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PAPER

THE ROLE OF PROTECTED DESIGNATION OF ORIGIN IN CONSUMER PREFERENCE FOR IBERIAN DRY-CURED HAM IN SPAIN

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ABSTRACT

This paper analyses the importance of Protected Designation of Origin (PDO) in shaping consumer preferences for Iberian dry-cured ham, one of the most typical and appreciated meat products in Spain. Conjoint Analysis was used to estimate the importance of selected attributes affecting consumer preferences for this product. Results showed that consumers granted the highest importance to price and type of ham, although PDO was of some value for all consumers. A segmentation with PDO-related variables produced three clusters (pro-PDO consumers, average consumers, and price-sensitive consumers). These clusters were later used to determine the premium that consumers were willing to pay for the PDO attribute, which was then compared to the extra costs of producing ham within a PDO scheme.

- Key words: Conjoint Analysis, Iberian ham, preferences, Protected Designation of Origin, Segmentation -

INTRODUCTION

Protected Designation of Origin (PDO) is one of the most important programmes designed by the European Union to promote and protect the names of regional foods. These programmes encourage varied agricultural production, protect the trademark of the PDOs and help consumers by giving them information concerning specific product characteristics. Specifically, PDO covers agricultural products and foodstuffs which are produced, processed and prepared in a given geographical area. Such is the case of the Iberian dry-cured ham, one of the most typical and appreciated meat products in Spain.

Spain is the world's largest producer and consumer of dry-cured ham; more than 36 million hams were produced in 2004 (CRUZ, 2005), with a yearly consumption of 4.6 kg per capita (MAPA, 2007). In Spain, there are two main types of dry-cured ham: Serrano ham and Iberian ham. The first is produced from white pigs (a term which refers to all the improved pig breeds such as Large white or Landrace) that are usually fed an intensive cereal-based diet. Iberian ham is produced from an indigenous breed of pig (Iberian pig), native to the southwest part of the Iberian Peninsula. Iberian pigs have adapted over generations to the harsh environmental conditions of the Spanish Mediterranean woods (*dehesas*), where they graze freely on pastures and mainly eat acorns.

Iberian pigs can be classified into three quality categories depending on the amount of acorns they are fed during their fattening stage: "*Bellota*" or "Mast-fed" (Iberian pigs reared extensively in the *dehesas*, where they mainly eat acorns and pasture); "*Recebo*" (Iberian pigs reared extensively for some time, although fed with fodder in their last fattening stage), and "*Cebo*" (Iberian pigs reared in pig sheds, with an intensive production system and only fed fodder). Both "*Recebo*" and "*Cebo*" Iberian hams are known on the market indistinctly as Iberian ham or Iberian fodder-fed ham, to differentiate them from the higher quality Iberian mast-fed ham.

Iberian hams have a very limited market, and their consumers, considered as an elitist and demanding niche, are looking for quality and are ready to pay higher prices. In fact, most of the cured hams sold on the Spanish market are *Serrano* hams (89%), which are a cheaper product and more appropriate for mass consumption. For the remaining 11% of the market, which is Iberian, only 6% is certified under any of the existing PDOs.

In general, there are no relevant differences between PDO and non-PDO products within the supply chain until the distribution stage. The only difference lies in the last stage, retailing, since non-PDO hams can usually be found in supermarkets and hypermarkets, whereas PDO hams are frequently sold by small retailers, such as specialized delicatessen shops.

Although greatly appreciated on the market, the Iberian (mast-fed and fodder-fed) hams are facing serious challenges: the producer sector is highly fragmented, and the market is complex and opaque, with a great diversity of products (depending on the production system, the degree of purity used in the Iberian breed, the different types of feeding, etc.), which sometimes result in consumer distrust and lack of loyalty towards the products.

These drawbacks have to be analyzed taking into consideration consumer perception of quality, which is considered a multifaceted concept related to the requirements necessary to satisfy consumer needs and expectations (MOSKOWITZ, 1995; PERI, 2006). ESPEJEL *et al.* (2007) stated that consumers evaluate food quality based on its intrinsic attributes (aroma, flavour, smell…) and extrinsic attributes (brand name, price, country of origin…). As mentioned above, for a varied food product such as the Iberian ham, it is especially significant to be able to provide the consumer with a guarantee about the extrinsic and intrinsic parameters of quality, since consumers may find these aspects difficult to fully understand and check by themselves.

Based on this duality of factors, PDO certification may raise the quality perception of consumers both intrinsically and extrinsically (ESPEJEL *et al.*, 2007). Specifically, a PDO enhances the intrinsic attributes of the product as a result of performing stricter quality controls on ingredients, processing methods and product guarantees. In addition, the perceived quality of extrinsic attributes is also improved under a PDO scheme, since brand image is reinforced by its association with the generic PDO brand (BONETTI, 2004; FANDOS and FLAVIÁN, 2006). With respect to Iberian ham, PDO would provide consumers with certified information about origin, breed and feeding of the pigs, together with a guarantee that the ham was produced following the traditional methods of the region.

Although Iberian ham has been widely studied, researchers have mainly focused on the manufacturing technology and improvements in the production processes (CAVA *et al.,* 2000; ANDRÉS *et al.,* 2001) and on sensory assessment using trained panelists (ANDRÉS *et al.,* 2004; RUIZ *et al.,* 1998). There is a shortage of studies dealing with consumer preferences towards dry-cured ham (including Iberian) in different contexts. Some related literature can be found in studies by CANNATA *et al.* (2010), who analyzed the preferences for dry-cured hams in Italy and France, but mainly considered sensory attributes, by CILLA *et al.* (2006), MORALES *et al.* (2008) and RESANO *et al.* (2009), who used both Serrano and Iberian dry-cured hams in their studies, which were focused on consumer beliefs, attitudes and acceptability towards

these products, and by MESÍAS *et al.* (1997), who studied retailer preferences for Iberian and Serrano hams.

From the above, it is clear that researching into consumer preferences towards Iberian hams is of great interest, especially when considering the role Protected Designation of Origin plays in shaping these preferences. It is obvious that issues such as price, product origin, animal welfare, rearing type, quality labelling, and even cultural and social factors, can significantly modify the purchase decision. It is therefore necessary to know the importance granted by the consumers to those attributes, using appropriate research tools, such as Conjoint Analysis.

The aim of the present work was to determine, within the structure of consumer preferences for Iberian ham, the role of the attribute Protected Designation of Origin, its relative importance within the framework of preferences and the monetary value for different types of consumers of the presence/absence of this attribute.

MATERIALS AND METHODS

Data collection

The data were obtained by personal interviews of a representative sample of regular consumers of Iberian ham in the southwestern Spanish region of Extremadura. This region can be considered a typical Spanish region for the purposes of this study since it is the most important region regarding consumption of Iberian meat products in Spain (MIMARM, 2009), thus ensuring that the consumers have a good level of knowledge of Iberian ham, the types and characteristics. Furthermore, there is a good range of Iberian ham of different qualities and PDOs, together with quality Iberian ham from the region, itself, available in local shops.

Respondents had to be primarily responsible for food shopping within the household. The survey was carried out between April and May 2006. The design was a random stratified sampling weighted in proportion to the population of each town. In every town[1], households were selected through a random computerized procedure, in which each household had the same probability of being selected. In case of refusal, replacement households were drawn and interviewed. Although 480 questionnaires were handed out, 63 had to be discarded, mainly for incomplete response. The maximum error was 4.89% for a 95% confidence level (k=2).

Conjoint Analysis

Conjoint Analysis (CA) is a multivariate research technique developed within the field of psychology (LUCE and TUKEY, 1964) and widely used in the agri-food sector, as can be seen in some publications dealing with the analysis of preferences for eggs (NESS and GERHARDY, 1994), wine (MARTÍNEZ-CARRASCO *et al.*, 2006) or honey (MURPHY *et al.*, 2000).

CA starts from the assumption that purchasing behaviour can be interpreted as a choice among different products or brands which possess a set of differentiating attributes or characteristics (VARELA *et al.*, 1997). According to NESS and GERHARDY (1994), the main assumptions in the CA model are: i) alternative products can be defined by a series of specific levels of a common set of attributes; and ii) a product's total utility to a consumer is given by the partial utilities (part-worths) of each attribute level.

Two important aspects of the model that need to be specified prior to carrying out the estimation are the choice of the composition rule and the relationship between the utilities. The composition rule adopted in this study is additive; according to HAIR *et al.* (1999) it is the one that takes into account most (80 or 90%) of the variation of the preference in almost all cases and is thus sufficient for most applications.

Analytically, the additive composition rule is the following:

$$Y = \sum_{i=1}^{n} X_{ij} \sum_{j=1}^{m} V_{ij}$$

where Y is the total utility, Vij is the utility associated with level j (j=1,2...,m) of attribute i (i=1,2...,n) and Xij is a dummy variable that takes the value of 1 (or 0) in the case of presence (or absence) of the jth level of the ith attribute.

Concerning the relationship between the utilities, a linear-less model was used for the attribute price, as, in general terms, higher prices correspond to lower utilities or preferences (GIL and SÁNCHEZ, 1997; KUPIEC and REVELL, 2001; SOUZA and VENTURA, 2001). For the rest of the attributes (qualitative) a part-worth model was used, which implies no defined relationship between the different levels of the attributes and their part-worths. This model is the most general and flexible and is frequently used for qualitative attributes (HAIR *et al.*, 1999; GREEN and SRINIVASAN, 1978).

The following step in the research design concerns the identification of appropriate attributes and, subsequently, the specification of feasible attribute levels (HAIR *et al.*, 1999). These attributes must reflect the key characteristics that consumers use to evaluate products (BRETTON-CLARK, 1987). All attributes that potentially create or detract from the overall utility of the product or service should be included, and the objective would be to include the attributes that differentiate most between the products (HAIR *et al.*, 1999).

After a previous review of studies on consumer preferences for ham and meat prod-

ucts, the research team selected a list of attributes[2], which were subjected to a double evaluation. First, they were presented to a panel of 25 consumers, who were asked to rate them according to their importance in the purchasing decision, and to indicate the levels that they considered feasible. Finally, the most highly valued attributes and their levels were presented to a panel of dry-cured ham experts[3], who chose those they considered most influential in the Iberian ham purchasing decisions. They also selected the definitive levels. The attributes and levels finally included in the study were: type of ham (Iberian Mast-Fed Ham, Iberian Ham – fed with fodder –, Serrano Ham), Protected Designation of Origin (with or without), price (6, 16, 26 or 36 €/kg), and purchasing format (over the counter, sliced and packed, whole ham). The relatively small number of attributes selected led to the decision to use the traditional additive conjoint methodology.

It has been mentioned by several authors that it is important to attempt to balance or equalize the number of levels across factors, due to the fact that the estimated relative importance of a variable increases as the number of levels increases, even if the end points stay the same (GREEN and SRINIVASAN, 1990; HAIR *et al.*, 1999). Nevertheless, it is common to find conjoint studies with a similar range in the number of levels to the one used in this work (STEENKAMP, 1987; RUIZ DE MAYA and MUNUERA, 1993; BEHE *et al.*, 1999; COX *et al.*, 2008).

In spite of discrepancies about the usage of price as an attribute (STEENKAMP, 1987; TULL and HAWKINS, 1990; HAIR *et al.*, 1999), it was decided to keep price as one of the attributes in this study. Since a wide range of prices was found on the ham market, it was assumed that some consumers would select the type of ham mostly based on the price.

Once the factors and their levels were selected, they were combined to create hypothetical products that would be presented to the consumers for evaluation. In this case, and using a fractional factorial design, ten profiles were produced (Table 1). Then, by using the full-profile method[4], written descriptions of all the different products were handed to the respondents, so that they could be rated from 1 to 9, where 1 was the lowest preference and 9 was the highest.

Cluster analysis

A cluster analysis was conducted to identify segments of consumers with respect to their behaviour towards PDO. A two-step cluster procedure (SPSS, 2003) was performed by using the part-worths of the PDO attribute, together with other variables dealing with consumers' knowledge about PDO, appreciation of the PDO attribute and willingness to pay for this attribute. This procedure was selected since it is able to handle both continuous and categorical variables. This algorithm also determines the appropriate number of clusters, generating a three-cluster solution in this study.

RESULTS

Part-worths and relative importance of the attributes

The model was estimated using ordinary least squares regression analysis, and the relative importance of the attributes, as well as the part-worth of each level of the attributes was established. To test the accuracy of the model, the Pearson correlation coefficient was calculated between the original ratings given by the respondents and those determined by the model. The high value of this coefficient (0.933) indicates that the model provides good prediction of the consumer preferences.

Table 2 shows the relative importance of the attributes and part-worths for the overall sample and the three clusters.

Table 1 - Final ham profiles presented to the consumers.

Product	Type of Ham	Designation of origin	Price (€/kg)	Purchasing format
1	Iberian	Without	16	Over the counter
2	Serrano	Without	16	Sliced & Packed
3	Iberian	With	26	Over the counter
4	Iberian Mast-fed	Without	26	Whole ham
5	Iberian Mast-fed	With	36	Sliced & Packed
6	Serrano	With	6	Over the counter
7	Iberian	Without	16	Sliced & Packed
8	Iberian Mast-fed	Without	36	Over the counter
9	Iberian	With	26	Sliced & Packed
10	Serrano	With	16	Whole ham

Table 2 - Part-worths and relative importance of the attributes for the overall sample and the three clusters.

Attributes and levels	Overall Sample	Pro-PDO consumers (n=132)	Average consumers (n=177)	Price-sensitive consumers (n=83)
Type of ham				
Iberian Mast-fed	1.138	1.034	1.281	1.335
Iberian fodder-fed	0.152	0.282	0.171	-0.141
Serrano	-1.920	-1.318	-1.451	-1.194
Relative importance	*43.5%*	*46.2%*	*47.5%*	*32.8%*
Protected Designation of Origin				
Yes	0.347	0.423	0.348	0.289
No	-0.347	-0.423	-0.348	-0.289
Relative importance	*12.4%*	*16.6%*	*12.1%*	*7.5%*
Price				
6 €/kg	-0.431	-0.262	-0.433	-0.847
16 €/kg	-1.149	-0.699	-1.155	-2.258
26 €/kg	-1.867	-1.136	-1.877	-3.669
36 €/kg	-2.585	-1.573	-2.599	-5.080
Relative importance	*38.5%*	*25.7%*	*37.7%*	*54.9%*
Purchasing format				
Sliced and packed	-0.160	-0.241	-0.099	-0.167
Over the counter	0.010	-0.102	0.044	0.206
Whole ham	0.150	0.343	0.055	-0.038
Relative importance	*5.6%*	*11.5%*	*2.7%*	*4.7%*

The value of each level's part-worth indicates if the presence of that level of the attribute adds (positive sign) or lessens (negative sign) that amount of utility to the product. Regarding one of the most important attributes, the price, it can be seen that for the majority of the samples, as price increases, the utility for the consumer decreases, which conforms to various Conjoint Analysis studies of food products (MURPHY *et al.*, 2000; MESÍAS *et al.*, 2005).

By analyzing the values for the PDO, it can be seen that this attribute was only granted 12% of relative importance for the overall sample, as contrasted with the values obtained in comparable studies for other food products, including 23% for beef (MESÍAS *et al.*, 2005), 40% for cheese (SOUZA and VENTURA, 2001), and 17% for dry-cured ham (MESÍAS *et al.*, 1997). These studies used a similar number of attributes (4 or 5). These differences may derive from the nature of Iberian dry-cured ham, whose main characteristic is the original Iberian breed. With this characteristic alone, consumers take a certain quality of ham for granted, and thus the PDO may not be considered so important. Nevertheless, for all the clusters, the presence of Protected Designation of Origin added some utility to ham consumers.

With respect to the clusters, the first one has been called "Pro-PDO consumers". This group showed the highest preference for the Protected Designation of Origin, the highest knowledge about this factor, and also greater willingness to pay a higher price (up to 15%) just for the presence of this attribute.

The second cluster was termed "Average consumers", since their preference function was very similar to the one of the overall sample. Most of the consumers in this group had a medium knowledge of Designations of Origin, and although they appreciated this attribute, they were willing to pay just up to 11% for a PDO product over a conventional food product.

Finally, Cluster 3 was found to be the one that gave the highest importance to price, and the lowest to type of ham and to Protected Designation of Origin. Most of the consumers in this cluster had no knowledge of Designations of Origin and 89% of them were not willing to pay for the PDO attribute (average willingness to pay for this group was 1%). According to the above, this cluster was designated as "Price-sensitive consumers".

Table 3 shows a description of the socio-demographic characteristics of the sample and the clusters. It also shows the level of significance obtained in a X-square test carried out for the three clusters. Significant differences among the clusters were found for most of the socio-demographic variables. Cluster 1 (Pro-PDO consumers) had the greatest percentage of individuals under 50, with mid-income levels and with a majority of high-school or university studies. Cluster 2 is similar to Cluster 1, but it mainly consisted of men. Finally, Cluster 3 is mainly characterized by the lowest income level (which justifies its previous designation as Price-sensitive consumers); it had the lowest level of education and the highest percentage of individuals over 50.

Table 3 - Socio-demographic characteristics by cluster and overall sample (%).

		Pro-PDO consumers	Average consumers	Price-sensitive consumers	Overall Sample	Significance[a]
Level of study	No formal education	2.3	6.8	8.4	5.6	**
	Primary education	19.7	18.6	43.4	24.2	
	High school	31.8	27.1	30.1	29.3	
	University	46.2	47.5	18.1	40.9	
Income level	<1,500 €/month	28.9	38.4	65.2	40.9	**
	1,500-2,500 €/month	46.3	31.4	29.2	36	
	>2,500 €/month	24.8	30.2	5.6	23.1	
Family size	1-2	28.8	23.9	37.3	28.4	n.s.
	3-4	50	53.4	39.8	49.3	
	≥5	21.2	22.7	22.9	22.3	
Sex	Man	40.9	52	37.3	45.2	*
	Woman	59.1	48	62.7	54.8	
Age of the respondent	18-35 years	34.5	31.7	26.3	31.5	**
	36-50 years	34.4	33.3	23	31.5	
	51-65 years	21.6	21.5	31.3	23.6	
	>65 years	9.5	13.5	19.4	13.4	
Residence	Rural	41.7	43	56.6	45.2	n.s.
	Urban	58.3	57	43.4	54.8	

[a]Differences significant at: *p < 0.05, **p < 0.01; n.s.: not significant.

Simulation of market shares

Once the preference functions were determined, a simulation analysis was carried out (both for the sample and for each of the three clusters) in order to quantify, in monetary terms, the value of presence of the Protected Designation of Origin in Iberian ham. The simulation consisted of estimating market shares for different hypothetical products. These market shares were calculated using the maximum utility model, which considers that a consumer will select the product that provides the highest utility. The following four products were selected to be used in the simulation, trying to represent broadly what can be found in the supermarkets or butcher shops of the area:

Product 1: Iberian mast-fed ham with Protected Designation of Origin, 36 €/kg

Product 2: Iberian ham without Protected Designation of Origin, 19 €/kg

Product 3: Iberian Ham with Protected Designation of Origin, 26 €/kg

Product 4: Serrano ham without Protected Designation of Origin, 8.6 €/kg

In order to allow the comparison between the different products, the attribute "Purchasing format" was set as "Whole Ham".

Table 4 shows the initial market shares for the selected products, which reflect the relative im-

Table 4 - Initial market shares (%).

	Overall sample	Pro-PDO consumers	Average consumers	Price-sensitive consumers
Product 1	43.23	55.68	43.50	26.51
Product 2	14.16	9.85	16.10	18.07
Product 3	17.36	20.08	18.64	4.82
Product 4	25.25	14.39	21.76	50.60

Product 1: Iberian mast-fed ham with Protected Designation of Origin (PDO), 36 €/kg
Product 2: Iberian ham without PDO, 19 €/kg
Product 3: Iberian ham with PDO, 26 €/kg
Product 4: Serrano ham without PDO, 8.6 €/kg

Table 5 - Final market shares (%) and economic value of Protected Designation of Origin attribute.

	Overall sample	Pro-PDO consumers	Average consumers	Price-sensitive consumers
	Price prod. 2= 17.8 €/kg	Price prod. 2= 15.1 €/kg	Price prod. 2= 17.8 €/kg	Price prod. 3= 20.0 €/kg
Product 1	42.00	53.41	42.37	24.10
Product 2	17.12	16.67	19.49	14.46
Product 3	17.24	17.05	18.93	14.46
Product 4	23.65	12.08	19.21	46.98
Economic value of Protected Designation of Origin attribute[a]	8.2 €/kg	10.9 €/kg	8.2 €/kg	1.0 €/kg

Product 1: Iberian mast-fed ham with Protected Designation of Origin (PDO), 36 €/kg
Product 2: Iberian ham without PDO, 19 €/kg
Product 3: Iberian ham with PDO, 26 €/kg
Product 4: Serrano ham without PDO, 8.6 €/kg
[a]The economic value of the Protected Designation of Origin attribute was calculated as the difference between the prices of Product 2 and Product 3.

portance that each cluster gave to the attributes. For the sample as a whole, the relative importance given to the attributes led to the preferred product being Iberian Mast-Fed (the attribute Type of Ham had a relative importance of 43.5%, and Mast-Fed level had the greatest part-worth). The relative importance of price (38.5%, with a negative utility for increasing prices) led to Product 4 as the second preferred, as it was a cheap cured ham. The market share of Product 2 was almost the same as that of Product 3, as both products are very similar (although Product 3 is covered by a PDO). This difference reflects the small importance that the overall sample gives to the attribute PDO (although it is an attribute that adds utility to the consumer).

The low value for Product 2 shown by "Pro-PDO consumers" is explained by the fact that they granted the highest importance to the PDO attribute, with low importance given to price (a reduced price does not compensate for the lack of another attribute). Both aspects also explain the high value for Product 1.

Finally, among the "Price-sensitive consumers", the main attribute was price (54.9% of relative importance). This, together with the small importance given to Protected Designation of Origin, led to higher market shares for Products 2 and 4 (they preferred cheaper hams and without a PDO label).

Willingness to pay for the PDO attribute

After the previous estimation of the initial market shares, other simulations were run to determine the extra price a consumer was willing to pay for Iberian ham with PDO compared with Iberian ham without PDO. Products 2 and 3 were modified through the simulations, since both are Iberian hams, although the first one does not have a PDO. In this case, the price of the less preferred ham was reduced until a new price was reached for which the market share values for both hams were almost the same. At this point, the difference in price between Products 2 and 3 was considered to be the willingness to pay for the PDO, the only difference between the two hams. After several repetitions, the final price for each cluster was determined, and the results are shown in Table 5.

"Pro-PDO consumers" were ready to pay 10.9 extra €/kg for the PDO ham, as compared to only 1.0 extra €/kg in the case of "Price-sensitive consumers". "Average consumers" are in an intermediate zone, since they were willing to pay 8.2 €/kg extra. These results are in line with the characteristics of each cluster: "Pro-PDO consumers" granted the highest importance among the three groups to Protected Designation of Origin and the lowest to price, while "Price-sensitive consumers" gave the lowest importance of the three clusters to PDO, and the highest to price. Consequently, "Pro-PDO consumers" were more willing to purchase a PDO Iberian ham than the other consumers, even though this type of ham is more expensive.

Willingness to pay *vs* extra costs derived from PDO

The price premium that consumers were willing to pay has to be compared with the extra costs of producing an Iberian ham within a PDO scheme. The main differences are found at the farming level, due to the longer period of time that Iberian pigs are kept in "*montanera*" (a traditional method in which animals are free to roam in the *dehesa* countryside, eating mainly



Fig. 1 - Location of Extremadura, Spain.

acorns and pasture) and due to the certification costs of the Ruling Council (EUROPEAN COMMISSION, 2006).

As mentioned in the introduction section, depending on the time period that an Iberian pig is kept in "*montanera*", it will be labelled as "*Bellota*", "*Recebo*" or "*Cebo*". For example, in Extremadura's PDO, only those pigs that have put on up to 60% of their initial weight during the "*montanera*" period are labelled as "*Bellota*" (www.dehesa-extremadura.com). Those non-PDO pigs can be labelled as "*Bellota*" by just putting on 40% of their weight during the "*montanera*" stage.

The PDO Ruling Council charges a certification fee of about 5 €, per pig (EUROPEAN COMMISSION, 2006). This price covers inspections at the farms and processing plants, together with analytical laboratory tests about ham quality.

In addition, Iberian PDO hams have to be kept longer in "*bodegas*" (natural warehouses where hams are cured and matured) than non-PDO hams. For example, PDO mast-fed hams are not sold until they have aged over 2.5 years (www.dehesa-extremadura.com), as compared to 1.2 years for non-PDO mast-fed hams.

All these concepts are reflected in Fig. 2, that shows the price evolution for Iberian ham (mast-fed and fodder fed) with and without Protected Designation of Origin at the retail level. As can be seen, the difference in price between a fodder-fed Iberian ham with Protected Designation of Origin and without it is about 6.4 €/kg (the price difference for Iberian mast-fed ham is just 4.4 €/kg). These values imply that both "Pro-PDO consumers" and "Average consumers" will always be willing to purchase an Iberian PDO ham, while "Price sensitive consumers" will consider it as an expensive product, and therefore they will not purchase it.

These results could provide information, both for the producers of Iberian ham and for the PDO Ruling Council, in order to target their potential consumers. In this sense, "Pro-PDO consumers" would be their natural market segment, mainly

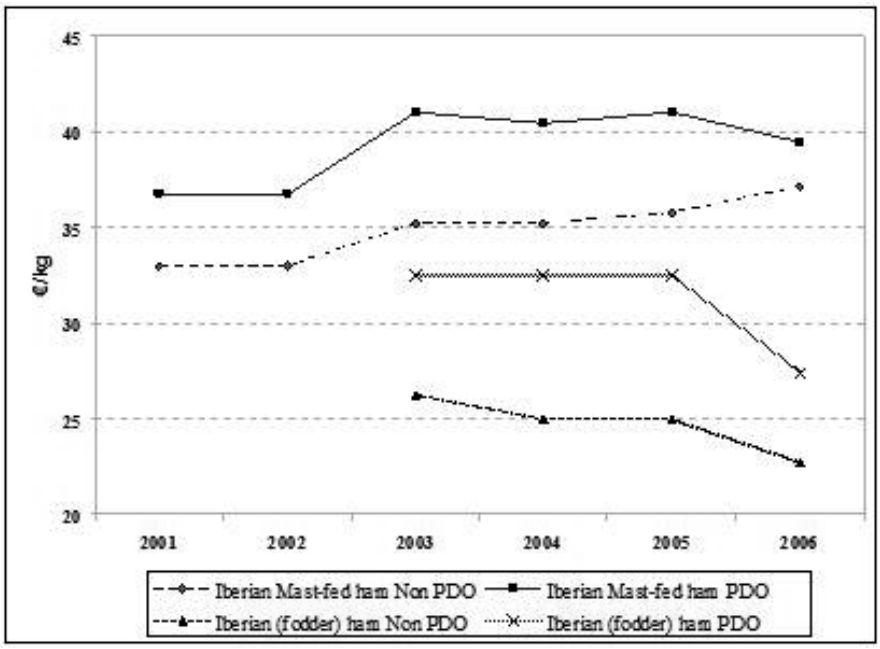


Fig. 2 - Price evolution (2001-2006) for Iberian Mast-fed hams and Iberian hams with and without PDO at the retailer level (Source: EUROPEAN COMMISSION, 2006).

due to their preferences. Moreover, this group contains the highest percentage of families with medium-high income levels and of young families. "Average consumers" is another interesting segment, with a socio-demographic structure similar to "Pro-PDO consumers", and a good but lower appreciation of Protected Designation of Origin attribute.

Actions to promote the consumption of PDO Iberian ham should be focused on the abovementioned consumers, since "Price sensitive consumers" are not willing to pay an extra price for the Protected Designation of Origin attribute. With two-thirds of their members in the lowest income level, the income structure of these consumers makes them very concerned about their expenditures. It is unlikely that they will purchase delicatessen or high-priced products, such as the Iberian ham (with or without PDO).

CONCLUSIONS

Protected Designation of Origin has been found to be an attribute whose presence in the Iberian dry-cured ham adds utility, in different amounts, for all consumers. Thus, the consumer prefers Iberian hams produced under a PDO scheme, which guarantees some characteristics of the Iberian ham (such as the degree of purity in the Iberian breed or the different types of feeding) that are difficult for the consumers to fully understand and check.

Although consumers have granted much more importance to other attributes, like price or type of ham, than to the Protected Designation of Origin (in fact price and type of ham accounted for more than 75% of the importance within the preference structure for all the consumers), it has to be borne in mind that, in a typical and heterogeneous product such as the Iberian ham, there are other attributes (for example maturing time, or fat content) that could improve the definition of the preference structure. Although these attributes were not included in the study in order to facilitate answering the questionnaire, they could be taken into account in further studies. Nevertheless, with a greater number of factors, an increased number of stimuli are needed in order to maintain statistical efficiency and reliability of the results.

Segmentation analysis with PDO-related variables produced three well-defined groups of consumers with different socio-demographic profiles and preference structures. Regarding their appreciation for the Protected Designation of Origin, only one of the clusters granted very low importance to this attribute, caused mainly by the importance these consumers gave to price.

The use of simulation techniques facilitated the analysis of consumer willingness to pay a premium for Iberian ham covered by a Protected Designation of Origin (since PDO implies extra production costs, such as higher feeding and production costs and certification fees). As only some consumers are willing to pay a premium, Protected Designation of Origin might become a useless or even counterproductive tool for Iberian ham producers, unless a proper selection of market segments is carried out or information campaigns are developed to improve consumer appreciation of this attribute.

NOTES

[1] Due to budget restrictions, only population centres of more than 3,000 inhabitants were considered for the study (they represent more than 75% of the total population of the region).

[2] Price, PDO, maturing time, fat content, color, type of ham, packaging, place of purchase, brand name, feeding of the pigs, origin, salt content.

[3] Consumers were selected among the customers of several traditional shops selling dry-cured ham. The experts were selected among the members of an association of dry-cured ham producers.

[4] The full-profile method presents respondents with a series of full descriptions of the product concepts and requires them to rank (using car-sorting techniques) or score each concept according to their preference or willingness to buy (NESS and GERHARDY, 1994; WITTINK and CATIN, 1989).

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PAPER

THE LIPID COMPOSITION AND TEXTURE PROFILE OF DRY-CURED LOIN AS AFFECTED BY FEEDING LEVEL IN THE PERIOD PRIOR TO THE LATE FATTENING PHASE AND BY REARING SYSTEM OF IBERIAN PIGS

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ABSTRACT

The effect of a moderate feed restriction (L, 50 g feed/kg $LW^{0.75}$) and a high feeding level (H, 70 g feed/kg $LW^{0.75}$) during the period prior to the late fattening phase was studied on Iberian pigs reared in free-range (FR) or in confinement (C). The degree of proteolysis, fat content, fatty acids and texture profile of Iberian dry-cured loins was investigated. Products from free range animals showed the lowest hardness and the highest adhesiveness and degree of proteolysis, whereas those raised in confinement had the lowest hypocholesterolemic/hypercholesterolemic and the highest PUFA/SFA ratios.

- Key words: fatty acid, feeding level, free-range, Iberian dry-cured loin, rearing system, texture -

INTRODUCTION

The rearing system used for Iberian pigs is a good example of tradition adapted to the current quality requirements of the meat industry. During the free-range fattening phase, animals are fed on acorns and grass which give the meat and meat products special characteristics related to fat content, fatty acid and volatile profiles (LÓPEZ *et al.*, 1992; HOZ *et al.,* 1996; ORDÓÑEZ *et al.*, 1996). In the 4 to 5 month period prior to the fattening stage, animals are reared in confinement with mixed diets (LÓPEZ-BOTE, 1998).

Feeding restriction prior to fattening is usually applied to pigs reared in free-range because it is assumed that this procedure leads to a higher consumption of acorns during free-range fattening, and consequently, to higher fat accumulation during this fattening period, which in turn seems to lead to a higher proportion of oleic acid in the backfat of pigs at slaughter (LÓPEZ-BOTE *et al.*, 2001; FREITAS *et al.*, 1995). There are previous reports in the literature concerning the effects of feeding restriction prior to free-range fattening on carcass and fat quality traits in Iberian pigs (DAZA *et al.*, 2008), as well as the importance of the feeding level during the growing and fattening phases on fatty acid composition (outer and inner subcutaneous layers) and lipogenic enzyme activities (DAZA *et al.*, 2007).

DAZA *et al.* (2008) stated that animals raised without restriction had longer and wider-perimeter hams ($p < 0.10$) and tended to have higher slaughter and carcass weights, carcass inner length and loin and sirloin weights than those subjected to high restriction feeding. Animals reared with high restriction feeding had lower proportions of total saturated fatty acids (SFA) and higher proportions of total monounsaturated fatty acids in the inner backfat layer than the non-restricted animals. Total polyunsaturated fatty acids (PUFA) in intramuscular neutral lipids were higher in high restriction pigs. DAZA *et al.* (2007) indicated a marked effect of the feeding system on pig productivity and fatty acid composition since animals under a low feeding system during the growth period showed a significantly ($p<0.05$) higher proportion of C18:1*n*-9 and total monounsaturated fatty acids in the outer and inner layers of the subcutaneous backfat. In general, the fatty acid composition of the meat products remained unchanged after processing and curing (KRISTINSSON *et al.*, 2001; HOZ *et al.*, 2004). Bearing this in mind, the fatty acid composition of the dry-cured loin should reflect the fatty acids present in the pig feed.

In Iberian dry-cured products (loin and ham), higher quality has been associated with higher contents of oleic and monounsaturated fatty acids, which are typical of animals fed in free-range conditions. The higher quality of meat products from free-range Iberian pigs is attributed to a high consumption of acorns, which are rich in oleic acid (CANTOS *et al.*, 2003).

The present study was conducted to assess the effect of a moderate feeding restriction during the period prior to the late fattening phase on the degree of proteolysis, fat and fatty acid composition of Iberian dry-cured loins from free-range animals and those fed in confinement. The curing of these products, which takes 80-120 days, is a good experimental model for determining the effect that rearing system (effect of exercise and diet) and the prior feeding level have on meat product quality.

MATERIAL AND METHODS

Animals, experimental design and diets

Forty Iberian castrated male Torbiscal line pigs (El Dehesón del Encinar, Oropesa, Toledo, Junta de Comunidades de Castilla-La Mancha, Toledo, Spain) with an average initial live weight of 55.07 kg (standard error = 0.80 kg) were used and randomly distributed into two groups. One group of twenty pigs, was fed with 70 g feed/kg $LW^{0.75}$(high feeding level, H) and another group of twenty pigs was fed with 50 g feed/kg $LW^{0.75}$ (low feeding level, L) for 164 days, from May to November. In this growing period, the pigs were fed in confinement with a conventional commercial diet. The ingredients, chemical composition and major fatty acids of the diets are reported in Table 1. To begin the fattening period, ten pigs on H feeding level (pigs weighing about 105-115 kg) and ten pigs on L feeding (pigs weighing about 80-90 kg) were fed under free-range (FR) conditions for a period of 95 days (from November to February) in a *Quercus ilex* forest with acorns and grass fully available to them (batch FRH and FRL). The stocking rate in the FR feeding phase was 0.7 pigs/ha and the average temperature was 8°C.

The remaining twenty pigs were kept under confinement (C) in individual cages of 8 m^2 (batch CH and CL) with concentrate diets, that imitated the free-range diet, during the whole fattening period. The average indoor temperature was 14°C. Water was provided *ad libitum*. All pigs were weighed weekly and the amount of feed provided to pigs kept in confinement was calculated in order to achieve a similar growth rate as the pigs fed under FR conditions.

Ingredients, chemical composition and the main fatty acids of the concentrate diet used during the fattening period are also presented in Table 1. The chemical composition of feeds (by triplicate) was determined according to AOAC (1995). The fatty acids of diets were extracted and quantified by the SUKHIJA and PALMQUIST (1988) one-step procedure on freeze-dried samples, and the fatty acid me-

Table 1 - Ingredients and chemical composition of the diet used for feeding of Iberian pigs in confinement during the growing and fattening period.

Growing period		Fattening period	
Ingredients	(g/kg feed)	**Ingredients**	(g/kg feed)
Barley	704.6	Barley	533
Wheat bran	25.5	Wheat	311
Sunflower meal-30	121.7	High-oleic sunflower seed	50
Soybean meal-44	86.8	Soybean meal-44	81
Calcium carbonate	7	Calcium carbonate	8
Dicalcium phosphate	13	Dicalcium phosphate	9
Sodium chloride	3	Sodium chloride	4
Mineral/vitamin mix	7	Mineral/vitamin mix	4
Lard	30		
Lysine supplement	1.3		
Composition	(g/100 g feed)	**Composition**	(g/100 g feed)
Water	10.5	Water	10.4
Crude protein	16	Crude protein	12.6
Crude fat	4.9	Crude fat	3.6
Major fatty acids	(% methyl esters)	**Major fatty acids**	(% methyl esters)
C16:0	0.24	C16:0	15.4
C18:0	3.14	C18:0	3.43
C18:1*n*-9	25.1	C18:1*n*-9	31.2
C18:2*n*-6	61.3	C18:2*n*-6	45.7

thyl esters were analyzed according to LÓPEZ-BOTE *et al.* (2003).

Animals were stunned, slaughtered and exsanguinated on the same day at a local slaughterhouse (Mafisa, Guijuelo, Salamanca, Spain) with an average weight of 166.2 kg for FRH, 138.4 kg for FRL, 170.2 kg for CH and 143.60 kg (SEM = 4.9 kg) for CL pigs. Pork loins were obtained after 24 h of refrigeration at 4°C.

Preparation of the experimental dry-cured loins

For each of the four batches (FRH, FRL, CH and CL), ten dry-cured loins were prepared using the whole left loin (*Longissimus dorsi*). All dry-cured loins were manufactured the same day using the same technology, ingredients and formulation (SOTO *et al.*, 2008). The raw material was whole pork loin (*Longissimus dorsi*) weighing 1.5-2.0 kg, after removal of the surface fat and connective tissue. Additives and other ingredients (g/kg of raw material) were water (25), NaCl (25), glucose (5), curing salts composed of $NaNO_3$ (2) and $NaNO_2$ (1), ascorbic acid (1.0), sweet paprika (6), hot paprika (2), powdered garlic (1.5) and oregano (0.5). All the additives and other ingredients were mixed. This mixture was evenly distributed on the loin surface and then the loins were left at 4°C for 48 h. Afterwards, each loin was stuffed into a synthetic case (90 mm in diameter). The resulting forty pieces were all cured together in an Ibercex curing cabinet, model G-28 (ASL, San Fernando de Henares, Spain). The meat products were kept at 23°C and 94% relative humidity (RH) for 48 h. Then, temperature and RH were slowly reduced to 7°C and 84%, respectively, within 72 h. These curing conditions were used until the end of the curing process (a total of 100 days). At that time, water activity (a_w) was lower than 0.90 and the final product was vacuum-packed and maintained at 4°C (less than a week) until sampling.

All animals used for this study, as well as the dry-cured loin manufacturing process, fulfilled the requirements of the Spanish regulation (MINISTERIO DE LA PRESIDENCIA, 2001).

Sampling and analysis

Once the dry-cured loin was cured (about 100 days), the external case and the outer layer (1 cm) of the dry-cured loin samples were discarded.

Samples (without the case and the outer layer) used for chemical determinations were vacuum packed just after sampling, frozen at –18°C and stored until analysis. Analysis for degree of proteolysis (amino acids and ammonia) and fatty acids were performed within a maximum time of four months. Samples for texture analysis were vacuum-packed, stored at 4°C and analyzed within ten days.

Chemical analysis

The dry matter (oven air-drying method) and ash (muffle furnace) were analyzed following AOAC (1995) procedures. For the estimation of

the degree of proteolysis, the nitrogen content from free amino acids and ammonia was estimated. To do this, free amino acids were extracted in an aqueous solution according to YANG and SEPULVEDA (1985) and their phenylisothiocyanate (PITC) derivatives were analyzed by HPLC as described by BRUNA *et al.* (2000). Ammonia levels were determined using the Boehringer kit for enzyme analysis (Boehringer Mannheim, Mannheim, Germany). The nitrogen content (mmols/100 g of defatted dry matter) from the degree of proteolysis was calculated taking in account the molecular weights of free amino acids, ammonia and nitrogen, and the fat content.

Lipids from dry-cured loin samples were obtained using the method of Bligh and Dyer described by HANSON and OLLEY (1963). Fatty acid methyl esters were prepared using the method of BAUBLITS *et al.* (2006). To do this, transmethylation was performed using 1 mL of 1.09 M HCl in methanol and 1 mL of methanol added to each lipid sample, which was then followed by heating at 80°C for 30 min and vortex-mixing every 5 min. Upon cooling, 1 mL of H_2O and 2 mL of hexane were added to each tube. Tubes were capped and vortex-mixed for about 15 s and then centrifuged for 3 min at 900 *g*. The hexane layer was transferred to gas liquid chromatography (GLC) vials containing anhydrous Na_2SO_4. The methyl esters were extracted with 3 mL of petroleum ether. Then, 1 µL was analyzed using a Perkin Elmer 8420 gas chromatograph (Perkin Elmer, Beaconsfield, UK) equipped with a flame ionization detector and a capillary column HP-Innowax (30 m × 0.32 mm i.d., 0.25 µm film thickness). Helium at 2.0 mL/min was used as a carrier gas and the split/splitless injector was used with a split ratio of 10/1. The temperature program was as follows: injector and detector temperature was 250°C, the initial column temperature was 200°C, which was kept for 2 min; this was followed by a programmed rise to 245° at 3.5°C/min and, then held for 7 min.

Fatty acid methyl esters were identified by comparison with commercial standards purchased from Sigma Chemical Co. (Saint Louis, MO, USA), except C18:2*n*-7Δ*cis* 9, *trans* 11 which was supplied by Nu-Chek-Prep, Inc. (Elysian, MN, USA). These standards were previously run in the same conditions. Samples and commercial standards were injected at least twice and quantified (g/kg of total methyl esters).

The calculated Iodine Index (II) was estimated according to CAMBERO *et al.* (1991), using the expression:

$$II = SII \times FA$$

Where: II = total Iodine Index; SII = specific Iodine Index of every fatty acid; FA = concentration of the specific fatty acid obtained by gas chromatography.

The hypocholesterolemic/Hypercholesterolemic ratio (h/H) was estimated (SOTO *et al.*, 2009) according to the expression h/H ratio= [(sum of C18:1*n*-9, C18:1*n*-7, C18:2*n*-6, C18:3*n*-3, C18:3*n*-6, C20:1*n*-9, C20:3*n*-6, C20:4*n*-6, C20:5*n*-3, C22:4*n*-6, C22:5*n*-3 and C22:6*n*-3)/(sum of C12:0, C14:0 and C16:0)].

Texture measurements

The texture profile analysis (TPA) was performed using a TA.XT2i SMS Stable Micro Systems Texture Analyser (Stable Microsystems Ltd., Surrey, England) with the Texture Expert programmes. Textural tests were carried out at about 22°C.

For analysis, a cylindrical probe P/25 was used. This procedure involved the preparation of four cylinders (1.5 cm high and 2 cm wide) for each sample. A double compression cycle test up to 50% compression of the height of the original portion was performed with an aluminium cylinder probe of 2 cm diameter. A time of 5 s was allowed to elapse between the two compression cycles. Force-time deformation curves were obtained with a 25 kg load cell applied at a crosshead speed of 2 mm/s. The following parameters were quantified (SANTOS *et al.*, 2004): hardness (N), maximum force required to compress the sample; springiness (m), ability of the sample to recover its original form after deforming force was removed; adhesiveness (N x s), area under the abscissa after the first compression; cohesiveness, extent to which the sample could be deformed prior to rupture; gumminess (N), force to disintegrate a semisolid meat sample for swallowing (hardness x cohesiveness); and chewiness (J), work required to masticate the sample before swallowing.

Statistical analysis

An individual dry-cured loin was the experimental unit for analysis of all data. To check the normal distribution (90% confidence) of samples, the Shapiro-Wilks test was applied. When samples fit the normal distribution, the one-way ANOVA analysis was performed. When samples did not fit the normal distribution, the Kruskal-Wallis test was used to determine the null hypothesis that the medians of the variable within each of the sample levels were the same. Duncan's test for multiple mean comparisons was performed to ascertain differences among means. Data are presented as the means and the standard deviations (SD) of each dry-cured loin batch.

In order to describe the effects of the degree of proteolysis (mmols N/100 g defatted dry matter) and the fat content (g/100 g dry matter) on hardness values, a simple and multiple regression and the estimated response surface were carried out.

The principal component analysis (PCA) was

used to group the dry-cured loins according to their fat content and fatty acid profile characteristics (PUFA/SFA and PUFA n-6/n-3 ratios). The statistical analyses were performed using Statgraphics Plus version 5.0.

RESULTS AND DISCUSSION

The degree of proteolysis (mmols of nitrogen from free amino acids and ammonia/100 g of defatted dry matter) and the fat content (g/100 g dry matter) of the dry-cured loins are shown in Fig. 1. The products from the free range (FRL and FRH) pigs showed a higher degree of proteolysis ($p < 0.05$), with values of about 73 mmols N/100 g defatted dry mater, than those of dry-cured loins from animal fed in confinement (CL and CH) which had values of about 40 mmols N/100 g defatted dry matter. No significant differences ($p > 0.05$) were found in FRL *versus* FRH or CL *versus* CH. On the other hand, the fat content showed significant differences ($p < 0.05$), the dry-cured loins from batch CL showed lower fat content than those from the FRL and FRH batches. The products from animals fed in confinement (CL *versus* CH) had similar fat content ($p > 0.05$). These results might indicate a priority effect of the rearing system (free-range or confinement) in the fattening phase compared to that of the feeding regime (high or low) in the previous period with animal exercise favouring the final degree of proteolysis and fat accumulation in dry-cured products. Similar (SOTO *et al.*, 2010) and related (DAZA *et al.*, 2007) findings have previously been described. DAZA *et al.* (2007) reported that, at slaughter, the pigs fed with a low feeding system during growth and fattening periods had higher values of loin width and lower values of backfat thickness and *Longissimus dorsi* muscle intramuscular fat percentage. The relationship between a pig's physical activity and meat quality characteristics is still unclear. While some studies find no differences in performance or meat quality (GENTRY *et al.*, 2002), others report some exercise effects on meat color, drip-loss and carcass length (GEVERINK *et al.*, 1998; KARLSSON *et al.*, 1993). It is still controversial whether exercise affects the overall rate of muscle protein breakdown (HENRIKSSON, 1991). On the other hand, there is literature concerning the interaction of proteolytic enzymes with the generation of final protein degradation compounds and other meat quality characteristics (FLORES *et al.*, 2000; TOLDRÁ and FLORES, 1998). LOPEZ-BOTE *et al.* (2008) reported that exercise induced the inhibition of dipeptidyl peptidases II and III and arginyl aminopeptidase, as well as the activation of dipeptidyl peptidases IV and leucyl aminopeptidase. On the other hand, the concentration of branched-chain amino acids decreased in the free-range pig probably due to an increase in physical activity. This increased proteolytic enzyme activity could be related to the observed higher degree of proteolysis of products from the free-range (FRL and FRH) pig (Fig. 1). Although in post-mortem muscles (*Psoas major*), no effects on total free amino acids were observed between free-range and confinement pigs, the curing period of dry-cured loins (80-120 days) makes it possible to observe the different proteolytic enzyme activities.

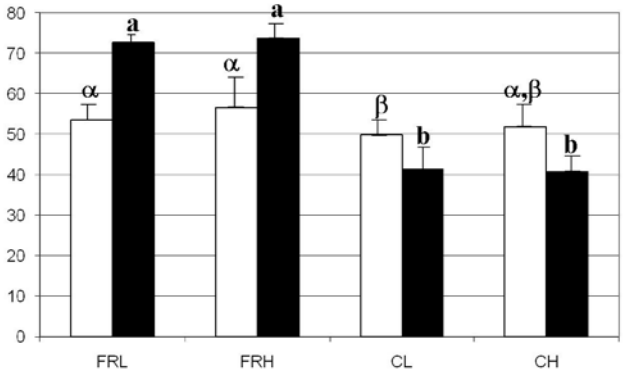


Fig. 1 - Nitrogen from free amino acids and ammonia (■, mmol/100 g defatted dry matter) and fat content (□, g/100 g dry matter) of Iberian dry-cured loins.
a, b or α, β: columns with different letters are significantly different ($p < 0.05$).

FRL, FRH, CL, CH: Iberian dry-cured loins from animals reared in free-range (FR) or confinement (C) during the fattening phase. Animals were fed with low (L) or high (H) regime diets during the period prior to the fattening phase.

The fatty acid C16:0, C18:0, C18:1*n*-9, C18:1*n*-7, C18:2*n*-6, C18:3*n*-3, C20:0, C20:1*n*-9, C20:3*n*-6 and C20:4*n*-6 percentages (Table 2) showed significant differences ($p < 0.05$) among batches. These differences ($p < 0.05$) were reflected in the total saturated, monounsaturated and polyunsaturated fatty acids. Dry-cured loins from free-range animals (FRL *vs* FRH) showed similar fatty acid composition and generally, no differences ($p > 0.05$) between them (they only differed in C18:3*n*-3 and C20:1*n*-9 contents). However, dry-cured loins from animals fed in confinement (CL *vs* CH) showed important differences ($p < 0.05$). Oleic acid was the most abundant fatty acid in all the batches studied; batch CH had the lowest concentrations of oleic acid and total monounsaturated fatty acids (MUFA) and the highest concentrations of saturated fatty acids (SFA, Table 2). When animals were reared in free-range, no effect related to the growing phase was observed, while the effect of this phase was noticeable with the animals fed in confinement. The meat products from CL showed similar fatty acid composition to those of free-range (FRL and FRH) products. Animals from the CL batch probably had a higher fat turnover than those of CH. Thus, the products from CL showed more similarity with respect to the fatty acid composition of diet ingested during the last period of fattening (Ta-

ble 1). The absence of significant differences between FRL and FRH fatty acid composition could be attributed to the effect of pig exercise during the free-range rearing system which overlapped with the effect of feeding restriction during the period prior to the fattening phase.

Related (DAZA *et al.*, 2009) findings have been previously described on the fatty acid composition of subcutaneous and intramuscular fat from fattening pigs. This research indicated that the subcutaneous fat and neutral lipids of muscle from exercised pigs had higher C18:1n-9, MUFA, C18:1/C18:0, MUFA/SAT values and lower C16:0 and SFA values when compared with the fat from the sedentary pigs (DAZA *et al.*, 2009). Several researchers have attempted to establish the benefit of exercise and thus indicate that exercise might play an important role in lipid metabolism, such as lipolytic activity and oxidation, as well as elongation and desaturation of fatty acids (BERGOUIGNAN *et al.*, 2006; PETRIDOU *et al.*, 2005; VOTRUBA *et al.*, 2005); this may affect fatty acid composition in animal tissues (SZABÓ *et al.*, 2002). DAZA *et al.* (2009) has also indicated that exercise could increase delta-9-desaturase activity. However, PETRIDOU *et al.* (2005) reported that exercise training decreased the estimated delta-9-desaturase activity in muscle and adipose tissue of rats. These contrasting results are probably due to the different use of the fatty acids to produce energy, depending on the exercise level; MUFA is preferentially oxidized compared with PUFA or SAT (LEYTON *et al.*, 1987). DAZA *et al.* (2009) found that the exercised pigs had higher acid and neutral esterase levels and lower neutral lipase activity than sedentary pigs. In the present study, the polyunsaturated (PUFA) *n*-6/*n*-3 and PUFA/SFA ratios (Table 2) varied considerably depending on the dry-cured loin batches. No differences ($p > 0.05$) were found between batches FRL *vs* FRH or CL *vs* CH. However, significant differences ($p < 0.05$) were observed for *n*-6/*n*-3 and PUFA/SFA ratios between products from free-range (10.7 and 0.21, respectively) and confinement (14.6 and 0.24, respectively). The results suggest that the rearing system (free-range or confinement) in the fattening phase is more im-

Table 2 - Fatty acids (percentage of total methyl esters) of Iberian dry-cured loins.

	FRL	FRH	CL	CH
C10:0	0.04±0.01	0.04±0.005	0.04±0.001	0.04±0.001
C12:0	0.06±0.01	0.06±0.01	0.05±0.03	0.06±0.01
C14:0	1.09±0.10	1.05±0.09	1.06±0.16	1.04±0.11
C16:0	23.59±0.78^{b}	23.36±0.78^{b}	23.01±0.15^{b}	24.20±0.77^{a}
C16:1*n*-7	2.34±0.28	2.44±0.46	2.22±0.10	1.94.±0.47
C18:0	11.13±0.51^{b}	11.33±0.37^{b}	12.41±0.26^{b}	14.03±0.67^{a}
C18:1*n*-9	48.63±0.68^{a}	49.56±0.85^{a}	48.54±0.29^{a}	45.30±0.83^{b}
C18:1*n*-7	3.69±0.60^{a}	3.24±0.29^{a}	2.52±0.14^{b}	2.44±0.31^{b}
C18:2*n*-6	6.49±0.48^{c}	6.25±0.65^{c}	7.31±0.31^{b}	8.02±0.41^{a}
C18:2*n*-7Δ *cis*9, *trans*11	0.08±0.01	0.08±0.01	0.09±0.01	0.08±0.02
C18:3*n*-3	0.45±0.05^{a}	0.36±0.05^{b}	0.32±0.06^{b}	0.33±0.07^{b}
C18:3*n*-6	0.05±0.01	0.04±0.01	0.05±0.01	0.05±0.01
C20:0	0.19±0.03^{a,b}	0.17±0.01^{b}	0.19±0.02^{a,b}	0.21±0.01^{a}
C20:1*n*-9	1.21±0.16^{a}	1.01±0.07^{b}	1.10±0.06^{a,b}	1.10±0.10^{a,b}
C20:3*n*-6	0.36±0.05^{b}	0.32±0.07^{b}	0.41±0.04^{a,b}	0.45±0.04^{a}
C20:4*n*-6	0.29±0.04^{b}	0.31±0.02^{b}	0.42±0.01^{a}	0.31±0.04^{b}
C20:5*n*-3	0.03±0.01	0.03±0.01	0.02±0.01	0.02±0.01
C22:4*n*-6	0.09±0.01	0.10±0.01	0.11±0.02	0.11±0.02
C22:5*n*-3	0.17±0.04	0.17±0.02	0.15±0.03	0.16±0.05
C22:6*n*-3	0.07±0.03	0.06±0.03	0.06±0.02	0.07±0.02
TOTAL SFA	36.09±0.97^{b}	36.01±0.94^{b}	36.68±0.10^{b}	39.65±0.57^{a}
TOTAL MUFA	55.87±0.73^{a}	56.25±0.40^{a}	54.38±0.37^{a}	50.78±0.55^{b}
TOTAL PUFA	8.00±0.52^{b,c}	7.64±0.72^{c}	8.94±0.32^{a,b}	9.58±0.41^{a}
n-6/*n*-3	10.11±0.37^{b}	11.32±1.47^{b}	14.26±1.62^{a}	15.02±0.87^{a}
h/H	2.49±0.05^{a}	2.51±0.09^{a}	2.36±0.08^{b}	2.18±0.13^{b}
II	66.04±1.17^{a}	65.74±1.07^{a}	66.38±0.30^{a}	64.19±0.63^{b}
PUFA/SFA	0.22±0.01^{b}	0.21±0.01^{b}	0.25±0.01^{a}	0.24±0.01^{a}

FRL, FRH, CL, CH: Iberian dry-cured loins from animals reared in free-range (FR) or confinement (C) during the fattening phase. Animals were fed with low (L) or high (H) regime during the period prior to the fattening phase.
a, b, c: columns with different letters are significantly different ($p < 0.05$).
h/H: hypocholesterolemic/Hypercholesterolemic ratio = [(sum of C18:1*n*-9, C18:1*n*-7, C18:2*n*-6, C18:3*n*-3, C18:3*n*-6, C20:1*n*-9, C20:3*n*-6, C20:4*n*-6, C20:5*n*-3, C22:4*n*-6, C22:5*n*-3 and C22:6*n*-3)/(sum of C12:0, C14:0 and C16:0)].
II: Iodine index.

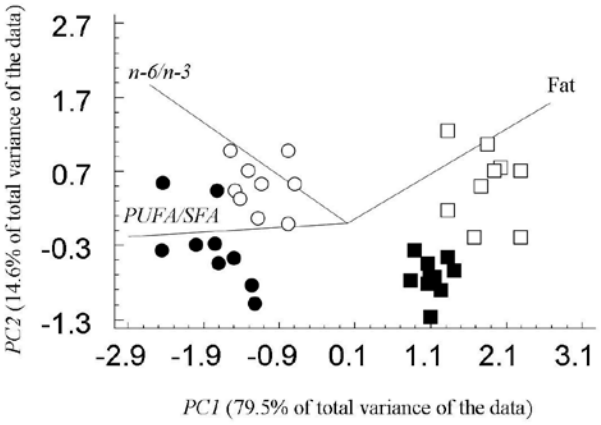


Fig. 2 - Loading plot describing the relationship among Iberian dry-cured loin fat characteristics derived from a principal component (*PC*) analysis based on proportion of fat content (g/100 g dry matter), polyunsaturated/saturated fatty acids (*PUFA/SFA*) and PUFA *n-6/n-3* (*n-6/n-3*) ratio from animals fed with low (■,●) or high (□,○) regime during the growing period prior to the fattening phase in free-range (■, □) or confinement (●, ○).

PC1 = 0.568 (fat) – 0.553 (*n-6/n-3*) – 0.610 (*PUFA/SFA*)
PC2 = 0.653 (fat) + 0.753 (*n-6/n-3*) – 0.075 (*PUFA/SFA*)

portant than the feeding regime in the previous period (high or low) with respect to the fatty acid composition in the dry-cured loin. Likewise, DAZA *et al.* (2009) reported that exercised pigs had higher PUFA in intramuscular neutral fat from *Psoas major* than those from sedentary pigs. Moreover, dry-cured loins originating from the formulated diet had a lower C18:1*n*-9 content than the loins from free-range pigs (SOTO *et al.*, 2009). This is a typical difference found in meat, meat products and subcutaneous fat from pigs fed in free-range *versus* those fed in confinement and fed with a formulated diet; the oleic acid content is always the highest in free-range animals (REY *et al.*, 2006). The products from animals raised on a formulated diet in confinement had high C18:2*n*-6 values, while the C18:3*n*-3 values did not show significant differences (SOTO *et al.*, 2009). REY *et al.* (2006) found a similar trend with C18:2*n*-6 values although they found the highest C18:3*n*-3 value in the subcutaneous fat of free-range pigs.

A principal component analysis (PCA) was carried out (Fig. 2) using fat content (g/100 g dry matter), PUFA/SFA and PUFA *n*-6/*n*-3 (*n*-6/*n*-3) ratios as variables. Two principal components (PC1 and PC2) were extracted from linear combinations of the three variables [PC1 = 0.568 (fat) – 0.553 (*n*-6/*n*-3) – 0.610 (PUFA/SFA) and PC2 = 0.653 (fat) + 0.753 (*n*-6/*n*-3) – 0.075 (PUFA/SFA)]. The two PC together accounted for 94% of the variability in the dry-cured loin data (79.5% by PC1 and 14.6% by PC2). This analysis separated the samples (Fig. 2) into two different groups: (1) dry-cured loins from pigs reared in free-range (FR) during the fattening period, with the lowest *n*-6/*n*-3 and PUFA/SFA ratios and the highest fat content and (2) products from animals fed in confinement (C), with the lowest fat content and the highest *n*-6/*n*-3 and PUFA/SFA ratios. In these two groups, the meat products were separated according to the pig feeding regime during the growing period prior to the late fattening phase. In this way, it was possible to observe two associations: one formed by dry-cured loins from pigs fed with high (H) regime during the growing period and another related to the products from animals fed with low (L) regime in this phase (Fig. 2). This data association is due to the combination of the three variables (fat content, PUFA/SFA, *n*-6/*n*-3) which accounts for most (94%) of the variability of the values from the L and H batches: products from L pigs tended to have higher PUFA/SFA and lower *n*-6/*n*-3 ratios, and lower fat content than those from the H animals (Table 2 and Figs. 1 and 2).

Textural properties of dry-cured loins are shown in Table 3. Results for hardness and adhesiveness showed significant differences ($p < 0.05$) between FR and C samples, hardness values fluctuated from 162-167 (confinement) to about

Table 3 - Textural profile analysis of Iberian dry-cured loins.

	FRL	FRH	CL	CH
Hardness (N)	129.29±19.5^{b}	140.7±18.3^{b}	166.7±18.4^{a}	162.29±15.5^{a}
Adhesiveness (N s)	-0.66±0.14^{a}	-0.65±0.17^{a}	-0.49±0.20^{b}	-0.50±0.28^{b}
Springiness (m10^{-3})	0.29±0.03	0.28±0.05	0.27±0.12	0.28±0.09
Cohesiveness	0.49±0.02	0.50±0.03	0.43±0.04	0.44±0.06
Gumminess (N)	64.1±8.12	70.6±11.33	71.1±18.17	71.2±21.23
Chewiness (J)	0.018±0.003	0.020±0.005	0.019± 0.014	0.020±0.007

FRL, FRH, CL, CH: Iberian dry-cured loins from animals reared in free-range (FR) or confinement (C) during the fattening phase. Animals were fed with low (L) or high (H) regime during the period prior to the fattening phase.
a, b: columns with different letters are significantly different ($p < 0.05$).

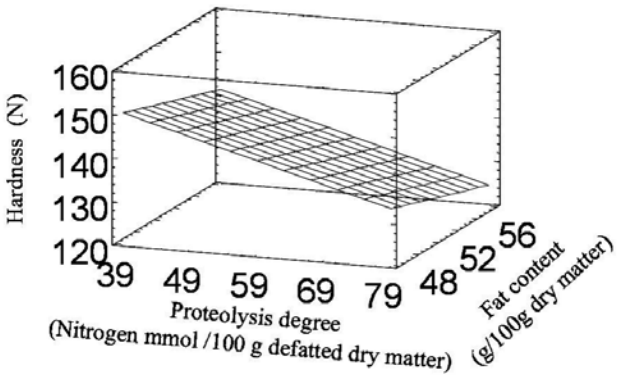


Fig. 3 - Estimated response surface[a] for the effect of proteolysis degree and fat content on hardness values of dry-cured loins from Iberian pigs.

[a]Hardness = 206.40 – 0.43 * X_1 – 0.82 * X_2 ; $p < 0.03$
Where:
X_1= Proteolysis degree
X_2= Fat content

135 N (free-range). No differences ($p > 0.05$) between dry-cured loin batches from animals fed in free range (FRL *vs* FRH) or in confinement (CL *vs* CH) were found. The most probable explanation for the differences in the textural profile is the combination of effects due to degree of proteolysis and fat content (Fig. 3). A significant negative correlation between hardness and degree of proteolysis [Hardness = 169.1 – 0.54 (degree of proteolysis), $p < 0.01$] and fat content [Hardness = 292.80 – 2.92 (fat content), $p < 0.02$] was found. The estimated response surface relating hardness to degree of proteolysis and fat content of the dry cured loins is plotted in Fig. 3. It may be observed that the hardness of dry cured loins increased as degree of proteolysis and fat concentration decreased ($p < 0.03$). On the other hand, the meat products showed less adhesiveness when the degree of proteolysis and fat content decreased ($p < 0.05$). Similarly, ENFALT *et al.* (1993) and GENTRY *et al.* (2002) found higher ($p < 0.05$) shear force values in fresh meat from indoor-finished pigs than from outdoor-finished animals. GENTRY *et al.* (2002) also stated that outdoor-reared pigs gave more ($p < 0.05$) tender pork, although both sample means were acceptable in pork tenderness and no differences were detected in sensory panel tenderness scores. After analyzing dry-cured hams, RUIZ-RAMIREZ *et al.* (2006) found similar trends for the hardness of *Biceps femoris* muscle, since this parameter diminished as the index of proteolysis increased. These results may again suggest a priority effect of the rearing system (free-range or confinement) in the fattening phase when compared to that of the feeding regime (high or low) in the previous period with animal exercise favouring the decrease of the final hardness because of an increase in degree of proteolysis and fat accumulation in dry-cured products.

CONCLUSIONS

The results obtained in this research suggest that the rearing system (free-range or confinement) during the fattening phase exerts a more important role than the feeding regime in the previous period (high or low) in relation to the dry-cured loin characteristics by influencing the degree of proteolysis, fat content, fatty acid composition, hardness and adhesiveness. Free-range products showed the lowest hardness values and the highest adhesiveness values, and degree of proteolysis. This texture behaviour is associated with high fat content, low SFA and high unsaturated fatty acid content, in the free-range products. The products from animals fed in confinement had the lowest hypocholesterolemic/Hypercholesterolemic ratio and the highest PUFA/SFA ratio, whereas those that originated from free-range conditions had the lowest *n*-6/*n*-3 ratio. The similar fatty acid composition in the products from free-range animals may be attributed to the effect of pig exercise in the free-range rearing system, which overlapped with the effect of the feeding restriction during the period prior to the fattening phase. However, the animals reared in confinement with a low feeding regime (CL) in the period prior to the fattening phase had a higher fat turnover than those with a high level feeding (CH) in the same period. Thus, the products from CL pigs had a fatty acid composition that was more similar to the diet ingested during the last period of fattening.

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PAPER

INFLUENCE OF HUNTING-SEASON STAGE AND RIPENING CONDITIONS ON MICROBIOLOGICAL, PHYSICOCHEMICAL AND SENSORY CHARACTERISTICS OF VENISON (*CERVUS ELAPHUS*) CHORIZO SAUSAGES

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ABSTRACT

This study was performed to determine the influence of hunting stage and ripening conditions on the microbiological, physicochemical and sensory characteristics of venison chorizo sausages. Hunting-season stage was correlated with variation in venison. The meat obtained at the start of hunting season contained less fat and more protein than the meat obtained at the end. All venison chorizo batches complied with the microbiological standard established for raw dry-cured sausages. Lactic acid bacteria counts increased after the first week of ripening and were the dominant flora throughout the ripening process. Regarding the organoleptic characteristics, venison chorizo batches ripened under controlled conditions had a more perceptible aroma, greater taste intensity, more pungency and flavour aftertaste and greater hardness than naturally-ripened batches.

- Key words: dry venison sausages, hunting season, microbiological, ripening conditions, sensory and physicochemical characteristics -

INTRODUCTION

In the last years, the consumption of traditional meat products and consumer interest in game meat has increased. Game is further distinguished by the characteristic texture and taste of the meat, which differs from that of poultry and farmyard animals. Game meat is generally darker, stronger tasting and often tougher, depending on the type and age of the animal. Concerning chemical composition, game species have very low muscle fat content with a high proportion of polyunsaturated fatty acids (HOFFMAN and WIKLUND, 2006). In addition, consumers are increasingly concerned about animal welfare and the environmental aspects of the animal production systems. HOFFMAN and WIKLUND (2006) showed that the intensive production of cervids on farms and the associated practices (castration, velvetting, feeding...) may have a negative impact on the consumer's perception of these animals in the near future.

There is a tendency to produce processed game meat products (sausages, cans, pates...) that are ready-to-eat and are suitable for home consumption (SALGUETTI, 1991). The presence of game products on the market is very restricted, and depends on the season, for example, for deer and wild boar the presence depends on the hunting season and the culling quotas. In Spain, cured sausage production amounted to 193,000 tons in 2006 (AICE, 2008), and game preparations and canned meat have increased notably in recent years. Game hunting is a very important rural activity from an economic viewpoint in some Spanish regions such as Castilla-La Mancha. On average, the total annual income is 240 million euros; hunters take 40,000 big game animals and 3,000,000 small game animals (GAME PRODUCERS ASSOCIATION OF CASTILLA-LA MANCHA, 2007).

Several studies have been carried out to characterise the physicochemical composition of venison (STEVENSON *et al.,* 1992; SEMIADI *et al.*, 1993; PEÑA *et al.,* 1993; ZOMBORSZKY *et al.,* 1996); to determine the influence of deer feeding on the composition of fatty acids (VOLPELLI *et al.,* 2003), and the sensorial quality (WIKLUND *et al.,* 2001; 2003); to analyse the influence of the slaughter method on the quality of the meat (WILKUND *et al.,* 2001); and to study the optimal conditions for packing deer meat (VERGARA *et al.*, 2003). However, very few studies have been reported on microbiological, physicochemical, sensory and rheological characteristics of dry sausages made with game meat (VIOQUE *et al.*, 2003; SORIANO *et al.,* 2006).

The aim of this study was to determine the effect of hunting-season stage and sausage ripening conditions on the behavior of the microbiological, physicochemical, and sensory characteristics of venison chorizo sausages during ripening and following vacuum packing.

MATERIALS AND METHODS

Chorizo sausage preparation and ripening process

Venison chorizo sausages were obtained from two companies in the Castilla-La Mancha region (Spain). One factory prepared chorizo using a natural drying process, in which the only drying mechanism was the opening and closing of windows. The other company used a ripening system in which chorizo was dried in a room under controlled temperature and relative humidity conditions.

Each company prepared venison chorizo sausages at the start of the hunting season (November) and at the end of the same season (March). Four treatments were coded as follows: A: made at the start of the hunting season and ripened under controlled conditions; B: made at the end of the hunting season and ripened under controlled conditions; C: made at the start of the hunting season and ripened under natural conditions; D: made at the end of the hunting season and ripened under natural conditions.

The venison used in the manufacture of each treatment came from several animals killed on the same day in the same area. Meat from deer legs (obtained from hunts) was stored at -18°C until processed. Venison and pork back fat (1:1) were ground in a grinder (Mainca, Granollers, Spain) fitted with a plate containing 12 mm-diameter holes. Ground meat and pork back fat were mixed in a mixer (Fuerpla, Valencia, Spain) with the following ingredients: a Ceylamix CH-L (Ceylan, S.L., Valencia, Spain) (90 g/kg) prepared formula containing salt, lactose, dextrose, dextrin, spices, polyphosphates, sodium ascorbate, potassium nitrate and paprika; garlic (1.7 g/kg) and sodium nitrite (1 g/kg). No starter cultures were added. The mixture was kneaded for 4 min and then stuffed into 30 mm artificial casings using a stuffing machine (Fuerpla, Valencia, Spain). Casings were hydrated prior to being immersed in brine for 15-20 min.

Treatments A and B were ripened in a drying room at a relative humidity of 76-80% and a temperature of 10°-12°C for 3 weeks. Treatments C and D were ripened in a natural drying room in which relative humidity and temperature varied according to outdoor weather conditions. The temperature fluctuated between 3°-14°C and the relative humidity between 41-97% in the natural drying room of batch C. Batch D was ripened at a temperature of 7°-15°C and a relative humidity of 60-84%.

Three samples from each treatment were analyzed at 0 (just after stuffing), 7, 14 and 21 days of ripening. At the end of the ripening period, chorizo sausages were vacuum-packed and stored at room temperature; further analyses were performed 45 days after packing.

Sample preparation
for physicochemical analysis

All samples were deskinned prior to analysis. Samples were ground in a household blender, placed in hermetically-sealed containers and stored at -20°C for future analysis. Samples were analyzed in duplicate.

Physicochemical analysis

Water activity (a_w) was measured directly using a dew-point hygrometer (Decagon Devices, model CX-2, Pullman, WA, USA). The pH was determined directly using an Ingold electrode probe connected to a Crison model 2001 pH-meter (Crison Instruments S.A., Barcelona, Spain). Moisture, fat, sodium chloride and ash content were analyzed according to AOAC methods (1990). Total nitrogen (TN) and protein nitrogen (PN) were determined following the Kjeldahl method, with the protein nitrogen being calculated by multiplying the total nitrogen by 6.25 (AOAC, 1990).

Sample preparation
for microbiological analysis

Chorizos were skinned under sterile conditions and 10 g aliquots were weighed and put into stomacher bags (IUL-Instruments Masticator); 90 mL of 2% sodium citrate solution (w/v) (Panreac) were added, and samples were homogenized for 2 min at 30-second intervals. The appropriate decimal solutions were prepared from this homogenate. A further 25 g of chorizo were weighed under sterile conditions and put into stomacher bags and homogenized with 225 mL peptone water (Difco) to check for *Salmonella* spp. and *Listeria monocytogenes.*

Microbiological assays

Aerobic mesophilic bacteria were incubated at 30°C for 72 h and then analyzed on Plate Count Agar (PCA) (Difco). Lactic acid bacteria were analyzed following the method described by DE MAN *et al.* (1960). Non pathogenic staphylococci were determined following the method of BUTTIAUX (1974). Total *Enterobacteriaceae,* total coliforms and moulds and yeast were analyzed according to APHA (1976). *Escherichia coli* was determined following the method described by DE MAN (1983). Enterococci were analyzed according to MOSSEL *et al.* (1978). *Salmonella* was determined by the standard ISO 3565 method (ISO, 1983). *Listeria monocytogenes* was analyzed by the method developed by VAN NETTEN *et al.* (1989). The results are expressed as log cfu/g for all organisms except *Salmonella* and *L. monocytogenes*, which are expressed as presence or absence in 25 g of sample.

Sample preparation for sensory analysis

Venison chorizo sausages were analyzed after 45 days of vacuum-packed storage at room temperature. Samples were coded and served at room temperature as 3 mm thick slices.

Sensory analysis

Sensory evaluation was performed by a panel of 10 tasters (aged 23 to 40 years old), who had extensive experience in sensory analysis of different foods. The tasting room met the requirements of Spanish Standard UNE 87-004 (1979). Each panellist participated in the generation and selection of venison chorizo attributes. Once the list of attributes was generated, synonyms and confusing terms, that were not representative, were rejected by consensus of all panellists. The sensory profile of the venison chorizo included appearance attributes: the amount and colour of the fat (from white to yellow) and colour of the lean meat (from light red to brown); odour attributes: intensity and quality of odour, paprika, spice and ripened; taste attributes: intensity of taste, acidity, saltiness, sweetness, bitterness, pungent flavour, intensity and quality of aftertaste; texture attributes: hardness, juiciness, chewiness and fat mouthfeel. The panellist were trained how to use the scales for each attribute.

The evaluation of chorizo samples was performed by Quantitative Descriptive Sensory Analysis (UNE Spanish Standard 87-020, 1993). The intensity of tested attributes was scored using unstructured 10 cm scales anchored at the extremes with the terms “weak” and “strong” in most cases. The sensory panel tested 4 samples per session using all possible presentation orders. All samples were analyzed in duplicate.

Statistical analysis

One-way ANOVA was performed to determine the effect of the ripening and storage time on the microbiological and physicochemical parameters of the four treatments of venison chorizo, and to determine the effect of the treatment on the sensory characteristics of venison chorizo. The Student-Newman-Keuls test was applied to discriminate among means. The statistical analysis was performed by SPSS program version 15.0 for Windows XP (SPSS, Inc, Chicago, Illinois).

RESULTS AND DISCUSSION

Microbiological composition

Table 1 shows the mean values for the microbiological composition of raw meat and venison chorizo sausages during ripening and after 45 days of being vacuum-packed.

Mesophilic aerobic bacterial counts were vir-

Table 1 - Mean values (log cfu/g) for microbiological composition of raw meat and venison chorizo during ripening and storage.

	Ripening time (days)					Storage
	Meat	0	7	14	21	
			A			
Aerobic mesophilic bacteria	5.52	6.70	9.21	9.28	9.15	8.94
Lactic acid bacteria	4.93	5.97	8.79	8.81	9.02	8.86
Staphylococci non-patogenic	4.70	5.64	4.54	3.92	4.52	3.15
Enterobacteriaceae	4.33	4.22	4.43	3.08	3.08	0.00
Total coliforms	4.26	3.80	4.24	2.85	1.00	0.00
Moulds and yeast	6.12	4.67	6.00	5.00	5.00	2.00
			B			
Aerobic mesophilic bacteria	5.78	7.01	8.50	9.07	9.12	8.39
Lactic acid bacteria	5.48	7.39	8.76	8.74	8.96	7.99
Staphylococci non-patogenic	4.05	3.48	3.66	2.30	2.78	2.74
Enterobacteriaceae	4.59	4.73	3.51	2.45	1.78	0.00
Total coliforms	4.38	4.60	2.87	2.50	1.65	0.00
Moulds and yeast	4.21	5.05	4.00	4.00	4.00	3.00
			C			
Aerobic mesophilic bacteria	5.70	5.11	7.70	7.88	8.18	7.70
Lactic acid bacteria	3.85	3.85	7.32	7.93	7.91	7.88
Staphylococci non-patogenic	5.50	4.93	5.94	6.40	6.48	5.69
Enterobacteriaceae	4.48	4.52	4.19	3.24	3.26	0.00
Total coliforms	4.23	4.51	4.24	3.03	2.96	0.00
Moulds and yeast	5.40	4.10	5.89	7.10	6.00	5.89
			D			
Aerobic mesophilic bacteria	5.55	5.66	8.68	8.95	8.72	8.95
Lactic acid bacteria	3.99	4.19	8.56	8.82	8.76	8.26
Staphylococci non-patogenic	3.48	4.48	4.91	6.14	6.73	4.08
Enterobacteriaceae	2.99	2.62	3.86	5.46	3.95	2.61
Total coliforms	3.70	3.18	3.83	5.53	4.45	2.22
Moulds and yeast	4.40	4.71	5.50	6.50	6.00	5.00

A: made at the start of the hunting season and ripened under controlled conditions; B: made at the end of the hunting season and ripened under controlled conditions; C: made at the start of the hunting season and ripened under natural conditions; D: made at the end of the hunting season and ripened under natural conditions.

tually the same for all four treatments, regardless of hunting-season stage. The counts showed similar trends, with a marked increase during the first week of ripening, coinciding with an increase in lactic acid bacteria; thereafter, values remained constant or declined slightly in the final stages of ripening. Similar results have been reported by DOMÍNGUEZ *et al.* (1989); GARCÍA *et al.* (1998), FERREIRA (2001) and FERNÁNDEZ *et al.* (1995) for fermented pork sausages, but higher than those recorded by SANZ *et al.* (1997) for sausages fermented without the addition of starter culture (6.0 log cfu/g).

Overall, there was a gradual decline in the Gram-negative bacteria, counts which are largely responsible for meat spoilage, and a marked increase in cocci and lactobacilli.

At day 0 and after, lactic acid bacteria counts for naturally-ripened treatments (C and D) remained practically constant, while the counts gradually increased in batches ripened under controlled conditions (A and B). This was particularly apparent in batch B, suggesting that fermentation had already begun in this batch when the count was performed. Thus the bacteria were in the exponential growth stage. Lactic acid bacterial counts increased in all four treatments after the first week, when these organisms were established as the dominant flora; thereafter, the values remained stable until the end of ripening and into packaging. Similar findings have been reported by GARRIGA *et al.* (1988), SANZ *et al.* (1988), DOMÍNGUEZ *et al.* (1989), SARASÍBAR *et al.* (1989), DÍAZ *et al.* (1993), SAMELIS *et al.* (1994), BELLO and SÁNCHEZ-FUERTES (1995), FERNÁNDEZ *et al.* (1995), COCOLIN *et al.* (2001), FERREIRA (2001) and NATOLA *et al.* (2002) in naturally fermented sausages with no starter culture added.

The non-pathogenic staphylococcus counts ranged from 3.5 to 5.5 log cfu/g. Counts for meat obtained at the end of the hunting season were lower than those obtained at the start of the season. Counts tended to decline over time in treatments ripened under controlled conditions (A and B), while under naturally-ripened batches the values rose steadily throughout the first three weeks of ripening. Similar results were reported by FERREIRA (2001) in *salchichon*. This difference could be due to paste acidification,

which is the main cause of *Staphylococcus* inhibition in dried fermented sausages (JOHANSSON *et al.,* 1994; SAMELIS *et al.,* 1994).

Total enterobacteria counts ranged from 2.9 log cfu/g in treatment D to 4.5 log cfu/g in the other treatments, highlighting the better hygiene quality of the meat used in treatment D. After mixing the ingredients together, the counts remained similar to those of meat, matching those reported by BALDINI *et al.* (1981), MENDOZA *et al.* (1983), LIZASO *et al.* (1999) and NATOLA *et al.* (2002) in pork sausages. Enterobacteria counts in treatments A, B and C started to decline after the first week of ripening.

Coliform counts in all treatments approached 4.0 log cfu/g, regardless of hunting-season stage. After the first week of ripening, values followed a pattern similar to that displayed by enterobacteria, similar to those reported by MENDOZA *et al.* (1983), HUERTA (2000) and FERREIRA (2001) for cured sausages. Counts decreased during storage, reaching zero for all treatments except batch D, which still displayed counts of 2.2 log cfu/g after vacuum packaging; a similar pattern was observed for the enterobacteria counts.

Initial mould and yeast counts (Table 1) were higher in venison meat obtained at the start of the hunting season. Over the first two weeks of ripening, values increased steadily in naturally-ripened treatments, falling thereafter to final values of 6 log cfu/g. In treatments ripened under controlled conditions, counts remained fairly constant throughout ripening (4-5 cfu/g). During packaging, counts remained stable in treatments C and D, but decreased in treatments A and B; naturally-ripened batches thus displayed much higher values.

E.coli and enterococci counts decreased during ripening and storage in all four treatments, meeting the requirements of the Spanish Microbiological Quality Standard (COMMISSIONS REGULATION 1441/2007).

Neither *Salmonella* nor *Listeria monocytogenes* were detected in the samples tested. Thus the requirements of Spanish Microbiological Quality Standard, which stipulates the absence of these organisms in a 25 g sample were met (COMMISSIONS REGULATION 1441/2007).

Physicochemical composition of venison

Table 2 shows the mean values (±SD) for the physicochemical parameters in the venison used for the four chorizo treatments analyzed.

No significant differences were noted as a function of hunting-season stage for a_w, dry matter (DM), salt and ash content. In all cases the a_w values were greater or equal to 0.980. Dry matter values roughly matched those recorded by PALEARI *et al.* (2003) for venison and the ash content values roughly matched those reported by ZOMBORSZKY *et al.* (1996) in the *Semimembranosus* and *Longissimus dorsi* muscles of 10-month-old deer, and by PALEARI *et al.* (2003) in venison.

Venison pH ranged from 5.48 to 5.83, and was therefore suitable for the production of raw cured sausage (5.4 < pH < 6.2); similar values were reported for venison by PALEARI *et al.* (2003).

Total protein content was higher in early-season meats. ZOMBORSZKY *et al.* (1996) reported a total protein content of 93.9% DM for the *Longissimus dorsi* muscle and 92.7% DM for the *Semimembranosus* in 10-month-old deer, while PALEARI *et al.* (2003) recorded a total protein content of 86.8% DM in venison.

The fat content of the venison used ranged from 1.0 to 2.6% DM for the meat used to make the early-season treatments and from 8.3 to 11.6% DM for end-of-season treatments. The difference could be due to a number of factors, including: sex (WENHANG and PENNIE, 1986), age (GREGSON and PURCHAS, 1985), feeding regimen (SUTTIE *et al.*, 1983; HOGG *et al.,* 1990), physiological and sexual status (WALLACE and DAVIES, 1985; HOOG *et al.,* 1990; STEVENSON *et al.*, 1992), and age (PEÑA *et al.*, 1993). ZOMBORSZKY

Table 2 - Mean values and standard deviations of the physicochemical composition of venison used to make the four chorizo sausage treatments.

	A	B	C	D
a_w	0.991±0.002	0.988±0.001	0.994±0.003	0.980±0.007
pH	5.77±0.37	5.48±0.01	5.83±0.11	5.59±0.02
DM (g/100 g sample)	21.97±0.33	22.87±0.19	22.31±0.39	22.36±1.54
Protein (g/100 g DM)	98.22^{a}±0.80	83.27^{c}±3.86	94.09^{b}±1.00	$87.09^{b,c}$±3.12
Fat (g/100 g DM)	1.04^{c}±0.43	8.26^{b}±0.02	2.55^{c}±0.63	11.57^{a}±1.43
NaCl (g/100 g DM)	1.06±0.09	1.28±0.05	1.17±0.17	0.91±0.12
Ash (g/100 g DM)	6.36±0.87	5.22±2.48	5.04±1.35	3.31±1.35

Different superscripts in the same row indicate significant differences (P<0.05).
DM: dry matter.
A: venison obtained at the start of the hunting season and used for sausages ripened under controlled conditions; B: venison obtained at the end of the hunting season and used for sausages ripened under controlled conditions; C: venison obtained at the start of the hunting season and used for sausages ripened under natural conditions; D: venison obtained at the end of the hunting season and used for sausages ripened under natural conditions.

et al. (1996) reported a fat content of 2.6% DM in the *Longissimus dorsi* of 10-month-old deer, and 4.5% DM in the *Semimembranosus* in late winter, while PALEARI *et al.* (2003) recorded a fat content of 8.0% DM in venison.

The meat used for early-season treatments probably contained less fat and more protein, since the rutting season was either still going on or had just finished, and animal fat reserves would therefore have been depleted.

Weight loss and physicochemical composition of venison chorizo sausages

The most evident change during drying was the weight loss caused by the elimination of water. Table 3 shows the mean values (±SD) for weight loss over the 21-day ripening period in all four chorizo treatments. After the first week, weight loss was greatest in treatment B; after the second week, treatment D displayed almost no weight loss. After the third and final week of ripening, batch D displayed its greatest weight loss, reaching a final weight similar to that of batch C. Weight loss values for controlled-ripened treatments (A and B) were higher than those of the two treatments dried under natural conditions (C and D).

Table 4 shows mean values (±SD) for the physicochemical parameters in the four chorizo treatments analyzed, throughout ripening and after 45 days of being vacuum packed. The four treatments differed significantly during ripening, in terms of water activity, pH and dry matter content.

After the first week, the pH values fell in all treatments; thereafter, and in the packaged product, pH values remained constant or increased, possibly due to enzyme-mediated formation of non-protein nitrogen and ammonium ions. By the end of ripening, both treatments dried under controlled conditions displayed the same pH value (4.96), which was lower than those of the naturally-ripened treatments (C: 5.13 and D: 5.40). Values and variations in pH were similar to those reported by other authors for chorizo made without added starter cultures or with sugar added (MENDOZA *et al.,* 1983; GARRIGA *et al.,* 1988; LIZARRAGA *et al.*, 1989; DOMÍNGUEZ *et al.,* 1989; FERNÁNDEZ *et al.*, 1995). VIOQUE *et al.* (2003) recorded pH values of 5.1-5.5 in commercial venison *salchichon* and 5.3-5.7 in commercial venison chorizo, while values reported by SORIANO *et al.* (2006) for commercial venison chorizo and *salchichon* ranged from 4.9-6.0 to 5.4-5.9, similar to those found here for packaged chorizo.

The a_w values declined, due to the solutes added (e.g. salt, carbohydrates), and especially due to the partial dehydration during ripening. Initial values approached 0.970 (after adding ingredients), falling thereafter in all treatments to below 0.877-0.903 after 21 days of ripening. Treatment B displayed the lowest a_w values throughout, and at the end of ripening (as well as treatment C), due to greater weight loss in the course of the process (Table 3). VIOQUE *et al.* (2003) reported a_w values of 0.89-0.78 and 0.90-0.76 for commercial venison *salchichon* and chorizo, respectively. SORIANO *et al.* (2006) recorded a_w values of 0.91-0.80 and 0.81-0.87 for commercial venison chorizo and *salchichon*, respectively.

Table 3 - Mean values and standard deviations for the weight loss (%) in venison chorizo sausages during ripening.

	Ripening time (days)		
	7	14	21
A	9.21^{c}±6.70	25.17^{b}±1.80	41.00^{a}±2.17
B	24.57^{b}±2.11	34.09^{b}±1.62	45.20^{a}±4.28
C	16.67^{b}±6.32	32.57^{a}±8.32	39.81^{a}±2.74
D	14.83^{b}±3.54	18.95^{b}±6.38	38.88^{a}±3.34

Different superscripts in the same row indicate significant differences ($P<0.05$).
A: made at the start of the hunting season and ripened under controlled conditions; B: made at the end of the hunting season and ripened under controlled conditions; C: made at the start of the hunting season and ripened under natural conditions; D: made at the end of the hunting season and ripened under natural conditions.

Finally, comparison of the a_w values with the microbiological results shows that the growth of spoilage microorganisms was inhibited as the a_w values decreased. This is to be expected, since in the early stages of chorizo ripening, salt and nitrites are the first obstacles to the growth of pathogenic and spoilage organisms. Due to the growth of other microorganisms which consume the oxygen present in the meat mass, there is a drop in redox potential, inhibiting the growth of aerobic organisms and favouring the selection of lactic acid bacteria, which, as competitive flora, grow rapidly, acidifying the medium, thus lowering the pH. During prolonged maceration, the main factor responsible for the stability of ripe sausage is a_w, which intensifies with time due to drying. Here, dry matter content rose progressively throughout ripening in all treatments; the final values were similar to those reported by VIOQUE *et al.* (2003) and SORIANO *et al.* (2006) for commercial venison chorizo and *salchichon* during the shelf-life of these products.

Although the meat of deer slaughtered at the start of the hunting season had a lower fat content, the percentage fat in the four chorizo treatments was fairly similar, because the fat content of the added pork rind differed among treatments: A (46.62±5.52 g/100 g DM), C (43.59±3.16 g/100 g DM), B (32.93±0.86 g/100 g DM) and D (32.17±4.89 g/100 g DM). Chorizo fat content ranged from 27.4 to 37.6% DM; these values were lower than those reported by VIOQUE *et al.* (2003) (44-58% DM) and SORIANO *et al.* (2006) (32-52% DM) for commercial venison chorizo and *salchichon*.

Protein content in the four chorizo treatments was around 46.3-51.2% DM, and these values remained relatively stable throughout the process. Sodium chloride values in treatments dried in controlled conditions were around 5.7% DM, compared to 7.2% DM in naturally-ripened treatments. Ash content was higher in naturally-ripened treatments, due to higher sodium chloride levels. Values for the protein, salt and ash contents were in line with those reported by VIOQUE *et al.* (2003) and SORIANO *et al.* (2006) for commercial venison sausages.

Sensory characteristics

Table 5 shows the scores given by tasters for the organoleptic parameters. All treatments presented a similar intensity and quality of odour, and the same appearance: light yellow fat, dark red lean meat and the same amount of visual fat, except treatment C that had the highest amount of visible fat. All treatments were similar with respect to juiciness. On the other hand, treatments dried under controlled conditions differed from those ripened naturally with respect to several sensory characteristics. Thus, treatments dried under controlled conditions (A and B) were scored significantly higher for spice and ripened odour, taste intensity, pungency, aftertaste intensity and hardness, since constant temperature and relative humidity cause greater moisture loss than natural drying conditions. Scores for attributes such as pungency and bitterness, which might be considered defects, were lower than 3.7 in all treatments. Flavour intensity and aftertaste intensity received scores of

Table 4 - Mean values and standard deviations of the physicochemical characteristics and composition of the venison chorizo sausages during ripening and after 45 days of vacuum-packed storage.

	Ripening time (days)				Storage
	0	7	14	21	
			A		
pH	5.32^{a}±0.061	4.91^{b}±0.02	4.81^{c}±0.01	4.96^{b,A}±0.046	4.99^{b}±0.049
a_w	0.973^{a}±0.003	0.950^{b}±0.005	0.924^{c}±0.001	0.903^{d,B}±0.013	0.849^{e}±0.010
DM (g/100 g sample)	35.53^{e}±0.62	44.81^{d}±0.21	54.88^{c}±1.20	59.90^{b,A,B}±2.96	67.27^{a}±0.57
Fat (g/100 g DM)	37.30±1.19	34.55±2.06	36.96 ±1.19	36.19±3.90	34.12±0.92
Protein (g/100 g DM)	50.73±3.01	46.31±0.97	49.31±2.82	48.03±2.99	47.47±2.32
NaCl (g/100 g DM)	5.95±0.16	5.71±0.04	5.61±0.18	5.83^{A}±0.23	5.55 ±0.12
Ash(g/100 g DM)	7.63±1.07	7.67±0.82	7.68±0.35	7.99^{A}±0.32	7.95±0.07
			B		
pH	5.20^{a}±0.04	4.95^{c}±0.04	4.94^{c}±0.02	4.96^{c,A}±0.04	5.11^{b}±0.06
a_w	0.966^{a}±0.001	0.940^{b}±0.003	0.915^{c}±0.007	0.877^{d,A}±0.008	0.827^{e}±0.004
DM (g/100 g sample)	38.61^{e}±0.26	48.53^{d}±1.63	56.79^{c}±2.02	64.28^{b,A}±1.54	69.46^{a}±1.04
Fat (g/100 g DM)	37.56±2.80	36.35±2.32	32.98±2.23	30.93±2.40	32.49±2.92
Protein (g/100 g DM)	46.40±0.75	47.84±1.04	48.69±3.03	48.78±0.86	49.93±0.56
NaCl (g/100 g DM)	5.18±0.15	5.95±0.46	5.56±0.15	5.78^{A}±0.06	5.86±0.12
Ash (g/100 g DM)	8.30±0.82	8.17±0.52	8.39±0.40	8.52^{A}±0.28	8.52±0.33
			C		
pH	5.72^{a}±0.03	5.09^{c}±0.17	5.23^{b,c}±0.07	5.13^{b,c,B}±0.22	5.32^{b}±0.01
a_w	0.972^{a}±0.001	0.946^{b}±0.005	0.911^{c}±0.004	0.887^{d,A}±0.025	0.879^{d}±0.003
DM (g/100 g sample)	34.18^{d}±1.12	42.76^{c}±2.66	52.71^{b}±0.91	59.23^{a,A,B}±4.01	59.58^{a}±0.18
Fat (g/100 g DM)	32.07±4.46	27.36±3.61	30.37±0.38	30.78±1.56	29.18±3.77
Protein (g/100 g DM)	50.72±1.93	50.99±2.62	50.97±1.94	46.94±3.71	51.17±1.24
NaCl (g/100 g DM)	7.55±0.44	7.23±0.08	7.40±0.24	7.12^{B}±0.14	7.15±0.28
Ash (g/100 g DM)	10.02±0.39	9.69±0.35	9.45±0.77	10.16^{B}±0.75	9.67±0.51
			D		
pH	5.68^{b}±0.01	5.07^{e}±0.02	5.32^{d}±0.06	5.40^{c,C}±0.11	5.76^{a}±0.03
a_w	0.969^{a}±0.002	0.952^{b}±0.002	0.944^{c}±0.004	0.902^{d,B}±0.004	0.849^{e}±0.003
DM (g/100 g sample)	35.10^{e}±0.19	41.46^{d}±0.74	43.15^{c}±0.88	56.26^{b,B}±2.20	65.82^{a}±0.86
Fat (g/100 g DM)	33.29±2.94	35.05±1.11	36.57±1.25	34.45±0.63	33.54±1.62
Protein (g/100 g DM)	47.38±2.30	47.19±1.58	50.94±2.35	50.03±0.76	49.37±1.34
NaCl (g/100 g DM)	6.86±0.06	6.65±0.21	7.18±0.09	7.38^{B}±0.04	7.01±0.19
Ash (g/100 gDM)	8.98±0.27	8.72±0.25	9.42±0.41	9.67^{B}±0.51	9.21±0.19

Different superscripts in the same row indicate significant differences (P<0.05).
Different superscripts in capital in the 21 day column and for the same parameter indicate significant differences (P<0.05).
DM: dry matter.
A: made at the start of the hunting season and ripened under controlled conditions; B: made at the end of the hunting season and ripened under controlled conditions; C: made at the start of the hunting season and ripened under natural conditions; D: made at the end of the hunting season and ripened under natural conditions.

Table 5 - Mean values and standard deviation of the sensory characteristics of venison chorizo sausages.

	A	B	C	D
Appearance				
Amount of fat	2.96^{c}±0.19	3.68^{b}±0.77	5.82^{a}±0.16	3.14^{b}±0.25
Fat colour	4.14±0.39	5.44±2.16	3.84±0.08	2.82±0.59
Lean colour	8.75±0.68	8.80±0.08	7.38±0.17	8.81±0.02
Odour				
Odour intensity	7.14±1.59	5.17±0.30	6.20±0.27	5.67±0.63
Odour quality	6.72±0.30	4.81±0.67	4.74±0.50	5.44±1.34
Paprika odour	1.20±0.11	2.31±1.40	0.50±0.30	0.00±0.00
Spice odour	6.48^{a}±0.51	5.89^{a}±0.27	3.49^{b}±0.09	1.25^{c}±0.81
Ripened odour	7.61^{a}±0.65	6.91^{a,b}±0.38	4.99^{b}±0.10	6.54 ^{a,b}±0.93
Taste				
Taste intensity	7.77^{a}±0.16	7.23^{a}±0.57	5.92^{b}±0.42	5.92^{b}±0.01
Acidity	6.22^{a}±0.40	4.71^{b}±0.85	1.74^{c}±0.21	3.56^{b}±0.22
Saltiness	5.41^{a,b}±0.50	4.69^{b}±0.00	5.73^{a}±0.09	4.53^{b}±0.08
Sweetness	0.00^{b}±0.00	1.37^{a}±0.62	0.26^{a,b}±0.02	0.49^{a,b}±0.24
Bitterness	3.65^{a}±0.34	0.00^{b}±0.00	0.00^{b}±0.00	1.11^{b}±0.66
Pungent flavour	3.18^{a}±1.06	3.53^{a}±0.23	0.00^{b}±0.00	1.23^{a,b}±0.81
Aftertaste intensity	7.53^{a}±0.03	7.26^{a}±0.62	5.42^{b}±0.16	6.14^{b}±0.05
Aftertaste quality	6.35±0.54	5.21±0.14	4.70±0.05	5.19±0.89
Texture				
Hardness	7.44^{a}±0.31	5.96^{b}±0.16	7.73^{a}±0.21	4.53^{c}±0.66
Juiciness	5.68±0.80	3.57±0.04	2.66±0.22	4.19±0.14
Chewiness	2.49^{b}±0.56	6.87^{a}±0.05	3.34^{b}±1.46	3.17^{b}±0.35
Fat mouthfeel	5.19^{a}±0.29	3.39^{b}±0.24	3.23^{b}±0.08	4.05^{b}±0.45

Different superscripts in the same row indicate significant differences (P<0.05).
A: made at the start of the hunting season and ripened under controlled conditions; B: made at the end of the hunting season and ripened under controlled conditions; C: made at the start of the hunting season and ripened under natural conditions; D: made at the end of the hunting season and ripened under natural conditions.

5.5-7.8. Off-odours and off-flavours were not detected in any of the treatments.

Proteolysis (data shown in GARCÍA RUIZ *et al.*, 2007a,b) was less intense in treatment C than in the other treatments, possibly due to a higher salt content and lower temperatures during ripening. This batch received lower scores for cured odour, quality odour, acidity, intensity and quality aftertaste.

CONCLUSIONS

The fermentation of venison chorizo sausages, prepared without the addition of starter cultures and ripened under controlled or natural conditions for 21 days, is mainly due to lactic acid bacteria, which establish as dominant flora after the first week of ripening. On the other hand, the evolution of the control parameters, pH, a_w and moisture content, during ripening is similar to other types of dried sausages made with pork and/or beef. The venison chorizo sausages dried under controlled conditions differed from those that were naturally-ripened with respect to a lower pH and lower moisture content during curing, and a greater weight loss at the end of ripening. With respect to the sensorial characteristics, venison chorizos ripened under controlled conditions had a more perceptible aroma, greater taste intensity, and more pungency and aftertaste flavour, and a greater hardness than naturally-ripened chorizos.

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PAPER

FRUIT AND OIL CHARACTERISTICS OF THE MAIN SYRIAN OLIVE CULTIVARS

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ABSTRACT

In the three-year period 2004-06, a study was carried out to characterise the fruit and oil of the main Syrian olive cultivars: Kaissy and Zaity in the Aleppo area, Dan, Hemplasi and Souri in the Damascus area, Insassy, Karamani and Sorani in the Idleb area, Khodeiri in the Lattakia area, Safrawi in the Hama (Mousiaf) area and Doebli in the Tartous area. Olive samples were collected in October or November and the fruit weight, epicarp and mesocarp pigmentation and oil content were determined. Oil was also extracted from the samples and the acidity, peroxide value, spectrophotometric absorbance at 232 and 270 nm (K_{232} and K_{270}) and ΔK, fatty acid composition, sterol composition and content and erythrodiol + uvaol content were determined. Marked differences were recorded in the cultivars for all the fruit characteristics studied. The oleic acid content was around 70% in most of the oils. The Karamani cultivar had a low oleic acid value (60%) and high palmitic and linoleic acid values. The average values of the parameters (acidity, peroxide value, absorbance in ultraviolet, fatty acid composition, sterol composition and content, erythrodiol + uvaol content) used to evaluate oil quality were within the International Olive Council (IOC) trade standards for extra virgin olive oils, with the exception of Δ-7-stigmastenol values which were higher than 0.5%, the maximum value established by such standards, in the cultivars Khodeiri and Doebli. The average Δ-7-stigmastenol values were as high as 0.5% or very close to 0.5% in the cultivars Insassy and Sorani, respectively, indicating that, also in some replicates of these cultivars, the Δ-7-stigmastenol values were higher than 0.5%.

- Key words: apparent β-sitosterol, erythrodiol + uvaol content, fatty acid composition, olive germplasm, *Olea europaea* L., Δ-7-stigmastenol, sterol compostion and content, Syrian olive oil characteristics -

INTRODUCTION

Syria, being part of the original habitat of *Olea europaea* L., has a very rich olive germplasm. Although there are about seventy varieties cultivated in the different areas of the country (NSEIR *et al.*, 1985; GCSAR, 2007), there are very few that have been extensively cultivated. In fact, five cultivars, Zaity, Sorani, Doebli, Khodeiri and Kaissy, represent about 90% of the total olive trees cultivated in Syria (about 33, 30, 12, 10 and 5%, respectively) (AL IBRAHEM, 2006). The others are local varieties that have a limited distribution. Up to now, very few studies have been carried out to evaluate the agronomic and productive characteristics of Syrian olive cultivars (NSEIR *et al.*, 1985; TUBEILEH *et al.*, 2008a,b), and very little is known about the characteristics of the oils (TUBEILEH *et al.*, 2008b). This last aspect has become increasingly more important, since consumers are more and more sensitive about the quality of olive oil. Moreover, as a result of the large number of new olive orchards that have been planted in Syria in the last decades, an increasing amount of oil is available for export, that must meet the qualitative standards required by the international market.

More knowledge about the productive characteristics and oil quality of Syrian olive cultivars is useful for making the best choice of varieties for new olive orchards. Moreover, it is useful to determine if the oils produced by the different cultivars meet the qualitative standards required by the international market.

The aim of this study was to evaluate the fruit characteristics and oil quality of the main Syrian olive cultivars, by evaluating them in the areas where they have the largest distribution. The investigation was carried out as a part of a larger study aimed at fully characterizing the morphological, biological, agronomic and oil characteristics of the main Syrian olive cultivars (ABDINE *et al.*, 2007). Oil characteristics were also evaluated by comparing them with the International Olive Council (IOC) trade standards for extra virgin olive oils (COI/T.15/NC no. 3/Rev. 3 - November 2008).

MATERIAL AND METHODS

The study was carried out in the three-year period 2004-06. The cultivars taken into account were: Kaissy (also known as Qaisi) and Zaity in the Aleppo area (where about 30% of Syria's olive cultivation is concentrated), Dan, Hemplasi and Souri in the Damascus area (about 3.0%), Insassy, Karamani and Sorani in the Idleb area (about 22%), Khodeiri in the Lattakia area (about 7%), Safrawi in the Hama (Mousiaf) area (about 6%) and Doebli in the Tartous area (about 13%). The areas taken into account differ with respect to environmental conditions, especially annual rainfall: 350-450 mm in Aleppo and Hama, 200-250 mm in Damascus, 450-500 mm in Idleb and 800-900 mm in Lattakia and Tartous (AL IBRAHEM, 2006). The olives of all these cultivars are also used as table olives in amounts depending on the cultivar (ABDINE *et al.*, 2007).

In each area, 3-5 trees per cultivar were selected from germplasm collections or from private farms and labelled. Fruit samples were harvested within the normal harvesting times (October – December) in Syria: in November, for the cultivars Dan, Doebli, Hemplasi, Insassy, Karamani, Safrawi, Sorani, Souri and Zaity, and in October, for the cultivars Doebli and Khodeiri (that are harvested earlier). The samples from the labelled trees were used to determine: fruit weight (50 drupes/tree); pigmentation (50 drupes/tree) by using the "Jaèn pigmentation index", from 0 to 7, with 0 for green olives and 7 for olives with superficial pigmentation on 100% of the epicarp and 100% pigmentation on the pulp; water content by drying the samples used for fresh weight determination in an oven until constant weight; oil content (1 sample/tree) by using a Soxhelet apparatus.

The oil was extracted from 2-3 kg of olives (2 samples/cultivar/year) using a lab hammer mill; the paste was malaxed for 30 min, then centrifuged and the oil was separated from the water and filtered through a filter plate. On the oil, acidity (ISO 660), peroxide value (ISO 3960), spectrophotometric absorbance at 232 and 270 nm (K_{232} and K_{270}) and at 264, 268 and 272 nm (K_{264}, K_{268}, and K_{272}) to determine $\Delta K = K_{268} - (K_{264} + K_{272})/2$ (COI/T.20/Doc.n.19), fatty acid composition (COI/T.20/Doc.n.24), sterol composition and content and erythrodiol + uvaol (triterpene alcohols) content (COI/T.20/Doc.n.10) were determined according to the procedures indicated within parentheses.

As far as oil characteristics are concerned, the IOC trade standard values for extra virgin olive oils (COI/T.15/NC no. 3/Rev. 3 - November 2008) are reported in the tables.

As a preliminary statistical analysis of data did not show substantial year effects, data is reported in tables as means of the three-year period 2004-06±standard error.

RESULTS

The cultivars in the different environments showed notable differences in all the fruit characteristics taken into account (Table 1). Fresh fruit weight was high for the Kaissy and Safrawi cultivars, medium for Zaity, Dan, Hemplasi, Souri, Karamani, Khodeiri, Sorani and Doebli and medium-low for Insassy. The level of pigmentation was very different among the cultivars. The oil content was high in Zaity and Sorani, medium-high in Khodeiri, medium in Insassy, Karamani and Doebli and relatively low in the other cultivars.

The acidity and peroxide value of the oils of all the cultivars were very low (Table 2). K_{232}, K_{270}

Table 1 - Fruit characteristics of eleven Syrian olive cultivars. Average values of the three-year period 2004-06±standard error. Olive samples were collected in November for all the cultivars with the exception of the cultivars Khodeiri and Doebli which were collected in October.

Area	Cultivar	Fresh weight (g)	Pigmentation index (0-7)	Water content (%)	Oil content (% f.w.)	Oil content (% d.w.)
Aleppo	Kaissy	4.3± 0.8	1.3±0.5	54.9±3.8	15.7±1.2	34.8±5.1
	Zaity	2.3±0.3	2.7±0.8	41.4±2.7	27.2±4.3	46.4±8.0
Damascus	Dan	2.7±0.4	2.4±0.4	47.2±2.3	14.8±0.7	28.0±2.0
	Hemplasi	2.3±0.3	2.6±0.4	52.8±2.3	14.5±1.2	30.7±1.8
	Souri	2.7±0.4	2.2±0.4	47.4±2.1	17.5±1.9	33.3±4.4
Idleb	Insassy	1.9±0.3	2.3±0.3	45.5±1.9	23.2±2.0	42.2±3.5
	Karamani	3.2±0.4	1.8±0.3	53.3±2.4	19.7±1.7	42.2±2.3
	Sorani	3.0±0.4	1.9±0.4	45.0±2.6	25.8±2.6	46.9±2.8
Lattakia	Khodeiri	2.1±0.3	1.0±0.2	41.0±2.3	25.3±1.4	42.9±2.0
Mousiaf	Safrawi	5.0±0.9	2.9±0.4	48.4±2.3	18.2±2.1	35.3±3.1
Tartous	Doebli	3.3±0.3	1.2±0.4	46.7±1.9	21.9±1.8	41.1±2.8

and ΔK values were also low. All the cultivars had an oleic acid content around 70%, with the exception of Karamani, which had a value of about 60% associated with relatively high amounts of palmitic and linoleic acids (Table 3). The sterol composition and content and erythrodiol + uvaol content were quite different (Table 4). The Khodeiri and Doebli cultivars had relatively high Δ-7-stigmastenol values, followed by the cultivars Insassy and Sorani.

The average values of all the parameters (acidity, peroxide value, absorbencies in ultra-violet, fatty acid composition, sterol composition and content, erythrodiol + uvaol content) used to evaluate oil characteristics were within the IOC trade standards, with the exception of those of the Δ-7-stigmastenol in the cultivars Khodeiri and Doebli that were higher than 0.5%, which is the maximum value allowed by IOC trade standards. It should be noted that the average values of the cultivars Insassy and Sorani were as high as 0.5% or very close to 0.5%, respectively, indicating that, also for these cultivars, in some cases (replicates) the values were higher than 0.5%. In all the oils, on average, the content of apparent β-sitosterol (β-sitosterol + Δ-5-

Table 2 - Free acidity, peroxide value, spectrophotometric absorbencies (K_{232}, K_{270}, ΔK) of oils of eleven Syrian olive cultivars. Average values of the three-year period 2004-06±standard error. The IOC trade standard (TS) values for extra virgin olive oils are reported in the last line. Oils were extracted from olive samples collected in November for all the cultivars with the exception of those of the cultivars Khodeiri and Doebli which were extracted from olive samples collected in October.

Area	Cultivar	Free acidity (%)	Peroxide value (meq O_2/kg oil)	K_{232}	K_{270}	ΔK
Aleppo	Kaissy	0.3±0.08	6±0.9	1.66±0.07	0.17±0.03	0.002±0.001
	Zaity	0.3±0.04	5±0.9	1.68±0.09	0.13±0.03	0.002±0.001
Damascus	Dan	0.2±0.20	5±0.8	1.90±0.07	0.15±0.02	0.002±0.001
	Hemplasi	0.2±0.02	5±0.4	1.76±0.06	0.15±0.02	0.005±0.002
	Souri	0.3±0.01	6±1.8	1.99±0.01	0.15±0.01	0.004±0.002
Idleb	Insassy	0.3±0.04	7±1.1	1.77±0.17	0.15±0.02	0.005±0.001
	Karamani	0.4±0.06	8±1.6	1.95±0.10	0.20±0.03	0.004±0.002
	Sorani	0.4±0.02	7±1.6	1.89±0.07	0.19±0.01	0.004±0.001
Lattakia	Khodeiri	0.4±0.04	8±1.5	1.77±0.08	0.17±0.02	0.005±0.002
Mousiaf	Safrawi	0.6±0.01	4±0.2	1.62±0.11	0.12±0.02	0.004±0.002
Tartous	Doebli	0.4±0.06	6±0.2	1.63±0.04	0.17±0.01	0.004±0.001
IOC-TS		≤0.8	≤20	≤2.50	≤0.22	≤0.01

Table 3 - Fatty acid composition of oils of eleven Syrian olive cultivars. Average values of the three-year period 2004-06±standard error. The IOC trade standard (TS) values for extra virgin olive oils are reported in the last line. Oils were extracted from olive samples collected in November for all the cultivars with the exception of those of the cultivars Khodeiri and Doebli which were extracted from olive samples collected in October.

Area	Cultivar	Palmitic (%)	Palmitoleic (%)	Stearic (%)	Oleic (%)	Linoleic (%)	Linolenic (%)	Arachidic (%)
Aleppo	Kaissy	11.9±0.4	0.69±0.17	3.07±0.26	72.4±1.0	9.7±0.2	0.65±0.03	0.44±0.03
	Zaity	13.8±0.2	0.75±0.05	3.65±0.13	71.8±1.8	8.8±0.2	0.61±0.04	0.54±0.01
Damascus	Dan	13.7±0.4	0.60±0.03	2.14±0.02	71.2±0.9	10.1±0.5	0.71±0.04	0.35±0.02
	Hemplasi	12.7±0.3	0.88±0.03	2.86±0.13	73.7±0.9	7.8±0.1	0.69±0.08	0.45±0.01
	Souri	12.7±0.4	0.67±0.13	2.90±0.21	72.7±1.7	9.4±0.3	0.61±0.09	0.41±0.03
Idleb	Insassy	11.5±0.3	0.36±0.02	3.66±0.12	71.6±0.9	11.5±0.2	0.55±0.01	0.46±0.01
	Karamani	18.4±0.3	1.66±0.11	2.33±0.15	59.9±1.9	14.4±0.6	0.79±0.03	0.42±0.03
	Sorani	14.6±0.5	0.72±0.07	3.40±0.19	68.2±1.9	11.3±0.7	0.70±0.04	0.47±0.03
Lattakia	Khodeiri	13.4±0.3	0.50±0.05	3.93±0.32	71.4±1.7	9.1±0.5	0.55±0.05	0.53±0.02
Mousiaf	Safrawi	12.9±0.4	0.98±0.08	2.69±0.09	70.6±1.2	11.1±0.4	0.50±0.03	0.50±0.01
Tartous	Doebli	14.6±0.2	0.73±0.04	2.97±0.02	69.7±0.8	11.0±0.4	0.53±0.05	0.43±0.02
IOC-TS		7.5- 20.0	0.3-3.5	0.5-5.0	55.0-83.0	3.5-21.0	≤1.0	≤0.6

Table 4 - Sterol composition and content and erythrodiol + uvaol content in the oils of eleven Syrian olive cultivars. Average values of the three-year period 2004-06±standard error. The IOC trade standard (TS) values for extra virgin olive oils are reported in the last line. Oils were extracted from olive samples collected in November for all the cultivars with the exception of those of the cultivars Khodeiri and Doebli which were extracted from olive samples collected in October.

Area	Cultivar	Cholesterol (%)	Brassicasterol (%)	Campesterol (%)	Stigmasterol (%)	β-sitosterol (%)	Δ-7-Stigmastenol (%)	Apparent β-sitosterol (%)	Total sterol (mg/kg oil)	Erythrodiol + Uvaol (% total sterols)
Aleppo	Kaissy	0.05±0.03	0.00±0.00	3.48±0.30	0.67±0.13	86.7±0.4	0.32±0.01	95.0±0.2	1,361±45	1.20±0.10
	Zaity	0.05±0.02	0.00±0.00	3.66±0.31	0.72±0.12	86.4±0.8	0.25±0.03	93.9±0.3	1,270±62	1.38±0.28
Damascus	Dan	0.04±0.02	0.00±0.00	2.76±0.31	0.88±0.16	89.4±0.2	0.30±0.12	95.5±0.3	1,398±132	1.80±0.16
	Hemplasi	0.04±0.02	0.01±0.00	3.28±0.22	0.44±0.11	89.2±0.4	0.28±0.10	95.0±0.4	1,704±161	1.69±0.15
	Souri	0.02±0.01	0.00±0.00	2.30±0.12	0.70±0.10	88.8±0.3	0.37± 0.06	95.7±0.3	1,105±82.2	1.10±0.15
Idleb	Insassy	0.05±0.02	0.00±0.00	3.55±0.45	0.78±0.02	88.8±1.1	0.50±0.02	93.3±0.3	1,382±55	1.90±0.15
	Karamani	0.05±0.02	0.00±0.00	2.61±0.28	0.72±0.15	85.8±0.2	0.30±0.02	95.6±0.2	1,219±85	1.78±0.16
	Sorani	0.05±0.02	0.00±0.00	3.67±0.22	0.71±0.08	88.6±0.3	0.46±0.02	94.4±0.2	1,363±105	1.90±0.50
Lattakia	Khodeiri	0.01±0.01	0.00±0.00	2.55±0.23	0.80±0.19	87.0±0.8	0.57±0.03	95.2±0.2	1,126±60	2.08±0.20
Mousiaf	Safrawi	0.05±0.02	0.00±0.00	3.45±0.2	0.72±0.06	90.1±0.2	0.28±0.06	94.6±0.2	1,770±111	1.70±0.70
Tartous	Doebli	0.04±0.01	0.00±0.00	2.60±0.25	0.84±0.31	88.7±0.2	0.57±0.05	94.3±0.2	1,449±182	1.08±0.30
IOC-TS		≤0.5	≤0.1	≤4.0	<campesterol		≤0.5	≥93.0	≥1,000	≤4.5

avenasterol + Δ-5-23-stigmastadienol + clerosterol + sitostanol + Δ-5-24-stigmastadienol) was higher than 93.0%, the IOC trade standard for this parameter.

DISCUSSION AND CONCLUSIONS

The fresh fruit weight and oil content in Zaity, Sorani and Kaissy were substantially similar to values reported in the literature for the same cultivars (TUBEILEH *et al.*, 2004, 2008a,b). The cultivar Dan showed a lower oil content than that reported by TUBEILEH *et al.* (2004); this difference could be due to harvesting time/ripening stage of the olives, since significant increases in oil content can be obtained by delaying harvesting (ABDINE *et al.*, 2007).

The high-medium oil content in Zaity, Sorani, Khodeiri and Doebli indicates their high efficiency in accumulating oil in the fruit which is in agreement with the fact that these cultivars are the main ones for oil production in Syria (these 4 varieties cover about 85% of the total area cultivated with olive) (AL IBRAHEM, 2006). The medium or high fresh fruit weight, as well

as the moderate oil content of the varieties Kaissy, Karamani and Safrawi, justify their use as dual purpose cultivars, with Kaissy being the main Syrian cultivar used for the production of table olives (ABDINE *et al.*, 2007). The relatively good fruit weight of the cultivar Sorani justifies the use of the drupes also as table olives. Indeed, while most of the fruit of the variety Sorani is used for oil production, Sorani is also among the main Syrian cultivars used for the production of table olives, especially black ones (ABDINE *et al.*, 2007). The dry environmental conditions of the Damascus area have probably contributed to determining the relatively low oil content in the cultivars Dan, Hemplasi and Souri.

As far as oil characteristics are concerned, low acidity and peroxide values were also observed in another study concerning the cultivars Kaissy, Zaity and Sorani (TUBEILEH *et al.*, 2008b). With respect to the present work, TUBEILEH *et al.* (2008b) reported slightly higher values for palmitic acid and lower values for oleic acid for the cultivars Kaissy and Zaity; these differences could be due to the different environmental conditions, especially temperature, and fruit ripening stage, that can affect fatty acid composition (INGLESE *et al.*, 2009).

The Karamani cultivar had relatively low oleic acid values and high palmitic and linoleic acid values. It is known that high environmental temperatures tend to reduce the oleic acid content and increase the palmitic and linoleic acids (INGLESE *et al.*, 2009) but, since the Idleb area is not the warmest among those considered, it seems that the observed fatty acid composition is mainly a characteristic of the variety.

The sterol composition and content as well as the erythrodiol + uvaol content differed in the cultivars; this is in agreement with the fact that these compounds are greatly dependent on cultivar (APARICIO *et al.*, 1997; RANALLI *et al.*, 2002; SÁNCHEZ CASAS *et al.*, 2004; QUESLATI *et al.*, 2009).

All the oils produced by the olive cultivars considered meet the IOC trade standards applied to extra virgin olive oils, except the cultivars Khodeiri and Doebli, which showed average of Δ-7-stigmastenol values that were higher than the IOC limit. Also in some replicates of the cultivars Insassy and Sorani the values were higher than 0.5%; indeed, the reported average values were 0.5% or very close to this value. In this regard, also other measurements for the cultivar Sorani, recorded in 2008 and 2009, were between 0.4 and 0.6, and, therefore, in some cases the values were higher than 0.5% (Jbara, unpublished data). It is important to note that this value is used as an indicator of the purity of the oil, because it can indicate possible adulteration (admixtures with other vegetable oils), but some varieties naturally have relatively high contents. Apart from the cultivars taken into account in the present work, values higher than 0.5% have been observed in oils of other varieties, such as Nebbio (RANALLI and ANGEROSA, 1996), Empeltre (GÓMEZ, 2001), especially in extremely dry seasons, Cornicabra (SALVADOR *et al.*, 1998; RIVERA DEL ÁLAMO *et al.*, 2004), Nociara and Termite di Bitetto (LOMBARDO *et al.*, 2004) and Colombaia (LANTERI *et al.*, 2003; BOGGIA *et al.*, 2005). Besides being affected by the variety, the sterol content and composition have also been reported to be affected by fruit ripening (DE LA TORRE-BORONAT *et al.*, 1985; GUTIÉRREZ *et al.*, 1999; SALVADOR *et al.*, 2001) and agro-environmental conditions (STEFANOUDAKI *et al.*, 2001; CECI and CARELLI, 2007; BEN TEMIME *et al.*, 2008). In the hot, arid region of Catamarca in Argentina oils from Coratina showed a Δ-7-stigmastenol value that was higher than the IOC limit (CECI and CARELLI, 2007), whereas this value was within the IOC limit in the oils obtained in Italy (LOMBARDO *et al.*, 2004). With reference to environmental conditions and olive ripening, the cultivars Doebli and Khodeiri showed average Δ-7-stigmastenol values that were higher than 0.5% also in oils obtained from olives produced in different areas in Syria (Jbara, unpublished data) or from olives harvested in periods different from that considered in the present work (ABDINE *et al.*, 2007). However, in 2009, a cool and rainy year, the Khodeiri cultivar showed relatively lower values (Jbara, unpublished data). In other studies, values exceeding the upper limits established by the IOC standards were also found for other sterol compounds, such as campesterol (RIVERA DEL ÁLAMO *et al.*, 2004).

The overall results regarding the oil characteristics are very important because of the increasing amounts of Syrian olive oil that will be available for export in the coming years.

It should be noted that the different cultivars were studied in order to obtain information about characteristics of the olives and the olive oils obtained in Syria. The study considered all the main Syrian olive varieties in the areas where they are mostly located. The olives were harvested according to the normal local practices. The different cultivars were cultivated in different environments and the ripening stage of olives was not uniform. Therefore, the results reported are dependent on the cultivar and could also have been affected by the differences in the environmental conditions in the different areas considered as well as the ripening stage of the olives.

In conclusion, the results of the study show that good fruit and oil can be produced with the main olive cultivars cultivated in Syria; the olive oil produced meets the IOC standards. One concern regards the Δ-7-stigmastenol content; some cultivars had values higher than 0.5%, the maximum value established

by the IOC trade standards. The results of this study should be considered when updating the international rules/standards regarding the trading of olive oil.

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PAPER

FRUIT RIPENING IN RELATIONSHIP TO OIL QUALITY AND SOME QUALITY CHARACTERISTICS OF THE GREEK OLIVE CULTIVAR KORONEIKI

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ABSTRACT

Extra virgin olive oil obtained from the olive cultivar Koroneiki from different regions of southern Greece was analysed for acidity, peroxide value and specific extinction coefficients (K_{232}, K_{270}). Fatty acids, total phenols and o-diphenols, the phenolic profile and oxidative stability were determined. Sensory analysis of the oil was carried out. The oils had fatty acid compositions similar to a commercial extra virgin olive oil. The oxidative resistance and the content of phenols and o-diphenols were higher in the olive oils produced by the low-scale mill compared to the commercial extra virgin olive oils produced by the industrial plant. Determination of sterols was also carried out on olive oil obtained from mature and immature fruit. Acidity, K_{232} and K_{270} and peroxide values were within the limits of extra virgin olive oil. The sterol content of the extra virgin olive oils from November 2008, December 2008 and January 2009 all fell within permissible levels. Hence, olive oil produced in 2009 and 2008 came from fruit that contained fully developed oil since the sterol fractions were balanced. No qualitative differences were reported in extra virgin olive oil samples obtained from different regions of southern Greece, showing a good homogeneity of the fruit. With reference to orthonasal perceptions, extra virgin olive oils from the Koroneiki variety, (either mature or immature) were well balanced between grassy (39%) and green tomato (16.2%). Considering the flavors retronasally perceived, the Koroneiki oils showed a good balance between grassy and green almond (10%) and green tomato (25%), with the presence of artichoke flavor (approx. 15%).

- Key words: Koroneiki cultivar, olive oil, quality parameters, sensory evaluation, sterol determination -

INTRODUCTION

Olive oil is one of the most precious products in Greece. Over 350,000 families earn their living from olive oil production, with 200,000-300,000 tons produced annually, amounting to about 18% of the total world production. 30% of the Greek olive oil is produced on the island of Crete Island, 26% in Peloponnisos, 10% on the island of Lesvos, 10% on the Ionian Islands and the remaining 24% is scattered around the rest of Greece. Greeks are listed as the highest per capita consumers of olive oil at 20 liters per person per year, twice as much as Italians and Spanish (KIRITSAKIS, 1998).

Of the many oil producing cultivars of *Olea europaea* in Greece, the most common and commercially important ones are Koroneiki, Manaki, Mastoidis, Megaritiki, Valanolia Chondrolia with Koroneiki being the most popular and widely grown (KIRITSAKIS, 2007). In the Messinia area every year approximately 50,000 tonnes of olive oil are manufactured, with Koroneiki being the most common cultivar of the region (92%), followed by Mavroelia, Mastoidis and the table olive cultivar, Chondrolia. Koroneiki is a cultivar widely distributed around the world, producing olive oil of a shiny green colour with a rich aroma, delicate odour and low acidity. Of the oil produced, 95% is extra virgin, since the acidity is much lower than the maximum permissible level (0.8% oleic acid) of the EU (COUNCIL REGULATION (EC) No 865/2004) and other parameters such as the peroxide number and the specific extinction coefficients which are also below the maximum permissible EU levels. Fatty acid analysis of Messinian olive oil is 70-80% mono-unsaturated, whereas only 10% is polyunsaturated, which corresponds to the composition of maternal milk. The European Union recognized the special characteristics of Messinian virgin olive oil and awarded the distinction of protected designation of origin (EC REGULATION 1065/97).

Four principal classes of steroidal alcohols can be found: 4-desmethylsterols (also called sterols), 4-monomethylsterols, 4,4'-dimethylsterols (also called triterpenic alcohols) and triterpenic diols. According to VLAHAKIS and HAEZBROEK (2000), evaluation of the total sterol content and the sterol profile is a suitable tool to assess the authenticity of fats and oils. There are three major phytosterols (β-sitosterol, stigmasterol, campesterol), two of which, β-sitosterol ($C_{29}H_{50}O$) and stigmasterol ($C_{29}H_{48}O$), are Δ^5-sterols. The former is the principal sterol of a large number of vegetable oils, while the latter is an important sterol for authenticity assessment and is present in several oils, e.g. soybean, castor, grape seed, palm, palm kernel, rice bran, safflower and sunflower.

According to KIRITSAKIS (1998, 2007), Greek olive oils contain traces of cholesterol, campesterol, sigmasterol, β-sitosterol (90% of the total sterols) and anemasterol. The total percentage of sterols in olive oil ranges from 180 to 265 mg per 100 g of oil. In the case of olives, stigmasterol and Δ^5-avenasterol increase as the fruit matures up to a point which coincides with the maximum oil content of the olive fruit (CIMATO, 1990).

The aim of this work was to analyse a number of olive oil samples of the Koroneiki variety cultivated in different regions of Messinia in Peloponnisos (Greece) and describe the quality profile of these oils in terms of acidity, peroxide value and extinction coefficients, C18:1/C18:2, oleic acid and the linoleic acid ratio; OSI, oxidative stability index; TP, total phenols; *o*-diphenols determined by spectrophotometry; SP, simple phenols (hydroxytyrosol and tyrosol) analyzed by HPLC.

Sterols were also determined in both mature and immature olive oil samples and mature and balanced extra virgin olive oil from 2008 was compared with that of 2009.

MATERIALS AND METHODS

Sampling

Olive samples were collected from the northwestern part of Messinia to the southern part of the Koroni area. Sampling took place in two stages, one between the 18th of November and 15th of December 2008 and the other one from the 16th of December 2008 until the 20th of January 2009, with a total number of samplings of 262. Three replicates were taken for each different sample.

Olive fruit of the Koroneiki variety (*Olea europaea* var. *microcarpa alba*) (THERIOS, 2009) was collected at the mature stage only. The olives were picked by hand from three trees at each location. Only undamaged, fresh and healthy drupes, were selected. From all olive trees of each region, a representative 5 kg sample was collected and immediately transported to the laboratory mill where they were processed into oil within 24 h.

Oil extraction

Olives were processed in a low-scale extraction plant (Olio Mio Baby 50, Toscana Enologica Mori, Firenze, Italy) at the Technological Institute of Kalamata, Greece. The unit consisted of the mill, the thermo-mixer, and the pulp centrifuge. The low-scale mill was equipped with a hammer crusher, a vertical malaxer (at a temperature of 18°C), and a two-phase decanter. After processing in the mill, the oil was separated in the two-phase decanter, transferred into dark glass bottles, and stored in the dark at 4°C until analysis. Malaxation lasted for 40 minutes between 20°- 22°C.

Analytical methods

Measurements refer to K_{232} and K_{270} coefficients, acidity and peroxide values (expressed as meq O_2 /kg). Sterol determination was also carried out on oil extracted from early harvested olives on the 18th November 2008 and mature olives on the 15th January 2009.

Finally, determinations were carried out on the commercially available olive oil named "DANAI" (Lot L05354 and L06345) produced by the Association of Agricultural Union of Messinia, comparing samplings from 2008 with that of 2009.

Acidity, peroxide value
and specific extinction determination

Titratable acidity, peroxide value, and the coefficients of specific extinction at 232 and 270 nm were determined according to EC methods (COMMISSION REGULATION (EC) No 1065/97; KIRITSAKIS and MARKAKIS, 1991; KIRITSAKIS and CHRISTIE, 1998).

Sterol determination

The identification and determination of sterols by GLC was carried out according to the method reported by LALAS and TSAKNIS (2002). Five replications for each sample were carried out.

Sensory evaluation

Sensory analysis was performed by a fully trained analytical taste panel recognized by the International Olive Oil Council (IOOC). A panel test was established using a standard profile sheet (IOOC, 2000). The sensory profile of the oil was determined. According to this methodology both defects and merits of the olive oil are reported on a scale between 0 and 100 mm, where 0 mm represents the absence of organoleptic characteristics, and 100 mm represents the highest value. Different pleasant flavour intensities perceived by orthonasal, retronasal routes were obtained.

Spectrophotometric determination
of total phenols

The total phenol (TP) content was determined using Folin-Ciocalteau reactive substance by spectrophotometry (UV-VIS Uvikon 1031 Spectrophotometer (Contron, Milano, Italy)) at 750 nm (PIRISI *et al.*, 2000). The total phenols are expressed as mg gallic acid/kg oil (calibration curve with r^2 = 0.996). The spectrophotometric analysis was repeated three times for each type of extract.

The gallic acid standard used for the spectrophotometric phenol quantification and the 2,2-diphenyl-1-picrylhydrazyl (DPPH) were obtained from Sigma-Aldrich, Inc. (St. Louis, MO, USA).

O-diphenols

A 0.5-mL sample of each phenolic extract was dissolved in 20 mL methanol/water (1:1, v/v). Of the resulting solution, 4 mL was added to 1 mL of a 5% solution of sodium molybdate dihydrate in ethanol/water (1:1, v/v) and shaken vigorously. After 15 min, the absorbance at 370 nm was measured using gallic acid for the calibration curve (r^2=0.998). The results are expressed in mg gallic acid/kg oil. The spectrophotometric analysis was repeated three times for each type of extract.

Fatty acid methyl esters analysis

Fatty acid methyl esters (FAME) from the oil samples were obtained by alkaline treatment with 1 M KOH in methanol (CHRISTIE, 1998). Gas chromatographic analyses were carried out according to CERRETANI *et al.* (2004) using an HP 7890A gas chromatograph (Agilent Wallborn, Germany). The average was calculated from four replications for each sample.

Oxidative stability
by oxidative stability instrument index

The oxidative stability index (OSI) was measured using a Rancimat apparatus (Metrohm Ltd., CH-10101, Herisau, Switzerland). The instrument was set at 110°C and 20 L/h air flow rate. The OSI was determined five times for each sample, and the mean value is expressed as OSI time in hours.

Chromatographic analysis

HPLC analyses were carried out in reverse phase using a C-18 Luna™ column, 5 mm, 25 cm x 3.00 mm i.d. (Phenomenex, Torrance, CA, USA), according to CERRETANI *et al.* (2004). The average of phenolic compounds was calculated from five replications for each sample and results are expressed as area in mAU at 280 nm at room temperature.

Gas-chromatography
mass spectrometry (GC-MS)

Analyses were carried out using a HP 5975C mass quadrupole spectrometer coupled to a HP 7890A gas chromatograph with a G1888 network headspace sampler (Agilent, Wallborn, Germany) located at the Dept. of Food Technology, Quality Control Lab, Kalamata. Helium was used as the carrier gas with a linear velocity of 38 mL/sec. Sample aliquots of 1 μL were injected. GC injector temperature was 250°C and transfer line temperature was held at 320°C. Prior to GCMS dried methanolic extracts were derivatized by addition of 100 μL BSTFA at room temperature for 15 min.

RESULTS AND DISCUSSION

Determination of acidity, K_{232}, K_{270} and peroxide number

Table 1 showed that there were small differences in the values of acidity, K_{232}, K_{270} and peroxide number for olives sampled in 2008-2009. Most of the values were within the limits set by the EU with acidity <0.8% and peroxide values (expressed as meq O_2/kg of oil) <20, K_{270}<0.22, K_{232}<2.5 for extra virgin olive oil (COMMISSION REGULATION (EC) No 2568/91). The small discrepancy shown for some values could be due to the altitude of some regions or the different cultivation techniques. However, there was a similarity in all measured which is evidence of the homogeneity of the olive variety Koroneiki throughout the region of Messinia.

Moreover, in Table 1 a descriptive statistical analysis is shown for the above-mentioned parameters. The low values of SD show the small differences in the measurements of the acidity, K_{232}, K_{270} and peroxide values. All acidity values were below 0.5%, and 90% of them were between 0.280 and 0.390%. The peroxide values were below 10.65, 90% of which were between 5.534 and 9.633. All K_{232} values were below 1.75 with 90% of them being between 1.450 and 1.660. All K_{270} values were below 0.16 with 90% of them falling between 0.110 and 0.140.

Sterols in mature and immature olive oil

Table 2 shows the analysis of the sterol content of extra virgin olive oil produced from early harvested olives in 2008 (mature olives harvested at the end of December 2008) and from fully mature olives harvested at the end of January 2009. Values reported are means of 5 measurements ± SD.

Sterol content values fell within permissible limits. Hence, it can be concluded that mature olive oil samples produced both in 2008 (December) and 2009 came from fully formed olive fruit. Mature olive oil means oil fully formed and with the characteristic of dark colour. It would be expected that the mature or immature olive oil produced in 2008 would not be so comparable regarding the sterol fraction compared to the mature olive oil produced in 2009 because it is well known that ripening affects the phenolic content (KIRITSAKIS, 1998, 2007). However, the content of sterols seemed unaffected by the time of harvesting.

TEMIME *et al.* (2008) studied the sterol profile of Tunisian virgin olive oils produced from the Chetoui cultivar, the second main variety cultivated in the north of the country. As expected for virgin olive oil, the main sterols found in all Chetoui olive oils were β-sitosterol, Δ^5 avenasterol, campesterol and stigmasterol. Total sterol amounts were higher than the minimum limit set by legislation, ranging from 1,017 to 1,522 mg/kg. Two triterpenic dialcohols (erythrodiol and uvaol) were also detected besides the sterolic components. Several factors are known to affect the quantitative sterolic profiles of olive fruits. Among these factors, the ripening cycle of the fruit and the nature of the cultivar (GUTIERREZ *et al.*, 1999; KOUTSAFTAKIS *et al.*, 2000), oil extraction and refining procedures and storage conditions (GRACIA, 2001). The effects of agronomic and climatic conditions were also studied by STEFANOUDAKI *et al.* (2001).

The mature olive oil produced in 2009 was shown to be a fully balanced mature olive oil. Despite this, it was believed that further analysis should be carried out on all the samples taken throughout the whole region of Messinia to confirm the conclusions from sterol determination.

β-sitosterol and campesterols, which are structurally related to cholesterol, dominated

Table 1 - Descriptive statistics for acidity, peroxide value and coefficients K_{232} and K_{270} for olive oil samples during 2008-2009, N=270.

	Mean (95% Conf. Interval)	Min	Max	SD	Percentiles						
					5	10	25	50	75	90	95
Acidity %	0.320 (0.318, 0.323)	0.25	0.50	0.033	0.280	0.280	0.300	0.320	0.340	0.350	0.390
Peroxide value	7.387 (7.299, 7.475)	5.18	10.65	1.251	5.534	5.840	6.398	7.325	8.183	9.186	9.633
K_{232}	1.579 (1.574, 1.584)	1.39	1.75	0.065	1.450	1.490	1.540	1.590	1.630	1.650	1.660
K_{270}	0.125 (0.124, 0.126)	0.10	0.16	0.011	0.110	0.110	0.120	0.120	0.130	0.140	0.140

Table 2 - Sterol content, organoleptic traits, C18:1/C18:2, oleic acid and linoleic acid ratio; OSI, oxidative stability index; TP, total phenols; *o*-diph, *o*-diphenols determined by spectrophotometry; SP, simple phenols (hydroxytyrosol and tyrosol) analyzed by HPLC comparison between extra virgin olive oil produced from mature and immature olive fruit.

Sterol	IMMATURE olive oil (November 2008) % in the unsaponifiable part	MATURE olive oil (End December 2008) % in the unsaponifiable part	MATURE extra virgin olive oil (Mid January 2009) % in the unsaponifiable part
Cholesterol	0.5±0.05	0.50±0.05	0.50±0.05
Brassicasterol	0.10±0.01	0.10±0.01	0.10±0.01
Campesterol	3.80±0.41	3.87±0.4	3.70±0.4
Stigmasterol	0.82 ±0.9	0.85±0.9	0.84±0.9
Δ7-stigmasterol	0.31±0.03	0.35±0.03	0.30±0.03
β-sitosterol	93.63±10.1	94.12±10.1	94.89±10.2
Organoleptic traits			
Fruiting (mm)	23	22	24
Bitterness (mm)	6	4	4
Pungency (mm)	10	8	7
C18:1/C18:2	10.8±0.1	11.2±0.1	11.2±0.1
OSI Time [h]	38.5±0.2	38±0.3	38.5±0.2
TP [mg of gallic acid/kg]	350±18	380±18	380±18
o-diph [mg of gallic acid/kg]	200±10	210±10	210±10
SP [Area in mAU]	800±80	1200±100	1200± 100

Values are means of 5 determinations ± SD.

the sterol fraction as can be seen in Table 2. The content of sterols in the extra virgin olive oil samples was comparable (ANOVA), a conclusion in line with findings reported by GORINSTEIN *et al.* (2003). The highest mean per cent value of β-sitosterol was observed in mature extra virgin olive oil (95%, January 2009) comparable to Testour oil (84.4%) as reported by TAMIME *et al.* (2008). These values are similar to those reported for other olive oil varieties (PARDO *et al.*, 2007; RIVERA DEL ALAMO *et al.* 2004). According to GUTIERREZ *et al.* (1999), the content of β-sitosterol generally decreases during ripening, while Δ^5 avenasterol increases. KOUTSAFTAKIS *et al.* (1999) reported that β-sitosterol is minimal and Δ^5 avenasterol is at a maximum when olives are harvested at their optimum.

Low levels of stigmasterol were detected which is an indication that the oil came from healthy fruit (KOUTSAFTAKIS *et al.*, 1999). High levels correlate with high acidity and low organoleptic quality (GRACIA, 2001).

In the Chetoui variety, the highest phytosterol levels were found for β-sitosterol, followed by Δ^5 avenasterol (TEMIME *et al.*, 2008). All the olive oil samples analysed showed a low level of campesterol ranging from 2.45% (Bou Arada) to 2.84% (Testour). The campesterol content was below the threshold established by EU Regulations (4%) in all of the oils studied. In our oils, campesterol levels fell within the range of 3.70 to 3.87%, indicating a close similarity to the Tunisian oils.

Comparison of commercial olive oil "DANAI" produced in 2008 and 2009

Comparing the results from the analysis of olive oil samples produced in both years (Table 3), there was an increase in K_{232} for olive oil produced in 2008 compared to that produced in 2009, along with an increase in the peroxide values. Both results are absolutely normal and are due to the level of oxidative degradation of the olive oil during the year.

Sensory results

Sensory characteristics tested by IOOC methodology showed no defects in the oils and they are classified as extra virgin olive oils. Fruity sensation, bitterness and pungency had similar values for both immature and mature extra virgin olive oils. The Koroneiki olive variety gave to the extracted oil a well-balanced sensory profile between bitterness and sweetness, together with a pleasant flavor of grassy, green tomatoes and artichoke. There was an increase in the percentage of assessors who perceived a grassy aroma in the oils processed in the low-mill scale (Table 4).

With reference to orthonasal perceptions, extra virgin olive oils from the Koroneiki variety, (either mature or immature) showed a good balance between grassy (39%) and green tomato (16.2%). Considering the flavors retronasally perceived, the Koroneiki variety extra virgin olive oils showed a good balance between grassy

Table 3 - Analytical results of the commercially produced virgin olive oil "DANAI" from 2008 and 2009.

Analytical parameters	"DANAI" lot L05354 2008 (%)	"DANAI" lot L06345 2009 (%)
Acidity	0.38	0.31
K_{232}	1.95	1.64
K_{270}	0.16	0.13
ΔK	0.004	0.005
Peroxide value (meq O_2/kg)	14	6.5
C18:1/C18:2	11.2±0.1	11.1±0.1
OSI time (h)	33.5±0.2	35.5±0.2
TP [mg of gallic acid/kg]	230±10	250±15
o-diph [mg of gallic acid/kg]	100±5	110±5
SP [Area in mAU]	400±50	500±50

Values are means of 3 determinations ± SD.

and green almond (10%) and green tomato (25%), with the presence of artichoke flavor (approx. 15%) (Table 4). An apple flavour was also detected by some panelists. Results are in good agreement with data presented by (CERRETANI *et al.*, 2005).

Oxidative stability results

It is important to highlight that in the Koroneiki olive oil samples obtained from the low-scale mill, the OSI time values were particularly high (5 h higher) compared to those of the commercial extra virgin olive oils (Danai) (Tables 2 and 3). The high oxidative stability of the samples may be due to both the higher total phenol and *o*-diphenol contents.

It is important to observe that the production of a typical olive oil in a low-scale mill permits the exaltation of the characteristics related to the phenolic content and the shelf life of an oil (higher stability to the accelerated oxidation). The difference can be explained by the different conditions of malaxation (see Materials and Methods). In fact, in the low-scale mill, both the use of lower temperature and lower contact surface with the air cause a lower tendency to oxidative processes.

Fatty acid composition

The olive oils originating from the Koroneiki cultivar showed similar fatty acid compositions (particularly the C18:1/C18:2 ratio) compared to the commercial extra virgin olive oils. The results are in good agreement with those presented by CERRETANI *et al.* (2005) and STEFANOUDAKI *et al.* (1999). The latter determined the fatty acid composition of 105 virgin olive oil samples of the two dominant Cretan olive cultivars, Koroneiki and Mastoides, harvested from different areas of production at different maturity stages. The oils of the Koroneiki cultivar were characterized by lower concentrations of oleic and decaheptanoic and higher concentrations of linoleic and palmitic acids. They also proved that fatty acid compositional data have a significant discriminating power even within close geographical areas.

CONCLUSIONS

The chemical characteristics of Messinian olive oil are more or less similar throughout the whole region of Messinia due to the same olive cultivar (Koroneiki variety) found everywhere and due to the similar climatological conditions. There was a small discrepancy in the peroxide values which is due to the altitude of some regions or the different cultivation techniques that might have been used. However, there is homogeneity in all other parameters showing the homogeneity in the olive cultivar Koroneiki throughout the region of Messinia.

Sterol content values fell within permissible limits showing that mature olive oil samples produced both in 2008 (December) and 2009 came from fully formed olive fruit. Similar results for non mature olive oil produced in 2008 showed that the oil inside the fruit mass was also fully formed. Comparing mature olive oil produced in 2009 with non mature olive oil produced in 2008, it was shown that the content of sterols in all extra virgin olive oils was not statistically different (ANOVA, p=0.05).

Regarding the analysis of peroxide number and K_{232} of olive oil "DANAI" produced in 2008 and 2009, there was an increase in both peroxide number and K_{232} values. These differences were expected due to olive oil oxidation during bad storage conditions. However, the values fell within the limits of extra virgin olive oil. The very high percentage in mono-unsaturated fatty acids indicates that 'DANAI' olive oil is a well balanced olive oil of high quality.

Table 4 - Percentages of different attributes orthonasally and retronasally perceived in extra virgin olive oils from the Koroneiki variety extracted from a low-scale mill.

Perception	Artichoke	Grassy	Green almond	Green tomato	No perception
Orthonasal	5.2	39.1	5.0	16.0	34.7
Retronasal	15.1	10.2	10.0	25.0	39.7

The oils originating from the Koroneiki cultivar of olive fruit showed similar fatty acid compositions (particularly the C18:1/C18:2 ratio) to the commercial extra virgin olive oils. The oxidative resistance and the content of phenols and *o*-diphenols were higher for the olive oils produced by the low-scale mill compared to the commercial extra virgin olive oils produced by the industrial plant.

With reference to orthonasal perceptions, extra virgin olive oils from the Koroneiki variety, (either mature or immature) balanced well between grassy (39%) and green tomato (16.2%). Considering the flavors retronasally perceived, the Koroneiki variety oils balanced well between grassy and green almond (10%) and green tomato (25%), with the presence of artichoke flavor (approx. 15%)

In conclusion the Koroneiki olive variety affects the phenolic content and sensory profiles to a great extent, whereas the different extraction systems (low-scale or large-scale) exert a minor effect, although this needs to be confirmed in future studies.

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PAPER

CHANGES IN ANTIOXIDATIVE COMPONENTS IN FRUIT OF FIVE STRAWBERRY CULTIVARS DURING THREE HARVEST TIMES

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ABSTRACT

During the harvest season of two consecutive years, the total phenols, flavonoids, anthocyanins and antioxidant capacity of five strawberry cultivars ('Arosa', 'Elsanta', 'Marmolada', 'Miss' and 'Raurica'), grown in the continental part of the Republic of Croatia, were examined. Each year, fruit was harvested three times, at the beginning, at the midpoint and at the end of the harvesting season. Results showed that in both years, differences in the phenolic compound content depended on cultivar and harvest time. In both years, fruit of cultivar 'Arosa' had the highest total phenol content. Over three consecutive harvest times, the quantity of phenolics did not show any regularity, but most of the cultivars at early harvest time had the highest amounts. Most of the cultivars had the lowest quantity of total anthocyanins at the third harvest time.

- Key words: antioxidant capacity, *Fragaria* × *ananassa* Duch., strawberry cultivars, polyphenolic compounds -

INTRODUCTION

Strawberry (*Fragaria* x *ananassa* Duch.) is a very popular and widely consumed fresh fruit and is used in various food products such as preserves, jams, yogurts and ice-creams. It is reported that strawberries have antioxidant, anticarcinogenic, anti-inflammatory and anti-neurodegenerative biological properties. Determination of the phytochemical content in strawberries is necessary to evaluate the effect that the consumption of strawberries has on human health (LAUGALE and BITE 2006; RUTKOWSKI *et al.*, 2006; SEERAM *et al.*, 2006; KAFKAS *et al.*, 2007; BATTINO *et al.*, 2009).

Strawberries are a good source of natural antioxidants. In addition to the usual nutrients, such as vitamins and minerals, strawberries are rich in anthocyanins, flavonoids and phenolic acids (AYALA-ZAVALA *et al.*, 2004; TAVARINI *et al.*, 2008). Phenolic substances such as flavonoids are the most common compounds in fruits and vegetables; they have a high antioxidant capacity (SCALZO *et al.*, 2005; VOCA *et al.*, 2008; KOVAC *et al.*, 2010). Phenolic compounds are abundant in highly colored berry fruits, and because of their great popularity and vast consumption, these berries are one of our most important dietary sources of phenolics (SEERAM *et al.*, 2006; WANG *et al.*, 2002; YURDUGÜL, 2008). Berry fruits are reported to contain a wide spectra of phenolics, including hydroxybenzoic and hydroxycinnamic acid derivatives, anthocyanins, flavonols, flavanols, condensed tannins (proanthocyanidins) and hydrolyzable tannins (LAUGALE and BITE 2006; RUTKOWSKI *et al.*, 2006; SEERAM *et al.*, 2006; VOCA *et al.*, 2009a). Anthocyanins are water-soluble pigments that are located in the vacuoles and are responsible for a wide range of colors, from orange to purple. The colorless phenolic compounds can act as co-pigments and provide protection against UV radiation and insect attack, but they can also be substrates for enzymatic browning reactions of strawberry fruit flesh (GIL *et al.*, 1997).

Many authors have studied factors which could have an effect on the quantity of total phenols in different strawberry cultivars. They discovered that the most influential factors are cultivar, climatic conditions, cultivation systems and harvest times (CORDENUNSI *et al.*, 2005; SCALZO *et al.*, 2005; ZHENG *et al.*, 2007). TÖRRÖNEN and MÄÄTTÄ (2002) examined different strawberry cultivars and reported total phenol values that ranged from 96 to 133 mg/100 g f.w.

Antioxidative capacity has been studied by several authors on many fruit and vegetable species using different methods for determining antioxidative capacity (CORDENUNSI *et al.*, 2005; SCALZO *et al.*, 2005; HANSAWASDI *et al.*, 2006, JAKOBEK *et al.*, 2007a,b; VOCA *et al.*, 2009b).

Strawberry is a non-climacteric fruit and, to achieve the maximum quality in terms of fruit flavor and color, it must be harvested at full maturity. The main changes in fruit composition that are associated with ripening, take place when the fruit is still attached to the mother plant. Consequently, strawberries should be harvested when ready for consumption. This means that there is a very short time when the fruit is at its best quality (CORDENUNSI *et al.*, 2003).

In this study changes in the quantity of total phenols, flavonoids, anthocyanins and the antioxidant capacity of five strawberry cultivars were investigated during the harvest season. In an attempt to prolong the consumption season of fresh strawberries and still maintain high quality fruit, five different cultivars, from very early ripening ('Miss'), to late ripening ('Raurica'), were included.

MATERIALS AND METHODS

Cold-stored (frigo) strawberry plants of cultivars 'Arosa', 'Elsanta', 'Marmolada', 'Miss' and 'Raurica' were planted in black plastic foil in August 2004. Fertilizers and water were provided by fertirigation. The strawberries were planted at a planting density of 40,000 plants/ha in an open field on alluvium soil in the Zagreb area. The experiment was designed as a random block design with three blocks. Each block consisted of five plots and each plot consisted of 50 strawberry plants per cultivar.

Fruits were harvested at the beginning, the midpoint and at the end of the harvest season, during June 2005 (with 29.9 mm precipitation) and June 2006 (with 85.8 mm precipitation). At full maturity (when fruit had reached full red color) 500 g of randomly selected fruit samples were harvested from each plot. Fresh fruit samples were analyzed and the following quality parameters were determined: total phenols, total flavonoids, total anthocyanins and total antioxidant capacity.

Determination of total phenolic content

Total phenolics (TP) were determined using the Folin-Ciocalteu colorimetric method described by OUGH and AMERINE (1998) with some modifications. Fruit phenolics were extracted from 10 g of fresh samples using 40 mL of 80% (by volume) aqueous ethanol. The mixture was extracted (in a water bath at 80°C), kept for 20 min in inert atmosphere, and filtered through a Whatman filter paper using a Büchner funnel. Extraction of the residue was repeated under the same conditions. The filtrates were combined and diluted to 100 mL in a volumetric flask with 80% aqueous ethanol. This extract was used to determine the total phenolics (TP) and total flavonoids (TF).

Determination of total flavonoids

Formaldehyde precipitation was used to determine total flavonoids (TF) in fruit samples (KRAMLING *et al.*, 1969). A mixture of 10 mL strawberry fruit extract, 5 mL of 25% HCl aqueous solution (1:4) and 5 mL of formaldehyde was prepared in a 25 mL flask. In order to remove air, nitrogen gas was injected and the stoppered flask was left in the dark for 24 h at 22°C. The next day it was filtered; and the clear liquid was used in the same procedure as the one used to prepare samples for TP determination.

The TP content was measured as follows: 0.5 mL of diluted extract was added to a 50 mL volumetric flask containing 30 mL of double distilled water. Then 2.5 mL of Folin-Ciocalteu reagent was added to the mixture and shaken. After 5 min, 7.5 mL of 7% Na_2CO_3 solution were added with stirring; this solution was then diluted with double distilled water to 50 mL. After 2 h of incubation at 20°C, the absorbance of the solution was measured at 760 nm. TP and TF are expressed as mg of gallic acid equivalents (GAE)/kg of fresh mass of edible fruit. The extract of total phenolics was also used for the DPPH assay.

Determination of anthocyanin content

The total anthocyanin content in the extract was determined using the bisulphite bleaching method (PELLEGRINI, 1965). Fruit anthocyanins were extracted from 2 g of fresh samples using 2 mL of 0.1% HCl (by volume) in 96% ethanol and 40 mL 2% aqueous HCl (by volume). This mixture was then centrifuged at 5500 rpm for 10 min. The supernatant was used for the determination of total anthocyanins. The total anthocyanin content was measured as follows: 10 mL of extract were put into two test tubes, and then 4 mL of 15% sodium bisulphite were added to one tube and 4 mL of double distilled water to the other. After 15 min of incubation at room temperature, the absorbance of each mixture was measured at 520 nm. The molar absorbance value for cyanidin-3-diglucoside was used as the standard value. Results are expressed as mg of cyanidin-3-diglucoside equivalents/kg of fresh mass of edible fruit.

Determination of antioxidant capacity

The free radical scavenging capacity of fruit phenolic extracts was determined using the stable 2,2-diphenyl-1-picrylhydrazyl (DPPH$^\bullet$) radical scavenging method (BRAND-WI LLIAMS *et al.*, 1995.) The method is based on the reduction of stable DPPH$^\bullet$ nitrogen radicals in the presence of antioxidants.

The antioxidant capacity of fruit extracts was measured spectrophotometrically with a UV-Vis spectrophotometer (Shimadzu UV 1650 PC, Kyoto, Japan). Five dilutions of fruit extract were analyzed with the DPPH$^\bullet$ method. Fifty μL of diluted fruit extract were mixed with 300 μL of methanolic DPPH$^\bullet$ solution (1 mmol/dm^3) and brought to 3 mL with methanol. The solution was left in the dark for 15 min at room temperature. The absorbance ($A_{extract}$) was read against the prepared blank (50 μL of diluted fruit extract, 2,950 μL of methanol) at 517 nm. DPPH$^\bullet$ blank solution was prepared fresh each day (300 μL of 1 mmol/dm^3 DPPH$^\bullet$, 2.7 mL of methanol) and its absorbance (A_{DPPH}) was measured daily. Trolox standards of the final concentration of 0-2,500 μmol/dm^3 were prepared in methanol and assayed under the same conditions. Fifty μL of Trolox were mixed with 300 μL of methanolic DPPH$^\bullet$ solution (1 mmol/dm^3) and topped to 3 mL with methanol. After 15 min, the absorbance (A_{Trolox}) was read against the prepared blank at 517 nm. A calibration curve of Trolox was calculated by linear regression of the absorbance value (A_{Trolox}) vs. concentration. The Trolox calibration curve was used to calculate the antioxidant capacity of each diluted fruit extract and to express the antioxidant activity in mmol of Trolox equivalent (TE) per kg of fruit. Using the curve obtained, the results are expressed as mmol of TE per kg of fruit needed to reduce DPPH$^\bullet$ radical by 50%.

The ability of fruit extract to act as a free radical scavenger against DPPH$^\bullet$ radical was tested by measuring the disappearance of the absorbance at 517 nm after the addition of fruit extract, and by comparing it with the disappearance of the absorbance produced by the addition of known amounts of Trolox, a water-soluble vitamin E analogue, under the same conditions.

The results are expressed as mmol Trolox equivalents per kg of fresh mass of the edible fruit.

Statistical analysis

According to a randomized block design (with three replicates), ANOVA and Duncan's multiple range test were performed to determine the significance of differences within the factors examined (harvest time and cultivar) and between the combination of factors (where interaction was proven significant), using the commercial software SAS 8.0 (SAS Institute: Cary, NC). Values are presented as the mean±SD of three replications. *P*-values lower than 0.05, either from ANOVA or Duncan's multiple range test were considered statistically significant.

RESULTS AND DISCUSSION

Climatic data were provided by the Croatian Meteorological and Hydrological Service. The average air temperature during the strawberry harvest was higher in the first year (2005) than in the second. And there was less rainfall during

the harvest season in the first year. The differences in the contents of the compounds studied could have been due, in part to the different climatic conditions (listed in Tables 1, 2 and 3).

The fruits of the strawberry cultivars had a high average total phenolic content, of which the total flavonoids made up a relatively small portion (Table 4). Cultivar 'Arosa' had the highest TP content in both years (248.86 in the first year and 352.34 mg/kg f.w. in the second year). It was followed by cultivars 'Elsanta' (237.96 mg/kg f.w.), 'Marmolada' (217.34 mg/kg f.w.), 'Raurica' (186.71 mg/kg f.w.) and 'Miss' (166.44 mg/kg f.w.) in the first year, and by cultivars 'Elsanta' (348.26 mg/kg f.w.), 'Marmolada' (345.02 mg/kg f.w.), 'Miss' (269.96 mg/kg f.w.) and 'Rauricа' (261.69 mg/kg f.w.) in the second year. The literature data report that the quantities of TP in strawberry fruits of different cultivars range from 276.90 to 285.45 mg/kg f.w. (DRAGOVIĆ-UZELAC *et al.*, 2007).

In the first year of the study the amounts of total phenols were similar at all three harvest times in fruits of cultivar 'Elsanta', 'Marmolada' and 'Miss', while a somewhat higher value was measured in fruits of cultivar 'Arosa' at the third harvest time (288.21 mg/kg). Values obtained in the second harvest time were similar in the varieties 'Arosa', 'Marmolada' and 'Elsanta' cvs, while those of cultivars 'Miss' and 'Raurica' were somewhat lower. At the third harvest time, the total phenolic content was similar among

Table 1 - Harvest date of the strawberry cultivars in two years of study.

Cultivar	Year of research					
	2005			2006		
	Harvest time			Harvest time		
	1.	2.	3.	1.	2.	3.
Arosa	02 June	10 June	17 June	29 May	06 June	12 June
Elsanta	02 June	10 June	17 June	24 May	31 May	07 June
Marmolada	02 June	10 June	17 June	29 May	06 June	12 June
Miss	02 June	10 June	17 June	24 May	31 May	07 June
Raurica	02 June	10 June	17 June	01 June	07 June	12 June

Table 2 - Precipitation (mm) at the experimental field.

Year	Ten days Harvest time	Jan.	Feb.	Mar.	Apr.	May	Jun.
2005	I	2.4	11.0	13.4	37.5	40.5	24.4
	II	7.5	26.7	9.7	21.3	42.4	3.4
	III	17.8	36.2	33.1	12.0	0.0	2.1
	Total	**27.7**	**73.9**	**56.2**	**70.8**	**82.9**	**29.9**
2006	I	53.1	0.6	27.6	52.5	23.5	54.5
	II	0.6	7.2	24.9	37.4	2.6	17.1
	III	0.1	33.4	19.6	40.6	68.4	14.2
	Total	**53.8**	**41.2**	**72.1**	**130.5**	**94.5**	**85.8**

Table 3 - Average air temperatures (°C) measured at the experimental field.

Year	Ten days Harvest time	Jan.	Feb.	Mar.	Apr.	May	Jun.
2005	I	2.8	-6.6	-3.8	10.0	14.1	15.7
	II	-1.7	1.2	7.5	11.5	14.8	20.1
	III	-3.5	-1.0	11.3	13.5	21.2	23.9
	Mean	**-0.7**	**-2.2**	**5.0**	**11.4**	**16.7**	**19.9**
2006	I	0.4	-2.3	1.4	10.6	13.2	13.4
	II	-1.5	3.9	2.6	12.2	17.8	22.3
	III	-3.8	2.1	10.4	14.2	18.0	25.8
	Mean	**-1.7**	**1.2**	**5.0**	**12.3**	**15.8**	**20.5**

Table 4 - Effects of cultivar and harvest time on average total phenolic content (TP), total flavonoids (TF) and total anthocyanins (TA) in strawberries in the first and second years of the study.

	Total phenolic content (mg/kg)		Total flavonoids (mg/kg)		Total anthocyanins (mg/kg)	
	1st year	2nd year	1st year	2nd year	1st year	2nd year
Cultivar	**P<0.0001**	**P<0.0001**	**P<0.0001**	**P<0.0001**	**P<0.0001**	**P<0.0001**
Arosa	248.9a±36.76	352.3a±32.83	53.3a±39.2	106.1a±70.87	233.2a±76.04	266.3c±27.74
Elsanta	238.0b±10.28	348.3b±25.38	57.1a±33.4	38.3c±15.68	193.2d±89.89	280.1b±25.31
Marmolada	217.3c±14.57	345.0b±9.25	40.4b±37.3	57.3b±15.79	218.3b±60.56	299.0a±41.94
Miss	166.4e±17.44	270.0c±35.59	27.6c±19.1	28.8d±9.32	141.7e±38.03	284.2b±33.23
Raurica	186.7d±28.23	261.7d±4.79	22.0d±22.8	54.9b±27.03	199.5c±71.21	186.2d±32.51
Harvest time	**P<0.0001**	**P<0.0001**	**P<0.0001**	**P<0.0001**	**P<0.0001**	**P<0.0001**
1	214.7c±29.11	335.5a±48.51	78.6a±24.6	84.7a±46.43	222.6a±66.12	288.3a±38.01
2	198.9b±34.69	310.1b±54.21	27.7b±16.1	62.8b±40.17	217.1b±55.95	271.7b±51.40
3	220.8a±48.26	300.7c±33.18	14.1c±8.9	23.8c±9.88	151.8c±77.69	229.5c±45.79
Int. C×Ht	**P<0.0001**	**P<0.0001**	**P<0.0001**	**P<0.0001**	**P<0.0001**	**P<0.0001**

C = cultivar; Ht = harvest time; Int. C×Ht = interaction Cultivar×Harvest time.
Within each column and effect, different letters indicate significant differences between means at P≤0.05 according to Duncan's multiple range test.

Table 5 - Average total phenolic content (mg/kg) in five strawberry cultivars during three harvest times during two years of study. Values are the means of three replicates ± SD.

	1st year Harvest time			2nd year Harvest time		
Cultivar	**1**	**2**	**3**	**1**	**2**	**3**
Arosa	254.4b±1.50	204.0fg±0.60	288.2a±4.22	389.9a±4.61	352.8c±1.45	314.4g±2.60
Elsanta	238.7c±0.10	248.2b±8.49	227.0d±3.45	378.5b±5.54	344.9d±8.32	321.3f±1.17
Marmolada	202.8g±1.31	213.9e±5.49	235.3c±0.73	336.8e±1.73	357.0c±1.33	341.3de±0.08
Miss	176.8h±4.65	178.7h±5.99	143.8i±1.97	310.9g±7.89	229.3k±0.79	269.6h±1.70
Raurica	200.7g±4.84	149.7i±3.94	209.7ef±1.01	261.4ij±1.33	266.6hi±4.19	257.0j±2.01

Within each year, different letters indicate significant differences between means at P≤0.05 according to Duncan's multiple range test.

cultivars 'Elsanta', 'Marmolada' and 'Raurica', while 'Arosa' had the highest value (288.21 mg/kg) and cultivar "Miss" had the lowest (Table 5).

The TP content in all the cvs was lower in the first year of the study. The values ranged from 143.8 to 288.2 mg/kg in the first year, and from 229.3 to 389.9 mg/kg in the second year. Fruits of cultivars 'Elsanta', 'Marmolada' and 'Miss' had uniform TP contents at all three harvest times. Somewhat higher values were noted in cultivar 'Arosa' at the third harvest time in the first year of the study. In the second year the cultivars 'Marmolada' and 'Raurica' had very similar values at all three harvest times, while cultivars 'Arosa', 'Elsanta' and 'Miss' had the highest values at the first harvest time. On average, the highest TP values were recorded at the first harvest time, with the exception of cultivar 'Raurica' which had uniform values at all three harvest times. TP values at the second harvest time were uniform for all cultivars, except for cultivars 'Miss' and 'Raurica' that had significantly lower values. Statistical analysis showed that the TP content differed significantly within cultivars independent of the year and harvest time (Table 4). The analysis also showed that harvest time had no statistically significant influence on TP content, but the differences between the years were statistically significant.

The quantity of TF was different for the various harvest times, with substantial differences between the cultivars. In both years, the highest values were recorded at the first harvest time. The average TF values in the first year were the highest for cultivar 'Elsanta' (57.13 mg/kg f.w.), followed by cultivars 'Arosa' (53.26 mg/kg f.w.), 'Marmolada' (40.42 mg/kg f.w.), 'Miss' (27.64 mg/kg f.w.) and 'Raurica' (22.04 mg/kg f.w.). In the second year, the highest values were noted for cultivar 'Arosa' (106.08 mg/kg f.w.), followed

by cultivars 'Raurica' (69.88 mg/kg f.w.), 'Marmolada' (57.19 mg/kg f.w.), 'Elsanta' (38.30 mg/kg f.w.) and 'Miss' (28.83 mg/kg f.w.).

Changes in the quantity of total flavonoids (TF) followed the same pattern in both years. For all the cultivars, the highest values were noted at the first harvest time, and subsequently decreased in the second and third harvest times. At the first harvest time in the first year, the TF values ranged from 50.08 mg/kg for cultivar 'Miss' to 103.87mg/kg for cultivar 'Arosa'. However, the TF content in cultivars 'Miss' and 'Raurica' were significantly lower. The total flavonoid contents were significantly lower at the second and the third harvest times. They ranged from 11.27 to 49.93 mg/kg at the second harvest time, and from 3.58 to 23.80 mg/kg at the third harvest time. In the second year the highest total flavonoid values were recorded in the fruits of cultivar 'Arosa', while the TF content in the other cvs. was somewhat lower, ranging from 39.05 to 84.81mg/kg. At the third harvest time the TF content decreased, and ranged from 13.38 to 41.02 mg/kg. In both years the lowest TF values were noted at the third harvest for all five cultivars (Table 6).

Analysis of the data showed that the TF content within selected cultivars had significant differences independent of year and harvest time, while both harvest time and year influenced the TF content among the cultivars in a statistically significant manner (Table 4).

The quantity of total anthocyanins (TA) is shown in Table 7. The quantity of total anthocyanins varied with respect to harvest time and cultivar. The data are consistent with the data of other researchers (GIL *et al.*, 1997, LOPEZ DA SILVA *et al.*, 2007; ZHENG *et al.*, 2007; JAKOBEK *et al.* 2007a,b). In the first year relatively large and statistically significant differences among cultivars were observed, the total anthocyanin content was dependent on harvest time; in the second year those differences were somewhat smaller but still statistically significant. Lower values for total anthocyanin content were noted at the third harvest time in both years, except for cultivar 'Marmolada' in the first year of the research. Values for the other cultivars ranged from 76.6 to 175.0 mg/kg. In the second year at the third harvest time the TA values were similar for cultivars 'Arosa', 'Miss' 'Marmolada' and 'Elsanta' and ranged from 232.4 to 272.5 mg/kg, while the TA values in cultivar 'Raurica' at the third harvest time were significantly lower (145.50 mg/L). Values obtained at the first harvest time in the first year were scattered widely and ranged from 139.46 do 334.16 mg/kg, while in the second year they were more similar and ranged from 219.05 to 320.86 mg/kg. The results are in agreement with the results of other researchers. GIL *et al.* (1997) studied the total anthocyanin content in fresh strawberry fruits and obtained a value of 120.2 μg/g f.w. LOPEZ DA SILVA *et al.* (2007) extracted the anthocyanins from five different strawberry cultivars and the total anthocyanin content ranged from 200 to 600 mg/kg. Very similar results (from 150 to 350 mg/kg, f.w.) were obtained by CLIFFORD (2000). Studies of different strawberry cultivars show that the anthocyanin content differs among cultivars. MEYERS *et al.* (2003) studied eight different strawberry cultivars and found an average anthocyanin concentration of 414 mg/kg, with marked differences among cultivars; the cultivar 'Earliglow' had twice the concentration of 'Allstar'. Cultivar 'Camarosa' was also studied by other researchers; and their anthocyanin content values were somewhat lower (482.00 mg/kg) (CASTRO *et al.*, 2002). Differences in concentration of certain compounds within each cultivar can be explained by the influence of climatic conditions and by the use of different extraction methods.

Statistical analysis showed that there was a significant difference in total anthocyanin content between selected cultivars, independent of year and harvest time. From the all that has been stated above, it can be concluded that the harvest date has a considerable influence on the concentration of total anthocyanins (Table 7).

The antioxidative capacity was higher in the first year and it ranged from 3.25 to 4.46 μmol

Table 6 - Average total flavonoids (mg/kg) in five strawberry cultivars during three harvest times during two years of study. Values are the means of three replicates ± SD.

	1st year Harvest time			2nd year Harvest time		
Cultivar	**1**	**2**	**3**	**1**	**2**	**3**
Arosa	103.9a±6.56	37.1d±2.83	18.8ef±8.20	168.0a±3.24	136.8b±2.76	13.4h±2.84
Elsanta	97.7ab±2.61	49.9c±12.84	23.8e±8.40	55.3e±5.20	39.4f±5.17	20.2gh±0.19
Marmolada	90.0b±0.99	15.7efg±4.50	15.6efg±4.86	76.1d±4.07	54.8e±3.94	41.0f±5.16
Miss	50.1c±9.01	24.3e±7.51	8.6gh±2.31	39.1f±9.13	26.6g±1.33	20.8gh±1.51
Raurica	51.3c±7.32	11.3fgh±6.77	3.6h±2.02	84.8c±2.88	56.4e±8.87	23.4g±2.27

Within each year, different letters indicate significant differences between means at P≤0.05 according to Duncan's multiple range test.

Table 7 - Average total anthocyanins (mg/kg) in five strawberry cultivars during three harvest times during two years of study. Values are the means of three replicates ± SD.

	1st year Harvest time			2nd year Harvest time		
Cultivar	**1**	**2**	**3**	**1**	**2**	**3**
Arosa	334.2a±4.46	190.5fg±1.28	175.0h±1.96	291.5d±0.82	274.8e±16.81	232.4gh±3.40
Elsanta	227.6d±2.52	275.4c±3.24	76.6m±1.54	310.4bc±15.28	257.5f±9.13	272.5e±4.31
Marmolada	220.8e±4.18	147.2i±1.46	286.9b±1.71	299.7cd±3.22	346.5a±10.48	251.0f±8.51
Miss	139.5j±0.93	186.6g±1.37	98.9l±1.91	320.9b±10.88	285.7de±3.20	246.1fg±9.73
Raurica	191.2f±2.53	285.5b±1.62	121.7k±1.44	219.0h±5.03	194.2i±1.23	145.5j±0.97

Within each year, different letters indicate significant differences between means at P≤0.05 according Duncan's multiple range test.

Table 8 - Average total antioxidant capacity (μmol/g) in five strawberry cultivars in three harvest times during two years of study. Values are the means of three replicates ± SD.

	1st year Harvest time			2nd year Harvest time		
Cultivar	**1**	**2**	**3**	**1**	**2**	**3**
Arosa	4.44a±0.025	4.36bc±0.020	4.46a±0.030	3.19a±0.211	2.80cd±0.045	2.86cd±0.040
Elsanta	4.39b±0.020	4.35bc±0.040	4.35bc±0.030	2.61f±0.000	2.89c±0.031	2.63f±0.032
Marmolada	4.05d±0.046	4.33c±0.035	4.32c±0.010	2.91bc±0.010	2.82cd±0.057	3.02b±0.084
Miss	3.54g±0.045	3.61f±0.010	3.30h±0.030	2.61f±0.006	2.62f±0.006	2.75de±0.006
Raurica	3.95e±0.055	3.25i±0.040	4.02d±0.026	2.47g±0.072	2.59f±0.026	2.66ef±0.015

Within each year, different letters indicate significant differences between means at P≤0.05 according to Duncan's multiple range test.

TE /mL. In the second year it ranged from 2.47 to 3.19 μmol TE /mL (Table 8). The values for antioxidative capacity were uniform among the cultivars independent of harvest time. A somewhat lower value of antioxidative capacity was noted for cultivar 'Miss' in the first year of the study. Values recorded in the second year were more uniform. Research conducted on different fruit species (strawberry, sour cherry, cornelian cherry, blackthorn), confirms the results of this study and shows that the antioxidative capacity measured depends greatly on the method of determination (DRAGOVIĆ-UZELAC *et al.*, 2007).

Table 9 - Effects of cultivars and harvest time on average total antioxidant capacity (TAC) in strawberry in the first and second years of study.

	Total antioxidant capacity (μmol/g)	
	1st year	2nd year
Cultivar	**P<0.0001**	**P<0.0001**
Arosa	4.42a±0.050	2.95a±0.213
Elsanta	4.36b±0.034	2.71b±0.139
Marmolada	4.23c±0.142	2.91a±0.101
Miss	3.48e±0.144	2.66b±0.065
Raurica	3.74d±0.371	2.57c±0.091
Harvest time	**P<0.0001**	**P=0.3481**
1	4.07a±0.338	2.76±0.282
2	3.98b±0.481	2.75±0.126
3	4.09a±0.436	2.78±0.152
Int. C×Ht	**P<0.0001**	**P<0.0001**

C = cultivar; Ht = harvest time; Int. C×Ht = interaction Cultivar×Harvest time. Within each column and effect, different letters indicate significant differences between means at P≤0.05 according to Duncan's multiple range test.

The statistical analysis showed that the antioxidant capacity significantly depended on cultivar but not on year or harvest time. Harvest time had no significant influence on the antioxidative capacity of the strawberry cultivar fruit studied, but there was a significant difference between the research years (Table 9).

CONCLUSIONS

Strawberry fruits are a good source of antioxidative compounds. The results of this study confirm that climatic conditions during the ripening of fruits (mainly frequency and quantity of rainfall) significantly effected on the concentration of antioxidative compounds. Statistical analysis of differences between the cultivars showed that cultivar has the main impact on the concentration of antioxidative compounds and their antioxidative activity, and that the dif-

ferences between cultivars did not depend on harvest time. It was also shown that strawberry fruits with higher phenolic compound values, especially those with a higher anthocyanin fraction, had higher antioxidant activity. Strawberry fruits can therefore be considered to be a good source of natural antioxidant compounds.

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PAPER

CHEMICAL AND PHYSICAL CHARACTERIZATION OF CACTUS CLADODE (*OPUNTIA FICUS-INDICA*) POWDER

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ABSTRACT

Some physical and chemical characteristics of cactus cladode powder and the influence of concentration, temperature and pH on the viscosity of a water suspension were studied. The powder showed low water activity (0.53). The total dietary fiber content was 42.99%, and the ratio of insoluble fiber to soluble fiber was 2:1. The pH significantly influenced the viscosity of the powder suspensions at low concentration and temperature. The concentration significantly affected the viscosity at all temperatures and pH values. The IDF/SDF ratio suggests that the main physiological effect would be to regulate intestinal functioning, depending on other factors such as daily intake.

- Key words: cactus cladodes, cactus pads, cactus powder, dietary fiber, *Opuntia ficus-indica*, semiarid plants -

INTRODUCTION

In recent years, interest in healthy foods has increased markedly. Consumers prefer low-cholesterol, low-fat, low-calorie and high-fiber foods. There are many studies about the effects of fiber in humans (GALLAHER and SCHNEEMAN, 2001; CHAU *et al.,* 2004; NAS, 2005; NELSON, 2001). Consumers are aware of the relationship between fiber consumption and the control of cholesterol and the prevention of illnesses such as diabetes and obesity. Dietary fiber can also contribute to the prevention, or treatment of illnesses, like gastrointestinal disorders, that are associated with a lack of dietary fiber intake. Thus, the trend is to search for new, natural sources of dietary fiber for the development of food ingredients. Cactus cladode (*Opuntia ficus-indica*) powder appears to be a promising source to obtain this kind of ingredient. This powder could be considered a natural food supplement to be used in solid or liquid foods. The age or size of the cladodes and the climatic conditions prevailing when they are harvested have some influence on their physical and chemical composition (RODRÍGUEZ-GARCÍA *et al.,* 2007). BETANCOURT-DOMÍNGUEZ *et al.* (2006) observed that during the development of young cladodes (called "nopalitos" in Mexico), the insoluble fiber increased from 29.87 to 41.65% and the soluble fiber decreased from 25.22 to 14.91% in cladodes from 60 to 200 g, respectively. PIMIENTA (1990) reported an increase in crude fiber as a function of their maturation, from 12 to 17% in 1- and 3-year-old cladodes, respectively.

ZAMBRANO *et al.* (1998) studied some properties of young cladodes (1-3 months), as a source of fiber. The dried product had 20.4% dietary fiber as well as other interesting physico-chemical characteristics: water absorption (5.8 mL/g), water retention (4.7 g/g), organic molecule absorption (0.69 g/g), and cation exchange (0.49 Meq H^+/g), which could explain the role of "nopalitos" in intestinal health. HERNÁNDEZ *et al.* (1998) and DUQUE *et al.* (1998) tested the effects of blanched "nopalitos" (11% fiber) consumption in rats and observed that while the amount of fiber in the diet increased, the deposition production increased, but the rats lost weight and showed some signs of malnutrition. HERNÁNDEZ *et al.* (1998) concluded that the consumption of fresh "nopalitos" must be carefully controlled in humans. The use of cactus cladode powder in food is completely different depending if the prepared food is a solid or a liquid. SÁENZ *et al.* (2002a) replaced a portion of wheat flour with nopal powder (from old cladodes) in biscuits and got good results with up to 15% addition, but also observed that the use of nopal powder in preparing a vegetable soup had a negative effect on the viscosity of the soup when the amount of powder approached 15% (SÁENZ, 2006). Other studies in powdered dessert formulations showed that the use of up to 16% of nopal powder gave products with good texture (SÁENZ *et al.,* 2002b). Recently, in México, young cladodes ("nopalitos") have been included in typical "tortillas" (a thin bread made with corn flour), in order to increase the dietary fiber intake because this product is massively consumed, instead of bread, in this country (SÁENZ *et al.,* 2006). In a complete review about the chemistry of cactus stems, STINTZING and CARLE (2005) concluded that much research is needed to get an insight into the multitude of bioactivity properties reported in the literature.

The objectives of this study were to determine the physical and chemical characteristics of cactus cladode powder, from mature pads, as a source of natural dietary fiber for foods and the effect of the concentration, temperature and pH on the viscosity of the powder suspensions in water.

MATERIAL AND METHODS

Cactus cladodes (1-, 2- and 3-year-old, blends in the same ratio) from *Opuntia ficus-indica* from an orchard located in the semiarid central region of Chile were used. The flour was obtained according to the method described by SEPÚLVEDA *et al.* (1995), which involves drying the cladodes in a forced air tunnel (70°-80°C for 6-8 h; 2 ms^{-1}) and milling. The milling was done first, using a knife mill with a 1 mm diameter sieve and then in a lab mill (Mills 3800) with a 150 μm sieve.

Physical and chemical analysis

The following analyses were performed on the cactus cladode powder: particle size using the official method of the A.A.C.C. (1989); moisture (A.O.A.C.,1996), water activity (a_w), with a Lufft equipment model 5830 (Fellbach, Germany); color parameters (L*, a*, b*, C* and h°) with a reflectance colorimeter Minolta CR-200b (Osaka, Japan); water absorption index (W.A.I.) according to the method described by QUINN and PATTON (1978); total dietary fiber, soluble (SDF) and insoluble (IDF) (LEE *et al.*,1992); calcium, sodium, potassium and phosphorus (A.O.A.C, 1996); and calorie content using the FAO Atwater factors (FAO, 2002).

All the physical and chemical analyses were done in triplicate and the coefficient of variation was calculated.

Microbiological analyses

A total count of aerobic microorganisms, total yeast and molds were determined (PASCUAL, 1982).

Viscosity measurements

The effect of the concentration (2.5, 5.0 and 7.5%), temperature (10°, 20°, 40° and 70°C) and pH (4.0, 6.0 and 7.0) on the viscosity of a cactus cladodes powder/water suspension was measured with a Brookfield viscometer model RVT (Middleboro, MA, USA), with different spindles according to the concentration.

The experimental design for the viscosity effects was totally random, with a factorial arrangement (3x3x4) with 4 replications. The results were analyzed by ANOVA applying a Duncan Test when differences were detected ($p \geq 0.05$).

RESULTS AND DISCUSSION

The cactus powder had 77.5% of the particles between 150 and 190 μm and about 7.5%, of the particles larger than 190 μm. These results agree with LARRAURI (1994), who reported that products with high fiber content generally have particle sizes between 150 and 430 μm.

The water activity (Table 1) was 0.53, similar to that reported by LAHSASNI *et al.* (2003) for dehydrated cladodes. The low a_w value indicates that this powder will be stable during storage mainly against microbiological attack.

The color parameters (Table 1), represented by L*, a*, b*, C* and h°, show that the powder is light green with a high L* value; similar to that reported by SEPÚLVEDA *et al.* (1995) in a powder prepared with mature cladodes as in this study. The chroma (C*) and the h° value indicate a pale, yellow-greenish color. These parameters are important when the powder is used as a food ingredient. This green color may need to be masked in some foods, such as biscuits, in order to make them more acceptable. However, if the powder is used in a liquid, the pale color is consistent with the color of other vegetable fibers.

The water absorption index (5.6 mL/g) was lower than that reported by ROSADO and DÍAZ (1995) for dehydrated cactus cladodes and for cactus cladode fiber isolates 11.1 and 7.1 mL/g, respectively. This can be attributed to the different raw material used, the cladode age, or to the particular analytical techniques applied in this study.

Table 1 - Physical characteristics of cactus cladode powder.

Analysis	Value	c.v.
a_w	0.53	0.3
Color		
L*	73.37	0.13
a*	-5.20	0.9
b*	26.1	0.02
C*	5.6*	---
h°	101.3*	---
W.A.I.** (mL/g)	5.6	3.46

*Determined by calculus;
**W.A.I. = Water Absorption Index.

Table 2 - Chemical characteristics of cactus cladode powder (g 100 g^{-1}).

Analysis	Value	c.v.
Water	7.14	0.29
Protein (x 6.25)	3.87	7.42
Fat	1.85	3.86
NNE* (x difference)	28.29	0.41
Crude Fiber	6.76	3.29
Total dietary fiber	42.99	6.21
Insoluble dietary fiber (IDF)	28.45	5.79
Soluble dietary fiber (SDF)	14.54	13.08

*Non-nitrogen extract.

Table 2 shows some chemical characteristics of cactus cladodes powder. The water content (7.14%) was similar to that reported by SEPÚLVEDA *et al.* (1995) and assures a good blend with other kinds of flour. Wheat flour commonly has a water content close to 12-13%, while flour from legumes has a lower water content (9.2-9.6%), suggesting the possibility of mixing cactus powder with legume flour, rather than with wheat flour, without caking or microbiological problems.

The total dietary fiber of cactus cladode powder was 43%, where 28.45% was insoluble dietary fiber (IDF), and 14.5% was soluble dietary fiber (SDF). The insoluble/soluble dietary fiber ratio was 2:1; which is a little lower than the 3:1 reported by SALAS-SALVADÓ *et al.* (2006) for most vegetable products and for a powder prepared with from 2- to 3-year-old Tunisian varieties cladodes reported by AYADI *et al.* (2009). It is generally accepted that fiber suitable for use as a food ingredient should have an IDF/SDF ratio close to 2:1 (JAIME *et al.*, 2002; SCHEENAM, 1987). RODRÍGUEZ-GARCÍA *et al.* (2007) reported that dried young cladodes (7.31% moisture) from *Opuntia ficus-indica*, Redonda variety, harvested after 65 days had a total dietary fiber content of 56.56%, with 41.65% IDF and 14.91% SDF. The IDF content was greater than that observed in the present study with mature cladodes; the SDF contents were similar. The IDF/SDF ratio was close to 2.8:1, greater than that observed in the present study.

Total dietary fiber was similar to the 41.8% reported by AYADI *et al.* (2009) for dehydrated spineless cladodes. ROSADO and DIAZ (1995) reported values of 50.4% and near 56.7%, in dehydrated cactus cladodes and isolated cactus cladodes, respectively. The variability between these values can be attributed to the climatic conditions where the plants were grown and

also to the methods of preparation, as well as to the age and variety of the *Opuntia* cladodes used. The ratio between insoluble and soluble fiber was similar between the dehydrated cactus cladodes (ROSADO and DÍAZ, 1995) and the cactus cladode powder obtained in this study. The isolated cactus cladode fiber ratio was high (9:1), probably due to the ethanol used to extract the fiber which caused a loss of some soluble fiber. The cactus powder showed a higher value of dietary fiber compared with whole wheat flour (12.57%) and wheat flour (2.78%). There are many products with much higher dietary fiber contents, for example: *Psillium plantago* (87.3%), glucomannan (83.4%), and soybean extracts (68.8%) (ROSADO and DÍAZ, 1995). Among these three sources of dietary fiber, only glucomanann provides an appreciable proportion of soluble fiber, with an insoluble and soluble fiber ratio of 32:1. FIGUEROLA *et al.* (2005) reported total fiber contents between 60.7 and 89.8% for concentrated apple pomace, of which 4.14 to 14.33% was soluble fiber. The physiologic effect of cactus powder is not easy to define because it depends on many factors, such as the amount consumed, and also the other food constituents of the diet. IBAÑEZ-CAMACHO *et al.* (1983), using another species of *Opuntia*, *Opuntia streptacantha*, reported a hypoglycemic effect in rabbits, associated with the soluble fiber content. More recently, GALATI *et al.* (2007) proposed the protective effect of carbohydrate polymers from *Opuntia* cladodes against ethanol-induced ulcers in rat.

Dietary fiber is associated with several physiological effects, such as decreasing cholesterol levels in the blood GALLAHER and SCHNEEMAN (2001). There is a widespread conviction that the effect of dietary fiber on plasma cholesterol concentration may be largely mediated by enhanced fecal excretion of bile acids. Many types of dietary fibers show hypocholesterolemic activity in human, increasing fecal bile acid output. However, increased bile output due to fiber is not always accompanied by a reduction in plasma cholesterol. Therefore, the mechanism by which fiber exerts its hypercholesterolemia effect remains an intriguing question (GALLAHER and SCHNEEMAN, 2001, CHAU *et al.,* 2004, MC PHERSON, 1992). Dietary fiber is also associated with glucose control and thus with diabetes (GALLAHER and SCHNEEMAN, 2001; IBAÑEZ-CAMACHO *et al.,* 1983; GALATI *et al.,* 2003) and may play a role in reducing the risk of certain types of cancer, particularly colon cancer, and reduce symptoms of chronic constipation, diverticular disease and hemorrhoids (MC PHERSON, 1992; ANDERSON *et al.*, 1994).

There have been several studies on the possible uses of fresh young cactus cladodes ("nopalitos") in medicine (FRATI-MUNARI *et al.,* 1992), as well as some studies directly related to the consumption of "nopal" powder capsules (FRATI-MUNARI *et al.,* 1989). Studies on the use of cactus cladode powder in foods are very scarce. Nevertheless, this possibility is an interesting alternative for increasing fiber consumption, which is known to be frequently below the recommended dietary intake in most countries.

Table 3 - Ash and mineral content in cactus cladode powder.

Analysis	g 100 g^{-1}	c.v.
Ash	15.86	1.96
K	2.05	0.00
Ca	3.43	0.55
Na	0.02	0.00
P	0.11	1.30

Other nutritional components in the cactus powder studied were ash and minerals (Table 3). The calcium content could be considered high and similar to that reported by RODRIGUEZ-GARCÍA *et al.* (2007), 3.30 mg/100 g in of 65-day harvested cactus pads (200 g). However, the availability of this mineral is not clear; some authors report that calcium is in the form of calcium oxalate in the cladodes (PIMIENTA, 1990), which would limit its bioavailability. MCCONN and NAKATA (2004) suggested that although the cactus pear pads are enriched in a number of minerals, the calcium is not freely available because it is present as calcium oxalate crystals. RODRÍGUEZ-GARCÍA *et al.* (2007) reported that when the maturity stage of cladodes increases, calcium oxalate content decreases and calcium increases. This would favor calcium absorption. On the other hand, in studies on the bioavailability of calcium salts cited by WEAVER (2003), it was reported that calcium does not need to be dissociated from low molecular salts, such as oxalates, to be absorbed.

The caloric value of the powder was 6.9 kJ/g. This value was less than that of wheat flour or legume flour 15.33 and 14.95 kJ/g, respectively (FAO/LATINFOODS, 2002). The caloric value of cladode powder was about 40.0% that of wheat flour and is in the range suggested by LARRAURI (1999) for commercial dietary fiber powder, close to 8.00 kJ/g.

The microbiological analysis showed that the total aerobic mesophylic count was 3.3 c.f.u./g and the total yeast and mold count was 4.6 c.f.u./g. These values are low. In addition, the "nopal" powder will not be consumed directly, but rather will be used as an ingredient in foods that are generally thermally treated.

The viscosity behavior of cactus cladode powder suspensions and the effect of pH, concentration and temperature can be observed, respectively, in Figs. 1, 2 and 3, along with the corresponding regression equations. The property of certain types of dietary fiber (pectins, mucilages, gums, carrageenan, etc.) to form

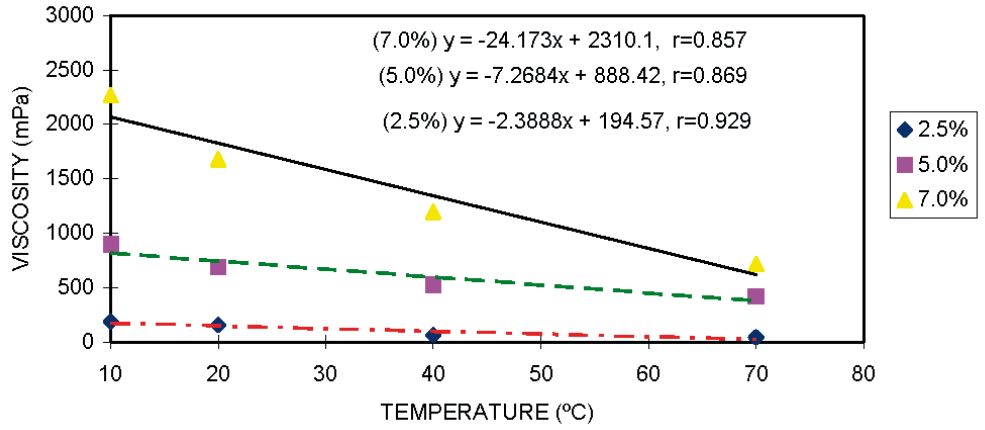


Fig. 1 - Viscosity behavior of a cactus cladode powder (water suspension) at different temperatures and concentrations at pH 4.

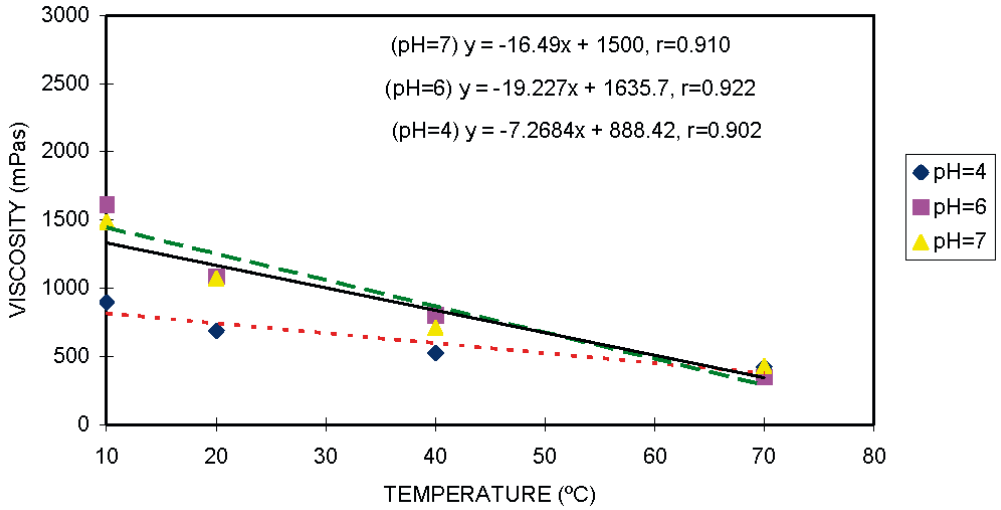


Fig. 2 - Viscosity behavior of a cactus cladode powder (water suspension) at different temperatures and pHs at 5.0% concentration.

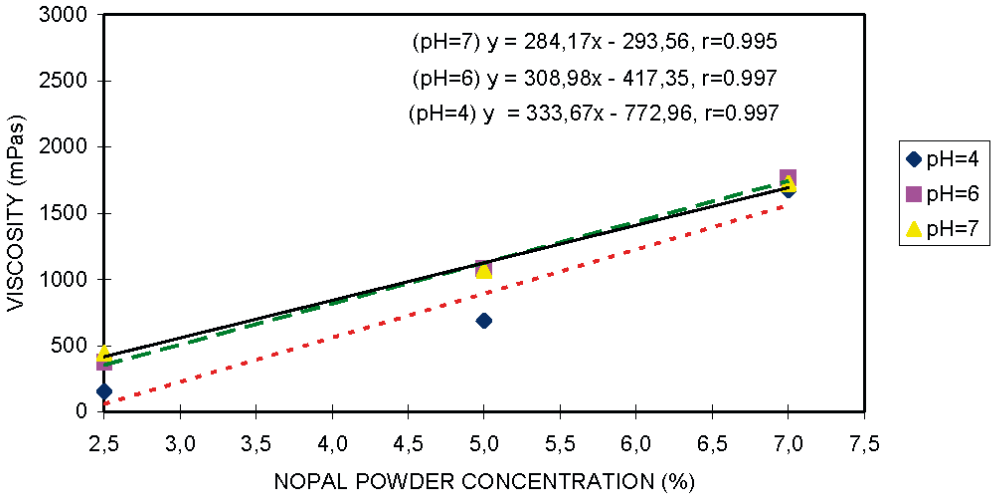


Fig. 3 - Viscosity variation of a cactus cladode powder (water suspension) at different concentrations and pHs at 20°C.

highly viscous solutions is widely known (GALLAHER and SCHNEEMAN, 2001). Viscosity is an important characteristic for the formulation of foods and for their influence on intestinal bowel formation and transit. KENDALL and JENKINS (2004) recommended increasing consumption of viscous fibers to promote heart health. The viscosity of a nopal powder suspension, as in other types of polymer solutions, is affected significantly by temperature (Fig. 1). At higher temperatures the effect on viscosity is less affected by the concentration of the nopal powder, the slopes of the curves are less at higher temperatures (70°C) than at lower temperatures (10°C). The viscosity is also influenced by the concentration of the powder and the pH of the sample (Fig. 2). According to SÁENZ *et al.* (1992), the change observed at different pH values can be attributed to the ionization of carboxylic groups at high pH and the repulsion of negative charges, maintaining the mucilage molecule extended with the consequent increase in the viscosity. The slopes at pH 6 and 7 were similar (19.23 and 16.49, respectively) and greater than that at pH 4 (7.27). This behavior gives useful information for the potential addition of this kind of fiber to some foods because many foods have a pH close to 4.0. The effect of concentration on the viscosity (Fig. 3) shows that independent of temperature, the viscosity increased when the concentration increased. This behavior was also observed in cereal and legume flour suspensions (6.8 and 5.8%, respectively), where the viscosity ranged from 12 to 405 mPa s depending on whether the flours were uncooked or extruded. But when the concentration was increased from 7.5 to 12%, the viscosity values increased significantly, from 1,000 to 14,000 mPa s (REYES, 1984).

The temperature always affected the viscosity at all pH and concentrations assayed. As can be expected, the lower viscosity values are associated with higher temperatures. The nopal powder concentration influenced the viscosity at all the temperatures and pH values assayed, showing a direct relationship between concentration and viscosity.

At a constant pH, as the concentration increased, the viscosity also increased. In a 7% nopal powder water suspension, the viscosity reached a high value (1726.1 mPa s at 20°C); a product with these characteristics could be difficult to manage. For this reason, the recommended concentration in fluid food formulations should be close to 5%. This amount will contribute only a proportion of the recommended daily dietary fiber intake, but it would help increase the low dietary fiber intake of many people. This study and those cited above suggest that a purified cladode powder, using cladodes without the epidermis, could be used as a food additive that would give a different final product having other physical characteristics such as viscosity, color and aroma, allowing for greater addition to foods.

In conclusion, the cactus powder obtained from mature cladodes is a low calorie food ingredient with high fiber content, making it a good source of alternative dietary fiber. The IDF/SDF ratio suggests that the main physiological effect could be to regulate intestinal functioning, depending on other factors, such as the amount of intake. This aspect needs further study. The viscosity produced by this powder is an important property to be considered during the formulation and preparation of foods, especially liquids.

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PAPER

EVALUATION OF ATTRIBUTES ASSOCIATED WITH THE QUALITY OF NOPALITO (*OPUNTIA* SPP. AND *NOPALEA* SP.)

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ABSTRACT

The aim of this work was to evaluate some attributes associated with the quality of four species of nopalito (*O. robusta*, *O. ficus-indica*, *O. undulata* x *O. tomentosa* and *N. cochenillifera*) at three growth stages produced under hydroponic and greenhouse conditions. The following attributes were evaluated: resistance to longitudinal and transversal flexion and penetration, color, mucilage content and pH at the time of harvest. *O. robusta* ssp. *larreyi* showed the highest values of longitudinal and transversal flexion, while *N. cochenilliefera* presented the highest resistance to penetration, mucilage content and pH.

- Key words: color, flexion, growth stage, mucilage, pH -

INTRODUCTION

Nopalitos are vegetative sprouts or young cladodes of cactus pear that are from 15-days to 4-months old (FIGUEROA, 1984). Depending on the production system, season of the year and climate, nopalitos need 20 to 30 days to reach commercial size, which ranges from 10 to 26 cm in length in *Opuntia* and from 5 to 20 cm in *Nopalea* (RODRÍGUEZ-FÉLIX and CANTWELL, 1988; OCHOA *et al.*, 2004; RAMÍREZ-TOBÍAS *et al.*, 2007). The main species used for nopalito production are: *Nopalea cochenillifera*, *Opuntia albicarpa*, *O. ficus-indica*, *O. fuliginosa*, *O. hyptiacantha*, *O. leucotricha*, *O. lindhemeiri*, *O. megacantha*, *O. undulata* x *O. tomentosa*, *O. robusta* and *O. strepthacantha* (RODRÍGUEZ-FÉLIX and CANTWELL, 1988; FLORES-HERNÁNDEZ *et al.*, 2004; BELLÓN *et al.*, 2009).

In Mexico, nopalito has been part of the national cuisine since pre-Columbian times. It is generally served boiled or in a variety of dishes. It is consumed both for its good taste and its beneficial health properties (RODRÍGUEZ-FÉLIX and VILLEGAS-OCHOA, 1997). Nopalitos have a high dietary fiber content and therefore the relationship to the control of cholesterol and the prevention of some diseases like diabetes and obesity have been developed and investigated in recent years (SÁENZ, 2000; SÁENZ *et al.*, 2004; PAÍZ, 2007). This has fostered an increase in nopalito production and commercialization which in turn, has increased the demand for a high-quality product. Hence it is necessary to identify the characteristics associated with consumer quality and production processes.

The term "food quality" comprises a set of features that are real, assumed or perceived by the consumer, which enable him or her to judge and score a given product as equal, better or worse than other similar products (WALDRON *et al.*, 2003). According to FIGUEROA (1984), nopalito quality depends on consumer preferences which are evident in the selection of certain species with characteristics that are seemingly more attractive than those of others.

Nopalitos grown in hydroponics are an important source of protein, fiber and minerals; they contain 19.2% crude protein, 29.5% neutral-detergent fiber (NDF), 16.3% dietary fiber (ADF) and 29.4% ash (RAMÍREZ-TOBÍAS *et al.*, 2007). However, consumer acceptance also depends on other quality indicators such as the amount of fiber (soluble or insoluble), flavor and color, which, similar to the chemical composition, vary according to growth stage (RODRÍGUEZ-FÉLIX and CANTWELL, 1988; RAMÍREZ-TOBÍAS *et al.*, 2007; BETANCOURT-DOMÍNGUEZ *et al.*, 2006). Mucilage content can be considered as an indicator of soluble fiber, whereas resistance to flexion and penetration may indirectly indicate the amount of insoluble fiber. Nopalito taste also depends on acidity, which varies throughout the day, due to the Crassulacean Acid Metabolism (CAM) (RODRÍGUEZ-FÉLIX and CANTWELL, 1988; CORRALES-GARCÍA *et al.*, 2004). The consumer also considers color as a visual quality indicator; CANTWELL (1995) pointed out that high-quality nopalitos must be bright green in color. Thus, the aim of this study was to assess some quality indicators of four nopalito species (*N. cochenillifera*, *O. robusta* ssp. *larreyi*, *O. ficus-indica* and the hybrid *O. undulata* x *O. tomentosa*) at three growth stages and grown in hydroponics and greenhouse conditions.

MATERIAL AND METHODS

Four variants of cactus pear, cultivated intensively to produce nopalitos throughout various climatic regions of Mexico, were assessed: *N. cochenillifera cv.* Tamazunchale, from warm and humid regions; *O. robusta* ssp. *larreyi cv.* Tapón pelón, from areas with a temperate and semi-arid climate; *O. ficus-indica* cv. Tlaconopal, from regions with a temperate and sub-humid climate, and the hybrid *O. undulata* x *O. tomentosa cv.* Valtierrilla, grown in an area with a semi-warm to temperate climate. Nopalitos were collected from these different plants, growing in a hydroponic medium in a tunnel-shaped greenhouse with an east-west orientation, at San José de la Peña, Villa de Guadalupe County in San Luis Potosí State, Mexico. A nutrient solution was applied through sub-irrigation. Irrigation was supplied twice (09:00 and 13:00 h) on alternate days. The pH of the nutrient solution was kept at 5.8 by adding sulfuric acid. The nutrient solution was recycled for 15 days and then totally replaced (OLMOS *et al.*, 1999; RAMÍREZ-TOBÍAS *et al.*, 2007). The region has a semiarid climate with rain in the summer and extremely hot temperatures and cool winters. The mean temperature inside the greenhouse (measured with a hygrothermograph) was 22°C, with an 8°C range, and 61.5% relative humidity.

Nopalitos were harvested upon reaching a certain growth stage (GS) depending on the species and treatment (Table 1). The growth stage was established by dividing the mean length of mature cladodes by four for each species. This number, when multiplied by 1, corresponded to GS1, to GS2 when multiplied by 2, and to GS3 when multiplied by 3 (Table 1). For example, in *O. ficus-indica* the mean length at maturity was 40 cm, with a quarter being 10 cm; thus, the GS1 was 10±1.5; the GS2 was 20.0±1.5 and GS3 was 31.0±1.5. The nopalitos were harvested between 13:00 and 14:00 because *Opuntia* and *Nopalea* have an acid crassulacean metabolism and their acidity changes during the day. With the increase in acid content during cladode development, the pH decreases (RODRIGUEZ-FELIX and CANTWELL, 1988), so the measure of pH was used as an indirect method to determine

Table 1 - Growth stage (cm) of nopalitos in four species of *Opuntia* spp. and *Nopalea* sp. (RAMÍREZ-TOBÍAS *et al.*, 2007).

Growth stage	Species			
	N. cochenillifera	*O. robusta* ssp. *larreyi*	*O. undulada x O. tomentosa*	*O. ficus-indica*
GS1	6±1.5	8±1.5	8±1.5	10±1.5
GS2	12±1.5	17±1.5	16±1.5	20±1.5
GS3	17±1.5	25±1.5	24±1.5	31±1.5

the acidity. The variables assessed were resistant to flexion and penetration, mucilage content, color and pH. Resistance to flexion and penetration measurements were carried out the day after harvesting, using a universal testing machine (Instron® model 1000, Grove City, PA, USA). Resistance to flexion was assessed as the strength needed to bend the nopalito lengthwise (longitudinal flexibility) and widthwise (transverse flexibility) by 18.5% (RODRÍGUEZ-FÉLIX and VILLEGAS-OCHOA, 1997). The fiber arrangement is in a rhomboid form, therefore the resistance is different in each of the longitudinal and transverse planes. Resistance to penetration was measured at the base, center and apex with a 1.5 mm diameter awl working at a speed of 100 mm/min (RODRÍGUEZ-FÉLIX and VILLEGAS-OCHOA, 1997; GUEVARA *et al.*, 2003; ANZUREZ-SANTOS *et al.*, 2004), and a mean resistance was calculated for each nopalito. To determine mucilage content, nopalitos were chopped into small pieces, kept in separate bags inside an ice cooler with a refrigerant gel during transportation, and were then kept at -20°C in the lab. These were then dried in a laboratory lyophilizer (ilShin® Europe model TFD5505, Ede, The Netherlands) and ground in a mortar. Mucilage extraction was conducted according to the procedure described by PEÑA-VALDIVIA and SÁNCHEZ-URDANETA (2006). To this end, 1 g of freeze-dried sample was placed in a test tube with 10 mL water in a water bath for 15 min. The tube was centrifuged (Thermo IEC model 120, Waltham, MA, USA) at 1951.6 G for 15 min and the liquid was transferred to another test tube. The precipitate was re-suspended in 5 mL hot water and centrifuged again. Cold ethanol was added and acidified (to reach a pH of 5.8) using a 4:1 ratio, and refrigerated for 24 h. The substance was then centrifuged until the liquid was clear. To remove ethanol residues, the material obtained was dehydrated in an oven at 85°C to achieve a constant weight. The final residue is expressed as percent of mucilage in dry matter.

Color was determined the day after harvest according the Hunter system (MACDOUGALL, 2002) using a Hunterlab colorimeter (AccuProbe™ model HH06, Pittsford, NY, USA). The measurements were taken at the apex, center and base of the nopalito. Lightness values (L^*) were obtained, using the red-green (a*) and yellow-blue (b^*) dimensions and an average was calculated for each of these measurements for each nopalito; the hue angle [h^o= arctan (b^*/a^*)] and chroma [$C^*= (a^{*2} + b^{*2})^{0.5}$] were also calculated (SÁENZ, 2000; MACDOUGALL, 2002).

The pH was evaluated *in situ* with a portable potentiometer (Oacton®, Ayer Rajah Crescent, Singapore); to this end, the glass electrode and the temperature probe were introduced through an incision into a piece of nopalito (FLORES-HERNÁNDEZ *et al.*, 2004). Measurements were taken between 13:00 and 14:00 h (CORRALES-GARCÍA *et al.*, 2004).

A random full-block experimental design was used, with a factorial arrangement of treatments (12) with four replicates. Factors and levels were: cactus pear species (four) and growth stage (three). A total of 48 experimental units were assessed. The GLM procedure and the LS-MEANS test for comparing means were used to analyze the results (SAS, 1990).

RESULTS AND DISCUSSION

The species growth-stage interaction was statistically significant ($p < 0.05$) for resistance to longitudinal and transversal flexion, whereas it was highly significant for the other variables ($p < 0.001$) (Table 2). Thus, the results obtained for each attribute depended on both the species and the developmental stage of the nopalito. Maturation of nopalitos resulted in an increased resistance to longitudinal flexion in *O. robusta* ssp. *larreyi* ($p < 0.05$) from GS1 to GS3, whereas nopalitos from other species did not require a greater force to bend (Fig. 1A). Lateral flexibility was statistically similar ($p > 0.05$) in GS1 and GS2 nopalitos of the four species; while, GS3 nopalitos of the four species required a greater force to bend transversally (Fig. 1B).

Resistance to flexion is related to turgidity as well as to the arrangement of fibers associated with vascular bundles. Similar relations were observed by BETANCOURT-DOMÍNGUEZ *et al.* (2006). Greater turgidity might result in greater resistance, since parenchyma cells are filled with water in the absence of hydric stress. The fibers run diagonally from one cladode edge to

Table 2 - Effect of species and growth stage on attributes related with the nopalito quality. Means with different letters in a column are statistically different (p<0.05).

Factor	Attribute							
	Flexion (N)				Color			
	Long[a]	Trans[b]	Pen[c] (N)	Muc[d] (%)	*h**[e]	*C**[f]	*L**[g]	pH
Species								
Noco[h]	2.94b	6.77b	2.77a	8.27a	111.14c	30.18a	43.20a	4.77a
Ficu[i]	2.19bc	12.22a	2.91a	5.34c	112.90b	20.46c	38.96c	4.52bc
Robu[j]	5.20a	10.70a	2.39b	6.76b	114.15a	21.13c	41.05b	4.63ba
Unxt[k]	1.40c	5.51b	2.52b	6.93b	113.51ab	24.26b	40.20b	4.47c
Growth stage								
GS1	2.88ab	5.72b	2.58b	6.87a	111.30c	27.73a	41.49a	4.69a
GS2	2.32b	6.80b	2.41c	7.31a	113.16b	24.23b	41.48a	4.43b
GS3	3.44a	14.62a	3.00a	6.36a	115.67a	19.20c	38.61b	4.63a
Significance								
SE[l]	0.2	0.49	0.03	0.22	0.16	0.34	0.19	0.02
SP[m]	**	***	***	***	***	***	***	***
GS[n]	***	***	***	NS	***	***	***	***
SP*GS[o]	*	*	***	***	***	***	***	***

[1]Long = resistance to the longitudinal flexion expressed in Newtons (N); [2]Trans = resistance to the transversal flexion in N; [3]Pen = resistance to the penetration in N; [4]Muc = content of mucilage expressed in percentage referred to the dry matter; [5]*h** = hue angle expressed in degrees; [6]*C** = chroma; [7]*L** = luminosity; [8]Noco = *N. cochenillifera*; [9]Ficu = *O. ficus-indica*; [10]Robu = *O. robusta* ssp. *larreyi*; [11]Unxt = *O. undulata* x *O. tomentosa*; [12]SE = standard error; [13]SP = species; [14]GS = growth stage; [15]SP*GS = interaction species*growth stage.
***, p≤0.001; **, p≤0.01; *, p≤0.05; NS, p≥0.05.

the other, crossing over one another, forming a net with diamond-shaped spaces (GIBSON and NOBEL, 1986). Likewise, the length and width of these spaces are seemingly associated with the width:length ratio of the cladode. Thus, in *O. robusta* ssp. *larreyi* nopalitos, characterized by a width:length ratio that is close to 1 (0.66), fibers form nets with the smallest spaces relative to those of the other species studied. In an investigation by RODRÍGUEZ-FÉLIX and VILLEGAS-OCHOA (1997) of 20 cm *O. ficus-indica* nopalitos (equivalent to GS3) grown outdoors, the resistance to flexion (longitudinal and transversal) was greater than that in this study (Table 2). The greater resistance of field-grown nopalitos, which lack a continuous water supply and are subjected to greater mechanical stress, is explained by the fact that, under those conditions, fibers associated with vascular bundles are more robust than those of nopalitos grown in a hydroponics system in a protective greenhouse environment.

The resistance to penetration (Fig. 1C) observed in *O. ficus-indica* and *N. cochenillifera* nopalitos (GS1 and GS2) was similar ($p > 0.05$) and higher ($p < 0.05$), respectively, than the one in *O. undulata* x *O. tomentosa* and *O. robusta* ssp. *larreyi*. *Opuntia ficus-indica* GS3 nopalitos displayed the highest resistance to penetration ($p < 0.05$), while there were no significant differences among the other species. This overall increase in resistance to penetration while developing from GS2 to GS3 might have derived from an increase in cellulose and lignin content (GARCÍA and PEÑA, 1995). Indeed GUEVARA *et al.* (2003) observed a lower resistance to penetration in *O. ficus-indica* nopalitos associated with a lower crude fiber content (consisting of lignin, mostly cellulose and, in some cases, a small portion of hemicellulose, according to GARCÍA and PEÑA, 1995). Resistance to penetration can also be associated with the hydric stress of a plant, since stored water causes a reduction in resistance to penetration (GIBSON and NOBEL, 1986). In fact, RODRÍGUEZ-FÉLIX and VILLEGAS-OCHOA (1997) found a higher resistance to penetration in partially dehydrated nopalitos.

BETANCOURT-DOMINGUEZ *et al.* (2006) suggested that hardness of nopalitos could be associated with their anatomy. To observe the effect of the cuticle on the resistance to penetration, its thickness was measured in 1-year-old cladodes from each species. The cellulose and lignin distribution was also observed in transversal sections after staining with safranin and fast green respectively (RUZIN, 1999). Microscopic measurements were made; *N. cochenillifera* had the thinnest cuticle (4.10±0.99 μm), while *O. ficus-indica* (17.45±3.53 μm) has the thickest. Hypodermis cell walls displayed the greatest safranin staining; furthermore, there were five to six rows of hypodermal cells, and

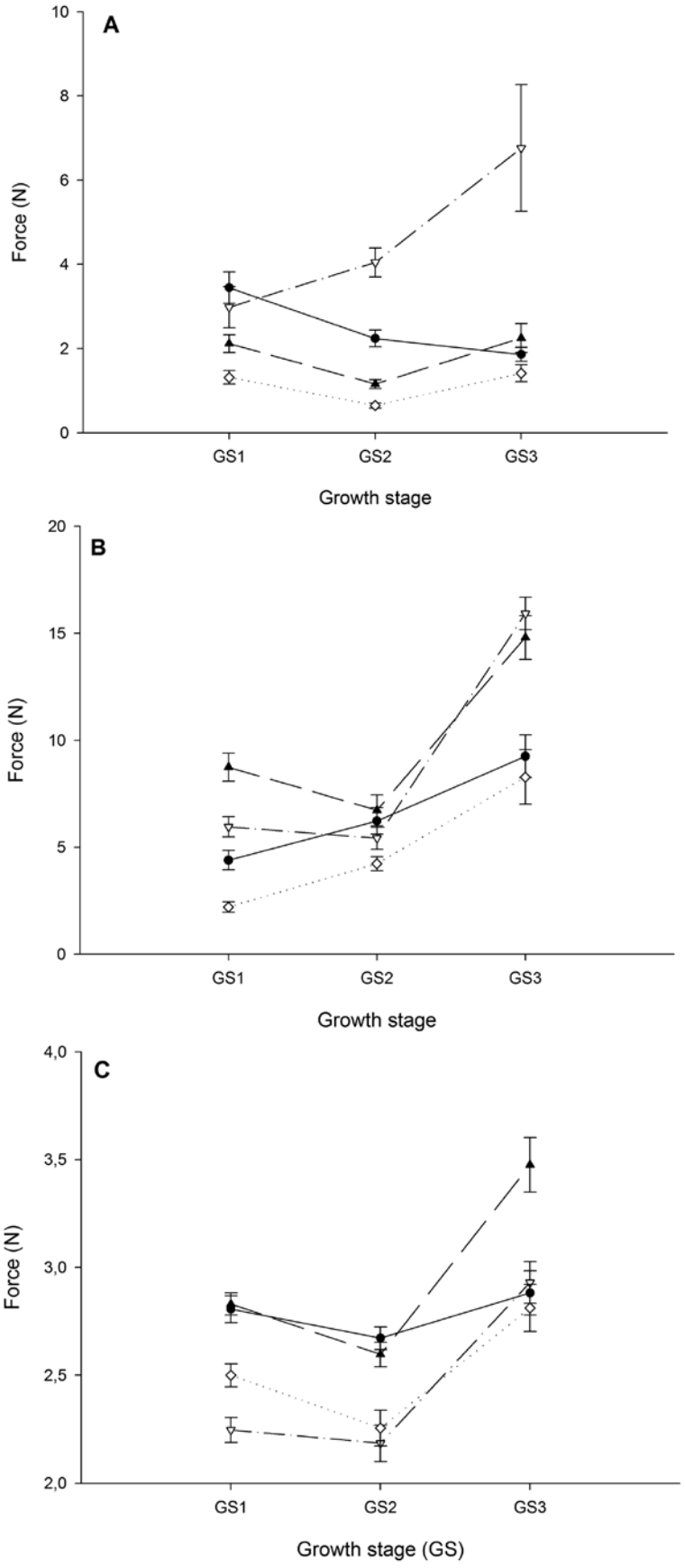


Fig. 1 - Resistance to the longitudinal (A) and transversal flexion (B) and penetration (C) in four species of nopalitos at three growth stages produced in hydroponics. (●) *N. cochenillifera*, (▲) *O. ficus-indica*, (▽) *O. robusta* ssp. *larreyi* and (◇) *O. tomentosa* x *O. undulata*.

druses (calcium oxalate crystals) in cells in the row closest to the epidermis. Likewise, parenchyma cells appeared to have the highest cellulose content. The resistance to penetration was lower than the values recorded in nopalitos grown in the field without irrigation (RODRÍGUEZ-FÉLIX and VILLEGAS-OCHOA, 1997; GUEVARA *et al.*, 2003). Thus, resistance to penetration seems to depend more on hypodermal cell-wall lignification and the chain of druses than on the characteristics of the cuticle and epidermis.

Mucilage content in the *Opuntia* species studied was similar to that reported previously (PEÑA-VALDIVIA and SÁNCHEZ-URDANETA, 2006; SÁENZ *et al.*, 2004); however, each displayed a different pattern in relation to the stage of maturity (Fig. 2). *O. undulata* x *O. tomentosa* nopalitos showed an increase in mucilage content that corresponded directly to the maturity level. As for the other species, *O. ficus-indica* showed the lowest mucilage content ($p < 0.05$) in the three growth stages. Mucilage content in *O. robusta* ssp. *larreyi* and *O. ficus-indica* GS1 and GS2 nopalitos was statistically similar, and decreased in GS3 nopalitos in both species. This decrease might have been due to the increase in the parenchyma/chlorenchyma ratio and fiber content (RODRÍGUEZ-FÉLIX and CANTWELL, 1988). *N. cochenillifera* nopalitos had a higher mucilage content than those of *Opuntia*, but the values were lower than those reported for the same species in the literature (NERD *et al.*, 1997). No significant differences were observed between the three GS. *N. cochenillifera* showed the highest neutral-detergent insoluble fiber content (RAMÍREZ-TOBÍAS *et al.*, 2007) and an increase in dietary fiber from GS2. Other studies have shown that nopalitos contain little insoluble fiber in contrast to soluble fiber (PEÑA-VALDIVIA and SÁNCHEZ-URDANETA, 2006). The above seems to indicate that the soluble fiber content decreases as the content of insoluble fiber content increases with maturity. Mucilage content may influence consumer acceptance, which may vary regionally or according to cultural factors, but no data are available on the optimum mucilage content.

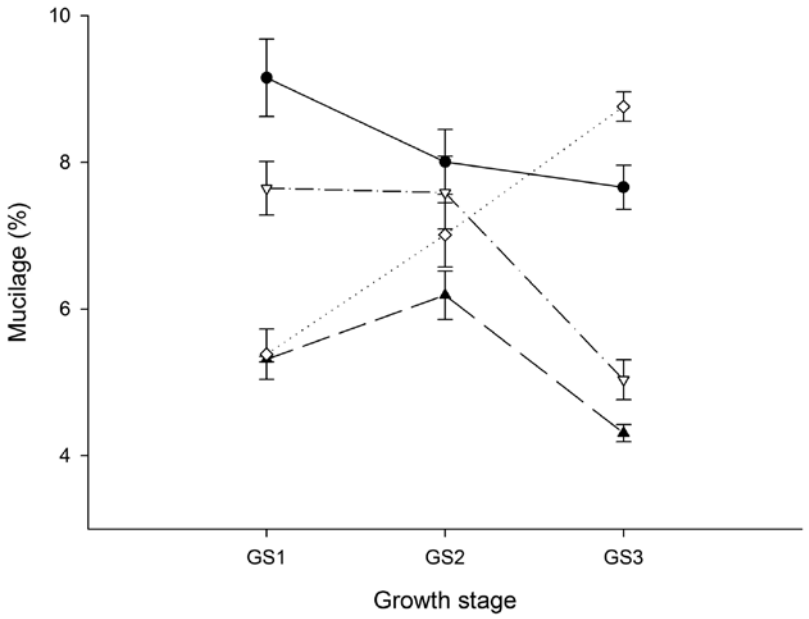


Fig. 2 - Content of mucilage in four species of nopalitos at three growth stages produced in hydroponics. (●) *N. cochenillifera*, (▲) *O. ficus-indica*, (▽) *O. robusta* ssp. *larreyi* and (◇) *O. tomentosa* x *O. undulata*.

The color of the four species of nopalitos increased in hue angle from GS1 to GS3 (Fig. 3A). *N. cochenillifera* nopalitos had the lowest hue angle in the three GS; however, these also had the highest chroma (Fig. 3B) and luminosity (Fig. 3C) values. In contrast, *O. robusta* ssp. *larreyi* nopalitos had the highest hue angle and the lowest intensity from GS2; both attributes displayed intermediate values in GS1. The shift in hue angle from yellow to green observed in nopalitos from GS1 to GS3 could be related to the increase in chlorophyll content (LADYGIN *et al.*, 2004; GIBSON AND NOBEL, 1986). The shift in cladode color seems to be correlated to the increased chloroplast content and rate of photosynthesis, which, in turn, might be related to cladode development. GEORGE *et al.* (2004) reported that consumers prefer nopalitos with a hue angle near 114° and a chroma and luminosity of 30 and 43, respectively. In this investigation, none of the species displayed the color attributes that GEORGE *et al.* (2004) considered to be indicative of the highest quality. Only two species had similar values in the color attributes, *O. robusta* ssp. *larreyi* in hue and *N. cochenillifera* in chroma and lightness. The waxy cuticle and pubescence in *O. robusta* spp. *larreyi* and *O. undulata* x *O. tomentosa*, respectively, probably influenced the chroma and luminosity values, which may explain the greener hue angle and the lower chroma.

The pH values recorded were similar to those obtained by GEORGE *et al.* (2004), OCHOA *et al.* (2004) and BETANCOURT-DOMINGUEZ *et al.* (2006), but were lower than those reported by RODRÍGUEZ-FÉLIX and CANTWELL (1988) and FLORES-HERNÁNDEZ *et al.* (2004). The pH values decreased from GS1 to GS2 and then increased from GS2 to GS3 in *N. cochenillifera*, *O. ficus-indica* and *O. undulata x O. tomentosa* nopalitos (Fig. 4). These values were significant in the latter two species. BETANCOURT-DOMINGUEZ *et al.* (2006) reported similar trends. In contrast, the pH values in the nopalitos of *O. robusta* ssp. *larreyi* remained constant and were not significantly different ($p > 0.05$) during development. A study on pH RODRÍGUEZ-FÉLIX and CANTWELL, 1988 reported that the pH decreased in nopalitos as growth progressed; a different pattern was observed in this investigation.

A shift from the CAM to C_3 metabolic pathway was observed in nopalitos grown under irrigation (PIMIENTA-BARRIOS *et al.*, 2005) and the pH values increased. This metabolic shift from CAM to C_3 has already been related to water availability (CUSHMAN, 2001). GS1 and GS3 showed C_3 metabolism, which may have led to a higher pH in these stages; the decrease in pH in GS2 in three of the four species may have been due to a hydric stress caused by an increase in temperature. Studies on other species (*Phaseolus vulgaris*, *Lolium temunentum*, *Zea mays* and *Commelina communis*) as well as in cactus pear under non-irrigation and irrigation, by NIEDDU *et al.* (1997) and THOMPSON *et al.* (1997), showed that the hydric and acidity levels in plants were related to hormonal function, specifically in relation to auxins and abscisic acid (ABA). NIEDDU *et al.* (1997)

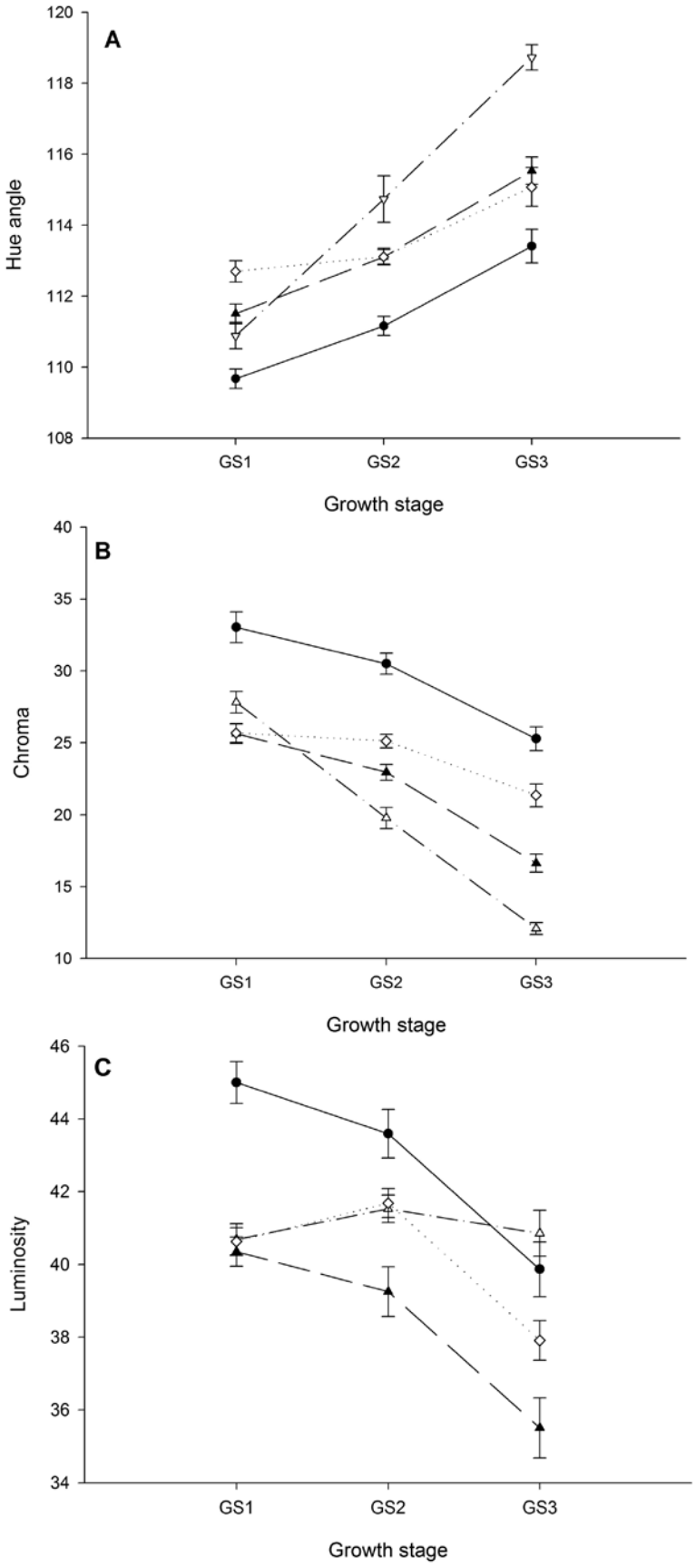


Fig. 3 - Hue angle (A), chroma (B) and luminosity (C) in four species of nopalitos at three growth stages produced in hydroponics. (●) *N. cochenillifera*, (▲) *O. ficus-indica*, (▽) *O. robusta* ssp. *larreyi* and (◇) *O. tomentosa* x *O. undulata*.

and THOMPSON *et al.* (1997) observed that plants developed under adequate irrigation have a lower pH in the leaves compared to plants grown under drought conditions. GS2 nopalitos from *N. cochenillifera, O. ficus-indica* and *O. undulata* x *O. tomentosa* showed a decrease in ABA concentration in xylem fluids (HARTUNG and RADIN, 1989, cited in THOMPSON *et al.*, 1997; NIEDDU *et al.*, 1997), since, according to the findings of RAMÍREZ-TOBÍAS *et al.* (2007), GS1 showed the highest percentage of dry matter, which dropped significantly in GS2. GS1 had the highest relative water content coupled with the highest nopalito quality is determined by features perceived through the senses; it is also influenced by anatomical and physiological differences inherent in each species, as well as by its availability and abundance in different regions (FIGUEROA, 1984). In other words, nopalito quality is determined by a set of anatomical and physiological features that result in perceptible attributes, which allow the consumer to develop certain preferences for the various nopalito species available in a particular region.

CONCLUSIONS

Resistance to flexion and penetration, mucilage content, chroma, hue angle, lightness and acidity in nopalitos depends mostly on the species, stages of development and hydric status.

The quality features preferred by consumers have been determined in the regions where the species are found. *N. cochenillifera* nopalitos are moderately firm, with a high mucilage content. They have an intense and luminous color and a lower pH. *O. ficus-indica* nopalitos have a greater resistance to penetration, lower mucilage content, a medium green color, a lower intensity and lightness and mean pH values. *O. robusta* ssp. *larreyi* nopalitos have a greater resistance to flexion, a lower resistance to penetration, a medium mucilage content, a more greenish color with an intense and luminous color, and a medium pH. *O. undulata* x *O. tomentosa*, have a lower resistance to flexion and pen-

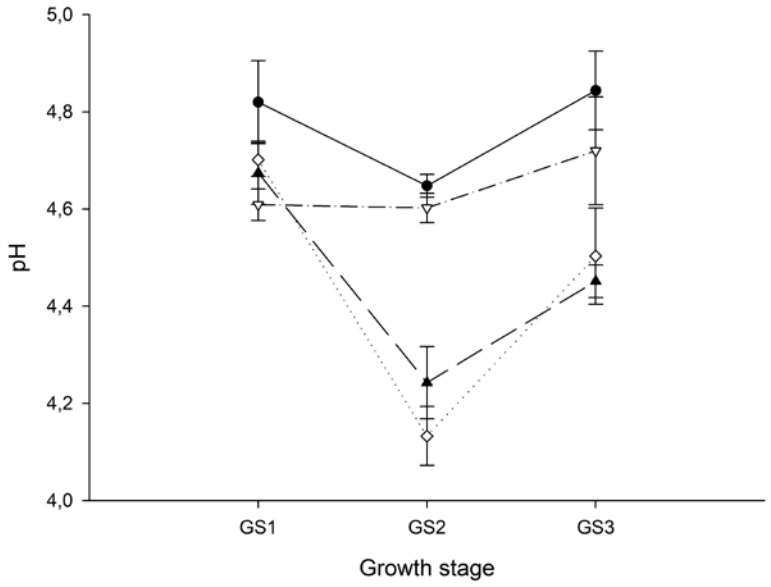


Fig. 4 - pH in four species of nopalitos at three growth stages produced in hydroponics. (●) *N. cochenillifera*, (▲) *O. ficus-indica*, (▽) *O. robusta* ssp. *larreyi* and (◇) *O. tomentosa* x *O. undulata*.

etration, an intermediate mucilage content, and a relatively intense, luminous green color and lower pH.

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PAPER

EFFECT OF DRYING METHOD ON THE SORPTION ISOTHERMS AND ISOSTERIC HEAT OF PERSIMMON PULP POWDER

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ABSTRACT

Moisture equilibrium data of persimmon pulp powders with 50% maltodextrin (dry basis) obtained with different drying methods were determined at 20, 30, 40 and 50°C. The spray-dryer gave a dry product with a higher adsorption capacity than the other methods. The vacuum- and freeze-dried products had the same adsorption capacity. The highest isosteric heat of sorption was observed for powders produced by spray-drying. The isokinetic temperature (T_B) calculated for persimmon pulp powder obtained by vacuum-, spray- and freeze-drying were 541.4 K, 616.3 K, 513.2 K, respectively. The sorption process was spontaneous and enthalpy controlled.

- Key words: freeze-drying, spray-drying, thermodynamic properties, vacuum-drying, water activity, water sorption -

INTRODUCTION

The world's persimmon production in 2008 was 3,627,575 tons, which was an increase of about 7% with respect to 2007. China produced about 70% of the world annual crop (2,533,899 tons), followed by Korea (430,521 tons), Japan (244,800 tons) and Brazil (around 169,000 tons from an area of 8,650 ha) (FAOSTAT, 2010).

A number of persimmon varieties have a marked astringency due to their highly soluble tannin contents, but this undesirable attribute can be removed by several methods, such as exposing the fruit to anaerobic conditions or to products of anaerobic respiration, or even by immersion in water at moderate temperatures (40° to 60°C). The relatively high temperatures commonly applied during convective drying also lead to tannin degradation, whereas sugars present in the fruit exude to the surface where they crystallize. The result is a sweet, tasteful, and non-astringent dried product, which is already traditionally consumed in oriental countries (NICOLETI *et al.*, 2007).

Persimmon is rich in ascorbic acid (15 mg/100 g of edible portion) and vitamin A (2710 IU/100 g of edible portion) (TOUS and FERGUSON, 1996). It is also rich in phenolic compounds, other than tannins, and antioxidant effects have been associated with these compounds (GORINSTEIN *et al.*, 2000).

The development of adequate drying techniques has received special attention focused on preserving the sensorial and nutritional quality of the dried fruit. In addition to adding commercial value to the fruit, drying reduces waste and post-harvest losses, and could allow them to be marketed for extended periods of time, with minor dependence on seasonal conditions (MARQUES *et al.*, 2007). Powders obtained from fruit pulp are interesting products because they are good alternatives for convenient and health food products or ingredients in formulated foods. Their physical form provides a stable, natural ingredient, which may be easily dosed and used to impart color and taste to food products (BHANDARI *et al.*, 1993).

Several types of dryers can be used in food drying and they are selected based on a number of factors, including process characteristics and the desired form of the final product. For paste drying, the diversity of physical characteristics of the materials makes it difficult to recommend a specific type of dryer (FREIRE, 1992). In accordance with TSAMI *et al.* (1999), the final product characteristics such as water sorption ability, porosity, and color depend on the drying method used. Several changes can occur during drying, including changes in solubility, rehydration and shrinkage, alteration in shape, size, porosity and density reduction, which can influence the adsorption capacity of the dried products. Spray-dryers are used to dry solutions, suspensions, emulsions, pulps and pastes. Fruit powders produced by spray-drying usually present small (10-100 μm), amorphous particles that are susceptible to glass transition related changes. These changes include stickiness, caking and collapse that lead to poor handling and reconstitution properties (FUCHS *et al.*, 2006; CANO-CHAUCA *et al.*, 2005). Vacuum-drying has been successfully applied to many fruits and vegetables. Vacuum-dried products are characterized by a better quality retention in relation to hot air-drying at atmospheric pressure (JAYA and DAS, 2005; WANG *et al.*, 2007). Freeze-drying results in high-quality dehydrated products due to the absence of liquid water and the low temperatures required in the process. The solid state of water during freeze-drying protects the primary structure and minimizes changes in product shape, with minimal reduction of volume, providing a dry product with porous structure (RATTI, 2001). Fruit juices and pulps produced by freeze-drying have high contents of amorphous sugars that favor high water adsorption (CARLOS *et al.*, 2005).

Water sorption isotherms are useful thermodynamic tools for predicting the interaction between food components and water. Sorption isotherms can also be used to investigate the structural features of a food product, such as specific surface area, pore volume, pore size distribution and crystallinity. Such data can be used when selecting appropriate storage conditions and packaging systems that optimize aroma retention, color, texture, nutrients and biological stability and that optimizes the drying or rehydration conditions of a product (RIZVI, 1995). Sorption isotherms of some fruits and vegetables dried by different methods have been studied, including freeze-drying and vacuum shelf drying (DEBNATH *et al.*, 2002; GABAS *et al.*, 2007; MARTINELLI *et al.*, 2007), spouted bed drying (COSTA *et al.*, 2003), and foam-mat drying (ALEXANDRE *et al.*, 2007).

The net isosteric heat of sorption (q_{st}) is a thermodynamic function that can be calculated from water sorption isotherms obtained at three or more temperature levels; it is related to the energy needed to remove water from a solid matrix, i.e., it is related to the water-solid binding strength. Knowledge of the net isosteric heat of sorption is of practical value when completing the drying calculations and modeling the energy requirements. In addition, it provides important information about the state of water in food products (McLAUGHLIN and MAGEE, 1998; AL-MUHTASEB *et al.*, 2004; KAYA and KAHYAOGLU, 2005).

The enthalpy-entropy compensation or isokinetic theory investigates physical and chemical phenomena involved in water sorption (AGUERRE *et al.*, 1986; MADAMBA *et al.*, 1996). Changes in enthalpy (ΔH) may provide a measure of the energy changes that occur during sorption processes. Entropy changes (ΔS) may also be associated with the binding or repulsive forces in the system.

Gibbs free energy (ΔG) may be indicative of the affinity of sorbents for water and provides a criterion as to whether water sorption occurs as a spontaneous ($-\Delta G$) or non-spontaneous process ($+\Delta G$).

Considering that sorption isotherms are generally affected by drying methods, the objective of this work was to determine and compare sorption isotherms and thermodynamic properties of persimmon pulp powder produced by freeze-drying, vacuum-drying and spray-drying in order to obtain information about the effect of drying method on the sorption properties of the powdered fruit pulp.

Mathematical development

The Guggenheim, Anderson and de Boer (GAB) isotherm equation has been widely used to describe the sorption behavior of foods, especially fruit (LOMAURO *et al.*, 1985; IGLESIAS and CHIRIFE, 1995; MAROULIS *et al.*, 1998; GABAS *et al.*, 2000; TELIS *et al.*, 2000). Having only three parameters, the GAB equation adequately represents the experimental data in the range of water activity (a_w) which is of most practical interest in food, i.e., $0.10 \leq a_w \leq 0.90$. It is a relatively simple model with parameters that have a physical meaning. Mathematically, it is expressed as follows (RIZVI, 1995):

$$X_{eq} = \frac{(C-1)Ka_wX_m}{1+(C-1)Ka_w} + \frac{Ka_wX_m}{1-Ka_w} \quad (1)$$

where X_{eq} is the equilibrium water content (dry basis) at water activity a_w, X_m is the water content (dry basis), corresponding to an adsorbed monolayer (BET), and C and K are the GAB model constants related to the temperature effect.

The net isosteric heat of sorption (q_{st}) is defined as the difference between the isosteric heat of sorption (Q_{st}) and the heat of vaporization of pure water (λ_w). It can be determined from the following equation, which is derived from the Clausius-Clapeyron equation (RIZVI, 1995):

$$\left.\frac{\partial \ln(a_w)}{\partial(1/T)}\right|_X = -\frac{Q_{st}-\lambda_w}{R} = -\frac{q_{st}}{R} \quad (2)$$

where q_{st} is the net isosteric heat of sorption (J/mol), Q_{st} is the isosteric heat of sorption (J/mol), λ_w is the heat of vaporization of pure water (J/mol), R is the universal gas constant (8.314 J/mol K) and T is the absolute temperature (K).

The change in molar entropy can be calculated according to RIZVI (1995):

$$\Delta S = \frac{\Delta H - \Delta G}{T} \quad (3)$$

where ΔS is the change in specific entropy (J/mol K), ΔH is the change in enthalpy (J/mol), and ΔG is the free energy (J/mol) calculated as

$$\Delta G = RT \ln a_w \quad (4)$$

Substituting equation (4) in (3) and rearranging results in:

$$\ln a_w = \frac{\Delta H}{RT} - \frac{\Delta S}{R} \quad (5)$$

The changes in enthalpy and entropy can be calculated from equation (5) by plotting $\ln(a_w)$ versus $(1/T)$ for a given water content, with the slope of the regression line $(-\Delta H/R)$ providing a measure of the sorption enthalpy, whereas the intercept $(\Delta S/R)$ gives the sorption entropy. This procedure is repeated for several values of moisture content in order to detect the dependence of these quantities on water content (TSAMI, 1991).

A plot of ΔH versus ΔS using the values obtained from equation (5) for the persimmon pulp powder can be built for each set of sorption data. These values can be correlated according to the following equation:

$$\Delta H = T_B(\Delta S) + \Delta G_B \quad (6)$$

where T_B is the so-called isokinetic temperature that corresponds to the temperature at which all reactions involved in the sorption process proceed at the same rate. Since there is a high degree of linear correlation between enthalpy and entropy, the compensation theory was assumed to be valid for sorption (MADAMBA *et al.*, 1996). KRUG *et al.* (1976) recommended a statistical analysis test for the compensation theory, which involves comparing the isokinetic temperature with the harmonic mean temperature T_{hm} as defined by:

$$T_{hm} = \frac{n}{\sum_{i=1}^{n} 1/T} \quad (7)$$

where n is the number of available isotherms. If $T_B > T_{hm}$, the process is enthalpy controlled, while if the opposite condition is observed, the process is controlled by entropy (GOULA *et al.*, 2008).

MATERIALS AND METHODS

Raw material

Samples of persimmon (*Diospyros kaki)* with moisture content of (84.4±1.3)% (wet basis) were obtained at a local market (São José do Rio Preto, SP, Brazil) and stored at 7°C prior to their use. A batch of persimmon pulp was prepared

in a pilot plant finisher and sieved through a 1.6 mm-mesh.

An aqueous solution with 50% solids (mass basis) was prepared by dispersing commercial maltodextrin MOR-REX® 1910 (Corn Products, Mogi Guaçu, Brazil) in distilled water at 40°C, using a mechanical stirrer. This solution was added to persimmon pulp and the mass ratio between maltodextrin solution and pulp was adjusted to give a final ratio of 1.0 kg maltodextrin/1.0 kg soluble solids (50% maltodextrin in a dry solids basis).

Drying methods

Spray-dryer

The spray-dryer (model SD 5.0, Labmaq do Brasil Ltda., Ribeirão Preto, Brazil) operates with concurrent flows and has a spray nozzle with an orifice of 2 mm in diameter. The inlet air temperature was about 140°C and the outlet air temperature was about 102°C. The liquid feed to the dryer was between (1.27±0.16) L/h. The drying air-flow was about 12 m^3/h (MARTINELLI, 2008). The experiments were performed at constant process conditions. The dry powder was carried out of the drying chamber along with the exhausted air, and collected in a cyclone. The material obtained was placed in plastic bags, which were stored in a desiccator containing silica gel until later use.

Freeze-dryer

The persimmon pulp was put into three stainless steel trays and frozen before drying in a freezer (LIOTOP, FV500, LIOBRAS, São Carlos, Brazil) at -40°C. Freeze-drying was carried out in a freeze-dryer model L101 (LIOBRAS, São Carlos, Brazil) at a total pressure and temperature of 500 μHg and –45°C, respectively, in the vacuum chamber for 48 hours.

The freeze-dryer is made up of a cylindrical transparent acrylic drying chamber (35 cm in diameter) covered with a stainless steel top with eight silicone faucets to fit glass bottles with round bottoms (vacuum relief system). In order to connect bottles to faucets there is a silica adapter and a stainless steel pipe that connects the faucets and bottles. Inside the drying chamber there is a shelf that supports four stainless steel trays. Samples can be placed on the trays or in the bottles. The vacuum is produced by a vacuum pump connected to the drying chamber by a hose. At the end of the process, the dried product is removed and ground in a hammer mill.

Vacuum chamber dryer

Samples of pulp were dried on a stainless steel tray inserted in a vacuum chamber (MA 030, MARCONI, Piracicaba, Brazil) at 60°C for 48 h (GABAS *et al.*, 2007). The dried product was ground in a hammer mill.

Sorption isotherms

Equilibrium moisture contents of pulp powder at several water activity values were determined by the static gravimetric method (JOWITT *et al.*, 1983) at 20°, 30°, 40° and 50°C. Ten saturated salt solutions [NaOH, LiCl, $KC_2H_3O_2$, $MgCl_2$, K_2CO_3, $Mg(NO_3)_2$, $NaNO_2$, NaCl, KCl and $BaCl_2$] were prepared that corresponded to a range of water activities from 0.06 to 0.90. Each solution was transferred to separate jars in a quantity sufficient to occupy a space of about 1.5 cm depth at the jar bottom. Triplicate samples of about 1 g of persimmon powder were weighed and put into small plastic receptacles and placed on tripods in the jars, which were then tightly closed and placed in a temperature-controlled chamber. The required equilibration time was 4 to 5 weeks. It was based on the change in sample weight expressed on a dry basis, which did not exceed 0.1% (0.001 g/g dry solids). The equilibrium moisture content was determined by the gravimetric method, in a vacuum oven at 60°C for 48 h (AOAC, 1990).

RESULTS

Sorption isotherms

The experimental moisture sorption data determined at 20°, 30°, 40° and 50°C for persimmon pulp powder with 50% of maltodextrin (dry basis) obtained using different dryers are shown in Table 1. The moisture content for each level of water activity represents the mean value of three replicates. The standard deviations between triplicates were within a maximum limit of 4% from the average X_{eq} value, except for values corresponding to the lowest a_w, where the standard deviations attained up to 9% from the average X_{eq} value.

The isotherms obtained are of type III, according to Brunauer's classification, for most of the temperatures and all drying methods examined. The sorption isotherms demonstrate an increase in equilibrium water content with increasing water activity (Fig. 1).

The typical effect of temperature on the isotherms is shown for powders produced in the spray-dryer (Fig. 1). The shape of the curves is characteristic of foods with high sugar content, which sorbs small amounts of water at low water activity and large amounts of water at high water activity. The explanation for the nature of the isotherms may be that at low water activities, physical sorption on strongly active sites occurs, since water can be sorbed only to surface – OH sites of crystalline sugar (GOULA *et al.*, 2008).

The effect of the equilibration temperature on the sorption isotherms is of great importance given that foods are exposed to a range of temperatures during storage and processing, and

Table 1 - Equilibrium water content (% dry basis) of persimmon pulp powders with 50% (dry basis) maltodextrin 10 DE using different dryers.

		Vacuum-dryer	Spray-dryer	Freeze-dryer
Temperature (°C)	a_w	X_{eq}	X_{eq}	X_{eq}
20	0.070	0.048	0.076	0.049
	0.113	0.056	0.094	0.053
	0.246	0.081	0.123	0.081
	0.331	0.089	0.138	0.088
	0.446	0.108	0.167	0.109
	0.547	0.125	0.202	0.124
	0.655	0.167	0.252	0.172
	0.754	0.221	0.347	0.219
	0.853	0.311	0.518	0.300
	0.907	0.378	0.544	0.400
30	0.069	0.041	0.067	0.041
	0.112	0.048	0.080	0.048
	0.223	0.083	0.132	0.081
	0.324	0.082	0.137	0.080
	0.439	0.101	0.168	0.102
	0.526	0.110	0.184	0.111
	0.635	0.183	0.290	0.183
	0.756	0.211	0.348	0.211
	0.835	0.264	0.438	0.266
	0.9	0.398	0.703	0.395
40	0.066	0.036	0.060	0.037
	0.111	0.047	0.074	0.046
	0.206	0.067	0.107	0.066
	0.319	0.081	0.127	0.078
	0.432	0.100	0.164	0.100
	0.506	0.111	0.178	0.111
	0.615	0.147	0.242	0.148
	0.753	0.215	0.345	0.214
	0.82	0.266	0.422	0.265
	0.893	0.361	0.603	0.359
50	0.059	0.033	0.053	0.034
	0.110	0.041	0.067	0.040
	0.189	0.058	0.095	0.057
	0.314	0.070	0.113	0.066
	0.432	0.101	0.162	0.102
	0.489	0.107	0.173	0.106
	0.599	0.149	0.242	0.150
	0.746	0.206	0.333	0.205
	0.809	0.254	0.412	0.253
	0.884	0.376	0.609	0.373

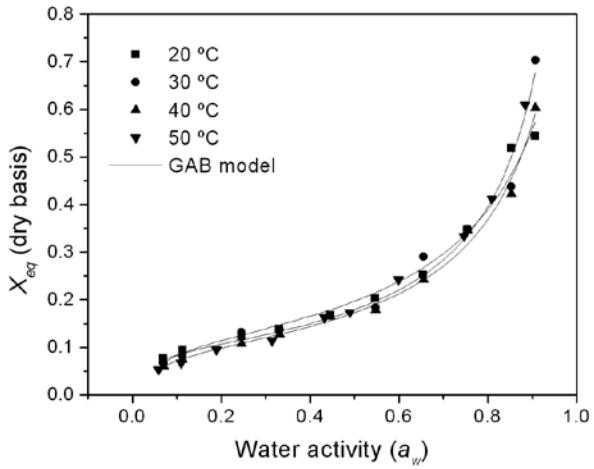


Fig. 1 - Influence of temperature on sorption isotherms of persimmon pulp powder containing 50% (dry basis) maltodextrin 10 DE produced by spray-drying.

water activity for the same moisture content changes with temperature (GOULA *et al.*, 2008). The equilibrium moisture content tends to decrease with increasing temperature at constant water activity. The extent of the decrease depends on the nature or constitution of the food (RIZVI, 1995). For persimmon pulp powder, this behavior was not very pronounced, and was independent of the dryer used to obtain the powder. This result was in agreement with results for passion fruit pulp obtained by using different dryers (PEDRO, 2009). GOULA *et al.* (2008) reported that for spray-dried tomato pulp the temperature effect was not dependent on water activity but rather that it might be due to the fact that tomato powder contains high levels of protein and insoluble solids. GABAS *et al.* (2007) compared isotherms of vacuum-dried pineapple pulp powder and MARTINELLI *et al.* (2007) compared isotherms of vacuum-dried lemon pulp powder, both were determined between 20 and 50°C. They reported that the sorption data was only slightly dependent on temperature.

A marked intersection of the isotherms and the inverse temperature effect were observed for persimmon pulp powders above 0.8. Some researchers have reported this inversion in the temperature effect at water activities above 0.7 for products with high sugar content, such as fruit (TELIS-ROMERO *et al.*, 2005; TSAMI *et al.*, 1990). This can be explained by an increase in sugar solubility in water as a result of increasing temperature.

The results of the fitting procedure of the GAB model to the experimental data of the equilibrium water content versus water activities at different temperatures are presented in Table 2, which shows the values of the fitting parameters calculated by non-linear regression. The adequacy of GAB models was evaluated by the determination coefficient (R^2) and the magnitude of the Sum of Square of Residuals (*SSR*) that are also reported.

The value of the monolayer moisture content (X_m) is of particular interest because it indicates the amount of water that is strongly adsorbed to specific sites on the food surface; it is considered the optimum value to assure food stability. Below this water content level, the rates of deteriorative reactions, except the oxidation of unsaturated fats, are minimal (GOULA *et al.*, 2008). Estimated values of X_m for persimmon pulp powders were found to be lower than the values reported for other fruit, which varied from 10 to 15% (dry basis) (KIRANOUDIS *et al.*, 1993). The X_m values obtained by vacuum- and freeze-drying were lower than those for spray-dried persimmon. The monolayer values for persimmon pulp powder obtained by the different drying methods were not temperature dependent.

TELIS *et al.* (2000) obtained monolayer values of 0.109 and 0.113 (dry basis), respectively, for persimmon pulp and skin without previous drying of samples. SOBRAL *et al.* (2001) de-

Table 2 - Estimated GAB parameters at different temperatures for persimmon pulp powders obtained with different drying methods.

Parameter	Temperature			
	20°C	30°C	40°C	50°C
		Vacuum-dryer		
C	22.326	23.709	14.710	13.609
K	0.900	0.927	0.913	0.941
X_m	0.070	0.064	0.067	0.063
R^2	0.997	0.985	0.999	0.997
SSR	0.235E-03	0.169E-02	0.678E-04	0.256E-03
		Spray-dryer		
C	18.047	30.997	16.387	13.732
K	0.8787	0.949	0.925	0.941
X_m	0.118	0.100	0.105	0.102
R^2	0.982	0.987	0.999	0.997
SSR	0.467 E-02	0.446 E-02	0.259E-03	0.671E-03
		Freeze-dryer		
C	26.593	21.835	14.192	13.609
K	0.916	0.924	0.912	0.941
X_m	0.067	0.065	0.067	0.063
R^2	0.998	0.986	0.999	0.997
SSR	0.193E-03	0.151 E-02	0.788E-04	0.256E-03

termined a monolayer value of 0.131 (dry basis) for freeze-dried pieces of persimmon. The differences between these values and those obtained in the present work could be attributed to compositional differences in the raw material, mainly the sugar content.

Parameter C showed distinct trends for the different drying methods. For vacuum- and freeze-dried materials, the C values decreased as temperature increased. For spray-dried products, the C parameter showed no clear temperature dependence. In accordance with GOULA *et al.* (2008) the K values were smaller than unity as dictated by the GAB equation. Parameter K was practically unaffected by the drying method.

The effect of dryer types on the sorption isotherms at 20°C is shown in Fig. 2, and at 50°C in Fig. 3. The effect of drying method on the behavior of the isotherms was the same at both temperatures.

The spray-dryer provides a dry product with higher adsorption capacity than the other methods. Freeze- and vacuum-dried products had the same adsorption capacity. These drying methods have some similarities. Both are based on drying in the absence of air, which gives products with a very porous structure. Nevertheless, the adsorption capacity may not be directly related to porosity, since in order to obtain powders, the products obtained from the freeze- and vacuum-drying processes were ground. This factor could also be responsible for the similar adsorption capacity observed between powders obtained by these two drying methods.

TONON *et al.* (2008) reported that the materials produced by spray-drying were made up of spherical particles, which had a larger surface area than the other irregular shaped particles (for the same particle volume). This is probably the reason for the highest adsorption capacity shown by spray-dried materials.

Sorption thermodynamic properties

Isosteric heat of sorption

The dependence of the net isosteric heat of sorption on the moisture content of persimmon pulp powder with 50% maltodextrin is shown in Fig. 4. The results illustrate a progressive increase of the heat of sorption in relation to the decreasing moisture content. AL-MUHTASEB *et al.* (2004) stated that this behavior may be due to the fact that at the initial stages of sorption (low moisture content), there are highly active polar sites available on the surface of the food material, which are

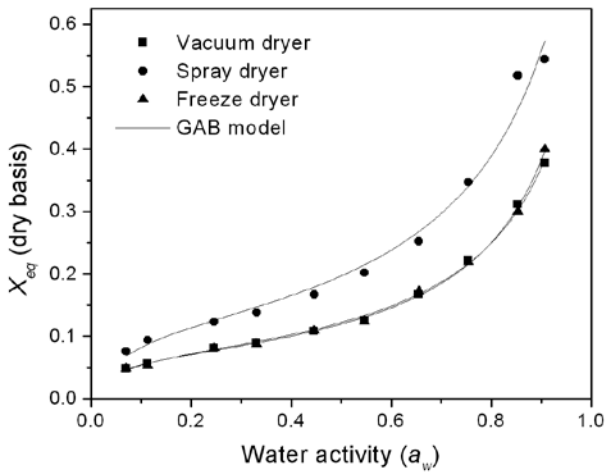


Fig. 2 - Influence of different drying methods on sorption isotherms of persimmon pulp powder determined at 20°C.

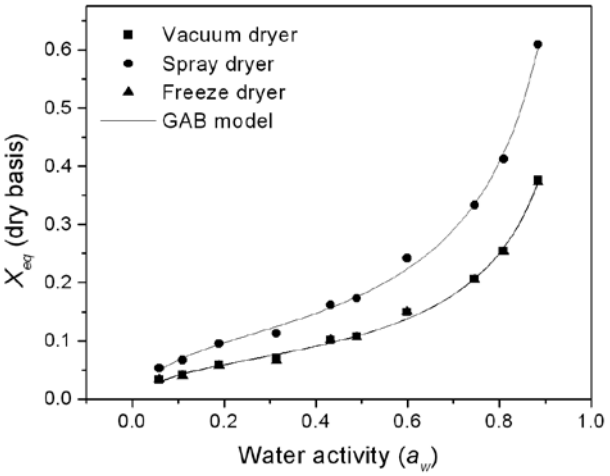


Fig. 3 - Influence of different drying methods on the sorption isotherms of persimmon pulp powder determined at 50°C.

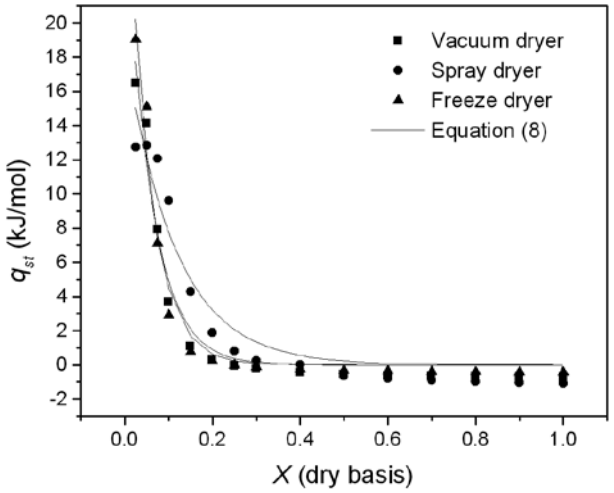


Fig. 4 - Influence of drying methods on the net isosteric heat of sorption of persimmon pulp powders.

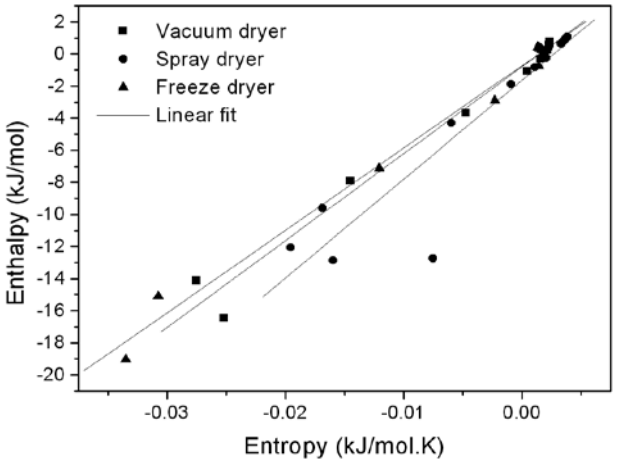


Fig. 5 - Enthalpy-entropy relationship for water sorption in vacuum-, spray- and freeze-dried persimmon pulp.

covered with water molecules to form a monomolecular layer. This results in stronger water-solid interactions. GOULA *et al.* (2008) reported that the main water-adsorbing constituents in foods are various polymers (proteins, starch, cellulose, hemicellulose, etc.) and sugars. The different polar groups of the polymers provide energetically different sites for sorption. As the water content increases, the covering of less active adsorption sites and the formation of multilayers are manifested by the decrease in the heat of sorption (PÉREZ-ALONSO *et al.*, 2006).

Over almost the entire range of moisture content, the highest q_{st} values corresponded to the powders produced by spray-drying. This suggests that the spray-dried product has more polar surface sites than products obtained by the other drying methods. These results are in agreement with those for passion fruit pulp powders obtained using different dryers (PEDRO, 2009). The elevated heat of sorption values of water at low moisture content are an indication of strong water-food component interactions in the fruit.

The q_{st} values were correlated with Equation (8) (TSAMI *et al.*, 1990); the q_0 and X_0 constants and the determination coefficient values (R^2) are shown in Table 3. The determination coefficient of the adjustment was found to be satisfactory ($R^2 > 0.94$).

$$q_{st} = q_0 \exp\left(\frac{X}{X_0}\right) \qquad (8)$$

where X is the water content (dry basis).

Enthalpy-entropy compensation theory

The plots of differential enthalpy (ΔH) and differential entropy (ΔS) of sorption for persimmon pulp powder obtained by using different dryers are shown in Fig. 5. Linear relationships were exhibited by the correlation of these variables and the compensation theory was assumed to be valid for water adsorption by persimmon pulp powder.

The T_B and ΔG_B parameters were calculated by linear regression based on equation (6). The isokinetic temperatures for water sorption by dehydrated persimmon pulp are shown in Table 4. Persimmon pulp powder obtained by vacuum- and freeze-drying presented lower isokinetic temperatures than persimmon pulp produced by spray-drying. MCMINN *et al.* (2005) reported that the isokinetic temperature usually varies with the material composition, and does not depend on whether the material is undergoing adsorption or desorption. There is not much information about the isokinetic temperature for fruit pulp powders in the literature. GABAS *et al.* (2000) reported that the isokinetic temperature for the desorption process in plum

Table 3 - Estimated parameters of the empirical Equation (8) for samples dried with different methods.

Drying Method	q_0 (kJ/mol)	X_0 (dry basis)	R^2
Vacuum-dryer	27.263	0.058	0.967
Spray-dryer	18.755	0.113	0.940
Freeze-dryer	33.429	0.049	0.973

Table 4 - Isokinetic temperatures and Gibbs free energy for vacuum-, spray-, and freeze-dried persimmon pulp.

Drying Method	T_B (K)	ΔG_B (kJ/mol)
Vacuum-dryer	541.4	-0.796
Spray-dryer	616.3	-1.631
Freeze-dryer	513.2	-0.725

pulp was 473.1 K, and TELIS *et al.* (2000) obtained approximately the same isokinetic temperature (475 K) for persimmon pulp during the desorption process. TELIS-ROMERO *et al.* (2005) reported the isokinetic temperature for the adsorption and desorption of mango, 463 K and 501 K, respectively.

The harmonic mean temperature (T_{hm}) was calculated from equation (7) and resulted in 307.7 K. This value is significantly different from all T_B values reported in Table 4. KRUG *et al.* (1976) pointed out that a linear chemical compensation pattern exists only if $T_B \neq T_{hm}$ and, according to LEFFLER (1995), if $T_B > T_{hm}$ the process is enthalpy-driven, whereas if the opposite condition is observed ($T_B < T_{hm}$), the process is considered to be entropy-controlled. Comparison of the results presented in Table 4 and the resulting value for T_{hm} thus confirms the applicability of the isokinetic theory. It also indicates that the sorption processes for persimmon pulp powder obtained by vacuum-, spray- and freeze-drying are enthalpy-driven.

TELIS *et al.* (2000) suggested that the sorption process for persimmon pulp might be enthalpy-controlled. GABAS *et al.* (1999) also reported an enthalpy-controlled mechanism in the sorption behavior of grapes and plums. Moreover, McMINN *et al.* (2005) suggested that sorption behavior of starchy materials was likewise enthalpy-controlled. Most of the sorption processes can therefore be considered to be enthalpy-controlled.

Finally, Table 4 shows the negative values of ΔG_B for persimmon pulp powder obtained by using the three different drying methods: vacuum-, spray- and freeze-drying. The results indicate that the sorption processes are spontaneous in the studied materials. McMINN *et al.* (2005) reported a spontaneous sorption process for starchy materials, whereas McMINN *et al.* (2007) suggested that water sorption by oatmeal and oat flakes was not spontaneous.

CONCLUSIONS

The results obtained in the present work offer evidence of the effects of different drying methods on the water sorption behavior of persimmon pulp powder. The drying method significantly affected the sorption ability of the dried products and spray-drying provided powdered persimmon pulp that had a higher adsorption capacity than the other methods studied. On the other hand, the water sorption ability was similar in the products obtained by vacuum- and freeze-drying.

The sorption isotherms and thermodynamic properties determined for persimmon pulp powder produced by vacuum-, freeze- and spray-drying processes provide information that is useful for defining the packaging and storage requirements for each type of dried pulp powder. This information should be taken into account when choosing the most suitable drying method for the desired product.

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PAPER

INSTRUMENTAL TEXTURE OF *TORRONE* OF TAURIANOVA (REGGIO CALABRIA, SOUTHERN ITALY)

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ABSTRACT

Torrone is a typical Italian confection prepared from almonds, honey, sugars and/or chocolate. A textural attribute assessment of puncture and cutting forces, hardness, adhesiveness, cohesiveness, springiness and chewiness was performed on two types of Italian *torrone*, "vaniglia duro" and "gianduja tenero", by means of puncture test, cutting test and Texture Profile Analysis to characterize and identify the particular aspects of these local products. Besides the softer texture of the "gianduja tenero", there was a remarkable manufacturing variability; these differences were probably due to different manufacturing conditions (heating times and/or temperatures).

- Key words: adhesiveness, almond, chewiness, hardness, honey, springiness, torrone -

INTRODUCTION

Torrone is a typical Italian confectionery product, very well-known and appreciated by Italian consumers living in Italy and abroad. It is made from high quality toasted almonds, honey, sugar and egg-whites, and is still manufactured in a traditional way. It is considered a purely seasonal product and, in fact, it is the most widespread Christmas sweet in Italy. For this reason, over 60% of *torrone* consumption is concentrated in December (TAVERNA, 2009).

All Christmas confections yielded a turnover of around 650 million Euro in the 2001-2002 season, with *torrone* representing approximately 10% or 65 million Euro (SPERLARI, 2009). It is very interesting to note that there has been a renewed interest in *torrone*, with an increase in sales of about 9.4% between the 2000 and the 2001 season (ACNIELSEN, 2003). The increase in the sales of this traditional Italian confection is due, at least in part, to the rediscovery by consumers of traditional foods. Local raw materials (almonds and honey) are used in the manufacturing of this confection. In Taurianova (Reggio Calabria, Southern Italy), *torrone* quality depends mainly on both the quantity and quality of the almonds and honey used to make it. The most popular raw materials are orange honey and almonds from Sicilia or Puglia (cultivar Pizzuta d'Avola) (TAVERNA, 2009; VOLPE, 2007).

In the manufacturing of Italian *torrone*, honey and sugars (glucose and sucrose) are warmed in a copper container at low temperature and mixed at uniform speed for 2 h in the "*torroniera*", with the addition of fresh egg-whites. After this time, the caramel (previously prepared in another copper container), natural vanilla extract, pistachios, and toasted almonds (toasted at 60°C for 48 h) are added. When all the ingredients are properly mixed, the mass is transferred to a marble table and manually mixed to confer the desired shape using wood frames or appropriate moulds. To obtain the so-called *torrone vaniglia* (vanilla *torrone*) the product is covered on both surfaces with wafer and is cut and then packed. If the mixture is covered with chocolate, then the resulting product is called *torrone gianduja* (chocolate *torrone*). In general, there are compositional and operational differences between these two types of torrone. For instance, the vanilla *torrone* is heated for a longer time and has a higher almond content, which leads to a harder texture, while the chocolate *torrone* is cooked for only 3 h with no addition of caramel (TAVERNA, 2009).

Typical confectionery products similar to torrone can be found throughout the Mediterranean countries such as Spain, with *turrón* (VERDU *et al.*, 2008; MARTINEZ-NAVARRETE *et al.*, 2006) and France with *nougat*, (BLANCHET and TZOUKERMANN, 1979; BERNARDIN, 1981). Such products have a structure that leads to particular properties (LAZPITA *et al.*, 1991; MARTINEZ-NAVARRETE *et al.*, 1996; VASQUEZ-ARAÚJO *et al.*, 2006). The textural properties of commercial *torrone* are therefore quite heterogeneous (LAZPITA *et al.*, 1991) due to several causes:

- Each manufacturer uses different temperatures and times during the heating steps, according to their own experience, leading to different final textures. The manual control over operational conditions (temperature and time) also leads to different final textures.
- The micro-structural parameters of the product and therefore its textural properties are affected by the degree of homogenization of the final product (a highly viscous mass), that depends on the industrial equipment and the operation variables (e.g. rotation speed).
- Compositional factors also influence the final texture of the products. Small variations in moisture content and sugar composition will affect the structural characteristics of vanilla and chocolate *torrone*.

Even though most *torrone* samples have the same composition and are manufactured in a similar way, their final textures are quite different. Therefore, the aim of this study was to increase the knowledge about typical southern Italian torrone products by analyzing the influence of the manufacturing company (6 companies) and the manufacturing year (2 seasons: 2007 and 2008) on the final texture of commercially available samples of vanilla and chocolate *torrone*.

MATERIALS AND METHODS

Materials

In this study, two different types of *torrone* were studied: 1) "*vaniglia duro*" or hard *torrone* with vanilla flavor (type 1: hard vanilla *torrone*), and 2) "*gianduja tenero*" soft *torrone* covered with a layer of chocolate (type 2: soft chocolate *torrone*). *Torrone* samples were provided by 6 different artisanal manufacturers of Taurianova (Reggio Calabria, Southern Italy). Samples were letter coded (A, B, C, D, E and F) to avoid using company names. The last producer (F) only manufactures vanilla *torrone*. The samples, consisting of 6 samples of type 1 and 5 samples of type 2 torrone, were collected during the 2007 and 2008 seasons (September-December: time of the year when the largest stocks of these products are available in Italy). Samples were of different sizes, with mean dimensions of 52 mm × 34 mm × 33 mm, and a mean weight of 20 g.

Texture

Final product texture was assayed on 10-mm cubes, obtained by cutting the product with a

sharp knife, using a Texture Analyzer TA-XT2i (Stable Micro Systems, Surrey, UK) equipped with a 25 kg load cell capacity.

In order to select the most appropriate test for this special type of confection, three different tests were used with commercial samples of *torrone*: a) puncture test (PT), b) cutting test (CT), and c) Texture Profile Analysis (TPA).

Puncture Test (PT) is an empirical indicator of food hardness, particularly of the food surface, and measurements are recorded using a 2-mm diameter cylindrical probe (P/2). This test included both compression and cutting efforts. The penetration rate was 0.3 mm/s for 5 mm after contacting the surface of the confection cube and results are expressed in N (STABLE MICRO SYSTEMS, 1996b).

Cutting Test (CT) is an empirical indicator of the force needed to cut a particular food and measurements are recorded using a knife blade (HDP/BS). This test only included cutting effort. Cutting was performed at 1 mm/s until the confection cube was fully cut; results are expressed in N (STABLE MICRO SYSTEMS, 1996a).

Texture Profile Analysis (TPA). In this case, two full compression cycles were performed to give an instrumental texture profile as described by BOURNE *et al.* (1978) and BOURNE (1978). All instrumental texture analyses were conducted at room temperature 24°±2°C. At least 15 cubes were measured for each type of sample (6 manufacturing companies x 2 manufacturing seasons). The samples were compressed to 25% of their original height with a 10 cm diameter cy-

Table 1 - Main compositional characteristics of vanilla *torrone* as affected by manufacturing season and company.

Product	Chemical parameters					
	Dry Matter	Moisture	Fat	Protein	Sugars	Almond
			(%)			
A 2007	97.6±0.1†	2.37±0.04	33.7±0.1	10.0±0.5	53.8±0.1	45.25±1.51
A 2008	98.2±0.1	1.72±0.14	31.1±0.3	12.3±0.4	49.1±2.0	56.56±3.10
B 2007	98.5±0.1	1.54±0.08	27.2±1.9	7.82±0.13	57.2±7.1	42.38±0.75
B 2008	97.5±0.1	2.52±0.09	37.5±0.3	11.0±0.1	47.8±1.1	52.97±2.77
C 2007	97.1±0.1	2.90±0.12	35.3±4.6	12.2±0.2	48.1±2.0	51.13±2.04
C 2008	97.3±0.1	2.63±0.09	35.6±0.1	13.6±0.4	35.5±0.3	63.91±1.71
D 2007	97.0±0.1	2.96±0.04	33.2±0.7	10.1±0.2	59.9±0.8	40.95±1.33
D 2008	97.6±0.1	2.21±0.00	39.0±0.3	10.3±0.2	45.9±1.5	51.19±0.95
E 2007	98.5±0.1	1.55±0.06	25.6±0.1	7.28±0.17	70.4±1.2	31.74±0.21
E 2008	98.0±0.1	1.81±0.08	26.9±0.2	9.47±0.10	55.9±0.5	39.67±0.23
F 2007	96.4±0.4	3.64±0.35	30.4±0.9	7.40±0.10	70.2±1.9	35.96±0.45
F 2008	97.9±0.1	1.94±0.14	31.8±0.5	9.80±0.13	55.6±0.4	44.95±0.17
			ANOVA Test			
Variation Source						
Year	**‡	***	**	***	***	***
Manuf. Company	***	***	***	***	***	***
Year x Company	***	***	**	***	***	***
			Tukey's Multiple Range Test			
Year						
2007	97.5 b¥	2.49 a	30.9 b	9.13 b	60.0 a	41.2 b
2008	97.7 a	2.14 b	33.6 a	11.1 a	48.3 b	51.5 a
Manuf. Company						
A	97.9 a	2.04 b	32.4 ab	11.1 b	51.4 b	50.9 ab
B	98.0 a	2.03 b	32.3 ab	9.40 cd	52.7 b	47.7 b
C	97.2 b	2.77 a	35.4 ab	12.9 a	42.2 c	57.5 a
D	97.3 b	2.59 a	36.1 a	10.2 c	52.5 b	46.1 b
E	98.2 a	1.68 b	26.2 c	8.37 e	63.2 a	35.7 c
F	97.1 b	2.79 a	31.1 b	8.60 de	62.9 a	40.5 bc

† Treatment means of the ANOVA test (values are the mean value of 15 replications); "± values" are standard errors.

‡ N.S. = not significant F ratio ($p < 0.05$); *, **, and ***, significant at $p < 0.05$, 0.01, and 0.001, respectively.

¥ Values followed by the same letter, within the same source of variation, are not significant different ($p < 0.05$), Tukey's multiple-range test.

Table 2 - Main compositional characteristics of chocolate torrone as affected by manufacturing season and company.

Product	Chemical parameters					
	Dry Matter	Moisture	Fat	Protein	Sugars	Almond
	(%)					
A 2007	96.3±0.1†	3.69±0.07	29.2±1.9	9.26±0.10	59.1±1.0	32.7±1.8
A 2008	95.7±0.1	4.25±0.20	33.7±1.1	10.5±0.3	39.1±1.3	40.8±3.1
B 2007	97.2±0.1	2.84±0.13	29.6±2.1	6.94±0.13	57.6±1.4	26.4±1.6
B 2008	97.7±0.2	2.04±0.21	35.3±0.1	9.23±0.37	44.0±0.1	33.0±2.8
C 2007	95.8±0.1	4.25±0.08	36.5±0.2	8.32±2.55	40.0±0.6	31.1±0.5
C 2008	96.2±0.1	3.62±0.07	35.5±0.2	10.9±0.3	39.8±0.5	38.8±1.1
D 2007	98.3±0.1	1.67±0.04	31.1±1.0	7.93±0.07	41.9±0.7	29.8±1.3
D 2008	97.7±0.2	2.10±0.15	40.4±2.4	8.53±0.09	40.5±0.4	37.2±0.6
E 2007	97.6±0.1	2.43±0.07	28.3±0.3	9.03±0.23	71.0±2.6	21.8±0.9
E 2008	96.8±0.3	3.03±0.30	28.8±0.4	6.76±0.08	55.1±3.2	27.3±0.7
	ANOVA Test					
Variation Source						
Year	N.S.‡	N.S.	***	*	***	***
Manuf. Company	***	***	***	**	***	***
Year x Company	***	***	**	**	***	***
	Tukey's Multiple Range Test					
Year						
2007	97.0 a¥	2.98 a	30.9 b	8.30 b	53.9 a	28.3 b
2008	96.8 a	3.00 a	34.7 a	9.18 a	43.7 b	35.4 a
Manuf. Company						
A	96.0 c	3.97 a	31.4 bc	9.89 a	49.1 b	36.8 a
B	97.4 b	2.44 b	32.4 ab	8.08 b	50.8 b	29.7 ab
C	96.0 c	3.93 a	35.9 a	9.60 ab	39.9 c	34.9 a
D	98.0 a	1.88 c	35.7 a	8.23 b	41.2 c	33.5 a
E	97.2 b	2.73 b	28.5 c	7.89 b	63.0 a	24.5 b

† *Treatment means of the ANOVA test (values are the mean value of 15 replications);"± values" are standard errors.*
‡ *N.S. = not significant F ratio ($p < 0.05$); *, **, and ***, significant at $p < 0.05, 0.01$, and 0.001, respectively.*
¥ *Values followed by the same letter, within the same source of variation, are not significant different ($p < 0.05$), Tukey's multiple-range test.*

lindrical probe at a speed of 10 mm/min. Similar test conditions were used by MARTÍNEZ-NAVARRETE *et al.* (1996) and VÁZQUEZ-ARAÚJO *et al.* (2006). Texture profile parameters were determined as described by BOURNE *et al.* (1978), BOURNE (1978) and ROSENTHAL (1999):

- Hardness (N) is defined as the maximum force required to compress a sample and is calculated as the peak force of the first compression of the product.

- Adhesiveness (N x s) is defined as the work needed to detach a product stuck to the compression plate; it is assumed to measure the adhesiveness of a sample to palate and teeth and is calculated as the negative area between the first and second compression cycles.

- Cohesiveness (%) is a measure of how well the product withstands a second deformation relative to how it behaved under the first deformation (A_2/A_1, A_1 being proportional to the total energy required for the 1st compression and A_2 being proportional to the total energy required for the 2nd compression).

- Springiness (%) is a measure of how well a product physically springs back after it has been deformed during the first compression and is calculated as the ratio between the distance of the second compression peak/distance of the first compression peak.

- Chewiness (N) is assumed to be a measure of the work to masticate the sample for swallowing (springiness x hardness x cohesiveness, with springiness and cohesiveness being expressed as fractional numbers between 0 and 1).

Chemical analyses

The AOAC Official Methods (HORWITZ, 2000) were used for moisture (925.40), protein (950.48), oil (963.15) and sugar (977.20) analyses, respectively. Samples were ground in a grinder before being used for chemical analysis.

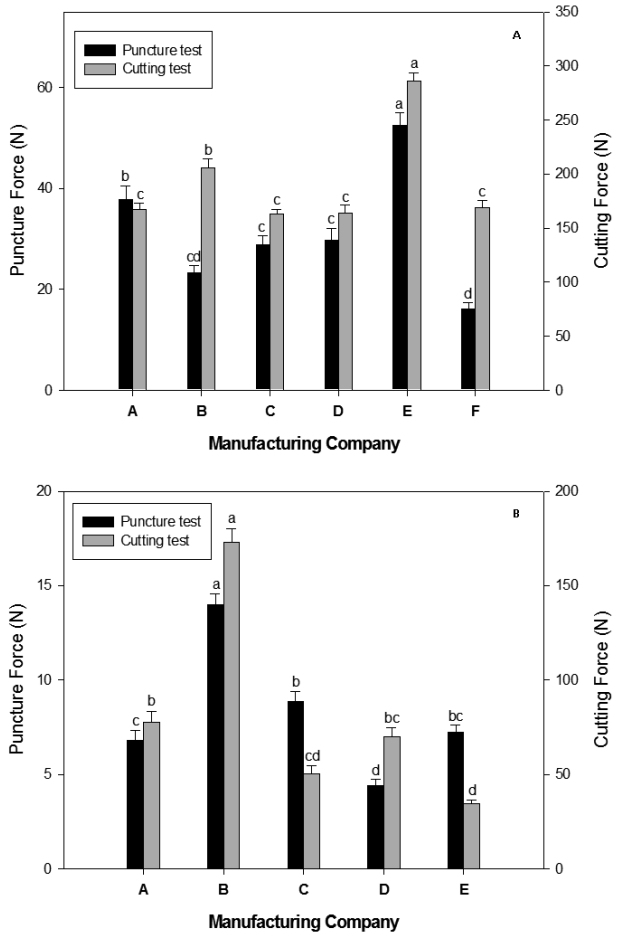


Fig. 1 - Puncture and cutting forces of vanilla (A) and chocolate (B) *torrone* from different manufacturing companies. Values represented here are the means of samples from 2007 and 2008[†].
[†] *The letters on the bars are used to explain the grouping of values for similitude in the Tukey's multiple-range test.*

The equipment used for sugar analyses was an HP Series 1100 HPLC (Hewlett Packard, Waldbronn, Germany), with a refractive index detector (HP Model 1100 Series); the column was a Supelcogel C-610H column, 30.0 cm x 7.8 mm (id) with a Supelguard C-610H guard column, 5.0 cm x 4.6 mm (id). Fructose, glucose, sucrose, and maltose standards were used for sugar quantification. Almond content was analysed in both types of *torrone* (vanilla and chocolate) because it is considered one of the key ingredients; besides, fat and protein content depend mainly on the almond content. All chemical analyses were run, at least, in three replicates.

Statistical analyses

All data were subjected to analysis of variance, ANOVA [two factors: 1) year: 2007 and 2008, and 2) manufacturing companies: A, B, C, D, E and F]. Tukey's least significant difference multi-comparison test was used to determine significant differences among treatments. Significance of differences was represented as $p \leq 0.05$. The statistical analyses were done using Statgraphics Plus 5.0 software (Manugistics, Inc., Rockville, MD, USA.).

RESULTS AND DISCUSSION

Tables 1 and 2 report the chemical composition of the samples. In general and during both seasons, protein, almond and sugar contents were higher in the vanilla samples. Besides, 2007 samples of both types of *torrone*, vanilla and chocolate, showed lower contents of fat, protein and almond and higher content of sugars, indicating that fewer almonds and more sugars were used in 2007.

Puncture and cutting force

As expected, both the puncture strength and the cutting force were significantly higher in vanilla *torrone* compared to chocolate *torrone* (Fig. 1); this is due to the longer cooking time during caramelization of the sugars and honey. This is the reason why the vanilla *torrone* is also classified as <u>hard</u> *torrone*, while the chocolate *torrone* is classified as <u>soft</u> *torrone*.

In vanilla *torrone*, the puncture and cutting forces were significantly affected by both manufacturing season and company (Fig. 2A). The 2008 products were significantly harder and more difficult to cut than those from 2007 (Table 3). This could be due, at least in part, to two different factors: a) longer cooking times at a similar temperature were used in the 2008 season,

Table 3 - Statistical analysis of texture data of *torrone vaniglia*.

Variation Source	Force	
	Puncture	Cutting
	(N)	
ANOVA Test		
Year	***	***
Manufacturing Company	***	***
Year x Company	***	***
Tukey's Multiple Range Test		
Year		
2007	23.3 b	175 b
2008	39.5 a	210 a
Manufacturing Company		
A1	37.8 b	167 c
B1	23.3 cd	206 b
C1	28.9 c	163 c
D1	29.8 c	164 c
E1	52.6 a	286 a
F1	16.1 d	169 c

Table 4 - Statistical analysis of texture data of *torrone gianduja*.

Variation Source	Force	
	Puncture	Cutting
	(N)	
ANOVA Test		
Year	***	N.S.
Manufacturing Company	***	***
Year x Company	***	**
Tukey's Multiple Range Test		
Year		
2007	7.03 b	86.5
2008	9.49 a	75.7
Manufacturing Company		
A2	6.78 c	77.9 b
B2	14.0 a	173 a
C2	8.87 b	50.4 cd
D2	4.40 d	69.9 bc
E2	7.25 bc	34.4 d

and/or b) there was a higher almond content in the 2008 products (this statement is supported by sensory data).

In vanilla *torrone* and regarding the manufacturing companies, the hardest and most difficult samples to cut were those manufactured by company E (Fig. 2A and Table 3). The hardness of these samples could be related to their low moisture, fat and protein contents and high sugar content. This specific composition plus long heating times could be the main reasons for the hardness of the E samples. On the other hand, companies F, C and D manufactured the softest and easiest-to-cut products.

The puncture strength was significantly affected by both manufacturing season and company in chocolate *torrone* (Fig. 2B); however the cutting force was only dependent on the manufacturing company. The 2008 products were significantly harder than those from 2007; however, the energy needed to cut the products was statistically equal in products from both seasons (Table 4). Again, higher almond contents and longer heating times could be the reasons behind the hardness of the 2008 products.

Table 5 - Texture Profile Analysis (TPA) parameters of chocolate torrone samples from different manufacturing companies during the 2007 and 2008 seasons.

Variation Source	Hardness (N)	Adhesiveness (N s)	Cohesiveness (%)	Springiness (%)	Chewiness (N)
A 2007	43.7±6.5[†]	-1.24±0.17	24.2±2.3	34.6±3.6	3.73±0.73
A 2008	31.1±7.6	-0.38±0.07	15.3±0.9	30.5±1.6	1.59±0.55
B 2007	127±27	-3.49±0.57	12.6±1.5	88.0±28.7	11.1±2.8
B 2008	273±22	-0.63±0.11	20.9±1.0	42.2±2.4	25.0±3.4
C 2007	42.2±11.3	-1.53±0.43	13.6±1.1	64.0±2.7	5.00±1.61
C 2008	36.8±13.9	-0.28±0.14	19.3±1.9	48.3±2.7	3.65±1.54
E 2007	15.1±1.7	-2.20±0.53	18.5±1.4	69.6±2.7	1.79±0.18
E 2008	59.4±12.4	-0.74±0.14	15.0±0.7	39.5±2.7	3.55±0.78
ANOVA Test					
Year	***[‡]	***	N.S.	**	*
Manufacturing Company	***	**	N.S.	*	***
Year × Company	***	N.S.	N.S.	N.S.	***
Tukey's Multiple Range Test					
Year					
2007	57.0 b[¥]	-2.11 b	17.2	64.0 a	5.40 b
2008	100 a	-0.51 a	17.6	40.1 b	8.46 a
Manufacturing Company					
A	37.4 b	-0.81 a	19.7	32.6 b	2.66 b
B	200 a	-2.06 b	16.7	65.1 a	18.1 a
C	39.5 b	-0.90 a	16.4	56.1 ab	4.33 b
E	37.2 b	-1.47 ab	16.7	54.6 ab	2.67 b

[†] Treatment means of the ANOVA test (values are the mean value of 15 replications); "± values" are standard errors.

*[‡] N.S. = not significant F ratio ($p < 0.05$); *, **, and ***, significant at $p < 0.05$, 0.01, and 0.001, respectively.*

[¥] Values followed by the same letter, within the same source of variation, are not significant different ($p < 0.05$), Tukey's multiple-range test.

Results for chocolate *torrone* were more difficult to interpret and perhaps the only conclusion to be drawn is that the hardest and most difficult to cut samples were those manufactured by company B (Fig. 2B and Table 4).

As previously explained, both composition and manufacturing conditions played predominant roles in determining the puncture and cutting forces of *torrone*; however, further studies are needed to study these factors in detail. For instance, composition was not included in the product labels and manufacturing companies did not provide detailed information about the manufacturing conditions or product compositions.

The external layer of chocolate in the *gianduja torrone*, without any doubt, influenced the textural tests. This influence was particularly important in the puncture test, because the cylindrical probe used in this analysis penetrated only 5 mm into the sample (the chocolate layer plus the external surface of the internal *torrone*). Consequently, the puncture and cutting forces depended on both the processing conditions and the composition (including the nature of the chocolate layer).

Texture Profile Analysis (TPA)

This test was only carried out on samples of chocolate *torrone* (companies A, B, C and E) because the samples of vanilla *torrone* and even chocolate *torrone* from company D proved to fall outside the operating range of the 25 kg texture analyser load cell (Table 5).

Samples from company B were characterized by the highest values of hardness, (which completely agreed with results previously discussed on puncture and cutting forces), adhesiveness, springiness and chewiness, while samples from company A had the lowest values for these parameters.

Hardness. There were differences in hardness for both year and manufacturing company (Table 5). Hardness was higher in samples from 2008 and in both years, samples from company B were significantly ($p < 0.001$) higher (200 N) than those of the other manufacturing companies (mean of 38.0 N). No significant differences were found for products from the other three manufacturing companies.

Adhesiveness. Adhesiveness is defined as the "work required to pull the food away from a surface", particularly teeth and/or palate (ROSENTHAL, 1999). It is quite reasonable that this parameter would be better correlated with the degree of heating during the initial concentration of sugars, honey and water than with compositional factors, such as protein or fat contents. These ingredients are cooked to a lower degree in chocolate *torrone* than in vanilla *torrone* and perhaps the cooking conditions (lower temperature and/or shorter cooking time) led to more adhesive products (company B). In addition, it is well-known that honey is a hygroscopic material and adsorbs water from the surrounding environment, making the products stickier.

Cohesiveness. ROSENTHAL (1999) defined this attribute as "the strength of the internal bonds making up the food". In this particular study, neither season nor manufacturing company significantly affected the values of this texture parameter. However, samples from company A showed slightly higher values.

Springiness. Springiness is defined as "the extent to which a compressed food returns to its original size when the load is removed" (ROSENTHAL, 1999). Samples from 2007 showed significantly ($p < 0.01$) higher springiness values than 2008 products. The springiness values of samples from company B (65.1%) were significantly higher than those of the other companies (mean of 47.8%). Both composition and experimental conditions of the main unit operations could be responsible for these differences.

Chewiness. Both year and the manufacturing company significantly affected chewiness of the confections. A similar pattern to those previously discussed for hardness and springiness was also observed for chewiness. Samples of company B and company E obtained for 2008 presented higher values of chewiness than those of 2007, and without any doubt, samples of company B can be considered as the most chewable ones (18 N) compared with the rest of products (mean of 3.2 N).

CONCLUSIONS

In general, both factors under study (production year and manufacturing company) significantly influenced the instrumental texture of *torrone.* The results of puncture (PT) and cutting (CT) tests showed that samples of vanilla *torrone* were significantly harder than those of chocolate *torrone* in both 2007 and 2008. Samples of company B had the highest values of puncture and cutting forces and TPA also demonstrated that these samples were the hardest and the most adhesive, springy and chewable. TPA appears to be, among the three procedures used to study the texture of the *torrone*, the most comprehensive testing procedure, because it allows the examination of many different textural parameters, such as hardness, adhesiveness, cohesiveness, springiness, chewiness, while PT and CT only measure hardness. This study also showed that texture attributes are mainly affected by manufacturing conditions (especially heating times and/or temperatures). However, further study is required to establish clear relationships among texture parameters, composition, and experimental conditions of the main unit operations involved

in *torrone* manufacture. Local *torrone* manufacturers desire to increase their international market shares; this could be better accomplished with greater control over manufacturing conditions, in order to obtain more homogeneous products.

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PAPER

EFFECT OF MICROBIOLOGICAL TRANSGLUTAMINASE ON SELECTED PHYSICOCHEMICAL PROPERTIES OF TVAROG

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ABSTRACT

The effect of the microbiological transglutaminase (mTGase) treatment of milk on the main physicochemical and sensory properties of acid curd cheese (tvarog) was analysed. Protein polymerisation was carried out prior to the fermentation process. Three variants of tvarog were produced: (i) one from milk containing inactivated mTGase (the enzyme was added to the base milk in active form and subsequently inactivated by heating), (ii) one with active mTGase and (iii) a control sample (without mTGase). The tvarog samples produced under laboratory conditions were vacuum-packed in polyethylene foil and stored for 21 days at 5°C (±1°C). The organoleptic properties of the cheeses were assessed. Changes in the water, fat, titratable acidity and pH were measured. Moreover, the cheese hardness was determined by means of a double compression test (TPA). During storage, the cheese that was produced from milk containing inactivated mTGase was judged as having better organoleptic attributes, although it also had more whey leakage than tvarog made with active mTGase or the control sample. The storage time and treatment of the base milk containing mTGase (active or inactivated) did not have a significant effect on the fat content in the cheeses produced. The water content was only affected by storage time. The titratable acidity, pH and hardness of the samples analysed depended significantly on how the base milk was prepared for the tvarog (the presence of an active or inactive enzyme in the milk) and on the storage time.

- Key words: acid curd cheese, enzyme, inactivation, physiochemical properties -

INTRODUCTION

The most important stage in the production of acid curd cheese (ACC) production is obtaining a curd that has the appropriate consistency. This process is influenced by many factors including the quality of the base milk, the production conditions and the use of certain additives. During the production and storage of ACC, certain defects may occur such as an unusual taste and/or aroma in the final product, excessive whey leakage and an unsuitable texture or loose consistency. According to consumer opinion, the most important defects are a lack of typical taste and aroma, a non-typical aftertaste and a defective texture. In practice, controlling the texture of dairy products is often associated with controlling the chemical composition. Texture is also correlated with the degree of interaction among the chemical components (ŻBIKOWSKI and ŻBIKOWSKA, 2003).

Proteins impart the structural basis for the various properties of a wide range of foodstuffs, and by modifying them, several physicochemical characteristics can be manipulated. In the last few years, enzymatic cross-linking of proteins has gained increasing importance in food technology (DICKINSON, 1997; GERRARD, 2002; RASIAH *et al.*, 2005; HINZ *et al.*, 2007; LANTTO *et al.*, 2007). In particular, TGase is an enzyme that has recently received much attention due to its ability to crosslink proteins (SCHORSCH *et al.*, 2000a,b; O'SULLIVAN *et al.*, 2002b; WALSH *et al.*, 2003; VASBINDER *et al.*, 2003; KOŁODZIEJSKA *et al.*, 2004; YOKOYAMA *et al.*, 2004; ÖZRENK, 2006; SMIDDY *et al.*, 2006; HILLER and LORENZEN, 2009; WRÓBLEWSKA *et al.*, 2009). This enzyme catalyses the acyl transfer reactions between the γ-carboxyamide groups of peptide-bound glutamine residues and the ε-amino groups of lysine residues, leading to the formation of intra- and intermolecular isopeptide bonds. The enzyme reaction results in the formation of covalently cross-linked protein polymers (MILANOVIĆ *et al.*, 2007). The introduction of additional covalent cross-linkages by using TGase is a promising tool for improving certain functional properties, such as solubility, water-binding or emulsifying capacity, viscosity, elasticity, gelation, and the rheological or renneting properties of food proteins where the use of chemical reagents for modification is not acceptable (MOTOKI and SEGURO, 1998; FAERGEMAND and QVIST, 1999; JAROS *et al.*, 2006; HINZ *et al.*, 2007; TRESPALACIOS and PLA, 2007).

The cross-linking of proteins is determined by the molecular structure and accessibility of glutamine and lysine residues. Therefore, the mTGase-catalysed reaction can be influenced by various parameters, such as pressure, chemical reagents and heat. In studies on the effect that the enzyme has on the quality attributes of dairy products, researchers have focused their attention on various procedures applied in dairy technology that may affect mTGase-induced reactions. Some of these are: incubating the milk with mTGase prior to fermentation followed by inactivation (or no inactivation) (MAUTNER *et al.*; 1999, LAUBER *et al.*, 2000; LORENZEN *et al.*, 2002a; JAROS *et al.*, 2010), the concomitant addition of the enzyme and the starter (FAERGEMAND *et al.*, 1999; NEVE *et al.* 2001; JAROS *et al.*, 2006), prolonging the time of incubation of the base milk with mTGase (LORENZEN and NEVE, 2003), adding varying amounts of mTGase (FARNSWORTH *et al.*, 2006), applying mTGase prior to and concomitantly with rennet to milk cheese (HAN and SPRADLIN, 2000; LORENZEN, 2000a; LORENZEN *et al.*, 2002b; HAN *et al.*, 2003) and adding mTGase after clotting and curd cutting (COZZOLINO *et al.*, 2003).

A review of the available literature shows that TGase is a relatively universal food modifier; it cross-links most plant and animal proteins (PIETRASIK, 2003; MYLLÄRINEN *et al.*, 2007). There are many patents on the use of mTGase in plant and animal technologies, e.g., in bread, fish, meat and dairy products (HAN and SPRADLIN, 2000; HAN *et al.*, 2003; SCHAEFFER and FUNDA, 2004; KUMAZAWA and MIWA, 2005). However, there is little information in the literature about the influence of cross-linking on the properties of dairy products and acid cheese curd. Hence, the aim of the present study was to examine the use of mTGase in the production of ACC.

MATERIALS AND METHODS

Cheese preparation

Three variants of ACC produced under laboratory conditions were used. The base milk, containing 3.2% fat, 3% protein and 4.3% lactose, was homogenised (15 MPa/55°C) and pasteurised at a high temperature (85°C/15 s).

The first and second variants of the ACC (A and B, respectively) were obtained by using a lyophilised and concentrated mesophilic culture for direct vat inoculation (DVI) (Danisco Biolacta Ltd., Olsztyn, Poland) known as CHOOZIT TP°3 LYO 300 DCU, and TGase of microbiological origin in the preparation of Activa MP®. The enzyme (Ca^{+2}-independent transglutaminase), derived from *Streptoverticilium moberansae* (1% TGase, 99% maltodextrin), was obtained from Ajinomoto Co. Inc. (Tokyo, Japan). The enzyme preparation was used in the original form without any further purification. The inoculum cultures used contained the following bacterial strains: *Lactococcus lactis* ssp. *cremoris, L. lactis* ssp. *lactis, L. lactis* ssp. *lactis var. diacetylactis,* and *Leuconostoc mesenteroides* ssp. *mesenteroides*.

The third type of ACC (variant C) was used as the control. It was produced by using the tradi-

tional technology of ACC production (Manufacturing Instruction CZSM Nr 342/88), using only a lyophilised dairy culture (the same as in variants A and B). The production of cheeses A and B differed with respect to milk processing. The method for preparing the base milk was in accordance with the producer's recommendations (AJINOMOTO, 2000), data from the literature (LORENZEN, 2000b; LORENZEN *et al.*, 2002a, b; LORENZEN, 2007) and the author's own research.

In the first variant, the pasteurised base milk was heated to 40°C, and a 0.06% Activa MP® preparation was added; the milk was then incubated at this temperature for two hours. Afterwards, the milk was heated to 80°C, and the temperature was maintained for 1 min to inactivate the enzyme. After cooling to 23°C, the milk was inoculated with a starter (2.5% of the milk volume). Variant B cheese was produced in a similar fashion, except that after incubation at 40°C, the processed milk was not subjected to mTGase inactivation; the milk was cooled to 23°C and inoculated by adding a 2.5% starter. The control ACC was made without the addition of mTGase (variant C) and was prepared by adding a starter (2.5%) to the pasteurised base milk that had been previously heated to 23°C. All of the cheeses were kept at this temperature until curd formation began (approximately ten hours). The matured curd (pH 4.6) was gently heated to separate it from the tank walls; then it was cut into cubes (120 x 120 mm). After flipping them over, it was warmed and gently stirred; the temperature was raised at a rate of 1°C/min. The warming process was stopped after the temperature in the centre reached 45°C. The slurry obtained after straining out the whey with a sieve was poured into aseptic single-use PE sacks (FPH Piotr Szymczak, Kępice, Poland). The samples were then pressed for 1 h with a 1-kg load for every kg of tvarog.

Packaging and storage of cheese

The cheese samples, (about 150 g each) were vacuum-packed (Turbovac SB-420, HFE vacuum systems by Hs-Hertogenbosch, The Netherlands). The vacuum packing system used a vacuum pressure of 15 mbar for 2.5 s and a 400 mbar 'soft-air' option. The foil used was 40 μm of PE/PA foil with EVOH (PPH Gąsior Sp z o.o., Bydgoszcz, Poland). The packaged ACC (60 wedge-shaped portions) was kept at 5°±1°C for 21 days.

Organoleptic assessment

The samples were subjected to an organoleptic assessment using a five-point scale (PN-ISO 6658, 1998; PN-ISO 4121, 1998). The structure, consistency, colour, taste and aroma of the cheeses were evaluated by a nine-member panel trained in the sensory evaluation of ACC. The samples used for the analysis were selected randomly. The organoleptic assessment was performed using scales, categories and centre-punching. The results for particular variants of tvarog were added together and are expressed as an arithmetic mean (PIECZONKA, 1995; DMYTRÓW *et al.*, 2007; 2009). The evaluation was carried out in a room that was free of any foreign odours; each panellist had a separate test stand and distilled water to rinse their mouths.

Characterisation of the product

The following characteristics of the tvarogs were analysed in accordance with Polish Standards (PN-73/A-86232): water content was measured by using a drying method, fat content was determined by the Gerber's method, and titratable acidity was measured in°SH and pH. The whey leakage was determined in relation to the ACC weight by using percentages. Samples were weighed in their wrapping (with a precision of 0.01 g) and weighed again after unwrapping. The wrapping was dried with a paper-towel. Based on the differences in weight, the percentage of whey outflow was determined (ZIÓŁKOWSKI *et al.*, 2004).

Rheological analysis

The rheological analysis of the experimental ACC was based on an evaluation of hardness. This analysis was conducted by means of a double compression test TPA using a texture analyser TA.XT Plus (Stable Micro System Ltd., Godalming, UK.). The samples were penetrated with a pressure of 1G and a velocity of 5 m/s up to a depth of 20 mm. The diameter of the aluminium pin was 6 mm (DMYTRÓW *et al.*, 2007; 2009).

All analyses were performed on the day of production and then on the first, third, seventh, fourteenth and twenty-first days of storage.

Statistical analysis

The results were subjected to a statistical analysis carried out with Microsoft Excel using a two-factor analysis of variance with repetition and significance tests for dependent means. All of the tests were performed at a significance level of $\alpha = 0.05$.

RESULTS AND DISCUSSION

The scores from the organoleptic assessments clearly show that the presence of mTGase and base milk processing with the enzyme influenced the organoleptic attributes of the ACC. The cheeses were characterised by appropriate organoleptic attributes, with the greatest differenc-

es being found in the taste, structure and sample consistency (Fig. 1).

According to the test panel, the ACC produced from milk in which mTGase had been inactivated by heating (tvarog A) was the most desirable one. As storage time increased, the organoleptic attributes of the samples gradually deteriorated. The colour of the ACCs stayed uniformly white and received a maximum score (5 points) until the end of the storage period. Similarly, the aroma of the samples was described as very good, clean and aromatic, even after 21 days of storage (5°±1°C). In the first week of the study the taste of all of the variants was rated very good. After 14 days of storage, the taste of variant A (with inactivated mTGase) was still rated very good (due to its softness), but variants B (active mTGase) and C (control sample) were slightly worse in this regard and were described as typical but quite sour (4.5 points). On the last day of the study variant B had the highest rated flavour. The remaining two samples (A and C) were given 4 points each.

The best structure and consistency was observed in the ACC made with inactivated mTGase (variant A). During the study, variant A consistently received a maximum score of 5 points. The structure and consistency of the remaining variants worsened slightly over time. On the last day of the analysis, the consistency and structure of variants B and C obtained 4 points each. It should be noted that the structure and consistency of variant B (with active mTGase) were judged as being too hard and gummy.

Several authors (BOROWSKI, 2006; SZPENDOWSKI *et al.*, 2005; ŚMIETANA *et al.*, 2003; STEINKA and PRZYBYŁOWSKI, 1997) have observed that the organoleptic features of ACCs remain very stable during cool storage. The appearance of tvarog does not change, but its consistency becomes more compact; the colour becomes less uniform, and the structure becomes grainier. Moreover, the intensity of the taste and the aroma become increasingly sour and slightly acidic. The gradual worsening of the organoleptic characteristics of ACC has also been described by KORNACKI *et al.* (1999). Their organoleptic evaluation showed that the tvarogs analysed immediately after production had a pleasant but slightly sour taste and a concise and easy-to-smear consistency. These characteristics gradually worsened over time.

The literature lacks information on the influence of mTGase on the organoleptic features of ACC. Most of the available reports on using mTGase in dairy technologies refer to the use of this enzyme in yoghurt production (LORENZEN 2002a, b; MILANOVIĆ *et al.*, 2007). According to the above-mentioned authors, when the enzyme is used, the appearance of the product changes from a typical acid-induced casein gel to that of heat-induced whey protein gels. The hard and gummy texture of variant B could have been caused by continued cross-linking that occurred during the cooling period (NEVE *et al.*, 2001). An increased softness in the cheese containing inactivated mTGA, in contrast, could be explained by the fact that acid gels from the mTGase-treated milk had a finer protein network with thinner protein particles than the untreated samples (FAERGEMAND and QVIST, 1997).

During ACC storage (5°±1°C), a general decrease in whey leakage was observed for variant A, a slight increase was observed for variant B, and a two-fold increase was recorded for the control sample, variant C (Fig. 2).

Whey leakage from tvarogs is due to syneresis, which occurs as a result of local stresses in the gel network, leading to rearrangements and the

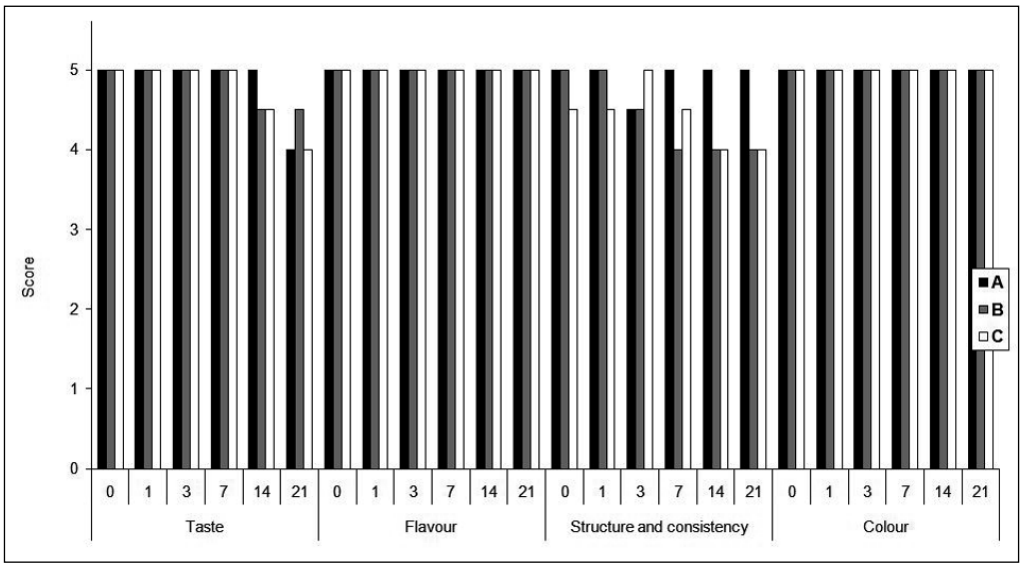


Fig. 1 - Results of organoleptic assessment scores for acid curd cheese stored at 5°±1°C (5-point scale).
(A) Tvarog with mTGase with inactivation, (B) Tvarog with mTGase without inactivation, (C) Tvarog without mTGase (control).

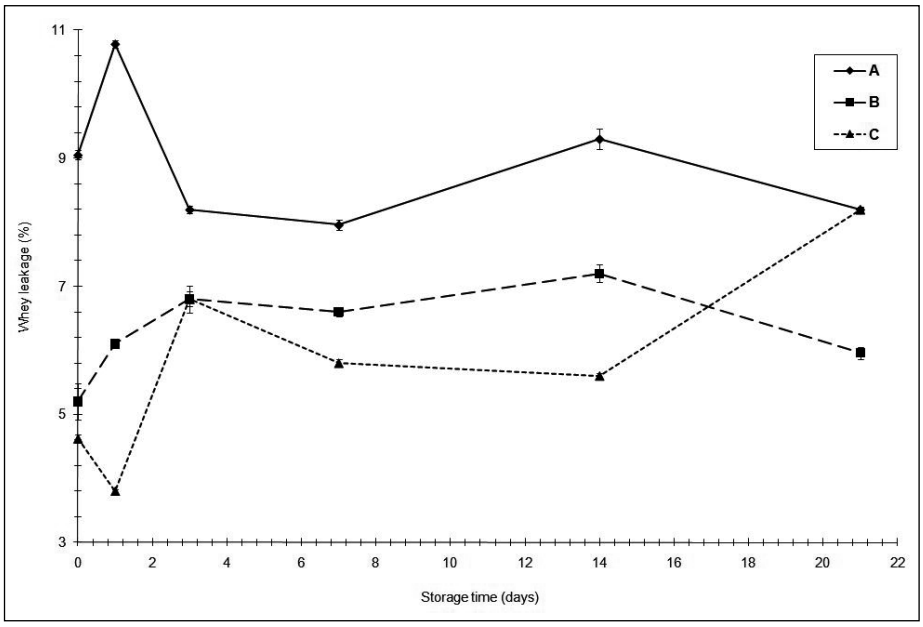


Fig. 2 - Changes in the whey leakage from acid curd cheese stored at 5°±1°C.
(A) Tvarog with mTGase with inactivation, (B) Tvarog with mTGase without inactivation, (C) Tvarog without mTGase (control). Error bars show standard deviations.

local expulsion of whey. GERTNER *et al.* (1980) reported that whey leaks during refrigeration and transport. It is caused by changes in the colloid system of the tvarog and the self-pressing of the cheese mass. An increase in whey leakage can also be due to a decrease in the cheese's ability to retain water and the progressive drying out of the surface (LITWIŃCZUK *et al.*, 2003). The whey outflow is very often caused by excessive vacuum pressure during packaging (PLUTA *et al.*, 2003). However, the whey leakage found in the samples is not associated with the packaging conditions because this effect was minimised by using the "soft air" packaging option, which circumvents the unfavourable influence of packaging on product quality. According to ZANDER (2005), vacuum packaging of cheese does not significantly affect water content or the amount of whey leakage during storage. The decreased permeability of curd caused by enzyme activity and the finding that protein networking strengthens the cheese protein matrix and reduces syneresis were probably the cause of the decrease in whey loss during storage of the tvarog that contained inactivated mTGase. This phenomenon was confirmed by FAERGEMAND and QVIST (1997), who proved that there was less permeability in gels from enzyme-treated milk. The slight whey leakage in the sample containing the active enzyme could be associated with the continued cross-linkages that took place during the cooler storage phase of the final product. When there is excessive networking, the milk gel may show symptoms of syneresis (NONAKA *et al.*, 1992; NEVE *et al.*, 2001).

The analysis of the basic chemical composition of the ACC examined showed that all three variants conformed to the Polish Norm (PN-91/A-86300/A2) with respect to the water and fat contents (Figs. 3 and 4). According to this Norm, with respect to the class of ACC (I or II), the water content must be within a 65-75% range, and the fat content should be between 15-45% range. In the case of skim tvarog, the Norm for fat content is not defined.

Storage time and the method of treating the base milk containing mTGase did not have a significant effect on the fat content of the ACC, whereas the water content depended significantly on the storage time (Table 1). Statistically significant increases in the water content in all of the samples analysed and a decrease in the fat content of sample B were observed. The changes observed in the fat content of tvarogs A and C were not statistically significant.

Several authors have affirmed that success in cheese production depends on how well the maximum admissible moisture content is maintained. However, they have also indicated that small increases in the water content of the product and the joining of protein masses have a favourable influence on cheese yield and economic effectiveness. In the literature, a reported increase in the water content in some analysed tvarogs could have been caused by protein swelling and free water absorption (ŚMIETANA *et al.*, 1998, 2003; CAIS and WOJCIECHOWSKI, 1997). The cross-linking of proteins may lead to increased water content in the product because, as stated by LAUBER *et al.* (2000), mTGase can form a stable protein network by immobilising water.

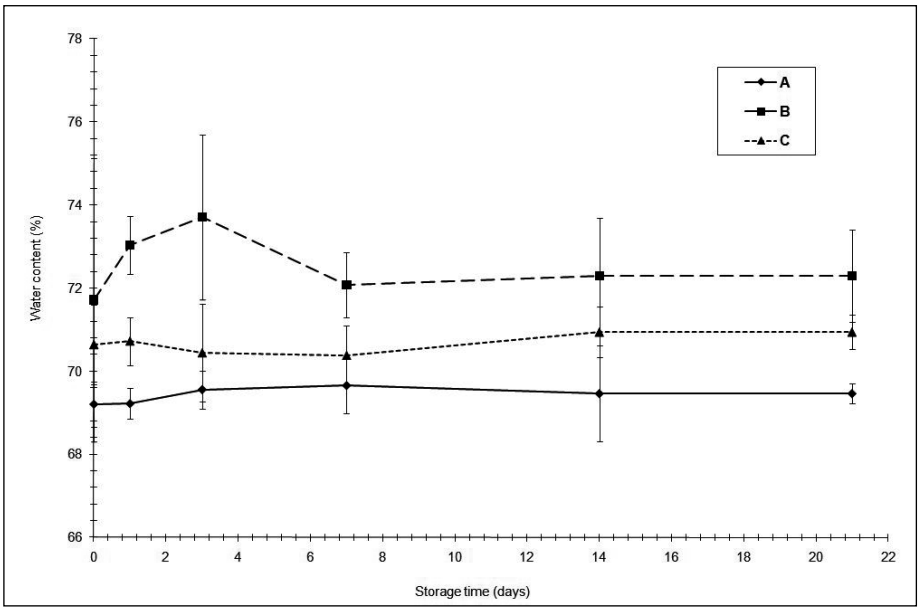


Fig. 3 - Changes in the water content changes in acid curd cheese stored at 5°±1°C.
(A) Tvarog with mTGase with inactivation, (B) Tvarog with mTGase without inactivation, (C) Tvarog without mTGase (control).
Error bars show standard deviations.

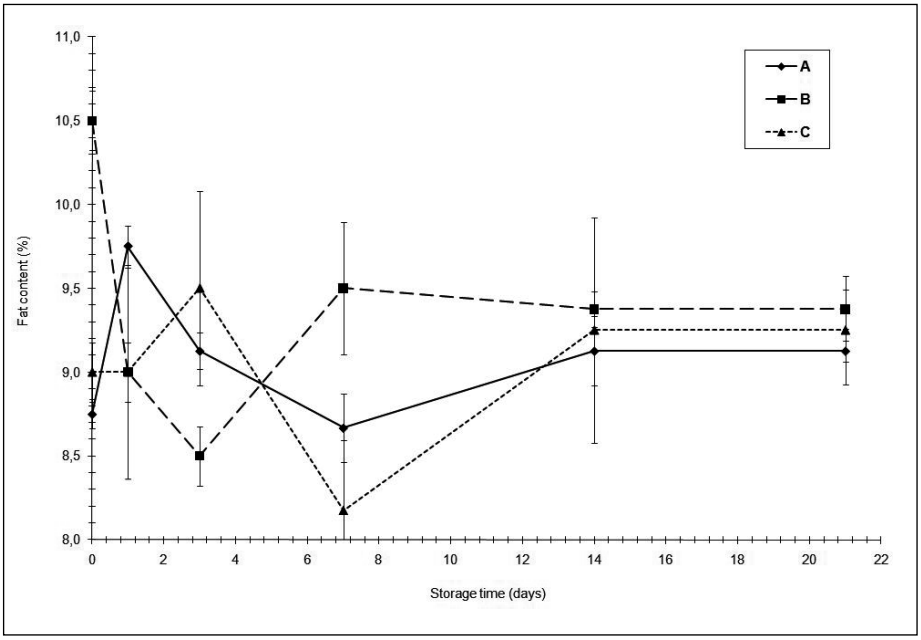


Fig. 4 - Changes in the fat content changes in acid curd cheese stored at 5°±1°C.
(A) Tvarog with mTGase with inactivation, (B) Tvarog with mTGase without inactivation, (C) Tvarog without mTGase (control).
Error bars show standard deviations.

The cool storage of the samples was associated with changes in the titratable acidity in all of the variants of ACC (Fig. 5). Statistical analysis showed that the storage time of the samples and the treatment of the base milk containing mTGase significantly influenced the titratable acidity. Moreover, the combined influence of these two factors was significantly greater than each of them taken separately (Table 1).

The highest acidity value recorded during the study was observed in variant A (inactivated mTGase). The curves showing changes in the titratable acidity were essentially the same for the cheeses made from milk with mTGase. The acidity of these samples decreased rapidly on the first day of storage and then fluctuated during further storage. The acidity of the control sample, after a similar initial decrease, increased steadily until the end of the storage period. Despite the observed fluctuations in acidity, a statistically significant increase in this parameter was recorded for variant A (by about

Table 1 - Results of analysis of variance for the effects of microbiological transglutaminase (mTGase) and storage time on selected qualitative attributes of acid curd cheeses (tvarogs).

Source of variation	*p* values				
	pH	Titrable acidity	Water content	Fat content	Hardness
Storage time	1.30E-025*	2.07E-041*	2.82E-012*	0.353	1,36E-085*
Cheese type	1.38E-006*	1.40E-028*	0.0263	0.283	5.63E-146*
Interaction	3.86E-023*	3.86E-023*	3.01E-009*	0.023*	1.09E-063*

Where: *p* - probability of the first-kind error (significance level $\alpha = 0.05$);
* - $p \leq \alpha$, significant influence of source of variation on an attribute.

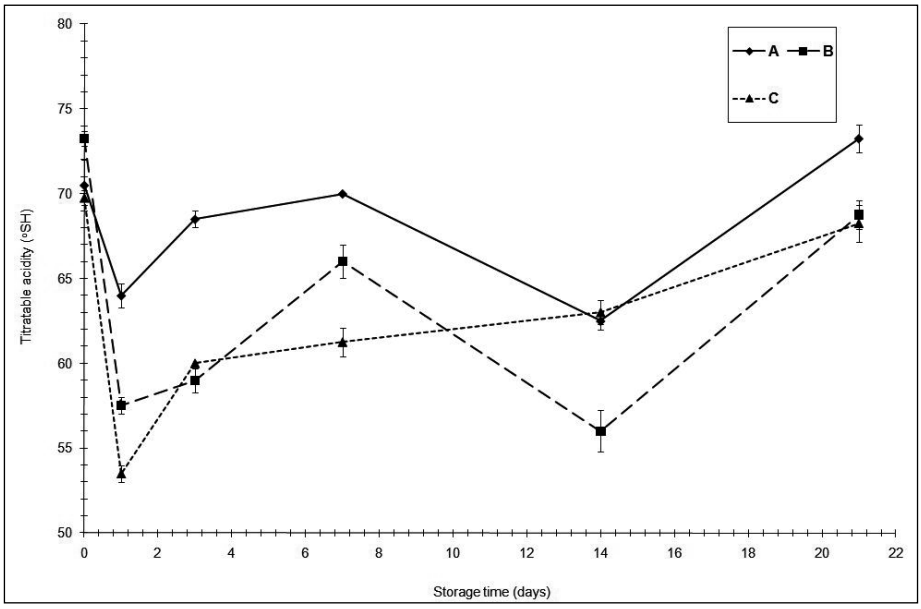


Fig. 5 - Changes in the titratable acidity of acid curd cheese stored at 5°±1°C.
(A) Tvarog with mTGase with inactivation, (B) Tvarog with mTGase without inactivation, (C) Tvarog without mTGase (control). Error bars show standard deviations.

3.9%) while the acidity decreased for variant B (about 6.1%). The change in the potential acidity of the control sample (variant C) was not statistically significant (Table 2).

An increase in pH was observed in all of the variants (Fig. 6, Table 2). The highest pH was recorded in the tvarog that was produced from milk containing active mTGase (variant B), while the lowest value was observed in variant A, containing inactivated mTGase. The correlation between pH and the use of mTGase and storage time was statistically significant. The interaction between these factors was also statistically significant (Table 1).

A similar increase in pH was explained by MOLSKA *et al.* (1996), with protein changes and the formation of ionised groups. Decreases in the titratable acidity of ACC during storage under cooling conditions were described by CAIS and WOJCIECHOWSKI (1997) and GERTNER *et al.* (1980). The potential acidity of ACC was associated with the inhibition of the lactic streptococci activity, and even with their partial extinction during the cooling storage of tvarog. In their study, after the third day of storage, an initial increase was followed by a steep decrease in acidity, which was associated with the peptonisation of casein. The decrease in acidity was accompanied by an increase in pH and a disappearance of lactose. FAERGEMAND *et al.* (1999) stated that the growth and vitality of lactic acid bacteria introduced with a starter in the base milk depends on the presence of various amino acids that arise from the degradation of milk proteins. The addition of mTGase results in a decrease in the availability of low-molecular peptides that are essential for the production of proteinases by starter bacteria. NEVE *et al.* (2001) and FAERGEMAND and QVIST (1999) stated that the addition of mTGase to base milk

Table 2 - Results of the statistical analysis of the relative mean values (Student's t-test).

		Tvarog A (inactivated mTGase)		Tvarog B (active mTGase)		Tvarog C (control)	
		Storage time (days)		Storage time (days)		Storage time (days)	
		0	21	0	21	0	21
Titrable acidity (°SH)	$\bar{x}$	70.50	73.25	73.25	68.75	69.75	68.25
	t	4.371		9.000		2.324	
	t_α	3.182*		3.182*		3.182	
pH	$\bar{x}$	4.28	4.42	4.28	4.56	4.25	4.49
	t	3.417		13.236		10.556	
	t_α	3.182*		3.182*		3.182*	
Water content (%)	$\bar{x}$	68.20	68.46	63.72	72.29	70.64	70.94
	t	3.532		3.917		3.811	
	t_α	3.182*		3.182*		3.182*	
Fat content (%)	$\bar{x}$	8.75	8.13	10.50	9.38	9.00	9.25
	t	2.324		3.576		0.322	
	t_α	3.182		3.182*		3.182	
Hardness (N)	$\bar{x}$	1.95	6.49	3.67	10.99	1.41	1.84
	t	43.628		20.769		2.495	
	t_α	2.228*		2.228*		2.228*	

Where: * - on the basis of Student's t-test, change of analysed attribute is significant, $\bar{x}$ - mean value of the analysed indicator, t - value of testing statistics, t_α - critical value for significance level $\alpha = 0.05$.

causes a minor imbalance in the associative growth of bacteria, as well as the viable lactobacilli counts, which are slightly less than in products that are made from untreated milk. The influence of cross-linking on the process of acidification was also observed in the case of mesophilic starter cultures used in the manufacture of cottage cheese. Higher enzyme-to-substrate ratios resulted in a slower decrease in the pH. Accordingly, it can be stated that the differences in the acidities of ACC were due to the presence of an enzyme and by using different methods of treatment for the base milk containing mTGase. The decrease in titratable

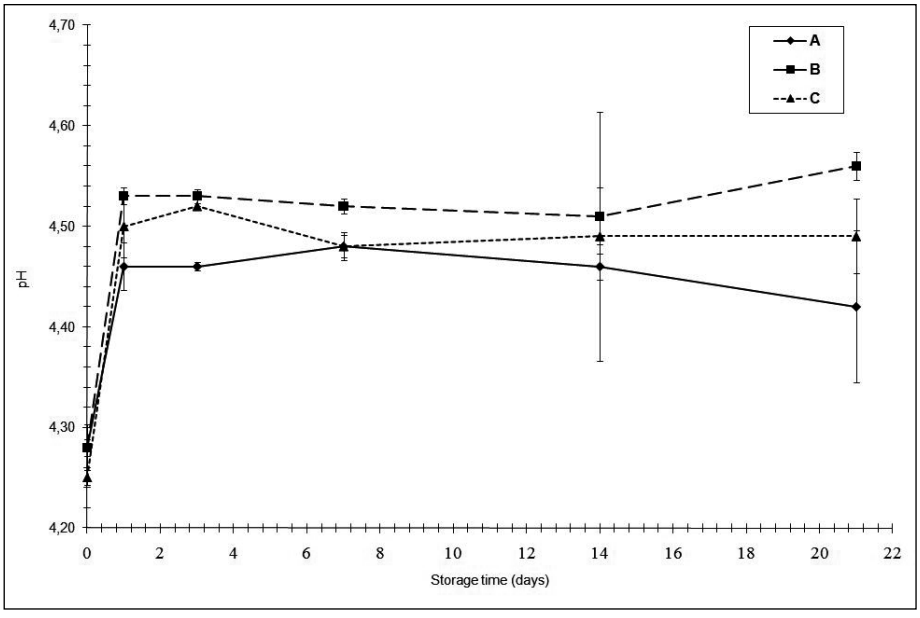


Fig. 6 - Changes in the active acidity (pH) of acid curd cheese stored at 5°±1°C.
(A) Tvarog with mTGase with inactivation, (B) Tvarog with mTGase without inactivation, (C) Tvarog without mTGase (control). Error bars show standard deviations.

acidity in tvarog B (active mTGase) may be explained by the fact that, apart from the cross-linking reaction, two other reactions are catalysed by mTGase: deamidation and amine incorporation. All three reactions result in an increase the amount of free ammonia (DE JONG and KOPPELMAN, 2002; SHARMA *et al.*, 2001; MOTOKI and SEGURO, 1998). By increasing this amount, acidity (increase of pH) is reduced.

A rheological analysis showed a statistically significant increase in the hardness of all of the variants of ACC during storage as well as a statistically significant influence of mTGase on the hardness of the samples. Throughout the study, the highest hardness values were observed in tvarog that was manufactured with active mTGase (Fig. 7, Table 1). These phenomena are confirmed in the literature. It is well known that milk protein gels made by traditional methods are stabilised mainly through weak non-covalent interactions. Microbiological transglutaminase can form covalent bonds between protein molecules. The introduction of these new covalent bonds leads to gels that are different in structure (SCHORSCH *et al.*, 2000a).

The increased hardness of tvarog observed in the experiment was also confirmed by ZIÓŁKOWSKI *et al.* (2004); the observed phenomena were explained by whey leakage. They also emphasised that the leakage was slight, and therefore, the differences in hardness were minimal. According to PRENTICE (1987), the rheological properties of different cheeses depend mostly on their protein and water contents. The author states that when protein content in cheeses increases, the hardness of the cheese increases.

The study performed by DMYTRÓW *et al.* (2007, 2009) and BONCZAR and WALCZYCKA (2001) showed that water content was negatively correlated with almost all texture parameters, including hardness. This means that as the water content in the cheeses decreases, the hardness of the product increases. Unfortunately, for the tvarogs, the inversely proportional relationship between water content and hardness was not observed.

There is a lack of information about the influence of the enzyme on the rheology of the ACC; however, according to JAROS *et al.* (2006), the polymerisation of milk proteins by TGase results in an increased water holding capacity, increased gelation and gel strength, and higher rheological and emulsifying properties. Moreover, various authors have reported enhanced gel strength and firmness for the yoghurt made from mTGase-treated milk (MAUTNER *et al.*, 1999, LAUBER *et al.*, 2000, KURAISHI *et al.*, 2001).

The application of mTGase without inactivation had a greater impact on the texture of the final product compared to the product that was produced after enzyme inactivation (NEVE *et al.*, 2001). The greater hardness of tvarog B could be ascribed to increased cross-linking during cold storage. O'SULLIVAN *et al.* (2002a) demonstrated that mTGase treatment makes casein micelles extremely stable in the presence of a range of disruptive forces. Therefore they are suitable for applications in which the increased physical stability of dairy products is required. LORENZEN *et al.* (2002a, b) and RODRIGUEZ-NOGALES (2006), for example, concluded that gel strength in the mTGase-treated samples was approximately double that in

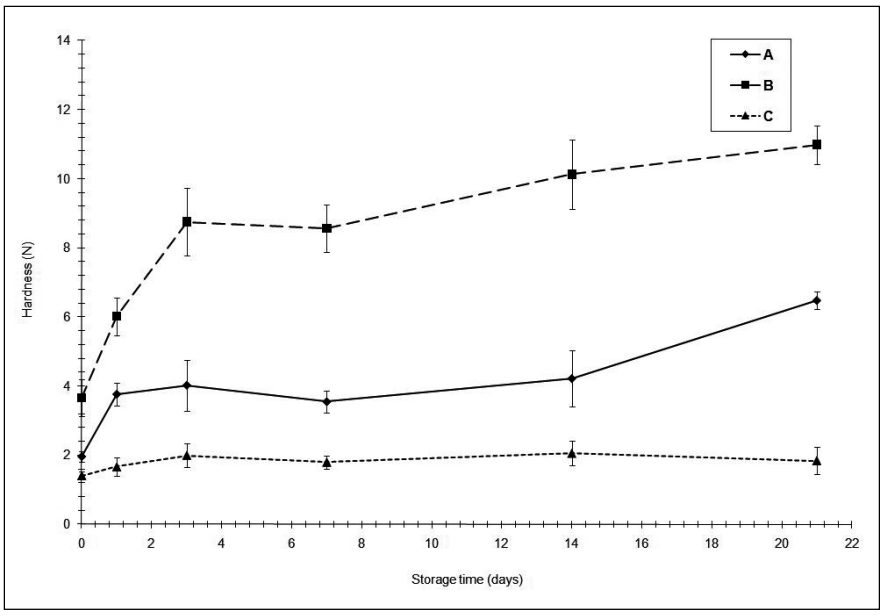


Fig. 7 - Changes in the hardness of acid curd cheese stored at 5°±1°C.
(A) Tvarog with mTGase with inactivation, (B) Tvarog with mTGase without inactivation, (C) Tvarog without mTGase (control). Error bars show standard deviations.

the untreated samples. In addition, the cross-linking of proteins in milk prior to lactic acid fermentation resulted in improved gel firmness and a reduction in whey syneresis of the acid–induced gel (FAERGEMAND *et al.*, 1999; LORENZEN *et al.*, 2005).

The rheological properties of goat milk yoghurt with mTGase added were studied by FARNSWORTH *et al.* (2006). They ascertained that, along with an increase upon addition of mTGase (after inactivation), a significant increase in the viscosity of the samples was also observed. FAERGEMAND and QVIST (1997) investigated the effects of mTGase-catalysed cross-linking on the rheology and structure of acidic skim milk gels acidified chemically with glucono-delta-lactone (GDL). They found that the enzymatic cross-linking of skim milk prior to acidification gave much stronger gels than those created in the absence of the enzymatic treatment. Furthermore, cross-linked gels had much lower permeabilities than the non-treated acidic skim milk gels. This was correlated with a much finer microstructure, with smaller pores in the protein network of the cross-linked gels. The simultaneous addition of bacteria and mTGase (without inactivation) had a greater influence on the texture of the final product than the addition of starter bacteria (inoculum) after a previous inactivation of the enzyme (NEVE *et al.*, 2001).

The results of this study show that mTGase mainly affected the organoleptic attributes of ACCs, its pH, potential acidity and the hardness. Summing up, the use of mTGase provides an interesting variation in the production of ACC.

CONCLUSIONS

- The examined ACCs had the appropriate organoleptic attributes and the proper chemical compositions.
- The ACCs produced with mTGase were not significantly different from those produced without mTGase with respect to fat and moisture content.
- ACCs made with mTGase, and subsequently inactivated, was judged to have the best structure and consistency. However they also had the greatest whey leakage.
- ACCs containing mTGase after its inactivation had the highest titratable acidity and the lowest pH.
- The duration of storage and the use of mTGase significantly affected the acidity of the ACCs.
- The rheological analysis confirmed a statistically significant increase in the hardness of all analysed ACC variants.
- The tvarog manufactured with mTGase without inactivation had the highest hardness levels for the entire study period.

ABBREVIATIONS

TGase - transglutaminase; mTGase - microbiological transglutaminase; ACC - acid curd cheese.

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PAPER

QUALITY MAINTENANCE PERFORMANCE AND RESISTANCE TO *TRIBOLIUM CASTANEUM* AND *PLODIA INTERPUNCTELLA* PENETRATION OF AN ALTERNATIVE PACKAGING MATERIAL FOR SEMOLINA

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ABSTRACT

Insect attacks to food packages are a major concern for food producers, who are often blamed for being responsible for the occurrence of contamination which seriously affects the image of the company. Wheat semolina is commonly packed in paper bags, which do not always offer sufficient protection against insect pests. The present research evaluated the performance of a new packaging alternative, consisting of paper laminated with polypropylene (P+CPP), in terms of resistance to penetration by two common cereal product pests, namely *Tribolium castaneum* and *Plodia interpunctella*, and quality maintenance of the packaged product. Neither of the two pest species tested were able to pierce the P+CPP, while no differences were observed in the time required to penetrate other materials. Moreover, quality parameters, namely pH, acidity and color, did not differ significantly among samples packed in different materials. The new P+CPP combination is a promising alternative for the packaging of semolina, because it offers improved protection against pests compared with conventional materials, while maintaining the paper-appearance of traditional packaging.

- Key words: insect pests, packaging, *Plodia interpunctella*, quality parameters, shelf-life, *Tribolium castaneum*, wheat semolina -

INTRODUCTION

Packaging is critical for preserving food quality and is the final defence against insect pests. Insect infestations are the main cause of loss in packaged foods both in terms of direct damage (MULLEN, 1994) and the extra costs required to preserve and package foods (HANLON *et al.*, 1998). Consumers tend to think that food manufacturers are responsible for any insect contamination, independent of their real involvement (HOU *et al.*, 2004). The argument has been addressed by several Authors, who evaluated the ability of insects to penetrate packaged foods (ESSIG *et al.*, 1943; GERHARDT and LINDGREN, 1954, 1955; BATTH, 1970; CLINE, 1978a) and to assess the resistance of various packaging materials (SREENATHAN *et al.*, 1960; DOMENICHINI and FORTI, 1975).

Packages have several points of weakness and almost every packaging material can be perforated by insects. Penetration depends on various factors, depending on the insect and the packaging variables. Among the former, species and life stage of the insects involved must be considered (CLINE, 1978a; 1978b; BOWDITCH, 1997; ROBERTSON, 1993; RIUDAVETS *et al.*, 2007). Regarding the packaging features, the nature and structure of the packaging material, the combination of materials and the package design play a key role with respect to insect penetration. Package design parameters, such as folds and seals, can be points of weakness and may facilitate the formation of holes which offer preferential access to the package contents. Consequently, the design of a suitable package for each type of product is crucial for quality maintenance and an extended shelf-life. Recently, there has been an increased availability of different materials for food packaging, particularly multilayer films, which combine various materials (e.g. aluminium and paper with different polyolefines). Researchers continuously try to improve the quality of complex multilayer films to guarantee food safety (RIUDAVETS *et al.*, 2007). The quality of the package seal is also fundamental for protecting foods from insect infestations. Even packages that are perfectly sealed can be attacked by insects, that are attracted to the product by volatiles that permeate through the material (BROWN, 1992; NAVARRO *et al.*, 2007). A study by YERINGTON (1978) demonstrated the direct correlation between the package seal and the level of infestation.

The most destructive insect pests of stored products belong to the Orders Coleoptera (beetles) and Lepidoptera (moths) (ROBERTSON, 1993) and can be subdivided into "penetrators" and "invaders" (HIGHLAND, 1984). Penetrators bore holes through packaging materials. An example is the Indian meal moth, *Plodia interpunctella* (Hübner) (Lepidoptera Pyralidae), which is considered responsible for 98% of food infestation worldwide (SÜSS and LOCATELLI, 2001). Invader insects enter packages through existing holes; the red flour beetle *Tribolium castaneum* (Herbst), is an example (HIGHLAND, 1991). The presence of this pest in flour for 2 to 3 months permanently alters its quality (SÜSS and LOCATELLI, 2001). The above-mentioned subdivision however, should be considered with caution, because in some conditions invaders can behave as penetrators and vice versa (CLINE, 1978a; CLINE and PRESS, 1990; BOWDITCH, 1997).

Packaged foods can be attacked during the production phase or in the subsequent phases of distribution, such as transportation and storage in warehouses, retail stores (TREMATERRA, 2009), and the home.

Durum wheat semolina is the main ingredient of pasta, and is used in the Mediterranean area for bread-making and in the preparation of pizza. It is commonly commercialized in kraft paper which, despite being the most widely used material for flours and semolina, does not provide sufficient protection against pest infestations and absorption-desorption of moisture.

The aim of the present work was to assess the resistance of various materials commonly used to preserve flour and semolina, to the penetration of *P. interpunctella* and *T. castaneum*. In addition, a new combination of packaging materials for durum wheat semolina was evaluated. This alternative to the traditional kraft paper bags could extend the shelf-life of semolina and ensure resistance to pest attacks. The new bilayer material (paper laminated with cast polypropylene) was developed to satisfy the technical requirements of producers (i.e. printability, machinability, etc.), to fulfil the expectations of consumers, who are accustomed to packages with a "paper look", and to improve the overall performance in terms of quality preservation and resistance to external agents.

MATERIALS AND METHODS

Rearing conditions

Tests were carried out using adults of *T. castaneum* and third and subsequent instar larvae of *P. interpunctella*. The colonies of the above-mentioned insects were established in the laboratory of the Dipartimento di Scienze e Tecnologie Fitosanitarie of the Università di Catania in 2001, from specimens collected from infested wheat flour. The colonies are routinely reared in glass jars (25 × 17 cm), maintained in climatic chambers with a constant temperature of 23°±1°C, 70±5% RH and a L8:D16 photoperiod and are fed wheat flour. Three months before the experiment, a part of the *T. castaneum* colony was reared on durum wheat semolina to accustom the insects to this diet. *P. interpunctella* were continuously fed on durum wheat semolina.

To obtain similar experimental conditions,

mature larvae of *T. castaneum* were collected and groups of 100 insects were put into plastic Petri dishes (9 cm diameter × 2 cm high), in which they were maintained until the adult stage. Second instar larvae of *P. interpunctella* were isolated in the same way and used in the experiment when they had reached the third instar.

Packaging materials

The packaging materials used for the tests were: kraft paper alone (P), bioriented polypropylene laminated with coextruded polypropylene (BOPP+PP), cast polypropylene alone (CPP), and kraft paper coupled with cast polypropylene (P+CPP). The materials tested with their thickness and water vapour transmission rate (WVTR) values are reported (Table 1). Paper bags were used as received and were identical to the ones commonly used for marketing semolina. The other bags were made in our laboratory and had the same dimensions as the kraft paper ones (13 cm × 12 cm × 4 cm). Packages, made in our laboratory, were made from reels of test materials; three seals were applied, one for each short side (top and bottom) and one on the back side of the bags, using an industrial machine. The bags were filled with 250 g semolina, bought from a local mill and visually inspected to exclude the presence of insects and, then, hermetically sealed using a method commonly used for commercial packaging.

Penetration tests

The test evaluated the ability of adult *T. castaneum* and *P. interpunctella* larvae, to pierce and consequently penetrate the packaging materials. Insects collected from the colonies were grouped in plastic Petri dishes (40 individuals of each species). Thirty adults of *T. castaneum* and third instar larvae of *P. interpunctella* were randomly chosen and transferred into transparent plastic cages (30 cm × 18 cm × 6 cm), each containing the semolina packages made with the different materials. Penetration tests were carried out using five cages, each containing 30 insects of each species. Experiments were repeated three times, at 35-day intervals. The investigation was carried out from October 1st 2008 to the end of January 2009.

The tops of the cages were covered with a fine mesh nylon screen to assure ventilation. Each container was provided with 15 g of wheat flour to feed the insects after the start of the trial and to stimulate them to search further food in order to survive. All cages were kept in a climatic chamber at a constant temperature of 23°±1°C and 70±5% RH and in the dark to provide a favourable environment for pests.

Packages were examined daily for the first month and 3 times/week thereafter and the test was considered finished when the first hole was observed. The viability of the insects and their activity towards the packages were monitored. The pouches were visually inspected through the cages; there was no manipulation in order to avoid disturbing the insect activity. This procedure was effective because most of the pest attempts were made at the same places on the bags, i.e. seals and folds, which were exposed to the observer's view. The packages were monitored for a few days after the end of the trial in order to assess pest behaviour in package colonization. Insects that died within the first 72 h were replaced in order to maintain a constant number.

Shelf-life test

The assessment of the resistance of the new material to pest penetration should be considered together with its performance in terms of quality maintenance which was carried out by a parallel shelf-life simulation test of semolina packed in the various test materials. Samples were stored for five months at 22°C and 22.7% relative humidity. The following chemical-physical parameters were monitored at 30-day intervals: moisture content, pH, acidity and colour. Moisture content was determined by drying 10 g semolina at 130°C until constant weight. The pH was measured on 4 g semolina to which 100 mL of previously neutralized 50% ethanol were added, mixed and decanted for 3 h before filtration through filter paper. Acidity, expressed as mL of 1 N NaOH necessary to neutralize 100 g semolina, was determined by titration of 50 mL filtrate with 0.5 N NaOH, using phenolphthalein as indicator. CIE Lab colour parameters were determined with a portable colorimeter NR-3000 (Nippon Denshoku Ind. Co. Ltd., Japan) with an illuminant type C/2°, previously calibrated with a white tile.

Table 1 - Packaging materials tested, with relative water vapour transmission rate (WVTR), density (for paper) and thickness values.

Material	WVTR	Density/Thickness
P	8482 g/(m^2 24 h)	30 g/m^2
CPP	13.18 g/(m^2 24 h)	30 µm
BOPP+PP	3.06 g/(m^2 24 h)	25 µm + 30 µm
P+CPP	1.19 g/(m^2 24 h)	30 g/m^2 + 30 µm

Statistical analysis

The time of penetration into the different packages of the species tested was analyzed using a one-way-analysis of variance (ANOVA) and the Student's t-test ($p \leq 0.05$) was used for post-hoc comparison. Statistical analysis was performed using STATISTICA 8 for Windows (StatSoft Inc., 2008).

RESULTS AND DISCUSSION

Microscopic inspection of the package surfaces carried out at the end of each trial, revealed numerous abrasions on every material tested, indicating the attempts of insects to pierce and penetrate the packages. On the whole, the coupled materials were more resistant to penetration. Table 2 reports the results relative to the time required for the insects to penetrate the semolina packages made with the different materials. Kraft paper was pierced by both insect species considered: *T. castaneum* needed a minimum of 12 days with an average of 16.0±3.6 days, to penetrate the packages. In one replicate, *T. castaneum* did not find a suitable point of surface weakness for a successful attack. In one case, the larvae of *P. interpunctella* needed 9 days to pierce the packages, but on the average the species needed more days to penetrate (20.2±7.4) than *T. castaneum*. However, the difference in time needed for penetration among the species was not statistically significant ($F=1.45$; $df=5.77$; $p>0.05$). CPP packages showed little resistance to pests. Even if the times were different, both insect species were able to penetrate the packages. Larvae of *P. interpunctella* were quicker (minimum 9 days) than *T. castaneum* (15 days), but on the whole, there were no significant differences ($F=1.76$; $df=6.60$; $p>0.05$). This finding disagrees with that of CLINE (1978b), who found PP to be the most resistant to the attack of *P. interpunctella* larvae and *T. castaneum* adults. This contrasting result cannot be explained by the thickness of the package, given that the PP used by CLINE (1978b) was thinner (24 μm) than the one used in the present experiment (30 μm). With regards to *T. castaneum*, a possible explanation could be that the observation time was less, but this is only a hypothesis considering that the Author did not specify the length of time of the trial with the Coleoptera adults. Moreover, the Author reported that PP was bored only by adults of *Lasioderma sericorne F.* and *Trogoderma variabile* Ballion (Coleoptera Dermestidae).

Table 2 - Time required (days) for the insects to penetrate the semolina pouches made with different materials. No significant differences were found.

	Tribolium castaneum			
	P	**CPP**	**BOPP+PP**	**P+CPP**
1	12	15	13	-
2	-	24	18	-
3	19	18	16	-
4	18	22	15	-
5	14	17	20	-
	16.0±3.6	19.2±3.7	16.4±2.7	-
	Plodia interpunctella			
	P	**CPP**	**BOPP+PP**	**P+CPP**
1	29	9	-	-
2	9	19	-	-
3	19	11	-	-
4	24	17	-	-
5	20	23	-	-
	20.2±7.4	15.8±5.8	-	-

Only *T. castaneum* succeeded in penetrating the BOPP+PP bags. On the average, penetration was achieved in 16.4±2.7 days, with a minimum of 13 days required to complete their attack on the material, which was pierced in correspondence with the seal zone. This was evidently a point of weakness of the package made with this material which was probably due to the change in structure caused by the sealing temperatures. This species showed a typical behaviour, in which the individuals scout the whole area carrying out isolated attempts to pierce the surface. DOMENICHINI (1996) observed that if an individual finds a suitable place, other individuals would be drawn to it and together they would concentrate their efforts to penetrate the package. Conversely, *P. interpunctella* did not succeed in getting into the packages, which demonstrates that this material can protect the food from this species.

The results of the experiment showed that the P+CPP bi-layer was the most resistant to the insect attacks. The package surfaces and seal zones showed some abrasions but no species succeeded in piercing them. This result could be due to the lack of release of volatile odours, the primary factor of attraction for insect pests (BARRER and JAY, 1980; DAVIS and PETTITT, 1999) that are fundamental in the selection of a favourable place of development. The scarce release of volatiles from the new packaging material could prevent infestations.

In assessing the suitability of a new material or packaging solution, all of the aspects involved in food preservation should be considered with special regard to protection from external agents and quality maintenance of the packaged product. The shelf-life test of packaged semolina considered three main quality parameters: pH, acidity and colour. The shelf-life test highlighted similar performances for all the plastic materials tested. With the exception of the pa-

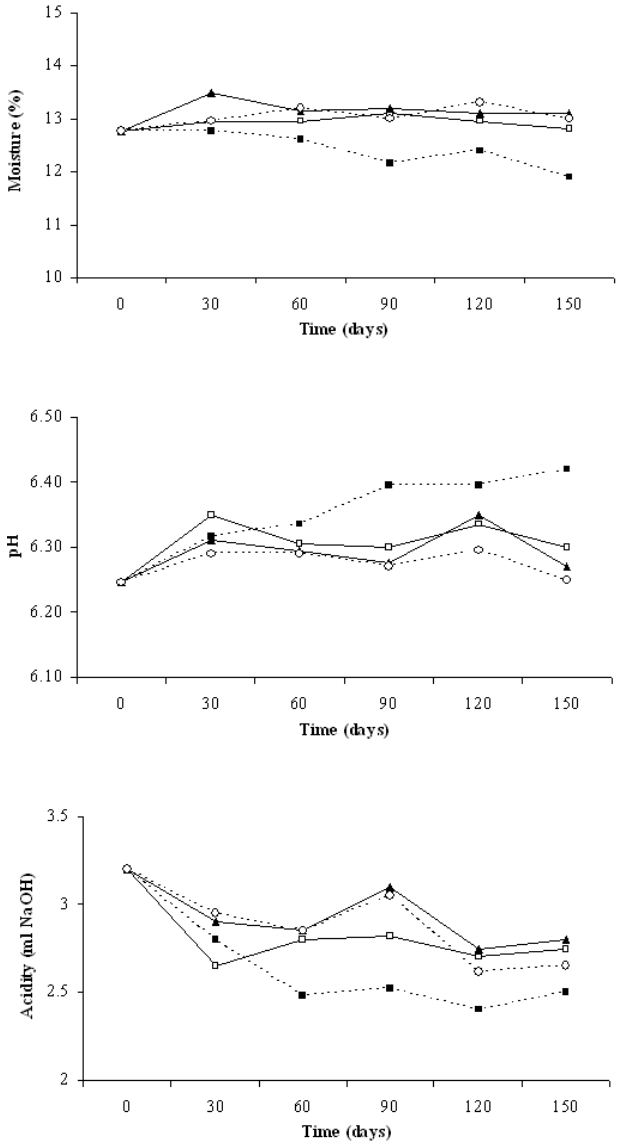


Fig. 1 - Changes in the values of moisture content, pH and acidity of semolina packed in different materials, as a function of storage time. P(■), CPP(○), BOPP+PP(□), P+CPP(▲).

per bags which, as expected, showed the highest moisture loss (i.e. around 6.5%), due to the highest WVTR (Table 1), all of the other materials preserved the initial moisture content of the semolina (Fig. 1). The final moisture content of semolina packed in paper bags (11.9%) was significantly lower than that of semolina packed in the plastic materials (12.8-13.1%); no significant differences were observed among the latter. The pH and acidity values (Fig. 1) did not vary significantly throughout the storage time for samples packed in plastic packages, with only slight decreases in the acidity values. The pH and acidity values were practically the same among all the samples packed in plastic throughout the sampling period. Only semolina packed in paper bags showed significantly higher pH values after 60 days until the end (150 days) of storage, compared to the other samples. Differences observed for samples packed in paper bags compared to CPP, BOPP+PP and P+CPP could be attributed to the much higher permeability of the former material to organic acids, CO_2 and low-molecular-weight compounds, which caused the increase in pH. Finally, the colour parameters (Fig. 2) were characterized by a similar trend (i.e. slight increase) during shelf-life, and no significant differences were observed among the samples packed in different materials.

CONCLUSIONS

Studies on food packaging and materials used to prevent insect infestations are of particular relevance for the food industry and the distribution system. Insect-proof packaging can help reduce the use of insecticides to lower the risk

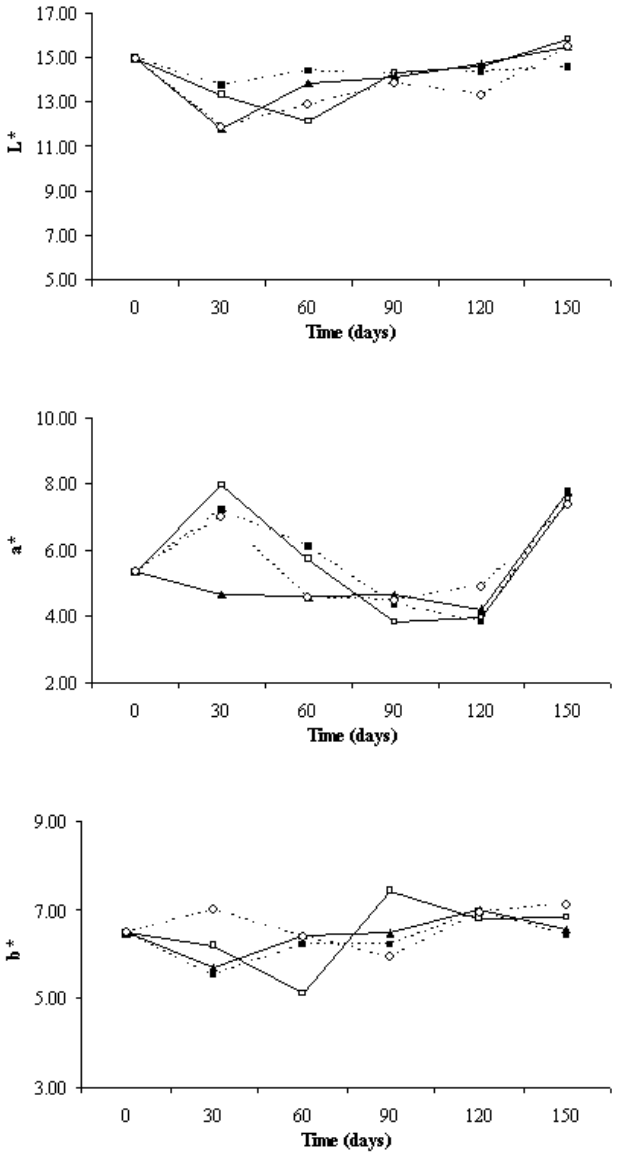


Fig. 2 - Changes in the CIE Lab colour parameters L* (lightness index), a* (red index) and b* (yellow index) in semolina packed in different materials, as a function of storage time. P(■), CPP(○), BOPP+PP(□), P+CPP(▲).

of food losses due to insect contamination. Until recently, pesticides were the most frequently used method to avoid it, but these are dangerous and have been banned in most parts of the world. The present study focused on the ability of *P. interpunctella* larvae and *T. castaneum* adults to pierce materials used for the packaging of semolina, considering that these two species are the most common pests in stored food. The results show differences in the resistance of materials but not in the time required for the two different species to pierce packages. No significant difference was observed among the materials pierced (P and CPP) in terms of time required by the insects to penetrate the packages. On the other hand, BOPP+PP was pierced only by *T. castaneum*, while the new bi-layer P+CPP was not pierced by the species tested. Quality parameters did not differ significantly among samples packed in the different materials. The new P+CPP combination could be a promising alternative for the packaging of semolina, offering an improved protection against pests compared with conventional materials while maintaining the paper-appearance of the traditional packages.

The trials performed in the present research are useful for testing the resistance of packaging materials to insects and choosing the best materials for the packaging of pasta, cereals and other products that are subject to insect attacks, such as bakery products, powdered milk, tea, etc. Further work is needed to determine the permeability of materials to volatiles and to study the correlations between this parameter and the susceptibility of packages to insect attacks. Among the technical specifications of a material for food packaging, data concerning the permeability to aroma compounds are needed.

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SHORT COMMUNICATION

MULTIPLE ANTIBIOTIC RESISTANCE AMONG *LISTERIA MONOCYTOGENES* IN RETAIL FOODS, IN ADANA, TURKEY

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ABSTRACT

A total of 178 food samples (fresh soft cheese, raw milk, ice cream, sausage, chicken meat and minced beef meat) were investigated for *Listeria monocytogenes.* Of these, 29 (16.3%) were contaminated. 102 strains of *L. monocytogenes* were isolated and the susceptibility to 13 different antibiotics was determined. Most of the isolates (57.8%) were resistant to amdinocillin; in contrast, all isolates were sensitive to chloramphenicol. Multiple antibiotic resistance (MAR) index values ranged from 0.21 to 0.43. Results show that antibiotic resistant *L. monocytogenes* strains were easily isolated from selected foods and antibiotic resistance may become a potential food poisoning concern for public health.

- Key words: Antibiotics, food *Listeria monocytogenes*, MAR index -

INTRODUCTION

In the last decade the Gram-positive, motile, bacteria *Listeria monocytogenes* has become a major concern for the food industry and public health authorities in developed countries. This organism can persist within food processing environments for long periods of time, due, in part, to its ability to grow at a wide range of temperatures and pH (0.4° to 45°C, pH 4 to 9.6) (BERESFORD *et al.*, 2001). It is ubiquitous in the environment (GUDBJÖRNSDÓTTIR *et al.*, 2004), and in different kinds of food (MIYASAKI *et al.*, 2009; AURORA *et al.*, 2009).

L. monocytogenes is a model organism used in studies on the pathogenesis of intracellular parasites. It can penetrate, multiply and propagate in various types of eukaryotic cells and is also able to overcome the three main barriers encountered in the host: the intestinal barrier, the blood-brain barrier and the placenta (POPOWSKA and MARKIEWICZ, 2004). Most patients with listeriosis have an underlying condition which predisposes them to infection by interfering with T cell-mediated immunity (HOF *et al.*, 1998). Infection causes mild symptoms including fever, febrile gastroenteritis, nausea, vomiting and diarrhea in healthy individuals, while the symptoms may be severe, e.g. meningitis, encephalitis or septicemia in infants, elderly and immuno-compromised persons. It may also cause intrauterine infection or spontaneous abortion in pregnant women (SWAMINATHAN and GERNERSMIDT, 2007).

Although the incidence of listeriosis in Europe is low, 2-3 cases/million inhabitants/year, the high mortality rate (up to 40%) requires early diagnosis and appropriate antimicrobial therapy (JONES and MACGOWAN, 1995). In 2005, listeriosis was reported in a 49-year-old female patient in Turkey (AKTAS *et al.*, 2005). In 2007, a local health officer in central Massachusetts contacted the Massachusetts Department of Public Health (MDPH) to report listeriosis in an 87-year-old man (MMWR, 2008). The number of *Listeria* infections in humans in 2007 remained at the same level as in 2006 with 1,554 confirmed cases; *Listeria* also showed the highest mortality rate, especially among vulnerable groups (EFSA-ECDC, 2009).

Although *Listeria* spp. have been reported to be susceptible to antibiotics that are active against Gram-positive bacteria (HAWKINS *et al.*, 1984), there have been recent reports of antibiotic resistance (ABRAHIM *et al.*, 1998; MARTÍNEZ and RODRÍGUEZ, 2005; YÜCEL *et al.*, 2005). These increases in antibiotic resistance among *Listeria* spp. are in line with a general worldwide trend of an increasing prevalence of antibiotic resistance, including multiple antibiotic resistance among many groups of bacteria.

Listeriosis usually occurs sporadically, although at times it affects numerous patients simultaneously. Outbreaks of listeriosis occur most frequently after the consumption of contaminated milk, soft cheese, salads, and meat; and less often after the consumption of contaminated fruit or fish (DE VALK *et al.*, 2000; LYYTIKÄINEN *et al.*, 2000; VITAS *et al.*, 2004). A large, multi-province outbreak of listeriosis associated with ready-to-eat meat products contaminated with *Listeria monocytogenes* serotype 1/2a occurred in Canada in 2008 (GILMOUR *et al.*, 2010).

Turkey has a very long tradition of producing a diversity of fermented dairy products, among which white cheese is one of the most significant. The average annual consumption of white cheese was estimated to be around 243,000 tons in 2001 (DPT, 2001). In Turkey, white cheese is usually manufactured from raw (unpasteurized) milk in villages, small dairies and farms, with only 10% of the production occurring in big plants. Such traditional cheese manufacture from raw milk is a very common practice in many countries and the associated high incidence of *L. monocytogenes* could pose a health hazard (ARSLAN and ÖZDEMIR, 2008).

Little information is available on the cases of listeriosis in Turkey. Although 32 cases of human listeriosis with a mortality rate of 33% were reported for the period 1987 to 2001 in Turkey (DOGANAY, 2003), information about the source of the infections was omitted. The present study is the first to determine the prevalence and resistance to antimicrobial agents of *L. monocytogenes* isolated in Adana, Turkey.

The objectives were: (i) to identify the *L. monocytogenes* strains isolated from foods; (ii) evaluate the susceptibility patterns of these bacteria against widely used antibiotics in Turkey; and (iii) to evaluate multiple-antibiotic resistance (MAR) index for selected isolates.

MATERIALS AND METHODS

A total of 178 food samples were collected from May to August 2008 from dairies, supermarkets, retail stores, and local butcher shops in Adana, in southern Turkey. The samples included fresh soft cheese 48, raw milk 32, ice cream 21, raw sausage 23, raw chicken meat 27 and raw minced beef meat 27. All samples were brought to the laboratory in an ice chest, and were processed within 4 h of collection.

Examination of foodstuffs

Food samples (25 g) were homogenised with a stomacher in 225 mL Fraser pre-enrichment broths (Merck, Darmstadt, GERMANY) supplemented with ½ *Listeria* enrichment broth (Merck). After 48 h of incubation at 30°C, a culture was streaked in triplicate on Oxford- *Listeria* selective agar (MERCK) containing selective supplement (MERCK), and all plates were incubated at

37°C for 48 h. The plates were examined for typical *Listeria* colonies (black colonies with black sunken areas) and five suspicious colonies on each Oxford agar were sub-cultured on Tryptic soy agar (ACUAMEDIA) supplemented with 0.6% yeast extract (DIFCO). After further incubation at 37°C for 24 h, the following biochemical identification tests were performed: Gram staining, nitrate reduction, beta haemolysis reaction, oxidase reaction, catalase reaction, motility in SIM medium, hydrolysis of esculin, MR/VP and CAMP test, acid production from glucose, mannitol, rhamnose, xylose, and α-methyl-D-mannoside. The isolates were identified using the Becton Dickinson Crystal GP ID System and identification software (BBL, Sparks, MD, USA).

Antibiotic resistance testing

Susceptibility testing was performed for all strains (102) with an agar diffusion test (NCCLS, 1993), using Mueller–Hinton agar (DIFCO) and 13 antibiotic discs representing 7 classes of antibiotics. The antibiotics and their sensidisk concentrations were: aminoglycosides: amikacin (AN, 30 μg), gentamicin (GM, 10 μg), kanamycin (K, 30 μg), streptomycin (S, 10 μg); carbapenems: meropenem (MEM, 10 μg); cephalosporins: cefazolin (CZ, 30 μg), cefepime (FEP, 30 μg), ceftizoxime (ZOX, 30 μg), cefuroxime (CXM, 30 μg); chloramphenicols: chloramphenicol (C, 30 μg); penicillins: amdinocillin (AMD, 10 μg); tetracyclines: tetracycline (TE, 30 μg); and trimethoprim-sulphamethoxazoles: trimethoprim-sulphamethoxazole (SXT, 1.25 and 23.75 μg). The turbidity of the bacterial suspension was then compared with 0.5 MacFarland's Barium Sulfate standard solution (corresponding to $1.5X10^8$ cfu/mL). If the bacterial suspension was turbid, autoclaved distilled water (0.85% saline) was added drop by drop; if less turbid, more bacteria were added to the tube or the inoculated tube was further incubated. After 24 h of incubation at 30°C, the strains were characterized to determine if they were sensitive, intermediate or resistant, based on the size of the inhibition zones around each disc according to the manufacturer's instructions. (BBL, Sparks, MD, USA).

Reference strains of *Staphylococcus aureus* ATTC 29213 and *Enterococcus faecalis* ATTC 29212 as recommended by NCCLS (1997) were used as control organisms to verify the antibacterial effect of the discs on Mueller-Hinton agar plates. All discs were purchased from Becton Dickinson (BBL, Sparks, MD, USA).

Multiple antibiotic resistance (MAR) index

The MAR index values were calculated for all isolates, (a/b, where 'a' represented the number of antibiotics the isolate was resistant to, and 'b' represented the total number of antibiotics the isolate was tested against). A MAR index value >0.2 is observed when isolates are exposed to high risk sources of human or animal contamination, where the use of antibiotics is common. In contrast a MAR index value ≤ 0.2 is observed when antibiotics are seldom or never used (KRUMPERMAN, 1985).

RESULTS AND DISCUSSION

L. monocytogenes in foods

A total of 102 isolates of *L. monocytogenes* were recovered from the six types of food. The percentage of positive samples varied markedly between the different foods as follows: fresh soft cheese 12.5%, raw milk 3.1%, ice cream 4.8%, sausage 21.7%, chicken meat 40.7%, and minced beef meat 18.5% (Table 1).

A high incidence of *L. monocytogenes* in meat and meat products was also found by AURELI *et al.* (2003) (35.8% in poultry, and 33.1% in raw and cured meat samples), BRACKETT (1988) (55.3% in raw chicken samples in Switzerland), and by VITAS *et al.* (2004) (36.1% in poultry, and 34.9% in minced pork and beef). Food processing plants may become contaminated with *L. monocytogenes* from contaminated raw materials, equipment, personnel and traffic GUDBJÖRNSDÓTTIR *et al.* (2004). Furthermore, contamination may occur during processing, be-

Table 1 - Incidence of *Listeria monocytogenes* in different foods in Turkey.

Type of food	N. of samples	N. and (%) of positive samples	N. of *L. monocytogenes* strains
Fresh soft cheese	48	6 (12.5)	26
Raw milk	32	1 (3.1)	3
Ice-cream	21	1 (4.8)	5
Sausage	23	5 (21.7)	23
Chicken meat	27	11 (40.7)	29
Minced beef meat	27	5 (18.5)	16
Total	178	29 (16.3)	102

cause of the release of blood and tissue during cutting, deboning and grinding of meat, along with the large quantities of water used which favor the growth of *Listeria* GUDBJÖRNSDÓTTIR *et al.* (2004). Since moist environments create favorable conditions for survival and growth of this bacterium, it is important to keep the processing plants as dry as possible (SUTHERLAND and PORRIT, 1996). Although, *L. monocytogenes* has been isolated from many different types of raw and processed food (YÜCEL and ÇITAK, 2005; AURELI *et al.*, 2003; KELLS and GILMOUR, 2004; JALALI and ABEDI, 2008), the main sources and routes of contamination are still not fully understood.

There was a relatively low incidence of *L. monocytogenes* contamination in fresh soft cheese, raw milk and ice cream in the present study. Similar findings were also reported by AURELI *et al.* (2003) (contamination in 24.3% in soft fresh cheese, and 6.8% in raw milk), BAEK *et al.* (2000) (6.1% in ice cream), SAGUN *et al.* (30) (1.2% of raw milk), and VITAS *et al.* (2004) (6.8% in raw cow milk and 3% in raw sheep milk).

Antibiotic resistance patterns

In the present study, there was a common resistance to certain antibiotics (Table 2); 57.8% of the *L. monocytogenes* isolates were resistant to amdinocillin, 35.3% were resistant to streptomycin, 32.4% to amikacin and 31.4 and 30.4% to ceftizoxime and cefuroxime, respectively. *Listeria* resistance has previously been reported for streptomycin (CHARPENTIER *et al.*, 1995) and tetracycline (18.6% from processed bison meat) (LI *et al.*, 2007).

Antibiotic resistance in *Listeria* species is due to the acquisition of self-transferable and mobiliszable plasmids, and conjugative transposons (CHARPENTIER *et al.*, 1995). In the present study, all strains were susceptible to chloramphenicol (100%), which is consistent with the findings of DHANASHREE *et al.* (2003), and similar to those of LI *et al.* (2007). Resistance to other antibiotics was relatively infrequent: cefazolin (8.8%), trimethoprim-sulphamethoxazole (8.8%), gentamicin (5.9%), and meropenem (2.9%). Trimethoprim-sulphamethoxazole is used in the treatment of food-borne *L. monocytogenes* in patients who are allergic to penicillin (LI *et al.*, 2007)

There were some marked differences in the degree of antibiotic resistance between samples. Isolates from fresh soft cheese and raw milk showed a higher resistance to amikacin (76.9% and 66.6%, respectively) than did those from other samples, including the isolates from ice cream, none of which were resistant. Isolates from minced beef meat showed high resistance to amdinocillin (75.0%) and ceftizoxime (68.8%), and isolates from raw milk showed a high resistance to streptomycin (66.6%). All isolates from fresh soft cheese, raw milk, ice cream and minced beef meat were susceptible to meropenem and trimethoprim-sulphamethoxazole. All of the isolates from raw milk, ice cream and minced beef meat were susceptible to gentamicin and cefazolin; and all those from raw milk, ice cream and sausage were susceptible to tetracycline. In contrast, isolates from chicken meat showed variable resistance to the antibiotics (Table 2).

The use and misuse of antibiotics contribute

Table 2 - Source and percentage of *Listeria monocytogenes* strains resistant to 13 antibiotics.

Classes of antibiotics	Antibiotics	Fresh soft cheese N=26	Raw milk N= 3	Ice-cream N=5	Sausage N=23	Chicken meat N=29	Minced beef meat N= 16	Average Resistances N=102
Aminoglycosides	Amikacin (AN, 30 µg)	76.9	66.6	-	17.4	13.8	18.8	32.4
	Gentamicin (GM, 10 µg)	11.5	-	-	4.3	6.9	-	5.9
	Kanamycin (K, 30 µg)	38.5	33.3	-	8.7	20.7	6.3	19.6
	Streptomycin (S, 10 µg)	61.5	66.6	20.0	-	58.6	6.3	35.3
Carbapenems	Meropenem (MEM, 10 µg)	-	-	-	4.3	6.9	-	2.9
Cephalosporins	Cefazolin (CZ, 30 µg)	3.8	-	-	4.3	24.1	-	8.8
	Cefepime (FEP, 30 µg)	7.7	33.3	80.0	30.4	10.3	18.8	20.6
	Ceftizoxime (ZOX, 30 µg)	3.8	-	-	52.2	20.7	68.8	31.4
	Cefuroxime (CXM, 30 µg)	7.7	33.3	80.0	39.4	20.7	50.0	30.4
Chloramphenicol	Chloramphenicol (C, 30 µg)	-	-	-	-	-	-	-
Penicillin	Amdinocillin (AMD, 10 µg)	50.0	33.3	20.0	60.9	62.1	75.0	57.8
Tetracycline	Tetracycline (TE, 30 µg)	60.0	-	-	-	24.1	12.6	23.5
Trimethoprim-sulphamethoxazole	Trimethoprim-sulphamethoxazole (SXT, 1.25 and 23.75 µg)	-	-	-	34.8	3.4	-	8.8

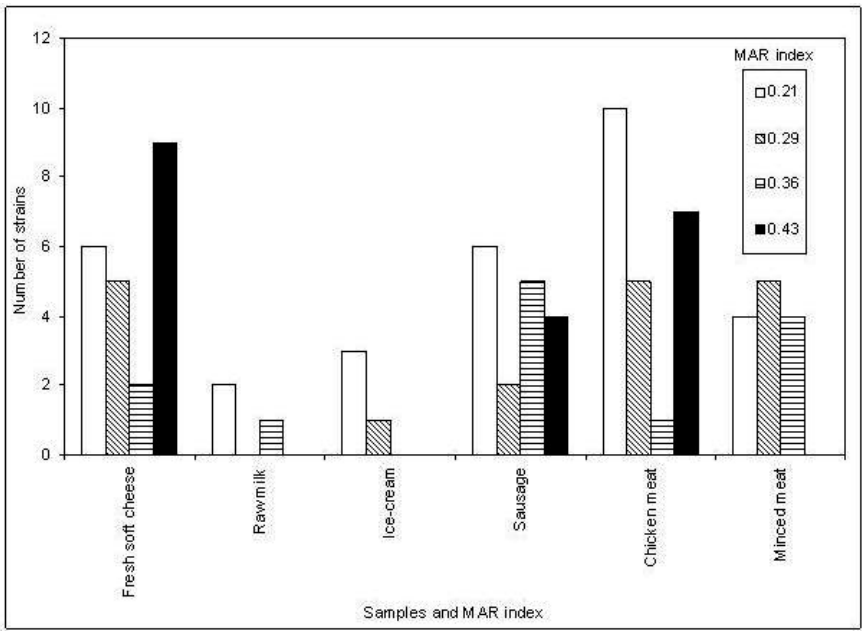


Fig. 1. Multi-antibacterial resistance of *Listeria monocytogenes* isolated from 6 different foods in Adana, Turkey.

to the development of resistance among bacteria including food-borne pathogens.

Multiple antibiotic resistance index

The multiple antibiotic resistance (MAR) index values ranged from 0.23 to 0.38 for the isolates. Resistance to five antibiotics was most common among the isolates, 20 isolates (19.6%) were resistant to five antibiotics (9, 7, 4, from fresh soft cheese, chicken meat, and sausage samples, respectively); furthermore, 18 (17.6%) isolates were resistant to three antibiotics and 13 (12.7%) to four antibiotics (data not shown).

There has been an increase in antibiotic resistance among *Listeria* spp. (BARBUTI *et al.*, 1994; PAPA *et al.*, 1996); this is in line with a general worldwide pattern of an increasing prevalence of antibiotic resistance, including multiple antibiotic resistance among many groups of bacteria (ARSLAN and ÖZDEMİR, 2008). High percentages of resistant strains of *L. monocytogenes* have been reported in dairy and meat products (ROTA *et al.*, 1996), and since the incidence of contamination of this bacteria is high in meat products in Adana, Turkey, these foods are of particular concern. Since the detection in 1988 of the first clinical strain of *L. monocytogenes* multi-resistance to antibiotics, other strains of *Listeria* spp. resistant to antibiotics have been reported (CHARPENTIER and COURVALIN, 1999).

The markedly different distribution of resistance to antibiotics, among isolates from the six different foods, is shown in Fig. 1. The multiple antibiotic resistance (MAR) index values ranged from 0.23 to 0.38 for fresh soft cheese, chicken meat and sausage isolates; from 0.23 to 0.31 for minced beef meat isolates; and the MAR index value for milk and ice cream isolates was 0.31. The highest numbers of antibiotic-resistant isolates were detected in cheese, chicken meat and sausage samples, 9 out of 26 isolates from fresh soft cheese, 7 out of 29 isolates from chicken, and 4 out of 23 isolates from sausage, were resistant to five antibiotics; no other isolates were resistant to so many antibiotics.

This is the first report of antibiotic-resistant *L. monocytogenes* strains isolated from foods in Adana, Turkey where *L. monocytogenes*-contaminated food resistance to antibiotics is common. There have been numerous multiple antibiotic resistant isolates identified which is a major public health concern because of the high mortality rates associated with Listeriosis. *L. monocytogenes* can be avoided by vigorous scrupulous cleaning and sanitation practices; contamination prevention programs, food safety and HACCP programs must be required in all food-processing plants. Strict standards for food safety practices are urgently needed. It is also important to reduce or eliminate the incorrect therapeutic use of antimicrobial agents in veterinary practice and human medicine. Furthermore, studies are needed to elucidate the mechanisms of antibiotic resistance among *L. monocytogenes* isolates.

ACKNOWLEDGEMENTS

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SHORT COMMUNICATION

INVESTIGATION OF THE ANTIGENOTOXIC PROPERTIES OF THE PROBIOTIC *LACTOBACILLUS RHAMNOSUS* IMC 501® BY GAS CHROMATOGRAPHY-MASS SPECTROMETRY

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ABSTRACT

A method was set up to study the antigenotoxic property of the probiotic *Lactobacillus rhamnosus* IMC 501® against 4-nitroquinoline-1-oxide (4-NQO). The method is based on liquid-liquid extraction followed by gas chromatography-mass spectrometry (GC-MS) analysis. The GC-MS analysis was used to determine the quantitative change of 4-NQO before and after co-incubation with *Lactobacillus rhamnosus* IMC 501®. The results from GC-MS analysis showed that 4-NQO genotoxicity was inhibited and then converted to less toxic compounds. These preliminary results from GC-MS analysis indicate that the method described is able to detect physicochemical modifications of the genotoxic agent, which occur after co-incubation with bacteria cells. The method, therefore, is a novel alternative to biological assays for detecting the antigenotoxicity of lactobacilli.

- Key words: antigenotoxicity, functional foods, GC/MS analysis, *Lactobacillus*, 4-nitroquinoline-1-oxide, probiotics -

INTRODUCTION

Food contaminants entering the body through the oral route are directly exposed to the action of the gut microflora. Normal healthy intestinal microflora contains many strains of lactic acid bacteria (LAB), some of which have been identified, and termed probiotic strains with ascribed health benefits (SALMINEN *et al.*, 1998). Probiotic bacteria have generated much scientific and commercial interest as ingredients in several foods. This interest is due to a range of possible health benefits of these bacteria, which include the improvement and balance of intestinal microflora, suppressing enhanced inflammatory responses, immune system stimulation, cholesterol lowering and prevention of cancer recurrence (BURNS and ROWLAND, 2000; MARTEAU, 2001). The protective effect of LAB against food mutagens such as heterocyclic amines, *N*-nitroso compounds, and aflatoxins has also been reported (ORRHAGE *et al.*, 1994; EL-NEZAMI *et al.*, 2000). Consequently, the antigenotoxicity and antimutagenicity of LABs begin to be considered when characterizing the functional properties of probiotic bacteria. Remarkably, the inhibitory activity of LABs against some genotoxins has been shown for various strains of *L. rhamnosus*, *L. casei*, *L. plantarum*, *L. brevis*, *L. acidophilus* and *Lactobacillus spp.* isolated from dairy and pharmaceutical products or obtained from culture collections (CENCI *et al.*, 2005; CALDINI *et al.*, 2008). In these studies the antigenotoxicity was demonstrated by short-term biological assays (SOS-Chromotest and Comet assay) and was strain-dependent. The mechanism of antimutagenic activities of probiotics is not clearly understood. There is, however, evidence to associate some known modes of activity such as binding of mutagens to microbial cells (ORRHAGE *et al.*, 1994), mutagen conversion or direct inhibition of tumorogenesis by these metabolites (HIRAYAMA and RAFTER, 2000) to explain the mechanism of action. The antigenotoxicity of probiotics as evaluated by short-term biological assays only reflects not more than the relative activity of probiotics, but no information about antigenotoxicity mechanisms could be obtained. In particular, it could not differentiate between the absorption or the conversion of the genotoxin.

The aim of the present work was to set-up a gas chromatography-mass spectrometry method that is able to delineate the mechanisms involved in the LAB inhibition of 4-NQO, a direct-acting agent which produces strand scission and formation of charge-transfer adducts on DNA. Specifically, the 4-NQO antigenotoxicity of a LAB probiotic strain (*Lactobacillus rhamnosus* IMC 501®) revealed by short-term biological assays (data not shown), was evaluated by GC-MS analysis.

MATERIALS AND METHODS

Bacterial strain

Lactobacillus rhamnosus IMC 501® strain was used in this study. The bacterial strain was isolated in our laboratory from faecal samples of elderly Italian subjects during an EU project named Crownalife, as previously described (SILVI *et al.*, 2003; MUELLER *et al.*, 2006). *L. rhamnosus* IMC 501® has been studied for its probiotic properties such as gastro-intestinal transit (acid and bile tolerance), *in vitro* adhesion to cell line, antimicrobial activity, antibiotic susceptibility, plasmid profile and *in vivo* colonization and persistence in the human gut. The bacterial strain was phenotypically and genotypically characterized and deposited in the culture collection Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Germany) with number DSM 16104 and it was assigned Italian patent no. RM2004A000166 (VERDENELLI *et al.*, 2009).

Viability

The viable number of *L. rhamnosus* IMC 501® after co-incubation with 4-NQO was determined by plate count on MRS agar (Oxoid Ltd, Basingstoke, Hampshire, UK) under aerobic incubation conditions. The experiment was conducted in triplicate.

Chemicals

The genotoxin, 4-nitroquinoline-1-oxide, was obtained from Sigma (CAS no. 56-57-5, Sigma-Aldrich, St. Louis, MO, USA). Stock solution (1 mg/mL) was prepared in dimethyl sulphoxide (DMSO) and aliquots were stored at -20°C, until the test. Working solutions were obtained with suitable dilution in saline.

Cell preparation and genotoxin-cell co-incubation

Cultures were grown overnight in MRS-broth at 37°C and harvested by centrifugation (6,000 g for 15 min). The pellet was resuspended in physiological saline (10^8-10^9 CFU/mL) and 4-NQO was added at a final concentration of 0.1 mM. Co-incubation was carried out at 37°C for 15, 30 and 135 min under agitation. The supernatant was recovered by centrifugation followed by filtration through a 0.45 μm membrane filter (Sartorius AG, Göttingen, Germany).

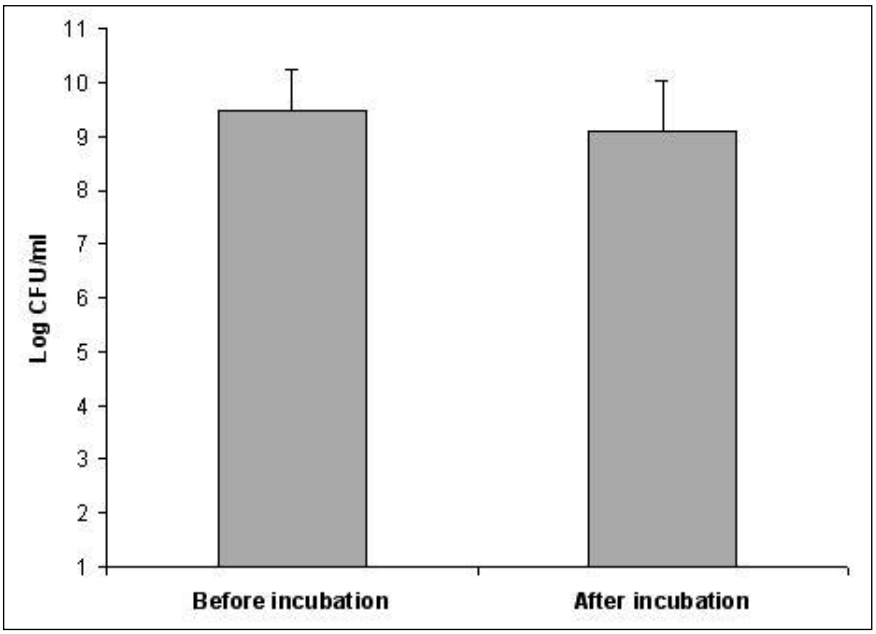


Fig. 1 - The bacterial count of *Lactobacillus rhamnosus* IMC 501® after co-incubation with 0.1 mM 4-NQO at 37°C for 135 min. Mean values ± standard deviation of three measurements.

Physicochemical analyses

Supernatants of 4-NQO-treated cultures were examined by both absorbance profiles (Shimadzu UV-1204 spectrophotometer, Columbia, MD, USA) and gas chromatography/mass spectrometry.

Fifty mL of 4-NQO-treated culture supernatant was extracted three times in a separatory funnel with dichloromethane by using 15, 15 and 20 mL, respectively. The solvent was evaporated under vacuum and dried samples were re-dissolved in 1 mL of dichloromethane. The GC-MS analyses were performed on a gas chromatography (Agilent Technologies 6890N, Santa Clara, CA, USA) coupled to a mass spectrometer (Agilent Technologies 5973N). A HP-5MS Agilent Techonologies (30 m - 0.25 mm I.D. - 0.1 µm film) column was used with helium as carrier gas (flow rate 1 mL/min). The sample (2.0 µL) was injected in the splitless mode at 250°C (purge time 2.0 min). The GC oven was operated with the following temperature program: initial temperature 70°C held for 3 min, increased to 320°C at 30°C/min and held at 320°C for 10 min. The ionizing energy was 70 eV and all data were obtained by collecting full-scan mass spectra at a scan range of 29-350 amu. The compounds were identified with NIST 08 mass spectra library using the chemStation Software.

Statistical analysis

The mean values and the standard deviation were calculated from the data obtained in three separate experiments. A Student *t*-test was used to determine the significance of differences in bacterial counts before and after genotoxin-cell co-incubation.

RESULTS

Survival of probiotic bacteria in the presence of 4-NQO

It was observed that the viability of the probiotic bacteria, *L. rhamnosus* IMC 501®, remained comparatively high after co-incubation with the genotoxin. In fact, cell survival remained above 90% (Fig. 1) and there were no significant differences.

Spectroscopic characteristics of 4-NQO before and after cell co-incubation

Bacteria-mutagen co-incubation was accompanied by modifications in the spectroscopic characteristics of the supernatants. Representative absorbance profiles indicate that the maximum absorbance peak of 4-NQO was shifted to a short wavelength (hypsochromic shift), compared to the normality typical of the compound (Fig. 2).

Fig. 3 illustrates mass spectral data from supernatants of the strain *L. rhamnosus* IMC 501® treated with 4-NQO at different incubation times. The spectra showed the gradual disappearance of 4-NQO and the appearance of 4-aminoquinoline and 4-nitroquinoline at 15 min as depicted in the standards shown in Fig. 4. The concentration of these two products also showed a gradual reduction at 30 and 135 min. Fig. 5 shows the decrease of 4-NQO peak areas at different times of genotoxin-cell co-incubation.

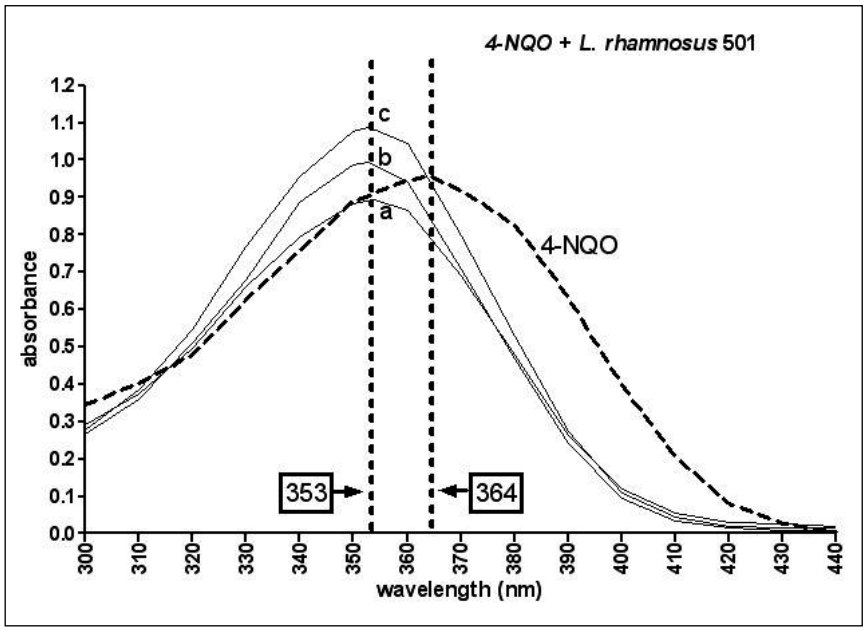


Fig. 2 - Representative absorbance profiles of 4-NQO without and with *Lactobacillus rhamnosus* IMC 501® co-incubation at different times. (a) 15 min, (b) 30 min, (c) 135 min. The dashed profile represents genotoxin spectrum without bacteria co-incubation.

CONCLUSIONS

The data from the study of the co-incubation of *L. rhamnosus* IMC 501® with the reference genotoxin 4-NQO shows that this probiotic bacterial strain has potential inhibitory activity against this genotoxin as revealed by GC mass spectrometry. Lactic acid bacteria have been reported to have antimutagenic/anticarcinogenic properties in vitro and in vivo. The mechanisms for such effects have been hypothesized to include the physical binding of the mutagenic compounds to the bacteria, genotoxin bioconversion or conjugation (BURNS and ROWLAND, 2000; COMMANE *et al.*, 2005). The GC-MS protocol set-up in this study has demonstrated the inhibition of 4-NQO genotoxicity by the strain *L. rhamnosus* IMC 501®, revealing physicochemical modifications of the genotoxic agent that originated from bacterial preincubation. In fact, the spectra show appearance of 4-aminoquinoline as a bioconversion product, which is a widely known inactive compound. First of all, the modification seen in the absorbance profile of 4-NQO after co-incubation with *L. rhamnosus* IMC 501® proves the involvement of chemico-biological interactions. It is notable that the antigenotoxicity is a strain-dependent charac-

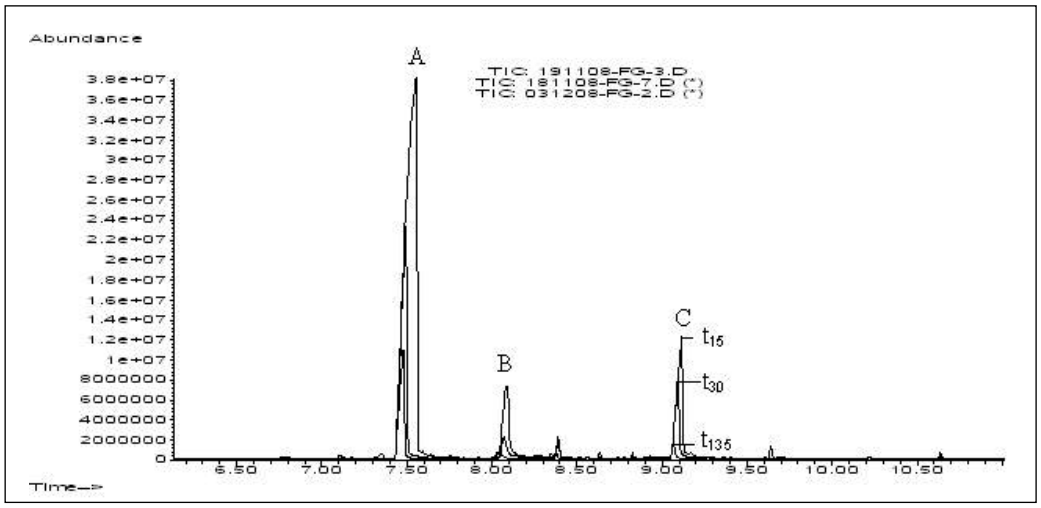


Fig. 3 - Mass spectra of supernatants from the *Lactobacillus rhamnosus* IMC 501® strain co-incubated with 4-NQO for different times of incubation (15, 30 and 135 min). A, 4-nitroquinoline; B, 4-aminoquinoline; C, 4-nitroquinoline-1-oxide.

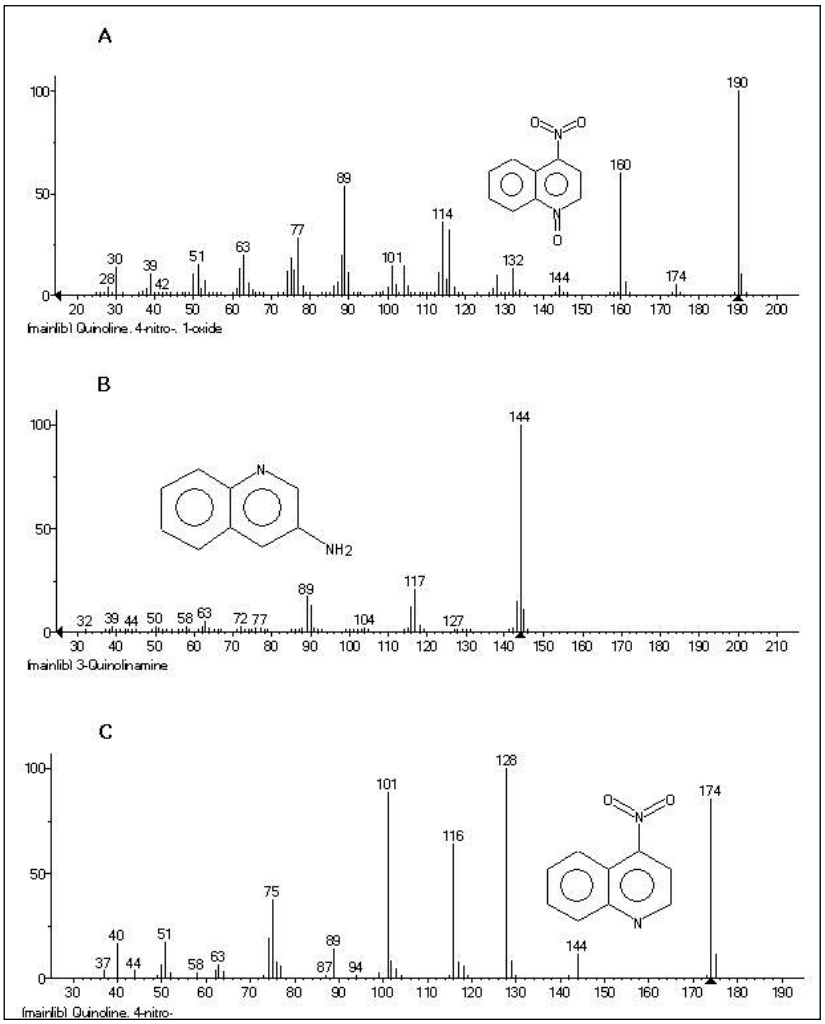


Fig. 4 - Mass spectra of 4-nitroquinoline-1-oxide (A), 4-aminoquinoline (B) and 4-nitroquinoline (C).

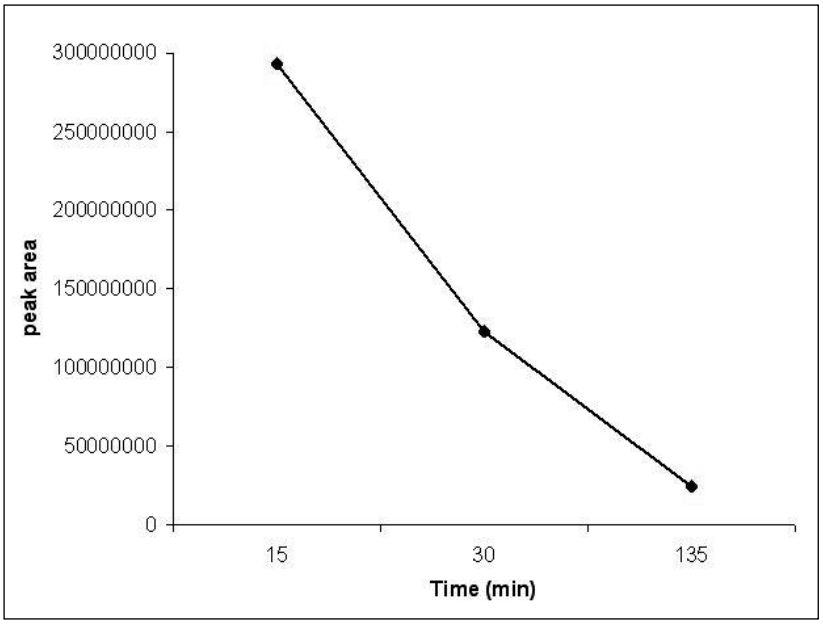


Fig. 5 - Peak area reduction of 4-nitroquinoline-1-oxide after 15, 30 and 135 min of co-incubation with *Lactobacillus rhamnosus* IMC 501®.

teristic as demonstrated by the observable spectroscopic modification profile of the genotoxins during co-incubation with different bacterial strains (CORSETTI *et al.*, 2008). The results obtained in this study are in accordance with those of CALDINI *et al.* (2002) who demonstrated a blue shift in the maximum absorbance wavelength of 4-NQO after co-incubation with *Bacillus* strains. Moreover, the GC-MS method was able to monitor the disappearance of the toxic agent and the appearance of other possible metabolic products, as also shown by other authors (CALDINI *et al.*, 2002). In this study GC-MS spectra showed that the antigenotoxic process was accompanied by a decrease in the content of 4-NQO and a simultaneous appearance of a new product peak. It could be deduced from the GC-MS results that the mechanism of antigenotoxicity for *L. rhamnosus* IMC 501® was the conversion of 4-NQO to an inactive end product, and not the simple absorption or binding onto cell wall components. In this context, it is interesting to highlight that the inhibition of the tested genotoxin was related to the maintenance of cell viability after co-incubation. This fact, together with the capability of resistance to gastrointestinal stress by *L. rhamnosus* IMC 501® suggests the possibility of using this probiotic strain as an ingredient in functional foods where it could serve as a source of protection against genotoxic risk in the human gut. These results are of considerable interest with the increasing demand for functional foods, especially dairy products, such as yoghurts and fermented milks, containing *Lactobacillus* and *Bifidobacterium*. Results suggest that GC-MS may be considered an excellent methodology for revealing genotoxin deactivation by probiotic bacteria. The GC-MS analysis is in fact applicable to different genotoxic compounds which may be present in foods or originate

from cooking (exogenous genotoxins) and/or be produced by host metabolism (endogenous genotoxins). Finally, with regard to the microorganism used, the results of this in vitro study suggest that the incorporation of *L. rhamnosus* IMC 501® in the diet might be able to suppress or reduce the genotoxic activity of potentially harmful compounds. In this context it is important to emphasize the excellent tolerance to gastro-intestinal transit demonstrated by this microorganism (VERDENELLI *et al.*, 2009). The evidence from the above-described *in vitro* approach, although not directly transferable to humans, is in line with the hypothesised efficacy of probiotics in providing a protective effect against genotoxins in the gut. Further trials are in progress to confirm *in vivo* antigenotoxicity of *L. rhamnosus* IMC 501® using an animal rat model.

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SHORT COMMUNICATION

RAPID GC/MS TEST FOR IDENTIFICATION OF *OLEA EUROPAEA* L. LEAVES IN GROUND OREGANO

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ABSTRACT

Various ethanol extracts of dried oregano samples purchased from international wholesale traders and from retail markets have been examined by GC/MS: the same procedure was conducted with various samples of olive leaves. The extracts were studied in the chromatographic region where the volatile compounds of high mass can be eluted. The experiments revealed that two markers derived only from olive leaves were identifiable in oregano extracts containing olive leaves, also at very low levels. This paper describes the operating conditions useful to realize the test which permits to evidence the adulteration of commercial batches of ground oregano containing olive leaves added as bulking agents.

- Key words: adulteration, GC/MS test, olive leaves, oregano, quality control -

INTRODUCTION

Various species of commercially named "oregano" or "origanum" have achieved economic interest and they are used indiscriminately for culinary purposes. Oregano plant parts and biochemical extracts (herb, leaf, essential oil, etc.) are also commonly used in the food industry as a spice. In Europe and throughout the world, the most commonly used oregano species belong to the genus *Origanum*. This genus is represented by various species and subspecies and many analytical investigations and morphological studies of oregano have been undertaken to discriminate between numerous subspecies. The genus *Origanum* (Labiatae family) includes about 70 species, subspecies, varieties and hybrids, characterized by considerable morphological and chemical diversity (AZIZI *et al.*, 2009; BARANSKA *et al.*, 2005; BASER *et al.*, 1992; BASER *et al.*, 1993; CARMO *et al.*, 1989; ESEN *et al.*, 2007; KINTZIOS, 2002; NAPOLI *et al.*, 2009; OZKAN *et al.*, 2010; RUSSO *et al.*, 1998; TUCKER and MACIARELLO, 1994).

Origanum vulgare L. ssp. *hirtum* is widely distributed in the Mediterranean Basin and is termed "Greek oregano" while *Origanum onites* L. is commonly known as "Turkish oregano" and also other subspecies of *Origanum vulgare* L. such as *gracile, viride, virens, glandulosum* are represented. The volatile compounds of ground oregano have been shown to vary significantly and the composition of these volatile substances has not been clearly correlated with individual genotypes. Indeed, the presence of some herbs of different genus in ground oregano cannot be easily identified or confirmed by evaluation of the volatile fraction conventionally considered.

The detection of *Cistus incanus* L., *Rubus caesius* L. and *Rhus coriaria* L. has been indicated by MARIESCHI *et al.* (2010) by SCAR markers and by RAPD-PCR techniques, while by RAPD-PCR MARIESCHI *et al.* (2009) previously proposed the identification of *Satureja montana* L. and *Origanum majorana* L. These earlier publications suggested the identification of the aforementioned dried plants in Mediterranean oregano, but there is actually no evidence of SCAR markers useful to detect the adulteration of herbs with *Olea europaea* L. leaves. Moreover, as reported in a recent paper, the SCAR markers have been developed for only a few medicinal herbs (KIRAN *et al.*, 2010).

Morphological and botanical methodologies can often be conveniently adopted for quality control and we have previously reported sufficiently extended data, obtained by a stereo zoom microscope system, to identify ground leaves of *Olea europaea* L. in commercially prepared ground oregano samples (BONONI *et al.*, 2010).

Preliminary HPLC analyses also evidenced the possibility to differentiate between the alcoholic extract of *Olea europaea* L. leaves, where oleuropein represented the main phenolic component, and the alcoholic extracts of oregano. In fact, the phenolic fraction is conveniently studied by High Performance Liquid Chromatography (HPLC) or Liquid Chromatography/Mass Spectrometry (LC/MS) (JAPÓN-LUJÁN *et al.*, 2006; LAGUERRE *et al.*, 2009; PAPOTI and TSIMIDOU, 2009). On the contrary, the characterization of the compounds having molecular weight higher than 280 Da by Gas Chromatography/Mass Spectrometry (GC/MS) in olive leaves has not been studied before. However, in this paper, the authors describe an easy preliminary GC/MS test for the rapid identification of two markers probably derived from the phenolic fraction of *Olea europaea* L. leaves and sufficiently identified by their mass spectra.

An evaluation of phenolic compounds in virgin olive oil by GC/MS was realized by ANGEROSA *et al.* (1995) but these Authors evidence the difficulty of the clear identification of the most of phenolic compounds with high molecular weights. The aim of this paper is not the characterization of the phenolic fraction by GC/MS, but is to demonstrate that *Olea europaea* L. leaves, when added to oregano, produce clear modifications of the GC/MS trace of the alcoholic extract of the mixture: in fact, the presence of two characteristic markers can unequivocally put in evidence the presence of olive leaves used as bulking agents.

In the near future we will publish two methods characterized by generalized applicability and based on the identification of oleuropein by HPLC/DAD and LC/MS/MS-ESI(-) in dried herbs containing *Olea europaea* L. leaves.

MATERIALS AND METHODS

Chemicals and reagents

Ethyl alcohol (99%) was purchased from Sigma-Aldrich (Milan, Italy). Filters GH Polypro (Hydrophilic Polypropylene Membrane Filter) 0.45 μm Pall were purchased from VWR International (Milan, Italy).

Plant material

Dried samples of different species of oregano were prepared from fresh plant material by drying at 40°C for ten days. Other dried samples of Mediterranean oregano were purchased from international wholesale traders. The samples were ground to a particle size diameter of 1.0-5.0 mm as usually found in the retail market. Various samples of ground and dried oregano were also purchased from retail markets.

Dried leaves of *Olea europaea* L. of different origin, treated as previously described or collected in various regions of Italy and dried in not controlled conditions, were studied. These

leaves were grounded to similar dimensions of oregano.

Extract production

All dried samples were treated separately as follows: ~1 g of each herb, ground as previously described, was mixed with 5 mL of ethyl alcohol at 20-25°C. After magnetic stirring for 10 h, the extract was centrifuged at 500 rpm for 3 min and the supernatant was filtered through a GH Polypro 0.45 μm filter. An aliquot (1 μL) of the extract was injected for the GC/MS analysis.

GC/MS analysis

GC/MS analyses were carried out with a Shimadzu 2010 gas chromatograph coupled to a Shimadzu 2010 MSD quadrupole mass spectrometer (Shimadzu, Italy). Helium was used as the carrier gas at a flow rate of 1 mL/min. Suitable identification of the analytes was achieved using an Equity™-5, a poly(5% diphenyl - 95% dimethylsiloxane) stationary phase column, 30 m × 0.25 mm i.d., 0.25 μm film thickness (Supelco, Milan, Italy). The oven temperature program was initially 60°C (held for 1 min.) then increased to 240°C at a rate of 3°C/min (held for 5 min). The injector temperature was 230°C and the split injection mode (1:5) was used. The ion source and the transfer line temperatures were 200° and 250°C, respectively. The MS instrument was operated in positive electron ionization mode (EI+) with automatic gain control, with 70 eV of electron energy and 250 mA of emission current. The mass spectrometer was operated in the full scan mode from 40 to 300 m/z.

RESULTS AND DISCUSSION

All the *Olea europaea* L. extracts produced GC/MS chromatograms that were strictly similar from the qualitative point of view over the time range 35.0-50.0 min (Fig. 1). The two peaks A and B were characterized by MS spectra (Fig. 2) and were considered as markers of the *Olea europaea* L. leaves, because the traces produced by GC/MS from all extracts of oregano examined until today did not show similar trends and did not contain these two peaks. The two markers

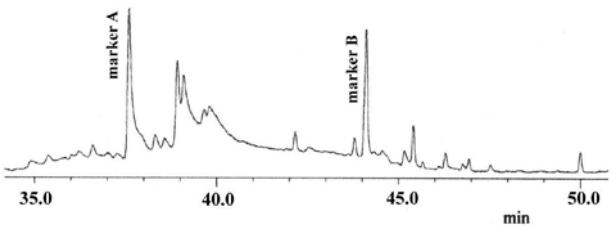


Fig. 1 - Typical GC/MS trace over the time range 35.0-50.0 min of an alcoholic extract of *Olea europaea* L. leaves. Two peaks indicated with markers A and B are used to identify the presence of olive leaves in oregano.

A and B eluted at reproducible retention times showing very characteristic mass spectra with the heaviest detectable m/z values 263 and 266, respectively. The mass spectra produced in the full scan mode from 100 to 400 m/z didn't show heaviest fragments.

The two markers are probably generated from the biophenol fraction, well known to be present in olive leaves, as reported by various Authors which studied the biophenol fraction by HPLC and LC/MS (JAPÓN-LUJÁN *et al.*, 2006; LAGUERRE *et al.*, 2009; PAPOTI *et al.*, 2009).

The identification of biophenols with high molecular weights, as mentioned in the Introduction section, is difficult since the fragmentation of complex phenolic compounds has been not yet studied by GC/MS and consequently lacks in the literature. In fact, the resulting GC/MS spectra derive from various fragmentations of complex phenolic compounds present as glycosides and a lot of masses could be justified by the existence of the fragments that can be formed through Mc Lafferty rearrangements.

Fig. 3 compares the extracts of three samples of oregano purchased from the retail market with one extract of *Olea europaea* L. leaves and clearly shows the absence of the two marker peaks in the three chromatograms of samples of oregano (traces 2, 3 and 4).

In the GC/MS traces corresponding to three extracts of different samples of *Olea europaea* L. leaves (Fig. 4), the quantitative responses for the two marker peaks varied in intensity, but were strictly similar and reproducible from the qualitative point of view. The samples prepared for these studies were derived from olive leaves from Lake Garda region and treated as described in the "Plant material" section. Other samples derived from the regions of Italy Apulia, Tuscany and Liguria were collected in August and December and dried under different drying conditions. In some cases the drying treatment was spontaneous, as declared by suppliers, in other cases the drying treatment was realized in dryer for herbs at not strictly controlled conditions.

Mixtures of both olive leaves and oregano showed evidence of the presence of olive leaf markers A and B: a sample of oregano containing 12% olive leaves (Fig. 5, "Mix 12") proved the usefulness of this test to identify adulterated preparations of the herb.

The two markers of olive leaves are adequately characteristic to permit the selection of two ions, one for each marker, sufficient by a multiple ion detection MID-GC/MS mode for the identification of olive leaves addition in oregano. The ions selected, previously termed "selected ion marker" in Fig. 2, have m/z values of 233 (from peak marker A) and 192 (from peak marker B). These ions were never present in GC/MS traces obtained with the described method in samples of only oregano leaves, but both were present in extracts of olive leaves. Research previously

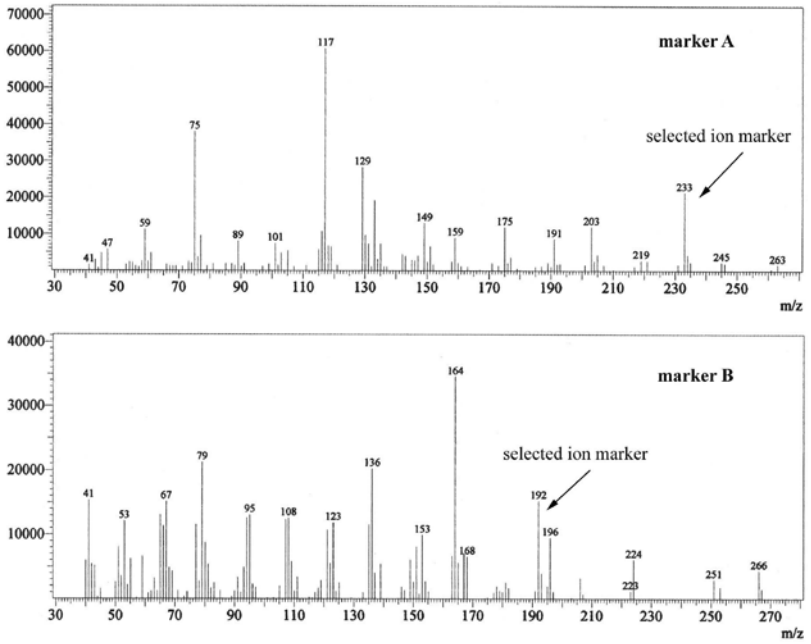


Fig. 2 - GC/MS spectra of two markers A and B evidenced in the olive leaves alcoholic extract.

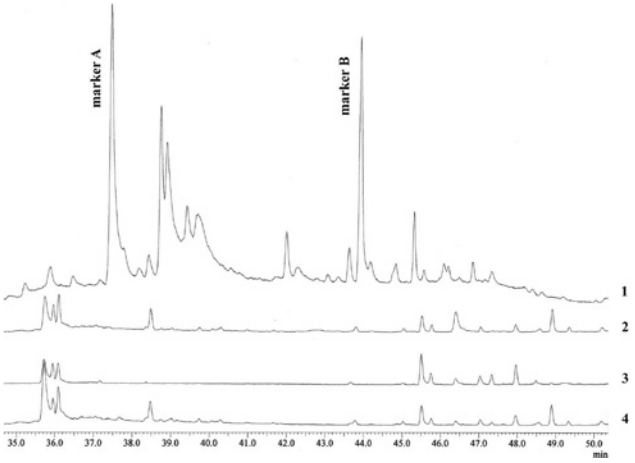


Fig. 3 - Comparison of GC/MS traces of an olive leaves extract (1) with three oregano extract traces (2, 3 and 4) produced from "pure" samples derived from retail market.

mentioned demonstrated the large presence of phenolic compounds and of main glycosides in olive leaves as oleuropein, ligstroside, verbascoside, demethyloleuropein and others similar molecules identified in alcoholic extracts (JAPÓN-LUJÁN *et al.*, 2006 ; LAGUERRE *et al.*, 2009). Therefore, considering the ion masses of the compounds extracted in the proposed test with ethanol and their elution in the range 35.0-50.0 min, it is probably possible to assign generically to biophenols derivatives the structure of the markers A and B.

During the quality control conducted in 2009 on commercial batches of both ground and dried oregano, we identified various samples shipped mainly from foreign producers to the EU as being adulterated with olive leaves at different percentages, as evaluated by manual microscopic

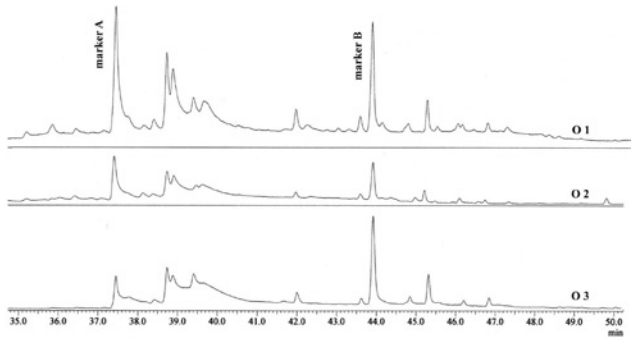


Fig. 4 - GC/MS traces of three extracts derived from different sources of ground olive leaves collected from the trees from various Italian regions, in different seasons and obtained with different drying treatments (O1, O2, O3).

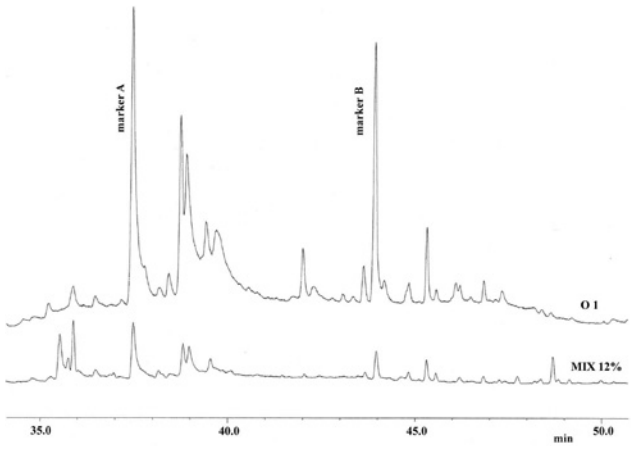


Fig. 5 - GC/MS traces confirming the presence of olive leaves in a mixture of oregano containing 12% (w/w) added olive leaves (mix 12%). Chromatogram O1 is from an extract of olive leaves alone.

screening at levels of 10-20% (w/w). The method we present here can detect adulteration of oregano samples by olive leaves without microscopic screening.

CONCLUSIONS

The GC/MS test described in this paper permits the confirmation and the documentation of the irregularities in commercial batches of ground oregano due to the presence of ground olive leaves. The detection limit of the test, confirmed by analysis of different mixtures made with olive leaves of many origins and drying levels, was as low as 1% and has also been applied to other studied cases to detect the adulteration of other herbs (example "*Salvia officinalis* L.") with the same extraneous matter considered in this paper. In particular, until today the use of *Olea europaea* L. leaves as adulterant has been detected only in oregano and sage.

The commercial significance of the test results is obviously higher in all cases where the adulteration reached levels of 5% or more, and the experience of the analyst often permits the production of a minimum quantitative evaluation of the adulteration level. A more specific study on the identity of biophenols by liquid chromatography is possible, but for a more rapid and routinary application for quality control purposes, the nonspecific identification of markers by GC/MS technique appears to be easy to conduct. As reported before, the reliability and reproducibility of the results from the proposed test has been supported by other contemporaneous experiences conducted by the same Authors. Using HPLC/DAD and LC/MS/MS-ESI(-) techniques it is possible to identify the oleuropein, a coumarin-like compound abundant in Oleaceas, which is a very specific molecule deriving from olive leaves.

ACKNOWLEDGEMENTS

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First Announcement

10th International Symposium on Supercritical Fluids
San Francisco, CA, USA
13-16 May 2012

A continuation of the 20+ year symposia series – the 10th International Symposium on Supercritical Fluids (ISSF 2012) will be held at the Embarcadero Hotel on the Bay in San Francisco, California, USA, May 13-16, 2012. A full traditional 3-day format will feature plenary lectures, invited lectures, multiple poster sessions, a book of abstracts with proceedings on a CD disk, gala dinner, and much more.

Each day of the symposium will begin with a topical plenary lecture followed by parallel sessions and all- day poster sessions to expose participants to the current trends and applications of SCF technology.

Meeting participants will have the opportunity to interface with companies engaged in high pressure and critical fluid technology, instrumentation vendors, and journal and book publishers that will exhibit their latest offerings during the symposium.

Some Selected Topical Areas: Material Science and Nanotechnology, Biomass Conversion, Food Processing and Nutraceuticals, Thermodynamics and Fluid Phase Equilibria, Polymer Processing, Chromatography, Reaction Chemistry, CO_2 Sequestration, Ionic Liquids, Simulations and Process Economics, Integrated Unit Operations

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GUIDE FOR AUTHORS

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Acknowledgments. Acknowledgments of assistance are appropriate provided they are not related to analyses or other services performed for a fee. Financial support, thanks for assistance, artic1e number or thesis fulfilment may be included.

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