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PAPER

PRELIMINARY SURVEY ON COMPOSITION AND QUALITY OF CONVENTIONAL AND ORGANIC WHEAT

INDAGINE PRELIMINARE SULLA COMPOSIZIONE E LA QUALITÀ DI FRUMENTO BIOLOGICO E CONVENZIONALE

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ABSTRACT

Nutrient content, insects, mycotoxins and heavy metal contamination were determined on 35 samples of organic and conventional wheat (*Triticum aestivum*). A higher crude protein level was observed in conventional wheat (129.6 vs 107.5 g kg-1; P<0.01). Organic wheat was more infested by pests than the conventional one. Ochratoxin

RIASSUNTO

Trentacinque campioni di frumento tenero biologico o convenzionale sono stati analizzati per il contenuto in nutrienti e la presenza di insetti, micotossine e metalli pesanti. Il frumento convenzionale ha mostrato maggiore contenuto proteico (129,6 vs 107,5 g kg^{-1}; P<0,01), mentre il grano biologico ha mostrato maggiore presenza da inset-

- Key words: heavy metals, mycotoxins, organic agriculture, wheat -

A levels were low and not different between organic and conventional wheat. The deoxynivalenol level was significantly lower in organically grown than in conventionally grown wheat (2.85 vs 99.67 μg kg-1). Cadmium concentration was higher in conventional wheat (27.87 vs 19.41 μg kg-1; P<0.01), while lead was higher in the organic samples (29.63 vs 8.47 μg kg-1; P<0.05).

ti e minore contaminazione da deossinivalenolo (2,85 vs 99,67 μg kg^{-1}). Nessuna differenza per i livelli di ocratossina A. Rispetto al grano convenzionale, quello biologico ha mostrato livelli di Cd inferiori (27,87 vs 19,41 μg kg^{-1}; P<0,01) e di Pb superiori (27,38 vs 4,80 μg kg^{-1}; P<0,05).

INTRODUCTION

Organic agriculture is steadily becoming more widespread in terms of cultivated area (TOSI, 2003; ZAKOWSKA BIEMANS, 2003; FIRTH and SCHMUTZ, 2004), as it is becoming more sought after by the consumer (ISMEA, 2005). Since organic farmers do not use fertilizer, manure is the main nutrient supplier and crop rotation is used to preserve organic matter in the soil and reduce pest and fungi infestation. In organic farming only natural pesticides and fungicides are allowed, synthetic products are banned. Consumers perceive that organic products are healthier and more environmentally-friendly (STEPTOE and POLLARD, 1995; LINDEMAN and VÄÄNÄNEN, 2000). Research supports the idea that this production technique is, to a large degree, environmentally sustainable (REGANOLD *et al.*, 2001). The main characteristic of this category of products is that it is practically without pesticide residues. However, there is concern that the reduced protection against pests may lead to an increased microbiological risk, in particular to higher levels of fungal growth with consequent synthesis of mycotoxins. The studies available do not all agree; some report higher mycotoxin concentrations in organic cereals, while others report higher levels in conventional ones (PERENZIN *et al.*, 2001; SOLFRIZZO *et al.*, 2002).

Regarding the presence of arthropods in flour produced from organic or conventional wheat, there is little information about the differences that may exist between the two flours. The lack of chemical protection of organic products means that there must be more prevention strategies and more physical methods used. As far as the protein level is concerned, organic cereals tend to have lower protein content than conventional cereals (WOESE *et al.*, 1997), which is probably a consequence of less nitrogen fertilization.

The aim of this study was to determine the quality of organic versus conventional wheat (*Triticum aestivum*) lots produced in the same area, by analyzing the composition and determining the level of pest infestation of the kernels and mycotoxin and heavy metal-contamination. Factors affecting the quality of the cereals were not considered.

MATERIALS AND METHODS

The wheat lots investigated were produced on different farms located in the province of Piacenza (northern Italy; 2600 hectares). Fifteen lots of conventional wheat and 20 lots of organic wheat stored in two mills in the area were sampled according to the Europe-

an rules for the official control of ochratoxin levels (EUROPEAN COMMISSION, 2002). Samples came from the same adjacent towns (nine), and according to the local Soil Service, the soils are classified as Inceptisol. They are homogeneous for heavy-metal content, with a Cd content ranging from 0.3 to 0.6 ppm, while Pb was ranged from 7.3 to 30 ppm.

Technical assistance in organic farming methods was supplied by the same Farmer Association. The weather conditions in the area where the wheat was cultivated were not different, and during heading total rainfall was 70 mm in 8 days of rain and the average temperature was 16.4°C with a maximum of 28°C.

The wheat varieties used in conventional and organic farming were different; for organic wheat Bolero was the most widely used variety, while Mieti was the most preferred by the conventional farmer, this cultivar and the cv. Colfiorito were also widely used for the production of organic wheat.

According to the local Crop Production Service, Bolero and Mieti are quite similar, having low sensitivity to pathogenic fungi and a similar protein content.

In this area the rotation for organic farming is: wheat, barley and three years of alfalfa or grass. Fertilization is based on manure and no phosphatic fertilizers are applied. Some farmers spread by-products of the leather industry (hydrolyzed leather and bovine skins); strobulins and fungicides were not applied.

In conventional wheat 150 kg N ha^{-1} were top dressed in two applications and no manure was applied. Fungicide treatments were applied if necessary.

No pesticides were applied during storage of organic wheat, while in conventional wheat pesticides were applied at least once a year, if necessary. In both cases the temperature was checked by means of probes and was maintained within the range 10°-35°C by means of forced air.

In order to compare the quality of the organic products versus the conventional ones, the samples were subjected to the following chemical and physical analyses:

- moisture, 103°C for 5 hours (AOAC 1995, method 925.09B);
- crude protein, Kjeldahl method (AOAC 1995, method 979.09);
- starch, polarization method (AOAC 1995, method 945.37);
- dietary fibre, enzymatic-gravimetric method (AOAC 1995, method 985.29);
- ash, burn at 550°C (AOAC 1995, method 923.03).

Ergosterol and ochratoxin A (OTA) were analyzed by high performance liquid chromatography (HPLC); the trichothecenes (TCT), deoxynivalenol (DON), 3-acetyl-DON (3-Ac-DON), 15-acetyl-DON (15-Ac-DON), nivalenol (NIV), HT-2 toxin and T-2 toxins, were analyzed by gas chromatography-mass spectrometry (GC-MS). Cadmium and lead levels were determined by atomic absorption spectroscopy (AAS).

Analysis for mycotoxins

Ochratoxin A

Apparatus. HPLC analysis was performed using a Perkin Elmer liquid chromatograph equipped with a model LC-200 pump and ISS-200 autosampler (Perkin Elmer, Norwalk CT, USA) and a Jasco FP-920 fluorescence detector (Jasco, Tokyo, Japan). The system and data acquisition were controlled by Borwin 1.5 PC software (Jasco Ltd., Great Dunmow, Essex, UK).

Standard. OTA standard was purchased from Sigma (St. Louis, MO, USA). An OTA stock solution of 40 mg mL^{-1} was prepared in benzene:acetic acid (99:1). The concentration was measured by UV spectrophotometry at 333 nm, using the value 5.550 l mol^{-1} cm^{-1} for the molar absorptivity (ε). The working standard was prepared after evaporation of the stock solution (100 mL) under nitrogen and redissolution in 10 mL of acetonitrile:water (50:50).

Procedure. OTA analysis was performed in subdued light. OTA was extracted from samples (10 g) with 100 mL methanol-3% aqueous sodium hydrogen carbonate (50:50); the extract was filtered and an aliquot of 5 mL was diluted to 50 mL with a phosphate-buffered saline solution (pH 7.4) and the whole diluted extract was applied to an immunoaffinity column (Ochraprep, Rhône Diagnostics Technologies, Glasgow, UK), at a slow steady flow rate of 2-3 mL/min. OTA was eluted with 2 mL of methanol-acetic acid (98:2) into a graduated glass cylinder; the eluate was concentrated under a stream of nitrogen at 40°C and brought to 1 mL with the HPLC mobile phase. The extract was filtered (Millipore Corporation, Bedford, MA, USA, HV 0.45 mm) before HPLC analysis. Ochratoxin A was quantified by reversed-phase HPLC with fluorescence detection (λ_{ex} 333 nm, λ_{em} 470 nm) using a Select-B RP-8 column (5 μm particle size, 125x4 mm i.d., Merck, Darmstadt, Germany) at room temperature with a mobile phase of acetonitrile-2% aqueous acetic acid (43:57) at 1.2 mL/min. The detection limit for OTA was 0.020 μg kg^{-1}; the recovery of the method was 92.3±3.7%.

Trichothecenes

Apparatus. GC-MS analysis was carried out using a ThermoQuest Model TraceGQ coupled with Polaris Q ion trap mass spectrometry (ThermoQuest, Austin TX, USA). Data acquisition was performed by ThermoQuest Excalibur 1.2 software.

Standards. DON, 3-Ac-DON, 15-Ac-DON, NIV, HT-2, T-2 and diacetoxyshirpenol (DAS) were purchased from Sigma (St. Louis, MO, USA). Single trichothecene stock solutions of 500 mg/mL were prepared in acetonitrile and, only for DON and NIV, the concentrations were measured by UV spectrophotometry at 218 nm using ε=6,400 l $mol^{-1}cm^{-1}$ (WIDESTRAND and PETTERSSON, 2001). The concentrations of the other trichothecenes were calculated from the weight of the toxin in the vial supplied by the manufacturer. DAS was used as the internal standard. Trichothecene working solutions of 25-500 μg/L were prepared by diluting with methanol.

Procedure. TCT were extracted from samples (25 g) with 100 mL of acetonitrile-water (86:14); an aliquot (6 mL) of the filtrate was slowly pressed through a MycoSep 227 column (Romer Labs, Washington DC, USA). An aliquot (200 μL) of the internal standard (DAS 10 mg/L) was added to 2 mL of the eluate. The solution was evaporated to dryness and derivatized with 200 μL trimethylsilylimidazole:trimethylchlorosilane (1+0.2) (Sigma, St. Louis, MO, USA) for 15 minutes in subdued light. Then 0.8 mL of hexane were added, the solution was washed with 1 mL 0.2M phosphate buffer pH 7.5, and the hexane phase was used for GC-MS. The analysis was carried out using a capillary column Rtx-5MS, 30 m x 0.25 mm i.d., 0.25 mm film thickness (Restek Corporation, Bellefonte, PA, USA). Helium was the carrier gas with a column head pressure of 55 kPa. The sample was injected (2 μL, split ratio 1:30) into the GC-MS by a programmed temperature vaporisation (PTV) injector. The PTV temperature was raised from 70° to 250°C (held for 2 min) at 10°C sec^{-1}. The oven temperature programming was from 70°C (held for 1 min), to 245° at 10°C min^{-1} and then to 300°C (held for 1 min) at 30°C min^{-1}. MS transfer-line and ion source temperature were at 300° and 200°C, respectively. Electron ionization at 70 eV and selected ion monitoring (SIM) were used for detection. Fragment ion peaks monitored were 393, 407, 422, 512 for DON, 377, 392, 467 for 3-Ac-DON, 350, 377, 392, 407, 467 for 15-Ac-DON, 377, 392, 407, 467, 510, 585 for NIV, 350, 377, 392 for DAS, 287, 347, 350, 377, 466 for HT-2, 290, 347, 377, 436 for T-2; ion peaks used for the quantification were 393 for DON, 377 for 3-Ac-DON, 392 for 15-Ac-

DON, 510 for NIV, 350 for DAS, 466 for HT-2 and 290 for T-2.

The detection limit was 2.0 μg kg^{-1} for DON, 3-Ac-DON, 15-Ac-DON and NIV; 10.0 μg kg^{-1} for HT-2 and T-2. The recovery for DON was 90.1±2.4%.

Analysis for ergosterol

Apparatus. The HPLC system consisted of a Thermo Separation Products (TSP) instrument, equipped with a TSP-2000 pump, an AS-3000 sampling system and a Spectra Focus UV-Vis detector (San Jose, CA, USA) The system was controlled by TSP PC1000 software.

Standard. Ergosterol was purchased from Sigma (St. Louis, MO, USA). Twenty mg were dissolved in ethanol; the concentration was determined by UV spectrophotometry at 282 nm (ε=11.900 L mol^{-1} cm^{-1}). Working solutions of 1-10 mg mL^{-1} were prepared by dilution with n-hexane:isoamyl alcohol 98+2.

Procedure. Ergosterol analysis was performed using the method of SCHWADORF and MÜLLER (1989) with slight modifications according to AFNOR (1991). The whole analysis was performed in subdued light. Briefly, 150 mL of ethanol, 50 mL methanol, and 20 g potassium hydroxide were added to 25 g of sample. The mixture was refluxed for 30 min at 80°C, cooled to 20°C, and filtered through a folded filter-paper; 20 mL of the filtrate were transferred to an Extrelut column (Merck) and after 15 min ergosterol was slowly eluted with 90 mL n-hexane in a round-bottomed flask. The eluate was evaporated to dryness at 35°C by rotary evaporation, quantitatively transferred to a 10 mL volumetric flask and brought to volume with n-hexane-isoamyl alcohol (98:2). A Superspher Si-60 column (4 μm particle size, 125x4 mm i.d., Merck) was used at ambient temperature, with a mobile phase of n-hexane-isoamyl alcohol (98:2) at 1.0 mL min^{-1}. The UV detector was set at 280 nm. Ergosterol standards between 20 and 200 ng were injected; the retention time was about 7.1 min. The detection limit for ergosterol was 0.3 mg kg^{-1} with a recovery of 89.0±3.4%.

Analysis for lead and cadmium

Apparatus. Atomic absorption spectrophotometry analysis was performed by a Perkin Elmer AAS Analyst 600 (Wellesley, MA, USA) equipped with a Zeeman effect corrected graphite furnace.

Standards. The standards used for lead and cadmium were National Bureau Standard - Standard Reference Material 1567 wheat flour with a reference value of 20 μg kg^{-1} for Pb and 32 μg kg^{-1} for Cd.

Procedure. Samples were previously dehydrated at 65-70°C, ground in a mill with a 0.5 mm sieve, and processed as described; 500 mg of the sample were weighed and placed in an HPV80 Milestone microwave mineralization unit (Milestone Inc., Shelton, CT, USA) and submitted to digestion in an acid environment by the following method: 3 mL of HNO_3 (695 g kg^{-1} Fluka "Trace select") were added to 0.5 mL of H_2O_2 (100 vol. 300 g kg^{-1} BDH "Aristar") and to 1 mL of double-distilled H_2O. The HPV80 unit was hermetically closed with its own top (equipped with a Hp100 breaking valve), by the ACM100 automatic closing and opening module and placed in an MLS 1200 Milestone microwave mineralization unit, connected to the appropriate fume extraction apparatus Milestone EM5. Then, a 34 min mineralization program, purchased from the microwave manufacturer, was activated. It was divided into 5 steps of different length and emission power: 1st step 250 W for 6 min; 2nd step 0 W for 2 min; 3rd step 250 W for 6 min; 4th step 500 W for 10 min; 5th step 0 W for 10 min.

After taking the unit out of the microwave, the solution was transferred to a graduated measuring tube, washing the

unit sides with small quantities of double-distilled H_2O and bringing it to a 10 mL volume. This solution was used for AAS analysis.

The detection limit of the method was 5 μg kg^{-1} for Pb and 2.5 μg kg^{-1} for Cd. The reliability of the method was evaluated by 10 determinations for each metal on reference material standard NBS SRM 1567 wheat flour, with a reference value of 20 μg kg^{-1} for Pb and 32 μg kg^{-1} for Cd. The recovery was 97±0.9% for Pb (mean value 19.4 μg kg^{-1}) and 98±1.2% for Cd (mean value = 31.4 μg kg^{-1}).

Entomological analysis

Infestations were evaluated using radiography. The count of damaged grains using the method suggested by the Italian Ministry of Agriculture and Forestry (MAF, 1967), suggests the same execution procedures as the ISO Standard Method (1980). The investigation served to ascertain:

- external infestation (live or dead arthropods, visible either to the naked eye or using magnifying lenses);
- the presence of live or dead arthropods inside the kernels;
- the number of insect damaged kernels counted as reported in the FDA (1984) Technical Bulletin.

To determine whether there were any insects inside the kernels, a radiographic examination of the grains was carried out. Each X-ray photographed about 2500 kernels, equal to a sample of about 115 g, placed in a single layer on a glass plate. A General Electric X-ray Grain Inspection Unit (Fairfield, CT, USA), regulated at 21 kV and 5 mA, with an exposure time of 5 min was used. The level of infestation is expressed according to Italian Law, referring to 1 kg of grain on the basis of the number of live arthropods found in the sample: 0 no infestation; 1 slight; 2-3 noticeable; 4-10 significant; >10 major.

Statistics

Statistical analysis was performed using the SAS 6.0 statistical package (SAS, 1990). A completely randomized design was adopted, with the sample as experimental unit. First, the normality of the data distribution was checked using PROC UNIVARIATE. With a normal distribution, the analysis of variance was performed with the PROC GLM using the following experimental model:

$$y = \mu + \alpha + \varepsilon$$

where

y = analytic parameter;
μ = general average;
α = effect of the treatment;
ε = experimental error.

When distribution was not normal, a Kruskal-Wallis test was carried out using the PROC NPAR1WAY.

RESULTS AND DISCUSSION

Analytical composition

No significant differences were observed between the two types of wheat as far as percentages of starch, ash and dietary fiber were concerned (Table 1). The crude protein level was statistically higher in the conventional than in the organic wheat (Table 1), probably because industrial nitrogen fertilizers cannot be used in organic farming (VARGA *et al.*, 2003; DAVID *et al.*, 2004). This result is in accord with data obtained by PERENZIN *et al.* (2001) and with the results of 19 experiments reported by WOESE *et al.* (1997), but not with the data of RYAN *et al.* (2004), who did not observe any differences in protein content between organic and conventional wheat. The moisture level was higher in the organic wheat compared to conventional wheat (Table 1).

Table 1 - Means of moisture, crude protein, starch fibre and ash content (% on dry matter) in conventional and organic wheat.

Wheat	n	Moisture	Crude Protein	Starch	Soluble fibre	Insoluble fibre	Total Fibre	Ash
Organic	20	9.73±1.8^{b}	10.75±1.9^{A}	56.76±3.5	2.01±0.9	10.85±1.1	12.86±1.6	1.61±0.2
Conventional	15	8.79±1.4^{a}	12.96±1.7^{B}	56.62±1.9	2.35±1.4	11.14±1.1	13.48±1.6	1.57±0.1
SEM*		1.664	2.140	0.391	0.799	0.322	0.438	0.109

^{a,b} (P<0.05); ^{A,B} (P<0.01); *SEM = standard error of the mean.

Mycotoxin content

The OTA concentration found in wheat samples (Fig. 1) was very low, independent of the cultivation technique (organic or conventional). This result agrees the results of other studies that, although OTA-producing *Aspergillus* can be detected in Mediterranean climates (ACCENSI *et al.*, 2004), OTA is not closely related to cultivation techniques because it is a storage-growing rather than a field-growing mycotoxin.

All data were well below the 3 μg kg^{-1} limit set by the European Union (2001). The higher OTA content observed in a conventional sample (0.53 μg kg^{-1}) was far below the legal EU limit. There were no significant differences between the mean OTA levels of organic and conventional grains (0.064 *vs* 0.042 μg kg^{-1}). CZERWIECKI *et al.* (2002a) found higher OTA levels in Polish organic soft-grain wheat in 1997, but the following year they found a greater incidence of contaminated samples and higher toxin levels in the conventional grain (CZERWIECKI *et al.* 2002b). Contrasting results regarding the presence of OTA in organic and conventional cereals were found by BERETTA *et al.* (2002) and BIFFI *et al.* (2004); their surveys on baby foods showed that OTA levels were higher in organic rice than conventional rice products, while the opposite was found with wheat. It should be emphasized that in these surveys the products with the lowest mycotoxin contamination came from integrated pest management cultivation.

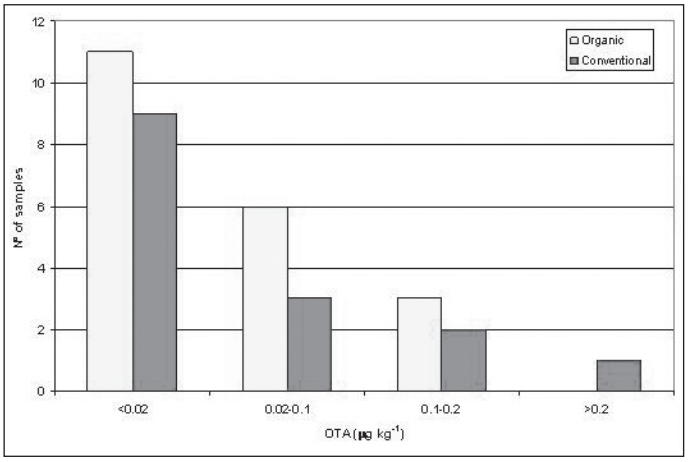


Fig. 1 - Distribution of OTA content in organic and conventional wheat samples. Means of OTA concentration are 0.042±0.055 μg kg^{-1} for organic and 0.064±0.138 μg kg^{-1} for conventional samples. (Level of detection 0.02 μg kg^{-1}). OTA was not normally distributed and was analyzed by means of a non-parametric procedure (Kruskal-Wallis test).

DON was the only trichothecene found (Fig. 2), and the levels were generally very low; the average values calculated for the organic wheat were lower with respect to conventional wheat samples (2.85 vs. 99.67 μg kg^{-1}, respectively, P<0.01).

Only 25% of the organic wheat was found to be contaminated by DON (max. value 17.1 μg kg^{-1}), while all

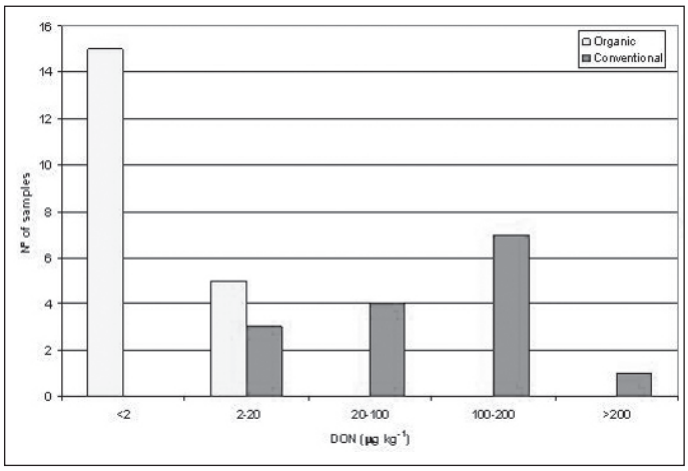


Fig. 2 - Distribution of DON content in organic and conventional wheat samples. Means of DON concentration are 2.85±5.78 µg kg^{-1} for organic and 99.67±91.15 µg kg^{-1} for conventional samples and are different for P<0.01. (Level of detection 2 µg kg^{-1}). DON was not normally distributed and was analyzed by means of a non-parametric procedure (Kruskal-Wallis test).

of the conventional samples were contaminated (max. value 219.1 µg kg^{-1}). After examining 274 samples of soft-grain wheat in 1999 and 2000, SOLFRIZZO *et al.* (2002) found a higher presence of DON in the conventional samples than in organic samples. BIRZELE *et al.* (2002) and DOLL *et al.* (2002) also observed lower values of fungal contamination in organic cereals compared to conventional ones. BERLETH *et al.* (1998), on the other hand, did not find any differences in the fungal contamination in conventional cereals compared to those grown according to the organic regulations.

PASCALE *et al.* (2000) compared organic and conventional soft-grain wheat samples that had not been subjected to pesticide treatment; they reported higher percentages of DON-contaminated samples in the organic wheat (51%) than in the conventional (12%) wheat. However, the average mycotoxin levels were higher in the conventional wheat (146 µg kg^{-1}) than in the organic (70 µg kg^{-1}) samples.

The higher DON levels generally found in conventional samples could be due to the fact that nitrogen fertilizers, used in traditional cereal cultivation, increase the susceptibility of the plant to attack by DON-producing fungi, like *Fusarium culmorum* and *F. graminearum* (PEREZ MARCO 2000; LEMMENS *et al.*, 2004); however the ban on fungicide treatment in organic farming can also result in an increased presence of *Fusarium* spp.

DON level is also affected by weather (MOSCHINI *et al.*, 2001; HOOKER *et al.*, 2002). According to HOOKER *et al.* (2002) DON levels increase if the number of days of rain is greater than 3 or if precipitation totals 5 mm around heading, while the optimal temperature range is 10°-35°C. In the area were wheat plots were cultivated, the rainfall was 70 mm with 8 days of rain around heading and the average temperature was 16.4°C with a maximum temperature of 28°C. The weather conditions in our area were less favorable to DON synthesis compared to the data reported by HOOKER *et al.* (2000) and trichothecene contamination in our samples was consequently lower. In the province of Piacenza the area cultivated with wheat was about 2600 hectares and was grouped, so the weather should have been similar in the 35 fields considered.

The quality of both organic and conventional wheat samples can be evaluated as good, even considering the ergosterol level (Table 2). In fact, all the samples examined in this survey showed concentrations of this fungal marker that were lower than 10 mg kg^{-1}, a value that is generally accepted as an upper limit of an "acceptable" quality of wheat (CAHAGNIER, 1988). The mean

Table 2 - Average value of ergosterol content (mg kg^{-1} on dry matter) in organic and conventional wheat.

Wheat	n	Ergosterol
Organic	20	2.78±1.2
Conventional	15	2.63±1.5
SEM*		0.917

*SEM = standard error of the mean.

values of the two wheat sample types were very close.

Pest infestation

The purpose of preventing infestation in stored cereals is to reduce product loss as well as to impede development of fungi, which is more likely in damaged kernels.

In this study, infestations were more frequent in the organic grains (3 samples out of 20) than in the non-organic samples (1 out of 15) (Table 3).

Moreover, the 3 infected samples of organic wheat had infestation levels of more than 10 insects kg^{-1} ("major" infestation), while the infested conventional wheat sample had a "significant" infestation level.

Considering the data as a whole, there was no correlation between pest infestation, mechanical damage to the kernels caused by insects, and ergosterol and mycotoxin contents (Table 4). DON content showed a significant positive relationship with OTA content, but only in conventional samples.

If the data are evaluated according to the cultivation technique, a strong link is observed between the number of damaged kernels and the presence of live arthropods in the organic grain. There was no such correlation in the conventional grain. However in the conventional grain the number of damaged kernels was correlated with the presence of ergosterol. The higher levels of infestation in the organic wheat could explain the strong correlation (r = 0.98) between damaged kernels and the presence of live arthropods.

Table 3 - Pest infestation in organic and conventional wheat (mean ± SD).

	Damaged kernels/2700 kernels		Live pests kg^{-1}	
	Overall Mean	n. of damaged samples	Overall mean	n. of positive samples
Organic (n = 20)	2.4±5.5	9	16.05±48.3	3
Conventional (n = 15)	61.6±232*	7	0.6±2.3	1
	Single data of damaged samples only		Single data of positive samples only	
	Organic	Conventional	Organic	Conventional
	2	1	17	9
	13	1	113	
	22	6	191	
	2	13		
	1	1		
	3	2		
	1	900		
	1			
	3			

*1 sample out of 15 had 900 damaged kernels.

Table 4 - Pearson correlation coefficients between fungal contamination and pest infestation in organic and conventional wheat.

	Ergosterol	Ochratoxin	DON	Damaged kernels	Live pests
All samples					
Ergosterol	1.00				
Ochratoxin	-0.14	1.00			
DON	-0.26	0.66**	1.00		
Damaged kernels	-0.08	-0.06	0.14	1.00	
Live pests	-0.14	-0.07	-0.14	-0.02	1.00
Organic					
Ergosterol	1.00				
Ochratoxin	0.15	1.00			
DON	0.08	0.18	1.00		
Damaged kernels	-0.24	0.12	-0.06	1.00	
Live pests	-0.27	0.04	-0.16	0.98**	1.00
Conventional					
Ergosterol	1.00				
Ochratoxin	-0.05	1.00			
DON	0.38	0.62*	1.00		
Damaged kernels	0.57*	-0.13	0.02	1.00	
Live pests	-0.07	-0.13	0.04	-0.07	1.00

*(P<0.05); **(P<0.01).

OTA and DON were not correlated with ergosterol, probably because the levels of both mycotoxin levels were very low.

The merceological quality of the wheat samples under consideration cannot in any case, be regarded as adequate. In addition, the infestations noted in the samples analyzed, with the relative damage to the kernels, could enhance fungal development.

Presence of heavy metals

The concentrations of Cd and Pb in all the samples was below the maximum levels for cereals fixed by the European Union (2001), i. e. 100 and 200 μg kg^{-1}, respectively, Pb levels were significantly higher in organically-grown wheat, while the opposite was true for Cd (Table 5).

These results are in part similar to those of PETR *et al.* (1999) and JORHEM and SLANINA (2000). The latter observed lower levels of Cd in organic cereals than in conventional ones in one locality, but the opposite results were observed in the products from another farm. Considering the results as a whole, these authors did not observe any clear trend between Cd levels in the grain and cultivation technique.

As far as Pb is concerned, organic farming techniques reduce (PETR *et al.*, 1999) or do not modify (JORHEM and SLANINA, 2000) the Pb content in the kernels.

Table 5 - Cd and Pb content (mean ± SD expressed as μg kg^{-1} dry matter) of organic and conventional wheat[1].

Wheat	Cd	Pb
Organic (n=20)	19.41 ± 15.64^{A}	29.63 ± 39.24^{b}
Conventional (n=15)	$27,87\pm7.34^{B}$	8.47 ± 6.91^{a}

^{A,B} (P<0.01); ^{a,b} (P<0.05).
[1]Cd and Pb were not normally distributed and were analyzed by means of a non-parametric procedure (Kruskal-Wallis test).

Determination of the causes for the different heavy metal content was not within the scope of our work. Use of mineral fertilizers or manure, soil organic matter content as well as road traffic are all factors that affect Cd and Pb concentration in the soil and consequently in the wheat. Further research is needed to explain any of these single factors.

CONCLUSIONS

While the results of this research, do not clearly affirm the superiority of either the organic or conventional cultivation techniques, we can focus on some critical points for obtaining healthy raw materials that are suitable for processing into food for humans.

In particular, the lower protein level found in organic wheat probably results in worse rheological properties. The use of less synthetic nitrogen fertilizers led to less protein synthesis, but also reduced the susceptibility of the plant to fungal attack by the genus *Fusarium*, which is responsible for DON production. This underlines the importance of ensuring adequate pest protection of conventional crops, so as to reduce fungal contamination without having to give up the use of synthetic fertilizers. In any case, it is not possible to conclude that conventional wheat is more susceptible to fungal contamination than organic, because other studies have shown higher levels of contamination in organic cereals. What emerges, then, is the need to find the critical points of fungal contamination in the cultivation of wheat so as to organize adequate agronomic counter-measures.

While the levels of heavy metals are not worrying, the link between manure, environmental pollution and heavy-metal accumulation in foods deserves further study.

Finally, the data relative to contamination show the need to constantly check for the presence of insects in wheat because, even though the organic product was more contaminated in this study, the risk of contamination in the conventional wheat was also high.

ACKNOWLEDGEMENTS

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PAPER

THERMOPHYSICAL PROPERTIES OF FREEZE-CONCENTRATED PINEAPPLE JUICE

PROPRIETÀ TERMOFISICHE DEL SUCCO D'ANANAS CONCENTRATO PER CONGELAMENTO

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ABSTRACT

Pineapple juice obtained from pineapple (*Ananas comosus*), variety smooth Cayenne, was concentrated by freeze concentration which was carried out at different temperatures (-15°, -20°, -30° and -40°C). Juice separation from the ice-concentrate phase was carried out by centrifugation using stainless steel centrifuge tubes. Physicochemical determinations were carried out on the fresh and freeze-concentrated juice which included thermophysical properties such as density, viscosity, ther-

RIASSUNTO

Il succo estratto dall'ananas (*Ananas comosus*), varietà liscia Cayenne, è stato concentrato tramite concentrazione per congelamento effettuata a diverse temperature (-15°, -20°, -30° e -40°C). La separazione del succo dalla fase concentrata ghiacciata è stata effettuata per centrifugazione utilizzando tubi per centrifughe di acciaio inox. Le determinazioni fisico-chimiche, effettuate sul succo fresco e sul succo concentrato per congelamento, comprendevano le proprietá termofisiche

- Key words: aroma profile, freezing, rheological properties, water activity -

mal conductivity, total soluble solids, freezable and unfreezable water content, titrable acidity, pH, water activity and redox potential. Aroma profiles were obtained for fresh pineapple juice, an evaporation-concentrated pineapple juice and the -40°C freeze-concentrated pineapple juice. The loss of volatile compounds was significantly (64%) lower for the freeze-concentrated juice when compared with pineapple juice obtained by the evaporation process. All pineapple juice concentrates exhibited shear thinning properties and the Herschel-Bulkley equation was the one that best fitted the experimental data. Both, concentration and temperature greatly influenced the thermophysical properties, particularly viscosity.

quali densitá, viscositá, conducibilitá termica, solidi solubili totali, il contenuto di acqua congelabile e non congelabile, l'acidità titolabile, il pH, l'attività dell'acqua, il potenziale d'ossidoriduzione. I profili aromatici sono stati effettuati su di un succo d'ananas fresco, su di un succo d'ananas concentrato per evaporazione e su di un succo d'ananas concentrato per congelamento a -40°C. La perdita di composti volatili era significativamente piú bassa (64%) per il succo concentrato per congelamento paragonato al succo d'ananas ottenuto tramite il processo di evaporazione. Tutti i concentrati di succo d'ananas ottenuti per congelamento mostravano riduzione di viscosità da sforzo e l'equazione di Herschel-Bulkley era l'unica che riproduceva con maggiore esattezza i dati sperimentali. La concentrazione e la temperatura influenzavano significativamente le proprietà termofisiche, in particolare la viscosità.

INTRODUCTION

Multi-stage vacuum evaporation is the classical technology used in the juice industry, even though this process always reduces juice quality. Temperatures higher than 50°C degrade sensorial characteristics, leading to a partial loss of the fresh juice flavour, even when the evaporator is equipped with an aroma recovery system (DORNIER *et al.*, 2005).

In order to limit thermal damage, other processes that concentrate juices at low temperatures, such as freeze-concentration and membrane processes have been developed, including those involving osmotic evaporation. The latter is an emerging membrane technique that until recently, has received little attention as a technique for concentrating fruit juices (SHAW *et al.*, 2001).

Freeze-concentration is a choice method for obtaining high quality concentrated juices. In this processing method, water is frozen, and flavor, aroma, color and nutritional losses are minimized, compared to technologies based on thermal processing (SCHWARTZBERG, 1990). In this method, temperature control is required to achieve a desired concentration (CHEN, 1993). Knowledge of the physical properties of food is important for process design and optimization, and product research and development (CONSTENLA *et al.*, 1989). These properties change

during processing and are good indicators of product quality (RAMOS and IBARZ, 1998). Knowledge of the thermophysical properties such as density, viscosity, specific heat and thermal conductivity over a wide range of concentrations and temperatures is of great interest to the food industry. Thermophysical properties of foods are used in the efficient and economic design of frozen food products (MCDONALD *et al.*, 2002). Modeling, optimization and automatization of products and processes are very difficult due to the large range of thermophysical properties involved (TELIS-ROMERO *et al.*, 1999; HAMDAMI *et al.*, 2004).

Redox potential is a thermodynamic measurement that provides information about the actual capacity of a molecule to be oxidized or reduced and about its prevalent form (oxidized or reduced) in the system. Given its thermodynamic nature, a redox potential value does not provide information regarding the rate at which a reaction may occur, but it is recognized as an important barrier against spoilage that consequently helps extend the shelf-life of food (NICOLI *et al.*, 2004).

Knowledge about the flow behavior of concentrated fruit juices is pertinent to quality control, sensory evaluation and food processing and handling operations (transport, mixing, homogenization, sterilization, concentration). Likewise, it is important to know the effect of temperature and concentration on flow properties (RAO *et al.*, 1984).

The objective of this work was to determine the effect on the thermophysical properties, flow properties and aroma profile of pineapple juice by concentrating it from 12° to 51°Brix using the freeze-concentration method.

MATERIALS AND METHODS

Pineapple (*Ananas comosus*) fruit, of the variety smooth Cayenne and with a maturity index of 5 (PANTÁSTICO, 1979), was purchased from a local market at Xalapa, State of Veracruz, México. Juice was obtained using an electric juice extractor (Moulinex mod. 140-1-03, Naucalpan, State of México, México). Samples of fresh juice were concentrated by two methods: (a) freeze-concentration where fresh juice was placed in a stainless steel vessel and frozen in an air circulation vertical freezer model MDF-U5411 (Sanyo Electric Biomedical Co. Ltd., Tokyo, Japan) followed by centrifugation (IEC Chemical Centrifuge, International Equipment Co., Anaheim, CA, USA) using 8 cm long x 8 cm wide stainless steel centrifuge tubes to separate ice from unfrozen concentrated juice. Freezing was done by carrying out successive freezing (-15°, -20°, -30° and -40°C) -centrifuging cycles in order to obtain increased soluble solids concentration, or by (b) evaporating water from fresh juice using a digital rotavapor (Ika-Werke RV06-ML, IKA Works, Inc., Wilmington, NC, USA) operated at 60°C.

Physicochemical analyses

The freezable and unfreezable water content was calculated with the following equations:

$$\text{Freezable water} = \frac{\text{Experimental pineapple juice melting enthalpy (J/g)}}{\text{Pure water melting enthalpy (J/g)}} \quad (1)$$

$$\text{Unfreezable water} = \text{Moisture content (\%)} - \text{freezable water (\%)} \quad (2)$$

The experimental pineapple melting enthalpy was determined with a TA Instruments calorimeter (model 2910, New Castle, DE, USA) using a cooling temperature sweep from 20° to -60°C, followed by a temperature heating sweep from -60° to 25°C, using a 5°C/min ramp. The calorimeter was previously calibrated with indium and mercury. Pure water melting enthalpy at 0°C is 333.6 (J/g). Concentrated juices were analyzed for °Brix as a measure of soluble solids with an ATAGO NAR-1T refractometer (Atago Co., Ltd., Tokyo, Japan) with a digital thermometer. Titrable acidity and pH (AOAC, 1995) were measured with an Orion 720A pH meter (Beverly, MA, USA). Water activity was determined using an Aqualab series 3 TE water activity meter (Decagon Devices, Inc., Pullman, WA, USA). Redox potential was determined following the procedure proposed by MANZOCCO et al. (1998). Density was determined with a Paar digital densimeter DMA 35 (Anton Paar K.G., Graz, Austria) (MATISSEK *et al.*, 1992).

All measurements were carried out at 25°C unless otherwise specified.

Aroma profile

The aroma profiles of the concentrated juices obtained by evaporation and freeze-concentration were obtained with a gas chromatograph (HP 6890, GMI, Inc., Ramsey, MN, USA) operated under the following conditions: 2 mL sample; open capillary column HP5 (60 m x 0.25 mm) with packing diameter of 320 μm, thermostat at 90°C for 10 min; flame ionization detector at 250°C, column temperature programmed from 60°C to 200°C with heating ramp of 1°C/min at 64°C and of 2.5°C/min at 155°C. Carrier gas was helium at a flow rate of 1 mL/min. Samples were withdrawn from the headspace with a syringe and injected into the chromatograph. Results are expressed as area units/g dry solids.

Apparent viscosity

The apparent viscosities of the juices concentrated to different °Brix were determined with a Modular Compact Rheometer Physica MCR 300 (Physica Metechnik GmbH, Stuttgart, Germany) using the DG 26.7 concentric cylinder measuring system and the TEZ 150 P-C Peltier system for temperature control. Firstly, the apparent viscosity dependence upon shear rate was studied by applying a shear rate sweep from 10^{-3} to 10^{3} 1/s at 20°C. Different models contained in equipment software were fitted to experimental data to determine which explained the flow properties the best. Secondly, the apparent viscosity change with temperature was determined by applying a temperature sweep from -10° to 90°C at a constant shear rate of 10 1/s.

All experiments were done in duplicate.

Statistical analyses

Analysis of variance (ANOVA) and Tukey's test ($P \leq 0.05$) were performed on the thermophysical and rheological results using the Statgraphics 7 statistical analysis system (Statistical Graphics Corp. Manugistics Inc., Cambridge, MA, USA).

RESULTS AND DISCUSSION

Physicochemical properties

The physicochemical properties of the fresh and freeze-concentrated pineapple juices concentrated at different temperatures are given in Table 1. No significant differences were found for pH ($P > 0.05$), but there were significant differences for thermal conductivity, °Brix and density (data not shown). Degrees Brix (soluble solids) increased as the freezing temperature decreased from 12°Brix for the fresh juice up to 51°Brix

Table 1 - Physicochemical properties of pineapple juice at 25°C corresponding to samples at different freeze-concentrated temperatures.

Physicochemical property	Fresh juice	Freezing temperature -15˚C	-20˚C	-30˚C	-40˚C
Soluble solids (ºBrix)	12.0	21.7	40.7	47.9	51.0
Water activity	0.992[a]	0.990[a]	0.944[b]	0.930[c]	0.914[d]
pH	3.57[a]	3.52[a]	3.45[a]	3.43[a]	3.42[a]
Titrable acidity (% anhydrous citric acid)	0.63[a]	1.19[b]	1.89[c]	2.15[d]	2.36[d]
Thermal conductivity (W/mºC)	0.581	0.534	0.440	0.404	0.390

Different superscripts in rows indicate significant differences at P≤0.05.

at -40°C. This solids content was greater than that reported for pineapple juice concentrated by reverse osmosis (MATSUURA *et al.*, 1975), as well as those reported for orange and passionfruit juice concentrated by osmotic evaporation (SHAW *et al.*, 2001). Increasing solids content induced a reduction in the water vapor pressure that lowered the water activity from 0.992 for the fresh juice to 0.914 for the concentrated juice at 51°Brix. The pH of the freeze-concentrated juice decreased, but titrable acidity increased as the solids concentration increased. Both phenomena can be attributed to the high content of organic acids in the juice. Other researchers have reported pH and titrable acidity values for apple, orange, pineapple and tangerine juices that are in accord with those reported here, but the reported values for grape juice were slightly lower (ROS *et al.*, 2004, BRADDOCK and MARCY 1985). Using the equation reported by SWEAT (1974), the calculated thermal conductivity decreased linearly as the ºBrix increased (ZAINAL *et al.*, 2000, SHAMSUDIN *et al.*, 2005). The effect of total soluble solids on the density of the juices showed a linear relationship as also reported for apricot, apple and orange juices (RAMOS and IBARZ 1998, CONSTENLA *et al.*, 1989).

Freezable and unfreezable water

The increase in the soluble solids content (°Brix) in juices produced a decrease in the melting and freezing temperature of the freeze-concentrated juices (Table 2). HANDAMI *et al.* (2004) reported non-freezable water contents of 43.6 and 44.3% for an unspecified porous vegetable having a water content of 60%; these values fall within those found for our juices with water mass fractions of 0.882 and 0.444.

Flow properties

All juices, irrespective of °Brix concentration, exhibited shear thinning properties (Fig. 1). The freeze-concentrated juices showed higher apparent viscosity over the whole shear rate range as the soluble solids contents were higher. However, unexpectedly, the fresh juice had a higher apparent viscosity at low shear rates (~0.02-0.3 1/s) and lower apparent viscosities than all of the freeze-concentrated juices at shear rates above ~110 1/s. Fresh pineapple juice contains pectins, which have low viscosities compared to other plant gums, but nevertheless they increase the viscosity of aqueous solutions. Thin pectin solutions are close to Newtonian in their flow properties, but this changes to pseudoplastic when the

Table 2 - Frozen and unfrozen water for fresh and freeze-concentrated (-40°C) pineapple juice.

Sample	Water mass Fraction	*Freezing enthalpy (J/g)	*Melting enthalpy (J/g)	Frozen water (%)	Unfrozen water (%)
Fresh pineapple juice	0.882	297.6	273.2	81.89	6.34
Freeze-concentrated juice at -40°C	0.444	111.1	79.90	23.95	20.53

*Note: The observed difference between the freezing and melting enthalpy of the juices can probably be attributed to water being redistributed within the juice during the freezing-melting process, giving rise to different water-juice component interactions, which in practical terms is equivalent to having juices with different composition.

pectin concentration increases (PEDERSEN, 1980). The pectin concentration must have been sufficiently high in the fresh pineapple juice for it to exhibit a higher viscosity at low shear rates, and a lower viscosity at higher shear rates. The pectin gel-like structure was deformed by shear compared to the concentrated juices. The best fit for all flow curves was achieved with the Herschel-Bulkley model (r>0.999, Table 3)

$$\tau = \tau_0 + k\dot{\gamma}^n \qquad (3)$$

where τ = shear stress (Pa), τ_0 = yield stress (Pa), k = is the consistency index (Pa.s^n), $\dot{\gamma}$ = shear rate (1/s), and n = flow index (dimensionless). All of the juices required the application of a finite stress to induce flow, but the relative low values of τ_0 tend to indicate that the gel-like structures were rather weak. As expected, the consistency index (k) increased as the soluble solids contents in juices increased. However, the flow index (n) showed a more complex behaviour, exhibiting a minimum value at 21°Brix.

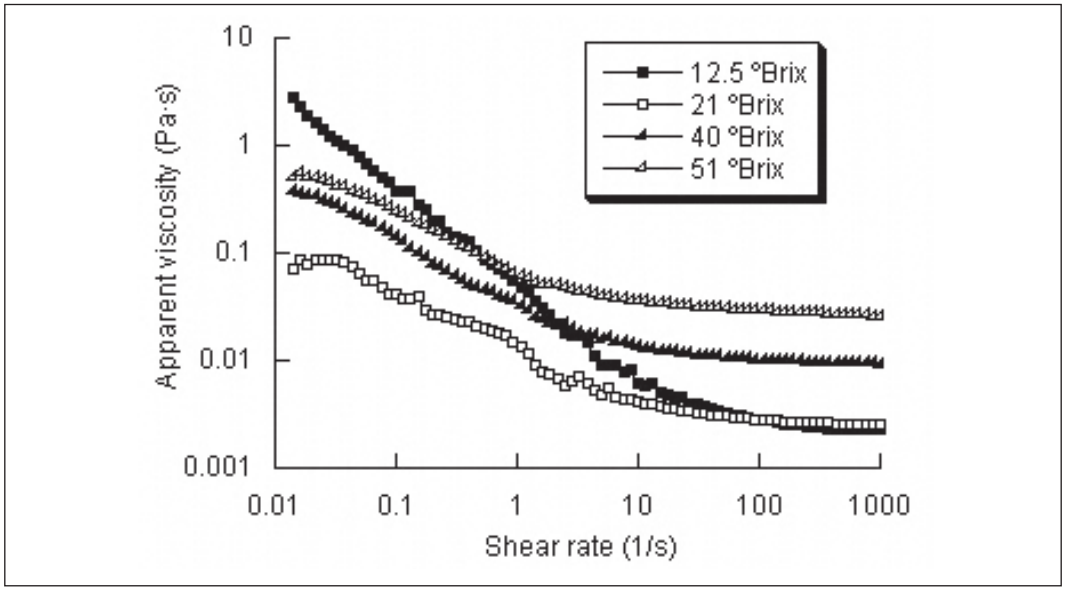


Fig. 1 - Apparent viscosity as a function of shear rate of fresh and freeze-concentrated pineapple juice at different soluble solids concentrations.

Table 3 - Herschel-Bulkley rheological parameters for the different pineapple juices.

Soluble solids content (°Brix)	Yield stress, τ_0 (Pa) ± SD	Consistency index, k ($Pa \cdot s^n$) ± SD	Flow index, n (dimensionless) ± SD
12.5	$2.594x10^{-2} \pm 5.8x10^{-4}$	$3.603x10^{-3} \pm 6.3x10^{-7}$	$0.907 \pm 3.9x10^{-3}$
21	$1.2994x10^{-3} \pm 8.1x10^{-8}$	$1.008x10^{-2} \pm 3.6x10^{-6}$	$0.723 \pm 1.9x10^{-3}$
40	$9.678x10^{-3} \pm 8.3x10^{-6}$	$1.841x10^{-2} \pm 1.2x10^{-5}$	$0.872 \pm 1.2x10^{-3}$
51	$1.088x10^{-2} \pm 3.0x10^{-7}$	$4.713x10^{-2} \pm 6.8x10^{-5}$	$0.894 \pm 1.2x10^{-3}$

This behaviour seems to have been due to a combination of two simultaneously occurring phenomena, one of which prevails over the other at a given time. As the juice was freeze-concentrated at different temperatures, the lower the temperature, the higher was the amount of water eliminated, and the greater the leaching out of water-soluble pectin, causing a decrease in shear thinning behaviour. But as water was removed, the concentration of other less water-soluble solids increased, causing the formation of flocs, whose effective volume fraction was greater than the sum of the volume fractions of the individual solids, due to the presence of continuous phase trapped within it (McCLEMENTS, 1999). Shearing of the flocs led to their disruption and to a decrease in viscosity (GARZA, 1996). The apparent viscosity dependence on temperature of pineapple juice is shown in Fig. 2. Below 0°C freezing can occur for 12.5° to 40°Brix juices with sharp increase in viscosity. A relatively constant apparent viscosity behavior was observed in the temperature range between 10°-60°C, whereas the 51°Brix juice showed a relatively constant apparent viscosity throughout the applied shear range interval. Appar-

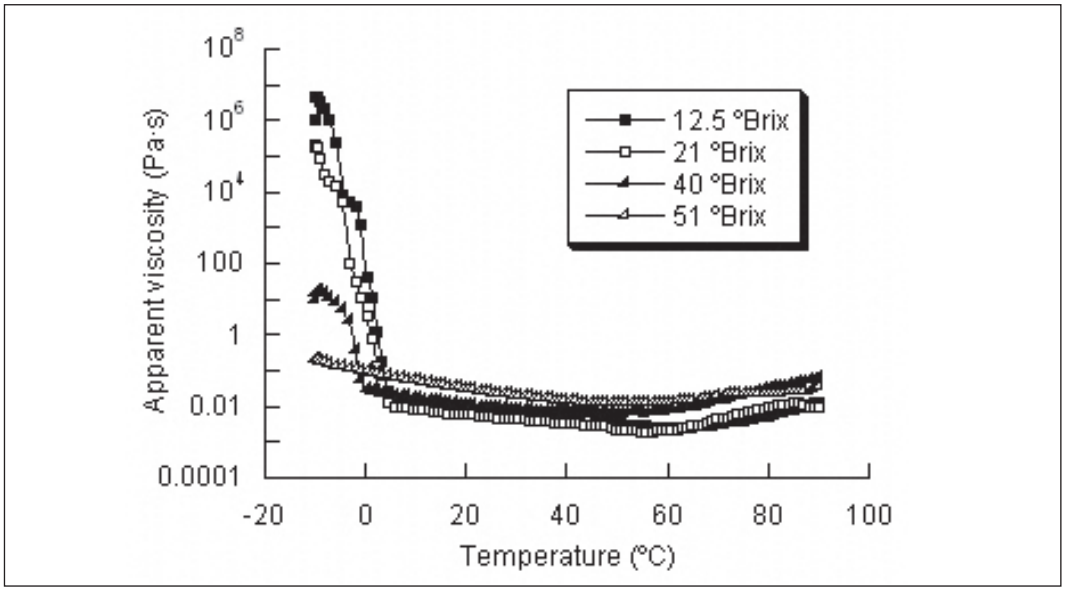


Fig. 2 - Apparent viscosity as a function of temperature and a constant shear rate of 10 1/s of fresh and freeze-concentrated pineapple juice at different soluble solids concentrations.

ent viscosity in the temperature range from 10°-60°C increased as the soluble solids contents of the juices increased. As can be observed in Fig. 1, all the juices exhibited a high shear limiting Newtonian viscosity at around 10 1/s and higher shear rates. This behaviour was seemingly independent of temperature, but dependent on solids concentration. All of the juices exhibited an increase in apparent viscosity in the shear range between 60°-90°C. This behaviour was probably due to a restructuring of juice solids due to configurational changes that are induced by temperatures above 60°C and that tend to form flocs. As temperature increases, more flocs are formed, which effectively entraps the continuous phase between their interstitial spaces. This results in an increase in viscosity, as less continuous phase is available for the flow of the flocs.

Redox potential

Redox potential varied with juice concentration (Fig. 3). As the soluble solids concentration increased, the redox potential decreased. Nevertheless, the processing method did not seem to have an effect on redox potential. Redox potential values for the -40°C freeze-concentrated juice (245.0 and 241.8 mV) were higher than those reported by MANZOCCO *et al.* (1998) for black tea extracts, and lower than those reported by (REYES, 2005) for purple maguey tea extract.

Aroma profile

The aroma profiles of the fresh juice, the freeze concentrated juice at -40°C and the evaporated juice are shown in Fig. 4. It is evident that the freeze-concentration method provides juice with higher aroma compound contents than the traditional evaporation method, in which there is a significant loss of aroma compounds during the first minutes of processing which irreversibly alters the aroma profile of the juice (PETROTOS and LAZARIDEZ, 2001). In addition, the high temperatures (between 45° and

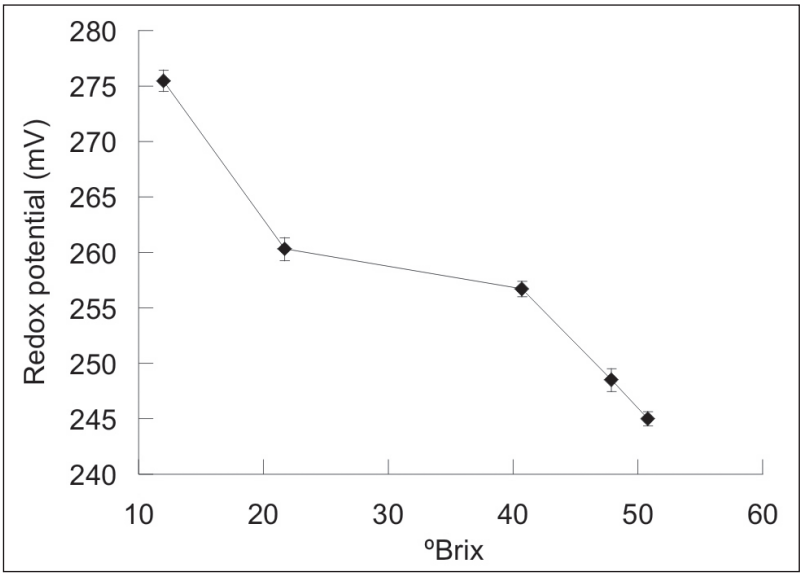


Fig. 3 - Redox potential variation as a function of soluble solids concentration in the fresh and freeze-concentrated pineapple juices.

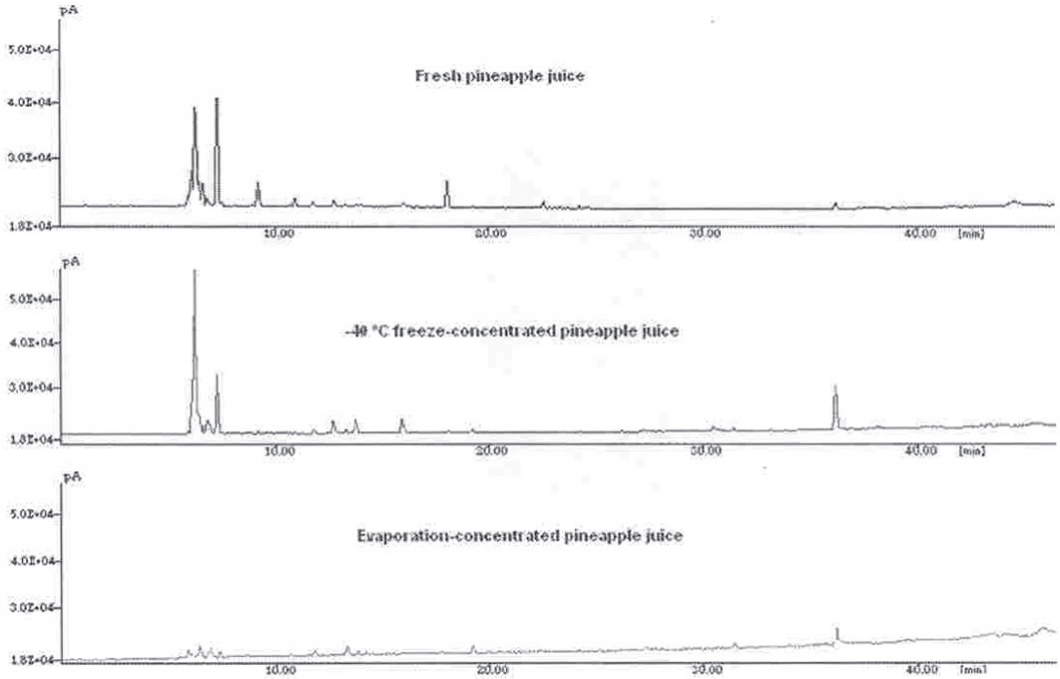


Fig. 4 - Aroma profiles of fresh, freeze-concentrated at -40°C and evaporation concentrated pineapple juices.

90°C) caused a colour change and a reduction in the nutritional value (ÁLVAREZ *et al.*, 2000).

CONCLUSIONS

The freeze-concentration method using centrifuge tubes gives concentrated pineapple juice that has a better aroma profile. Using this method, the yield was approximately 50%. All pineapple juice concentrates exhibited shear thinning properties. The rheological properties, and to a lesser degree, the thermophysical properties were affected by their solids content and temperature. This work provides interesting insights for the processing of pineapple juice, and other juices that involve fluid flow and heat transfer.

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PAPER

THE DEVELOPMENT OF A METHOD FOR DETERMINING AVERMECTIN B1A RESIDUES IN HONEY BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

SVILUPPO DI UN METODO PER LA DETERMINAZIONE DI RESIDUI DI AVERMECTINA B1A NEL MIELE PER CROMATOGRAFIA LIQUIDA AD ALTA PRESTAZIONE

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ABSTRACT

A new method has been developed for determining avermectin B1a residues in honey. The analyte was extracted by means of liquid-liquid extraction with methanol/ethyl acetate/hexane and extract clean-up in Silica SPE cartridges. Determination was carried out by RP-HPLC using acetonitrile:methanol:water as the mobile phase and the wavelength set at 245 nm. Results indicate that the method is exact and pre-

RIASSUNTO

È stato messo a punto un nuovo metodo per la determinazione di residui d'avermectina B1a nel miele. L'analita è stato estratto dalla matrice per estrazione liquido-liquido con metanolo/etilacetato/esano e l'estratto è stato lavato in cartucce di silice SPE. La determinazione dell'analita è stata effettuata attraverso HPLC a fase inversa utilizzando come fase mobile una miscela acetonitrile:metano-

- Key words: avermectin B1a, chromatography, honey -

cise, with average recoveries between 73.4 and 97.45% and RSD varying from 0.91 to 13.7%. The LOD and LOQ found were 0.002 and 0.007 mg/kg, respectively. The analysis conditions might even allow the 8,9-Z-isomer that results from the photo degradation of avermectin B1a to be determined.

lo: acqua ed alla lunghezza d'onda di 245 nm. I risultati indicano che il metodo è esatto e preciso, con recuperi medi compresi tra il 73,4 e il 97,4% e una DSR dallo 0,91 al 13,7%. I LOD e LOQ ottenuti erano rispettivamente 0,002 e 0,007 mg/kg. Le condizioni dell'analisi potrebbero permettere anche la determinazione dell'isomero 8,9-Z, risultante dalla fotodegradazione dell'avermectina B1a.

INTRODUCTION

The determination of pesticide residues in food is important, not only as far as public health is concerned, but also because it allows environmental conditions to be monitored.

The avermectins, including abamectin, were discovered in the 1970s. They were extracted from *Streptomyces avermitilis* actinomycete mycelium, a microorganism found in the soil (HAYES and LAWS, 1991; ALBERS-SCHONBERG *et al.*, 1981; SHOOP *et al.*, 1995). While they have little antibiotic and antifungal activity, they possess a potent action against helminths and arthropods. Abamectin is used as a pesticide and acaricide in many countries throughout the world (ELBETIEHA and DA'AS, 2003; HSU *et al.*, 2001). It has a wide range of action at low dosage levels. The chemical structure of abamectin is shown in Fig. 1. Series *a* and *b* are homologous and differ from each other by a single methylene of the alkyl radical bonded to carbon 25 (VALENZUELA *et al.*, 2001), sec-butyl and isopropyl, respectively. Abamectin consists of a mixture containing not less than 80% of the homologue avermectin B1a, and not more than 20% avermectin B1b (CROUCH *et al.*, 1992; SHOOP *et al.*, 1995).

The MRLs (maximum residue limits) established for abamectin are calculated by the sum of avermectin B1a and its 8,9-Z-isomer that is formed by photoisomerization (PRABHU *et al.*, 1992). Once in the environment, abamectin has a relatively short half-life when compared to other pesticides. It degrades rapidly in the soil, where it undergoes photodegradation and microbial decomposition. By estimating its decomposition rates, abamectin residues found in certain foods might indicate recent contact with it or its excessive use.

The use of abamectin is steadily increasing, due to its attractive properties. This is why the number of analytical methods for its determination is increasing; most of them are chromatographic methods. In general, these analytical approaches are based on liquid-liquid extraction and pre-purification by solid-phase extraction cartridges in Florisil packed columns (SUNDARAM and CURRY, 1997), aminopropyl (PRABHU *et al.*, 1992; COBIN and JOHNSON, 1995; 1996), silica (VUIK, 1991), octadecilsylanol (YOSHII *et al.*, 2000) or octasylanol (PRABHU *et al.*, 1991).

Many of the methods for determining pesticide residues in honey involve liquid-liquid extraction, while other ana-

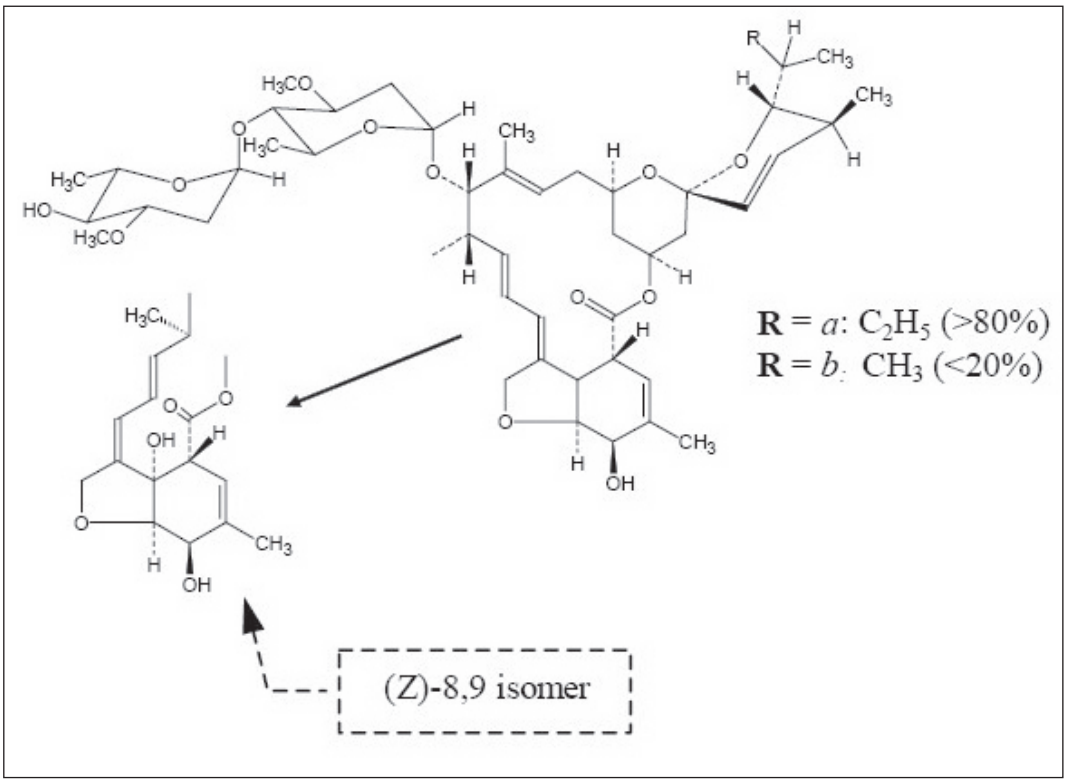


Fig. 1 - The structure of abamectin and its 8,9-Z-isomer.

lytical approaches have been successfully used either to extract the analytes or for clean-up steps (ATIENZA *et al.*, 1993; BERNAL *et al.*, 1996; JIMÉNEZ *et al.*, 1998; SÁNCHEZ-BRUNETE *et al.*, 2002; KORTA *et al.*, 2003; ALBERO *et al.*, 2004; RISSATO *et al.*, 2004). Analysis of these extracts usually involves chromatographic techniques, with detection fitting the purpose and sensitivity of the method (FERNÁNDEZ *et al.*, 2002). Although less sensitive than mass spectrometry, UV detectors are still important for monitoring the presence of pesticides in honey (KORTA *et al.*, 2001).

The detection of abamectin in honey is important considering its range of use and economical importance, as well as the role of pollination played by bees. In addition, research on pesticide residues in apicultural products can also serve as environmental indicators, and reveal the nature of pesticides used in the area around the hives (FERNÁNDEZ *et al.*, 2001; 2002; BLASCO *et al.*, 2004).

To the best of our knowledge, there are no methods for determining abamectin residues in honey. Thus, one aim of this paper is to propose a methodology for determining one of its homologous forms, avermectin B1a in honey. This should contribute to giving a clearer understanding of the consequences associated with the use of abamectin as a pesticide in agriculture. In so doing, this is the first step towards monitoring the impact of its use on this food matrix.

MATERIALS AND METHODS

Reference materials and reagents

The reference material used was Abamectin (Merck®) in glycero-formal at 0.873%, with 0.819% (93.8%) of avermectin B1a and 0.0545% (6.2%) in avermectin B1b. Since the ratio of avermectin B1b is small and is not included in the MRL calculations, the residue study only focused on the B1a homologe. The relationship of the two homologous forms, however, was used to establish suitable chromatographic conditions.

The solvents used were: acetonitrile and standard spectroscopic methanol, ethyl acetate, hexane and acetone for residue analysis. Samples were extracted and pre-purified with solid-phase extraction cartridges with two different adsorbents: Silica-OH (Applied Separations, Allentown, PA, USA) and Florisil (Altech, Deerfield, MI, USA). All the cartridges contained a 6.0 mL reservoir and 500 mg of adsorbent. Stock solutions were dissolved in methanol, while intermediary and fortification solutions were diluted in mobile phase.

Chromatographic conditions

Chromatographic conditions were determined by taking as a point of reference the pharmacopeical method for ivermectin with the mobile phase composed of acetonitrile:methanol:water (51:34:15); the flow rate was adjusted to 1.2 mL/min, detection at a wavelength of 245 nm, at room temperature and an injection volume of 100 μL . Two reversed-phase columns were tested for the analyses: a) Nucleosil C18 250x4.6 mm 100A (Phenomenex, Torrance, CA, USA), and Zorbax® C8 250x4.6 mm (Agilent Technologies, Palo Alto, CA, USA). For each column, 40 μg/mL reference solution replicates were applied to compare their resolution and efficacy for the abamectin homologue. Simultaneously, a 10 μg/mL reference solution was used to measure its UV absorption spectra using a scanning spectrophotometer.

Injection repeatability was determined by applying 11 μg/mL of abamectin reference solution in ten replicates. Linearity was evaluated by applying duplicates of reference solutions at six concentration levels. The regression equation was considered to be linear for correlation coefficients higher than 0.995. The linearity interval of the method was studied with the application of reference solutions in concentrations varying from 0.38 to 19.46 μg/mL.

Extraction and clean-up procedures

Aliquots of 50 g of honey in an amber flask were fortified with aliquots of the reference solution at three concentration levels and left to rest for at least 30 min before analysis. Ten mL of methanol were added to the samples; they were heated at 40°C and sonicated for 5 min. Twenty-five mL of ethyl acetate were added and the solution was shaken horizontally for 5 min. Ten mL of hexane were added and the solution was shaken for 3 min. The flasks were put in a freezer at -17°C for 20 min to complete phase separation. The lower phase containing the honey was submitted to a new extraction.

The extracts obtained in the two stages of extraction were evaporated to complete dryness under nitrogen flow and shaken in a 45°-50°C water bath. Extract residues were dissolved by adding 2 mL of ethyl acetate, followed by 3 mL of hexane. The contents were transferred to previously cleansed SPE cartridges. Avermectin B1 was eluted from the cartridges with a 12 mL acetone:ethyl acetate mixture (5:1) into amber tubes. The eluate was evaporated to dryness and the residue obtained was resuspended in 1 mL of mobile phase.

The two different SPE cartridges were tested. Extractions were performed for

each adsorbent with fortified samples at a 0.19 mg/kg level. Chromatographic profiles of pre-purified extracts were evaluated considering the resolution of the peaks and the presence of interfering substances co-eluting with the analytes, as well as, the analyte recovery.

Recovery and precision

In order to determine the recovery and precision of the method, extractions in triplicate were performed for the 3 concentration levels: 0.016, 0.023 and 0.039 mg/kg of avermectin B1a.

RESULTS AND CONCLUSIONS

Chromatographic conditions

The resolution of two peaks indicates the capacity of a column to separate chromatographic peaks. Both columns, Nucleosil and Zorbax® C8, when tested with repetitions of 10 μg/mL reference solutions, under a 1.5 mL/min flow rate, were capable of separating the two homologues of avermectin B1. However, the Nucleosil column (C18) showed more resolved peaks with the Zorbax® column (C8), a result that was determined by the estimated value for the resolutions of each column for the two homologues, 1.07 and 0.45 for Nucleosil and Zorbax®, respectively. Further, under such conditions, the Nucleosil column was more efficient and had better reproducibility. Based on these initial results, the Nucleosil column was preferred to the Zorbax® column.

The UV spectra of abamectin dissolved in the mobile phase proved that this medium did not influence absorption in this UV range; its absorption spectra showed the same peaks described in the literature (BUDAVARY, 1989). Furthermore, the use of a ternary mobile phase provided better resolution for the abamectin homologues.

Repeatability is a measurement of the precision of the analytical system under normal operating conditions over a short time interval (SHABIR, 2003). The results of the applications of the reference solution replicates under previously described conditions were accurate; the RSD values were lower than 1% for the homologues, 0.37 and 0.52% for avermectin B1a and B1b, respectively. These results are in accord with the commonly accepted values for instrumental accuracy (SHABIR, 2003). These values indicate that, under the conditions used for each analysis, the instrument did not influence the responses for the samples in each of the analytical runs.

The analysis of the interval of linearity demonstrated that the detection was linear. The correlation coefficient ($r = 0.9993$) obtained for the data indicated an adjustment of the data to the line of regression for amounts of the analyte in concentrations as low as 0.008 mg/kg. This sensitivity for the method is in the same range as most of the MRLs regulated by Brazilian legislation (ANVISA, 2002) for different biological matrices.

Extraction and clean-up

Sorbents and adsorbents used in the development of the method were selected considering the large amount of polar compounds in the sample. Honey solubilization in methanol allowed for the diffusion of the sorbent throughout the matrix to solubilize the analyte of interest. The subsequent addition of ethylacetate and hexane with an increasing apolar character, gave rise to the formation of two separable phases.

In addition, the use of different temperatures over the matrix was shown to be efficient in the extraction. This peculiarity of honey may be quite favorable for the determination not only of avermectin, but also of other pesticides. It could be an alternative to liquid-liquid extrac-

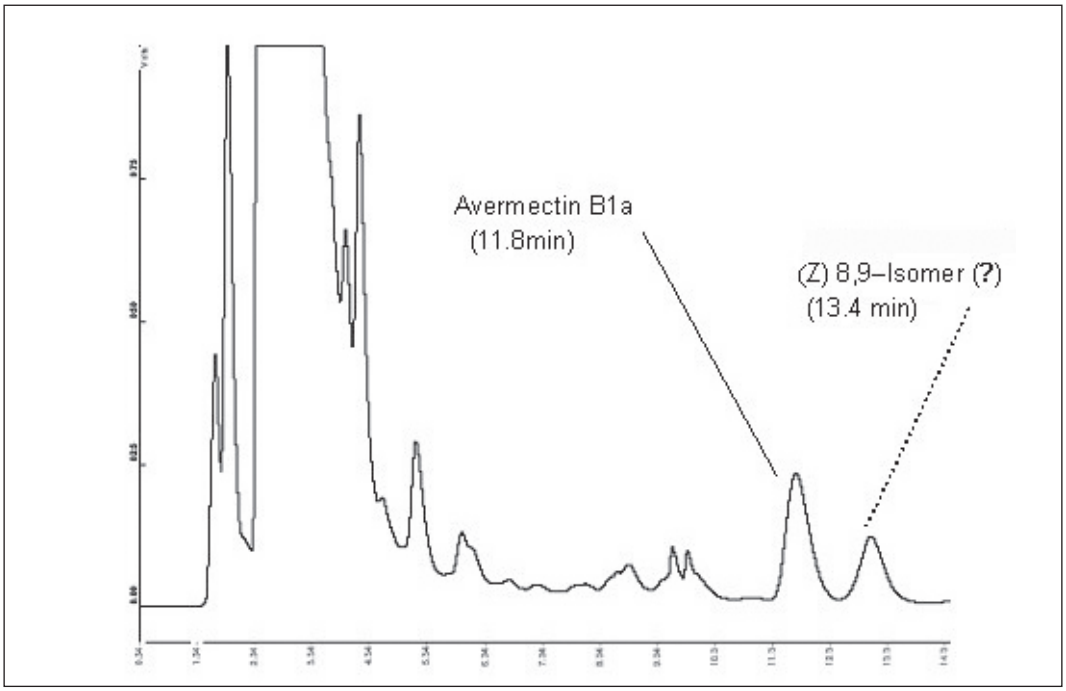


Fig. 2 - Chromatogram of a sample extract fortified at a concentration level of 0.19 mg/kg of the analyte, after elution with Florisil solid-phase extraction cartridges.

tion, since the addition of water is not necessary to solubilize the honey.

The two SPE adsorbents gave acceptable recoveries, with means of 89.52% and 94.99% for the Florisil and Silica-OH cartridges, respectively. Considering, the selectivity of each adsorbent, Silica-OH cartridges were chosen because they gave chromatograms with fewer interfering substances that eluted with retention times close to avermectin B1a (Figs. 2 and 3).

The low values observed in the recoveries of both cartridge replicates cannot be considered to be a direct influence of the adsorbent used. The occurrence of a peak with a retention time immediately after the elution of avermectin, that was not detected in the blank sample, allows us to infer that this peak represents the 8,9-Z-isomer avermectin B1a, that may have been formed as a result of the sample being exposed to light and heating of the extract in the evaporation stages (Fig. 1). These findings are in agreement with those of other studies on avermectin photodegradation (MROZIK *et al.*, 1988). It was not possible to characterize the suspected peak as being that of the isomer due to a lack of corresponding reference material. However, its identity may be assumed because it matched the expected retention time and was not found in the sample replicates. The analyses were conducted in the dark and at temperatures lower than 50°C to avoid photodegradation.

About two hours are required for each extraction and clean-up procedure for up to 10 samples. Overall, this technique does not require much work, is rapid and does not require sophisticated equipment; it is therefore feasible for routine analysis.

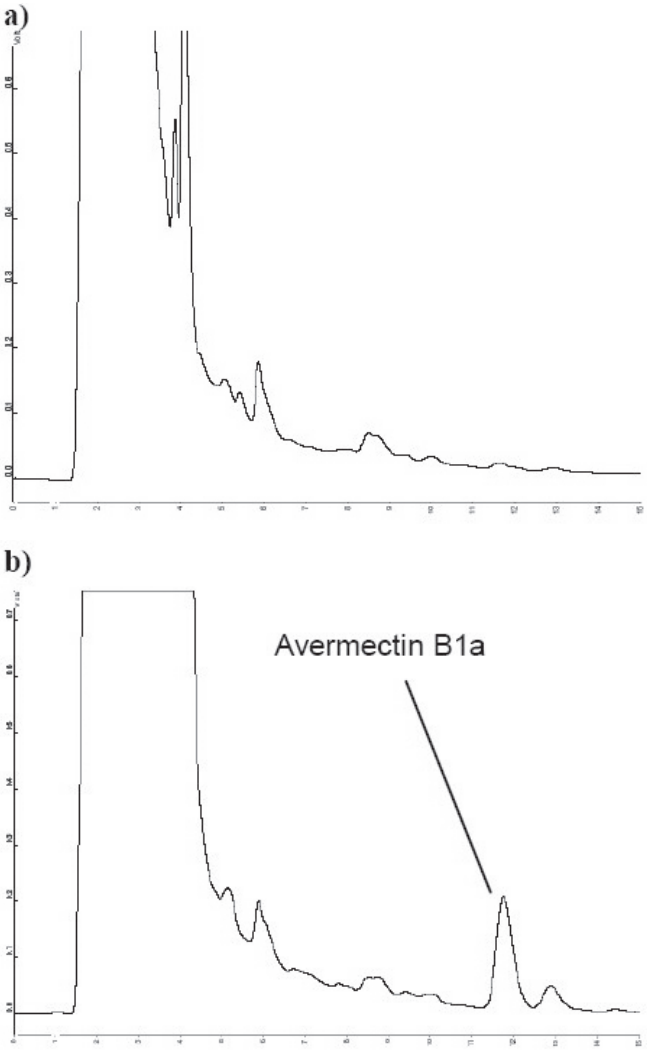


Fig. 3 - Chromatograms of a honey sample extract after elution with silica-OH solid-phase extraction cartridges: a) blank sample, b) silica-OH (sample fortified with abamectin 10 μg/mL reference solution).

Recovery

The recoveries of the analyte of interest for each of the analytic runs are reported in Table 1. It can be seen that, for the three fortification levels and in the three different analytical runs, the recovery values for the analyte are within the limits accepted by Brazilian legislation (MINISTÉRIO DA AGRICULTURA, 2003). The mean intra-day recovery percentages varied from 73.40 to 97.45%, among the different levels of fortification. When analyzing the mean inter-day recoveries, it should be noted that values for the three levels were relatively close (85.53, 84.98 and 92.59%) and are within the variation interval acceptable for the concentration levels of the analyte.

Concerning the individual results of each replicate, only one value was below 70% (68.70%), at the 0.016 mg/kg level. Although this might indicate that such a value is close to the Limit of Quantification (LOQ) for the method, this hypothesis was not confirmed, because the values for this concentration level were satisfactorily higher in subsequent analytical runs.

The limit of detection (LOD) and (LOQ) were calculated on the basis of the signal-to-noise ratio. The LOD and LOQ values were 0.002 and 0.007 mg/kg, respectively. These values indicate that the method can detect residual values of avermectin B1a at levels close to the MRLs established for many food matrices.

The analysis of variance of the regression of the analytical runs revealed a significant difference among the angular coefficients of all the analyses, except for runs 1 and 2. These results imply that there were differences in the degree of the response expressed by the instruments for the same concentration range of avermectin B1a on different days. This difference could be due to variations in room temperature, mobile phase composition, or to disturbances in the baseline. Although there were significant varia-

Table 1 - Recoveries and relative standard deviations for the different levels of avermectin B1a fortified samples (%).

Level of Fortification		Analytical run											
		1			2			3			4		
0.016 mg/kg	% Recovered	80.71	70.78	68.70	83.61	85.75	96.93	91.18	97.67	*	-	-	-
	Mean Intra		73.40 (8.8)			88.76 (8.1)			94.43 (4.9)			-	
	(RSD-intra)												
	Mean Inter					85.53 (12.72)							
	(RSD-inter)												
0.023 mg/kg	% Recovered	84.29	87.65	82.76	76.70	89.30	86.24	-	-	-	96.37	73.18	88.32
	Mean Intra		84.90 (2.9)			84.08 (7.8)			-			85.96 (13.7)	
	(RSD-intra)												
	Mean Inter					84.98% (1.11)							
	(RSD-inter)												
0.039 mg/kg	% Recovered	89.48	83.80	*	-	-	-	91.12	95.58	94.33	98.08	96.82	*
	Mean Intra		86.64 (4.6)			-			94.33 (2.5)			97.45 (0.91)	
	(RSD-intra)												
	Mean Inter					92.59 (5.93)							
	(RSD-inter)												
Slope	4092.3		3993.3			1885.4			2506.37				
Intercept	74.16		254.30			146.36			-188.488				
Correlation Coefficient	0.9997		0.9980			0.9995			0.9999				

Where:
SD-intra: standard deviation intra-day;
SD-inter: standard deviation inter-day;
* Not determined.

tions for the analyte responses, the study of linearity and precision demonstrate that they do not influence the final result, since the impact on both reference solutions and on the analyte present in sample extracts is the same.

The accuracy of a bioanalytical method is a measurement of the random error and is defined by the agreement of the replicate values of the same sample (CAUSON, 1997). Taking into consideration the RSD values in order to analyze the precision, the intra-day RSD values for the three levels were within the variation interval that is acceptable for concentrations in biological matrices, ranging from 0.91%, for the 0.039 mg/kg level, to 13.7%, for the 0.023 mg/kg level. Except for this latter value, all values were less than 10%. As for the inter-day RSDs, the values for the three concentration levels were below 15%. In general, the values obtained for the recovery and precision of the method are in agreement with other studies described in the literature in which the presence of avermectin B1 was determined in biological matrices by chromatography (PRABHU *et al.,* 1992*;* COBIN and JOHNSON, 1995; 1996; VUIK, 1991).

Since there is no regulation on the limits of avermectin residue in honey, it was impossible to correlate the results with the MRLs for the honey matrix. Nonetheless, it can be postulated that the method is precise and exact enough to determine dosages of avermectin B1a residues over this concentration range, giving reliable results for both scientific and routine analyses of avermectin residues in honey samples.

Considering the rapid decomposition of avermectins in the environment, it can be assumed that the presence of avermectin in honey would indicate that there had been recent contact with it, either by direct application to the hives, or because it was applied near the hives. The proposed method can also discriminate the original molecule from its isomer, which should increase its application; this hypothesis has yet to be confirmed.

In conclusion, under the given experimental conditions, the matrix extracts gave resolved peaks for avermectin B1a and, possibly, for its 8,9-Z-isomer. The accuracy and precision of the methodology were satisfactory in concentrations between 0.016 and 0.039 mg/kg. The percentage recoveries were higher than 70% but less than 100%. Based on these results, the proposed method is useful for the detection of avermectin B1a in honey, as it allows residual levels of avermectin B1to be monitored in routine analyses.

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PAPER

MICROBIOLOGICAL CONDITION OF CARCASSES AND EQUIPMENT IN A PIG SLAUGHTERHOUSE AND EVALUATION OF A STEAM DECONTAMINATION SYSTEM

CONDIZIONI MICROBICHE DELLE CARCASSE E DELL'AMBIENTE DI LAVORAZIONE IN UN MACELLO SUINO E VALUTAZIONE DI UN SISTEMA DI DECONTAMINAZIONE MEDIANTE VAPORE

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ABSTRACT

The microbiological conditions of the environment and carcasses in a commercial pig slaughter facility were examined. Environmental samples collected during the slaughtering, and after the cleaning and disinfection procedures showed that sanitization did not always significantly decrease the number of bacteria. Microbial counts on carcasses were higher than those indicated in the Decision 2001/471/CE. These data suggest that general hygiene standards alone are not suf-

RIASSUNTO

È stato condotto uno studio sulle condizioni microbiche delle carcasse e dell'ambiente di lavorazione in un macello suino. I campioni ambientali raccolti durante la macellazione e dopo le operazioni di pulizia e disinfezione hanno dimostrato che l'efficacia dei sistemi di sanificazione non sono sempre stati in grado di ridurre efficacemente la carica microbica nell'impianto considerato per questo studio. Anche le conte microbiche ottenute sulle carcasse si sono rivelate più alte rispetto a quanto ri-

- Key words: contamination, *Listeria monocytogenes*, pig carcass, *Salmonella* spp., slaughterhouse, steam decontamination -

ficient to ensure food safety. To evaluate the effectiveness of steam pasteurization, a pilot-plant process which exposes the carcasses to steam was developed and tested. Eighty-six carcasses were sampled. After treatment, mesophilic counts on carcasses were significantly reduced (up to 2.26 $\log_{10}$ CFU/cm^2). The current study verifies that the scaled-up commercial decontamination system is very effective in reducing the levels of natural mixed flora on surfaces of commercially slaughtered pig carcasses.

portato dalla Decisione 2001/471/CE. Questi dati suggeriscono che da sole, le classiche operazioni di pulizia e disinfezione, non sono in grado di garantire la sicurezza dei prodotti alimentari. Per valutare l'efficacia del trattamento di decontaminazione mediante vapore, è stato allestito un impianto prototipo che ha permesso di esporre le carcasse suine al vapore saturo. Dopo il trattamento le conte dei batteri mesofili sono state significativamente ridotte, fino ad un massimo di 2,26 $\log_{10}$ UFC/cm^2. Questo studio, quindi, evidenzia l'efficacia del trattamento di decontaminazione delle carcasse suine durante la normale attività di macellazione.

INTRODUCTION

The swine slaughtering process can lead to the contamination of the pig carcass by potentially pathogenic bacteria. The main source of microbial contamination during the processing of pigs in the slaughterhouse is the digestive tract and its contents (EUROPEAN COMMISSION, 2000). The hygienic conditions of the walls, floors and ceilings and the presence of hard-to-clean equipment can facilitate spreading of bacteria (RIVAS *et al.*, 2000).

In 2002, the European Union (EU, 2001) introduced a HACCP system for fresh meat slaughter, including pigs, which gives performance criteria for measuring the effectiveness of the controls during the slaughter process. The system is based on a three-class attribute sampling plan; the criteria to be measured are the aerobic mesophilic counts (AMC) and the *Enterobacteriaceae* or *Escherichia coli* counts. Unacceptable values obtained for AMC and *E. coli* on carcasses, according to the criteria of the Decision 2001/471/CE (EU, 2001), indicate that the slaughter process is not being carried out correctly. The finding of unsatisfactory results should lead to decisive action to review the control procedures, determine the causes of contamination and make the necessary changes to avoid repetition of such results. Microbiological data on carcasses should be the basis for establishing programs to protect public health, determining the needed interventions and evaluating the effectiveness of such action (EUROPEAN COMMISSION, 2000; WHO, 2001).

Some steps of the slaughtering process decrease the bacterial populations on carcasses (scalding, singeing) but scalded carcasses are probably recontaminated during the dehairing and polishing operations (BERENDS *et al.*, 1997; GILL *et al.*, 2000). Intensive production, in some cases, worsens the microbiological conditions of the raw materials, and mechanization does not always improve the hygienic or microbiological conditions of the food (ROBERTS, 1996).

Carcass decontamination technologies, including chemical treatments, have been investigated to reduce bacte-

rial contamination (SOFOS and SMITH, 1998; HUFFMAN, 2002) and some commercial applications have been found. Chemical decontamination of carcasses, however, is not permitted in the European Union. Studies using hot water washes of pig carcasses have been shown to reduce bacterial counts by 1 to 2 $\log_{10}$ cycles (GILL *et al.*, 1997, 1998).

A logical extension of hot water washing is the use of steam to thermally destroy bacteria on the pig carcasses. Although there have been many studies on the use of steam to reduce bacterial contamination of meat (GILL and LANDERS, 2003; KOZEMPEL *et al.*, 2003), few studies have evaluated the effectiveness of this treatment in commercial processing situations (DORSA, 1997; NUTSCH *et al.*, 1997 and 1998; MINIHAN *et al.*, 2003). To the best of our knowledge, no data are available in the literature on the application of this technology in pig processing facilities.

The aim of the present study was to obtain baseline data on the types and levels of bacterial contamination in an abattoir environment and on pig carcasses, and to determine the effectiveness of the cleaning and disinfecting procedures used. A steam decontamination system was then applied to reduce the naturally occurring populations of aerobic bacteria on the carcasses of commercially slaughtered pigs.

MATERIALS AND METHODS

The microbiological condition of carcasses and the slaughtering environment were examined in a commercial pig slaughter facility with a maximum slaughter capacity of 200 animals/h. Animals were slaughtered at around 160 kg live weight to produce typical dry cured products, such as Parma hams. The slaughter facility was divided into the stunning-singeing area (ground floor), and the evisceration and sectioning area (first floor). Pigs were held in lairage, stunned using low voltage electrical stunning tongs and immediately exsanguinated. Exsanguinated animals were scalded for approximately 4-5 minutes using a "scald tank" (64±1°C), dehaired and hung on an overhead conveyor belt by hooking the hind legs. Carcasses were then passed through a singeing device operating at approximately 1,200°C for 15 s. Singed carcasses were polished by passing them through a series of horizontal and vertical flails; they were then sent to the first floor.

The steam pasteurization pilot-plant, developed by REA (Torino, Italy), is situated on the ramp that leads from the ground floor to the first floor and precedes the evisceration step on the processing line. The apparatus was designed to expose the carcasses to nearly saturated steam, in a non-pressurized enclosed on-line chamber, followed by a cold-water spray to remove surface heat induced during pasteurization. The cabinet is a stainless steel sheet-metal tunnel (3.00 m long, 1.03 m wide, and 2.55 m tall) that houses both the steam chamber and the chilling system. The chamber is divided into four compartments by silicone walls which open and close as the carcasses pass through. This is to prevent steam from escaping into the environment during treatment. The steam boiler delivers steam through nozzles, at rates ranging from 700 to 1,000 kg/h. The carcasses pass through the chamber in 8 seconds during which time, fresh steam enters at the base of the cabinet and spent steam leaves at the top. Immediately after exposure to the steam, the carcasses are cooled with a cold water spray to remove the surface heat. The overall process takes 10 seconds. The system can also be turned off to allow carcasses to pass through the tunnel cabinet without being steam treated.

Subsequently, the carcasses are submitted to debunging with a rectal pistol; the belly is opened and the connective

tissues joining the bung and viscera to the carcasses are cut. The diaphragm, heart, lungs, trachea and the digestive tract are removed and the carcasses are split along the midline from the hind to the fore using an automatic splitter, and, finally, the head is removed. Carcasses are not trimmed but are spray-washed with cold (15°C) potable water to remove bone dust and blood clots before being moved to the sectioning room.

Cleaning and disinfection

The large equipment (dehairing device, dehairing conveyor, scraping device, walls and floors) was cleaned and disinfected at the end of the workday. The process included the (i) removal of organic detritus using pressurized water at 40°C; (ii) application of alkaline detergent (left to act for 10 min) with foam projection equipment; (iii) rinsing with pressurized water; (iv) application of quaternary ammonium disinfectant (left to act for 10 min); and (v) rinsing with pressurized water and allowing the equipment to dry.

Sampling, preparation of samples and enrichment procedures

Environment

When possible, 15 environmental sites were sampled, (i) 4 h after the beginning of the work day and (ii) after cleaning and disinfecting procedures at the end of the day. A rayon swab moistened with 10 mL of Ringer solution (aerobic mesophilic count, AMC; coliforms; *E. coli*) was used to swab 100 cm^2 (floor, wall, conveyor belt, table, etc.) or the entire surface (hook, knives). For pathogen detection, the swabs were moistened in 10 mL of buffered peptone water (BPW; Oxoid, Basingstoke, England) (*Salmonella* spp.) or half Fraser broth (Biomérieux, Marcy d'Etoile, France) (*Listeria monocytogenes*). The "Ringer swabs" were submitted to serial dilutions, whereas the others were incubated directly at the appropriate temperature (BPW: 37±1°C for 18-24h; half Fraser: 30±1°C for 24h). Two-hundred and seventy samples were taken during the pig slaughtering and cutting process, and 101 samples were taken after cleaning and disinfection.

Carcasses

To evaluate carcass contamination during the working day, 108 half-carcasses were examined immediately after the evisceration step (before the last shower). All the samples were obtained from randomly selected carcasses, in the morning, 3 hours after the beginning of the slaughtering process and on 15 different slaughtering occasions. For each carcass, four sites were investigated: three external skin sites (loin, shoulder and jowl), and one internal muscle site (leg).

To evaluate the effectiveness of the steam treatment, 86 carcasses were sampled in 4 trials performed on 4 different slaughtering days, under the same conditions as described above. In these cases, the three external locations were investigated, while the internal muscle site was excluded. Carcasses were tagged and tracked through the evisceration process, and were then sampled at the end of the slaughter line. Fifty-four carcasses were treated with steam, while 32 carcasses were used as negative controls.

Samples were collected using the destructive method, as recommended in the Decision 2001/471/CE (EU, 2001). Tissue samples representing a total of 80 cm^2 were obtained by cutting a slice of maximum thickness of 5 mm. Samples were then placed aseptically into a plastic dilution bag, transferred to the laboratory and analyzed immediately. Ten cm^2 of each sample were used to enumerate AMC, coliforms and *E. coli*.

Microbiological analyses

Samples were stomached with 90 mL of Ringer solution (Oxoid) in a

Stomacher BagMixer (Interscience, St. Nom La Breteche, France). Tenfold serial dilutions were prepared using the same diluent. AMC were performed on plate count agar (Oxoid); coliforms and *E. coli* were determined by using Violet Red Bile Lactose Agar (Oxoid) and using the selective and chromogenic medium Coli ID (Biomérieux); *E. coli* were also enumerated by using the selective medium Gram negative PMK Agar MUG (Biolife, Milano, Italy). All plates were incubated at 37±1°C for 18-24 h.

Regarding *Salmonella*, after pre-enrichment, 0.1 mL and 1 mL of BPW cultures were added to 10 mL Rappaport Vassiliadis (RV) broth (Oxoid) and to 9 mL of selenite cystine broth (Biomérieux), respectively. RV broth was then incubated at 42±1°C, whereas selenite cystine broth was incubated at 37±1°C (selective enrichment). After 24 h, both broths were streaked onto Hektoen Enteric Agar (Oxoid) and Brilliant Green agar (Oxoid) and were incubated at 37±1°C for 18-24 h. Suspected colonies were submitted to an agglutination latex test (Microgen-Salmonella, Microgen Bioproducts Ltd., Camberley, England) and tested biochemically (API 20E, Biomérieux).

Regarding *L. monocytogenes*, 1 mL of half Fraser broth was added to 9 mL Fraser broth and then incubated at 30±1°C for 24 h (selective enrichment). Enrichment broth was streaked onto Palcam Agar (Oxoid) and onto the chromogenic medium Rapid-L-Mono (Biorad, Hercules, CA, USA). All plates were incubated at 37±1°C for 24 h. Suspected colonies were submitted to an agglutination latex test (Microgen-Listeria, Microgen Bioproducts Ltd.) and tested biochemically (API Listeria, Biomérieux).

Statistical analysis

Microbiological counts were transformed into logarithms before the means were obtained and statistical analysis was carried out. The minimum detectable counts (detection limit) for the carcass samples and for the environmental samples on a 1 cm^2 basis were 10 CFU/cm^2 (1 $\log_{10}$ CFU/cm^2) and 0.1 CFU/cm^2 (-1 $\log_{10}$ CFU/cm^2), respectively. For statistical analysis, a value of one-half of the detection limit was used for any sample in which a population was not detected (NUTSCH *et al.*, 1998).

Means of the data were compared using a two-tailed *t*-test. The SPSS ver. 11.5 software package (SPSS Inc. Chicago, IL, USA) was used to carry out computations.

RESULTS

Environmental samples

During the working day, 28.0% (n=100) of the environmental samples were *Salmonella* spp.-positive and 18% (n=61) were *L. monocytogenes*-positive. After cleaning and disinfection procedures, these percentages dropped to 10.8% (n= 74) and 9.8% (n=51), respectively.

The lairage floor sampling site showed the highest *Salmonella* spp. isolation rate (57.1%), followed by the tray used to collect viscera during the evisceration step (50.0%) and the ground floor, where the steps from stunning to flaming were performed (37.5%) (Fig. 1). The prevalence of *Salmonella* spp. in the environment was generally lower after the cleaning and disinfecting procedures, although in some cases, it remained relatively high, e.g. the ground level floor (33.3%), on the splitting machine (20.0%) and on the dehairing conveyor.

The highest percentages of *L. monocytogenes* were found in samples taken from the floor of the ground level (50.0%), the floor of the first level and from a table in the cutting room (33.3%). Sanitizing procedures generally reduced the *L. monocytogenes* lev-

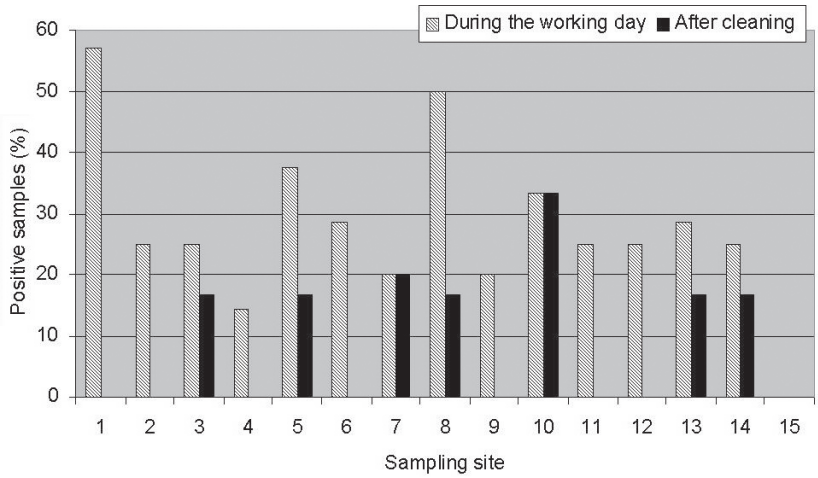


Fig. 1 - Incidences of *Salmonella* spp. at selected environmental sites. (1) lairage; (2) killing conveyor; (3) dehairing conveyor; (4) wall of the ground floor; (5) floor of the ground floor; (6) evisceration knives; (7) splitting machines; (8) trays for viscera; (9) pluck hook; (10) floor of the first floor; (11) knives of the cutting room; (12) breaking table; (13) belly environment; (14) floor of the cutting room; (15) shoulder table.

els but, higher percentages were noted on samples from the tray receiving the viscera (from 16.7 to 20.0%) and on the killing conveyor (from 0 to 20.0%). After cleaning and disinfection, *L. monocytogenes* were detected at four sampling points (lairage, floor of the ground floor and of the cooling room and table in the cutting room) (data not shown).

Statistical analysis of environmental bacterial counts showed that, after cleaning and disinfection, AMC were significantly reduced in almost all sampling points (Fig. 2). On the contrary, cleaning and disinfection procedures did not always significantly decrease the number of *E. coli* and coliforms (data not shown).

Carcasses and steam decontamination

A total of 108 pig carcasses were analyzed during the ordinary slaughtering process on 15 slaughtering days. AMC

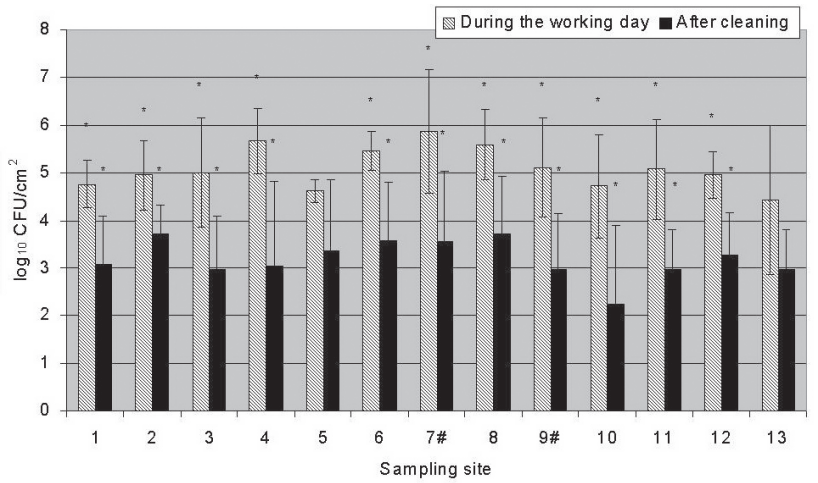


Fig. 2 - Mean aerobic mesophilic counts (log_{10} CFU/cm^2) on environmental samples. (1) killing conveyor; (2) dehairing conveyor; (3) wall of the ground floor; (4) floor of the ground floor; (5) splitting machines; (6) trays for viscera; (7) pluck hook; (8) floor of the first floor; (9) hook cutting room; (10) breaking table; (11) belly environment; (12) floor of the cutting room; (13) shoulder table. * means having a symbol in common are significantly different ($P<0.05$). # log_{10} CFU/entire surface.

were recovered from all carcass samples. The mean log number of total aerobes was 5.89±0.35 $\log_{10}$ CFU/cm^2. The estimated mean log numbers of coliforms and *E. coli* were 2.86±0.78 and 2.31±0.60 $\log_{10}$ CFU/cm^2, respectively. *Salmonella* was isolated in 29 out of the 108 slaughtered pigs (26.9%), whereas 6 out of 70 samples (8.6%) were found positive for *L. monocytogenes*.

A total of 86 carcasses were analyzed to evaluate the effectiveness of steam decontamination: 54 carcasses were treated with steam, while 32 were used as the negative control. In the first two trials (24 carcasses), the unit was tested to evaluate its capability of reducing microbial contamination. Although AMC were significantly reduced (P<0.05) after pasteurization, the difference was not as great as expected (0.58 $\log_{10}$ CFU/cm^2); the coliform and *E. coli* counts were not significantly reduced (Fig. 3). The modest effectiveness of the exposure of the carcasses to steam was probably due to a blast of air that funnelled through the passage from the ground level to the first floor, as confirmed by the difference in the temperature between the two areas. The ventilation system in the cabinet was then improved in order to prevent the steam from escaping. Following these modifications, the third and fourth series of samples (30 treated carcasses) were collected, and the reduction of the mean AMC, coliforms and *E. coli* counts were 2.00, 1.07 and 0.78 $\log_{10}$ CFU/cm^2, respectively (Fig. 3) with the highest AMC reduction (2.26 $\log_{10}$ CFU/cm^2) obtained in the fourth trial. All of these reductions are statistically significant (P<0.05).

DISCUSSION AND CONCLUSIONS

The results of the present study show that the microbial load of some equipment and machinery used in the slaughtering process did not decrease after routine cleaning and disinfection procedures. Other authors have reported that there were no statistically significant differences between the microbial counts for dirty and clean equipment (GILL *et al.*, 1999; RIVAS *et al.*, 2000) and that cleaning and disinfection do not always completely eliminate the pathogens from such environments (SWANENBURG *et al.*, 2001; HALD *et al.*, 2003). In particular, *L. monocytogenes* seems more resistant to the disinfectants used than *Salmonella* spp., as shown by the number

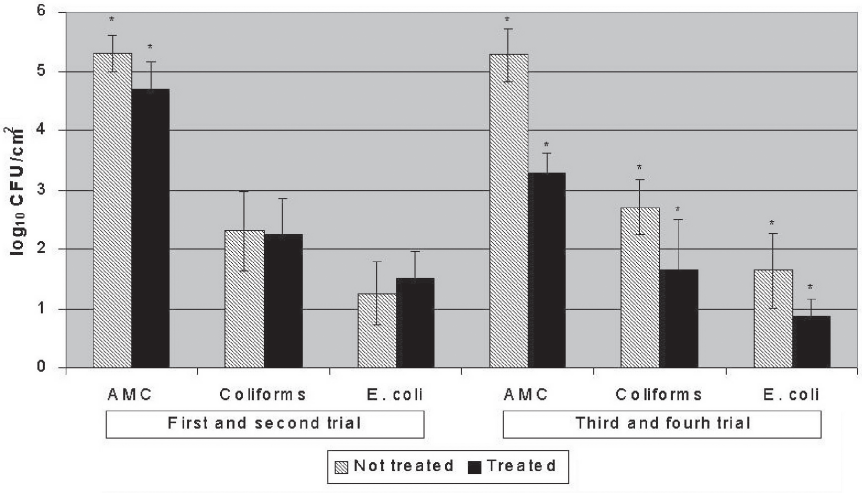


Fig. 3 - Mean AMC, coliform and *E. coli* counts ($\log_{10}$ CFU/cm^2) on carcasses treated and not treated with steam. * means having a symbol in common are significantly different (P<0.05).

of positive samples after cleaning and disinfection, that decreased from 28 to 13.5% for *Salmonella* spp. and from 18 to 9.8% for *L. monocytogenes*. The isolation of pathogens from the environment even before the slaughtering process started indicates that cross contamination between animals and the environment can occur. These results coincide with a World Health Organization Expert Committee report (WHO, 1990) that pointed out that one of the causes of the contamination of carcasses in pig slaughterhouses is due to the fact that some equipment and machinery are difficult to clean because of their design and state of wear. In addition, the high standard deviation of the microbial counts of the environmental samples (Fig. 2) indicates a great variability in the effectiveness of sanitation procedures. These data suggest that the normal cleaning and disinfection processes need to be improved in the facility considered in this study.

In this study, 26.9% of the carcasses sampled at the end of the slaughter line were positive for *Salmonella* spp. This frequency is in agreement with similar studies in other European countries (DAVIES *et al.*, 2000; BOTTELDOORN *et al.*, 2003). *L. monocytogenes* was found at every point along the pork processing line. It was also confirmed at the farm level, although it was less prevalent. The incidence of this micro-organism increases along the processing chain, from the slaughterhouse to the cutting room (NESBAKKEN and SKJERVE, 1996; GIOVANNACCI *et al.*, 1999). In the slaughterhouse examined in this study, 8.6% of the carcasses were *L. monocytogenes* positive.

The results obtained for microbial counts, together with the high prevalence of *Salmonella* spp. and *L. monocytogenes*, suggest that overall hygiene standards alone are not sufficient to ensure food safety and reduce the risk of foodborne illness. For this reason, it is important to consider some processes to obtain carcass decontamination. Since no chemical treatment has yet been sanctioned in the EU, and only potable water is allowed to come into contact with carcass meat, steam decontamination provides a very desirable critical control point (CCP) during pork processing. As a matter of fact, steam treatment offers a genuine opportunity to significantly reduce the surface microbial contamination of the carcasses and prevent pathogens such as *Salmonella* spp. from contaminating edible carcass tissue. Steam treatment does not require the use of chemicals or specialized waste water handling, and, when used properly, with commercially installed safeguards, it is not hazardous to plant personnel or equipment. Contamination does not need to be visible to be treated, because steam vapour uniformly covers irregularly shaped surfaces over the entire carcass surface area, theoretically providing total and complete carcass decontamination. A distinct advantage of steam decontamination is the reduced potential for selecting resistant bacterial populations.

The current study verifies that the scaled-up commercial steam decontamination system is very effective in reducing the levels of natural mixed flora on surfaces of commercially slaughtered pig carcasses. Steam treatment reduced the numbers of natural flora on pig carcasses by about two orders of magnitude thereby aiding the hygienic processing of pigs. Even the strictest good manufacturing practices cannot prevent contamination that occurs during steps, such as dehairing or polishing (BERENDS *et al.*, 1997), while the evisceration process, if carried out properly, may not contribute to carcass contamination (RIVAS *et al.*, 2000; WARRINER *et al.*, 2002). Despite the possible recontamination after treatment, steam decontamination should take place before the evisceration step in order to reduce the negative effect of heat on meat

appearance. Furthermore, steam decontamination of fully-dressed pig carcasses is not a commercially viable option because the appearance of muscle surfaces can be permanently degraded even by mild heat treatment (GILL and BADONI, 1997). The risk of damage to the appearance of pork muscles is even more important if the meat is to be used in the manufacture of dry cured meat products, such as Parma ham. Decontamination must be considered to be supplemental to an already hygienically optimized slaughter procedure. While decontamination treatments cannot eliminate the consequences of slaughtering under poor hygienic conditions, they can improve the effectiveness of an already implemented HACCP system.

In conclusion, routine cleaning and disinfection should be carried out properly, considering the role that machinery plays in cross-contamination. A high prevalence of pathogens on carcasses and high AMC values show the need for improved slaughtering systems and the introduction of new decontaminating steps. Steam decontamination significantly reduced the microbial flora of pig carcasses and could be an effective tool for improving hygienic quality.

ACKNOWLEDGMENTS

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PAPER

ASSESSMENT OF THE MICROBIOLOGICAL QUALITY OF RETAIL FRESH PORK MEAT IN CENTRAL ITALY

DETERMINAZIONE DELLA QUALITÀ MICROBIOLOGICA
DI TAGLI FRESCHI DI CARNE DI MAIALE IN ESERCIZI COMMERCIALI
DELLA REGIONE LAZIO

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ABSTRACT

An assessment of the microbiological quality of retail fresh pork meat was conducted with two microbial isolation methods (swab, disruptive) on 108 samples from two muscle types (*Longissimus thoracis* and masseter), collected from 18 outlets of two retail types (supermarkets and butcher's shops) in three provinces of the Latium re-

RIASSUNTO

È stata determinata la qualità microbiologica di tagli di carne di maiale fresca con due metodi di isolamento (tampone e distruttivo) su 108 campioni da due tipi di muscolo (*Longissimus thoracis* e massetere) prelevati da 18 esercizi commerciali di due tipi (supermercati e macellerie) in tre province del Lazio. I conteggi hanno fornito valori compresi

- Key words: fresh pork meat, microbiological quality assessment, microbial isolation method, reference values -

gion (central Italy). Counts ranged from 2.2 to 5.4 log cfu/g for total mesophilic bacteria, from 0.09 to 5.6 log cfu/g for thermophilic bacteria, and from 1.3 to 4.3 log cfu/g for coliforms. On the average, the disruptive method gave higher colony counts by a factor of 2.4. Residual variability was mostly due to muscle type and only to a limited extent, to the retail outlet or sampling visit. The frequency of positive samples detected with the disruptive method was 19% for *E. coli*, 16% for *Staphylococcus* spp., 19% for *Salmonella* spp., 29% for *Campylobacter* spp., 11% for *B. thermosphacta* and 20% for lactic acid bacteria; in general the method was two to five times more efficient than the swab method. Method agreement was quite low for both analytical determinations and overall quality assessment. Compared with EU reference values, the swab method was stricter in assessing microbiological quality, signalling over one-tenth of the samples as unacceptable on mesophilic counts and about two thirds on coliforms counts, while 6% on coliform counts and 19% on *Salmonella* tests were rated as unacceptable according to the Italian Zooprophylactic Service reference based on the disruptive method.

fra 2,2 e 5,4 log cfu/g per i batteri mesofili totali, fra 0,09 e 5,6 log cfu/g per quelli termofili e fra 1,3 e 4,3 log cfu/g per i coliformi. In media, il metodo distruttivo ha dato un numero di colonie maggiore di un fattore 2,4. La variabilità residua è dovuta principalmente al tipo di muscolo e solo per una entità limitata al tipo di esercizio commerciale e alla replica del campionamento. La frequenza di campioni risultati positivi con il metodo distruttivo è stata del 19% per *E. coli*, 16% per *Staphylococcus* spp., 19% per *Salmonella* spp., 29% per *Campylobacter* spp., 11% per *Brochotrix thermosphacta* e del 20% per batteri acidi lattici, essendo il metodo distruttivo da due a cinque volte più efficiente del metodo con tampone. L'accordo fra i due metodi per entrambe le determinazioni (conteggi ed isolamenti), ed in generale per la definizione della qualità, è risultato abbastanza basso. Usando come valori di riferimento quelli previsti dalla normativa UE, il metodo del tampone è risultato più stringente nella definizione di qualità microbiologica, segnalando come non accettabili più del 10% dei campioni per la carica di mesofili totali e circa i due terzi dei campioni per la carica di coliformi, mentre in riferimento al tipo di microrganismi da esaminare, al metodo di isolamento (distruttivo) ed ai valori limite della metodica messa a punto dagli Istituti del Servizio Zooprofilattico italiano, il 6% dei campioni risulta non accettabile per la carica dei coliformi ed il 19% risulta contaminato da *Salmonella*.

INTRODUCTION

During 2004, approximately 900,000 t of pork meat were imported into Italy to fulfil the internal demand. More than half of the imported pork meat was fresh thighs for ham production; the remainder was made up of half carcasses, shoulders and loins for fresh retail (BORELLI, 2005). In Italy, most swine rearing, destined for typical salami and ham production is subject to strict rules,

according to the Regulations for Protected Designation of Origin (DELLA CASA *et al.*, 2004).

Generally speaking, the aspects of concern for meat quality include chemical, physical and sensory characteristics. Furthermore, microbiological properties of all food products, including meat, have to comply with hygiene, safety and processing suitability requirements. Several groups of microorganisms that are hosted on freshly slaughtered or stored meat include processing agents (protechnological), health promoters (probiotic), spoilage and technologically undesirable agents involved in spoilage reactions and pathogens, which may cause toxic and toxic-infective states (OTTOGALLI and COPPOLA, 1996; OTTOGALLI, 2004).

Microbial contamination of meat is caused mostly by improper livestock rearing and processing practices, unclean processing equipment and environments and poor worker hygiene.

Zoonotic microorganisms frequently cause human diseases, such as intoxications by *Staphylococcus* and gastroenteritis by *Cryptosporidium* spp., *Salmonella* spp. and *Campylobacter* spp., generally known to have a benign course. Listeriosis (*Listeria monocytogenes*), botulism (*Clostridium botulinum*) and the hemolytic uremic syndrome (enterohemorrhagic *Escherichia coli*) can also be lethal. About 40% of the cattle population is estimated to be healthy carriers of enterohemorrhagic *E. coli* (VTEC, STEC) (LARDY, 2002). Pigs are asymptomatic carriers of *Salmonella* spp., hosting it in several sections of the gastrointestinal and lymphatic systems (CANTONI, 2005). *Campylobacter* spp. are present in the gastrointestinal tract of poultry (positive up to 98% depending on the flock), beef, pork and sheep (positive values less than 20%) and are transmitted to people through eating infected animal products (MOVIA, 2004; OTTAVIANI, 2005). Potential pathogens can be haematogenically spread (diapedesis) from the gastrointestinal system to muscles as a consequence of shock received by the animal during culling and/or transport to the slaughterhouse.

Spoilage microorganisms like lactic acid bacteria, *Staphylococcus* spp. and others alter color, taste and odour, thereby reducing product shelf life (SNEATH and JONES, 1986; CANTONI and COZZI, 2002), technologically undesirable microorganisms, like *Brochothrix thermosphacta*, *Listeria* and *Bacillus*, can also damage processing and preservation processes (BORCH *et al.*, 1996).

Among meat microorganisms there are some notable antagonists of pathogens and/or harmful microflora. For instance, *Leuconostoc mesenteroides* L124 and *Lactobacillus curvatus* L442, both bacteriocin-producers, are antagonists of *Brochothrix thermosphacta* and of several enterococci, while *Leuconostoc carnosum* (LA54a e TA26b) and *L. mesenteroides* subspecies *dextranicum* (TA33a) produce bacteriocins against *Listeria* sp. (PAPATHANASOPOULOS *et al.*, 1994; METAXOPOULOS *et al.*, 2002; KANG *et al.*, 2002), which, in all cases compete for nutrients as well as for space.

The purpose of this study was to gather information on the microbial species which may survive or proliferate on meat after slaughtering and sectioning of retail pork meat sold in the provinces of Latina, Rome and Viterbo in the Latium region (Central Italy) and to compare and assess the performance of two isolation methods used for microbiological analysis and quality evaluation of the meat.

MATERIALS AND METHODS

Meat

Samples of pork meat kept in aerobic, refrigerated conditions (i.e. counter or cold storage in supermarkets and butcher shops) of two muscle types

(*Longissimus thoracis* and masseter) were collected in three successive visits to 18 outlets selected from the two types of stores in the three provinces of Latina (LT), Rome (RM) and Viterbo (VT) (108 in total). The origin (foreign, Italian DOP, non-DOP) of the raw samples was not declared.

Microbiological analysis

The 108 samples, kept between 0° and +4°C, were transported to the laboratory in labelled sterile plastic bags and were analysed in triplicate after being processed by each of the two processing methods (swab and disruptive), totalling 648 determinations.

The swab method was applied by taking three smears per sample with sterile swabs on an area of 100 cm^2 defined with a delimiter, adding suitable volumes of isotonic eluent (Tampoexpress + Diluexpress, PBI International, Milano, Italy) for decimal dilutions and plating 1 mL per dilution on a sheet medium culture containing chromogenic substrates (e.g. magenta-gal) for colony differentiation (Chromogenic Compact Dry EC, PBI International, Milano, Italy) (MIZUOCHI and KODAKA, 2000; MIZUOCHI *et al.*, 2000).

With the disruptive method, three 10 g subsamples, taken under aseptic conditions after surface sterilisation with U.V. light (2 min for each side of the sample) in a biohazard hood, were treated in a stomacher (Lab blender 400 stomacher, Seward, London) with sterile peptone water (0.1% peptone and 0.85% NaCl in a 1/10 ratio), followed by suitable decimal dilutions and plating (1 mL/plate) on specific agar media.

Culture media were chosen with reference to the official testing methods and the ISO rule 7218:1996. Counts of colony forming units (cfu) were expressed on the usual decimal log scale (log cfu/g or log cfu/mL).

To sample a wide range of microorganisms, count determinations for mesophilic (TMC), thermophilic and coliform bacteria with *Escherichia coli* distinction and qualitative assays (for *E. coli*, *Campylobacter* spp., *Salmonella* spp., *Brochothrix thermosphacta,* coagulase positive *Staphylococcus* spp., *Bacillus stearotermophilus*, Lactic Acid Bacteria (LAB) were made using both processing methods.

Mesophilic and thermophilic bacteria were counted on Dextrose Tryptone Agar (Oxoid, Garbagnate Milanese (MI), Italy), after incubation at 32°C for 72 hours and 55°C for 48 hours, respectively (AMERICAN PUBLIC HEALTH ASSOCIATION, 1992).

Coliforms with *E. coli* distinction were counted on Chromogenic Compact Dry EC (PBI, Milano, Italy) after incubation at 35°C for 20 hours (MIZOUCHI *et al.*, 2000).

Coagulase positive *Staphylococcus* spp. (*S. aureus*, *S. intermedius*, *S. hyicus*, etc.) were detected with Baird-Parker with RPF (Rabbit Plasma Fibrinogen) added in and incubated at 35°C for 48 hours (ISO 6888-2, 1999).

For *Campylobacter* spp. detection, rule ISO 10272 (1995) was followed, adding suitable volumes of eluent to *Campylobacter* Enrichment Broth (BHI + *Campylobacter* Growth Supplement and modified Karmali Selective Supplement, OXOID, Garbagnate Milanese (MI), Italy), plated on selective media (Skirrow agar, Karmali agar, Preston Agar, OXOID, Garbagnate Milanese (MI), Italy) and incubated for 48 hours at 42°C in microaerophilic conditions ("Campygen" system, OXOID). After incubation, catalase-positive colonies were treated with the latex agglutination test (*Campylobacter* test kit, OXOID, Garbagnate Milanese (MI), Italy).

The Simple Pre-enrichment and Rapid Isolation New Technology kit (SPRINT, OXOID, Garbagnate Milanese (MI), Italy) and the Salmonella Latex Test (OX-

OID, Garbagnate Milanese (MI), Italy) for enrichment were used to detect *Salmonella* spp.

STAA agar base and the selective supplement (CM0881+ SR0151 OXOID, Garbagnate Milanese (MI), Italy), incubated at 22°C for 48 hours (NCFA, 1991), were used to detect *B. thermosphacta*.

Lactic acid bacteria were determined on MRS broth pH 5.6 (De Man, Rogosa, Sharpe OXOID, Garbagnate Milanese (MI), Italy), incubated for 48-72 hours at 25°C and 5-10% CO_2 in an anaerobic system (BBL Becton Dickenson Co.) according to rule ISO/TC 34/SC 6/WG 15, 3 E 5 (1984).

Statistical analysis

Populations of total mesophilic, thermophilic and coliform bacteria were converted to log cfu/g and subjected to statistical analyses by a linear model to test for relevant effects of processing method after accounting for province, retail outlet type and muscle effects. Binomial responses of the qualitative assays for pathogens and LAB were fitted with an analogous linear logistic model, bootstrapping coefficients to obtain confidence intervals for the expected probabilities of positives. Inter-method agreement was assessed by Pearson correlation coefficient for continuous responses and by the kappa statistic (COHEN, 1960) for binomial ones; the overall quality was assessed using the two methods according to the respective reference values. Graphical and numerical analyses were performed with the open source R environment (R CORE TEAM, 2005), using functions of several packages: lattice (SARKAR, 2005) and Hmisc (HARREL *et al.*, 2005) for graphics; MASS (VENABLES and RIPLEY, 2002) for model fitting; effects (FOX., 2005) for model effects display and Zelig (IMAI *et al.*, 2005) for estimating expected probabilities.

RESULTS

Bacterial counts

The sample distribution of TMC ranged from 2.2 to 4.7 log cfu/g with the swab method and from 2.5 to 5.4 log cfu/g with the disruptive method. Corresponding ranges were 0.09-4.7 vs 0.21-5.6 log cfu/g for thermophilic bacteria and 1.30-4.30 vs 2.10-4.11 log cfu/g for coliforms (Fig. 1). Samples from the Latina province had higher loads of coliforms and thermophilic bacteria, and samples from the Rome province had higher loads of mesophilic bacteria. Supermarket samples generally showed higher coliform and thermophilic counts, except for two samples (one from Latina, the other from Viterbo), while butcher shop samples tended to have higher levels of coliforms.

The linear model fits for TMC, thermophilic and coliform bacteria were rather poor, with large residuals due to variation among outlets within the retail type and province and among sampling visits. About one-fifth of the variance was accounted for by the significant factors for thermophilic bacteria and coliforms, and about one-third for mesophilic bacteria. However, sample processing method was significant for all groups, along with province and retail type. With the disruptive method 2.5 times more (0.4 log cfu/g) mesophilic and coliform bacteria and about 3 times more (0.5 log cfu/g) thermophilic bacteria were counted (Fig. 2). TMC were significantly higher for the province of Rome, particularly the supermarkets, while little difference was found between retail types and the provinces of Latina and Viterbo. Thermophilic counts were higher for the supermarkets and the Latina province; the coliform counts were higher in the supermarket samples, compared with the butcher shops, for Latina and were lower for Rome.

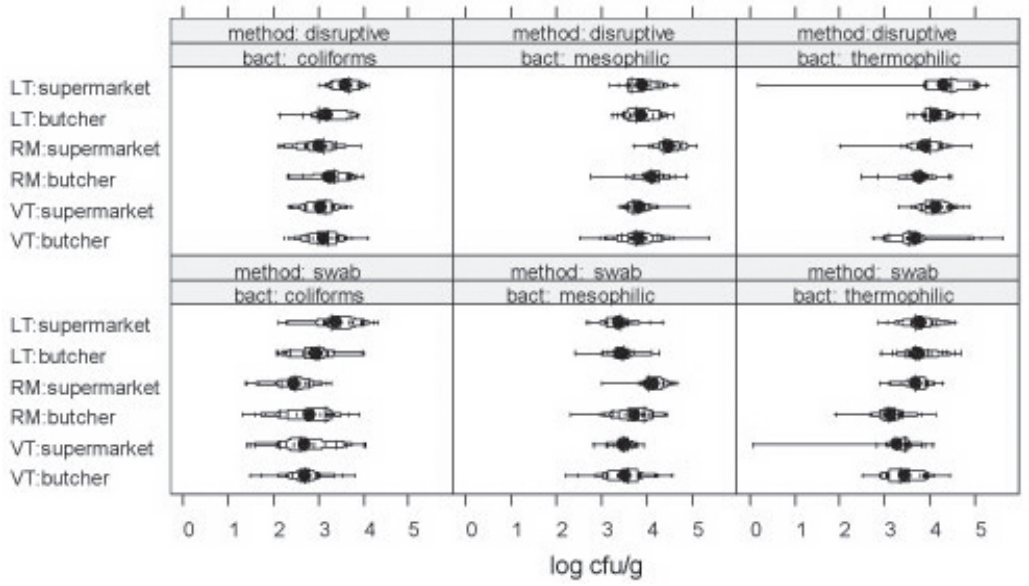


Fig. 1 - Box-percentile plots (ESTY and BANFIELD, 2003) at decile resolution of the distributions of thermophilic, mesophilic and coliform colony counts found on 108 samples of fresh pork meat, from a factorial combination of two muscle types (*L. thoracis* and masseter), three outlets for each of two types (supermarket vs butcher), in the provinces of Latina, Rome and Viterbo, three visits per outlet, partitioned by isolation method (swab vs disruptive), retail type and province (VT, RM and LT). Distributions are over muscle types, outlets within type and sampling visits.

Tests for pathogens and spoilage microorganisms

Microorganisms whose presence disqualifies fresh or refrigerated pork meat destined for catering were found in about half of the samples, and spoilage bacteria were found in about one fifth of the samples. The frequency of positive samples was significantly influenced by the isolation method for the pathogens *E. coli*, *Staphylococcus* spp. and *Campylobacter* spp. (Fig. 3), by method and province for LAB (*Carnobacterium* spp., *Leuconostoc* spp., *Lactobacillus* spp.) and the spoilage agents (*B. stearothermophilus* and *B. thermosphacta*), and by method for muscle type and for retail type interactions for *Salmonella* sp (Fig. 4).

With the disruptive method more samples resulted positive for *E. coli*, *Campylobacter*, *Staphylococcus* and *B. thermosphacta*; the odds of detecting positives increased by a factor between 2 and 5 (Fig. 3). In contrast, more samples tested positive for *B. stearothermophilus* and lactic acid bacteria with the swab method. Samples from Rome and Latina, compared with those from Viterbo, showed higher frequencies of positives for *B. thermosphacta* and LAB and a lower frequency of *B. stearothermophilus* positives.

Compared to the swab method, the disruptive method detected more *Salmonella* positives among the *L. thoracis* samples and fewer among the masseter ones, and many more among the samples from supermarkets compared with those from butcher shops (Fig. 4).

Method agreement

Method agreement was rather poor, for both continuous and binomial responses. The coefficient of linear correlation for log-counts between methods ranged

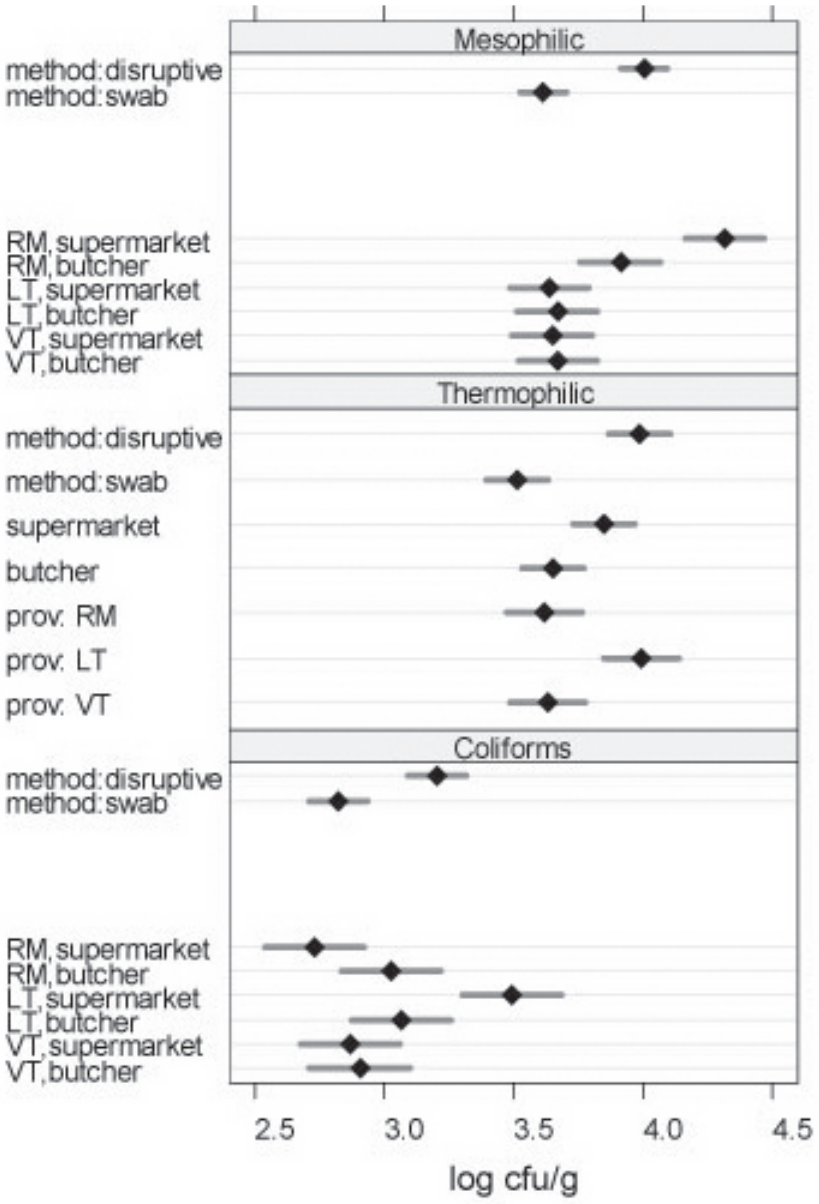


Fig. 2 - Significant effects of market province, retail outlet type and isolation method (swab and disruptive) on predicted colony counts (with 95% confidence intervals) of total mesophilic, thermophilic and coliform bacteria found on 108 samples of fresh pork meat, from a factorial combination of two muscle types (*L. thoracis* and masseter), three outlets for each of two types (supermarket and butcher), in the provinces of Latina, Rome and Viterbo, three visits per outlet.

from 0.58 (TMC) to 0.68 (thermophilic bacteria). Inter-method agreement for each binomial response was tested with the kappa statistic (COHEN, 1960), a ratio of the proportion of observed agreements to total observations, both adjusted for the proportion of agreements expected by chance, on a scale from zero (no more than chance agreement) to one (perfect agreement), and negative values indicating less than chance agreement. This latter case was observed for the *Campylobacter* and *B. thermosphacta* tests, chance agreement for *E. coli* and *Salmonella*, minimal agreement for LAB and moderate agreement for *B. stearo-*

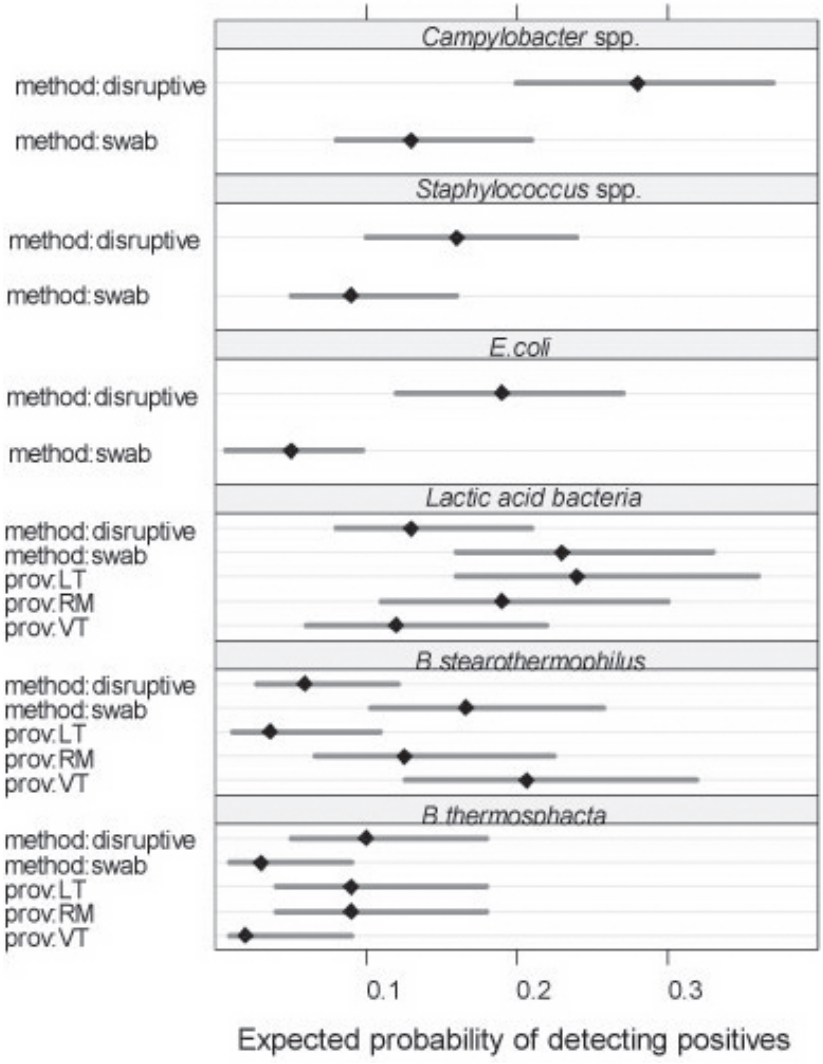


Fig. 3 - Significant effects of market province, retail outlet type and isolation method (swab and disruptive) on the expected probability (with 95% confidence interval) of detecting some pathogenic and lactic acid bacteria on 108 samples of fresh pork meat, from a factorial combination of two muscle types (*L. thoracis* and masseter), three outlets for each of two types (supermarket and butcher), in the provinces of Latina, Rome and Viterbo, three visits per outlet.

thermophilus (Table 1). The overall rate of agreement for all the tests amounted to little more than chance.

Quality assessment

Results of the microbial counts and detection tests gave a representative idea of meat contamination from the point of view of an integrated microbial analysis, (OTTOGALLI, 2004). Reported values have to be compared with some suitable reference values for hygiene assessment. Two existing sets of reference values were used, established for two different stages of the overall process of raw material handling, from slaughtering to the final consumer.

The EC Decision 2001/471/CE of 8 June 2001 provides reference values for the assessment of general hygiene carried out following Directive 64/433/

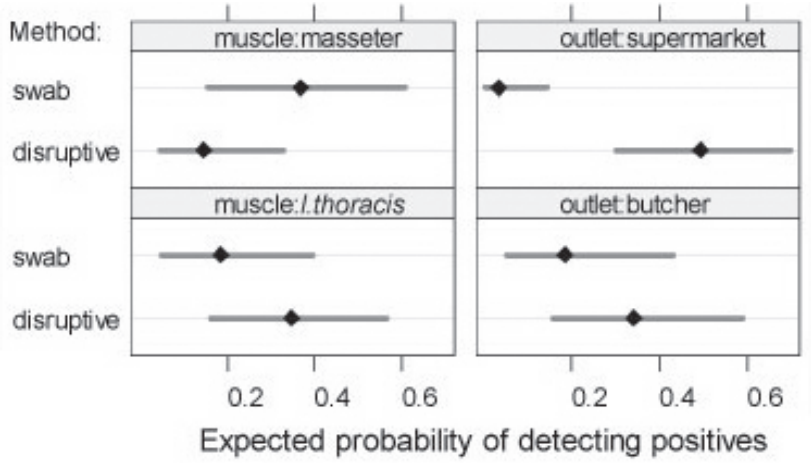


Fig. 4 - Effects of isolation method (swab and disruptive), retail outlet type and muscle type on the expected probability (with 95% confidence interval) of detecting *Salmonella* sp. on 108 samples of fresh pork meat, from a factorial combination of two muscle types (*L. thoracis* and masseter), three outlets for each of two types (supermarket and butcher), in the provinces of Latina, Rome and Viterbo, three visits per outlet.

EEC on health conditions for the production and marketing of fresh meat and Directive 71/118/EEC on health problems related specifically to fresh poultry meat (ANONYMOUS, 2001), to apply to carcasses or half carcasses at the slaughterhouse, in the earlier stages of handling. The two sampling methods were assigned different reference values for calibration. The rates of their respective assessment of the meat samples showed little agreement for the TMC criterion but were in slightly better agreement for the Enterobacteria criterion, according to the kappa statistic (Table 2). The rating obtained with the referenced swab method was more severe for both criteria, accepting less than one-fourth of the samples according to the TMC criterion and none according to the Enterobacteria criterion, compared to half and none for the corresponding ratings with the disruptive method. Moreover, 11% vs 2% of samples were rated as unacceptable on account of TMC and 73% vs 61% on account of Enterobacteria using the swab vs the disruptive method.

Table 1 - Pearson correlation coefficient and kappa statistic estimates of inter-method agreement for continuous and nominal responses, respectively.

Microorganisms	Pearson correlation	Cohen's kappa
Mesophilic	0.58	
Thermophilic	0.68	
Coliform	0.60	
E. coli		0.01
Campylobacter spp.		-0.12
Salmonella spp.		0.08
Staphylococcus spp. cps		0.20
Bacillus stearothermophilus		0.42
Brochotrix. thermosphacta		-0.06
Lactic acid bacteria		0.13
All qualitative tests		0.05

Using the reference values, lower down in the raw material handling process, specified by the Italian Zooprophylactic Service regarding the suitability of meat for public catering (TONUCCI *et al.*, 2005), no samples were rated as unacceptable on account of TMC, a very low proportion (6%) on account of Enterobacteria and 19% did not meet the *Salmonella* absence criterion.

DISCUSSION

In the meat industry, microbiological criteria are fundamental for assessing the quality, safety and hygienic compliance of raw and processed products, as well as processing procedures and environments. For identifying pathogenic and toxic-in-

Table 2 - Microbiological quality assessment by two isolation methods (swab and disruptive), compared with current EC and Italian criteria, on 108 samples of fresh pork meat, from a factorial combination of two muscle types (*L. thoracis* and masseter), three outlets for each of two types (supermarket and butcher), in the provinces of Latina, Rome and Viterbo, three visits per outlet.

	Disruptive method			Swab method		
	Reference	samples		Reference	samples	
Microorganisms and criteria	value	N.	%	value	N.	%
Total Mesophilic Count (TMC)						
ref. EC Decision 2001/471						
acceptable	<4 log cfu/g	51	47.2	<3.3 log cfu/g	25	23.1
marginal	4-5 log cfu/g	55	50.9	3.3-4.3 log cfu/g	71	65.7
not acceptable	>5 log cfu/g	2	1.9	>4.3 log cfu/g	12	11.1
Kappa coefficient of agreement	*0.15*					
ref. It. Zooprophylactic Service						
acceptable	<7 log cfu/g	108	100.0			
Enterobacteria (coliforms)						
ref. EC Decision 2001/471						
acceptable	<2 log cfu/g	0	0.0	<1.3 log cfu/g	0	0.0
marginal	2-3 log cfu/g	42	38.9	1.3-2.3 log cfu/g	29	26.9
not acceptable	>3 log cfu/g	66	61.1	>2.3 log cfu/g	79	73.1
Kappa coefficient of agreement	*0.40*					
ref. It. Zooprophylactic Service						
acceptable	<4 log cfu/g	102	94.4			
***Salmonella* spp.**						
ref. It. Zooprophylactic Service						
acceptable	absent in 25 g	87	80.6			

fective or spoilage bacteria, plate count methods are limited in terms of time and specificity. Serological methods also have problems, for example, loss of antigenicity in *E. coli* O157 strains in different survival conditions can invalidate the immunological method for detecting the verotoxic strain (HARA-KUDO *et al.*, 2000).

To monitor the microbiological quality of highly perishable products such as fresh meat, rapid identification and characterization methods are needed, so that unacceptable products can be promptly withdrawn from the market (HOCHKOEPPLER, 2004; MANFREDA, 2004). If the shelf-life assigned to refrigerated minced meat is five days, as in Italy, the search for some pathogens by traditional ordinary plate count methods might only give results only at the end of product's shelf life. However, quantitative determination of spoilage microflora and tests for pathogens are significant elements in the official controls, and should also be taken into consideration when drafting autotesting plans. The evolution of the European juridical framework has motivated the establishment of testing laboratories within production facilities. In practice, however, labour and equipment are often preferably assigned to other analytical tasks that have greater business relevance. Assessment of pathogen presence (*Salmonella spp., Listeria monocytogenes, Campylobacter, Yersinia enterocolitica*) and risk evaluation for toxins (*Staphylococcus aureus, Bacillus cereus, Clostridium perfringens*) should be based on molecular diagnostics.

The contamination levels detected by the disruptive method indicated the presence of gastrointestinal microorgan-

isms released in muscle cavities by diapedesis.

Besides the differences between the microbial isolation methods, the differences found among provinces do not allow a clear ranking in terms of overall microbiological meat quality; depending on the variables measured, the sample source had no effect, or the effect seemed randomly distributed.

Because the meat origin was unknown (foreign, Italian D.O.P. or non-D.O.P.), most of the total variation was residual, due to the fluctuating contributions from the particular retail outlets, sampling occasion and muscle type, that were confounded with effects of different regulations in force in all the rearing facilities. The overall results showed a rather low level of quality for the fresh pork meat marketed in these provinces: the large number of borderline and beyond limit samples indicates the need for a thorough review of the current monitoring and control procedures.

Assessment rates determined by the two isolation methods, upon calibration with the EU reference values, which give greater weight to swab results, were not well correlated between the two and were even less correlated with the rates resulting from reference values given by the Zooprophylactic Service. This is a cause for concern. A reliable methodology for official microbial evaluation is needed as well as a tool for implementing the legally mandated HACCP system at the production and processing levels.

The molecular identification and characterisation of microbial species reported in this paper are in progress.

ACKNOWLEDGEMENTS

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PAPER

DETERMINATION OF THE POLYPHENOLIC CONTENT IN SICILIAN RED WINES OF PROTECTED GEOGRAPHICAL INDICATION

DETERMINAZIONE DEL CONTENUTO FENOLICO IN VINI ROSSI SICILIANI A MARCHIO DI INDICAZIONE GEOGRAFICA TIPICA

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ABSTRACT

Thirty-one red wines of "Protected Geographical Indication", including most of the wine cultivars grown in Sicily, were assayed for their phenolic content. Individual phenolic compounds were identified and quantified by HPLC with a photo-diode array (PDA) coupled on-line with a MS system. The total phenolic content was evaluated by the Folin-Ciocalteau method and the antioxidant capacity was measured by the DMPD (N,N-dimethyl-*p*-phenylendiamine) method. The results provide

RIASSUNTO

Lo scopo di questo studio è stato quello di determinare la composizione qualitativa e quantitativa in polifenoli in vini Siciliani a marchio IGT, peculiari della produzione vitivinicola dell'isola. Ciascun composto fenolico è stato identificato e quantificato utilizzando un sistema HPLC-DAD interfacciato on-line con uno spettrometro di massa. È stato valutato, inoltre, il contenuto in polifenoli totali con il metodo di Foling-Ciocalteau mentre l'attività antiossidante è stata misurata con

- Keywords: Antioxidant capacity, commercial wines, HPLC/MS, polyphenols -

new insight into the polyphenolic composition of commercial Sicilian wines, and highlight some interesting points about the different wine typologies. In particular, the Merlot wines produced in Sicily, similar to those produced by other allochthonous grapes, generally have a higher polyphenolic content. Based on the data given, Merlot wines appear to be an important source of dietary polyphenolic antioxidants. It may be supposed that wines produced from this grape typology in Sicily are endowed with properties that have potential health benefits.

la DMPD (N,N-dimetile-*p*-fenilendiammina). Lo studio ha consentito la raccolta di una prima serie d'informazioni sul profilo polifenolico dei vini commerciali siciliani, differenziando, così, le diverse tipologie. In particolare è emerso che i vini da cv. Merlot, prodotti in Sicilia, così come quelli prodotti da altre uve di cv. alloctone, mostrano un contenuto dei singoli polifenoli mediamente più alto, il che fa supporre che da questa tipologia di uve si possano ottenere, in Sicilia, vini dotati di buone proprietà salutistiche.

INTRODUCTION

Polyphenolic compounds are ubiquitous in foods of plant origin, and are an integral part of the human diet. Interest in polyphenols has increased greatly, because it has been reported that they suppress the rate of degenerative processes such as cardiovascular disorders and cancer (BRAVO, 1998; DUTHIE *et al.*, 2000). Wine is an excellent source of various classes of polyphenols, including benzoic and cinnamic acid derivatives, flavan-3-ols, flavonols, stilbenes and anthocyanins.

The phenolic compounds are derived from the skin, seeds, stems and pulp of the grape which are transferred to the wine during the first stage of wine fermentation when they are kept with the grape juice. The concentrations of these compounds may depend on a number of factors such as grape variety, climatic conditions, ripening stage of the grapes, agronomic practices and wine-making technology, the year and the length of time of the wine storage (KLAUTSKY and ARMSTRONG, 1993).

Different chromatographic methods are used to identify the phenolic compounds in wine. Since the UV-Vis spectra of the different groups (flavonoids, non-flavonoids and their derivatives) are very similar, their identification is often difficult. The HPLC-MS technique is a rapid method to obtain structural information about phenolic compounds that have similar UV-Vis spectra or that co-elute without any sample pre-treatment.

In this study, the phenolic composition of commercial Sicilian wines of "Protected Geographical Indication" was investigated by HPLC-MS, using an ESI probe. To further elucidate the effect of wine consumption, and to aid epidemiological studies, the antioxidant properties were determined and the polyphenol content of the wines was measured.

MATERIALS AND METHODS

Chemicals

Acetonitrile and H_2O (solvent HPLC grade) were purchased from Carlo Erba (Milano, Italy).

Formic acid, (-)-epicatechin, (+)-catechin, gallic acid, 3,4-dihydroxybenzoic acid (protocatechuic acid), 4-hydroxy-

3-methoxybenzoic acid (vanillic acid), 4-hydroxy-3,5-dimethoxybenzoic acid (syringic acid), 3,4-dihydroxycinnamic acid (caffeic acid), 4-hydroxy-3-methoxycinnamic acid (ferulic acid), 4-hydroxycinnamic acid (*p*-coumaric acid), tyrosol (2-(4-hydroxyphenyl) ethylalcohol), *trans*-resveratrol and Folin-Ciocalteau reagent were purchased from Sigma-Aldrich (Milano, Italy).

The other phenolic compounds, procyanidin B1, procyanidin B2, ethylgallate, quercetin, isoquercitrin (quercetin-3-O-glucoside), kaempferol, kaempferol 3-O-glucoside, rhamnetin, isorhamnetin, isorhamnetin 3-O-glucoside, rutin (quercetin-3-O-rutinoside), myricetin and malvidin-3-glucoside were purchased from Extrasynthese (Genay, France).

Stock solutions of the individual standards were prepared by dissolving 100 mg of standard in aqueous formic acid (pH=3)/methanol (90:10). All the solutions were stored in the dark at -4°C. The stock solution of *cis*-resveratrol was produced by UV irradiation of *trans*-resveratrol in methanol for 120 min at 366 nm (TRELA and WATERHOUSE, 1996). A five-point calibration curve for the quantitative analysis of *cis*-resveratrol was obtained as described by ROMERO-PÉREZ *et al.* (1996).

All the solutions were filtered through a 0.45 μm glass-microfiber GMF Whatman chromatographic filter (Aldrich, Milano, Italy) before HPLC analysis and mobile phase solvents were degassed before use.

Wine samples

All the wines analyzed were of "Protected Geographical Indication". The samples included most of the wine cultivars that are commercially available and widely consumed in Sicily. All wines were stored in the dark at 4°C, and each one was opened just prior to analysis. The analyses were carried out on samples without any prior purification; an aliquot was filtered through a 0.45 μm glass-microfiber GMF Whatman chromatographic filter, prior to HPLC analysis.

Total phenol content

The total phenol content was analyzed using the Folin-Ciocalteau method (SINGLETON and RED, 1965) and the results are expressed as gallic acid equivalents (GAE).

Antioxidant capacity

The antioxidant capacity of the wine samples was determined according to the method of FOGLIANO *et al.* (1999). The assay is based on the use of a N,N-dimethyl-*p*-phenylenediamine (DMPD) solution, $FeCl_3$ and Trolox [(S)-6-methoxy-2,5,7,8-tetramethylchromane-2-carboxylic acid]. The results are expressed as Trolox antioxidant equivalent capacity (TAEC).

Analysis of anthocyanin fraction in red wines

The individual anthocyanins in the wines were determined by HPLC-MS, by using a direct-injection chromatographic method (DUGO *et al.*, 2004). The red wine samples were filtered through 0.45 μm filters and diluted with water (1:10 v/v). The peaks were identified by micro HPLC-ESI-MS analysis using a Shimadzu HPLC system (Milano, Italy) equipped with two HPLC pumps (LC-10AD), a UV-Vis detector (SPD-10A), a degasser (DGU-14A) and a controller SCL-10A. The HPLC system was coupled on line with a Shimadzu QP-8000 API mass spectrometer. UV and MS data were acquired and processed using the Class 8000 software.

The flow rate was reduced by placing an accurate flow splitter (LC Packings) between the pump and the injector. A U-Z view™ capillary flow cell, 35 nL, 8 mm path length (LC Packings) was fitted with the Shimadzu SPD-10A detector.

The column used was a Waters Symmetry C18, 150x1 mm i.d., 3.5 μm particle diameter. Flow rate: 40 μL/min; injection volume: 0.64 μL. Injection was performed using a Rheodyne injector model 7725i. High pressure binary gradient using solvent A (H_2O:HCOOH 9:1) and solvent B (H_2O:HCOOH:CH_3CN, 4:1:5). 0-2 min: 12% B; 2-20 min: from 12 to 50% B; 20-25 min: from 50 to 90% B with a final hold of 5 min. UV-Vis detection: 518 nm. MS conditions: ESI in positive mode. Nebulizer gas (N_2) flow rate: 4.5 L/min. Probe voltage: 4.5 kV. Curved desolvation line (CDL) voltage -45V. Deflector voltages: 40 or 55 V. Since anthocyanins have similar spectroscopic properties and comparable molecular masses, the total anthocyanin content is expressed in terms of malvidin-3-glucoside, available as pure standard. A calibration curve was obtained by injecting different amounts of malvidin-3-glucoside standard solution and measuring the absorbance at 518 nm.

Analysis of other phenolics in red wines

The phenolics in the wine samples were identified using a direct-injection chromatographic method (LA TORRE *et al.*, 2006). The analyses were performed on a Shimadzu system (Milano, Italy) equipped with two pumps LC10-A, a controller SCL-10A, with a photodiode array detector SPD-M10Avp equipped with a semi micro-cell and operating at wavelengths between 200 and 600 nm. The system was coupled with a MS detector Shimadzu 2010 with an ESI interface. The compounds were then separated through a 150x2.1 mm i.d., 5 μm particle size, Supelco (Milano, Italy) Discovery C18 column; a Supelco guard column packed with the same stationary phase was also used. The mobile phase was a gradient consisting of water (pH=3 by formic acid, solvent A) and acetonitrile (pH=3 by formic acid, solvent B): 0.01-20.00 min 5% B isocratic; 20.01-50.00 min, 5-40% B; 50.01-55.00 min, 40-95% B; 55.01-60.00 min 95% B isocratic. The flow rate was 0.2 mL/min and the analyses were performed at 20°C. The samples were injected directly into the column using a Rheodyne (model 7725i) injection valve. ESI source and negative ionisation mode was used with different fragment voltages. Nitrogen was used as the nebulizing and drying gas.

The MS acquisition with the ESI interface was performed under the following conditions: probe high voltage, 4 kV; nebulizing gas (N_2) flow rate, 4.5 L/min; curved desolvation line (CDL) voltage, 10 V; CDL temperature, 250°C; acquisition mode, SCAN, 50-700 m/z and deflector voltage at -20 and -80 V. The SIM (selected ion monitoring) mode was used when there was a search for some particular ions.

RESULTS AND DISCUSSION

Anthocyanins

The qualitative analysis of the wine samples was based on the relative retention times of malvidin-3-glucoside and on MS spectra information. The quantitative analysis was carried out using a calibration curve prepared by injecting standard solutions of malvidin-3-glucoside at different concentrations.

The analysis was not carried out on all the commercial red wines studied. The wines were grouped according to grape variety. Tables 1-4 report the compounds identified and the quantitative data for all the red wines analysed are expressed as malvidin-3-glucoside. The analyses were carried out in triplicate, and the results are the average value. The coefficient of variation (CV%) of the three analyses was always less than 5%.

All of the wine samples contained the 3-glucoside derivatives of delphinidin, petunidin, peonidin, and malvidin. Cy-

Table 1 - Quantitative values of anthocyanins (mg/L) in commercial red wines of cv. Nero d'Avola.

	Corvo Rosso 2000	Cantine Montalto 2000	Cellaro cantine Cellaro 2000	Basileus cantine Greco 2001	Portale Duca di Salaparuta 2001	Morgante cantine Morgante 2000	Paolini cantine Paolini 2001	Zangara cantine Zangara 2001	Corvo Novello 2002
dp-3-glucoside	3.15	4.79	3.91	5.50	3.53	14.06	11.14	3.42	4.58
cy-3-glucoside	8.04	0.48	0.54	-	0.85	4.39	3.35	0.20	1.20
pt-3-glucoside	5.28	9.41	5.63	1.96	6.03	13.35	12.77	5.16	7.90
pn-3-glucoside	3.35	8.77	7.40	2.96	3.28	6.11	5.42	4.96	3.91
mv-3-glucoside	41.82	58.86	55.99	63.44	54.86	19.13	30.22	56.63	54.94
dp-3-(6"-acetyl)-glucoside	1.46	2.46	3.13	8.18	0.64	5.28	4.13	3.61	1.92
cy-3-(6"-acetyl)-glucoside	6.43	0.54	1.17	-	0.35	-	4.71	1.93	1.47
pt-3-(6"-acetyl)-glucoside	8.21	0.55	1.03	0.81	6.98	6.87	4.72	1.47	1.16
pn-3-(6"-acetyl)-glucoside	0.70	0.78	1.22	0.76	0.57	5.30	3.49	1.43	0.48
mv-3-(6"-acetyl)-glucoside	7.69	5.06	7.25	7.17	9.32	6.50	7.08	15.31	9.88
mv-3-(6"-caffeoyl)-glucoside	0.52	0.84	1.32	1.43	0.40	2.68	1.57	0.16	0.38
pt-3-(6"-coumaroyl)-glucoside	0.27	0.81	0.66	1.43	0.56	2.54	2.68	0.21	0.58
pn-3-(6"-coumaroyl)-glucoside	0.02	0.80	0.25	1.53	0.43	2.04	1.40	0.39	0.92
mv-3-(6"-coumaroyl)-glucoside	13.06	5.87	10.52	4.83	12.22	11.77	7.32	5.13	10.67

With dp = delphinidin, cy = cyanidin, pt = petunidin, pn = peonidin, mv = malvidin.

anidin-3-glucoside was only found in a few samples. In particular, low or nothing quantities of cyanidin-3-glucoside were found in Basileus, Zangara 2001 and Cabernet Zangara wines, while a high cyanidin-3-glucoside level was found in the Syrah Planeta wine sample (25.62 mg/L). The levels of malvidin-3-glucoside ranged from 19.13 mg/L (Morgante wine) to 220.60 mg/L (Syrah Fici

Table 2 - Quantitative values of anthocyanins (mg/L) in commercial red wines of cv. Merlot.

	Planeta 2000	Lithos 2001	Istituto Agrario 2001	Fici 2001	Fici 2002
dp-3-glucoside	6.70	5.99	3.22	3.45	3.55
cy-3-glucoside	4.76	1.32	0.33	2.63	2.68
pt-3-glucoside	12.65	6.07	6.45	6.69	6.07
pn-3-glucoside	6.57	5.40	6.23	6.11	5.93
mv-3-glucoside	21.54	37.90	56.21	35.07	38.02
dp-3-(6"-acetyl)-glucoside	6.90	2.32	6.63	3.71	4.60
cy-3-(6"-acetyl)-glucoside	7.99	2.87	4.13	3.89	2.98
pt-3-(6"-acetyl)-glucoside	5.72	2.17	3.26	4.51	3.55
pn-3-(6"-acetyl)-glucoside	3.34	1.47	1.73	3.05	3.01
mv-3-(6"-acetyl)-glucoside	7.25	12.08	4.98	12.56	12.03
mv-3-(6"-caffeoyl)-glucoside	2.03	0.98	0.69	1.85	1.99
pt-3-(6"-coumaroyl)-glucoside	1.87	1.45	0.77	1.78	1.88
pn-3-(6"-coumaroyl)-glucoside	1.37	2.65	0.43	3.68	3.49
mv-3-(6"-coumaroyl)-glucoside	11.31	16.33	4.96	11.02	11.13

With dp = delphinidin, cy = cyanidin, pt = petunidin, pn = peonidin, mv = malvidin.

Table 3 - Quantitative values of anthocyanins (mg/L) in commercial red wines of cv. Cabernet, Syrah and Petit-Verdot.

	Cabernet			Syrah		Petit-Verdot
	Planeta Burdese 2000	Fici 2002	Zangara 2001	Fici 2002	Planeta 2000	Fici 2002
dp-3-glucoside	1.18	8.54	2.43	10.82	19.54	2.07
cy-3-glucoside	7.53	6.43	-	10.39	25.62	1.99
pt-3-glucoside	1.58	20.55	3.51	28.71	35.80	5.50
pn-3-glucoside	1.96	13.91	3.46	18.70	24.04	3.58
mv-3-glucoside	22.38	156.60	61.50	220.60	74.20	42.28
dp-3-(6"-acetyl)-glucoside	3.35	17.07	3.09	14.99	21.75	2.87
cy-3-(6"-acetyl)-glucoside	0.18	16.20	-	23.83	31.71	4.57
pt-3-(6"-acetyl)-glucoside	7.40	18.04	1.97	20.14	20.50	3.86
pn-3-(6"-acetyl)-glucoside	0.70	7.74	1.66	15.66	12.89	3.00
mv-3-(6"-acetyl)-glucoside	6.88	67.40	19.95	98.05	40.22	18.79
mv-3-(6"-caffeoyl)-glucoside	0.69	4.31	0.10	7.17	8.63	1.37
pt-3-(6"-coumaroyl)-glucoside	0.15	8.73	0.32	13.81	9.98	2.65
pn-3-(6"-coumaroyl)-glucoside	0.25	4.42	0.28	-	7.91	-
mv-3-(6"-coumaroyl)-glucoside	22.66	23.39	6.94	38.85	47.43	7.45

With dp = delphinidin, cy = cyanidin, pt = petunidin, pn = peonidin, mv = malvidin.

Table 4 - Quantitative values of anthocyanins (mg/L) in commercial red wines of cv. in mixture.

	Terre di S. Maria	Planeta		Corvo	Crepuscolo
	Paliotto 2000	Cerasuolo di Vittoria 2001	La Segreta 2001	Megara 2000	Cantine Mozia-Garibaldi 2000
dp-3-glucoside	4.17	2.07	6.33	8.63	4.28
cy-3-glucoside	0.31	0.48	0.79	5.48	0.70
pt-3-glucoside	7.05	4.30	3.84	11.12	3.40
pn-3-glucoside	4.66	4.55	0.61	13.81	4.61
mv-3-glucoside	58.43	56.28	58.60	49.51	56.97
dp-3-(6"-acetyl)-glucoside	6.05	2.52	2.50	4.21	18.49
cy-3-(6"-acetyl)-glucoside	2.26	1.87	1.08	3.86	7.44
pt-3-(6"-acetyl)-glucoside	1.68	1.21	0.83	3.47	-
pn-3-(6"-acetyl)-glucoside	1.84	1.28	0.55	-	-
mv-3-(6"-acetyl)-glucoside	5.27	14.81	15.88	-	1.08
mv-3-(6"-caffeoyl)-glucoside	0.83	0.68	0.39	-	-
pt-3-(6"-coumaroyl)-glucoside	-	0.47	0.37	-	-
pn-3-(6"-coumaroyl)-glucoside	-	0.53	0.18	-	-
mv-3-(6"-coumaroyl)-glucoside	7.43	8.95	8.04	-	11.02

With dp = delphinidin, cy = cyanidin, pt = petunidin, pn = peonidin, mv = malvidin.

wine). All of the samples had high levels of petunidin and peonidin 3-glucosyl derivatives, with the Petit Verdot (Fici) wine samples showing the highest concentrations of these two components.

The delphinidin-3-glucoside concentration values were between those of petunidin-3-glucoside and peonidin-3-glucoside for almost all the samples except Basileus, Nero d'Avola (Morgante) and La Segreta (Planeta) samples, that had much higher concentrations of delphinidin-3-glucoside.

The acetyl derivatives of anthocyanidins were detected, and malvidin-3-(6"-acetyl)-glucoside was found in all the samples. The delfinidin-3-(6"-acetyl)-glucoside content (average value 6.35 mg/L) was higher than that of petunidin-3-(6"-acetyl)-glucoside, except for Corvo Rosso 2000, Nero d'Avola (Paolini) and Nero d'Avola (Morgante), Portale 2001, Corvo Novello 2001, Merlot Fici, Cabernet-Sauvignon Fici, Syrah Fici and Petit-Verdot Fici wine samples. The cyanidin-3-(6"-acetyl)-glucoside content was also higher than that of petunidin-3-(6"-acetyl)-glucoside, particularly in the Syrah Planeta sample (31.71 mg/L). The highest level of peonidin-3-(6"acetyl)-glucoside was found in the Syrah Fici wine sample (15.66 mg/L).

Malvidin-3-(6"-caffeoyl)-glucoside (mean value 2.40 mg/L) was detected in all the wines except three: Nero d'Avola (Cellaro), Crepuscolo and Petit-Verdot Fici; the highest value was found in Syrah Planeta wine (8.63 mg/L).

As regards the coumaroyl derivatives, malvidin-, petunidin- and peonidin- glucosides acylated with coumaric acid were found. The malvidin derivative was the most abundant coumaroyl compound in all the wine samples. The highest concentration was found in the Syrah Planeta (Fici) sample (47.43 mg/L). The coumaroyl derivatives of petunidin and peonidin were detected in all the samples; their concentration ranged from 2 to 3 mg/L.

Phenolics

The qualitative analysis of the phenolics in the wine samples was based on the relative retention times and the MS spectral information of the individual standards. Quantitative analysis was carried out using a calibration curve prepared by injecting four standard solutions, of different dilutions. These standard solutions, obtained from stock solutions, were injected for the linearity range and detection limit tests. The analyses were carried out in triplicate, and the results are the average value. The coefficient of variation (CV%) of the three analyses was always less than 5%.

To analyse the total resveratrol in wine, an enzymatic hydrolysis method was used (LA TORRE *et al.*, 2004). The wine resveratrol-O-glycosides (*trans*- and *cis*-piceid) were completely hydrolysed in about 9 hours after incubation with β-glucosidase at 50°C; the *trans*- and *cis*-aglycones were then measured by high-performance liquid chromatography/mass spectrometry.

Tables 5-8 show the concentrations of single polyphenolic compounds in the different wine samples, grouped according to grape variety. The data show that, on the basis of the grape variety, the qualitative composition of all the samples is quite similar, while quantitative differences are notable.

Gallic acid (mean value 65.46 mg/L) was the most abundant phenolic compound found in all the commercial wines. The procyanidin B1 level ranged from 8.06 mg/L (Syrah Planeta wine) to 127.50 mg/L (Merlot Fici 2002). All the samples had high levels of (-)-epicatechin (mean value 39.30 mg/L) and (+)-catechin (mean value 26.28 mg/L). The Merlot Fici 2002 wine sample had the highest concentration of (+)-catechin and the Petit Verdot Fici wine sample had the highest concentration of (-)-epicatechin. All the samples contained tyrosol in concentrations ranging from 2.65

Table 5 - Quantitative values of phenols (mg/L) in commercial red wines of cv. Nero d'Avola.

	Santa Cecilia Planeta 2000	Corvo Rosso 2000	Cantina Montalto 2000	Cellaro Cantine Cellaro 2000	Duca Farese Bucca 2000	Basileus Cantine Greco 2001	Porale Duca di Salaparuta 2001	Morgante Cantina Morgante 2001	Paolini Cantine Paolini 2001	Zangara Cantine Zangara 2001	Corvo Novello 2002
Gallic Acid	54.80	64.85	75.29	75.89	100.65	28.34	47.83	70.60	63.45	63.53	46.63
Protocatechuic Acid	2.80	1.67	2.67	1.64	0.90	1.90	1.19	0.85	3.19	2.48	2.60
Tyrosol	4.01	26.96	24.89	5.37	39.05	15.09	30.01	33.69	64.79	31.67	37.07
Vanillic Acid	6.72	8.50	7.12	6.06	11.19	8.10	6.26	10.51	6.83	7.52	7.99
Syringic Acid	4.59	4.80	4.13	3.62	4.65	4.11	4.07	5.08	4.86	7.46	4.17
Caffeic Acid	4.49	4.97	3.04	4.22	3.16	3.69	4.25	2.25	3.07	8.55	24.42
Ferulic Acid	1.96	1.11	0.52	0.13	0.52	0.14	0.70	0.47	1.09	0.85	1.38
p-Coumaric Acid	0.22	1.12	0.56	0.32	0.18	0.46	1.73	0.29	0.39	1.15	0.21
Procyanidin B 1	17.68	45.36	42.61	38.95	16.88	37.48	56.81	37.89	60.20	61.30	59.68
Procyanidin B 2	10.83	20.40	13.23	10.76	4.66	20.41	30.72	8.71	37.65	31.66	13.83
(+)-Catechin	19.56	24.68	21.44	32.37	17.72	22.53	29.24	26.53	24.16	41.87	38.63
(-)-Epicatechin	28.97	36.20	30.52	32.06	23.20	32.30	35.95	35.33	37.01	33.21	25.95
Ethyl gallate	11.71	15.17	17.37	17.54	18.84	6.37	12.58	16.08	13.78	13.48	7.27
Rutin	27.25	1.86	13.25	3.60	5.90	3.68	13.42	10.22	21.53	16.26	18.84
Isoquercitrin	15.76	14.71	23.58	22.11	9.06	11.96	14.75	17.54	21.32	21.63	25.59
Isorhamnetin-3-O-glucoside	13.32	2.09	4.66	0.61	0.96	3.12	1.60	0.98	0.88	0.96	2.71
Kaempferol-3-O-glucoside	44.86	27.63	5.58	1.22	3.94	5.70	4.07	6.88	5.96	7.35	2.59
Myricetin	6.18	15.26	8.29	10.09	6.11	24.37	11.94	12.62	4.72	2.86	8.02
Quercetin	7.43	12.65	12.39	11.92	6.87	11.08	11.82	11.64	6.42	3.54	12.02
Kaempferol	0.32	0.39	0.41	0.37	0.22	0.35	0.33	0.57	0.39	0.14	0.38
Isorhamnetin	0.17	0.47	0.32	0.32	0.23	0.38	0.35	0.41	0.14	0.16	0.33
Rhamnetin	-	-	-	-	-	0.08	0.08	0.08	-	-	-
trans-Resveratrol	0.12	0.48	0.30	0.51	0.23	0.19	0.43	0.26	0.40	0.33	0.60
cis-Resveratrol	0.04	0.21	0.24	0.05	0.11	0.08	0.11	0.07	0.14	0.09	0.09
trans-Piceid	0.41	2.16	1.50	1.27	1.43	0.25	1.43	1.57	2.15	1.51	0.90
cis-Piceid	0.05	0.27	0.25	0.43	0.33	0.17	0.23	0.34	0.25	0.37	0.21

Table 6 - Quantitative values of phenols (mg/L) in commercial red wines of cv. Merlot.

	Planeta 2000	Lithos 2001	Istituto Agrario 2001	Fici 2001	Fici 2002
Gallic Acid	79.15	101.16	58.13	84.86	107.83
Protocatechuic Acid	3.98	2.47	1.12	3.28	4.05
Tyrosol	25.51	23.30	33.03	40.33	43.77
Vanillic Acid	4.60	8.00	6.07	6.57	4.96
Syringic Acid	9.90	3.71	6.03	4.32	6.31
Caffeic Acid	7.71	4.83	2.64	7.66	8.61
Ferulic Acid	0.39	0.69	0.08	1.11	0.18
p-Coumaric Acid	1.25	0.78	0.34	2.78	0.56
Procyanidin B 1	60.04	69.80	39.54	87.49	127.50
Procyanidin B 2	44.57	29.23	24.90	42.48	80.98
(+)-Catechin	64.04	43.13	14.24	62.14	78.67
(-)-Epicatechin	49.02	69.27	34.74	99.85	107.08
Ethyl gallate	16.75	31.83	17.53	16.54	16.76
Rutin	8.50	29.28	15.65	6.23	4.78
Isoquercitrin	58.44	50.44	17.26	21.15	23.21
Isorhamnetin-3-O-glucoside	2.04	3.22	1.24	2.34	1.60
Kaempferol-3-O-glucoside	6.89	11.96	12.46	4.76	9.14
Myricetin	6.74	1.91	8.91	8.43	11.87
Quercetin	11.05	3.19	13.37	13.14	16.70
Kaempferol	0.44	0.17	0.54	0.33	0.45
Isorhamnetin	0.27	0.10	0.52	0.53	0.90
Rhamnetin	0.13	-	-	-	0.22
trans-Resveratrol	0.61	0.62	1.29	1.88	2.44
cis-Resveratrol	0.09	0.11	0.27	1.45	1.81
trans-Piceid	1.48	2.20	2.35	2.95	3.70
cis-Piceid	0.22	0.73	0.31	1.53	1.97

mg/L (La Secreta Planeta) to 75.66 mg/L (Petit Verdot Fici). The procyanidin B2 concentrations were lower than those of procyanidin B1 for almost all the samples, except those of Syrah Planeta and La Secreta Planeta that had higher concentrations of procyanidin B2.

Regarding the cinnamic acids, vanillic acid was the major compound (mean value 7.13 mg/L), followed by ferulic acid (mean value 0.70 mg/L) and *p*-coumaric acid (mean value 0.83 mg/L). All the samples had high levels of ethyl gallate, with the Merlot Lithos wine sample having the highest.

All the analysed wines contained rutin, isoquercitrin, isorhamnetin-3-O-glucoside, kaempferol-3-O-glucoside, myricetin, quercetin and kaempferol. On the basis of grape variety, quantitative differences were detectable. Isoquercitrin was the main component, followed by rutin, myricetin and quercetin that often showed comparable values. In addition, the glucosidic derivatives of isorhamnetin and kaempferol were a little less abundant, with the exception of the Santa Cecilia Planeta and Cabernet Burdese Planeta samples that had much higher concentrations. The concentrations of kaempferol and isorhamnetin were very low and were not detected in the Vallovin Edonè 1999 wine samples. The concentration of rhamnetin never exceeded 0.22 mg/L and was absent in some samples.

The mean concentrations of *trans*- and *cis*-resveratrol were 0.42 and 0.14 mg/L,

Table 7 - Quantitative values of phenols (mg/L) in commercial red wines of cv. Cabernet, Syrah and Petit-Verdot.

	Cabernet Burdese Planeta 2000	Cabernet Fici 2002	Cabernet Zangara 2001	Syrah Fici 2002	Syrah Planeta 2000	Petit-Verdot Fici 2002
Gallic Acid	72.66	101.70	62.72	39.07	64.35	106.66
Protocatechuic Acid	2.57	1.23	1.48	1.22	2.07	2.18
Tyrosol	64.08	49.20	40.20	23.31	3.10	75.66
Vanillic Acid	5.89	9.02	6.50	5.38	9.55	6.22
Syringic Acid	4.53	5.52	7.35	4.53	3.76	5.64
Caffeic Acid	4.26	8.34	19.65	6.51	6.12	7.48
Ferulic Acid	0.04	1.05	1.06	1.02	0.73	1.34
p-Coumaric Acid	0.30	2.42	1.67	1.52	0.29	2.75
Procyanidin B 1	55.67	102.38	77.95	42.67	8.06	73.42
Procyanidin B 2	36.64	58.61	36.38	21.26	17.54	57.58
(+)-Catechin	46.91	99.00	5.45	21.07	35.27	67.60
(-)-Epicatechin	72.46	136.01	52.46	31.65	46.23	109.97
Ethyl gallate	22.25	16.86	20.49	5.45	13.66	17.27
Rutin	29.75	8.52	22.17	5.06	5.93	6.24
Isoquercitrin	29.44	20.34	34.60	14.86	25.27	14.68
Isorhamnetin-3-O-glucoside	21.89	3.61	2.05	5.45	6.10	4.84
Kaempferol-3-O-glucoside	37.72	7.73	12.63	0.87	19.97	9.12
Myricetin	5.36	16.68	2.84	8.62	5.00	30.92
Quercetin	9.73	15.53	4.63	8.49	13.80	16.63
Kaempferol	0.48	0.36	0.21	0.13	0.39	0.35
Isorhamnetin	0.30	0.52	0.15	0.78	0.55	0.56
Rhamnetin	0.11	-	-	0.11		-
trans-Resveratrol	0.10	0.45	0.27	0.23	0.15	0.88
cis-Resveratrol	0.05	0.28	0.05	0.19	0.06	0.17
trans-Piceid	0.36	3.35	3.74	3.01	1.14	3.27
cis-Piceid	0.18	0.24	0.09	0.16	0.21	0.54

respectively. In these samples *cis*-resveratrol was present in a lower concentration, which is in accord with GOLDBERG *et al.* (1995) who observed that *cis*-resveratrol is usually present in lower concentrations than the *trans*-form. The two Merlot Fici wine samples had the highest levels of *cis*-resvesratrol (1.45 and 1.81 mg/L); the *trans*-resveratrol levels were between 0.08 and 2.44 mg/L. The highest *trans*-resveratrol levels were found in the Merlot Fici wine samples (2.44 and 1.88 mg/L), while the two Edonè Vallovin wine samples had the lowest values (0.08 and 0.10 mg/L).

Concerning *trans* and *cis*-piceid, the *cis*-piceid concentrations were usually lower than those of *trans*-piceid. On average, the amount of *trans*-piceid was greater than that of *trans*-resveratrol, ranging from 0.36 to 3.74 mg/L. The concentrations of *trans*-piceid and *trans*-resveratrol in Paneta La Secreta and Basileus (Cantine Greco) wines were very similar. Cabernet Zangara wine showed the highest concentration of *trans*-piceid (3.74 mg/L).

The *cis*-piceid levels ranged from 0.04 to 1.97 mg/L, and the concentration was usually greater than that of its aglycon (*cis*-resveratrol). The Merlot Fici 2002 wine sample showed the highest concentration of *cis*-piceid; the two Merlot Fici wine samples had the highest total-resveratrol content.

Table 8 - Quantitative values of phenols (mg/L) in commercial red wines of cv. in mixture.

	Terre di S. Maria Paliotto	Planeta		Corvo	Birgi	Cantine Mozia-Garibaldi		Vallovin	
		Cerasuolo di Vittoria	La Secreta	Megara	Birgi rosso	Borromeo rosso	Crepuscolo	Edonè	Edonè
	2000	2001	2001	2000	2000	2001	2000	1999	2000
Gallic Acid	63.36	48.49	47.02	76.49	31.74	67.50	79.37	38.61	29.38
Protocatechuic Acid	1.18	3.27	0.63	2.88	1.24	0.68	1.95	1.63	1.21
Tyrosol	38.96	21.83	2.65	28.23	4.49	17.11	26.36	36.58	16.90
Vanillic Acid	8.27	2.32	5.36	8.62	7.23	7.92	5.66	7.21	5.95
Syringic Acid	3.57	9.18	5.12	4.81	2.85	2.01	4.81	3.06	2.60
Caffeic Acid	5.64	11.24	12.50	5.10	2.89	2.79	2.99	1.82	1.69
Ferulic Acid	0.37	0.87	0.12	1.90	0.92	0.77	0.23	0.45	0.16
p-Coumaric Acid	0.51	1.69	0.38	1.17	0.36	0.45	0.62	0.15	0.09
Procyanidin B 1	37.55	31.58	8.80	51.96	16.75	35.40	45.41	13.78	10.20
Procyanidin B 2	13.54	28.37	12.21	26.47	3.97	15.33	23.21	5.09	4.81
(+)-Catechin	29.07	29.67	33.88	31.07	17.11	22.16	27.52	6.70	9.02
(-)-Epicatechin	39.58	29.64	43.99	43.67	29.52	33.48	36.60	14.63	13.02
Ethyl gallate	15.14	6.24	10.73	17.94	6.25	18.68	18.81	7.10	5.60
Rutin	4.25	2.02	3.49	2.40	14.83	20.75	5.15	8.72	5.70
Isoquercitrin	15.76	11.76	17.33	18.07	19.92	34.44	7.25	10.93	8.59
Isorhamnetin-3-O-glucoside	1.83	5.70	1.19	0.90	0.92	2.66	2.81	0.13	0.10
Kaempferol-3-O-glucoside	8.45	-	11.76	1.81	5.08	4.05	2.28	3.18	3.41
Myricetin	19.27	16.62	10.12	13.06	12.24	4.01	3.99	0.56	1.32
Quercetin	12.68	15.99	11.23	9.96	14.82	10.89	3.93	0.67	0.92
Kaempferol	0.36	0.20	0.30	0.17	0.74	0.52	0.15	-	0.06
Isorhamnetin	0.49	0.95	0.54	0.43	0.29	0.26	0.11	-	0.07
Rhamnetin	0.07	-	0.11	-	-	-	-	-	-
trans-Resveratrol	0.91	0.15	0.12	0.83	0.27	0.51	0.20	0.08	0.10
cis-Resveratrol	0.13	0.02	0.02	0.24	0.11	0.18	0.17	0.07	0.01
trans-Piceid	2.62	1.05	0.17	2.96	2.24	1.28	1.64	1.30	0.64
cis-Piceid	0.19	0.13	0.13	0.29	0.21	0.27	0.25	0.07	0.04

The total flavan-3-ols content (amount of procyanidin B1, procyanidin B2, (-)-epicatechin and (+)-catechin) was higher than that of the total flavonoids (amount of quercetin, isoquercitrin, kaempferol, kaempferolo-3-O-glucoside, rhamnetin, isorhamnetin, isorhamnetin-3-O-glucoside, rutin, myricetin), for each wine sample.

Total phenol content and antioxidant activity

The total phenols differed in the different types of wine and depended on the grape variety. The level determined by the Folin-Ciocalteu method varied from 1,794 mg/L GAE (Cerasuolo di Vittoria Planeta) to 4,614 mg/L GAE (Cabernet Zangara 2001) (Table 9). These values are in accord with the values reported by other authors (KANNER *et al.*, 1994; SIMONETTI *et al.*, 1997; SÁNCHEZ-MORENO *et al.*, 2000.). The wines from Nero d'Avola grapes showed a higher average total phenol content (3,607 mg/L GAE) than that of the other wine samples.

Table 9 shows the antioxidant capacity of the wines, expressed as Trolox antioxidant equivalent capacity (TAEC) and determined using the FOGLIANO *et al.* (1999) method. The antioxidant capacity levels of these wines ranged from

Table 9 - Total phenolic content and antioxidant capacity of commercial red wines.

	Wine Samples	Total Polyphenols (mg/L GAE)	Antioxidant Capacity (mg/mL TAEC)
Nero d'Avola	Cellaro 2000	4209	23.7
	Paolini 2001	4074	22.8
	Cantina Montalto	3225	22.0
	Zangara 2001	3804	21.6
	Morgante 2001	4209	21.6
	Duca Farese 2000	3669	21.3
	Basileus 2001	3534	20.6
	Portale 2001	3596	19.7
	Corvo Novello 2002	3540	18.6
	Santa Cecilia Planeta 2000	2563	18.1
	Corvo Rosso 2000	3254	15.9
Merlot	Planeta 2000	3130	27.2
	Ist. Agrario 2001	4074	26.9
	Lithos 2001	2995	25.3
	Fici 2002	3540	21.1
	Fici 2001	3251	14.1
Cabernet-Sauvignon, Petit-Verdot, Syrah	Cabernet Fici 2002	3663	25.7
	Cabernet Zangara 2001	4614	24.5
	Syrah Planeta 2000	3900	21.1
	Syrah Fici 2002	3110	20.6
	Cabernet Burdese Planeta 2000	3521	20.1
	Petit-Verdot Fici 2002	3387	20.1
cv mixture	Birgi 2000	3669	24.5
	Crepuscolo 2000	3614	23.2
	La Segreta Planeta 2001	2320	21.7
	Edonè 2000	3130	21.5
	Paliotto 2000	4344	21.4
	Edonè 1999	2350	20.3
	Borromeo rosso 2001	3135	20.0
	Megara Corvo 2000	3601	18.5
	Cerasuolo di Vittoria Planeta 2001	1794	14.1

14.1 mg/mL TAEC (Cerasuolo di Vittoria Planeta) to 27.2 mg/mL TAEC (Merlot Planeta). The Merlot varieties had the highest antioxidant capacity (average 22.6 mg/mL TAEC). The classification by wine types according to antioxidant capacity showed that the Nero d'Avola wines had the lowest activity (average 20.0 mg/mL TAEC), but the values for these wines were still within the range for red wines.

The results of the analysis of the interdependence between the antioxidant capacity and the total phenol content of these wines show that there is no correlation. The best *r* value was found for the Nero d'Avola wines (r = 0.62). Nevertheless, it should be mentioned that, while a correlation is often found between total phenol content and TEAC values, the determination of total phenols with the Folin-Ciocalteau reagent is not a selective method of analysis because it identifies every potentially oxi-

dizable compound present in the wine. The concentrations of the phenolic constituents determined by HPLC-MS and grouped according to structural characteristics were also correlated with the antioxidant capacity. No correlation was found for total flavan-3-ols, gallic acid, (+)-catechin, (-)-epicatechin, procyanidin B1, procyanidin B2, anthocyanins or TEAC values. While the values related to the antioxidant activity of the wines examined were not correlated with the total phenol content, the antioxidant properties of the wines could be linked to a parameter or parameters not considered in this investigation.

CONCLUSIONS

The direct analysis procedure applied to wine samples of "Protected Geographical Indication" was more precise, faster and technically simpler than other methods. The results of this study indicate differences in the phenolic composition and antioxidant activity of wines from different cultivars.

The results of the study provide new insights into the polyphenolic composition of commercial Sicilian wines, and highlight some interesting points with regard to the different wine typologies. The Merlot wines produced in Sicily, as well as those produced from other allochthonous grapes, generally have the highest polyphenolic content. Based on the data, Merlot wines appear to be an important source of dietary polyphenolic antioxidants. It may be supposed that Sicilian wines, produced from this grape typology, are endowed with characteristics that are potentially good for one's health.

Finally, considering the results related to the antioxidant activity and polyphenol content, it is difficult to explain the relationship between the phenolic compounds and the antioxidant activity. The effect of possible interactions (synergism and antagonism) of these phenolic compounds must also be considered (MEYER *et al.*, 1998). Future studies will investigate other compounds that may contribute to the antioxidant properties in order to better explain the mechanism that gives wine its antioxidant properties.

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SHORT COMMUNICATION

EFFECT OF SPECTRAL PRE-PROCESSING METHODS ON THE EVALUATION OF THE COLOR COMPONENTS OF RED WINES USING FOURIER-TRANSFORM INFRARED SPECTROMETRY

ANALISI DI COMPONENTI DEL COLORE DEI VINI ROSSI MEDIANTE SPETTROSCOPIA NEL MEDIO INFRAROSSO CON TRASFORMATA DI FOURIER

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ABSTRACT

A rapid method for measuring the (i) free anthocyanins, (ii) copigmented anthocyanins, (iii) polymeric pigment fractions and (iv) actual red color at 520 nm from the Fourier transform infrared (FT-IR) spectrum was evaluated. Twenty wines, mostly one-year old, with red

RIASSUNTO

La spettroscopia nel medio infrarosso (FT-IR) è stata utilizzata per valutare rapidamente i principali parametri del colore dei vini rossi: antociani liberi, antociani copigmentati, pigmenti polimerici a basso ed elevato peso molecolare e intensità colorante a 520 nm.

- Key words: anthocyanin, FT-IR, PLS, red wine color, spectral data pre-processing, wine analysis -

color ranging from almost zero to 10 absorbance units (AU) at 520 nm were analyzed by reference methods for their levels of the selected color components. Partial least squares (PLS) regression, obtained from several spectral data preprocessing methods, was used for multivariate calibration to relate the chemical variables to the FT-IR spectra. The performance of different models was compared in terms of root mean square error of prediction for cross-validation (RMSECV) and coefficient of determination (R^2). The best results were obtained using vector normalization and no-preprocessing of spectral data. FTIR measured the copigmentation of wine with 5.7% RMSECV only.

Lo studio è stato condotto su venti vini giovani, la maggior parte di undici mesi di età, selezionati in modo tale da coprire un ampio intervallo di intensità a 520 nm: da circa zero fino a 10 unità di assorbanza (AU). I vini sono stati analizzati sia mediante dei metodi di riferimento reperiti in bibliografia che tramite tecnica FT-IR. I risultati dei due metodi sono stati confrontati mediante la regressione lineare ai minimi quadrati parziali (Partial Least Square, PLS). La copigmentazione è il parametro dei vini misurato con più precisione (errore 5,7%).

INTRODUCTION

The color of young red wine is due principally to free monomeric anthocyanins and their enhancement due to copigmentation with other non-colored phenolics derived from the grape berry (BOULTON, 2001). The grape berry also contains small and large polymeric pigments (SPP and LPP, respectively) which contribute to the color of red wine (HARBERTSON *et al.,* 2003). During fermentation and especially during aging, the monomeric anthocyanins are gradually incorporated into polymeric pigments and this confers color stability to a wine (SOMERS, 1971; SOMERS and EVANS, 1974; RIBEREAU-GAYON, 1982). The most important color parameters of red wine (i.e. free anthocyanins, copigmented anthocyanins, small and large polymeric pigments and actual red color at 520 nm) are currently analyzed by UV-Vis spectrophotometry using separate methods for each parameter.

There is increasing interest in analytical techniques for rapid, reproducible, nondestructive, multicomponent analysis, with minimal or no sample preparation. According to COZZOLINO *et al.* (2004) the concentrations of anthocyanins, pigmented polymers and tannins in red wine can be predicted by using Visible-near infrared (Vis-NIR) scanning, the Vis and the NIR spectra explain 85 and 15% of the variation in the concentration of the different components, respectively.

Fourier transform infrared (FT-IR) spectrometry, in combination with multivariate data analysis, is an interesting alternative to UV-Vis and NIR techniques. The suitability of FT-IR spectrometry for the analysis of several wine components has already been demonstrated (BELLON, 1993; SCHINDLER *et al.*, 1998; PATZ *et al.*, 1999; DUBERNET *et al.*, 2001; KUPINA and SHRIKHANDE, 2003; PATZ *et al.*, 2004; CUADRADO *et al.*, 2005). Today, little information is available on its application for evaluating the color components of red wine (ROSSEAU *et al.*, 2002). Apart from choosing the appropriate spectral interval of measurement, selecting the

spectral data pre-processing method plays an important role in spectroscopy application. The application of pre-processing to FT-IR spectra for quantification purposes is not a new approach in wine analysis (URTUBIA *et al.*, 2004), but, to date, a comparison of the different spectral data pre-processing methods has not been reported, yet.

The aim of this study was to evaluate several pre-processing methods of the FT-IR spectra of red wines for their ability, in conjunction with Partial Least Squares regression, to design calibration sets for the quantification of color components currently determined in the laboratory by the respective reference methods.

MATERIALS AND METHODS

Wine samples

Twenty young red wines were selected for a wide range of color density ranging from almost zero to 10 absorbance units (AU) at 520 nm. These included two Sangiovese (Atlas Peak Winery, Napa Valley, CA), four Merlot (Robert Mondavi Winery, Napa Valley, CA; Trefethen Vineyards, Napa Valley, CA), two Cabernet Sauvignon (Robert Mondavi Winery, Trefethen Vineyards), one Cabernet Franc (Trefethen Vineyards), nine Pinot Noir (Robert Mondavi Winery, Saintsbury, Napa Valley, CA; UCDavis Winery, Davis, CA), one Cagnulari, and one Cannonau red wine (Santa Maria la Palma, Alghero, Italy). At the time of these analyses eighteen wines were 11 months old and two wines were 23 months old.

Analytical methods

For each wine the following components were measured by reference UV-Vis methods described in the literature: total color, total anthocyanins, copigmentation (BOULTON *et al.,* 1999), small and large polymeric pigments (SPP and LPP, respectively), and tannins (HARBERTSON *et al.*, 2002; 2003). For the determination of total color, total anthocyanins and copigmentation (BOULTON *et al.*, 1999), the wines were filtered through a 0.45 μm polytetrafluoroethylene membrane filter (PTFE, Acrodisc CR, Pall Corporation, Corvina, CA) and then the wine pH was adjusted to 3.6 using HCl or NaOH as needed, before analysis. Measurements for tannins and polymeric pigments were carried out in a buffer containing 200 m*M* acetic acid and 170 m*M* NaCl adjusted to pH 4.9 with NaOH. The amount of tannin in the wine was calculated using a calibration curve with (+)-catechin standard solution with eight concentration levels ranging from 50 to 500 mg/L ($R^2 \geq 0.999$). The UV-Vis spectra in the range of 200-700 nm for each wine was also measured using a HP8543 diode array spectrophotometer (Hewlett-Packard, Palo Alto, CA). Data from spectral analysis were expressed as absorbance units (AU) with 1 cm path length and corrected for dilutions, unless otherwise specified. All chemicals were of analytical grade from commercial sources (Fisher Scientific, Fair Lawn, NJ; Extrasynthése, Geney, France).

FT-IR analyses were carried out using a WineScan FT120 spectrometer (Foss, Hilleroed, Denmark) equipped with a pyro-electric detector. Measurements were carried out at 40°C using a liquid flow-through cell equipped with CaF_2 windows and a 37 μm optical path length. A HeNe frequency laser with a 632 nm signal beam was used with a reference interferometer. The spectra were recorded in duplicate for each sample from 926 to 5,012 cm^{-1} and the mean of the two measurements was used.

Spectral data processing and statistical analysis

A preliminary Principal Component Analysis (PCA) was used to visualize the

occurrence of grouping among the red wines by means of the color components measured by the reference methods.

The three main steps in handling the spectroscopic data are (i) optimization of spectral data pre-processing, (ii) wavenumber selection, and (iii) component quantification (BEEBE *et al.*, 1998). The effect of eleven spectral data pre-processing methods (No spectral data preprocessing, First derivative, Second derivative, Vector normalization, Constant offset elimination, Min-Max normalization, Multiple scattering correction, Straight line subtraction, and some of them jointly) was investigated. The purpose of this comparison was to minimize the root mean square error of cross-validation (RMSECV) for a quantitative PLS regression model predicting the colored component content in red wines.

In spectroscopy applications it is often appropriate to improve the model by selecting appropriate wavenumbers. In practice, the *B*-coefficients (regression coefficients) give the accumulated picture of the most important wavenumbers (ESBENSEN *et al.*, 1998).

The method of full-cross-validation (i.e. internal validation) was used to determine the maximum number of significant factors (PCs) (MARTENS and NÆS, 2001). The optimum number of wavenumbers for inclusion in the calibration equations was determined by comparing the regression results in terms of the root mean squared error of prediction for cross-validation (RMSECV), which should be minimized. Statistical analyses were performed using two commercially available chemometric softwares (Uncrambler 7.6, CAMO ASA, Oslo, Norway; OPUS 4.2, Bruker Optics, Milano, Italy).

RESULTS AND DISCUSSION

When designing a calibration set, besides selecting the number of samples, the concentration range to be covered by the samples and the distribution of samples within this range should also be taken into consideration. Although the total number of samples was limited, the wines covered a wide range of values (Table 1) that are representative of most of the commercial young red wines. Fig. 1 shows the distribution of the total color (AU@520 nm) for a set of 145 commercial red wines, made from twenty different grape varieties (VERSARI, 2006). The wines analysed in this study included three Pinot Noir wines made with skin contact time of between 0 and 7 days, to provide a reference "white wine" with essentially no color, and several low colored samples. Each single grape cultivar has a limited variation in the color values, thus the need to include more cultivars in the study. FT-IR instruments that are used for quality control often contain a single calibration curve for each grape cultivar. Thus, from an analytical point of view, the presence of wines from different grape cultivars probably affects the results to some extent. However, this can lead to a better understanding of the complex chemistry of grape and wine color components.

The PCA plot of unprocessed spectra shows a lack of grouping of wines according to their cultivar (Fig. 2). This finding suggests that the color components of red wines vary depending on several factors, including grape cultivar, origin and winemaking conditions.

The full IR spectra of wines showed the presence of some regions that introduce noise to the calibration due to the presence of interfering compounds, especially water that absorbs at 1,717-1,543 and 3,627-2971 cm^{-1}. Moreover, the spectral interval from 5,012-3,627 cm^{-1} contains very little useful information (REH, 2001). Thus, only the following IR spectral regions were considered for further analysis: 1,543-965, 2,280-1,717, and 2,971-2,435 cm^{-1} (Fig. 3).

Table 1 - Statistics for the red color components of wine.

Red color component	Concentration range	Spectral pre-processing technique	PCs[a]	R^{2b}	RMSECV[c]	RMSECV (%)
SPP[d]	0.01-0.99 AU520nm	Vector normalization	10	0.977	0.043	8.5
		First Derivative[f] + Vector normalization	10	0.973	0.046	9.2
		Constant Offset Elimination	10	0.966	0.052	10.4
		No Spectral Data Pre-processing	10	0.965	0.053	10.6
LPP[e]	0.0.87 AU520nm	No Spectral Data Pre-processing	9	0.955	0.056	12.5
		Vector normalization	10	0.935	0.068	15.0
		Second Derivative[f]	9	0.934	0.068	15.1
		First Derivative[f]	10	0.924	0.073	16.2
Copigmentation	0.14-1.98 AU520nm	Vector normalization	10	0.989	0.060	6.0
		Constant Offset Elimination	10	0.987	0.065	6.5
		Straight Line Subtraction	10	0.987	0.067	6.6
		Multiplicative Scattering Correction (MSC)	9	0.986	0.070	6.9
Total anthocyanin	0-3.37 AU520nm	No Spectral Data Pre-processing	10	0.988	0.105	6.3
		Second Derivative[f]	10	0.985	0.119	7.1
		Vector normalization	10	0.984	0.122	7.3
		First Derivative[f]	10	0.984	0.123	7.4
Total color	0.03-10.61 AU520nm	No Spectral Data Pre-processing	10	0.990	0.319	5.8
		Vector normalization	10	0.986	0.368	6.6
		Second Derivative[f]	10	0.985	0.384	6.9
		Min-Max Normalization	10	0.984	0.392	7.1
Tannin	0-1,655 mg/L	Second Derivative[f]	10	0.978	63	6.6
		No Spectral Data Pre-processing	10	0.969	76	7.9
		First Derivative[f] + MSC	10	0.965	80	8.3
		First Derivative + Straight Line Subtraction	10	0.965	81	8.4

[a]Number of principal components chosen in PLS regression model explains most of the variation in red color components; [b]Coefficient of determination for the calibration model (n=20); [c]Root mean square error of cross-validation; [d]Small Polymeric Pigments; [e]Large Polymeric Pigments; [f]First and second derivative used 17 smoothing points.

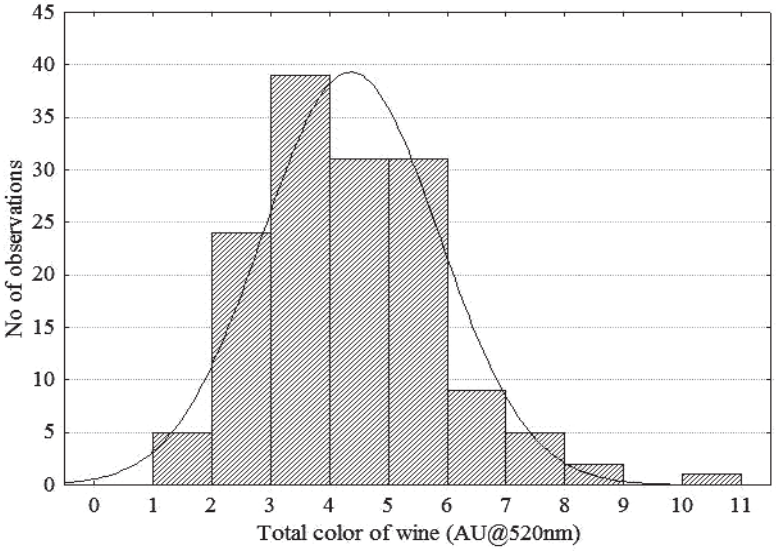


Fig. 1 - Distribution of the total color of wine (AU at 520 nm) for 145 commercial red wines, including Aglianico, Barbera, Bardolino, Cabernet Sauvignon, Cannonau, Cagnulari, Chianti, Ciliegiolo, Gutturnio, Lagrain, Lambrusco di Sorbara, Merlot, Montepulciano, Nero d'Avola, Pinot Noir, Sangiovese, Salice salentino, Marzemino, Teroldego, and Lacryma Christi.

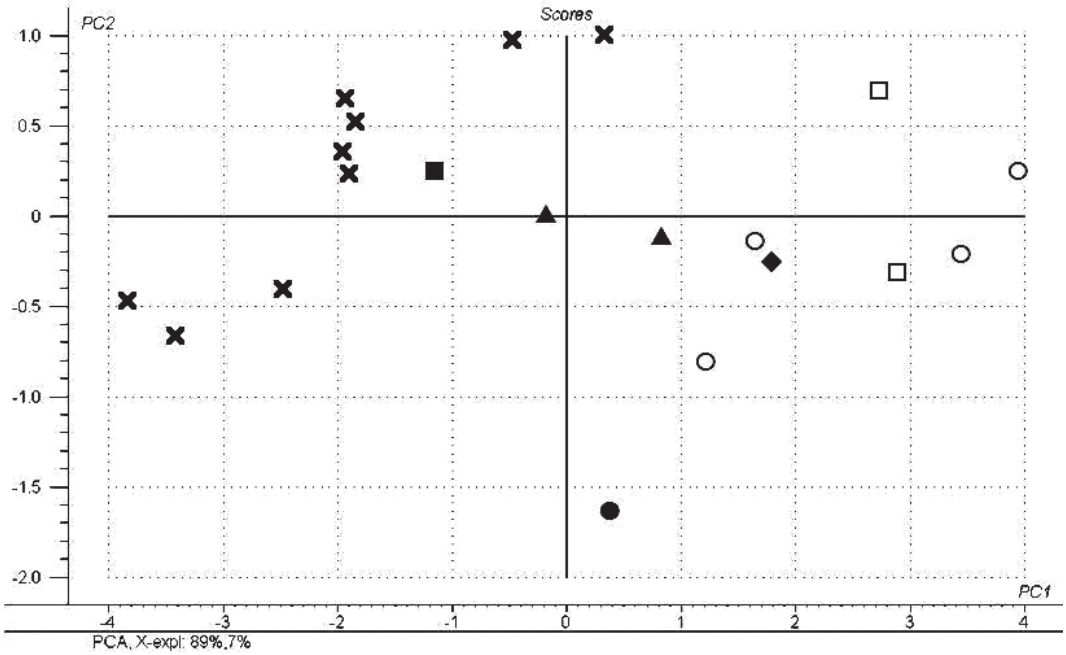


Fig. 2 - PCA plot of the first two PCs of red wines. Legend: (▲) Sangiovese; (○) Merlot; (□) Cabernet Sauvignon; (♦) Cabernet Franc; (×) Pinot Noir; (●) Cagnulari; (■) Cannonau.

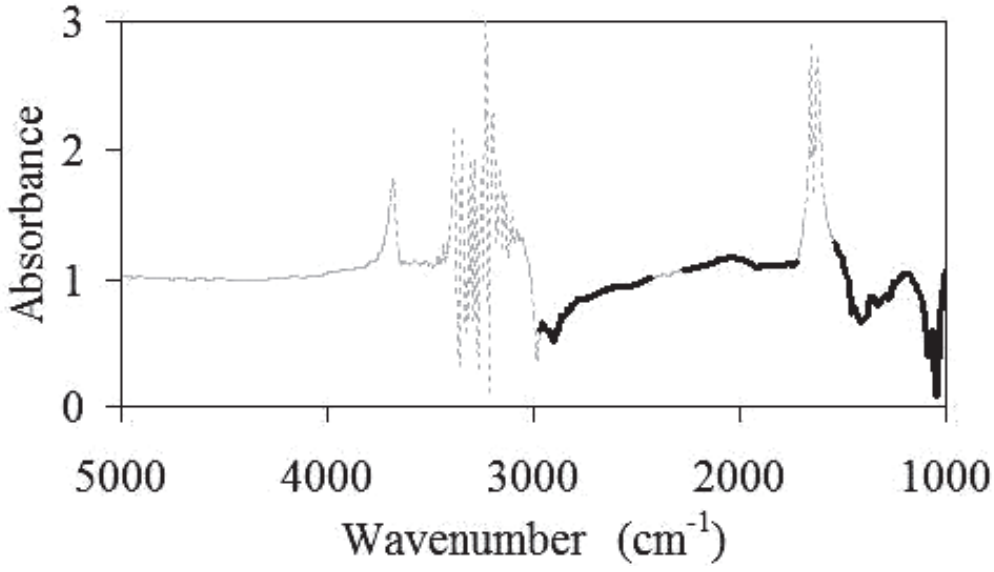


Fig. 3 - Example of a FT-IR spectrum of wine. The thin line shows the spectral region discarded (noise and interference), whereas the bold line indicates the spectral region with useful information (see Results and Conclusion section for explanation).

Among them, the early region of the FT-IR spectra (1,539-926 cm^{-1}) provided the most useful information. No single wavenumber showed any selective signal for color component in red wine, and only through the empirical combination of several wavelengths was it possible to get selectivity.

The residual Y-variance for both calibration and validation decreased regularly as the number of PCs increased. A similar trend was shown by the prediction error in *y* (RMSECV). For practical purposes, the color properties of a compositionally complex system, such as a red wine, can be explained by an optimal model having 9-10 PCs (Table 1).

When working with FT-IR spectra, an important decision is whether a pre-processing method is necessary. A number of pre-processing methods are available and the key to deciding upon the most appropriate pre-processing method is usually based on an empirical approach. The spectral data pre-processing methods can, in general, be divided into scaling, filtering, baseline correction and derivatization categories.

The overall best prediction results, in terms of RMSECV, were obtained using vector normalization, followed by no pre-processing and second derivative of spectroscopic data (Table 1). Application of the vector normalization algorithm resulted in a 16% reduction in the RMSECV when compared with the raw data model for the PLS prediction of gibberellic acid concentrations in a bioprocess (JARVIS and GOODACRE, 2005). To perform a vector normalization the average *y*-value is calculated first. The average value is then subtracted from the spectrum, which has the effect of centering the spectrum around $y = 0$. The sum of the squares of the *y*-values is then calculated, and the spectrum is divided by the square root of this sum (CONZEN, 2003).

No pre-processing provided a fitting performance especially for total color at 520 nm with R^2 of 0.990 and RMSECV of 5.8%, thus proving the suitability of FT-IR for measuring the color of red wines. PALMA and BARROSO (2002) found that no pre-processing performed best for the classification of wines. The use of raw spectral data is probably most used for quantification purposes, considering that many studies on FT-IR analysis of wines do not mention the spectral data pre-processing method (PATZ *et al.*, 1999; DUBERNET and DUBERNET, 2000; MOREIRA *et al.*, 2002; KUPINA and SHRIKHANDE, 2003).

Derivative is used to reduce baseline features, i.e. low-frequency sources of variation that are not related to the chemistry under investigation (BEEBE *et al.*, 1998). This pre-processing method has already been used in FT-IR spectroscopy (URTUBIA *et al.*, 2004).

The RMSECV, which expresses the error expected in future predictions, was satisfactory as ≤8.5% for all the parameters tested (Table 1), except large polymeric pigments (RMSECV = 12.5%) whose calibration model needs to be improved (Fig. 4).

CONCLUSION

In conclusion, this short communication reports a preliminary result of a study aimed at evaluating the phenolic compounds in red wine by spectroscopic techniques. In this view, SKOGERSON *et al.* (2006) recently used UV-Vis spectroscopy to predict the major phenolic compounds responsible for the red color of 414 wines with a coefficient of determination (R^2) ranging from 0.78 to 0.88. Additional FT-IR analysis on a large number of wines is in progress in the USA and Italy. Based on the result of this study the vector normalization, no pre-processing and second derivative of spectroscopic data are the best spectral

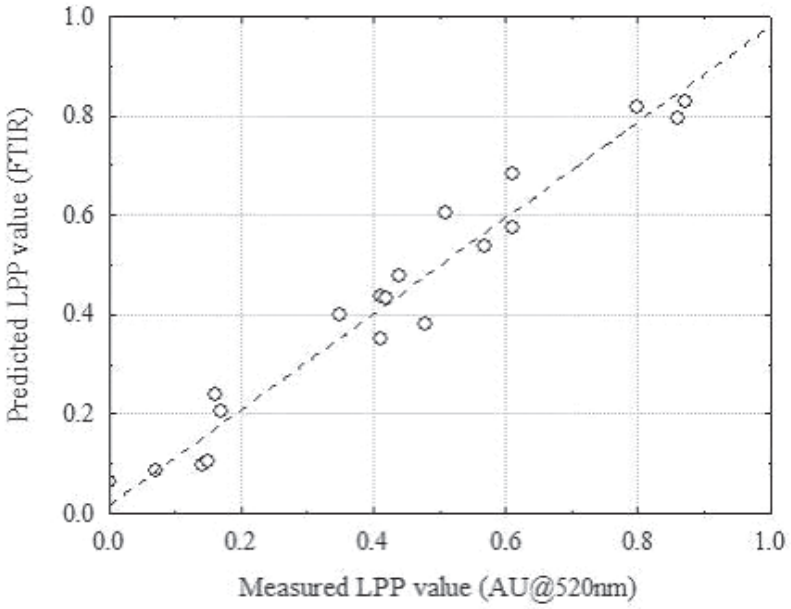


Fig. 4 - Scatter plot for the value measured with the reference method and predicted value with FT-IR for the large polymeric pigments (LPP).

pre-processing methods and will be tested in future analyses.

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SHORT COMMUNICATION

α-AMYLOLYSIS OF NATIVE CORN, POTATO, WHEAT AND RICE STARCH GRANULES

α-AMILOLISI DI GRANULI DI AMIDO DI MAIS NATURALE, PATATA, GRANO E RISO

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ABSTRACT

A partially purified α-amylase produced by a novel *Bacillus subtilis* was used for the hydrolysis of native corn, potato, wheat, and rice starches at 50° and 60°C. The hydrolyses were carried out for 1, 2, 3 and 4 hours. For each process, the degree of hydrolysis (D.H.%) and residual starch concentrations were determined. The D.H.% values for the starches after 4 hours of hydrolysis at 50°C were: 5.4% (potato), 4.8% (rice), 3.9% (wheat) and 2.3% (corn). The results after 4 hours

RIASSUNTO

Una α-amilasi parzialmente purificata prodotta da un nuovo *Bacillus subtilis* è stata utilizzata a 50° e 60°C per l'idrolisi di amido di mais naturale, di patata, di grano e di riso. Le idrolisi sono state condotte per 1, 2, 3 e 4 ore. Per ogni processo sono stati determinati il grado di idrolisi (D.H.%) e le concentrazioni di amido residuo. I valori di D.H.% per l'amido dopo 4 ore di idrolisi a 50°C erano: 5,4% (patata), 4,8% (riso), 3,9% (grano) e 2,3% (mais). I risultati dopo 4 ore a 60°C erano molto più alti per la

- Key words: α-amylase, scanning electron microscopy, starch hydrolysis -

at 60°C were much higher for potato (18.5%) and wheat (14.3%), but did not change significantly for rice (4.2%) and corn (2.4%). Loss of starch was also determined after each time interval using an iodine method. Untreated and digested corn and potato starch granules were observed using a scanning electron microscope. The micrographs showed some holes and cracks (corn starch) or scratches (potato starch) on the surface of the granules.

patata (18,5%) ed il grano (14,3%), ma non variavano significativamente per il riso (4,2%) ed il mais (2,4%). La perdita di amido veniva anche determinata dopo ogni intervallo di tempo utilizzando il metodo allo iodio. Granuli di amido, non idrolizzati e digeriti, sono stati osservati al microscopio elettronico a scansione. Le microfoto mostravano alcuni fori e fessure (amido di mais) o scalfitture (amido di patata) sulla superficie dei granuli.

INTRODUCTION

The hydrolysis of starch catalyzed by an α-amylase is one of the most important commercial enzyme processes and the products are used in many branches of industry. Enzyme hydrolysis is also used to study starch ultrastructure (GALLANT *et al.* 1997). α-amylases have been produced from various sources (animals, plants and microbes) and have different properties. The most abundant microbial source of this enzyme is the genus *Bacillus*, especially *B. subtilis, B. amyloliquefaciens* and *B. licheniformis* (NIZIOŁEK, 1998; SARIKAYA *et al.*, 2000). α-amylase specifically catalyses the hydrolysis of α-1,4-glycosidic linkages in starch.

The susceptibility of starch to hydrolysis by amylases varies according to starch origin and enzyme source (PLANCHOT *et a*l. 1995; HOOVER, 2001; SŁOMIŃSKA *et a*l., 2003). In general, starch granules are less susceptible to α-amylolysis than gelatinized starch (KONSULA and LIAKOPOULOU-KYRIAKIDES, 2004). According to GALLANT *et al.* (1992) potato starch is slightly eroded by exocorrosion, while wheat starch has specific susceptible zones which become pitted and these pits enlarge due to endocorrosion. Deep pitted canals form randomly on native corn and rice starch granules, which then enlarge and penetrate the interior of the granule. Numerous researchers have investigated enzyme hydrolysis of native starch (KIM *et al.*, 1989; YAMADA *et al.*, 1995; PLANCHOT *et al.*, 1995; HAMILTON *et al*, 1998; ZHANG and OATES, 1999; SARIKAYA *et al.*, 2000; KONSULA and LIAKOPOULOU-KYRIAKIDES, 2004; LI *et al.*, 2004) and among them native potato starch was the most resistant to enzyme attack. In general, hydrolyses have been carried out for 72 hours or at high temperatures.

The aim of this work was to compare the susceptibility of four native starches toward α-amylase produced by a novel *B. subtilis* isolated from rotting cocoyam (*Discorea esculenta*) tubers obtained from Nigeria. This α-amylase was able to hydrolyze native starches of different origin, especially potato starch. The influence of temperature and time on the extent of hydrolysis and changes of the starch granule surfaces were also examined.

MATERIALS AND METHODS

Rice, potato, wheat and corn starches (Sigma Chemicals Co., St. Louis, MO, USA) and partially purified *Bacillus subtilis* α-amylase were used. After enzyme

production, cells were removed by centrifugation and the supernatant was precipitated with 80% ammonium sulphate. The precipitate was resuspended in 0.01 M acetate buffer (pH 6), dialyzed at 4°C for 24 h against the same buffer and centrifuged for 15 min at 10,000×g. Enzyme activity was assayed by the dinitrosalicylic method (MILLER, 1959). One unit (U) is defined as the amount of enzyme that releases 1 μmol of reducing end groups per minute in 0.01 M acetate buffer (pH 6) with 1% (w/v) soluble starch as substrate at 60°C. Glucose was used as a standard.

Hydrolysis reactions were conducted in a total volume of 20 mL in 250 mL Erlenmeyer flasks in a thermostated rotary shaker at 150 rpm (Water Bath Shaker Elpan type 357, Lubawa, Poland). Samples of each starch (200±0.1 mg) were suspended in 0.01 M acetate buffer (pH 6) containing 5 mM $CaCl_2$, 0.02% sodium azide and 0.1 mL toluene, and placed in a thermostated rotary shaker at 50° and 60°C. α-amylase was then added to the starch slurries (100 U/200 mg of starch). Hydrolyses were carried out for 1, 2, 3 and 4 h. After each time interval, the reaction mixtures were boiled for 3 min to stop the enzyme activity (HAMILTON *et al.*, 1998) and then cooled to room temperature. Blank controls without enzyme were made using the same process. Reducing sugars liberated in the reaction mixture were determined according to the DNS method (MILLER, 1959). The concentrations of reducing sugars formed were calculated according to calibration curves drawn for known concentrations of glucose solutions. At least three measurements were made for each temperature and the data are an average of these results.

Degree of hydrolysis (D.H.%) is defined as:

$$D.H.\% = (A_1/A_0) \times 100\%,$$

where:

A_1 - molar concentration of reducing sugars liberated by enzymatic hydrolysis,

A_0 - molar concentration of reducing sugars liberated by acid hydrolysis.

Acid hydrolysis was carried out by treating starch (100 mg) with 1N HCl (20 mL) at 100°C for 2 h.

Residual starch was determined according to the Heinkel method (KRAWCZYŃSKI and OSIŃSKI, 1967): 0.15 mL of 0.9% NaCl solution, 0.25 mL of 20% sulfosalicylic acid solution, and 9.5 mL of I_2/KI solution (2 g KI and 1 g I_2) diluted 150 times were added to 0.1 mL of starch solution. The absorbance was measured at λ_{max}560 nm. A standard curve was drawn by measuring the absorbance of known concentrations of each starch solution prepared after sterilizing the starch at 121°C for 1 h.

The dried starch samples were directly mounted on circular aluminum stubs and coated with gold using Sputter Coater CS 100. The preparations were then examined and photographed in a BS-300 (Tesla, Brno, Czech Republic) scanning electron microscope at an accelerating voltage of 10 kV.

RESULTS AND DISCUSSION

The partially purified α-amylase produced by *B. subtilis* was used to hydrolyze native corn, potato, wheat and rice starches at 50° and 60°C. Figs. 1 and 2 show the degree of hydrolysis (D.H.%) of the starches at 50° and 60°C, respectively. The control experiments carried out under the same conditions did not yield any reducing sugars. The final degradation values for native potato, rice, wheat and corn starches after 4 h of hydrolysis at 50°C were 5.4, 4.8, 3.9, and 2.3%, respectively. The results at 60°C were: 18.5% (potato), 14.3% (wheat), 4.2% (rice) and 2.4% (corn). At both temperatures potato starch was degraded more efficiently than the oth-

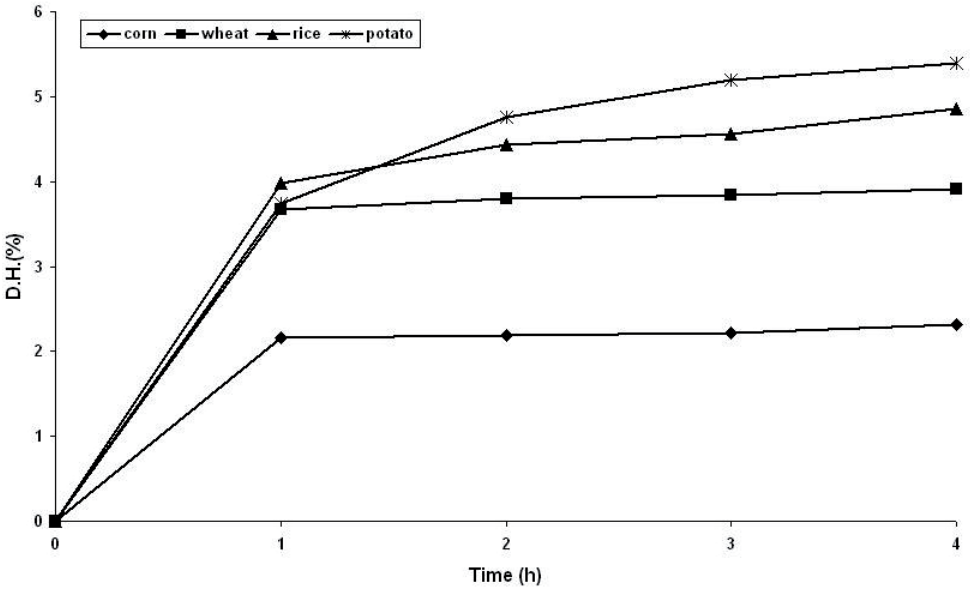


Fig.1 - Hydrolysis of different starches by α-amylase from *B. subtilis* at 50°C.

er starches. The potato starch degraded rapidly from the onset of the process, whereas the wheat, corn and rice starches degraded very slowly. Potato starch is reported to be resistant to enzyme hydrolysis (OATES, 1997). Since this starch is one of the most important sources in many branches of industry, enzymes capable of digesting raw potato starch are of great interest. Recent-

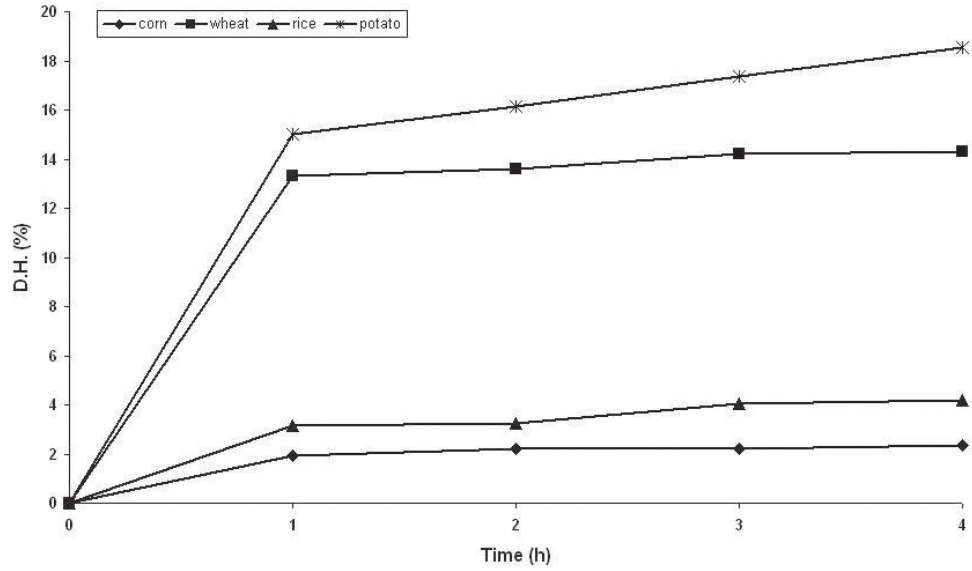


Fig. 2 - Hydrolysis of different starches by α-amylase from *B. subtilis* at 60°C.

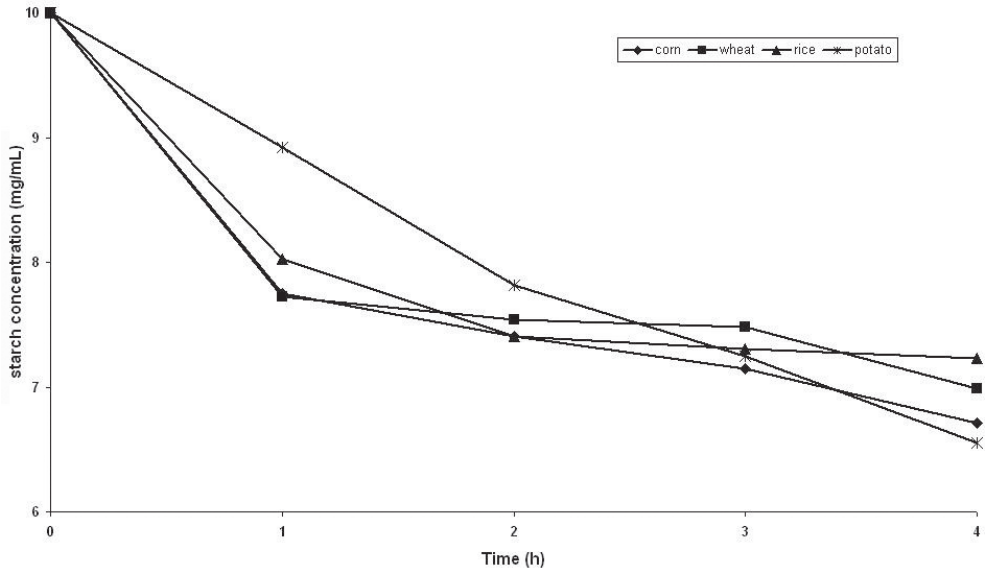


Fig. 3 - Residual starch after 1, 2, 3 and 4 h of hydrolysis at 50°C.

ly, GOYAL *et al.* (2005) reported the existence of an α-amylase from *Bacillus* sp. I-3, that very efficiently digests raw potato starch at 60° and 70°C. However, no information was given about its ability to digest starches of different origin.

Figs. 3 and 4 show residual starch concentrations versus time of hydrolysis at 50° and 60°C, based on the io-

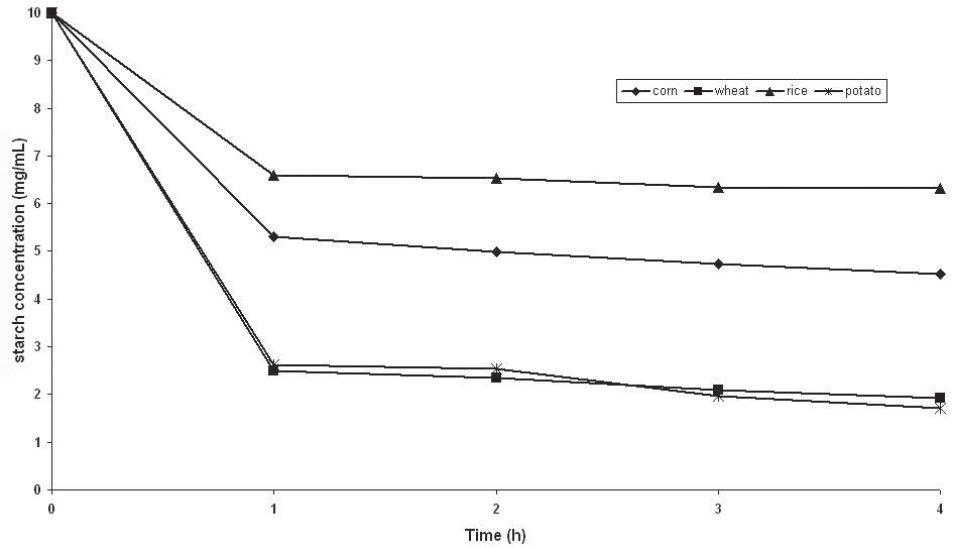


Fig. 4 - Residual starch after 1, 2, 3, and 4 h of hydrolysis at 60°C.

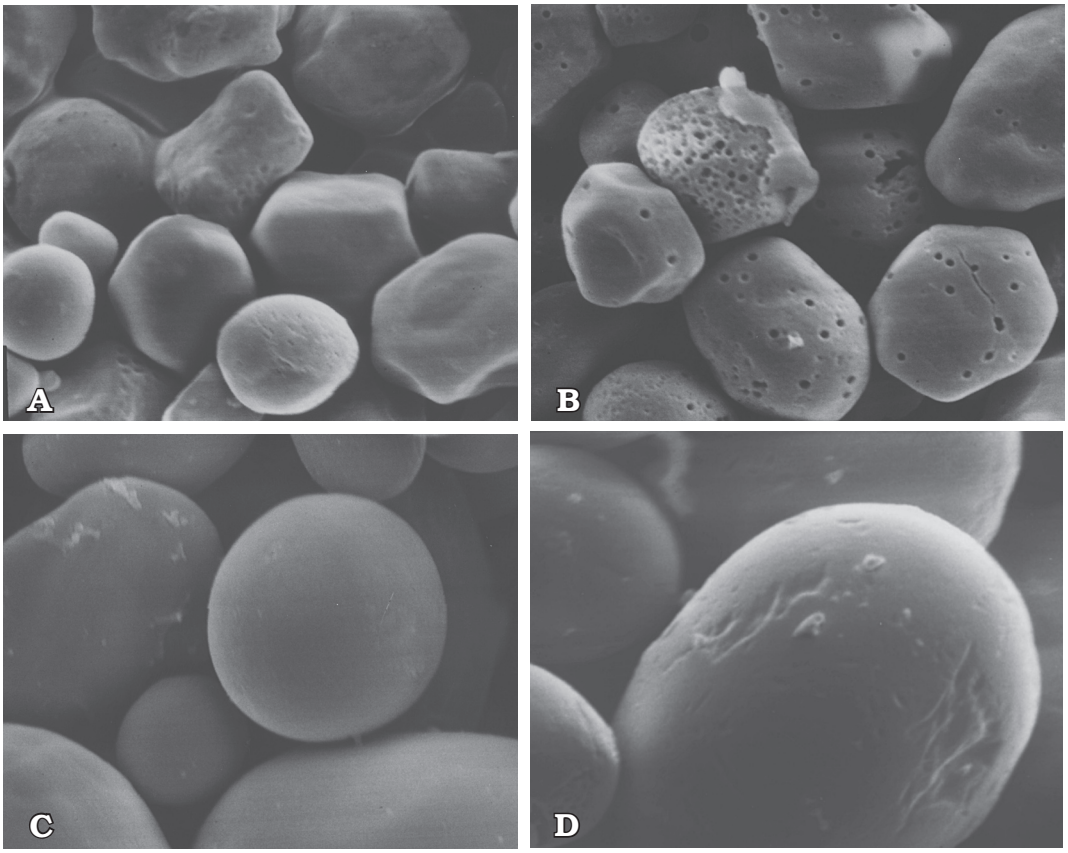


Fig. 5 - Scanning electron micrographs of native corn (A) and potato (C) starch granules and granules hydrolyzed by α-amylase at 50°C for 1 h: corn (B) and potato (D) (magnification x 5000).

dine method. These data partly confirm the previous results. α-amylolysis of the four examined starches reduced the initial wheat and potato starch concentrations from 10 mg/mL to about 2 mg/mL after 4 h of hydrolysis at 60°C. These results are not directly proportional to the amount of reducing sugars liberated after each time interval. Gel solutions formed after boiling for 3 min in each of the control experiments at both temperatures. This differed markedly from the enzyme-treated starches of different origin. These differences in the examined starches confirm the action of the α-amylase on these substrates, which is evident based on the analyzed reducing sugars and the residual starch contents. The observed intermediate iodine color intensity for enzyme-treated starches indicates a mixture of dextrins and residual starches. In fact, the loss of starch was much higher than indicated by the D.H.% results.

Scanning electron micrographs of native and hydrolyzed starch granules are presented in Fig. 5. The granule surface of corn and potato starches became roughened after 1 h of hydrolysis at 50°C. Some holes and cracks could be observed on the surface of corn starch granules. In the case of potato starch, characteristic scratches appeared.

CONCLUSIONS

This investigation showed that native potato starch could be hydrolyzed effectively by the α-amylase from *B. subtilis* at 50° and 60°C. Wheat starch was also a good substrate at 60°C. Granules of native starches were degraded differently by the enzyme, but its action was always evident after 1 h of α-amylolysis at 50°C. Increasing the hydrolysis temperature from 50° to 60°C generally resulted in the production of more reducing sugars and higher losses of starch. The fact that this α-amylase hydrolyzed the normally enzyme-resistant potato starch granules better than the other examined starches, makes it an enzyme of choice in the food industry.

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SHORT COMMUNICATION

EFFECT OF COOKING AND STORAGE TIMES ON GENTAMICIN RESIDUES IN EGGS

EFFETTI DEI TEMPI DI COTTURA E DI CONSERVAZIONE SUI RESIDUI DI GENTAMICINA NELLE UOVA

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ABSTRACT

The aim of this study was to determine the effects of different cooking (boiling, frying) and storage (+4°C, 15°-20°C) procedures on the gentamicin levels in eggs obtained from laying hens that had received subcutaneous application of gentamicin. Gentamicin residues in eggs from 50 ISA brown laying hens were analysed using HPLC-fluorescence. The recovery study showed that approximately 90% of the gentamicin could be recovered from the egg. The lowest detection limit was 0.01

RIASSUNTO

Lo scopo di questo studio era la determinazione degli effetti di differenti metodologie di cottura (bollitura e frittura) e di stoccaggio (+4°C, 15°-20°C) sui livelli di gentamicina in uova provenienti da galline ovaiole cui è stata somministrata, per via sottocutanea, gentamicina. I residui di gentamicina nelle uova provenienti da 50 galline ovaiole della specie ISA brown sono stati determinati via HPLC-fluorescenza. Gli studi di recupero mostravano che, approssimativamente, era possibile recuperare dal-

- Key words: cooking procedure, egg, gentamicin, HPLC-fluorescence, residues, storage times -

μg/g in the whole egg. The retention times of the combined peaks of three components of gentamicin were 4.0 to 4.1 min. Their initial concentrations of the gentamicin residues were retained for up to fourteen days at both refrigerated (+4°C) and room temperatures (15°-20°C), but started to lose strength on day 21 of storage. On the 28th day, gentamicin residues in the eggs kept at room temperature were lower than those kept at the refrigerated temperatures. The different cooking procedures did not affect the gentamicin levels in the eggs. Therefore it is concluded that if gentamicin is used to treat laying hens, withdrawal times should be taken into account and eggs should not be offered for human consumption if gentamicin residue concentrations exceed regulatory tolerances.

le uova il 90% di gentamicina. Il limite inferiore di rivelazione era 0.01 μg/g nell'uovo intero. I tempi di ritenzione dei picchi combinati dei tre componenti della gentamicina erano da 4.0 a 4.1 minuti. Le concentrazioni iniziali dei residui di gentamicina restavano inalterate fino a 14 giorni, sia per le uova refrigerate (+4°C) sia per quelle conservate a temperatura ambiente (15°-20°C), ma al ventunesimo giorno di stoccaggio iniziavano a diminuire. Al ventottesimo giorno, i residui di gentamicina nelle uova conservate a temperatura ambiente erano minori di quelli mantenuti nelle uova refrigerate. Le differenti tipologie di cottura non influenzavano i livelli di gentamicina nelle uova. Si è quindi concluso che se la gentamicina viene impiegata per il trattamento di galline ovaiole, dovrebbero essere tenuti in considerazione i tempi di ritiro e le uova non dovrebbero essere destinate al consumo umano se le concentrazioni del residuo di gentamicina superano i valori tollerati.

INTRODUCTION

Veterinary medicines are used for various reasons but priority is given to combating diseases and increasing animal growth. At least 60% of all animals that were used for food production in the 1980s had been exposed to antibiotics some time during their lives and now the level of exposure is probably even higher (NORCROSS and BROWN, 1991; ŞANLI, 1999). Antibiotics have an important role among these medicines and may produce residues in edible tissues. In addition to their positive effects, they can also cause health problems, like hypersensitivity (PAIGE *et al.*, 1999). Antibiotics are not only threatening food security, but are also causing some resistant strains to develop among the sensitive bacteria when used in moderate doses for long periods (CORCIA and NAZARI, 2002; PAIGE *et al.*, 1999; SCHENCK and CALLERY, 1998; ŞANLI, 1999). In an attempt to reduce these health risks, Food and Agriculture Organization (FAO) and World Health Organization (WHO) stated that it is compulsory to meet the legal waiting times that are specific for each medicine (KAYA and ŞAHAL, 1989; NOUWS, 1981). The misuse of antibiotics may produce residue concentrations in edible food tissues that exceed the regulatory safety tolerance levels (DONOGHUE, 2003; NORCROSS and POST, 1990).

Gentamicin is an amino glycoside-structured antibacterial agent, effective as a bactericide that is effective against

Gram negative aerobic bacteria. *In vitro*, 99% of 2,788 isolates from dogs, cats, horses and cattle were found to be sensitive to gentamicin (PEDERSOLI *et al.*, 1990; POSYNIAK *et al.*, 2001). For poultry, gentamicin is administered orally or by intramuscular injection to treat birds infected with *Salmonella typhimurium* (OLESIUK *et al.*, 1973).

Gentamicin is the most commonly used antimicrobial agent in Turkey, especially for laying hens. However, few studies have been conducted to determine the residue levels in eggs. In a previous study (FILAZI *et al.*, 2005), gentamicin was detected in eggs after therapeutic use in laying hens. The aim of this study was to determine the effects of different cooking (boiling, frying) and storage procedures on gentamicin residues in eggs obtained from gentamicin treated hens.

MATERIALS AND METHODS

Birds

Fifty 30-week-old ISA brown laying hens, weighing between 1.6 and 2.0 kg were kept individually in fibre cages (30x35x45 cm), in a ventilated, heated room (20°C), with 14 h of daylight. The hens were fed a standard commercial layer mash (120 g/day) and water *ad libitum*. The hens were fed for a week and their eggs collected. After establishing that no antibacterial agent contaminated the eggs, the trials were begun.

Trials

All hens were subcutaneously injected with 25 mg/kg of the drug containing 50 mg/mL gentamicin (Gentavet, Vetaş Company, İstanbul-Turkey) which is the common practice. The injection was given between 10.00 and 11.00 in the morning. Eggs were collected daily after the gentamicin injection. The effect of storage procedures on the residues was determined on the first day using 44 eggs. The effects of cooking procedures were determined on the second day using 32 eggs. Twenty of the eggs collected on the first day were kept at 4°C in a refrigerator, while 20 were kept at 15°-20°C (room temperature). Four eggs were analyzed per day on the 4^{th}, 7^{th}, 14^{th}, 21^{st} and 28^{th} days of storage to determine gentamicin residues. On the first day, four uncooked eggs were analyzed and used as the control; of the eggs collected on the second day, 8 uncooked, 8 fried (in oil), 8 undercooked (1.5 min in boiling water) and 8 overcooked (5 min in boiling water) were then analyzed.

Analytical methods

The gentamicin residues in the eggs were detected by modifying the HPLC method recommended by the European Union to detect the quantity of gentamicin residues in sheep, pig and beef muscles and calf offal (HEITZMAN, 1994). According to this method, yolk and albumen are mixed and 5 g samples are taken so that the antibiotic content in the whole egg is analyzed. Trichloracetic acid (5% w/v)/ 1 mM EDTA solution was added to the sample in a tube; the samples were homogenized and then centrifuged to precipitate the proteins. The pH was then adjusted to 7.0 and they were filtered using a Sephadex G-25 (Bakerbond Spe Kolon, Griesheim, Germany) ion exchange column. The samples were analyzed by HPLC with a post-column derivation (Phenomenex Luna C-18 column; 5 μm, 250 mm x 4.6 mm, Aschaffenburg, Germany), using o-phthalaldehyde in a Rigas Lab. Prometheus 300 Marathon plus system (Thessaloniki, Greece). The o-phthalaldehyde (0.8% containing 0.2% 2-mercaptoethanol and 0.1% Brij) was pumped at 0.5 mL per min and the fluorescent peaks were detected at 400 nm. The total concentration of the gentamicin (C_1, C_2, and C_{1a}) was then calculated.

Recovery studies

The gentamicin sulfate analytical standard (Sigma G1914, Lot: 070K1038, Munich, Germany), with purity of 665 μg/mg was added to the whole egg at concentrations of 0.01, 0.05, 0.08 μg/g, respectively, and homogenized to allow extraction of the gentamicin from the eggs and to establish the limit of detection. It was extracted using the above-stated analytical method, and analyzed by HPLC.

Statistical analysis

Analysis of Variance was applied to all the data and a multiple range test was used to determine if there were differences among the groups (SPSS Release 10.1).

RESULTS AND CONCLUSIONS

The recovery studies showed that approximately 90% of the gentamicin could be recovered from the egg. The lowest detection limit was 0.01 μg/g in the whole egg. The retention times of the combined peaks of three components of the gentamicin were 4.0 to 4.1 min. The effects of different storage temperatures and times on gentamicin residue levels in eggs are shown in Table 1. There was no significant difference in gentamicin residue concentrations in the eggs after fourteen days of storage at the two temperatures. However, by the 21st and 28th days of storage at both temperatures, the residue concentrations had decreased.

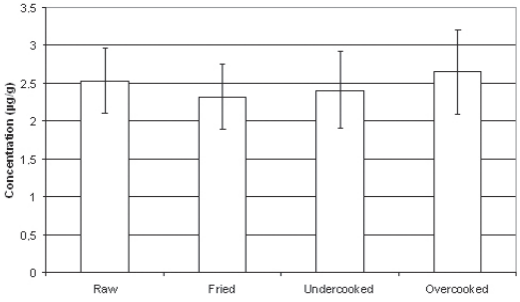


Fig. 1 - Amount of gentamicin residues after different cooking procedures (μg/g). Bar represent (Mean±S.E).

The effects of different cooking procedures on the gentamicin content are shown in Fig. 1. The gentamicin residues did not undergo any change after the eggs were fried, undercooked (1.5 min) and overcooked (5 min).

In studies carried out with streptomycin (same class as gentamicin), the residue concentrations in veal stored for 5-7 days or eggs subjected to boiling or frying did not change (INGLIS and KATZ, 1978; O'BRIEN *et al.*, 1980). Similarly, neomycin residues were not affected by cooking or storage at 4°C for 21 days (INGLIS and KATZ, 1978; KATZ and LEVINE, 1978).

Table 1 - Concentration of gentamicin residues after being kept at different temperatures (μg/g).

Storage	DAY (n=4) Mean ± SE*					
	0	4	7	14	21	28
Refrigerator (+4°C)	1.46±0.41^{ax}	1.35±0.42^{ax}	1.39±0.39^{ax}	1.44±0.46^{ax}	1.11±0.26^{ay}	1.14±0.31^{ay}
Room temperature (15°-20°C)	1.46±0.41^{ax}	1.41±0.44^{ax}	1.32±0.43^{ax}	1.29±0.37^{ax}	1.01±0.22^{ay}	0.92±0.22^{by}

*SE: Standard Error.
ab: The differences among the values with different letters in the same columns are significant ($p<0.01$).
xy: The differences between the values with different letters in the same rows are significant ($p<0.01$).

BAYDAN *et al.* (2000a, b) reported that the concentrations of enrofloxacin and danofloxacin in tissues and liver of chicken did not change after being grilled or boiled; the levels began to decrease after the 20th day after having been frozen. In another study, BAYDAN *et al.* (2002) determined that the sulphonamid residue levels in chicken meat were not effected by freezing (-18°C) but were reduced by grilling and boiling. These authors also determined that fluoroquinolone residues in chicken tissues were not affected by cooking but decreased after 20 days of being frozen.

When penicillin G was kept in a deep-freezer for 10 days, the level decreased by half in the gluteal muscles, 20% in the kidneys and only slightly in the plasma (BOISON *et al.*, 1992). Penicillin G in the kidneys remained stable when stored at 4°C for a week (NOUWS and ZIV, 1976). The penicillin residues were also quite resistant during storage at -76°C (BOISON *et al.*, 1992).

Sulphadimidine, used in veterinary medicine for disease prevention and treatment and to hasten growth, was not affected by roasting procedures or by being stored for 7 weeks in a deep-freezer (O'BRIEN *et al.*, 1980) and residues in the kidneys were resistant for up to 20 min. However the residue levels in the kidneys decreased 15% by frying at 140°C for 10 min and 30% by baking at 200°C for 20 min (HASSETT *et al.*, 1990). Sulphametazin levels decreased approximately 50% with heating procedures (KATZ and LEVINE, 1978). These results show that cooking time and method are important factors that influence the levels of sulphonamide residues.

CONCLUSIONS

The results of this study shown that gentamicin residue levels in eggs from treated laying hens were not reduced by different cooking procedures or by storage up to 14 days. Therefore, if gentamicin is used to treat laying hens, the treatment withdrawal times must follow regulatory guidelines (e.g., WHO) in order to ensure that consumers are not exposed to residue levels that exceed safety tolerance levels.

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SHORT COMMUNICATION

SAFETY EVALUATION OF FOOD SERVED IN TOURIST ACCOMMODATION ENTERPRISES BY DETECTION OF PATHOGENS AND HYGIENIC INDICES

VALUTAZIONE DELLA SICUREZZA DI ALIMENTI SERVITI DA IMPRESE SISTEMAZIONI PER TURISTICHE ATTRAVERSO LA DETERMINAZIONE DI INDICI PATOGENI E IGIENICI

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ABSTRACT

The legal framework in Greece which ensures that food operations provide safe foods to consumers is described. The level of hotels' compliance with food safety legislation is reported along with the reasons that affect conformity with regulatory requirements, particularly the implementation of HACCP. The safety of foods served in hotels was assessed using the microbial quality of hot and cold meals as indicator. Food was examined for the presence of *Salmonella* spp., *Staphylococcus* coag-

RIASSUNTO

Viene descritta la struttura legale che, in Grecia, garantisce che le operazioni sugli alimenti forniscano alimenti sicuri per il consumatore. Viene riportato il livello di aderenza degli hotels agli standards di sicurezza alimentare ed alla relativa legislazione, insieme alle ragioni che influenzano la conformità con i requisiti regolamentari, in particolare con l'implementazione del sistema HACCP. La sicurezza alimentare offerta negli hotels è stata accertata utilizzando come in-

- Key words: *Escherichia coli,* food safety legislation, GHP, Greece, HACCP, hotels, *Salmonella* spp., *Staphylococcus* coagulase positive -

ulase positive and *Escherichia coli*. The colonies of the last two microorganisms were also enumerated.

dicatore la qualità microbiologica di alimenti caldi e freddi. Negli alimenti è stata valutata la presenza di *Salmonella* spp., la reazione positiva alla coagulasi da *Staphylococcus* e la presenza di *Escherichia coli*. Sono state inoltre contate le colonie degli ultimi due microrganismi.

INTRODUCTION

In Greece, law 487 that came into force in 2000 and the European law (ER 852/2004) require that all food businesses, except those involved in primary production, implement Good Hygienic Practice (GHP) in combination with the HACCP system. The Codes for Good Hygiene are published by Eniaios Foreas Elegxou Trofimon (EFET, the national Authority for Food Control, the equivalent of FSA in the UK), while the national standard for HACCP, ELOT 1416, was issued by the Hellenic organisation for standardization (ELOT). In September 2005, the new International Standard ISO 22000 was published which describes the requirements for all businesses participating in the food chain production.

Food safety is supervised by four authorities, which conduct the official controls:

- the General Veterinary Commission under the control of the Ministry of Rural Development and Food;
- the General Commission of Public Hygiene under the control of the Ministry of Health;
- the State Chemical Laboratory under the control of the Ministry of Economy and Finance;
- EFET under the control of the Ministry of Development.

Of the above-mentioned authorities, EFET is responsible for the co-ordination of the four ministries and cooperation among them.

The Department for Surveillance and Intervention (DSI) of the Hellenic Center for Infectious Disease Control (HCDSI) is responsible for the surveillance and control of food-borne infections and intoxications. The numbers of studies on the extent of HACCP implementation and the effect of HACCP on the quality of food served (BATA *et al.*, 2006; DROSINOS *et al.*, 2005) has been increasing.

The aim of this study was to determine the microbiological quality of food served by hotels, the level of their compliance with the laws 487 and ER 852/2004 and the reasons that food businesses do not comply with food regulations. One reason why this particular area of safety of foods was chosen is the number of tourists who visit Greece annually. According to the WHO Surveillance Programme for Control of Foodborne Diseases in Europe (WHO, 2002), data collected from more than 30,000 investigated outbreaks, involving a total of 17,960 cases that were reported by 42 European countries from 1993 to 1998, showed that mass catering kitchens in restaurants, cafeterias and catering services accounted for 22% of the outbreaks, being the second most frequent location where most food-borne outbreaks occur.

MATERIALS AND METHODS

Sampling and pre-treatment of samples

Two samples, one hot cooked meal (scrabbled eggs, fried eggs, fried bacon-sausage, boiled eggs, cooked beans) and one cold meal (Greek feta cheese, tomato and cucumber salad and smoked ham), chosen from among the most common foods known to cause foodborne diseases (WHO, 2002), were collected three times (12-2004, 3-2005, 5-2005) from each of nine hotels in Athens. They were examined for the presence of *Salmonella* spp., *Staphylococcus* coagulase positive and *Escherichia coli*. For the latter two microorganisms, their colonies were also counted. The temperatures of the samples during display in each establishment were measured with a digital thermometer (Ebro Electronics GmbH & Co., Kologne, Germany).

All samples were taken aseptically from the buffet, placed in sterilised plastic bags, transported to the laboratory in ice chests and analysed within three hours after sampling. The samples (25 g) were weighed aseptically in sterile stomacher bags, diluted with 225 mL buffered peptone water (BPW) (Biolife, Milan, Italy) to detect *Salmonella* spp. or with 225 mL peptone water (PW) (Biolife, Milan, Italy) to detect and enumerate *Staphylococcus* coagulase positive and *Escherichia coli* colonies and then homogenised in a Stomacher (Bagmixer 400, Interscience, Lyon, France) for 2 min.

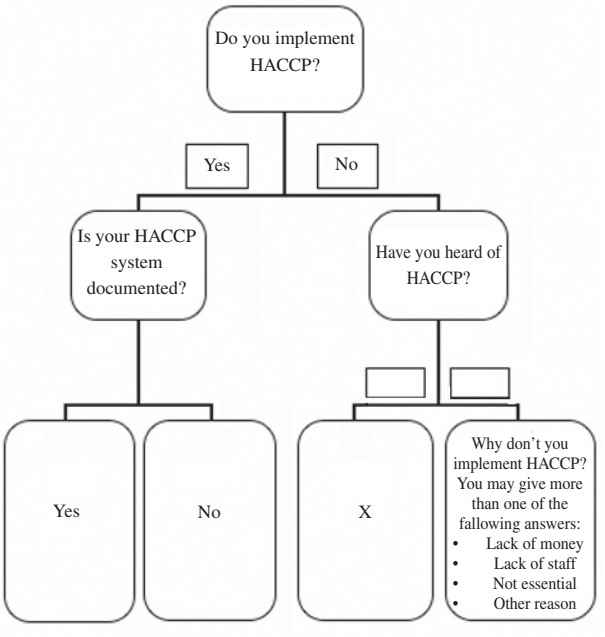


Fig. 1 - Questions for the manager.

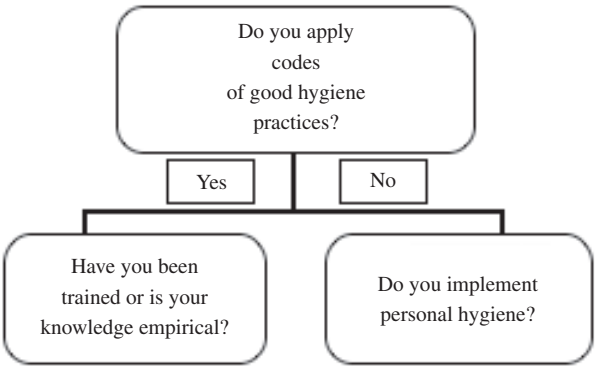


Fig. 2 - Questions for chef and personnel.

To determine the level of the hotels' compliance with laws 487 and ER 852/2004 and the reasons for limited application of the food regulations, each hotel's manager and personnel were asked to complete a questionnaire during the first sample collection (Figs. 1 and 2).

Microbiological methods

The ISO 16649-2:2001 method was used to detect and enumerate β-glucuronidase-positive *Escherichia coli*. A 1 mL portion of the homogenate in PW was inoculated in duplicate onto Tryptone Bile X-glucuronide medium (TBX) (bioMérieux, Lyon, France) according to the pour plate technique and the plates were incubated at 44°C for 24 h. The methods illustrated in the ISO 6888-2:1999

were used to detect and enumerate coagulase positive *Staphylococci*. Another 1 mL of the homogenate in PW was transferred onto sterile Petri dishes, in duplicate; molten Baird-Parker (BP) agar (bioMérieux, Lyon, France) was then added according to the pour plate technique and, after setting, the plates were incubated at 37°C for 48 h. The methods illustrated in the ISO 6579:2002 were used to detect *Salmonella*. The homogenate in BPW was incubated for enrichment at 37°C for 24 h and then subcultured either in Rappaport-Vassiliadis broth (bioMérieux, Lyon, France) and in Muller Kauffman tetrathionate/novobiocin broth (bioMérieux, Lyon, France). The tubes which contained the Rappaport-Vassiliadis broth were incubated at 41.5°C for 24 h; a portion was then streaked with a loop onto brilliant green /phenol red lactose agar (BGA) (bioMérieux, Lyon, France) and xylose lysine deoxycholate agar (XLD) (bioMérieux, Lyon, France), in duplicate. The tubes which contained the Muller Kauffman tetrathionate/novobiocin broth were incubated at 37°C for 24 h and then transferred onto the abovementioned selective solid culture mediums (BGA, XLD).

RESULTS AND DISCUSSION

The responses to the questionnaires led to the assumption that the personnel applied personal hygiene and good hygienic practices in all the hotels but in only 67% of the hotels had they attended food hygiene training courses.

Furthermore only 22% (i.e. two out of nine) of the hotels had a documented HACCP system in place and 11% (i.e. one out of nine) had an undocumented one, even though its implementation is law. While all managers had heard of the HACCP, some of the reasons given for not applying HACCP were lack of money (56% of the cases), lack of staff (11% of the cases) as well as the belief that HACCP was not essential in their business to assure the safety of their food (67% of the cases).

Regarding the results of the microbiological analysis, all samples were found to be of satisfactory quality. The numbers of *Staphylocoocus* coagulase positive and *Escherichia coli* were below the safety standard levels of the PHLS guidelines and *Salmonella spp*. was not detected in any of the samples (GILBERT *et al.*, 2000).

It is also worth mentioning that the hot and cold meals were not displayed at the temperatures recommended (>60°C for hot display, <5°C for cold display) in the Codes of Good Hygienic Practices of the National Health Authority. The measured temperatures (given as range and average with s.d. for 95% confidence limits) are reported in Table 1.

Even though temperature misuse was involved in 44% of 17960 outbreaks investigated by the joint FAO/WHO Surveillance Programme for Control of Foodborne Diseases in Europe (WHO, 2002), in the present study it did not adversely affect the microbiological quality of the food since the food was consumed in less than 4 hours and the left-overs were discarded.

The results of this study show that the majority of the "stand-alone" hotels in Greece do not apply HACCP. Nevertheless, the quality of the food served in them is high, a fact that indicates that hygienic food preparation, processing and service are crucial lines of defence in the prevention of most types of foodborne illness. This fact was also reported by BLACK *et al.* (1981).

The results of this study are in agreement with those from audits that have been carried out by EFET (EFET, 2002) on ready-to-eat meals provided by retail markets and foodservice establishments. This research which begun in 2002, is on-going. To date 350 samples have been examined for *Salmonel-*

Table 1 - Temperatures measured (range and mean values with s.d. for 95% confidence limits) in food samples.

Food	Measured temperature (in °C)	
	Range	Mean values with s.d.
Ham	9.1°C-16.5°C	13.4±1.5°C
Feta	6.2°C-20.1°C	12.9±4.5°C
Scrabbled eggs	44.0°C-89.0°C	67.1±10.1°C
Fried eggs	60.3°C-79.8°C	71.2±5.6°C
Boiled eggs	45.5°C-58.7°C	52.5±3.9°C
Bacon-sausage	57.3°C-83.2°C	69.9±9.9°C
Tomato and cucumber salad	15.7°C-19.9°C	18.0±1.7°C
Bean soup	57.6°C-70.8°C	64.3±4.3°C

la spp., *Listeria monocytogenes*, *Staphylococcus aureus* and *Escherichia coli*. *Salmonella* spp. have not been detected in any of the samples. *Listeria* was found in one cooked meat meal and as far as *Escherichia coli* and *Staphylococcus aureus* are concerned, their colonies ranged from less than the detection limit to 240,000 cfu/g and from less than the detection limit to 470 cfu/g, respectively.

Then again, the absence of pathogens and/or the below limit levels of index microorganisms is common for the hotel enterprises, which have been related to some outbreaks of foodborne diseases. The incidence of pathogenic microorganisms in the final ready-to-eat product in foodservice establishments is rare, since the application of codes for good hygiene procedures is usually sufficient. Yet the complexity of the production process and/or the lack of knowledge of food microbiology by food handlers may lead to a problematic product even if there is no such historical background. The HACCP system reduces the possibility of a sudden outbreak of foodborne illness since it establishes critical control points where hazards can be eliminated. On these grounds it is concluded that the development and the application of the HACCP system (as mandated by law 487/2000) are essential for hotel enterprises. According to the literature, as a result of the application of the HACCP system in foodservice establishments, the microbial quality of the meals served has improved considerably (LAMBIRI *et al.*, 1995; SORIANO *et al.*, 2002).

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GUIDE FOR AUTHORS

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