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PAPER

CHANGES IN QUALITY PARAMETERS AND VOLATILE AROMA COMPOUNDS IN 'FAIRTIME' PEACH DURING FRUIT DEVELOPMENT AND RIPENING

EVOLUZIONE DEI PARAMETRI QUALITATIVI E DELLE COMPONENTI VOLATILI NEI FRUTTI DI PESCO DELLA CULTIVAR 'FAIRTIME' DURANTE L'ACCRESCIMENTO E LA MATURAZIONE

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ABSTRACT

Variation in the quality characteristics and flavor compounds of 'Fairtime' peach (*Prunus persica* L. Batsch) fruit were evaluated during development and maturation. Quality parameters were monitored on six peaches collected weekly or bi-weekly from each of ten trees starting at the end of pit hardening (12 July) until commercial harvest (13 and 18 September). In order to evaluate the quality of tree ripened

RIASSUNTO

Scopo del presente lavoro è lo studio sulla variazione dei caratteri qualitativi e dei componenti volatili di aroma delle pesche (*Prunus persica* L. Batsch) della cultivar 'Fairtime'. I parametri qualitativi sono stati determinati su gruppi di sei frutti raccolti con frequenza settimanale o bisettimanale da dieci alberi iniziando da frutti immaturi (12 Luglio) fino alla raccolta commerciale (13 e 18 Settembre). Per valutare la qualità dei

- Key words: 'Fairtime', GC/MS, peach flavor profile, soluble solids, SPME, tree ripe -

fruit, sampling was repeated 3 days after commercial harvest (September 21). The evolution of volatile flavor compounds in the fruit was determined by Solid Phase Micro-Extraction technique in Head Space followed by Gas Chromatography/Mass Spectrometry (HS-SPME-GC/MS). The aromatic composition is enriched in tree-ripened fruit, especially ester compounds, determining the typical flavor of mature peach fruit. The aromatic profile of 'Fairtime' peach fruit was similar to that of 'Guglielmina' fruit, another late-ripening, yellow-flesh peach cultivar.

frutti maturati sulla pianta (tree ripe) sono stati studiati anche campioni raccolti tre giorni dopo la raccolta commerciale (21 Settembre). L'evoluzione della componente volatile del frutto è stata determinata tramite la SPME in spazio di testa, abbinata alla Gas Cromatografia/Spettrometria di Massa (HS-SPME-GC/MS). I dati dimostrano che la composizione aromatica si arricchisce nei frutti tree ripe, specialmente la componente esterea, determinando l'aroma tipico della pesca matura. Questo metodo analitico è stato utilizzato per confrontare il profilo aromatico di questa cultivar con quello della 'Guglielmina', evidenziando forti analogie.

INTRODUCTION

Volatile compounds can be regarded as one essential component of fruit quality. In fact, aroma makes an important contribution to the flavor and eating attractiveness of fruit. It is determined by many, often over 200, volatile substances belonging to different chemical classes, i.e. esters, alcohols, terpenes, eters aldehydes, etc., present in very small concentrations (NURSTEIN, 1970). Aroma of peach (*Prunus persica* L. Batsch) fruit is not attributable to one or several compounds, but is considered to be the integrated response of the olfactory organ to a series of contributing flavor compounds (SEVENANTS and JENNINGS, 1966). Their influence on the aroma is not strictly proportional to their absolute quantity or volatility, but also depends on the sensorial impact and their interaction with other compounds (ARENA *et al.*, 2001; BELITZ and GROSCH, 1999; BUTTERY *et al.*, 1988, 1971; BUTTERY, 1981; POLESELLO, 1980; PYYSALO *et al.*, 1977; SALO, 1970).

In several investigations on the volatile fraction of peach flavor, approximately 70 compounds belonging to lactones (SEVENANTS and JENNINGS, 1966; CHAPMAN *et al.*, 1991; ENGEL *et al.*, 1988 a,b), esters and alcohols (VISAI and VANOLI, 1997) have been identified. Lactones represent the characteristic impact compounds, whereas C_6 aldehydes, alcohols and terpenes are responsible for the spicy, floral and fruity components of the peach aroma (SPENCER *et al.*, 1978; ENGEL *et al.*, 1988 a). Minor constituents include xylene, naphthalene, benzyl alcohol, α-terpineol, benzothiazole, 2-pentylfuran, (E, E)-2,4-decadienal, methyleugenol, dihydroionone, ionone, and α-bergamotene (HORVAT *et al.*, 1990 a,b).

The development of aroma in peach fruit is a dynamic process. Volatile substances are continuously synthesized and accumulated during fruit growth and maturation. Hence, the pattern of qualitative and quantitative volatile constituents varies greatly during fruit development. C_6 aldehydes and alcohols are the major components isolated from "green fruit" (ENGEL *et al.*, 1988 b) and

generally show a decreasing trend during fruit growth (VISAI and VANOLI, 1997) whereas the concentration of lactones, and specifically γ-decalactone, δ-decalactone, benzaldheyde and linalool, increases reaching the highest amounts in mature fruit (CHAPMAN *et al.*, 1991).

Generally, the concentrations of most of the impact compounds increase as the fruit matures, therefore peach fruit should be harvested when completely mature in order to have the best quality in terms of typical flavor (VISAI *et al.*, 1993), color, soluble solids (SS) and titratable acidity (ECCHER ZERBINI *et al.*, 1990). Peach fruit quality at consumption depends strictly on the degree of maturation at harvest because all the major quality parameters, such as SS, organic acids, and pectins, change during fruit development, but the changes are more rapid in proximity to fruit maturation, at which time optimal values are reached (ECCHER ZERBINI *et al.*, 2002). Many studies have shown significant improvements in quality when harvest is delayed to some extent (FORLANI *et al.*, 2001; ECCHER ZERBINI *et al.*, 1991). In particular, tree-ripened fruit of different cultivars has a higher (3 to 6 fold) lactone content than fruit harvested according to commercial specifications (VENTURA, 1992). Delayed harvesting also increases the extent and intensity of the red color in the fruit peel (ECCHER ZERBINI *et al.*, 1992), which is particularly appreciated by the consumer (IVASCU *et al.*, 2002). Weight and sugar content also increase during the final stages of fruit development (LO BIANCO *et al*,. 1999; PAVEL and DEJONG 1993). Hence, harvest timing becomes a determining factor for final fruit quality and consumer appreciation. In addition to the full expression of the potential fruit quality, peach marketing and consumer appreciation also depend on the possibility of maintaining such characteristics at a sufficient level until consumed.

The composition of the volatile fraction in peach fruit has been investigated extensively, by different methods. Several studies have shown qualitative and quantitative differences in the constituents found due to the different techniques used. Many lactones have been identified by Vacuum Steam Distillation in peach (CHAPMAN *et al.,* 1991; ENGEL *et al.,* 1988a) and nectarine (TEKEOKA *et al.,* 1988), whereas esters and alcohols have been recognized in nectarine and peach by Headspace Sampling (VISAI and VANOLI, 1997). Differences in the composition of the fruit peel and flesh have also been observed as well as the enzymatic formation of secondary volatiles caused by rupturing fruit tissues (TEKEOKA *et al.,* 1988).

A rapid and reproducible analytical method that uses less sample tissue (AGOZZINO *et al.*, 2005) and exploits the Head Space Solid Phase Micro-Extraction (HS-SPME) followed by Gas-Chromatography/Mass-Spectrometry (GC/MS) analysis (VAS and VÉCKEY, 2004; ARTUUR *et al.*, 1992) was used in this study.

Variations in the volatile fraction during fruit maturation have been already investigated, whereas knowledge about the evolution of aroma components in tree-ripened fruit is limited. The aim of this research was to study the changes in volatile compounds in immature, commercial harvest (CH) and tree-ripened (TR) fruit of the late-ripening peach cultivar 'Fairtime'. Flavor components were also related to the physical (weight, firmness, color) and chemical (soluble solids, titratable acidity) properties of the same peach fruit.

MATERIALS AND METHODS

The study was conducted in a commercial orchard located near Riesi, in south-central Sicily (37°17'N, 14°04'E). The soil was a sandy loam (pH 7.3) with low active carbonates. In summer, 10

eight-year-old peach trees of the yellow-flesh late-ripening cultivar 'Fairtime' and five eight-year-old peach trees of the yellow-flesh late-ripening cultivar 'Guglielmina', both grown on GF677 (*Prunus persica* x *Prunus amigdalus*) rootstock, were selected. Trees were trained to a delayed vase (central leader was removed the third year after planting) and spaced at 4.5-5 m. Routine horticultural care (pruning, thinning, irrigation, fertilization and pest control) was applied to all trees throughout the season. Fruit were thinned approximately 30 days after full bloom by removing doubles and leaving about one fruit every 15 cm of shoot, and summer pruned once at the beginning of July.

Six peaches were collected weekly or bi-weekly from each tree starting on July 12 (after pit hardening) until September 13 and 18 (commercial harvest). In order to evaluate the quality of tree-ripened fruit, sampling was repeated 3 days after commercial harvest (September 21). From July 12 until September 13, the same number of fruit were collected from 'Fairtime' and 'Guglielmina' peach trees in order to compare the volatile fraction of the two cultivars. Sample fruit were placed in paper bags and transported to the laboratory where the weight, diameter, flesh firmness, SS, titratable acidity, percentage of red colored peel and volatile compounds were determined. Weight, diameter, red colored peel, and firmness were determined on each fruit, while SS and titratable acidity were determined on groups of six fruit (one per plant and per date of sampling). Volatile compound analysis was carried out separately on flesh and peel of two groups of five fruit each (one per plant and per date of sampling). Flesh firmness was measured with a manual pressure tester (TR di Turoni & Co., Forlì, Italy) by mounting an 8 μm tip on two opposite sides of the peeled fruit, SS was determined using an Atago Palette PR-32 digital refractometer (Atago Co., Ltd., Tokyo, Japan), and the titratable acidity was determined by using a Crison S compact titrator (Crison Instruments, SA, Alella, Barcelona, Spain). Percentage of red colored peel was determined by visual scale only on commercial harvest and tree-ripened fruits.

The analysis of volatile compounds was carried out by homogenizing 2 g of fruit flesh or peel and placing 50 mg into 2 mL vials with a pierceable silicone rubber septa coated with polytetrafluoroethylene (PTFE) film. The vials were stirred in a water bath for 15 min at controlled temperature (27±0.5°C) in order to reach equilibrium. Sampling was carried out by using a SPME (Supelco, Bellafonte, PA) fiber, coated with a 100 μm film of polydimethylsyloxane (PDMS), in head space. An 85 μm Carboxen/PDMS fiber was also tested but a less complete aromatic profile was obtained. On the basis of the preliminary tests, a 15 min exposure time was suitable for fiber saturation and for the reproducibility of the extraction procedure (Fig. 1).

Desorption time in the chromatograph injector at 250°C was fixed at 5 min in splitless mode. Separation was performed with a GC-MS Varian Saturn-3 Ion Trap System, equipped with a 30 m x 0.25 mm i.d. fused-silica capillary column Supelcowax-10 (Supelco, Bellafonte, PA) coated with 0.25-μm polyethylene glycol film. The carrier gas (helium) pressure was fixed at 12 psi on the column head. The transfer line and ion trap temperatures were 180°C. The GC was programmed for a starting temperature of 35°C (5-min hold), a first ramp of 5°C/min to 130°C (10-min hold) and a second ramp of 10°C/min to 220°C (5-min hold). In the ion trap mass spectrometer, the electron ionization mode (EI) was set at 70 eV, the mass range at 40-400 Thompson and the frequency at 3 scans per second.

The data were processed with the instrument data system and the chromatographic and spectrometric results

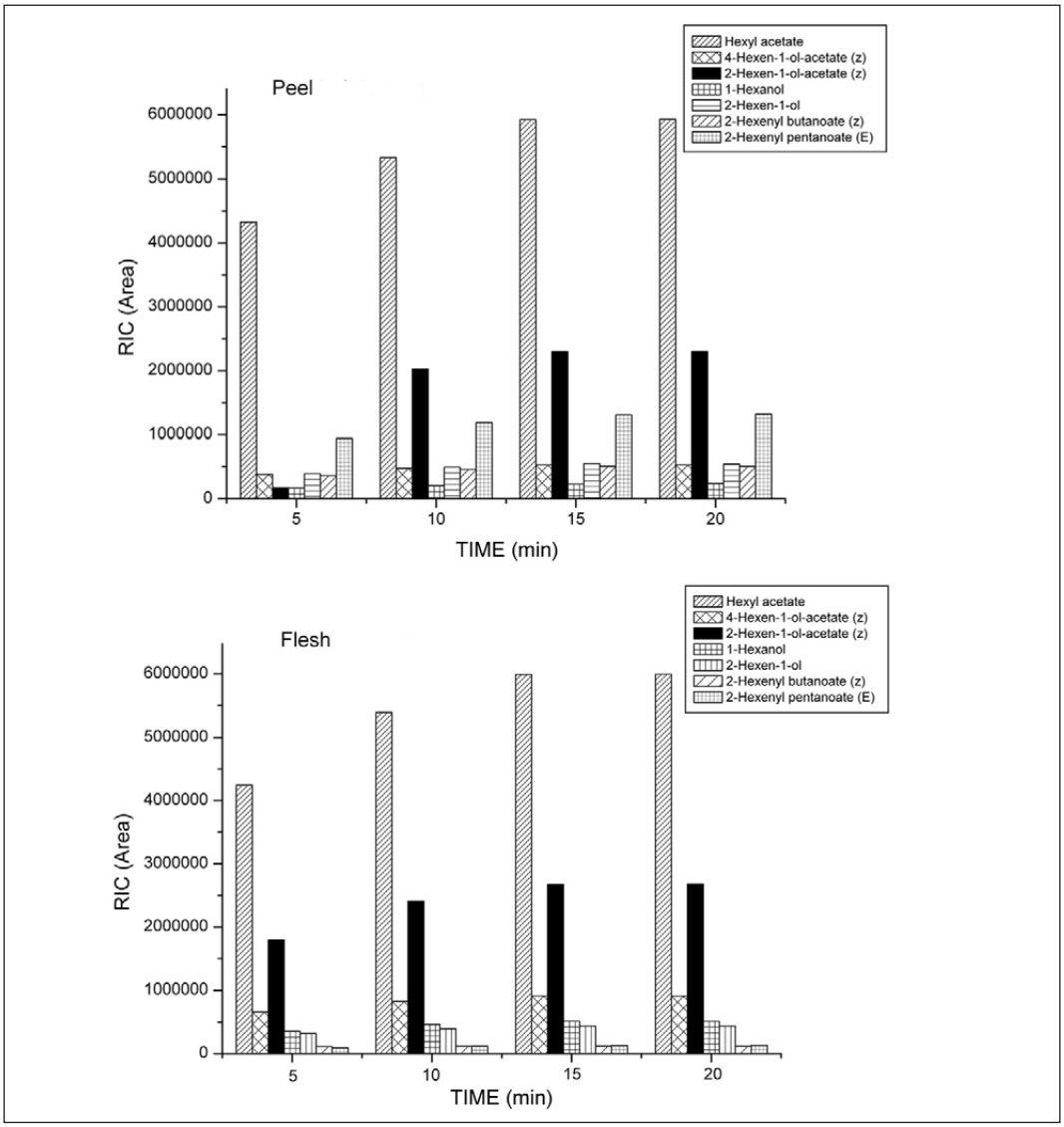


Fig. 1 - Reconstructed Ion Current (RIC) of volatile compounds from peel and flesh of 'Fairtime' peach fruit at different exposure times (5, 10, 15 and 20 min).

showed excellent reproducibility (SD ≤ 4%). Each determination was repeated three times.

The compounds were identified by linear retention indexes (RI) calculated using the Van Den Dool and Kratz's equation (VAN DEN DOOL and KRATZ, 1963) and by mass spectra through a critical and reasoned comparison within NIST 2002 library of mass spectral data; the presence of 1-hexanol, 2-hexen-1-ol, limonene and hexyl acetate was also verified using standard compounds (Fluka, Buchs, Switzerland).

The means (and standard errors for fruit quality parameters) of the measure-

ments taken on each fruit and for each date are reported. The trends of the volatile compounds during fruit development and maturation were determined by non-linear regression analysis using SigmaPlot (Systat Software, Inc., Richmond, California, USA); the *P* values are reported in the text.

RESULTS AND DISCUSSION

All of the quality parameters, with the exception of pH, showed substantial differences between immature and commercial harvested fruit (Table 1). In addition, soluble solids increased in tree-ripened fruit compared to commercially harvested fruit, whereas weight, pH, acidity, and red peel color remained fairly constant. During the same time, flesh firmness decreased but remained within the range acceptable for manipulation and commercial needs.

The SPME technique was used mainly for qualitative or semi-quantitative (screening) studies. HS-SPME involves multi-phase equilibrium processes and careful consideration must be given to the physicochemical properties of the compounds to be extracted.

Qualitative optimization of the SPME parameters should be applied to determine the best fiber, sampling conditions and instrument calibration. Gas chromatograms of the fresh fruit, peel and flesh, are shown in Fig. 2.

Reproducibility and precision can be improved with SPME fiber by carefully controlling and monitoring the time and temperature (which should be very constant) during sample extraction. The extraction time is a critical parameter in the SPME sampling process, but once the extraction equilibrium is reached, no change in time results in adsorbed analytes. By standardizing all these parameters, it is possible to use RIC% as a quantitative approach. While this semi-quantitative method has severe limitations, as is evident in the peak areas that do not reflect the real amounts of the different compounds, it is a very useful tool for making comparisons, especially for simple non-complex matrices such as gases or HS.

The percentage of reconstructed total ion current (RIC) and the RI of each compound obtained by HS-SPME/GC-MS are reported in Tables 2 and 3, which show the quantitative composition and evolution of aroma according to the stage of fruit development. The results show different qualitative and quantitative patterns of the aroma compounds in the commercially harvested fruit compared

Table 1 - Quality parameters (mean ± SE) of 'Fairtime' peach fruit collected when immature (IM), at commercial harvest (CH), and tree-ripened (TR).

	Date	Weight (g)	Flesh firmness (kg/cm^2)	Soluble solids (°Brix)	pH	Acidity (g/L)	Peel red color (%)
IM	07/12	56.7±1.6	10.8±0.77	10.9±0.15	3.73±0.07	0.48±0.02	-
	07/26	72.6±1.8	10.1±0.24	10.0±0.62	3.73±0.02	0.46±0.01	-
	08/03	82.2±1.0	8.54±0.15	11.2±0.07	4.04±0.06	0.96±0.01	15.5±1.27
	08/20	82.2±1.0	7.0±0.13	12.0±0.10	3.49±0.19	0.47±0.01	38.5±2.14
	08/30	164.9±3.6	6.1±0.18	12.6±0.17	3.77±0.03	1.00±0.01	38.5±2.14
CH	09/13	221.0±5.3	5.8±0.16	13.0±0.11	3.58±0.03	1.02±0.01	46.1±1.70
	09/18	251.6±2.6	5.4±0.07	12.7±0.06	3.65±0.06	1.09±0.01	50.0±2.67
TR	09/21	249.1±5.4	5.2±0.12	13.0±0.10	3.70±0.02	1.08±0.01	50.3±1.45

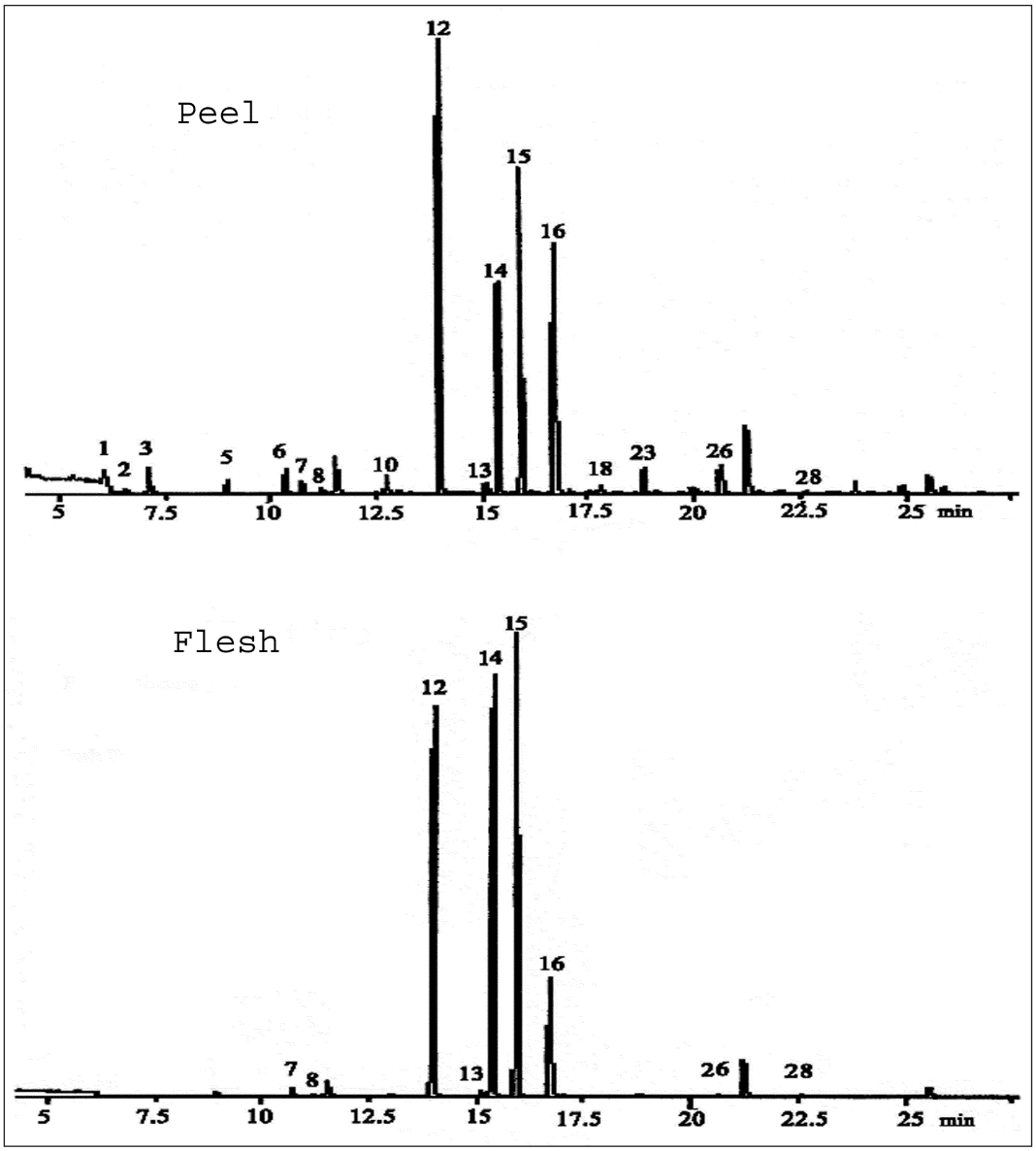


Fig. 2 - Gas chromatograms of volatile compounds in the peel and flesh of 'Fairtime'. (see Table 1 and 2 for compounds corresponding to the numbers).

to immature fruit, both for peel and flesh. Furthermore, the content of volatile compounds was higher in the peel (Table 2) than in the flesh (Table 3).

In fruit peel, compounds 12 (33.94%) and 15 (30.81%) were the most abundant regardless of developmental stage, while compound 14 was relatively low (5 to 9%) in immature fruit and increased exponentially (over 20%; P = 0.0001) in mature fruit (Table 2). Compound 26 (initially at 23%) decreased linearly (P =

Table 2 - Retention Index (RI) and Reconstructed Ion Current (RIC)% of volatile compounds from peel of 'Fairtime' peach fruits collected from July 12 (immature, IM) to September 21 (tree-ripened, TR). Samples analysed by SPME/GC-MS.

			IM					CH		TR
			7/12	7/26	8/03	8/20	8/30	9/13	9/18	9/21
N.	RI	FAIRTIME PEEL COMPOUNDS	Reconstructed Ion Current (RIC)%							
1	1039	Ethyl butanoate		1.37	0.57	0.60	0.67	0.88	1.10	
2	1056	2-Methyl-ethyl butanoate				0.66	0.68	0.30	0.37	
3	1071	3-Methyl-ethyl butanoate		0.57	0.30	1.19	1.08	0.98	1.74	1.43
4	1078	Hexanal			0.50					
5	1108	1-Butanol, 3-methyl acetate						0.43	0.79	0.37
6	1144	Ethyl 2-butenoate				0.54	0.61	0.25	0.54	
7	1165	Pentyl acetate						0.29	0.50	0.25
8	1173	Limonene						0.58	0.52	
9	1202	2-Hexenal (E)								
10	1234	Ethyl Hexanoate				1.17	1.72	1.23	1.46	0.44
11	1245	β-cis-Ocimene	0.55							
12	1282	Hexyl acetate	33.94	51.51	48.17	51.57	52.58	49.79	48.89	47.60
13	1319	3-Hexen-1-ol-acetate (z)	0.39	1.87	0.44	0.42	0.44	0.36	0.60	0.56
14	1329	4-Hexen-1-ol-acetate (z)	4.94	8.80	5.07	5.31	6.60	15.20	20.56	20.29
15	1348	2-Hexen-1-ol-acetate (z)	30.81	17.09	20.19	17.63	14.69	22.88	16.61	24.32
16	1362	1-Hexanol	0.53	1.48	3.39	3.26	3.84	0.24	0.68	
17	1379	Heptyl acetate				0.13	0.08	0.22	0.33	0.22
18	1393	3-Hexen-1-ol					0.24	0.29	0.32	
19	1398	Cis-3 hexenyl piruvate	1.51	0.80	0.65	0.72	1.23	0.45	0.39	
20	1408	2-Hexen-1-ol	0.73	0.55	2.48	1.23	0.70	0.22	0.21	
21	1414	Hexyl butanoate		0.51	1.20	0.64	0.72	0.24	0.21	
22	1428	2 Methyl-hexyl butanoate		0.21	0.13	0.31	0.62	0.05	0.17	
23	1441	Ethyl octanoate		0.21	0.26	0.50	0.95	1.46	1.41	1.71
24	1452	Hexyl pentanoate	1.41	1.14	1.29	1.17	1.88	0.45	0.47	0.51
25	1483	2-Hexenyl butanoate (z)	2.18	0.79	3.15	1.21	1.45	0.78	0.50	0.96
26	1511	2-Hexenyl pentanoate (E)	23.00	12.71	11.53	10.54	7.87	1.80	1.14	1.34
27	1548	Ethyl nonanoate							0.33	
28	1611	Hexyl hexanoate			0.14	0.27	0.34	0.15		
29	1641	Ethyl decanoate							0.15	
30	1664	2-Hexenyl hexanoate		0.40	0.55	0.93	1.02	0.48		

0.0002) until 1.34% at TR. The six-carbon alcohols (1-hexanol, 2-hexen-1-ol and 3-hexen-1-ol) were present in small quantities during ripening and disappeared at TR. Compound 23 was not detected at the beginning and increased linearly (P = 0.0002) throughout the sampling period.

Many esters increased during ripening, some (2, 6, 10, 12) appeared later and others (7, 5) only appeared at complete ripening. Small amounts of terpenes were recovered (compound 11) in immature fruit and at complete ripening (compound 8). At TR, only esters were present in the volatile fraction of the fruit peel and the principal compounds were 12, 14 and 15, which accounted for 93% of the total.

Initially the most abundant compounds in the fresh fruit were 26 (39.65%), 12 (31,96%), 15 (16.04%) and 16 (3.10%) (Table 3), but compound 26 decreased exponentially (P < 0.0001) and disappeared

Table 3 - Retention Index (RI) and Reconstructed Ion Current (RIC)% of volatile compounds from flesh of 'Fairtime' peach fruits collected from July 12 (immature, IM), to September 21 (tree-ripened, TR). Samples analysed by SPME/GC-MS.

			IM					CH		TR
			7/12	7/26	8/03	8/20	8/30	9/13	9/18	9/21
N.	RI	FAIRTIME FLESH COMPOUNDS	Reconstructed Ion Current (RIC)%							
1	1039	Ethyl butanoate								
2	1056	2-Methyl-ethyl butanoate								
3	1071	3-Methyl-ethyl butanoate		0.57						
4	1078	Hexanal			10.22	0.18				
5	1108	1-Butanol, 3-methyl acetate						0.42	0.52	0.42
6	1144	Ethyl 2-butenoate								
7	1165	Pentyl acetate						0.30	0.54	0.31
8	1173	Limonene		0.29				0.14	0.27	
9	1202	2-Hexenal (E)			11.08	0.32	0.25			
10	1234	Ethyl Hexanoate								0.36
11	1245	β-cis-Ocimene	2.10							
12	1282	Hexyl acetate	31.96	55.95	42.53	57.44	52.58	39.77	30.31	28.07
13	1319	3-Hexen-1-ol-acetate (z)	0.82	1.28	0.74	0.93	0.50	0.35	0.39	0.93
14	1329	4-Hexen-1-ol-acetate (z)	2.55	11.67	10.60	9.62	16.36	23.66	35.74	29.39
15	1348	2-Hexen-1-ol-acetate (z)	16.04	15.33	13.83	24.66	27.00	33.16	30.47	40.25
16	1362	1-Hexanol	3.10	1.70	3.65	2.49	2.31	1.41	1.76	
17	1379	Heptyl acetate				0.20	0.17	0.13		
18	1393	3-Hexen-1-ol			0.16	0.14	0.11			
19	1398	Cis-3 hexenyl piruvate								
20	1408	2-Hexen-1-ol	2.51	1.76	2.77	2.31	0.76	1.33		
21	1414	Hexyl butanoate								
22	1428	2 Methyl-hexyl butanoate								
23	1441	Ethyl octanoate				0.07				
24	1452	Hexyl pentanoate	1.27	1.31	0.32	0.24	0.08			
25	1483	2-Hexenyl butanoate (z)			0.16	0.15				
26	1511	2-Hexenyl pentanoate (E)	39.65	10.14	3.95	1.24	0.16			
27	1548	Ethyl nonanoate								
28	1611	Hexyl hexanoate								
29	1641	Ethyl decanoate								
30	1664	2-Hexenyl hexanoate								

in September. The concentrations of 14 and 15 ranged from 13.83 to 40.25% and from 2.55 to 35.74%, respectively, both followed a quadratic increase (P = 0.0006 and P = 0.0002, respectively). The typical aldehydes, hexanal (10.22%) and 2-hexenal (11.08%) were detected starting in the third week and then disappeared again toward maturation. These aldehydes originate from unsaturated fatty acids and have been reported to be the main volatile compounds in unripe peach (ZHANG and JIA, 2005; DE SANTIS and MENCARELLI, 2001) and are responsible for the "green flavor" (VENTURA, 1992). They decreased until they disappeared at CH. Small but detectable quantities of 5 and 7 esters were present after September 13. Among the other esters, 24 decreased in a steep linear fashion (P = 0.046) during ripening. Alcohols like 16 and 20 were present during ripening but disappeared completely by TR.

At TR, the volatile fraction composition

was greatly simplified; in fact, only esters (mainly 12, 14 and 15) were found; this followed the same trend observed in the fruit peel. Only a few esters increased in the flesh of tree-ripened fruit, and these contribute to the typical flavor of mature peach fruit (VENTURA, 1992).

As expected, substantial changes were observed in several quality parameters during the transition from immature fruit to commercial harvest. On the other hand, only soluble solids increased in tree-ripened fruit as compared to commercial harvested fruit. The volatile compound content was usually higher in the fruit peel than in the flesh.

We also studied the evolution of aroma compounds in the cultivar Guglielmina during ripening. Qualitatively the aroma profile is similar to that of 'Fairtime'. No typical compound could be identified that would to differentiate between the two cultivars.

In contrast, quantitative differences for some compounds of the two cultivars were observed, but the differences may have been due to various environmental factors as well as to the genetic make up.

CONCLUSION

The HS-SPME-GC/MS method may be a suitable tool for the qualitative and semi-quantitative determination of volatile aroma compounds in peach. It is able to extract, isolate, detect and identify a series of volatile compounds. The results obtained in this first screening provide information on substantial quantitative changes in volatile constituents during peach fruit ripening. Application and further improvement of this method for the analysis of the volatile fraction of fruit may allow for additional quantitative investigations.

Although improvements of peach fruit quality parameters observed in this study from the time of commercial harvest to tree-ripened seem to be minor, some relevant differences in the volatile fraction (particularly of the fruit peel) may contribute to significant flavor improvements for consumer appreciation of tree-ripened fruit which would justify a three-day harvest delay. 'Fairtime' and 'Guglielmina' are two late-ripening peach cultivars with similar pomological characteristics, that have very similar aroma profiles, at least in the early stages of fruit maturation. Information obtained by using this analytical approach could provide an understanding of the formation and evolution of flavor compounds and/or provide a method for distinguishing between similar commercial fruit varieties.

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PAPER

OLIVE OIL-FRIED SARDINE CONSUMPTION INCREASES FAT, CHOLESTEROL AND BILE ACID EXCRETIONS AND ACCELERATES THE NORMALIZATION OF THE LIPOPROTEIN PROFILE AND PEROXIDATION IN HYPERCHOLESTEROLAEMIC RATS

IL CONSUMO DI SARDINE FRITTE IN OLIO D'OLIVA INCREMENTA L'ESCREZIONE DI GRASSI, COLESTEROLO E ACIDO BILIARI ED ACCELERA LA NORMALIZZAZIONE DEL PROFILO LIPOPROTEICO E LA PEROSSIDAZIONE IN RATTI AFFETTI DA IPERCOLESTEROLEMIA

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ABSTRACT

The effects of omega-3 fatty acids and oily fish on *in vivo* plasma lipids and oxidant status are not well defined and understood. The effect of olive oil-fried sardine consumption on serum and lipoprotein levels and thiobarbi-

RIASSUNTO

Gli effetti degli acidi grassi omega-3 e dell'olio pesce, *in vivo*, sui lipidi plasmatici e sul loro stato ossidativo non sono né ben definiti né completamente compresi. È stato testato l'effetto del consumo di sardine fritte in olio d'oliva

- Key words: bile acids, faecal cholesterol, olive oil-frying, sardine, rats, serum and lipoprotein lipids, serum and lipoprotein peroxidation -

turic acid-reactive substances (TBARS) and on faecal fat, bile and cholesterol excretions was tested in hypercholesterolemic rats. Cholesterol plus bovine bile was used as a serum cholesterol-raising agent in a casein plus olive oil (COC) diet for 3 weeks. Groups of seven rats were switched for 2 weeks to diets containing casein plus olive oil (CO) and olive oil-fried sardines (S). Seven rats received the CO diet throughout the whole study and served as the control (R). TBARS, cholesterol, triglycerides and phospholipids were determined in serum and high density lipoproteins (HDL) while fat, bile acid and cholesterol levels were measured in the faeces. Cholesterol withdrawal decreased serum cholesterol and TBARS in S and CO rats ($p<0.01$). The S rats had cholesterol and TBARS values similar to those of the R rats. The S diet significantly increased the daily fat, bile acid and cholesterol excretions (all, $p<0.05$), while the cholesterol and TBARS contents in serum, HDL and non-HDL (VLDL+LDL) (all, $p<0.05$) decreased with respect to the CO rats. The cholesterol/phospholipid ratio was lower in serum and HDL of the S rats than in the CO rats ($p<0.05$). These findings show that the inclusion in diets of olive oil-fried sardines as the only source of fat and protein in diets increases faecal cholesterol and bile acid excretions that normalise cholesterolaemia and serum lipid peroxidation.

sul siero, sui livelli di lipoproteine e sostanze reattive all'acido tiobarbiturico (TBARS) e sui grassi, acidi biliari e colesterolo escreti con le feci, in ratti affetti da ipercolesterolemia. Del colesterolo addizionato di bile bovina è stato utilizzato come agente per innalzare il colesterolo nel siero in una dieta a base di olio di oliva addizionato di caseina (COC), per la durata di tre settimane. Gruppi di sette ratti sono stati sottoposti alternativamente, per 2 settimane, a diete consistenti in caseina addizionata all'olio d'oliva (CO) e sardine fritte in olio di oliva (S). Un gruppo di sette ratti (R), che andava a costituire il gruppo di controllo, seguiva la dieta CO dall'inizio dello studio. Sono stati determinati nel siero le TBARS, il colesterolo, i trigliceridi ed i fosfolipidi e le lipoproteine ad alta densità (HDL). Il contenuto in grassi, acidi biliari e colesterolo è stato invece determinato nelle feci. L'eliminazione di colesterolo diminuisce il colesterolo serico e le TBARS nei ratti dei gruppi S e CO ($p<0,01$). I ratti del gruppo S presentavano valori di colesterolo totale e di TBARS simili ai ratti del gruppo R. La dieta S incrementava significativamente l'escrezione giornaliera di grassi, acidi biliari e colesterolo ($p<0,05$), mentre il contenuto di colesterolo e TBARS nel siero, di HDL e di non-HDL (VLDL+LDL) ($p<0,05$) diminuiva se confrontato con i ratti del gruppo CO. Il rapporto colesterolo/fosfolipidi risultava minore nel siero e nelle HDL per i ratti S rispetto ai ratti CO ($p<0,05$). Questi risultati dimostrano che includere nella dieta sardine fritte in olio d'oliva, come unica fonte di grassi e proteine, incrementa l'escrezione di colesterolo fecale e acido biliare, normalizzando la perossidazione dei lipidi del siero e la colesterolemia.

INTRODUCTION

Consumption of fish oil with a high omega-3 polyunsaturated fatty acid content (n-3 PUFA) has been associated with a reduced risk of cardiovascular disease (ASCHERIO *et al.*, 1995; HARPER and JACOBSON, 2001). Data from reviews by HARRIS (1996) and NESTEL (1998) indicate that the usefulness of fish oil in treating hypercholesterolaemia is doubtful except when the excess cholesterol is in the very low density lipoprotein (VLDL) fraction of the serum. It has been suggested that reduction of high density lipoprotein (HDL) levels after n-3 PUFA administration may be related to an increase in the reverse cholesterol transport (RCT) pathway (MONTOYA *et al.*, 2002). As known, SR-BI mediates the binding of HDL and the selective uptake of its cholesterol content by hepatocytes and its utilization for biliary secretion (RIGOTTI *et al.*, 1997). Cholesterol is eliminated from the body by converting it into cholic acid and other bile acids (HOFMANN, 1999). The bile acid production increases significantly in cholesterol-fed animals as a compensatory mechanism to decrease the hypercholesterolaemic effect of dietary cholesterol (MADANI *et al.*, 1998). On the other hand, it has been suggested that lipoprotein peroxidation plays a central role in the early stages of atherosclerosis (ESTERBAUER *et al.*, 1992), and hypercholesterolemia appears to be associated with an increase in serum lipid peroxidation (REILLY *et al.*, 1998). GARRIDO-POLONIO *et al.* (2004) found that HDL is an important carrier of thiobarbituric acid reactive substances (TBARS) in rats fed thermally oxidized sunflower-seed oil.

The effect that fried fish consumption has on the prevention and treatment of dietary hypercholesterolaemia has been studied by our group (SÁNCHEZ-MUNIZ *et al.*, 1992b; 1996; 2003), but to the best of our knowledge no data concerning the effects of fried-fish consumption on serum lipid peroxidation are available.

In the present study it was hypothesised that the consumption of olive oil-fried sardines increases the excretion of cholesterol and bile acids and decreases the serum HDL-cholesterol as a mechanism to decrease serum cholesterol and lipid peroxidation in hypercholesterolaemic animals. The aims of this study were to assess the effect of a diet containing olive oil-fried sardines, as the only source of fat and protein, on (i) fat, cholesterol and bile acid faecal excretions; (ii) serum lipids and HDL composition and level; and (iii) serum and HDL-TBARS with respect to other diets containing casein and olive oil.

MATERIALS AND METHODS

Frying performance and diet preparation

Sardines (600-700 g) were cut open in a fan-shaped fashion and fried in 3-L domestic fryers with olive oil for 4 min at 180°C. After frying, the sardines were freeze-dried and included in the diet as the only source of protein and fat. According to previous studies (SÁNCHEZ-MUNIZ *et al.*, 1992b; 1996, 2003) diets containing casein were supplemented with 0.2% DL-methionine; the sardine diet was not supplemented with this amino acid. The hypercholesterolemic diet contained cholesterol (20 g/kg diet) and bovine bile (5 g/kg diet, consisting of a mixture of free and conjugated bile acids, with a minimum of 50% bile acids).

Other basic dietary components were the following: crude fibre (microcrystalline cellulose) 50 g/kg, minerals mix 37.7 g/kg, vitamin supplements 1.2 g/kg, sucrose 250 g/kg, BHT 1 g/kg and BHA 1 g/kg. Wheat starch (roughly 620 g/kg in the casein and olive oil diet (CO) and in the diet containing sardines fried in olive oil (S)) was used to complete

the diet composition. The composition (mg/kg diet) of the mineral mix was KI, 0.21; Na_2SeO_3, 0.24; $Na_2CrO_4.4H_2O$, 1.58; NaF, 2.43; $CuSO_4.5H_2O$, 24.72; $ZnCO_3$, 25.50; $MnSO_4.H_2O$, 169.20; $FeSO_4.7H_2O$, 199.00; $MgCO_3$, 769.70; NaCl, 906.30; and $MgSO_4.7H_2O$, 2250; $Na_2H_2PO_4.2H_2O$, 2930; $KHCO_3$, 6100; $CaHPO_4.2H_2O$, 8590; KH_2PO_4, 8200; $CaCO_3$, 10000. The composition (mg/kg diet) of the vitamin mix was choline, 1111.11; folic acid, 1.11; niacin, 22.22; calcium pantothenate, 8.88; riboflavin, 3.33; thiamin, 4.44; vitamin B_6, 6.66; vitamin K, 0.055; vitamin E, 33.33, vitamin D_3, 0.028; vitamin A, 0.0015 (retinol acetate). The energy content of the diets was 6.9 MJ/kg for COC, 7.0 MJ/kg for SC, 7.2 MJ/kg for S, and 7.2 MJ/kg for CO.

Animal and dietary treatment

Male Wistar rats weighing 65 g were housed individually in metabolic cages (20°-23°C, 12 h light/dark cycle). The study protocol was approved by a Committee for Animal Studies of the Universidad Complutense de Madrid and performed in accordance with the Guide for the Care and Use of Laboratory Animals (NATIONAL RESEARCH COUNCIL, 1985). During a 3-day period adapting to the environmental conditions, rats were fed commercial rat pellets (Sander, Madrid, Spain). Following the adaptation period the rats were introduced to the hypercholesterolaemic diet containing casein + olive oil + cholesterol + bovine bile (COC). After three weeks, blood samples were collected from rats that had fasted for 16-18 h. The samples were taken from the tail vein of COC fed rats following vasodilatation in a water bath at 37°C. Following the analysis, the animals were divided into two groups of 7 rats each that had similar mean cholesterol levels and standard deviations. The rats were then switched to the experimental diets. During the experimental period, 7 rats were given the CO diet and the other 7 rats received the S diet. A reference group of seven rats (R) received the CO diet throughout the entire study. Food and water were provided *ad libitum* (Fig. 1). Food intake was checked daily and the rats were weighed on alternate days. At the end of the experiment, R, CO and S rats were anaesthetised by intraperitoneal injection with sodium pentobarbital (60

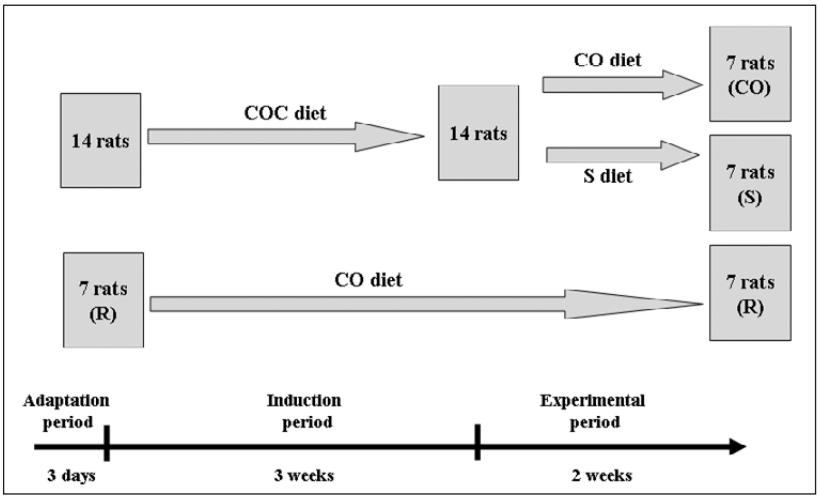


Fig. 1 - Experimental design. COC rats: Rats in which hypercholesterolaemia was induced by diet containing casein plus DL-methionine + olive oil + bovine bile + cholesterol; CO rats: Rats fed casein plus DL-methionine + olive oil diet; S rats: Rats fed a diet containing olive oil fried sardine as the only protein and fat sources; R rats: Rats fed the CO diet during both the induction and experimental periods and used as the reference group.

mg/kg body weight) and blood was extracted by carotid puncture.

Analytical determinations

Moisture was determined by oven drying at 105°C to constant weight (AOAC, 1993). Protein was determined by the KJELDAHL procedure (AOAC, 1993). Sardine, diet, and faecal fats were extracted by the BLIGH and DYER (1959) method. The fatty acid methyl esters of olive oil, fish fat, and dietary fat were analysed in a a Hewlett Packard 5890 Series II chromatograph (Palo Alto, CA) equipped with a 50 m (i.d. 0.22 mm) capillary column (BPX70, 0.25 μm film thickness (SGE, Austin TX). The content of different fatty acids in the diet was calculated on the basis of the percentage in the fat and the proportion of fat in the fish, using the conversion factors for fried sardines found previously (SÁNCHEZ-MUNIZ *et al.*, 1992a). Cholesterol in faeces was determined after solubilising the faecal fat extracts with isopropanol/water (95:5, by vol) (MONTES *et al.*, 1978). Serum was separated by low-speed centrifugation at 1,500 x *g* at 4°C for 30 min. VLDL and LDL were precipitated with dextran sulphate (Mw 50,000) and magnesium chloride (FINLEY *et al.*, 1978). Total cholesterol, HDL-cholesterol, triglycerides, total phospholipids and HDL-phospholipids were analysed according to standard enzymatic methods (Boehringer Mannheim, Germany). Faecal bile acids were extracted by the DE WAEL *et al.* (1977) method and determined by spectrofluorometry (CODOCEO *et al.*, 1980) using the Sterognost-3α^R Pho commercial kit (Nyegaard & Co. A/S, Norway). TBARS levels in serum and HDL were measured at 532 nm following the method of YAGI (1993). The U-Mann Whitney non-parametric test was used to compare data. The SPSS 11.0 statistical package was used. P values of <0.05 were considered significant.

RESULTS AND DISCUSSION

Proximate diet composition

The major fatty acids (in % w/w of total fatty acids) in the olive oil used for frying were C16:0, 12.3; C18:0, 3.7; C18:1, 76.5; C18:2, 4.7, while that of the raw sardines were C16:0, 25.6; C18:0, 9.0; C18:1, 19.5; C18:2, 3.5; C20:5, 4.9; C22:6, 17.2. The diets had similar protein and fat contents but differed in the amounts of saturated and polyunsaturated fatty acids (PUFA) (Table 1).

Food consumption and body weight gain

The effect of experimental diets on food consumption was tested. Food consumption was not significantly different ($p<0.1$) in the different dietary groups which suggests that the fried sardine diet was well accepted by the rats. However, more fat and fatty acids (e.g. saturated, PUFA n-3) were consumed by the S rat group than by the CO or R groups (Table 2). Body weight gain was not significantly affected by the different diets.

Serum lipids

The hypercholesterolaemic induction period led to moderate hypercholesterolemia (Table 3). Consumption of the CO or S diets resulted in significantly lower total cholesterol ($p<0.01$) and phospholipid ($p<0.05$) levels than those observed in the COC group; the S diet group had ($p>0.1$) total cholesterol levels similar to those in the R group (Table 3). These results are similar to those previously observed (SÁNCHEZ-MUNIZ *et al.*, 2003), although, in the present study, the assayed sardines contained only ~5% n-3 PUFA (Table 1). Thus, according to present results a relatively low amount of n-3 PUFA seemed to normalise serum cholesterol in rat in a 2-wk period. Fish protein, as well as n-3 PUFA, may have a significant hy-

Table 1 - Composition of semi-synthetic diets containing casein plus DL-methionine and olive oil (CO), casein plus 0.2% DL-methionine, olive oil, bovine bile and cholesterol (COC), and sardines fried in olive oil (S).

	CO	S	COC
Protein (g/100 g dry matter)	14.54±0.46	14.75±0.14	13.92±0.68
Lipids + cholesterol (g/100 g dry matter)	9.08±0.22 *	9.52±0.28 a*	11.80±0.24
Cholesterol (2%) and bovine bile (0.5%) added	NO	NO	YES
Fatty acids (g/100 g total fatty acids)			
C16:0	12.3±0.3	13.1±0.2	11.9±0.1
C16:1 n-7	0.7±0.02	1.1±0.1 a	0.7±0.04
C18:0	3.7±0.0	3.8±0.1	3.6±0.0
C18:1 n-9	76.5±0.3	70.1±0.7 a	77.1±0.3
C18:2 n-6	4.7±0.1	4.4±0.1	4.7±0.0
C18:3 n-3	0.9±0.06	1.6±0.2	0.7±0.06
C20:5 n-3	ND	1.0±0.16 a*	ND
C22:6 n-3	ND	2.2±0.4 a*	ND
Total MUFA	77.3±0.2	71.5±0.7 a	77.9±0.3
Total SFA	16.9±0.3	18.1±0.4	16.4±0.2
Total PUFA n-3	0.9±0.06	5.3±0.3 a*	0.7±0.06
Total PUFA n-6	4.9±0.1	5.1±0.2	5.0±0.1
PUFA/SFA	0.34±0.00	0.57±0.02 a*	0.35±0.01
PUFA n-6/PUFA n-3	5.3±0.4	0.97±0.06 a*	6.9±0.1

Data are mean ± standard deviation of three determinations. ND: Not detected. Values bearing the letter a indicate significant differences between CO and S groups. CO and S data with an asterisk were significantly different with respect to COC (U-Mann Whitney test, $p<0.05$).

Table 2 - Food, fat and major fatty acid intakes and body weight gain during the experimental period.

Rats	CO	S	R
Food intake (g/day)	15.81±0.62	17.40±0.38	15.79±0.58
Fat intake (g/day)	1.41±0.06a	1.70±0.04b	1.41±0.05a
Fatty acid intake (g/day)			
C16:0	0.16±0.01a	0.21±0.00b	0.16±0.01a
C18:0	0.05±0.00a	0.06±0.00b	0.05±0.00a
C18:1 n-9	1.03±0.04	1.10±0.02	1.03±0.04
C18:2 n-6	0.06±0.00	0.07±0.00	0.06±0.00
C18:3 n-3	0.01±0.00a	0.02±0.00b	0.01±0.00a
C20:5 n-3	0.00±0.00a	0.02±0.00b	0.00±0.00a
C22:6 n-3	0.00±0.00a	0.03±0.00b	0.00±0.00
Total MUFA	1.04±0.04	1.13±0.02	1.04±0.04
Total SFA	0.23±0.01a	0.28±0.01b	0.23±0.01a
Total PUFA n-3	0.01±0.00a	0.08±0.00b	0.01±0.00a
Total PUFA n-6	0.06±0.00a	0.08±0.00b	0.06±0.00a
Body weight gain (g/day)	5.44±0.59	5.56±0.56	4.64±0.29

CO rats: Rats fed casein plus DL-methionine + olive oil diet; S rats: Rats fed a diet containing olive oil fried sardine as the only protein and fat sources; R rats: Rats fed the CO diet during both the induction and experimental periods, and used as the reference group.
Data are mean values ± standard deviations of seven rats. Values in the same row bearing different letters are significantly different (U-Mann Whitney tests, $p<0.05$). MUFA: monounsaturated fatty acids, SFA: saturated fatty acids, PUFA: polyunsaturated fatty acids.

pocholesterolaemic effect (VAZQUEZ and SÁNCHEZ-MUNIZ, 1994; AIT YAHIA *et al.*, 2003; SANCHEZ-MUNIZ *et al.*, 2003). The S rats tended to have lower triglyceride levels ($p<0.1$) than their CO counterparts, these results are in line with the hypotriglyceridaemic effect of n-3 PUFA reported in other studies (HARRIS, 1996; NESTEL, 1990). The R group had the highest triglyceride levels. The S and R rats had the lowest total cholesterol/phospholipid ratio (Table 3). The total cholesterol/total phospholipid ratio has been used as hypercholesterolaemic and atherogenic markers (KANNEL *et al.*, 1971). According to their total cholesterol/total phospholipid ratio, the atherogenic risk of S rats is similar to that of R group animals.

Lipoprotein-cholesterol and phospholipids

The non-HDL-cholesterol) and non-HDL-phospholipid contents in serum decreased ($p<0.01$ and $p<0.05$, respectively) in the rats that had the S and CO diets due to the cholesterol withdrawal (Table 3). More significant ($p<0.05$) decreases in the non-HDL-cholesterol levels were noted in the rats on the S diet. These results can be explained by the fact that the production of liver β-VLDL is inhibited by n-3 PUFA (NESTEL, 1990). In agreement with MORGADO *et al.* (2005) HDL-cholesterol tended to be lower in n-3 PUFA-enriched diets which suggests that there was an active RCT pathway in the rats that were fed the S diet.

Serum and lipoprotein-TBARS

The S and CO diets decreased serum and HDL-TBARS ($p<0.01$) values compared to the COC values, but the S diet caused the greatest decrease in serum and non-HDL-TBARS. No significant differences ($p>0.1$) were found in the TBARS values between the S and R groups (Table 3). HDL is known to carry a large amount of cholesteryl ester

Table 3 - Serum, HDL and non HDL-lipids (mmol/L) and peroxidation levels of rats fed semi-synthetic experimental diets.

Rats	COC	CO	S	R
Serum cholesterol (mmol/L)	4.79±0.39	2.43±0.13a*	1.85±0.08b*	1.84±0.09b*
Serum phospholipids (mmol/L)	1.55±0.06	1.31±0.09a*	1.17±0.04a*	1.34±0.12a
Serum triglycerides (mmol/L)	0.44±0.09	0.75±0.09ab*	0.58±0.04a*	1.02±0.12b*
Serum cholesterol/Phospholipids (mol/mol)	2.97±0.17	1.86±0.19a*	1.51±0.08b*	1.49±0.08b*
Serum HDL-cholesterol (mmol/L)	0.59±0.06	0.65±0.04a	0.49±0.03b	1.04±0.11c*
Serum HDL-phospholipids (mmol/L)	0.68±0.06	0.78±0.01a*	0.70±0.02b	0.93±0.09a*
Serum HDL-cholesterol/Total cholesterol (mol/mol)	0.12±0.02	0.27±0.01a*	0.27±0.02a*	0.58±0.05b*
Serum HDL-phospholipids/Total phospholipids (mol/mol)	0.44±0.04	0.62±0.02a*	0.60±0.02a*	0.72±0.03b*
Serum non-HDL-cholesterol (mmol/L)	4.20±0.32	1.78±0.12a*	1.36±0.08b*	0.80±0.10c*
Serum non-HDL-phospholipids (mmol/L)	0.87±0.06	0.52±0.05a*	0.47±0.05a*	0.30±0.09b*
Serum TBARS (MDA µmol/L)	3.26±0.45	1.63±0.20a*	1.10±0.12b*	0.85±0.12b*
Serum HDL-TBARS (MDA µmol/L)	1.00±0.10	0.65±0.07*	0.72±0.09 *	0.62±0.10*
Serum non-HDL-TBARS (MDA µmol/L)	2.25±0.34	1.01±0.18a*	0.40±0.06b*	0.20±0.05c*

COC rats: Rats in which hypercholesterolaemia was induced by diet containing casein plus DL-methionine + olive oil+ bovine bile + cholesterol; CO rats: Rats fed casein plus DL-methionine + olive oil diet; S rats: Rats fed a diet containing olive oil fried sardine as the only protein and fat sources; R rats: Rats fed the CO diet during both the induction and experimental periods, and used as the reference group.
Data are mean values ± standard deviations of seven rats. Values in the same row bearing different letters are significantly different (U-Mann Whitney tests, $p<0.05$).
* Significantly different (U-Mann Whitney tests, $p<0.05$) with respect to COC group.

hydroperoxides via the RCT pathway (MACKNESS and DURRINGTON, 1995). Thus, the present results are relevant because fried sardine consumption had a hypolipaemic effect and the TBARS levels decreased.

Moreover, the results should be attributed to the lipid, rather than the protein, fraction of the fried sardines because, according to AIT YAHIA *et al.* (2003) plasma and VLDL + LDL lipid peroxidation are not significantly affected by dietary fish protein or casein. Furthermore, while n-3 PUFA have several double bonds and are, thus, susceptible to free radical attack, there are conflicting reports on the effect of fish oil supplementation on the lipid peroxidation status in both animals and humans. ERDOGAN *et al.* (2004) found that the TBARS levels decreased in rats when fish oil was ingested for 30 days, suggesting that n-3 fatty acids may enhance resistance to lipid peroxidation. NALSEN *et al.* (2006) found that the antioxidant status was not affected by supplementation of n-3 PUFA, but the concentration of a biomarker of non enzymatic lipid peroxidation, 8-iso-$PGF_{2\alpha}$, decreased significantly in healthy humans. However, SONG and MIJAZAWA (2001) reported that lipid peroxidation increased in rats fed dietary docosahexaenoic acid-rich oil.

Cholesterol and bile acid faecal excretions

S and CO rats excreted significantly ($p<0.01$) more bile acids than the R rats. Daily faecal excretions of cholesterol and fat were also significantly higher ($p<0.05$) in S rats than those of R rats (Table 4). These results suggest that these excretions actively contribute to reducing plasma cholesterol due to less liver cholesterol being available for VLDL synthesis. IRITANI *et al.* (1985) found that, compared with dietary casein, fish and soybean protein significantly increased the excretion of cholesterol and its metabolites (coprostanol and bile acids) and decreased the plasma cholesterol levels. Using rats kept on a standard low-fat diet, the cholesterol from chylomicrons enriched with n-3

Table 4 - Moisture, fat, cholesterol, and bile acid contents in faeces of rats fed semi-synthetic experimental diets.

Rats	CO	S	R
Faeces			
g/day (dry matter)	1.40±0.08a	1.89±0.10b	1.35±0.07a
Fat (% dry matter)	4.28±0.34a	3.84±0.35a	4.26±0.29a
Bile acid			
mg/g faeces (dry matter)	13.63±0.46b	12.27±1.22b	3.27±0.50a
mg/day	19.15±0.82b	25.27±2.62c	4.32±0.53a
Fat			
mg/g faeces (dry matter)	43.16±3.45a	38.71±3.51a	43.0±2.87a
mg/day	62.04±8.62ab	74.08±8.19b	58.77±6.47a
Cholesterol			
mg/g faeces (dry matter)	4.02±0.45a	3.57±0.28a	3.07±0.19a
mg/day	5.75±0.88ab	6.79±0.72b	4.15±0.32a

CO rats: Rats fed casein + olive oil diet; S rats: Rats fed a diet containing olive oil fried sardine as the only protein and fat sources; R rats: Rats fed the CO diet during both the induction and experimental periods and used as the reference group.
Data are mean values ± standard deviations of seven rats. Values in the same row bearing different letters are significantly different (U-Mann Whitney tests, $p<0.05$).

PUFA (from fish oil) was removed from the blood and excreted via the bile more rapidly than that from chylomicrons enriched with saturated or monounsaturated fatty acids (palm or olive oil, respectively) (BRAVO *et al.*, 1995).

It has been proposed that taurine plays a significant role in cholesterol metabolism by increasing cholesterol excretion and reducing serum and liver cholesterol concentrations in rats fed a high cholesterol diet (YOKOGOSHI *et al.*, 1999). According to the nutritional composition of sardines described by SHIRAI *et al.* (2002), 1 kg of the S diet contains about 1.3 g of taurine, which explains the present results, at least in part.

The cholesterol excretion via the bile acids also helps keep the hepatic free cholesterol pool low, while the gene expression of lipoprotein liver receptors would be high which would help decrease the concentration of serum atherogenic lipoproteins (DIETSCHY, 1998). Cholesterol used to synthesize bile acids and excrete free cholesterol into the bile is derived mainly from the hepatic uptake of plasma HDL in the final step of the RCT pathway (BOTHAN and BRAVO, 1995). According to MORGADO *et al.* (2005) the reduction in plasma HDL-cholesterol associated with increased biliary cholesterol and bile acids may be surrogate markers of an accelerated RCT pathway in fish oil-fed rats. This concurs with the low HDL-cholesterol levels found in rats fed the S diet.

In conclusion, hypercholesterolaemic rats that consumed olive oil-fried sardines increased their excretion of bile acids and cholesterol. By the end of the study, these rats had lower atherogenic lipoprotein profiles and the serum cholesterol and serum and lipoprotein TBARS levels were normal. However, long-term studies of experimental animals and healthy and dislipaemic human volunteers are needed to ascertain the usefulness of consuming large amounts of fried oily fish.

ABBREVIATIONS:

HDL: high density lipoproteins; LDL: low density lipoproteins; PUFA: polyunsaturated fatty acids; SR-BI: Scavenger receptors BI type; TBARS: thiobarbituric acid reactive substances; VLDL: very low density lipoproteins.

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PAPER

DETERMINATION OF TRACE METALS AND PESTICIDES IN MUST DURING FERMENTATION IN A VINIFICATION PROCESS

DETERMINAZIONE DI METALLI IN TRACCIA E PESTICIDI NEL MOSTO DURANTE PROCESSI DI VINIFICAZIONE

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ABSTRACT

Selected trace metals (copper, lead, and zinc), an ultra-trace metal (cadmium) and pesticides (metalaxyl, penconazole, procymidone and parathion) were determined during the fermentation step in a vinification process. Two laboratory-scale fermentations were carried out, with and without skins (for the production of red and white wine, respectively), and each day samples of must were collected and analysed. Levels of metals were determined by two analytical techniques: Inductive-

RIASSUNTO

Al fine di valutare il comportamento di alcuni elementi in traccia (rame, piombo, cadmio e zinco) e pesticidi (metalaxyl, penconazole, procymidone e parathion) durante la fermentazione in un processo di vinificazione, sono state effettuate due vinificazioni in scala-laboratorio, con e senza bucce, ed ogni giorno sono stati analizzati campioni di mosto. I metalli sono stati analizzati utilizzando due tecniche analitiche: ICP-MS e SV, mentre i pesticidi sono stati analizzati in GC-MS. Rame e zinco hanno

- Key words: anodic stripping voltammetry, GC-MS, heavy metals, ICP-MS, pesticides, wine -

ly Coupled Plasma Mass Spectrometry (ICP-MS) and Stripping Voltammetry (SV) with a Thick-Film Modified Graphite-Containing Electrode (TFMGE). For the analysis of pesticides, a liquid-liquid extraction and silica gel clean up followed by Gas Chromatography with Mass Spectrometry detection (GC-MS) was used. Copper and zinc concentrations decreased in a continuous manner throughout the two wine-making processes, while lead and cadmium concentrations dropped sharply during the first two days and stabilised afterwards. The reduction in pesticide concentrations was more evident in red wine vinification than in the white one. Parathion decreased quite rapidly, while metalaxyl was the most persistent.

mostrato una diminuzione costante durante i due processi, mentre le concentrazioni di piombo e cadmio hanno avuto una repentina iniziale diminuzione e poi si sono stabilizzati, a livelli comunque molto bassi. La diminuzione dei pesticidi è più evidente nella vinificazione in rosso che in quella in bianco, il parathion decresce più rapidamente mentre il metalaxyl è più persistente.

INTRODUCTION

Wine is one of the most important agricultural/food industry products in the world. In 2000, vineyards made up 5.4% of the total cultivated land in Italy (ISTAT, 2002), covering over 700,000 hectares. Moreover, the production of DOC (Denominazione di Origine Controllata or "controlled denomination of origin") and DOCG (Denominazione di Origine Controllata e Garantita or "controlled and guaranteed denomination of origin") wines is continuously increasing. In the last decades results from several medical studies have indicated that a moderate consumption of wine could provide some health benefits due to the presence of antioxidant compounds at significant concentration levels and of essential elements such as Co, Cu, Se and Zn at adequate concentration levels. On the other hand, several potentially toxic elements, such as Pb and Cd, can also be present in wines at low but significant levels [e.g. 3.3 μg L^{-1} for Cd (MENA *et al.*, 1996) and 73 μg L^{-1} for Pb (WHO, 1996)]. It is therefore important to determine their concentration levels in order to evaluate the dietary intake of these toxic elements through wine consumption.

The presence of toxic trace metals in wine may be due to metal-containing insecticides and fungicides, environmental pollution (water, soil and atmospheric contamination) and poor cellar practices such as contact with wine production equipment and oenological products used in the wine-making process (PEDERSEN *et al.*, 1994). Lead concentration in wine is regulated in most countries and the maximum acceptable values range between 50 and 300 μg L^{-1}. Because of its toxicity, lead and other elements can be a potential trade barrier

for importing wine if the concentration levels do not meet the tolerance limits set by the importing country (GONZALEZ HERNANDEZ *et al.*, 1996; OIV, 1990).

Cadmium is highly toxic, tends to accumulate in biological systems and has a long half-life. Generally, the cadmium content in wine is low (<1 μg L^{-1}) but, when wine is consumed in large quantities, the dietary intake can reach physiologically significant levels.

Copper may be carried over into wine mainly from copper sulphate-based applications to control fungus. Certain countries authorize the use of copper sulphate (0.02 g L^{-1}) to eliminate non-desirable sulphur derivatives. Copper is desirable in wine up to 0.5 mg L^{-1}, because it regulates numerous reactions during the ripening of wine. The maximum copper concentration permitted in the EU is 1 mg L^{-1}. Similar to iron, high quantities of copper give wine a metal-like taste. Must contains a relatively high amount of copper (up to 5 mg L^{-1}) but fermentation cuts the copper content in the young wine by about one order of magnitude (RIBEREAU-GAYON *et al.*, 2000; CASTINEIRA *et al.*, 2004). Analogously, high levels of zinc may affect the fermentation process (SCOLLARY, 1997). Italian regulations have established that the maximum acceptable levels of copper and zinc in wine are 1 mg L^{-1} and 5 mg L^{-1} , respectively (D.M. MINISTERO AGRICOLTURA E FORESTE, 1986).

Pesticides, mainly fungicides and insecticides, are used on grapes to prevent the attack of numerous parasites such as the grape moth (*Lobesia botrana*), downy mildew (*Plasmopara viticola*), powdery mildew (*Uncinula necator*) and gray mold (*Botrytis cynerea*). If growers do not follow the regulations regarding pesticide quantity and preharvest intervals, the grapes become contaminated and the pesticides end up in the wine. It has been shown that the vinification process decreases pesticide levels (CABRAS *et al.*, 1999; CABRAS and ANGIONI, 2000; FERNANDEZ *et al.*, 2005; NAVARRO *et al.*, 1999; SALA *et al.*, 1996). However, since the various pesticides have different chemical properties, their behaviour should be tested individually.

The aim of this work was to perform a simple vinification on a laboratory-scale and to evaluate the trace elements (Cu, Pb and Zn), an ultra-trace element (Cd), fungicides (metalaxyl, penconazole, procymidone), and an insecticide (parathion) during the fermentation process. Paraoxon, which is not present in formulates, but has to be determined and summed up with parathion according to Italian regulations (D.M. MINISTERO DELLA SALUTE, 2004), was also investigated in a parallel experiment on the use of biosensors for the determination of organophosphorous pesticides.

Levels of metals were determined employing two analytical techniques: Stripping Voltammetry (SV) with a Thick-Film Modified Graphite-containing Electrode (TFMGE) (BALDO *et al.*, 1997, 1998; BRAININA *et al.*, 2004; WIESE and SCHWEDT, 1997) and Inductively Coupled Plasma Mass Spectrometry (ICP-MS) (ALMEIDA and VASCONCELOS, 1999; CASTINEIRA *et al.*, 2001; PEREZ-JORDAN *et al.*, 1999). Must samples were not pretreated in SV, but a thick-film modified graphite-containing electrode (BRAININA *et al.*, 2004) was used instead.

Several analytical methods with different sample pre-treatments can be found in the literature for the analysis of pesticide residues in must and wine. They often employ rapid extraction procedures, such as SPE (Solid Phase Extraction) (JIMENEZ *et al.*, 2001; WONG *et al.*, 2003) or SPME (Solid Phase Micro Extraction) (CORREIA *et al.*, 2001; ZAMBONIN *et al.*, 2002). Other methods rely on more traditional techniques such as liquid-liquid extraction usually followed by some clean-up steps (FERNANDEZ *et al.*, 2005; OLIVA *et al.*, 2000; SALA *et al.*, 1997). In this paper we adopted a method based on liquid-liquid partition, fol-

lowed by silica gel clean-up and GC-MS detection. The other types of extraction (SPE and SPME), though more rapid and solvent-saving than the liquid-liquid partition, require careful standardization that is difficult to achieve with the must, because the composition of the matrix continuously changes during the experiment. This results in a continuous change in the solid/liquid equilibria of analytes in the must, which is not replicable in the calibration matrix. Moreover, this method is also applicable to the residues from the centrifugation of must.

MATERIALS AND METHODS

Wine-making

About 20 kg of grapes (*Vitis vinifera*, var. *Cesanese di Affile,* supplied by the Experimental Institute of Enology of Velletri, Rome) were pressed and the stems removed. The crushed fruit was divided into two parts, with and without skins (for red and white wine production, respectively). Each sample was further divided into two parts to obtain two replicates for each kind of vinification. The four vessels were conventionally labeled A and B for "white" must and C and D for "red" must. For fermentation purposes, 250 mg L^{-1} of yeast, 300 mg L^{-1} of an activating medium, 40 mg L^{-1} of SO_2 for A and B, and 30 mg L^{-1} for C and D were added, according to the oenologist's recommendation. The must was then fortified with 0.5 mL of a methanolic solution of pesticides to obtain a concentration of each analyte of about 1 mg L^{-1}: parathion, metalaxyl, penconazole and procymidone were added to replicates A and C, while only paraoxon was added to replicates B and D. All of the pesticides studied were stable to hydrolysis at the pH of the must (pH 3.0-3.3). The experiment was carried out at room temperature for six days, until the end of the fermentation phase (young wine).

Each day a sample of must was collected from each vessel and centrifuged for 5 min at 3,000 rpm. Concentrations of trace metals and pesticides were determined in triplicate with each technique as described below. Centrifugation was performed because the aim was to follow the soluble fraction of metals and not the fraction complexed to insoluble compounds and suspended matter.

Chemicals

Concentrated HNO_3 Aristar (BDH, Poole, England), 30% H_2O_2 Aristar (BDH, Poole, England) and ultrapure water obtained by a MilliQ Elment System (Millipore, Billerica MA, USA) were used for sample preparation and dilution for ICP-MS analysis. Single-element ICP-MS standard solutions, 1,000 mg L^{-1} (BDH, Poole, England), were used for the preparation of calibration solutions for trace element analyses. A rhodium ICP-MS standard solution, 1,000 mg L^{-1}, (BDH, Poole, England) was used as internal standard to correct for residual matrix interferences and compensate for plasma variations during ICP-MS analysis. Reagents of the extra pure and chemically pure grades (HCl and HNO_3 acids; CH_3COONa, NaCl and $Ga(NO_3)_3 \cdot 8H_2O$ salts; 30% H_2O_2) were used for SV analysis. Standard solutions of Cu, Pb, Cd and Zn (Aldrich, St. Louis MU, USA) containing 1,000 mg L^{-1} of metal ions and tri-distilled water were used for the preparation of the calibration solutions.

All organic solvents were of pesticide analysis grade (J.T. Baker, Deventer, The Netherlands). Silica gel 60 extra pure, 60-230 mesh (Merck, Darmstadt, Germany), was activated at 250°C for four hours before use. Anhydrous sodium sulphate was analytical grade (Carlo Erba, Milan, Italy). Parathion, metalaxyl, paraoxon, penconazole, procymi-

done and p-terphenyl (used as injection standard) were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Stock solutions were prepared in methanol from the individual standards at an approximate concentration of 6 g L^{-1}. Working solutions were prepared by diluting the stock solutions in ethyl acetate.

Stripping voltammetry (SV) analysis

SV measurements were made using an IVA-5 voltammetric analyzer (IVA Co. Ltd., Ekaterinburg, Russian Federation) and a three-electrode system consisting of TFMGE as the working electrode, an Ag/AgCl (saturated KCl) reference electrode and a glassy carbon rod as the auxiliary electrode. Prior to analysis, the TFMGE working surface was electrochemically pretreated by keeping the electrode at potentials -0.8 V and -1.5 V sequentially applied for 60 s and 180 s in an agitated solution containing 0.5 M HCl. This procedure decreased the competitive hydrogen emission on the graphite surface and provided a potential sufficient for overcoming the overvoltage of calomel reduction and mercury evolution. The electrode surface was regenerated between measurements by sweeping the potential between the accumulation potential and -0.05 V. Before TFMGE is discarded, it is necessary to convert the mercury film to non-toxic calomel by polarization of the electrode at a potential of 0.3 V applied for 3-5 min in an agitated solution containing 0.5 M HCl. The maximum current of the metal oxidation, which was observed in the I-U dependence (the integral curve), or the amplitude of the current derivative with respect to the potential (the differential curve) was taken as the metal response.

An aliquot of a must sample and desired reagents were poured into the cell. TFMGE, the reference electrode and the auxiliary electrode were immersed into the cell, the stirrer was switched on, and accumulation potentials were applied. Anodic voltammograms were recorded at a scan rate of 0.5 Vs^{-1} after the rest period. Optimal conditions for determination of each metal in the wine samples are shown in Table 1. The concentration of metal ions in the wine samples was determined using the method of standard additions.

ICP-MS analysis

ICP-MS measurements were made using a Perkin-Elmer ELAN 6100 ICP-MS spectrometer (PerkinElmer Inc., Wellesley, MA, USA) equipped with a cross-flow nebulizer. A microwave system (Mile-

Table 1 - Instrumental operating conditions for SV measurements of heavy metals in wine.

Element	V_{wine}, mL	Reagents added to the cell	E_{acc}, V	t_{acc}, s	Sweep range, V	$E_{response}$, V
Cu			-0.8	30	-0.6 – 0.05	-0.15±0.05
Pb	9.0	1 mL of 5 M HCl	-1.2	120	-0.8 – -0.1	-0.35±0.05
Cd			-1.2	120	-0.8 – -0.1	-0.65±0.05
Zn	0.2	1 mL of 3.5 M NaCl (pH= 2) 1 mL of 1 M CH_3COONa 7.8 mL H_2O $Ga(NO_3)_3$*	-1.4	10	-1.2 – -0.6	-0.90±0.10

*A solution of $Ga(NO_3)_3$ (1 g L^{-1}) was added to the cell in order to ensure that the gallium response was not smaller than the zinc response in the recorded voltammogram.

stone 1200 Mega, Italy) was used for digestion of samples.

Two mL aliquots of samples were put in TFM vessels and digested with a mixture of 2 mL of HNO_3 and 2 mL of H_2O_2. After two hours at room temperature, the vessels were closed and kept in a microwave system according to the following heating programme:

Step	Time (min)	Power (W)
1	2	250
2	2	0
3	5	250
4	2	0
5	2	400
6	2	0
7	2	500

The resulting solution was cooled, transferred to a 25 mL volumetric flask, and brought to the final volume with ultrapure water. The final solutions were analyzed for Pb, Cd, Cu and Zn by ICP-MS. The best instrumental operating conditions for determining trace metals in must and wine samples are given in Table 2.

Calibration curves from standard acidified water solutions, covering a concentration range of 100-1 μg L^{-1} for Pb, Cu, Zn and 10-0.1 μg L^{-1} for Cd, were performed. Rh was added to blanks, calibration solutions and samples up to the Rh concentration of 10 μg L^{-1}.

Table 2 - Instrumental operating conditions for ICP-MS measurements of heavy metals in acid digested wine.

Operating parameters	
RF power	1,250 watts
Nebulyzer gas-flow	0.85 L min^{-1}
Auxiliary gas-flow	1.2 L min^{-1}
Plasma gas-flow	15 L min^{-1}
Lens setting	9.25 volts
Nebulizer type	Cross-flow
Interface cones	Platinum
Dwell time	100 ms
Number of sweeps	60
Number of replicates	3
Scan mode	Peak hopping

GC-MS analysis

The pesticides were determined by GC-MS using a Thermo Trace GC 2000 gas chromatograph coupled to a Trace MS mass selective detector, split-splitless injector and AS 2000 auto sampler (all from Thermo Electron Corporation, Waltham, MA, USA). A fused silica capillary column DB-5 MS (Agilent Technologies, Santa Clara, CA, USA), 60 m by 0.25 mm i.d., and film thickness 0.25 μm was used. The injector and interface temperatures were 250° and 280°C, respectively. The carrier gas was helium at a flow of 1 mL min^{-1}. One μL of sample was injected in splitless mode (60 s) and the oven temperature was programmed as follows: 80°C for 1 min, 80°-205°C at 25°C min^{-1}, 205°C for 6 min, 205°-225°C at 2°C min^{-1} and 225°-280°C at 15°C min^{-1}. The mass spectrometer was operated in the selected ion monitoring (SIM) mode.

Five mL of centrifuged must were extracted with 4 mL of a mixture of hexane and ethyl acetate 1:1. The organic phase was filtered through a funnel containing anhydrous sodium sulphate. The extraction procedure was repeated twice with 3 mL + 3 mL of the extracting mixture and the organic phases were collected together and concentrated with rotary vacuum evaporator to about 0.5 mL. The extract was cleaned-up on a glass column (i.d. 1 cm) containing 1 g of silica gel, eluted with 15 mL of ethyl acetate and concentrated to 0.5 mL. p-Terphenyl was added as injection internal standard to all the samples and standard solutions, at a concentration of 1 mg L^{-1}.

GC/MS instrumental parameters and method performance details are given in Table 3.

Table 3 - GC/MS method parameters: pesticide name, molecular weight (MW), target ion (T) and qualifier ions (Q1 and Q2), percentage of qualifier to target ratios (Q1/T and Q2/T), limit of detection (LOD), regression coefficient (r^2), linearity range (range) and limit of quantification (LOQ).

Compound	MW	Ret times min	T target ion	Q1 qualifier ion	Q1/T %	Q2 qualifier ion	Q2/T %	LOD pg	r^2	range pg	LOQ ($\mu g\ L^{-1}$)
metalaxyl	279.3	16.29	206	192	57	160	96	4	0.995	10-5,000	0.5
paraoxon	275.2	16.50	149	109	249	275	37	8	0.998	25-5,000	1.0
parathion	291.3	18.58	291	139	83	155	62	7	0.999	25-5,000	1.0
penconazole	284.2	20.48	248	250	33	159	124	5	0.997	15-5,000	0.5
procymidone	284.1	21.34	283	285	65	267	288	4	0.998	10-5,000	0.5

RESULTS AND DISCUSSION

Method optimisation – ICP-MS

Different procedures were studied and optimised for the analysis of must samples in ICP-MS, considering both sample pre-treatment and analytical technique. Wine is a quite complex matrix which can produce severe spectral interferences. The huge amounts of organic compounds introduced into ICP-MS affects the plasma stability and worsens detection limits with respect to aqueous solutions. The high carbon load can lead to carbon deposition on the metallic cones of the interface and in the injector tube of the torch. Sometimes interferences cannot be overcome by simple dilution of the sample. Moreover, aged wines or high sugar-content wines generally present difficulties in subsampling the aliquots to be analyzed due to their lack of homogeneity. A significant percentage of the element to be measured could also be strongly bound to organic compounds or particulate matter.

Considering that the chemical composition of the organic matrix changes continuously during the vinification process and for the reasons described above, the procedure that includes acid digestion in a microwave oven was chosen for ICP-MS. In this way the organic matrix is destroyed and the different samples throughout the fermentation process can be compared because any possible matrix interference on any of the isotopes due to organic species is eliminated.

Accuracy and precision of ICP-MS and SV methods

Precision was evaluated by analyzing five different aliquots of a wine sample. Since reference materials for wine are not available, method accuracy was tested by adding known amounts of the element to five different aliquots of the wine sample. Precision and accuracy data (expressed as % recovery) are shown in Table 4 for ICP-MS and SV.

Pesticide recovery test

To evaluate the recovery, samples of red must and red wine were spiked with pesticides at two different levels: 0.5 and 0.05 mg L^{-1}. The samples were analysed according to the procedure described above. Four replicates of each level were analysed. The recovery values in the must and wine were greater than 86% for both levels with a standard deviation of 9%, in the most unfavourable case, as reported in Table 5.

Trace and ultra-trace metals

The results of the monitoring of trace metals during a 6-day fermentation process are given in Tables 6 and

Table 4 - Recovery (%) and precision of concentration measurements (RSD, %) of copper, lead, cadmium and zinc in spiked samples of a red wine by ICP-MS and SV analysis methods (n = 5, P = 0.95).

Elements	ICP-MS			SV		
	Precision (%)	Fortification level - spiking (up to µg L^{-1})	Recovery (%)	Precision (%)	Fortification level - spiking (up to µg L^{-1})	Recovery (%)
Cu*	4.4	0.6	98	6.7	31	101
Pb	3.3	72.5	95	4.7	16	101
Cd	4.7	5.5	93	2.9	0.9	95
Zn*	0.6	2.6	99	1.3	506	103

* results are given in µg mL^{-1}.

Table 5 - Recovery (%) and precision of concentration measurements of pesticides in spiked samples of red must and red wine (n=5, P=0.95).

Pesticide	Fortification level (mg L^{-1})	Mean recovery % (red must)	Precision % (red must)	Mean recovery % (red wine)	Precision % (red wine)
Parathion	0.5	92.5	8.1	94.2	2.8
	0.05	87.0	4.8	91.7	3.1
Penconazole	0.5	94.0	7.8	94.7	3.8
	0.05	88.5	6.0	90.5	3.2
Procymidone	0.5	97.0	9.0	98.0	3.0
	0.05	93.5	7.7	91.6	3.3
Metalaxyl	0.5	88.0	6.9	88.5	4.0
	0.05	86.5	9.2	87.6	4.6
Paraoxon	0.5	93.6	7.4	96.8	3.2
	0.05	94.1	8.3	96.3	3.8

7. Data shown in parentheses in Table 7 were excluded from all the subsequent calculations because there was simultaneous disagreement between the results from the two analytical techniques and between the two independent experiments. Some discrepancies in the results obtained by the ICP-MS and SV methods are probably attributable to relative inhomogeneity of the aliquots.

It is known that zinc, lead and copper are used by yeast enzyme during the fermentation process and precipitate out of solution as the yeast die and decay (SALA *et al.*, 1996). Moreover, fermentation can be accompanied by the formation of heavy metal insoluble compounds (e.g. zinc sulphides) (GONZALEZ HERNANDEZ *et al.*, 1996). The copper and zinc concentrations decreased continuously during the fermentation of the white must (Table 6). At the end of the process the Cu and Zn concentrations had been reduced by 95 and 80%, respectively (Figs. 1 and 2). In the white must the cadmium concentration dropped sharply after the first day of fermentation, while the lead concentration decreased in the first two days and then stabilised afterwards (Figs. 3 and 4).

Major problems were experienced trying to obtain representative samples from the red must so it is very difficult to interpret the results in this case.

Table 6 - Trace metal concentrations (mean ± sd from triplicate analyses) in the white must measured by two analytical techniques (ICP-MS and SV).

		EtOH %	Pb µg L^{-1} ICP	Pb µg L^{-1} SV	Zn mg L^{-1} ICP	Zn mg L^{-1} SV	Cu mg L^{-1} ICP	Cu mg L^{-1} SV	Cd µg L^{-1} ICP	Cd µg L^{-1} SV
Day 0	Sample A	0.23	49.0±1.3	52.4±1.3	0.59±0.04	0.62±0.02	1.59±0.07	1.52±0.10	1.44±0.05	1.60±0.05
	Sample B	0.38	35.8±1.2	59.1±1.3	0.64±0.04	0.59±0.01	1.63±0.08	1.47±0.09	2.00±0.04	1.40±0.03
Day 1	Sample A	0.78	43.9±2.0	39.4±1.4	0.64±0.05	0.47±0.02	1.04±0.04	0.65±0.03	0.63±0.05	0.49±0.03
	Sample B	0.80	32.1±1.2	28.7±1.1	0.42±0.03	0.32±0.02	1.07±0.07	0.74±0.04	0.44±0.02	0.36±0.03
Day 2	Sample A	3.25	19.1±0.9	24.0±1.1	0.26±0.03	0.43±0.02	0.63±0.05	0.45±0.03	0.23±0.03	0.44±0.02
	Sample B	3.43	25.4±1.2	26.1±1.2	0.25±0.02	0.25±0.02	0.59±0.04	0.46±0.03	0.49±0.02	0.39±0.02
Day 3	Sample A	4.38	14.5±1.0	13.4±0.9	0.18±0.02	0.27±0.02	0.17±0.02	0.15±0.01	0.36±0.02	0.29±0.02
	Sample B	4.50	25.6±1.3	21.0±1.1	0.18±0.02	0.19±0.01	0.17±0.01	0.19±0.01	0.43±0.01	0.36±0.03
Day 4	Sample A	8.08	18.9±1.3	17.8±0.9	0.25±0.02	0.25±0.02	0.19±0.01	0.15±0.01	0.44±0.03	0.40±0.04
	Sample B	7.89	17.4±1.2	20.1±1.2	0.22±0.02	0.24±0.02	0.28±0.02	0.11±0.01	0.38±0.02	0.32±0.03
Day 6	Sample A	9.77	18.6±1.0	17.4±1.0	0.17±0.01	0.14±0.01	0.07±0.01	0.07±0.01	0.51±0.03	0.40±0.03
	Sample B	10.19	15.8±0.9	15.6±0.9	0.10±0.01	0.08±0.01	0.07±0.01	0.07±0.01	0.18±0.03	0.20±0.03

Table 7 - Trace metal concentrations (mean ± sd from triplicate analyses) in the red must measured by two analytical techniques (ICP-MS and SV).

		EtOH %	Pb µg L^{-1} ICP	Pb µg L^{-1} SV	Zn mg L^{-1} ICP	Zn mg L^{-1} SV	Cu mg L^{-1} ICP	Cu mg L^{-1} SV	Cd µg L^{-1} ICP	Cd µg L^{-1} SV
Day 0	Sample C	0.56	23.0±1.1	26.3±1.1	1.20±0.08	1.16±0.05	3.14±0.12	2.47±0.19	0.54±0.03	0.51±0.04
	Sample D	0.76	22.5±1.2	24.9±1.1	0.87±0.05	0.75±0.03	3.06±0.14	2.40±0.21	0.55±0.04	0.80±0.06
Day 1	Sample C	1.00	22.5±1.2	27.4±1.0	0.78±0.06	0.42±0.02	1.64±0.09	1.53±0.09	0.40±0.04	0.32±0.03
	Sample D	1.04	26.6±1.0	26.2±1.1	0.66±0.04	0.60±0.03	1.45±0.08	1.38±0.08	*(1.56±0.78)	*(0.41±0.19)
Day 2	Sample C	4.66	29.4±0.9	28.2±1.0	0.53±0.04	0.51±0.02	1.07±0.10	1.10±0.09	0.46±0.03	0.35±0.03
	Sample D	4.62	26.9±0.9	31.0±0.9	0.34±0.02	0.38±0.01	0.74±0.06	0.90±0.08	0.41±0.02	0.40±0.02
Day 3	Sample C	6.27	25.1±1.2	18.3±1.0	0.71±0.03	0.62±0.03	1.25±0.09	1.19±0.09	0.47±0.04	0.38±0.03
	Sample D	6.46	*(99.8±33.5)	*(9.0±1.2)	0.50±0.03	0.42±0.02	0.76±0.05	0.65±0.06	0.77±0.04	0.60±0.03
Day 4	Sample C	10.42	21.3±1.0	18.4±0.9	0.24±0.02	0.48±0.02	0.37±0.03	0.33±0.02	0.41±0.03	0.40±0.02
	Sample D	11.25	34.0±1.2	26.4±1.0	0.23±0.02	0.35±0.02	0.41±0.03	0.44±0.03	0.33±0.02	0.48±0.03
Day 6	Sample C	12.73	21.8±0.8	17.0±0.9	*(0.84±0.25)	*(0.50±0.15)	0.17±0.01	0.18±0.01	*(1.38±0.55)	*(0.42±0.14)
	Sample D	12.49	22.1±0.9	24.1±1.1	0.29±0.01	0.31±0.02	0.10±0.01	0.10±0.01	0.39±0.02	0.43±0.03

*Data shown in parentheses were excluded from all the subsequent calculations because there was simultaneous disagreement between the two analytical techniques and between the two independent experiments.

However, the concentrations of copper and zinc decreased (Figs. 1 and 2, respectively). The trend was very similar to that in the white must, despite the unstable effect of the solid/liquid phase equilibria of the dissolved ions. Fluctuations of lead and cadmium concentrations during fermentation were less significant; this could have been due to the effect of solubilisation, absorption and/or precipitation. The different trends observed in the two fermenta-

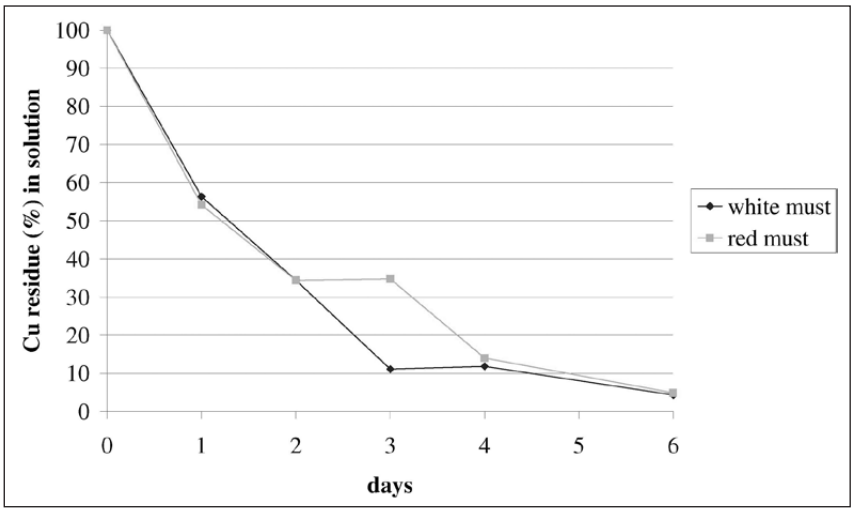


Fig. 1 - Comparison of copper concentration trend in the two fermentation processes.

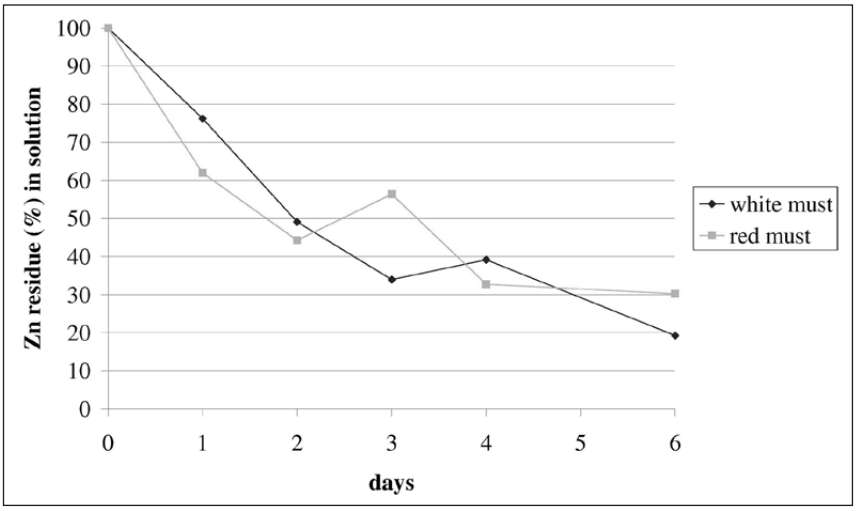


Fig. 2 - Comparison of zinc concentration trend in the two fermentation processes.

tions for lead and cadmium (Figs. 3 and 4), can be explained by the fact that the absolute concentrations of cadmium and lead at the beginning of fermentation were lower in the red must than in the white one (Tables 6 and 7) due to the drastic initial decrease related to co-precipitation with the skins. In the fermentation without skins the precipitation of the residual aliquots of these elements requires more time, which is probably due to the small quantity of solid material. Moreover, lead and cadmium form very stable complexes with organic and inorganic materials (GARRIDO *et al.*, 1997).

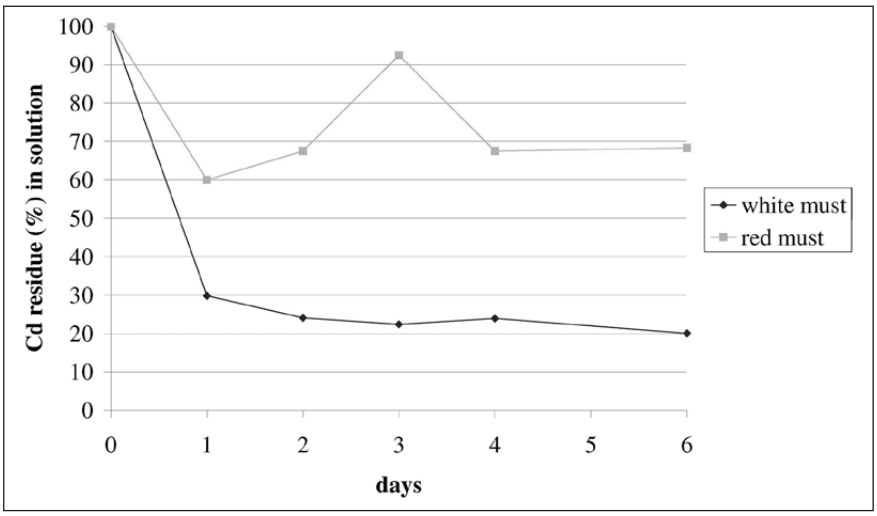


Fig. 3 - Comparison of cadmium concentration trend in the two fermentation processes.

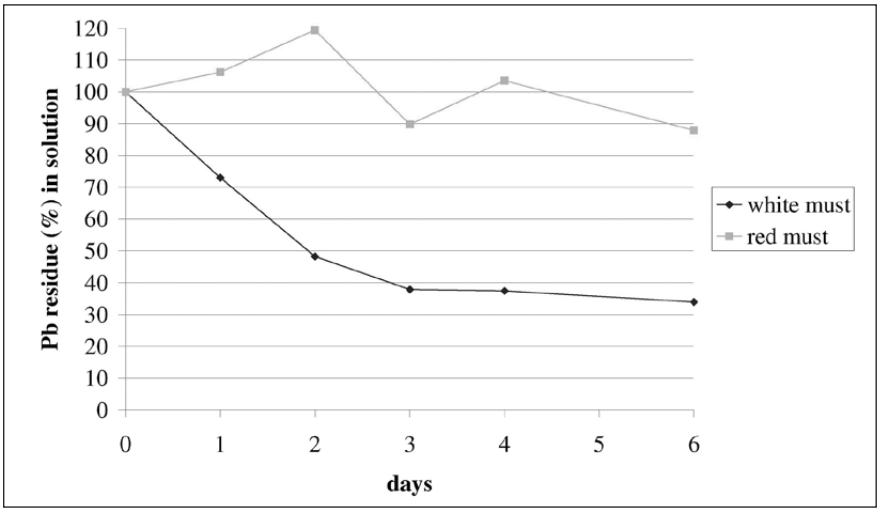


Fig. 4 - Comparison of lead concentration trend in the two fermentation processes.

Pesticides

Two hours after spiking the fermentation vessel with pesticides, the first samples were collected. At the time of spiking the concentration of pesticides was considered to be 100%, and this value was used to evaluate the trend of the analytes during fermentation. All the samples were centrifuged and the liquid phase was extracted immediately after sampling.

Figs. 5 and 6 show the percentage of residual pesticides in the must for the two types of vinification. The dissipation rate for all the analytes was high-

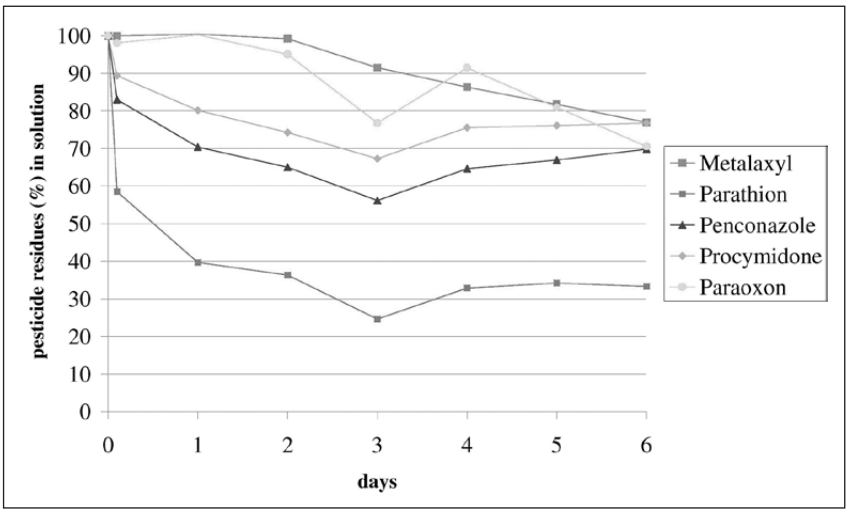


Fig. 5 - Residual pesticide concentration trends throughout the fermentation process in white must.

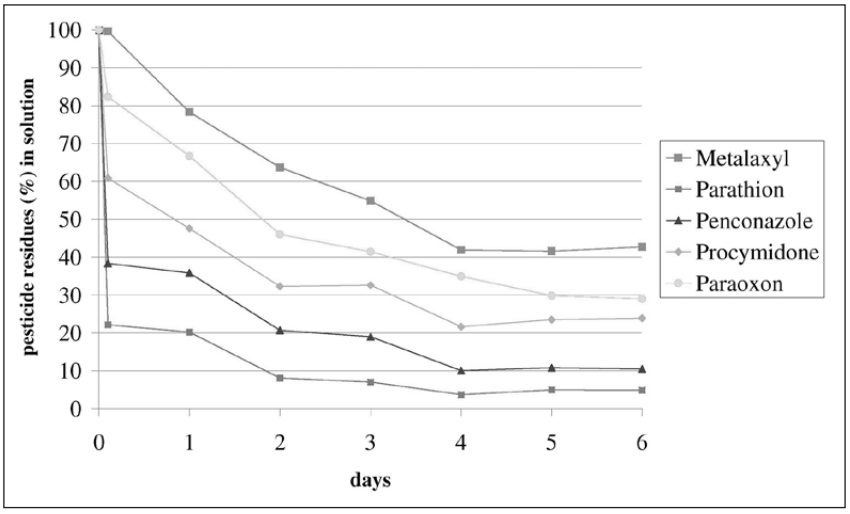


Fig. 6 - Residual pesticide concentration trends throughout the fermentation process in red must.

er in the vinification with skins than in that without skins. The affinity of the analytes for the suspended and solid matter can be noted in the sharp decrease in their concentration, which took place almost immediately after spiking. After 4 days the pesticide concentrations were less than 42% of the initial levels in the red wine vinification. In contrast, in the white wine vinification the pesticide residues were still over 60% of the initial level at the end of the fermentation, with the exception of parathion which disappeared at a faster rate. After seven days 33 and 5% remained in the white and red must, respectively. These values are

consistent with other studies (CABRAS *et al.*, 1995; SALA *et al.*, 1996). In samples taken from the vessel spiked with parathion, paraoxon was detected at 0.1 to 0.5% of the initial value of the parent compound, suggesting that the "degradation" of parathion to paraoxon was not evident during fermentation.

The remaining percentages of the fungicides penconazole and metalaxyl were 70 and 10% and 77 and 43% in the white and red wines, respectively, while the remaining percentages of procymidone were 77 and 23% in the white and the red wines, respectively. These data are substantially in agreement with the data of NAVARRO *et al.* (1999) and SCARPONI and MARTINETTI (1992).

CONCLUSIONS

The data obtained in this study give specific information about the fate of the selected trace and ultra-trace elements and pesticides during the fermentation process.

Obviously the interpretation of these data must take into account that the fermentation processes were performed under ideal laboratory conditions and the differences induced by the drastic reduction in scale, in comparison with the commercial processes, can hardly be estimated (CASTINEIRA *et al.*, 2004).

In conclusion there is a limited decrease in the lead concentration during the first two days of the fermentation process, and then it becomes quite stable in the must. Lead can therefore be used to determine the origin of wines (CASTINEIRA *et al.*, 2004), if contamination during vinification can be excluded. The zinc concentration decreases more than lead. Cadmium is found at very low levels and the concentration of copper, which is largely used in agronomical treatments, is reduced by approximately one order of magnitude. Therefore zinc, cadmium, and copper can only be used to check for contamination in the final product.

Moreover, from a strictly analytical point of view, the SV method is an interesting and original approach and demonstrates its suitability even for complex matrices such as musts, in addition to its former application on wine samples (BRAININA *et al.*, 2004).

Data obtained in this work showed that the fermentation step in the wine-making process contributes to a significant reduction in pesticide concentrations, due to absorption by suspended matter. Dissipation was evident in the vinification with skins (red wine). However, pesticide concentrations still present at the end of fermentation ranged from 33 to 77% and 5 to 43% in the white and red must, respectively. After fermentation, wine undergoes several other "clarification" procedures, including racking and filtration and, as a general rule, the concentration of pesticides continues to drastically reduce. In some cases, pesticide residues are completely eliminated.

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PAPER

PROTEIN FINING AGENTS: CHARACTERIZATION AND RED WINE FINING ASSAYS

AGENTI PROTEICI CHIARIFICANTI: CARATTERIZZAZIONE E PROVA DI CHIARIFICAZIONE DEL VINO ROSSO

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ABSTRACT

The physico-chemical characteristics of protein fining agents are important for optimizing the fining treatment which affects wine quality. The aim of this study was to characterize nineteen commercial fining products. Furthermore, a fining trial was done to evaluate the influence of different fining proteins on some phenolic characteristics of red wine. The results show that the molecular weight (MW) distribution of caseins and potassium caseinate products are characterized by

RIASSUNTO

Le caratteristiche chimico-fisiche degli agenti chiarificanti proteici sono importanti per l'ottimizzazione del trattamento di chiarificazione che influenza la qualità del vino. Lo scopo di questo studio era la caratterizzazione di diciannove chiarificanti commerciali. È stata inoltre effettuata una prova di chiarificazione allo scopo di valutare l'influenza di differenti proteine chiarificanti su alcuni composti fenolici caratterizzanti il vino rosso. I risultati hanno mostrato che la distribuzione del peso molecolare

- Key words: fining agents, isoelectric point, phenolic compounds, protein, SDS-PAGE, surface charge density, wine -

a band at 30.0 kDa and egg albumins by a band close to 43.0 kDa. Isinglass (swim bladder) has bands at 20.1, between 94.0-43.0 and above 94.0 kDa. In addition, two of the gelatins studied do not have any band in the MW range studied. The other fining agents displayed polydispersion. The isoelectric point (IEP) of the proteins ranged from 4.20 to 6.48. The effects of egg albumin (AS_1), isinglass (IL_1 and IS_4), potassium caseinate (CKS_1), casein (CS_4) and gelatin (GS_2, GS_4 and GL_1) on red wine phenolic compounds are discussed.

(PM) della caseina e del caseinato di potassio è caratterizzata da una banda di 30,0 kDa e quella di albumina di uovo da una banda di 43,0 kDa. La colla di pesce (vescica natatoria) presenta bande a 20,1, comprese tra 94,0-43,0 e sopra i 94,0 kDa. Inoltre, due delle gelatine esaminate non presentano alcuna banda nell'intervallo di PM considerato. Gli altri agenti di chiarificazione hanno rivelato polidispersione. Il punto isoelettrico delle proteine studiate variava tra 4,20 a 6,48. Sono stati discussi gli effetti dell'albumina d'uovo (AS_1), della colla di pesce (IL_1 e IS_4), del caseinato di potassio (CKS_1), della caseina (CS_4) e della gelatina (GS_2, GS_4, e GL_1) sui composti fenolici del vino rosso.

INTRODUCTION

The oenological fining agents are very diverse and complex. They are usually made from non-modified animal proteins or from protein extracts obtained after adequate treatment of animal tissue (AMATI and MINGUZZI, 1976). Recently, other protein sources, such as cereals and legumes, have been studied as wine fining agents (MARCHAL *et al.,* 2000a; b; 2002; PANERO *et al.,* 2001; MAURY *et al.,* 2003). Gelatin, isinglass, casein, potassium caseinate and egg albumin are the most commonly used proteins in wine fining. They can be used separately or with mineral fining agents (MACHADO-NUNES *et al.,* 1998), such as bentonite or silica gel.

Proteins used as wine fining agents have different physico-chemical characteristics mainly molecular weight (MW) distribution, isoelectric point (IEP) and surface charge density. Several authors have shown that these characteristics influence the properties of fining agents (HRAZDINA *et al.,* 1969; PAETZOLD and GLORIES, 1990; LAGUNE and GLORIES, 1996a; b; VERSARI *et al.,* 1998). It has been pointed out that the molecular weight of gelatin influences the amount and type of phenolic compounds removed from red wine (HRAZDINA *et al.,* 1969; YOKOTSUKA and SINGLETON, 1987; RICARDO-DA-SILVA *et al.,* 1991; LAGUNE and GLORIES, 1996b; SCOTTI and POINSAUT, 1997; VERSARI *et al.,* 1998; LEFEBVRE *et al.,* 1999; SARNI-MANCHADO *et al.,* 1999; MAURY *et al.,* 2001). For example, MAURY *et al.* (2001) showed that more hydrolysed gelatins eliminate more polymerized tannins than less hydrolysed ones.

Gelatins have been the most studied fining agents (PAETZOLD and GLORIES, 1990; MARCHAL *et al.,* 1993; 2002; LAGUNE and GLORIES, 1996a; b; VERSARI *et al.,* 1998; 1999). These products, obtained by enzymatic hydrolysis, showed that most of the protein fractions had MWs lower than 13.7 kDa (PAETZOLD and GLORIES, 1990); several authors have also verified that liquid and hot soluble gelatins show polydispersion in the MW distribution (PAETZOLD and GLORIES, 1990; MARCHAL *et al.,* 1993;

2000a, b; 2002; VERSARI *et al.,* 1998; 1999). The IEP of gelatin depends on the technological processes. When the insoluble collagen is transformed into soluble gelatin by either an acid or basic process, a gelatin of Type A or B, respectively, is obtained (KAUFMANN, 1988; LAGUNE and GLORIES, 1996c): The IEP of Type A gelatin ranges from 7.5 to 9.5 and of Type B from 4.7 to 5.0 (PAETZOLD and GLORIES, 1990). MARCHAL *et al.* (2000a) reported that the electrophoretic pattern of solid isinglass presented individualized bands with a MW between 17 and 80 kDa, and another isinglass revealed bands with MWs between 110 and 220 kDa. The casein fining agent showed a band close to 30 kDa (MARCHAL *et al.,* 2000a; b), with some other bands with lower MWs (10-23 kDa), as well as some with higher MWs (50-80 kDa). Milk casein is a heterogeneous group of four principal phosphoproteins and phosphoglycoproteins (α_{s1}-casein, α_{s2}-casein, κ-casein and β-casein) whose MW ranges from 11.6 to 24.1 kDa with an average isoelectric point of 4.6 (EVANS, 1982; FOX *et al.,* 1982). Similarly, egg white is a mixture of different proteins, where ovalbumin (phosphoglycoprotein) makes up about 54% of the total proteins, with a MW of 45 kDa and an isoelectric point of 4.6 (CHEFTEL *et al.,* 1985; FRONING, 1988). Other proteins in egg white showed antimicrobial factors such as conalbumin (MW 76 kDa; pI 6.1), lysozyme (MW 14.3 kDa; pI 10.7) and avidin (MW 68.3 kDa; pI 10.0) or enzyme inhibitors including ovomucoid (MW 28 kDa; pI 4.1), ovoinhibitor (MW 49 kDa; pI 5.1) and ficin (MW 12.7 kDa; pI 5.1) (FRONING, 1988). The electrophoretic pattern of solid egg albumin fining agent had a band close to 43-45 kDa, along with two other bands at 15 and 90 kDa, as well as several minor bands between 25 and 100 kDa (MARCHAL *et al.,* 2002).

Protein fining agents exhibit different surface charge densities when evaluated in a model solution like wine. Depending on the type of gelatin and the pH of the medium, the surface charge density ranged from 0.02 to 1.2 meq/g (PAETZOLD and GLORIES, 1990; LAGUNE and GLORIES, 1996a; b; LAMADON *et al.,* 1997). Surface charge density for different isinglasses, evaluated at a pH between 2.8 and 3.8 ranged from 0.32 to 0.83 meq/g, and for egg albumin (solid and fresh) at a pH between 3.0 and 4.0 ranged from 0.22 to 0.96 meq/g. The surface charge density of potassium caseinates estimated at pH 7 was close to 0.5 meq/g (LAMADON *et al.,* 1997).

Wine fining agents are added exogenous products that should not contribute compounds such as lead (Pb) and cadmium (Cd) to the wine (OIV, 2006a; b). The technology of making high-quality wines includes an accurate quantitative knowledge of the presence of these elements and their continuous monitoring (BRAININA *et al.,* 2004). These elements need to be quantified due to their high toxicity and potentially by adverse health effects. In order to protect consumer health, Pb and Cd levels are limited by regulations (for fining agents and wine) (MENA *et al.*, 1996; LEMOS *et al.,* 2002).

Given the important role that protein fining agents play in wine quality and safety, it is important to characterize them. To our knowledge, there are no data in the literature concerning the electrophoretic patterns for MW distribution of potassium caseinate, liquid isinglass and liquid egg albumin. To our knowledge, the surface charge densities of casein, liquid isinglass and liquid egg albumin have not been published, nor have the isoelectric points of solid and liquid isinglass or of solid and liquid egg albumin. Consequently, the main objectives of this study were: 1) to describe and compare the characteristics such as molecular weight distribution, surface charge density, isoelectric point, and protein, Pb and Cd contents of several protein fining agents present

on the market and 2) to increase the understanding of the action of these proteins on wine limpidity, monomeric anthocyanins and flavonoid and non-flavonoid compounds during the wine fining process.

MATERIALS AND METHODS

Fining agent characterization

Protein fining agents: Two potassium caseinates, two caseins, four egg albumins, four isinglasses and seven gelatins from different companies were characterized (Table 1).

Protein quantification: Total nitrogen was determined by the Kjeldahl method based on mineralization, distillation and titration with 0.1 N HCl (MANFREDINI, 1989; OIV, 2006b). Total protein content was estimated as Kjeldahl nitrogen multiplied by the following factors: 6.38 (OIV, 2006b), for casein and potassium caseinate, 5.55 (LEES, 1971) for gelatin, 6.68 (LEES, 1971) for egg albumin and 6.25 (MACKIE, 1983) for isinglass.

Protein concentration was also determined by the Bradford method modified by READ and NORTHCOTE (1981) to reduce the variation in the response of different proteins. The assay was performed by adding different proteins [protein fining agents and standard protein (bovine serum albumin)] to a dye reagent [Coomassie brilliant blue G-250 (Acros Organics, New Jersey, NJ, USA), ethanol, phosphoric acid and deionized water], which resulted in an increased absorbance at 595 nm, due to the formation of a protein-dye complex (READ and NORTHCOTE, 1981).

Protein molecular weight distribution

Table 1 - Protein fining agents characterized and used in this study.

Product	Code	Concentration[a] (g of commercial fining agent [wet weight])	Producer information
Egg albumin solid	AS_1	12.5 g/hL	-
Egg albumin solid	AS'_1	-	With lysozyme
Egg albumin solid	AS_4	-	-
Egg albumin liquid	AL_4	-	-
Isinglass solid	IS_1	-	Collagen hydrolysis contained in fish skin
Isinglass solid	IS_4	2.25 g/hL	Obtained from swim bladder
Isinglass liquid	IL_1	50 mL/hL	Collagen hydrolysis contained in fish skin
Isinglass liquid	IL_4	-	Collagen hydrolysis contained in fish skin
Potassium caseinate solid	CKS_3	-	-
Potassium caseinate solid	CKS_1	40 g/hL	-
Casein solid	CS_2	-	-
Casein solid	CS_4	40 g/hL	-
Gelatin solid	GS_3	-	Cold soluble
Gelatin solid	GS_2	8 g/hL	Hot soluble
Gelatin solid	GS_4	8 g/hL	Cold soluble
Gelatin liquid	GL_1	50 mL/hL	High concentrated
Gelatin liquid	GL_2	-	-
Gelatin liquid	GL_5	-	-
Gelatin liquid	GL_4	-	Pig source

A-Egg albumin, C-Casein, CK-Potassium caseinate, I-Isinglass, G-Gelatin, S-Solid, L-Liquid.
1, 2, 3, 4 and 5 different fining agent suppliers. a-used in the wine fining trials.

characterized by SDS-PAGE: Molecular weight distributions of oenological protein fining agents were studied by the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) method as suggested by LAEMMLI (1970) and adapted for protein fining agents by MARCHAL *et al.* (2000a; b; 2002). Standard proteins covering a 14.4 to 94.0 kDa range were used to evaluate the molecular weight [Low Molecular Weight (LMW) Amersham Biotech, London, U.K.]. Samples and standard proteins were treated with buffer [(0.125 M Tris-Cl, 4% SDS, 20% glycerol, 2% 2-mercaptoetanol, pH 6.8)] (v/v) and denatured at 100°C for 5 minutes. A 5 μL sample was loaded in each electrophoresis well, which corresponds to a protein content (determined by the modified Bradford method) of 2.7-2.8 μg for potassium caseinates, 1.5-3.0 μg for caseins, 1.3-6.0 μg for isinglasses and 1.0-5.8 μg for gelatins. The gel with 0.75 mm thickness was run in a mini-vertical gel electrophoresis unit (Mighty-Small II SE 250, Hoefer, San Francisco, CA, USA) at a constant voltage (75 V) at 20°C until the bromophenol blue reached the bottom of the gel. After migration, proteins were stained in a solution made up of one part Coomassie blue R-350 (Amersham Bioscience, Uppsala, Sweden) and nine parts of a solution with methanol: acetic acid: water (3:1:6) and destained in a mixture of acetic acid: methanol: water (1:2:7) (MARCHAL *et al.*, 2000a; b; 2002).

pH: The pH was measured on a 1% solution of initial product (w/v) of solid gelatin, solid isinglass and solid egg albumin. The pH was measured on a 5% solution of initial product (w/v), of solid potassium caseinate and on a 10% solution of initial product (w/v) of solid casein. The pH determination was based on the International Codex of Oenology (OIV, 2006b). The pH was measured directly in the colloidal solution of the liquid fining agents (gelatin, isinglass and egg albumin).

Weight loss on drying: The weight loss was determined according to the International Codex of Oenology (OIV, 2006b) at 100°-105°C on a 2 g sample of the following proteins: casein, potassium caseinate, egg albumin, solid gelatin and solid isinglass. In the case of a colloidal solution of gelatin, egg albumin or isinglass, a 10 g sample was used, which was dried over water at 100°C for four hours, and then dried in an oven at 100°-105°C for three hours.

Ash: Ash was evaluated by progressive incineration at 500°-550°C of the residue that remained after the determination of loss during drying, according to the International Codex of Oenology (OIV, 2006b).

Lead and Cadmium: Lead and cadmium were determined by graphite furnace atomic absorption spectrometry using Zeeman background correction according to CATARINO and CURVELO-GARCIA (1999). These analyses were performed at the "Estação Vitivinicola Nacional" laboratory, Dois Portos, Portugal.

Surface charge density: Surface charge density of protein fining agents was measured with a particle charge detector – produced by MÜTEK (Herrsching, Germany) model PCD 03 pH – by titration with a charge compensating polyelectrolyte 0.001 N electropositive-polydiallyldimethylammonium [polyDADMAC (Herrsching, Germany)] or 0.001 N electronegative-sodium polyethylensulfate [PES-Na (Herrsching, Germany)] (PAETZOLD and GLORIES, 1990; DIETRICH and SCHÄFER, 1991) until the streaming potential was 0 mV, which corresponds to the point where all charges are neutralized. The volume of polyelectrolyte needed for the neutralisation allowed the surface charge density of the product, to be evaluated; it is expressed in milliequivalents of polyelectrolyte per gram of fining agent

(meq/g). All determinations were done at 20°C.

The fining agents – gelatin, isinglass and egg albumin – were dispersed in a model solution similar to wine but lacking ethanol (VERNHET *et al.*, 1996).

Caseins and potassium caseinates were first dissolved in 0.1 N KOH and then dispersed in the model solution. The surface charge density of these fining agents was measured at the pH of dissolution and at pH 3.4 (adjusted with 50% HCl and centrifuged at 4.000 rpm for 15 min).

Isoelectric point: The isoelectric point from the protein fining agents dispersed in distilled water was evaluated with a model PCD 03 pH particle charge detector (MÜTEK, Herrsching, Germany) by titration with an acid or basic solution until the streaming potential was 0 mV. The pH measured corresponds to the isoelectric point.

Wine fining trials

Protein fining agents: One egg albumin (AS_1), two isinglasses (IL_1, IS_4), one potassium caseinate (CKS_1), one casein (CS_4) and three gelatins (GL_1, GS_2 and GS_4) were added to a young red wine. All these protein fining agents were previously characterized.

Red wine: The young red wine (vintage 2003) used in this study was produced from different grapevine varieties from the Estremadura Region (North of Lisbon) and had the following chemical characteristics: alcohol content 8.7% (v/v), density 0.9972, titratable acidity 7.6 g/L expressed as tartaric acid, volatile acidity 0.76 g/L expressed as acetic acid, pH 3.31, free sulphur dioxide 10 mg/L and total sulphur dioxide 46 mg/L.

Fining trials: Fining experiments were carried out by adding protein fining agents (isinglass, egg albumin, casein, potassium caseinate and gelatin) at the average levels and prepared as recommended by the producers (Table 1) to 250 mL of wine. An untreated sample was used as a control. The fining agents were thoroughly mixed and allowed to remain in contact with the wine for 7 days at 20°C. All experiments were done in duplicate.

Limpidity: Limpidity was evaluated by measuring the optical density at 650 nm of the centrifuged and non-centrifuged wine as described by FEUILLAT and BERGERET (1966).

Monomeric anthocyanins: Monomeric anthocyanin analysis was performed by High Performance Liquid Chromatograph (HPLC) according to DALLAS and LAUREANO (1994). The equipment used for the HPLC analysis was a Perkin-Elmer (Norwalk, CT, USA) system, equipped with a model L-7100 Lachrom Merck Hitachi-High-Technologies pump (Tokyo, Japan), a model LC-95 UV-Vis detector set at 520 nm coupled to a version 6.2 Konikrom data chromatography treatment system (Konik Instruments, Konik-Tech, Barcelona, Spain). The column was a reversed-phase C_{18} Lichrosphere 100 (5 μm packing, 250x4.6 mm i.d.) (Merck, Darmstadt, Germany) protected with a guard column of the same material. The separation was performed at room temperature. The elution conditions for monomeric anthocyanins was as followed: 0.7 mL/min, flow rate, solvent A was 40% formic acid, solvent B was CH_3CN and solvent C was double distilled water. The initial conditions were 25% of A, 6% of B and 69% of C for 15 min followed by a linear gradient to 25% of A, 25.5% of B 49.5% of C during 70 min, and 20 min of 25% A, 25.5% of B and 49.5% of C. Wine samples were analysed in duplicate after filtration.

Quantification of monomeric anthocyanins in wine was carried out by means of standard curves prepared by using different concentrations of malvidin 3-glucoside chloride in methanol 0.1% HCl. The peak area was converted to mg/L of

malvidin 3-glucoside equivalent. Twenty μL of each concentration were injected in triplicate.

Chromatic characterization: The absorption spectra of the wine samples were recorded with a Unicam UV-vis UV4 spectrophotometer (Unicam, Cambridge, U.K.), scanned over the range 380 to 770 nm using quartz cells of 1-mm path length. Data were collected at 10 nm intervals, and referred to a 1-cm path length, in order to calculate L* (lightness), a* (measure of redness) and b* (measure of yellowness) coordinates using the CIELab method (OIV, 1990). The spectrophotometer has the required software to calculate the CIELab parameters directly (Chroma version 2.0 Unicam, Cambridge, U.K.). To differentiate the colour more precisely, the colour difference was obtained using the following expression: $\Delta E^* = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2}$, in CIELab units. It quantifies the overall colour difference of a given sample when compared to a reference sample (non-treated sample). The mean visual perception of colour difference between two solutions will be assumed as a value of $\Delta E^* = 1$ (GONNET, 1998). All samples had been clarified by centrifugation and were analysed in duplicate.

Quantitative estimation of flavonoid phenols and non-flavonoid phenols: Determination of the phenol content before and after precipitation of the flavonoids through reaction with formaldehyde was done according to KRAMLING and SINGLETON (1969). All samples were analysed in duplicate.

Statistical analysis

The data are presented as the mean±SD. Analysis of variance and comparison of treatment means (LSD, 5% level) were performed using ANOVA Statistica 5.1 software (StatSoft, Tulsa, OK, USA) in that it compared the effect of the protein fining agents.

RESULTS AND CONCLUSIONS

Characterization of fining agents

Protein content

Total nitrogen values of the protein fining agents ranged from 11.1 to 22.8% (w/w) expressed in dry weight (Table 2). Regarding the protein content estimated by the total nitrogen, the liquid fining agents in general had the highest values when expressed in dry weight. In the case of solid gelatins, the values [88-98% (w/w)] agree with previously published data (VERSARI *et al.,* 1998). The protein content obtained by a modified Bradford method (READ and NORTHCOTE, 1981) were lower than those estimated by converting total nitrogen to protein. This can probably be explained by the fact that Coomassie Blue G Dye reacts poorly with proteins whose MW ranges from 3 to 10 kDa (BOULTON *et al.,* 1995; MARCHAL *et al.,* 1997). The fining agents that are intensely hydrolyzed during production include many low MW protein fractions, which have a reduced response to this method, as can be seen for gelatin. Due to the large range in protein concentrations observed, different quantities of fining agent should be added in order to obtain the same final concentration in the wine.

Protein molecular weight distribution

The MW distributions of potassium caseinate (CKS_1 and CKS_3) and casein (CS_2 and CS_4) observed in the SDS-PAGE electrophoretic patterns (Fig. 1), differed among these fining agents, but were similar within each group (potassium caseinate or casein). The potassium caseinates (CKS_1 and CKS_3) and caseins (CS_2 and CS_4) both presented a major band at 30.0 kDa with other bands at lower and higher MWs. The potassium caseinates however had more bands with MWs less than 30.0 kDa, particularly CKS_3.

Table 2 - Total nitrogen, surface charge density, isoelectric point, protein, lead and cadmium content of the fining agents.

Product	Total Nitrogen [a] (% N) (% w/w, dry weight)	Protein content [a] as % Nxk (% w/w, dry weight)	Protein content [a] by Bradford method (% w/w, dry weight)	Surface charge density [a] (meq /g product at pH 3.4)	Isoelectric point [a]	Pb [a] (mg/kg dry weight)	Cd [a] (mg/kg dry weight)
AS_1	11.9±0.2	78±1	52.6±2.0	0.73±0.01	5.00±0.02	0.25±0.003	n.d.
AS'_1	14.1±0.2	94±2	54.1±0.8	0.72.±. 0.02	4.96.±.0.01	0.22±0.003	n.d.
AS_4	14.5±0.2	97±1	65.7±0.7	0.79±0.01	5.01±0.03	0.16±0.002	n.d.
AL_4	15.9±0.3	106±2	55.8±0.8	0.07±0.01	5.14±0.05	0.64±0.027	0.01±0.0003
IS_1	17.0±0.2	106±1	2.0±0.2	0.20±0.02	4.21±0.05	0.44±0.016	n.d.
IS_4	11.6±0.4	73±3	26.4±1.0	0.41±0.01	6.48±0.03	0.82±0.003	0.01±0.0003
IL_1	17.9±0.6	112±4	1.8±0.3	0.04±0.00	4.55±0.02	0.38±0.018	n.q.
IL_4	19.1±0.7	119±4	6.2±0.1	0.09±0.00	5.24±0.04	0.16±0.007	n.q.
CKS_3	14.5±0.2	93±2	58.8±1.6	0.09±0.01	4.53±0.02	0.78±0.008	n.q.
CKS_1	13.3±0.2	85±2	59.5±5.5	0.04±0.00	4.51±0.04	0.62±0.002	0.01±0.0003
CS_2	14.2±0.2	91±1	65.9±5.3	0.25±0.03	4.65±0.01	0.35±0.006	0.01±0.0006
CS_4	11.1±0.2	71±1	33.4±1.2	0.09±0.01	4.64±0.06	0.47±0.032	n.d.
GS_3	15.9±0.3	88±2	4.0±0.1	0.28±0.00	4.65±0.06	0.22±0.003	0.01±0.0012
GS_2	17.7±0.2	98±1	15.0±4.1	0.74±0.02	4.74±0.00	0.40±0.019	n.q.
GS_4	16.3±0.7	91±4	4.8±0.1	0.26±0.00	4.50±0.00	0.28±0.031	n.d.
GL_1	16.5±0.4	92±2	4.8±0.3	0.11±0.00	4.20±0.01	1.10±0.057	n.d.
GL_2	18.1±0.1	100±1	2.8±0.3	0.07±0.00	4.41±0.03	0.55±0.007	0.09±0.0014
GL_5	20.0±0.7	111±4	7.6±0.1	0.07±0.00	5.46±0.01	0.22±0.025	n.d.
GL_4	22.8±0.5	126±3	14.1±0.2	0.10±0.00	5.31±0.00	0.18±0.006	n.q.

A-Egg albumin, C-Casein, CK-Potassium caseinate, I-Isinglass, G-Gelatin, S-Solid, L-Liquid.

1, 2, 3, 4 and 5 different fining agent suppliers.

k - Multiplication factor, which was 6.68 for egg albumin; 6.25 for isinglass; 6.38 for casein and potassium caseinate; 5.55 for gelatin.

a - mean values of triplicate determinations ± Standard Deviation (SD).

nd-not detected (values below the limit of detection), nq-not quantified (values below the limit of quantification).

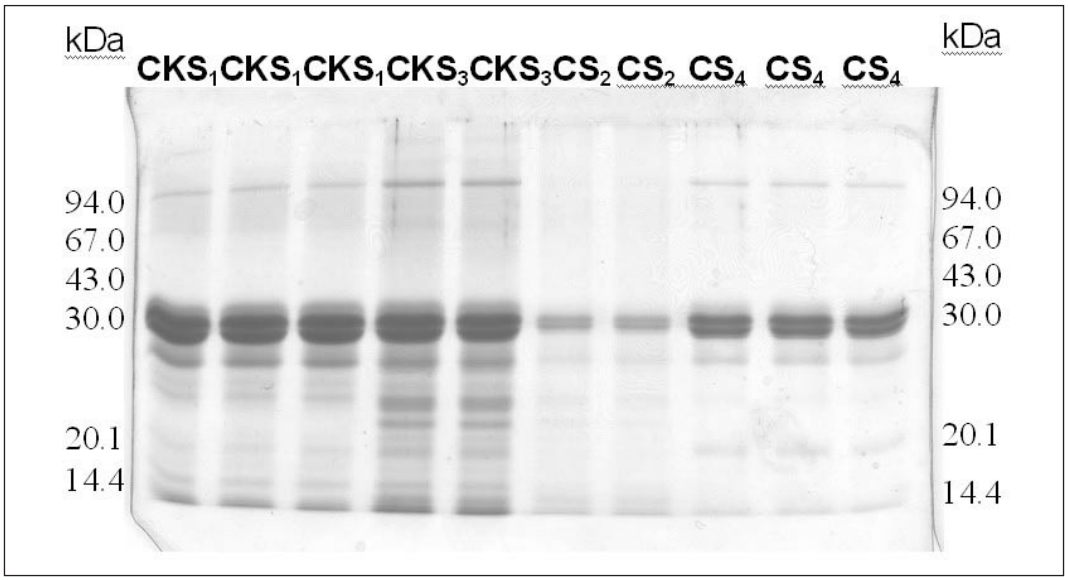


Fig. 1 - Electrophoretic patterns of potassium caseinates – CKS_1, CKS_3 and caseins – CS_2, CS_4.

No relevant differences were detected in the MW distribution among the egg albumins (AS_1, AS'_1, AS_4 and AL_4) (Fig. 2). They were characterized by bands at 43.0 kDa and at 14.4 kDa, with other bands between 67.0 and 94.0 kDa and between 20.1 and 43.0 kDa, as reported by MARCHAL *et al.* (2002) for solid egg albumin.

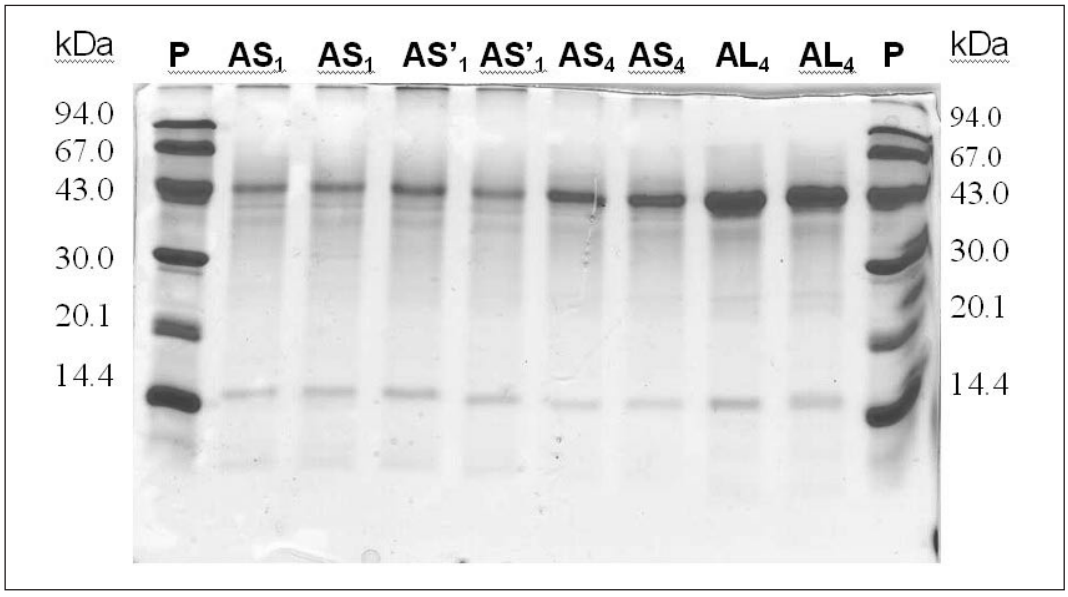


Fig. 2 - Electrophoretic patterns of egg albumins – AS_1, AS'_1, AS_4 and AL_4. MW standard – P, are given on the left and right side.

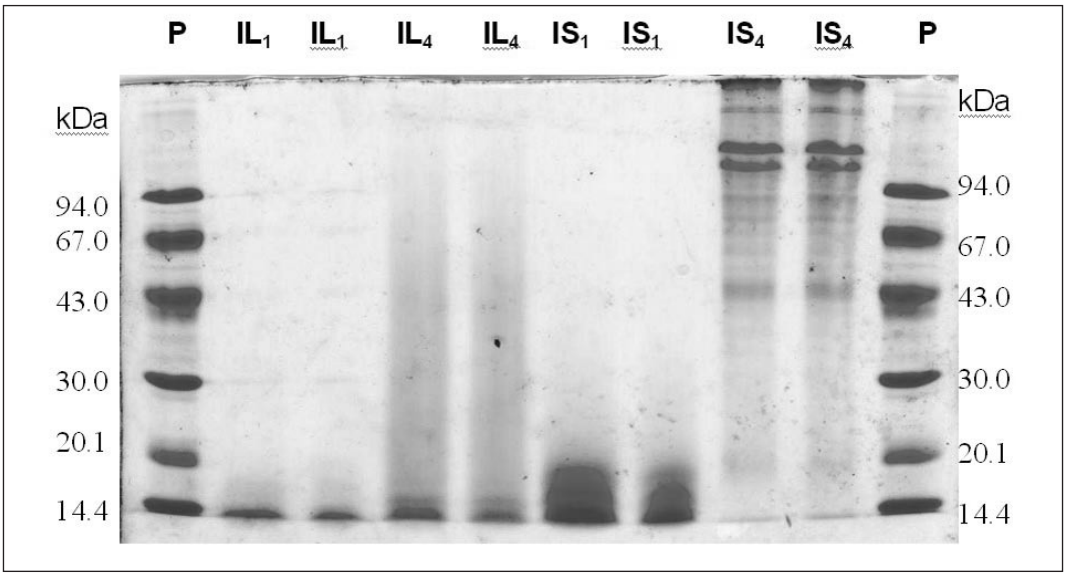


Fig. 3 - Electrophoretic patterns of isinglasses – IL_1, IL_4, IS_1 and IS_4. MW standard – P, are given on the left and right side.

However, in the case of several isinglasses (IS_1, IL_1, IS_4 and IL_4) the electrophoretic patterns were not similar (Fig. 3). IL_1 and IS_1 showed a polydispersion in the low MW range (20.1-14.4 kDa), as did, IL_4 (94.0-14.4 kDa). In contrast, IS_4 presented several individual bands, namely: one at 20.1, several above 94.0 and some between 94.0 and 43.0 kDa. To our knowledge, the literature has only reported electrophoretic patterns of isinglasses with individualized bands (MARCHAL *et al.,* 2000a; BONERZ *et al.,* 2004).

The electrophoretic patterns of gelatins (GS_3, GS_4, GS_2, GL_1, GL_2, GL_4 and GL_5) are illustrated in Figs. 4 and 5. No bands were detected in the MW range (94.0-14.4 kDa) for gelatins GS_3 and GS_4 (Fig. 4). These results are in accordance with PAETZOLD and GLORIES (1990) who indicated that gelatins obtained by enzymatic hydrolysis present several polypeptides with MWs lower than 13.7 kDa. The gelatins GS_2, GL_1, GL_2, GL_4 and GL_5 showed polydispersion in the MW distribution, which has also been confirmed by other authors (MARCHAL *et al.,* 1993; 2000a; b; 2002). The polydispersion with respect to molecular weight is a result of the breakdown of intact collagen to produce commercial gelatins (SIMS *et al.,* 1997). While, GS_2 showed more protein fraction with high MW (>43.0 kDa) (Fig. 4), GL_1, GL_2, GL_4 and GL_5 presented very similar electrophoretic profiles, with MWs lower than 43.0 kDa (Fig. 5). According to the literature (LAGUNE and GLORIES, 1996a; VERSARI *et al.,* 1999), the electrophoretic profiles are directly related to the particular elaboration processes.

Surface charge density

The highest surface charge densities were found in solid egg albumin (AS_1, AS'_1 and AS_4) and gelatin GS_2, which had the highest value within the solid gelatins studied (Table 2). These results can be associated with the lower degree of hydrolysis of these proteins (SCOTTI and POINSAUT, 1997; LAMADON *et al.,*

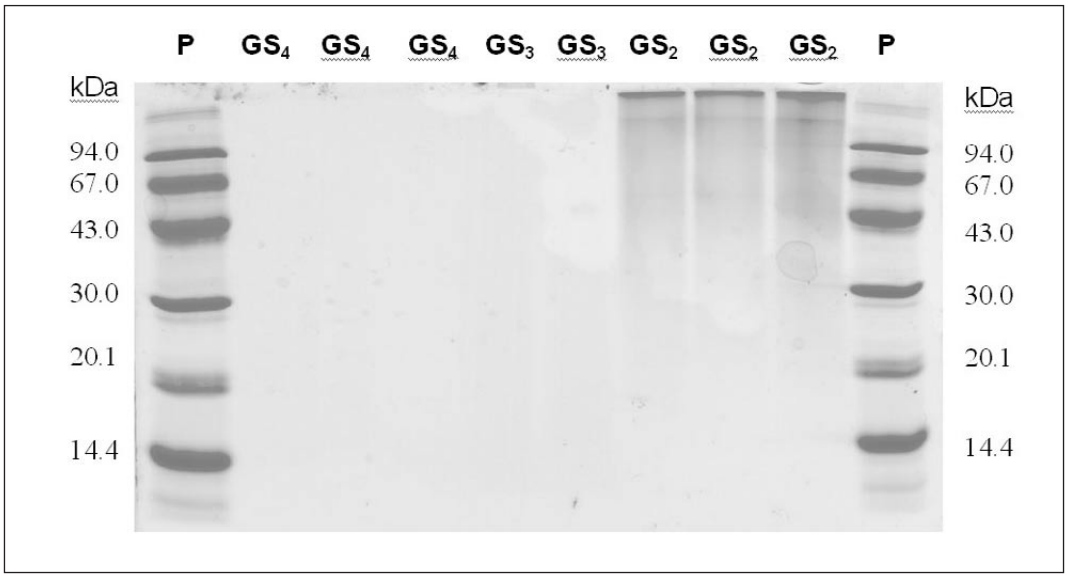


Fig. 4 - Electrophoretic patterns of gelatins – GS_4, GS_3 and GS_2. MW standard – P, are given on the left and right side.

1997) as shown by the electrophoretic profiles.

As previously described, casein and potassium caseinate were first dissolved in KOH and then dispersed in a model solution without ethanol. The surface charge densities of these fining agents were measured at the pH of dissolution

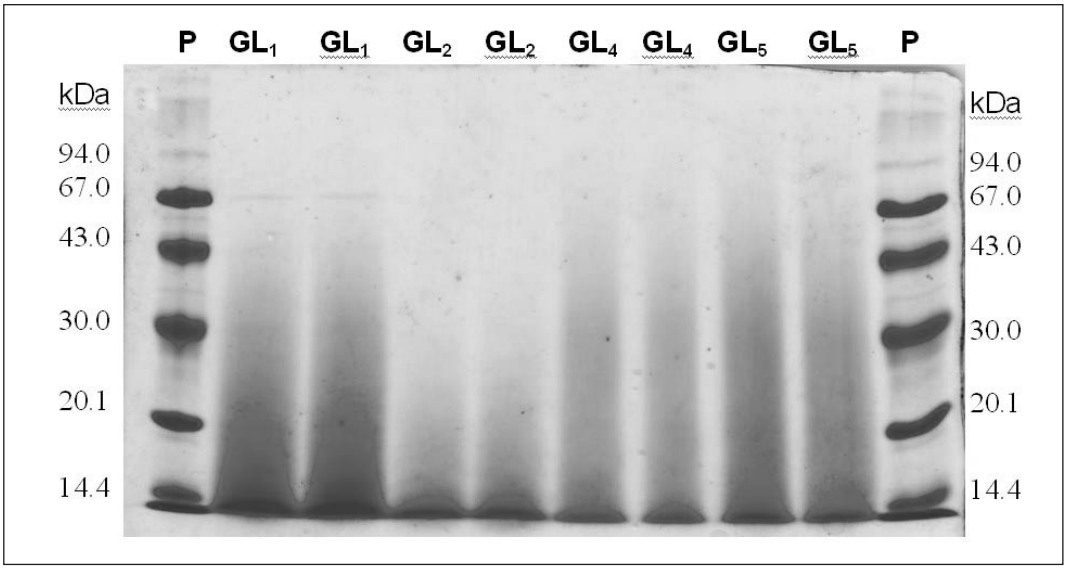


Fig. 5 - Electrophoretic patterns of gelatins – GL_1, GL_2, GL_4 and GL_5. MW standard – P, are given on the left and right side.

(CS_2 – pH 5.30, CS_4 – pH 6.80, CKS_1 – pH 5.48 and CKS_3 – pH 5.30) and afterwards, at pH adjusted to 3.4. The surface charge density of CKS_1, CKS_3 and CS_4 decreased after the pH adjustment (from 0.32 to 0.04; 0.33 to 0.09 and 0.61 to 0.09 meq/g of product, respectively), but the surface charge density of CS_2 remained constant (0.25 meq/g of product).

It was also observed that the shape of the titration curve was related to the electrophoretic pattern. If the fining agent showed an electrophoretic pattern with individualized bands, the titration curve was constant during a certain volume of titration and then presented a sudden decrease. In contrast, for the fining agents that had an electrophoretic pattern that was a polydispersion, the titration curve showed a continuous decrease. DIETRICH and SCHÄFER (1991) suggested that the sample conductivity may influence the shape of the titration curve.

Isoelectric point

The IEP of the studied fining agents ranged from 4.20 to 6.48 (Table 2). Potassium caseinate (CKS_3 and CKS_1) and casein (CS_2 and CS_4) had very similar IEP values that are in accord with the data reported by MANFREDINI (1989) and STOCKÉ and ORTMANN (1999) for potassium caseinate. The egg albumin IEP values (AS_1, AS'_1, AS_4 and AL_4) were 4.96-5.14 and the isinglass IEP values varied from 4.21-6.48 in solid or liquid state. The gelatins had IEP values ranging from 4.20 (GL_1) to 5.46 (GL_5), which suggests that the gelatins studied are of Type B - basic hydrolysis (PAETZOLD and GLORIES, 1990).

Lead and Cadmium

The level of Pb was below 0.5 mg/kg in 68% of the fining agents studied. The average Pb content was about 0.43 mg/kg, ranging from 0.16 to 1.10 mg/kg (Table 2).

In thirteen samples the Cd levels were below the quantification and detection limits (QL = 0.15 μg/L, DL = 0.05 μg/L). In the other six samples the Cd content was less than 0.01 mg/kg, except gelatin GL_2, which had 0.09 mg/kg (Table 2).

All of the Pb and Cd values measured in the protein fining agents were below the limits recommended by the International Organization of Vine and Wine [(fining agents: Pb < 5 mg/kg for casein, potassium caseinate, gelatin, isinglass and egg albumin, Cd < 1 mg/kg and < 0, mg/kg for casein and gelatin, respectively); (wine: Pb < 200 μg/L; Cd < 10 μg/L)] (OIV, 2006a; b) which guarantees wine production without metal enrichment.

Loss during drying, ash and pH

The loss of solid fining agents during drying was 6.6 to 12.0% (w/w); as expected the values were lower than those for the liquid agents in which water loss was 52.1 to 89.5% (w/w) (Table 3). The lowest ash content was observed for gelatin GS_4 and isinglass IS_1, respectively, 0.3 and 0.8% (w/w) (Table 3). An unexpected value of 22.1% (w/w) was found for casein CS_4. According to MANFREDINI (1989) the ash content of casein could indicate the manner of production in that the low values indicated that casein was coagulated by mineral acid

All of the fining agents studied had acidic or almost neutral pH (Table 3). Potassium caseinate with higher pH values had a better solubilization but a lower flocculation capacity (MANFREDINI, 1989).

Wine fining trials

Limpidity

With respect to limpidity, it was shown that proteins with higher surface charge density increased wine limpidity. A linear correlation was found between total surface charge density and decrease of turbidity (Fig. 6). So, among the fining proteins assayed, the best results were ob-

Table 3 - Weight loss on drying, ash and pH of fining agents.

Product	Weight loss [a] (%w/w)	Ash [a] (% w/w, dry weight)	pH [a]
AS_1	7.3±0.0	3.6±0.3	6.15±0.01
AS'_1	8.2±0.1	2.6±0.3	5.94±0.01
AS_4	8.4±0.1	4.1±0.2	6.23±0.02
AL_4	88.0±0.0	6.3±0.1	6.64±0.04
IS_1	6.8±0.2	0.8±0.1	5.06±0.03
IS_4	9.9±0.1	2.0±0.3	3.58±0.09
IL_1	71.6±0.1	1.7±0.0	5.32±0.01
IL_4	83.7±0.1	1.9±0.1	4.47±0.02
CKS_3	7.7±0.0	3.3±1.1	6.04±0.02
CKS_1	6.6±0.0	5.1±0.2	7.83±0.03
CS_2	8.7±0.2	3.3±0.0	5.72±0.03
CS_4	9.7±0.0	22.1±0.0	5.86±0.01
GS_3	8.2±0.1	1.1±0.1	5.47±0.03
GS_2	12.0±0.9	2.3±0.1	4.68±0.03
GS_4	10.1±0.0	0.3±0.0	5.51±0.02
GL_1	52.1±1.4	2.3±0.6	6.08±0.01
GL_2	53.5±0.7	2.1±0.1	5.66±0.02
GL_5	89.5±0.0	2.9±0.7	3.87±0.01
GL_4	85.1±0.2	4.6±0.8	4.22±0.01

A-Egg albumin, C-Casein, CK-Potassium caseinate, I-Isinglass, G-Gelatin, S-Solid, L-Liquid.
1, 2, 3, 4 and 5 different fining agent suppliers.
a - mean values of triplicate determinations ± Standard Deviation (SD).

tained with gelatin GS_2 (MW > 43.0 kDa) and egg albumin AS_1 (band close to 43.0 kDa) and the worst with isinglass IL_1 (MW < 20.1 kDa). These results are in accord with SCOTTI and POINSAUT (1997) and VERSARI *et al.* (1998; 1999), in which assays made with gelatin, showed an increase in precipitation due to the increase of the surface charge density and therefore greater limpidity in the wine.

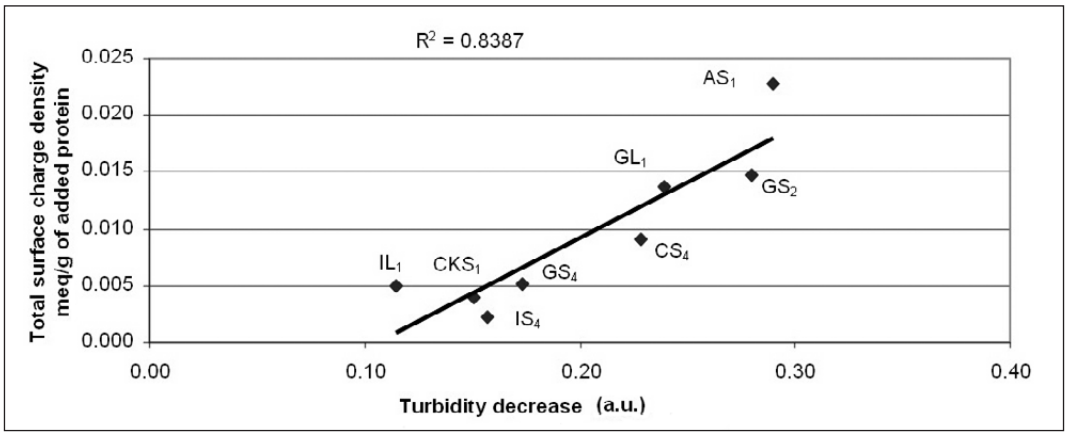


Fig. 6 - Total surface charge density of protein fining agents versus turbidity decrease. Isinglass (IL_1), isinglass (IS_4), casein (CS_4), potassium caseinate (CKS_1), egg albumin (AS_1), gelatin (GL_1), gelatin (GS_2), gelatin(GS_4). The turbidity of the unfined wine (T) was 0.421.

Influence of the fining protein on chromatic characteristics and monomeric anthocyanins

The results obtained with the CIELab method for determining the chromatic characteristics of the unfined and fined wine with different proteins showed that there were significant changes after fining (Table 4). In all fined wines, the lightness (L*) increased significantly, therefore the unfined wines were darker. The increase in lightness (L*) of the fined wine seemed to be correlated with less redness a* (NEGUERUELA *et al.,* 1995), due to the removal of pigments as previously observed by GIL-MUÑOZ *et al.* (1997). These data are in agreement with the results obtained for monomeric anthocyanins (Table 5) and for total and polymeric pigments (molecules that result from the condensation of anthocyanins with tannins) (COSME *et al.*, 2006). This confirms that the different protein fining agents promote a decrease of these compounds. With regard to the values obtained for the colour difference (ΔE), between each wine and the unfined wine (Table 4), with exception of wine fined with IL_1 all the others had values higher than one cielab unit, indicating that the colour differences can be detected visually (GONNET, 1998).

The isinglasses had the least effect on the total monomeric anthocyanin content (<5% decrease); similar results were reported by BONERZ *et al.* (2004). However, isinglass IS_4 obtained from swim bladder (with bands at MW > 94.0, 94.0-43.0 and at 20.1 kDa) had a different effect when compared with isinglass IL_1 (MW < 20.1 kDa). This fining agent induced a minor decrease in peonidin-3-glucoside, peonidin-3-p-coumarylglucoside, malvidin-3-glucoside, malvidin-3-acetylglucoside and malvidin-3-p-coumarylglucoside. Gelatin GS_4, casein and potassium caseinate removed the total monomeric anthocyanins to the greatest extent (28.2, 20.2 and 11.9%, respectively). These findings agree with LOVINO *et*

Table 4 - Flavonoids, non-flavonoids, total phenols and chromatic characteristics of both fined and unfined red wine (means ± SD).

Fining Treatment	Flavonoid phenols (mg/L gallic acid)	Non-flavonoid phenols (mg/L gallic acid)	Total phenols (mg/L gallic acid)	L*(%)	a*	b*	Δ E*
T	3816±56 a	361±4 a	4177±63 a	46.3±0.2 a	59.40±0.31 a	5.80±0.06 a	
IL_1	3705±2 b	361±5 a	4066±7 bc	46.8±0.1 b	58.19±0.02 c	4.72±0.07 de	1.72±0.10 b
IS_4	3649±35 bc	319±9 c	3969±45 de	46.7±0.1 bc	58.89±0.28 b	5.50±0.04 b	0.97±0.17 a
CS_4	3605±4 c	297±5 d	3901±0 ef	47.9±0.3 d	56.00±0.13 f	4.66±0.19 e	3.94±0.24 f
CKS_1	3658±14 bc	348±5 b	4005±8 cd	48.5±0.1 e	56.90±0.07 e	4.89±0.06 d	3.46±0.11 e
AS_1	3787±60 a	323±7 c	4110±69 ab	47.0±0.1 bc	58.14±0.04 c	5.07±0.01 c	1.69±0.11 b
GL_1	3710±9 b	306±1 d	4017±9 cd	47.2±0.1 c	57.86±0.08 c	4.65±0.01 e	2.13±0.08 c
GS_2	3810±32 a	322±5 c	4132±27 ab	47.7±0.2 d	57.40±0.28 d	5.75±0.00 a	2.46±0.15 d
GS_4	3489±32 d	361±5 a	3850±27 f	48.8±0.1 e	55.42±0.16 g	5.74±0.05 a	4.70±0.17 g

Unfined (T), isinglass (IL_1), isinglass (IS_4), casein (CS_4), potassium caseinate (CKS_1), egg albumin (AS_1), gelatin (GL_1), gelatin (GS_2), gelatin (GS_4); L* - lightness, a* - redness, b*- yellowness, ΔE - total colour difference. The values corresponding to ΔE were obtained taking as a reference the unfined wine (T). Means (n=2) within a column followed by the same letter are not significantly different (LSD, 5%).

Table 5 - Monomeric anthocyanins (mg/L malvidin-3-glucoside) for both fined and unfined red wine (means ± SD).

	T	IL_1	IS_4	CS_4	CSK_1	AS_1	GL_1	GS_2	GS_4
Delphinidin-3-glucoside	16.7±0.7 a	14.5±2.1 abc	14.5±0.2 abc	11.3±0.7 cd	11.8±0.7 bcd	15.4±2.0 ab	14.4±0.1abc	14.1±0.1 abc	8.9±1.6d
Cyanidin-3-glucoside	1.8±0.0 a	1.8±0.4 a	1.8±0.5 a	1.3±0.1 a	1.4±0.1 a	1.6±0.2 a	1.5±0.1 a	1.5±0.1 a	1.3±0.1 a
Petunidin-3-glucoside	19.8±0.2 a	18.9±2.9 a	18.9±2.6 a	16.1±0.7 ab	16.3±0.5 ab	18.1±0.2 a	17.6±0.1 ab	17.5±0.1 ab	13.6±0.0 b
Peonidin-3-glucoside	131.3±2.4 a	124.4±14.2 a	128.9±15.2 a	94.1±1.4 b	109.1±5.4 ab	124.5±4.5 a	121.7±5.5 a	119.7±0.4 a	93.5±1.1 b
Malvidin-3-glucoside	351.7±6.2 a	342.9±37.9 a	347.2±38.9 a	298.6±16.5 ab	326.8±8.5 ab	337.9±9.3 a	327.6±1.7 ab	321.3±1.6 ab	266.5±3.8 b
Malvidin-3-acetylglucoside	11.6±0.3 a	10.9±1.9 a	11.5±1.7 a	8.3±0.2 bc	9.6±0.1 abc	11.2±0.5 a	10.2±0.0 ab	9.9±0.0 ab	7.6±0.1 c
Peonidin-3-p-coumarylglucoside	10.3±0.0 a	9.3±1.5 ab	9.8±1.5 a	6.6±0.4 d	7.4±0.2 bc	8.7±0.1 abc	9.2±0.0 ab	8.6±0.0 abc	5.0±0.0 d
Malvidin-3-p-coumarylglucoside	31.9±0.3 a	28.8±6.0 a	29.9±6.1 a	22.7±1.2 ab	23.8±0.1 ab	26.3±1.6 ab	26.7±0.0 ab	25.1±0.0 ab	17.9±0.2 b
Σ monomeric anthocyanins	575.5±10.2 a	551.5±18.1 abc	562.5±14.6 ab	459.2±16.0 de	509.2±18.4 cd	543.2±18.0 abc	528.5±17.7 abc	519.7±11.0 bc	414.1±13.4 e

Unfined (T), isinglass (IL_1), isinglass (IS_4), casein (CS_4), potassium caseinate (CKS_1), egg albumin (AS_1), gelatin (GL_1), gelatin (GS_2), gelatin (GS_4). Means (n=2) within a line followed by the same letter are not significantly different (LSD, 5%).

al. (1999) who found that fining red wine with casein decreased the monomeric anthocyanin levels and with RICARDO-DA-SILVA *et al.* (1991) who observed lower concentrations of total anthocyanins in the treated wine. Our results show that the more hydrolysed gelatin GS_4 (MW < 14.4 kDa) always decreased the monomeric anthocyanins more than GL_1 (MW < 43.0 kDa) and GS_2 (MW > 43.0 kDa). Those effects were not statistically different. While potassium caseinate decreased the concentration of total monomeric anthocyanins (66 mg/L) and casein (116 mg/L), these two fining agents had identical electrophoretic profiles and isoelectric points. It should be noted that the reduction observed for casein was mainly due to a decrease in peonidin-3-glucoside and malvidin-3-glucoside.

Influence of the fining protein on flavonoid and non-flavonoid phenols

Regarding the total phenolic content, fining agents induced a reduction from 1.1 to 7.8%; the greatest decrease was observed on gelatin GS_4 (7.8%) followed by casein (6.6%). Fining with higher molecular weight proteins and agents with higher surface charge density such as gelatin GS_2 (MW > 43.0 kDa) and egg albumin (MW close to 45.0 kDa) resulted in a decrease in total phenolic compounds (1.1 and 1.6%, respectively) (Table 4). These results were not statistically significant.

In contrast, significantly different results were observed among the three gelatins (GL_1, GS_2 and GS_4). The gelatin with the lowest MW [GS_4 (MW < 14.4 kDa)] removed significantly more total phenolic compounds than the gelatin with the highest MW [GS_2 (MW > 43.0 kDa)]. With respect to the non-flavonoid and flavonoid compounds, gelatin GL_1 (MW < 43.0 kDa) mainly reduced the non-flavonoid compounds, while gelatin GS_4 (MW < 14.4 kDa) mainly reduced the flavonoids which was probably due to the decrease in anthocyanins (Table 5).

The results for isinglass were similar to those for gelatin. Isinglass IS_4 (with bands at MW > 94.0, 94.0-43.0 and at 20.1 kDa) exerted a greater effect on the total phenolic compounds than IL (MW < 20.1 kDa), by removing a significant amount of non-flavonoid compounds.

While casein and potassium caseinate have analogous protein MW distributions and isoelectric points, the results regarding the reduction of flavonoids and non-flavonoids differed. The casein mainly removed non-flavonoid compounds (decrease of 17.8%), while the potassium caseinate only induced a 3.6% decrease of these compounds.

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PAPER

KINETICS AND THERMODYNAMICS OF HEAT INACTIVATION OF SORGHUM PEROXIDASE

CINETICA E TERMODINAMICA DELL'INATTIVAZIONE TERMICA DELLA PEROSSIDASI DI SORGO

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ABSTRACT

Crude peroxidase was extracted from sorghum seeds four days after germination. It was purified 86.30-fold by DEAE-Cellulose ion exchange chromatography. The kinetics of thermal inactivation was first order in the temperature range of 50°-80°C. At 80°C, the activation energy (ΔEa) for inactivation determined from the Arhrenius plot was -2.983 kcal/mole. The free energy change (ΔG) was 64,416.69 kcal/mol; enthalpy (ΔH) and entropy (ΔS) changes for inactivation were -2,937.83 kcal/

RIASSUNTO

La perossidasi grezza è stata estratta da semi di sorgo quattro giorni dopo la loro germinazione. È stata purificata 86,30 volte con cromatografia a scambio ionico in DEAE-Cellulosa. La cinetica di inattivazione termica era di primo ordine nell'intervallo di temperature compreso fra 50° ed 80°C. Ad 80°C, l'energia di attivazione (ΔEa) per l'inattivazione dell'enzima, determinata dal diagramma di Arhrenius, era di -2,983 kcal/mole. La variazione di energia libera (ΔG) era 64.416,69 kcal/mol; le va-

- Key words: kinetics, peroxidase, regeneration, sorghum, thermodynamics, thermoinactivation -

mol and -190.81 kcal/mol, respectively. Regeneration of activity after inactivation was observed, with the regenerated enzyme molecule being less stable than the native enzyme.

riazione di entalpia (ΔH) ed entropia (ΔS) per l'inattivazione erano, ripettivamente, -2.937,83 kcal/mol e -190,81 kcal/mol. È stata studiata la rigenerazione dell'attività enzimatica dopo l'inattivazione; in tale studio la molecola di enzima rigenerata risultava meno stabile dell'enzima nativo.

INTRODUCTION

Peroxidase (EC 1.11.1.7) contains ferriprotoporphrin IX as a prosthetic group (CONROY *et al.*, 1982) and is one of the most heat-stable enzymes. Regeneration of activity has been widely reported in peroxidases and many other enzymes (MCLELLAN and ROBINSON, 1981), which regain their native structure and biological activity upon removal of the inactivating conditions (LU and WHITAKER, 1974). Regeneration of peroxidase activity after heat inactivation has been associated with enhanced lipid oxidation that can, in turn, lead to deterioration of processed food products during storage (CANO *et al.,* 1995; CHILAKA *et al.,* 2002a). This is a cause for concern in the food processing industry.

Sorghum, a raw material for beer brewing has been shown to contain high amounts of tannins (JAMBUNATHAN *et al.*, 1986) and lipids especially linoleic acid (PALMER *et al.,* 1989). The oxidation of malt lipids and tannins during brewing is undesirable because the products, especially the aldehydes and quinines, are responsible for the development of off flavour and off colours during storage.

NWANGUMA and EZE (1995) have demonstrated the presence of peroxidase in sorghum and noted that the activity of peroxidase increased about 14-fold during malting of sorghum. Although the thermal inactivation and regeneration of peroxidase from many plants has been investigated, there is limited literature on the thermodynamics of thermoinactivation and the mechanism of the regeneration process.

This study was carried out to investigate the thermal inactivation of a partially purified peroxidase preparation and to suggest an explanation for regeneration.

MATERIALS AND METHODS

Materials

Improved Nigerian sorghum variety (KSV 8) was obtained through the seed service of Pioneer seeds, Kano State, Nigeria. o-Dianisidine was obtained from BDH (Poole) England. All other reagents were of analytical grade.

Enzyme extraction and purification

The method used here was adapted from that of EZE and CHILAKA (2002) with the following modifications. Sorghum seeds were steeped in water for 24h at 28°C.The end of steeping was regarded as day zero of germination. The seeds were germinated at 28°C for 4 days in a dark cupboard. The plumules and radicles of the germinated seeds were removed, and the seeds were then washed

and ground to paste with a pestle and mortar. The paste was extracted at 4°C with 0.025M Tris HCl buffer pH 8.0. After 1h, the suspension was filtered through three layers of cheesecloth. The filtrate was centrifuged at 10,000 g for 10 min at 4°C. The supernatant was stored in the refrigerator as the crude enzyme extract.

The crude enzyme extract was made to 70% $(NH_4)_2SO_4$ saturation by dissolving solid $(NH_4)_2SO_4$ in the crude enzyme solution. The solid $(NH_4)_2SO_4$ was added gradually at 4°C and stirred gently using a magnetic stirrer. The solution was kept at 2°C for 12h. After centrifugation at 10,000 g for 10 min at 4°C, the precipitate was dissolved in 0.025M Tris-HCl buffer pH 8.0 and dialyzed overnight against the same buffer. The dialyzed enzyme was redisolved in Tris-HCl buffer pH 8.0 and introduced onto a DEAE-cellulose (DE 52 Whatman) column (2.5x17 cm) pre-equilibrated with buffer. After washing off unbound protein with buffer, the column was then eluted with a linear gradient of 250 mL of 0-0.5M of sodium chloride dissolved in the same buffer. The linear gradient was generated with a gradient mixer (Pharmacia gradient mixer, GMI, Pharmacia, IL, USA). Active fractions (140 mL) were pooled and dialyzed in 0.05M acetate buffer pH 5.4.

Enzyme assay

Peroxidase activity was assayed by the method of MCLELLAN and ROBINSON (1981). The change in absorbance at 460nm due to the oxidation of o-dianisidine in the presence of H_2O_2 and enzyme at 30°C was monitored using a Pye Unicam 5P 460 spectrophotometer (Pye Unicam Ltd, Cambridge, UK) The standard assay solution contains 0.3 mL of o-dianisidine, 2.6 mL of 0.1M sodium phosphate buffer pH 6.0, 0.1 mL of enzyme extract, in a total volume of 3.0 mL. One unit of enzyme activity was defined as the amount of enzyme that gives an absorbance change of 0.1/min at 30°C.

Protein determination

Protein content of the enzyme was determined by the method of LOWRY *et al.* (1951). Protein concentration during chromatography was followed by reading the absorbance at 280 nm on the Pye Unicam 5P 460 spectrophotometer used for the enzyme assay. Both the enzyme assay and the protein assay were performed on both the crude and the partially purified enzyme.

Kinetic studies

Km and Vmax of the enzyme were determined from Lineweaver-Burk plots of initial velocity data at different concentrations of H_2O_2 (0-13.7 mM) and at fixed concentrations of thiourea (0-2.0 mM).

Inactivation and regeneration studies

Heat inactivation and regeneration were carried out as in MCLELLAN and ROBINSON (1981), with some modifications as in CHILAKA *et. al.* (2002a). Solutions of the peroxidase were subjected to heat treatment at various temperatures (30°-80°C) for periods of time up to 15 min, in a temperature controlled water bath. To minimize lag phase, 4 mL of acetate buffer pH 4.5 were preincubated to the required temperature and 1 mL of the enzyme was added and rapidly mixed (CHILAKA *et al.*, 2002b). Samples (0.1 mL) were removed after suitable time intervals and the residual peroxidase activity was determined immediately. Samples were also heat inactivated at 60°C in the presence of 8M urea. Thermodynamic analysis was based on the method of MAINER *et al.* (1997), VITA and FONTANA (1982) and ZALE and KLIBANOV (1986). Values reported are the means of triplicate experiments.

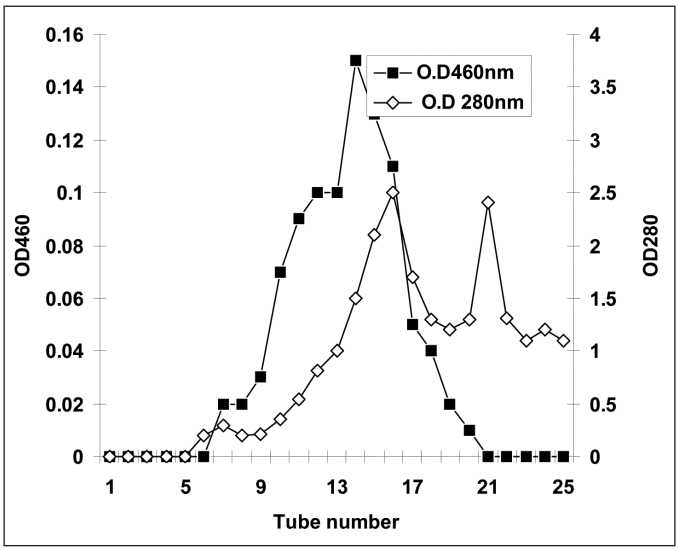


Fig. 1 - DEAE – cellulose ion-exchange chromatography of sorphum peroxidase. Linear gradient of 0-0.5M NaCl was used for elution. Ten mL fractions were collected.

For regeneration studies, samples of peroxidase heat-inactivated at various temperatures, as stated above, were used. After inactivation, the samples were then incubated at 30°C for different periods of time and the activity was monitored.

RESULTS AND DISCUSSION

Crude peroxidase extract from a four-day germinated sorghum seeds when saturated to 70% with $(NH_4)_2 SO_4$ resulted in the precipitation of most of the enzyme with 3.54-fold purification. The elution profile of the partially purified sorghum peroxidase on DEAE-Cellulose ion-exchange chromatography (Fig. 1) showed two enzyme activity peaks, which in each case did not coincide with the protein peak. This suggests the existence of a peroxidase isoenzyme because the assay showed that the enzyme activity was that of peroxidase. It is possible that there could have been contaminants in the preparation because the enzyme was partially purified. The purification profile of sorghum peroxidase by ion-exchange chromatography of the enzyme resulted in a 86.30-fold purification (Table 1), which is by far less than the 10,490-fold purification of peroxidase from *Narcisses pseudonarcissus* (ALFRED *et al.*, 1981). SESSA and ANDERSON (1981) showed a 384-fold purification of soybean peroxidase. In this experiment, the sorghum peroxidase may have bound poorly to the DEAE-Cellulose during ion-exchange chromatography.

Analysis of the initial velocity data using a Lineweaver-Burk plot (Fig. 2a) showed that the enzyme had a Vmax of 1.54 units and a Km of 3.13 mM for H_2O_2. Thiourea was a competitive inhibitor. The slope replot (Fig. 2b) of the data in Fig. 2a, shows that the Ki for thiourea was 1.1 mM.

Table 1 - Purification profile for sorghum peroxidase.

Procedure	Total Protein (mg)	Total enzyme (units)	Specific *activity (unit/mg prog)	Activity yield (%)	Purification fold
Crude Enzyme	27,120	190,800	7.0	100	1.00
$(NH_4)_2SO_4$ fractionation	27,120	148,800	24,80	78	3.54
ion-exchange chromatography	5,300	31,800	600	17	86.30

* One unit of activity is defined as the amount of enzyme that gives an absorbance change of 0.1/min at 30°C at 460 nm.

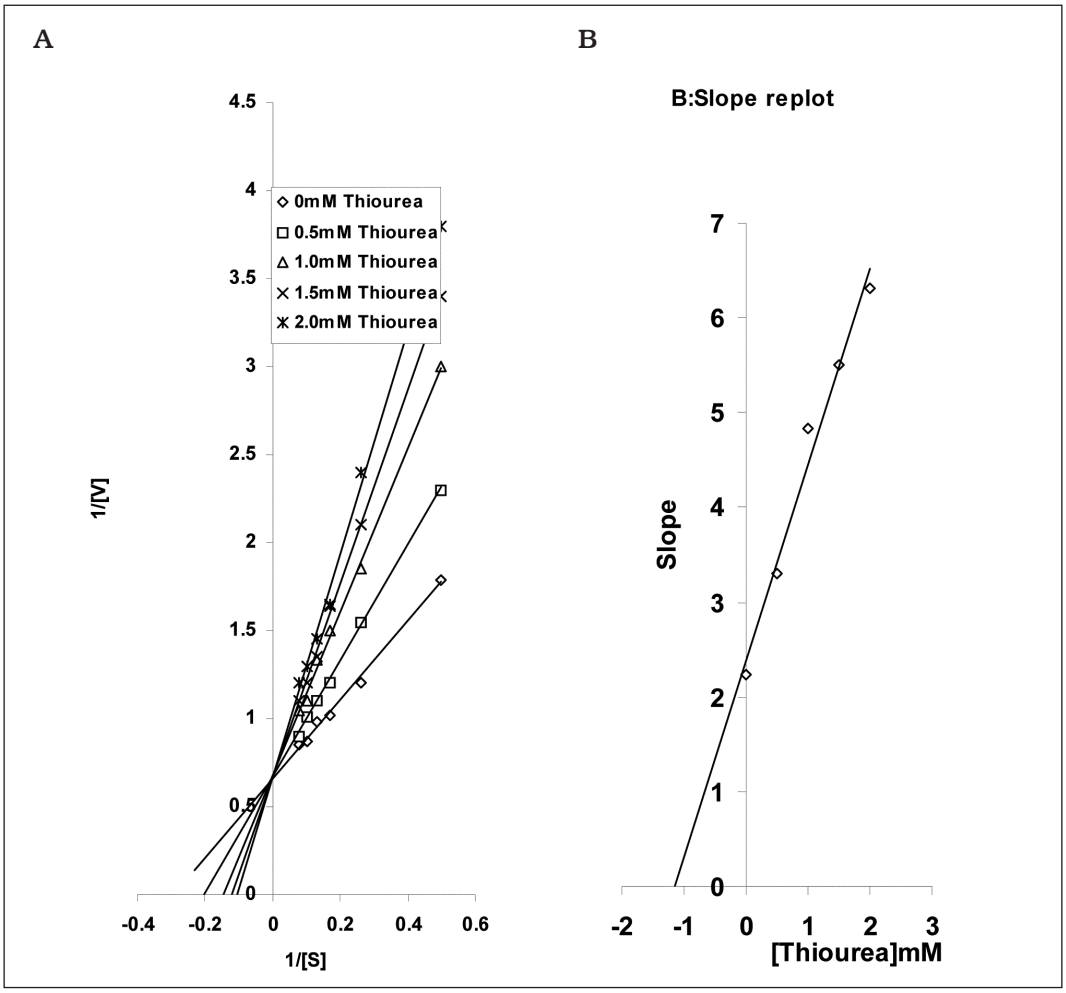


Fig. 2 - (A) Lineweaver-Burk plot of initial velocity data for the inhibition of sorghum peroxidase by thiourea and (B) the slope replot.

The denaturation curves for the inactivation of sorghum peroxidase are shown in Fig. 3a. The t½ values were 23, 56 and 106 min at 80°, 70° and 60°C, respectively. These results showed that inactivation of the enzyme increased with temperature.

The rate constants, k = 2.3/D for the thermal inactivation of peroxidase are shown in Table 2. The D-value, which reflects the time needed for a 90% reduction of enzyme activity at a given temperature, was derived from the equation, 1/

Table 2 - Rate constants (k) and corresponding half-lives for thermal inactivation of sorghum peroxidase at different temperatures.

Heating temp. (°C)	Slopes	1/slope = D	K = 2.3/D	($t_{½}$)(min)
50	1.908	0.524	4.38	nd
60	1.818	0.550	4.182	106
70	1.592	0.628	3.662	56
80	1.273	0.786	2.926	23

nd = not detected.

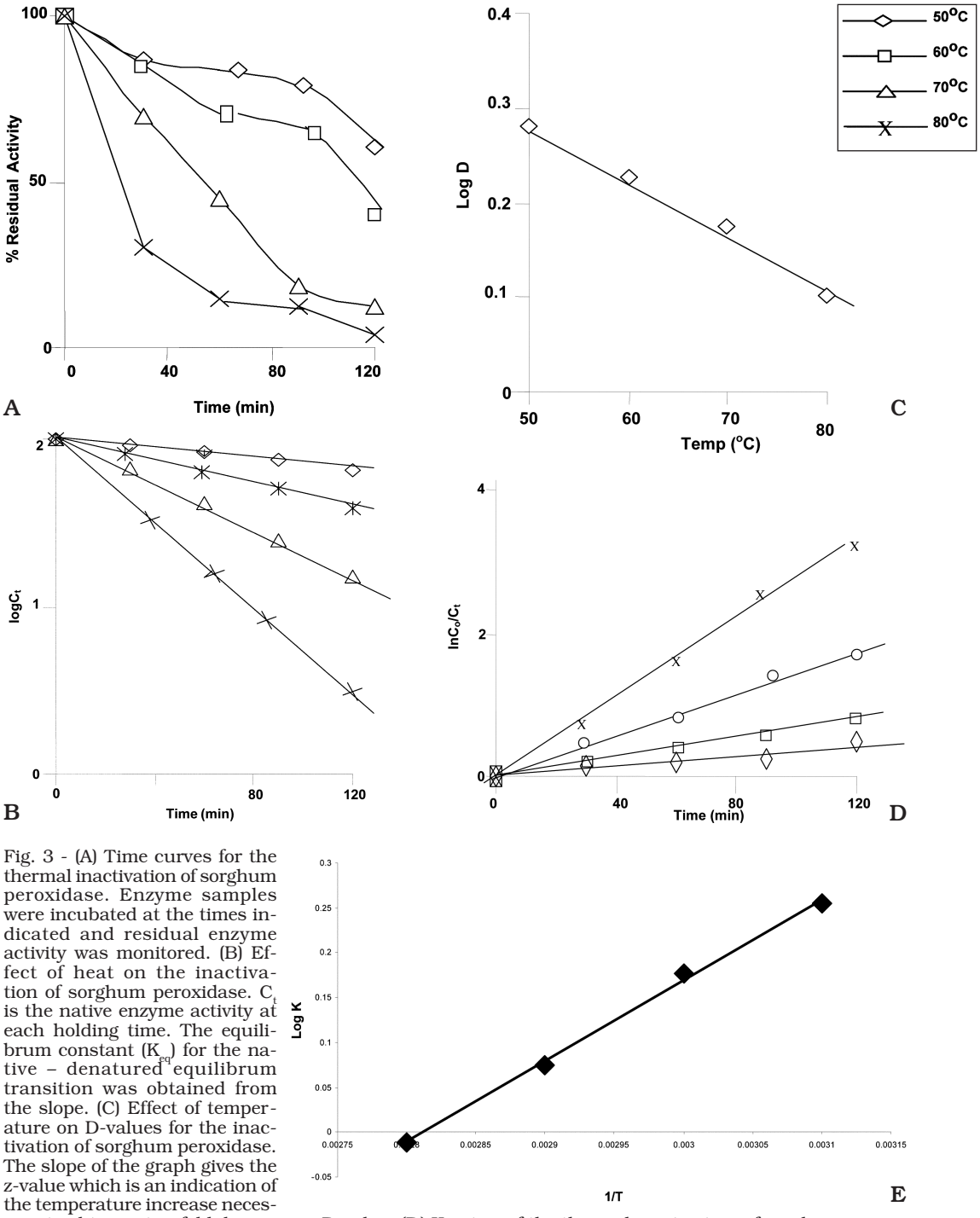


Fig. 3 - (A) Time curves for the thermal inactivation of sorghum peroxidase. Enzyme samples were incubated at the times indicated and residual enzyme activity was monitored. (B) Effect of heat on the inactivation of sorghum peroxidase. C_t is the native enzyme activity at each holding time. The equilibrum constant (K_{eq}) for the native – denatured equilibrum transition was obtained from the slope. (C) Effect of temperature on D-values for the inactivation of sorghum peroxidase. The slope of the graph gives the z-value which is an indication of the temperature increase necessary to obtain a ten-fold decrease in D-value. (D) Kinetics of the thermal inactivation of sorghum peroxidase. Aliquots were withdrawn at the times shown and residual activity C_t measured. C_o is the initial activity of the enzyme. (E) Arrhenius plots of rates of thermal inactivation of soghum peroxidase.

slope=D. It could also be derived from a plot of 1/slope vs time, t. The slope (k) was calculated from the plot of log C_t vs time, where C_t is the activity of the enzyme at each holding time at a given temperature [1/slope=D] (Fig. 3b). A plot of log D at various temperatures vs temp (°C), gave a straight line graph with a slope of 0.238. This is the thermal destruction time, known as the Z-value (Fig. 3c). This is an indication of the temperature increase needed to obtain a ten-fold decrease in the D-value.

A plot of ln C_o/C_t vs time, where C_o is the original activity of the enzyme (at time t = o) and C_t the activity of enzyme at each holding time, showed a straight line graph passing through the origin (Fig. 3d). This further demonstrated that the inactivation process was first-order. When log k was plotted against the reciprocal of the actual temperature, 1/T (in Kelvin) (Fig. 3e), a linear function was obtained. The slope of the regression line was used to calculate the experimental inactivation energy, (Ea), of 0.165 kcal/mole. The changes in enthalpy (ΔH), entropy (ΔS) and free energy (ΔG) (Table 3) were determined using the following equations,

$$\Delta H = Ea - RT$$
$$\Delta S = R\,(\ln A - \ln (K_B/h_p) - \ln T)$$
$$\Delta G = \Delta H - T\Delta S$$

where K_B = Boltzman constant, h_p = planck's constant, R = the gas constant, T is temperature in Kelvin and A is the Arrhenius constant.

Table 3 - Thermodynamic parameters for thermal inactivation of sorghum peroxidase at different temperatures. Free energy change (ΔG), enthalpy change (ΔH), entropy change (ΔS) and the inactivation energy.

Temp (°C)	Ea kca/mol	ΔH kcal/mol	ΔS kcal/mol	ΔG kcal/mol
50	-2.983	-2,688.41	-190.08	58,705.82
60	-2.983	-2,771.55	-190.32	60,606.35
70	-2.983	-2,854.69	-190.57	62,512.20
80	-2.983	-2,937.83	-190.81	64,416.69

A high negative value of the entropy change (-190.81 kcal/mol) at 80°C is indicative of an increased rate of inactivation at higher temperatures. Low values of ΔH showed decreased intramolecular stabilizing interactions during inactivation process (Table 3). Sorghum peroxidase is a globular protein since a plot of ln K vs the reciprocal of actual temperatures ($^1/_T$) (in Kelvin) gave a slight curvature (Fig. 4A) (CARL and KIN-PONG, 1980). This indicates that the ΔH of the unfolding reaction varies with temperature. A small change in heat capacity (ΔCp), 3.0 kcal/mol/deg was also obtained from a plot of ΔH vs temperature (T°C) (Fig. 4b). Similar results were obtained by MAINER *et al.* (1997) for globular proteins.

CONCLUSIONS

About 80% activity was regenerated by sorghum peroxidase after complete inactivation by heat (Fig. 5), showing that reversible inactivation occurred. The inability of the enzyme to regenerate 100% activity suggests that this inactivation involves a reversible unfolding process followed by an irreversible modification, which may be of a chemical or conformational nature. Conformational inactivation arises from the disruption of non-covalent interactions while maintaining the 3-dimensional structure of the enzyme (CARL *et al.*, 1980; HONG *et al.*, 2005). Denaturants, such as urea and guanidine hydrochloride, allow for fast renaturation by disrupting the imperfectly formed non-covalent interactions in the reconfigured structures, and thus protect the enzymes against irreversible conformational inactivation (HONG *et al.*, 2005; TAVAKOLI *et al.*, 2005). The inability of urea to offer 100% protection to the enzyme (Fig. 6) showed that irreversible non-conformational (covalent) change

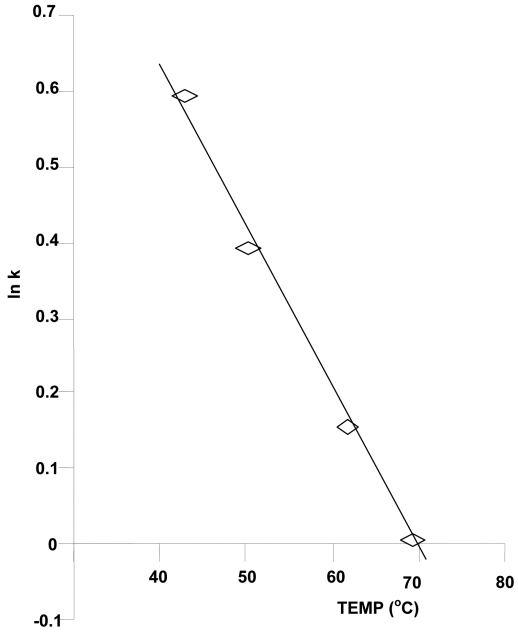


Fig. 4 (A) - Arrhenius plots for the thermal inactivation of sorghum peroxidase.

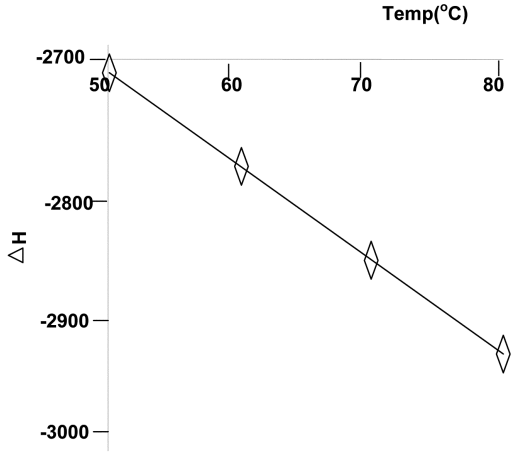


Fig 4 (B) - Dependence of the enthalpy of heat denaturation (ΔH) of sorghum peroxidase on temperature. ΔH values were obtained from ΔH = Ea – RT. The slope of the line indicates the change in heat capacity (ΔCp) associated with heat denaturation.

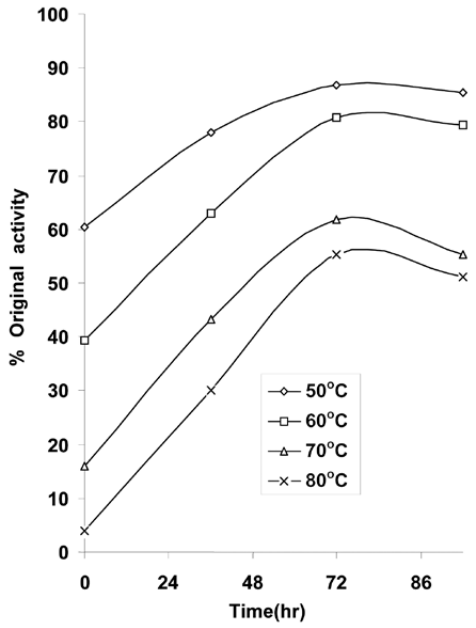


Fig. 5 - Regeneration of sorghum peroxidase.

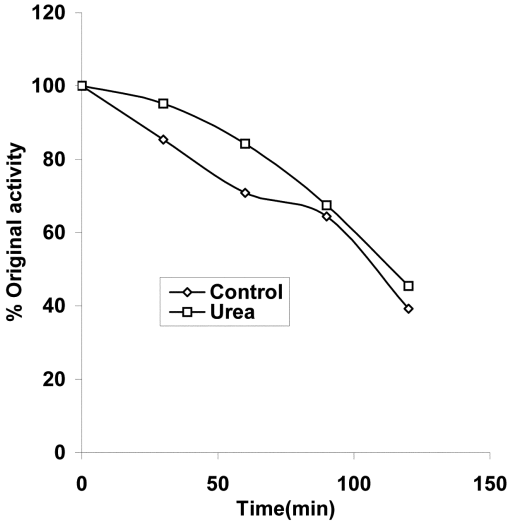


Fig. 6 - Effect of urea on heat inactivation of sorghum peroxidase.

was also involved in the inactivation process. However, reversible inactivation was the major pathway during heat inactivation. Regeneration of activity after inactivation was observed; with the regenerated enzyme molecule was less stable than the native enzyme (Fig. 6).

A thermal destruction time, or Z-val-

ue, of 0.238 seconds showed that complete inactivation may be achieved within a short period of time, and thus the preservation of processed sorghum products may be based on specific enzyme inactivation. In this study, peroxidase denatured by rapid heating for 0.238 seconds was reactivated after 86h. During storage of food products, it is recommended that, this being a complex matrix where conditions for reactivation may well pertain, enatioselectivity of this peroxidase at different temperatures, as well as before and after inactivation, be studied is further. Also, further understanding of the inactivation process would require use of some physical techniques such as CD-spectroscopy to follow the inactivation process, which is the focus of our next paper.

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PAPER

APPLICATION OF THE HAZARD ANALYSIS AND CRITICAL CONTROL POINT (HACCP) SYSTEM IN THE PROCESSING LINE OF BY-PRODUCTS FOR THE PREPARATION OF ANIMAL FEED IN A SLAUGHTERHOUSE

APPLICAZIONE DEL SISTEMA HAZARD ANALYSIS AND CRITICAL CONTROL POINT (HACCP) NELLA LINEA DI PROCESSO DEI SOTTOPRODOTTI PER LA PREPARAZIONE DEI MANGIMI IN UN MATTATOIO

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ABSTRACT

Hazard Analysis and Critical Control Point (HACCP) is a system which attempts to guarantee food safety and harmlessness. It ensures the protection of products and the correction of failures which decrease the costs for quality defects and practically eliminates the need for a final super control. In this study, the possible hazards in

RIASSUNTO

L'Hazard Analysis and Critical Control Point (HACCP) è un sistema che permette di garantire la sicurezza alimentare. Questo sistema assicura la tutela dei prodotti e la correzione degli errori che svalutano i prodotti per mancanza di qualità ed elimina praticamente la necessità di un controllo finale. In questo studio si descrivono i possibili

- Key words: by-products, feed, HACCP, hygiene, safety, self-control, slaughterhouse -

a processing line of slaughterhouse by-products destined for animal feed (material in category 3) are described as well as the preventive measures that are applied, the monitoring systems used, the corrective actions that need to be taken, and records that are kept in the plant. The implementation of the HACCP System will make it possible for any slaughterhouse to monitor the production of a by-products line for the preparation of animal feed.

rischi in una linea di processo di sottoprodotti di mattatoio destinati alla mangimistica (materiale di categoria 3) ed anche le misure preventive applicate, il sistema di monitoraggio utilizzato, le azioni correttive che devono essere intraprese ed i documenti conservati nell'impianto. L'implementazione di un sistema HACCP renderà possibile, a qualunque mattatoio, il monitoraggio della produzione di una linea di sottoprodotti destinati alla mangimistica.

INTRODUCTION

The guiding principle of any White Book is that food safety policies must be based on a global and integrated scheme comprising the entire food chain (from farm to table), in any food sector within the Member States of the European Union (EU) and beyond their borders. This includes those involved in decision-making in the international forum, as well as those responsible for establishing policies at each stage of the process (CCE, 2000).

This global and integrated approach throughout the food chain includes animal feed which will eventually become food for the final consumer. Feed processing plants and companies producing raw materials for feed are both involved in the business of producing animal feed.

In 2002, the European Parliament and Council passed Regulation (EC) N. 1774/2002, which established the health and safety rules regarding animal by-products not destined for human consumption. These animal by-products are raw materials for use in animal feed production (category 3 materials). Article 17 of this Regulation emphasizes the need to establish and apply monitoring methods and critical control points in order to obtain authorization for these processing plants. The Hazard Analysis and Critical Control Point (HACCP) system is the method of choice since it allows specific known hazards and actions to be identified which should, in turn, help to guarantee consumer safety.

The HACCP system should be applied throughout the food chain, from the primary producer to the final consumer, and its application must be based on scientific evidence of human health hazards (FAO, 2003).

In addition to improving food safety, the HACCP system offers other significant advantages, e.g. inspection by the regulatory officers.

This system was first presented in the USA during the National Conference on Food Protection, in 1971 (APHA, 1972). Subsequently, guidelines were drawn up by the Food Hygiene Committee of the Codex Alimentarius Commission (CODEX, 1995). Afterwards, the system has been successfully employed and has been implemented in different sectors of the meat industry (MORENO, 1988; ADAMS, 1990; PARDO *et al.*, 1998, 1999, 2001; ALVARRUIZ *et al.*, 1999; RONCERO *et al.*, 2002). In the near future it will be implemented for animal by-products to be used for feed production. The lack of

information on this subject in the literature justifies the present study.

The HACCP system is based on the following seven steps: (i) conduct a hazard analysis, (ii) determine the critical control points (CCPs), (iii) establish the critical limits, (iv) establish monitoring procedures, (v) establish corrective actions, (vi) establish verification procedures and (vii) establish record-keeping and documentation procedures.

The purpose of this paper is to show the implementation of the HACCP system in the processing line of by-products for the preparation of animal feed in a slaughterhouse, with the aim of improving the hygienic quality of the final product.

MATERIALS AND METHODS

The implementation of the HACCP system was carried out in the Montes de Toledo Meat Processing Co-op, located in Pulgar, Toledo Province (Spain). The main activity of this Co-op is the slaughtering of cattle, and to a lesser extent pigs and sheep. From the different final by-products obtained, the fat is used in the production of feed for livestock, while the flour is used for pet food.

In this company, the processing line of meat by-products for the preparation of raw materials for feed production, also known as a processing plant for category 3 material, attempts to complete the slaughtering process within the slaughterhouse and thus improve the management of animal by-products unsuitable for human consumption.

The company facilities were visited several times during the course of the study. During the first stage, information was gathered about the process and the physical-chemical and microbiological characteristics of the by-products and the raw materials produced. Information concerning management instructions, transportation and storage conditions was also collected.

A decisive step in the methodology was the preparation of a flow chart for the complete process. Once this chart was defined, revised and verified, each stage was then reviewed for possible hazards (biological, physical or chemical) that could pose a threat to food safety.

Once the hazards were identified, the preventive measures which could be implemented to decrease or eliminate the hazard had to be defined. Subsequently, the critical control points (CCP) were determined by using a decision tree (CODEX, 1995), where a logical reasoning approach was used, as recommended by several International Agencies (ICMSF, 1991; FAO, 2003).

When a stage was considered a CCP, critical limits for some parameters were established, and the value limits for these parameters were established. If these limits were exceeded, the process was considered unacceptable. A monitoring or surveillance system, which measures the parameter values and compares the values with the CCP, was established in order to detect possible deviations. Specific corrective actions for each CCP were then formulated in order to cope with possible deviations.

A wide procedure for the verification of the process was established in order to confirm that the HACCP system is operating efficiently. The quality manager will checks the daily values for temperature, pressure and time recorded during the loading of the digester. The results of the fat and flour analyses are also checked in order to ensure that they comply with the quality parameters required for the products. The analyses are carried out in a laboratory with a certification by the Spanish National Accreditation Institution (ENAC). The cleaning and disinfection program as well as the maintenance program are also checked regularly.

Finally, a system for documenting

and recording every procedure included in the HACCP system was established, and all records were kept for the correct implementation of the HACCP system.

RESULTS AND DISCUSSION

In this section the process flow chart (Fig. 1) is explained, from the input of raw material to the storage of the fat and flour, based on the actual process carried out at the Montes de Toledo Co-op.

As shown in Table 1, a summary table of hazard analysis is established. The foreseeable main hazards (microbiological, chemical and physical) are described for each stage, as well as the preventive measures to be taken in order to minimize or eliminate such hazards. The table also indicates the stages that were considered CCPs, after the decision tree was implemented. The answers to the different questions raised on the decision tree for each stage, as well as the comments explaining the reasons for not being included as a CCP, are shown in Table 2. The critical levels, monitoring system, including the frequency of surveillance, corrective actions and records taken for each stage are also described in the summary table.

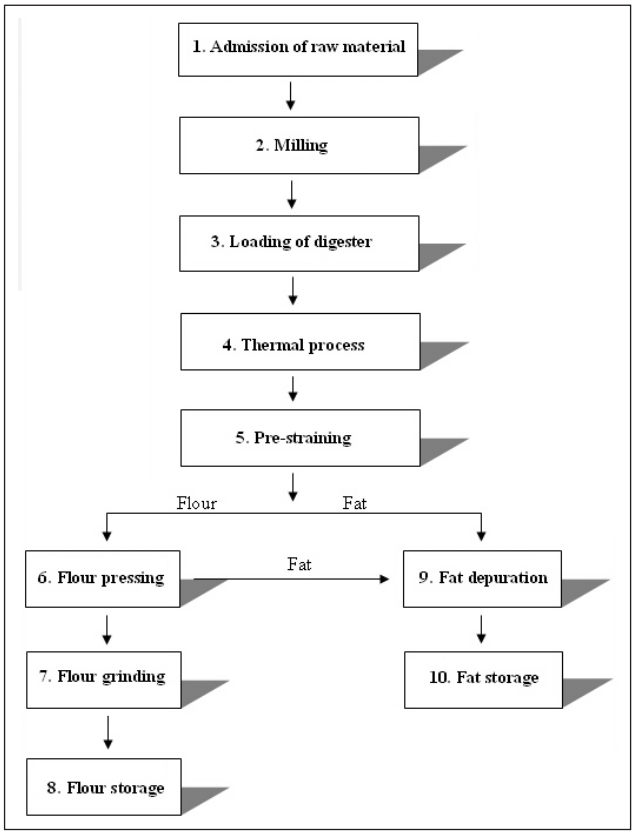


Fig. 1 - Flow chart of the processing line of by-products from a slaughterhouse used for the production of feed (category 3 material).

DETAILED DESCRIPTION OF THE SUMMARY TABLE

Stage 1. Admission of raw material

Regulation (EC) N. 1774/2002 describes the animal by-products that are unsuitable for human consumption (category 3 materials) but can be used as raw material for producing animal feed. Materials for category 1 and category 2 are also produced in the slaughterhouse, but require a different process and are described in the Regulation:

- Materials in category 1 comprise any piece of a slaughtered animal suspected of being infected by Transmissible Spongiform Encephalopathies (TSEs), according to Regulation (EC) n. 999/2001.

- Materials in category 1 (or 2) include any part of a dead animal not slaughtered for human consumption, including animals sacrificed to eradicate an epizootic illness and animal products with residues of veterinary medications.

The materials in category 3, the ob-

ject of this study, include the following by-products:

- Pieces of slaughtered animals, considered suitable for human consumption, but not destined for this aim due to commercial reasons.
- Pieces of slaughtered animals, turned down for human consumption, but without any sign of illness which could be transmitted to humans, or pieces from dressed carcasses suitable for human consumption, according to EC Regulations.
- Skins, hoofs, horns, bristles and feathers from animals slaughtered in a slaughterhouse after being subjected to ante-mortem inspection and being declared suitable for slaughtering for human consumption, according to EC Regulations.
- Blood from non-ruminant animals, slaughtered in a slaughterhouse, which has been declared suitable, according to the EC Regulations and after being subjected to ante-mortem inspection.
- Animal by-products from the processing of products destined for human consumption, including defatted bones and pork scraps.

All of the category 3 materials produced in the slaughterhouse are transported to the processing plant, either directly to the feeding hopper or in metallic tins carried by the workers.

The feeding hopper is situated in an unclean area of the processing plant that is physically isolated from the clean area. The steel hopper is equipped with two worm gears at its base to carry the material towards the hammer mill, while slightly reducing the volume.

Hazards: The main hazard at this stage could be the contamination of category 3 material with category 1 or 2 materials. This contamination could occur in the slaughterhouse as a result of defective separation. In that case, all the material must be considered and treated as category 1 and cannot be treated in the plant for category 3 materials.

Preventive measures: The main preventive measure would be the proper handling of the materials, by putting each material into a different container and dyeing the category 1 materials blue.

Critical control point (CCP): This was not considered as a CCP, since each of the by-products had their own independent container and, the category 1 materials were dyed blue.

Stage 2. Milling

Before thermal treatment in the digester, the by-products pass through a hammer mill where their particles size is reduced to less than 50 mm in order to increase the performance of the system. The hammer mill is situated next to the admission hopper, in the unclean area of the plant.

Hazards: A physical hazard at this stage would be the presence of particles larger than 50 mm after milling, which would decrease the efficacy of the treatment. It would also be a violation of Regulation (EC) N. 1774/2002, which specifies that the particles size of any raw materials in this treatment process must be less than 50 mm.

Preventive measures: The main preventive measure requires the proper maintenance of the hammer mill. At the end of the process, there is another milling operation to ensure that the particles size is suitable for shipping.

Critical Control Point (CCP): This was not considered a CCP, since there was adequate maintenance of the milling facilities. In addition there was another milling operation at the end of the process with a hammer mill to ensure that all the particles were the right size.

Stage 3. Loading of digester

From the hammer mill, the product is transported by a worm gear toward the upper entrance of the digester, where it

Table 1 - Summary of the hazard analysis for a processing line of by-products from a slaughterhouse for feed prod

STAGE	TYPE OF HAZARD			HAZARDS	PREVENTIVE MEASURES
	Physical	Biological	Chemical		
1. Admission of raw material		X		*Mixing with material from category 1	*Different colored e containers for each material type
2. Milling	X			*Physical: faulty milling	*Proper maintenance plan for equipment A second milling after the treatment is performed
3. Loading of digester				*None	
4. Thermal process	X			* Physical: unachieved process conditions	* Proper maintenance plan *Proper calibration of measurement equipment
5. Pre-straining	X			*Physical: soiled sieve or faulty filtration	*Proper maintenance plan
6. Flour pressing	X	X		*Microbiological: Flour recontamination *Physical: Fat with impurities	*To ensure air tightness of the pressing machinery and equipment * Separation of soiled and clean areas *Proper maintenance plan
7. Flour grinding		X		* Microbiological: Flour recontamination	* To ensure air tightness of the grinding machinery and equipment * Separation of soiled and clean areas
8. Flour storage		X		*Microbiological: microbial contamination and recontamination of flour	*Storage in hygienic conditions. * To ensure air tightness of the storage equipment. * Separation of soiled and clean areas
9. Fat depuration	X			*Chemical: impurity content > 15%	*Maintenance plan and proper hygienic conditions
10. Fat storage	X	X		* Microbiological: microbial contamination *Physical: impurities by contamination or by sedimentation	*Storage hygienic conditions *Ensure air tightness *Proper maintenance of tanks

uction (category 3 material).

CCP	CRITICAL LEVELS	MONITORING/ FREQUENCY	CORRECTIVE ACTIONS	RECORDS
NO				
NO				
YES	*Temperature > 133ºC *Pressure > 3 bar *Time > 20 minutes	*Visual *In each digester batch	*Reprocessing of material * Direct shipment of material to incinerating plant	*All corrective actions. * Temperature, pressure and time conditions in each batch
NO				
NO				
NO				
YES	*Low microbial contamination, absence of *Salmonella* and *Clostridium perfringens*	*Analytical *Twice a month	*Reprocessing of material.	*Corrective actions and flour analyses
YES	*Impurity content < 0.15%	*Analytical *Twice a month	*Use this fat as fuel for the boiler	*Corrective actions and fat analyses
YES	*Comply with microbiological criteria, absence of *Salmonella* and *C. perfringens* *Impurity content < 15%	* Analytical *Daily for microbiological analysis *Weekly for impurity analysis	*Reprocessing of material in the processing plant	* Corrective actions and fat analyses

Table 2 - Determination of the processing stages as Critical Control Points (CCP), according to the answers given

STAGE	HAZARD	Q1*
1. Admission of raw material	Microbiological: Contamination with materials from categories 1 or 2.	YES
2. Milling	Physical: Insufficient size reduction of particles	YES
3. Loading of digester.		
4. Thermal process	Physical: Defective process conditions	YES
5. Pre-straining	Physical: Impurity pass through sieve	YES
6. Flour pressing	Microbiological: Material recontamination	YES
	Physical: Contamination of fat with impurities	YES
7. Flour grinding	Microbiological: Recontamination of material	YES
8. Flour storage	Microbiological: Recontamination of material	YES
	Bacterial contamination	YES
9. Fat depuration	Physical: Fat with high impurity content due to faulty operation of decanter	YES
10. Fat storage	Microbiological: Bacterial contamination	YES
	Physical: Fat with high impurity content due to faulty operation of decanter Contamination of fat with soil of tanks Sedimentation of fat in tanks	YES

*__Q1:__ Are there preventive measures for the identified hazard?; *__Q2__: Is this stage specifically designed to eliminate or reduce app
*__Q4__: Will a later stage eliminate or reduce the hazard to an acceptable level?

in the decision tree.

Q2*	Q3*	Q4*	CCP	COMMENTS
NO	NO	-	NO	The by-products have their own independent containers and the materials from category 1 are painted in blue
NO	NO	-	NO	A proper maintenance of the milling equipment is carried out. In the end, another milling process with a hammer mill is performed to ensure a uniform particle size
YES	-	-	**YES**	
NO	YES	YES	NO	This fat passes through the centrifugal decanter where the impurities are eliminated to a lower limit of 15%
NO	NO	-	NO	The soiled and clean areas of the plant are separated. A rigorous cleaning and disinfection plan is carried out and the storage elements have a proper air tightness
NO	YES	YES	NO	This fat passes through the centrifugal decanter where the impurity content is reduced to a lower level of 15%
NO	NO	-	NO	The soiled and clean areas of the plant are separated. A rigorous cleaning and disinfection plan is carried out
NO	NO	-	NO	The soiled and clean areas of the plant are separated. A rigorous cleaning and disinfection plan is carried out and the storage elements have a proper air tightness
NO	YES	NO	**YES**	
YES	YES	NO	**YES**	
NO	YES	NO	**YES**	
NO	YES	NO	**YES**	

earance of the hazard to an acceptable level?; ***Q3**: Could the contamination appear or increase to reach unacceptable levels?;

is thermally processed. The loading operation is discontinuous and is carried out when the reception hopper is full. At this stage there is no hazard which could alter the processing of the by-products.

Stage 4. Thermal process

The digester in the Montes de Toledo slaughterhouse is a dry fusion type. It is made up of a steam jacketed tank, where the by-products are subjected to high temperature and pressure for a certain period of time. Inside the digester there are a number of blades that rotate during the process to homogenize the material and improve heat transfer. The digester works according to the conditions described in Method 1 of Regulation 1774/2002:

- Temperature > 133°C.
- Pressure > 3 bar.
- Time: 20 uninterrupted min.

When the process is finished, a flour-like product with a high fat content is obtained.

Hazards: The main physical hazard is the non-compliance with the conditions required for the process, e.g. temperature, pressure and/or time are not as specified in Regulation 1774/2002.

Preventive measures: The main preventive measure involves the proper maintenance and calibration of the digester and ancillaries.

Critical Control Point (CCP): This stage was considered as a CCP, and therefore the critical levels, monitoring systems, corrective actions and necessary records were determined.

Critical levels: The temperature inside the digester must be above 133°C and the pressure above 3 bar; both conditions must prevail for at least 20 min.

Monitoring: Visual surveillance of the graphical records after each digester batch is required, to ascertain that the processing conditions were correct. The measuring equipment is calibrated yearly by a company certified by ENAC. The thermostat, barometer and timer precisions are ±2°C, ±0,1 bar and ±4 s, respectively.

Corrective actions: If there is any deficiency in the treatment of the material, one of the following corrective actions must be taken:

- Reprocessing;
- Direct shipment to an incinerating plant.

The application of corrective actions implies that the material and facilities that have been in contact with the defectively processed material must be sanitized.

Records: All corrective actions that are taken must be recorded, as well as the conditions for each digester batch.

Stage 5. Pre-straining

Once the digestion and sterilization of the product is completed, it is loaded into a pre-strainer (stainless steel tank equipped with a sieve at the bottom), where the liquid fat is separated (by passing through sieve holes) from the solid particles which are retained by the sieve and sent to a screw press, to obtain the maximum quantity of fat. The fat which passes through the pre-strainer goes to the collecting compartment situated below it and is subsequently sent to an intermediate tank.

Hazards: The main hazard (physical) is the possible contamination of the fat by impurities caught in the sieve, or by faulty filtration which allows debris to pass.

Preventive measures: These will be based on a preventive maintenance plan, where the sieve is checked weekly, and on a proper cleaning and disinfection plan for the plant.

The following stages can be established for the cleaning and disinfection process:

- Preliminary removal of coarse dirt without applying any product, to facilitate the detergent action.

- Rinsing with warm or hot water to help remove grease.

- Application of a detergent or degreasing product. Regardless of the application method, the application time and concentration must be taken into account. These two aspects are usually described in the technical specifications or labels of the detergent products.

- Rinsing to remove the remaining detergents and dirt. An effective rinsing is of the utmost importance to prevent the detergent or dirt from contaminating the product. The pH should be measured to confirm this.

- Application of a disinfectant. Once again, the application time and concentration is of utmost importance.

- Rinsing is needed for some products, such as chlorinated disinfectants. Some other disinfectants do not require subsequent rinsing, although enough time must be allowed to ensure that no residues remain on the surfaces that could contaminate the food.

- Drying is required for some surfaces in order to limit the quantity of water available to microorganisms which could eventually contaminate the product.

Critical Control Point (CCP): This was not considered a CCP, since the fat goes to a centrifugal decanter, where impurities are separated and the values remain below the 0.15% level.

Stage 6. Flour pressing

Solid products, comprised of flour and fat residues which are captured in the pre-sieving net, are transported by a worm gear to a screw press where most of the remaining fat is recovered. There are two outlets on the press, one for the defatted flour which is transferred to the mill and the other for the fat, which is then transferred to the intermediate tank where the fat from the pre-straining step is also stored.

Hazards: In this stage there is a microbiological hazard due to the possibility of the flour or fat coming into contact with unprocessed category 3 materials. There is also a physical hazard that could be caused by any defect in the press, which could produce a high percentage of impurity in the fat.

Preventive measures: To avoid possible recontamination of flour or fat during the pressing operation, the following preventive measures must be taken:

- Good manipulation practices will be followed during the pressing operation;

- Air tightness of pressing machinery and equipment will be ensured;

- A perfect separation between the unclean and clean areas will be ensured.

To avoid the contamination of fat with impurities, an adequate preventive maintenance plan for the press must be followed.

Critical Control Point (CCP): From a microbiological point of view, this was not considered a CCP, since the unclean and clean areas are already separated in the plant and a rigorous cleaning and disinfection program is being carried out. Also, from a physical point of view, this stage was not considered a CCP, since the fat goes through the centrifugal decanter, where the impurities are reduced to a level below 0.15%.

Stage 7. Flour grinding

Two products are obtained from the flour press: the fat which was included in category 3 materials and the defatted flour. The latter exits from the press in a compact form and with a grain size distribution which makes manipulation difficult. To alleviate this, the flour is ground with a mill situated under a regulation silo, which, in turn, is fed by a worm gear from the flour press. With this process, the flour acquires its final properties and aspect before shipment. The grinding operation ensures a particle size under 50 mm, measured by a sieve situated at the exit of the mill.

Hazards: During this stage, as in the

previous one, a recontamination of the flour can occur if it comes into contact with the unprocessed category 3 materials. To avoid such recontamination, the following measures will be followed:

- A proper preventive maintenance plan for the mill;
- Air tightness of milling machinery and equipment;
- Perfect separation between the unclean and clean areas.

Critical Control Point (CCP): This was not considered a CCP, since the unclean and clean areas are already separated and a rigorous cleaning and disinfection program is being carried out.

Stage 8. Flour storage

The defatted and ground flour is stored in a silo, situated inside the bay, until it is shipped to a pet food processing plant.

Hazards: In this operation the flour could undergo recontamination if it comes into contact with unprocessed category 3 materials. An undesirable microbial contamination could also occur.

Preventive measures: To avoid possible recontamination of the flour or an undesirable microbial contamination, the following preventive measures will be taken:

- Storage under adequate hygienic conditions;
- Air tightness of the storage machinery and equipment;
- Perfect separation between the unclean and clean areas.

Critical Control Point (CCP): This stage was considered a CCP, with regard to undesirable microbial contamination, and therefore the critical levels, monitoring systems corrective actions and records must be determined. Note that the flour recontamination does not determine the CCP property of this stage, since, as in previous stages, the unclean and clean areas are separated, a rigorous cleaning and disinfection program is carried out and the storage elements have perfect air tightness.

Critical levels: The microbial contamination will be low, with a total absence of *Salmonella* and *Clostridium perfringens*.

Monitoring: An analytical control of the flour will be carried out by taking a sample for analysis twice a month.

Corrective measures: In case of bacterial contamination, the product will be reprocessed in the processing plant for category 3 materials.

Records: The corrective actions applied and all the analyses performed on the flour will be recorded.

Stage 9. Fat depuration

All the fat from the pre-strainer and from the flour press passes from the intermediate tank to a horizontal centrifugal decanter, where the solids are separated from the fat. The fat obtained, with an impurity content below 0.15%, is sent to the storage tanks.

Hazards: The main (physical) hazard in this stage is the presence of impurities in the fat higher than 0.15%, due to faulty operation of the decanter.

Preventive measures: A proper maintenance plan for the centrifugal decanter is the primary preventive measure.

Critical Control Point (CCP): This stage was considered a CCP, and therefore the critical levels, monitoring systems, corrective actions and necessary records must be determined.

Critical level: The critical level of an impurity content in the fat is less than or equal to 0.15%.

Monitoring System: The state of the centrifugal decanter will be surveyed, and a fat sample will be taken monthly at the exit, to analyze the impurity content in order to corroborate the correct operation.

Corrective actions: When the fat produced shows an impurity content above

0.15%, it will be used as fuel for the steam boiler.

Records: The preventive maintenance plan for the centrifugal decanter and the results of the fat analysis will be recorded.

Stage 10. Fat storage

The fat obtained in the centrifugal decanter is stored in two 50,000 L stainless steel tanks until it is shipped to a feed processing plant.

Hazards: The main hazard is the microbial contamination of the fat due to incorrect hygiene in the tank or in the inlet to the tank. There is also a physical problem of fat contamination due to a variety of factors: lack of hygiene in the tanks, the possible entry of fat from the centrifuge with a high impurity content, or sedimentation of the fat in the tanks, which in turn, may alter the impurity content along the height gradient.

Preventive measures: The main preventive measures will be to have an effective cleaning and disinfection program, an adequate preventive maintenance program for the tanks and the guarantee of air tightness of the storage elements.

Critical Control Point (CCP): This stage was considered a CCP, and therefore the critical levels, monitoring systems, corrective actions and records must be determined.

Critical levels: The microbiological criteria established for this product must be met: i.e., the absence of *Salmonella* and *Clostridium perfringens*. Another critical level to consider is the impurity content, which should always be below 0.15%.

Monitoring: Fat samples are taken daily for microbiological analysis. A weekly sample is taken for a physical-chemical analysis.

Corrective actions: In the case of a bacterial contamination or an impurity content above the allowed level, the fat will be reprocessed in the processing plant for category 3 material.

Records: The corrective actions applied and the results of the fat analysis will be recorded.

Traceability system

The HACCP system works well for known hazards, but when the hazards are unknown, a study of the product traceability must be carried out, as recommended by Regulation (CE) 178/2002. This regulation sets the basis for assuring a high level of protection for human health and consumer well being. It also establishes the common principles and responsibilities, as well as the means for providing a solid scientific base and efficient organizational procedure upon which decision-making for food and feed safety is based.

The traceability system carried out by this company is described below, in complement to the implementation of the HACCP system.

The traceability of the processing line begins with the admission of the animals to the slaughterhouse where the individual traceability of the dressed carcasses is carried out and is linked to the traceability of the by-products processing plant. This traceability is managed in daily lots, so the traceability of the by-products is known each day, from the stockbreeder to the final use of the carcass by-products. After each daily lot of by-products has been processed, it is directed to the storage tanks or silos. The quantity of fat and flour produced in the plant is estimated from the daily slaughter through pricing. This information is linked to the sales of fat and flour and a record is kept of all the customers and the quantities sold. Since the performance of the digester is precisely known, the amount of fat and flour produced in each batch of the digester can be determined quite accurately. Each lot that is ready for sale is assigned a code which

appears on the shipping document that shows the daily lot information and the amount stored. In the case of a health crisis that requires the withdrawal of the lot, the lots from the previous and subsequent day will also be withdrawn or directed to a treatment plant for category 1 material, as a safety margin.

Therefore, all the fat and flour sales are identified with a lot code, which is the date when the by-products went to the treatment plant, i.e. the day when the animals were slaughtered. This coincides with the lot code which appears on the delivery note of the by-products.

As regards TSEs, traceability in the slaughterhouse is carried out individually for each dressed carcass, so it is impossible that by-products originating from TSE-contaminated animals could be admitted into the plant. Nevertheless, analyses are carried out daily by the health authorities and are received before the sale of any lot; the information can be used in case of any problem or doubt.

In conclusion, the carcasses are individually identified when the animals are slaughtered. The category 3 by-products go to the processing plant and generate a batch of fat and flour. The traceability of that batch is kept until shipping, by using the data from the fat and flour yields and the stored quantities. A lot code with the slaughter date is given at the time of shipping and printed on the delivery note. The slaughterhouse keeps a daily record of the quantities produced, stored and shipped.

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SHORT COMMUNICATION

MICROBIAL AND SAFETY QUALITIES OF PDO GALOTYRI CHEESE MANUFACTURED AT THE INDUSTRIAL OR ARTISAN SCALE IN EPIRUS, GREECE

QUALITÀ E SICUREZZA MICROBIOLOGICA
DEL FORMAGGIO DOP GALOTYRI, OTTENUTO IN EPIRO (GRECIA)
SU SCALA INDUSTRIALE ED ARTIGIANALE

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ABSTRACT

Microbiological quality and safety attributes of Galotyri, a traditional Greek soft acid-curd cheese of Protected Denomination of Origin, were evaluated. Fresh retail cheese (12 batches) manufactured at either an industrial or artisan scale contained lactic acid bacteria at levels of 8 log cfu/g, while pseudomonads, enterobacteria and coagulase-positive staphylococci were <2 log cfu/g and *Salmonella* spp. were absent in 25 g of cheese. However, one industrial and two artisan batches har-

RIASSUNTO

Sono state studiate la qualità e la sicurezza microbiologica del Galotyri, un formaggio DOP Greco molle a coagulazione acida. I formaggi freschi venduti al dettaglio (12 lotti), prodotti sia su scala industriale sia artigianale, contenevano, su 25 g di campione, batteri lattici a livelli di 8 log cfu/g, mentre i livelli di *Pseudomonas*, enterobacteri e stafilococci positivi alla coagulasi erano <2 log cfu/g, mentre i ceppi di *Salmonella* spp. erano assenti in 25 g di formaggio. Tuttavia un lotto industria-

- Key words: Galotyri cheese, lactic acid bacteria, *Listeria monocytogenes*, PDO cheese -

boured *Listeria monocytogenes.* Artisan cheese had higher (P<0.05) enterococci, enterobacteria and yeast populations than the respective industrial cheese. When stored aerobically at 4°C, none of the Galotyri cheese was stable for more than two weeks; surface-growing yeasts were the main spoilage agents under simulated retail distribution and storage conditions. Although natural *Listeria* contaminants at the time of purchase (<10 cfu/g) were undetectable by culture enrichment after 7 days of cheese storage at 4°C, this survey indicated that commercially marketed Galotyri may contain low viable counts of *L. monocytogenes*. This should be a concern to cheese manufacturers and consumers; the hygienic measures along the processing line and the current practice in most Greek dairies to minimize or reduce ripening and/or in-plant chill-holding times of freshly prepared Galotyri should be carefully reconsidered.

le e due artigianali ospitavano *Listeria monocytogenes.* I formaggi artigianali presentavano livelli maggiori di enterococci, enterobatteri e lieviti rispetto ai formaggi prodotti su scala industriale. Quando conservati a 4°C in presenza di ossigeno nessuno dei formaggi Galotyri risultava stabile per più di due settimane; i lieviti cresciuti sulla superficie dei formaggi rappresentavano il principale deterioramento in una simulata vendita al dettaglio ed in condizioni di stoccaggio. Sebbene il contaminante naturale *Listeria* al momento dell'acquisto (<10 cfu/g) risultava non rilevabile dalla coltura di arricchimento, dopo 7 giorni di stoccaggio del formaggio a 4°C questa scoperta indicava che il Galotyri commercializzato può contenere conte poco vitali di *L. monocytogenes.* Questa osservazione dovrebbe essere una preoccupazione dei produttori e dei consumatori; nella maggior parte dei caseifici Greci dovrebbero essere riconsiderate le misure igieniche lungo la linea di processo e le procedure correnti per minimizzare o ridurre la maturazione e/o i tempi di conservazione a freddo di Galotyri appena preparato nell'impianto.

INTRODUCTION

Cheeses of Protected Denomination of Origin (PDO) or Registered Designation of Origin (RDO) are authentic traditional dairy products characterised by desirable microecological complexity and biodiversity. They generally have a high sensorial quality, and probably have greater probiotic potential in the human gut compared to industrial cheeses. In addition, PDO/RDO cheeses are economically, socially and culturally important foods in Europe, particularly in Italy, Greece, France, Spain and the other Mediterranean countries. For this reason there is increasing interest in microbiological research of these cheeses (TSAKALIDOU *et al.* 1998; HATZIKAMARI *et al.,* 1999; BIZZARRO *et al.*, 2000; BOUTON *et al.*, 2002; DI CAGNO *et al.*, 2004; DUTHOIT *et al.,* 2005; PIRAINO *et al.,* 2005).

Galotyri is one of the 20 Greek cheeses that currently have PDO recognition (ANON., 2004); it is traditionally produced in the regions of Epirus and Thessaly. It is a clean-white, typically acid-curd, spreading cheese that has no surface skin, eyes or cracks. Its maximum

permitted water content is 75% and its minimum permitted fat content in dry matter is 40% (ANON., 1994). It has a pleasant, slightly acidic, refreshing taste and aroma, and is made from ewes' or goats' milk, or their mixtures. The milk should be collected from breeds raised in the above-mentioned regions, at least 10 days after giving birth, and should be of good quality, full fat, raw or pasteurised. Addition of rennin, enzymes, edible sea salt, and lactic acid bacteria (LAB) starters is allowed; however, addition of condensed milk, milk powder, milk proteins, casein salts, colourings, and preservatives is prohibited (ANON., 1994). The traditional manufacturing method of Galotyri and its current modifications to adopt modern processing practices and satisfy economic needs in Greek dairy plants have been reported (ROGGA *et al.,* 2005). Briefly, the commercial Galotyri currently found in the Greek market is a fresh (non-ripened) acidic (pH *ca.* 4.0), moist (*ca.* 75% water), and low salt (<2% NaCl) soft cheese (ROGGA *et al.,* 2005), while the traditional artisan cheese is reported aged, drier (*ca.* 70%) and saltier (*ca.* 3%); it used to be ripened in leather sacks or barrels for up to 3 months before consumption (ZYGOURIS, 1952; ANIFANTAKIS, 1991; ANON., 1994).

Although Galotyri is one of the oldest Greek cheeses (ANIFANTAKIS, 1991), there has been little research on its microbiology in relation to its shelf life, safety, probiotic potential and sensory quality. There are no data in the literature regarding the microbial and safety qualities of Galotyri. Recent studies in our laboratory have shown that important pathogens, such as *Listeria monocytogenes* and *Escherichia coli* O157:H7, which are frequently carried on small ruminants and transmitted to milk processing plants, may tolerate Galotyri acidity and survive during retail storage under refrigeration (4°C) or abusive temperature (12°C) conditions (ROGGA *et al.,* 2005; LEKKAS *et al.,* 2006). Because the raw milk for Galotyri production was traditionally boiled, and nowadays it is pasteurized in most dairies, cross contamination with the above pathogens is more likely to occur after heating. For instance, the heated milk may be cross-contaminated from natural starters that may contain pathogen survivors, or the curdled milk may be cross-contaminated from dirty utensils, cheese clothes, drainers and/or containers, or from various other contamination sources. Therefore, the aim of this study was to evaluate the microbial and safety qualities of commercial PDO Galotyri cheeses marketed in Epirus, Greece, and their shelf-life and safety during aerobic storage at 4°C.

MATERIALS AND METHODS

Galotyri cheese samples

Samples of commercially manufactured Galotyri were purchased from the retail outlets of two local dairy plants, A and B, and were from cheese lots ready for distribution in the market after a short (4-5 days) holding period at 4°C. Retail samples represented twelve commercial batches, six of which were manufactured in plant A, which is an industrial plant, while the other six were manufactured in plant B, a small traditional dairy producing artisan cheeses. Both types of Galotyri, hereafter named industrial or artisan, were produced from ewes' milk. Standard manufacturing practices were used in both plants; commercial (plant A) or natural (plant B) starters were added to the milk after pasteurization at 72°C for 15 sec or heating to 85°-90°C according to the traditional method, and cooled to 30°-35°C. Samples were received in insulated polystyrene boxes, divided into 500 g portions in 1 kg flex plastic containers with a lid used for retail distribution of Galotyri, and stored at 4°C. Samples were tak-

en to the laboratory for microbiological analyses and pH determination on the day of transport (day 0 of storage) and at days 3, 7, 14, 21 and 28 of storage. The pH was measured with a Crison digital pH meter (model Micro-pH 2001, Alella, Spain) equipped with a glass electrode, which was immersed in the soft cheese mass after sampling for microbiological analyses, as described below.

Microbiological analyses

Cheese samples (25 g) were transferred aseptically to stomacher bags, 225 mL of 0.1% w/v buffered peptone water (BPW; Merck, Darmstadt, Germany) were added and the mixture was homogenised in a stomacher (Lab Blender, Seward, London, UK) for 60 sec at room temperature. Serial decimal dilutions in 0.1% BPW were prepared and duplicate 1 or 0.1 mL samples of appropriate dilutions were poured or spread on agar plates. Unless otherwise stated, all media and supplements were purchased from Merck. Total viable counts (TVC) were determined on casein-peptone, soymeal-peptone (CASO) agar with 0.6% yeast extract, incubated at 30°C for 72 h; lactic acid bacteria (LAB) on de Man, Rogosa, Sharpe (MRS) agar, incubated at 30°C for 72 h under anaerobic conditions (GasPack System, BBL, Becton Dickinson, Sparks, MD, USA); presumptive lactococci on M-17 agar, incubated at 37°C for 48 h; enterococci on kanamycin aesculin azide agar, incubated at 37°C for 48 h; pseudomonads on *Pseudomonas* agar base (LAB M, Bury, Lancashire, UK) supplemented with cephalothin-fucidin-cetrimide (CFC; supplement X108, LAB M), incubated at 25°C for 48 h; total enterobacteria on violet red bile glucose agar, overlayed with 5 mL of the same medium and incubated at 37°C for 24 h; coagulase-positive staphylococci on Baird-Parker agar base with egg yolk tellurite, incubated at 37°C for 48 h, followed by rapid confirmatory testing for agglutination (Staph Microscreen, Microgen Bioproducts, Camberley, UK) of the colonies showing lecithinase activity; yeasts on rose bengal chloramphenicol agar, incubated at 25°C for 5 d. The lowest detection limit of the above analyses was 1 and 2 log cfu/g for the poured (LAB and enterobacteria) and spread (all other microbial types) agar media, respectively.

The electivity of the above microbiological media was checked with appropriate rapid tests according to SAMELIS *et al.* (1998). Specifically, the types of predominant LAB grown on CASO, MRS and M-17 agar plates were tentatively characterized by checking for gram-positive and catalase-negative reactions of the colonies. Five LAB colonies were then randomly isolated from each of countable MRS and M-17 plates per batch; this resulted in a total of 120 isolates, 60 from the six industrial batches and another 60 from the six artisan batches. All isolates were checked for cell morphology by phase contrast microscopy, growth at 15° and 45°C, growth in 2, 4 or 6.5% NaCl, and gas production from glucose and arginine hydrolysis (SAMELIS *et al.*, 1998).

The presence of *Salmonella* and *Listeria* spp. was determined by culture enrichment of 25 g cheese samples in appropriate broth media followed by streaking on selective agar media and confirmation of 3 to 5 typical colonies by the API 20E or the API *Listeria* identification kits (BioMerieux, Marcy l'Etoile, France), respectively (SAMELIS *et al.*, 1998; ROGGA *et al.*, 2005). Culture enrichment for the detection of naturally occurring salmonellae and listeriae was done in all cheese batches at day 0 of storage. Cheeses were also analyzed in parallel by direct plating, particularly for *Listeria*: 1 mL of the first dilution in 0.1% BPW of each cheese sample was plated on tetraplicate PALCAM agar plates, by spreading 0.25 mL/plate, to achieve a detection limit of 10

cfu/g (ROGGA *et al.*, 2005); when after incubation at 30°C for 48h no typical *Listeria* colonies were detected on those tetraplicate plates, *Listeria* counts were <10 cfu/g and their survival was monitored by culture enrichment. Only *Listeria*- or *Salmonella*-positive batches at day 0 were further evaluated for survival/growth of the pathogens during storage at 4°C.

Statistical analysis

A total of 12 commercial (6 industrial and 6 artisan) Galotyri batches were analyzed. For each cheese type, storage trial experiments were replicated at least twice by analyzing duplicate samples per replicate. Microbiological counts were converted to log cfu/g and subjected to analysis of variance along with the pH values (Statistical Graphics Corp., Rockville, IL, USA). Means and standard deviations were calculated, and when F-values were significant at the P<0.05 level, mean differences were separated by the Least Significant Difference (LSD) procedure.

RESULTS AND DISCUSSION

Microbiological attributes of commercial Galotyri cheeses and their shelf-life at 4°C

Populations of LAB in commercial Galotyri cheeses at purchase ranged from 7.5 to 8.1 log cfu/g on M-17 and MRS agar plates, while the total viable counts (TVC) enumerated on CASO agar plates were at similar levels (Table 1). There were no significant differences (P>0.05) between the industrial and artisan cheeses with regard to the populations of LAB grown on each of the above media. However, there were major differences in the predominant LAB groups in each cheese type, which were probably due to the different type of starters used in each plant. Specifically, the vast majority (96.7%) of the 60 isolates from either MRS or M-17 agar plates of the industrial Galotyri cheeses were homofermentative, arginine-negative, thermophilic (growth at 45°C; not at 15°C) cocci in chains, which showed weak or no growth in MRS broth with 2% salt; thus, they were thermophil-

Table 1 - Microbial populations (log cfu/g) present in commercial PDO Galotyri cheeses at the time of purchase from retail outlets of plant A (industrial) and plant B (artisan).

Microorganism	Galotyri cheese type	
	Industrial	Artisan
Total viable count	8.03±0.33[a]	7.87±0.33[a]
Lactic acid bacteria (MRS agar; 30°C)	7.55±0.88[a]	7.58±0.74[a]
Lactic acid bacteria (M-17 agar; 37°C)	8.11±0.49[a]	8.01±0.56[a]
Enterococci	<2.00[b]	4.62±1.00[a]
Enterobacteria	<1.00[b]	1.66±1.19[a]
Pseudomonads	<2.00[a]	<2.00[a]
Coagulase-positive staphylococci	<2.00[a]	<2.00[a]
Yeasts	2.93±0.78[b]	4.79±0.98[a]
Salmonella spp.	0/6	0/6
Listeria spp./*Listeria monocytogenes*	1/6*	2/6*

Mean values ± s.d. of 6 commercial batches per cheese type.
Means in each row with a different superscript letter differ significantly (P<0.05).
For *Salmonella* and *Listeria* (detection by culture enrichment per 25 g of cheese): Number of positive batches/number of batches tested. The asterisk indicates detection of *L. monocytogenes* by the API method.

ic dairy streptococci, probably *Streptococcus thermophilus*. In contrast, the respective agar plates of the artisan cheese samples contained mainly homofermentative, arginine-negative mesophilic (growth at 15°C; not at 45°C) lactobacilli (66.7% of the 60 isolates) and lactococci (26.7%), which were able to grow in 4 and 6.5% salt. Gas-producing LAB groups made-up a minor part of the technological flora in both the industrial (3.3%) and the artisan (6.6%) Galotyri, and were represented by few isolates of *Leuconostoc*-like bacteria (arginine-negative elongated cocci which grew at 15° and 45°C and in 6.5% salt). Interestingly, thermophilic dairy lactobacilli were not isolated from either type of Galotyri cheese, suggesting that this group might have been subdominant or absent. In fact, populations of thermophilic lactobacilli might have been overtaken by mesophilic lactobacilli on the MRS agar plates used for colony isolation following their incubation at 30°C, and not at 37°C which is a more favourable growth temperature for thermophilic lactobacilli.

Psychrotrophic milk spoilage bacteria, such as pseudomonads, were <2 log cfu/g in all the samples at the time of purchase (day 0), probably because of their increased sensitivity to the acid pH of the cheese. Conversely, yeast populations were considerably higher in the artisan cheeses ($P<0.05$) than in the industrial cheeses (Table 1).

Populations of LAB did not undergo major changes ($P>0.05$) during storage at 4°C when enumerated on M-17 (Fig. 1) or MRS agars, or as TVC on CASO agar plates (data not shown). Pseudomonads and closely-related gram-negative psychrotrophic milk spoilage bacteria remained <2 log cfu/g of cheese throughout storage (data not shown). Yeasts were the only microorganisms that grew significantly ($P<0.05$) in both types of commercial Galotyris, exceeding 7 log cfu/g after 14 to 21 days of storage at 4°C (Fig. 1). Yeast growth was associated with an increase in pH of the artisan cheese from pH 4.1±0.1 by day 14 to pH 4.4±0.2 by day 28 of 4°C storage. The pH of the industrial cheese remained at 3.8-4.0 throughout the storage time. Yeast growth in both cheeses resulted in slimy

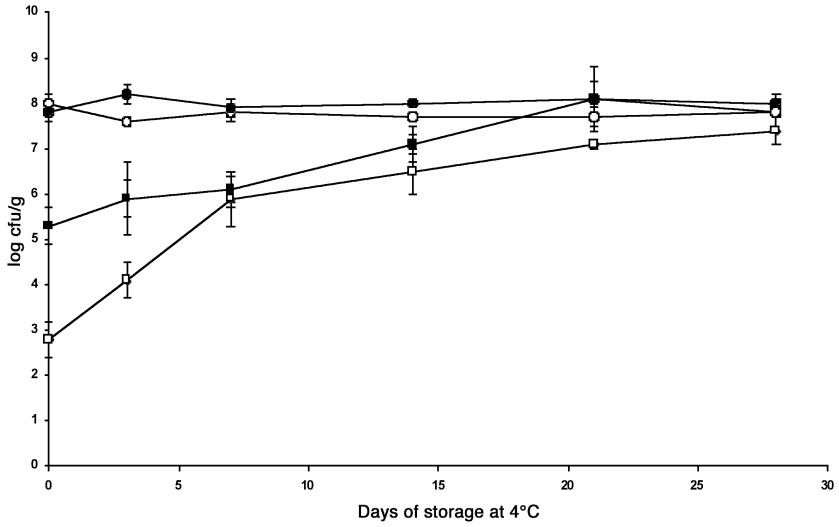


Fig. 1 - Changes in populations of lactic acid bacteria enumerated on M-17 agar (circle) and yeasts (square) during storage at 4°C of commercial Galotyri cheeses purchased from the retail outlets of industrial (opened) and artisan (closed) Greek dairy plants. Values are the means ± s.d. of two replicate cheese storage trials with two samples analyzed per replicate (n = 4).

yellowish yeast colonization spots on the cheese surface, which initiated at 14 and 21 days at 4°C in the artisan and industrial cheeses, respectively. The artisan and industrial cheese samples became macroscopically unfit for human consumption after 21 and 28 days, respectively. It was concluded that neither type of Galotyri was stable for more than two weeks when stored aerobically at 4°C.

These results indicate that commercial Galotyris, either industrial or artisan products, at the time of purchase contained viable LAB populations (ca. 8 log cfu/g) that were well above the minimum levels (6 to 7 log cfu/g) required for milk products with an active beneficial flora, such as yoghurt (IDF, 1992; MARAGKOUDAKIS *et al.,* 2006). Further results showed that the greatest differences in the technological LAB flora between the industrial and artisan cheeses were compositional rather than numerical. The fact that thermophilic dairy streptococci were contained almost as a mono-culture in all of the industrial cheese samples suggested that plant A always uses a commercial, single-strain dairy starter, probably *S. thermophilus*. Conversely, the natural starter preparations used to make artisan Galotyri in plant B were mainly mesophilic lactobacilli and lactococci. Mesophilic LAB species have been found to be dominant in many other artisan cheeses and generally dominate during ripening (HATZIKAMARI *et al.*, 1999; DE ANGELIS *et al.*, 2001; PIRAINO *et al.*, 2005). Additional biochemical and molecular studies are in progress in our laboratory to identify the LAB isolates from Galotyri at the species level and determine their origin (e.g., the milk, the starters used, the plant equipment, etc.).

Hygienic quality and safety of commercial Galotyri cheeses

With regard to the bacteria associated with plant hygiene, artisan cheeses generally contained significantly ($P<0.05$) higher populations of enterococci and enterobacteria than the respective industrial cheeses, while populations of coagulase-positive staphylococci were <100 cfu/g, and salmonellae were absent in 25 g of all the batches at day 0 (Table 1). Subsequently the number of these bacteria remained below or close to the above-cited levels throughout storage (data not shown). Based on these findings, the hygienic quality of commercial Galotyri cheeses was in compliance with the microbiological criteria of the most current EC Regulation 2073/2005 for cheese products, with the industrial cheese being slightly better than the artisan cheese. In particular, total enterobacterial counts in 83% of the batches tested were <100 cfu/g, which is the lowest level permitted for *Escherichia coli* in cheeses made from pasteurized milk. The coagulase-positive staphylococci were always below the highest permitted level (100 cfu/g) in soft non-ripened cheeses made from pasteurized or boiled milk. These results, along with the absence of *Salmonella* from all cheeses, were positive safety indices. Enterococci never exceeded 6 logs in the artisan Galotyri. Similar or higher enterococcal populations are often found in traditional Greek cheeses (TZANETAKIS and LITOPOULOU-TZANETAKI, 1992; HATZIKAMARI *et al.*, 1999; SARANTINOPOULOS *et al.,* 2001). There are still some safety concerns and scientific controversy with regard to the risks versus the benefits from the prevalence of enterococci in cheese (FRANZ *et al.*, 1999; FOULQUIE MORENO *et al.*, 2006), and their potential for safe use as starter and/or protective anti-listerial cultures in traditional cheeses (GIRAFFA, 1995; CENTENO *et al.*, 1999; LAUKOVA and CZIKKOVA, 2001; SARANTINOPOULOS *et al.*, 2002). Additional studies are needed to evaluate the significance, if any, of enterococci in artisan Galotyri.

In this survey, the only safety concern was the detection (by culture en-

richment, not by direct plating) of *L. monocytogenes* in 3 of the 12 commercial cheese batches analysed, including one industrial batch (Table 1). Specifically, 8 out of 12 total listerial colonies picked post-enrichment from PALCAM plates of naturally contaminated batches were identified as *L. monocytogenes* by the API method; the remaining colonies were *Listeria innocua*. Low detectable levels (<10 cfu/g by direct plating) of natural *Listeria* contaminants in those fresh cheeses were undetectable by culture enrichment after 7 days of storage at 4°C (data not shown). This positive result, however, should not lessen the concern associated with the presence of viable *L. monocytogenes* cells in 25% of cheese samples at purchase (Table 1). It appears that *L. monocytogenes* frequently contaminates Galotyri post-processing, which may indicate a poor hygienic level even at the industrial plants. *Listeria* spp., particularly *L. monocytogenes*, should not be present on the cheese processing lines in plants that apply effective cleaning procedures and use sanitation tools implemented in HACCP programs. If not, this is the first safety measure that should be taken. Additional tools should then be used, such as monitoring of moisture and salt content in cheese, use of effective starter or protective cultures or natural antimicrobials, ripening of cheeses, etc. Based on this survey, (i) measures should be taken to minimize *L. monocytogenes* from fresh cheese, and (ii) the common practice should be reconsidered to minimize or reduce ripening and/or in-plant chill-holding times of Galotyri in most Greek dairies. Survival of *L. monocytogenes* may be enhanced in fresh acid-curd cheeses that have a higher moisture and lower salt content, regardless of their low pH. Indeed, previous challenge studies have shown survival of *L. monocytogenes* in cottage cheese, the best known acid-curd cheese (HICKS and LUND, 1991; PICCININ and SHELEF, 1995). More recently, it was shown that artificially inoculated (3 log cfu/g) *L. monocytogenes* could survive for up to 28 d at 4°C in non-ripened industrial (pH 3.8) and artisan (pH 4.0) Galotyris, which had 76-77% moisture and 1.8% salt (ROGGA *et al.*, 2005) as compared to *ca.* 70% moisture and 3-4% salt reported for traditionally ripened Galotyri (ANIFANTAKIS, 1991; ANON., 1994). It should be stressed, however, that *L. monocytogenes* survived the 100-fold artificial contamination of Galotyri (ROGGA *et al.*, 2005) compared with the natural contamination (<10 cfu/g) with the pathogen in this study. Moreover, according to the new 2073/2005 EC microbiological regulatory criteria, 100 cfu/g of *L. monocytogenes* is considered the safe limit in all ready-to-eat foods with a pH≤4.4, such as Galotyri, because these foods do not support growth of the pathogen during storage. Although the observed death of *L. monocytogenes* in Galotyri after one week at 4°C storage confirmed the above regulation, we maintain that efforts in the cheese plants should continue to minimize the presence of *L. monocytogenes* in commercial Galotyri at the time it is to be sold. If viable cells of the pathogen survive in the fresh cheese, there is a potential scenario for the recovery and growth of such dormant *L. monocytogenes* cells as the pH increases in spoiling cheese during aerobic storage. Indeed, the commercial shelf-life of fresh Galotyris when sold unpacked in retail, and then stored in domestic refrigerators was shown to be limited, with yeasts being the main spoilage agents (Fig. 1). Yeasts generally increase the pH of acid foods at spoilage and may create micro-environments of pH>4.4 underneath their colonization spots on Galotyri surface where *L. monocytogenes* survivors may recover and increase before the cheese is considered unfit for consumption. This potential risk needs to be addressed in future studies. Further research is also

needed to study the microbial dynamics of the main LAB, yeast or other species involved in artisan Galotyri, determine their technological role and potential probiotic activities, and better monitor industrial manufacturing processes in order to preserve traditional sensorial characteristics of this popular Greek PDO cheese.

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SHORT COMMUNICATION

THE USE OF REFLECTANCE FOR MONITORING PHENOLIC MATURITY CURVES IN RED GRAPES

UTILIZZO DELLA RIFLETTANZA PER IL MONITORAGGIO DELLE CURVE DI MATURAZIONE FENOLICA NELLE UVE ROSSE

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ABSTRACT

A new laboratory method is discussed for monitoring phenolic quality in grape during maturation in the vineyard. Spectroscopy on whipped grapes was used to determine an index of phenolic maturity (Phenolic Maturity Trend - PMT) derived from the elaboration of the reflectance signals from six light emitting diodes in the spectral range 525-880 nm. The PMT index allows the trend of the overall maturity of polyphenols in grape berries to be qualitatively defined in laboratory and

RIASSUNTO

In questo lavoro di laboratorio viene studiata una metodica nuova per il monitoraggio della qualità fenolica dell'uva nel corso della maturazione. Attraverso una apparecchiatura spettroscopica si giunge alla determinazione, direttamente sul frullato di acini, di un indice di maturità (Maturity Trend) ricavato dall'elaborazione dei segnali di riflettanza provenienti da sei leds nel range spettrale compreso tra 525 e 880 nm.

L'indice MT consente di definire qualitativamente, in laboratorio, l'anda-

- Key words: analysis, phenolic maturity, polyphenols, red grape, reflectance, spectroscopy -

supplies information equivalent to that obtained with the traditional methods used to identify polyphenols. Since the method does not require polyphenol extraction, many more analyses can be done daily.

mento di maturazione globale delle sostanze polifenoliche contenute nelle bacche, fornendo informazioni equivalenti a quelle ottenute con le metodiche tradizionali in uso per la determinazione dei polifenoli. La metodica non prevede l'utilizzo di alcun estraente né tempi di preparazione del campione, e consente così di aumentare notevolmente il numero di analisi giornaliere.

INTRODUCTION

Methods for defining grape quality and determining the harvesting date are of fundamental importance for the enologist. In recent years, methods have been proposed and developed to meet these needs.

There are two main types of methods to evaluate maturity, one refers to the sensorial characteristics and the other to the analytical characteristics of the grapes. Approaches also exist which exploit plane and satellite imaging of the physiological state of the vineyard, with special spectrometric systems which analyse the signal coming from the surfaces of the leaves (JOHNSON *et al.*, 1996; PERNET *et al.*, 2003). This approach gives information on the state of leaf nutrition and, indirectly, the maturity level of the grapes. However, this approach, which has not yet been fully developed, requires specialised personnel training and costly instruments. Furthermore, the plane and satellite system only provides general information on the maturity level, without giving significant details about the grape composition.

One of the two most widely used categories of analysis is the sensorial one. It involves testing the grapes during maturation and evaluating the principal organoleptic characteristics in order to decide the date of harvest and for other technological purposes (DELTEIL and ROUSSEAU, 2000). The limitations of this approach are the sample size and the high number of judges required. These problems can be overcome by employing specialised personnel.

There are many laboratory procedures including some systems that analyse only a single analyte and those that evaluate groups of molecules with overall analytical indexes (chromatography, electro-analysis, infrared, etc.). The use of a light source in IR has been widely used in the food sector (BELTON *et al.*, 1995; PEARSON, 1999; BAUGMARTNER *et al.*, 2001; DAMBERGS *et al.*, 2003; 2006) and more recently in the enological sector for the qualitative determination of some compounds in musts and wines (BERGER *et al.*, 2003; BOUVIER, 2001; COPE, 2000; DUBERNET *et al.*, 2000a, b, 2001; PATZ *et al.*, 1999; ROUSSEAU *et al.*, 2002). The analyses are always carried out on samples of juice obtained from grapes after separation from the solid matrix, or after skin and pips have been removed by centrifugation (BERGER and HUCHETTE, 2003; GRANDJEAN *et al.*, 2004). Some experimental applications use infrared spectroscopy for non-destructive analyses for the appraisal of the polyphenols (DESSEIGNE *et al.*, 2003; DESSEIGNE, 2005; ROUSSEAU, 2003).

Spectrophotometry is also used in UV and VIS-transmittance approaches after extraction of the phenolic fractions (DI STEFANO *et al.*, 1991; GLORIES and AUGUSTIN, 1993; PEYRON, 1998; CELOTTI *et al.*, 1999; CELOTTI and CARCERERI, 2000; MATTIVI *et al.*, 2003).

The first analytical approaches were aimed at studying the content and type of polyphenols present in the grapes (DI STEFANO *et al.*, 1991) and their extractability (SINGLETON and DRAPER, 1964; RIBÉREAU-GAYON, 1971). Other methods have since been developed to classify the grapes (GLORIES and AUGUSTIN, 1993; GRAY *et al.*, 1997) in an attempt to develop the maceration methods during vinification.

These methods, some of which are very complex and not very practical, either economically or for the timely use of the information, have been combined with faster and more economical ways of testing phenol maturity.

Most of these methods evaluate the anthocyanin content by decolouration (GLORIES, 1984), after suitable extraction of the samples. The type of sample, solvent and extraction time varies from method to method but the evaluation of the decolouration by transmittance spectrophotometry is the same and is mainly limited to anthocyanin content.

Analysis times can be very long and depend on: 1) the type of sample e.g. a homogeneous sample of whole grapes or analysis of skins, pulp and pips separately; 2) the mode of action of the extractant: alcoholic, acidic, or enzymatic; 3) the length of time of the extraction, which may take several hours.

The information obtained from the various indexes is precise enough but requires lengthy and laborious procedures. The indexes give quantitative indications, even of the single analytes when suitable calibration curves are used, and qualitative information about the trends of the phenol accumulation in the grapes.

The results can be used by the viticulturist and enologist to determine the time of maximum polyphenol accumulation in order to decide the best date for the grape harvest.

The method proposed by GLORIES (1993) is the most widely used method, despite some deficiencies in both the original procedure and in that modified according to the requirements of the laboratories. The type of sample (whipped grapes) also allows it to be used in the wineries, where the process of skin-pulp separation (PEYRON, 1998; MATTIVI *et al.*, 2003) is impractical. Although the analysis is simple, the amount of work involved and the extraction times do not allow a large number of samples to be analysed daily.

In order to rectify these drawbacks, an attempt was made to develop a laboratory method that could produce the same qualitative information as that obtainable with these methods, but with simple sample preparation and faster times. Therefore the aim of this study was to determine the curves of accumulation of total polyphenols in grapes.

From the experience gained during the optimisation of colourimetric reading procedures for the on-line determination of an index of Phenolic Quality (PQ) of the grapes at delivery (CELOTTI and CARCERERI, 1999; 2000; CELOTTI *et al.*, 2001), laboratory and field equipment were developed to be used for the rapid determination of phenolic maturity in the vineyard. The aim was to obtain quick and precise estimation of the phenolic potential of red grapes to be used for agronomic and technological purposes in the winery.

MATERIALS AND METHODS

The equipment used consisted of a series of leds (light emitting diodes) covering the reflectance spectrum between 525 and 880 nm. Spectrophotometry,

which exploits measurement principles in the field of reflectance, can be used to carry out measurements on cloudy liquids. Therefore colourimetric information can be obtained from unrefined matrixes without any prior treatment of the samples.

Unlike all the other methods used so far, the measurements were carried out, directly on the whipped grape berries. All the information from each of the leds was suitably analysed by a photodiode, weighted and calibrated until the same information was obtained as that from a classical polyphenol extraction curve.

Sample preparation

Grape samples were made up of batches of 300 intact berries without stems, that were whipped immediately after harvesting.

The laboratory preparation procedure was standardised as follows: 200 g of grape berries were whipped, while the rest were centrifuged for the analyses of pH, total acidity and sugar content.

A normal kitchen mixer was used and the equipment parameters were standardised (treatment time was two minutes, sample loading was always at the same level, constant and at standard speed for two minutes) in order to guarantee the repeatability of the operation and to grind the whole grapes homogeneously.

Approximately 35 g of the whipped berries were used for the reflectance analysis, while the remainder, approximately 25 g, were used for the reference analyses that included extraction using solvents at pH 1.0 and pH 3.2 (according to GLORIES and AUGUSTIN, 1993) and methanol acidified to 1% with HCl (CELOTTI *et al.*, 1999). After extraction and separation of the solid particles, the anthocyanins were analysed by decolouration with SO_2 and total phenols by Abs 280 nm.

The equipment used for the reflectance analysis consists of an optical body on which six leds are mounted which direct their light rays onto a surface upon which a glass cuvette (volume 35 mL) is placed. The cuvette has a lid to avoid any interference from external light. The entire apparatus is connected to a computer.

A standard volume of whipped berries was placed in the cuvette and a reading of the reflectance generated by each led was taken using a photodiode. The time between sample treatment and reading was less than a minute and the reading of the reflectance from the sample took just a few seconds.

RESULTS AND DISCUSSION

Light source calibration

The equipment was calibrated by inserting two discs, one grey and one black, in the cuvette to be used as a background scale, in order to obtain a standardised and repeatable optimal electrical tension for the various leds. The calibration process was carried out using whipped grape berries of the varieties analysed, diluted with a whipped sample of white grapes, so that the natural evolution of polyphenols was reproduced from veraison to maturity (gradient). The response of each led was then compared with the variations in the percentage of red grapes maturation and the complete spectra of the signal traceable to these percentages (Fig. 1).

After correcting the incoming signal for the effect of darkening, using the ratio between each signal and the sum of all the signals (Fig. 2), it was possible to study the complete spectra for each percentage and analyse the signals in their entirety.

Percentages of white grapes were added to the red grapes being tested, starting with a concentration of 100% of the red grapes, until reaching the concentration of 50%, in six or seven steps.

The red grape concentrations varied approximately as follows: 100, 90, 85,

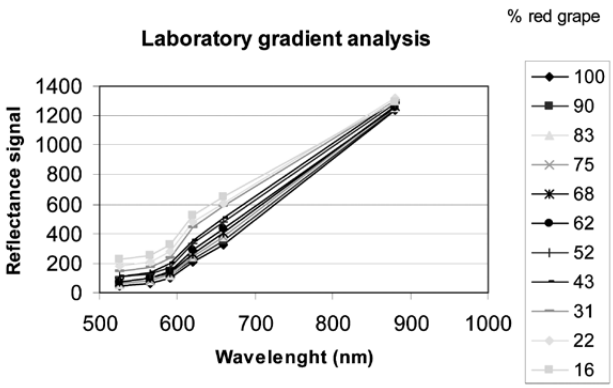


Fig. 1 - Reflectance signal from whipped grape berries in laboratory gradient.

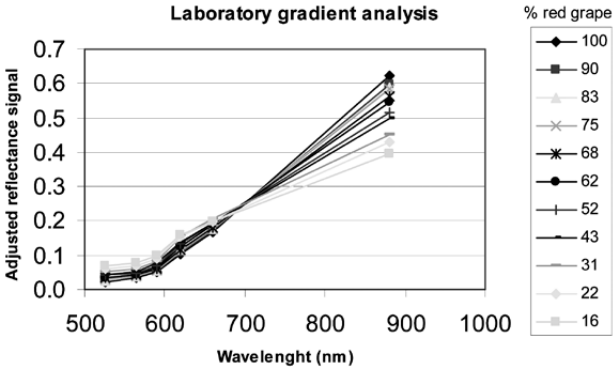


Fig. 2 - Adjusted reflectance signal from whipped grape berries in laboratory gradient.

80, 75, 70, 60 and lastly 50%, which represents veraison. In the case of Merlot, the lowest percentage of red grape was 15%. The main international cultivars were used: Merlot, Cabernet Franc, Cabernet Sauvignon and Pinot Noir as well as some native ones: Rondinella, Corvina, Corvinone, Montepulciano and Sangiovese.

A trend was identified from each led, which can be traced back to the increases or rather reductions of the signal. This finding gave the idea to use the chosen light sources because they could provide a uniformly variable reflectance signal.

Multiple regression analysis was applied in order to identify the LED variables that were significantly correlated to the percentage of phenolic maturity. Table 1 shows a few examples related to some of the gradients obtained. The data recorded for each variety used to construct the gradients allowed different weights to be attributed to each LED according to the *beta* values. Such a study could lead to

Table 1 - Main parameters of multiple regression applied to reflectance signals of different leds.

Cultivars	Multiple R^2	R^2	Adjusted R^2	*Beta*
Cabernet Sauvignon	0.8724	0.7612	0.7346	880 nm: 1.4 620 nm: -0.93 565 nm: -3.0 525 nm: -1.63
Merlot	0.8275	0.6847	0.6560	880 nm: 2.51 660 nm: 0.685 525 nm: 1.46
Montepulciano	0.7886	0.6218	0.6002	880 nm: -0.30 590 nm: -1.0
Sangiovese	0.9716	0.9439	0.9353	660 nm: -1.3 590nm: -0.75 565 nm: 2.54 525 nm: -1.7

the definition of a maturity index specific for each variety. The polyphenolic composition is undoubtedly due to both the genetic make-up of the plant and the soil and cropping characteristics. An attempt was therefore made to identify an algorithm by taking into account, in a weighted measurement, all the signals, minimising the sources of error due to external light, so that it could be used for all varieties without distinction.

Thus it was an attempt to identify an index that can summarise, in some way, all the variability of the signals obtained from the six leds, which almost completely describe the reflectance signal. This was done by optimising their contributions with the aim of drawing up an index with an appreciable and significant increase during the course of maturation.

The maturity index was obtained, by first weighting the leds until they reached the highest values of correlation with the gradients reproduced (Table 2 and Fig. 3) and then comparing them with the trends of phenol maturity obtained by using the traditional analysis systems. The trial was carried out on different varieties during their maturation.

Acting in this way, it was possible to reach an analytical compromise which provided an algorithm that defines the index of overall phenol maturity of the berry: the phenolic maturity trend (PMT).

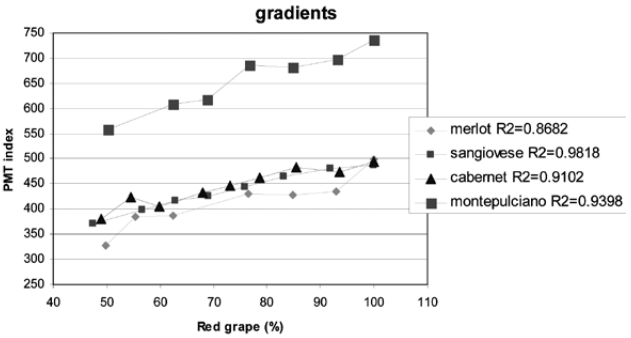


Fig. 3 - Relationship between % red grape and PMT index for Merlot, Cabernet Sauvignon, Montepulciano and Sangiovese.

The good relationship between the PMT index and the gradient with different percentages of red grape (maturation simulation) validates the use of the new index of reflectance for estimating a phenolic maturation curve that provides a good compromise between anthocyanins and tannins during maturation. The results obtained justified the use of the formula proposed for evaluating the phenolic maturity trend.

$$PMT = R880*5 / (R660*45 + R620*40 + + R590*5 + R565*5 + R525*5)$$

PMT = phenolic maturity trend index

R = reflectance value

The use of white grapes, prepared in the same way as the red, i.e. by stand-

Table 2 - Correlation parameters between the PMT index calculated from reflectance analysis and % of red grapes in the laboratory gradient.

Cultivars	Determination coefficients (R^2) of gradients between PMT index and red berry percentage				average	SD	RSD
Merlot	0.9554	0.9920	0.9703	0.8682	0.9465	0.0470	4.97
Cabernet Franc	0.7976	0.9239	0.8120	0.7768	0.8276	0.0570	6.89
Cabernet Sauvignon	0.9102	0.8438	0.9170	0.8750	0.8865	0.0294	3.31
Pinot Noir	0.9852	0.9791	0.9775	0.9878	0.9824	0.0042	0.43
Rondinella	0.8980	0.8920	0.9849	0.9279	0.9257	0.0368	3.97
Corvina	0.9485	0.9186	0.8694	0.8791	0.9039	0.0317	3.50
Corvinone	0.8980	0.8920	0.9849	0.9279	0.9257	0.0368	3.97
Montepulciano	0.9875	0.9397	0.8982	0.9489	0.9436	0.0317	3.36
Sangiovese	0.9817	0.9676	0.9603	0.9689	0.9696	0.0077	0.79

ardised sample treatment, provides a matrix in which the colourants (anthocyanins and tannins) are mainly derived from the contribution made by the percentage of red grapes. The supply of tannins from the white grapes is irrelevant in comparison to that due to the markedly higher concentration of the red grapes.

Taking these observations into account, the whipped sample of white grapes is nothing other than a plant matrix identical to that being studied and, as such, useful for constructing the gradients. These latter, being made of grapes at a fixed state of phenol maturity to which white grape is added, have the limitation of not considering any possible variation in the tannin/anthocyanin ratio during maturation.

The phenol maturity trend applied to the gradients is only useful for identifying a suitable index to represent the gradient increase regardless of the variety on which it was done.

To reduce errors linked to the variability of the tannin/anthocyanin ratio during maturation, gradients were also produced using grapes at different stages of maturity.

Analysis of the practical use of PMT in laboratory

Having proven that PMT is a reliable method for reproducing the increase in colour, the maturity trend was also measured on the whipped grapes on which the chemical analyses had been done after polyphenol extraction in order to compare the data obtained using the maturity index and data from phenolic extractions.

The phenolic maturity curves obtained by analysing the data from more than 40 vineyards were then compared with those obtained from the index trends.

The coefficient of determination (R^2) was also evaluated for each vineyard in order to identify how reliable the new index was in estimating phenol maturity (Figs. 4, 5).

The PMT index is significantly correlated with the percentage of polyphenols (see gradients) and is a valid compromise between anthocyanins and tannins. Therefore it is an ideal marker for expressing the evolution of the polyphenols in a maturing red grape.

The existence of the correlation between the classic maturity indexes (anthocyanins and total phenol analysis) and the PMT index proposed, means that the PMT could be used in laboratory to de-

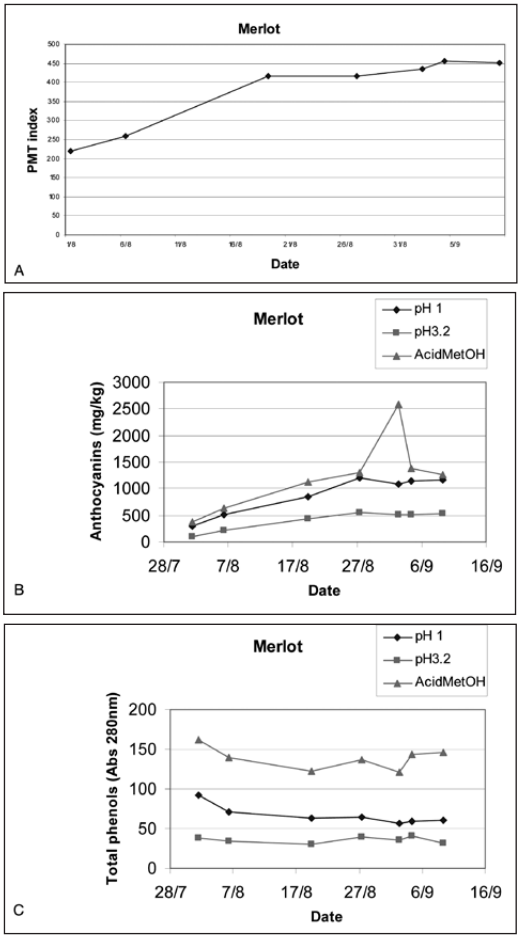


Fig. 4 - Maturation curve of Merlot using the PMT index, anthocyanins and total phenols (Abs 280 nm) extracted with different solvents (pH 1.0; pH 3.2; acidified methanol) from whipped berries.

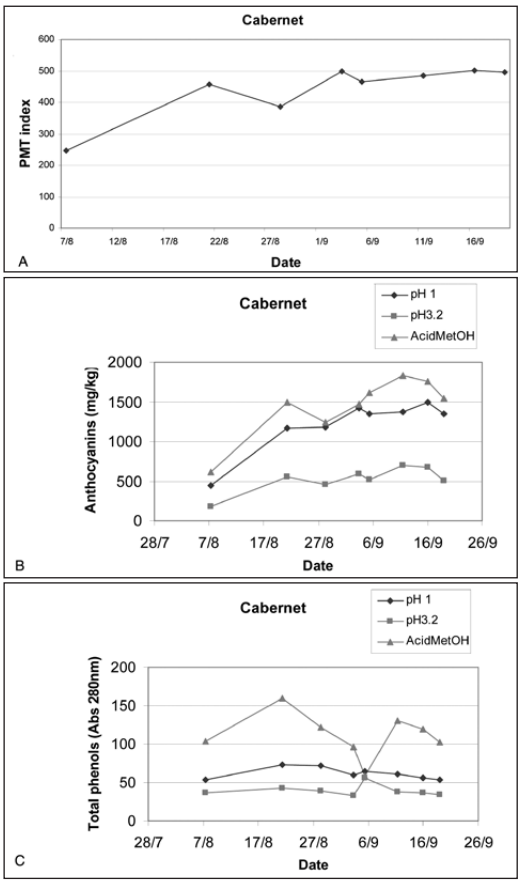


Fig. 5 - Maturation curve of Cabernet using the PMT index, anthocyanins and total phenols (Abs 280 nm) extracted with different solvents (pH 1.0; pH 3.2; acidified methanol) from whipped berries.

termine the curve of maturity in the vineyard. The PMT therefore not only corresponds to gradients produced in the laboratory, in which the phenols are fixed, but also to the evolution (change in the tannin-anthocyanin ratio) during maturation. The gradients have been produced from mature grapes as well as from grapes at different maturity levels which would have different anthocyanin/tannin ratios.

In the case of Merlot (Fig. 4) the anthocyanin curves obtained with three different extraction systems were very similar, but the best extraction was obtained with acidified methanol, that represents the real anthocyanin content of the skins. The PMT index derived from the direct reflectance measurement is well correlated to the anthocyanin trends (R^2 of PMT index vs. anthocyanins are 0.9167, 0.9404 and 0.9579, respectively for pH 1.0, pH 3.2 and acidified methanol extractions). If we consider the total phenols evaluated at 280 nm, the trends with the different solvents are similar but very different from the anthocyanin trends (R^2 of PMT index vs. total phenols at 280 nm are 0.7990, 0.0016 and 0.3010, respectively for pH 1.0, pH 3.2 and acidified methanol extractions).

The results for Cabernet (Fig. 5) showed a very similar trend between the PMT index and the anthocyanin analysis. Again, the total phenol trend at 280 nm was different from the anthocyanins and PMT index. The R^2 of PMT index vs. anthocyanins were 0.9354, 0.8478 and 0.8900, respectively for pH 1.0, pH 3.2 and acidified methanol extractions. Regarding total phenols at 280 nm, the R^2 with the PMT index were 0.0025, 0.0037 and 0.0007, respectively for pH 1.0, pH 3.2 and acidified methanol extractions.

Regarding the reference analytical methods, the acidified methanol extractant used had more effect than the pH 1.0 and pH 3.2 proposed by GLORIES and AUGUSTIN (1993). There was also a certain objective difficulty in identifying a single reference method for the rapid measurement of the total polyphenols. Currently Abs 280 nm is used most often, but it provides no information about the ratio between total polyphenols and anthocyanins.

The very good relationship between the PMT applied to the gradients in the laboratory allows this parameter to be used as an estimate of potential polyphenol accumulation in the berry. The results obtained by comparing the traditional maturity curves demonstrate that the new PMT is applicable in prac-

tice, particularly for anthocyanin estimation. One limitation is that PMT provides a qualitative evaluation (curve of maturity) but no quantitative data on the polyphenols.

CONCLUSIONS

The trend of phenol maturity reconstructed using the PMT index was significantly correlated with the quantitative evaluations of the total polyphenols. Use of the laboratory gradients permitted a generally good calibration of the equipment for defining the PMT in all of the cases analysed.

The PMT index is significantly correlated with polyphenol percentage (see gradients) and is a valid compromise between anthocyanins and tannins. It is therefore an ideal marker for expressing the evolution of potential polyphenols in red grape during maturity.

While the proposed PMT index, does not provide quantitative information about the polyphenolic concentration, it does allow the trend of phenol maturity to be qualitatively defined very quickly in laboratory. After the treatment to obtain the whipped grape berries, it only takes seconds to memorise and determine the index. The method is simple to use, provides rapid analysis and allows traditional analysis techniques to be eliminated.

Some possible applications are that it can be used to obtain a qualitative definition of the trend of phenol maturity, determine the optimal harvesting date, compare vineyards of the same variety, compile a databank that would be useful for optimising vineyard management, improve the management of large consignment deliveries to the winery and lastly, given the absence of laborious analysis phases, allow many more samplings to be done and therefore increase the number of vineyards checked.

The quantification of polyphenols using traditional laboratory systems could therefore be limited to just one sampling close to the expected harvest time. The data obtained could be used to decide the harvest date.

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SHORT COMMUNICATION

PHENOLIC CHARACTERIZATION OF GRAPEVINE CULTIVARS FROM GALICIA (SPAIN): BRANCELLAO, MERENZAO AND MENCÌA (*VITIS VINIFERA* L.)

CARATTERIZZAZIONE FENOLICA DI VITIGNI DELLA GALIZIA (SPAGNA): BRANCELLAO, MERENZAO E MENCÌA (*VITIS VINIFERA* L.)

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ABSTRACT

The polyphenolic composition of Brancellao, Merenzao and Mencìa grapes from Galicia (northwestern Spain) was studied using spectophotometric and High Performance Liquid Chromatographic (HPLC) methods. The results showed significant differences between these cultivars. The total anthocyanins ranged from 215 mg/kg berries in Brancellao to 422 mg/kg berries in Mencìa. The three cultivars were characterized by an interesting anthocyanin profile for winemaking

RIASSUNTO

La composizione polifenolica di tre vitigni provenienti dalla Galizia (Nord Ovest, Spagna) è stata valutata per la prima volta con l'utilizzo di metodi analitici spettrofotometrici e HPLC.

I risultati mostrano differenze tra le diverse cultivar. L'indice di antociani totali varia tra i 215 mg/kg uva del Brancellao e i 422 mg/kg uva della Mencìa. Le cultivar sono caratterizzate da un profilo antocianico interessante dal punto di vista tecnologico per la prevalenza di malvidina-3-glucoside. Sia i vi-

- Key words: Anthocyanin profile, flavonoids, Galician grapevine, grape skins, HPLC, seeds -

with a prevalence of malvidin-3-glucoside. The skins and seeds, both had small amounts of flavonoids. In contrast to Brancellao and Mencìa, Merenzao had more flavonoids in the seeds (64%) than in the skins. The most important parameters for differentiating between the cultivars were the peonidin and malvidin derivative forms, and the total amounts of acetyl-glucosides.

naccioli, sia le bucce mostrano un basso contenuto in flavonoidi. Contrariamente al Brancellao e alla Mencìa, il Merenzao possiede più flavonoidi nei vinaccioli (64%) che nelle bucce. I più importanti componenti di differenziazione tra le cultivar studiate risultano le forme esterificate della peonidina e malvidina e le forme acetil-glucosilate.

INTRODUCTION

The phenolic composition of wines depends mainly on the phenolic content of the grapes and on the numerous reactions that occur during juice extraction, winemaking and wine aging. The phenolic content of the berries is of great ampelographic and taxonomic importance in viticulture for classifying grape cultivars (CRAVERO *et al.*, 1994; DI STEFANO, 1996; GUIDONI *et al.*, 2003; MATTIVI *et al.*, 1993) because it varies greatly between species and cultivars (CRAVERO *et al.*, 1994; GONZALEZ-NEVES, 2001; 2005; MATTIVI *et al.*, 2003).

Moreover, the synthesis and concentration of phenols in red grapes depend on a number of environmental factors, as well as vineyard management practices. Of particular importance are fruit ripeness, climatic conditions, soil features and crop load (DI STEFANO *et al.*, 1994; GUIDONI *et al.*, 2002; JACKSON and LOMBART, 1993; PEREZ-MAGARINO and GONZALEZ-SAN JOSE, 2006; ROSON and MOUTOUNET, 1992; ROUBELAKIS-ANGELAKIS and KILEWER, 1986).

Many authors have reported on the anthocyanin profile in the grape skins of several grape cultivars, on the anthocyanin evolution during grape ripening (CLIMENT and PARDO, 1997; HMAMOUCHI *et al.*, 1995; GERBI *et al.*, 2003; MAZZA *et al.*, 1999; TAMBORRA *et al.*, 2003; RYAN and REVILLA, 2003; LOVINO *et al.*, 2006) and on the tannins in skins and seeds (HARBERTSON *et al.*, 2002; ROSON and MOUTOUNET, 1992; TAMBORRA and DI BENEDETTO, 1991). Such information can also be used to evaluate enological potential (CHEYNIER *et al.*, 1997; GERBI *et al.*, 2002; OTTENEDER *et al.*, 2004).

In recent years interest in autochthonous or rare European cultivars and in the unique wines that can be produced from them has increased and many heirloom varieties have been rescued from potential extinction. Many studies on the recovery and qualitative evaluation of minor grapevine cultivars have been carried out in recent years (MARTINEZ and PÉREZ, 2000; SANTIAGO *et al.*, 2005a,b; SCHNEIDER *et al.*, 2001; ZEPPA *et al.*, 2001). There has been particular interest in mountainous viticultural areas. Despite the difficulty of cultivating them because of steep slopes and pedoclimatic conditions, many mountainous areas have preserved a wealth of biodiversity.

A European project, entitled "Sustainable Enhancement of Autochthonous Wine Grapes in Mountain Areas", is aimed at the recovery, conservation and exploitation of the viticultural patrimony of mountain areas. Within the framework of this project, the three indige-

nous cultivars, Mencìa, Brancellao and Merenzao, cultivated in Galicia (northwestern Spain), have been studied (ORRIOLS *et al.*, 2006). The aim of this work was to analyze the phenolic composition of these cultivars in order to assess the most important parameters that characterize and differentiate them.

MATERIALS AND METHODS

Grapes of *Vitis vinifera* cvs. Brancellao, Merenzao and Mencía were collected from nine Ribeira Sacra valley (Galicia, Spain) vineyards located at altitudes over 300 m a.s.l. in 2003 and 2004. The vines had similar characteristics (age, pruning system, amount of buds) and were grown under controlled pedoclimatic conditions.

The samples were harvested at technological ripeness and picked in a statistically representative way from different parts of the bunch. In each vineyard, 300 berries were collected, and three replicates of 10 berries each were then weighed before phenolic extraction.

A total of 18 samples were analyzed for each cultivar; three replicates from each of the three vineyards, were analyzed during the two years.

Samples for analyzing berry skins and seeds were prepared according to the DI STEFANO and CRAVERO (1991) method which reproduces vinification extraction conditions.

The berry skins were removed manually from the pulp and dried with paper. They were then quickly immersed in 25 mL of a buffer solution containing 12% ethanol, 600 mg/L of sodium metabisulfite, 50 mg/L NaN_3, 5 g of tartaric acid and titrated to pH 3.20 by adding 1N NaOH. After homogenization with an Ultraturrax T25 (IKA Labortechnik, Staufen, Denmark), the extract was centrifuged for 10 min at 3,000 rpm at 20°C. The supernatant was then used for analysis.

The seeds were removed from the mesocarp, placed in 50 mL of the same buffer solution used for the skin extraction at pH 3.20 and then put in a temperature-controlled room at 25°C for a week. The extract was then used for analysis.

Analysis

The analytical parameters of technological ripeness (reducing sugars, total acidity, pH) were estimated with official methodologies EC (EEC 1990).

Spectrophotometric methods were used to evaluate the total anthocyanin and flavonoid content in the berry skins and seeds (DI STEFANO, 1996; DI STEFANO and CRAVERO, 1991; DI STEFANO *et al.*, 1994). Analysis of individual anthocyanins was performed after the berry skin extract was placed in a 300 mg SEP-PAK C18 cartridge (Waters Corporation, Milford, MA, USA) and eluted with methanol. The cartridge was preconditioned with methanol (2 mL), water (5 mL) (DI STEFANO *et al.*, 1989) and 0.01N H_2SO_4 (2 mL) before elution. A P100 chromatograph was used that was equipped with an AS3000 auto-sampler (Spectra Physics Analytical, Inc, San Jose, CA, USA) and a 20 mL Rheodyne sample loop.

A LiChroCART analytical column (25x0.4 cm i.d.) from Merck (Darmstad, Germany) was used. It was packed with LiChrosphere 100 RP-18 5-μm particles from Alltech (Deerfield, IL, USA) and was equipped with a Spectra Focus Diode Array Detector (Spectra Physics Analytical, Inc, San Jose, CA, USA) operating at 520 nm.

The following conditions were used: solvent A = 10% formic acid in water. Solvent B = 10% formic acid with 50% methyl alcohol in water. These solvents were filtered through a 0.20 μm filter. Solvent flow rate was 1 mL/min. The solvent program used was 72% A to 55% A over 15 min; to 30% A over 20 min; to 10% A over 10 min; to 1% A over 5 min; to 72% A over 3 min. An equilibri-

um time of 10 min was used (Guidoni *et al.*, 2002). The data treatment was carried out using the ChromQuest™ chromatography data system (ThermoQuest, Inc, San Jose, CA, USA).

The anthocyanins in the berry skin extract were identified by matching UV-Vis spectra, the retention times of each chromatographic peak with available data in the literature and using authentic standards purchased from Extrasynthèse (Genay, France).

The single anthocyanin concentrations were determined by comparing the area of the individual peak with the total peak area; the data are expressed in percentages (DI STEFANO and CRAVERO, 1991; HEBRERO *et al.*, 1988).

The statistical differences between the quantities of the various phenolic compounds of the three cultivars analyzed were determined by analysis of variance (ANOVA) using STATISTICA for Windows Release 6.0 (StatSoft Inc., Tulsa, OK, USA).

RESULTS AND DISCUSSION

The average technological ripeness parameter values for the two years in the three vineyards are reported in Table 1. The pH values for the three cultivars were the same. Merenzao had the highest sugar content and the lowest total acidity, while Brancellao had the lowest sugar content and the highest total acidity.

The total amounts of anthocyanins and flavonoids in berry skins and seeds of Brancellao, Mencìa and Merenzao are reported in Table 2.

The results of the analysis of variance showed that the three cultivars differed

Table 1 - Technological ripeness of Brancellao, Mencìa and Merenzao grapes. Values are the means (± standard deviations) of 3 vineyards and 2 years.

	Brancellao	Mencìa	Merenzao
Reducing sugars (g/L)	199±6	200±31	219±14
pH	3.6±0.4	3.6±0.0	3.6±0.1
Total acidity (g/L tartaric acid)	6.5±0.5	4.7±0.4	5.0±0.8

Table 2 - Polyphenolic parameters of the Brancellao, Mencìa and Merenzao berry skins and seeds. The values are the means (± standard deviations) of 18 samples representing three replicates picked from each of the three vineyards and analyzed in the two years. The average values with the same superscript letter are not statistically different at the 5% level.

		Brancellao	Mencìa	Merenzao	Significance
Berry skins	Total anthocyanins (mg malvidin-3-O-glucoside chloride/kg berries)	215^{a}±53	422^{b}±111	$347^{a,b}$±26	*
	Total flavonoids (mg (+) catechin/kg berries)	$3,296^{b}$±511	$2,370^{a}$±168	$1,940^{a}$±140	**
Seeds	Total flavonoids (mg (+) catechin/kg berries)	$2,158^{b}$±189	$1,803^{a}$±188	$3,492^{c}$±136	***
Berry skins and seeds	Total flavonoids (mg (+) catechin/kg berries)	$5,454^{b}$±689	$4,173^{a}$±355	$5,432^{b}$±5.6	**
	% seeds total flavonoids	40	43	64	
	% berry skin total flavonoids	60	57	36	

*: p≤0.05; **: p≤0.01; ***: p≤0.001.

significantly with respect to the flavonoid and anthocyanin contents in both berry skins and seeds.

With regards to the total anthocyanin concentration, Brancellao and Mencìa were completely different while Merenzao had an intermediate behavior. The anthocyanin concentrations in the berries varied from 215 mg/kg in Brancellao to 422 mg/kg in Mencia. The latter value is approaching that of Nebbiolo (550 mg/kg berries) (GERBI *et al.*, 2003), but is low compared to 1,577 mg/kg of Cabernet Sauvignon or 1,541 mg/kg of Sangiovese (TAMBORRA and DI BENEDETTO, 1991).

The total flavonoid concentrations in the berry skins of the three cultivars were higher in comparison with some French and Italian cultivars such as Cabernet sauvignon and Sangiovese (TAMBORRA and DI BENEDETTO, 1991) at harvest but approached that of Nebbiolo (GERBI *et al.*, 2003; GUIDONI *et al.*, 2002). The total flavonoid concentrations in the seeds were also high compared to Cabernet sauvignon (1,479 mg/kg berries) and Sangiovese (1,388 mg/kg berries) which were analyzed by TAMBORRA and DI BENEDETTO (1991).

Based on their flavonoid concentrations, the three cultivars have different skin and seed profiles. Indeed, there were more flavonoids in Brancellao berry skins (3,296 mg/kg berries) than in Mencìa or Merenzao. Whereas Merenzao was the cultivar with the highest flavonoid content in the seed (3,492 mg/kg berries).

The total flavonoid concentrations (sum of berry skins and seeds) for Brancellao and Merenzao were nearly the same as Merenzao (5,454 mg/kg berries and 5,432 mg/kg berries respectively), but the distribution between the berry skins and seeds varied. The flavonoids in Brancellao and Mencìa were primarily located in the skins (60 and 57%, respectively). In contrast, 64% of the flavonoids were located in the Merenzao seeds.

The anthocyanin profiles of Brancellao, Mencìa and Merenzao grapes are shown in Fig. 1. In Table 3, the anthocyanins are grouped according to the acylation (acetyl-glucosides, cinnamoyl-glucosides and free-form monoglucosides) and anthocyanidin (malvidin, delphinidin, petunidin, cyanidin and peonidin) compounds. The cinnamoyl-glucosides included both p-coumaroyl and caffeoyl anthocyanin forms.

The monoglucoside group made up the highest proportion of the anthocyanin forms (72% in Brancellao, 71% in Merenzao and 50% in Mencìa). The anthocyanin distribution in Brancellao was very similar to that in Merenzao, with nearly the same proportions of monoglucosides, acetyl-glucosides (8 and 10% respectively) and cinnamoyl-glucosides (19.5 and 20%, respectively). In contrast, Mencìa was characterized by a smaller proportion of monoglucosides (50%) and higher proportions of acetyl derivatives (21%) and cinnamoyl derivatives (29%) (Table 3).

Malvidin derivative forms were the most abundant anthocyanin group in Brancellao, Merenzao and Mencìa, as in Cabernet sauvignon, Cabernet franc, Merlot and Tempranillo (NAGEL and WOLF, 1979; MAZZA *et al.*, 1999; HEBRERO *et al.*, 1988). Brancellao had the lowest proportion of malvidin derivatives (64%), followed by Merenzao (73%) and Mencìa (85%). The percentages of delphinidin and cyanidin derivative forms in the three cultivars were low (not more than 2%). Merenzao had a slightly higher proportion of petunidin derivative forms (5%), followed by both Brancellao and Mencìa (2.5%). The percentages of peonidin derivative forms, however, were distinctly different among the cultivars: Brancellao (31%), Merenzao (18%) and Mencìa (10%).

Monagas *et al.* (2003) established that in wines made from Graciano, Tempranillo and Cabernet sauvignon grapes, the ratios between cinnamoyl and acetyl-glu-

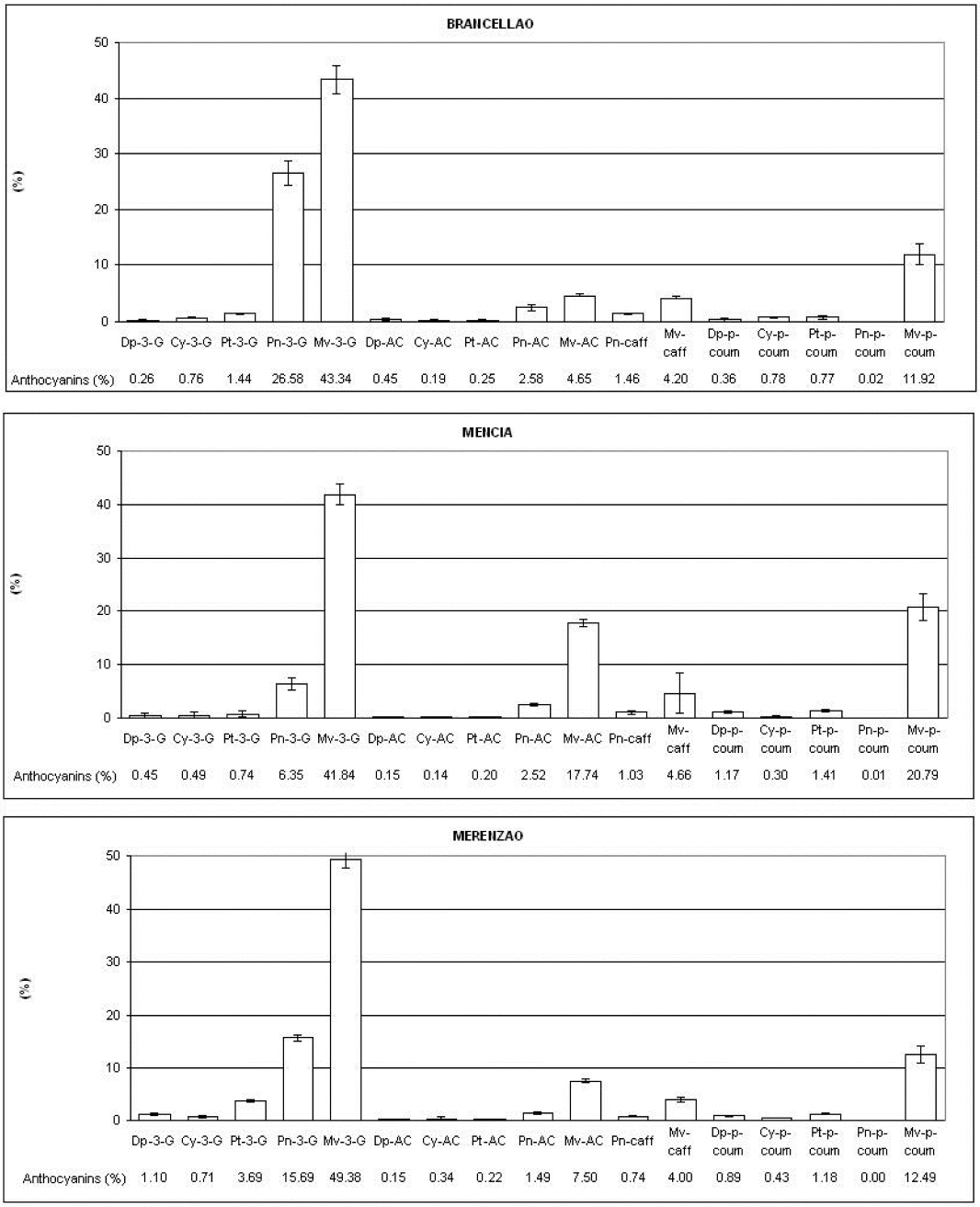


Fig. 1 - Anthocyanin profiles of Brancellao, Mencìa and Merenzao grapes. The amount of each anthocyanin is expressed as the mean of the percentages of the total anthocyanins of 18 samples representing three replicates picked from each of the three vineyards and analyzed in the two years. Key: Dp delphinidin; Cy cyanidin; Pt petunidin; Pn peonidin; Mv malvidin; G glucoside; AC acetyl-glucoside; p-coum p-coumaryl-glucoside; caff o-caffeoyl-glucoside. Vertical bars denote standard deviation computed for each mean value, n = 18.

Table 3 - Percentages and ratios of anthocyanins in Brancellao, Mencìa and Merenzao grapes. The values are the means (± standard deviations) of 18 samples representing three replicates picked from each of the three vineyards and analyzed in the two years. The percentage values with the same superscript letter are not statistically different at the 5% level.

	Brancellao	Mencìa	Merenzao	Significance
Monoglucosides	72.4^{b}± 1.8	50.2^{a}± 2.9	70.6^{b}±1.2	***
Acetyl-glucosides	8.1^{a}± 0.7	20.8^{c}±1.0	9.7^{b}± 0.4	***
Cinnamoyl-glucosides	19.5^{a}± 2.4	29.0^{b}± 3.4	19.7^{a}± 1.5	**
Total anthocyanidins (%)				
Sum of malvidin derivative forms	64.1^{a}± 2.4	85.0^{c}± 1.7	73.4^{b}± 0.7	***
Sum of delphinidin derivative forms	1.1^{a}±0.7	1.8^{a}± 0.4	2.1^{a}± 0.5	ns
Sum of petunidin derivative forms	2.5^{a}± 0.3	2.4^{a}± 0.6	5.1^{b}± 0.2	***
Sum of cyanidin derivative forms	1.7^{a}±0.2	0.9^{a}± 0.5	1.5^{a}± 0.4	ns
Sum of peonidin derivative forms	30.6^{c}±2.8	9.9^{a}± 1.3	17.9^{b}± 0.6	***
Cinn/Ac	2.4±0.5	1.4±0.2	2.0±0.2	
Mvs/Pns	2.1±0.3	8.5±1.3	4.0±0.1	

Cinn: cinnamoyl-glucosides; Ac: acetyl-glucosides; Mvs: malvidin derivative forms; Pns: peonidin derivative forms; Sign: significance; ns: not significant; *: p≤0.05; **: p≤0.01; ***: p≤0.001.

cosides (Cinn/Ac) and between the malvidin derivative forms and the peonin derivative forms proportions (Mv/Pn) could be used as varietal markers. These ratios were calculated for the Brancellao, Mencìa and Merenzao grapes (Table 3); the (Cinn/Ac) ratios for Brancellao and Merenzao were very similar but were different from Mencìa (1.4). On the other hand, the (Mv/Pn) ratio was lower in Brancellao (2.1) than in Merenzao (4.0) and Mencìa (8.5). The presence of more stable molecules, such as the trisubstituted anthocyanins such as malvidin and its acylated forms, would give more stability to the wine color during winemaking. Malvidin is more resistant to oxidation (RIBEREAU-GAYON *et al.*, 2000).

The anthocyanin profile of Cabernet franc was analyzed by MAZZA *et al.* (1999). In Mencìa, delphinidin-3-glucoside accounted for only 0.4% of the anthocyanins while in Cabernet franc it constitutes 15% of the anthocyanins. The cinnamoyl derivatives accounted for 29% of the anthocyanins in Mencìa but only 6.7% in Cabernet franc. Even if malvidin derivative forms were the most abundant anthocyanin group in the two cultivars, the proportion was higher in Mencìa than in Cabernet franc (85 and 35%, respectively). This result is in accord with MARTINEZ and PÉREZ (2000) who found that Mencìa grapes were completely different from Cabernet franc grapes.

The values reported in Table 3 and the analysis of variance of the corresponding data indicated the decreasing order of importance of the acetyl-glucosides, monoglucosides and the cinnamoyl-glucosides. These could be used to differentiate the three Galician varieties. But the best parameters for differentiating were the total derivative forms of anthocyanidins, especially of malvidin-3-glucoside and peonidin-3-glucoside. These results are in accord with CALÒ *et al.* (1994) who showed that they were more important than the petunidin-3-glucoside derivatives in differentiating grapevine cultivars. As GONZALEZ-NEVES *et al.* (2001) reported, the situation in wines differs and is based on petunidin and malvidin glucosides, with the acetyl esters of the petunidin, peonidin and malvidin

glucosides. This indicates the importance of methoxylation in varietal differentiation.

CONCLUSIONS

It can be confirmed that parameters such as total anthocyanins, total flavonoids and the anthocyanin profile show important significant differences between Brancellao, Mencìa and Merenzao grapes. Other indicators such as the cinnamoyl-glucoside/acetyl-glucoside and the malvidin derivative/peonidin derivative ratios, can also be used to differentiate between the three cultivars. However, more studies with grapes from different production areas should be carried out to complete these observations. In addition, a detailed study in the future on the phenolic composition of wines produced from the Brancellao, Mencìa and Merenzao grapes would be interesting to check the differentiating parameters in wine.

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SHORT COMMUNICATION

INCIDENCE OF *AEROMONAS* SPP. IN MARINE FISH AND THE ENVIRONMENT OF FISH MARKETS IN NORTHERN GREECE

INCIDENZA DI *AEROMONAS* SPP. NEL PESCE MARINO E NELL'AMBIENTE DEI MERCATI DEL PESCE NELLA GRECIA DEL NORD

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ABSTRACT

Samples (fish, personnel and environmental) were collected from retail fish markets in Thessaloniki (Northern Greece) and tested for the presence of *Aeromonas* spp. They consisted of 120 fish samples representing four types of fish: mackerel, bogue, horse mackerel and whiting. In addition, 20 samples each were taken from the personnel workers' hands, workers' knives, work surfaces, wooden boxes and floor surfaces. Of the fish samples examined 65, 28, 24 and 12% harboured *Aeromonas*

RIASSUNTO

Sono stati raccolti dei campioni (di pesce, del personale addetto e nell'ambiente) in un mercato di pesce al dettaglio di Tessalonicco (Grecia del nord) e testati per verificare la presenza di *Aeromonas* spp. I campioni consistevano di 120 pesci di 4 tipologie: il maccarello; la boga; il sugarello e il merlango, 20 campioni sono stati raccolti dalle mani del personale, 20 dai coltelli, 20 dai piani di lavoro, 20 dalle scatole di legno e 20 dal pavimento. Delle 4 tipologie di pesce esaminate il 65, il 28,

- Key words: A*eromonas* spp., environment, fish, personnel -

spp., *Aeromonas hydrophila, Aeromonas caviae* and *Aeromonas sobria*, respectively. Of the personnel and environmental samples examined, 69, 25, 26, and 13% harboured *Aeromonas* spp., *A. hydrophila, A. caviae* and *A. sobria*, respectively. *A. hydrophila* and *A. caviae* were the dominant species detected in the fish, personnel and environmental samples.

il 24 e il 12% rispettivamente ospitava *Aeromonas* spp., in particolare *Aeromonas hydrophila*, *Aeromonas caviae* e *Aeromonas sobria*. Dei campioni del personale e quelli ambientali, il 69, il 25, il 26 e il 13% conteneva rispettivamente *Aeromonas* spp., *A. hydrophila*, *A. caviae* e *A. sobria*. *A. hydrophila e A. caviae* erano le specie dominanti ritrovate nel pesce, nel personale e nell'ambiente.

INTRODUCTION

Aeromonas hydrophila and other motile aeromonads are widely distributed in the aquatic environment, in both fresh and saline waters (PALUMBO *et al.*, 2000). Water is the likely source of these bacteria in food, with fish and seafood often being contaminated (PALUMBO *et al.*, 2001). Due to the nature of their normal habitat, *Aeromonas* spp. are found in many other types of food, especially food of animal origin such as red meat, poultry, etc. (PALUMBO *et al.*, 2000), raw milk and dairy products (MELAS *et al.*, 1999). In addition, motile aeromonads can be isolated from a wide range of other food such as vegetables, spices, and fresh and processed food (PALUMBO *et al.*, 2000). *Aeromonas* spp. are also associated with the spoilage of refrigerated food products (BUCHANAN AND PALUMBO, 1985) and it can grow competitively in food held at 5°C (PALUMBO *et al.*, 1985). In some food, motile aeromonads occur at a level of 10^5 cells/g or mL (RAY, 2004). This group of bacteria can be recovered from food that has been stored at -20°C for many years and successfully stored in culture media at -70°C (KIROV, 2001).

Some *Aeromonas* spp. are considered to be important pathogens to humans, amphibians, reptiles and fish (AUSTIN and ALLEN-AUSTIN, 1985; GOODWIN *et al.*, 1983). These bacteria have been implicated as the cause of gastroenteritis, septicemia and meningitis, particularly, in young children, the elderly and the immuno-compromised, when consumed in large numbers (ABEYTA *et al.*, 1986; VARNAM AND EVANS, 1991). Cases of endocarditis, urinary tract infections, pulmonary infections, arthritis, and osteomyelitis involving *Aeromonas* spp. have also been reported (KROVACEK AND FARIS, 2003).

Aeromonas (principally *A. hydrophila*) has the status of a food-borne pathogen of emerging importance. Since fish may be a vehicle for this group of bacteria it is important to have information on the incidence of this pathogen. Information on the incidence of *Aeromonas* spp. in Greek fish is rather limited. Thus, the purpose of the present study was to investigate the presence of *Aeromonas* spp. in fresh fish, on the personnel and in the environment of fish markets in Thessaloniki.

MATERIALS AND METHODS

One-hundred and twenty fish samples (flesh and skin), including 30 from mackerel (*Scomber scombrus*), 30 from bogue (*Boops boops*), 30 from horse mackerel

(*Trachurus trachurus*) and 30 from whiting (*Merlangius merlangus*), were bought at the fish markets in Thessaloniki. Fish were caught in the North Aegean Sea, covered with ice and brought to the fish markets the same day. Also, one-hundred environmental and personnel (swabbed) samples, including 20 from workers' hands, 20 from workers' knives, 20 from work surfaces (wooden board), 20 from containers (wooden boxes) and 20 from the floors, were taken from the fish markets. About 100 cm^2 of plane surfaces and whole workers' hands and knife surfaces were swabbed 2-5 times by the wet-dry double swab technique using sterile cotton swabs moistened with 0.1% sterile peptone water containing 0.85% sodium chloride. The same sampling procedure was repeated using a dry swab. The 2-5 swabs were pooled as one sample. All samples were transported to the laboratory inside cold portable insulated boxes and processed within 1 h of collection. All samples were collected during the nine-month period from September 2003 to May 2004. Swab samples were directly inoculated in tubes containing 10 mL of sterile tryptone soy broth (TSB, Biokar Diagnostics) containing 30μg/mL of ampicillin (Sigma Chemical Co., St. Louis, Mo.) and incubated at 28°C.

For fish samples, 25 g portions consisting of skin and flesh were taken from the anterior-dorsal region and placed in sterile stomacher bags containing 225 mL of TSB; samples were blended for 2 min in a stomacher (Lab Blender 400, A.J. Seward and Co. Ltd., London) and incubated at 28°C for 18 h (enrichment). One loopful of the enriched culture was streaked onto Starch Ampicillin (SA) agar and incubated at 28°C for 24 h. SA agar was prepared according to PALUMBO *et al.*, (1985). SA agar plates were flooded with ca. 5 mL Lugol iodine solution (PALUMBO and BUCHANAN, 1988). The yellow to honey coloured colonies surrounded by a clear zone were scored as presumptive *Aeromonas* spp. Five presumptive *Aeromonas* colonies were selected from each plate and transferred to nutrient agar (Biokar Diagnostics) slants and incubated at 28°C for 24 h for further studies.

Only those isolates that were gram-negative rods, motile, oxidase positive, glucose fermenting, and O/129 resistant were considered aeromonads (CARNAHAN *et al.* 1991). The classification into the species *A. hydrophila, A. sorbia (A. veronii* biotype *sorbia*) and *A. caviae* was done according to the criteria of POPOFF (1984), using additional biochemical tests. These included gas from glucose, aesculin/esculin hydrolysis, fermentation of arabinose, salicin and arbutin, the production of elastin and growth in nutrient broth containing 0 or 6% sodium chloride. Isolates differing in more than one test described above were characterized as "nonclassified" (KIROV *et al.*, 1986; 1993). The API-20E kit (bioMérieux, Marcy-l'Etoil, France) was used as well.

RESULTS AND DISCUSSION

Aeromonas hydrophila and other motile aeromonads are indigenous to the aquatic environment (HUSS, 1997) and therefore may be expected to occur in fish. *Aeromonas* spp. have been isolated from such other aquatic animals as shellfish, crustaceans, and amphibians. Both finfish and shellfish are known reservoirs of this bacterium (ABEYTA *et al.* 1994). The results of this study clearly show a high incidence of *Aeromonas* spp. in all fish samples examined. *Aeromonas* spp. isolated from 78 (65%) of the 120 fish samples analysed. *Aeromonas* spp. were isolated from 20 (67%) of the 30 mackerel, from 24 (80%) of the 30 bogue, from 18 (60%) of the 30 horse mackerel and from 16 (53%) of the 30 whiting samples. Of the fish samples examined, 33 (28%), 29 (24%) and 14 (12%) har-

boured *A. hydrophila*, *A. caviae* and *A. sobria*, respectively. One sample of bogue (3%) and one sample of horse mackerel (3%) were contaminated with other unclassified isolates. *A. hydrophila* and *A. caviae* were the dominant species in all fish samples examined (Table 1).

DAVIES *et al.* (2001) reported an overall incidence of 40% of *A. hydrophila* in European fish; they observed incidences of *A. hydrophila* of 65, 77, 25 and 25% in fish samples from France, Great Britain, Portugal and Greece, respectively. None of the 4 bogue and 3 out of the 7 horse mackerel samples examined in Greece harboured *A. hydrophila* (DAVIES *et al.*, 2001). In a study on the prevalence of aeromonads in catfish, 228 channel catfish fillets from three processing plants in the Mississippi Delta were examined, and both *A. hydrophila* and *A. sobria* were found in 36% of the samples, while *A. caviae* was found in 11% (WANG and SILVA, 1999). RADU *et al.* (2002) reported that 69, 55, 11.5 and 2.3% of the fish samples examined harboured *Aeromonas* spp., *A. veronii* biovar sobria, *A. hydrophila*, and *A.caviae*, respectively. As also reported by other authors, mesophilic aeromonads have been isolated from 37.3% of finfish and 36.6% of prawn (THAYUMANAVAN *et al.*, 2003), 72% of fish and shrimps (NEYTS *et al.*, 2000), 93% of fish, 100% of fish-egg, 16% of shrimp samples and 100% of freshwater samples (HÄNNINEN *et al.*, 1997). A total of 82 strains of *Aeromonas* spp. were also isolated from 250 samples of frozen fish (CASTRO-ESCARPULLI *et al.*, 2003).

High incidences of *Aeromonas* spp. were also observed in both the personnel and environmental samples taken from the fish markets. Table 2 shows that *Aeromonas* spp. were isolated from 69 (69%) of the 100 personnel and environmental samples analysed. *Aeromonas* spp. were isolated from 7 (35%) out of 20 samples taken from the hands of workers, from 10 (50%) out of 20 samples taken from the knives of workers, from 13 (65%) of the 20 samples from wooden boxes, from 19 (95%) of the 20 work surfaces (wooden board) and from 20 (100%) of the 20 floor samples. Of the personnel and environmental samples examined, 25 (25%), 26 (26%) and 13 (13%) harboured *A. hydrophila*, *A. caviae* and *A. sobria*, respectively. One sample taken from workers knives (5%), two samples (10%) of work surfaces and two (10%) floor samples were contaminated with other unclassified *Aeromonas* spp. As in the case of fish samples, *A. hydrophila* and *A. caviae* were also the dominant species in the personnel and environmental samples examined.

The present study highlights a notable incidence of *A. hydrophila* in fish samples as well as in personnel and envi-

Table 1 - Incidence of *Aeromonas* spp. in fish samples.

Fish	Number of samples	Number and (%) of samples positive for				
		Aeromonas spp.	*A. hydrophila*	*A. caviae*	*A. sobria*	Not classified
Mackerel (*Scomber scombrus*)	30	20 (67)	11 (37)	6 (20)	3 (10)	-
Bogue (*Boops boops*)	30	24 (80)	9 (30)	10 (33)	4 (13)	1 (3)
Horse mackerel (*Trachurus trachurus*)	30	18 (60)	6 (20)	8 (27)	3 (10)	1 (3)
Whiting (*Merlangius merlangus euxinus*)	30	16 (53)	7 (23)	5 (17)	4 (13)	-
Total	120	78 (65)	33 (28)	29 (24)	14 (12)	2 (2)

Table 2 - Incidence of *Aeromonas* spp. in environmental and personnel samples.

Location	Number of samples	Number and (%) of samples positive for				
		Aeromonas spp.	*A. hydrophila*	*A. caviae*	*A. sobria*	Not classified
Workers' hands	20	7 (35)	3 (15)	3 (15)	1 (5)	-
Workers' knives	20	10 (50)	3 (15)	4 (20)	2 (10)	1 (5)
Containers (wooden boxes)	20	13 (65)	6 (30)	5 (25)	2 (10)	-
Work surfaces (wooden board)	20	19 (95)	7 (35)	7 (35)	3 (15)	2 (10)
Floor surfaces	20	20 (100)	6 (30)	7 (35)	5 (25)	2 (10)
Total	100	69 (69)	25 (25)	26 (26)	13 (13)	5 (5)

ronmental samples. It appears that the high incidence of *A. hydrophila* represent a risk of foodborne disease, under certain circumstances, since it is considered an opportunistic pathogen. The potential risk is magnified by the fact that motile aeromonads, because of their psychrotrophic nature, can grow at refrigeration temperatures. Therefore, certain measures, such as adequate cooking of the fish and preventing post-heat contamination, will completely eliminate the risk, because the bacteria and their toxins are heat sensitive (FELDHUSON, 2000).

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