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PAPER

CREMOSO CHEESE: PREDICTION OF RIPENING TIME USING PHYSICOCHEMICAL PARAMETERS AND MULTIVARIATE STATISTICAL TECHNIQUES

PREDIZIONE DEL TEMPO DI MATURAZIONE DI FORMAGGI CREMOSI IMPIEGANDO PARAMETRI CHIMICO-FISICI E TECNICHE STATISTICHE MULTIVARIATE

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ABSTRACT

Physicochemical parameters were used to study the ripening evolution in Cremoso cheese. Twenty-eight cheeses manufactured in different seasons and ripened for 30 days were obtained from two nearby factories. Using principal components analysis (PCA), cheese samples were grouped by ripening time, mainly according to PC_1. The high loading values of %pH4.6-SN/TN, %TCA-SN/TN, %PTA-SN/TN and the α_{S1}-I-casein/(α_{S1}-casein + α_{S1}-

RIASSUNTO

Per lo studio della evoluzione della maturazione del formaggio Cremoso sono stati impiegati parametri chimico-fisici. 28 forme di formaggio stagionati per 30 giorni e prodotti in differenti epoche dell'anno sono stati ottenuti da due caseifici vicini. L'analisi per componenti principali (PCA) ha evidenziato un raggruppamento tra campioni in funzione del tempo di maturazione, principalmente su PC_1. Gli elevati loadings trovati per %pH4,6-SN/TN,

- Key words: Cremoso cheese, multivariate statistical methods, physicochemical parameters, ripening time -

I-casein) ratio on factor 1 indicate that these variables are the most important for grouping cheeses according to ripening time. Using Partial Least Squares Regression (PLS) based on physicochemical parameters selected by PCA, a good model for predicting the ripening time of Cremoso cheese was developed. This model includes parameters of moderate, simple, and fast analyses and has an error of estimation of 2 days.

%TCA-SN/TN, %PTA-SN/TN e per la relazione α_{S1}-I-casein/(α_{S1}-casein + α_{S1}-I-casein) sul fattore 1, hanno mostrato che queste variabili sono le più importanti per riunire i formaggi in relazione al loro tempo di maturazione.

Impiegando la Partial Least Squares Regression (PLS) basata in parametri chimico-fisici selezionati per il PCA, è stato svilupato un buon modello per la predizione del tempo di maturazione del formaggio Cremoso. Il modello si basa su parametri analitici semplici e rapidi. Il modello, inoltre, indica un errore di valutazione pari a due giorni.

INTRODUCTION

Proteolysis is of great importance in most cheese varieties and is carried out by various enzymes during cheese ripening. It is thought to have a profound impact on the development of cheese texture, taste and aroma.

In soft cheese, proteolysis is responsible for the softening of the cheese mass and the consequent development of a typical creamy texture. In this type of cheese, the main proteolytic agent is the residual milk-clotting enzyme because the high moisture content as well as the absence of cooking enhance its retention in the curd and its activity on proteins. The hydrolysis of α_{s1}-casein in the Phe_{23}-Phe_{24} bond by this enzyme is a very important transformation during the ripening of this cheese (HYNES *et al.,* 1999). The α_{s1}-I-casein peptide is more hydrophilic than the original α_{s1}-casein and, for this reason, the cheese has a greater water retaining capacity and this allows the creamy texture typical of this variety to develop.

The analytical methodologies used to evaluate proteolysis have been described in depth and include both classical physicochemical methods (pH, Kjeldahl, moisture, dry matter, etc.) and more sophisticated approaches such as electrophoresis and chromatography.

For most cheese, proteolysis is commonly used as an index of maturity. Most, if not all, nitrogenous compounds that contribute to cheese flavour are soluble in aqueous solvents. Solubility at pH 4.6 is widely used for the initial fractionation of cheese nitrogen (N) or as a crude index of proteolysis. Values for nitrogen which is also soluble in both 12% tricloroacetic acid and 2.5% phosphotungstic acid are also used as indices of maturity, but secondary proteolysis is not very important in soft cheese. Polyacrylamide gel electrophoresis (PAGE) is widely used to monitor primary proteolysis in cheese and is mostly related to the activities of residual coagulant and plasmin. Residual α_{s1}-casein is a good index of the level of general proteolysis in relatively young cheeses (FOX, 1993).

The results from proteolysis analysis are often rather complicated and multivariate in their nature. Multivariate statistical techniques have therefore been successfully used to analyse proteolysis data.

Ripening time can be determined by applying multivariate regression analysis to physicochemical and proteolysis data obtained during cheese ripening.

GARCIA-RUIZ *et al.* (1998) and POVEDA *et al.* (2004) predicted the ripening time of Manchego cheese with an error close to 11 days by using PLS regression on the results from classical analysis. Peptides and amino acid profiles obtained by HPLC and PLS regression have been successfully used to differentiate the age of Ragusano cheese (FALLICO *et al.*, 2004). DOWNEY *et al.* (2005) predicted the maturity and sensory attributes of Cheddar cheese using near-infrared spectroscopy with an accuracy sufficient for industrial use. MARTIN-DEL-CAMPO *et al.* (2007) determined the ripening stages of Camembert cheese with an error of 1 day by applying mid-infrared spectroscopy and PLS.

Soft cheese is very important among the Argentinean cheeses with Cremoso being the most important. According to the Código Alimentario Argentino (CAA), Cremoso is a cow milk cheese produced with a thermophilic starter. It is enzymatically-coagulated and has a creamy aroma and taste, neutral white color, with no holes and a slight elastic texture. The minimum ripening time is 30 days; however, cheeses with different ripening times are available on the market.

Currently, there are no analytical procedures for determining the ripening time of Argentinean cheeses. Given the major effect that ripening has on the final quality of cheese, information about the progress of proteolysis would be useful to the cheese-maker in order to be able to check the ripening process. Moreover, the determination of the degree of ripening is an important part of cheese quality evaluation and is currently done by trained sensory panellists. This approach is time-consuming and expensive. Consequently, there is considerable interest in developing instrumental techniques that would enable more objective, faster and less expensive assessment of cheese quality.

In the present study the physicochemical characteristics of Cremoso cheese were used to develop statistical models for predicting the ripening time.

MATERIALS AND METHODS

Cheese samples

Twenty-eight cheeses from different seasons (8 in autumn, 6 in winter, 14 in spring) were supplied by two nearby dairy plants in Santa Fe, Argentina (two brands). These cheeses were produced according to standard Cremoso technology (ZALAZAR *et al.*, 1999). Ripening was carried out at 7°C and 80% relative humidity over 30 days in the Instituto de Lactología Industrial (INLAIN-FIQ) located in Santa Fe (Argentina). Considering that proteolysis indices are influenced by factors other than ripening time, such as temperature of ripening, moisture content of the cheese and the milk coagulant and starter used, these conditions were held strictly constant in the production of these cheeses and throughout the present study.

Cheeses were sampled (IDF, 1995) for individual analysis at different ripening times (Table 1). The physicochemical parameters were determined at all the ripening times, whereas the overall composition was only performed after 30 days of ripening.

Cheese composition

The overall composition was assessed by determining moisture (IDF, 1982), fat (BRADLEY *et al.*, 1993) and protein content (IDF, 1993).

Cheese analysis

The physicochemical parameters analysed were: moisture content, pH, soluble nitrogen at pH 4.6 (pH 4.6-SN), in 12% tricloroacetic acid (TCA-SN), in 2.5% phosphotungstic acid (PTA-SN) and the α_{S1}-I-casein/(α_{S1}-casein + α_{S1}-I-casein) ratio. Cheese ripening was monitored for 30 days because the sensory characteristics of this cheese decrease significantly after this length of time.

pH. Grated cheese (10 g) was thoroughly blended with 10 mL H_2O using a mortar and pestle. The pH of the resulting slurry was measured with a pH meter (ES16 Titriskop, Metrohn Herisau, Switzerland).

Soluble nitrogen. Cheese samples were treated to obtain a crude citrate extract (HYNES *et al.*, 2001). This extract was obtained by adding 20 mL of 0.5M sodium citrate to 10 g of cheese and grinding to homogeneity using a pestle. Deionised water was added to ~90 mL and the pH was adjusted to 4.6. After centrifugation (3,000 g/15 min), the volume of the soluble fraction was adjusted to 100 mL. 12% TCA and 2.5% PTA soluble fractions were obtained from the pH 4.6 soluble fraction (GRIPON *et al.*, 1975). Nitrogen contents were determined in duplicate by the macro-Kjeldahl method (IDF, 1993). These values are expressed as percentage of total nitrogen.

α_{S1}-I-casein/(α_{S1}-casein + α_{S1}-I-casein) ratio. Samples of cheese caseins were prepared by precipitation at pH 4.6, purification and lyophilisation. The insoluble residue at pH 4.6 was analysed by Urea-PAGE in a Mini Protean II cube (Bio-Rad Laboratories, California, USA) using the method of ANDREWS (1983) with 7.5% acrylamide (HYNES *et al.*, 1999). Proteins were stained with Coomassie Blue G-250. The protein bands were quantified by densitometric analysis using a Minidensit densitometer (SEAC, Firenze, Italy) at 632 nm.

Sensory evaluation

Sensory examination was performed on all cheeses at the end of ripening to establish if their general characteristics were typical of Cremoso cheese, according to Argentinean Regulations (CAA, 2007).

Statistical analysis

Analysis of variance (ANOVA) with Statgraphics Plus 3.0 (Manugistics, Inc., Rockville, MD, USA) was used as a first step to determine whether treatment (ripening time, season and brand) had a significant impact on the physicochemical parameters. When differences were found ($p \leq 0.05$), means were compared by the least significant difference (LSD) test using the same statistical tool.

Table 1 - Number of cheeses sampled at each ripening time.

Days	0	9	15	22	30	Total
Cheeses sampled	8	4	4	4	8	28

Subsequently, the data matrix was analysed with multivariate techniques (Principal component analysis-PCA and Discriminant analysis-DA) using the software SPSS 10.0 (SPSS Inc., Chicago, USA) in order to evaluate the evolution of the physicochemical parameters. PCA was performed after standardising the variables (mean = 0; SD = 1).

Multiple linear regression (MLR), principal component regression (PCR) and PLS are the conventional statistical methods used for modelling quantitative relationships between two blocks of variables Y and X. MLR delivers the optimal fit according to the least-squares criterion when there is no multicollinearity between the X variables. PCR and PLS may be used when multicollinearity is present among the variables in the X block or when the number of samples is small (CARPINO *et al.*, 2002; HAIR *et al.*, 1999). PCR finds components (t_i) that capture the greatest amount of variance in the predictor variables of the X block without using the information contained in the response variables. However, PLS calculates components (t_i) which capture the maximum variance of block X and simultaneously achieves maximum correlation with response variables (MARTENS and NAES, 1993).

In this study, MLR could not be used because there was a strong correlation (multicollinearity) between the predictor variables, thus violating one of the main assumptions of MLR. With regard

to PCR and PLS, the latter was chosen because of its emphasis on predicting the responses and not necessarily on trying to understand the underlying relationship between the variables.

PLS regression (Unscrambler 7.6, Camo, ASA, Olso, Norway) was applied to the cheese samples (n = 28). This procedure typically uses cross-validation, a method of internal validation using the original data set, to test for the optimal number of principal components to be used. The data matrix was divided into calibration samples (n = 19) and validation samples (n = 9); this latter set was chosen by random selection. Calibration samples were used to study the relationship between ripening time and physicochemical parameters in order to obtain a prediction model using the following equation:

$$t = b_0 + \sum_{j=1}^{n} b_j x_j$$

where t was the ripening time, x_j were the physicochemical parameters and b_0 and b_j were the regression coefficients in the models.

The validation samples were used to test the predictive ability of the models obtained. The quality of the regression models was evaluated by the coefficient of regression (R) and the root mean square error of prediction (RMSEP) defined as follows:

$$RMSEP = \left(\sum_{i=1}^{n} (t_i - t_{(i)})^2/n\right)^{1/2}$$

Table 2 - Overall composition values obtained at the end of ripening.

	Moisture content	Protein content	Fat content
X	48.6%	20.6%	25.5%
SD	1.3	1.3	1.6

where, t_i is the real ripening time, $t_{(i)}$ the predicted ripening time obtained with the model for the *i*th validation sample and *n* the number of cheese samples of the validation set.

RESULTS AND DISCUSSION

Physicochemical parameter evolution

Table 2 shows the gross composition values obtained for all cheeses at 30 days of ripening. These values were within the normal ranges established by the Argentinean Regulations for Cremoso cheese (CAA, 2007). Furthermore, all of the cheeses showed a satisfactory and typical sensory quality for this cheese variety. Therefore, it was valid to use these cheeses to develop a prediction model.

Electrophoretic urea-PAGE patterns of pH 4.6 insoluble fractions for some calibration cheeses at 0, 9, 22 and 30 days are presented in Fig. 1. The α_{s1}-casein degradation was extensive and showed a concomitant increase of the α_{s1}-I-casein fraction, a peptide resulting from the breakdown of casein by residual chymosin. For this reason, the α_{S1}-I-casein/(α_{S1}-casein + α_{S1}-I-casein) ratio is expected to increase significantly with cheese age.

The ANOVA and LSD analysis showed that all the variables studied, with the exception of moisture content, changed significantly with ripening time ($p \leq 0.05$) over the ripening period (Table 3). Changes in the values of these variables with ripening time were evaluated. For example, %pH4.6-SN/TN and the α_{S1}-I-casein/(α_{S1}-casein + α_{S1}-I-casein) ratio in cheese sampled at 0, 9, 15, 22 and 30 days is shown in Figs. 2A and 2B and is used to describe the changes throughout ripening. The %pH4.6-SN/TN shows a strong linear progression (R = 0.95) with the ripening time for all the studied ripening periods (Fig. 2A). The α_{S1}-I-casein/(α_{S1}-casein + α_{S1}-I-casein) ratio showed a similar evolution over the

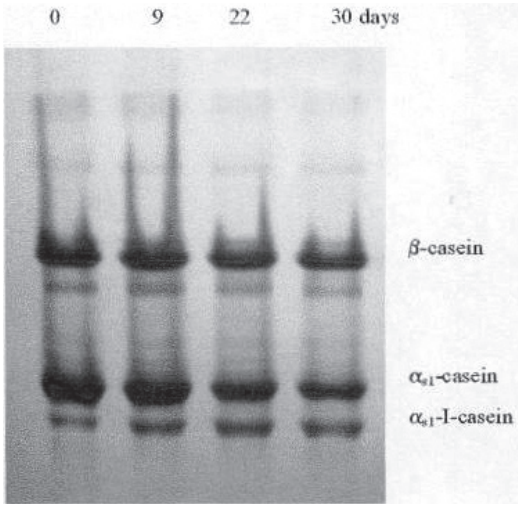

Fig. 1 - Urea polyacrylamide gel electrophoresis of 0- 9- 22- and 30-days-old Cremoso cheeses.

ripening period (Fig. 2B). These changes are in agreement with the fact that the hydrolysis of α_{s1}-casein in the Phe_{23}-Phe_{24} bond is the most important transformation of this cheese variety during ripening and consequently these fractions increase with the cheese age. Increase of the α_{S1}-I-casein/(α_{S1}-casein + α_{S1}-I-casein) ratio with ripening is, however, limited to the first days of ripening because the α_{s1}-I-casein peptide is usually hydrolysed for longer periods. Since Cremoso cheese has a very short ripening period (30 days), the relationship between the α_{S1}-I-casein/(α_{S1}-casein + α_{S1}-I-casein) ratio and ripening time can be considered linear. This statement however may not be true for cheeses with longer ripening times. The %TCA-SN/TN increased slowly up to day 9 and then more quickly to the end of ripening (result not shown). An important increase in %PTA-SN/TN was observed up to day 22 (result not shown). These two nitrogen fractions contain medium- and small-size peptides and free amino acids which appear at the end of ripening as products of secondary proteolysis. The pH showed a weak progression throughout ripening (result not shown).

The ANOVA analysis of all the studied variables with seasons (data not shown) showed that Cremoso cheeses manufactured in different seasons differed significantly in moisture content (p=0.03), %TCA-SN/TN (p=0.02) and %PTA-SN/TN (p=0.01); however, these differences may not be practically significant because the p-values were very close to the significance level. The %TCA-SN/TN and %PTA-SN/TN values from spring were slightly higher than those from winter and autumn; these results may have been related to the higher psychrotrophic bacterial counts found in raw milk in Argentina during the hotter seasons (REINHEIMER *et al.*, 1985). The endo- and exo-enzymes of the psychrotrophic bacteria may survive normal pasteurisation and sterilisa-

Table 3 - Physicochemical parameter values of cheeses during ripening (Means and SD).

Parameters	*p-value*	Ripening time (days)				
		0	9	15	22	30
%Moisture	0.27	49.82±0.96	49.17±1.03	49.27±1.58	48.56±1.18	48.57±1.25
pH	0.02	5.18±0.07^{a}	5.24±0.07^{a-b}	5.21±0.07^{a-b}	5.34±0.05^{b-c}	5.26±0.09^{b-c}
%pH4.6-SN/TN	0.00	5.06±0.34^{a}	6.72±0.55^{b}	7.26±0.18^{b}	8.81±0.42^{c}	10.01±0.70^{d}
%TCA-SN/TN	0.00	2.38±0.38^{a}	2.96±0.20^{a}	3.59±0.68^{b}	4.58±0,75^{b}	5.28±0.46^{c}
%PTA-SN/TN	0.00	0.81±0.26^{a}	1.00±0.14^{a-b}	1.21±0.38^{b}	1.24±0.17^{b}	1.65±0.25^{c}
α_{S1}-I-Cn/(α_{S1}-Cn + α_{S1}-I-Cn)	0.00	0.05±0.04^{a}	0.16±0.07^{b}	0.18±0.01^{b}	0.31±0.09^{c}	0.44±0.10^{d}

$^{a-d}$ Means in rows with different superscripts differ (P≤0.05) according to the LSD test.

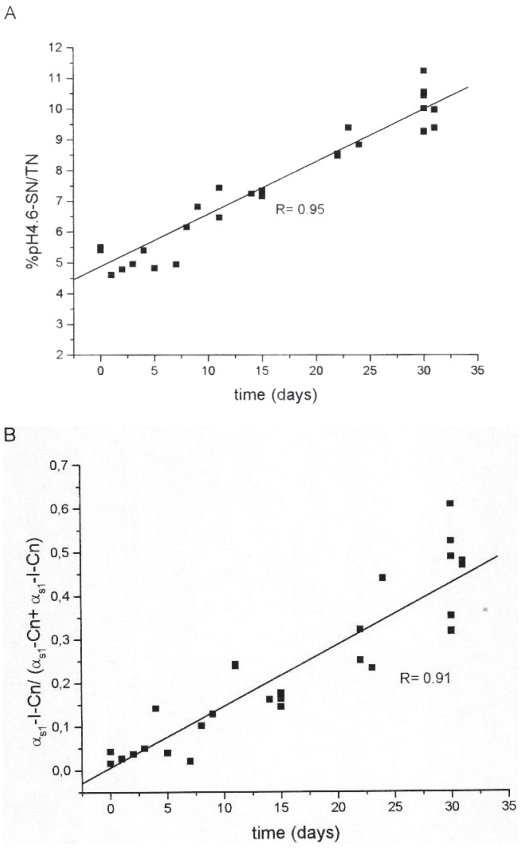

Fig. 2 - Changes in physicochemical parameters in cheese samples during ripening: (A) %pH4.6-SN/TN and (B) α_{S1}-I-Cn/(α_{S1}-Cn + α_{S1}-I-Cn) ratio.

tion temperatures, and are likely to remain active in cheeses throughout the ripening period (ZALAZAR *et al.,* 1986). Significant differences in moisture content with seasons could be attributed to differences in manufacturing technology.

The ANOVA results of all variables studied showed no significant differences between brands (data not shown).

Multivariate data analysis: PCA and DA

The results of the physicochemical parameters of the twenty-eight cheese samples were used in the construction of the multivariate data matrix, resulting in a data matrix of 28 samples and 6 variables. Exploratory data analysis of physicochemical parameters was performed to evaluate the influence of age, season and brand on ripening of Cremoso cheese. The Kaiser-Meyer-Olkin value of sampling adequacy obtained was > 0.5 (0.823), therefore, the application of PCA on the data matrix was justified (HAIR *et al.,* 1999). The number of required components according to the Kaiser criterion (eigenvalue ≥ 1) was 2 but three factors were chosen because the information regarding pH was contained in PC_3. These three components accounted for 94% of the total variability (PC_1 66.7%, PC_2 16.7% and PC_3 10.6%). All variables studied were considered in the PCA analysis.

Fig. 3 shows the score plot, defined by PC_1 and PC_2, and the samples identified by ripening time. The factorial map shows the differentiation of cheese samples based on ripening time, mainly according to PC_1. In this factor, the highest loading values belonged to the three nitrogen fractions and the α_{S1}-I-casein/(α_{S1}-casein + α_{S1}-I-casein) ratio; the grouping of cheeses on this axis indicates that it was largely on the basis of these variables.

The score plot of PC_2 vs PC_3 (results not shown) showed that the cheese samples were loosely grouped by season along PC_2 and PC_3. Regarding the PC_2 and PC_3 loadings, moisture content and pH had medium loading values, so the information about these variables was divided between these two PCs.

No grouping of cheese samples was observed in relation to brand.

In order to maximise the loading of each variable in only one factor, the rotated solution (VARIMAX) was performed. This rotation only affects the distribution of the proportions of the total variance explained by each factor; the cumulative proportion of the total variance explained for all factors does not change. The factorial map of rotated factors shows that the cheese samples were again grouped along PC_1 on the basis of ripening time (results not shown). The three nitrogen

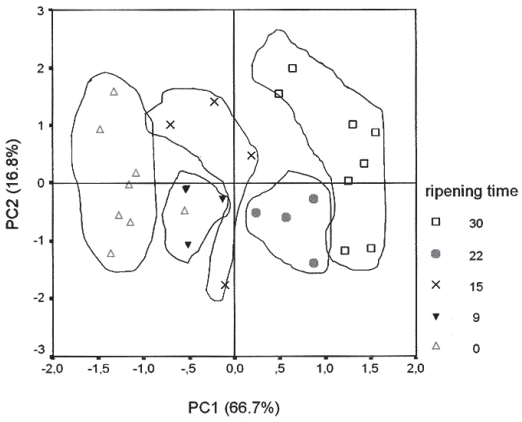

Fig. 3 - 2-dimensional representation of scores on PC1 and PC2 after PCA of the physicochemical parameters.

fractions and the α_{S1}-I-casein/(α_{S1}-casein + α_{S1}-I-casein) ratio had the highest loadings in this PC (Fig. 4) and were positive. This means that these variables increased during ripening.

It can be observed that cheeses tended to cluster on the basis of production season along the rotated PC_2 and only moisture content had a high loading value in this PC (Fig. 4). Therefore, according to multivariate analysis, moisture content was the most important variable in grouping the samples based on seasons. These results were in accord with the univariate analysis even though the differences in moisture content observed between seasons were weak.

No grouping by brand was observed.

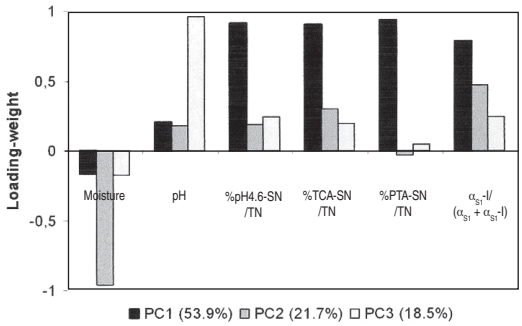

Fig. 4 - Loading weight values of rotated PC_1, PC_2 and PC_3 after PCA of the physicochemical parameters.

pH had a high loading in PC_3 which did not give information on any treatment studied (Fig. 4).

In order to validate the previous conclusion based on the visual observation of scores, the discriminant model was applied using PC_1, PC_2 and PC_3 scores as predictors. Ripening time and seasons were used as classification factors. Samples were classified according to the group for which they had the best match. This was expressed in terms of the percentage of samples assigned to the correct class. A significant discriminant function ($p \leq 0.05$) was found with respect to the time factor. This function allowed 5 pre-defined groups (0, 9, 15, 22 and 30 days of ripening) to be classified; 86% of the cases were classified correctly.

The situation was not the same for the seasons. No significant discriminant function was observed; it was not possible to distinguish between the 3 pre-determined seasons (autumn, winter and spring) when a PCA analysis was made for all the samples.

In summary, there was no grouping of cheese samples according to brand. The grouping of samples by seasons was poor and did not influence differentiation based on ripening time. Consequently, cheese samples of different brands and seasons could be used to obtain only one more robust ripening time prediction model. Moreover, this multivariate analysis allowed appropriate variables to be selected for subsequent application to other statistical method. These variables were the three nitrogen fractions and the α_{S1}-I-casein/(α_{S1}-casein + α_{S1}-I-casein) ratio which grouped the samples according to cheese age on the basis of the biochemical processes that occur during the ripening of this cheese variety.

Prediction of ripening time by PLS regression

PLS regression was applied to the calibration sample set (n = 19) to get a rip-

ening time prediction model for Cremoso cheese. The moisture content, pH, %pH4.6-SN/TN, %TCA-SN/TN, %PTA-SN/TN and the α_{S1}-I-casein/(α_{S1}-casein + α_{S1}-I-casein) ratio were used as predictor variables to obtain the full model. A reduced model based on the last four variables was studied. These models were then applied to the validation set (random selection validation).

Table 4 reports the statistical parameters from the PLS regression. These results include the intercept *(b_0)*, standardised regression coefficients *(b_j)*, the non-standardised regression coefficients (b_{uj}), the correlation coefficient (*R*), and the root mean squares error of prediction (RMSEP). As can be seen, the reduced model had a higher correlation and lower error (RMSEP) than the full model. This result was in agreement with the PCA in that the three nitrogen fractions and the α_{S1}-I-casein/(α_{S1}-casein + α_{S1}-I-casein) ratio were the variables that grouped the cheese samples according to ripening time.

A plot of the real cheese age and that predicted from the reduced model is shown in Fig. 5, there is a high correlation between the real and predicted ripening time (R = 0.99).

In the reduced PLS model, %pH4.6-SN/TN and the α_{S1}-I-casein/(α_{S1}-casein + α_{S1}-I-casein) ratio were the variables with the highest non-standardised regression coefficients. These variables were the most important for predicting the ripening time

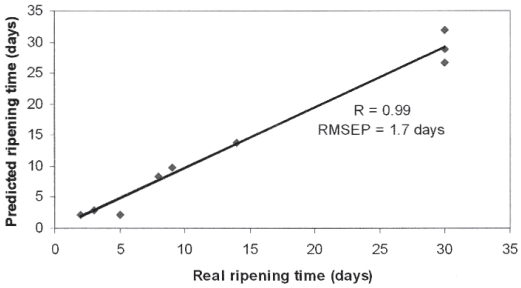

Fig. 5 - Correlation between the predicted ripening time, obtained using a reduced PLS model, and the real ripening time in the cheese samples (n = 9).

of Cremoso cheese, in agreement with the fact that these fractions are indicators of primary proteolysis, the principal event that occurs in this cheese variety. %TCA-SN/TN and %PTA-SN/TN were the next most important factors; this is in accord with the previously obtained results. Moreover, the regression coefficients were positive, which indicated that these variables increased during the ripening process.

CONCLUSION

ANOVA and PCA of the physicochemical parameters changed during ripening time; this was due to the previously described biochemical changes that occur in Argentinean soft cheeses. The %pH4.6-SN/TN and the α_{S1}-I-casein/(α_{S1}-casein + α_{S1}-I-casein) ratio were the most impor-

Table 4 - Statistical parameters from full and reduced PLS models.

	b_j (b_{wj})								
	b_0	Moisture	pH	%pH 4.6-SN/ TN	%TCA-SN/ TN	%PTA-SN/ TN	α_{S1}-I/(α_{S1} + α_{S1}-I)	R	RMSEP
full model	-75.3	0.23 (0.28)	9.31 (0.86)	1.82 (3.54)	1.78 (2.33)	6.22 (2.44)	16.36 (2.75)	0.98	2.4
reduced model	-14.61	-	-	2.48 (4.82)	1.45 (1.91)	0.68 (0.27)	24.10 (4.05)	0.99	1.7

b_0: intercept; b_j: standardised regression coefficients; b_{wj}: non-standardised regression coefficients; R: correlation coefficient; RMSEP: root mean squares error of prediction.

tant variables for predicting the ripening time; this is in agreement with the fact that these fractions are indicators of primary proteolysis, the principal biochemical event that occurs in this cheese variety. By applying PLS regression to variables selected by PCA, the ripening time of Cremoso cheese could be predicted with a prediction error of 2 days. These parameters can be measured rather simply and quickly so the ripening time of the cheeses is very easy to calculate. The models suggested in this study were developed for two specific local products of Argentina; their applicability for similar cheeses made in other processing plants has yet to be established.

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PAPER

PRELIMINARY CHARACTERIZATION OF STAPHYLOCOCCAL, MICROCOCCAL AND YEAST ISOLATES OBTAINED FROM RAW COW MILK CHEESES CURRENTLY PRODUCED IN GALICIA (NW SPAIN)

CARATTERIZZAZIONE PRELIMINARE DI ISOLATI DI STAFILOCOCCHI, MICROCOCCHI E LIEVITI OTTENUTI DA FORMAGGI DI BOVINI PRODOTTI NEL NORD OVEST DELLA SPAGNA (GALIZIA)

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ABSTRACT

One-hundred-sixty-one microbial isolates were obtained from the predominant halotolerant microflora of 20 raw-milk cheeses selected on the basis of their sensorial profiles. Of these, twenty-six isolates were identified as *Staphylococcaceae*, 17 as *Micrococcaceae* and 50 as yeasts. The remaining 68 isolates belonged to other bacterial groups and

RIASSUNTO

Centosessantuno microrganismi alotolleranti sono stati isolati da 20 formaggi da latte crudo selezionati in base ad un preciso profilo sensoriale. Di questi, ventisei ceppi sono stati identificati come appartenenti al genere *Staphylococcus*, 17 come *Micrococcaceae* e 50 come lieviti. I 68 ceppi rimanenti appartengono agli altri gruppi batterici, essendo 49

- Key words: cheese flavour, *Micrococcaceae*, raw-milk cheeses, *Staphylococcaceae*, yeasts -

most (49) were probably lactic acid bacteria. Twenty-seven of the 93 staphylococcal, micrococcal and yeast isolates produced butyric or rancid flavours in pasteurised whole milk. Flavours defined as fruity, "must-wine" or "bakery" were imparted by 13 yeast isolates. Among the 40 (27+13) selected isolates, 23 showed exocellular proteolytic activity, 21 showed exocellular lipolytic activity and 23 were able to grow at 8°C. It was concluded that selected halotolerant strains should be included in adjunct cultures to enhance the flavour profiles, and therefore, the typical characteristics of Galician cheeses.

degli stessi probabilmente batteri lattici. Ventisette dei 93 isolati di stafilococchi, micrococchi e lieviti hanno prodotto aromi descritti come butirrico o rancido in latte intero pastorizzato. Tredici lieviti hanno prodotto aromi descritti come fruttato, "mosto-vino" e "forno". Su 40 (27+13) ceppi selezionati, 23 hanno mostrato attività proteolitica extracellulare, 21 hanno mostrato attività lipolitica extracellulare e 23 erano idonei a crescere a 8°C. Si può concludere che i ceppi alotolleranti selezionati potrebbero essere aggiunti alle colture starter per migliorare il profilo aromatico e quindi la tipicità dei formaggi della Galizia.

INTRODUCTION

A large proportion of the secondary microflora (non-lactic acid bacteria) present in non-smear-ripened raw-milk cheeses is constituted by halotolerant and usually psychrotrophic microorganisms. Among such microorganisms, *Staphylococcaceae* and *Micrococcaceae* appear to predominate; in some cases, Coryneform bacteria and yeasts are also present in large numbers. These microbial groups may play an important role in the biochemical processes that take place during cheese ripening, because of their complex enzymatic activities (MASSA and TURTURA, 1989; BHOWMIK and MARTH, 1990; BINTSIS *et al.*, 2003; FADDA *et al.*, 2004).

In a previous study (CENTENO *et al.*, 1995), presumptive micrococcal isolates were obtained from raw-milk Arzúa cheese (renamed Arzúa-Ulloa in 1997) and selected strains were used in the manufacture of pasteurised-milk Arzúa and Tetilla cheeses (CENTENO *et al.*, 1996; MENÉNDEZ *et al.*, 2004). Typical raw-milk cheese flavours were detected, particularly in the experimental batches characterised by more acute lipolysis.

The aim of the present study was to obtain new isolates of microorganisms from the secondary microflora of the four Protected Designation of Origin (PDO) raw-milk starter-free cheeses currently recognized in Galicia (Arzúa-Ulloa, Tetilla, Cebreiro and San Simón da Costa), in order to increase the available microbial isolate pool with characteristics that are of interest for manufacturing these cheeses.

MATERIALS AND METHODS

Cheese sampling

A total of 60 raw-milk cheeses belonging to the Arzúa-Ulloa, Tetilla, Cebreiro and San Simón da Costa PDOs (15 cheeses of each variety), manufactured without added starters in different dairies in the spring of 2004 and 2005, were judged by a 5-member panel trained in testing traditional raw-milk cheeses. Six Arzúa-Ulloa cheeses, 4 Tetilla cheeses, 6 Cebreiro cheeses and 4 San Simón da Costa cheeses were selected for the study on the basis of their typical flavours. The cheese that remained after the sensorial test (500-750 g of each) was transported to the laboratory under refrigeration and

stored at 4±1°C until analysis (within 24 h). Microbiological analyses were carried out on 20 g samples removed aseptically with a sharp sterilised knife from the central part (pointed end) of 100-150 g wedges of cheese. Samples included the surface, which was previously wiped with 70% (v/v) undenatured ethanol.

Microbiological analysis and determination of cheese pH

The samples (20 g) for microbiological analysis were homogenized in a Masticator® peristaltic blender (IUL, Barcelona, Spain). Sample preparation and decimal dilutions were carried out in accordance with IDF Standard 122C (IDF, 1996). Corresponding dilutions were surface-inoculated in duplicate on tryptone soy agar (TSA) (Oxoid, Basingstoke, UK) with the addition of 6% NaCl; plates were incubated at 30±1°C for 5 days.

The pH of each cheese was measured in duplicate on 10 g samples taken as described for microbiological analysis but without aseptic handling. Samples were homogenised in a peristaltic blender in 20 mL of distilled water preheated to 70±1°C and then cooled to room temperature before the pH was measured electrometrically.

Isolation, maintenance and phenotypic identification of isolates

Three to six colonies were selected at random from plates corresponding to the highest dilutions at which growth occurred; these were purified by subculturing in tryptone soy broth (TSB) (Oxoid, Basingstoke, UK) and TSA. Yeast isolates and gram-positive, catalase-positive cocci grouped in pairs, tetrads or irregular clusters, were maintained at -20°C in a milk-based medium with the addition of glycerol (25% v/v) (CENTENO *et al.*, 1995).

Isolates of gram-positive, catalase-positive cocci were phenotypically assigned to the family *Staphylococcaceae* or *Micrococcaceae* based on the results of the following tests: a) growth on furazolidone (FTO) agar (VON RHEINBABEN and HADLOK, 1981); b) resistance to lysostaphin (LACHICA *et al.*, 1971) and c) resistance to bacitracin (BAKER *et al.*, 1986). Selected (technologically interesting) micrococcal or staphylococcal isolates were phenotypically identified after checking pigment production on TSA, by using the ID 32 Staph micromethod (bioMérieux, Marcy l'Etoile, France); selected yeast isolates were identified by the RapID Yeast Plus System micromethod (Remel, Lenexa, KS, USA).

Evaluation of properties of technological interest

Isolates of *Staphylococcaceae* (27), *Micrococcaceae* (17) and yeasts (50) were tested for flavours produced in (100 mL) pasteurised (100±1°C, 15 min) whole (3.8% fat) milk inoculated at 1% with (24 h) cultures in supplemented milk (skim milk +0.5% tryptone +0.5% yeast extract) and incubated at 30±1°C for 48 h. Flavours were perceived by smelling by a panel of seven judges – including three connoisseurs of raw-milk cheeses – proposed by the PDO Regulating Councils. The panel members had been trained for sensory evaluation of cheese in accordance with IDF Standard 99C (IDF, 1997), and were specifically trained in the test carried out in the current study, in accordance with the methodology proposed by GUERRA *et al.* (1999). The assessors were initially requested to propose the terms that best described the flavours of (100) microbial cultures prepared in whole pasteurised milk, as previously indicated. The cultures used for training (10 cultures per session) included lactic acid bacteria (20 lactococci, 10 mesophylic lactobacilli and 10 enterococci), staphylococci (20), micrococci/kocuria (20), and yeasts (20), all of which were previously isolated from raw-milk cheeses. Several chemical compounds were also used to compare the sensorial perceptions:

diacetyl (20 ppm, in whole milk) for buttery flavour; isoamylic alcohol (20 ppm) for malty flavour; butyric acid (20 parts per thousand) for butyric flavour; and isovaleric acid (100 ppm) for barny flavour. New descriptors (must-wine and bakery) were also introduced during the evaluation sessions performed in the current study. A final list of 20 flavour descriptors (the 12 listed in Table 2 as well as: sour, yoghurt, vanilla, nutty, acetic acid, sharp, barny and putrid) was drawn up.

Selected isolates of *Staphylococcaceae*, *Micrococcaceae* and yeasts were characterised for the following: a) haemolysis on sheep blood agar (CENTENO *et al.*, 1995); b) exocellular proteolytic activity on pH 6.0 calcium caseinate agar (CENTENO *et al.*, 1995); c) exocellular lipolytic activity on pH 6.0 tributyrin agar supplemented with arabic gum powder (1 g/L) (DURLU-OZKAYA *et al.*, 2001); and d) ability to grow at 8±1°C on pH 6.0 TSA (CENTENO *et al.*, 1995). All tests were carried out in duplicate.

RESULTS AND DISCUSSION

Microbial counts and pH of cheese

The results obtained for the halotolerant microflora counts and pH of the selected cheeses are shown in Table 1. Microbial counts for Arzúa-Ulloa and Tetilla cheeses, which are manufactured by very similar methods (from rennet curds), but differ in shape, were similar to those reported in previous studies (i.e. 6-7 log units) (CENTENO *et al.*, 1994; MENÉNDEZ *et al.*, 2001). Microbial counts in Cebreiro cheese, obtained mainly from an acid curd, were higher than the counts on manitol salt (7.5%) agar reported in a previous study (mean value 4.7 log units for 30 cheeses) (CENTENO *et al.*, 1996a). This may be explained by the higher concentration of salt in the culture medium and the lower pH (mean value 4.4) of the cheeses analysed in the previous work. Counts of halotolerant microflora in San Simón da Costa cheese (until 1999 known as San Simón), ripened for at least 45 days and then smoked, were lower than those reported by GARCÍA *et al.* (2001) for cheeses younger than those analysed in the present study (6-7 log units for 5 batches ripened for 6 weeks). The San Simón da Costa cheeses had the highest mean pH values of the cheeses under study, which may have been due to the longer ripening period and probably, more intense proteolysis. It should also be pointed out that halotolerant microflora counts were generally higher in the cheeses with shorter ripening periods; this is consistent with the previously reported decrease in the number of these microorganisms throughout the ripening period (CENTENO *et al.*, 1994; GARCÍA *et al.*, 2001).

Phenotypic classification of isolates

Of the 161 purified isolates obtained in the present study, 43 were gram-positive, catalase-positive cocci (26 were identified as *Staphylococcaceae* and 17 as *Micrococcaceae*) and 50 were identified as yeasts (Table 2). The rest of the isolates (data not shown) included 31 gram-positive, cata-

Table 1 - Halotolerant microflora counts (log cfu/g) and pH of the selected raw-milk cheeses (values expressed as mean ± standard deviation).

Type of Cheese	Number of samples	Ripening time (days)	Halotolerant microflora count	pH
Arzúa-Ulloa	6	14-60	6.42±0.61	5.04±0.22
Tetilla	4	14-60	6.57±0.27	4.98±0.17
Cebreiro	6	14-28	6.88±0.34	4.80±0.30
San Simón da Costa	4	60-120	5.56±1.08	5.33±0.20

lase-negative cocci (presumably enterococci), 19 gram-positive, catalase-positive rods and 18 gram-positive, catalase-negative rods (presumably lactobacilli).

The high proportion of yeast isolates obtained in the present study, especially from Cebreiro cheese (34/42), is noteworthy. It has been suggested that halotolerant yeasts may play an important role in the ripening of different types of non-smear-ripened raw-milk cheeses (BINTSIS *et al.*, 2003; CORBO *et al.*, 2001; FOSCHINO *et al.*, 2006).

Technological characterisation and phenotypic identification of micrococcal, staphylococcal and yeast isolates

All 93 micrococcal, staphylococcal and yeast isolates obtained in the present study were initially evaluated by smelling the flavours produced in pasteurised whole milk after 48 h of incubation at 30°C. The results are reported in terms of the predominant flavour or combination of flavours produced by each strain of the milk cultures detected by at least four of the seven assessors (Table 3).

The most frequently detected lactic flavour was buttery (a flavour reminiscent of ripe butter or diacetyl), which was predominantly perceived by itself or in combination with other flavours in 18 of the milk cultures. It has been reported that *Micrococcaceae* strains isolated from milk products can produce different amounts of diacetyl in milk (TOURNEUR and DAUDIN, 1983). Some lactose-fermenting yeasts use citrate as a substrate, with the possible formation of diacetyl (CORBO *et al.*, 2001).

Table 2 - *Staphylococcaceae*, *Micrococcaceae* and yeast isolates obtained from the selected cheeses (percentages of the total in brackets).

	Staphylococcaceae	*Micrococcaceae*	Yeasts	Other microbial groups	Total n. of isolates
Arzúa-Ulloa	17	5	8	34	64
Tetilla	8	6	7	18	39
Cebreiro	-	4	34	6	42
San Simón da Costa	1	2	1	12	16
Total n. of isolates	26	17	50	68	161
(%)	(16%)	(11%)	(31%)	(42%)	(100%)

Table 3 - Predominant flavour or combination of flavours (perceived by at least four of the seven assessors) produced by the micrococcal, staphylococcal and yeast isolates cultured in whole pasteurised milk for 48 h.

Main flavour	*Staphylococcaceae*	*Micrococcaceae*	Yeasts
Buttery	5	2	4
Buttery + Malty	2	2	1
Buttery + Sulphide	-	-	2
Malty	2	-	4
Butyric	4	5	6
Rancid	4	5	3
Alcoholic + Fruity	-	-	8
Alcoholic + Must-wine	-	-	3
Yeasty + Bakery	-	-	2
Sulphide	2	1	12
Cooked milk	7	2	5

The malty flavour, probably caused by the presence of aldehydes and alcohols derived from the metabolism of branched-chain amino acids (URBACH, 1993), was described as the most intense flavour produced by 2 staphylococcal and 4 yeast isolates. Production of malty compounds, which has also been described for yeast isolates (ARFI *et al.*, 2004), should be considered negative for the selection of cheese cultures, since an excess of these compounds in cheese is generally perceived as an off-flavour (CENTENO *et al.*, 2002; WOUTERS *et al.*, 2002).

Twenty-seven of the isolates imparted butyric or rancid (a flavour reminiscent of ripe ewe's cheese) flavours, probably due to lipolytic activity with the release of free fatty acids. Flavours defined as alcoholic and fruity or "must-wine" (or "wine cellar"; a flavour reminiscent of must fermentation) were produced by 11 yeast isolates, and a characteristic flavour defined as "bakery" (also described as "raw-milk cheese rind"; a flavour reminiscent of flour fermentation) was imparted by two of the yeast cultures. These flavours were probably caused by esters formed by free fatty acids reacting with alcohols, and the must-wine nuance may be related to the presence of methyl-ketones produced by β-oxidation of free fatty acids (LECRERCQ-PERLAT *et al.*, 2004).

Sulphide flavours were predominant in 15 of the milk cultures, 12 of which were yeast cultures, and cooked milk was the main flavour for 7 staphylococcal, 2 micrococcal and 5 yeast cultures. Sulphide flavours may be derived from the metabolism of L-methionine (BONNARME *et al.*, 2001), and cooked milk flavour may have occured as a result of the heat treatment of the milk without any enzymatic intervention of the microorganisms.

Regarding technological performance, 56 isolates that predominantly produced buttery, malty, sulphide or cooked milk flavours were excluded from further analysis, because the connoisseurs of traditional cheeses did not consider these flavours to be typical of good-quality raw-milk cheeses. Thus, only 8 *Staphylococcaceae*, 10 *Micrococcaceae* and 22 yeasts, which produced flavours related to their lipolytic activities, were considered for further identification and characterisation.

The selected isolates were phenotypically identified as follows: 2 *Staphylococcus epidermidis*, 1 *S. equorum*, 1 *S. lentus* and 1 *S. xylosus* among the staphylococcal isolates, 5 *Kocuria varians* and 2 *Micrococcus luteus* among the micrococcal isolates, and 1 *Candida kefir*, 1 *C. lambica*, 1 *C. zeylanoide*, 2 *Candida* spp., 3 *Kluyveromices* spp., 1 *Rhodotorula rubra* and 1 *Saccharomyces* spp. among the yeast isolates. The rest of the isolates could not be correctly identified (data not shown). None of the selected isolates showed β-haemolytic activity on sheep blood agar (data not shown).

Among the 40 selected isolates, 23 showed exocellular proteolytic activity on casein, 21 showed exocellular lipolytic activity on tributyrin and 23 were able to grow at 8°C with all culture media adjusted to pH 6.0 (Table 4). Two staphylococcal isolates (identified as *S. epidermidis* and *S. equorum*) and 3 micrococcal isolates (all identified as *K. varians*) displayed strong proteolytic and lipolytic activities (hydrolysis halos > 2 mm width) and were able to grow at low temperatures.

High proportions of staphylococci isolated from raw-milk cheeses showing caseinolytic activity and lipolytic activity on tributyrin have been reported (CÁCERES *et al.*, 1997). Exocellular proteolytic and lipolytic activities have also been reported for 40 and 42 of the 88 presumptive micrococcal isolates obtained from Arzúa cheese (CENTENO *et al.*, 1995). In further studies in which several of these isolates were used (CENTENO *et al.*,1996b; MENÉNDEZ *et al.*, 2004), the

Table 4 - Exocellular proteolytic and lipolytic activities (pH 6.0) and ability to grow at low temperature of the selected staphylococcal, micrococcal and yeast isolates.

Microbial group	N. of strains	Proteolytic activity*			Lipolytic activity*			Growth at 8ºC
		+	++	+++	+	++	+++	
Staphylococcaceae	8	1	3	2	1	2	1	4
Micrococcaceae	10	1	4	3	1	4	1	6
Yeasts	22	4	4	1	9	2	-	13

*+: hydrolysis halos < 2 mm width; ++: halos between 2 and 5 mm; +++: halos > 5 mm.

authors concluded that selected lipolytic micrococcal strains should be used as adjunct cultures in the manufacture of Arzúa and Tetilla cheeses, because of the desirable effects they have on the cheese flavour.

Among the yeast isolates, 5 showed strong exocellular proteolytic activity and 2 others showed strong exocellular lipolytic activity; only 2 isolates (one *Kluyveromyces* spp. and a *Saccharomyces* spp.) displayed both types of activity and grew at 8°C. Both yeast isolates predominantly produced alcoholic and fruity flavours in pasteurised whole milk. High production of esters and a global fruity flavour in a model cheese medium have been reported for *K. marxianus* (LECRERCQ-PERLAT *et al.*, 2004).

Psychotrophic behaviour has been reported in a number of yeast strains isolated from cheese (CARRASCO *et al.*, 2006). It has also been reported that a large proportion of cheese yeasts produce esterases that release short-chain fatty acids, and proteases with caseinolytic activity (BINTSIS *et al.*, 2003; LECLERCQ-PERLAT *et al.*, 2004). The extent of lipolytic activity and casein breakdown patterns appear to be strain-dependent (LANCIOTTI *et al.*, 2005). Several authors (BINTSIS *et al.*, 2003; FADDA *et al.*, 2004; FERREIRA and VILJOEN, 2004; CARRASCO *et al.*, 2006) have proposed using selected yeasts as adjunct cultures for the manufacture of different types of cheese, in order to accelerate ripening and intensify typical cheese flavours.

CONCLUSIONS

Large numbers (6-7 log cfu/g) of halotolerant microorganisms identified as *Micrococcaceae*, *Staphylococcaceae* and yeasts were detected in raw cow milk cheeses produced in Galicia (Spain). Among these populations, many isolates produce interesting flavours in cheese-milk. Selected strains, especially those that show strong lipolytic activity and can grow at low (ripening) temperatures, could be an important source of microorganisms that could be included in adjunct cultures in the manufacture of Galician PDO cheeses. The use of such microorganisms could help enhance the typicality of the cheeses, particularly their flavour profiles.

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PAPER

FLOW PROPERTIES OF PETIT SUISSE CHEESES: USE OF CHEESE WHEY AS A PARTIAL MILK SUBSTITUTE

FLUIDITÀ REOLOGICA DEI FORMAGGI PETIT SUISSE:
UTILIZZO DI SIERO DI FORMAGGIO IN SOSTITUZIONE AL LATTE

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ABSTRACT

Petit Suisse cheeses were manufactured using whole milk (control cheese) or formulated milk in which whole milk was partially substituted by 30% (cheese 1) or 20% (cheese 2) whey retentate (WR) obtained by ultrafiltration. The physicochemical characteristics of whole milk, WR and milk-WR blends were evaluated. The flow properties, instrumental texture characteristics and syneresis index of the cheeses were also evaluated. The apparent viscosity of the cheeses was determined as

RIASSUNTO

I formaggi "Petit Suisse" sono stati ottenuti a partire da latte intero (formaggio di controllo) o latte formulato nel quale avveniva una sostituzione parziale del latte intero con 30% (formaggio 1) o 20% (formaggio 2) di siero ritentato di latte ottenuto tramite ultrafiltrazione. Sono state determinate le caratteristiche fisico-chimiche del latte intero, del WR e dei formulati di latte con WR. Sono state inoltre valutate la fluidità reologica, e gli indici di consistenza e di sineresi dei formaggi. La viscosità apparente è stata

- Key words: Petit Suisse cheese, rheology, syneresis, texture, whey retentate -

a function of an ascending - descending shear rate sweep (0.2-0.85 s^{-1}) in 1 h and as a function of time using a constant shear rate of 0.85 s^{-1} in a Brookfield viscosimeter at 8±1°C. All cheeses showed thixotropic behavior. Experimental data fitted both the Weltman and the Abu-Jdayil structural kinetic models well. The textural characteristics of hardness and consistency and the syneresis index of the cheeses incorporating WR were lower than that of the control cheese.

determinata come funzione ascendente (upward)-discendente (downward) della velocità di taglio (0,2-0,85 s^{-1}) nel corso di un'ora e come funzione del tempo usando una velocità di taglio costante di 0,85 s^{-1} utilizzando un viscosimetro Brookfield a 8±1°C. Tutti i formaggi hanno mostrato un comportamento tixotropico. I dati sperimentali coincidono con quanto indicato in entrambi i modelli cinetici strutturali di Weltman e Abu-Jdayil. Le caratteristiche di durezza, di consistenza e l'indice di sineresi dei formaggi ottenuti con l'uso di WR sono risultati ridotti rispetto al formaggio di controllo.

INTRODUCTION

Whey proteins are commonly used as ingredients in many types of food due to their excellent technological functionality and high nutritional value. They are used as textural ingredients in food preparations to increase firmness or cause gel formation after heating. The use of whey into milk derivatives would help the dairy industry reduce problems related to its disposal. Petit Suisse is a product that originated in France; cream, sugar and fruit are added to fresh quark (soft fresh cheese). Petit Suisse, with a smooth, creamy texture and mild dairy flavor, is ideal for snacks and desserts.

Ultrafiltration (UF) is a membrane screening technology used to concentrate or separate whey constituents, resulting in a retentate or concentrate (particles larger than the membrane pores) and a permeate or filtrate (particles smaller than the membrane pores) (ROSENBERG, 1995). Whey retentate (WR) has been used in yogurt, cheese and other dairy derivatives, resulting in various products of high nutritional and functional value, with high consumer acceptability (CASTRO and GERLA, 2005; ROSENBERG, 1995). The addition of WR affects the rheological properties and, consequently, the texture of these derivatives (AICHINGER *et al.*, 2003).

Fresh-type cheese has non-Newtonian fluid behavior, with viscoelasticity and thixotropy (HEMAR *et al.*, 2004; OMAR *et al.*, 1995), where viscosity depends on temperature, composition, stress, shear rate and time (ABU-JDAYIL, 2003). The Weltman model (WELTMAN, 1943) and the structural kinetic model proposed by ABU-JDAYIL (2003) are used to investigate dairy products whose viscosities are time-dependent.

Texture is an important parameter in the evaluation of cheese quality during storage; it is affected by composition (lipids, proteins and moisture), pH, temperature, salt content and the degree of structural breakdown (proteolysis and lipolysis) of the curd during the coagulation and fermentation stages (ATTAIE, 2005; KULMYRZAEV *et al.*, 2005). Ripened soft cheeses are classified as viscoelastic; their behavior can also be influenced by changes caused by incorporating raw materials that may interact

with the casein matrix in the curd (LOBATO-CALLEROS *et al.*, 2000). The textural characteristics can be evaluated by sensorial or instrumental methods (TRUONG *et al.*, 2002). The instrumental methods are based on the deformation of the material through a compression force that can be converted into stress-strain curves. Parameters are obtained that represent the physical properties of the material (KULMYRZAEV *et al.*, 2005) including hardness, consistency, force of adhesion and adhesiveness and recoverable energy (KONSTANCE, 1993).

During the storage of fermented dairy products, syneresis can be observed (AICHINGER *et al.*, 2003; LUCEY, 2001; HINRICHS *et al.*, 2004), which is the gradual expulsion of whey caused by the instability and contraction of the gel network (AICHINGER *et al.*, 2003). It is caused by low acidity (pH ≥ 4.6), the total solids content and the high incubation temperature of the product, as well as others factors (CASTILHO *et al.*, 2006; LUCEY *et al.*, 1997). Syneresis can influence the quality of the end product and consumer acceptability (CASTILHO *et al.*, 2006; TIJSKENS and DE BAERDEMAEKER, 2004; AICHINGER *et al.*, 2003).

The objective of this study was to evaluate the physicochemical characteristics, flow properties, instrumental texture characteristics and the syneresis index of two Petit Suisse cheeses manufactured from blends composed of 70 and 80% milk with 30% and 20% WR, respectively, in comparison to those of a control Petit Suisse cheese manufactured from whole milk.

MATERIALS AND METHODS

Materials

Skimmed milk, sucrose and milk cream (30% w/w of fat) were obtained commercially; the cheese whey was obtained from the enzymatic coagulation of milk with rennet (Chr. Hansen, Valinhos, SP, Brazil). A culture of mesophilic *Lactococcus lactis* subsp. *cremoris* and *Lactococcus lactis* subsp. *lactis* (R704, Chr. Hansen, Valinhos, SP, Brazil), guar (Kerry) and xanthan (Kerry) gums were used in the manufacture of Petit Suisse chesses. All the reagents used in the physicochemical analysis were of analytical grade.

Manufacturing of milk whey

Milk whey was manufactured in our laboratory by enzymatic coagulation of whole fat by adding 0.8 mL of rennet per liter of milk, and then incubating at 37°C for 40 min. The curd was cut with knives and the milk whey was obtained by filtration of the remaining mass. The milk whey obtained had a pH of 6.60.

Ultrafiltration (UF)

Cheese whey was submitted to UF until a Volume Reduction Factor (VRF) of 5.0. The UF process was carried out in an ultrafiltration unit (Reginox, São Paulo, SP, Brazil), using an organic membrane (Romicon®) in spiral conformation, with 4.7 m^2 of filter area, with an average retention cutoff point of 20,000 daltons. The operating temperature was 45°C, entrance and exit pressures were 3 bar and 1 bar, respectively, and the permeation flow was around 15 $L.h^{-1}.m^{-2}$. These values were obtained in preliminary tests.

Petit Suisse cheese production

The Petit Suisse cheese was manufactured with pasteurized skimmed milk (71°C/15 sec) followed by the addition of 0.5 mLL^{-1} mesophilic culture (R704, Chr. Hansen, Valinhos, SP, Brazil), 0.4 mL $CaCl_2$ (50%L^{-1}), 0.8 mL rennet (Chr. Hansen, Valinhos, SP, Brazil) and incubated at 30°C until pH 4.3. The curd was cut and added to 12% sucrose (w/w), 19% milk cream (w/w) (with 30% w/w

of fat), guar (0.07% w/w) and xanthan gums (0.03% w/w), to obtain a cream cheese. For the test samples, 30% of the milk was substituted with pasteurized WR (67°C/30 min) (cheese 1) and 20% of the milk for WR (cheese 2) before thermal treatment (71°C /15 sec).

Physicochemical characteristics

Skimmed milk, WR, blends of milk-WR and Petit Suisse cheeses were submitted to the following analyses: moisture (% w/w), ash (% w/w), total solids (% w/w), fat (% w/w), total proteins (% w/w) and acidity (%) according to AOAC (2005) and pH with pH meter (Metler Toledo, Model MP 220, Barueri, SP, Brazil). Carbohydrate values (% w/w) were obtained by taking the difference. All the analyses were performed in duplicate.

Physical properties of Petit Suisse cheeses

Flow property measurements

The flow property measurements of cheeses were conducted at 8°C using a Brookfield rotational rheometer (Brookfield Engineering Laboratories model DV III Ultra, Stoughton, MA, USA), with conical geometry (*spindle* SC 27) and collected using the Rheocalc 32 software version 2.5 (Brookfield Engineering Laboratories, Inc., Middleboro, MA, USA). The samples remained at rest for 15 min (8°C) prior to measuring their viscosity. The shear rate increased linearly from 0.2 to 0.85 (s^{-1}) in 30 min with a return of 0.2 s^{-1} in the subsequent 30 min. The measurements were performed in triplicate. Hysteresis was determined by calculating the area between the ascending and descending curves. The apparent viscosity of the cheeses as a time function was determined using the Weltman model (1) (WELTMAN, 1943) and the structural kinetic model proposed by ABU-JDAYIL (2003) (2), with a constant shear rate of 0.85 s^{-1} (equilibrium state) and using Origin® software version 6.0 (Microcal Software Inc., Northampton, MA, USA). The values of k and n were obtained by linear regression of equation 2. The Weltman model is based on an empirical equation (1) for constant shear rate,

$$\sigma = A - B.\log t \quad (1)$$

where σ is the shear stress (Pa), t is time (s), A and B are constants (Pa) which refer to the required initial stress and to the time coefficient of thixotropic breakdown, respectively (WELTMAN, 1943).

The structural kinetic model is presented in equation 2 for constant shear rate,

$$(\eta - \eta_\infty/\eta_o - \eta_\infty)^{1-n} = (n-1)\ kt + 1 \quad (2)$$

where: η is apparent viscosity at time t; η_∞ is the apparent equilibrium viscosity as $t \rightarrow \infty$ (non-structured state); η_o is the apparent initial viscosity at $t = 0$ (structured state); n is the order of the structure breakdown reaction and k can be considered to be a measure of the rate of structural breakdown, that is, the degree of thixotropy (ABU-JDAYIL, 2003).

Instrumental texture analysis

A universal testing machine (texturometer Stevens LFRA, Model TA1000, UK, England), with Interface software, was used to determine the parameters for hardness, consistency, force of adhesion, adhesiveness and recoverable energy from the penetration curve of cheeses 1, 2 and the control. A 30 mm acrylic conical sensor was used and the analysis was performed in a 50 mL sample container at 8°C. The measurements were made in triplicate, where the operation distance and speed were equal to 2.5 cm and 2.0 $mm.s^{-1}$, respectively. Hardness is defined by the maximum force during the cycle of the initial penetration and consistency; the area under this curve is the work required to achieve the deformation

that indicates the internal resistance of the product (AHMED *et al.*, 2005). The adhesion force is the maximum force of the return of the sensor (ERDEM, 2005; KONSTANCE, 1993) and the adhesiveness is the area of this curve, which represents the work required to remove the sensor from the sample (AHMED *et al.*, 2005). The recoverable energy, in turn, is the energy recovered during the decompression resulting from the first cycle of penetration (KONSTANCE, 1993).

Syneresis index

The syneresis index of cheeses 1, 2 and control were evaluated according to AICHINGER *et al.* (2003) with modifications. After 2 days of storage at 6°C, a 15 g sample was centrifuged at 5,700 g (Janetzki K-24 centrifuge, Jena, Germany) for 60 min, under refrigeration, in duplicate. The percentage of syneresis was calculated by the mass of whey separated from the gel network during centrifugation, divided by the initial mass of the cheese multiplied by 100 (AICHINGER *et al.*, 2003).

Statistical analysis

The mean values, standard deviation and analysis of variance (5% significance) were calculated with Statistic software version 6.0 (StatSoft, Inc. 1984-2001, Tulsa, OK, USA).

RESULTS AND DISCUSSION

Physicochemical composition

The physicochemical composition of milk, WR and blends of milk-WR used in the production of the Petit Suisse are listed in Table 1. The total solids, moisture, protein, fat and carbohydrate values in the milk samples were significant ($p>0.05$), as were the values obtained for the milk-WR blends. Only the WR samples had values that were lower ($p<0.05$) than those of the milk samples.

Rennet coagulation of milk in combination with fermentation is an effective way to dehydrate the resulting curd (HINRICHS, 2001); thus, the cheeses had higher total solids content ($p<0.05$) and lower moisture values ($P<0.05$) compared to the raw material used (Table 1 and Table 2). This resulted in increased proteic and carbohydrate contents ($p<0.05$) in the cheese samples. The increased lipid content of cheeses 1, 2 and the control were the result of the addition of milk cream (30% w/w of fat) in the Petit Suisse production process.

The protein content ($p<0.05$) in cheeses 1 and 2 (Table 2) decreased when WR was substituted. In the control cheese, the casein content was greater than the milk whey proteins because this cheese was made from milk only.

Table 1 - Mean (standard deviation) values for the composition of milk, WR and blends 1 and 2.

	Milk	WR	Blend 1	Blend 2
Total solids (% w/w)	8.98 (0.01) [b]	8.53 (0.02) [a]	8.99 (0.01) [b]	9.00 (0.02) [b]
Moisture (% w/w)	91.02 (0.01) [b]	91.47 (0.05) [a]	91.01 (0.02) [b]	91.00 (0.04) [b]
Ash (% w/w)	0.69 (0.01) [c]	0.62 (0.01) [a]	0.66 (0.01) [b]	0.67 (0.01) [b c]
Protein (% w/w)	3.22 (0.03) [b]	2.87 (0.01) [a]	3.13 (0.03) [b]	3.14 (0.04) [b]
Lipids (% w/w)	0.48 (0.01) [b]	0.34 (0.01) [a]	0.45 (0.01) [b]	0.46 (0.01) [b]
Carbohydrates (% w/w)	4.6 (0.03) [a b]	4.72 (0.03) [a]	4.75 (0.06) [b]	4.73 (0.08) [a b]
Acidity (% lactic acid)	1.30 (0.01) [a]	1.50 (0.00) [b]	1.37 (0.03) [a]	1.38 (0.03) [a]
pH	6.70 (0.00) [c]	6.47 (0.00) [a]	6.62 (0.00) [b]	6.62 (0.00) [b]

Whey Retentate (WR) VRF 5.
Blends: Blend 1 (70% milk and 30% WR); Blend 2 (80% milk and 20% WR).

Table 2 - Mean (standard deviation) values for the composition of Petit Suisse cheeses 1, 2 and Control.

	Cheese 1	Cheese 2	Control cheese
Total solids (% w/w)	24.45 (0.05)[a]	23.95 (0.10)[b]	23.78 (0.10)[b]
Moisture (% w/w)	75.53 (0.06)[b]	76.50 (0.10)[a]	76.22 (0.10)[a]
Ashes (% w/w)	0.73 (0.01)[a]	0.71 (0.02)[a]	0.72 (0.02)[a]
Protein (% w/w)	6.22 (0.02)[b]	6.30 (0.07)[b]	6.71 (0.02)[a]
Lipids (% w/w)	4.60 (0.20)[a]	4.45 (0.13)[a]	4.30 (0.11)[a]
Carbohydrates (% w/w)	12.90 (0.08)[a]	12.03 (0.16)[b]	12.08 (0.19)[b]
Acidity (% lactic acid)	10.41 (0.01)[a]	10.26 (0.00)[a]	10.79 (0.12)[a]
pH	4.55 (0.01)[a]	4.56 (0.00)[a]	4.57 (0.01)[a]

Cheese 1 = 30% of milk substituted by Whey Retentate (WR); cheese 2 = 20% of milk substituted by WR; control cheese with 100% milk; replicate means followed by the same letter are significantly different at the 0.05 level.

The cheese yield potential of milk is largely dependent on milk composition, particularly fat and protein. The casein fraction of milk protein is the dominant factor that affects curd firmness and syneresis rate (ZENG *et al.*, 2007). In cheeses that were made with the addition of milk WR, the structure was more fragile, which resulted in a greater loss of proteins into the whey after the curd was cut. No differences ($p>0.05$) among the cheeses (Table 2) were observed with respect to ash content, acidity, lipid content and pH.

Physical property of Petit Suisse cheeses

Flow property measurements

The rheological behavior (Fig. 1) of the Petit Suisse cheese samples (1, 2 and control) showed hysteresis that is time-dependent. Similar results in which the flow properties were time-dependent were obtained for *labneh* (concentrated yogurt) (MOHAMEED *et al.* (2004), stirred yogurt (O'DONNELL and BUTLER, 2002) and double cream cheeses (SANCHEZ *et al.*, 1996).

Thixotropic behavior can be evaluated through hysteresis (TÁRREGA *et al.*, 2004), where the greater the area, the greater the thixotropic effect (BRANCO and GASPARETTO, 2003; HALMOS and TIU, 1981). The hysteresis values (Table 3) obtained from the area between the ascending and descending curves of the apparent viscosity-shear rate plots also demonstrated that the use of WR in the production of these cheeses reduced hysteresis ($p<0.05$). According to MOHAMEED *et al.*, (2004), the increased concentration of total solids contributed to an increase in the thixotropic effect, where small changes in the concentration of solids can exert a strong effect on the rheological properties. The predomi-

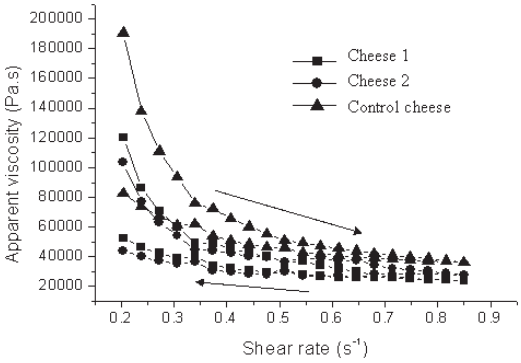

Fig. 1 - Apparent viscosity x shear rate relationship flow curves for Petit Suisse cheeses 1, 2 and control at 8±1°C, where cheese 1 = 30% of milk substituted by Whey Retentate (WR); cheese 2 = 20% of milk substituted by WR; control cheese with 100% milk.

Table 3 - Mean (standard deviation) values for hysterisis and rheological parameters of Petit Suisse cheeses 1, 2 and Control obtained by the Weltman model and structural kinetic model.

	Cheese 1	Cheese 2	Control cheese
Hysterisis (Pa)	7.956 (0.673)[a]	9.029 (0.753)[a]	13.102 (0.656)[b]
		Weltman model	
A	33.60 (0.35)[a]	40.71 (1.57)[b]	56.10 (0.30)[c]
-*B* (Pa)	8.22 (0.77)[a]	12.58 (1.08)[b]	19.75 (0.24)[c]
R	0.968 (0.030)[a]	0.950 (0.01)[a]	0.990 (0.00)[a]
		Structural kinetic model	
k x 10^{-3} (s^{-1})	2.779 (0.013)[a]	3.164 (0.006)[b]	3.598 (0.003)[c]
η_0 (Pa.s)	41.40 (3.197)[a]	44.27 (2.195)[a]	66.00 (2.757)[b]
η_0/η_∞	1.524 (0.03)[a]	1.522 (0.025)[a]	1.823 (0.038)[b]
R	0.971 (0.017)[a]	0.922 (0.024)[a]	0.990 (0.005)[a]

Cheese 1 = 30% of milk substituted by Whey Retentate (WR); cheese 2 = 20% of milk substituted by WR; control cheese with 100% milk; replicate means followed by the same letter are significantly different at the 0.05 level.
A = initial shear stress;
B = time coefficient of thixotropic breakdown;
R = correlation coefficient;
k = rate of structure breakdown;
η_0 = apparent initial viscosity at t = 0 (structured state);
η_0/η_∞ = extent of thixotropy.

nance of caseins in the control cheese led to the formation of compact structured gel (AHMED *et al.*, 2005) that resulted in greater structural failure (thixotropy).

Fig. 2 shows the apparent viscosity of Petit Suisse cheese samples over time. At a constant shear rate, the apparent viscosity of the evaluated samples decreased with time. The control sample presented a greater decrease in viscosity, confirming a greater thixotropic effect.

The parameters of the Weltman model and structural kinetic model are shown in Table 3. In the Weltman model, parameter *A* is the initial shear stress and parameter *B*, the time coefficient of thixotropic breakdown, that is, "the product of the rate in breakdown of thixotropic structure and the time of agitation at constant rate of shear" (WELTMAN, 1943). ANOVA results showed that the use of WR in the production of these cheeses was significant for both parameters (F = 296.47, p = 0.000357 for parameter *A* and F = 112.25, p = 0.001514

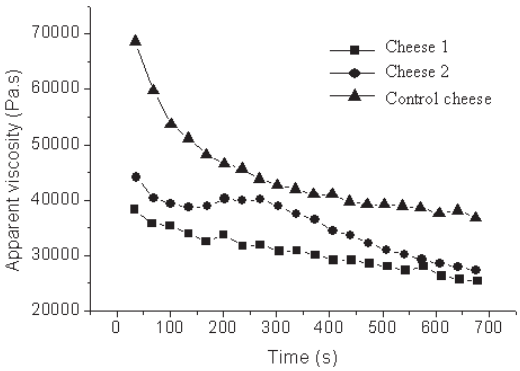

Fig. 2 - Apparent viscosity x time relationship flow curves for Petit Suisse cheeses 1, 2 and control at constant shear rate = 0.85 s^{-1} at 8±1°C, where cheese 1 = 30% of milk substituted by Whey Retentate (WR); cheese 2 = 20% of milk substituted by WR; control cheese with 100% milk.

for parameter *B*). It is interesting to note that the observed variations in the values of the Weltman parameters *A* and *B* between the control cheese, cheese 1 and cheese 2 (Table 3) followed the same pattern as the thixotropic area values (Fig. 3 and Table 3).

The control cheese showed the highest value for parameter *B* ($p<0.05$) of the Weltman model (coefficient of thixotropic breakdown), which in turn correlated with the largest area of the thixotropy hysteresis loop. According to AGUILAR *et al.*, (1991), the values of the Weltman model parameters depend on the size distribution of the solid particles suspended in the continuous phase.

The addition of milk whey to the cheeses lowered the stress values required for the onset of product degradation (A) (Table 3). A stronger and much denser gel structure would be expected for the control cheese as a result of more protein-protein interactions at higher protein levels (OZER *et al.*, 1988). The correlation coefficients obtained (Table 3) demonstrated that the Weltman model could be adapted to these semi-solid cheeses.

When applying the structural kinetic model, a 0.75 order at a constant shear rate ($\gamma = 0.85\ s^{-1}$) was found, indicating that the model was adequate ($R>0.92$) to describe the thixotropic behavior of the semi-solid cheese samples (Table 3). Fig. 4 shows the applicability of the structural kinetic model to the rheological data of the cheeses, where plots of $[(\eta_o - \eta_\infty)/\eta - \eta_\infty)]^{0.25}$ versus time are shown. ABU-JDAYIL (2003) obtained linearity when applying values of viscosity over time in $(\eta - \eta_\infty/\eta_o - \eta_\infty)^{1-n}$ (as a second order), at a constant shear rate and constant temperature using the structural kinetic model for mayonnaise and tahini.

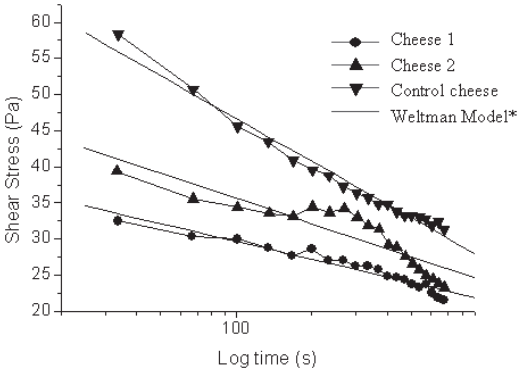

Fig. 3 - Shear stress x time relationship flow curves for Petit Suisse cheeses 1, 2 and control at constant shear rate of 0.85 s^{-1} at 8±1°C, where cheese 1 = 30% of milk substituted by Whey Retentate (WR); cheese 2 = 20% of milk substituted by WR; control cheese with 100% milk. *Continuous lines indicate adjustment to the Weltman model.

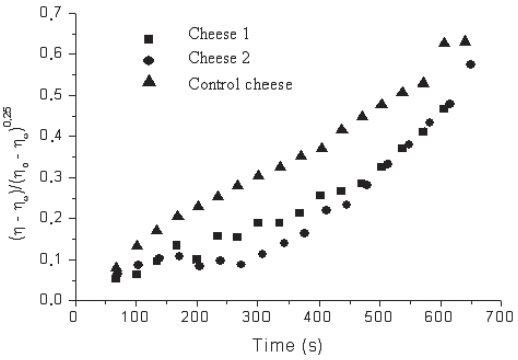

Fig. 4 - Structural kinetic model in order $n = 0.75$ for Petit Suisse cheeses 1, 2 and control at constant shear rate of 0.85 s^{-1} at 8±1°C, where cheese 1 = 30% of milk substituted by Whey Retentate (WR); cheese 2 = 20% of milk substituted by WR; control cheese with 100% milk.

At a constant shear rate (γ) and constant temperature, the structural kinetic model considers *k* as the measure of the rate of internal structural breakdown of the material, i. e. the degree of thixotropy. On other hand, under the same conditions this model suggests that the ratio of initial viscosity to equilibrium viscosity (η_o/η_∞) can be considered as a relative measure of the amount of structure breakdown, that is, the relative measure of the extent of thixotropy (ABU-JDAYIL, 2003). In the kinetic structural model, the values of *k* and η_o/η_∞ observed (Table 3) were lower ($p< 0.05$) in the cheese samples with

Table 4 - Mean (standard deviation) values for instrumental texture analysis parameters of the Petit Suisse cheeses at (8±1) °C.

Parameters	Control Cheese	Cheese 1	Cheese 2
Hardness (g)	57.14 (0.90) [a]	48.15 (0.90) [b]	45.58 (1.82) [b]
Consistency (g.s)	316.75 (9.15) [a]	274.48 (6.14) [b]	278.56 (0.32) [b]
Adhesion force (g)	9.95 (0.45) [a]	9.95 (1.36) [a]	7.84 (1.11) [a]
Adhesiveness (g.s)	58.65 (8.29) [a]	66.58 (16.11) [a]	50.35 (7.14) [a]
Recoverable energy (g.s)	0.23 (0.01) [a]	0.20 (0.02) [a]	0.22 (0.00) [a]

Cheese 1 = 30% of milk substituted by Whey Retentate (WR); cheese 2 = 20% of milk substituted by WR; control cheese with 100% milk; replicate means followed by the same letter are significantly different at the 0.05 level, respectively.

the greatest WR content. These results suggest that the shear rate and extent of network structure breakdown in the Petit Suisse cheese under shear, decreased ($p<0.05$) as the WR content increased.

The results obtained by the structural kinetic model are in agreement with the results obtained using the Weltman model. The quantity of structural degradation of the Petit Suisse samples decreased with the use of WR in the elaboration of the cheeses.

The correlation coefficients obtained (Table 3) demonstrated that the models that were evaluated could be adapted to these semi-solid cheeses. In both models, the samples presented time dependent flow properties at a constant shear rate (γ) equal to 0.85 s^{-1}.

Instrumental texture analysis

The use of WR in the Petit Suisse cheeses resulted in cheeses with less hardness and consistency ($p<0.05$) (Table 4). The gel protein matrix is responsible for dairy product hardness (RAWSON and MARSHALL, 1997), which is dependent on the total solids content (LUCEY and SINGH, 1998), system protein content and protein type (ABU-JDAYIL, 2003; HINRICHS, 2001). Therefore, the addition of WR conferred lower values to these parameters because it diminished the protein content (Table 2), reducing the force that is required to break the protein network. Another factor that could have influenced the cheese texture was the proteolytic activity of yoghurt bacterial culture. Whey proteins are hydrolyzed in limited amounts during the fermentation process (BERTRAND-HARB *et al.*, 2003); the increase in α-lactalbumin and β-lactoglobulin content in the system decreased protein hydrolysis, resulting in a weaker gel.

The use of WR showed no influence ($p>0.05$) on the force of adhesion and recoverable energy values. The use of polysaccharides in all the cheeses could have contributed to the production of more stable gels (KONSTANCE, 1993) by recovering the energy consumed in compression. The use of xanthan gum and guar

Table 5 - Mean (standard deviation) values for syneresis index Petit Suisse cheeses samples 1, 2 and control at 8±1°C.

Cheese	Syneresis index (% w/w)
1	40.92 (0.86)[a]
2	39.28 (0.31)[a]
Control	34.69 (0.20)[b]

Cheese 1 = 30% of milk substituted by Whey Retentate (WR); cheese 2 = 20% of milk substituted by WR; control cheese with 100% milk; replicate means followed by the same letter are significantly different at the 0.05 level, respectively.

gum in the production of these cheeses could have contributed to this result.

Syneresis index

Petit Suisse cheeses manufactured with WR had a greater syneresis index ($p<0.05$) (Table 5). This behavior can be attributed to the greater protein content present in the control cheese (Table 2). A lower syneresis index indicates the formation of a gel with a greater water - holding capacity, which compromises quality and homogeneity (CASTILHO *et al.*, 2006). The increase of syneresis in fermented dairy derivatives with WR was also observed by MAGENIS *et al.* (2006) and MODLER and KALAB (1983).

CONCLUSIONS

A significant difference between the physicochemical compositions was observed in cheeses manufactured with or without whey retentate. The flow property measurements of the cheeses showed hysteresis, which indicated thixotropic behavior, where η is time-dependent. A lower hysteresis was observed in samples to which whey retentate was added. The correlation coefficients obtained demonstrated that the Weltman and structural kinetic (0.75 order) models can be applied to describe the flow properties of semi-solid cheese samples. In both models the samples showed time-dependent flow properties at a constant shear rate (γ) equal to 0.85 s^{-1}. The use of whey retentate in the elaboration of Petit Suisse cheeses resulted in cheeses with less hardness, less consistency and higher syneresis ($p<0.05$).

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PAPER

IMPROVEMENT AND VALIDATION OF A METHOD FOR DETERMINING LOW-MOLECULAR-WEIGHT PHENOLS IN GRAPE SKINS

OTTIMIZZAZIONE E VALIDAZIONE DI UN METODO PER LA DETERMINAZIONE DEI COMPOSTI FENOLICI A BASSO PESO MOLECOLARE NELLA BUCCIA DI UVA

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ABSTRACT

The aim of this study was to optimise and validate a method for analysing eleven phenolic compounds belonging to different subclasses (phenolic acids, stilbenes and flavonoids) in grape skins. The method proposed includes a solid phase extraction step of purification and concentration prior to HPLC analysis. Under the optimised conditions, the total HPLC run time was reduced from 150 to 75 min. The method showed high precision (3-12%). There

RIASSUNTO

Scopo del presente studio è ottimizzare e validare un metodo di analisi che consenta l'identificazione di 11 composti fenolici suddivisi in differenti subclassi (acidi fenolici, stilbeni e flavonoidi) nelle uve. La procedura di separazione degli analiti prevede uno step di purificazione e concentrazione mediante estrazione in fase solida prima dell'analisi HPLC. Il metodo proposto ha consentito una riduzione della durata delle corse HPLC da 150 a 75 min. Esso presenta

- Key words: clean-up procedure, grape skins, HPLC, low-molecular-weight phenols, method validation, SPE -

was a high linearity of response for all the compounds analysed ($r^2 \geq 0.998$) over the normal range for grape. The method was used to analyse the phenolics in Aglianico and Uva di Troia grape skins. Greater amounts of all the flavonoids and stilbenes were found in the Uva di Troia grape skins with respect to the Aglianico ones.

una elevata precisione (3-12%). Per tutti i composti identificati la risposta è lineare ($r^2 \geq 0{,}998$) all'interno dell'intervallo di concentrazione rilevato per tali composti nell'uva. Tale metodo è stato applicato per la determinazione dei composti fenolici delle bucce di uva Aglianico ed Uva di Troia. Le bucce dell'uva di Troia sono caratterizzate da un maggior contenuto di flavonoidi e di stilbeni rispetto a quelli dell'uva Aglianico.

INTRODUCTION

Grape skin is an important source of complex phenolics that belong to different classes having pharmacological activity both *in vitro* and *in vivo* (KATALINIĆ *et al.*, 2004; WOOD *et al.*, 2004). Unfortunately the analysis of grape phenolics is difficult to perform due to the large number of structures involved, their different polarities and their high reactivities. Determination of individual phenolics is usually performed by HPLC under reverse-phase conditions. However, different sample preparation steps are needed prior to HPLC injection to extract phenolics from skin tissues and to purify and concentrate them. Acidified methanol has been reported to be the best solvent for extracting stilbenes from skins (SUN *et al.*, 2006). The same mixture is often used to extract other phenolics such as phenolic acids and procyanidins from grape skins (RODRÌGUEZ *et al.*, 2006). In the purification and concentration step, the solid-phase extraction (SPE) is used owing to the high selectivity, speed and ease of automation (KAMMERER *et al.*, 2004). The clean-up procedures and long elution programs may result in an extended time of analysis which is often inadequate for checking grape quality. The aim of this work was to develop a simple and rapid method for determining and quantifying, in a single analysis, 11 phenolics in grape berries including: a) phenolic acids: gallic acid, caffeic acid and p-coumaric acid; b) flavonoids: procyanidin B_1, (+)-catechin, procyanidin B_2, (-)-epicatechin and quercetin and c) stilbenes: *trans*-piceid, *trans*-resveratrol and *cis*-resveratrol.

MATERIAL AND METHODS

Sample

Grape berries of *Vitis vinifera* cvs Uva di Troia and Aglianico were used in this study. Uva di Troia and Aglianico grapes were obtained from vineyards in the DOC Daunia area (Puglia region, Italy) and from vineyards in the DOC Taburno area (Campania region, Italy) respectively.

Extraction method

The polyphenols were extracted as described by KAMMERER *et al.* (2004) with slight modifications. Frozen grape pomace samples were manually separated into skins and seeds, lyophilized (5PASCAL LIO-5P 4P, Trezzano sul Naviglio, Italy) overnight and finely ground using a Waring blender. Aliquots (4 g) of pulverized skins were weighed and extract-

ed with 100 mL of methanol/0.1% HCl (v/v) for 2 h under stirring after flushing with nitrogen. The extracts were centrifuged (10 min, 12,857 g) and the material was re-extracted with 50 mL of methanol/0.1% HCl (v/v) (15 min). The combined supernatants were evaporated to dryness under vacuum at 35°C and the residue was dissolved in 20 mL of acidified water (pH 3.0). Aliquots (5 mL) of the skin extracts were made up to 20 mL. After the pH was adjusted to 1.5, the solution was extracted four times with 50 mL of ethyl acetate each time. The combined extracts were evaporated to dryness, dissolved in 5 mL of water after the pH had been adjusted to pH 7.0 and then applied to the C_{18} cartridges, which were activated with 10 mL of methanol and rinsed with 10 mL of deionised water (pH 7.0). Phenolic acids were subsequently eluted with 10 mL of deionised water (pH 7.0) and 10 mL of 0.01% HCl (fraction I), flavonoids and stilbenes were eluted with 20 mL of ethyl acetate (fraction II). The elutes were evaporated to dryness and were dissolved in 2.5 mL of 2% acetic acid (fraction I) and in 5 mL of deionised water (pH 3.0) (fraction II) respectively, membrane-filtered (Teknokroma PTFE 0.45 μm and Advantec MFS PTFE 0.2 μm Dublin, CA, USA) and then used for HPLC analyses.

Chemicals

All chromatographic solvents were high-performance liquid chromatography (HPLC) ultra-gradient grade and were purchased from J.T. Baker (Mallinckrodt Baker, Inc., Phillipsburg, NJ, USA), deionised water (<18 MΩ cm resistivity) was obtained from a Milli-Q element water purification system (Millipore, Bedford, MA, USA). *trans*-Resveratrol (purity > 99%), quercetin dehydrate (purity min. 98%) and (+)-catechin hydrate (purity min. 98%) standards were purchased from Sigma (Milan, Italy) and (-)-epicatechin (purity 90%) was purchased from Aldrich (Milan, Italy), while p-coumaric acid, gallic acid, caffeic acid, procyanidin B_1 and procyanidin B_2 were purchased from Extrasynthese (Genay, France). *trans*-Piceid was purchased from Polyphenol AS (Bergen, Norway). C_{18} Hydra reversed-phase cartridges (Chromabond, 1,000 mg) were obtained from Macherey-Nagel (Düren, Germany).

Preparation of standard solutions

Five mixed standards were prepared and named MS_1, MS_2, MS_3, MS_4 and MS_5. Mixed standard solution 1 (MS_1): 100 mL of mixed standard solution containing 2.0 g/L (+)-catechin, 1 g/L (-)-epicatechin, 0.5 g/L quercetin, 2.0 g/L procyanidin B_1 and 2.0 g/L procyanidin B_2 was prepared by diluting the standards in methanol. Mixed standard solution 2 (MS_2) was 100 mL made up of 0.8 g/L gallic acid, 0.085 g/L p-coumaric acid and 0.084 g/L caffeic acid and was prepared by diluting the standards in methanol. Mixed standard solution 3 (MS_3) was 100 mL containing 0.1 g/L *trans*-resveratrol and was prepared by diluting the standards in ethanol-water (50:50, v/v). For the preparation of a standard solution containing *cis*-resveratrol (MS_4) the MS_3 solution was subjected to UV irradiation at 254 nm for 47 min (SUN *et al.*, 2006). Under these conditions the *trans*-resveratrol was converted into the *cis*-isomer with high yields (72-92%). For the quantification of the *cis*-resveratrol, a standard curve was obtained with known amounts of *trans*-resveratrol submitted to isomerisation. Mixed standard solution 5 (MS_5) was 100 mL made up of 0.1 g/L *trans*-piceid and was prepared by diluting the standard in ethanol-water (50:50, v/v). The solutions containing the mixed standards were kept in the dark at -20°C until used. The calibration curves were determined by their respective standards covering the range of linearity.

High-performance liquid chromatography

The polyphenols were separated and quantified by HPLC as described by KAMMERER *et al.* (2004) with slight modifications. The HPLC used was a Shimadzu apparatus (Shimadzu Italy, Milan) LC10 ADVP, consisting of a SCL-10AVP system controller, two LC-10ADVP pumps, a SPD-M 10 AVP detector, and an injection system full Rheodyne model 7725 (Rheodyne, Cotati, CA, USA) equipped with a 50 μL loop. The column used for this separation was a Nova-Pak C_{18} column (3.9x150 mm, 4 μm particles diameter) equipped with a Nova-Pak Sentry C_{18} guard column (3.9x20 mm, 4 μm) (Waters Corporation, Milford, MA, USA). Fifty μL samples of extract or calibration standards were injected directly into the column.

Fraction I (phenolic acids). The mobile phase consisted of 2% (v/v) acetic acid in water (eluent A) and 0.5% acetic acid in water and acetonitrile (50:50, v/v; eluent B). The elution program was as follows: 0 min, 95% A, 5% B at a flow rate of 1 mL/min; 5 min, 90% A, 10% B at a flow rate of 1 mL/min; 6.5 min, 85% A, 15% B at 1 mL/min; 12 min, 75% A, 25% B at a flow rate of 1 mL/min; 25 min, 45% A, 55% B at a flow rate of 1 mL/min; 25.5 min, 100% B; 28.1 min, 95% A, 5% B until the end of analysis at 30 min. Detection was performed by monitoring the absorbance signals at 280 nm (gallic acid) and 306 nm (caffeic acid and p-coumaric acid). The retention times (RT) of the three phenolic acid compounds, identified by comparing the results with the UV-visible spectra of pure reference standards and by injecting of co-eluted samples (sample + standard), were as follows: gallic acid (RT = 2.1 min), caffeic acid (RT = 9.9 min) and p-coumaric acid (RT = 14.5 min). For each sample of extract, extractions were carried out in duplicate and analyses were carried out in triplicate.

Fraction II (flavonoids and stilbenes). The mobile phase consisted of the same eluents described for fraction I. The gradient program was as follows: 0 min, 90% A, 10% B at a flow rate of 1 mL/min; 10 min, 76% A, 24% B at a flow rate of 1 mL/min; 20 min, 70% A, 30% B at 1 mL/min; 30 min, 45% A, 55% B at a flow rate of 1 mL/min; 37.5 min, 100% B; 42.5 min, 90% A, 10% B until the end of analysis at 45 min. Detection was performed by monitoring the absorbance signals at 280 nm (procyanidin B_1, (+)-catechin, procyanidin B_2, (-)-epicatechin and *cis*-resveratrol), 306 nm (*trans*-resveratrol), 320 nm (*trans*-piceid) and 369 nm (quercetin). The retention times of the eight phenolic compounds, identified by comparing the results with the UV-visible spectra of pure reference standards and by injecting co-eluted samples (sample + standard), were as follows: procyanidin B_1 (RT = 4.8 min), (+)-catechin (RT = 6.4 min), procyanidin B_2 (RT = 8.4 min), (-)-epicatechin (RT = 10.5 min), *cis*-resveratol (RT = 31.5 min), *trans*-resveratrol (RT = 26.7 min), *trans*-piceid (RT = 19.5min) and quercetin (RT = 31.5 min). For each sample of extract, extractions were carried out in duplicate and analyses were carried out in triplicate.

Calibration curves, detection limit, and reproducibility of the HPLC method

The calibration curves obtained by injecting mixed standard solutions containing (+)-catechin, (-)-epicatechin, *trans*-resveratrol, quercetin, procyanidin B_1, procyanidin B_2, caffeic acid, p-coumaric acid, gallic acid, *cis*-resveratrol and *trans*-piceid were characterised by a correlation coefficient (r^2) > 0.998. The linearity range of the calibration curves is reported in Table 1. The precision of the method used was tested in 10 replicate analyses on two skin extract samples. The limit of detection (signal-to-noise ratio 3:1) and limit of quantification (signal-to-noise 10:1) were obtained by injecting a standard solution at in-

creasing standard concentrations. To validate the method, skin extract was fortified with standards. Each fortified sample was subjected to the extraction procedure and HPLC analysis.

Statistical analysis

All the data are expressed as the arithmetic average ± standard deviation of six replicates. The equation of the calibration lines ($p<0.05$) was calculated by linear regression; analysis of variance and the Students' t-test were used to interpret any differences in the means, at the 95% confidence level. Data were elaborated by means of XLSTAT-Pro 7.5.3 (Addinsoft, New York, NY, USA).

RESULTS AND DISCUSSION

Extraction and clean up

During method development, the procedure described by KAMMERER *et al.* (2004) was applied to the control grape sample but the resolution of fraction II (containing flavonoids and stilbenes) was poor and the analytical sensitivity was low. This problem was overcome by concentrating, under vacuum the eluate obtained after fractionation by SPE and dissolving it in acidified water (pH = 3.0) so that the composition of the sample solvent was close to that of the mobile phase. This allowed the baseline disturbance to be minimised and increased the sensitivity (SNYDER *et al.*, 1997). Other set-up parameters were also optimised: i) an octadecyl silica (C_{18}) Hydra sorbent was used to limit the loss of polar phenols such as gallic acid that could not be retained in the non-polar C_{18} cartridges; ii) an additional step of drying the cartridges after the elution of fraction I and before the elution of fraction II was introduced. Figs. 1-3 show the resulting chromatograms. Fig. 3 reports the sample obtained by dissolving fraction II in methanol (Fig. 3a) and the same sample obtained by performing the extraction stage as reported (Fig. 3b).

Table 1 - Linearity of assay for eleven phenolic constituents of grape skin assessed by regression analysis (y = concentration in mg/L; x = area).

	Equation of calibration lines	n	*P*	r^2	linearity range (mg/L)
Phenolic acid					
caffeic acid	*y = 0.0243 + 1E-05*x*	6	0.14	1.000	0.084-8.400
p-coumaric acid	*y = 2.370E-02 + 5E-06*x*	7	0.006	1.000	0.034-8.500
gallic acid	*y = 0.1495 + 1E-05*x*	7	0.42	0.999	0.080-80.000
Flavonoids					
procyanidin B_1	*y = 2.435E-03 + 1E-04*x*	6	0.99	0.999	2.000-200.000
(+)-catechin	*y = -0.4552 + 1E-04*x*	7	0.43	0.999	1.000-200.000
procyanidin B_2	*y = -0.4381 + 1E-04*x*	7	0.39	0.999	1.000-200.000
(-)-epicatechin	*y = -0.4178 + 9E-05*x*	6	0.15	0.999	1.000-100.000
quercetin	*y = 0.1037 + 9E-06*x*	7	0.46	0.999	0.062-50.000
Stilbenes					
trans-resveratrol	*y = -4.080E-02 + 5E-06*x*	7	0.28	1.000	0.050-50.000
cis-resveratrol	*y = 0.5809 + 4E-06*x*	6	0.01	0.998	0.090-39.500
trans-piceid	*y = -0.1303 + 1E-05*x*	6	0.48	0.999	0.500-50.000

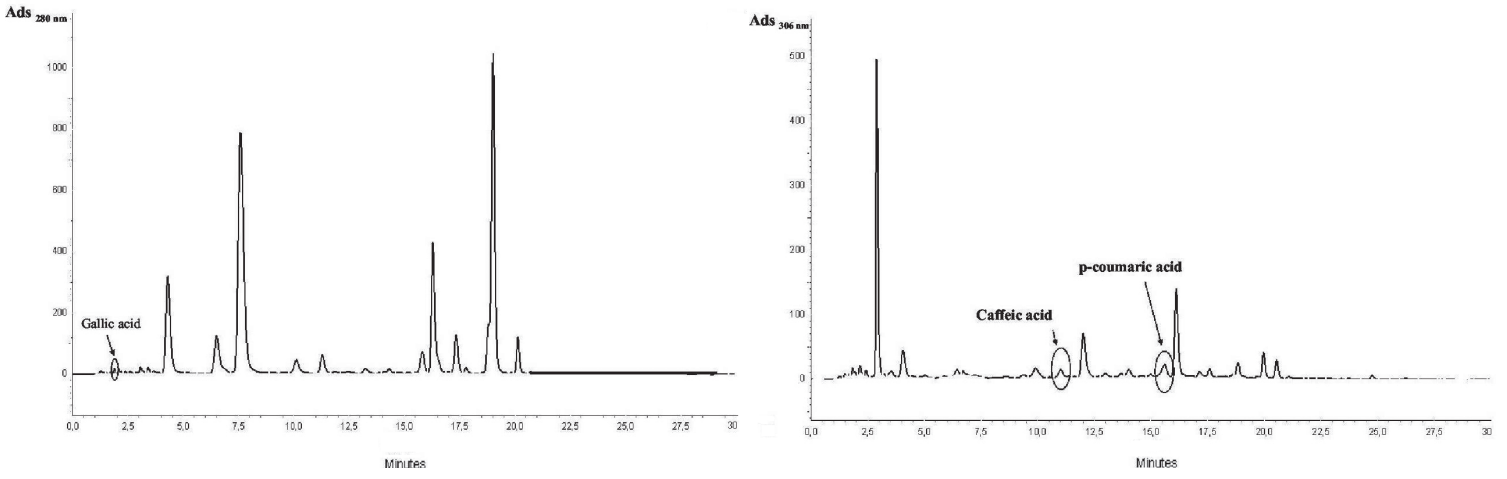

Fig. 1 - HPLC separation of grape skin extract obtained by dissolving fraction I in 2% acetic acid detected at 280 and 306 nm.

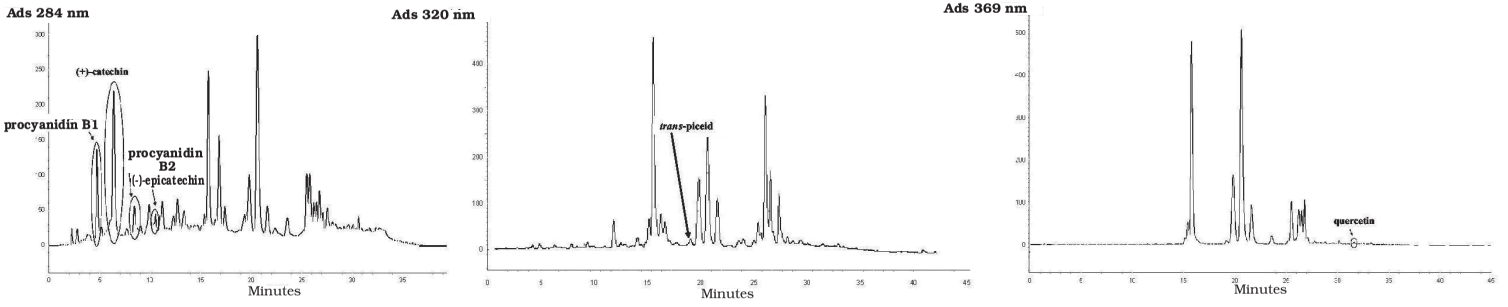

Fig. 2 - HPLC separation of grape skin extract obtained by dissolving fraction II in acidified water (pH = 3.0) detected at 280, 320 and 369 nm.

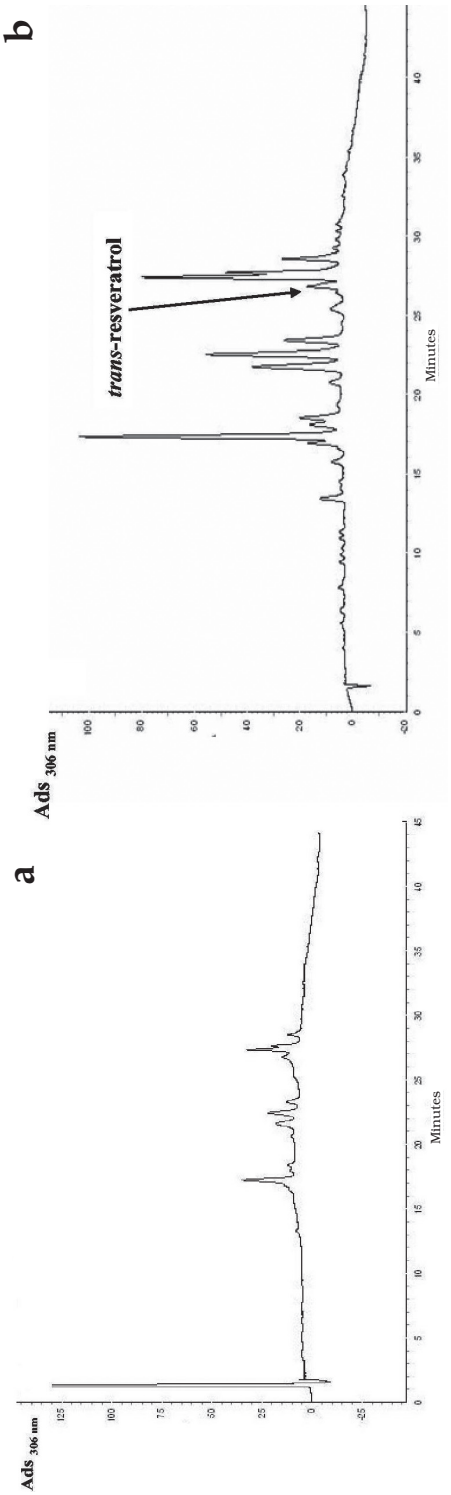

Fig. 3 - HPLC separation of grape skin extract obtained by dissolving fraction II in methanol (a) and in acidified water (pH = 3.0) (b) detected at 306 nm.

Method validation

Linearity and sensitivity

A total of eleven calibration curves (relative area vs. relative concentration) were prepared and assayed in triplicate. The calibration intervals covered the normal range of occurrence for each of the phenolics in grape (DE FREITAS *et al.*, 2000; KAMMERER *et al.*, 2004; YILMAZ AND TOLEDO, 2004). In Table 1, it can be seen that the coefficients of correlation (r^2) for caffeic acid, p-coumaric acid and *trans*-resveratrol, differed from unity only in the fourth decimal place, while they were ≥ 0.998 for the other analytes considered. The inverse of the slope is a measure of the factor response for each compound. Thus the factor responses were very high for all the compounds detected. All calibration curves had intercepts that were close to zero ranging from -0.4552 to +0.5809. The intercepts differed significantly from zero ($p<0.05$) only for p-coumaric acid and *cis*-resveratrol. These results indicate that their concentrations were probably overestimated as a result of background or baseline interference.

Spiking/recovery data

Recoveries were higher than 61% for all target compounds except for gallic acid, procyanidin B_2 and (-)-epicatechin (Table 2). Gallic acid had the lowest recovery and the results were similar to those reported by ALONSO GARCIA *et al.* (2004) (6% for gallic acid in alcohol-free beer). These results were probably due to the higher polarity of gallic acid.

Precision

The precision of the procedure was evaluated by carrying out ten replications of the same skin sample with two different operators (A and B) at two different times (Table 3). The relative standard deviation (RSD) was less that 12% and less that 6% for six compounds. According to SNYDER *et al.* (1997), these re-

Table 2 - Precision, recovery, limit of detection (LOD) and quantification (LOQ) of the optimised method for determining phenolic compounds in grape skin extract.

	Precision[a] (%)	Method recovery [b]				LOD[c] (mg/L)	LOQ[c] (mg/L)
		Spiked mg/L	Recovery %±S.D.	Spiked mg/L	Recovery %±S.D.		
Phenolic acids							
caffeic acid	4	38	155±0.01	76	189±0.03	0.0168	0.084
p-coumaric acid	4	41	145±0.01	83	175±0.02	0.0085	0.034
gallic acid	8	11	26±0.10	22	40±0.10	0.0160	0.080
Flavonoids							
procyanidin B_1	10	9	91±0.13	18	98±0.15	0.500	2.000
(+)-catechin	6	14	77±0.13	29	84±0.06	0.500	1.000
procyanidin B_2	11	6	13±0.24	11	29±0.30	0.500	1.000
(-)-epicatechin	9	5	13±0.10	9	20±0.03	0.250	1.000
quercetin	6	19	75±0.10	37	79±0.14	0.025	0.0625
Stilbenes							
cis-resveratrol	3	5	68±0.13	10	22±0.08	0.045	0.09
trans-piceid	5	29	63±0.14	57	112±0.03	0.1	0.50
trans-resveratrol	12	78	109±0.11	156	61±0.08	0.02	0.05

[a] n = 10; [b] n = 6; [c] n = 3.

Table 3 - Precision data determined by ten-replicate analyses of the same grape skin extract (mg/kg dry skin) by two operators (A and B) on different days.

	A Mean±S.D.	B Mean±S.D.
Phenolic acid		
caffeic acid	9.7±0.6 a	10±0.1 a
p-coumaric acid	1.5±0.1 a	1.5±0.01 a
gallic acid	10±0.4 a	9.0±0.7 a
Flavonoids		
procyanidin B_1	723±15 a	631±74 a
(+)-catechin	1,910±39 a	1,913±163 a
procyanidin B_2	891±20 a	784±105 a
(-)-epicatechin	377±9 a	415±46 a
quercetin	3.3±0.2 a	3.4±0.2 a
Stilbenes		
trans-resveratrol	30±2.2 a	28±2.5 a
cis-resveratrol	18±0.1 a	17±0.7 a
trans-piceid	177±2.9 a	128±2.0 b

Data determined by different operators sharing the same letters are not significantly different ($p<0.05$).

Table 4 - Polyphenol content of grape skin extract (mg/kg dry skin).

	Uva di Troia Mean±S.D.	Aglianico Mean±S.D.
Phenolic acids		
caffeic acid	10±0.4 b	28±0.2 a
p-coumaric acid	1.5±0.1 a	4.1±0.1 b
gallic acid	9.6±0.8 a	12±0.2 b
Flavonoids		
procyanidin B_1	677±70 b	37±3 a
(+)-catechin	1,912±112 b	12±1 a
procyanidin B_2	838±91 b	362±25 a
(-)-epicatechin	396±37 b	40±9 a
quercetin	3.3±0.2 b	1.6±0.1 a
Stilbenes		
cis-resveratrol	17±0.5 b	14±0.4 a
trans-piceid	153±26 b	17±0.9 a
trans-resveratrol	29±2.6 b	6.4±0.8 a

n = 6.
Varieties sharing the same letters are not significantly different ($p<0.05$).

sults are satisfactory for the complexity of the sample and the levels at which the compounds are found in skins. A significant ($p<0.05$) loss of *trans*-piceid was detected when the sample was analysed by operator B after 1 week (recorded at -20°C protected from light). This result may be due to both the capability of operator B and the possible degradation of this molecule.

Method detection limits

The limit of detection (LOD) was estimated by analysing standard samples and was calculated as the concentration at which the signal-to-noise ratio becomes 3. The limit of quantification (LOQ), set as signal-to-noise ratio of 10, is well below the concentrations usually found in grape extracts.

Application to the analysis of grape skin phenolics

The validated method was used to determine the phenolics in two grape varieties from southern Italy: Aglianico and Uva di Troia (Table 4). The flavonoid fraction was mainly dominated by the flavanols in both red grape skins. A different distribution of phenolics detected in the relative classes has been observed for the two grape varieties. Uva di Troia grape skins had higher amounts of all the flavonoids and stilbenes detected with respect to those of Aglianico. The skins of Aglianico showed higher concentrations of p-coumaric and gallic acid. Since flavonoids and stilbenes are formed from p-coumaric acid via the shikimic acid and phenylpropanoid pathways (DEWIK, 2001), the behaviour observed may be due to less expression of this pathway in Aglianico with respect to Uva di Troia. However the levels of phenolics found are in agreement with phenolic concentration levels reported by other authors. The (+)-catechin and (-)-epicatechin concentrations detected in Uva di Troia grape skins were higher than those reported by YILMAZ AND TOLEDO (2004) for Merlot grape and by DE FREITAS *et al.* (2000) for Cabernet Sauvignon grape. The concentration of *trans*-resveratrol in Uva di Troia was similar to that detected in Cabernet Mitos (11.1 mg/kg of dry skins) by KAMMERER *et al.* (2004) and was less than that detected in Nero d'Avola (27.5 μg/kg of dry skins) by CARERI *et al.* (2004). Unfortunately a wider comparison cannot be made because the method of analysis and expression of the results are not the same.

CONCLUSION

The optimised method ensures the quantification of eleven phenolic compounds in grape skins and provides good performance in terms of precision, linearity and limits of quantification. The use of a C18 column (3.9x150 mm, 4 μm particles diameter) reduced the total run time. It is a useful analytical research tool for studying the roles of grape cultivar, environment and agricultural practices in controlling these important metabolites of the shikimic acid pathway. The optimised experimental conditions used in this method may also be used to determine other grape phenolics such as anthocyanins and glycosilated flavonols.

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PAPER

ANTIOXIDANT AND ANTIMUTAGENIC ACTIVITIES OF THE ESSENTIAL OIL AND METHANOL EXTRACT FROM TUNISIAN *NIGELLA SATIVA* L. (RANUNCULACEAE)

ATTIVITÀ ANTIOSSIDANTI ED ANTIMUTAGENICHE DELL'OLIO ESSENZIALE E DELL'ESTRATTO METANOLICO DI *NIGELLA SATIVA* L. (RANUNCULACEAE)

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ABSTRACT

Tunisian *Nigella sativa* seeds were studied for their biological activities. Essential oil was analysed by gas chromatography and gas chromatography/mass spectrometry, whereas the polyphenolic composition was de-

RIASSUNTO

I semi della *Nigella sativa* tunisina sono stati analizzati per scoprire le loro attività biologiche. L'olio essenziale é stato analizzato con gas cromatografia e gas cromatografia/spettrometria di massa. Le componenti fenoliche

- Key words: antimutagenic activity; antioxidant activity; chemical composition; essential oil; GC-MS; methanol extract; *Nigella sativa* L. seeds; Ranunculaceae; RP-HPLC -

termined by reverse phase high performance liquid chromatography. *p*-cymene (56.8%) was the major compound in the essential oil, while vanillic acid was the predominant phenolic compound (190±0.34 mg/100 g dry weight) in the methanol extract. Antioxidant activity was evaluated using 1, 1-diphenyl-2-picrylhydrazyl (DPPH) and superoxide anion radical scavenging. Methanol extract and essential oil were both strong radical scavengers with the essential oil having the highest activity. Antimutagenic activity was determined by the Ames test. Essential oil and methanol extract showed similar important antimutagenic effects; the inhibition percentages were 58.5 and 60%, respectively.

sono state identificate mediante cromatografia liquida ad alta prestazione in fase inversa. La maggior presenza all'interno dell'olio essenziale è costituita da *p*-cymene (56,8%), mentre l'acido vanillico è il componente predominante nell'estratto metanolico (190±0,34 mg/100 g PS). L'attività ossidante è stata valutata usando il sistema di 1, 1-diphenyl-2-picrylhydrazyl (DPPH) e ha rivelato che più l'olio essenziale che l'estratto metanolico presenta poteri antiradicali. Il test di Ames, utilizzato per determinare l'attività antimutagenica, ha rivelato che l'olio essenziale e l'estratto metanolico presentano importanti attività antimutageniche, rispettivamente in percentuale d'inibizione del 58,5 e 60%.

INTRODUCTION

Oxygen can have harmful effects due to the formation and activity of reactive oxygen species (ROS) that act as oxidants. They can damage all types of cellular macromolecules and have been implicated in human pathologies such as cardiovascular and degenerative diseases (YOO *et al.*, 2004). The exposure of DNA to ROS leads to the formation of various lesions on the DNA, many of which are known to cause mutations, which may contribute to carcinogenesis (MARON and AMES, 1983). Several synthetic antioxidants have been widely used as food additives, but due to increasing information about possible carcinogenic and toxic effects, the current trend is to decrease their use (FERON and GROTEN, 2002). Therefore it is necessary to discover sources of natural compounds that have antioxidative and anticarcinogenic potency (KAUR and SAINI, 2000).

Recent studies have demonstrated the chemoprotective action of numerous plant extracts and essential oils against many oxidation-related diseases (RICCI *et al.*, 2005). Essential oils have been widely used as flavoring additives in foods and beverages, as fragrances in cosmetics and as intermediates in the manufacture of perfume chemicals. Their effectiveness as antioxidant and antimicrobial agents has been gaining attention (DORMAN *et al.*, 2000). Several terpenic compounds, such as farnesol, carvacrol and limonene have been shown to inhibit tumorigenesis (ZEYTINOGLU *et al.*, 2003). Phenolic compounds constitute a large group of organic compounds that are widely distributed in plants; they show a broad spectrum of biological activities (BELL and CHARLWOOD, 1980). Phenolic acids and flavonoids are the most commonly occurring antioxidant polyphenols acting as radical scavengers, hydrogen donors and reducing agents (RICE-EVANS *et al.*, 1996). Epidemiological evidence has shown an inverse relationship between the intake of polyphenol-rich foods and the risk of

coronary heart diseases and some types of cancer (KNEKT *et al.*, 1996).

Nigella sativa L. (Ranunculaceae), black cumin, is an annual herbaceous plant used as a popular aromatic herb and culinary spice. Its seeds are frequently added to bread or pickles as a flavoring agent and are also commonly eaten alone or in combination with honey on many other foods. Traditionally, *N. sativa* seeds have been used as a natural remedy for a number of illnesses such as asthma, cough, diabetes, headache, inflammation and other diseases.

The antioxidant activity of *N. sativa* seeds has been reported (RAMADAN and MORSEL, 2004; BURITS and BUCAR, 2000), but to date, the Tunisian variety has not been studied. To the best of the authors' knowledge, neither the phenolic composition nor the genotoxic activity of *N. sativa* seeds have been characterized. Thus, the aim of the present work was to evaluate the antioxidant capacity of the essential oil and methanol extract obtained from *N. sativa* seeds by determining the DPPH radical and superoxide anion scavenging properties and to screen their mutagenic and antimutagenic activities. The chemical composition of the essential oil was analyzed by GC and GC/MS and individual phenolic compounds were identified and quantified by RP-HPLC.

MATERIALS AND METHODS

Chemicals

Folin-Ciocalteu reagent, butylated hydroxytoluene (BHT), nitroblue tetrazolium (NBT), phenazine methosulfate (PMS), nicotinamid-adenin-dinucleotid (NADH), 1, 1-diphenyl-2-picrylhydrazyl (DPPH) and sodium azide were purchased from Sigma-Aldrich (GmbH, Sternheim, Germany). Hydrocarbon series (C_8-C_{22}) was purchased from Fluka. Authentic standards of phenolic compounds were purchased from Sigma and Fluka (Buchs, Switzerland). Stock solutions of these compounds were prepared in HPLC grade methanol, then wrapped in aluminium foil and stored at 4°C. All other chemicals were of analytical grade.

Plant material

Seeds of *Nigella sativa* L. were collected in July 2005 from cultivated plants from the region of Menzel Temime (Northeastern Tunisia). The collected material was identified and a voucher specimen was deposited in the herbarium of the Biotechnologic Center of Borj Cédria Technopol.

Essential oil extraction

Essential oil was isolated from the seeds (20 g) by hydrodistillation for 1.5 h in a Clevenger type apparatus. Oil was then dried over anhydrous sodium sulphate and stored at -20°C in darkness until tested and analyzed.

Analysis and identification of essential oil compounds

The essential oil was analysed using a Hewlett-Packard 6890 gas chromatograph (Agilent Technology, Palo Alto, CA) equipped with a flame ionization detector (FID) and electronic pressure control (EPC) injector. The HP-Innowax PEG column was 30 m in length, 0.25 mm i. d., and 0.25 μm in thickness (Agilent Technology, Palo Alto, CA). The injector and detector temperatures were 250° and 300°C, respectively. Nitrogen was used as a carrier gas with a flow rate of 1.6 mL min^{-1}. The split ratio was adjusted to 60:1, and the temperature program of the oven was 35°-205°C min^{-1}. For GC-MS analysis, a HP 5890 series II gas chromatograph coupled to a HP 5972 mass spectrometer with electron impact ionization (70 eV) was used. The capillary column was an HP-5MS (30 m x 0.25 mm x

0.25 μm). The oven temperature was programmed to rise from 50° to 240°C at a rate of 5°C min^{-1}. The carrier gas used was helium, at a flow rate of 1.2 mL min^{-1}.

The compounds were identified by comparing the retention indices relative to the homologous series of n-alkanes (C_8-C_{22}) with those of the literature or those of authentic compounds available in our laboratory. Further identification was made by matching their recorded mass spectra with those stored in the Wiley/NBS mass spectral library of the GC-MS data system.

Preparation of methanol extract

The extract was obtained from air-dried, finely ground seeds (2.5 g) by stirring the powder with 25 mL of absolute methanol at room temperature for 30 min. The extract was kept for 24 h at 4°C, and then filtered through Whatman filter paper. The extract was then evaporated under vacuum to dryness and stored at 4°C until used in biological assays.

Hydrolysis and identification of phenolic compounds

Dried samples were hydrolyzed according to a slightly modified method of PROESTOS *et al.* (2006). Methanol (40 mL) containing BHT (1 gL^{-1}) was added to 0.5 g of dried *N. sativa* sample; 10 mL of 6 M HCl were then added. The mixture was stirred carefully, sonicated for 15 min and then refluxed in a water bath at 90°C for 2 h. The mixture was then filtered through a 0.45 μm membrane filter and injected into the HPLC. The phenolic compound analysis was carried out using an 1100 series reverse phase-high performed liquid chromatograph (Agilent Technologies, Palo Alto, CA) coupled with a UV-Vis multiwavelength detector. The separation was carried out on a 250x4.6 mm, 4 μm Hypersil ODS C18 reversed phase column at ambient temperature. The mobile phase consisted of acetonitrile (solvent A) and water with 0.2% sulphuric acid (solvent B).The flow rate was kept at 0.5 mL min^{-1}. The gradient program: 15% A/85% B 0-12 min, 40% A/60% B 12-14 min, 60% A/40% B 14-18 min, 80% A/20% B 18-20 min, 90% A/10% B 20-24 min, 100% A 24-28 min. The injection volume was 20 μL and peaks were monitored at 280 nm. Peaks were identified by congruent retention times compared with standards. Analyses were performed in triplicate.

DPPH assay

The ability of the seed extracts to donate a hydrogen atom or electron was measured by bleaching purple-colored solution of the 1, 1-diphenyl-2-picrylhydrazyl radical (DPPH) according to the method of HANATO *et al.* (1988). Extracts (2 mL, 10-1,000 $\mu g.mL^{-1}$) were added to 0.5 mL of 0.2 mM methanol solution of DPPH. After incubating for 30 min at room temperature. The absorbance was determined against a blank at 517 nm. Percentage inhibition (PI %) of free radical DPPH was calculated as follow:

$$PI\ \% = [(A_{blank} - A_{sample})/A_{blank}] \times 100$$

where A_{blank} is the absorbance of the control reaction and A_{sample} is the absorbance in the presence of plant extract. The antiradical activity was expressed as IC_{50} ($\mu g.mL^{-1}$), i.e. the extract concentration required to cause 50% inhibition. A low IC_{50} value corresponds to a high extract activity. Samples were analyzed in triplicate.

Determination of anion superoxide-scavenging activity

Superoxide anion is generated in a PMS-NADH system by oxidation of NADH and assayed by the reduction of NBT. The method described by DUH *et al.* (1999) was used to determine the superoxide anion radical scavenging ac-

tivity. The mixture, containing samples (10-1,000 μg.mL^{-1}) in distilled water, 0.2 mL PMS (60 μM), 0.2 mL NADH (677 μM), and 0.2 mL NBT (144 μM) was incubated at room temperature for 5 min. The absorbance was then read at 560 nm. All solutions were prepared in phosphate buffer (0.1 M, pH 7.4). The scavenging activity was calculated as follows:

$$PI\ \% = [(A_{blank} - A_{sample})/A_{blank}] \times 100$$

where A_{blank} is the absorbance of the control reaction and A_{sample} is the absorbance in the presence of plant extract. The antioxidant activity was expressed as IC_{50} (μg.mL^{-1}). A low IC_{50} value corresponds to a higher plant extract activity. Samples were analyzed in triplicate.

Determination of total polyphenol content

The total phenolic content of *N. sativa* methanol extract was determined using the Folin-Ciocalteu (F-C) reagent according to the method described by DEWANTO *et al.* (2002). Diluted extract (0.12 mL) was added to 0.5 mL of distilled water and 0.12 mL of the (F-C) reagent. After shaking, the mixture was incubated for 3 min at room temperature; 0.12 mL of 7% Na_2CO_3 solution was then added. The volume was adjusted to 3 mL, mixed vigorously and held at ambient temperature for 90 min. The absorbance of the solution was then measured at 760 nm against a blank. The total phenolic content was expressed as mg of gallic acid equivalents (GAE) per gram of dry weight using the calibration curve of gallic acid. The sample was analyzed in three replications.

Mutagenic and antimutagenic tests

The mutagenicity assay was performed with *Salmonella typhimurium* strain TA 1535 using the plate incorporation procedure described by MARON and AMES (1983). Bacterial stock (15 μL) was incubated in 5 mL of Oxoid Nutrient for 16 h at 37°C on an orbital shaker. Then, 100 μL of the overnight bacteria culture (approximate cell density 10^8 cells.mL^{-1}) and 100 μL of extracts dissolved in DMSO (0.05-0.5 mg.mL^{-1}) were added to tubes containing 2 mL of top Agar (supplemented with 0.5 mM l-histidine and 0.5 mM d-biotine). The mixture was then poured onto minimal agar plates previously prepared as described by MARON and AMES (1983). The plates were incubated at 37°C for 48 h and the revertant bacterial colonies of each plate were counted. Negative control cultures gave the number of revertants per plate that were within the normal limits found in the laboratory. An extract was considered mutagenic if the number of revertants per plate was at least doubled. Since the spontaneous revertant frequency is known to be responsive to sodium azide (MARON and AMES, 1983), this mutagen was chosen to study the antimutagenic activity. The mutagenic agent (dissolved in DMSO) was added to a tube of top agar containing the bacterial strain and the extract. The resulting mixture was poured onto the minimal agar plate. The plates were incubated at 37°C for 48 h and the revertant bacterial colonies of each plate were counted. Percentage inhibition of mutagenicity (PI) was calculated according to the following formula:

$$PI\ (\%) = [1 - (\text{number of revertants on test plates/number of revertants on control plates})] \times 100$$

RESULTS AND DISCUSSION

Chemical composition of the essential oil

The yield of essential oil isolated from *N. sativa* seeds was 0.36% (w/w). GC-FID and GC-MS analysis of the oil resulted in the identification of twenty eight com-

pounds, representing 98.13% of the essential oil compounds (Table 1). The major component was *p*-cymene (56.83%). The other important constituents were γ-terpinene, α-thujene, thymol and octen-3-ol with percentages of 11.39, 6.71, 5.34 and 4.84, respectively. The predominant class was monoterpene hydrocarbons (83.31%), followed by oxygenated monoterpene derivatives (14%) and sesquiterpenes (0.81%). The Tunisian *N. sativa* essential oil had a different chemical profile compared to *N. sativa* plants of different geographical origins. Two main chemotypes reported in the literature are: *trans*-anethole (NICKAVAR *et al.*, 2003) in Iranian *N. sativa* and an intermediate chemotype with 33% *p*-cymene and 26.8% thymol for *N. sativa* from Morocco (MORETTI *et al.*, 2004). When compared to the Moroccan type, our type had a lower percentage of thymol and a greater amount of its precursor *p*-cymene. The changes in the essential oil composition could have arisen from differences such as geographical origin, ecological factors and extraction procedures (KESKITALO *et al.*, 2001).

Table 1 - Chemical composition of *N. sativa* essential oil.

Compound	RI[a]	RI[b]	(% w/w)
α-thujene	930	1035	6.71
α-pinene	939	1030	1.21
Camphene	954	1102	0.02
Sabinene	976	1126	0.6
β-pinene	978	1118	1.94
Myrcene	990	1154	0.11
α-phelandrene	1003	1160	0.20
α-terpinene	1015	1164	1.78
o-cymene	1020	1177	1.49
p-cymene	1026	1241	56.83
Limonene	1030	1181	0.12
E-β-ocimene	1047	1223	0.68
γ-terpinene	1062	1214	11.39
Terpinolene	1088	1249	0.23
Monoterpene hydrocarbons			83.31
Octene-3-ol	979	1263	4.8
Trans sabinene hydrate	1070	1416	0.15
Linalool	1090	1508	0.54
Borneol	1165	1681	0.04
Terpinene-4-ol	1176	1533	2.32
α-terpineol	1189	1659	0.07
Linalyl acatate	1258	1514	0.23
Anethole	1283	1845	0.21
Thymoquinone	1289	1690	0.23
Thymol	1293	2135	5.34
Carvacrol	1298	2142	0.08
Oxygenated monoterpenes			14.01
β-bourbonene	1392	1506	0.45
β-selinene	1490	1434	0.22
α-selinene	1493	1787	0.14
Sesquiterpenes			0.81
NI			1.86
Total			99.99

[a] Apolar HP-5 MS column; [b] Polar HP Innowax column.

Amount of total phenolics

The methanol extract yield was 38%. The Folin-Ciocalteu reagent was widely used to estimate the total phenolic content. The total amount of polyphenols in the seeds was 8.17 mg of GAE.g^{-1} dry weight; this value is double that reported by THIPPESWAMY and NAIDU (2005) for Indian *N. sativa.* This difference can be explained by the fact that extraction procedures affect the polyphenols yield (ZHOU and YU, 2004). In addition, the accumulation of phenolic compounds is affected by different factors such as climatic factors, maturity, cultivar, horticultural practices and post harvest storage conditions (YOO *et al.*, 2004).

Identification and quantification of phenolic compounds by HPLC

The free forms of phenolic compounds are rarely found in plants. More often, they occur as esters or glycosides or are bound to the cell wall. For this reason, acidic hydrolysis was used to release the aglycones in order to simplify the identification process (NUUTILA *et al.*, 2002). BHT, a powerful antioxidant, was add-

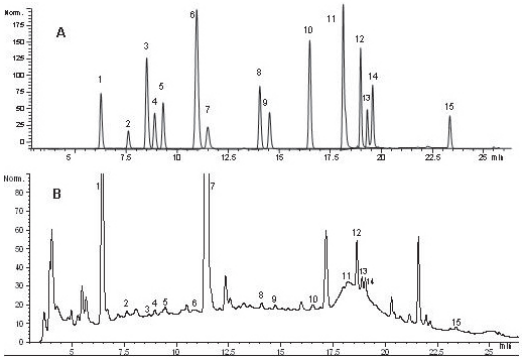

Fig. 1 - Chromatographic profiles of standard mixture (A) and *N. sativa* extract (B) monitored at 280 nm. The peaks correspond to: 1) gallic acid, 2) (-)-epicatechin, 3) *p*-dihydroxybenzoic acid, 4) chlorogenic acid, 5) (+)-catechin, 6) syringic acid, 7) vanillic acid, 8) *p*-coumaric acid, 9) ferulic acid, 10) *trans*-2-hydroxycinnamic acid, 11) quercetin, 12) *trans*-cinnamic acid, 13) apigenin, 14) amentoflavone, 15) flavone.

ed to prevent the phenolic degradation during hydrolysis (NARDINI and GHISELLI, 2004). Fig. 1 shows the chromatograms of authentic standards and *N. sativa* seeds. RP-HPLC successfully identified 15 phenolic compounds. These phenolics were identified according to their retention times and the spectral characteristics of their peaks compared with those of the standards, as well as by spiking the sample with standards. The total amounts of the various phenolic acids and flavonoids are shown in Table 2. Vanillic acid was the most abundant phenolic compound with a mean concentration of 190±0.34 mg/100 g, followed by gallic acid (40.9±0.92 mg/100 g). The *trans*-cinnamic acid, quercetin, amentoflavone and apigenin values were 10.84±0.76, 2.35±0.06, 1.23±0.17 and 1.06±0.3 mg/100 g, respectively. The reported values of the various compounds in our study are close to those reported in similar aromatic and medicinal herbs (ZHENG and WANG, 2001).

The total amount of phenolic compounds in *N. sativa* determined by HPLC was 2.26 mg.g^{-1} dry weight, that is about three times less than that obtained by the Folin-Ciocalteu method. This result was expected due to the low selectivity of the Folin-Ciocalteu reagent; it reacts positively with polyphenols as well as various non-phenolic compounds.

Antioxidant capacity

The assessment of the radical scavenging capacity of *N. sativa* by the DPPH test showed that both the essential oil and methanol extract exhibited strong and variable antiradical activity (Table 3). The essential oil effectively scavenged the DPPH radical; in fact, its IC_{50} value was 14 μg.mL^{-1} which is comparable to that of the synthetic antioxidant BHT (IC_{50} = 12 μg.mL^{-1}). The Tunisian *N. sativa* was a better hydrogen atoms or electrons donor than the Austrian species; its IC_{50} value was 460 μg.mL^{-1} (BUR-

Table 2 - Phenolic acid and flavonoid content of *N. sativa* methanol extract.

Phenolic compounds	Content (mg/100 g dry sample)*
Phenolic acids	
Gallic acid	40.9±0.92
p-dihydroxybenzoic acid	0.14±0.36
Chlorogenic acid	0.77±0.56
Syringic acid	0.76±0.59
Vanillic acid	190±0.34
p-coumaric acid	1±0.09
Ferulic acid	0.51±0.04
trans-2-hydroxycinnamic acid	0.76±0.04
trans-cinnamic acid	10.84±0.76
Flavonoids	
(-)- Epicatechin	0.95±0.46
(+)- Catechin hydrated	0.84±0.14
Quercetin	2.35±0.06
Apigenin	1.06±0.3
Amentoflavone	1.23±0.17
Flavone	0.49±1.04
Unknown peaks	40.64
Total	294.61

*Each value is the mean of three replications.

ITS and BUCAR, 2000). The antiradical capacity of the methanol extract was lower than that of the essential oil; the IC_{50} was 150 $\mu g.mL^{-1}$. The antiradical property of the methanol extract from Tunisian seeds was about 8 times higher than that of the Indian species (THIPPESWAMY and NAIDU, 2005). On the other hand, the capacity of the essential oil and extract to quench the superoxide anion was greater than the DPPH scavenging capacity. Furthermore, the essential oil exhibited a higher antioxidant activity than the methanol extract (the IC_{50} values were 6 and 24 $\mu g.mL^{-1}$ for the essential oil and the extract, respectively).

It is evident that *N. sativa* essential oil was more active than the methanol extract. The efficiency of essential oils to act as antioxidants was reported by RICCI *et al.* (2005). The strong antioxidant capacity of *N. sativa* essential oil could be related to the activity of some compounds present in the oil. RUBERTO and BARRATA (2000) studied the antioxidant activity of 96 pure essential oil components and reported that terpinolene, α-terpinene and γ-terpinene, present in *N. sativa* oil in significant amounts (Table 1), showed strong antioxidant capacity. It is well known, that the essential oil compounds that have the most effective antioxidant activities are oxygenated monoterpenes, especially phenols and alcohols (YANISHLIEVA *et al.*, 1999). Appreciable levels of these two classes were also found in *N. sativa* oil (7.82 and 5.64% respectively). On the other hand, when tested individually, some volatile compounds had a lower antioxidant capacity than that of the activity of the whole oil. In this context, *p*-cymene, the major compound of *N. sativa* oil, has been reported to have a weak antioxidant capacity (DORMAN *et al.*, 2000). These results suggest that there is a synergy among the different chemicals of the essential oil; this should be taken into consideration when studying the biological activity.

The phenolic compounds in the *N. sativa* methanol extract (Table 2) probably contributed to the potent antioxidant activity of the extract because there is a positive correlation between phenolic composition and antioxidant activity (FEI *et al.*, 2006). Moreover, the antioxidant property of seeds could be due to the activity of the benzoates, especially vanillic acid because a number of studies have shown it to be a potent antioxidant, capable of quenching radicals, singlet oxygen and hydrogen peroxide (NATELLA *et al.*, 1999; MANOURI *et al.*, 2005; TRIANTISA *et al.*, 2005; CHIOU *et al.*, 2007). Some minor phenolic compounds must also be considered because the molecular structure and differences in number and position of the hydroxyl group on the aromatic ring influences the antioxidant activity (RICE-EVANS *et al.*, 1996). The presence of the CH=CH–COOH group found in hydroxycinnamic acids (*p*-coumaric, ferulic, chlorogenic, *trans*-cinnamic acids) is thought to be essential for its antioxidative efficiency (GOMBAU *et al.*, 2006). In the present study, quercetin,

Table 3 - Amount of total phenolic compounds and effect of essential oil and methanol extract of *N. sativa* seeds on DPPH and superoxide scavenging activity.

Samples	Total phenolics (mg GAE.g DW^{-1})	DPPH*	Superoxide*
Methanol extract	8.17±0.01	150±0.02	24±0.09
Essential oil	NE	14±0.07	6±0.05
Positive control BHT	-	12±0.02	0.2

*IC_{50} values ($\mu g.mL^{-1}$); NE = not examined.

was found to be the most abundant flavonoid (2.35±0.06 mg/100 g). This flavonol is a good antioxidant, due to the presence of the *o*-dihydroxy and *o*-hydroxyketo groups (VINSON *et al.*, 1995).

Mutagenic and antimutagenic activity

Results of the Ames test are reported in Table 4. Eighteen spontaneous revertants were present and none of the tested extracts (essential oil and methanol extract), not even at high concentrations, significantly increased the number of revertants in the *Salmonella typhimurium* (TA1535) strain. At higher concentrations (250 and 500 $\mu g.mL^{-1}$), the essential oil became slightly toxic and inhibited the viability of the strain revertants. The number of revertants decreased to 15.33 and 16.33, respectively. The absence of mutagenicity within the extracts of *N. sativa* in the *Salmonella* strain tested indicated that DNA does not seem to be a relevant target. Sodium azide damages DNA and thus induces mutagenicity. A dose of 1.5 μg per plate of this mutagen was chosen for the antimutagenicity study, since this dose was not toxic and induced 1320 revertants in *S. thyphimurium* TA1535. The inhibitory effect of the methanol extract and the essential oil on the mutagenicity of sodium azide using the plate incorporation assay is illustrated in Table 4. The results indicate that the seeds had a significant dose-related effect on mutagenicity. The highest antimutagenic activity of *N. sativa* essential oil was obtained at a low concentration (50 ug/plates); higher doses failed to increase antimutagenicity. This type of response has also been reported in other antimutagenic studies (FERRER *et al.*, 2002). In contrast, the methanol extract gave higher activity at a higher concentration of 500 $\mu g.mL^{-1}$ than at a lower one.

Independent of dose concentrations, the essential oil and methanol extract exhibited an important and similar antimutagenic effect; the percentage inhibition was 58.54 and 60.78%, respectively. The relevant antimutagenic activity of *N. sativa* seeds associated with an absence of genotoxicity suggests that they contain interesting active compounds. Our data suggest that their antigenotoxic activity is mediated by their antioxidative property as reported by several authors (KAUR and SAINI 2000; KILANI *et al.*, 2005; BOUHLEL *et al.*, 2007). In fact, many carcinogens and/or mutagens produce reactive oxygen species by interacting with cellular macromolecules, thus, with their antiradical properties; the antioxidants inhibit DNA lesions that would be generated by oxidative stress, and prevent mutagenicity (KAUR and SAINI, 2000). In this study, the methanol extract contained a high level of phenolic

Table 4 - Mutagenic and antimutagenic effect of essential oil and methanol extract of *N. sativa* seeds on *S. typhimurium* TA1535.

	Dose (μg/plate)	No. of revertants/plate	No. of revertants/plate in presence of sodium azide	Antimutagenic activity (% inhibition)
Essential oil	50	24.33±2.3	561.67±1.9	58.54
	250	15.33±2.3	591.66±3.2	56.18
	500	16.33±1.7	891.33±4.7	33.06
Methanolic extract	50	22±1.1	878.33±3.2	34.07
	250	26.33±0.6	545.66±5.2	59.03
	500	26.33±0.6	532±3.3	60.78
Spontaneous revertants		18±1.13	1,320±18.3	

compounds (Table 2); several of the flavonoids and phenolic acids particularly the benzoic ones, have been reported to be powerful antimutagenic and antitumor agents (GICHNER *et al.*, 1987; CHOI *et al.*, 1994; BIROSOVA *et al.*, 2005). The antimutagenic capacity of Tunisian *N. sativa* essential oil can be attributed to the effect of its main compounds, *p*-cymene, γ-terpinene, α-thujene and thymol. With weak genotoxic potential, several volatile compounds have been reported to have a strong antimutagenic and antitumor effect (ZEYTINOGLU *et al.*, 2003).

CONCLUSION

The results of this study show that the essential oil and methanol extracts obtained from Tunisian *N. sativa* seeds exert significant antioxidant and antimutagenic effects by scavenging radicals and inhibiting induced mutagenicity in a modified *S. typhimurium* (TA1535) strain. These activities could be related to the high terpenic hydrocarbon content in the essential oil and the high phenolic acid and flavonoid content in the methanol extract. These findings suggest that Tunisian *N. sativa* seeds could be a potential source of natural antioxidant and antimutagenic compounds.

ABBREVIATIONS

Nigella sativa (*N. sativa*), gas chromatography (GC), gas chromatography/mass spectrometry (GC/MS), reverse phase high performance liquid chromatography (RP-HPLC), butylated hydroxytoluene (BHT), Folin-Ciocalteu (F-C), 1, 1-diphenyl-2-picrylhydrazyl (DPPH), phenazine methosulfate (PMS), nicotinamid-adenin-dinucleotid (NADH), nitroblue tetrazolium (NBT), dimethyl sulfoxide (DMSO), the extract concentration required to cause 50% inhibition (IC_{50}).

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PAPER

APPLICATION OF FOOD GRADE COATINGS TO TURKEY BUTTOCKS

USO DI DIVERSI INGREDIENTI ALIMENTARI
NELL'IMPANATURA DI COSCE DI TACCHINO

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ABSTRACT

Battering and breading control the level of moisture removal and fat absorption and also improve the physical and sensorial properties of deep-fried products. The effects of zein, soy protein isolate (SPI), guar and xanthan gums and corn and soy flours on the quality of turkey nuggets were studied using Response Surface Methodology. Turkey buttock chunks were predusted with combinations of zein and SPI (25:75, 50:50, 75:25%), then sprayed with combinations of guar and xan-

RIASSUNTO

L'impanatura e l'infarinatura controllano il livello di rimozione dell'umidità e l'assorbimento di grasso ed inoltre migliorano le proprietà fisiche e sensoriali dei prodotti fritti. Utilizzando la Response Surface Methodology è stato studiato l'effetto della zeina, isolati proteici della soia (SPI), gomme di guar e xantano e farine di mais e soia sulla qualità dei nugget di tacchino. Le cosce di tacchino pre-impolverate con combinazioni di zeina ed SPI (25:75, 50:50, 75:25), poi irrorate con combinazioni di gom-

- Key words: breading, coating, food grade film, frying, turkey buttock -

than gum (0.1:0.3, 0.2:0.2, 0.3:0.1%) after which they were breaded with corn and soy flour (25:75, 50:50, 75:25%). The samples were evaluated for the degree of adhesion, fat absorption, cooking yield and color attributes. The pre-dusting adhesion rate (PD-AR) was higher than 5% with increased zein in the blend. Film-forming properties of zein also reduced fat uptake during frying. Wetting stage altered the last coating adhesion rate (LC-AR). Analytical optimization of nuggets having a good coating, higher cooked yield and desired color were obtained with zein and SPI at the ratio of 73:27% as pre-dusting, guar and xanthan at the ratio of 0.3:0.1%, and corn and soy flour at the ratio of 35:75. Adhesion rate of the last coating and cooking yield were correlated with each other (*r=0.73*, P<0.001).

me di guar e xantano (0,1:0,3, 0,2:0,2, 0,3:0,1) dopodichè infarinate con farine di mais e soia (25:75, 50:50, 75:25). I campioni sono stati valutati per il grado di adesione, assorbimento di grassi, resa di cottura, e caratteristiche del colore. Il rapporto di pre-impolveramento (PD-AR) è risultato più alto del 5% con l'incremento della zeina nella miscela. Le proprietà di formazione di film della zeina hanno anche ridotto l'assorbimento di grassi durante la frittura. Lo stadio di bagnatura ha alterato il rapporto di adesione dell'ultimo rivestimento (LC-AR). L'ottimizzazione analitica dei nugget aventi un buon rivestimento, una migliore resa di cottura e colore desiderato è stata ottenuta con zeina e SPI in rapporto di 73:27% come pre-impolveramento, guar e xantano in rapporto di 0,3:0,1% e farina di mais e soia in rapporto di 35:75. Il rapporto di adesione dell'ultimo rivestimento e resa di cottura sono risultati correlati tra loro (*r=0,73*, P<0,001).

INTRODUCTION

Deep-fat frying is one of the most preferred procedures for food preparation because it leads to the development of desirable flavor and texture. Awareness of the need to reduce oil uptake in fried foods has increased (MOHAMED *et al.*, 1998; DOGAN *et al.*, 2005). To respond to the recent trends and consumer demand for low fat products, oil uptake must be reduced during deep-fat frying. The food industry has been responding to these challenges by introducing low-fat and reduced-fat products.

Battered and breaded products constitute the largest segment of the processed poultry market. The trend to use batter and breading on chicken has increased approximately 100% since the 1980s (MUKPRASIRT *et al.*, 2000).

Moisture migration in foods is a serious problem which can adversely alter the texture, flavor and nutritive value of food and it is also a critical factor that affects the sensory quality and shelf life of the products. Dry and crispy cereal foods may become soggy and lose their crispness upon water absorption and soft textured foods may become hard upon loss of water (YANG and PAULSON, 2000).

Studies on the development of coatings are receiving more attention as coatings add value to the fried products by controlling the moisture removal and oil uptake rate, and they also make food more attractive (MALLIKARJUNAN *et al.*, 1997; HOLOWNIA *et al.*, 2000; DOGAN *et al.*, 2005). Such coating must have sensorial properties that are desirable to the consumer such as crisp texture and appealing color.

Various ingredients such as alginate, powdered cellulose, methyl cellulose and soy protein isolates have been used because of their barrier properties (MALLIKARJUNAN *et al.*, 1997). Some newly developed low-fat products also include fat substitutes in their formulations.

MALLIKARJUNAN *et al.* (1997) demonstrated the potential of edible films for moisture retention and reduction in fat absorption during frying of poultry products. Compared to uncoated control samples, chicken nuggets coated with an edible hydroxypropyl methyl cellulose (HPMC) coating reduced oil absorption by 17.9 and 33.7% in the surface layer and core, respectively. Coated samples also improved moisture retention up to 8.6% in the surface layer and 16.4% in the core, compared to a control sample (BALASUBRAMANIAM *et al.*, 1997). Edible coatings can also be used as natural antioxidants on the surface because the coating has low oxygen permeability. It has been shown that the mechanism of protection exerted by the coatings was due to their action as an oxygen barrier (MATE and KOCHTA, 1997).

The objective of this study was to evaluate the moisture retention and fat reduction capabilities of different coating materials during deep-fat frying of turkey buttocks. Response Surface Methodology (RSM) was used to determine the effects of each coating on the quality attributes.

MATERIALS AND METHODS

Materials

Commercial turkey buttocks of 14-week-old turkeys were purchased from Van-Et Integrated Meat Co., Van, Turkey. All visible connective tissue, fat and skin were removed before processing. The buttocks were chopped into pieces of approx. 3x3 cm. The meat pieces were then weighed and 1 kg of samples was placed in each polyethylene bag and immediately frozen at -18°C until the experiment.

The following materials were used for breading. Corn-zein (Sunar Corn Products, Co., Adana, Turkey), soy protein isolate (Elit Mumessillik Export and Import, Incorp., Istanbul, Turkey), guar and xanthan gums (Asuka Chemical Co., Istanbul, Turkey), soy flour (Selka Food and Chemicals Incorp., Istanbul, Turkey), onion and garlic powder (Kurtsan Co., Istanbul, Turkey), and salt obtained from a local market. A catering-type hydrogenated palm olein from Paksoy Fat Co., Adana, Turkey was used as the frying oil. Some chemical characteristics and the coded and actual levels of the coating materials are reported in Table 1. Garlic powder (3%), salt (3%) and onion powder (2%) based on the weight of the last coating (LC) were added to the LC to enhance the flavor. The other chemicals used in the experiment were reagent-grade and purchased from Merck Chemical Company, Darmstad, Germany.

Method

Chopped and frozen turkey buttocks were thawed overnight at 3°C and taken to room temperature until reaching the core temperature of 17±1°C. The meat was first predusted, and then an aqueous solution of xanthan and guar gum was sprayed on the surface of the predusted samples for wetting. Samples were subjected to a brief air drying for 2 min. As a last step, the meat was coated with a combination of soy and corn flours. Coated meat samples were fried at 185±5°C for 5.5 min. Fried nuggets were cooled to room temperature and analyzed.

Physical and chemical analyses

The moisture content of the breading materials was determined gravimetrically by oven-drying at 104°C for 4 h while

Table 1 - Characteristics and coded levels of coating material combinations (g/100 g).

Combinations	Properties			Coded Levels				
	Moisture (%)	Fat (%)	Protein (%)	-1.682	-1	0	1	1.682
Pre-dusting (PD)								
Zein	6.6	0.8	62.7	15	25	50	75	85
Soy protein isolate (SPI)	8.4	0.6	87.4	85	75	50	25	15
Wetting Stage (WS)								
Guar				0.06	0.1	0.2	0.3	0.34
Xanthan				0.34	0.3	0.2	0.1	0.06
Last Coating (LC)								
Corn Flour (CF)	12.2	6.4	6.2	15	25	50	75	85
Soy Flour (SF)	8.4	3.6	51.0	85	75	50	25	15

the protein content of the breading materials was measured using Kjeldahl analysis for the pre-coating mixture and for the last coating (AACC, 1995). Total fat content in non-coated and coated samples was measured by the Soxhlet extraction method according to the Official Methods of the AACC (1995) and is expressed on dry basis (db). The free fatty acid (FFA) level in the frying fat and after five batches was determined according to the Official Methods of the AACC (1995) and is expressed in terms of oleic acid. Particle size distribution of soy protein isolate (SPI), corn-zein protein, soy flour (SF) and corn flour (CF) was determined in a ro-tap shaker with U.S. standard sieves. Adhesion rate and cooked yield were determined according to GIBNEY *et al.* (1999). Weights were recorded at pre-dusting, last coating and after frying. Adhesion rate is described as the percentage weight gain of the buttock after pre-dusting and last coating. Cooked yield is described as the ratio of fried and coated nugget weight to the initial weight, expressed as a percentage. The color of the fried coated samples was measured on each cooked nugget and is expressed according to CIELAB systems as L (lightness), a (redness) and b (yellowness) values as described by DOGAN (2006). A flatbed scanner was used to capture the color image of the samples. L, a and b values were quantitatively analyzed using Photoshop 6.0 Software, Adobe Systems (2000). Hue angles were calculated as defined by the following equation:

$$h = \arctan (b/a)$$

Statistical analysis

The study was conducted using a central composite design to model the response. Six replicates at the center and a total of 20 trial combinations were used to estimate variances. The experiments were carried out in duplicate. Analysis of variance (ANOVA) and estimates for the interactions were determined using Design Expert 7.0 (STAT-EASE, 2005). The best models offered by the software that explain relationships between input and response variables were used to calculate the regression equations in the study.

RESULTS AND DISCUSSION

The average moisture and fat content and pH level of turkey buttocks were 74.7, 5.20 and 5.8%, respectively. After frying those levels were 46.17, 17.70 and 6.2%, respectively, in the samples with the coating layer removed.

Table 2 - Particle size distribution of coatings (g/100 g).

Combinations	>224 μ	125-224 μ	<125 μ	>125 μ	75-125 μ	<75 μ
Pre-dusting (PD)						
Zein	87.85	7.85	4.15			
Soy protein isolate (SPI)				43.35	49.75	6.70
Last Coating (LC)						
Corn Flour (CF)	78.50	20.45	0.10			
Soy Flour (SF)				38.90	38.45	22.65

Particle size distribution

Particle size distribution varied depending on the types and ratios of the ingredients in the blends. It altered the adhesion rate of pre-dusting (PD) and last coating (LC) to the surface of the meats. Particle size of the gums used in the wetting stage (WS) was also an important factor in preparing homogeneous gum solution mixtures.

The ratio of corn flour sizing over 224 μ was 78.5%; the ratio of soy flour sizing over 125 μ was 38.9% (Table 2). As in commercial practice, each part of any breading is classified in three ranges; coarse, medium and fine. According to this classification, corn flour is coarse and soy flour is fine. While coarse particles increase weight and make the breaded meat more attractive to the consumer, fine particles have high area-to-volume ratios that increase the absorption rate and improve adhesion of coarser particles (DYSON, 1992). The size distribution is also critical for obtaining the desired browning rate and textural attributes such as smoothness.

Adhesion rates (AR)
Pre-dusting (PD-AR)

PD-AR expresses the adhesion rate to the meat surface. Pre-dusting was applied to give a dry and homogeneous surface. Pre-dusting also absorbed the moisture on the surface and improved the success of subsequent coatings. PD-AR ranged from 3.2-6.5% depending on the levels of zein and soy protein isolate (SPI) in the blend. PD-AR changed significantly ($P<0.01$), and increased with the level of zein. The prediction polynomial model to express the effect of zein and SPI in the blend is as follows:

$$PD\text{-}AR = 4.68 + 0.696*PD$$
$$(R^2=0.715,\ P<0.001)$$

PD-AR was higher than 5% when more than 75% zein was added to the blend (Fig. 1). Zein, as the corn prolamine, forms excellent films and is successfully used as a coating protein (KUNTZ, 1997). For the adhesion rate, not only protein level but also the source of pro-

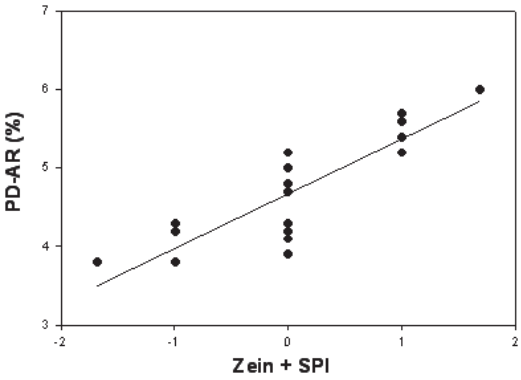

Fig. 1 - Effects of Zein/SPI on pre-dusting adhesion rate (PD-AR).
Codes, -1: (25:75); 0: (50:50); 1: (75:25), respectively.

teins is important. As shown in Table 1, zein has 25% less protein than SPI.

Last coating (LC-AR)

Last coating (LC), i.e. last breading stage, influences the level of absorbed fat and product attractiveness. The LC-ARs were between 12.1-25.5%. In all the tested combinations, the coating portion of turkey nuggets was less than 30% which is the maximum amount of coating permitted in some meat and poultry industries (KUNTZ, 1997).

The regression equation expressing the effects of coating applications on LC-AR is as follows:

$$\text{LC-AR} = 18.88 - 0.40*(PD) + 1.46*(WS) + 0.52*(LC) + 1.24*(PD*WS) - 0.24*(PD*LC) - 0.31*(WS*LC) + 0.21*(PD)^2 - 0.18*(WS)^2 - 0.97*(LC)^2$$

$$(R^2=0.751,\ P<0.05)$$

For LC-AR, the effects of WS were highly significant ($P<0.01$), the interaction of PD and WS and the quadratic effect of LC were significant ($P<0.05$). Addition of guar between 0.2-0.3%, LC-AR ranged from 19.5 to 21.0% (Fig. 2). Zein addition with increased guar level in the solution of guar/xantan mixtures slightly improved the LC-AR; however the difference was not significant ($P>0.05$). Increasing guar level from 0.1 to 0.3 in the wetting solution, and applying corn and soy flour at the ratio of 40:60 as LC material improved the LC-AR ($P<0.01$).

Guar gum and xanthan gum solutions are non-ionic and their solutions are not affected by the presence of salt (MEYERS, 1992). Xanthan gum is cold water-soluble and exhibits a synergistic interaction with guar gum. This interaction depends on the mixture ratio, pH and ionic strength (SWORN, 2000). In this study, a low concentration of xanthan (1%) in the mixtures gave a higher adhesion rate.

Fat Content (FC)

The mean fat absorption level of non-coated turkey samples was 17.7%. The absorption rates on coated nuggets were between 30.3-37.0%. The equation to express fat absorption levels of

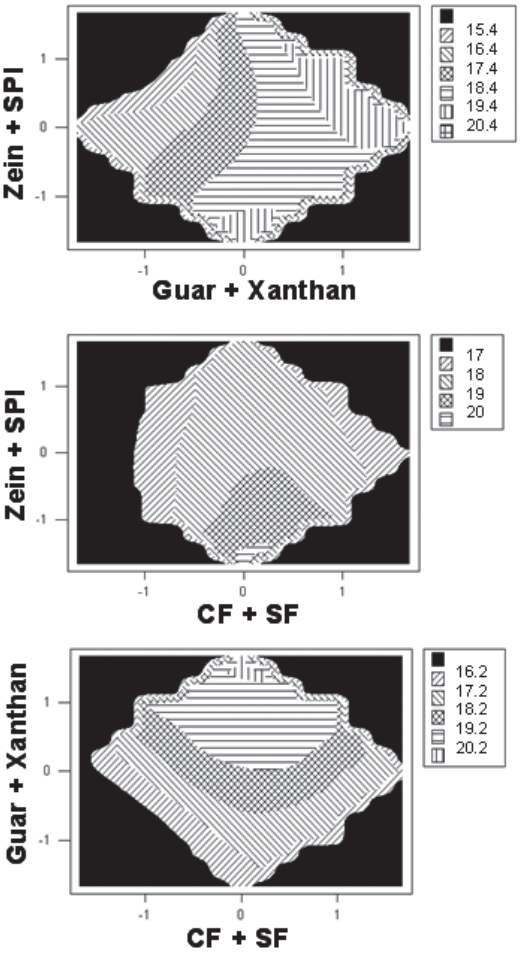

Fig. 2 - Effects of PD, WS and LC on last coating adhesion rate (LC-AR).
Codes of -1, 0 and 1% in the blends of zein/soy protein isolate (SPI) represent the ratios of (25:75), (50:50) and (75:25), respectively.
Codes of -1, 0 and 1% in guar/xanthan solution represent the ratios of (0.1:0.3), (0.2:0.2), and (0.3:0.1), respectively.
Codes of -1, 0 and 1% in the blends of corn flour (CF)/soy flour (SF) represent the ratios of (25:75), (50:50) and (75:25), respectively.

nuggets after deep-fat frying is as follows:

$$FC = 34.15 - 0.63*PD + 0.64*WS + 0.13*LC - 0.66*(PD*WS) - 0.39*(PD*LC) + 0.17*(WS*LC) - 1.18*(PD)^2 + 0.23*(WS)^2 - 0.28*(LC)^2 \quad (R^2=0.562,\ P>0.05)$$

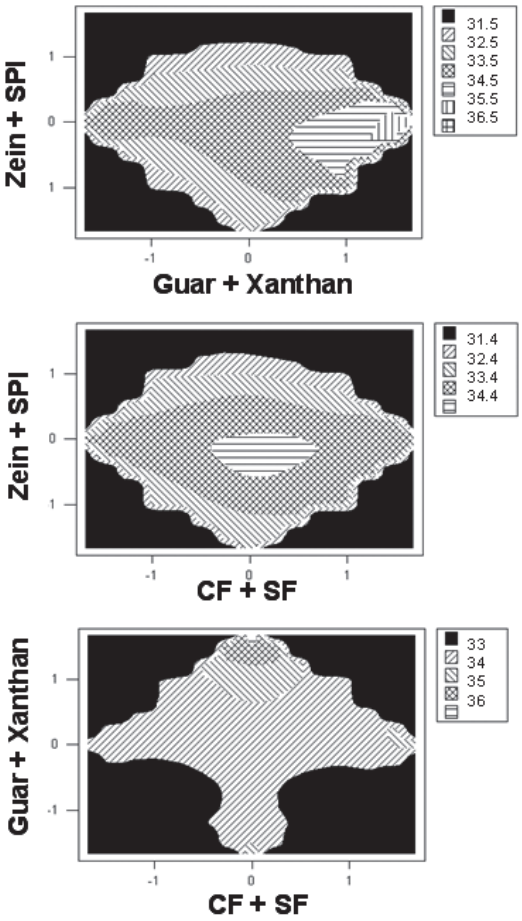

Fig - 3. Effects of PD, WS and LC on fat absorption level (%).
Codes of -1, 0 and 1% in the blends of zein/soy protein isolate (SPI) represent the ratios of (25:75), (50:50) and (75:25), respectively.
Codes of -1, 0 and 1% in guar/xanthan solution represent the ratios of (0.1:0.3), (0.2:0.2) and (0.3:0.1), respectively.
Codes of -1, 0 and 1% in the blends of corn flour (CF)/soy flour (SF) represent the ratios of (25:75), (50:50) and (75:25), respectively.

The quadratic effect of pre-dusting was significant ($P<0.05$). The fat absorption level tended to increase with a 50:50 ratio of zein/SPI mixtures. At this ratio, incorporation of more than 0.25% guar gum contributed to an increased fat absorption (Fig. 3). Increasing the zein level to 65% yielded less fat uptake because of the film-forming property of zein (FEENEY *et al.*, 1993).

Changes in Free Fatty Acids (FFA)

FFA formation is an indication of rancidity and is used to estimate the fat quality. The mean FFA value of fresh fat was 0.07% oleic acid. The FFA of the fat increased to 0.20-0.22% after the frying of five batches. The FFA level of the frying fat was below the range of 0.5-0.8% oleic acid as specified by WEISS (1970) and between 0.25-0.75 as given by WILLYARD (1993) indicating that the frying fat was still usable. The increase in FFA could be attributed to hydrolysis of frying fat (GOBURDHUN *et al.*, 2000).

GOBURDHUN *et al.* (2000) reported that the FFA level of frying fat of potato and chicken increased from 0.154% to 0.219 and 0.279%, respectively after 315 min of frying. The difference between the two products was not significant.

Cooked Yield (CY)

CY is a measure of weight change before and after frying. The CY changed during frying due to moisture removal and oil pick up. The regression equation to explain the effects of coating applications on CY is as follows:

$$CY = 88.45 - 0.40*PD + 1.89*WS + 3.26*LC + 1.94*(PD*WS) - 0.54*(PD*LC) - 0.11*(WS*LC) - 0.36*(PD)^2 + 0.46*(WS)^2 - 1.98*(LC)^2 \quad (R^2=0.858,\ P<0.001)$$

According to the effect of breading combinations on CY, linear effects of

LC (P<0.001) and WS (P<0.01), quadratic effect of LC (P<0.01) and an interaction term of PD and WS (P<0.05) were significant.

Increased levels of zein in PD and of guar gum in WS and corn flour in LC improved CY (Fig. 4). The highest coefficient of LC in the regression equation also indicates that LC is the most important breading stage. There was also a strong correlation between adhesion rate of LC and cooked yield (*r=0.73*, P<0.001). A higher corn flour level in LC increased the adhesion level. BURGE (1992) observed the same effect in a batter-type of breading.

The ratio of particles over 224 μ in zein and corn flour were 87.9 and 78.5%, respectively. Particle size distribution of PD and LC were also factors that contributed to CY as well as texture. GERDES (2001) indicated that a fine granulation of breading develops a smooth surface; using larger granulations results in open-textured crumbs. Both types are widely used to obtain more textural variety in breaded foods.

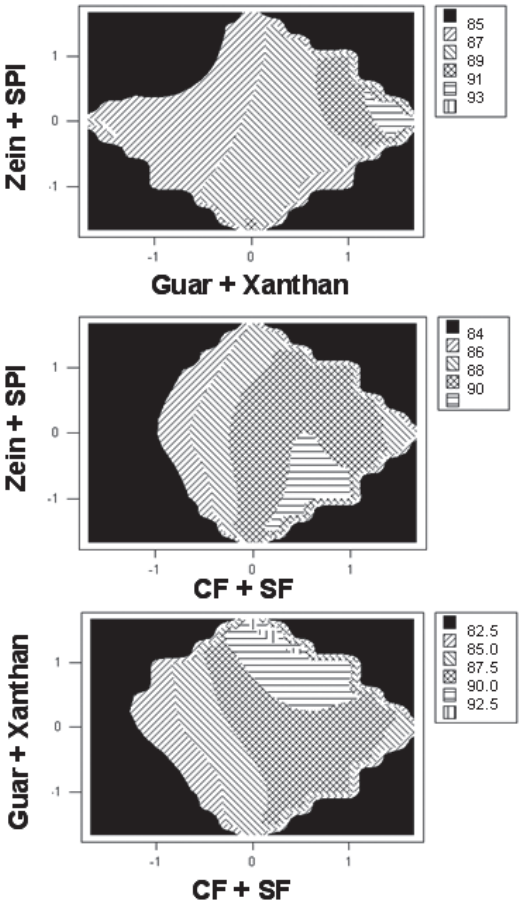

Fig. 4 - Effects of PD, WS and LC on cooked yield (%).
Codes of -1, 0 and 1% in the blends of zein/soy protein isolate (SPI) represent the ratios of (25:75), (50:50) and (75:25), respectively.
Codes of -1, 0 and 1% in guar/xanthan solution represent the ratios of (0.1:0.3), (0.2:0.2) and (0.3:0.1), respectively.
Codes of -1, 0 and 1% in the blends of corn flour (CF)/soy flour (SF) represent the ratios of (25:75), (50:50) and (75:25), respectively.

Breaded nugget colors

The color of deep-fried products changes depending on the coating materials and frying conditions. In the study, *L*, *a* and *b* values from the color parameters changed between 47.6-61.7, 10.6-16.5, and 32.5-40.0, respectively. Only *a* values, *i.e.* hue angles, were significantly affected by LC after frying (P<0.05). An increase in the *a* values enhanced color development. The best regression model for a value is given as follows:

$$a = 15.06 + 0.20*(PD) + 0.13*(WS) - 1.7*(LC)\ (R^2=0.41,\ P<0.05)$$

The hue angle values (*h*) of fried samples ranged from 1.108 to 1.295. Increasing the amount of soy flour in the LC provided more crust color development (lower *h* values). In the frying process the interactions between coating materials and the nature of the reactions are complex and need to be clarified (ESTELLER *et al.*, 2006). Conformity of breaded foods to certain specification should be provided by using color sorting charts.

Optimization

The optimization was performed through a numerical method using Design Expert 7.0 (STAT-EASE, 2005). The process is described by MYERS and MONTGOMERY (2002). To achieve the highest values of PD (5.45%), LC (20.96%) and CY (90.69%), desired hue angle (1.16) and FC (33%), optimization criteria for PD (Zein:SPI) at the ratio of 73:27%, for WS (Guar: Xanthan) at the ratio of 0.3:0.1%, for LC (Corn:Soy Flour) at the ratio of 35:75 resulted from the applied methodology. The highest desirability value for the suggested solution was 0.865. According to MYERS and MONTGOMERY (2002), a desirability of 1 defines an acceptable result, while 0 defines unacceptable results. Therefore, the value obtained satisfied the optimum objectives.

CONCLUSIONS

Pre-dusting, wetting stage and last coating as breading steps altered the adhesion rates, cooked yields and color of samples at varied levels. Zein, SPI, corn and soy flour blends affected adhesion rates as well as cooked yields. Zein was more effective in pre-dusting application due to film-forming properties. SPI increased the homogeneity of the last coating to a certain extent. By increasing the guar level to 3% and xanthan gum to 1% in wetting solutions and 37% corn flour in the last coating mixture there was gave a higher adhesion rate in the last coating and a greater cooked yield. The cooked yield is the most important factor in breading applications from the producer and consumers point of view. By selecting suitable pre-dusting, last coating and gum combinations, it is possible to control the moisture rate, fat absorption level, adhesion rate and cooked yield as well as all the desired attributes.

NOMENCLATURE

Adhesion rate ..... AR
Cooked yield ..... CY
Corn flour ..... CF
Fat content ..... FC
Last coating LC
Pre-dusting ..... PD
Soy flour ..... SF
Soy protein isolate ..... SPI
Wetting stage ..... WS

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PAPER

MEAT QUALITY OF BROILERS FROM DIFFERENT REARING SYSTEMS

QUALITÀ DELLA CARNE DI POLLI ALLEVATI IN DIFFERENTI SISTEMI DI ALLEVAMENTO

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ABSTRACT

Color, pH, shear force, water-holding capacity, chemical composition, cholesterol content, and fatty acid profile from conventional, free-range and alternative broiler breast meat were determined in order to evaluate differences in the quality of broiler meat produced under different systems. Broilers reared in a conventional system had the highest lipid content (1.3%) but lower proportions of polyunsaturated (17.3%) and ω-3 fatty acids (0.3%) ($p<0.05$) compared to free-range and alternative

RIASSUNTO

Alcune caratteristiche qualitative quali colore, pH, sforzo di taglio, potere di ritenzione idrica, composizione chimica centesimale, contenuto di colesterolo e profilo acidico sono stati determinati nella carne di polli allevati con tre sistemi: convenzionale, all'aperto e alternativo. Gli animali allevati con il sistema convenzionale hanno presentato valori maggiori di lipidi (1,3%) e minor proporzione di acidi grassi polinsaturi (17,3%) e ω-3 (0,3%) ($p<0,05$) rispetto ai sistemi all'aperto e alternativo. D'altra

- Key words: broiler production systems, meat quality -

broilers. On the other hand, free-range broilers had a lower cholesterol content (48.6 mg·100 g^{-1}) and lower pH (5.71) while broilers raised in an alternative system had a higher shear force (2.33 kgf) and lower yellowness value (b* value = 3.15) when compared to the other rearing systems ($p<0.05$).

parte, sul pollo allevato all'aperto sono stati osservati valori più bassi di colesterolo (48,6 mg·100 g^{-1}) e pH (5,71). I polli provenienti dal sistema alternativo hanno presentato i valori di sforzo al taglio più alti (2,33 kgf) e indice del giallo inferiore (b* = 3,15) quando confrontati con i campioni degli altri tipi di allevamento ($p<0,05$).

INTRODUCTION

The continuous technical evolution of Brazilian aviculture with respect to genetics, nutrition and handling has enabled low-cost, high quality meat to be produced that is competitive on the world market. With more market competitiveness and greater consumer awareness, the poultry sector has undergone some changes in its production chain in order to offer different and value-added products; the production of alternative broilers is an example (SOUZA, 2004).

In Brazil, broilers are currently produced in conventional, free-range and alternative systems. Standard systems are used on commercial farms with strains that are genetically selected for fast growth and excellent food efficiency. They are reared in intensive systems according to the health regulations in force, where the use of antibiotics, anticoccidians, growth promoters, chemotherapeutics and animal-source ingredients are permitted. The birds are slaughtered at 42 days of age, on average (MINISTRY OF AGRICULTURE, 2002).

The production of broilers in the free-range system is only allowed for specific strains. The use of growth promoters or animal-source ingredients in their feed is not permitted. The birds must have access to an outdoor area after 28 days of age. The minimum slaughter age is 85 days (MINISTRY OF AGRICULTURE, 1999).

Alternative poultry rearing systems have emerged as an option for meat production, and the main difference between alternative and conventional systems is that the former does not permit the use of antibiotics and antimicrobial growth promoters. It is defined as an intensive or non-intensive broiler production system with no strain restriction. The poultry are reared without the use of antibiotics, anticoccidians, growth promoters, chemotherapeutics or animal-source feed in their diets. The use of these substances is absolutely forbidden; if they are needed for therapeutic purposes, the lot will be marketed as conventional, implying the loss of the quality as alternative broilers (KODAWARA and DEMATTÊ FILHO, 2003).

According to FLETCHER (2002), the main quality characteristics of poultry are appearance, texture, juiciness, flavor and functional properties. Among these, appearance and texture are the most important parameters that influence the consumer in the initial selection of and final satisfaction with the product. Factors such as age, sex, strain, nutrition, handling, transportation, room temperature and fasting period affect the quality of the poultry products and must be checked at several stages along the production chain (FLETCHER, 1991).

Several researchers have demonstrated that diets containing fat sources with different unsaturation levels may affect on the lipid profile of meat and eggs, es-

pecially in relation to the fatty acid composition and cholesterol contents. The lipid metabolism in growing birds may also be affected (AN *et al.*, 1997).

Diets that are rich in ω-6 and ω-3 polyunsaturated fatty acids reduce the blood lipid levels (GRUNDY and DENKE, 1990). These fatty acids have different effects on the blood lipid concentration, ω-6 polyunsaturated fatty acids reduce the cholesterol levels, but the triacylglyceride concentration remains unchanged, while ω-3 polyunsaturated fatty acids reduce both the cholesterol and triacylglyceride levels (BERR *et al.*, 1993 and LEE *et al.*, 1992).

Information about the composition of the diets used in the different poultry production systems, as well as the effect that different diets have on the blood lipid levels and metabolism is quite limited.

The number of studies on the meat quality of alternative poultry has been limited so the objective of this study was to characterize the physicochemical properties of poultry meat raised in conventional, alternative and free-range systems.

MATERIAL AND METHODS

Sample collection

The animal density used in conventional, alternative and free-range systems was 16 birds·m^2, 12 birds·m^2 and 1 bird·3 m^2, respectively.

The conventional broilers diet included ground maize and soybean meal (phases starter, grower I, grower II and finisher), vitamin and mineral supplements in each rearing phase, as well as DL-methionine-99%, L-Lysine HCl 78%, choline and copper monosulfate. Other ingredients of animal-origin were poultry offal fat (5 and 1.8% in the grower II and finisher phases, respectively), meat and bone meal (7.2, 7.3, 6.3 and 5.5% in the four phases initially indicated above), and poultry by product meal (4.0 and 4.5%, in the grower II and finisher phases, respectively).

The alternative broilers feed was all plant-based with ground maize and soybean meal at the same rearing stages as those for conventional poultry, and supplemented with the same amino acids as in the diet for standard broilers with the addition of vitamins and minerals.

The free-range broilers were fed starter, grower and finisher diets based on maize, soybean meal, full fat extruded soybeans and supplemented with all of the nutrients to meet the nutritional requirements (the company responsible for the free-range rearing system did not authorize the publication of the diet used).

Breasts from broilers reared in conventional, free-range and alternative systems they had been slaughtered in commercial slaughterhouses were used.

Samples from the conventional production system (conventional broilers) were obtained directly from a slaughterhouse in Rio Claro/SP/Brazil. The breast meat was analyzed 24 h after slaughtering. Ross® AG 308 broilers were subjected to fasting for approximately 12 h before slaughtering and were slaughtered at 42-45 days of age.

Cobb 500 broilers were raised in the alternative system and slaughtered at 47-49 days of age. The meat from these broilers was obtained directly from a slaughterhouse in Ipeuna/SP/Brazil. The average feed withdrawal time was 9 h.

Breasts from female free-range broiler ISA JA57 and male SA77N.fr Rouge were obtained from the retail market 3 days after slaughtering. They came from a slaughterhouse in Veríssimo/MG/Brazil. Due to several difficulties and the lack of access to the slaughterhouse, samples could not be obtained directly from the producer nor was the producers disclose information about quantitative compo-

sition of the ration. These broilers were reared with free access to an outdoor area at a density of 3 birds·m^2, and were slaughtered at approximately 85 days of age after fasting for over 12 h.

Analytical determinations

The breast meat that was to be used in the physicochemical analyses was deboned and analyzed with no skin or apparent fat. The analyses were carried out with three replicates; samples were taken on three days between May and July 2005. Five breasts from each type of broiler were analyzed on each sampling day.

Color evaluation was performed using a MINOLTA Chroma Meter CR-508d color meter (Minolta, Sakai, Japan). The reading of the L* (lightness), a* (redness) and b* (yellowness) parameter values were carried out in the CIELab* system with the following characteristics: measuring area: 8 mm diameter, observation angle: 10°, illuminant D65 with specular component included. The color readings were taken in the inner part of the muscle and the mean value from four readings per breast was used.

The pH was determined using an electrode with a penetration probe with readings taken in four different spots of the muscle. A portable Oakton 300 series 35618 pH meter (Oakton Instruments, Vernon Hills, United States) was used. The measurements were made on samples from alternative and conventional broilers and free range-type broilers 24 h after slaughter.

The water-holding capacity (WHC) was measured according to NAKAMURA and KATOK (1985). In this method 1 g of raw muscle was put on filter paper, and then centrifuged at 1,500 x g for 4 min. After centrifugation, the sample was weighed and placed in an oven at 70°C for 12 h. The WHC was determined by the difference in weight of the sample after centrifugation and the weight of the dry sample divided by the initial weight; values are expressed as percentage.

To measure the shear force, the breast meat was wrapped in aluminum foil and cooked in an electric grill until the inner temperature was 82°C. The samples were then stored for 24 h at 2°C. The samples were then cut into the block-shaped pieces (2.0x1.0x1.0 cm), according to the method proposed by FRONING and UIJTTEENBOOGAART (1988). The samples with fibers perpendicularly-oriented to the blades were sheared with a FTC Texture Test System TP2 texture meter (Food Technology Corporation, Rockville, United States) coupled to a Warner Braztler-type model CW-1 Meat Shear Cell (FTC, Rockville, United States) accessory at a speed of 20 cm·min^{-1} and a load of 100 kg.

Breast meat moisture, protein and ash contents were determined according to AOAC specifications (1995). Moisture was determined in an oven at 105°C at constant weight. The protein content was quantified by the macro-Kjeldahl test, using a 6.25 for the nitrogen-protein conversion factor. The ash content was determined by incineration in a muffle furnace at 550°C. The total lipid value was determined using the acid hydrolysis method, according to PREGNOLATO and PREGNOLATO (1985). The chemical composition was determined in three replicates and the results were calculated on a wet basis and are expressed as g·100 g^{-1}.

To define the fatty acid profile, lipid extraction was performed according to the method described by FOLCH *et al.* (1957). Fatty acids were transformed into fatty acid methyl esters using the method of HARTMAN and LAGO (1973) and analyzed in a Variam 3900 gas chromatograph (Varian Inc, Palo Alto, United States) equipped with an automatic display, split ratio of 75:1, CP-SIL 88 capillary column, 100 m x 0.25 mm x 0.20 μm (Chrompack, Middelburg, The Netherlands), flame ionization detector (FID)

and a workstation with Star software. The chromatographic conditions were: temperature of the programmed column: initial temperature of 120°C for 5 min, increased to 235°C at 5°C min^{-1}, holding this temperature for 15 min; carrier gas: hydrogen at a flow of 1 $mL \cdot min^{-1}$, make-up gas: nitrogen at 30 mL; injector temperature: 270°C; detector temperature: 310°C. The quantification was carried out through area normalization and the results are expressed as % of area.

The cholesterol content was determined by the direct saponification method using 2% KOH in absolute ethanol (MAZALLI *et al.*, 2003), followed by analysis in an HP 6890 gas chromatograph (Agilent Technologies Inc, Santa Clara, United States) under the following conditions: column with stationary phase of 5% Phenyl and 95% dimethylpolysiloxane of 30 m x 0.25 mm x 0.25 μm; temperature of 160°C·1 min^{-1} – heating from 160° to 300°C (10°C·min^{-1}) – 300°C·7 min^{-1}; carrier gas (Helium) flow of 1 $mL \cdot min^{-1}$ (constant flow); split ratio of 1:50 and flow of 50 $mL \cdot min^{-1}$. The quantification (process, preparation) was obtained through internal standardization using 5-α-cholestane as reference. The results are expressed as $mg \cdot 100\ g^{-1}$.

Statistical analysis

The analyses of meat quality characteristics were determined on 15 replicates due to limitations in relation to the raw material and restrictions on obtaining samples such as those evaluated under the current experimental conditions.

The results of the physicochemical analysis were analyzed using the SPSS v. 13.0 software (SPSS Inc., Chicago, United States) (2004) and submitted to the Anderson-Darling's normality test ($\alpha = 0.05$). Analyses of variance (ANOVA) and multiple comparisons were then carried out using the Tukey test ($p<0.05$) on the data with a normal distribution. Data that did not show a normal distribution were submitted to a non-parametric analysis using the Kruskal-Wallis test. The means were compared determined using the Dunn test ($p<0.05$). Since there was no significant effect between evaluation days, this factor was excluded from the model.

RESULTS AND DISCUSSION

One difference observed with respect to the three types of rearing: was the 4-5 days in slaughtering age (between the conventional and alternative systems; 40-43 days for conventional and free-range systems and 36-38 days for the alternative and free-range systems).

The main difference between diets was the presence of copper monosulfate in the conventional diet which corresponded to the inclusion of 124 mg of Cu kg^{-1} of diet, as a growth promoting effect. Supplementating with high Cu levels (>8 mg Cu kg^{-1}) was sufficient to fulfill nutritional requirements (NRC, 1994). Another difference was that poultry offal fat was used in the conventional broiler diets, but not in the other rearing systems, while free-range and alternative broilers had an all plant-origin diet supplemented with other nutrients.

No significant differences were observed between the three types of broilers and the L* (lightness) and a* (redness) values. However, the b* (yellowness) value was significantly lower in the alternative broiler than in the others two types and which seemed much less yellow than the others (Table 1).

The L* values recorded in the present work ranged from 49.09 to 50.36 and are considered normal (Table 1). According to QIAO *et al.* (2001), L* values above 53.0 indicate that the broiler meat may have a quality problem known as pale meat. CASTELLINI *et al.* (2002) did not find any effect of the type of rearing system (or-

ganic and conventional) on the a* value. The meat yellowness may be influenced by the ingestion of xanthophyll; birds that ingest large quantities of this pigment tend to have yellower meat, skin and fat (UNITED STATES, 1998). This explains the higher b* value recorded in the free range broiler, compared to the other ones (Table 1). Although they were fed with rations made up of similar amounts of corn, the conventional broiler was also fed corn gluten meal, a xanthophyll-rich corn by-product used to give pigmentation to bird carcasses.

FANATICO *et al.* (2005) and CASTELLINI *et al.* (2002) observed a higher b* value (6.18 and 5.76, respectively) in broilers reared in systems with access to pastures (free-range and organic) than in indoor-reared broilers. The effect of ingesting carotenoids on the meat coloration was evident. The free-range birds may have ingested grass which would explain the b* value of 6.84 in the meat.

The pH value (Table 2) was significantly different ($p<0.05$) among the broilers. The free-range broilers had the lowest pH value which could have been due to the higher glycogen content of animals with a higher kinetic activity (MASOERO *et al.*, 2003). These results are in agreement which those of CASTELLINI *et al.* (2002), who found a lower pH value in the meat from organic broilers (5.75 to 5.80) compared to samples from conventionally-reared ones (5.96 to 5.98). According to these authors, the lower pH may have been due to better welfare conditions of animals reared in organic system (access to outdoor areas and slow growth), which reduces animal stress before slaughtering and decreases glycogen consumption.

In contrast, PELICIA *et al.* (2004) found

Table 1 - Color (L*, a* and b*) of broiler breast meat.

	Color		
Production system	L*	a*	b*
Conventional	50.01[a]±2.81	3.07[a]±0.86	5.99[a]±2.61
Free-range	50.35[a]±1.94	3.88[a]±1.37	6.84[a]±0.52
Alternative	49.09[a]±3.87	3.39[a]±1.04	3.14[b]±1.88
Pr > F	0.446	0.087	<0.001

[ab]Different letters in the same column indicate significant differences (P<0.05). Tukey test.
n = 15 samples; means ± standard deviation.

Table 2 - pH values, WHC and shear force of broiler breast meat.

Production system	pH	WHC %	Shear force kgf
Conventional	5.89[a] (0.17)	59.9[a] (3.09)	1.83[b] (0.40)
Free-range	5.71[b] (0.19)	60.5[a] (2.54)	1.86[b] (0.39)
Alternative	5.96[a] (0.16)	59.2[a] (2.67)	2.33[a] (0.48)
Pr > F	<0.001	0.468	<0.005

[ab]Different letters in the same column indicate significant differences (P<0.05). Tukey test.
n = 15 samples; means ± standard deviation.

no significant effect of conventional and free-range systems on pH values. They found the pH of the breast meat to be 5.98 for broilers reared in the conventional system, and 5.99 for those reared in the free-range system. This may be due to the fast that they used broilers reared up to 85 days of age in both systems. PAVAN *et al.* (2003) found no pH differences in Cobb and Ross strains, which were raised in the alternative and conventional poultry systems. They are the same strains used in the present study. The diversity of farming conditions such as type of strain, slaughtering age, feeding and rearing system found in the literature makes it difficult to compare the influence of these factors on meat quality.

There was a significant difference between meat from alternative broilers and that from the other systems in relation to the shear force (Table 2). The alternative broiler had a higher value, which means that the meat was less tender than that from conventional or free-range broilers. Shear force is influenced by several factors before and after slaughter such as stress, transport, fasting period, stunning and bleeding. As commercial samples were used in this study, the production and processing variables could not be controlled. Some of these factors may have influenced the shear force value of the alternative broilers.

PAVAN *et al.* (2003) did not observe a significant difference in relation to the shear force of the Cobb and Ross strains; the values reported by these authors (1.95 to 2.11 kgf) were similar to those found in the present study. CASTELLINI *et al.* (2002) found shear force values from 1.98 to 2.71 kgf·cm^{-2} for broilers produced in conventional and organic systems, respectively. They attributed this difference to the age (81 days) and greater physical activity of the birds reared in organic system.

SOUZA (2004) found no differences in tenderness between free-range and conventional (Cobb) strain broilers; they only found a significant increase in the shear force (2.25 kgf·cm^{-2}) of 110-day-old birds. According to this author, the glycolytic metabolism of the breast muscle decreases the effects of differences between rearing systems and, in addition, this muscle is not required when birds move.

In the present study, there were not significant, no significant differences between WHC values in the three rearing systems (Table 2). Similarly, NIELSEN *et al.* (2003) did not find differences in the WHC values of a fast-growing strain and a slow-growing one. In contrast, CASTELLINI *et al.* (2002) found a lower ($p<0.05$) WHC value in the breast meat of broilers produced in conventional system (52.0%) compared to that of organic broilers (53.2%).

There were statistical significantly differences ($p<0.05$) in the lipid and ash contents (Table 3) of the breast meat of the broilers. The broiler meat from the conventional system had a higher lipid and ash content than that from free-range broilers.

CASTELLINI *et al.* (2002) observed that birds raised in the organic rearing system had a significantly lower lipid content in their meat (0.7%) when compared to those from the conventional system (1.5%) due to more physical activity of the organic broilers. CHARTRIN *et al.* (2005) reported that the lipid value of broilers reared in the conventional system (1.3%) did not differ statistically from the Label Rouge system (1.2%). They reported that birds reared in the Label Rouge system were not allowed outdoors and, consequently, had no physical activity. Considering the results from both of these studies, the lower lipid value in the free-range broiler could be explained by the free access to the paddock which would result in more energy consumption.

The growth promoters added to the ration may have contributed to a higher

amount of lipids in the meat of the conventional broilers. Although the alternative broiler was reared indoors and fed with a ration that contained metabolizing energy like the conventional broiler (3.2 Mcal·kg^{-1}), the lipid content values in birds from the alternative system were similar to those of the free-range broilers. According to DIBNER and RICHARDS (2005), antibiotic growth promoters improve food conversion in birds because they decrease the microbial competition for nutrients and reduce metabolites produced by microorganisms that decrease animal growth.

The cholesterol content (Table 3) was significantly lower in free-range broilers. Some studies, such as those of KONJUCFCA *et al.* (1997) and PESTI and BAKALLI (1996) report that birds fed with high dietary Cu concentrations have lower breast and blood plasma cholesterol levels, while in the same research, they observed higher cholesterol content in broilers that were fed a standard diet. A reason for this higher cholesterol concentration could be the use of meat and bone meal in the diets. BRAGAGNOLO and RODRIGUEZ-AMAYA (1992) found 58±10 mg·100 g^{-1} of cholesterol in the white meat of broilers.

The fatty acid profiles of the broilers analyzed as well as the addition of saturated, monounsaturated, polyunsaturated and ω-3 fatty acids are reported in Table 4. The most abundant fatty acids found in the three types of broilers (in decreasing amounts) were oleic (C18:1), palmitic (C16:0) and linoleic (C18:2) acids, in agreement with LESKANIC and NOBLE (1997).

Birds that received a conventional diet which had meat meal and tallow added had palmitoleic acid contents that were higher in the breast meat than those that received rations that had soybean bran, soybean and ground yellow corn added. These results are in agreement with those found in the literature, in which the fatty acid profiles in the carcass are influenced by the fatty acid profile of the fat source added to the diet (BARTOV *et al.*, 1974).

Although most fatty acids found in nature are composed of an even number of carbon atoms (COBOS *et al.*, 1994), fatty acids composed of an odd number of carbon atoms were also found in the three types of broilers evaluated: Pentadecilic (C15:0) and margaric (C17:0) acids were found in proportions similar to those reported by RULE *et al.* (2002), who found 2.5% C15:0 and 0.2% C17:0 in broiler breast meat.

Saturated fatty acids include the sum of the myristic (C14:0), pentadecilic, palmitic, margaric and estearic (C18:0) fatty acids. These results demonstrate that the lipid source strongly influences the deposition of saturated fatty ac-

Table 3 - Chemical composition and cholesterol content of broiler breast meat.

Production system	Moisture	Protein g·100 g^{-1}	Lipids g·100 g^{-1}	Ash	Cholesterol mg·100 g^{-1}
Conventional	75.0^{a} (0.72)	23.8^{a} (0.74)	1.3^{a} (0.09)	1.1^{a} (0.04)	58.4^{a} (5.43)
Free-range	75.3^{a} (0.21)	23.7^{a} (0.69)	0.9^{b} (0.13)	1.0^{b} (0.02)	48.8^{b} (5.64)
Alternative	75.1^{a} (0.44)	24.2^{a} (0.79)	1.0^{b} (0.08)	1.1^{a} (0.02)	56.0^{a} (5.00)
Pr > F	0.338	0.304	<0.001	<0.001	<0.001

^{ab}Different letters in the same column indicate significant differences (P<0.05). Moisture by Tukey test and the others by Dunn test.
n = 9 samples; means ± standard deviation.

ids. Some authors, such as WOOD and ENSER (1997) have reported that most saturated and monounsaturated fatty acids are synthesized and therefore their concentrations are not readily influenced by diet.

Free-range broilers had the highest saturated fatty acid contents, followed by the conventional system.

CHARTRIN *et al.* (2005) observed a higher saturated fatty acid content in slow-growing broilers (certified – 34.0% and Label Rouge – 34.5%) when compared to the conventional broiler (29.4%).

The highest monounsaturated fatty acid content was found in the conventional broiler, which was statistically different from the others; the main monounsaturated fatty acid in all the samples was oleic acid (C18:1). EDWARDS *et al.* (1973) reported an increased deposition of oleic acid in the carcass of birds fed with viscera oil when compared to those fed with soybean and cotton oils. CHARTRIN *et al.* (2005), found 40.6% monounsaturated fatty acids in conventional broilers and 44.3% in Label Rouge broilers ($p<0.05$).

Among the polyunsaturated fatty acids, linoleic acid (C18:2) was the most abundant regardless of the rearing system. These results were expected based on the fatty acid profile, since the viscera oil has significantly loss linoleic acid (LARA *et al.*, 2006). The results obtained in this study demonstrate the influence of a soybean and corn-based diet (alternative and free-range broilers) on the unsaturated fatty acid profile.

When the types of broilers were compared, free-range and alternative broilers had significantly higher percentages of polyunsaturated and ω-3 fatty acids, compared to the conventional broiler.

Table 4 - Fatty acid profile of breast meat.

Fatty acid (%)	Conventional	Free-range	Alternative
C 14:0	0.6	0.4	0.4
C 15:0	1.9	3.7	2.4
C 16:0	24.9	24.7	22.3
C 16:1	5.3	2.6	2.8
C 17:0	0.7	1.1	0.7
C 17:1	0.5	0.7	0.7
C 18:0	8.3	9.3	8.5
C 18:1	39.3	29.4	31.7
C 18:2 ω-6	13.7	17.5	21.8
C 20:1	0.3	-	0.1
C 18:3 ω-3	0.3	0.5	0.9
C 20:2 ω-6	-	-	0.5
C 20:3 ω-6	0.7	0.6	0.7
C 20:3 ω-3	-	0.2	-
C 20:4 ω-6	2.6	6.3	4.1
C 22:5 ω-3	-	0.7	0.5
C 22:6 ω-3	-	0.6	0.2
Σ saturated[1]	$36.3^{ab}\pm0.86$[2]	$39.0^{a}\pm1.44$	$34.3^{b}\pm2.67$
Σ monounsaturated[1]	$45.3^{a}\pm0.63$	$32.7^{b}\pm3.19$	$35.4^{b}\pm1.40$
Σ total polyunsaturated[1]	$17.3^{b}\pm0.53$	$26.4^{a}\pm1.67$	$28.7^{a}\pm2.93$
Σ ω-3[1]	$0.3^{b}\pm0.19$	$2.0^{a}\pm0.39$	$1.6^{a}\pm0.64$

- number data equal to zero not due to rounding up; [1]n = 6; [2]standard deviation; Tukey test.
[ab]Different letters in the same column indicate significant differences ($P<0.05$).

JAHAN *et al.* (2004) observed that two of the organic broilers analyzed had lower ω-3 fatty acid contents, but higher amounts of polyunsaturated and ω-6 fatty acids, especially linoleic and arachidonic (C20:4) acids. This result may be explained by the capacity of birds to synthesize fatty acids through elongation and desaturation. Arachidonic acid belongs to the ω-6 family, and had linoleic acid as a precursor. This is probably the pathway that birds use to deposit higher amounts of this fatty acid in their tissues, as was observed in the results obtained for alternative and free-range rearing systems. These authors analyzed other conventional and organic broiler samples and the data varied regardless of the rearing system because the fatty acid profile is probably influenced by diet.

BRAGAGNOLO and RODRIGUEZ-AMAYA (1992) obtained results that were similar to those found in this study for the conventional broiler: 33% saturated fatty acids, 46% monounsaturated fatty acids and 21% polyunsaturated fatty acids in white chicken meat.

The main factors affecting the fatty acid profile are: feed, strain, sex and age of birds (COBOS *et al.*, 1994).

CONCLUSION

The results show significant differences in the physicochemical analyses of broilers reared in conventional, alternative and free-range systems, especially in relation to the fatty acid content, lipid content and cholesterol profiles. These differences may be attributed to feed, age, strain and handling, among others, which change according to the rearing system. All of the broilers analyzed had good quality characteristics, with values that were in agreement with those found in the literature. Further studies are needed to confirm these findings.

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SHORT COMMUNICATION

SENSORY PROFILING, VOLATILES AND ODOR-ACTIVE COMPOUNDS OF CANESTRATO PUGLIESE PDO CHEESE MADE FROM RAW AND PASTEURIZED EWES' MILK

PROFILO SENSORIALE, COMPOSTI VOLATILI E MOLECOLE ODOROSAMENTE ATTIVE DEL FORMAGGIO CANESTRATO PUGLIESE DOP PRODOTTO CON LATTE DI PECORA CRUDO E PASTORIZZATO

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ABSTRACT

Canestrato Pugliese is a traditional cheese produced in Puglia (Italy) which has the Protected Designation of Origin status. In this study the organoleptic properties which characterize this cheese, made with raw and pasteurized milk, were investigated and compared. Results on pasteurized samples were also compared with those obtained by

RIASSUNTO

Il Canestrato Pugliese é un formaggio tradizionale italiano prodotto in Puglia, dotato della Denominazione di Origine Protetta (DOP). Lo scopo di questo lavoro è stato quello di studiare e confrontare la proprietà organolettiche che caratterizzano questo formaggio prodotto da latte crudo e da latte pastorizzato. I risultati relativi ai campioni pastorizza-

- Key words: Canestrato Pugliese PDO cheese, GC/MS, GC/O, pasteurized ewes' milk, QDA, raw ewes' milk -

analyzing a similar pasteurized ewes' milk cheese (Canestrato Sardo) produced in a different geographical area. Sensory profiles, qualitative and quantitative analyses of the volatile fraction and gas-chromatography/olfactometry were carried out. Important differences were found between raw and pasteurized Canestrato Pugliese cheese, even though they have the same PDO status. On the other hand, there was a significant similarity between the two pasteurized cheese samples even though they were produced in different geographical areas and have a different designation of origin status.

ti sono stati confrontati con quelli ottenuti analizzando campioni di Canestrato Sardo, un formaggio simile prodotto da latte di pecora pastorizzato, ma proveniente da una diversa area geografica. Sono stati sviluppati i profili sensoriali, condotte le analisi qualitativa e quantitativa della frazione volatile e l'analisi gas-cromatografica/olfattometrica delle diverse tipologie di formaggio. I diversi approcci analitici hanno messo in evidenza importanti differenze tra i formaggi ottenuti da latte crudo e pastorizzato, nonostante fossero entrambi prodotti e commercializzati con la stessa appellazione Canestrato Pugliese DOP. Al contrario, sono state riscontrate alcune similitudini tra i formaggi prodotti da latte pastorizzato pur essendo prodotti in diverse aree geografiche sotto diverse appellazioni.

INTRODUCTION

There are 624 food products on the European register that are officially protected by the designation of origin (PDO/PGI) status. More than 20% of these (130) are produced in Italy which, together with France, is the leading country in the sector. Every typical product, even if produced in limited quantities in a small geographical area, is a great economic resource in its market niche and an asset to the producer country.

"Canestrato Pugliese" cheese is a traditional product from the region of Puglia in southern Italy. In 1985 it received the Denominazione di Origine Controllata (D.O.C.) status and in 1996 the Denominazione di Origine Protetta (D.O.P.) status. The conditions for producing Canestrato Pugliese cheese have remained faithful to the traditional ways. It is made from whole ewes' milk (raw, pasteurized or by heating the curd in hot whey) from the native "gentile di Puglia" breed. The milk is coagulated at 38°-45°C (15-25 min) using lamb rennet previously stored in contact with lemon peel, orange peel and nettle leaves. The curd is then broken and transferred to characteristic moulds for pressing and salting. The name Canestrato Pugliese is derived from the typical rush basket called "canestro" in which the cheese is ripened (3-12 months) and which imparts the characteristic wrinkled yellowish brown hard rind.

The microbiological and biochemical properties, the characterization of the composition after the main proteolysis events and some technological aspects of Canestrato Pugliese cheese have been investigated in recent years (ALBENZIO *et al.*, 2001; CORBO *et al.*, 2001; FACCIA *et al.*, 2003; DI CAGNO *et al.*, 2004). The organoleptic characteristics of Canestrato Pugliese cheese have been investigated and compared with only two other Italian Pecorino cheeses (DI CAGNO *et al.*, 2003).

To our knowledge, no previous studies have used both sensory and instrumental techniques to investigate the organoleptic properties which characterize Canestrato Pugliese cheese made from raw or pasteurized ewes' milk.

Milk pasteurization modifies the biochemistry and microbiology of cheese ripening. Therefore raw milk cheeses differ from those made with pasteurized milk with respect to the ripening process and sensory properties (GRAPPIN and BEUVIER, 1997).

Sensory characteristics are a direct parameter for identifying a food product, and must be preserved, particularly in products with a PDO status. The production and marketing of food products labelled with a specific PDO, but which have very different sensory characteristics, could favorize the production of imitation products.

The aim of the present work was to study the sensory profile, composition of the volatile fraction and odor-active compounds of Canestrato Pugliese PDO cheeses made from raw or pasteurized milk. Moreover, in order to evaluate the effect of pasteurization on the "sensory recognizability" of Canestrato Pugliese, the data from the pasteurized samples were compared to those obtained from the analysis of a similar pasteurized ewes' milk cheese (Canestrato Sardo) produced in a different geographical area.

MATERIALS AND METHODS

Samples

The study was conducted on Canestrato Pugliese cheese samples made from raw (CPR) and pasteurized (CPP) milk, labelled with the PDO status. The two kinds of cheese were produced by two different manufacturers, using the same milk and same technology (lactic starter: ~6.0 log cfu mL^{-1} *Lactobacillus deldrueckii* subsp. *Bulgaricus* and *Streptococcus thermophilus*; ripening: 10°-15°C with relative humidity at ~95%) except for pasteurization (~72°C for 30 sec). Results of CPP samples were compared to those obtained of Canestrato Sardo samples (CSP), a similar pasteurized ewes' milk cheese produced in Sardegna (central Italy). Samples of CPR and CPP cheeses were analyzed in triplicate. Each repetition consisted of 150 g of cheese (3 portions of 50 g cut from 3 different whole cheeses). The CSP cheese was bought in a market and analyzed in duplicate. All cheeses were analyzed at 6 months of ripening.

Sensory analysis

Eighty students from the Facoltà di Agraria of the Università degli Studi di Foggia were initially recruited; 54 were admitted to the first selection based on health, attitude toward cheese consumption, interest and time availability. The final selection of the panel was carried out after 18 preliminary sessions (discriminant and sensitivity tests) during which the olfactory and taste abilities of the candidates were tested using odor and taste standard references (ISO 8586/1-2). Twelve candidates (4 males and 8 females, 23 to 30 years of age) who attained an average score ≥0.32, calculated according to the method of GATTORDO and MOSCARELLA (1994), were selected to be judges on the panel that developed the sensory profiles (odor and flavor-taste) of the 3 cheeses, applying Quantitative Descriptive Analysis (STONE *et al.*, 1974). Six sessions were carried out: 3 training sessions and 3 replication sessions. Training and measuring sessions were conducted in exactly the same way; the judges were simultaneously served the three cheeses under investigation (CPR, CPP, CSP) grouped in a set of 6 samples (two of each type) presented according to a Latin-square design (MAC FIE *et al.*, 1989) and labelled with three-digit codes. During the first 3 training sessions, the judges became fa-

miliar with samples and procedures and learned to evaluate a sensory intensity on a 10 cm non-structured anchored scale (0 = absent perception; 10 = maximum perception). They developed a specific consensual vocabulary for Canestrato cheese odor (butter, ewes' milk, stable, mushroom, cream, rennet), flavour (butter, ewes' milk, stable, mushroom, cream) and taste (salty, pungent).

Extraction of the volatile constituents of cheese samples

Each cheese sample was ground, put into a 5 L round-bottomed flask (maintained at 35°C) and 100 mL of distilled water were added. After covering the internal wall of the flask with the cheese-water mix, the volatile components were distilled under vacuum at a constant pressure of $6 \cdot 10^{-1}$ Torr for 3h as described by DUMONT and ADDA (1972). The aqueous distillate containing the volatile components was recovered after condensation at -5°C in a first trap and in another two subsequent liquid nitrogen traps, placed between the sample and the vacuum generating system. Representativity of the extracts was evaluated on a 3 point scale (1 = the aroma extract is different from the aroma of the cheese sample; 2 = similar; 3 = very similar) by 5 internal laboratory technicians, trained in sensory analysis. Among the judges, 95% rated the first fraction of the distillate to be very similar to the cheese sample aroma, while the other two fractions were perceived to be different from the cheese sample aroma by 100% of the judges, even after addition to the first fraction. For this reason, only the first fraction of the aqueous distillate was submitted to the subsequent analyses. Sixteen μL of methyl decanoate as the internal standard were added to 110 mL of the distillate (MOIO and ADDEO, 1998) to allow quantitative GC analysis. The aromatic distillate was then extracted with 11 mL of dichloromethane for 1h under magnetic stirring. The emulsion was frozen at -20°C for one night, then the organic phase was recovered with a separator funnel and dehydrated with $(NH_4)_2SO_4$. Finally, 11 mL of the cheese aromatic extract were concentrated to 100 μL with a stream of nitrogen (0.5 mL/min). One μL of each extract was analyzed by gas chromatographic analyses (mix of three repetitions for GC/O and GC/MS).

Gas-Chromatography (GC/FID)

Quantitative analysis was performed with a 4890 Agilent Technologies gas chromatograph (Agilent Technologies, Avondale, PA) supplied with a split-splitless injector and a flame ionization detector (FID) both maintained at 250°C. The DBWax fused silica capillary column (30 m, 0.32 mm i.d., film thickness = 0.5 μm; J&W Scientific Inc., Folsom, CA) was directly connected to the detector. The oven temperature was programmed at 40°C for 3 min and increased up to 220°C at 3°C/min and then maintained for 10 min. The He carrier gas velocity was 37 cm/s. Peak area was calculated by an integrator HP 3395.

Gas-Chromatography/Mass Spectrometry (GC/MS)

Identification of volatile compounds was performed with an Agilent Technologies 5973 mass spectrometry detector directly coupled to a 6890 Agilent Technologies gas chromatograph. Analytical conditions were the same as described for GC/FID analysis; the same column was coupled directly to the electron impact ion source (energy: 70 eV; temperature: 280°C). Electron impact mass spectra were recorded with an HP Chemstation. Compounds were identified by comparing the experimental spectra with those of the WILEY and NIST '98 libraries and confirmed by injecting the corresponding pure standard references.

Gas-Chromatography/Olfactometry (GC/O)

Olfactory analyses were performed with a 5890 Agilent Technologies gas chromatograph supplied with a split-splitless injector, a flame ionization detector (FID) and a sniffing port, all maintained at 250°C. The DBWax fused silica capillary column (30 m, 0.32 mm i.d., film thickness = 0.5 μm; J&W Scientific Inc.) was directly connected to both the FID and the sniffing port. The column effluent was split equally between the electrochemical and the sensory detectors. The carrier gas (He) velocity was 37 cm/s. The oven temperature was programmed from 40° to 220°C at 3°C/min and then maintained for 10 min.

The GC/O analysis was performed according to the odor detection frequency method (POLLIEN *et al.*, 1997) by a panel of 6 judges selected and trained for descriptive analysis of Canestrato Pugliese cheese as described in a previous section. Three sniffing training sessions were held in order to familiarize the judges with the procedure. They were asked to smell the effluent at the end of the column and to record the retention time of each odor perception, the corresponding verbal description and of odor intensity score (1 = faint; 2 = medium; 3 = strong). Each odor-active region was then characterized by descriptor, retention time and intensity. For each extract, data provided by the 6 judges were first processed separately and then pooled to calculate the detection frequency of each odor characterized by a retention time and a descriptor.

Statistical analyses

QDA data were processed by analysis of variance (Tukey's test; $P<0.05$); quantitative data of volatile compounds were submitted to the Tukey's test ($P<0.01$) and Hierarchical Clustering Analysis (HCA); statistical treatment of olfactory detection frequencies were processed by Correspondence Analysis (CA). Gas chromatography/olfactometry data were processed by CA, a multivariate technique which is similar to principal component analysis (PCA) in that it reduces the dimensionality of data to a more easily interpretable number of dimensions, but which allows a finer distinction between samples (MCEWAN and SCHLICH, 1991). For this reason CA is a suitable analytical procedure for comparing chromatographic profiles obtained with quantitative (LE FUR, 1998) or olfactometric data (AUBRY, 1999), because it can distinguish a compound which characterizes a specific product, even at a low concentration or detection frequency. In this study CA was performed on a data contingency matrix where the rows represent the cheese samples being evaluated, and the columns represent the detection frequencies of odor-active regions detected during GC/O analysis. All statistical treatments were performed using JMP system software (version 8.1; SAS Institute).

RESULTS AND DISCUSSION

Sensory analysis

Odor and aroma-taste profiles of the cheese samples are reported as mean values of three repetitions (Fig. 1A, B). For each descriptor there were significant differences ($P<0.05$) between the 3 cheeses (CPR-CSP-CPP). The stable and cream odors were significantly different in the CPR pasteurized sample (Fig. 1A). The odor profile of CPR is more complex: it is dominated by the ewes' milk odor together with the more characteristic notes of stable, mushroom and rennet. In the aroma-taste profile (Fig. 1B) the CPR cheese showed the highest pungent and salty taste intensities with an aroma characterized mainly by ewes' milk, stable and mushroom notes. Moreover, the intensities of all seven descriptors which define the aroma-taste profile of

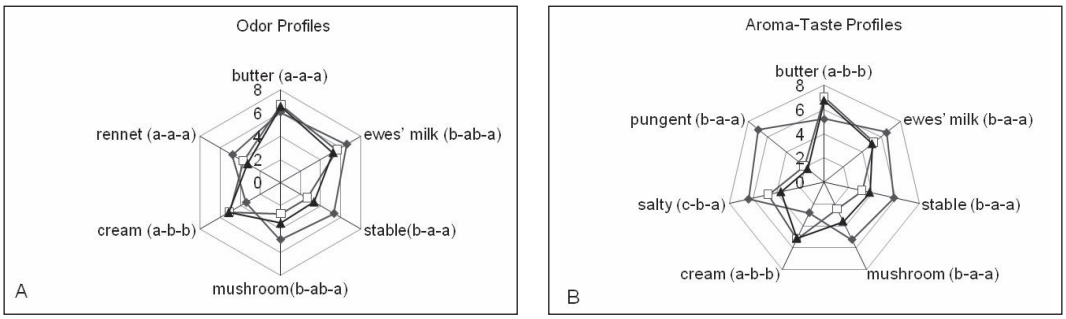

Fig. 1 - Quantitative descriptive analysis of odor (A) and aroma-taste (B) in cheese samples (CPR: —■—; CPP: —□—; CSP: —▲—). Letters in brackets refer to statistically significant differences (Tukey's test; $P<0.05$). (CPR: Canestrato Pugliese made with raw milk; CPP: Canestrato Pugliese made with pasteurized milk; CSP: Canestrato Sardo made with pasteurized milk).

this cheese, were significantly different from the CPP sample.

Sensory profiles of both cheeses made from pasteurized ewes' milk (CPP and CSP) were very similar. No odor descriptor intensity was significantly different between the CPP and CSP cheeses, which were both perceived to be dominated by descriptors implicitly associated with dairy products (butter, cream and ewes' milk notes).

The only significant difference in the aroma-taste profiles of the CPP and CSP cheeses was due to the salty taste, the intensity of which was also statistically different for the CPR sample (Fig. 1B). The salty taste cannot be considered as a characterizing sensory property because its intensity depends on the production technology. Nevertheless, differences in salt content influence the proteolytic development (PRIPP *et al.*, 2006), and indirectly affect aroma, taste and texture sensory properties of dairy products (SOUSA *et al.*, 2001). For this reason, this "salty effect" should be considered during the production of PDO cheeses. Accurate production procedures should be defined that will exalt the sensory properties that depend directly on the raw materials.

The CPP and CSP sample aromas were very similar and were dominated by the same nose-detected dairy notes: butter, cream and ewes' milk descriptors.

The sensory analysis highlighted a significant degree of heterogeneity between the two Canestrato Pugliese PDO cheeses; the cheese made with raw milk (CPR) was characterized by a more complex sensory profile in which some dairy notes were perceived along with the more specific stable and mushroom descriptors.

Identification and measurement of volatile components

The volatile fractions of the cheese samples were analyzed by GC/MS. Fifty-five volatile compounds were identified in the aroma extracts of CPR cheese, 58 in the volatile fraction of the CPP sample and 52 in that of the CSP sample. The 76 volatile components are grouped according to chemical class (Table 1). The quantity of each component was calculated with respect to the internal standard assuming that the extraction efficiency and the GC/FID response were identical for all the compounds. The average concentrations values were submitted to analysis of variance in order to evaluate significant quantitative differences between cheese samples.

Free fatty acids were the main volatile constituents of the three cheeses (Table 1). FFA developed during ripening from the hydrolysis of milk triglycerides by microbial and native milk lipas-

Table 1 - Volatile compounds found in the three kinds of cheeses analyzed.

Number	Compounds [a]	Concentrations (ppb) CP	CPP	CSP
21	**Free Fatty Acids**	X	X	X
	acetic acid	225.02 c	43.52 b	9.84 a
	propanoic acid	213.29 b	8.9 a	7.59 a
	2-methyl propanoic acid	83.68 a	162.78 b	80.63 a
	butanoic acid	16,673.34 c	2,385.28 b	677.1 a
	2+3-methyl butanoic acid	246.08 a	791.55 b	496.7 ab
	pentanoic acid	384.86 c	64.04 a	143.38 b
	4-methyl pentanoic acid	48.05 c	17.65 b	5.99 a
	hexanoic acid	25,447.8 c	7,467.55 b	2,297.86 a
	2-methyl hexanoic acid	7.94 a	3.52 a	nd
	4-methyl hexanoic acid	35.78 b	12.25 a	5.81 a
	heptanoic acid	483.86 c	172.81 b	45.5 a
	octanoic acid	12,962.1 b	6,129.51 a	2,959.75 a
	2,4-hexenedioic acid [b]	3.27 a	239.19 c	20.11 b
	nonanoic acid	122.49 c	72.37 b	47.43 a
	decanoic acid	4,491.64 b	2,638.21 a	2,348.99 a
	9-decenoic acid	195.88 b	105.06 a	90.89 a
	undecanoic acid	10.05 b	7.81 ab	5.74 a
	benzoic acid	54.12 b	14.5 a	16.29 a
	dodecanoic acid	123.45 b	69.19 ab	52.09 a
	tetradecanoic acid	38.31 a	40.55 a	39.22 a
	Total	**61,851.01**	**20,446.02**	**9,350.92**
13	**Alcohols**			
	2-butanol	43.64 b	26.24 a	15.45 a
	1-propanol	16.81	nd	nd
	2-pentanol	13.55	nd	nd
	1-butanol	103.85	nq	nd
	3-methyl-1-butanol	6.49 b	2.08 a	2.46 a
	2-methyl-3-pentanol	7.08 a	37.02 b	16.28 a
	1-butoxy-2-propanol	13.49 b	3.97 a	16.21 b
	1-hexanol	72.66	nd	nd
	2-butoxy ethanol	5.54 a	nd	5.63 a
	benzyl alcohol	6.72 b	1.12 a	4.23 b
	2-phenyl etanol	9.02 a	20.65 b	24.09 b
	2-phenyl isopropanol	nd	2.67 b	0.73 a
	α-terpineol	nd	nd	1.19
	Total	**298.85**	**93.75**	**86.27**
11	**Lactones**			
	γ-hexalactone	51.52 c	23.1 b	3.42 a
	δ-hexalactone	1.39 a	4.68 b	9.47 c
	lactone [b]	15.35	nd	nd
	γ-octalactone	135.47 b	nq	nq
	γ-nonalactone	9.88 b	4.85 a	9.66 b
	γ-decalactone	43.63 b	21.42 b	1.94 a
	γ-dodecalactone	37.23 b	23.32 a	22.88 a
	δ-dodecalactone	1.41 a	18.58 b	9.49 a
	γ-valerolatone	nd	nd	4.15
	γ-butyrolactone	nd	nd	7.29
	δ-decalactone	61.41 a	43.11 a	50.82 a
	Total	**357.29**	**139.06**	**119.12**

Number	Compounds [a]	Concentrations (ppb) CPR	CPP	CSP
10	**Ketones**	X	X	X
	2-heptanone	nq	7.88	nd
	3-hydroxy-2-butanone (acetoine)	117.58 a	1,326.48 b	196.46 a
	5,6-dihydro-4-methyl-2H-pyran-2-one	10.92 b	5.46 a	nd
	3-methyl-2-ciclohexen-2-one [b]	nd	9.68	nd
	3,5,5-trimethyl-2-ciclohexen-2-one [b]	nd	3.23	nd
	2-nonanone	nd	11.6	nd
	2,3-butanedione (diacetyl)	4.34 a	22.55 b	5.14 a
	2,5-hexadione	nd	2.82	nd
	2,3-pentadione	0.43 a	1.56 a	1.00 a
	2-(3H)-furanone	60.19 b	7.05 a	nd
	Total	**193.46**	**1,398.31**	**202.6**
6	**Esters**			
	ethyl butanoate	4.88	nd	nd
	ethyl hexanoate	7.06	nd	nd
	ethyl octanoate	tr	tr	tr
	butyl butanoate	150.77 b	nd	10.87 a
	isobutyl phtalate	nd	nd	9.75
	1-methyl butyl propanoate	nd	12.33	nd
	Total	**162.71**	**12.33**	**20.62**
5	**Volatile Phenols**			
	phenol	4.3 a	nd	5.42 a
	4-methyl phenol (*p*-cresol)	nd	27.58	nd
	3-methyl phenol (*m*-cresol)	nd	21.69 b	2.29 a
	2-ethyl phenol	3.81	nd	nd
	2-methoxyphenol (guaiacol)	nd	nd	1.79
	Total	**8.11**	**49.27**	**9,5**
4	**Pyrazines**			
	2,5-dimethyl pyrazine	nd	6.85	nd
	2,6-dimethyl pyrazine	nd	22.25 b	5.04 a
	trimethyl pyrazine	nd	23.43	nd
	tetramethyl pyrazine	nd	6.87	nd
	Total		**59.4**	**5.04**
3	**Sulphur Compounds**			
	3-methyl tio-1-propanal	25.42	nd	nd
	dimethylsulphone	nd	8.56	nd
	dimethylsulphide	2.58	nd	nd
	Total	**28**	**8.56**	
2	**Aromatic Compounds**			
	1,4-dimethyl benzene (*p*-xylene)	nd	3.15 a	3.13 a
	1,2-dimethyl benzene (*o*-xylene)	nd	7.51 a	7.72 a
	Total		**10.66**	**10.85**
1	**Aldehydes**			
	4-hydroxy-3-methoxy benzaldehyde (vanillin)	4.92 c	2.09 b	1.26 a
76	**Total Volatile Compounds**	**62,904.35**	**22,219.45**	**9,806.18**

X = Mean value of three repetitions, values with different letters within the same line are significantly different (P≤0.01);
[a] Identified on the basis of retention time and mass spectra of pure standard reference compounds and of MS database;
[b] Tentatively identified on the basis of MS databases; nd: not detected; nq: not quantified because coeluted; tr: traces.

es. This phenomenon is particularly important in raw milk cheeses where lipases are not deactivated by pasteurization. These compounds are predominant flavor components in many cheeses due to their strong and often sharp odors. They are also precursors of other odorants belonging to the methyl ketone, alcohol, lactone and ester chemical classes (URBACH, 1993). For this reason, milk pasteurization mostly affects the aroma character of dairy products. The relative proportions of FFA in cheeses is affected by climatic conditions and raw milk quality (microorganisms and relative quantitative composition of FFA which depends on the animal species, breed, feed and rearing conditions) (COLLOMB *et al.*, 1999; NÀJERA *et al.*, 1993; FERNÀNDEZ-GARCÌA *et al.*, 2006). These findings suggest that the composition of the FFA fraction in cheeses could be associated with the geographical area and manufacturing process. It could be a special feature of each specific cheese with PDO status if the cheese was produced with raw milk. In this study, hexanoic and butanoic acids were the most abundant in the CPR cheese. Different results have been reported for other PDO cheeses made with raw ewes' milk, such as Terrincho (octanoic and decanoic acids) (PINHO *et al.*, 2003) and Manchego (decanoic and octanoic acids) (GOMEZ-RUIZ *et al.*, 2002).

The highest total FFA concentration was found in the cheese made with raw milk (CPR), followed by CPP (~1/3 compared to CPR) and then the CSP sample (~1/6 compared to CPR). This result is in agreement with those reported for other ewes' milk cheeses, like the Spanish Manchego cheese for which the FFAs vary greatly during ripening in both the artisanal (raw) and industrial (pasteurized) cheeses. At the end of ripening, the FFA values were much higher in the samples made with raw milk (GOMEZ-RUIZ *et al.*, 2002).

Except for 2-methyl hexanoic acid, which was not detected in the CSP sample, the same FFAs were identified in the three cheeses, but with very different quantitative distributions (Table 1). Hexanoic, octanoic, butanoic and decanoic acids were the most abundant volatile acids, but the relative percentage varied greatly with each cheese. Together they represented ~96% of the total volatile acids of CPR, ~91% of CPP and ~88% of CSP. These acids are also found in other cheese types such as Grana Padano (MOIO and ADDEO, 1998), Gorgonzola (MOIO *et al.*, 2000) and Cheddar (CHRISTENSEN and REINECCIUS, 1995), and are considered important for the background aroma of the cheese. In general, short and medium straight-chain fatty acids (C_4-C_{12}) play a major role in cheese flavor, because their perception thresholds are much lower than those of long-chain fatty acids (>12 C). Hexanoic (~42%) and butanoic (~27%) acids characterized the CPR cheese.

Hexanoic acid, the main FFA in both CPR and CPP cheeses, was probably the product of butterfat lipolysis, but the presence of low levels of straight-chain fatty acids with odd numbers of carbon atoms such as pentanoic, heptanoic and nonanoic acids suggests a partial fermentative origin. The second main compound of CPP was octanoic acid, which was the most important FFA in the other pasteurized sample (CSP), followed by decanoic and hexanoic acids at similar percentages.

The total amounts of each chemical class in the 3 cheese samples (Table 1), show that CPR cheese is characterized by the highest concentrations of esters, alcohols and lactones (increasing order). The CPP and CSP samples had similar but lower total amounts of these compounds.

Esters and lactones, generally characterized by fruity odors and very low perception thresholds, contribute to the fruity character in cheese (CURIONI and BOSSET, 2002). Among the identified alcohols, 2-phenyl ethanol (significantly lower in CPR) and α-terpineol (detected only in CSP) are both characterized by pleasant flowery notes: the first is pro-

duced from phenylalanine by yeasts, the second is a terpene, which is thought to come from the forage eaten by the animal (MARIACA *et al.*, 1997). Some branched-chain alcohols were also identified in the cheese samples, and the presence of the primary alcohol 3-methyl-1-butanol (significantly higher in CPR) indicated the reduction of the aldehyde produced from leucine. In the 3 cheeses, vanillin was the only aldehyde identified. These compounds are transitory in cheese because they are rapidly reduced to primary alcohols or oxidized to the corresponding acids (CURIONI and BOSSET, 2002).

CPP cheese had a very high ketone concentration (~7 times higher that in CPR and CSP samples) and the highest amounts of pyrazines and volatile phenols. The important role of ketones in the volatile fraction of the CPP sample is essentially due to acetoine and diacetyl. The latter, responsible for a butter-like odor, is mainly due to the activity of lactic acid bacteria on lactose and citrate metabolism. An unbalanced high content of diacetyl was previously reported to be one of the principal sources for the sensory differentiation between Manchego cheese made from pasteurized "raw ewes" milk (FERNANDEZ-GARCIA *et al.*, 2002). Blue cheese notes are commonly associated with the two methyl ketones (only quantified in CPP) 2-heptanone and 2-nonanone; both are impact compounds of Gorgonzola cheese (MOIO *et al.*, 2000). 2-(3H)-Furanone was present (especially in CPR), but little is known about the influence of furans on cheese aroma. The volatile fraction of CPP cheese is characterized by four methyl pyrazines but none of these were detected in the CPR sample. The volatile phenol composition in CPP is also very different from that of CPR, particularly with respect to *p*- and *m*-cresol. Phenolic compounds originate from tyrosine and, if present at about threshold concentration, positively contribute to cheese flavor. They are responsible for very different sharp odors (medicinal, sweet, smoky, unpleasant).

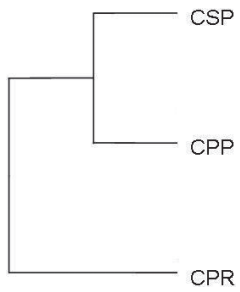

Fig. 2 - Dendrogram obtained by hierarchical clustering analysis of quantitative data. (CPR: Canestrato Pugliese made with raw milk; CPP: Canestrato Pugliese made with pasteurized milk; CSP: Canestrato Sardo made with pasteurized milk).

Sulphur compounds were only detected in the two Canestrato Pugliese cheeses, 3-methyl tiopropanal and dimethylsulphide in CPR and dimethyl sulphone in CPP. The degradation of methionine is the main source of these sulphur compounds (YVON and RIJNEN, 2001). Since the perception thresholds of their characteristic garlic and very ripe cheese odors (CURIONI and BOSSET, 2002) are very low, they generally play an important role in cheese flavor (MOLIMARD and SPINNLER, 1996). The two sulphur compounds detected in CPR, 3-methyl tiopropanal and dimethylsulphide, are the most common in cheese. The first is the product of the Strecker degradation and, is responsible for a boiled potato odor; it plays an active role in the aroma of several cheese varieties including Camembert, Cheddar, Emmental, goat cheese, creamy Gorgonzola, Grana Padano, Gruyère, Pecorino and Ragusano (CURIONI and BOSSET, 2002).

The quantitative data reported in Table 1 were submitted to hierarchical clustering analysis (HCA). The resulting dendogram (Fig. 2) shows a higher degree of similarity between the two cheeses obtained from pasteurized milk but having different labels (CPP and CSP), than between the two Canestrato Pugliese PDO cheeses. This result based on the quantitative composition of the volatile fractions is in

accord with the results obtained by sensory analysis and confirms a significant degree of heterogeneity between the two Canestrato Pugliese PDO cheeses.

Odor-active compounds detected in cheese samples

The results of Correspondence Analysis reported in Fig. 3 shows that the cheese samples occupy three different areas of the chart. The compounds which show the highest correspondence with CPR cheese are: γ-esalactone (34.11, hay/herbaceous), γ-nonalactone (49.34, coconut), hexanol (19.77, underwood) and an unknown compound characterized by a fatty odor (26.67). These odor-active compounds did not have high detection frequencies. Since they were only detected in this sample, they could be used to characterize

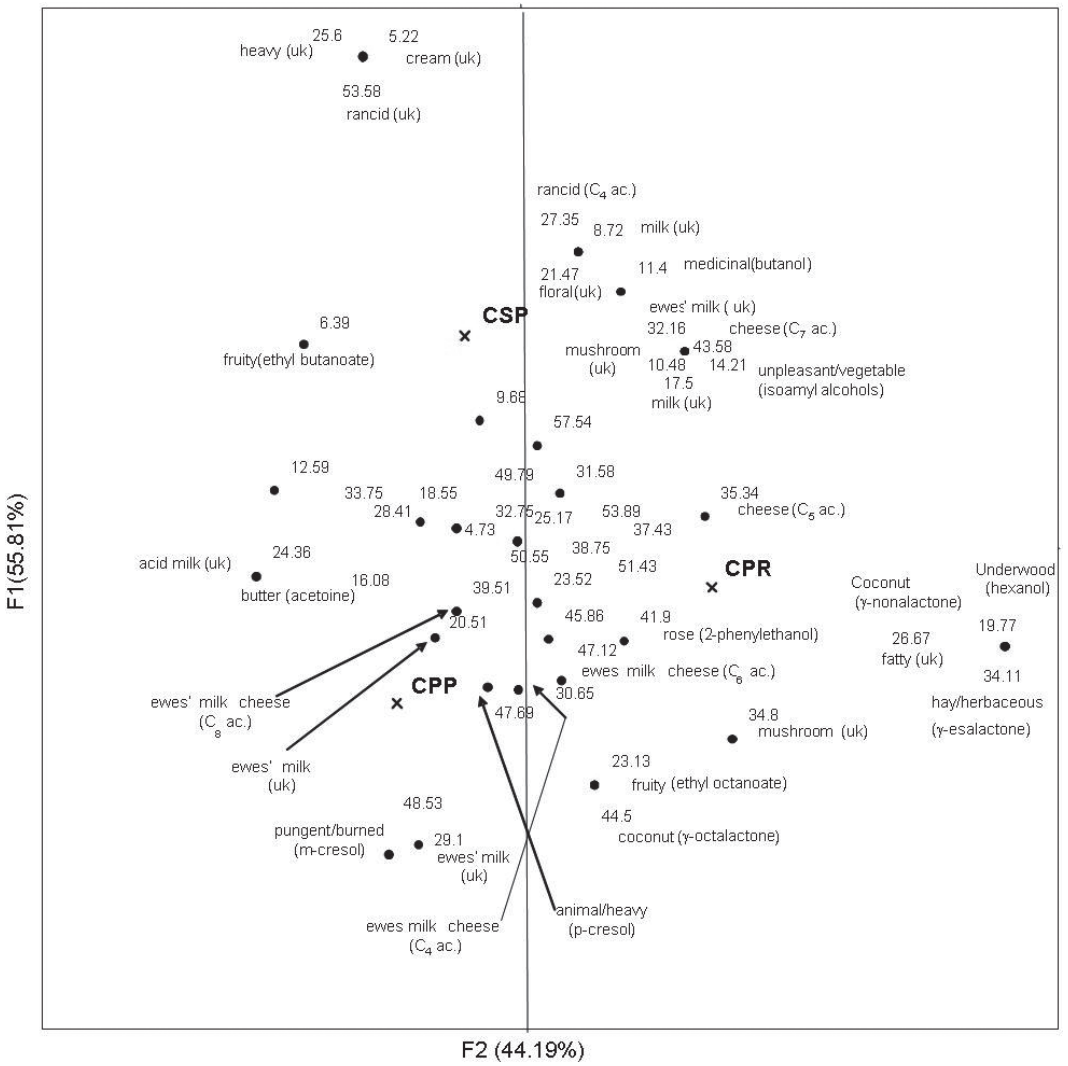

Fig. 3 - Correspondence analysis of detection frequencies of odor-active regions detected during gas-chromatography/olfactometry analysis of cheese samples (uk: unknown compound).

CPR cheese. During the olfactory analysis of both Canestrato Pugliese samples, butanoic acid (30.65, ewe's milk cheese) was the odor-active compound that had the highest detection frequency (100 and 83% for CPR and CPP, respectively). For this reason it shows a good correspondence with both samples on the CA map. Other compounds showed similar olfactory contributions to both CPR and CPP cheeses including: γ-octalactone (44.50, coconut), ethyl octanoate (23.13, fruity), an unknown compound (34.80, mushroom), octanoic acid (47.12, ewe's milk cheese) and 2-phenylethanol (41.90, rose). *m*-Cresol (48.53, pungent/burned) and an unknown compound (29.10) with a ewe's milk cheese odor showed the best correspondence with CPP cheese. *p*-Cresol (47.67, animal/heavy), an unknown compound (24.36, acid milk), acetone (16.08, butter) and another unknown compound (20.51, ewe's milk) contributed to CPP aroma. The CSP sample found at the top of the chart is mainly characterized by odor-active compounds with dairy and unpleasant odors (3 unknown compounds: 25.60 heavy, 5.22 cream, 53.58 rancid; propanoic acid: 27.35 rancid; two unknown compounds: 8.72 milk, 21.47 floral; butanol: 11.40 medicinal; ethyl butanoate: 6.39 fruity; eptanoic acid: 43.58 cheese; isoamyl alcohols: 14.21 unpleasant/vegetable; 3 unknown compounds: 32.16 ewe's milk cheese, 10.48 mushroom, 17.50 milk). These compounds were not the same as those responsible for similar notes which dominated the olfactory profile of CPP. This could explain the similarity of the sensory profiles of the two pasteurized cheeses.

CONCLUSIONS

The results obtained by using different analytical approaches did not allow specific characteristics to be identified that were common to the two PDO "Canestrato Pugliese" cheeses analyzed. This result depended on whether raw or pasteurized milk was used. The results also show that the sensory characteristics were indistinguishable. The composition of Canestrato Pugliese made with pasteurized milk was similar to a pasteurized cheese produced in a different geographical area under a different label (Canestrato Sardo). These first results suggest that the effect of pasteurization is stronger than that of the origin on the sensory characteristics of cheeses.

ALBENZIO *et al.* (2001) reported that Canestrato Pugliese cheeses produced from raw milk did not present any hygienic risks. Therefore, it is important to use raw milk because it provides the main microbiological and biochemical characteristics of the cheese, including the free amino acids and fatty acids which both affect cheese flavor. Therefore, the bio-diversity of raw milk should be preserved during the production of traditional PDO cheeses.

These results should help the appropriate authorities to define the production procedures that exalt the sensory properties which are directly dependent on the raw materials as well as on the manufacturing and ripening processes used in a defined geographical area. This would guarantee a minimum level of quality for PDO Canestrato Pugliese cheese.

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SHORT COMMUNICATION

DYNAMICS AND CHARACTERIZATION OF A YEAST POPULATION FROM AN ITALIAN FERMENTED SAUSAGE

DINAMICA E CARATTERIZZAZIONE DI UNA POPOLAZIONE DI LIEVITI PRESENTE IN UNA SALSICCIA STAGIONATA ITALIANA

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ABSTRACT

Yeast populations of twenty-one samples of an Italian fermented sausage were investigated during ripening in a pilot-scale chamber. Samples were analyzed in three manufacturing steps: fresh product (0 days), sausage after 15 days of maturation and finished product (60 days). In addition, 100 yeast strains were isolated and identified to the species level using a morphological and biochemical characterization procedure. With regard to the yeast population dynamics, the counts increased

RIASSUNTO

Le popolazioni di lieviti di ventuno campioni di un salume italiano fermentato sono stati studiati durante la maturazione in una camera su scala pilota. I campioni sono stati analizzati in tre stadi di preparazione: prodotto fresco (0 giorni), salume dopo 15 giorni di maturazione e prodotto finito (60 giorni). Inoltre, 100 ceppi di lieviti sono stati isolati ed identificati al livello di specie utilizzando una procedura di caratterizzazione morfologica e biochimica. Riguardo la dinamica della popolazione

- Key words: fungal biota, salamis, sausages, yeasts -

1.15 log CFU/g (2.75 to 3.90) during the ripening process. *Pichia carsonii* (33%) was the most frequently isolated species, followed by *Candida lipolytica* (anamorph of *Yarrowia lipolytica*) (13%), *Candida japonica* (anamorph of *Filobasidium capsuligenum*) (9%), *Debaryomyces hansenii* (8%), *Geotrichum candidum* (anamorph of *Galactomyces geotrichum*) (7%), *Pichia etchellsii* (6%) and *Lodderomyces elongisporus* (5%). *D. hansenii* was the only yeast species found in all the stages of the manufacturing process.

dei lieviti, le conte sono aumentate di 1,15 log CFU/g (da 2,75 a 3,90) durante il processo di maturazione. *Pichia carsonii* (33%) è stata la specie isolata con maggiore frequenza, seguita da *Candida lipolytica* (anamorfo di *Yarrowia lipolytica*) (13%), *Candida japonica* (anamorfo di *Filobasidium capsuligenum)* (9%), *Debaryomyces hansenii* (8%), *Geotrichum candidum* (anamorfo di *Galactomyces geotrichum*) (7%), *Pichia etchellsii* (6%) e *Lodderomyces elongisporus* (5%). *D. hansenii* è stata l'unica specie di lievito trovata in tutti gli stadi del processo di preparazione.

INTRODUCTION

Fermented sausages are generally produced as dry or semi-dry products. Dry or Italian-type sausages containing 30-40% moisture are not usually smoked or heat processed (PEDERSON, 1979). In the manufacture of traditional Mediterranean sausages, yeasts (*Debaryomyces hansenii*, *Candida famata)* and molds (*Penicillium nalgiovense*, *Penicillium chrysogenum* or *Penicillium camembertii*) are added to the surface of the casing. In northern and central Europe, fermentation is generally combined with smoking, which prevents the growth of yeasts and molds, and the drying period is shorter (MONTEL, 2000).

According to DILLON (1998), fermented sausages are considered to be good substrates for the growth of yeasts. In these products, competing microorganisms such as Gram-negative bacteria are inhibited by such properties as low pH, the presence of lactic acid, nitrite, and low water activity. The most ubiquitous yeasts found in meat and poultry are members of the genera *Candida* and *Rhodotorula* (JAY *et al.*, 2005). In fact, *Candida* and *Debaryomyces*, among other genera, are commonly found growing on the casings of sausages, mainly those made in southern Europe, which are ripened for a long time (MONTEL, 2000). In the opinion of PRAPHAILONG and FLEET (2000), species of *Debaryomyces* are commonly found in spoils, water, plants, foods and clinical specimens. For these authors, extensive studies have confirmed the predominance of *D. hansenii* in meat products compared with other yeasts.

The present paper describes the yeast population dynamics of an Italian fermented sausage during ripening in a pilot-scale chamber. A total of 100 yeast strains were isolated and identified to the species level.

MATERIALS AND METHODS

Sampling

Twenty-one samples of a traditional type of Italian salami from a pilot-scale ripening chamber were analyzed in three manufacturing steps: 3 samples of fresh sausages (0 days), 9 samples of sausage after 15 days of maturation and 9 samples of finished sausages (60 days). Pork

meat, lean (73%) and fat (27%), were used to manufacture the sausages. The minced meat was mixed with curing salts, spices, milk power, sugars and starter culture (*Lactobacillus sakeii*, *Lactobacillus curvatus*, *Staphylococcus carnosus* and *Staphylococcus xylosus*). The ingredients were mixed in a cutter until the particle size was 3.5 mm. Artificial (collagenic) casings (diameter of 90 mm) were filled with the mixture. After stuffing, the sausages were fermented and air-dried. Drying was carried out under pilot-scale ripening chamber conditions that were controlled during the whole process.

Three samples from each sampling step were collected and used for analysis: samples (10 g) of each sausage were taken aseptically, transferred to sterile pouches and homogenized for 2 min with 90 mL of sterile buffered peptone water, 0.1% w/v (Oxoid, Unipath Ltd., Basingstoke, UK) as diluent using a Stomacher (Lab Blender, Model 4001, Seward Medical, London, UK). Further decimal dilutions of the sample homogenates were prepared and inoculated in duplicate in growth media to estimate yeast counts.

Enumeration of yeasts

The yeast count was verified on potato dextrose agar, PDA (Merck, Darmstadt, Germany) adjusted to pH 3.50±0.1 by the addition of 10% tartaric acid solution. Plates were incubated at 25°C for 72 h. After counting, expressed as log CFU/g, means and standard deviations were calculated.

Identification and characterization of yeasts

A hundred yeast colonies were selected from the counting plates, picking representative colonies of each morphological type (the square root of the total number) present in the samples (fresh product, sausage after 15 days of manufacture and finished product). These strains were sub-cultured on oxytetracycline glucose yeast extract agar (OGYE, Oxoid) incubated at 25°C for 72 h. Isolates were identified and characterized to the species level for the following properties and tests for identification were as described by DEAK (1986). Tests included the study of colony and cell morphology, production of ascospores, assimilation of nitrate, erythritol, inositol (*myo*-inositol), raffinose, cellobiose, mannitol, maltose, galactose and xylose; fermentation of glucose and hydrolysis of urea. The growth at 37°C was evaluated by streaking the isolates onto OGYE plates incubated up to 3 days at 25°C. Identification criteria according to LODDER (1974) and BARNETT *et al.* (1990) were followed for final identification.

pH determination

Potentiometric measurements of pH were carried out with a pH-meter pin electrode (Sentron CMT, Roden, The Netherlands), inserted directly into two different positions in each sausage. Two independent measurements were made on each sample. Means and standard deviations were calculated.

RESULTS AND DISCUSSION

The yeast counts and pH values are reported in Table 1 and the yeast species identified from an Italian fermented sausage are listed in Table 2.

The average number of yeasts, initially and after 15 and 60 days of maturation were 2.75, 3.98 and 3.90 CFU/g, respectively. In the final product, the increase of the initial count was 1.15 log CFU/g (41.81%) (Table 1). In a previous study (LÓPEZ *et al.*, 2001), the yeast counts observed during the ripening process in that same sausage type made on an industrial scale were similar to these results (increase of 1.11 log CFU/g, 47.03%). According to BOISSONET *et al.* (1994), yeasts multiply up to counts of 3 to 4 log CFU/g

during sausage ripening (three weeks). Also, RANTSIOU *et al.* (2005) reported yeast counts above 2 log CFU/g in an Italian fermented sausage. In our case, the average counts observed, after 15 and 60 days of maturation, fell within this range. A study of the fungal biota of naturally fermented sausages in northern Italy revealed that the initial biota was made up 95% yeasts (ANDERSON, 1995).

ENCINAS *et al.* (2000) found mean counts of around 4 log CFU/g in a Spanish fermented sausage mixture. For these authors, the type of manufacture (artisanal or industrial) and sausage diameter were the variables that influenced the yeast counts the most. In a study on six different commercial European dry-sausages (LÓPEZ *et al.,* 2001), yeast counts during the ripening process showed an irregular development. The yeast counts observed were: 2.36 to 4.16 CFU/g (fresh product), 2.0 to 5.20 CFU/g (sausage after 15 days of maturation) and 2.55 to 5.49 CFU/g (finished product); moreover, there was no correlation between the sau-

Table 1 - Yeast counts and pH values from an Italian fermented sausage.

Ripening period (days)	n	pH	Initial count log CFU/g	Increase from initial count	
				log cycles	%
0	3	5.94 (0.03) [a]	2.75 (0.03)	-	-
15	9	5.13 (0.02)	3.98 (0.45)	1.23	44.72
60	9	5.07 (0.04)	3.90 (0.06)	1.15	41.81

(a) Mean with standard deviation in parentheses.

Table 2 - Yeast species identified from an Italian fermented sausage.

Species	Days of ripening			Total ripening period
	0	15	60	
Candida cantarelli	- [a]	-	3 (5.80) [b]	3 (3)
Candida catenulate	-	3 (8.80)	-	3 (3)
Candida diddensiae	-	1 (2.90)	-	1 (1)
Candida incospicua	-	1 (2.90)	1 (1.90)	2 (2)
Candida japonica	2 (14.30)	-	7 (13.50)	9 (9)
Candida lipolytica	-	9 (26.50)	4 (7.70)	13 (13)
Candida tenuis	-	-	2 (3.80)	2 (2)
Cryptococcus laurentii	2 (14.30)	1 (2.90)	-	3 (3)
Debaryomyces hansenii	4 (28.60)	2 (5.90)	2 (3.80)	8 (8)
Geotrichum candidum	-	3 (8.80)	4 (7.70)	7 (7)
Lodderomyces elongisporus	-	5 (14.70)	-	5 (5)
Pichia carsoni	-	10 (29.40)	23 (44.20)	33 (33)
Pichia etchellsii	1 (7.10)	-	5 (9.60)	6 (6)
Rhodotorula minuta	3 (21.40)	-	-	3 (3)
Rhodotorula mucilaginosa	1 (7.10)	-	-	1 (1)
Trichosporon beigelii	1 (7.10)	-	-	1 (1)
Total	14	34	52	100

[(a)] Not detected.
[(b)] No. of isolates (in parentheses, percentage of isolates).

sage diameter and yeast populations. The results reported in a study on Italian dry-sausages (DIAFERIA *et al.*, 1995) showed a variability in yeast counts that was similar to that found in our work. However, in naturally ripened Greek dry salami, SAMELIS *et al.* (1994) found higher yeast counts from the initial mixture to the end of the process.

Pichia carsonii (33%) was the most frequently isolated species, followed by *Candida lipolytica* (anamorph of *Yarrowia lipolytica*) (13%), *Candida japonica* (anamorph of *Filobasidium capsuligenum*) (9%), *D. hansenii* (8%), *Geotrichum candidum* (anamorph of *Galactomyces geotrichum*) (7%), *Pichia etchellsii* (6%) and *Lodderomyces elongisporus* (5%). The rest of the species identified (*Candida cantarellii*, *Candida catenulata*, *Candida diddensiae*, *Candida incospicua*, *Candida tenuis*, *Cryptococcus laurentii*, *Rhodotorula minuta*, *Rhodotorula mucilaginosa* and *Trichosporon beigelli*) were isolated in percentages below 5%. The number of types (species) of yeasts identified increased in the course of the maturation process: 7 in the fresh sausages (14 isolates), 9 in the sausages after 15 days (34 isolates) and 9 in the finished product (52 isolates) (Table 2). SIMONCINI *et al.* (2007) reported that the yeast counts and species distribution changed according to the stage of processing.

In the fresh product *D. hansenii* (28.60%) was the dominant yeast followed by *Rhodotorula* (*R. minuta*, 21.40% and *R. mucilaginosa*, 7.10%), and *C. japonica* and *Cr. laurentii* (14.30%). Members of *Candida* are the most common yeasts in fresh meat and *Cryptococcus* spp. and *Rhodotorula* spp. have been found in fresh beef (JAY *et al.*, 2005). This would explain the frequent occurrence of these yeast species in fresh sausage. In sausage after 15 days of maturation, predominance was shared by *P. carsonii* (29.40%) and *C. lipolytica* (26.50%); in the finished product the presence of *P. carsonii* significantly increased (44.20% of the isolates), whereas *C. lipolytica* dropped sharply (7.70%). On the other hand, the microflora of the final product included *Y. lipolytica* (teleomorph of *C. lipolytica*), but there are generally only qualitative reports of its occurrence (HEARD and FLEET, 2000).

D. hansenii was the only yeast species detected in all the stages of the manufacturing process, although its population fell noticeably during maturation (5.90% after 15 days and only 3.80% after 60 days) (Table 2). ENCINAS *et al.* (2000) also found the predominance of *D. hansenii* (and its anamorph, *C. famata*) and its presence in all the manufacturing stages of different types of Spanish fermented sausages, while in a study on the ecology of fresh sausages COCOLIN *et al.* (2004) found only *D. hansenii*. COCOLIN *et al.* (2006) and SIMONCINI *et al.* (2007) reported that *D. hansenii* was the main yeast species present in Italian sausages and dry-cured ham, respectively. This yeast is characterized by its tolerance to high concentrations of salt (NaCl) (JENSEN, 1995; PRAPHAILONG *et al.*, 2000; JAY *et al.*, 2005), and the presence of sodium nitrite and by its tolerance to refrigeration temperatures (JENSEN, 1995; PRAPHAILONG *et al.*, 2000). In our case, the behavior of *D. hansenii* in dry-sausages would seem to depend, to a great extent, on the presence or absence of other yeast species.

LÓPEZ *et al.* (2001) found the following yeast species in European dry-sausages (French, Italian and Spanish): *Candida* (*C. albicans, C. famata, C. guillermondii, C. krusei, C. parapsilosis, C. stellatoidea* and *C. zeylanoides*), *Geotrichum* (*G. candidum*), *Rhodotorula* (*R. rubra*) and *Trichosporon* (*T. capitatum* and *T. beigelli*). In addition to *D. hansenii* (*C. famata*), ENCINAS *et al.* (2000) detected the presence of *Trichosporon ovoides* (*T. beigelli*), *Y. lipolytica* (and its anamorph, *C. lypolitica*), *Candida intermedia/curvata, C. parapsilopsis, C. zeylanoides* and *Citeromyces matritensis* (teleomorph of *C. globosa*) in Spanish sausages. Other authors reported that *C.*

famata was the most frequently isolated yeast in different types of German (SMITH and HADLOK, 1976), Italian (GRAZIA *et al.*, 2001) and Greek (METAXOPOULOS *et al.*, 1996) sausage. PRAPHAILONG *et al.* (2000) reported the frequent isolation of *D. hansenii* from meat products, especially processed products, such as frankfurters, bacon, hams and fermented and unfermented sausages. Finally, according to GARDINI *et al.* (2001), the most frequently isolated yeasts, in "salsiccia sotto sugna" (a typical salami of the Lucania region, southern Italy), were *D. hansenii* and its anamorph *C. famata*, and *R. mucilaginosa*.

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SHORT COMMUNICATION

CHANGES IN MICROBIAL POPULATIONS IN READY-TO-EAT VEGETABLE SALADS DURING SHELF-LIFE

CAMBIAMENTI DELLA POPOLAZIONE MICROBICA NEI VEGETALI DI IV GAMMA DURANTE LA SHELF-LIFE

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ABSTRACT

The microbiological quality of 56 samples of packaged ready-to-eat vegetable salads was determined during the shelf-life at refrigerated temperature. *Listeria monocytogenes*, Salmonella, Vibrio, *Yersinia enterocolitica*, *Aeromonas* spp. were absent. As regards the other microorganisms investigated, the comparison with international guidelines revealed that the number of unsatisfactory samples increased during shelf-life. In conclusion, the absence of pathogens and the low incidence of *Escherichia coli*

RIASSUNTO

Sono state valutate le caratteristiche microbiologiche di 56 campioni di insalate di IV gamma durante il periodo di shelf-life. Li*steria monocytogenes*, Salmonella, Vibrio, *Yersinia enterocolitica*, *Aeromonas* spp. sono risultati assenti. Riguardo agli altri microrganismi ricercati, in base alle linee guida internazionali, il numero di campioni non soddisfacenti aumenta durante la *shelf-life.* L'assenza dei patogeni e la bassa incidenza di *Escherichia coli* (UFC/g $>10^2$-10^3 in 7,1%) evidenziava-

- Key words: contamination, microorganisms, ready-to-eat, shelf-life, vegetables -

(CFU/g >10^2-10^3 in 7.1%) indicate that in general the samples were in conformity with the conditions for health and safety. However the microbial changes found during storage indicate the need to improve the sanitization and preservation technologies in order to reduce deterioration and possibly increase the shelf-life of the product.

no le condizioni di salubrità e sicurezza dei campioni esaminati. Tuttavia lo sviluppo della carica microbica durante la conservazione indica la necessità di perfezionare le tecnologie di sanificazione e conservazione per ridurre il deterioramento e aumentare così la durabilità del prodotto.

INTRODUCTION

In recent years, there has been increasing interest in ready-to-eat fresh vegetables because they combine nutritional attributes with convenient and easy preparation. The minimally processed ready-to-eat vegetable industry was initially developed to supply restaurants, hotels and other institutions but, more recently, it has also been extended to home use. The demand for fresh-cut produce has led to an increase in the production of minimally processed vegetables. In Italy the sale of ready-to eat fruit and vegetables in 2004 amounted to € 375 million, 30% more than in 2003; in 2005 sales totalled over € 600 million (www.agricoltura.regione.lombardia.it).

A variety of vegetables including lettuce, carrots and celery are used in minimally processed products such as fresh salads. The preparation of minimally processed ready-to-eat vegetables involves cleaning, trimming, peeling, curing, slicing, shredding, washing and, finally packaging in air or modified atmosphere (VAROQUAUX and MAZOLLIER, 2002). The procedures use slight sanitizing measures which do not alter the sensory or nutritional characteristics but they do not eliminate microbial contamination.

The condition and shelf-life of ready-to-eat fresh vegetables depend on many factors such as vegetable quality, production technology and the interactions among microbial groups (GUERZONI *et al.,* 1996). The rate and extent of microbial growth in packaged products depend mainly on the initial microbial load and storage temperature (KING *et al.,* 1991).

The initial microbial load of ready-to-eat fresh vegetables is generally 10^5-10^7 CFU/g. It consists of bacteria belonging to *Pseudomonas, Erwinia, Corynebacterium* (LI *et al.*, 2001) genera, lactic acid bacteria (KELLY *et al.*, 1996) and pathogens such as Salmonella (SAGOO *et al.*, 2003) Shigella (AHVENAIN, 1996), *Yersinia enterocolitica* (AHVENAIN, 1996), *Listeria monocytogenes* (SIZMUR *and* WALKE, 1988; PINGULKAR *et al.*, 2001), *Aeromonas hydrophila* (MCMAHON and WILSON, 2001; LITTLE *et al.*, 1997), *Staphylococcus aureus* (AYCICEK *et al.,* 2005), and *Escherichia coli* (SAGOO *et al.*, 2001). Furthermore yeasts belonging to *Cryptococcus, Rhodotorula, Candida* genera and moulds such as *Fusarium, Penicillium, Mucor, Phoma, Rhizopus* genera have also been identified (GARCIA-GIMENO and ZURERA-COSANO, 1997; BABIC *et al.,* 1992; FLEET, 1992).

The possible sources of product contamination involve incoming raw vegetables, plant workers and the processing environment. When vegetables are cut, the release of cellular fluids provides a nutritive medium where microorganisms can grow. The high moisture content of fresh vegetables, the lack of lethal

processes to eliminate microorganisms and potential temperature abuse during preparation and distribution can result in increased microbial growth (BRACKET, 1987; NGUYEN-THE and CARLIN, 1994). In addition, low storage temperature does not prevent the development of psychrotrophic microorganisms. Thus, the microbial metabolism, together with the increased respiration and transpiration processes due to tissue breakdown caused by cutting, can cause a rapid decay of minimally processed vegetables and reduce of the shelf-life.

These foods can be a hazard to human health especially if they harbour psychrotrophic microorganism such as *A. hydrophila* and *L. monocytogenes*. Documents (GALLI and BERTOLDI, 1998) have been issued to help producers minimize microbial hazards and guidelines have been published that present criteria for the microbiological quality of ready-to-eat food (GILBERT *et al.*, 2000; www.foodstandards.gov.au/newsroom/publications/guidelinesformicrobi1306.cfm; CNERNA-CNRS, 1996). Several cases have been reported where vegetables were responsible for outbreaks of illness (SAGOO *et al.*, 2003; BEAUCHAT, 1996). Hence, there is concern about the influence of microbial growth on the quality and safety of fresh-cut vegetables.

Considering that ready-to-eat vegetable salads are used without washing or further cooking, analyses have been carried out in order to evaluate the microbiological quality of these products during their shelf-life. The results are discussed in light of the present microbiological guidelines.

MATERIALS AND METHODS

Fifty-six samples of mixed ready-to-eat vegetable salads were purchased in Urbino (Italy) from local markets on the same day of production. The salads were packaged in trays wrapped in plastic film or in polyethylene bags in non-modified atmosphere. Each pack weighed 250 g. The salads are normally consumed without cooking, further washing or preparation by the consumers. The composition and manufacturers of the products are reported (Table 1). The samples were stored at 5°C and examined for microbiological characteristics on the day of purchase (T_0), and on the expiry date (T_1), 7 days after.

Microbiological analysis

The enumeration and differentiation of microorganisms were performed by using the following media and culture conditions.

For the detection of total aerobic count, sporeformers, yeasts and moulds, lactic acid bacteria, coliforms, *S. aureus* and *Pseudomonas* spp., 25 g of each sam-

Table 1 - Composition of ready-to-eat vegetable salads.

Typology*	Manufacturer	Composition
1	A	carrot, red lettuce, rocket, cos lettuce, spinach
2	A	carrot, “chioggia” lettuce, curly lettuce, prickly lettuce, rocket
3	A	prickly lettuce, curly lettuce, red “radicchio” lettuce
4	B	carrot, “pan di zucchero” lettuce, “chioggia” lettuce, rocket
5	B	carrot, “pan di zucchero” lettuce, “chioggia” lettuce, curly lettuce
6	B	valerian lettuce, curly lettuce, carrot
7	B	“pan di zucchero” lettuce, carrot, lettuce

*For each typology 8 samples were examined.

ple were diluted with 225 mL of Buffered Peptone Water (0.1%) (Oxoid – Milan, Italy) and homogenised in a Stomacher 400 (International pbi – Milan, Italy) for 2 min at normal speed to obtain a 1:10 dilution. Additional 10-fold dilutions were carried out using the same diluent; for lactic acid bacteria the dilutions were made with the de Man, Rogosa and Sharpe MRS Broth (Liofilchem – Roseto degli Abruzzi, Italy). The presence of mesophiles and psycrophiles was detected as part of total aerobic count: 1 mL of each dilution was surface plated in duplicate on Plate Count Agar (Oxoid) with incubation at 30°C for 48 h for mesophilic microorganisms and at 7°C for 10 days for psychrotophs. For aerobic sporeformers, the initial dilutions were heat-treated at 80°C for 10 min to kill vegetative cells. One mL of appropriate dilution with Buffered Peptone Water was inoculated in duplicate using the pour plate method in Plate Count Agar with incubation at 30°C for 48 h. The detection of yeasts and moulds was performed by surface plating 1 mL of each dilution in quadruplicate on Rose-Bengal Chloramphenicol Agar (Liofilchem) with incubation at 25°C for 5 days. The microscopic examination was performed in order to identify the yeasts and moulds isolated. Lactic acid bacteria were enumerated using a pour plated method; volumes of 1 mL of each dilution were inoculated in duplicate into MRS Agar (Oxoid) and overlayed with the same medium; the plates were incubated at 28°C for 72 h. The isolates were confirmed using API 50 CHL strips (bioMérieux – Rome, Italy). The number of *Enterobacteriaceae* was determined by pour plating in duplicate 1 mL of each dilution into Violet Red Bile Glucose Agar (Liofilchem); after the inoculated medium was solidified, an overlay of 4 mL of the same medium was added. The plates were incubated at 37°C for 24 h. Characterisation of presumptive *Enterobacteriaceae* was carried out by Gram staining, catalase and oxidase reactions. For detection of *E. coli*, Violet Red Bile Lactose Agar was utilised with incubation at 44°C for 24 h. *S. aureus* was enumerated by surface plating 1 mL of each dilution in two plates of Baird-Parker Agar (Liofilchem) to which RPF Supplement (Liofilchem) was added with incubation at 37°C for 24-48 h. Colonies with typical morphology were stained with Gram's method and tested for catalase reaction. Coagulase test was confirmed with the Staphytect Plus Test (Oxoid). For *Pseudomonas* spp., 1 mL of each dilution was surface plated in duplicate onto Pseudomonas Agar Base (Liofilchem) with CFC Supplement (Liofilchem) and incubated at 37°C for 24-48 h.

To detect *Salmonella* spp., 25 g of each sample were homogenised in 225 mL of Buffered Peptone Water and incubated overnight at 37°C for 24 h as pre-enrichment; 10 mL of pre-enrichment suspension were inoculated into 90 mL of Selenite Cystine Broth (Oxoid) to which sodium biselenite and Salmonella Selective Supplement (Oxoid) were added and incubated at 37°C for 18 h. Subcultures were performed onto Salmonella Chromogenic Agar (Oxoid) with incubation at 37°C for 24 h. Suspected colonies were identified by appropriate biochemical and serological tests.

To isolate *Listeria* spp., 25 g of each sample were homogenised in 225 mL of Fraser Broth Base (Oxoid) half concentration to which Listeria Primary Enrichment Supplement (UVMLI) (Oxoid) was added and incubated at 30°C for 24±3 h as the first enrichment; at the same time, a loopful of the homogenate was streaked onto Listeria Oxford Agar (Liofilchem) with incubation at 37°C for 24 h. A second enrichment was performed inoculating 0.1 mL of the first enrichment in 10 mL of Fraser Broth normal concentration supplemented with Listeria Secondary Enrichment Supplement (UVMLII) (Oxoid) with incubation at 37°C

for 48 h. Subcultures were streaked onto Listeria Oxford Agar.

For *Y. enterocolitica* detection, 25 g of sample were homogenised with 225 mL of Peptone Sorbitol Bile Broth (PSBB); 1 mL of the homogenate was inoculated into 9 mL of 5% KOH in 0.5% salt solution and, after stirring for several seconds, surface plated onto Yersinia Selective Agar base (CIN) to which Yersinia Supplement (Liofilchem) was added and incubated at 28°C for 24-48 h. At the same time, 0.1 mL of homogenate was spread directly onto CIN Agar. Colonies were screened by Gram staining, motility assay at 22° and 37°C, glucose fermentation, utilisation of citrate and production of H_2S, urease, oxidase and API 20E (bioMérieux- Rome, Italy).

Aeromonas spp. were detected using 25 g of sample homogenised with 225 mL of Tryptone Soya Broth (TSB) (Oxoid) and serially diluted (1:10) with Ringer solution; 1 mL of each dilution was spread in duplicate onto m-Aeromonas Selective Agar Base (HAVELAAR) (Biolife – Milan, Italy) with Ampicillin Supplement (Biolife) and incubated at 28°C for 24 h. The colonies were identified as previously described (PIANETTI *et al.*, 2004).

To detect *Vibrio* spp. 25 g of sample were homogenised with 225 mL of Alkaline Peptone Water followed by surface plating onto two plates of Thiosulphate Citrate Bile Sucrose Agar (TCBS Oxoid) with 2% NaCl and incubation at 37°C for 24 h. The identification was performed as described elsewhere (PIANETTI *et al.*, 2004).

RESULTS AND CONCLUSIONS

The microbiological analysis carried out on ready-to-eat vegetable salads showed that the samples tested met health and safety conditions. In fact, pathogens such as *L. monocytogenes*, *Salmonella* spp., *Y. enterocolitica*, *Vibrio* spp. and *Aeromonas* spp. were always

Table 2 - Microbiogical parameters for hygienic quality (CFU/g) according to different guidelines.

EEC			CNERNA-CNRS				PHLS			
Microorganisms	Limits		Microorganisms	satisfactory[a]	acceptable[a]	limit[b]	Microorganisms	satisfactory	acceptable	unsatisfactory
	minimum (m)	Maximum (M)								
E.coli	10^2*	10^3*	Aerobic colony count	10^6	10^7	10^8	Aerobic colony count	10^6	10^6<10^7	$\geq 10^7$
			Lactic acid bacteria	10^3	10^4	10^7	*Enterobacteriaceae*	<10^2	10^2<10^4	$\geq 10^4$
			Yeast	10^3	10^4	10^5	*E. coli*	<20	20<10^2	$\geq 10^2$
							Listeria spp.	<20	20<10^2	$\geq 10^2$
							S. aureus	<20	20<10^2	10^2<10^4

*satisfactory: for values ≤m; acceptable: for values between m and M; unsatisfactory: for values ≥M;
[a] first day of production;
[b] last day of shelf-life.

absent. The absence of the above-mentioned microorganisms indicates that the overall agricultural, hygienic, harvesting and production practices were good. The detection of other microbial groups is important because it gives information about the hygienic quality, as well as product stability. In this regard, guidelines issued by different institutions consider different microbial groups as indices of process hygiene. The European Community (EC) guidelines only consider *E. coli* as indicator (EC n. 2005/2073). However, this parameter alone may be restrictive because it does not always correlate to other microbial groups which may be present in fresh vegetables. Therefore, for a more exhaustive evaluation of the hygienic quality of the vegetables other guidelines were also referred to, in particular, to the French CNERNA-CNRS (1996) and British PHLS (GILBERT *et al.*, 2000), which consider several microbiological parameters (Table 2). The CNERNA-CNRS guidelines provide for

Table 3 - Viable counts (CFU/g) of various bacterial groups detected in ready-to-eat vegetable salads during shelf-life.

Microorganisms	ND*		<20		20-10²		>10²-10³		>10³-10⁴		>10⁴-10⁵		>10⁵-10⁶		>10⁶-10⁷		>10⁷-10⁸		>10⁸	
	T_0	T_1	T_0	T_1	T_0	T_1	T_0	T_1	T_0	T_1	T_0	T_1	T_0	T_1	T_0	T_1	T_0	T_1	T_0	T_1
Mesophiles					2		12		28	6	5	11	6	3	38	13	6	26	6	14
					3.5%		21.4%		50.0%	10.7%	8.9%	19.6%	10.7%	5.3%	67.8%	23.2%	10.7%	46.4%	10.7%	25.0%
Psycrophiles					18	11	9	5	6	2			14		30		6	32	6	24
					32.1%	19.6%	16.0%	8.9%	10.7%	3.5%			25.0%		53.0%		10.7%	57.1%	10.7%	42.8%
Lactic acid bacteria					14		12		16	18	4	2	5	13	3	11				
					25.0%		21.4%		7.1%	2.1%	8.9%	23.2%	5.3%	19.6%						
Sporeformers	9	16	14	22	4	12			4		25	9	3	22	8	10		8		
	16.0%	28.5%	25.0%	39.2%	7.1%	21.4%			7.1%		44.6%	16.0%	5.3%	39.2%	14.2%	17.8%		14.2%		
Enterobacteriaceae	16	12	2		13	9	4		16	7	10	16	5	5		4				
	28.5%	21.4%	3.5%		23.2%	16.0%	7.1%		28.5%	12.5%	17.8%	28.5%	8.9%	8.9%		7.1%				
E. coli	47	40	5						16	27	4		3	7						
	83.9%	71.4%	8.9%						28.5%	48.2%	7.1%		5.3%	12.5%						
S. aureus	20	12	14	9	7	21.4%		7.1%												
	35.7%	21.4%	25.0%	16.0%	13	15	9	20												
Pseudomonas spp.					23.2%	26.7%	16.0%	5.7%												
					4													8		
					7.1%													14.2%		
Yeasts			5		11	1	9	3	16	27	10	16	5	5		4				
			8.9%		19.6%	1.7%	16.0%	5.3%	28.5%	48.2%	17.8%	28.5%	8.9%	8.9%		7.1%				
Moulds	5		8	4	13	5	15	25	8	15	4		3	7						
	8.9%		14.2%	7.1%	23.2%	8.9%	26.7%	44.6%	14.2%	26.7%	7.1%		5.3%	12.5%						

*Not Detected.

aerobic colony count, lactic acid bacteria and yeasts. Aerobic colony count is useful for indicating the overall microbial quality of a food product; generally it does not relate to food safety hazards but acts as an indicator for food quality and shelf-life duration. Lactic acid bacteria and yeasts, when present in high numbers, can contribute to spoilage and cause unpleasant odours due to the production of ethanol, organic acids, esters and CO_2 (BABIC *et al.,* 1992; FLEET, 1992).

Considering aerobic colony count (Table 3), the values ranged from 10^5 to $>10^8$ CFU/g on the first day and on the last day of the shelf-life, but the samples with the highest values ($>10^8$ CFU/g) increased from 6 (10.7%) to 14 (25.0%) for mesophiles and from 6 (10.7%) to 24 (42.8%) for psycrophiles at the expiry date. Lactic acid bacteria levels were lower. In fact, they ranged from 20 to 10^7 CFU/g on the first day of production and from $>10^5$ to 10^8 CFU/g on the last day. The growth of yeast during the shelf- life was limited; in fact, the initial and final levels ranged from <20 to 10^6 and from 20 to 10^7 CFU/g, respectively.

Based on CNRNA-CNRS microbiological criteria (Table 4), all three parameters were satisfactory or acceptable for most samples (about 75%) analysed on the first day of production. Considering the results obtained on the last day of storage, the numbers of unsatisfactory samples for aerobic colony count increased to 24 (42.8%), with 8 (14.2%) and 9 (16.0%) unsatisfactory samples for lactic acid bacteria and yeasts, respectively. As reported in the literature (BRACKET, 1987); BARRIGA *et al.*, 1991), the lesser increase of lactic acid bacteria and yeasts could be due to competition with other microorganisms which can grow rapidly at refrigeration temperatures.

Considering the typologies, only typology 1, supplied by manufacturer A, met the acceptability criteria for all three indices in all samples. In contrast, the

Table 4 - Microbiological quality of ready-to-eat vegetable salads based on CNERNA-CNRS guidelines.

Typology	Aerobic colony count			Lactic acid bacteria			Yeasts		
	satisfactory T_0	acceptable T_0	unsatisfactory T_0	satisfactory T_0	acceptable T_0	unsatisfactory T_0	satisfactory T_0	acceptable T_0	unsatisfactory T_0	unsatisfactory T_1
1	3 (37.5%)	5 (62.5%)		2 (25.0%)	6 (75.0%)		7 (87.5%)	1 (12.5%)		
2	1 (12.5%)	7 (87.5%)		2 (25.0%)	3 (37.5%)	3 (37.5%)	3 (37.5%)	2 (25.0%)	3 (37.5%)	2 (25.0%)
3		5 (62.5%)	3 (37.5%)	3 (37.5%)	4 (50.0%)	1 (12.5%)	2 (25.0%)	2 (25.0%)	4 (50.0%)	2 (25.0%)
4		7 (87.5%)	1 (12.5%)	3 (37.5%)	3 (37.5%)	2 (25.0%)	1 (12.5%)	7 (87.5%)		1 (12.5%)
5		4 (50.0%)	4 (50.0%)		4 (50.0%)	4 (50.0%)	1 (12.5%)	3 (37.5%)	4 (50.0%)	3 (37.5%)
6	1 (12.5%)	7 (87.5%)		2 (25.0%)	5 (62.5%)	1 (12.5%)	6 (75.0%)		2 (25.0%)	
7	1 (12.5%)	3 (37.5%)	4 (50.0%)	2 (25.0%)	3 (37.5%)	3 (37.5%)	5 (62.5%)	1 (12.5%)	2 (25.0%)	
Total	6 (10.7%)	38 (67.8%)	12 (21.4%)	14 (25.0%)	28 (50.0%)	14 (25.0%)	25 (44.6%)	16 (28.5%)	15 (26.7%)	9 (16.0%)

Table 5 - Microbiological quality of ready-to-eat vegetable salads based on PHLS guidelines.

Typology		Aerobic colony count						*Enterobacteriaceae*						*Escherichia coli*						*Staphylococcus aureus*					
		satisfactory		acceptable		unsatisfactory		satisfactory		acceptable		unsatisfactory		satisfactory		acceptable		unsatisfactory		satisfactory		acceptable		unsatisfactory	
		N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%
1	T_0	3	37.5	5	62.5			5	62.5			3	37.5	8	100					4	50.0	2	25.0	2	25.0
	T_1					8	100	5	62.5	1	12.5	2	25.0	8	100					3	37.5	2	25.0	3	37.5
2	T_0	1	12.5	7	87.5			5	62.5	3	37.5			8	100					8	100				
	T_1					8	100	4	50.0	3	37.5	1	12.5	5	50.0	3	37.5			4	50.0	2	25.0	2	25.0
3	T_0			5	62.5	3	37.5	6	75.0	2	25.0			8	100					7	87.5	1	12.5		
	T_1					8	100			6	75.0	2	25.0	6	75.0	2	25.0			4	50.0	1	12.5	3	37.5
4	T_0			7	87.5	1	12.5			4	50.0	4	50.0	4	50.0	4	50.0			4	50.0	1	12.5	3	37.5
	T_1					8	100			2	25.0	6	75.0	1	12.5	3	37.5	4	50.0	2	25.0	1	12.5	5	62.5
5	T_0			4	50.0			6	75.0	1	12.5	1	12.5	8	100					3	37.5	1	12.5	4	50.0
	T_1					8	100			1	12.5	7	87.5	5	50.0	3	37.5			2	25.0			6	75.0
6	T_0	1	12.5	7	87.5			4	50.0	4	50.0			8	100					8	100				
	T_1					8	100	3	37.5	4	50.0	1	12.5	8	100					6	75.0	2	25.0		
7	T_0	1	12.5	3	37.5	5	50.0	6	75.0	2	25.0			8	100							8	100		
	T_1					8	100			3	37.5	5	62.5	7	87.5	1	12.5					7	87.5	1	12.5
Total	T_0	6	0.71	38	47.8	12	21.4	32	57.1	16	28.5	8	14.2	52	92.8	4	7.1			34	60.7	13	23.2	9	16.0
	T_1					56	100	12	21.4	20	35.7	24	42.8	40	71.4	12	21.4	4	7.1	21	37.5	15	26.7	20	35.7

most contaminated samples were found in typology 5, supplied by manufacturer B, with 50% of the samples exceeding the limits set for the three parameters.

As hygienic quality indices, the PHLS guideline criteria include the aerobic colony count, *Enterobacteriaceae*, *E. coli* and *Listeria* spp. The number of samples with satisfactory or acceptable values of total aerobic count was the same according to the French criteria when the foods were examined on the same day of production (Table 5). Good results were obtained for faecal indicators. In fact, *Enterobacteriaceae* were at satisfactory levels in 32 (57.1%) samples, acceptable in 16 (28.57%) and unsatisfactory in 8 (14.28%). Very good results were obtained for *E. coli*, which was satisfactory in 52 (92.8%) samples and acceptable in 4 (7.1%). The presence of faecal indicators at lower levels than aerobic colony count indicates that they were not the dominant flora in any of the samples examined and are correlated with the absence of enteric pathogens such as Salmonella, *Vibrio,* etc. However, considering the results on the last day of storage, unsatisfactory levels were found in all the samples for aerobic colony count, in 24 samples (42.8%) for *Enterobacteriaceae* and in 4 samples (7.1%) for *E. coli*.

Finally *S. aureus*, included as a pathogen in the PHLS guidelines, was satisfactory in 34 (60.7%) samples, ac-

ceptable in 13 samples (23.2%), and unsatisfactory in 9 samples (16.0%) on the first day of production. Growth during the storage time caused the number of unsatisfactory samples to increase to 20 (35.7%). In any case the levels were always in the $>10^2$-10^3 CFU/g range (Table 5). These loads make such products unlikely sources of food poisoning. In fact, intoxication can take place following the ingestion of concentrations of enterotoxins which are produced only after a strong growth of the bacterium up to 10^6 cell/g or more in food kept at room temperature for at least 10-20 h (KRÄMER and CANTONI, 1994).

Considering the various typologies, only typology 6, supplied by manufacturer B, met the acceptability criteria for all four parameters in all samples throughout the storage time.

Regarding the other microorganisms investigated (Table 3), sporeformers were found at low levels; in fact, they were not detected in 9 (16.0%) and 16 (28.5%) samples at the first and last day of storage, respectively. In the other samples, they ranged from <20 to 10^4 CFU/g both at the first and last day. Moulds were absent in 5 (8.9%) samples on the first day of storage, present in very low numbers in 13 (23.2%) samples, while in about 30% they exceeded the limit values provided for fresh vegetables to be eaten uncooked by the REGION FRIULI-VENEZIA GIULIA GUIDELINES (1997). Finally, *Pseudomonas* spp., present at high concentrations ($>10^5$-10^7 CFU/g) in 11 (19.6%) samples on the first day, reached values $>10^7$-10^8 CFU/g in 8 (14.2%) samples at the end of the storage time. High numbers of pseudomonads are undesirable because they are often responsible for spoilage of fresh vegetables due to the production of pectinolytic enzymes which cause a breakdown of the peptic polymers in plant cells (MEMBRÉ and BURLOT, 1994).

In conclusion, the absence of pathogens and the low incidence of *E. coli* indicate that the overall agricultural, harvesting and production practices were good. However, the presence of a saprophytic microflora at high levels in a fair number of the samples, on the first day of production, points out the importance of the manufacturers selecting high quality raw materials. Furthermore, the existing sanitisation and conservation processes reduce, but do not eliminate the original microflora, which can grow during storage and cause deterioration. More efficient technologies that kill the naturally occurring microorganisms are needed to improve the shelf-life of the ready-to-eat vegetable salads. This could also provide greater safety for consumers and economic advantages for manufacturers and retailers. Finally, the fact that *E. coli* is the only parameter provided by the EC guidelines is insufficient to assess the hygienic quality of ready-to-eat vegetables. According to this criterion all samples tested were satisfactory, while the evaluation of the other microbiological parameters clearly indicated a number of unsatisfactory samples. Therefore it would be opportune to include other microbiological indicators in the European guidelines following the example of other guidelines.

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SHORT COMMUNICATION

INTERNAL FRUIT QUALITY OF FIGS (*FICUS CARICA* L.) IN THE NORTHERN MEDITERRANEAN REGION

QUALITÀ INTERNA DEL FRUTTO DEL FICO (*FICUS CARICA* L.) DELLA REGIONE DEL MEDITERRANEO SETTENTRIONALE

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ABSTRACT

The content of the most common sugars, organic acids and total phenols and antioxidative potential in fresh fig fruit were determined. Three cultivars were studied: 'Bela petrovka' – a white-type fruit, and 'Črna petrovka' and 'Miljska figa' – both dark-type fruit. The figs were picked in the summer and fall harvests and compared. There was no significant difference between the fruit from the two crops. While there were some significant differences in the sugar and organic acid

RIASSUNTO

Il frutto del fico è molto conosciuto e presente nell'alimentazione come frutto fresco o essiccato. In questa ricerca si è analizzato il contenuto degli zuccheri più comuni, degli acidi organici, dei polifenoli e degli antiossidanti del frutto fresco. All'uopo sono state utilizzate tre varietà di frutto di fico e cioè: 'Bela petrovka' che ha un frutto chiaro, 'Črna petrovka' e 'Miljska figa' che hanno entrambe il frutto di colore scuro. Sono stati raccolti i frutti estivi ed autunnali. Dai risultati non si sono emerse differen-

- Key words: antioxidative potential; fig; organic acids; sugars; total phenolic content -

contents, there were no significant differences among the three cultivars. The dark-fruit cultivars had higher total phenolic contents and therefore higher antioxidative potential. Given the important antioxidant character of the phenolic content and the high sugar levels, figs, either fresh or dried, are a healthy alternative to the consumption of sweets.

ze particolari tra i frutti di prima e di seconda fruttificazione. Anche per quanto riguarda i valori dei contenuti degli zuccheri e degli acidi organici non si sono rilevate grandi variazioni. Le due varietà di fico con frutto scuro contenevano una quantità maggiore di polifenoli e di conseguenza avevano anche una maggiore potenzialità antiossidante. Grazie all'elevato contenuto di polifenoli, importanti antiossidanti, e di zuccheri, i frutti del fico, sia freschi sia essiccati, possono rappresentare una sana alternativa al consumo dei dolci.

INTRODUCTION

Fig (*Ficus carica* L.) is a widespread species that grows wild or cultivated throughout the Mediterranean Basin. Other parts of the world that have a semi-arid climate and irrigation have ideal conditions for intensive fig cultivation.

In the parts of the world where figs are not produced, they are usually consumed as dried fruit, since drying prolongs their storage time. Fresh figs are typical seasonal fruits because they have a short, post-harvest shelf life of only 7-10 days. By combining cool conditions and a CO_2-enriched atmosphere, their post-harvest life can be extended to 2-4 weeks (SOZZI *et al.,* 2005).

Fresh figs are an important constituent of the Mediterranean diet (SOLOMON *et al.*, 2006), which is considered to be one of the healthiest diets and is associated with longevity (TRICHOPOULOU *et al.,* 2006). Besides fig fruit, other parts of the plant, such as the leaves and roots, have also been used in traditional folk medicine in the Middle East (RUBNOV *et al.,* 2001). Figs are an excellent source of minerals, vitamins and dietary fibre; they are fat- and cholesterol-free and contain large amounts of various amino acids (SLAVIN, 2006; SOLOMON *et al.*, 2006).

Sugars and organic acids are important constituents of the fruit. If their level varies the quality may be compromised (DOYON *et al.*, 1991). The composition of the sugars influences fruit sweetness. The phenolic substances in the fruit contribute to their taste and are very important because they have a positive effect on human health. They contribute to fruit quality and are usually influenced by the species and cultivar, as well as by growing conditions and orchard management practises (VEBERIC *et al.,* 2005).

In the northern Mediterranean region, fig trees produce one or two crops per year, depending on the cultivar. The first crop develops from flowers that were initiated in the previous year, and the fruit ripens at the beginning of summer. The second crop (the main one) is produced from flowers that emerge on shoots of the current season, and the fruit ripen in late summer. Therefore, the two crops develop under very different weather conditions. Fruit from the two crops can differ in size and shape (LODHI *et al.,* 1969).

Despite the fact that figs are an im-

portant fresh fruit in many countries and are commonly used as dried fruit in most parts of the world, there is only little information available regarding the content of different compounds that contribute to fruit quality.

The aim of this study was to determine the levels of selected sugars, organic acids and total phenolics as well as the total antioxidant capacity in figs grown in the Slovenian costal region, one of the northernmost fig-growing areas. The influence of the cultivar and crop timing on the contents of the different compounds in fresh figs was evaluated. The data obtained provide a good basis for evaluating the nutritional importance of fig fruit.

MATERIAL AND METHODS

Plant material

The figs were collected from orchards in the littoral area of Slovenia (northern part of the Mediterranean). All samples were picked at their commercially mature stage, determined by the softening of the fruit and development of their typical fruit taste and color.

The fig cultivars included in the study are well adapted to the environmental conditions of the northern Mediterranean climate and are probably closely related to other cultivars grown in the Mediterranean Basin. The cultivars included in the study were the following:

- 'Bela petrovka', a white-type fig with yellow flesh, sweet tasting and very juicy. The fruits are large and are quite resistant to rain.
- 'Miljska figa', a dark-type fig that is harvested in September. The fruit is round, purple and medium-sized. The red pulp is sweet and juicy.
- 'Črna petrovka' is a dark-type, medium-sized, purple fig with sweet, juicy fruit.

The figs were harvested at the optimal ripening time in 2005. Due to successive ripening, the cultivars were picked twice during the first harvest (at the beginning and middle of July) and twice during the second harvest (at the beginning and middle of September). Five repetitions were carried out (n=5) for each cultivar; each repetition included 10 figs taken from 5 trees. The figs were stored at -20°C until analysis.

Extraction and determination of sugars and organic acids

Fruit samples were first homogenized with a manual blender (Braun, Kronberg, Germany), and then with an Ultra-Turrax T-25 (Ika-Labortechnik, Staufen, Germany). Ten g of mashed fruit were dissolved in double-distilled water (50 mL) and left for 30 min at room temperature. After extraction, the homogenate was centrifuged (Eppendorf Centrifuge 5810 R, Hamburg, Germany) at 12,000 rpm for 7 min at 10°C. The supernatant was filtered through a 0.45 μm cellulose ester filter (Macherey-Nagel, Düren, Germany) and 20 μL of sample were transferred to a vial and used for the HPLC-analyses of sugars and organic acids.

The analyses of sugars (fructose, glucose, sucrose and sorbitol) and organic acids (malic, citric acid, fumaric and shikimic) were carried out using high-performance liquid chromatography-HPLC (Thermo Scientific, Waltham, USA). Sugars and sorbitol were separated by using a Rezex RCM-monosaccharide column (300x7.8 mm) operated at 65°C (Phenomenex, Torrance, USA). The mobile phase was double-distilled water and the flow rate was 0.6 mL min^{-1}; the total run time was 35 min, and a refractive index (RI) detector was used to monitor the eluted carbohydrates according to DOLENC-STURM *et al.* (1999) with minor changes.

Organic acids were analysed with HPLC, using an Aminex HPX-87H column (300x7.8 mm) (Bio-Rad, Hercules,

USA) in conjunction with a UV detector set at 210 nm, as described by DOLENC-STURM *et al.* (1999). The column temperature was set at 65°C. The elution solvent was 4 mM sulphuric acid in double-distilled water at a flow rate of 0.6 mL min^{-1} for 30 min.

Determination of total phenolic content

The fruit phenolics were extracted according to the method described by Escarpa and Gonzalez (1998): 10 g of whole fruit were extracted with methanol using a cooled ultrasonic bath. Samples were extracted with 10 mL of solvent for 1 h, 10 mL for 30 min and 5 mL for 30 min. The three fractions were combined to a final volume of 25 mL and filtered through a 0.45 μm membrane filter (Macherey-Nagel, Düren, Germany).

The total phenolic content of the extracts was assessed using the Folin-Ciocalteau phenol reagent method (SINGLETON and ROSSI, 1965). Six mL of double-distilled water and 500 μL of Folin-Ciocalteau reagent were added to 100 μL of the sample extracts and left to stand between 8 sec and 8 min at room temperature; 1.5 mL of sodium carbonate (20% w/v) was then added. The extracts were mixed and allowed to stand for 30 min at 40°C before the absorbance was measured with a spectrophotometer UV/VIS Lambada Bio 20 (Perkin Elmer, Waltham, USA) at 765 nm. A mixture of methanol and reagents was used as a blank solution. The total phenolic content is expressed as gallic acid equivalents (GAE).

Determination of antioxidant activity by the DPPH radical scavenging method

The samples used to determine the antioxidant activity were extracted according to the same protocol as described for the total phenolic content. The free radical scavenging activity of fig extracts was measured according to the DPPH (1,1-diphenyl-2-picrylhydrazyl) method reported by Brand-Williams *et al.* (1995), with some modifications. Fifty μL of fruit extract were placed in 96-well microplates, and 200 μL of 0.1 mM methanolic solution of DPPH were added and allowed to react in the dark at room temperature. The decrease in absorbance of DPPH at 520 nm was measured at 5-min intervals using a spectrophotometer MRX (Dynex Technologies, Chantilly, USA), until absorbance stabilized (30 min). Methanol was used as a blank solution and DPPH solution without test samples served as the control. All samples were prepared in triplicate. The DPPH radical scavenging activity of fruit extracts was expressed as mg of ascorbic acid (vitamin C) equivalents per kg of fresh fig fruits (AEAC = ascorbic acid equivalent antioxidant capacity) in 30 min reaction time. The AEACs of the samples at various concentrations were determined by using ascorbic acid standard curves (LEONG and SHUI, 2002).

Statistical analysis

The data were analyzed by analysis of variance (ANOVA) using the Statgraphics Plus 4.0 program (Manugistics Inc., Rockville, USA). The differences among the cultivars and picking times were estimated by using a multiple range test, the Tukey HSD test at $p<0.05$.

RESULTS AND DISCUSSION

Various chemical compounds in fruit, as well as the physical characteristics, like firmness and juiciness, determine the internal quality. In recent years, this type of quality has become more important to consumers, who no longer buy fruit based on outer appearance alone.

Sweetness and acidity are important aspects of fruit taste. Table 1 reports that glucose is the main sugar in the fruit an-

Table 1 - Content (g kg^{-1}) of glucose and fructose and the ratio of glucose content to fructose content in fresh fruit of different fig (*Ficus carica*) cultivars at different harvest times.

		Glucose		Fructose		ratio Glucose/fructose
Bela petrovka	J-beg	98.0±3.6	cd	81.2±2.9	cd	1.2
Bela petrovka	J-mid	93.2±3.4	bcd	75.6±2.2	ab	1.2
Bela petrovka	S-beg	104.0±3.1	cd	90.2±2.7	de	1.2
Bela petrovka	S-mid	90.1±5.9	abc	78.4±5.2	abc	1.1
Črna Petrovka	J-mid	110.5±4.8	d	91.6±4.9	de	1.2
Črna Petrovka	S-beg	89.5±7.5	abc	72.2±4.2	ab	1.2
Črna Petrovka	S-mid	74.1±7.7	a	66.4±7.0	a	1.1
Miljska Figa	S-beg	109.3±2.9	d	100.1±2.9	e	1.1
Miljska Figa	S-mid	76.9±6.1	ab	69.8±4.8	ab	1.1

Average values (n=5) and standard errors. Different letters in the columns indicate statistically significant differences at $p<0.05$.
J-beg: beginning of July; J-mid: middle of July; S-beg: beginning of September; S-mid: middle of September.

alysed in this study, followed by fructose. The sucrose content was very low and could not be clearly distinguished from that of other sugars. In juice obtained from figs, the sucrose level was also very low (MELGAREJO *et. al.*, 2003) or absent (SUGIYMA *et al.*, 1991). The glucose/fructose ratio was quite constant in the present study (1.1-1.2), regardless of the cultivar. A similar ratio between these two sugars was also reported by SUGIYMA *et al.* (1991) for the juice obtained from figs. The sugar composition can influence fruit sweetness. For example, fructose has a higher relative sweetness than glucose (SETSER, 1993). There were no major differences in sugar content among the cultivars. The 'Bela petrovka' and 'Miljska figa' cultivars had the highest values of the two sugars at the beginning of September, while 'Črna petrovka' had the highest values in the summer crop (mid July). The lower fructose values are comparable to those reported in apples and pears (COLARIC *et al.*, 2006). Similar values were also reported for some plums. The glucose content, which is higher than that of fructose, is also quite high compared to other fruit species of the temperate zone. The lowest values match the highest ones reported for sweet cherries (DOLENC and STAMPAR, 1998; USENIK *et al.*, 2005).

It has been shown that in peaches the citric acid content, even in small amounts, adds more to the sourness of fruit than malic acid. In relation to peaches, COLARIC *et al.* (2005) reported that the sweetness of fruit is correlated more (negatively) with the presence of certain acids, like citric and shikimic acid, than with the amount of various sugars. The presence of certain acids, even in small amounts, can influence the taste and make it less sweet, despite the presence of high amounts of sugars. A study by HECKE *et al.* (2006) showed comparable results for apples: some organic apples, which were classified as sour, contained more sugar than sweet apples that had a low organic acid content. In the present study the most abundant organic acid among those analysed was citric acid, followed by malic acid (Table 2); fumaric and shikimic acids were present in trace amounts. SUGIYMA *et al.* (1991) reported that the citric acid content was higher than that of malic acid in juice made from fig fruit. The highest citric acid values were found in the 'Miljska figa' cultivar. There was much less difference in the malic acid

contents among the cultivars. Only the September crop of the 'Črna Petrovka' cultivar showed a significantly lower malic acid content, in fact it was the lowest. The highest fumaric acid values were recorded in the summer crop of the 'Bela petrovka' cultivar. Significantly higher amounts of shikimic acid were recorded for cv. 'Črna Petrovka' in both crops. Because fumaric and shikimic acids are present in small amounts, they do not have a significant influence on the taste. Despite the fact that the differences were significant, the amounts among the cultivars were small. If we compare the malic acid content with that in other fruits, the amounts are less than those for most temperate zone fruit varieties; they are only comparable to some soft fruits like strawberries and raspberries (HERMANN, 2001). On the other hand, the citric acid content is quite high when compared to other fruit varieties; it is in approximately the same range as that reported for 'Williams' pear (COLARIC *et al.*, 2006) or various peach cultivars (COLARIC *et al.*, 2005). MELGAREJO *et al.* (2003) reported that citric acid was one of the main acids in juice made from figs from the first crop, while malic acid was one of the main acids in juice made from second-crop fruit. In the present study, no differences in composition of organic acids were found between the fruits of the first and second crop at cvs. 'Bela petrovka' and 'Črna petrovka'.

Phenolics make up another important group of chemical compounds due to their supposed beneficial health properties. In contrast to sugars and organic acids that are the result of primary metabolism, phenolics are synthesized in secondary metabolic pathways. Besides their numerous biological functions in plants, phenolics are important food ingredients because of their wide spectrum of biochemical activities that prevent various diseases.

In this study, the total phenolic content was measured and is expressed as equivalents of gallic acid. The highest total phenolic content was found in 'Miljska figa' and the lowest in 'Bela petrovka' (Table 3). In 'Bela petrovka', the phenolic content increased from the first to the second crop, while it decreased in 'Črna petrovka'. MARINOVA *et al.* (2005) compared the total phenolic content in 20 different Bulgarian fruit species and showed that figs had a higher phenolic content than peaches but less than sweet cherries, pears and apples.

Table 2 - Content of individual organic acids in fresh fruit of three fig (*Ficus carica*) cultivars at different harvest times.

		Citric acid (g kg^{-1})		Malic acid (g kg^{-1})		Fumaric acid (mg kg^{-1})		Shikimic acid (mg kg^{-1})	
Bela petrovka	J-beg	2.75±0.06	a	2.17±0.07	b	106.7±5.9	d	45.3±2.4	abc
Bela petrovka	J-mid	3.03±0.08	a	1.88±0.12	b	97.6±11.3	cd	40.5±1.1	a
Bela petrovka	S-beg	3.13±0.13	ab	2.10±0.09	b	47.8±1.3	b	52.5±1.9	cd
Bela petrovka	S-mid	2.96±0.26	a	2.06±0.16	b	33.7±3.3	ab	40.4±4.2	a
Črna Petrovka	J-mid	3.79±0.11	bc	1.88±0.10	b	82.9±6.5	c	54.7±1.5	d
Črna Petrovka	S-beg	2.91±0.28	a	1.29±0.10	a	32.4±3.6	ab	49.3±3.6	bcd
Črna Petrovka	S-mid	3.00±0.25	a	1.06±0.09	a	21.6±2.0	a	53.9±3.1	cd
Miljska Figa	S-beg	3.95±0.15	c	1.88±0.05	b	33.2±2.9	ab	39.5±2.3	a
Miljska Figa	S-mid	4.61±0.23	d	1.89±0.08	b	17.5±1.9	a	43.7±1.8	ab

Average values (n=5) and standard errors. Different letters in the columns indicate statistically significant differences at $p<0.05$.
J-beg: beginning of July; J-mid: middle of July; S-beg: beginning of September; S-mid: middle of September.

Table 3 - Ascorbic acid (vitamin C) equivalent antioxidant capacity (AEAC) and total phenolics in gallic acid equivalents (GAE) in fresh fruit of three fig (*Ficus carica*) cultivars at different harvest times.

		AEAC (mg vit. C kg^{-1})		GAE (mg kg^{-1})	
Bela petrovka	J-beg	29.3±1.5	a	151.5±6.9	a
Bela petrovka	J-mid	32.9±1.3	ab	170.6±8.7	ab
Bela petrovka	S-beg	34.2±2.0	b	190.4±10.7	ab
Bela petrovka	S-mid	38.5±1.5	c	223.8±15.4	bc
Črna Petrovka	J-mid	42.8±0.8	c	346.2±3.8	e
Črna Petrovka	S-beg	50.6±0.7	de	333.4±11.0	de
Črna Petrovka	S-mid	40.5±1.9	c	274.9±7.5	cd
Miljska Figa	S-beg	53.3±1.5	e	382.8±17.9	e
Miljska Figa	S-mid	47.2±1.9	d	454.6±5.4	f

Average values (n=5) and standard errors. Different letters in the columns indicate statistically significant differences at $p<0.05$.
J-beg: beginning of July; J-mid: middle of July; S-beg: beginning of September; S-mid: middle of September.

The total phenolic content in both dark fruit cultivars was somewhat higher than in the white-skin cultivar 'Bela petrovka'. This was probably due to the high anthocyanin content in the two dark purple skin cultivars. This assumption is supported by SOLOMON *et al.* (2006), who reported that an extract of the darker varieties contained more anthocyanins and therefore more total phenolics than that from the light coloured varieties. The results of the present study show a good correlation between the total phenolic content and total antioxidant capacity. The correlation coefficient was 0.79, indicating a moderately strong relationship between the variables at $p<0.01$. This supports the fact that the total antioxidant capacity of fruit in general and, in this case fig, is strongly dependent on the phenolic content. The 'Miljska figa' and 'Črna petrovka' cultivars had the highest antioxidant capacities in this study (Table 3). VINSON *et al.* (2005) reported that figs, especially dried ones, are an excellent source of nutrients and are *in vivo* antioxidants; the antioxidant capacity of plasma increased significantly for hours after consumption.

Based on the data obtained in the present study, as well as in other works, fig consumption should be encouraged as a healthy alternative to sweets, especially for children. Figs are not only sweet tasting fruit due to high amounts of sugars and low organic acid content, but they also contain medium-high amounts of phenolic compounds, especially if the red, anthocyanin-rich cultivars are considered.

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SHORT COMMUNICATION

STRUCTURAL CHARACTERISTICS OF THE TRIACYLGLYCEROL FRACTION FROM THE SEED FAT OF *MANGIFERA INDICA* L.

CARATTERISTICHE STRUTTURALI DELLA FRAZIONE TRIACILGLICEROLICA DEL GRASSO DEI SEMI DI *MANGIFERA INDICA* L.

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ABSTRACT

This paper reports the characterization of the triacylglycerols (TAG) from mango fat, one of the cocoa butter equivalents (CBEs) used to partially replace cocoa butter (CB) in chocolate. Several chemical parameters are proposed in order to evaluate the differences between CBEs and CB; among these, TAG and sterol compositions were considered. The stereospe-

RIASSUNTO

Il presente lavoro riporta una caratterizzazione dei triacilgliceroli (TAG) dei lipidi del mango, uno degli equivalenti del burro di cacao (CBE) usati per sostituire parzialmente il burro di cacao (CB) nel cioccolato. Molti parametri chimici sono stati proposti al fine di determinare l'aggiunta di equivalenti del burro di cacao al burro di cacao; fra questi possono essere citate la frazione TAG e

- Key words: Ag^+-HPLC, cocoa butter equivalents, mango lipids, stereospecific analysis, triacylglycerols -

cific analysis, coupled with silver ion high-performance liquid chromatography (Ag^+-HPLC), was used to characterise the TAG fraction from *Mangifera indica* L. (MAN) seed fat and its binary mixtures with CB. The results of the distribution of some fatty acids (FA) in the three *sn*-positions of the glycerol backbone for some TAG iso-unsaturated fractions showed differences between MAN fat and CB samples and also between the 5% MAN fat:95% CB w/w mixtures or 10% MAN fat:90% CB mixtures, and CB samples.

quella sterolica. La presente ricerca utilizza l'analisi stereospecifica accoppiata all'Ag^+-HPLC per caratterizzare la frazione TAG del grasso dei semi del mango *Mangifera indica* L. (MAN) e miscele binarie con campioni di CB. I risultati della distribuzione di alcuni acidi grassi (FA) nelle tre *sn*- posizioni dello scheletro glicerolico per alcune frazioni iso-insature TAG hanno mostrato differenze tra la frazione lipidica di MAN e CB; anche i risultati delle miscele costituite da CB e 5 o 10% di lipidi estratti dal MAN presentavano differenze, per quanto riguarda le composizioni acidiche intraposizionali di alcune frazioni TAG, rispetto ai campioni di CB.

INTRODUCTION

There are some fat matrices which, because of their chemical, physical and organoleptic features, are used to replace, at least partially, cocoa butter (CB) (COULTATE, 1984; NAWAR, 1985; SAMSUDIN and ALI RAHIM, 1996; LIPP and ANKLAM, 1998a). These substances are globally referred to as cocoa butter equivalents (CBEs) and the fat from mango (MAN) (*Mangifera indica* L.) fruit is considered among them.

Some significant problems arose after the foundation of the European Union (EU) and the ratification of the Schengen Treaty, since the CBEs were used in many countries up to a few years ago (EU, COUNCIL DIRECTIVE 73/241/EEC; EU, DIRECTIVE 2000/36/EC). In order to establish the regulations on the matter for all the countries belonging to the EU and to provide the consumer with correct, real and objective information, the European Parliament has set the maximum value of CBEs that can be added to chocolate; the last of these regulations (EU, DIRECTIVE 2000/36/EC) refers to the cocoa- and chocolate-containing products destined for human consumption. The regulation establishes that in each of the Member States vegetable fats other than CB can be added to the above-mentioned foodstuff to an extent not exceeding 5% of the final product; the fats permitted are derived from palm oil, illipe, sal, shea, kokum gurgi and mango kernel. The same law also states that all CBE-containing products can be sold only on the condition that their label contains a clear and unambiguous indication of the presence of these substitutes. For this reason, the problem regarding how to verify if the percentage of the added substitutes is below the limits sanctioned by the law is of particular significance.

Analysis of many classes of chemical compounds has been suggested in order to identify and quantify different CBEs added to foodstuffs to replace CB; triacylglycerol (TAG) and sterol fractions are among the most widely used (PADLEY and TIMMS, 1980; YOUNG, 1984; PALMER and PALMER, 1989; CHAISERI and DIMICK, 1995; CREWS *et al.*, 1997; LIPP and ANKLAM, 1998b; SIMONEAU *et al.*, 1999;

MACARTHUR *et al.*, 2000). High resolution gas chromatography (HRGC) analysis of TAG was recently used for the detection and quantification of CBEs in CB and plain chocolate and was sufficiently sensitivite (BUCHGRABER *et al.*, 2004a; 2004b). The method was collaboratively tested and standardised by the International Standard Organization (ISO 23275-1:2006; ISO 23275-2:2006).

Previous studies have been carried out on CB samples, different CBEs and on some of their mixtures (NERI *et al.*, 1998, 1999; DAMIANI *et al.*, 2006). The results of the percentage fatty acid (FA) composition of total TAG and its fractions, separated by silver-ion high-performance liquid chromatography (Ag^+-HPLC), together with those of TAG stereospecific analysis, have shown the significance of the fully saturated TAG fraction, as well as the potential use of stereospecific analysis of TAG fractions for the purpose of identifying and/or quantifying the CBEs added to CB.

The aim of this study was the characterisation of MAN fat in terms of the FA distribution in the three *sn*- positions of glycerol, considering both the total TAG and some iso-unsaturated TAG fractions, separated by Ag^+-HPLC. This procedure is not reported as an alternative to the above-cited HRGC analysis of intact TAG. However, a complete analysis not only of the mango fat TAG fraction but also of some CB samples and CB:CBE mixtures was carried out in order to compare the results of the different samples.

MATERIALS AND METHODS

Samples and reagents

- Six samples of genuine CB, from Brazil, Venezuela, Giava, Cameroun, Ghana and Togo. The composition of the samples was very similar to that of 43 CB samples examined in a previous study (data not published). The FA composition (% mol), mean values and standard deviations of the 43 samples, are reported in Table A.

Table A - Cocoa butter TAG, total FA composition (% mol).

	TAG (n=43)
C 14:0	0.1±0.05
C 16:0	26.2±0.92
C 16:1n-7	0.3±0.13
C 18:0	35.0±1.02
C 18:1n-9	34.2±1.27
C 18:2n-6	2.9±0.53
C 18:3n-3	0.1±0.13
C 20:0	1.0±0.45
C 20:1n-9	0.1±0.05

- Six samples of commercial mango fruit. MAN kernel fat was extracted with petroleum ether (40°-65°C) in a Soxhlet extractor for 12 h; prior to extraction, kernel samples were homogenised and mixed with anhydrous sodium sulfate.

- Two different groups of mixtures (95%CB:5% MAN fat and 90%CB:10% MAN fat, w/w), each containing six mixtures, prepared from the above-indicated CB and MAN fat samples. A random selection criterion was adopted in order to choose the samples to be used to prepare these two groups.

All the solvents and reagents, Analar or HPLC grades, were supplied by Sigma-Aldrich (St. Louis, MO, USA).

Standards

For the identification of the FAs (HRGC analysis), a standard mixture from Supelco (Bellafonte, PA, USA), named Supelco 37 component fatty acid methyl esters (FAME) mix, containing the methyl esters of 37 FAs (Catalog No. 47885-U) was used. The mix contained between 2 and 4% of each FA, except for palmitic acid methyl ester (6%).

For the identification of TAG (Ag^+-HPLC), five standard compounds (1,2-distearoyl-3-oleoyl-rac-glycerol; 1,2-diole-

oyl-3-stearoyl-rac-glycerol; 1-linoleoyl-2-oleoyl-3-stearoyl-rac-glycerol; glyceryl trioleate; 1,2-dioleoyl-3-linoleoyl-rac-glycerol), ≥99%, from Sigma-Aldrich (St. Louis, MO, USA) were used.

TAG isolation

Total TAG were separated from the lipid fraction of CB, MAN fat and their mixtures by TLC as previously described (NERI *et al.*, 1998), using silica gel plates, 0.25 mm, 20x20 cm (Macherey-Nagel, Düren, Germany) and the petroleum ether 40°-60°C/diethyl ether/formic acid mixture (70/30/1, v/v/v) as eluent.

Fatty acid % composition of TAG by HRGC

Total TAG and its fractions were subjected to transesterification catalysed by sodium methoxide to obtain the FAME; for the quantification of the TAG fractions separated by Ag^+-HPLC, an internal standard -methyl nonadecanoate- was added (SANTINELLI *et al.*, 1992). A Chrompack 9001 capillary gas chromatograph (Chrompack International B.V., Middelburg, The Netherlands), equipped with a split/splitless injection system and a flame ionisation detector was used for FAME analysis, with a fused silica capillary column coated with Supelcowax 10 (30 m x 0.25 mm i.d., 0.25 μm f.t.; Supelco, Bellafonte, PA, USA). The oven temperature was held at 165°C for 3 min; it was then raised 3°C/min to 240°C and held for 10 min. Helium was the carrier gas at a flow rate of 2 mL/min. The chromatograms were acquired and processed using the MOSAIC integration software (Chrompack International B.V., Middleburg, The Netherlands).

Ag^+-HPLC analysis

The HPLC system consisted of a Spectra-Physics Model 8700 solvent delivery system (Spectra-Physics, St. Albans, UK), a Cunow Model DDL21 light-scattering detector (Cunow SA, Cergy St. Cristophe, France) and a Spectra Physics SP 4290 integrator. An adjustable stream-splitter was installed between the column and the detector. An Ag^+ "Chromospher 5 Lipid" column – 250x4.6 mm, 5 μ - was used (Chrompack International B.V. Middleburg, The Netherlands). The conditions for the Ag^+-HPLC separation of TAG iso-unsaturated sub-fractions were previously described (CHRISTIE, 1988; NERI *et al.*, 1998, 1999). The experimental results obtained for the iso-unsaturated fractions were coincident with those obtained on the same samples using home-made software, capable of calculating the composition of the TAG molecular species using the experimental data of the stereospecific analysis of total TAG.

Stereospecific analysis of TAG by the *sn*-1,2-diacylglycerol kinase procedure

The Grignard deacylation of TAG, the preparations of *sn*-1,2-phosphatidic acids and *sn*-2-monoacylglycerols were carried out as previously described (DAMIANI *et al.*, 1994a and b; NERI *et al.*, 1998).

RESULTS AND DISCUSSION

The TAG from the MAN fat and CB samples were first analysed for the % mol total and intrapositional FA compositions; these latter compositions, obtained by stereospecific analysis carried out according to the above-reported procedure, describe the distribution of all the FAs in each of the three *sn*- positions on the glycerol backbone. The results are reported in Tables 1 and 2 for MAN fat and CB samples, respectively.

The data relative to the 95%CB:5%MAN fat and 90%CB:10%MAN fat mixtures were obtained using a home-made software, starting from the results of the

Table 1 - Mango fat samples. TAG total and intrapositional FA compositions, mean values % mol and standard deviations (n=6).

	TAG	*sn*-1-	*sn*-2-	*sn*-3-
C 14:0	0.1±0.01	0.3±0.02	n.d.	0.1±0.03
C 16:0	7.7±0.03	12.1±0.17	0.2±0.02	10.6±0.18
C 16:1n-7	0.1±0.01	0.4±0.20	n.d.	n.d.
C 18:0	36.2±0.14	50.3±0.10	0.5±0.01	57.8±0.35
C 18:1n-9	46.8±0.23	31.2±0.31	86.7±0.17	22.0±0.62
C 18:2n-6	6.8±0.06	4.0±0.05	11.5±0.14	5.0±0.11
C 18:3n-3	0.5±0.08	0.3±0.04	0.7±0.06	0.5±0.24
C 20:0	1.6±0.14	1.0±0.50	n.d.	3.9±0.48
C 20:1n-9	0.1±0.01	0.3±0.14	0.3±0.02	0.1±0.01

n.d. = not detectable, <0.1%.

Table 2 - CB samples. TAG total and intrapositional FA compositions, mean values % mol and standard deviations (n=6).

	TAG	*sn*-1-	*sn*-2-	*sn*-3-
C 14:0	0.2±0.11	0.2±0.11	n.d.	0.3±0.16
C 16:0	25.1±0.88	34.0±1.16	1.5±0.13	39.7±1.35
C 16:1n-7	0.4±0.17	0.6±0.42	0.1±0.04	0.6±0.42
C 18:0	35.5±1.03	54.3±1.47	2.0±0.38	53.0±1.43
C 18:1n-9	34.6±1.28	9.6±2.36	91.3±0.64	2.9±0.71
C 18:2n-6	2.3±0.42	1.0±0.57	4.9±0.48	0.9±0.51
C 18:3n-3	n.d.	n.d.	n.d.	n.d.
C 20:0	1.0±0.45	0.3±0.14	0.1±0.05	2.5±1.15
C 20:1n-9	n.d.	n.d.	n.d.	n.d.

n.d. = not detectable, <0.1%.

stereospecific analyses carried out on the CB and MAN fat samples.

Even if the total % mol FA compositions of the TAG fraction from CB and MAN fat show different values, especially for oleic, palmitic and linoleic acids, their 95%CB:5%MAN fat and 90%CB:10%MAN fat mixtures (data not shown) essentially behaved like CB.

Some important results were obtained when the intrapositional FA composition, % mol of the total TAG fraction was considered. In fact, some % molar values of palmitic and oleic acids in the TAG *sn*-1- and *sn*-3- positions for the CB:MAN fat mixtures considered (data not shown) were different in comparison with those

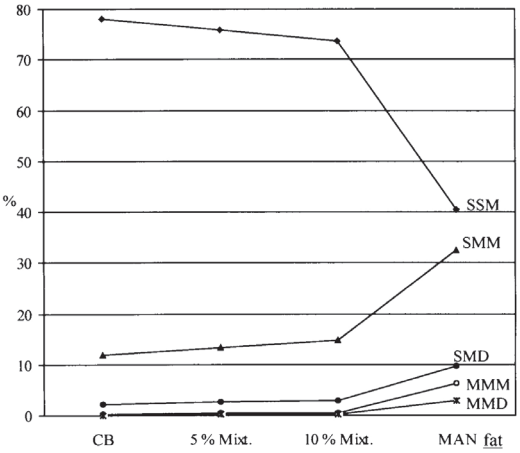

Fig. 1 - % content of some TAG iso-unsaturated fractions, mean values (n=6).

Table 3 - Intrapositional compositions, mean values % mol, of some iso-unsaturated TAG fractions separated by Ag^+-HPLC.

	SSM											
	CB			5% Mixt.			10% Mixt.			MAN fat		
FA	*sn*-1-	*sn*-2-	*sn*-3-	*sn*-1-	*sn*-2-	*sn*-3-	*sn*-1-	*sn*-2-	*sn*-3-	*sn*-1-	*sn*-2-	*sn*-3-
C 16:0	38.1		41.5	37.6		40.8	37.1		40.0	18.9		14.6
C 16:1n-7												
C 18:0	60.9		55.4	61.3		56.1	61.8		56.7	78.6	1.0	79.6
C 18:1n-9		99.3			99.3			99.3			99.0	
C 20:0			2.6			2.7			2.8	1.6		5.4
	SMM											
	CB			5% Mixt.			10% Mixt.			MAN fat		
FA	*sn*-1-	*sn*-2-	*sn*-3-	*sn*-1-	*sn*-2-	*sn*-3-	*sn*-1-	*sn*-2-	*sn*-3-	*sn*-1-	*sn*-2-	*sn*-3-
C 16:0	9.2		31.5	9.0		28.6	8.8		26.2	7.2		9.1
C 16:1n-7	4.4		4.2	4.0		3.6	3.6		3.2	1.0		
C 18:0	14.8		42.0	16.7		43.0	18.3		43.8	29.3		49.5
C 18:1n-9	71.4	99.8	20.1	70.1	99.8	22.3	68.9	99.8	24.3	60.4	99.8	37.8
C 20:0			2.0			2.2			2.3	1.0		3.3
C 20:1n-9										1.0		
	SMD											
	CB			5% Mixt.			10% Mixt.			MAN fat		
FA	*sn*-1-	*sn*-2-	*sn*-3-	*sn*-1-	*sn*-2-	*sn*-3-	*sn*-1-	*sn*-2-	*sn*-3-	*sn*-1-	*sn*-2-	*sn*-3-
C 16:0	15.1		25.1	13.9		21.9	13.0		19.5	8.7		7.9
C 16:1n-7	1.3		1.2	1.2		0.9	1.0		0.8			
C 18:0	24.1		33.6	26.3		35.4	28.0		36.7	36.1		43.1
C 18:1n-9	20.2	71.6	5.6	21.6	68.5	7.7	22.6	66.2	9.3	27.5	54.7	17.1
C 18:2n-6	39.1	28.1	32.8	36.7	31.3	32.0	34.9	33.6	31.5	26.1	45.1	28.8
C 20:0			1.6			1.9			2.0	0.7		2.9
	MMM											
	CB			5% Mixt.			10% Mixt.			MAN fat		
FA	*sn*-1-	*sn*-2-	*sn*-3-	*sn*-1-	*sn*-2-	*sn*-3-	*sn*-1-	*sn*-2-	*sn*-3-	*sn*-1-	*sn*-2-	*sn*-3-
C 16:1n-7	5.8		17.2	3.6		8.6	2.8		5.6	1.3		
C 18:1n-9	94.2	100.0	82.8	95.9	100.0	91.1	96.4	100.0	94.1	97.8	100.0	99.5
C 20:1n-9							0.8			0.9		
	MMD											
	CB			5% Mixt.			10% Mixt.			MAN fat		
FA	*sn*-1-	*sn*-2-	*sn*-3-	*sn*-1-	*sn*-2-	*sn*-3-	*sn*-1-	*sn*-2-	*sn*-3-	*sn*-1-	*sn*-2-	*sn*-3-
C 16:1n-7	4.5		6.0	2.9		2.9	1.9		1.7	1.0		
C 18:1n-9	72.2	86.5	30.8	72.1	79.0	42.8	72.8	76.1	47.0	72.6	72.3	53.2
C 18:2n-6	23.3	13.5	63.2	24.6	21.0	54.3	24.8	23.9	51.3	25.4	27.7	46.5
C 20:1n-9										0.8		

The % standard deviation values (n=6) were never over 7; some higher values refer to analytical results less than 5%.

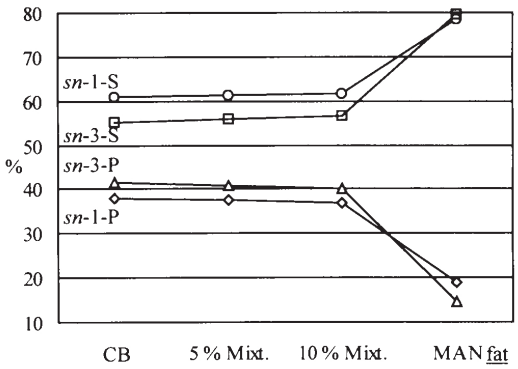

Fig. 2 - SSM fraction; trends of palmitic -P- and stearic -S- acids in *sn*-1- and *sn*-3- positions, mean values % mol (n=6).

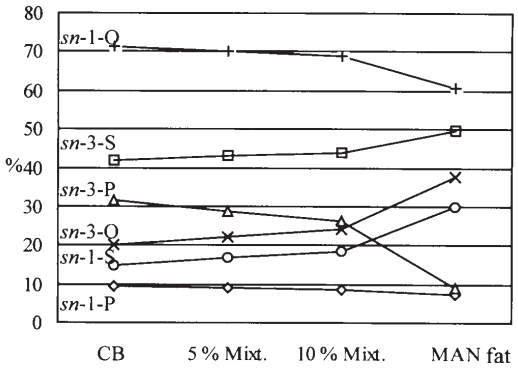

Fig. 3 - SMM fraction; trends of palmitic -P-, stearic -S- and oleic -O- acids in *sn*-1- and *sn*-3- positions, mean values % mol (n=6).

observed in the TAG of CB samples. For this reason, the intrapositional FA compositions were further investigated, in relation to total TAG and its Ag^+-HPLC fractions.

To this aim, a study was carried out to separate total TAG into fractions by Ag^+-HPLC according to the degree of unsaturation, as reported in other papers (DAMIANI *et al.*, 1997; NERI *et al.*, 1998, 1999). The % content of some iso-unsaturated sub-fractions (SSM, SMM, SMD, MMM and MMD, where S, M and D represent saturated, monounsaturated and diunsaturated acylic residues, respectively) are reported in Fig. 1. The reported values represent the mean values of six determinations; the % standard deviations were never more than 7, with the exception of some higher values for analytical results less than 5%.

Unlike the previous studies carried out on CB, some CBEs and relative mixtures (NERI *et al.*, 1998, 1999), in this study the completely saturated TAG fraction, SSS, was not considered because it is present in a very low % in MAN fat samples. In this study, other TAG fractions, such as the MMM and MMD, showed differences between the CB:MAN fat mixtures and the CB samples. The results of stereospecific analysis carried out on these TAG fractions are shown in Table 3, together with those relative to the SSM, SMM, SMD TAG fractions. The results that are most significant to the aim of this research are reported. The results relative to the *sn*-positional distribution of the most significant acids in SSM,

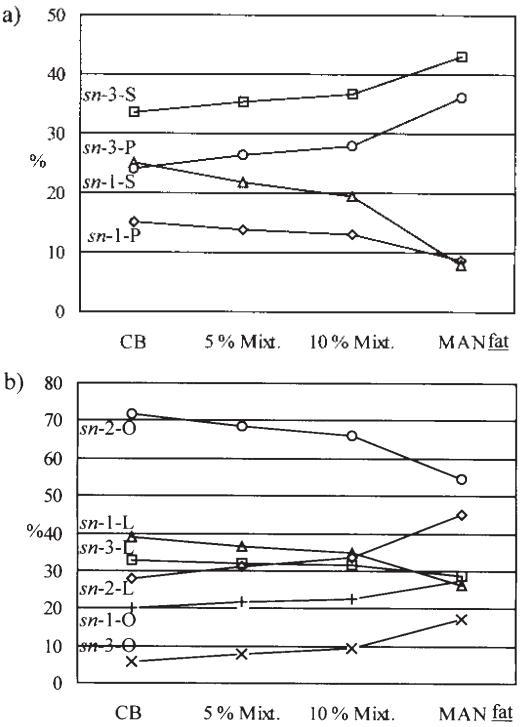

Fig. 4 - SMD fraction; trends of a) palmitic - P- and stearic -S- acids in *sn*-1- and *sn*-3- positions; b) oleic -O- and linoleic -L- acids in *sn*-1-, *sn*-2- and *sn*-3- positions, mean values % mol (n=6).

SMM, SMD, MMM and MMD are shown in the Figs. 2-6, respectively. The data represent the mean values of six determinations and the % standard deviation values were never more than 7; some higher values refer to analytical results less than 5%. Differences between CB and MAN fat samples can be observed in the intrapositional distributions of some FAs. With regard to the CB:MAN fat mixtures, the MMM and MMD TAG fractions were particularly interesting. The results for the MMM fraction showed differences in the in the *sn*-1- and *sn*-3 positions intrapositional composition of palmitoleic and oleic acids, while the MMD fraction showed differences in the *sn*-1- and *sn*-3 positions for palmitoleic acid and in the *sn*-2- and *sn*-3 positions for oleic and linoleic acids. These results show that the detailed analysis of TAG fraction is useful for characterising different lipid matrices and some differences can be observed also considering CB samples with only 5% of MAN fat added.

CONCLUSIONS

The reported analytical procedure can be used to obtain detailed results on the TAG fraction of MAN fat and CB and to characterise them. In particular, the results of stereospecific analysis of TAG show the intrapositional FA compositions. The complete TAG molecular species profile can be obtained from these data and thus all the possible isomers, positional and enantiomeric, can be evaluated. Further details can be obtained if the stereospecific analysis is carried out on TAG iso-unsaturated fractions, previously separated by Ag^{+}-HPLC analysis. In fact when MAN fat, CB and their mixtures are considered, the FA distribution in the three *sn*- positions of some TAG iso-unsaturated fractions presents some differences showing that these data have some interesting potential in characterization and differentiation of lipid matrices.

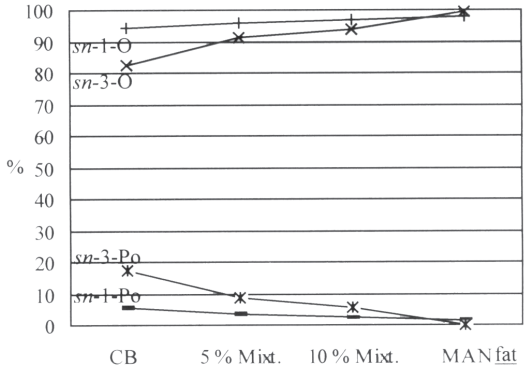

Fig. 5 - MMM fraction; trends of palmitoleic -Po- and oleic -O- acids in *sn*-1- and *sn*-3- positions, mean values % mol (n=6).

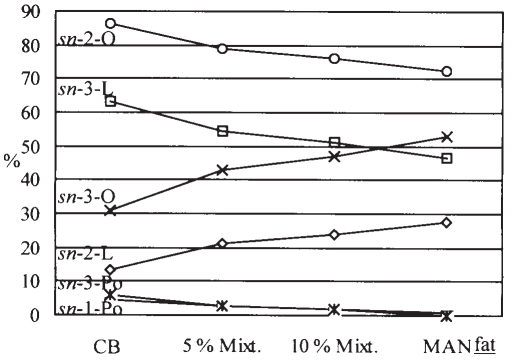

Fig. 6 - MMD fraction; trends of palmitoleic acid -Po- in *sn*-1- and *sn*-3- positions, oleic -O- and linolenic -L- acids in *sn*-2- and *sn*-3- positions, mean values % mol (n=6).

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SHORT COMMUNICATION

VARIATION OF FAT AND FATTY ACID COMPOSITION OF SOME PISTACHIO GENOTYPES

VARIABILITÀ DEL CONTENUTO IN GRASSI ED IN ACIDI GRASSI IN ALCUNI GENOTIPI DI PISTACCHIO

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ABSTRACT

The aim of the study was to determine the fatty acid composition of some selected pistachio types, two domestic pistachio cultivars (Uzun and Siirt), four Iranian cultivars (Ohadi, Vahidi, Mumtaz and H. Serifi) and an American cultivar (Kerman). The fat content of pistachio seeds ranged from 35.38% (Mumtaz) to 51.68% (Sel-5). The fatty acid composition was determined after harvesting. The fatty acid composition of the pistachio genotypes ranged from 7.45-10.14% saturated fatty ac-

RIASSUNTO

Lo scopo dello studio è stato quello di determinare la composizione in acidi grassi di alcuni tipi selezionati di pistacchio, due cultivar locali (Uzun e Siirt), quattro cultivar iraniane (Ohadi, Vahidi, Mumtaz e H. Serifi) e una cultivar americana (Kerman). Il contenuto in grassi dei semi di pistacchio è variato tra il 35,38% (Mumtaz) ed il 51,68% (Sel-5). La composizione in acidi grassi è stata determinata dopo la raccolta. La composizione in acidi grassi dei genotipi di pistacchio è va-

- Key words: fat content, fatty acid composition, pistachio, *Pistacia vera* L. -

ids (SFAs), 55.23-77.61% monounsaturated fatty acids (MUFAs) and 13.82-33.11% polyunsaturated fatty acids (PUFAs). The unsaturated/saturated fatty acid ratio varied between 8.74 and 12.34.

riata tra il 7,45-10,14% di acidi grassi saturi (SFA), il 55,23-77,61% di acidi grassi monoinsaturi (MUFA) ed il 13,82-33,11% di acidi grassi polinsaturi (PUFA). Il rapporto acidi grassi insaturi/saturi è variato tra 8,74 e 12,34.

INTRODUCTION

The genus *Pistacia* is a member of the *Anacardiaceae* family and consists of at least eleven species (ZOHARY, 1952) including *Pistacia vera* L., the cultivated pistachio that has edible nuts and is of considerable economical importance. Pistachio trees have been grown in south Anatolia since the 17th century B.C. (OZBEK, 1978). Turkey has a large population of wild pistachio and several pistachio cultivars because it is the genetic centre of pistachio. Iran, the United States, Turkey and Syria are the main pistachio producers in the world, making up over 90% of the world production (FAO, 2006).

Pistachio has a high nutritional value due to its high protein, vitamin, mineral and unsaturated fat content. The fruit can be consumed as salted or roasted appetizers and are widely used in the food industry, particularly for creamcakes, desserts, candies, chocolate and ice cream (OKAY, 2002). Recent studies on the mineral, vitamin, protein, fat and fatty acid contents show the nutritional value of pistachio. Pistachio seeds have a low carbohydrate content, approximately 10% (BARGHCHI and ALDERSON, 1989), a protein content of more than 20% (GARCIA *et al.,* 1992; AGAR *et al.* 1995; KUCUKONER and YURT, 2003) and the lipid content varies from 40 to 63% (GARCIA *et al.,* 1992; AGAR *et al.,* 1995, 1997; YILDIZ *et al.*,1998; KUCUKONER and YURT 2003; SATIL *et al.,* 2003), all on a dry weight basis. The nuts contain about 50% oil, with oleic acid being the dominant glyceride, followed by linoleic and palmitic acids (AGAR *et al.,* 1995).

Unsaturated fatty acids are known to have positive effects on human health; they prevent the accumulation of cholesterol and arteriosclerosis (ERSOY and BAYSU, 1986; MARTIN *et al.,* 1986). The fatty acid composition of pistachio oil in Turkish varieties has been found to vary (AGAR *et al.,* 1995; GARCIA *et al.,* 1992; SATIL *et al.,* 2003).

The aim of this study was to characterize the fat and fatty acid composition of some selected pistachio types and compare the results to some domestic and foreign pistachio cultivars.

MATERIALS AND METHODS

This study was carried out on 14 pistachio genotypes, including 7 standard cultivars and 7 selected types, grown at the Tektek Research Station of Soil and Water Resources Research Institute, located in the Sanliurfa province of Turkey (lat 37° 07' N., long 39° 15' E., altitude 530 m a.s.l.). Two standard domestic pistachio cultivars 'Uzun' and 'Siirt', and four Iranian cultivars, 'Ohadi', 'Vahidi', 'Mumtaz' and 'H. Serifi' and an American cultivar 'Kerman' were studied as well as 7 pistachio types selected from Turkey 'Sel-1 named as Tekin', 'Sel-2', 'Sel-5', 'Sel-10', 'Sel-11 named as Barak Yildizi', 'Sel-14' and 'Sel-15'. Fruit

samples of these cultivars were collected from pistachio trees grafted on *Pistacia khinjuk* rootstock in an irrigated experimental orchard.

Sampling

Fruit samples were collected at the same age from trees with similar vigour within the same orchard. Fruit of three trees of each genotype were picked at the ripe stage. The fruit was analysed after the pistachio fruit had been dried at 65°C for 48 h. All samplings were used to determine the fat and fatty acid compositions.

FAME analyses

Lipid extraction was carried out according to BLIGH and DYER (1959). Fatty acid profiles of fat extracted from fruit samples were determined by gas chromatography (GC) of methyl esters. Boron trifluoride/methanol was used to prepare the fatty acid methyl esters (AOAC, 1990).

Twenty-five mg of oil were extracted from samples, placed in a screw cap tube and 1.5 mL of 0.5 M methanolic sodium hydroxide, were added, mixed and heated at 100°C for 7 min. After cooling, 2 mL of boron trifluoride were added and heated again at 100°C for 5 min. The tube was then cooled to 30°-40°C, 1 mL of isooctane was added. The tube was capped and shaken using a whirl mix for 30 s. Five mL of saturated sodium chloride solution was immediately added and the tube was shaken again. The contents were allowed to separate and the top (iso-octane-containing fatty acid methyl esters) layer was removed and the lower layer was extracted again with an additional 1 mL of iso-octane. The two iso-octane extracts were combined and dried over anhydrous sodium sulphate and concentrated to approximately 1 mL with a stream of nitrogen.

Gas chromatographic condition

The fatty acid composition was analysed on a Clarus 500 gas chromatograph with autosampler (Perkin Elmer, Shelton, CT, USA) equipped with a flame ionisation detector and a fused silica capillary SGE column (30 m x 0.32 mm, ID x 0.25 μm, BP20 0.25 UM; SGE Analytical Science Pty. Ltd., Victoria, Australia). The oven temperature was 140°C, held for 5 min, raised to 200°C at a rate of 4°C/min and to 220°C at a rate of 1°C/min, while the injector and the detector temperature were set at 220°C and 280°C, respectively. The sample size was 1 μL and the carrier gas was controlled at 16 ps. The split used was 1:100. Fatty acids were identified by comparing the retention times of FAME with a standard 37 component FAME mixture (Supelco, Catalog No. 18919, Sigma-Aldrich Chemie GmbH, Münich, Germany). Triplicate GC analyses were performed and the results are expressed in % GC area as a mean value ± standard deviation.

Statistical analysis

The data were tested for statistical significance using the ANOVA (analysis of variance) test from the statistical package MSTAT (Michigan State University, Lansing), and differences between means were separated with the Duncan's multiple range test ($p \leq 0.05$).

RESULTS AND CONCLUSIONS

The total fat ratio of pistachio cultivars ranged from 35.38 to 51.68%; the highest value was recorded for 'Sel-5' (51.68%) followed by 'Vahidi' (50.56%) and the lowest values were found in 'Mumtaz' and 'Barak Yildizi' cultivars (35.38 and 38.83%, respectively, Table 1). In general, the total lipid values were lower than those reported in previous studies. OKAY (2002) reported that the total fat ratio of

Table 1 - Fat and saturated fatty acid content (%) of some pistachio cultivars and selected types.

Cultivars and Selected Types	Fat (%)	Saturated fatty acids (%)					
		Myristic C14:0	Palmitic C16:0	Margaric C17:0	Stearic C18:0	Arachidic C20:0	Behenic C22:0
Uzun	41.74±3.45 cd*	0.10±0.01 ab	8.53±0.10 b	0.03±0.00 bc	0.00±0.00 b	0.14±0.00 a	0.02±0.00 a
Siirt	42.11±4.46 cd	0.08±0.02 cd	7.23±0.13 f	0.00±0.00 e	0.76±0.00 a	0.10±0.02 e	0.00±0.00 c
Ohadi	42.12±3.49 cd	0.08±0.00 cd	8.56±0.02 b	0.03±0.01 bc	0.76±0.01 a	0.08±0.00 f	0.00±0.00 c
Vahidi	50.56±0.37 ab	0.07±0.01 de	8.96±0.09 a	0.03±0.00 bc	0.36±0.25 ab	0.08±0.00 f	0.00±0.00 c
Mumtaz	35.38±10.61 e	0.08±0.00 cd	8.55±0.15 b	0.02±0.00 cd	0.00±0.00 b	0.12±0.00 c	0.00±0.00 c
H. Serifi	45.59±3.67 ac	0.09±0.01 bc	9.15±0.04 a	0.02±0.01 cd	0.00±0.00 b	0.00±0.00 h	0.01±0.00 b
Kerman	44.27±0.40 bd	0.07±0.00 de	7.80±0.02 ce	0.03±0.00 bc	0.52±0.00 ab	0.10±0.00 e	0.00±0.00 c
Tekin	41.63±9.62 cd	0.07±0.00 de	7.60±0.06 e	0.52±0.01 a	0.00±0.00 b	0.14±0.01 a	0.00±0.00 c
Sel-2	45.80±0.25 ac	0.07±0.00 de	7.74±0.03 de	0.04±0.01 b	0.00±0.00 b	0.13±0.00 b	0.00±0.00 c
Sel-5	51.68±2.64 a	0.06±0.00 e	7.26±0.12 f	0.03±0.01 bc	0.00±0.00 b	0.10±0.01 e	0.00±0.00 c
Sel-10	46.61±1.03 ac	0.11±0.02 a	9.08±0.20 a	0.04±0.01 b	0.91±0.00 a	0.00±0.00 h	0.00±0.00 c
Barak Yildizi	38.83±5.52 de	0.09±0.01 bc	8.03±0.60 cd	0.03±0.00 bc	0.64±0.00 a	0.11±0.02 d	0.00±0.00 c
Sel-14	43.28±1.89 cd	0.08±0.01 cd	8.64±0.01 b	0.01±0.00 de	0.70±0.03 a	0.07±0.01 g	0.00±0.00 c
Sel-15	45.70±1.85 ac	0.09±0.01 bc	8.09±0.71 c	0.03±0.00 bc	0.71±0.50 a	0.00±0.00 h	0.01±0.00 b

*The letters following the numbers indicate different groups determined by Duncan's test ($p \leq 0.05$).

pistachio cultivars ranged from 55.9 to 59.7%, with the highest values found in 'Ohadi' (59.7%), followed by 'Kirmizi' (58.7%), 'Uzun' (56.4%), 'Halebi' (56.1%) and 'Siirt' (55.8%). ACAR (2004) reported that the fat ratio of 'Kirmizi', 'Siirt' and 'Ohadi' cultivars were 43.51, 44.16 and 48.00%, respectively. The fat ratio of 'Badami', 'Ohadi' and 'Mumtaz' cultivars ranged from 55.2 to 60.5% (KAMANGAR *et al.,* 1975). On the other hand, GARCIA *et al.* (1992) reported that the fat ratios of 'Mumtaz' 'Siirt' and 'Ohadi' were 67.2, 54.2 and 56.2%, respectively, while KAFKAS *et al.* (1995) reported values of 60.61, 51.77 and 54.70%, respectively. Differences in the fat content of pistachio cultivars may be due to differences in factors such as growing conditions, crop or season (OKAY, 2002).

The fatty acid content of the experimental genotypes and their ratios are reported in Tables 1 and 2, as well as the percentage of the mean value of 12 fatty acids for each pistachio genotype. Oleic (C18:1n9), linoleic (C18:2n6), palmitic (C16:0), stearic (C18:0), gadoleic (C20:1) and linolenic (C18:3n3) acids were the most abundant fatty acids.

The predominant saturated fatty acid in the cultivars was palmitic acid (7.23-9.15%), followed by stearic (0.00-0.91%), arachidic (0.00-0.14%) and myristic (0.06-0.11%) acids. Trace levels of margaric acid were found in the cvs except in 'Tekin' (0.52%), and behenic acid was only found in 'Uzun', 'H. Serifi' and 'Sel-15' (Table 1). The highest palmitic acid content was found in 'H. Serifi' (9.15%), followed by 'Sel-10' (9.08%), 'Vahidi' (8.96%). 'Siirt' and 'Sel-5' had the lowest palmitic acid ratio (7.23 and 7.26%, respectively). The highest stearic acid ratio was determined in 'Sel-10' as 0.91% with a stearic acid content of 0.00% in 'Uzun', 'Mumtaz', 'H. Serifi' and 3 selected types. The other saturated fatty acid values were low in all cultivars and selected types.

Unsaturated fatty acids were designated as monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids. Oleic acid (C18:1n9) was the main monounsaturated fatty acid in all pistachio gen-

otypes' seeds (Table 2). The oleic acid level was the highest in 'Siirt' (76.85%) and lowest in 'Ohadi' (54.75%). Oleic acid ratios were over 70% in 4 selected types ('Tekin', 'Sel-2', 'Sel-5' and 'Sel-10') and 2 domestic cultivars ('Uzun' and 'Siirt'). The palmitoleic acid content of the cultivars and selected types varied from 0.06 to 0.41%; 'Siirt' had the highest value, while 'Uzun', 'Tekin' and 'Sel-2' had the lowest. Linoleic acid (C18:2n6) had the highest percentage among the PUFAs and ranged from 13.60 to 32.75% for the cultivars and selected types, whereas the linolenic acid content was low (0.02-0.37%). The cultivars with the highest levels of linoleic acid were 'Ohadi' (32.75%) and 'Sel-14' (29.32%); those with the lowest levels were 'Siirt' (13.60%), 'Tekin' (15.35%) and 'Sel-10' (15.53%). Similarly, the cultivars with the highest linolenic acid levels were 'Vahidi' (0.37%), 'Ohadi', 'H. Serifi' and 'Sel-14' (0.36%); the lowest level was found in 'Tekin' (0.02%).

The results obtained in this study are in agreement with those reported in the literature; palmitic acid was the primary saturated fatty acid in pistachio cultivars and selected types, while palmitoleic acid was only found in trace amounts. Oleic acid was the most common monounsaturated fatty acid, while linoleic acid was the most common polyunsaturated fatty acid (SHOKRAII, 1977; GARCIA *et al.*, 1992; KAFKAS *et al.*, 1995; OKAY, 2002). Myristic acid in the pistachio cultivars was reported by OKAY (2002). ARENA *et al.* (2007) reported that Turkish and Iranish pistachio samples are completely different from Italian and Greek samples with respect to the distribution of fatty acids and sterols. The composition of pistachio oil could therefore be used as a marker to distinguish the geographic origin of the seed.

The total amount of saturated and unsaturated fatty acids and their ratios (unsaturated/saturated) are listed in Table 3. The fatty acid composition of pistachio genotypes led to the following re-

Table 2 - Unsaturated fatty acid content (%) of some pistachio cultivars and selected types.

Cultivars and Selected Types	Unsaturated fatty acids (%)					
	Monounsaturated fatty acids				Polyunsaturated fatty acids	
	Palmitoleic C16:1	Margaroleic C17:1	Oleic C18:1n9	Gadoleic C20:1	Linoleic C18:2n6	Linolenic C18:3n3
Uzun	0.06±0.00 b*	0.04±0.00 b	73.60±0.31 c	0.38±0.01 bc	16.22±0.23 ef	0.29±0.00 abc
Siirt	0.41±0.04 a	0.04±0.01 b	76.85±0.81 a	0.31±0.04 de	13.60±0.00 g	0.22±0.01 abc
Ohadi	0.07±0.00 b	0.05±0.00 ab	54.75±1.96 j	0.36±0.00 cd	32.75±0.16 a	0.36±0.00 ab
Vahidi	0.07±0.01 b	0.06±0.00 a	62.44±0.41 h	0.30±0.00 e	26.37±0.07 c	0.37±0.00 a
Mumtaz	0.09±0.02 b	0.04±0.01 b	67.18±0.45 f	0.39±0.02 bc	22.51±0.54 d	0.17±0.22 bcd
H. Serifi	0.40±0.45 a	0.04±0.01 b	62.95±0.54 gh	0.32±0.00 de	26.13±0.35 c	0.36±0.00 ab
Kerman	0.07±0.00 b	0.05±0.00 ab	63.72±0.45 g	0.45±0.01 a	26.22±0.26 c	0.19±0.25 a-d
Tekin	0.06±0.00 b	0.05±0.00 ab	75.41±0.03 b	0.45±0.01 a	15.35±0.02 f	0.02±0.00 d
Sel-2	0.06±0.00 b	0.05±0.00 ab	73.97±0.06 c	0.43±0.00 ab	16.72±0.07 e	0.15±0.18 cd
Sel-5	0.07±0.00 b	0.04±0.00 b	74.53±0.08 bc	0.38±0.01 bc	16.81±0.25 e	0.14±0.18 cd
Sel-10	0.07±0.01 b	0.05±0.01 ab	72.66±1.86 d	0.18±0.00 f	15.53±0.19 f	0.14±0.00 cd
Barak Yildizi	0.07±0.00 b	0.05±0.00 ab	68.19±0.78 e	0.40±0.04 abc	21.47±2.38 d	0.17±0.23 bcd
Sel-14	0.07±0.01 b	0.05±0.00 ab	59.65±0.08 i	0.32±0.01 de	29.32±0.05 b	0.36±0.00 ab
Sel-15	0.07±0.01 b	0.05±0.01 ab	67.44±0.35 ef	0.41±0.08 abc	22.09±2.13 d	0.18±0.23 a-d

*The letters following the numbers indicate different groups determined by Duncan's test ($p \leq 0.05$).

Table 3 - Total saturated (%) and unsaturated fatty acid content (%) of some pistachio cultivars and selected types.

Cultivars and Selected Types	Saturated fatty acids (SFA)	Unsaturated fatty acids (USFA)			Unsaturated/ Saturated
		Monounsaturated fatty acids (MUFA)	Polyunsaturated fatty acids (PUFA)	Total Unsaturated fatty acids (USFA)	
Uzun	8.82 bd*	74.08 d	16.51 fg	90.59 bc	10.27
Siirt	8.17 de	77.61 a	13.82 i	91.43 ab	11.19
Ohadi	9.51 ab	55.23 k	33.11 a	88.34 e	9.29
Vahidi	9.50 ab	62.87 i	26.74 c	89.61 cd	9.43
Mumtaz	8.77 bd	67.70 g	22.68 d	90.38 bc	10.31
H.Serifi	9.27 ac	63.71 hi	26.49 c	90.20 bc	9.73
Kerman	8.52 cd	64.29 h	26.41 c	90.70 bc	10.65
Tekin	8.33 ce	75.97 b	15.37 h	91.34 ab	10.97
Sel-2	7.98 de	74.51 cd	16.87 f	91.38 ab	11.45
Sel-5	7.45 e	75.02 c	16.95 f	91.97 a	12.34
Sel-10	10.14 a	72.96 e	15.67 gh	88.63 de	8.74
Barak Yildizi	8.90 bd	68.71 f	21.64 e	90.35 bc	10.15
Sel-14	9.50 ab	60.09 j	29.68 b	89.77 cd	9.45
Sel-15	8.93 bd	67.97 fg	22.27 de	90.24 bc	10.11

*The letters following the numbers indicate different groups determined by Duncan's test ($p \leq 0.05$).

sults: saturated fatty acids (SFAs) ranged in the interval of 7.45-10.14%; monounsaturated fatty acids (MUFAs) ranged in the interval of 55.23-77.61%; polyunsaturated fatty acids (PUFAs) ranged in the interval of 13.82-33.11%.

The unsaturated/saturated fatty acids ratios varied between 8.74 and 12.34, and these results are in agreement with previous studies (AGAR *et al.,* 1995, 1997; KAFKAS *et al.,* 1995; OKAY, 2002; SEFEROGLU *et al.,* 2006). It was also observed that the proportion of these fatty acids changed significantly among the genotypes. According to some researchers, the fatty acid composition of nuts can be affected by ecological conditions, variety, location, geographical origin, and technical and cultural practices (PARCERISA *et al.,* 1993; KOYUNCU *et al.,* 1997; BALTA *et al.,* 2006). In the present study, the fatty acid profile of pistachio nuts was generally dominated by MUFAs and PUFAs (Table 3).

Oleic and linoleic acid, the two major fatty acids in pistachio, are of interest due to their beneficial effects on human health. Consumers are especially interested in unsaturated FAs, because PUFAs are a natural preventive of cardiovascular disease (MELGAREJO and ARTES, 2000). Recently, it has been reported that MUFAs were as effective as PUFAs in reducting low-density-lipoprotein cholesterol in humans (MENSINK and KATAN, 1989). In this study, five potential health-promoting cultivars are identified: 'Ohadi', has the highest linoleic acid content, and 'Sel-5', 'Siirt', 'Sel-2' and 'Tekin', with the most USFAs. On the other hand, 'Sel-5' is potentially the most beneficial to human health because it has the lowest SFA value.

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MYCOTOXINS IN FEEDSTUFFS

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The term mycotoxin literally means poison from fungi. Mycotoxins are substances produced from fungal secondary metabolic processes, which impair animal health thereby causing great economic losses of livestock through disease. Feedstuff is any of the constituent nutrients of an animal ration. The plants used in feed, such as grains, oil seeds, nuts and root crops, are susceptible to mycotoxin contamination. An invaluable reference, *Mycotoxins in Feedstuffs* provides an overview of mycotoxins in feedstuffs and ingredients. It discusses whether a feedstuff ingredient is predisposed for a mycotoxin contamination and lists the degree of contamination, the concentration of the toxins, and the country of origin and/or detection of the contaminated food.

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