10 mg g^{-1} of total free amino acids. The concentrations of total free amino acids and, especially, of glutamic acid, lysine, leucine and valine increased throughout cheese ripening. Fully ripened cheese contained ca. 1,315 mg kg^{-1} of free fatty acids; butyric, caproic, palmitic, oleic and linoleic acids were found at the highest concentrations. In contrast to proteinase and lipase activities, peptidase, as well as esterase activities, increased progressively during ripening. The greatest increase in the peptidase activity occurred when the number of lactobacilli in the ripening cheese was at its highest level.

evidenziato modeste differenze. Dopo 150 giorni di maturazione, i formaggi erano caratterizzati dall'assenza di coliformi e dalla presenza di batteri mesofili, enterococchi e stafiloccchi ad una concentrazione di log 7,7, 4,6 e 4,0 ufc g^{1}, rispettivamente. Batteri lattic mesofili e termofili hanno evidenziato una variazione opposta. Le specie identificate durante la maturazione erano *Lactobacillus delbrueckii*, *L. fermentum*, *L. helveticus*, *Streptococcus thermophilus*, *Lactococcus lactis* subsp. *lactis*, *L. plantarum*, *L. casei* and *L. reuteri*. Le specie termofile sono probabilmente derivate dallo starter usato, quelle mesofite sono originate da una contaminazione ambientale. L'elettroforesi Urea-PAGE della frazione azotata insolubile in acqua e l'analisi RP-HPLC della frazione azotata solubile in acqua, hanno messo in evidenza le differenze dei formaggi durante la maturazione, come mostrato dall'analisi statistica multivariata dei formaggi dopo 90 e 150 giorni di maturazione. La concentrazione di aminoacidi liberi, in particolare acido glutammico, lisina, leucina e valina aumentava progressivamente con la maturazione sino a raggiungere nel formaggio di 150 giorni una concentrazione totale di 10 mg g^{-1}. I formaggi a fine maturazione erano caratterizzati da un contenuto di acidi grassi liberi pari a ca. 1.315 mg kg^{-1} ed in particolare gli acidi butirrico, caproico, palmitico, oleico e linoleico sono risultati preponderanti. Durante la maturazione i formaggi erano caratterizzati da un aumento delle attività peptidasiche ed esterasiche, a differenza delle attività proteinasica e lipasica. Il caratteristico aumento dell'attività peptidasica si identificava con il parallelo incremento dei lattobacilli mesofili durante la maturazione.

INTRODUCTION

Currently, thirty Italian cheeses have the "Protected Designation of Origin" (PDO) status. Of the 969,386 tonnes of cheese produced in Italy in 2000, 418,248 tonnes were of cheeses legally designated as PDO (ASSOCIAZIONE ITALIANA LATTIERO-CASEARIA, 2000). PDO cheeses are variously manufactured in the different regions of Italy but only a few of them (e.g., Parmigiano Reggiano, Grana Padano, Pecorino Romano, Taleggio and Gorgonzola) have been well characterized and are well known on the international market (BATTISTOTTI and CORRADINI, 1993; GOBBETTI *et al.*, 1997a, b).

Canestrato Pugliese cheese is a hard or semi-hard PDO Italian cheese made from ewes' milk that is produced in southern Italy (Apulia region). In 2000, eight hundred tonnes of ewes' milk were used to produce 150 tonnes of Canestrato Pugliese cheese, with a cheese yield of ca. 5.5 kg milk kg^{-1} cheese (ASSOCIAZIONE ITALIANA LATTIERO-CASEARIA, 2000). Although the PDO status is only legally recognized for the hard or semi-hard variants of Canestrato Pugliese cheese, another cheese ripened for less than one month and named "primo-sale" is also present on the market.

The name and traditional shape of Canestrato Pugliese cheese are derived from the rush basket "canestro", in which the curd is ripened. The cheese making is limited to two provinces, Foggia and Bari, of the Apulia region, and traditional technology is used exclusively. Ewes producing milk for the manufacture of PDO Canestrato Pugliese cheese must be fed fresh or semi-fresh forage and only on exception with pelleted fodder. Raw, whole ewes' milk from milking once or twice daily is generally used, but heated or pasteurised milk may also be used. Natural whey cultures, composed mainly of thermophilic lactic acid bacteria, may be added and liquid or powdered calf rennet, or lamb rennet paste are used. After cutting, the curd is held for 5-10 min at 42°-45°C in hot whey. This treatment is generally not considered cooking. The curds are dry salted or submerged in brine (20% NaCl) for several days, the actual time depends on the weight. During ripening (2-10 months) in the "canestro", the cheeses are turned regularly and rubbed with a mixture of oil and vinegar. Surface colonisation by mould from the environment becomes evident frequently during ripening; after a few months the mould is removed by brushing. The cheese has a cylindrical shape, 10-14 cm high, 25-34 cm diameter and weighs 7-14 kg. The rind is brown to pale yellow and the interior is compact with small eyes. The flavour is pronounced and tends to be moderately piquant.

To our knowledge, very few studies have been conducted on Canestrato Pugliese cheese. Recently, supplementation with bifidobacteria has been successfully used to produce a probiotic semi-hard Canestrato Pugliese cheese (CORBO *et al.*, 2001). The microbiological and biochemical characteristics of the Canestrato Pugliese cheese manufactured using raw milk, pasteurised milk or curd treated in hot whey have also been compared (ALBENZIO *et al.*, 2001). However, none of these studies considered the manufacturing process as described in the protocol for the PDO cheese and that used by a legally recognized dairy. A complete characterization of the microbiological, physico-chemical and biochemical aspects of Canestrato Pugliese cheese is needed so that they can be compared with some other hard and semi-hard cheeses. It will also help to identify the features that are typical of this particular cheese variety.

MATERIALS AND METHODS

Cheese making and samples

PDO Canestrato Pugliese cheese (ca. 7 kg) was produced by the Caseificio

Pugliese of Corato, Bari, Italy, according to the protocol shown in Fig. 1. The raw ewes' milk used to produce the cheese had an average pH of 6.5, contained 4.9% lactose, 5.5% protein and 7.6% fat and had ca. 550,000 mL^{-1} somatic cells.

Samples were taken from two batches (A and B) made on different days after 0, 15, 30, 60, 90, 120 and 150 d and analysed in triplicate. The 0 day of analysis corresponded to the first day of ripening. Except for the microbiological analysis, the cheese was grated and stored at -20°C until further analysis.

Microbiological analysis

The cheese (25 g) was dispersed in sterile 2% tri-sodium citrate solution, homogenised, diluted in peptone and saline solution and plated on specific media for viable counts. Total mesophilic bacteria were counted on Plate Count agar (Oxoid S.p.A. Garbagnate, Milano, Italy) and presumptive staphylococci (Baird Parker agar, Oxoid) were counted after incubation at 30°C for 48 h, presumptive mesophilic and thermophilic lactobacilli (MRS agar, Oxoid) were incubated at 30° and 45°C, respectively, for 72 h under anaerobiosis and then counted; presumptive mesophilic lactococci and thermophilic streptococci (M17 agar, Oxoid) were incubated at 30° and 45°C for 72 h, respectively, and counted; enterococci (Slanetz Bartley agar) were incubated at 37°C for 48 h, and counted; total and fecal coliforms (Violet Red Bile Lactose, Oxoid) were incubated at 37° and 45°C, respectively, for 24 h and counted; presumptive clostridia (Differential Reinforced Clostridial broth, Oxoid) were incubated at 30°C for 7 days under anerobiosis and then counted; presumptive *Listeria* spp. (Listeria Selective agar supplemented with Modified Listeria Selective Supplement, Oxoid) were incubated at 37°C for 24 h and then counted; and yeasts and moulds (Wort agar, Oxoid) were incubated at 25°C for 4 d and then counted.

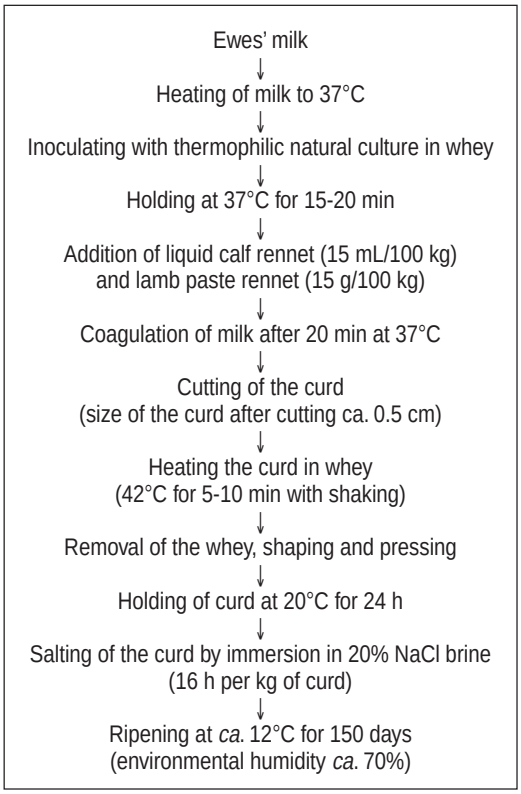


Fig. 1 - Protocol for the production of PDO Canestrato Pugliese cheese.

Gram-positive, catalase-negative rods and cocci growing on MRS and M17 media were randomly selected, cultured overnight in MRS and M17 broth at appropriate temperatures, frozen at -80°C and used for further characterization. The strains isolated were identified by amplification and sequencing of 16S rRNA genes as described by Cocconcelli *et al.* (1997). The DNA sequences were obtained using an ABI PRISM™ Big Dye Terminator Cycle Sequencing Kit (PE Applied Biosystems, Mona, Italy) and both forward and reverse primers for 16S rDNA were used. The reaction products were analysed on an Applied Biosystem 310 automated DNA sequencer (PE Ap-

plied Biosystems). The software package of the University of Wisconsin Genetics Group was used for analysing and comparing DNA sequences. Taxonomic strain identification and Similarity_rank (S_ab) calculations were performed, comparing the 16S rDNA sequences of Canestrato Pugliese cheese isolates with more than 30,000 unaligned sequences present in the Sequence Match of the Ribosomal Database Project II (RDP II), as described by MAIDAK *et al.* (2001).

Compositional analysis

Samples of cheese were analysed for protein (macro-Kjeldahl; IDF, 1964), fat (Gerber method; IIRS, 1955), moisture (oven drying at 102°C; IDF, 1982) and salt (FOX, 1963) content. The pH of the cheese was determined by direct insertion of a solid pH-meter.

Assessment of proteolysis

The pH 4.6-soluble extracts of the cheese and sub-fractionation of pH 4.6-soluble extracts using 70% ethanol were prepared according to the method of KUCHROO and FOX (1982). The nitrogen content of the extracts was determined by the macro-Kjeldahl method (IDF, 1964); results are expressed as the percentage of total cheese nitrogen. The pH 4.6-insoluble fraction of the cheese was analysed by urea polyacrylamide gel electrophoresis (Urea-PAGE) using a Protean II vertical slab gel unit (BIORAD Laboratories Ltd, Watford, HP2 7TD, UK) and the stacking gel system described by ANDREWS (1983). The gels were stained directly by the method of BLAKESLEY and BOEZI (1977) with Coomassie Brilliant Blue G250.

The peptide profiles of the 70% ethanol-soluble fractions were analysed by reversed phase HPLC using a Varian HPLC (Varian Associates Inc., Walnut Creek, CA, USA) consisting of an autosampler, a ProStar solvent delivery system with three pumps, a Prostar programmable multiwavelength spectrophotometric detector, interfaced with a PC with a software package for system control and data acquisition (Varian Star Workstation 5). Nucleosil RP-8 (250x4 mm, 5 μm particles, 300 Å pore size) analytical and guard (4.6x10 mm) columns (Capital HPLC Ltd., Broxburn, West Lothian, UK) were used. The mobile phase was comprised of buffer A (0.1%, v/v, trifluoroacetic acid, TFA in deionised water) and buffer B (0.1%, v/v, TFA in HPLC grade acetonitrile). The freeze-dried ethanol-soluble fraction (10 mg) was dissolved in 1 mL buffer A and filtered through 0.45 μm cellulose acetate filter (Sartorius GmbH, Gottingen, Germany). The filtrate (40 μL) was applied to the column with an eluent flow rate of 0.75 mL/min. The eluate was monitored at 214 nm. Peptide profiles of the ethanol-soluble fractions of the cheeses were analysed by using multivariate statistical techniques. The data for the factor reduction analysis were obtained by visually recognising the peaks and taking peak heights as variables. Factor reduction analysis was performed on the data using the covariance matrix for the determination of principal components (PRIPP *et al.*, 1999) using the SPSS statistical package (SPSS for Windows, Version 10.0, SPSS Inc, USA). In total, data from 84 peaks in each chromatogram were considered for the analysis.

Individual free amino acid analysis was carried out on the 12% TCA soluble fraction of the pH 4.6-soluble extracts after 60 and 150 days of ripening. Samples were analysed by a single injection into a Beckman model 6300 amino acid analyser (Beckman Instruments Ltd, High Wycombe, UK) using a Beckman P-N-338052 Na^+ cation exange column (12x0.4 cm, i.d.). A standard amino acid mixture (Beckman) was used to calibrate the column and norleucine (Sigma) was added to all samples before injection as

an internal standard. Samples were diluted in buffer (0.2 M sodium citrate, pH 2.2), filtered through Whatman 0.22 mm filters and 50 μL of filtrate were loaded onto the column. Amino acids were post-column derivatised with ninhydrin and detected by absorbance at 440 (proline and hydroxyproline) or 570 nm (all the other amino acids). Results were analysed using a VG Minichrom computer system with a chromatography data handling software package (LYNCH *et al.*, 1996).

Assessment of lipolysis

Total and individual free fatty acids were determined by the method of GARCIA *et al.* (1990), as modified by SOUSA *et al.* (1997). Cheese samples (2.5 g) were ground with 5.0 g of Na_2SO_4 and 100 μL of an internal standard solution of 0.1 g pelargonic acid (C9:0) and 0.2 g margaric acid (C17:0) made up to 10 mL in 1:1 methanol/chloroform solution and stabilised with 0.05% butylated hydroxyanisole (BHA). The mixture was repeatedly mixed and free fatty acids were extracted in diethyl ether. The total volume of extract was measured and 2.0 mL were taken and mixed with 2.0 mL of a 2 g L^{-1} solution of *p*-bromophenacyl-bromide in acetonitrile in a glass test tube with a sealed cap. Eighty microliters of a 5.0 g L^{-1} solution of 18-crown-6 ether were added followed by 0.2 g of dry K_2CO_3. The sample was mixed thoroughly and heated to 75°C for 30 min to promote derivatization. After the mixture had cooled to room temperature, 40 μL of a 40 g L^{-1} solution of formic acid in acetonitrile were added and the mixture was reheated to 75°C for 30 min. The mixture was then filtered through a 0.45 μm acrodisk membrane and 40 μL were used for analysis. The liquid chromatography system used consisted of a Waters Alliance 2690 Separation Module and 2487 Dual λ Absorbance Detector (Waters Corp., Milford, MA, USA). Data were collected and quantified using Millennium 32 software. The column used was a Kingsorb C18, 5 μm packing with 90 Å pore size (250x4.6 mm) with associated guard column. The flow rate used was 10 mL min^{-1} at a constant temperature of 33°C. Absorbance was measured at 245 nm. A gradient elution based on a combination of water, acetonitrile and methanol was used.

Assessment of enzymatic activity

Sterile, dialysed water-soluble extracts of cheeses, prepared according to a modification of the method of KUCHROO and FOX (1982) were assayed for enzyme activity. The modifications involved the dialysis (dialysis tubing, cut-off 12 kDa, Sigma, Milan, Italy) of the extracts for 24 h at 4°C against 0.05 M phosphate buffer, pH 7.0, to eliminate interference from salt and peptides in the samples, and sterile filtration (0.22 μm pore size, Syrfil Filter, Nucleopore, Costar Corporation, Cambridge, MA, USA) to avoid interference due to cellular activity (GOBBETTI *et al.*, 1997c).

Aminopeptidase (EC 3.4.11.11) and proline iminopeptidase (EC 3.4.11.9) activities were determined as described by GOBBETTI *et al.* (1997c), using Leu-*p*-NA, Glu-*p*-NA and Pro-*p*-NA as substrates. Leu-*p*-NA and Glu-*p*-NA were used as substrates to partially differentiate the activity of aminopeptidase type N (PepN) and A (PepA), respectively (CHRISTENSEN *et al.*, 1999).

A unit of enzymatic activity was defined as the amount of enzyme that caused an increase in absorbance at 410 nm of 0.01 min^{-1} at 37°C and pH 7.0. The pH and temperature used in this and subsequent analyses were selected as optimal for the enzyme activities in Canestrato Pugliese cheese.

Dipeptidase (EC 3.4.13.11) activity was measured by the Cd-ninhydrin method (FOLKERSTMA and FOX, 1992) using Leu-Leu as substrate. A unit of en-

zymatic activity was defined as the amount of enzyme that produced an increase in absorbance of 0.01 units min^{-1} at 505 nm.

Proteinase activity was measured by the method of TWINNING (1984), using fluorescence isothiocyanate-labelled casein as substrate, with incubation at 37°C for 12 h. A unit of proteinase activity was defined as the amount of enzyme that caused an increase in absorbance of 0.1 units min^{-1}at 405 nm.

Esterase (acetyl ester hydrolase, EC 3.1.1.6) and lipase (acylglycerol acylhydrolase, EC 3.1.1.3) activities were determined as described by GOBBETTI *et al.* (1996) using β-naphthyl butyrate or tributyrin as substrates, respectively. Esterase activity was expressed as moles of β-naphthol released per 20 min, while lipase activity was calculated as the milliequivalents of free fatty acids liberated from tributyrin 60 min^{-1}.

The specific activity for all enzymes was determined as units of total activity per gram of total cheese.

The methods and units of enzyme activity are the same as those used earlier by CORSETTI *et al.* (1998) and GOBBETTI *et al.* (1997, b, c; 1999a, b; 2002).

Data from microbiological, physico-chemical and enzymatic analysis were evaluated for their significance ($p< 0.05$) with one-way-Anova (SAS, 1985).

RESULTS AND DISCUSSION

Microbiological analysis

Fully ripened (150 d) PDO Canestrato Pugliese cheese contained a total of log 7.7 cfu g^{-1} mesophilic bacteria, log 4.6 cfu g^{-1} presumptive enterococci and log 4.0 cfu g^{-1} presumptive staphylococci. These cell numbers remained almost constant during ripening (Table 1). None of the *Staphylococcus* colonies were lecithinase positive. The number of total coliforms was at ca. log 4.2 cfu g^{-1} until 60 days of ripening and then decreased; these microorganisms were not detected in the fully ripened cheese. When present, faecal coliforms were at values less than log 2.0 cfu g^{-1}. The production protocol of PDO Canestrato Pugliese cheese does not include severe heat treatment but the hostile ripening conditions probably do not permit the survival of coliforms in the fully ripened

Table 1 - Levels of the main microbial groups[a] (log cfu g^{-1} cheese) found in PDO Canestrato Pugliese cheese.

Microbial groups	Days of ripening						
	0	15	30	60	90	120	150
Aerobic mesophilic bacteria	6.6	6.9	7.0	7.5	7.2	7.4	7.7
Total coliforms	3.2	3.7	4.3	4.5	2.8	2.7	n.d.
Staphylococci	4.1	4.0	4.3	3.8	4.3	4.0	4.0
Enterococci	3.6	5.3	5.9	4.8	4.0	3.9	4.6
Mesophilic lactobacilli	6.1	6.6	7.9	8.0	8.0	7.8	8.2
Thermophilic lactobacilli	7.0	6.1	5.7	5.9	5.2	4.8	4.5
Lactococci	7.2	7.5	7.0	7.6	8.0	8.0	7.9
Thermophilic streptococci	5.6	5.7	7.6	8.0	7.6	7.0	5.5
Yeasts	n.d.	2.3	2.0	4.0	3.8	3.9	3.3

[a] Average data from one batch analysed in triplicate; n.d., not detected in 1 g of cheese.

cheese. The number of presumptive thermophilic lactobacilli, as well as presumptive thermophilic streptococci, decreased during ripening, being log 4.5 and 5.5 cfu g^{-1} at the end of ripening, respectively. On the contrary, presumptive mesophilic lactobacilli increased until 30 days (ca. log 8.0 cfu g^{-1}) and then remained constant throughout ripening. The same was found for presumptive lactococci. Clostridia and *Listeria* spp. were not found in 25 g of cheese. From 15 days of ripening, yeasts were always present at cell numbers ranging from log 2.0 to 4.0 cfu g^{-1}. Few moulds (log 2.0 cfu g^{-1}) were present and were only detected at the end of ripening. Data from the microbiological analyses did not statistically differ ($p< 0.05$) between the two batches. With few exceptions, the above cell numbers approached those found by ALBENZIO *et al.* (2001) for Canestrato Pugliese cheese made from raw milk. In particular, high levels of presumptive thermophilic lactobacilli and streptococci were present in the PDO Canestrato Pugliese cheese, probably due to the use of a natural whey starter containing thermophilic bacteria.

The taxonomic position of some isolates of presumptive lactic acid bacteria was determined by means of the sequence analysis of at least 500 bp of the 5' region of the 16S rRNA gene. Only isolates with the highest cell counts during the whole ripening period were identified: *Lactobacillus delbrueckii*, *L. fermentum* and *L. helveticus* of the presumptive thermophilic lactobacilli, *Streptococcus thermophilus* of the presumptive thermophilic streptococci, *Lactococcus lactis* subsp. *lactis* of the presumptive lactococci and *L. plantarum*, *L. casei* and *L. reuteri* of the presumptive mesophilic lactobacilli. Thermophilic strains may derive from the natural culture in whey used as starter. This heterogeneous composition may be due to the empirical manipulation of this natural starter which may vary greatly in its microbial composition due to uncontrolled environmental and technological factors. A more in-depth study to characterise and standardise this natural starter is warranted. The species of mesophilic lactobacilli corresponded to those most frequently isolated within the adventitious milk microflora (non-starter lactic acid bacteria, NSLAB) of several cheeses (GOBBETTI *et al.*, 1997c, 1999b). Previously, *L. plantarum* was also identified in Canestrato Pugliese made from different types of milk (ALBENZIO *et al.*, 2001). NSLAB survive pasteurisation or enter the cheese milk or curd as post-pasteurisation contaminants and are the only microbial group which grows well in the hostile environment in the interior of the cheese which has a low pH, high salt content and lack of fermentable sugars, is anaerobic and may contain bacteriocins produced by starter bacteria. NSLAB may greatly contribute to secondary proteolysis during cheese ripening (FOX *et al.*, 1998).

Compositional analysis

The compositions of the two batches of PDO Canestrato Pugliese cheese were not statistically different ($p<0.05$). As ripening progressed, the pH increased gradually from 5.25 (0 d) to 5.91 (150 d). The moisture content of the cheeses decreased during the first 30 d of ripening (ca. 45 to 41%), then remained almost constant, but at 150 days the cheese had a slightly higher moisture content (39.5%) than at 120 d (37%); this increase was found in both batches. Since PDO Canestrato Pugliese cheese is ripened in typical baskets made of woven reed, the rate of moisture loss varies with the temperature and humidity of the surroundings, which is governed by atmospheric conditions. Absorption of moisture from more humid surroundings may explain the rise in the moisture at the end of ripening. The average salt content of the cheeses increased gradually

until 90 d (0.2 to 2.8%), after which it remained almost constant. Values for protein content were ca. 26.5%, and for fat ca. 30%, with no significant differences between the cheeses. These results agree with those reported by ALBENZIO *et al.* (2001) who characterised the effects of several heat treatments on the microbiological and biochemical characteristics of Canestrato Pugliese cheese.

Proteolysis

Patterns of proteolysis in PDO Canestrato Pugliese cheese were monitored throughout ripening. The level of pH 4.6-soluble nitrogen as % of total nitrogen increased from 10.14% at 0 d to 21.76% at 150 d with no significant differences between the two batches (not shown). The greatest relative increase was found from 30 to 60 d of ripening (12.45 to 16.34%).

Results of urea-PAGE of pH 4.6-insoluble fractions indicated a progressive breakdown of α_{s1}-casein (CN) and β-CN, indicating the role of chymosin and plasmin, respectively (Fig. 2). Similar results were found for the two batches. Ewes' milk casein contains two multiphosphorylated β-CN components designated β_1- and β_2-CN, which differ from each other in the degree of phosphorylation. β_1- and β_2-CN have the same polypeptide chain but the former contains 6 phosphates, while the latter contains 5 phosphates (RICHARDSON and MERCIER, 1979). Partial phosphorylation of the Thr_{12} residue in β_1-casein is responsible for the difference in the electrophoretic mobility between β_1 and β_2 caseins at alkaline pH. Hydrolysis of a_{s1}-CN of ewes' milk by chymosin resulted in bands with the highest electrophoretic mobility, while hydrolysis of β-CN by plasmin resulted in γ_1-CN, γ_2-CN, γ_3-CN and β_1-I-CN and β_2-I-CN respectively. β_1-I- and β_2-I-CN had greater electrophoretic mobilities

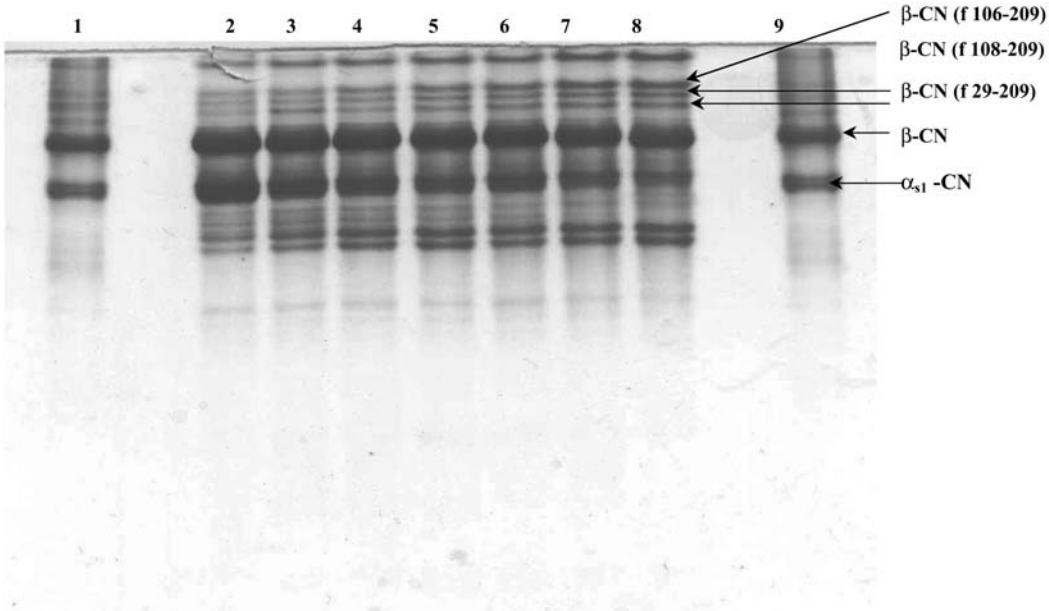


Fig. 2 Urea-polyacrylamide gel electrophoretograms of pH 4.6-insoluble fractions of PDO Canestrato Pugliese during cheese ripening. Lanes, 1 and 9, ewes' casein; 2, 0 d; 3, 15 d; 4, 30 d; 5, 60 d; 6, 90 d; 7, 120 d; and 8, 150 d of ripening.

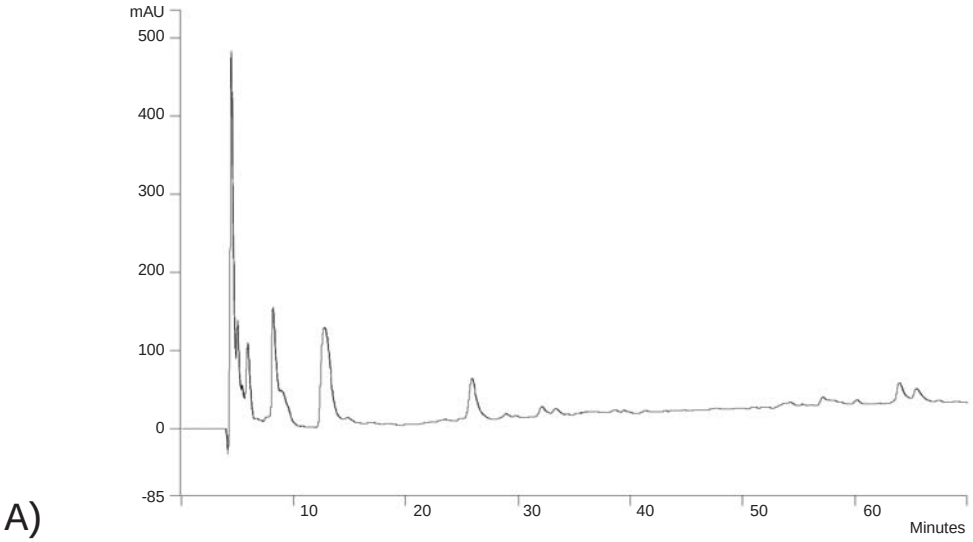
than β-CN but less than α_{s1}-CN (MACEDO and MALCATA, 1997). In agreement with ALBENZIO *et al.* (2001), α_{s1}-CN was hydrolysed faster than β-CN over time. As ripening progressed, the concentrations of γ_2-, γ_1-, γ_3- caseins and of α_{s1}-CN degradation products increased.

Complex peptide profiles were obtained by RP-HPLC of the 70% ethanol-soluble sub-fractions of the pH 4.6-soluble extracts from the cheeses. Differences in peptide profiles became apparent as ripening progressed, showing differences in the peak heights, which were used as variables for multivariate statistical analysis. The profile of the 70% ethanol-soluble peptides at 150 d of ripening is shown in Fig. 3A. Principal Component Analysis (PCA) was performed. It was observed that samples at the end of ripening (e.g., 90, 120, and 150 d) were separated from samples at the beginning of ripening on Principal Component (PC) 1, which explained 67.8% of the total variation (Fig. 3B). Similar trends in the peptide profile were obtained by urea-PAGE. As ripening progressed, there was a gradual breakdown of α_{s1}-CN and β-CN which resulted in intermediate- and small- sized peptides, as detected by RP-HPLC in the 70% ethanol-soluble fraction. On the score plot (Fig. 3B), the samples of the two batches of PDO Canestrato Pugliese cheeses differed at the beginning of ripening but, as ripening progressed, the samples grouped together. The variation at the initial stages of ripening may be attributed to slight variations in the microbial composition and environmental conditions of ripening.

The concentrations of most of the individual free amino acids increased during ripening (Fig. 4). This increase was similar to the increase of the pH 4.6-soluble nitrogen fraction (data not shown). Among all the individual free amino acids, glutamic acid, lysine, leucine and valine were found to be dominant at concentrations of 1.65, 1.48, 1.15 and 0.96 mg g^{-1} cheese, respectively. The total concentration of free amino acids at 150 d was found to be ca. 10 mg g^{-1} cheese. The results showed that the dominant amino acids at 60 and 150 d were similar for both batches. Glutamic acid, lysine, leucine and valine were found at the highest levels even in the Canestrato Pugliese cheese supplemented with bifidobacteria (CORBO *et al.*, 2001) and in the Canestrato Pugliese manufactured with raw milk, pasteurised milk or by heating the curd in the hot whey (ALBENZIO *et al.*, 2001). However, in the latter cheeses, the concentrations of the total and individual free amino acids were higher. The values found by ALBENZIO *et al.* (2001) for glutamic acid, lysine, leucine and valine in 63 day-old Canestrato Pugliese cheese made from pasteurised ewe's milk were 3.88, 1.75, 2.52 and 1.78 mg g^{-1} cheese. Similarly, IZCO *et al.* (2000) found that these free amino acids, along with asparagine and glutamine, were predominant in Ossau-Iraty cheese, which is also made from ewe's milk. Compared to other cheeses, the total concentration of free amino acids found in the PDO Canestrato Pugliese was higher than in 6-month-old Cheddar (ca. 3 mg g^{-1} cheese) (LYNCH *et al.*, 1996) and was considerably lower than that of Parmigiano Reggiano (70 mg g^{-1} cheese) (BATTISTOTTI and CORRADINI, 1993) and Fossa (pit) (20-38 mg g^{-1} cheese) (GOBBETTI *et al.*, 1999b) cheeses.

Lipolysis

The concentrations of individual and total free fatty acids in PDO Canestrato Pugliese cheese at the end of ripening are shown in Table 2. The free fatty acids butyric (C4:0), caproic (C6:0), palmitic (C16:0), oleic (C18:1) and linoleic (C18:2) acids were found at the highest concentrations. Lamb rennet paste contains pregastric esterase (PGE) which exhibits specificity for butyric and other short-chain fatty acids found at the sn^{-1} position (FOX and GUINEE, 1987). The high level of free



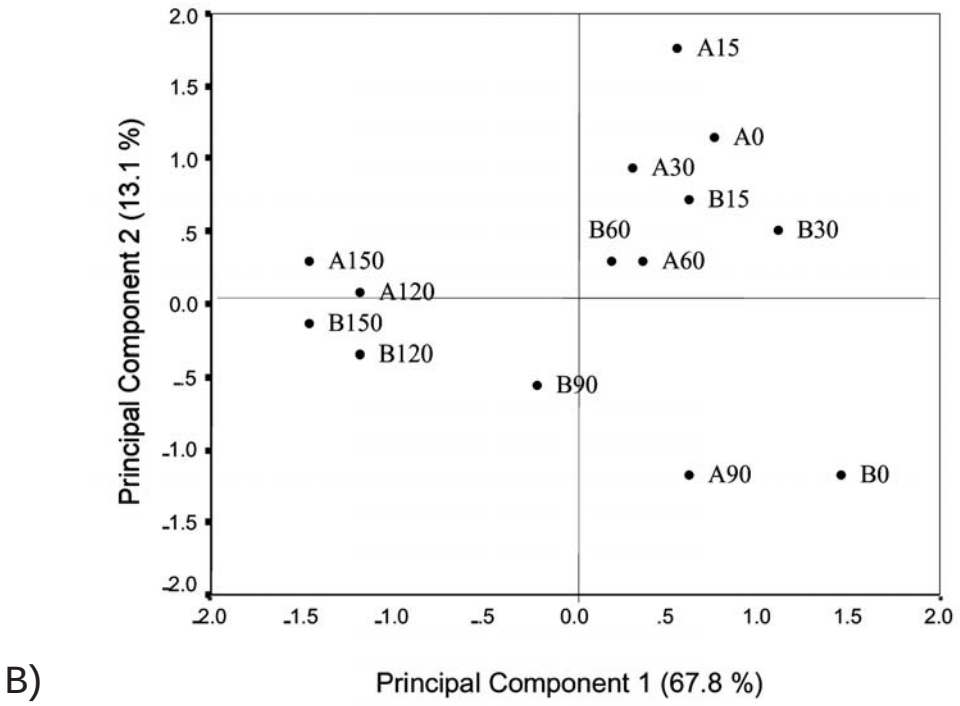


Fig. 3 (A) Reverse-phase (RP)-HPLC chromatograms of the 70% ethanol-soluble fractions of PDO Canestrato Pugliese cheese at 150 days of ripening. (B) Score plot obtained by Principal Component Analysis (PCA) of data obtained from RP-HPLC chromatograms of the 70% ethanol-soluble fractions of PDO Canestrato Pugliese cheese at time intervals of 0, 15, 30, 60, 90, 120 and 150 d for batches A and B.

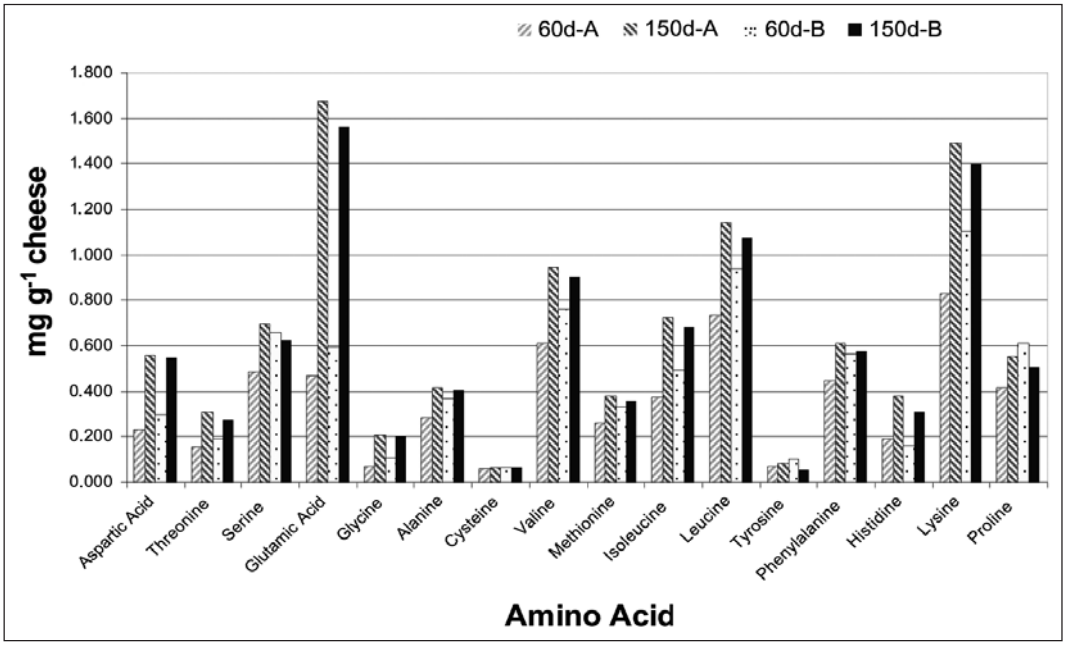


Fig. 4 Individual free amino acids of PDO Canestrato Pugliese cheese at 60 and 150 d of ripening for batches A and B.

Table 2 - Concentration of individual free fatty acids (mg kg^{-1} cheese)[a] in PDO Canestrato Pugliese cheese after 150 days of ripening.

Fatty acids	Concentration
Butyric acid (C4:0)	336
Caproic acid (C6:0)	195
Caprylic acid (C8:0)	65
Capric acid (C10:0)	92
Lauric acid (C12:0)	36
Myristic acid (C14:0)	73
Palmitic acid (C16:0)	144
Stearic acid (C18:0)	38
Oleic acid (C18:1)	165
Linoleic acid (C18:2)	106
Linolenic acid (C18:3)	65
Total free fatty acids	1,315

[a] Average data from one batch analysed in triplicate.

butyric acid is responsible for the moderately piquant flavour of the cheese. Lipases secreted by the cheese microflora may also contribute to the piquant flavour, especially by liberating long-chain free fatty acids. The total concentration of free fatty acids was found to be 1,315 mg kg^{-1} cheese. This is slightly lower than that reported by ALBENZIO *et al.* (2001) for Canestrato Pugliese made from pasteurised milk, but the profile of free fatty acids did not differ substantially. The profile of the free fatty acids was similar to those found in other hard and semi-hard Italian cheeses but the total concentrations differed (BOTTAZZI, 1993).

Enzyme activity

The activities of some of the enzymes in the curd which are liberated after cell lysis or which are derived from the coagulant and plasmin are shown in Table 3. Data did not differ significantly between the two batches. The kinetics of enzyme activities during ripening were different depending on the type of enzymes. Peptidase as well as esterase, activity increased

Table 3 - Enzymatic activities (U g^{-1} cheese)[a] contained in the water-soluble extracts of PDO Canestrato Pugliese cheese during ripening.

Enzymatic activity	Days of ripening						
	0	15	30	60	90	120	150
Aminopeptidase (Leu-*p*-NA)	3.6^{g}	10.7^{f}	23.5^{e}	43.2^{d}	56.8^{c}	72.7^{b}	82.9^{a}
Aminopeptidase (Glu-*p*-NA)	1.6^{g}	8.1^{f}	15.7^{e}	32.4^{d}	50.0^{c}	65.9^{b}	74.6^{a}
Proline iminopeptidase	1.2^{f}	3.4^{f}	10.5^{e}	27.4^{d}	42.2^{c}	55.8^{b}	65.2^{a}
Dipeptidase	18.6^{g}	28.4^{f}	37.5^{e}	56.3^{d}	69.8^{c}	87.0^{b}	96.4^{a}
Proteinase	22.3^{c}	28.7^{b}	30.5^{ab}	32.4^{a}	23.8^{c}	21.4^{c}	15.7^{d}
Esterase	12.5^{g}	18.4^{f}	24.7^{e}	42.3^{d}	55.0^{c}	59.6^{b}	68.7^{a}
Lipase	47.4^{a}	45.6^{a}	48.1^{a}	42.3^{b}	45.8^{a}	42.4^{b}	44.1^{ab}

[a] Average data from one batch analysed in triplicate; [a-g] Values in the same row without a common superscript are significantly different: P<0.05.

throughout ripening showing a relatively high increase between 30 and 60 d which coincided with the highest level of NSLAB in the cheese. Peptides from lysed thermophilic natural starter may also contribute. Studies by quadratic response surface methodology on the effect of cheese-like conditions on the microbial enzyme activity showed that peptidase activities of NSLAB retain a higher level of activity than those from starter lactic acid bacteria (GOBBETTI *et al.*, 1999a). The peptidase activities found at 150 days were *ca.* twice those at 60 days of ripening. Likewise, the concentrations of some amino acids (e.g., glutamic acid, leucine, lysine and valine) almost doubled during the same period (Fig. 4). The levels of the pH 4.6-soluble nitrogen also showed a similar trend. Proteinase and lipase activities did not continuously increase during ripening. A small increase in the proteinase activity, as detected on fluorescent casein, was found up to 30 days, after which it progressively decreased. It was presumed that there was no heat inactivation of chymosin during cheese making (Fig. 1), hence a major contribution to primary proteolysis by chymosin together with plasmin would be expected; this is in agreement with the results of the urea-PAGE electrophoretograms (Fig. 2). A certain inactivation of chymosin probably took place, especially during late ripening. Unlike esterase activity, lipase activity was constant throughout ripening, probably due to the PGE activity contained in the lamb rennet paste. Although PGE has both esterase and lipase activity, NSLAB mainly show an esterase-type activity on acylglycerols (GOBBETTI *et al.*, 1996) and this may explain the accumulation of a relatively high concentration of long-chain fatty acids at the end of ripening which are not pertinent to the PGE activity.

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PAPER

RELATIONSHIP BETWEEN PHYSICAL AND CHEMICAL ATTRIBUTES OF BEEF AND PORK MUSCLES AND PROCESSING SUITABILITY

RELAZIONE TRA CARATTERISTICHE CHIMICHE E FISICHE DI CARNI BOVINE E SUINE E IDONEITÀ ALLA TRASFORMAZIONE

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ABSTRACT

In order to characterise the suitability of raw meat for processing, commercial beef and pork muscles, foreign pork shoulders and Italian heavy pork *Semimembranosus* (SMp) muscles and shoulders, obtained from processing plants, were analysed. Foreign shoulders that were vacuum packed and frozen at -30°C for six months were also analysed. The extraction yields of sarcoplasmic proteins (WS) and pH values were higher ($p<0.001$) in the pork samples, while the cooking loss, extraction

RIASSUNTO

Per valutare l'idoneità alla trasformazione della materia prima, sono stati analizzati muscoli di bovino e di suino del commercio, spalle suine di provenienza estera, muscoli *Semimembranosus* (SMp) e spalle nazionali di suino pesante prelevati da ditte di trasformazione. Sono state, inoltre, analizzate spalle di provenienza estera, confezionate sottovuoto e congelate per sei mesi a -30°C. Le resa di estrazione delle proteine sarcoplasmatiche (WS) ed i valori dei pH sono risultati maggiori ($p<0.001$)

- Key words: beef and pork meat, sarcoplasmic and myofibrillar proteins and texture -

yields for salt-soluble proteins (SS), elasticity module (MY10) and maximum load (MAX) were higher ($p<0.01$) in the beef meat. Freezing reduced the SS ($p<0.001$) and increased the MAX ($p<0.01$). The PCA analysis provided a good separation between the beef and pork samples and between imported and Italian pork shoulders. It also grouped most of the Italian heavy pork samples, shoulder and SMp, on the basis of high extractability of sarcoplasmic proteins and good water retaining properties.

nei campioni di carne suina, viceversa il calo peso dopo cottura, la concentrazione delle proteine salino-solubili estraibili (SS), il massimo carico (MAX) e il modulo di Young (MY10) erano superiori nei campioni bovini ($p<0.01$). Le operazioni di congelamento hanno determinato una riduzione delle SS ($p<0.001$) e un incremento del MAX ($p<0.01$). L'analisi delle componenti principali (PCA) ha reso possibile una buona separazione dei campioni bovini e suini e delle spalle suine importate e nazionali e ha, inoltre, raggruppato la maggior parte dei campioni italiani di suino pesante (spalle e SMp) in funzione dell'elevata estraibilità delle proteine sarcoplasmatiche e di buone capacità di ritenzione d'acqua.

INTRODUCTION

Knowledge of the physical and chemical characteristics of meat is essential for the classification of raw materials destined for industrial processing and for the standardisation of the meat by-products. While industry focuses on perfecting the processing techniques in order to obtain high quality standards, very often the raw materials, whose properties cannot be modified during processing, are responsible for a lower quality and a great variability in the final meat products.

It is well know that the final pH value and its rate of decrease after slaughtering influence the fundamental properties of the raw material, such as water-holding capacity (WHC) and extractability of soluble proteins (MÜLLER, 1989). The genetic PSE (pale, soft and exudative) anomaly is the result of a rapid drop in muscle pH, while the muscle temperature is still high, causing the meat to lose its capacity to retain water and reducing the extractability of sarcoplasmic and myofibrillar proteins from the meat (KAUFFMAN *et al.*, 1998). PSE meat has pH values that are, on average, below 5.5, which makes the meat unsuitable for use in cooked meat products (SHEID, 1986; MÜLLER, I989). Muscle pH values above 6.2, on the other hand, are characteristic of dark, firm and dry meat (DFD) whose pH drop is slow and incomplete due to low glycogen reserves in the muscle. These meats are characterised by high WHC and protein extractability, which makes them more suitable for processing into cooked products, even though some problems may occur with salting (PIZZA and PEDRIELLI, 2000).

For whole and minced cooked meat products, the solubility and structure of muscular protein are very important. Myofibrillar proteins (60% of total protein), which are soluble in high-ionic strength saline solutions, have the capacity to bind water (HAMM, 1970). In fact, most of the water in meat is found within the myofibrils in the spaces between the thick and thin filaments. It seems very likely that changes in WHC are caused by changes in the volume of

the myofibrils which, in turn, are caused by changes in the interfilament spacing (OFFER and TRINICK, 1983). On the other hand, the sarcoplasmic proteins (≈30% of total protein weight) are partly responsible for the WHC (LAWRIE, 1987); the denaturation of sarcoplasmic proteins and their precipitation onto myofibrillar proteins could affect the WHC of raw meat (JOO *et al.*, 1999).

The texture of meat and meat products depends on the connective tissue, whose main component is collagen, and on the structure and changes that occur in the contractile myofibrills during maturation (CIERACH and MAJEWSKA, 1997) and processing (HAMANN, 1988). The water holding capacity also affects the mechanical properties of the products (PREZIUSO *et al.*, 1999).

In most cases, producers only control the pH, since it is the only parameter that can be measured instrumentally. Sometimes a sensory evaluation is performed at the slaughterhouse or more frequently at the processing plant. The sensory evaluation consists of a visual inspection of the colour of the fat and lean parts, and the hydration aspect of all the anatomical parts. Texture, which is one of the most important parameters for product evaluation, is never taken into account.

The objective of this study was to characterise some beef and pork muscles with respect to proximate composition, WHC (expressed through cooking loss), pH, sarcoplasmic and myofibrillar protein solubility and rheological parameters in order to group some raw meats with respect to their suitability for use in producing cooked products.

MATERIALS AND METHODS

Raw materials

Samples of beef (*Semimembranosus* (SMb) and *Psoas* (PS)) and pork (shoulder (SH-COM) and *Longissimus dorsi* (LD) were purchased from commercial outlets. The analyses were also carried out on a foreign commercial hybrid (SH F) and Duroc x Large White heavy (SH IT) pork shoulders obtained from Mortadella producers. Hybrid heavy pork *Semimembranosus* muscles, from two Italian breeds (SM1p) and (SM2p), obtained from a cooked ham industry, were also analysed. All the heavy pigs were reared according to the Protected Designation of Origin for Parma ham (G.U., 1990). The imported pigs were slaughtered in Italy with traditional stunning. All the samples were chilled at 4°C until analysed. The shoulders from foreign samples had been vacuum packed and frozen at -30°C for six months.

Slices were taken from the muscle, cutting perpendicular to the muscle fibre. Whole slices were used for the rheological tests; for the other analyses the slices, with the surface fat and connective tissue removed, were minced in a mixer and then homogenised.

Proximate composition and pH

The minced samples were analysed in duplicate for moisture, ash, fat and total proteins according to the AOAC methods (AOAC, 1995). In addition the non-protein nitrogen (NPN) was determined and is expressed as the percentage ratio between soluble nitrogen in trichloroacetic acid (5%) and total nitrogen, as well as the collagen, calculated according to the hydroxyproline content (AOAC, 1995).

Duplicate pH measurements were taken on the minced and homogenized meat samples using a pH-meter mod. HI 9321 Microprocessor (Hanna Instruments, Singapore) equipped with a Hamilton gel-electrolyte electrode.

Extraction of soluble proteins

Parallel extractions of the water-soluble proteins and total soluble proteins

were carried out on the minced sample using the modified Vega-Warmer method (VEGA-WARMER *et al.*, 1999). The water-soluble fraction was extracted by treating 6 g of meat with 90 mL of phosphate buffer at low ionic strength (0.03M) at pH 7.0 for 24 hours. The total soluble fraction was extracted treating 6 g of meat with a phosphate buffer at high ionic strength (0.1M with 0.7M NaCl) at pH 7.0 for 24 hours. After centrifuging (23,000 x g, 15 min, 4°C), the protein extracted was determined using the Kjeldhal method on weighted quantities of limpid supernatant. The amount of proteins which were not extracted in the pellet were also determined. The amount of extracted sarcoplasmic proteins was calculated by the difference between the water-soluble protein and the non-protein nitrogen (NPN).

The myofibrillar protein value was calculated as the difference between total soluble protein and water-soluble protein. The extraction yield was calculated for both the percent sample weight and percent total protein.

Cooking weight loss

Before the instrumental texture analysis, the weight loss after cooking was calculated for each sample according to the following formula:

[(weight before cooking-weight after cooking)/weight before cooking] x100.

Texture

Sections of muscle, about 4 cm thick, were vacuum-packed and cooked in a water-bath (Heto-Birkerød, Denmark) at 70°C for 40 min, then cooled in a refrigerator (approx. 2°C) for one day and then cut into two slices (about 1 cm thick). Two or three cylinders with a diameter of 11.5 mm, were taken from the central part of each slice, kept at constant temperature (4°C). The instrumental determinations were made using a material testing system, Instron mod. 5564 (Instron Universal Testing, U.K.), using a 100 N cell load. During the stroke, a graph of the force verses the distance was recorded.

Shear test

A Warner-Bratzler cell was used with a blade advance speed of to 50 mm/min (HONIKEL, 1998) with the force applied perpendicular to the direction of the muscle fibre. For each sample, the maximum load (MAX), expressed in Newton (N), was used.

Compression test

The compression head (diameter 57 mm) advanced at a speed of 20 mm/min in the same direction as the muscle fibre until it reached 60% of the original length of the meat cylinder (PIZZA *et al.*, 1982). The parameter considered was the Young or elasticity module (MY10) measured in the elastic performance area (from 0 to 10% deformation of the cylinder) of the samples.

Statistical analysis

The data obtained were processed using the statistical package SPSS/PC (NORUSIS, 1999). Differences between muscles, species and storage were evaluated by a one-way analysis of variance (ANOVA) using the LSD test to compare sample averages; Pearson's correlation coefficients were calculated using data from samples that were not frozen. In addition, PCA analysis was applied in order to determine the main components, which describe and discriminate groups of muscles. The Varimax option for rotating the PC1/PC2 plane was used. The group of pork shoulder samples was subdivided into commercial samples of unknown origin (SH COM), the samples from foreign sources (SH F) and samples from national sources (SH

IT) and the group of pork SM was divided according to breed (SM1p) and (SM2p).

RESULTS AND DISCUSSION

The results of the composition analysis and pH for all the chilled samples subdivided according to muscle-type are reported in Table 1. The group of pork shoulders included commercially available samples as well as imported and Italian chilled shoulders obtained from processing companies; the group of pork SM muscles included all the samples of the two Italian breeds. The differences between shoulders of various origin will be discussed later. The non-nitrogen protein index (NPN) of the LD pork samples and the moisture of the PS beef samples were significantly higher ($p<0.05$) than the other muscle samples analysed. Furthermore, the beef PS and pork LD samples had higher protein contents and lower intramuscular fat contents ($p<0.05$) while the pork SM had the highest collagen values ($p<0.05$). The pH values for all the chilled samples fell within the range for normal meat, between 5.5 and 6.2 (Table 1).

Table 2 shows a comparison of all the parameters between beef and pork meat. The pH values of the beef samples, whether PS or SM, were significantly lower ($p<0.001$) than the pork samples. This result is in agreement with a number of other studies, in which the differences in the speed and grade of post-mortem drop in beef and pork meat have been noted (HONIKEL, 1991). The final pH in pork and veal tends to be higher than that of adult beef and pork samples have a greater capacity to retain water (LAWRIE, 1987).

The extractability of sarcoplasmic proteins (WS), calculated as both percent sample weight (WS-W) and total proteins (WS-P), was significantly higher ($p<0.01$) for pork samples (Table 2), and for the LD pork samples, in particular (Table 3). The extraction yield for the salt-soluble proteins (SS), as well as total extractable proteins (TEP) was significantly higher ($p<0.01$) and ($p<0.05$) for beef meat (Table 2), especially for SM beef samples (Table 3). The results confirm the difference in protein solubility between the species due to differences in pH drop (HONIKEL, 1991) and between the muscles in terms of carcass position and specific function (XIONG, 1994). The data obtained for the frozen shoulder samples obtained from the Mortadella producers (Fig. 1) show that freezing and frozen storage, that result in the denaturation of the soluble proteins, have a negative

Table 1 - Proximate composition and pH of raw meat samples[a]. Comparison by one-way analysis of variance[b].

	Beef PS[a,b,c]	Beef SM	Pork LD	Pork Shoulder	Pork SM
Moisture (g/100 g)	74.93±0.38 a	74.20±0.03 b	73.90±0.08 b	74.04±0.43 b	74.99±1.01 a
Protein (g/100 g)	22.57±0.52 a	20.76±0.05 b	23.61±0.39 a	21.44±1.46 b	21.97±0.47
Ash (g/100 g)	1.11±0.01 a	1.09±0.01 a	1.15±0.03 b	1.16±0.01 b	1.09±0.03 a
Intramuscular fat (g/100 g)	1.39±0.91 a	3.88±0.11 b	1.38±0.20 a	3.23±0.91 b	2.02±0.69 c
NPN (g/100 g)	2.67±0.10 a	2.73±0.01 a	3.35±0.27 c	2.57±0.33 a	2.89±0.20 b
Collagen (g/100 g)	0.41±0.06 a	0.37±0.02 a	0.33±0.02 a	0.38±0.15 a	0.54±0.17 b
pH	5.61±0.06 a	5.67±0.01 a	5.81±0.17 b	5.85±0.08 b	6.02±0.23 c

[a] Each value represents the mean±standard deviation.
[b] Within the same row means with different letters are significantly different ($p<0.05$).
[c] PS=*Psoas*; SM=*Semimembranosus*; LD=*Longissimus dorsi*.

Table 2 - Comparison of pork and beef samples by one-way analysis of variance.

	Species Effect[a]	Pork Meat	Beef Meat
Moisture (g/100 g)		74.43±0.88	74.68±0.46
Protein (g/100 g)		22.28±1.18	21.96±0.97
Ash (g/100 g)		1.12±0.04	1.10±0.02
Intramuscular fat (g/100 g)		2.29±1.07	2.22±1.39
NPN (g/100 g)	°	2.88±0.38	2.69±0.08
Collagen (g/100 g)		0.44±0.17	0.39±0.05
pH	°°°	5.92±0.20	5.63±0.05
WS-W [b]	°°°	5.25±0.78	3.74±0.59
WS-P [c]	°°°	23.74±1.51	17.15±3.21
SS-W [b]	°°°	4.44±1.51	7.01±0.92
SS-P [c]	°°°	20.16±7.10	32.08±5.44
TEP-W [b]	°	12.58±1.71	13.44 ±1.16
TEP-P [c]	°	56.92±7.99	61.51±8.04
Cooking loss	°°°	16.75±2.57	22.13±4.51
Shear Test			
MAX (N)	°°	33.39±7.75	39.59±2.58
Compression Test			
MY10 (MPa)	°°	0.09±0.03	0.13±0.03

[a] Species effect:° significant at p<0.05; °° significant at p<0.01; °°° significant at p<0.001.
[b] X-W: grams protein extracted /100 grams of sample weight.
[c] X- P: grams protein extracted /100 grams of total muscle protein.

effect on the extractability of myofibrillar proteins as reported by many authors (AWAD *et al.*, 1968; LAN *et al.*,1993). The extraction yield of SS was in fact, reduced by one third (p<0.001).

The beef samples had significantly (p<0.01) higher shear maximum load value (less tender) and higher MY10 (p<0.01) (less springy) values than the pork meat (Table 2). This parameter, monitored in the elasticity area of the cylinder, is a measure of the rigidity of the sample (LUCISANO and CASIRAGHI, 1993). Species is one factor that has a great influence on meat tenderness, due to the structure; beef is coarser than pork (LAWRIE, 1987). However, MAX increased (p<0.01) with frozen storage (Fig. 1), thus confirming the results of BHATTACHARYA *et al.* (1988). No significant differences in MY10 (p>0.05) were found between the fresh and frozen samples of foreign shoulder.

The cooking loss was positively correlated (R=0.64, p<0.01) with the Young module (MY10) taken from the compression test. The samples with a high cooking loss were also characterised by low elasticity which confirms the relationship between WHC and the mechanical characteristics of the meat (HAMM, 1970). MY10 and cooking loss were both negatively correlated with pH (R=-0.38 p<0.01; R=-0.57 P<0.01).

Furthermore, MY10 and cooking loss were negatively correlated with intramuscular fat (Table 4). The lipid fraction contributes greatly to the tenderness of the muscle due to its rheological properties and its diluting effect on the collagen network, (LEPETIT and CULIOLI, 1994). A high capacity to retain water in muscles resulting in a better, juicier good quality meat is correlated with a higher content of intramuscular fat (LAWRIE, 1987). Cooking loss and MY10 were negatively correlated with all the parameters linked to the extraction of sarcoplasmic proteins (Table 4). The extractabili-

ty of these proteins is reduced when they are denatured by the formation of precipitating insoluble aggregates. The relationship observed could confirm the hypothesis of other authors that the WHC is also influenced by the denaturation of the sarcoplasmic proteins (JOO *et al.*, 1999), which are more sensitive to variations in pH and temperature conditions during the post-mortem phase (SAYRE and BRISKEY, 1963).

Table 4 also shows the correlation between the extractability of the sarcoplasmic protein and the pH, which increases as the pH increases. JOO *et al.* (1999) noted that the difference in the pH in meat belonging to different quality classes corresponded to differences in the sarcoplasmic proteins. No correlation was observed between the extractability of the myofibrillar proteins, which are more important for discriminating between the physical state of meat, both fresh and frozen (Fig. 1). For the pork meat, a negative correlation was observed between the myofibrillar fraction extraction and cooking loss (R=-0.52, p<0.01) and MY10 (R=-0.43, p<0.01) (Figs. 2, 3), respectively.

Among the linear correlations of the saline-soluble proteins, it is interesting to note that MAX from the shear test was positive. High extractability of the myofibrillar proteins corresponded to a higher cutting force. This parameter is a measure of the firmness of the sample being cooked, which is a quality factor for meats destined for industrial processing. In cooked meat products, the cohesion of the muscle fibres (LAN *et al.*, 1993) and the water holding capacity of the slice after cooking are very important (PIZZA *et al.*, 2002).

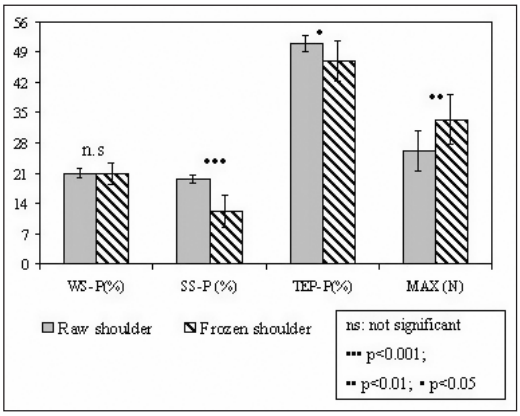


Fig. 1 - Yield of water-soluble (WS-P), salt-soluble (SS-P) and total extractable proteins (TEP-P) and maximum load (MAX) of imported shoulders obtained from the Mortadella producers.

Table 3 - Extraction yield of water-soluble (WS), salt-soluble (SS), total extractable proteins (TEP) and texture parameters from beef and pork muscles. Comparison by one-way analysis of variance[a].

	Beef PS[a,b]	Beef SM	Pork LD	Pork Shoulder	Pork SM
WS-W [c]	3.57±0.72 a	4.09±0.04 a, b	5.50±0.20 c	4.43± 0.75 b	5.75±0.29 c
WS-P [d]	15.89±3.53 a	19.68±0.12 b	23.33±0.91 c	20.73± 3.98 b, c	26.20±1.45 d
SS-W [c]	6.43±0.48 a	8.16±0.15 b	3.27±1.61 c	5.22± 1.79 d	4.43±0.72 e
SS-P [d]	28.48±1.49 a	39.29±0.81 c	13.81±6.69 b	24.32± 8.13 d	20.21±3.51 e
TEP -W [c]	12.68±0.37 a	14.7±0.13 b	12.11±1.75 a	12.22± 2.37 a	13.09± 0.9 b
TEP-P [d]	56.21±2.38 a	72.13±0.79 b	51.32±6.85 a	57.01±10.57 a	59.65±4.41 a
MAX (N)	39.72±2.18 a	39.35±4.26 a	32.81±6.81 b	34.35± 8.31 b	32.83±5.17 b
MY10 (MPa)	0.13±0.03 a	0.11±0.02 a, b	0.11±0.02 a, b	0.08± 0.03 b	0.09±0.03 b

[a] Within the same row means with different letters are significantly different (p <0.05).
[b] PS=*Psoas*; SM=*Semimembranosus*; LD=*Longissimus dorsi*.
[c] X-W: grams protein extracted /100 grams of sample weight.
[d] X- P: grams protein extracted /100 grams of total muscle protein.

Table 4 - Correlation coefficients for the relationship of technological parameters to proximate composition and protein solubility for all the chilled samples.

	pH	Cooking loss (%)	MY10 (Mpa)	MAX (N)
Moisture (g/100 g)	0.45**[a]	0.28**	0.05	-0.05
Protein (g/100 g)	0.29**	0.62**	0.30**	-0.16*
Intramuscular fat (g/100 g)	0.06	-0.58**	-0.30**	0.22**
Ash (g/100 g)	-0.10	-0.22	0.10	-0.22**
WS-P [b]	0.56**	-0.63**	-0.41**	-0.10
SS-P [b]	-0.20*	-0.13	-0.13	0.44**
TEP-P [b]	0.09	-0.63**	-0.41**	0.36

[a] p<0.01**; p<0.05*.
[b] X - P: grams protein extracted /100 grams of total muscle protein.

From the PCA analysis, a 4-factor system was obtained that accounted for 84% of the total variance, 59.9% of which was described by the first two components (PC1 and PC2). The projection of variables (Fig. 4) on the PC1-PC2 plane shows that all the parameters of saline solubility and MAX largely contribute to component 1. On the other hand, the percentages of extracted water-soluble proteins, pH, cooking loss and MY10 are correlated to PC2.

The projection of samples (Fig. 5) on the PC1-PC2 plane shows that the high variability in the raw meat prevented a good separation according to the muscles, with some exceptions.

Pork shoulders from producers were separated in function of their origin. In fact, the heavy pork shoulders are found in the upper right hand quadrant and are characterised by better processing

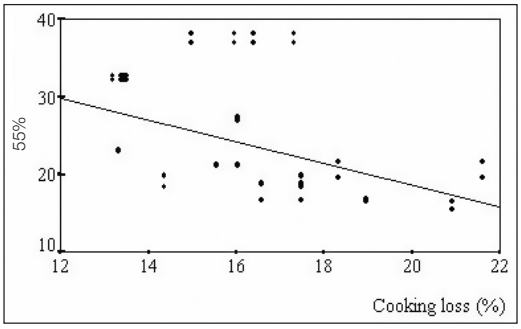


Fig. 2 - Linear correlation between myofibrillar proteins and cooking loss of pork samples.

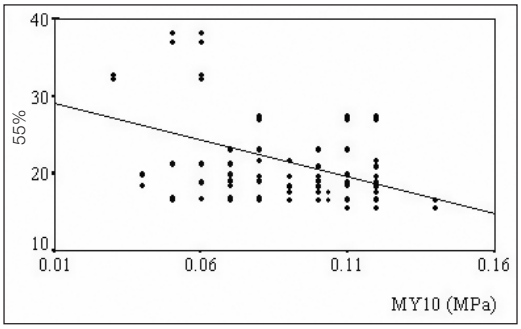


Fig. 3 - Linear correlation between myofibrillar proteins and MY10 of pork samples.

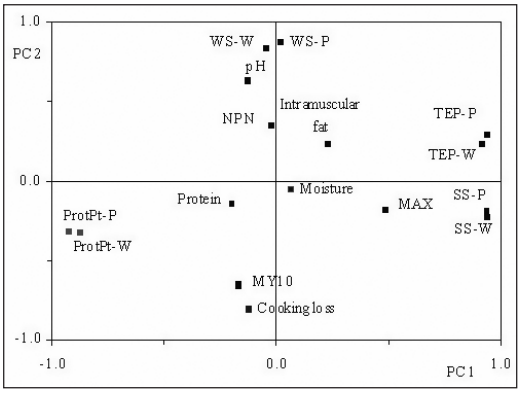


Fig. 4 - Projection of the variables relating to beef and pork samples onto the PC1-PC2 plane.

properties, such as high pH and extraction yield of myofibrillar and sarcoplasmic proteins and low cooking loss and MY10. The imported shoulders are, instead, found in the lower left quadrant of the graph.

Most samples from Italian heavy pork are found in the upper part of the graph, which indicates high extractability of water-soluble proteins and low cooking loss.

The beef samples (SMb and PS) are found in the lower right hand quadrant of the graph, they differ from the pork samples, because with a high extractability of SS, the cooking loss and elasticity module values are high. This separation has also been confirmed by other studies, which have shown that pork meat has a greater capacity of to hold water. Some authors have attributed this to be the quicker pH drop in pork meat during glycolysis. BENDALL (1978) hypothesized that this was the result of varying concentrations of intracellular free calcium ions, that could be caused by mitochondria. SAYRE and BRISKEY (1963) reported that a slower lowering of pH did not influence the solubility of saline-soluble proteins, but did cause sarcoplasmic denaturation.

The PCA analysis was also applied to the pork parameters. The projection of the variables (Fig. 6) onto the PC1-PC2 plane shows that by excluding bovine samples, a different composition of the two first components is obtained: the cooking loss and MY10 contributed negatively to component 1, in contrast to myofibril parameters to which they were more positively correlated than to soluble sarcoplasmic proteins.

CONCLUSIONS

In this study measurements of pH, proximate analysis and cooking loss were made and sarcoplasmic and myofibrillar protein solubility were determined.

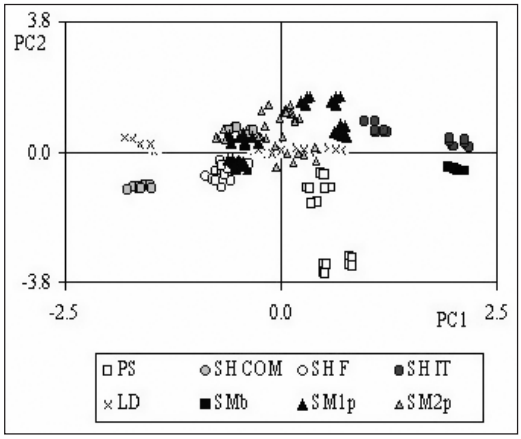


Fig. 5 - Projection of pork and beef muscles onto the PC1-PC2 plane.

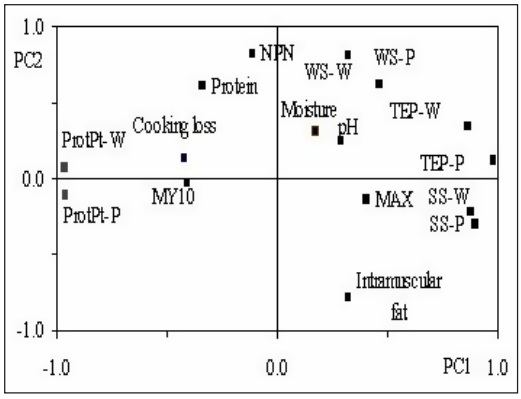


Fig. 6 - Projection of the variables relating to pork samples onto the PC1-PC2 plane.

Some texture data were obtained using the Instron material testing system. These descriptors proved to be valid for mapping the raw materials considered, and the functional and consistency characteristics could be grouped into two different components, which have a rather clear chemical and physical-chemical meaning. In fact, when the analysed samples are projected onto a plane the quality classes are clearly detectable in relation to industrial production.

The PCA analysis provided a good separation between beef and pork samples

and the pork shoulders obtained from producers were separated in function of their origin.

The data obtained for the foreign shoulder samples show that freezing and frozen storage have a negative effect on the extractability of myofibrillar proteins, reducing the extraction yield by one third.

The investigations performed on samples from both the market and processing plants showed some correlations between the various descriptors (pH, cooking loss, MAX, MY10 and yield of soluble proteins) and suitability for processing as a function of species, origin and storage of raw meat.

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PAPER

REVERSIBILITY OF THE CHANGES OF RABBIT MUSCLE FATTY ACID PROFILE

EFFETTO DELL'ALIMENTAZIONE SULLA REVERSIBILITÀ DEL PROFILO IN ACIDI GRASSI DELLA CARNE DI CONIGLIO

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ABSTRACT

Reversibility of the muscle fatty acid profile in rabbit was studied using two dietary fat supplementations. Thirty 4-week-old rabbits were fed a diet with 4% hydrogenated sunflower oil (SAT) for 4 weeks, which was changed to one with 4% sunflower oil (UNSAT) for a further 4 weeks. The reverse UNSAT-SAT feeding plan was also performed on another 30 rabbits. Muscle *Longissimus dor-*

RIASSUNTO

È stato studiato l'effetto dell'alimentazione sulla reversibilità del profilo in acidi grassi della carne di coniglio. Trenta conigli di 4 settimane di età hanno ricevuto per 4 settimane un mangime contenente il 4% di olio di girasole idrogenato (dieta SAT), sostituito nelle successive 4 settimane da un mangime contenente il 4% di olio di girasole insaturo (dieta UNSAT). Un piano alimen-

Key words: dietary fats, fatty acid profile, plasma total cholesterol, reversibility, skeletal muscle

si and blood samples were taken at 4, 5 and 8 weeks. Muscle fatty acid incorporation and reversibility were demonstrated. The SAT-UNSAT and UNSAT-SAT groups differed only in palmitic and linoleic acids at the end of the trial. Plasma cholesterol was temporarily decreased by changing the diet to UNSAT.

tare inverso (UNSAT-SAT) è stato applicato su altri 30 conigli. Campioni di sangue e di muscolo *L. dorsi* sono stati prelevati dopo 4, 5 e 8 settimane di trattamento alimentare. E' stata dimostrata l'incorporazione nel muscolo degli acidi grassi provenienti dalla dieta e la reversibilità di tale incorporazione, al variare del piano alimentare. I due gruppi di conigli appartenenti rispettivamente ai piani alimentari SAT-UNSAT e UNSAT-SAT si sono diversificati solamente per i livelli di acido palmitico e linoleico. Il colesterolo plasmatico totale è diminuito temporaneamente in seguito al passaggio alla dieta UNSAT.

INTRODUCTION

The composition of intramuscular fats (IMF) is an interesting factor both from the viewpoint of dietetics and physiology. The fatty acid (FA) profile of intramuscular fat, comprising multiple lipid classes (the most important being TAG and membrane phospholipids (PL)), depends on numerous parameters, such as the dietary fat source, age and genetic background (CAMBERO *et al.*, 1991). Dietary-induced changes in IMF have been widely studied, e.g. in pigs (WOOD *et al.*, 1999), cattle (ANDRAE *et al.*, 2001), broilers (HRDINKA *et al.*, 1996), rabbits (CAMBERO *et al.*, 1991; KESSLER and PALLAUF, 1994) and fish (BELL *et al.*, 2002). However, reversible modifications of the tissue fatty acid profile following dietary changes have only been reported for mice (SWANSON *et al.* 1987) and rats (AYRE and HULBERT, 1996; HELGE *et al.*, 1999). A trial was reported by Maldonado *et al.* (2002), where mice were fed a normal laboratory diet which included a two-week period on a fish oil supplemented diet. They reported exchange times between 3 and 14 days for different fatty acids, however the results mainly concerned tissue phospholipids.

SZABÓ *et al.* (2001) reported that after a four-week feeding period, the FA profile of whole muscle homogenates of rabbits effectively reflected that of the feed. MOREOVER, HUERTA-LEIDENZ *et al.* (1996) and FONTANILLAS *et al.* (1998) tracked the FA incorporation in swine and calf adipose tissue, respectively, by means of serial biopsy samplings. Thus, the present study aimed to demonstrate the exchange course of FA in *m. L. dorsi* (m. LD) total lipids, subsequent to the use of diets greatly differing in the degree of saturation of their FA. Two isocaloric diets (supplemented with intact sunflower oil (UNSAT) or with hydrogenated sunflower oil (SAT)) were fed because the FA incorporation in the different lipid stores is also influenced by the energy content of the diet. The main goal of this experimental design was to follow-up the dietary FA incorporation and the exchange in rabbit muscles. This was, in part, complemented with the investigation of blood lipid metabolites.

MATERIALS AND METHODS

Animals, feeding, sampling

Sixty Pannon White male rabbits were housed two-by-two in wire net cages of 500x800 mm. At weaning (28th day of life), the rabbits were randomly divided into two feeding groups and fed successively the following two experimental diets: UNSAT diet, containing 4% sunflower oil and SAT diet, containing 4% partially hydrogenated sunflower oil. Diets were offered *ad libitum*. Feed composition was analyzed according to the A.O.A.C. (1980) methods. The ingredients, chemical and FA composition of the diets are shown in Table 1.

The first group of 30 rabbits was fed SAT for 4 weeks when the diet was changed to UNSAT for a further 4 weeks. Rabbits were slaughtered after the first 4 weeks (n=10, 8th week), then after 1 (n=10, 9th week) and 4 (n=10, 12th week) weeks following the dietary change. The other 30 rabbits were treated analogously, only the UNSAT diet was fed for the first 4 weeks, and was then changed to SAT.

The m. LD samples were taken immediately after slaughtering, always from the same location (2nd to 3rd lumbar vertebrae) and were stored frozen (-80°C) until analysis. Blood samples were taken from the left ear vein prior slaughtering.

The trial was approved by the Animal Experimentation Ethics Committee of the University of Kaposvár.

Gas chromatography of the muscle FA composition

The total lipids were extracted from the muscle samples according the method of FOLCH *et al.* (1957). Transmethylation was carried out using a mixture of methanol, benzene and sulphuric acid (75:25:4). Gas liquid chromatography was performed on an automated instru-

Table 1 - Ingredients, chemical and fatty acid composition of the diets.

Ingredients *(%)*	SAT	UNSAT
Wheat	14.15	14.15
Barley	11	11
Extracted sunflower	13	13
Alfalfa (16% protein)	40	40
Sunflower oil	-	4
Partially hydrogenated oil	4	-
Bran	17	17
Limestone	0.3	0.3
MCP	0.55	0.55
Chemical composition *(%)*		
Dry matter	89.4	89.4
Crude protein	16.43	16.43
Ether extract	6.11	6.11
Crude fiber	14.85	14.85
Organic matter	83	83
DE rabbit (MJ/kg; calculated)	11.04	10.98
Fatty acid composition *(% of total)*		
<C12:0	0.70	0.00
C14:0	1.83	0.24
C15:0	0.08	0.00
C16:0	22.97	11.40
C17:0	0.09	0.07
C18:0	4.14	3.31
C20:0	0.28	0.16
C22:0	0.24	0.31
C16:1 n-7	0.12	0.12
C17:1 n-9	0.06	0.06
C18:1 n-7	1.38	0.79
C18:1 n-9	28.78	18.56
C20:1 n-9	0.41	0.22
C18:2 n-6	24.68	55.46
C18:3 n-3	10.82	8.24
C20:2 n-6	0.04	0.02
C20:3 n-3	0.02	0.00
C20:4 n-6	0.03	0.00
C22:2 n-6	0.85	0.53
C22:6 n-3	0.05	0.00
others	2.43	0.51
? Sat. /? Unsat.	0.44	0.18

ment (CE 8000 Top, Thermoquest, Milan, Italy), equipped with a flame ionization detector and a Supelco Omegawax 250 type capillary column (30 m x 0.25 mm ID). The operating conditions were the following: injector temperature: 250°C, detector temperature: 250°C; hydrogen flow: 1.60 mL/min. The heating program was started at 140°C initial value with a 4°C increment every 10 minutes until the final temperature of 220°C. FA were identified by comparing of the retention times to those of authentic FA methyl ester standards. Results are expressed as w/w% of the total FA methyl esters.

Determination of plasma total lipids, triacylglycerols and total cholesterol

The blood samples were collected in tubes with K-EDTA. Samples were immediately placed on ice and then centrifuged, decanted and the plasma was frozen. Plasma total lipids, triacylglycerol and total cholesterol content was determined by means of Randox® laboratory kits (TL100, TR210 and CH200, respectively, Randox Laboratories LTD, UK). Plasma TAG and cholesterol are expressed as mmol/L and total lipids as mg/mL.

Statistical methods

The comparisons were performed within the two different feeding groups (SAT-UNSAT and UNSAT-SAT). One-way analysis of variance was applied in each group at the three time points, while body weight was set as covariate and the LSD (least significant difference) post-hoc test was applied. The fatty acid composition at the end of the two feeding treatments was compared with the Student *t*-test. Linear regression models were developed from the individual data to demonstrate the changes in the measured fatty acids within the feeding periods of the two experimental groups. SPSS 10 for Windows (1999) was used for the analysis.

RESULTS

Muscle FA composition

The data obtained from the muscle FA analysis are given in Table 2. The change from SAT to UNSAT induced a significant ($P<0.05$) increase in linoleic (C18:2 n-6) acid already after one week; this increased even more after the second period of three weeks, according to the over twofold higher linoleic acid content of the UNSAT diet. Furthermore, after the second, four-week period the proportion of stearic (C18:0), eicosapentaenoic (EPA, C20:5 n-3) acid and that of the saturated to unsaturated fatty acid ratio decreased significantly. Bodyweight, as a covariate had significant effect on the margaric (C17:0), myristoleic (C14:1 n-5), palmitoleic (C16:1 n-7) and gondoic (C20:1 n-9) acids.

The UNSAT to SAT dietary change resulted in significant ($P<0.05$) increases in myristic (C14:0), palmitic (C16:0), vaccenic (18:1 n-7), oleic (C18:1 n-9) and _-linolenic acids and the saturated to unsaturated fatty acid ratio after the first week. Myristic and oleic acid continued to increase significantly in the second period. On the contrary, linoleic acid decreased significantly until the end of the experiment. Significant decreases were shown in arachidonic (C20:4 n-6), eicosadienoic (C20:2 n-6) and docosatetraenoic (C22:4 n-6) acids. Significant bodyweight effect was only shown for stearic acid.

The linear regression models were only taken into account above the R^2 value of 0.5. A proper linear fitting was obtained by eicosapentaenoic acid (EPA) after the SAT to UNSAT dietary change, while with the reverse feeding regimen myristic, oleic, linoleic, _-linolenic and docosadi-

Table 2 - Fatty acid composition of m. *L. dorsi* in the different feeding groups (means ± SD); n=10 for each time period.

Fatty acid	SAT-UNSAT change			sig. of weight	UNSAT-SAT change			sig. of weight	*t*-test between 4 weeks SAT+ 4 weeks UNSAT and 4 weeks UNSAT+ 4 weeks SAT
	4 weeks SAT	4 weeks SAT + 1 week UNSAT	4 weeks SAT+ 4 weeks UNSAT		4 weeks UNSAT	4 weeks UNSAT + 1 week SAT	4 weeks UNSAT+ 4 weeks SAT		
C14:0	2.07±0.63	2.05±0.5	2.35±0.69	NS	1.39±0.39	1.98±0.7	2.87±0.64	NS	NS
C15:0	0.40±0.05	0.41±0.08	0.40±0.07	NS	0.44±0.07	0.45±0.06	0.45±0.07	NS	NS
C16:0	28.86±1.35	27.52±1.5	25.97±2.64	NS	24.06±2.2	26.6±2.14	28.83±2.21	NS	0.017
C17:0	0.45±0.07	0.42±0.04	0.38±0.05	0.023	0.49±0.04	0.47±0.10	0.40±0.06	NS	NS
C18:0	7.67±0.65	7.40±0.68	6.44±0.92	NS	7.99±0.89	7.39±0.68	6.2±0.55	0.032	NS
C20:0	0.068±0.02	0.064±0.009	0.056±0.012	NS	0.062±0.004	0.059±0.008	0.063±0.001	NS	NS
C22:0	0.22±0.10	0.02±0.10	0.15±0.07	NS	0.15±0.08	0.22±0.17	0.16±0.07	NS	NS
C14:1 n-5	0.10±0.07	0.09±0.36	0.20±0.07	0.021	0.63±0.34	0.16±0.10	0.19±0.10	NS	NS
C16:1 n-7	1.43±0.87	1.60±0.66	2.86±0.74	0.002	1.19±0.51	1.89±1.09	2.83±1.05	NS	NS
C17:1 n-9	0.13±0.06	0.11±0.05	0.12±0.04	NS	0.081±0.043	0.094±0.038	0.095±0.039	NS	NS
C18:1 n-7	1.77±0.14	1.69±0.13	1.42±0.20	NS	1.21±0.14	1.46±0.15	1.58±0.17	NS	NS
C18:1 n-9	21.86±1.49	21.32±1.73	21.73±2.07	NS	16.98±0.89	19.8±1.22	22.92±1.02	NS	NS
C20:1 n-9	0.15±0.032	0.14±0.032	0.13±0.03	0.039	0.10±0.02	0.11±0.027	0.14±0.019	NS	NS
C18:2 n-6	20.78±0.7	23.36±2.7	25.78±2.85	NS	31.94±3.02	26.09±2.13	21.86±1.76	NS	0.002
C18:3 n-3	3.11±0.75	3.0±0.82	3.41±0.94	NS	2.64±0.53	3.02±0.44	3.97±0.64	NS	NS
C20:2 n-6	0.28±0.15	0.28±0.07	0.31±0.15	NS	0.37±0.12	0.36±0.07	0.17±0.10	NS	NS
C20:3 n-3	0.073±0.013	0.069±0.014	0.057±0.019	NS	0.064±0.012	0.065±0.018	0.066±0.021	NS	NS
C20:4 n-6	3.98±0.70	4.18±1.02	3.8±1.78	NS	4.53±0.55	4.35±0.96	3.38±1.17	NS	NS
C20:5 n-3	0.24±0.046	0.22±0.064	0.12±0.042	NS	0.11±0.029	0.15±0.051	0.14±0.058	NS	NS
C22:2 n-6	0.56±0.16	0.63±0.17	0.29±0.14	NS	0.58±0.12	0.62±0.19	0.26±0.07	NS	NS
C22:4 n-6	0.83±0.15	0.89±0.23	0.82±0.41	NS	1.14±0.17	1.03±0.2	0.69±0.24	NS	NS
C22:5 n-3	0.89±0.21	0.89±0.22	0.64±0.3	NS	0.54±0.09	0.65±0.24	0.55±0.23	NS	NS
C22:6 n-3	0.15±0.035	0.17±0.064	0.15±0.061	NS	0.16±0.053	0.15±0.051	0.12±0.049	NS	NS
others	4.08±2.05	3.45±0.74	2.93±1.60	NS	3.88±2.33	3.10±0.60	2.21±0.45	NS	NS
? Sat. /? Unsat	0.71±0.039	0.65±0.036	0.59±0.08	NS	0.56±0.064	0.62±0.052	0.66±0.07	NS	NS

a, b, c: values in the same row with different superscripts are significantly different, P<0.05

Table 3 - Linear regression equations on the fatty acid incorporation and washout.

Treatment	Fatty acid	sig.	R^2	B	constant	SEE*
UNSAT to SAT	C14:0	0.0001	0.534	0.206	1.05	0.58
	C18:1 n-9	0.0001	0.818	0.807	15.87	1.16
	C18:2 n-6	0.0001	0.689	-1.326	33.26	2.71
	C18:3 n-3	0.0001	0.541	0.19	2.26	0.53
	C22:2 n-6	0.0001	0.51	-0.05	0.738	0.15

enoic acids could be characterized (Table 3).

Blood plasma

The results of the blood plasma analysis are given in Table 4. The total lipid content of the blood plasma showed slightly increasing trends with both dietary treatments. After the SAT to UNSAT change during the last three weeks there was a significant increase. In the reverse treatment this increase occurred after the first week following the dietary change and then remained stable. The plasma triacylglycerol concentration increased throughout the trial, but was not significant

in either dietary treatment. The total cholesterol content of the plasma was modified by the SAT to UNSAT change: after the first week there was a significant decrease, though this decrease was only temporary.

DISCUSSION

Different studies have shown that after a four-week treatment the FA profile of rabbit muscles can effectively be modified when rabbits are fed diets with different FA supplementations (CAMBERO *et al.*, 1991; SZABÓ *et al.*, 2001). However, there is a lack of information about the exchange time or reversible incorporation of the dietary FA in rabbit muscle.

In the present study, after the SAT to UNSAT change, the amount of oleic acid in the muscle showed a decreasing trend that could be characterized linearly; similar results were reported by Fontanillas *et al.* (1998), feeding hydrogenated oil supplementation to pigs.

Oleic acid significantly increased just one week after the dietary UNSAT to SAT change, reflecting the SAT-dietary high level of this FA. However, after the SAT to UNSAT feed change, the concentration of oleic acid did not show any change, while its precursor, stearic acid, decreased significantly. This fact, at least in part, suggests an increase in the _9 desaturase activity. A similar finding was described by Bourre *et al.* (1997), as low (1.6%) and high (8.6%) dietary stearic acid supplementation did not alter the muscle oleic acid level in rats. They reported that endogenous oleic acid synthesis (from stearic acid) alone cannot produce the required amount of this acid, but dietary supplementation is also needed in rats.

On the other hand, enzymatic linkages of the mammalian FA synthesis could be clearly demonstrated. Since the diets did not contain ingredients of animal origin, they contained arachidonic acid only in traces, therefore, linoleic acid could be the only precursor of this FA in the muscle. The UNSAT to SAT change significantly lowered linoleic acid as well as the arachidonic acid in the muscle after four weeks. This agrees with that of ALESSANDRI *et al.* (1990), who report-

ed a decrease in arachidonic acid content in tissue lipids of rats when fed a restricted amount of linoleic acid. MALDONADO *et al.* (2002) reported that linoleic and arachidonic acids were the first two FA reflecting the dietary change in mouse liver and plasma, while other FA showed a longer washout time.

Moreover, enzymatic connections between precursor and product FA could be clearly recognized when the dietary amount of FA was measurably decreased, and not by dietary overload. This was seen after the SAT to UNSAT change, where the dietary stearic and oleic acid contents decreased. As a consequence, muscle stearic acid significantly decreased, while oleic acid was unaltered. A similar phenomenon was seen by the a-linolenic acid and EPA pair. In the change to UNSAT, a lowered dietary a-linolenic acid intake reduced only the amount of EPA in the muscle, but not that of a-linolenic acid. In the opposite treatment, a-linolenic acid was in excess (dietary overload), and its amount in the muscle increased, while no consequent change was seen in case of EPA.

Theoretically, when both SAT and UNSAT diets were fed in two equal four-week periods, irrespective of the sequence, there should have been no difference in the FA profile of the two groups at the end of the trial. This condition was nearly fulfilled. After the 8th week significant differences were found only in palmitic and linoleic acids. The diets contained strongly different amounts of these FA, therefore, it can be concluded that the dietary effect on the muscle FA composition was rather strong.

The blood plasma lipid metabolites were descriptive not only with regard to incorporation, but also with regard of the metabolism of the fats.

The slightly increasing trend in the plasma total lipid content may, at least in part, be related to the increasing body weight (Table 4) and the consequent elevated feed intake of the growing rabbits.

Table 4 - Changes in the blood plasma total lipid, triacylglycerol (TAG) and total cholesterol levels.

Blood plasma		4 weeks SAT	4 weeks SAT +1 week UNSAT	4 weeks SAT +4 weeks UNSAT	sig. of weight	4 weeks UNSAT	4 weeks UNSAT +1 week SAT	4 weeks UNSAT +4 weeks SAT	sig. of weight
Total lipid	mg/mL	2.60±0.48^{a}	2.96±0.58^{a}	3.13±0.78^{b}	0,057	2.67±0.34^{a}	3.28±0.59^{b}	3.23±0.78^{ab}	NS
TAG	mmol/L	1.04±0.28	1.23±0.40	1.30±0.42	NS	0.97±0.39	1.13±0.42	1.40±0.42	NS
Cholesterol	mmol/L	2.64±0.43^{a}	1.96±0.60^{b}	2.1±0.33^{ab}	0,06	2.58±0.34	2.28±0.57	2.18±0.55	NS
Bodyweight	kg	1.85±0.35^{a}	1.88±0.25^{a}	2.94±0.27^{b}	-	1.85±0.17^{a}	1.88±0.32^{a}	2.89±0.23^{b}	-

a, b: values with different superscripts are significantly different, P<0.05.

This was also supported by the borderline significant (P=0.057) bodyweight effect of the SAT-UNSAT change. On the contrary, the plasma total cholesterol level showed a temporary decrease (P=0.06) after the SAT to UNSAT change, though only in the first week following the dietary change. According to Hodson *et al.* (2001), an increase in the unsaturation of the dietary fat can strongly lower the plasma total cholesterol level. In addition, Maldonado *et al.* (2002) reported that in mice this decrease occurs in the first three days after the dietary change which is in agreement with our findings.

CONCLUSIONS

It can be concluded that the dietary fat source can effectively modify the skeletal muscle FA profile when fed in a proper time interval. The restitution of the tissue FA profile following the change of the dietary fat source can be perfectly achieved, however it also depends on the magnitude of the proportional change of the FA in the diet. The incorporation or the washout of certain fatty acids could be lineraly characterized. In blood lipids it was demonstrated that changing the diet to a more unsaturated one could temporarily reduce the plasma total cholesterol level.

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PAPER

VITAMIN C IN ORANGE JUICES DETERMINED BY HPLC: INFLUENCE OF THE WAVELENGTH OF DETECTION

DOSAGGIO DELLA VITAMINA C IN SUCCHI DI ARANCIA MEDIANTE HPLC: INFLUENZA DELLA LUNGHEZZA D'ONDA DI MONITORAGGIO

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ABSTRACT

Two different types of orange juice, ultrafrozen (UFOJ) and from concentrate (OJFC), were analysed to evaluate the applicability of a chromatographic method for analysing organic acids to quantify vitamin C. The 2,6-dichlorophenolindophenol titration method was used as reference. Detection at 214 nm allowed vitamin C to be quantified accurately in OJFC. For UFOJ, the levels of vitamin C obtained by means of the selected wavelengths were significantly different than those

RIASSUNTO

Sono stati analizzati due tipi di succhi d'arancia (da prodotto surgelati o da concentrato) con l'obiettivo di valutare la validità di un metodo cromatografico per la determinazione di acidi organici per la quantificazione della vitamina C. Prendendo come riferimento il metodo di valutazione con 2,6-diclorofenolindofenolo, il monitoraggio degli acidi organici a 214 nm permette di quantificare in modo esatto la vitamina C in esame. Nel caso dei succhi surgelati, le lunghezze d'onda di monito-

- Key words: ascorbic acid, 2,6-dichlorophenolindophenol, HPLC, orange juice, vitamin C -

obtained using the reference method, indicating that the HPLC method is not suitable for the determination of vitamin C in this juice.

raggio selezionate portano all'ottenimento di livelli di acido ascorbico significativamente differenti da quelli ottenuti con il metodo di riferimento, indicando che il metodo per HPLC non è adatto alla determinazione della vitamina C in questo tipo di succo.

INTRODUCTION

Vitamin C (L-ascorbic acid) is one of the most important organic acids in fruits and vegetables, in relation to their nutritional value. Its content is not only used as a nutritional index, but it is also used to evaluate processing effects, since vitamin C is highly unstable. Several analytical methods have been developed in the last decades to determine the vitamin C content in fruits and juices. One of the most frequently used methods is based on the reduction of the blue dye 2,6-dichlorophenolindophenol by ascorbic acid (AOAC, 1999). The endpoint of the titration is indicated by the appearance of the pink acid form of the dye. This is a simple and fast method (DE ASSIS *et al.*, 2001) and is usually used for validation of results. It is a suitable method to determine vitamin C not only in food or fruit juices but also in pharmaceutical products and biological samples (PARK *et al.*, 1983).

Determination of ascorbic acid by means of electrochemical and enzymatic methods are also well represented in the literature (AKYILMAZ and DINÇKAYA, 1999; IJERI *et al.*, 2001). However, the majority of the methods are based on high-performance liquid chromatography (HPLC) (MARINI and BALESTRIERI, 1994; ARENA *et al.*, 2001), that allows the simultaneous determination of ascorbic and dehydroascorbic acids (NISPEROS-CARRIEDO *et al.*, 1992) or the separation of ascorbic acid and isoascorbic acid (BUI-NGUYÉN, 1980). Attention has also been focused on methods for the simultaneous determination of organic acids and vitamin C in juices (LEE, 1993; CÁMARA *et al.*, 1994), since organic acids play an important role in the organoleptic properties of juices. These last methods are also based on HPLC, using a buffer solution as mobile phase in order to avoid ionization of the organic acids due to variations in pH.

Organic acids are usually monitored at wavelengths in the range of 206-220 nm (BLANCO GOMIS, 2000), although specific methods for ascorbic acid usually call for higher wavelengths, usually in the range of 245-260 nm (NISPEROS-CARRIEDO *et al.*, 1992; LEE and COATES, 1997).

The main objective of this study was to evaluate the suitability of an HPLC method for the analysis of organic acids to determine accurately the content of vitamin C in orange juices. Several wavelengths of detection were selected and the 2,6-dichlorophenolindophenol titration method was used as the reference method for the determination of vitamin C.

MATERIALS AND METHODS

Samples

Thirty-three samples of orange juice were selected: 14 ultrafrozen orange juice (UFOJ) samples and 19 orange juices

from concentrate (OJFC). UFOJ were obtained from Valencia late oranges during the 1999 season. Samples were provided by the CCH Zumos Vitafresh industry, (Almonte, Spain). After squeezing the oranges, the juice was cooled and immediately frozen by means of an industrial freezing tunnel. Hence, the juice had not undergone high temperature or low pressure treatment. The samples were kept in a freezer chamber between -18° and -21°C until analyses. Defrosting was carried out by means of a Samsung M1713 microwave oven at 800 watts for 11 min. OJFC samples were purchased from local markets as representative examples of commercially available orange juices from concentrates. Before analysis they were kept in a cool, dry place. OJFC were prepared by reconstituting either frozen concentrate orange juice (orange juices concentrated in a high vacuum evaporator and frozen) or concentrated orange juice.

Each sample was analysed in triplicate both by HPLC and by the titration method.

Methods

2,6-dichlorophenolindophenol titration

The titration method is based on the reduction of the sodium salt of the blue dye by ascorbic acid, resulting in the formation of a colourless derivative and dehydroascorbic acid. The endpoint of titration is indicated by the persistence of the pink colour of the solution (AOAC, 1999). The solution of 2,6-dichlorophenolindophenol was prepared by dissolving 50 mg of the sodium salt of the dye (Panreac, Montcada i Reixac, Spain) and 42 mg of sodium bicarbonate (Panreac, Montcada i Reixac, Spain) in 200 mL water, removing the insoluble material with Albet 243 filter paper. This solution was standardised daily against a standard solution of ascorbic acid and stored in a refrigerator.

The standard solution of ascorbic was prepared daily by dissolving 25 mg of high-purity L-ascorbic acid (Panreac, Montcada i Reixac, Spain) in 25 mL of metaphosphoric acid (Panreac, Montcada i Reixac, Spain) aqueous solution (3% w/v).

HPLC

The HPLC apparatus consisted of a Hewlett-Packard 1100 system (Hewlett-Packard, Palo Alto, CA, U.S.A.), equipped with a quaternary pump, a diode-array detector and a column temperature control module. A 20 μL loop was used for injection. The column was a C-18 ODS Hypersil (2504 mm, 5 μm packing) with a guard-column of the same material.

The chromatographic procedure used was based on the isocratic method reported by LEE (1993), although slight modifications were made. Two solvents: a 20 mM aqueous solution of KH_2PO_4 (pH = 2.8) and methanol were used. Elution was carried out according to the following program: 0 min 100% KH_2PO_4; 5 min 96% KH_2PO_4 + 4% MeOH; 15 min 100% KH_2PO_4.

The column was kept at 25°C and the flow rate was 0.7 mL/min. The eluate was monitored at 200, 210, 214 and 230 nm. The chromatograms were recorded using ChemStation software (Hewlett Packard; Palo Alto, CA, U.S.A). The column was washed with water at the end of each day.

Identification of ascorbic acid in the samples was made by comparison of the spectra and retention times with those obtained for the standard solutions. Peak purity was established by comparing the spectra which were obtained at the upslope (at peak half-height), apex, and downslope (at peak half-height) against a baseline reference. The spectra were normalized and overlaid for comparison. The limits of detection (LOD) of ascorbic acid at the different wavelengths selected were calculated according to the formula LOD = $\alpha + 2s_{y/x}$ (MILLER and MILL-

ER, 1993), where $s_{y/x}$ is the standard deviation of the calibration curve and a is the intercept.

Preparation of samples and standard solutions for HPLC

Samples were prepared as follows: 9 mL of juice and 1 mL of metaphosphoric acid solution (3% w/v) were mixed. The mixture was centrifuged at 5,000 rpm for 5 min and filtered through PVDF Millipore filters (13 mm, 0.45 μm).

The standard solutions of ascorbic acid (Panreac, Montcada i Reixac, Spain) were made from a 1 g/100 mL stock solution in 3% (w/v) metaphosphoric acid. Calibration curves at three concentration levels (100, 400 and 800 mg/L) were obtained for the different wavelengths monitored.

Statistical analysis

For statistical analyses Statistica v. 5.5 software (STATSOFT, 1999) was used. Significant differences were evaluated by means of analysis of variance (ANOVA) for repeated measurements ($\alpha = 0.05$).

RESULTS AND DISCUSSION

Table 1 shows the regression equations for the calibration curves for ascorbic acid, r values and LOD for the HPLC method at the different wavelengths tested.

Tables 2 and 3 show the concentration of vitamin C obtained by HPLC and the 2,6-dichlorophenolindophenol titration method, for OJFC and UFOJ, respectively. Determination at 200 nm led to the highest standard deviations for both juices, indicating that quantification at this wavelength was less accurate. The results obtained by the reference method showed that the vitamin C content in UFOJ was quite homogeneous. The average concentration was 360.85 ppm, and the minimum and maximum contents were 319.55 and 382.69 ppm, respectively. In OJFC, the range of vitamin C varied widely, since some of the samples were enriched juices. Most of the samples (n = 11) ranged from 400 to 800 ppm. The minimum and maximum contents were 125.80 and 1,191.84 ppm, respectively.

Analysis of variance (ANOVA) for repeated measurements ($\alpha = 0.05$) was applied, in order to test the same samples using different wavelengths. For OJFC (Table 2), an accurate quantification of vitamin C was obtained only at 214 nm, since the vitamin C levels were not significantly different from those of the reference method. According to the vitamin C levels determined with the reference method, the OJFC samples were divided into three groups: 0-400 ppm, 400-800 ppm and over 800 ppm (Table 4). For the first group, vitamin C levels were not significantly different from the reference method at any of the wave-

Table 1 - Regression equations and limits of detection (LOD) for ascorbic acid in OJFC and UFOJ using the HPLC method.

λ (nm)	Equations	r	LOD (ppm)
200	c = -3.358 + 0.049A	0.999	15.98
210	c = -7.984 + 0.067A	0.999	5.14
214	c = 4.31 + 0.03A	0.999	18.99
230	c = -15.204 + 0.021A	0.999	0.40

c refers to the ascorbic content (ppm) and A is the peak area of the chromatograms.

Table 2 - Vitamin C (ppm) in orange juices from concentrate (OJFC).

Samples	HPLC 200 nm	HPLC 210 nm	HPLC 214 nm	HPLC 230 nm	Reference
1	196.87±28.89	176.16±2.27	142.58±0.73	125.99±2.04	150.00
2	182.54±31.25	148.77±7.79	124.32±2.60	115.77±7.45	125.80
3	478.29±11.78	443.23±13.19	387.07±15.51	394.73±12.15	375.00
4	282.89±2.66	281.21±1.17	253.00±2.44	278.50±24.53	284.88
5	586.24±47.84	557.09±8.49	498.01±0.21	542.23±6.46	480.33
6	527.18±69.44	525.97±26.35	454.61±14.56	496.99±4.81	478.08
7	504.33±2.68	522.77±6.46	466.48±6.69	516.28±9.90	462.54
8	484.27±26.43	529.87±3.15	474.57±7.01	519.09±3.76	460.56
9	878.09±20.96	884.40±2.24	793.46±0.79	927.30±0.79	836.48
10	930.99±1.82	900.44±18.07	826.84±9.57	927.01±2.74	837.90
11	563.61±83.64	585.45±1.02	511.44±7.00	517.63±14.67	464.06
12	523.72±58.35	483.86±5.73	470.57±37.75	532.31±8.23	457.52
13	1,274.49±80.11	1291.74±32.43	1,148.75±45.84	1,291.85±31.93	1,149.10
14	1,229.05±18.78	1,223.28±5.89	1,126.80±8.29	1,282.13±16.30	1,191.84
15	688.23±49.10	660.89±6.97	601.98±2.50	664.21±16.19	598.08
16	739.66±5.60	734.10±12.96	627.16±12.49	679.67±3.08	631.80
17	452.79±35.90	465.77±13.81	398.40±2.04	440.71±0.36	423.64
18	531.92±25.21	485.79±27.08	436.72±2.00	460.49±16.17	421.20
19	616.96±7.04	643.19±1.46	575.77±1.70	636.46±0.38	550.08
Means	$614.32\pm295.79_{a}$	$607.58\pm301.20_{a}$	$543.08\pm275.43_{b}$	$597.33\pm321.29_{a}$	$546.26\pm284.76_{b}$

Means with different subscripts within a row are significantly different at $p < 0.05$.

Table 3 - Vitamin C (ppm) in ultrafrozen orange juices (UFOJ).

Samples	HPLC 200 nm	HPLC 210 nm	HPLC 214 nm	HPLC 230 nm	Reference
1	612.61±1.73	453.14±71.33	394.67±7.90	322.48±6.30	337.81
2	693.83±9.56	497.84±66.77	444.97±5.53	341.71±7.39	319.55
3	696.36±32.04	499.16±4.69	357.52±3.37	355.08±7.01	319.55
4	735.08±39.07	618.22±33.16	474.60±16.30	381.79±12.85	378.24
5	732.12±1.64	609.75±6.68	477.69±1.95	381.15±18.68	378.24
6	749.24±38.61	548.55±95.61	477.33±14.87	414.38±1.70	365.63
7	693.98±44.64	566.17±40.94	469.23±17.37	407.28±5.48	360.75
8	691.72±18.36	559.83±9.19	462.71±10.98	408.09±4.80	367.50
9	729.42±38.46	592.68±47.15	479.60±12.80	416.65±2.01	362.60
10	701.07±151.09	464.66±56.59	448.42±61.17	395.58±15.45	382.69
11	642.58±103.94	495.69±43.64	445.86±29.68	394.14±10.13	366.51
12	801.67±144.54	502.87±49.02	468.82±60.73	396.48±8.73	360.08
13	643.59±213.20	492.12±97.87	455.48±82.41	410.73±19.59	373.18
14	673.09±92.01	500.78±121.05	458.74±20.16	386.99±5.43	379.50
Means	$699.74\pm48.74_{a}$	$528.68\pm53.32_{b}$	$451.12\pm34.67_{c}$	$386.61\pm28.49_{d}$	$360.85\pm20.80_{e}$

Means with different subscripts within a row are significantly different at $p < 0.05$.

Table 4 - Average concentrations (ppm) of vitamin C in the different OJFC groups.

Group	HPLC 200 nm	HPLC 210 nm	HPLC 214 nm	HPLC 230 nm	Reference
0-400 ppm	285.15±136.17	262.34±133.42$_{a,c}$	226.74±121.06$_{b}$	228.75±133.35$_{b,c}$	233.92±117.24$_{a,b}$
400-800 ppm	565.36±87.10$_{a}$	563.16±84.61$_{a}$	501.43±71.73$_{b}$	546.01±79.65$_{a}$	493.44±69.27$_{b}$
> 800 ppm$_{a}$	1,078.16±202.48$_{a}$	1,074.97±212.73$_{a,c}$	973.96±189.86$_{b}$	1,107.07±207.79$_{a}$	1,003.83±193.21$_{b,c}$

Means with different subscripts within a row are significantly different at $p < 0.05$.

lengths of detection. For the second group (400-800 ppm), vitamin C was accurately quantified only at 214 nm. For juices with vitamin C levels over 800 ppm, both 214 nm and 210 nm were suitable for accurately determining vitamin C.

For UFOJ, detection at 200 nm led to the highest standard deviations (Table 3) and the higher the wavelength of detection the lower vitamin C levels determined. The vitamin C content at 200 nm was nearly double that obtained at 230 nm. This is due to partial peak overlapping, since in UFOJ at the lower wavelength of detection, there was higher partial peak overlapping (Fig. 1). This was not true for OJFC; while the profile of organic acids and, therefore, the chromatograms of UFOJ were quite constant, those corresponding to the OJFC juices analyzed were quite different among themselves. The results of ANOVA for repeated measurements showed that vitamin C levels in UFOJ obtained at all the wavelengths tested were significantly different among themselves ($p < 0.05$) and in comparison with the reference method. However, the vitamin C content calculated at 214 and 230 nm correlated well with those obtained with the titration method (r = 0.707 and 0.690, respectively) but poorly with those obtained at lower wavelengths (r = 0.170 and 0.352, for 200 and 210 nm, respectively). This indicates that detection at 200 nm or even at 210 nm is not suitable, probably due to the interference of compounds which either could not be detected or which do not occur in OJFC. With respect to the reference method, the vitamin C contents of UFOJ samples were all in the 0-400 ppm range. Whereas for OJFC in this group (Table 4) any of the wavelengths selected could be used to accurately determine vitamin C, but none of them were suitable for UFOJ.

In conclusion, the accuracy with which the vitamin C content can be determined using the HPLC method for organic acids depends on the wavelengths selected and on the type of sample. For OJFC with vitamin C contents in the range 400-800 ppm, the wavelength 214 nm should be selected. For lower vitamin C contents (lower than 400 ppm), any of the wavelengths tested in this study are suitable for an accurate quantification. However, the HPLC method studied overestimated the vitamin C content of UFOJ at all of the wavelengths tested, due to partial peak overlapping.

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PAPER

PRELIMINARY STUDY ON A COOLING PRACTICE OF GRAPE POMACE DURING STORAGE ON AN INDUSTRIAL SCALE

STUDIO PRELIMINARE SU SCALA INDUSTRIALE DELLA REFRIGERAZIONE DI VINACCIA INSILATA

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ABSTRACT

This study reports an estimation of the energy balance performed on vertical closed stainless steel containers (capacity 500 m^3) filled with pomace cooled by dripping wine at 0° and 4°C during storage. A comparison between the volatile composition of grappa low-wines at about 20% v/v ethanol obtained by distilling grape pomace stored for 3 months in stainless steel containers and in a plastic container is also re-

RIASSUNTO

Il presente lavoro riporta un bilancio di energia effettuato su silos verticali di acciaio inossidabile (capacità 500 m^3) riempiti con vinaccia raffreddata da vinello di sgrondo a 0° e a +4°C durante l'insilamento.

È stato effettuato un confronto fra la composizione volatile delle flemme di grappa ottenute dalla distillazione della vinaccia stoccata per 3 mesi nei silos di acciaio e nel tunnel di materiale plastico. Si è stimato che con il vinello

- Key words: distillation, ensilage, grape pomace, refrigeration, volatile compounds -

ported. Refrigeration at 0°C removed about 37% of the heat released in alcoholic fermentation, thus reducing the average grape pomace temperature increase from 11° to 6.5°C, while refrigeration at +4°C removed about 30% of the heat released reducing the average grape pomace temperature increase from 11° to 7.7°C. The cooling practice applied to the steel containers as well as storage in the plastic container resulted in lower concentrations of methanol, higher alcohols and short-chain ethyl esters in comparison to the volatile composition of grappa low wines from pomace stored in steel containers without refrigeration.

a 0°C, circa il 37% del calore di fermentazione è stato rimosso e che ciò ha ridotto da 11° a 6.5°C l'incremento della temperatura media della vinaccia. Invece a +4°C, circa il 30% del calore di fermentazione risulta rimosso, con una riduzione dell'incremento della temperatura media della vinaccia da 11° a 7.7°C. La composizione aromatica della flemma da vinacce refrigerate, analogamente a quella delle vinacce insilate in tunnel plastico, ha evidenziato la riduzione di concentrazione di metanolo, alcoli superiori ed esteri etilici a corta catena quando comparata alla flemma da vinaccia non refrigerata conservata in silos d'acciaio.

INTRODUCTION

Grappa is a spirit produced in Italy by directly distilling grape pomace (EC, 1989). Both unfermented grape pomace and fermented pomace (at about 2 to 4% v/v ethanol) are stored before distillation in industrial plants. Fermented grape pomace could be distilled immediately, but it is often stored to extend the use of industrial scale distilleries. Problems of setting up and managing both the distillation plant and the distillery wastewater plant give rise to this practice.

Various ensilaging systems are used for grape pomace storage. Traditionally, grape pomace is stored in large concrete earthen tanks, filled with 50-100 tons of pomace. Concrete rooms are also used. Usually they (20x6x5 m) are closed on three sides and the fourth side is opened to allow filling with 400-500 tons of grape pomace both by trucks and mechanical shovels.

At the end of the ensilaging operation, the pomace is covered with plastic sheets to limit the entrance of oxygen and the development of aerobic bacteria such as acetic bacteria (VERSINI and ODELLO, 1991). The fermentation temperature of grape pomace can often exceed 30°C, affecting yeast growth and metabolism. Besides stuck fermentation that can lead to the reduction of ethanol recovery, compounds (ethyl esters and volatile acids) detracting from overall grappa quality can be produced in appreciable amounts as a result of bacterial spoilage (DE ROSA and CASTAGNER, 1994). The poor heat transfer which occurs in grape pomace during ensilaging is due to various factors such as the high content of cellulose in the grape pomace and the material and size of the containers used (DA PORTO, 1998). During storage, bacteria or mould contamination of the pomace can take place, leading to off-flavour production in the resulting spirit (USSEGLIO-TOMASSET, 1971; WILLIAMS and STRAUSS, 1978).

In order to improve the quality of Grappa, the pomace may undergo several treatments such as acidification to lower the pH (VERSINI and INAMA, 1981), compression to reduce the presence of oxygen, addition of potassium metabisulfite usually just at the surface towards the outside, mostly to exploit the antimicrobial activity of sulfur dioxide.

Presently new systems of pomace ensilaging are used such as vertical closed stainless steel containers (capacity 200-500 tons of pomace) and salami-like containers made of plastic (capacity 300 tons) (DA PORTO, 2002).

The aim of this study was to perform an energy balance on vertical closed stainless steel containers filled with grape pomace cooled by dripping wine at 0° and +4°C and to compare the volatile composition of the grappa low-wines obtained by distilling pomace from different storage conditions.

Although several studies were conducted by USSEGLIO-TOMASSET (1971; 1985), VERSINI and MARGHERI (1978; 1979), VERSINI and INAMA (1981) and VERSINI and ODELLO (1991) with grappa and by ORRIOLS-FERNANDEZ *et al.* (1991), SOUFLEROS (1991), SILVA *et al.* (1995; 1996; 2000), SILVA and MAICATA (1998; 1999), CORTÉS (2002) and DIEGUEZ (2001) with similar spirits such as orujo, tsipouro and bagaceiras, there are no reports in the literature about the use of refrigeration for grape pomace storage on an industrial scale.

MATERIALS AND METHODS

Material

Prosecco grape pomace at 7°Brix was obtained from a distillery in Veneto (Italy), working continuously. The grape pomace was distilled during the winter of 2000-2001 after storing for 90 days, from October to December.

Storage conditions

Three vertical closed stainless steel tanks (diameter 8 m, height 10 m; capacity 500 m^3) externally cooled by water flow were used. They were equipped with some automatic devices for filling and emptying the pomace and each was filled with *ca.* 400 tons of raw material. Pomace was stored for 90 days, the average ambient temperature being 14.2°C in October, 9.6°C in November, 5.8°C in December. The grape pomace was stored as follows:

a) under refrigerated conditions (R): before filling the tank, an aqueous solution of potassium metabisulfite (15 g L^{-1}) at the rate of 187 g per ton was spread on the grape pomace. Then the dripping wine resulting from compressing the pomace, was cooled by a refrigeration system to 0°C and pumped to the top of the tank onto the pomace. The pump-over operation was carried out in two steps: 2 days after filling the tank and after 15 days. A volume of 300 hL of cooled dripping wine was pumped. Each pump-over operation lasted 24 hours. Using temperature probes put into various parts of the mass, the average temperature of the pomace at the beginning of the storage period was found to be 28°-32°C. The temperature of the dripping wine coming out of the tank was 19°-20°C at the end of the first pumping and 15°-16°C at the end of the second one;

b) under partially refrigerated conditions (PR): potassium metabisulfite ($9 \cdot 10^{-2}$ g L^{-1}) was added to the wine dripping from the pomace; the dripping wine was cooled to +4°C and pumped to the top of the tank. The pump-over operation and its duration were the same as reported for the refrigerated conditions (R). The average temperature of the pomace at the beginning of the storage period was 28°-32°C. The temperature of the dripping wine coming out of the tank was 22°-24°C at the end of the first pumping and 18°-19°C at the end of the second one;

c) without refrigeration conditions (WR): potassium metabisulfite ($9 \cdot 10^{-2}$ g L^{-1}) was added to the dripping wine; there was neither cooling nor pumping over;

d) using a salami-like container (S): in these conditions the grape pomace was pressed using a self-advancing de-

vice in a plastic tunnel (60x2.5 m diameter) filled with ca. 300 tons of pomace. An aqueous solution of potassium metabisulfite (15 g L^{-1}) at the rate of 187 g per ton was spread on the grape pomace before ensilage.

Although VERSINI and INAMA (1981) suggested acidifying the grape pomace before storage, this practice was impossible due to the processing of industrial amounts of raw material. It is hazardous to handle such quantities of acid solutions, the mixing technique is less effective in obtaining a homogenous treatment of the pomace, there are serious problems both of corroding the distillery equipment and the plant and of disposing of the waste liquids.

After the storage period in the previously reported conditions the grape pomace was continuously distilled. The entire mass from each storage trial (i.e. 400 tons of pomace stored and sprayed with the dripping wine cooled at 0°C and so on) was completely and separately introduced into the de-alcoholizing apparatus to obtain the grappa low-wine samples. The samples can be considered representative of the material obtained from steam-stripped heap-fermented pomace treated in various ways because each of them was taken from a tank containing all the distilled volume coming from that specific storage trial.

Energy balance on the steel containers

The most important heat fluxes were estimated, as well as formulating an energy balance for the pomace stored in the refrigerated conditions and in the partially refrigerated conditions. Heat released due to fermentation was estimated, and the consequent pomace temperature increase was calculated, comparing the (R), (PR) and (WR) storage conditions.

Chemical analyses

The ethanol content in the various grappa low-wines was determined by hydrostatic balance (Gibertini, Milano, Italy), measuring the density after distillation (AOAC, 1980).

GC-FID analysis: a Carlo Erba 8000 Top series gas chromatograph (CE Instruments, Milan, Italy) equipped with a Supelcowax 10 (Supelco, Milan, Italy) (30 m x 0.32 mm i.d., film thickness 0.3 μm) fused silica capillary column was used. The injection and detector temperature was 250°C; oven temperature program: 8 min at 60°C; 8°C min^{-1} to 170°C and 13°C min^{-1} to 240°C; carrier gas was: helium at 1.2 mL min^{-1}, with a split ratio of 1:20. Internal standard: n-dodecanol (3.94 g/L in ethanol; 100 μL in 5.0 mL sample). Peaks were identified by RT with a reference mixture of volatile compounds. Quantification is expressed as mg/100 mL a.a. (anhydrous alcohol).

The repeatability of the HRGC analysis was tested using a grappa low-wine sample analysed six times. The coefficients of variation were less than 8% (DA PORTO, 2002).

RESULTS AND DISCUSSION

This study which was carried out on an industrial scale is very difficult due to both the large amount of raw material and the availability of equipment required. It is not common, but it produces interesting results, which are usually very appreciated by the distillers. Indeed, working in this way, the results obtained take into account several variables that could be hardly anticipated or simulated in the laboratory.

For the calculation of the energy balance, the thermophysical properties of the pomace, estimated on the basis of its composition, were assumed to be: specific heat c_p = 3,350 J/(kg K) (DE

ROSA and CASTAGNER, 1994), thermal conductivity k = 0.245 W/(m K).

Estimation of the heat released during fermentation was performed as follows. From the change in the ethanol concentrations, an amount of 70 g/kg of sugar in the pomace was calculated to have fermented. On the basis of 550,000 J/kg heat generation for sugar fermentation (WILLIAMS, 1982), the total amount of heat released was calculated to be $15 \cdot 10^9$ J. In adiabatic conditions, such amount of heat could increase the average product (pomace and dripping wine) temperature by about 11°C. This value could be acceptable if the initial temperature was sufficiently low, and if the temperature inside the pomace was uniform. However, due to the conductive heat resistance of the pomace, the temperature distribution inside the storage volume is not uniform, and the maximum temperature is reached at the centre of the container.

An estimation of the increase in pomace temperature on the symmetry axis of the cylindrical container was then performed. Assuming that fermentation is usually completed in seven days, the average heat flux produced was 24,800 W, which is about 50 W/m^3 internal heat generation in the case of 500 m^3 tanks. The containers used in the storage tests were cooled by cold water flowing on the external vertical surface, which could have easily removed the entire fermentation heat flux, because of the large external surface area (250 m^2). It can be easily calculated that this task could be accomplished with a 10°C temperature difference between the pomace and the water and an overall heat transfer coefficient of 10 W/m^{2}K, quite easy to obtain with liquid flows. Unfortunately the low heat conductivity of grape pomace delays heat from reaching the most external layers of the pomace. It can therefore be assumed that water cooling was surely able to maintain the initial storage temperature of the product at least on the external layer. Due to the short duration of fermentation and to the cooling of the dripping wine, the heating process was presumably not able to reach temperature equilibrium, and took place in transient conditions. This prevents the use of the classical theory of steady state internal heat generation to evaluate the maximum temperature of the mass. Nevertheless, a typical parabolic temperature profile can be assumed for the pomace inside the container. In this case, and assuming adiabatic conditions, the temperature increase at the centre of the container was estimated to be 33°C, with respect to the initial temperature. Therefore, the pomace temperature at the centre of the container could easily exceed 50°C. From these calculations it is clear that cooling of the pomace is needed, with devices other than water flowing on the external surface of the container.

The use of cooled dripping wine is an efficient way to cool pomace, because wine is spread over the top of the pomace, and percolates through it with an efficient heat exchange. It is very difficult to measure the temperature after cooling with cold dripping wine because of the already mentioned uncertainties in the actual temperature profile and because of the random cold wine distribution. It is also difficult to correlate pomace temperature with the temperature of the dripping wine coming out from the tank. However, knowing the energy balance of the container, some interesting facts can be considered.

It was calculated that the total amount of heat released during fermentation is about $15 \cdot 10^9$ J. Taking into consideration the refrigerated (R) storage conditions, during the first pumping, 30,000 L of wine were cooled from an average temperature of 25° to 0°C. Therefore, from the specific heat of wine c_w = 4100 J/(kg °C), about $3 \cdot 10^9$ J were removed from the pomace through cooling with the wine. From the specific heat of pom-

ace c_p = 3,350 J/(kg °C), in adiabatic conditions it can be estimated that the average pomace temperature decrease was 2.5°C. During the second pumping, the wine was cooled from an average temperature of 20° to 0°C, leading to an average pomace temperature decrease of 2°C. Therefore, about 37% of the fermentation heat was removed by cooling dripping wine, and increase in the average pomace temperature in adiabatic conditions was reduced from the previous value of 11° to 6.5°C. The mechanical work for pumping the wine was neglected in these calculations, being that it is a minor part (about 0.5%) of the whole enthalpy change.

Taking into consideration the partially refrigerated (PR) storage conditions, with the same calculations it can be estimated that about 30% of the fermentation heat was removed by cooling dripping wine, and the average pomace temperature increase was reduced from 11° to 7.7°C.

The volatile composition of the low-wines obtained from the grape pomace is reported in Table 1. Data are for pomace stored in stainless steel containers without cooling of dripping wine (WR), with two pump-over operations at 0°C (R) and +4°C (PR), and for the plastic container (S). Being that the analytical error was about 8%, only variations in volatile compounds with a percentage difference higher than 8% can be considered significant (DA PORTO, 2002).

At the end of the storage period the ethanol content of pomace in (WR), (R), (PR) and (S) was 3.8% v/w, 3.9% v/w, 3.7% v/w and 3.7% v/w, respectively, whilst after the de-alcoholizing operation, sample WR had the lowest ethanol level (18.8% vol) and sample S the highest (24.5% vol). These results can be ascribed to the de-alcholizing process. In fact, with constant working conditions of the apparatus such as feed, initial feed temperature, steam pressure and waste composition, the heating of pomace should be applied homogeneously so as to promote the highest ethanol recovery. Ethanol losses are reduced only if pomace is placed in a regular and soft heap on the plate with holes of the de-alcholizing apparatus, because preferential ways to steam downflow are avoided.

The methanol content in the low-wines (S) and (R) decreased 75 and 81%, respectively, in comparison to the methanol concentration of the low-wine (WR). Reduction of methanol in sample PR was only 26%.

According to the data reported in a previous paper (DA PORTO, 2002) storing pomace in the plastic container resulted in a decrease in the level of methanol. Since prolonged contact time of grape skins with pectinesterase produces large amounts of methanol, it may be presumed that the environmental conditions created by the plastic tunnel are less effective for pectin methyl esterase activity than those created by the vertical closed stainless steel tank. The high percentage decrease of methanol under refrigerated conditions (R) might be due to the effect of temperature decrease on the pectin methyl esterase activity (KULP, 1975). There may also have been a skin washing effect by the dripping wine resulting in removal of the enzyme. This will be investigated on the lab scale in future work.

Obtaining low-wines with a low content of methanol is very advantageous, since in continuous distillation plants methanol is usually separated by the demethylization column with the loss of other volatile components. Otherwise, the same demethylization procedure might be limited to only a part of the low-wines then blended with untreated spirits in a certain ratio according to the desired methanol content. This could reduce the risk of excessive loss of volatile compounds of organoleptic interest.

Higher alcohols were in much lower amounts in the low-wines obtained cooling the pomace in the steel containers

Table 1 - Comparison of marc low-wines obtained using different methods and conditions of marc ensilaging (concentration of volatile compounds in mg/100 mL a.a.).

	Steel container			Salami-like container
	WR	PR (+4°C)	R (0°C)	S
Ethanol, % vol	18.8	23.3	22.8	24.5
Acetaldehyde	36.95	23.71	11.05	9.30
Methanol	701.69	517.82	127.61	173.27
Variation in methanol concentration with respect to WR, %		-26.20	-81.81	-75.31
2-Butanol	42.51	5.18	17.36	31.29
n-Propanol	56.74	43.03	17.11	25.78
n-Butanol	n.d.	n.d.	0.32	n.d.
Isoamyl alcohol, sum	173.42	133.69	50.64	56.92
2-Methylpentanol	n.d.	n.d.	1.14	n.d.
1-Hexanol	14.25	15.60	4.61	5.25
Cis-3-Hexen-1-ol	1.09	0.88	0.25	0.29
1-Nonanol	n.d.	n.d.	0.41	n.d.
Higher alcohols, sum	288.01	198.39	181.72	119.52
Variation in higher alcohols concentration with respect to WR, %		-31.12	-36.90	-58.50
Linalool	6.78	12.52	16.32	18.57
α-Terpineol	0.61	0.23	0.32	0.54
β-Citronellol	n.d.	n.d.	0.10	n.d.
Terpenes, sum	7.39	12.75	16.74	19.11
Variation in terpenes concentration with respect to WR, %		72.53	126.50	158.58
Methyl acetate	9.32	4.20	1.25	3.54
Ethyl acetate	339.13	240.13	97.45	145.00
Ethyl lactate	86.82	61.84	22.44	20.27
Ethyl caprylate	n.d.	n.d.	0.10	n.d.
Ethyl caprate	1.29	0.95	n.d.	0.47
Ethyl laurate + n.i.	3.27	0.75	1.74	0.92
Ethyl myristate	n.d.	n.d.	0.49	n.d.
Ethyl palmitate	1.24	2.60	n.d.	n.d.
Ethyl palmitoleate	1.94	1.00	0.73	0.44
Ehyl oleate	7.33	n.d.	0.25	1.66
Ethyl linoleate	5.99	0.72	0.97	n.d.
Diethyl succinate + n.i.	2.00	1.15	0.70	0.69
Esters, sum	458.33	313.34	126.12	172.98
Variation in esters concentration with respect to WR, %		-31.63	-72.48	-62.26

n.i. = not identified; n.d. = not detected;
WR = without refrigeration;
PR = partially refrigerated;
R = refrigerated.

(R; PR) and storing it in the salami-like container (S), with respect to the amount (288 mg/100 mL a.a) obtained in (WR) conditions. Since high temperature and aeration are factors which tend to stimulate the production of higher alcohols (BERRY, 1995), it may be presumed that cooling, in the case of steel containers, and the poor aeration in the plastic tunnel decreased the concentration of higher alcohols.

The terpenes were mainly represented by linalool. In the low-wines obtained from pomace stored in (R) and (PR) conditions, the terpene concentration was, respectively, 73 and 127% higher than the low-wine stored in (WR) conditions. Probably, the pump-over operation with the dripping wine gave the liquid better access to the pomace at the top, promoting extraction of the impact flavour compounds from the skins. In agreement with data reported previosly (DA PORTO, 2002) the low-wine obtained from the pomace stored in the salami like container had the highest content of terpenes.

Since the major contribution to total esters was given by ethyl acetate and ethyl lactate, it could be presumed that pomace is preserved from bacterial spoilage when pomace is cooled by the dripping wine at 0°C (-72.48%) rather than at +4°C (-31.63%) or when it is stored in the plastic container (-62.26%).

CONCLUSIONS

In tests performed on grape pomace stored in plastic salami-like containers and in cylindrical stainless steel tanks each containing 400 tons of pomace, traditional cooling of tanks was shown to be inadequate. By cooling two times with 300 hL of dripping wine at 0° and +4°C, about 37 and 30%, respectively, of heat fermentation was removed.

The composition of the low wines benefited from the cooling practice as well as from storage in the plastic containers. Therefore, they seem to be suitable methods to preserve grape pomace when it is stored for a long period, which is the usual practice in large distilleries. Further investigations about the heat released from grape pomace during storage on an industrial scale are in progress in order to improve the cooling practice. Application of the cooling practice could be advantageous from an economic point of view. Whilst a refrigeration system is always available in distilleries being that it is used throughout the sequence of spiritmaking operations, the disposable plastic tunnel and its disposal are very expensive.

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SHORT COMMUNICATION

EFFECT OF INDUSTRIAL MICROWAVE TREATMENT ON THE ANTIOXIDANT ACTIVITY OF HERBS AND SPICES

EFFETTO DEL TRATTAMENTO INDUSTRIALE A MICROONDE SULLA CAPACITÀ ANTIOSSIDANTE DI ERBE AROMATICHE E SPEZIE

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ABSTRACT

The effect of the industrial treatment with microwaves on the antioxidant activity of herbs and spices was evaluated. Samples of black pepper (*Piper nigrum* L.), oregano (*Origanum vulgare* L.), basil (*Ocimum basilicum* L.) and sage (*Salvia officinalis* L.) obtained before and after the microwave treatment were extracted with an acetone-water mixture (70:30). The extracts were analyzed for their phenolic content. The antioxidant activity was determined using the DPPH• (2,2-diphenyl-1-picrylhydrazyl)

RIASSUNTO

L'attività antiossidante di estratti di alcune erbe aromatiche e spezie è stata determinata per valutare l'effetto di un trattamento di sanificazione a microonde a cui vengono sottoposte nel corso della lavorazione industriale. Sono stati analizzati campioni di pepe nero (*Piper Nigrum* L.), origano (*Origanum Vulgare* L.), basilico (*Ocimum Basilicum* L.) e salvia (*Salvia Officinalis* L.) prelevati prima e dopo il trattamento di sanificazione. L'estratto in acetone/acqua 70% ottenuto da ciascun campio-

- Key words: Antioxidant activity, herbs, microwaves, phenolic substances, prooxidant capacity, spices -

and deoxyribose methods and the prooxidant activity was determined by evaluating the reducing power and the iron chelating capacity. The treatment did not modify the antioxidant properties of these herbs and spices as the majority of the differences between treated and non treated samples were not statistically significant.

ne è stato analizzato per valutare innanzitutto il contenuto fenoli totali mediante il noto metodo di Folin-Ciocalteu, sugli stessi estratti è stata quindi valutata l'attività antiossidante mediante i metodi del DPPH• (2,2-difenil-1-picrilidrazile) e del desossiribosio, e la capacità proossidante mediante la valutazione delle capacità riducente e chelante. Il trattamento a microonde sembra solo marginalmente influenzare l'attività antiossidante, infatti la maggior parte delle differenze riscontrate sono risultate statisticamente non significative.

INTRODUCTION

Natural antioxidants present in foods have attracted considerable interest because of their presumed safety and potential nutritional and therapeutic effects. The role of free radicals and active oxygen in the pathogenesis of certain human diseases including cancer, ageing and atherosclerosis is becoming increasingly recognised. Therefore, much attention has been focused on the use of antioxidants to inhibit lipid peroxidation or to protect from damage by free radicals. Food industries are interested in the use of antioxidants and food rich in antioxidant compounds as food preservatives or as dietetic supplements. Concerns about the safety of synthetic antioxidants, together with consumer preference for natural products, has resulted in increased research on natural antioxidants, and has led to the evaluation of antioxidants in a number of plant sources, in particular aromatic herbs and spices. Since ancient times, in fact, these substances have been added to different types of food to increase the shelf life and to improve flavour. In the food industry spices are used either as raw materials or extracts (MADSEN and BERTELSEN, 1995).

However, spices and dried herbs used for food preservation and flavoring are particularly exposed to microbiological contamination, and are often a potential source of microbial contamination to the food to which they are added. In fact, their level of contamination can be, in general, as high as 10^5-10^8 microorganisms per gram (FARAG *et al.*, 1995). Contaminating agents can be bacteria and moulds, with the consequent risk of contamination from mycotoxins, particularly if the food is preserved in humid and poorly aerated rooms, as well as insects and other small animals. Salmonella can be one of the main pathogenic bacteria (EMAM *et al.*, 1995). Therefore companies concerned with the processing and marketing of spices or with their use as sources of flavour and natural antioxidants must eliminate bacteria and any other forms of life that may contaminate spices and aromatic herbs.

One of the most common sanitizing treatments is the use of gamma rays which act on bacterial DNA, reducing viability, but remarkably altering the flavour. With directives 1999/2 and 1999/

3, the EUROPEAN UNION (1999 a;b) established that "...in the case of products intended for the ultimate consumer and mass caterers... the words 'irradiated' or 'treated with ionising radiation' shall appear on the label...". Another effective method could be the use of ethylene oxide, which,however, is prohibited for herbs and spices by the community regulations due to its carcinogenic and genotoxic properties (EUROPEAN UNION 1999b; THIER and BOLT, 2000; WELLER *et al.,* 1999). It is also possible to use pasteurization or sterilization by heating spices at appropriate temperatures. It is well known that the levels of many food antioxidants can be significantly reduced as a consequence of heat treatment. Probably because heating causes oil-based antioxidant compounds to evaporate, or, being phenolic in nature, to react with other compounds in the tissue. However, processing is not always responsible for depleting the antioxidant properties of foods. In some cases, it can induce the formation of compounds with novel antioxidant properties, which can maintain or even enhance the overall antioxidant potential of foods. (LINDLEY, 1998; MANZOCCO *et al.,* 1998; NICOLI *et al.,* 1997; 1999)

Recently the use of microwaves for the sanitization of spices has acquired increasing importance. The effectiveness of the microwave treatment for spices depends on the high penetrating power of radiation in these products, which have a low water content, usually 80 to 150 g/kg. This also causes moderate and uniform heating, while the higher water content of contaminants leads to higher, lethal temperatures. (EMAM *et al.,* 1995).

In two previous papers (PLESSI *et al.*, 2002; BERTELLI *et al.*, 2003) the effect of industrial microwave treatment on the volatile compounds of different herbs and spices was reported. In the present study the effect of the same treatment on the antioxidant properties of the extracts obtained from black pepper, oregano, basil and sage was evaluated.

MATERIALS AND METHODS

Samples

The samples were supplied by Cannamela Ozzano dell'Emilia (Bologna – Italy) who have developed a microwave treatment method for the sanitization of their products. This new process, improved by Cannamela consists in the continuous supply of spices and herbs to the pasteurization chamber where microwave oscillators sanitize the material with the maximum control of all relevant physical parameters. The treatment conditions are between 30 and 70 watt/kg of spices for 15 min depending on the kind of material.

Analyses were performed on 10 samples of black pepper (*Piper nigrum* L.), 8 samples of oregano (*Origanum vulgare* L.), 4 samples of basil (*Ocimum basilicum* L.) and 3 samples of sage (*Salvia officinalis* L.). For each herb/spice the company supplied samples taken before and after the microwave treatment. The same analyses were performed on treated (t) and non treated (nt) samples.

Extraction

Two grams of each sample (ground) were extracted with 20 mL of an acetone/water mixture (70:30) for 5 min. The extraction was conducted using an IKA-Ultra-turrax T25 homogenizer (Ika-Werke GmbH & Co. KG, Staufen, Germany). The extraction was repeated six times and all extracts were combined and evaporated under vacuum at low temperature (<40°C) until all the acetone was removed. The aqueous residue was transferred to a 50 mL volumetric flask and diluted to 50 mL with ultrapure water (Milli-Q plus 185 Reagent water system, Millipore Corp., Milford, MA,

U.S.A.). This solution was used for all the determinations.

Determination of total phenolics

The amount of total phenolics in treated and non-treated samples was determined according to the Folin-Ciocalteu procedure (SINGLETON and ROSSI, 1965) and data are expressed as mg/g of gallic acid equivalents.

Antioxidant activity

a) DPPH• free radical-scavenging method

The antioxidant activity of the various spice extracts was measured in terms of hydrogen donating or radical-scavenging ability using the stable radical DPPH• (BRAND-WILLIAMS *et al.*, 1995). To 300 μL of 1 mM DPPH• (2,2-diphenyl-1-picrylhydrazyl) in methanol (prepared daily), placed in a spectrophotometer cuvette, different volumes of each extract (20-350 μL) were added and the total volume was diluted to 3 mL with methanol. After 15 min at 25°C, the absorbance at 517 nm was measured. The percent of the stable free radical quenched by the different volumes of extracts was calculated according to the formula:

$$\% \text{ radical quenched} = [(A_{c(0)} - A_{a(15)})/ /A_{c(0)}]*100$$

Where $A_{c(0)}$ is the absorbance of the control at t=0 and $A_{a(15)}$ is the absorbance in the presence of the extracts at 15 min.

Plotting the % radical quenched versus the extract volumes, it was possible to extrapolate the amount of extract that quenched 50% of the DPPH• radical (EC_{50}). This value was used as the anti-radical efficiency parameter.

b) Deoxyribose assay

The deoxyribose method for determining the rate of reaction of the hydroxyl radical with the antioxidants present in the spice extracts was performed using a modification of the method described by HALLIWELL *et al.* (1987).

The sugar, 2-deoxyribose, is degraded on exposure to hydroxyl radicals generated by a mixture of Fe^{3+}, ascorbate, and H_2O_2 in the presence of EDTA at pH 7.4. If the resulting mixture is heated under acidic conditions, malonaldehyde is formed and may be detected by its ability to react with thiobarbituric acid to form a pink chromogen.

Different volumes of spice extracts (5-160 μL) were put into spectrophotometric cuvettes, diluted to 1 mL with KH_2PO_4 - KOH buffer (10 mM pH 7.4), then 1,380 μL of 2-deoxyribose (2.5 μM) in buffer, 200 μL of $FeCl_3$ (100 μM) premixed with 200 μL of EDTA (100 μM) were added. After 10 min the reaction was started by adding 20 μL of H_2O_2 (1 mM) and 200 μL of 100 μM ascorbic acid. The samples were then held in a water bath at 37°C for 1 hour. The color was developed by adding 1 mL of a 1% solution of thiobarbituric acid and 1 mL of 2.8% trichloroacetic acid solution to the samples and heating at 80°C for 30 min. The absorbance at 532 nm was then measured. The rate constant of the reaction between antioxidants and •OH ($K_S*10^9\ M^{-1}s^{-1}$) was calculated as reported in HALLIWELL *et al.* (1987) and was used as the antioxidant efficiency parameter.

Determination of the reducing power

The reducing power of the extracts was determined according to the method reported by OYAIZU (1986). One millilitre of each extract was added to 2.5 mL of sodium phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of 1% potassium ferricyanide solution. The reaction mixture was incubated at 50°C for 20 min, then 2.5 mL of 10% trichloroacetic acid solution

was added and the mixture was centrifuged at 3,000 rpm for 10 min, 5 mL of supernatant were diluted with 5 mL of ultrapure water and after adding 1 mL of 0.1% ferric chloride solution, the absorbance at 700 nm was recorded. A higher absorbance indicated a stronger reducing power and a high potential prooxidant capacity. The absorbance obtained for various extracts was used directly as the prooxidant capacity parameter.

Fe^{2+} Chelating activity

The Fe^{2+} chelating activity of the extracts was measured by the method of DINIS *et al.* (1994). To different volumes of extracts (1-3 mL), 200 μL of ferrous chloride (2 mM) solution and 200 μL of ferrozine (5 mM) were added, and the volume was made up to 3.5 mL with ultrapure water. After 10 min at 25°C the absorbance at 562 nm was measured. A lower level of absorbance indicated stronger chelating activity. The slope of the line obtained, plotting the absorbance versus volumes of extracts, was used as the parameter. The presence of chelating activity indicates a low prooxidant capacity.

Statistical analysis

Statistical analysis was performed using SPSS 10.0 for Windows. To evaluate the significance of the differences between treated and non treated samples and to eliminate the influence of the variations between different batches of spices, the paired T-test was used. The P-value (level of significance) was selected at 0.05. Principal component analysis (PCA) allows a large portion of total variance of the data to be expressed with a smaller number of variables, which can be used to graphically represent the population of samples and to identify the most significant original factors.

RESULTS AND CONCLUSIONS

The results are reported in Table 1. The herbs/spice with the higher phenolic content were oregano followed by sage, basil and black pepper. The total phenolic content in pepper, oregano and basil increased with microwave treatment, while in the case of sage, it decreased. Analyzing the results by paired T-test the differences were statistically significant for oregano and sage.

Except for oregano, the microwave treatment caused a reduction of EC 50 values and increased the antioxidant activity of the herb extracts, as determined by the DPPH• method. However, only the difference for basil was statistically significant.

As regards the results of the deoxyribose method, the same results as in the DPPH• test were observed. In fact, Ks values increased (higher antioxidant capacity) for black pepper, basil and sage, but not for oregano. The only significant difference was for basil.

The reducing power improved for all the extracts with microwave treatment, but none of the differences were statistically significant. In the case of chelating activity, black pepper and oregano showed an increase in slope, and the slopes of basil and sage decreased. None of the differences were statistically significant.

The results of PCA performed on all data are reported graphically in Fig. 1. Using this method, the samples were sufficiently grouped into four principal clusters. Only in the case of basil were the treated and non-treated samples clearly separated. The first two principal components explained 67% of the total variance. The first component, which explained 43% of the total variance, is related to the total phenolic content and DPPH•, while the second is particularly related to the chelating activity.

The microwave treatment of these herbs and spice only marginally influenced the

Table 1 - Results obtained for microwave treated and non-treated samples of black pepper, oregano, and sage basil.

Parameter	Black Pepper (n=10)		Oregano (n=8)		Basil (n=4)		Sage (n=3)	
	non treated	treated	non treated	treated	non treated	treated	Non treated	treated
Total phenolic content (gallic acid equivalents, mg/g)	2.648±0.76	3.300±0.44	112.1±39.9	135.3±43.8	40.05±8.82	44.68±13.3	52.12±5.62	48.29±5.59
	^{AA}P=0.065		^{A}**P=0.0005**		^{A}P=0.416		^{A}**P=0.010**	
DPPH• method ($EC_{50,}$ µL)	97.43±58.8	71.26±26.56	21.46±8.15	34.22±17.5	119.7±1.72	80.52±8.44	86.75±29	56.45±8.67
	^{A}P=0.222		^{A}P=0.061		^{A}**P=0.0014**		^{A}P=0.203	
Deoxyribose method (K_s * 10^9 $M^{-1}s^{-1}$)	3.256±1.99	3.720±2.74	38.51±30.8	21.28±13.6	33.74±11.4	81.66 ±4.06	11.07±4.74	20.12±22.8
	^{A}P=0.712		^{A}P=0.448		^{A}**P=0.007**		^{A}P=0.482	
Reducing power (A_{700})	0.503±0.11	0.543±0.1	0.782±0.28	0.792±0.25	0.420±0.14	0.518±0.11	0.659±0.02	0.868±0.17
	^{A}P=0.164		^{A}P=0.949		^{A}P=0.463		^{A}P=0.144	
Chelating activity (slope of regression)	-0.149±0.09	-0.098±0.07	-0.176±0.05	-0.144±0.05	-0.192±0.04	-0.216±0.02	-0.190±0.01	-0.212±0.04
	^{A}P=0.236		^{A}P=0.295		^{A}P=0.124		^{A}P=0.450	

Values = Mean±standard deviation; ^{A}= P values for paired T-test; Values in bold = statistically significant.

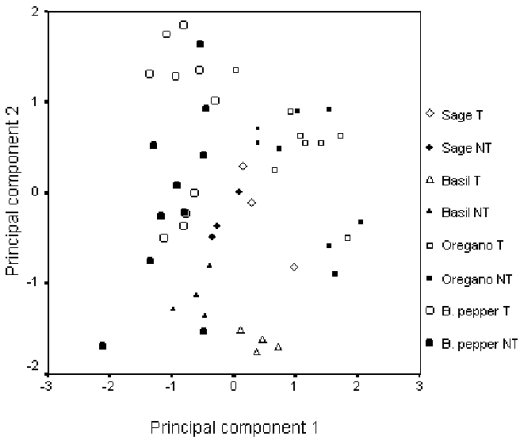


Fig. 1 - Plot of the first two principal components obtained from multivariate statistical analysis of the results.

antioxidant activity. The multivariate statistical analysis showed, in fact, that the slight differences found did not produce a real differentiation between treated and non treated samples except for basil. Results of the DPPH• and deoxyribose tests showed an increase in antioxidant activity with microwave treatment.

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SHORT COMMUNICATION

TRANSFER OF SELECTED YEASTS TO OIL THROUGH OLIVE INOCULATION

TRASFERIMENTO DI LIEVITI SELEZIONATI NELL'OLIO MEDIANTE L'INOCULAZIONE DELLE OLIVE

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ABSTRACT

This study was carried out to evaluate the possibility of increasing the presence of selected yeasts in freshly produced extra-virgin olive oil by inoculating olives prior to processing. New types of yeasts classified as *Williopsis californica* and *Candida boidinii* were found. A β-glucosidase producing strain of *W. californica*, used as starter in the olive inoculation trials, was able to increase the amount of oleuropeinolytic yeasts in olive oil during the entire sedimentation period. The increase in the

RIASSUNTO

È stato condotto uno studio sulla possibilità di arricchire l'olio extra vergine di oliva con lieviti selezionati inoculando le olive prima della loro trasformazione. Nuovi tipi di lieviti classificati come *Williopsis californica* e *Candida boidinii* sono stati isolati. Un ceppo di *W. californica*, capace di produrre una β-glucosidasi attiva sull'oleuropeina, è stato impiegato successivamente come starter per inoculare le olive prima della loro trasformazione, eseguita impiegando un impianto moderno di tipo

- Key words: inoculation, olives, olive oil, yeast -

yeasts in the olive oil compared to the un-inoculated control was 20 and 30%, respectively, for the continuous and discontinuous system used for processing the olives. Inoculation of the olives with selected yeasts was demonstrated to be a valid technique to enrich the oil with useful microorganisms without directly manipulating the product.

continuo ed uno tradizionale di tipo discontinuo. L'incremento dei lieviti nell'olio di oliva rispetto al controllo non inoculato, è risultato rispettivamente del 20 e 30%, a seconda se le olive venivano trasformate con il sistema continuo oppure con quello discontinuo. L'inoculazione delle olive con lieviti selezionati è risultata una tecnologia valida per arricchire l'olio con microrganismi utili, senza manipolare direttamente il prodotto.

INTRODUCTION

Olives can be used for direct consumption as table olives or processed to extract the oil. Freshly produced virgin olive oil is normally cloudy or slightly opaque due to the presence of suspended solid particles and micro-droplets of vegetation water in quantities which vary according to the variety, the ripeness of the fruit and the oil processing system used (BRUNI *et al.,* 1994). During storage, the suspended material sediments and the olive oil acquires greater clarity and new organoleptic properties.

Some authors have reported that the improvement in the chemical and organoleptic properties of the oil during the extraction and storage processes can be attributed to the activity of some enzymes originating in the fruit. In fact oleuropein, responsible for the highly bitter taste of newly produced olive oil, is hydrolysed by the endogenous β-glucosidase enzyme found in the fruit (ANGEROSA *et al.,* 1995; BOTÌA *et al.*, 2001; MONTEDORO *et al.,* 1993).

Nevertheless, it has been recently demonstrated that improvement in the quality of the olive oil can also be attributed to the processes of microorganisms trapped on the solid particles and in the micro-droplets of vegetation water suspended in the oil. CIAFARDINI and ZULLO (2002a) demonstrated, for the first time, that in freshly produced olive oil there is a rich micro-flora consisting principally of yeasts that survived in the oil for several months. The microorganisms present in olive oil can influence the physico-chemical and organoleptic properties of the oil through the production of enzymes. Amongst the microorganisms isolated were several strains of yeasts classifed as *Saccharomyces cerevisiae* and *Candida wickerhamii* (CIAFARDINI and ZULLO, 2002a). These species of yeasts re-inoculated in sterile olive oil were able to hydrolyse oleuropein by enzymatic means through the production of β-glucosidase, thereby modifying the organoleptic characteristics of the oil (CIAFARDINI and ZULLO, 2002a).

The presence in the oil of particular types of natural yeasts or the addition of selected yeasts originating from the same habitat are very important from a technological point of view since they allow an improvement in the quality and production of oils with specific chemical and organoleptic characteristics. However, knowledge on this subject is very limited and research on the role of selected yeasts for the production of oils of high organoleptic and nutritional quality are as yet non existent. Direct inoculation and mixing of a starter in the oil mass is detrimental to oil quality because the ox-

ygen and the water added with the starter can promote several chemical reactions. The aim of this study was to evaluate the effect of inoculating the olives before processing in order to increase the yeasts in the olive oil but without directly manipulating the product.

MATERIALS AND METHODS

Characterisation of yeasts

A freshly produced extra-virgin olive oil obtained from the "Leccino" variety harvested at early ripeness was used. The yeasts were isolated using a previously reported method (CIAFARDINI and ZULLO, 2002b). Fifty isolates were then randomly taken from approximately 500 colonies and transferred to Malt Yeast Glucose Peptone Agar (MYGPAgar) (WICKERHAM, 1953) and identified according to conventional methods used in yeast taxonomy (KURTZMAN and FELL, 1998). The β-glucosidase test was carried out by transferring the yeasts to the wells of a Tissue Culture Plate containing 150 μL of 0.1 M phosphate buffer (pH 7) containing 0.4% (w/v) of the synthetic substrate *p*-nitrophenyl-β-D-glucopyranoside (pNPG). After twenty min of incubation at 30°C the positivity of the reaction was observed through the appearance of a yellow colour (KENGEN *et al.*, 1993). The β-glucosidase producing yeasts were tested on oleuropein (Extrasynthese, Geney, France) as reported previously (CIAFARDINI and ZULLO, 2002a).

Starter preparation and olive inoculation

The starter used to inoculate the olives was prepared using the 1639 strain of *Williopsis californica* isolated from the olive oil and characterised from a physiological point of view. This yeast strain was chosen because it has specific markers, important from a technological aspect, which differentiate it from the other isolates. Among these specific markers are its ability to produce β-glucosidase, its growth at 35°C and its ability to ferment glucose of the 13 sugars reported in Table 1. These markers were used to recognise, in the oil, the strain of yeast inoculated on the olives before milling. The starter was prepared by multiplying strain 1639 of *W. californica* in 1 L flasks containing MYGP broth. After three days of incubation at 30°C, the yeast was collected using centrifugation at 5,000 g for 10 min and suspended in a volume of sterile, distilled water until reaching a final concentration of 10^9 cfu/mL. The starter produced was kept at 4°C for a maximum of 24 hours and used to inoculate the olives which had been washed in water immediately before inoculation. Two thousand kilograms of olives of the "Leccino" variety were divided into two equal parts and processed with a continuous system produced by the ALFA LAVAL company (Monza, Italy) and with a traditional discontinuous system produced by the Pieralisi company (Jesi, Ancona, Italy). Before processing, the olives were put into two plastic containers with a capacity of 500 kg, one of which was enriched with 1 L of starter (final concentration 0.2% v/w), while the other received 1 L of sterile, distilled water. The olives were inoculated by spraying the starter directly onto the fruit while the 500 kg containers were being filled. Immediately after inoculation, the olives were slightly mixed and then submitted to the extraction processes, using the control first for both of the extractions.

Evaluation of yeast movement to the olive oil

The oil from the control and the inoculated olives obtained from the two extraction systems was put into two 100 L stainless-steel covered containers and kept at 6°-7°C. The trials were carried out in a 2

Table 1 - Yeast species isolated from olive oil and comparison of some characteristics.

Description:	*Candida boidinii* white colonies multilateral budding simple and elaborate pseudohyphae	*Williopsis californica* white colonies multilateral budding no filaments
Enzymatic activity:		
Urease	-	-
Esterase	-	-
β-glucosidase	-	+
Growth on sugars:		
D (+)-Galactose	-	-
Maltose	-	-,+
Me α-D-glucoside	-	+
D (+)-Trehalose	-	-,+
Melibiose	-	-
Lactose	-	-
D (+)-Cellobiose	-	+
D (+)-Melezitose	-	-
D (+)-Raffinose	-	-
Inulin	-	-
D (-)-Arabinose	-	-
Starch	-	-
D (+)-Xylose	+	+
D (-)-Mannitol	+	+
Growth at:		
30°C	+	+
35°C	+,-	+
37°C	+,-	-
42°C	-	-
Growth on:		
Nitrate	+	+
Nitrite	+	+
Tolerance to:		
acetic acid 1% (v/v)	-	-
Resistence to:		
cycloheximide 0.01% (w/v)	+	-
cycloheximide 0.1% (w/v)	+	-
Sugars fermented:		
D (+)-Glucose	+	+
D (+)-Galactose	-	-
Maltose	-	-
Me α-D-glucoside	-	-
Sucrose	-	-
D (+)-Trehalose	-	-
Melibiose	-	-
Lactose	-	-
D (+)-Cellobiose	-	-
D (+)-Melezitose	-	-
D (+)-Raffinose	-	-
Inulin	-	-
Starch	-	-

+, positive reaction; -, negative reaction.

month time period (oil sedimentation time) taking a 500 mL sample at the beginning of the experiment (zero time) and subsequently once every ten days. Immediately after sampling, the samples were analysed for the presence of yeasts. The samples were emulsified using a vortex mod. Maxi Mixer (FAVS, Bologna, Italy) and part of each sample was diluted with sterile, distilled water and then used to inoculate Petri dishes containing MYGP Agar. After three days of incubation, the yeast colonies were counted and compared to the *W. californica* strain used as starter. This analysis took into account the following markers: growth at 35°C, production of β-glucosidase, ability to ferment only glucose of the sugars reported in Table 1. The yeast colonies that developed on the MYGP Agar, using small, sterile, wooden sticks, were put onto MYGP Agar, setting up masters each containing 50 colonies. After three days of incubation at 35°C, a part of the colonies was transferred to the wells of a Tissue Culture Plate and assayed for the production of β-glucosidase using the procedure described by KENGEN et al. (1993). Successively, the remaining part of the β-glucosidase positive colonies, were observed under a microscope and furthermore were used for the sugar fermentation test. Finally, the colonies containing all the characteristics similar to the *W. californica* strain used as a starter were recorded.

RESULTS AND DISCUSSION

The results of the microbiological analysis carried out on a freshly produced extra-virgin olive oil indicated the presence of a rich micro-flora formed principally of yeasts (data not shown). The taxonomical analysis of the isolated colonies, carried out on the basis of the carbohydrate fermentation patterns and other physiological characteristics, confirmed the presence of the *Saccharomyces cerevisiae* and *Candida wickerhamii* species in the olive oil as reported previously by CIAFARDINI and ZULLO (2002a). Furthermore new yeast isolates classified as *Williopsis californica* and *Candida boidinii* were identified and enzymatically characterized. In all of the isolates, there was no urease or esterase activity and only *W. californica* was able to synthesize β-glucosidase, active on the synthetic substrate pNPG and oleuropein (Table 1). This indicated the possibility of indirectly increasing the beneficial micro-flora in the oil by inoculating the olives before processing. The presence of yeasts in the oil increased when the inoculated olives were processed by both extraction systems (Fig. 1). However, the best results were obtained using the discontinuous system, where at zero time the increase in yeasts was equal to approximately 30% (Fig. 1). This could be due to the fact that with the discontinuous system the movement of the yeasts to the oil was promoted by the longer milling time which allowed a better homogeneity of the pulp mass before the successive separation of the oil. The yeasts in the oil obtained using the two extraction methods were found to remain vital throughout the entire storage period, even though their total number decreased with time, due to the precipitation of the solid and liquid particles suspended in the oil in which the microorganisms are entrapped. The oil extracted from the olives which were inoculated with *W. californica*, compared to the uninoculated control, was richer in yeasts throughout the 60 days of storage (Fig. 1). There was a higher percentage of β-glucosidase positive yeasts in the oil from both extraction systems from the olives inoculated with *W. californica* (Fig. 2). The β-glucosidase positive yeasts increased during storage of the olive oil, specially when the discontinuous system was used. This can be attributed to the predominance of the inoculated yeast over the other wild type yeasts normally

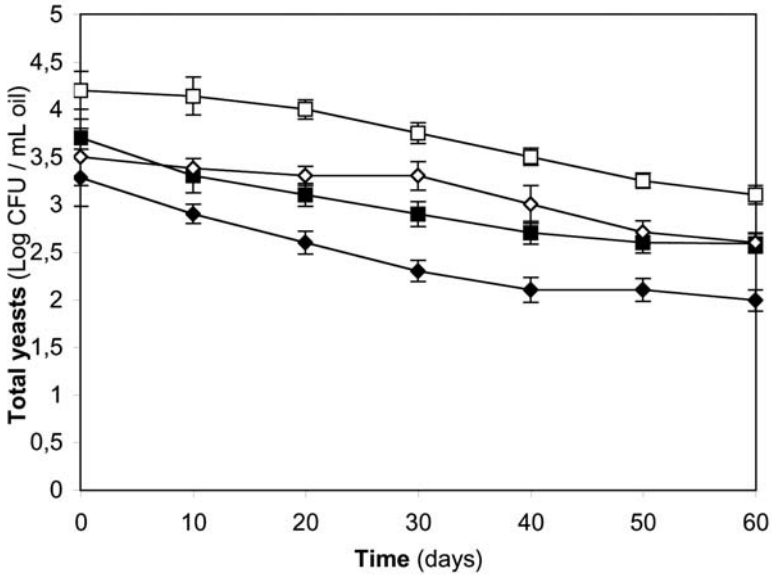


Fig. 1 - Total yeasts found in the olive oil obtained with the continuous and discontinuous milling system during storage. ◆, olive oil from un-inoculated olives (control) processed with the continuous milling system; ■, olive oil from olives inoculated with *W. californica* before processing with the continuous milling system; ◇, olive oil obtained from un-inoculated olives (control) processed with the discontinuous milling system; □, olive oil obtained from olives inoculated with *W. californica* before processing with the discontinuous system. Data are means ± standard deviation.

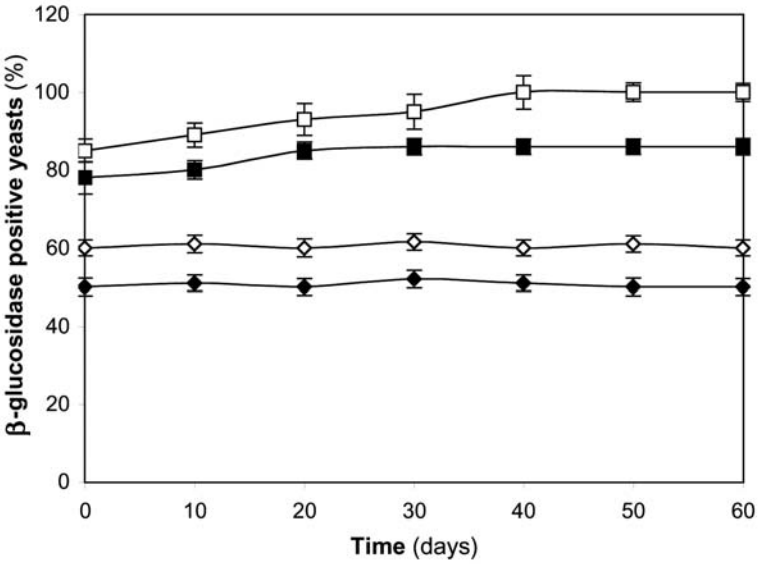


Fig. 2 - β-glucosidase positive yeasts found in the olive oil during storage. ◆, yeasts found in the olive oil extracted with the continuous system from un-inoculated olives; ■, yeasts found in the olive the oil extracted with the continuous system from olives inoculated with *W. californica*; ◇, yeasts found in the olive oil extracted with the discontinuous system from un-inoculated olives; □, yeasts found in the olive oil extracted with the discontinuous system from olives inoculated with *W. californica*. Data are means ± standard deviation.

present in olive oil. In fact, considering that this fraction of β-glucosidase-producing yeasts demonstrated their positivity also regarding the other typical markers of *W. californica*, it is plausible to attribute this increase, expressed as percent of the total yeasts present in the oil, to the 1639 strain of *W. californica*, inoculated on the olives before processing. The possibility of enriching the oil with selected yeasts by inoculating the olives, opens a whole new chapter in the sector of extra-virgin olive oil production. In fact, with this system it is possible to transfer selected yeasts to olive oil, eliminating any direct deleterious effect to the product which could cause oxidation or other undesirable alterations.

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SHORT COMMUNICATION

PHYSICAL AND CHEMICAL PROPERTIES OF DIFFERENT COMMERCIALLY AVAILABLE TYPES OF "HORCHATA DE CHUFA"

PROPIETÀ FISICHE E CHIMICHE DEI DIFFERENTI TIPI DI "HORCHATA DE CHUFA" DISPONIBILI IN COMMERCIO

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ABSTRACT

A physicochemical analysis was made of the different types of "horchata de chufa" sold in Spain. The analytical methods for the determination of total fat, total solids, ash, reducing sugars, starch and protein were adapted for these low-acid vegetable beverages. The contents found were: fat, 0.91-3.40 g per 100 g; total solids, 13.61-22.06 g per 100 g; ash, 0.079-0.298 g per 100 g; protein, 0.294-0.742 g per 100 g; starch, not detected-7.45 g per 100 mL; reducing sugars, 11.13-

RIASSUNTO

È stata condotta un'analisi chimico-fisica sui differenti tipi di "horchata de chufa" commercializzati in Spagna. I metodi analitici per la determinazione dei grassi totali, dei solidi totali, delle ceneri, degli zuccheri riducenti, dell'amido e delle proteine, sono stati adattati a questa bevanda vegetale. La composizione riscontrata è la seguente: grassi, 0,91-3,40 g%; solidi totali, 13,61-22,06 g%; ceneri, 0,079-0,298 g%; proteine, 0,294-0,742 g%; amido, non riscontrato-7,45 g%; zuccheri ri-

- Key words: chemical composition, horchata de chufa, physical properties -

17.86 g per 100 mL; pH, 6.37-7.56; and density, 1.039-1.067 g mL^{-1}. The fat content of all the sterilised horchatas was below the minimum value specified in the regulations.

ducenti, 11,13-17,86 g%; pH, 6,37-7,56; densità, 1,039-1,067. Il contenuto in grassi di tutte le horchata sterilizzate è risultato al di sotto del minimo previsto dalla legge.

INTRODUCTION

"Horchata de chufa" is a milky-looking Spanish dairy beverage, which is extracted from tiger-nut tubers (*Cyperus esculentus* L.) and to which sugar (sucrose) is added. It is a low-acid vegetable beverage of the Mediterranean area that has a high nutritional quality and therefore an important potential in the present food market, but is limited by a very short shelf-life (SELMA et al., 2003). Its high nutritional quality is due to its composition. The fat is rich in oleic acid (75% of total fat) and linoleic acid (9-10% of total fat), and arginine is the major amino acid, followed by glutamic acid and aspartic acid. With the exception of histidine, the essential amino acids in natural horchata de chufa are higher than the amount in the model protein proposed for adults by the FAO/OMS (MORELL and BARBER, 1983; NAVARRO et al., 1984).

The nutritional and sensory characteristics of horchata depend on many factors, including the chemical composition of the tuber and the manufacturing process (VARO et al., 1998a,b). The lipid and protein content can change without changes in their lipid and amino acid profiles.

Because of the importance of this non-alcoholic beverage, its manufacture, distribution and marketing are regulated by current legislation (BOE, 1988; 1991). However, completion of this legislation has been held back due to lack of information about the characteristics of the various types of horchata available on the market (CANTALEJO, 1996).

The appearance of sterilised horchata de chufa in Valencia in the eighties made it possible for this type of food to reach all parts of Spain, and in recent years sales have started to expand to other countries, such as France, Mexico, Panama, Venezuela, the Dominican Republic, the USA and some Mediterranean countries. Horchata consumption in Spain is about 50 million litres per year. Annual sales of this product amount to over 60 million euros and it has a very well established market (CONSEJO REGULADOR DENOMINACION ORIGEN "CHUFA DE VALENCIA", 2003).

Various food preservation techniques have given rise to the manufacture of horchata on an industrial scale, but heat treatment at temperatures above 75°C may gelatinise the starch and coagulate the proteins and so alter the organoleptic characteristics typical of horchata (CANTALEJO, 1996; MOSQUERA et al., 1996). The aim of the present study was to characterise the various types of horchata commercially available and establish the influence of the preservation treatment on its nutritional characteristics.

MATERIALS AND METHODS

Samples

Commercial products of the various types of horchata de chufa considered representative of the variability of this

kind of product on the Spanish market were selected. Three batches of each type of horchata with different manufacturing dates were purchased. The analyses were performed in duplicate on two subsamples from each batch and type of horchata.

The different types of horchata defined by Spanish legislation (BOE, 1988; 1991) are the following:

- Untreated horchata: this is prepared with the appropriate proportion of chufas, water and sugar. The starch and fat must come exclusively from the tubers used for preparing the horchata. The product may be sold as: refrigerated (A) or frozen (B).

- Pasteurised horchata: this is horchata de chufa subjected to a technological heat process that wholly or partially suppresses or transforms its starch content, and then it is submitted to a heat treatment that ensures the destruction of pathogenic organisms and most of the common flora. It may be sold as: refrigerated (C) or frozen (D).

- Sterilised horchata (E): this is similar to pasteurised horchata, but it is subjected to a heat treatment that ensures destruction of microorganisms and inactivation of their forms of resistance.

- UHT horchata (F): this is similar to sterilised horchata, but with the application of UHT heat treatment and aseptic canning.

All of these horchatas must have a minimum of 12 g of soluble solids per 100 g, expressed as °Brix at 20°C. Their minimum fat content, obtained exclusively from the tubers used, must be 2 g per 100 g. The minimum pH is 6.3, and total sugars expressed as sucrose must be more than 10 g per 100 mL.

- Condensed pasteurised horchata (G): this must be prepared with suitable proportions of chufa, water and sugar so that the resulting condensed product has a minimum of 60 g of soluble solids per 100 g, expressed as °Brix at 20°C, 3.5 g of starch per 100 mL, and 4.5 g of fat per 100 g, obtained exclusively from the chufas. It must have a minimum pH of 6. Total sugars must be at least 50 g per 100 mL, expressed as sucrose.

- Condensed pasteurised horchata without sugar (H): this must be prepared in the same way as condensed pasteurised horchata, but with the addition of fructose instead of sucrose.

Methods of determination

Total fat

The fat content of the horchata was determined by extraction with organic solvents in a Soxhlet-type apparatus (CARPENTER et al., 1993). As the sample was liquid, the method of FOLCH et al. (1958) and MANGLANO (1999) was used with some modifications. A mixture of chloroform and methanol (2:1, v/v) was chosen for the extraction of total fat. Fifty mL of horchata was placed in a decanting funnel, 50 mL of the extractant mixture was added, and the mixture was agitated for 2 min. The contents were separated into two tubes and centrifuged (3000 rpm, 15 min, 10°C). The chloroform phase was separated and the entire extraction process was repeated with the aqueous phase. The solvent was eliminated and the residue was heated in an oven at 80°C for 1 hour and then weighed. The intraday precision (RSD %) obtained was 2.2±0.7% and the interday precision was 3.3±1.1%, using four samples.

Total solids

It was seen that when the samples of horchata were dried to a constant weight at 100±4°C, they caramelised because of the high percentage of sugar. In order to overcome this difficulty, different tests were made with 5, 10, 15 and 20 mL of horchata, which was dried in a sand bath (60°C) until a pasty mixture was obtained. This was then placed in a forced air oven at different temperatures, ranging from 80 to 100°C, for a period vary-

ing from 2 to 48 hours. In all cases there was caramelisation of the carbohydrate present in the horchata. The tests were continued without drying the sample in the sand bath. The final conditions selected were 15 mL of horchata maintained at 95°C in a forced air oven for 2 hours.

Ash

The ash content of the horchata was determined in accordance with the official method (BOE, 1988), but varying the final temperature of the oven to 450°C. Different volumes of horchata (2, 5, 10 and 25 mL) were tested, and it was found that the percentage of ash was 0.238±0.033, 0.232±0.027, 0.273±0.008 and 0.281±0.005 (g per 100 g ± standard deviation) and the coefficient of variation was 13.7, 11.7, 2.5 and 1.9 for 2, 5, 10 and 25 mL of horchata, respectively. The method used was as follows: The organic matter of 25 mL of sample was destroyed by ashing in a temperature programmed furnace. The temperature was increased slowly, at a rate of 50°C/30 min, to a final temperature of 450°C. This temperature was maintained for 24-48 h. In order to complete the digestion, the residue was soaked with 1 mL concentrated nitric acid (d = 1.4 g mL^{-1}). From 48 to 72 h were needed to destroy the organic matter of the sample completely.

Reducing sugars

The volumetric method of Lane-Eynon was used. Four mL of horchata was centrifuged at 7,800 rpm for 10 min at 2°C (see determination of starch), and the supernatant was separated and collected in a 100 mL standard flask. To extract the sugars remaining in the sediment, different volumes of lukewarm water were added (10, 15 and 25 mL), the mixture was centrifuged at 4,300 rpm for 10 min at 20°C, and the supernatant was collected in a standard flask. After 4 extractions with 15 mL of lukewarm water, it was confirmed that the extraction of sugars was complete by means of the Molisch test (the solution to be tested was poured over sulphuric acid with some drops of α-naphthol at a rate of 15 g per 100 mL. The appearance of a red-violet ring in the contact area indicated the presence of sugar). A Carrez solution (4 mL) was then added, and the mixture was made up to 100 mL with deionised water and filtered.

Fifty mL of the filtrate was taken and placed in another 100-mL standard flask, 25 mL of deionised water and 5 mL of hydrochloric acid (d = 1.18 g mL^{-1}) were added, and the resulting mixture was placed in a water bath at 70°C. Various analysis times (3, 5, 7, 10, 12 and 15 min) were studied, and it was found that hydrolysis was complete after 7 min. The flask was cooled, neutralised with 2.5 N NaOH and made up to 100 mL with deionised water. The mixture was tested with 10 mL of Fehling's solution, which had been previously tested with a solution of inverted sucrose at 0.5 g per 100 mL.

In order to calculate the precision of the method, the sugar content was determined in 6 aliquots of a particular horchata, giving a coefficient of 1.4% (17.76±0.24 g per 100 mL of reducing sugars). To estimate the accuracy of the method, 6 recovery assays were prepared, giving a value of 98.1%.

Starch

The starch content of horchata varies in accordance with the preservation treatment to which it has been subjected. The method for the determination of starch in horchata was based on the method proposed by the MINISTERIO DE SANIDAD Y CONSUMO (1985). Four millilitres of horchata was taken and centrifuged (7,800 rpm, 10 min, 2°C), and one drop of iodine solution was added to the supernatant in order to ascertain whether starch was present or not. If starch was present in the supernatant,

the sediment alone could not be used to determine the starch content because it would give an underestimate, and so in this case a lyophilised sample of the horchata was analysed. If there was no starch in the supernatant, the sediment obtained by ultracentrifugation or a quantity of lyophilised sample equivalent to 4 mL of horchata was taken, 8 mL of ethanol:petroleum ether (1:3 v/v) was added, the mixture was centrifuged at 3,500 rpm for 5 min at 20°C, and the supernatant was discarded. In this way the fat was eliminated. The simple sugars were separated from the sediment by washing with lukewarm water as indicated in the section on the determination of reducing sugars. The starch in the sediment was then hydrolysed. A study was made with perchloric acid at 52 g per 100 mL using various volumes (1, 2, 4, 6 and 8 mL), times (15, 30 and 45 min) and temperatures (60°, 75° and 85°C) for the hydrolysis. The conditions finally selected were as follows: the sediment was dissolved in 5 mL of deionised water and then transferred to a 100 mL standard flask, 6 mL of perchloric acid (52 g per 100 mL) was added, the mixture was shaken and placed in a water bath at 75°C for 15 min with periodic agitation. In these conditions the hydrolysis of the starch was complete.

Protein

The official method (BOE, 1988) was followed, although adjustments were made to the amount of sample, the various amounts of reagents, the distillation time and the indicator most suitable for testing the protein content.

Different volumes (1, 2, 3 and 5 mL) of horchata were studied. The sample quantity selected was 2 mL, because it gave the smallest coefficient of variation. It was placed in a digestion tube, and 10 mL of concentrated sulphuric acid (d = 1.84 mL^{-1}), 6 drops of 2N $CuSO_4$, 3 g of K_2SO_4 and some glass beads were added. The mixture was placed on a heating blanket until mineralisation was complete. It was then distilled in a Kjeltec apparatus, with the automatic addition of 100 mL of water and 80 mL of NaOH (40 g per 100 mL). Different distillation times (5, 6.8 and 9.9 min) were tested, and the time finally chosen was 6.8 min. When distillation was complete, the NH_3 was tested with 0.1 N HCl until there was a change in the indicator (methyl red). A parallel test was performed with a blank.

pH and density

The determination of pH was based on the potentiometric measurement at 20°C (BOE, 1988). It was determined in a Crison GLP 21 pH meter equipped with a temperature compensation sensor at 20°C. The results are expressed to two decimal places.

Density was determined at 20°C with a pycnometer.

Statistical evaluation

The contents were compared using one-way analysis of variance (ANOVA). To determine differences between products and between samples of the same product the least significant difference (LSD) test ($p < 0.05$) was applied. The computer program employed was Statgraphics Plus for Windows 3.0.

RESULTS AND DISCUSSION

Tables 1 and 2 show the mean values of six determinations (two determinations for each subsample) of the chemical characteristics for each batch and type of horchata de chufa.

The fat content of all the sterilised horchatas and horchata F_1 (UHT) was below the minimum value specified in the regulations (2 g per 100 g) (BOE, 1988). The fat content was particularly high in condensed pasteurised horchata without sugar (H), with a value three

Table 1 - Chemical characteristics of each of the types of "horchata de chufa" commercially available in Spain (n=6).

Sample	Fat	Solids	Ash	Protein	Starch	Sugar
	(g per 100 g)				(g per 100 mL)	
A	2.95±0.09	20.03±0.28	0.265±0.021	0.730±0.198	4.31±0.16	17.86±0.13
B	2.84±0.09	19.24±0.34	0.246±0.001	0.438±0.001	2.88±0.06	16.47±0.20
C_1	2.79±0.24	22.06±0.95	0.266±0.027	0.631±0.018	6.04±0.09	17.05±0.08
C_2	3.40±0.18	21.56±0.58	0.240±0.006	0.691±0.010	5.57±0.34	17.24±0.73
C_3	2.75±0.14	21.17±0.42	0.298±0.006	0.742±0.026	0.85±0.05	16.95±0.62
D	2.76±0.19	21.05±0.18	0.242±0.001	0.693±0.023	3.66±0.19	16.35±0.34
E_1	1.09±0.05	15.10±0.09	0.227±0.006	0.378±0.025	6.07±0.52	11.49±0.06
E_2	1.50±0.05	15.55±0.19	0.159±0.004	0.418±0.021	N.D.	13.18±0.16
E_3	0.91±0.05	14.57±0.05	0.230±0.001	0.395±0.023	N.D.	12.89±0.10
E_4	0.92±0.05	15.09±0.03	0.200±0.021	0.366±0.042	1.55±0.03	11.95±0.20
E_5	1.27±0.06	14.62±0.01	0.195±0.001	0.434±0.018	2.83±0.11	11.21±0.03
F_1	1.74±0.04	13.61±0.02	0.217±0.001	0.437±0.004	0.15±0.06	11.13±0.10
F_2	2.24±0.08	13.63±0.39	0.265±0.002	0.446±0.005	N.D.	11.68±0.17
G	0.92±0.03	15.48±0.04	0.079±0.004	0.294±0.025	1.06±0.08	15.06±0.18
H	3.10±0.11	16.54±0.25	0.101±0.001	0.384±0.022	7.45±0.63	12.90±0.21

N.D.: not detected. Untreated horchata: refrigerated, A or frozen, B; Pasteurised horchata: refrigerated, C or frozen, D; Sterilised horchata: E; UHT horchata: F; condensed pasteurised horchata: G or without sugar, H.
All the values for the condensed horchatas (G and H) are expressed in terms of diluted horchata, so that the final result is of the same order as those of the other horchatas.

times greater than that of condensed pasteurised horchata (G), which differs in its sucrose content.

The smallest percentage (g per 100 g) of total solids was found in the sterilised horchatas (E_1 – E_5) and the UHT horchatas (F_1 and F_2), followed by the condensed pasteurised horchatas (all the values for the condensed horchata are expressed in terms of diluted horchata) (G and H) and then the remaining horchatas. These differences were statistically significant ($p < 0.05$).

The quantity of mineral salts in a sample was determined from the ash obtained after incineration of the sample. The mineral composition of horchata de chufa can vary because of agronomic factors such as climate, geographical location of the land, kind of soil, kind of fertiliser, etc.

The lowest percentage of ash was found in the condensed pasteurised horchatas (G and H), and these differences were statistically significant ($p < 0.05$). Sterilised and UHT horchata (E and F) had higher values than the condensed pasteurised horchatas and lower values than refrigerated untreated horchata (A), frozen untreated horchata (B), refrigerated pasteurised horchata (C) and frozen pasteurised horchata (D), but these differences were not statistically significant.

For each kind of horchata de chufa, the Technical Health Regulations (BOE, 1988) define the minimum starch content to be 1.9 g per 100 mL for frozen untreated horchata (B), refrigerated pasteurised horchata (C) and frozen pasteurised horchata (D), and 0.7 g per 100 mL for condensed horchatas (G and H) when diluted for consumption. However, the regulations in the Valencian Community (DOGV, 1995) establish slightly higher values of 2.2. and 0.85 g per 100

Table 2 - Determination of pH and density of each of the types of horchata de chufa commercially available in Spain (n=6).

Sample	pH	Density (g mL^{-1})
A	6.59±0.02	1.067±0.005
B	6.55±0.02	1.062±0.001
C_1	6.89±0.12	1.065±0.001
C_2	6.70±0.05	1.064±0.001
C_3	6.37±0.01	1.060±0.003
D	6.78±0.07	1.065±0.002
E_1	7.56±0.09	1.044±0.001
E_2	6.55±0.09	1.046±0.001
E_3	6.96±0.12	1.041±0.001
E_4	7.42±0.05	1.041±0.001
E_5	7.50±0.04	1.039±0.001
F_1	7.25±0.10	1.039±0.002
F_2	6.87±0.01	1.042±0.001
G	7.12±0.01	1.048±0.002
H	6.44±0.03	1.052±0.001

Untreated horchata: refrigerated, A or frozen, B; Pasteurised horchata: refrigerated, C or frozen, D; Sterilised horchata: E; UHT horchata: F; condensed pasteurised horchata: G or without sugar, H.
All the values for the condensed horchatas (G and H) are expressed in terms of diluted horchata, so that the final result is of the same order as those of the other horchatas.

mL, respectively. The starch content was quite variable, even within a given type of horchata. Only sample C_3 had values below those set by the regulations.

The sugar content of the condensed horchatas (G and H) is expressed in terms of diluted horchata, so that the final result is of the same order as those of the other horchatas. The Technical Health Regulations establish that total sugar, expressed as sucrose, should be at least 10 g per 100 mL, except in the case of condensed horchatas, in which it should be greater than 50 g per 100 mL (BOE, 1988; DOGV, 1995). The sterilised and UHT horchatas had the lowest content of reducing sugars, and these differences were statistically significant.

The total protein content was obtained by determining the total nitrogen using the Kjendahl method and multiplying by a correction factor of 6.25. As can be seen, the protein content varied according to the type of horchata, but in no case was it more than 1 g per 100 g.

In all the horchatas analysed, the pH (Table 2) satisfied the Technical Health Regulations (minimum pH of 6.3 except for condensed horchata, which must have a minimum pH of 6 after being reconstituted). The highest pH values (7.05 to 7.19) were found in the sterilised and UHT horchatas and condensed pasteurised horchata, and these horchatas were statistically different ($p < 0.05$) from the others, with the exception of UHT horchata, which did not differ statistically ($p < 0.05$) from pasteurised horchata.

The density varied from 1.041 g mL^{-1} for UHT horchata (F) to 1.067 for refrigerated untreated horchata (A) (Table 2).

Although many studies on chufa tubers have appeared, few studies on the physical and chemical properties of horchata de chufa have been published. The pH values found by MESTRES and PUJADAS (1980) for pasteurised and sterilised horchata (6.61 and 6.84 respectively) are slightly lower than those found in the present study for these two types of horchata. They obtained a mean pH value of 6.14 for natural horchata, lower than the minimum required in current legislation (BOE, 1988) and lower than the value found in the present study. The sugar contents found in pasteurised and untreated horchata, 8.16 and 9.4 g per 100 mL, respectively, was lower than that found in the present work, whereas the sugar content in sterilised horchata was of the same order. The same can be said for total solids, for which they obtained values of 14.7, 16.75 and 14.7 g per 100 g in pasteurised, untreated and sterilised horchata, respectively. The fat content (3.0 g per 100 g) that they found for pasteurised horchata is similar to the value found in the present work. For untreated and sterilised horchata they reported values of 3.24 and 2.26 g per

100 g, respectively, which are higher than the levels found in the present work, especially for sterilised horchata. The density values found in the present study were higher than those obtained by MESTRES and PUJADAS (1980) for untreated, pasteurised and sterilised horchata (1.034, 1.036 and 1.035 g mL^{-1}, respectively).

For untreated horchata, the sugar content found in this work is similar to the value obtained by NAVARRO et al. (1984) (18.3 g per 100 mL) and higher than the values found by VARO et al. (1998a), BENEYTO et al. (2000) and GOZALBO et al. (2000), who reported ranges of 14.5-16.9 g per 100 mL, 14.5-16.0 and 14.8-15.8 g per 100 g, respectively.

The total fat value for untreated horchata found in the present work was higher than the values reported by MORELL and BARBER (1983) and NAVARRO et al. (1984) (2.70 and 2.62 g per 100 mL, respectively) and similar to the values found by VARO et al. (1998a), BENEYTO et al. (2000) and GOZALBO et al. (2000).

In untreated horchata, the values found for solids were within the range obtained by VARO et al. (1998a) (19.7-21.6 g per 100 g) and are slightly below the values found by MORELL and BARBER (1983) and NAVARRO et al. (1984) (22.18 and 22.82 g per 100 g, respectively).

MORELL and BARBER (1983) and NAVARRO et al. (1984) obtained protein values of 1.23 and 0.93 g per 100 mL, respectively, in untreated horchata, higher than those found in the present study, and starch values of 3.44 and 2.42 g per 100 mL, respectively, lower than those found in this work.

The ash content found in untreated horchata in this study is similar to the values found by MORELL and BARBER (1983) and NAVARRO et al. (1984).

In untreated horchata the pH value found in this study and in the literature (MORELL and BARBER, 1983; NAVARRO et al., 1984; VARO et al., 1998a; BENEYTO et al., 2000 and GOZALBO et al., 2000) was higher than the minimum required by legislation (BOE, 1988).

MORELL and BARBER (1983) and VARO et al. (1998a) obtained a density value between 1.067 and 1.077 g/mL^{-1}, similar to the value found in the present study.

CONCLUSION

There were few differences between the different types of horchata (as verified by the statistical analyses performed), except for the condensed horchatas (G and H), which had lower values for total solids, ash and sugar than the other horchatas. Condensed pasteurised horchata also had lower values for fat and starch. It is important to note the higher protein content in untreated horchata. The Technical Health Regulations were satisfied in all cases, except in the fat content of all the sterilised horchatas and horchata F_1 (UHT), which was below the minimum value specified in the regulations.

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PASSING THE TEST FOR HEALTH CLAIMS

FFE 624/03/HP81

How can we be sure that claims made about functional foods are not untrue or misleading? The development of foods with benefits beyond their traditional nutritional value, so called functional foods, has led to a great deal of interest by health professionals and their patients.

Health professionals need to be confident in the scientific and regulatory processes that are used to support the health effects of functional foods, and that any health claims are based on good nutrition science.

The concept of functional foods and health claims was initially investigated in a Concerted Action on Functional Foods in Europe (FUFOSE) funded in the EU DG XII FAIR programme, and co-ordinated by ILSI Europe. The aim was to develop and establish a science-based approach for the development of food products that benefit health and well-being (British Journal of Nutrition 1999; 81 Suppl. 1:S1-27). The development of two types of health claims was proposed 'enhanced function' and 'reduction of disease risk'. FUFOSE stated that claims should always be valid in the context of the whole diet, must relate to the amounts of foods normally consumed, and be based on well designed studies using appropriately identified, characterised and validated biomarkers.

PASSCLAIM is an EC Concerted Action funded in the 5th Framework and built on the work of FUFOSE. It is formed by a network of Theme Groups and a Consensus Group, whose objectives are:

- To produce a tool for assessing the scientific support for health-related claims in foods;
- To evaluate critically the existing schemes that assess the scientific substantiation of claims;
- To select criteria for how biomarkers should be identified, validated and used in studies exploring the links between diet and health.

The first PASSCLAIM consensus reports have just been published. They cover diet-related cardiovascular disease, bone health and osteoporosis, physical performance and fitness, a review of existing codes, and a draft set of interim criteria

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for the substantiation of health claims (European Journal of Nutrition 2003; 42 Suppl. 1:1-119). Participants are now focusing on insulin sensitivity and risk of diabetes, diet-related cancers, mental performance and gut health and immunity.

Project No: QLK1-2000-00086 (PASSCLAIM) http://europe.ilsi.org/passclaim/

Project Co-ordinator: Dr. Laura Contor, International Life Sciences Institute - ILSI Europe, Avenue E. Mounier 83 bte 6, BE-1200 Brussels, Belgium, Tel. +32-2 771.00.14; Fax: +32-2 762.00.44, E-mail: dpannemans@ilsieurope.be.

This one-pager was written by Dr Gail Goldberg, British Nutrition Foundation, UK, in June 2003.

FOOD TRACEABILITY

FFE 646/03/SME88

Food quality and safety will be strongly improved by the EC legislation (178/2002) on food traceability, which will come into force in January 2005. The aim of this regulation is to improve food quality and safety, to protect consumers against false claims and product information and further to promote fair trade. Thus, the new system enables consumers to be provided with targeted and accurate information concerning the products.

This regulation requires that all stakeholders within the food supply chain (agriculture and feed producers, food manufacturers, retailers, etc.) must be able to identify the source of all raw materials and ingredients and also to whom the products have been sold. The food companies must develop and use new identification systems and data handling procedures and these must often be integrated into their quality management system and their HACCP system. Several product-specific traceability regulations have been prepared, for example on meat, fish and genetically modified products and more are in the pipeline. Further, international standardization organisations are working hard to develop standards to be used, such as Codex and CEN.

A group of 24 European scientists and industrialists established the EU-funded Concerted Action called FoodTrace, which aims to develop a practical framework for food traceability and develop the means to plan, model, validate and implement. This Action is working specifically on products, including animal feed, fish, meat, poultry, cereals, dairy, organic farming, processed foods, confectionery, wine, fresh produce, and supermarket supply as well as national and international trade.

The project partners are facing several problems, in particular: how to electronically identify the lots; selecting which data must / should be included; how to capture and store data. On the project website, www.eufoodtrace.org/, interested parties may read about the Action conferences, follow net meetings and read progress reports.

Another project (Tracefish, QLK1-2000-00164) has been working on farmed and captured fish traceability. Information on this project can be found in the internal section of the Flair-Flow website: http://www.flair-flow.com/industry-docs/ffe55702.html.

Project No: QLK1-2001-02202 (FoodTrace).

Project Co-ordinator: Ian G. Smith, Automatic Identification Manufacturers (Europe) Ltd, The Old Vicarage, Haley Hill, HX3 6DR Halifax, UK, Tel.: +44 1422 368368; Fax: +44 1422 355604, E-mail: ian@aimglobal.org.

This one-pager was written by Mr Finn. Holm, Food Group Denmark, in September 2003.

LINKING EARLY DIET AND CHILDHOOD OBESITY

FFE 660/03/HP93

Childhood obesity can lead to psychosocial distress as well as detrimental effects on cardiovascular disease risk factors such as dyslipidaemia, arterial hypertension and glucose intolerance.

It is clear, from studies across Europe, that the prevalence of obesity is increasing among children, with serious consequences in terms of quality of life and burdens on health care and social security systems. This is being investigated by the Chopin project with a view to finding effective strategies to prevent obesity at an early age.

Although genetics play some part in a person's predisposition to obesity, lifestyle factors are known to be of great importance. Furthermore, early nutrient supply during infancy is thought to have a lasting, programming effect on later obesity risk.

Infants fed formula milk are more likely to become obese than breastfed infants. Research has also shown a dose response effect; longer duration of breastfeeding has a marked effect on reducing later obesity risk. One hypothesised reason for this is the relative higher protein content of some infant formulae compared with breast milk. The Chopin project will test this theory.

Specifically, the project will include a double-blind randomised multicentre intervention trial of healthy infants in 5 European countries, comparing infant formulae, which are isocaloric, with high and low protein contents, balanced by fat. The project will also evaluate the effects of different feeding regimes on a novel anthropometric marker, namely the difference between body length at 2 years and length at birth, on later obesity development. Other measurements will include body composition, energy expenditure, protein metabolism, renal function, leptin and insulin-like growth factor-1.

In addition to promoting the benefits of breast-feeding, the project is expected to lead to the development of new infant foods and will break new ground in providing data for producing infant formula with adequate protein. The project website also includes a dedicated area for health professionals to access information, which will assist them in advising on infant feeding.

The project has already recruited babies, who will be studied over the first 2 years of life, and it is expected that the cohort will be followed up until the age of 8 years, enabling important conclusions to be drawn.

The study will be completed in 2005 and a large cluster meeting (Perinatal nutrition and its later consequences: New opportunities) is to be held in July 2004.

Project Reference: QLK1-2001-00389 (Chopin), http://www.childhood-obesity.org/index.php.

Project Contact: Ms. Julia Rosen, University of Munich, Div Metabolic Disorders and Nutrition, Lindwurmstrasse 4, 80337 Munchen, Germany, E-mail: jrosen@med.uni-muenchen.de; margaret@ashwell.uk.com.

This one-pager was written by Dr Frankie Phillips, British Nutrition Foundation, UK, in October 2003.

LESS STOMACH DISEASE

FFE 663/03/CG93

In the last decade of the twentieth century, the number of reported cases of gastro-enteritis, caused by bacteria named *Campylobacter*, has increased in Western industrialized nations, but there remains a lack of information on the prevalence of emerging *Campylobacteraceae*. Therefore, a joint European, US and South African project that started 7 months ago is aiming to develop methods capable of isolating and identifying these bacteria.

Many different *bacteria* are included in the family *Campylobacteraceae*. Most of them have been associated with disease in animals and man. For example, *Campylobacter jejuni* is the major cause of human gastro-enteritis worldwide, responsible for 400 to 500 million cases of diarrhoea each year. *Campylobacter* are easily destroyed by food processing because they are sensitive to heat, dehydration and acid environments. However, they can survive for long periods in frozen foods and moist environments.

Campylobacter are present in the intestinal tract of wild and domestic animals such as poultry, pork, beef and lamb, where they usually cause few or no symptoms. Raw or incompletely cooked food products, such as raw poultry, inadequately pasteurised milk and contaminated drinking water may be infectious agents. Usually the symptoms occur within 1-7 days after contamination. Typical symptoms include diarrhoea, high fever, headache, nausea and abdominal and muscle pain.

The routine detection methods are inadequate for a growing number of emerging *Campylobacteraceae*. In the CAMPYCHECK project improved isolation, detection and identification procedures for the analysis of *Campylobacteraceae* in the food and water chain are developed and the risk exposure is discussed with industry, consumers and other relevant interest groups.

Project Reference: QLK1-2002-02201 (Campycheck) http://www.campycheck.org/

Project Contact: Prof. C. William Keevil, Environmental Healthcare Unit, School of Biological Sciences, Biomedical Sciences Building, University of Southampton, Bassett Crescent East, Southampton SO16 7PX, UK, Tel.: +44 (0)2380 594726; Fax: +44 (0)2380 594459, E-mail: cwk@soton.ac.uk.

This one-pager was written by Mrs. Sirpa Karppinen, VTT Biotechnology, Finland, in October 2003.

ENGINEERED PLANTS HIGHER IN ANTIOXIDANTS

FFE 665/03/SME95

The quality of tomatoes, cereals and other crops may be improved in the future by using new genetically modified crops with a greater content of antioxidants, such as natural flavonoids or other phenolic compounds. Because of the healthy profile of these antioxidants, which are believed to have a protective effect against cardiovascular diseases and some forms of cancers, these new crops are considered as functional foods.

This tendency to improve food raw materials by traditional breeding, by genetic modification, or by changing the feeding of animals is often seen: health improved meat by improving the fatty acid profile of animal feed; health improved eggs by adding fish oils to the chicken feed; rice with higher content of vitamins and iron; and now tomatoes or cereals with high content of antioxidants, for example flavonoids, carotenoids or tocopherols (vitamin E). This is what could be called second generation functional foods.

Flavonoids are naturally present in most fruits and vegetables. They are as good antioxidants as carotenoids and vitamin E or C. These antioxidants are thought to work by scavenging oxygen radicals, thus protecting against oxidative breakdown of biopolymers such as DNA, proteins and lipids. Breakdown of DNA molecules in cells is believed to be the most prominent mechanism for initiation of cancer cells and oxidation of lipoproteins for increasing the risk of arteriosclerosis.

The scientists involved in the PROFOOD research project are trying to identify key genes responsible for flavonoid production and have started to grow the very first generation of tomato plants possibly high in flavonoids. After completion of this work, they intend to transfer their know-how to other crops, in particular cereals.

Project N.: QLK1-CT-2001-01080 (PROFOOD),

http://profood.ipk-gatersleben.de

Project Coordinator: Professor Dr. Uwe Sonnewald, Institute for Plant Genetics and Crop Plant Research, Molecular Cell Biology, Corrensstrasse 3, DE-06466 Gatersleben, Germany, Tel.: +49 394825214; Fax: +49 394825515, E-mail: sonnewald@ipk-gatersleben.de.

This one-pager was written by Mr Finn. Holm, Food Group Denmark, in November 2003.

PUSHING THE POTENTIAL OF PHYTOCHEMICALS

FFE 667/03/HP94

Diets that include plenty of plant-derived foods (e.g. fruits, vegetables, nuts and wholegrains) may help to prevent chronic diseases such as heart disease and some cancers, but the beneficial effects of these foods may be enhanced even further. An EU Concerted Action has examined the potential need for improving a selected range of nutrients and beneficial factors in plants, where the levels of these substances are possibly inadequate for protective effects in humans.

Understanding about the potential roles of phytochemicals in plants is still in its infancy and for many phytochemicals the benefits of improving their levels in crops is uncertain. Nonetheless, there are strong arguments for improving exposure through the diet, if there are health benefits associated with augmenting the intake of specific nutrients, rather than through the use of supplements. Real health benefits are likely to result from the enhancement of certain nutrients in plant foods, especially for the developing world where plant foods are the dietary staple.

Biotechnology has enabled specific foods to be modified to improve their nutritional content. Examples of these are:

- Tomatoes with increased amounts of β-carotene;
- Rice with increased iron content;
- Soybeans with increased or decreased amounts of isoflavones;
- Edible vaccines based on modified potato.

The NEODIET Concerted Action has brought together scientists, working in industry and academia, to review the nutritional enhancement of plant foods in the context of social, economic and political issues that need to be considered by those working in the field of biotechnology, if the potential health benefits are to be realised.

A major output of the project is the special edition of the Journal of the Science of Food and Agriculture (Issue 7, 2000), which includes position papers, prepared by the participants of NEODIET. These position papers review the current evidence on, among others, the examples of potential ways in which foods could be enhanced. Both increased content of nutrients and phytochemicals and removal, or reduction, of some "anti-nutritive" or undesirable factors have been reviewed.

The website includes information about the project, and project newsletters may be accessed, which provide more details on how the technologies for enhancing plant-derived foods have been developed for various products.

Project Reference: FAIR-CT97-3052 (NEODIET) http://www.ifrn.bbsrc.ac.uk/neodiet/

Project contact: Dr David Lindsay, Biochemistry Department, Institute of Food Research, Norwich Research Park, Norwich NR4 7UA, UK, Tel.: +44-1603-255224; Fax: +44-1603-505671, E-mail: dlindsay@bbsrc.ac.uk.

This one-pager was written by Dr Frankie Phillips, British Nutrition Foundation, UK, in November 2003.

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Abstract Deadline - February 20, 2004, www.supercritconf.org

Confectionery Manufacturing Expo (CME) Wiesbaden, Germany 27th to 29th April 2004

The show is the only pan-European major exhibition exclusively featuring suppliers to the confectionery industry. ZDS Solingen, The Central College of the German Confectionery Trade, will be organising the seminar programme. ZDS is the world's most prestigious training establishment for the confectionery industry.

The programme will include sessions on manufacturing technologies and the use of ingredients in different confectionery products. Details on the seminars will be published on the event website, www.cm-expo.com, in the next few weeks.

Confectionery Manufacturing Expo, takes place every other year and is organised by Simply Events, a leading international trade shows organiser and publisher.

For further information, please contact: Gillian Pattison, Show Press Office, Tel.: +44 (0)20 8542 9090, E-mail: press@simply-events.com.

Announcement of Competition

"PREMIO DEL MUSEO" EUROPEAN MUSEUM PRIZE XIII EDITION 2004

Section for journalism

The Board of Scientific consultants of the National Museum of Pasta, the only one of its kind in the world, will award a Prize for journalism in 2004.

The Prize aims to promote knowledge and appreciation of Italian pasta food, education in learning the nutritional value of this typical national food, which can contribute to alleviate the world-wide problem of hunger. The theme for 2004 is "La Pasta come conferma del valore di patria, in un mondo globalizzato in crisi".

For more information: Museo Nazionale della Pasta, Piazza Scanderbeg 114-120, 00187 Roma, Italy, Tel. +39 06 6991119-6991120 - Fax +39 06 6991109.

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ITALIAN JOURNAL OF FOOD SCIENCE - IJFS

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

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

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

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

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

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

Papers - The paper must follow manuscript preparation.

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

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Referees may not be from the same institution as the author. Referees should make their comments and questions in detail and return the paper to the Editor-in-Chief as soon as possible, usually within 4 weeks. The identity and the report of the referees are made known to the Editor-in-Chief, but only the anonymous report is routinely sent to the author. If all referees recommend acceptance or rejection, the decision stands. If the opinions of the referees tie, the Editor-in-Chief has the freedom to decide upon acceptance or rejection of the paper. Manuscripts will be edited in the order received and accepted papers will be published as closely as possible in this order. A letter announcing the issue of publication will be sent to the author after the manuscript has been accepted by the Editor-in-Chief. Each paper is accepted with the understanding that it is the sole document under active consideration for publication covering the work reported (it has NOT been previously published, accepted or submitted for publication elsewhere). Upon acceptance of the paper for publication, the author agrees to pay the page charges as published on the first page of each issue. Authors take full responsibility for all opinions stated in their papers and published in this journal.

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EXAMPLES of use in a Reference list are given below. The bold-faced parenthetical type of citation above the example is indicated ONLY for information and is NOT to be included in the reference list.

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CONTRIBUTORS

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