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PAPER

EFFECTS OF EXPOSURE TO GASEOUS OZONE AND NEGATIVE AIR IONS ON CONTROL OF EPIPHYTIC FLORA AND THE DEVELOPMENT OF *BOTRYTIS CINEREA* AND *PENICILLIUM EXPANSUM* DURING COLD STORAGE OF STRAWBERRIES AND TOMATOES

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ABSTRACT

The aim of this study is to compare the effects of gaseous treatments on the control on postharvest decay. Specifically, cold-stored tomatoes (*Solanum lycopersicum* L.) and strawberries (*Fragaria x ananassa* Duch.) were kept at 12° and 2°C, respectively, under 95% RH and exposed to Air (control), Ozone (0.5 ppm), Negative Air Ions (NAI, 6.1 x 10^6 ions/cm^3) and a NAI+Ozone mixture (0.5 ppm and 6.1 x 10^6 ions/cm^3) for 15 days. The effects of the gaseous treatments on control of epiphytic microflora and the development (disease incidence and aerial mycelial growth) of *Botrytis cinerea* Pers. and *Penicillium expansum* Link on the artificially inoculated fruits were evaluated. The examined treatments reduced superficial microbial contamination after 15 days of exposure. The best performance was achieved by the NAI+Ozone treatment combined with electrostatic filters; this treatment resulted in decreases in bacteria and yeast by 2.5 Log_{10} cfu/g. Visible mycelia and spore production on the artificially inoculated fruits were also considerably suppressed in the presence of ozone and/or NAI with a variable degree of success. The most promising results were obtained with the NAI+Ozone treatment; the disease incidence was considerably reduced, and the mean sporulation index never surpassed a value of 1, which completely inhibited spore formation. The fungistatic effect vanished when the fruits were removed from the atmosphere enriched with oxidizing agents.

- Keywords: *Botrytis cinerea*, *Penicillium expansum*, ozone, negative air ions, postharvest, strawberry, tomato -

INTRODUCTION

In recent years, increasing attention has been focused on the safety and quality of fruits and vegetables and specifically on postharvest treatments administered to reduce or to eliminate pathogens from fresh, raw produce and to increase the produce's shelf life. The numbers of produce-associated foodborne diseases and cases of illness due to food pathogens have risen significantly (TAUXE *et al.*, 1997). Moreover, the loss of fresh fruits and vegetables caused by microbial spoilage that occurs between the time of harvest and consumption is estimated to be as high as 30% (BEUCHAT, 1991).

Strawberries (*Fragaria x ananassa* Duch.) and tomatoes (*Solanum lycopersicum* L.) are important economic products in southern Italy. Italy yields 130,000 tons of strawberries every year, ranking among the most important producers of this crop in the world. These fruits are highly perishable and are susceptible to spoilage, which is primarily caused by *Botrytis cinerea* Pers. (SOMMER *et al.*, 1992; KITINOJA and KADER, 2002). Strawberries must be handled gently and stored at recommended temperatures under 90 to 95% relative humidity (RH) to prevent chilling injuries. According to common practice, high-quality fruit maintenance is ensured by using plastic film packaging and a high CO_2 concentration during a short storage period (WATKINS *et al.*, 1999; LI and KADER, 1989; WELLS and UOTA, 1970).

Additionally, Italy has produced approximately 1.85 million tons of tomatoes over the past three years. The postharvest storage of naturally ripened tomato fruits is limited, due to softening and disease development (BARKAI-GOLAN *et al.*, 1989). After tomatoes are harvested, they are more prone to bruising and subsequent attack by bacterial and fungal pathogens, such as *Botrytis cinerea*, *Penicillium expansum*, *Rhizopus stolonifer*, *Geotrichum candidum*, and *Fusarium oxysporum* (MAHOVIC *et al.*, 2002). Furthermore, tomatoes are notably sensitive to temperatures lower than 8°C, which may cause pitting and non-uniform ripening. Therefore, storage must be carried out at temperatures between 10° and 12°C under approximately 95% RH (WILLS *et al.*, 1998).

Microbial spoilage is most commonly controlled by decontaminating fruit (by washing with water containing chlorine-based disinfectants prior to storage or packaging) and/or modifying the storage and transit environment of fruits (e.g. through fungicide application) (SNOWDON, 1990). Chlorine and its derivatives are inexpensive and are effective against all types of vegetative cells. However, their use confers many serious disadvantages, such as being harmful and irritating to workers at high concentrations, being toxic to the environment (BELLAR *et al.*, 1974; TRUSSELL and UMPHRES, 1978) and demanding high water volumes for post-treatment rinses. Moreover, environmental and health organizations have expressed concerns regarding the formation of byproducts from their use, such as trihalomethanes and haloacetic acids, which are mutagenic and carcinogenic. Additionally, these substances can contaminate drinking water and food, which adversely affects public health and the environment (native aquatic and terrestrial species) (TRUSSELL and UMPHRES, 1978).

Some commodities, such as soft fruits, stone fruits, grapes, strawberries and other berries, are easily damaged by water dipping and drenching; therefore, they are more commonly gassed with antimicrobial agents, such as sulfur dioxide (for table grapes), which are toxic to consumers and the environment. In addition, large amounts of synthetic fungicides are currently used in orchards to prevent and treat pathogen infections. Many of these chemicals are now ineffective, due to the evolution of pathogen-resistant strains (SAPERS, 1998; SMILANICK, 2003). Furthermore, these chemicals leave residues on the fruit surface and ultimately affect public health. Therefore, there is growing interest in pesticide toxicity, due to health and environmental concerns. However, commercial losses remain significant because current procedures are ineffective (SPOTTS and PETERS, 1980; SAPERS, 1998).

Therefore, there is interest in developing safe and effective residue-free disease control strategies, including the application of gaseous or aqueous ozone (NEFF, 1998; RICE, 1999). In 1997, ozone was affirmed as being generally recognized as safe (GRAS) as a disinfectant for foods by an independent panel of experts (GRAHAM, 1997) sponsored by the Electric Power Research Institute (EPRI). In Europe and the US, ozone is currently used in potable water treatment plants. The US Food and Drug Administration has approved gaseous and aqueous ozone phases as a food additive for food treatment, storage and processing (Federal Register, 2001). Ozone is one of the most potent oxidants that are known. Ozone's standard reduction potential (E°) is +2,076 V (MANLEY and NIEGOWSKI, 1967), and it is effective against a much wider spectrum of microorganisms than are chlorine and other disinfectants (NEFF, 1998). Moreover, ozone leaves no detectable chemical residues inside or on the surface of treated products (GRAHAM, 1997; RICE, 1999). Ozone is safer to use than many other chemical treatments, due to its relatively short half-life (minutes in the aqueous phase and approximately 30 min in the gaseous phase). Furthermore, ozone decomposes into diatomic oxygen, it has a characteristic odor and is not recognized as a carcinogen or mutagen.

Among the other advantages of ozone, it is noteworthy that it can destroy pesticides, chemical residues (WU *et al.*, 2007; HWANG *et al.*, 2001; ONG *et al.*, 1996) and mycotoxins (CATALDO, 2008; INAN *et al.*, 2007; YOUNG *et al.*,

2006) that exist on food surfaces. Ozone can also slow the fruit and vegetable ripening process. Treatment of fruits and vegetables with gaseous phase ozone has principally been used to increase shelf-life (LOVINO *et al.*, 2007; RICE *et al.*, 1982; NORTON *et al.*, 1968).

Several studies have demonstrated that ozone gas prevents spore production and the germination of some fungi (HARDING, 1968; KRAUSE and WEIDENSAUL, 1977; PALOU *et al.*, 2002, 2003, 2006; NADAS *et al.*, 2003; KARACA and VELIOGLU, 2007; TZORTZAKIS *et al.*, 2007, 2008). The normal infection cycle resumes when fruits are removed from an ozone-enriched atmosphere. High ozone concentrations are required to kill rapidly fungal mycelia or pathogenic spores that cause various diseases (such as blue mold, sour rot, and green mold) (SMILANICK, 2003). However, excessive ozone concentrations oxidize food surfaces (especially in products that have a low ozone tolerance), which results in discoloration and deterioration of flavor and hedonic characteristics (RICE *et al.*, 1982). In addition, high ozone concentrations require corrosion-resistant facilities and appropriate safety measures (the current Threshold Limit Value - Short Term Exposure Limit that has been established by the US Occupational Safety and Health Administration for gaseous ozone is 0.3 ppm).

Although the sanitizing effects of Negative Air Ions (NAI) on living organisms have been debated, researchers agree that NAI can be bacteriostatic (KRUEGER and REED, 1976; CHARRY and KAVET, 1987; REITER, 1993; TANIMURA, 1997). NAI have been shown to reduce airborne microbial contamination in dental clinics (GABBAY *et al.*, 1990), to reduce Salmonella contamination in chicken hatcheries (GAST *et al.*, 1998) and to control the airborne spread of viruses in poultry houses (MITCHELL and KING, 1994). Additional studies (TANIMURA *et al.*, 1998, 1999; FAN *et al.*, 2002) have reported improvement of the sanitizing activity of ozone against certain bacteria when used in conjunction with NAI; however, the mechanism of this synergistic action was not explained. TUFFI *et al.* (2009) obtained promising results regarding the *in vitro* development of *B. cinerea* and *P. expansum*. This synergism could improve the efficacy of ozone treatment; equally promising, it could enable the use of lower ozone doses.

Finally, various food components have an important effect on the antimicrobial activity of ozone (and probably also NAI). Some authors (RESTAINO *et al.*, 1995 and GUZEL-SEYDIM *et al.*, 2004) have shown that the presence of such substances as fat and protein may compete with microorganisms for ozone. The precise mechanism that explains the protective effects of these substrates is unclear. However, it has been hypothesized that ozone more readily oxidizes substrate components than microbial cells. Therefore, the best results from using ozone and NAI are apparently achieved in water-rich substrates, such as fruits and vegetables.

The aims of this study are to compare the disinfecting action of ozone and NAI on epiphytic flora and two phytopathogenic fungi of fresh tomatoes and strawberries and to verify the existence of a synergistic sanitizing effect of the NAI+Ozone mixture in reducing food-borne disease and decay.

MATERIALS AND METHODS

Pilot plant

The experiments were conducted in a stainless steel pilot plant (prototype from Ionex s.r.l.), which is shown in Fig. 1. The plant had a volume of 1 m^3 and consisted of a treatment chamber and a fan to generate airflow. The input air was ambient air that was first enriched with ozone

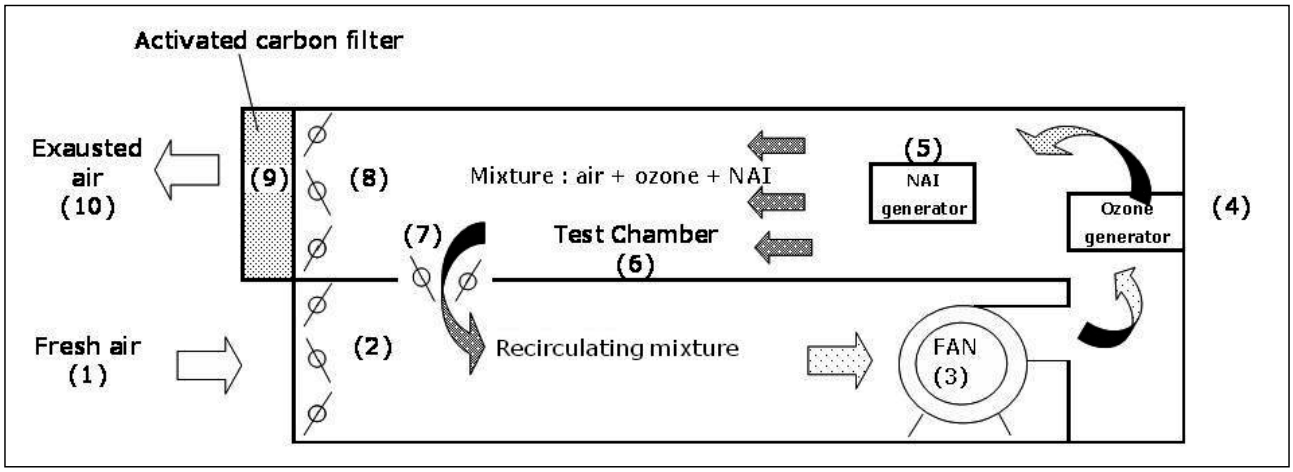


Fig. 1 - Pilot plant scheme: (1) inlet air, (2) inlet air gate, (3) ventilation fan, (4) -8 kV corona discharge ozone generator, (5) -12 kV corona discharge NAI generator, (6) test chamber, (7) recirculation air gate, (8) outlet air gate, (9) activated carbon filter and (10) outlet air.

using a corona discharge generator (-8 kV ozone generator) and subsequently enriched with NAI using a corona discharge generator (-12 kV NAI generator). The gas mixture was recycled into the test chamber using a fan. The output air was filtered through an activated charcoal filter. The ozone and NAI concentrations in the test chamber were set by regulating the inlet and outlet valves, which adjusted the quantity of ozone-free fresh air that was introduced into the pilot plant. The plant, which operated with partial air recirculation, was installed in a 20 m^3 industrial cold storage room maintained at 2°±1°C for strawberries and 12°±1°C for tomatoes with a high relative humidity (95 - 98% RH).

Inside the treatment chamber, a 0.7 m/s air flow was maintained, and the ozone and NAI concentrations were set at 0.5 ± 0.05 ppm (v/v) and approximately 6.1 x 10^6 ions/cm^3, respectively. The ozone concentration was monitored continuously using an ozone analyzer (model OS-4, Eco Sensors, Inc., Santa Fe, New Mexico, USA), while the NAI concentration was measured with an ion detector (Air Ion Counter, Alpha Lab, Inc., Salt Lake City, UT, Usa). A thermo-hygrometer (model 635, Testo, Milan, Italy) was used to control the temperature and air humidity in the pilot plant and in the cold storage room.

Fruit

Strawberry and tomato fruits were purchased from a local market (Andria, BA, Italy) and were stored for one day at 2° and 12°C, respectively, at 95 - 98% RH under plastic bags to prevent weight loss before the application of any postharvest treatment. The berries were selected based on quality, uniformity of size and ripeness. The berries were subsequently randomized and sorted in plastic trays before being placed into the treatment chamber of the pilot plant. Four replicates (trays) containing 20 fruits inoculated with *Botrytis cinerea* or *Penicillium expansum* and uninoculated berries (for the epiphytic flora experiment) were exposed continuously for 15 days to one of the following treatments: Air (control), Ozone, NAI and NAI+Ozone. Both the fruits inside each tray and the trays inside the treatment chamber were placed randomly. The experiments addressing *B. cinerea* and *P. expansum* were performed separately, and each treatment was conducted twice.

Epiphytic flora experiment

To observe the effect of the treatments on bacterial and yeast growth on the food surface, three samples (A, B and C, which contained 5 or 6 berries each) were evaluated at the beginning and end of each test. The samples were transferred into sterile bags, and a sterile physiologic solution (0.9% w/v) was added to produce a 1:3 dilution. The bags with the samples were subsequently shaken for 30 minutes at approximately 150 rpm. One milliliter of the shaken sample was serially diluted in 9 mL of sterile physiologic solution. The serially diluted samples were spread (0.1 mL) in duplicate onto plate count agar (PCA) dishes for the mesophilic bacteria and Rose Bengal Agar containing chloramphenicol for the yeast. The PCA plates were incubated at 30°C for 2 days, whereas the Rose Bengal Agar plates were incubated at 25°C for 5 days. Four experiments were performed for each treatment.

To investigate the effects of the NAI+Ozone treatment on tomatoes, an additional epiphytic flora experiment was performed in which the ozone and NAI concentrations remained constant. Two electrostatic filters (-9 kV per filter), which were assembled in series, were added to the pilot plant to verify whether it was possible to improve the system's performance.

Decay development experiments

Pure cultures of *Botrytis cinerea* Pers. and *Penicillium expansum* Link were kept refrigerated in test tubes. Before each experiment, young cultures were prepared by incubating the fungi in Petri dishes (90 mm in diameter) containing potato dextrose agar (PDA) medium at 25°C for 10 days (5 days under lamp light and 5 days in the dark). Eight milliliters of sterile water and 1-2 drops of Tween-80 were added to each dish, and spores were gently removed from the agar surface with a sterile loop. The resulting high-density spore suspension was filtered through a funnel containing two layers of sterile cotton lint in a flask after which the density of the suspension was measured with a hemocytometer (Blau Brand, Wertheim, Germany) and diluted with sterile water to yield a density of 10^7 spores/mL.

Strawberries and tomatoes were inoculated by briefly immersing a sterile needle in a test-tube containing either a *B. cinerea* or *P. expansum* spore suspension following which each berry was wounded once with the needle between its equator and calyx. The wound was approximately 1 cm long and 2-3 mm deep to prevent mold growth inside the berry where the gaseous agents could not penetrate (PALOU, 2001). The storage time for each experiment was fixed at 15 days, and samples were collected on days 2, 6, 8, 10, 12 and 15 for the strawberries and at days 2, 6, 9, 11, 13 and 15 for the tomatoes. Disease incidence (the percentage of infected berries) and severity were monitored at each sampling. A sporulation index was used to quantitatively determine the development of mycelial aerial growth on the surface of inoculated fruit. Values of 0, 0.5, 1 and 2 indicated the following conditions: the presence of soft lesion but the absence of spores and mycelium, the presence of mycelium but the absence of spores, and either < 5% or 5 - 30% of the fruit surface being covered with spores, respectively (ECKERT and BROWN,

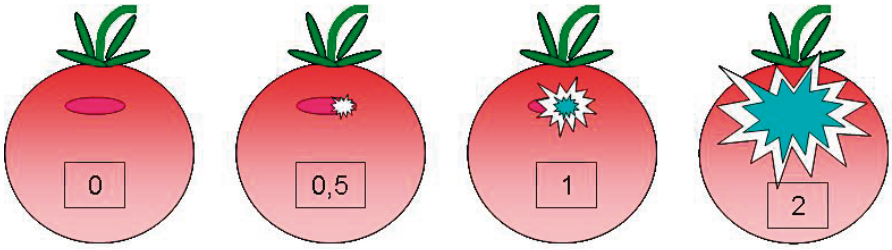


Fig. 2 - Example of the sporulation index for *P. expansum* on a wounded fruit at different stages of development.

1986; PALOU *et al.*, 2003) (Fig. 2). To evaluate post-treatment fungistatic effects, the strawberries that had been subjected to the NAI+Ozone treatment for 15 days were removed from the enriched atmosphere and stored for three additional days in a cold room under an air atmosphere with the same temperature and RH conditions as used in the experiment.

Statistical analyses

The data were subjected to analysis of variance (ANOVA), and the means were separated by Duncan's multiple range test (at $P<0.05$) to determine the presence of significant differences in disease incidence, the sporulation index and proliferation of epiphytic flora with respect to the mean value of the experiments. The percentage data were arcsine square-root transformed prior to ANOVA.

RESULTS

Epiphytic flora experiment

The results of the epiphytic flora evaluation are reported in Table 1. Increases in the microbial load in Air (control) depended on the proliferation of microorganisms on a berry's surface and on airborne contamination. In almost all cases, the treatments with sanitizers reduced microbial contamination. The yeasts were more resistant to the sanitizers, especially to NAI. Low temperature slowed the proliferation of mesophilic bacteria and yeast in Air and improved the antimicrobial activity of the gaseous treatments. The NAI+Ozone treatment acted synergistically in three of the four cases (underlined values). An even stronger result is achievable using electrostatic filters; in this case, the NAI+Ozone treatment reduced the numbers of epiphytic microorganisms present by 2.5 $\log_{10}$ cfu/g for both the bacteria and yeasts, which produced a notably lower value compared to the results reported in Table 1. There are two possible explanations for this positive result: the electrostatic filters prevented the airborne biological contaminants from reaching the surface of the berries and the removal of dust particles and/or microorganisms from the air stream increased the amounts of ozone and NAI present, which enhanced their action against the epiphytic flora.

Table 1 - Variations in epiphytic microorganism populations ($\Delta\log_{10}$ cfu/g = $\log_{10}$ cfu/g at the final time - $\log_{10}$ cfu/g at the initial time) after 15 days of exposure of strawberry and tomato fruits to Air, NAI, Ozone and NAI+Ozone. The values in the same row that share a common letter are not significantly different according to Duncan's test ($P<0.05$). The underlined values show a synergistic effect of the NAI+Ozone treatment.

		Air	NAI	Ozone	NAI+Ozone
Strawberry	Bacteria ($\Delta\log_{10}$ cfu/g)	+0.2 a	-1.1 b	-1 b	-1.3 b
	Yeasts ($\Delta\log_{10}$ cfu/g)	+0.2 a	+0.1 a	-0.1 b	<u>-0.7 c</u>
Tomato	Bacteria ($\Delta\log_{10}$ cfu/g)	+2.6 a	-0.1 b	-0.2 b	<u>-0.5 c</u>
	Yeasts ($\Delta\log_{10}$ cfu/g)	+1.6 a	+0.2 b	-0.1 c	<u>-0.4 d</u>

Decay development experiments – Strawberry

The results for the strawberries inoculated with *B. cinerea* during cold storage (2°C, under 92 - 98% RH) in the pilot plant are shown in Fig. 3. The graphs indicate the number of strawberries (expressed as a percentage) infected (Fig. 3a) and the mean sporulation index (Fig. 3b) of the different treatments as a function of the storage period. In the Air treatment, traces of gray mold had already appeared after two days of storage (Fig. 3a). The number of infected strawberries increased considerably on the sixth day (more than 30% of the strawberries were rotted), and more than 75% of the fruits were infected on the last sampling date. During storage, the percentage of rot was always lower in the NAI, Ozone and NAI+Ozone treatments; however, in the last control, 70% of the NAI-treated strawberries rotted (NAI was not statistically different from the control), while Ozone and the combination of NAI+Ozone reduced the incidence of rot to 40 and 20%, respectively. NAI+Ozone acted synergistically to reduce rot, which resulted in a detectable difference compared with the single treatments of NAI and Ozone. The mean sporulation index

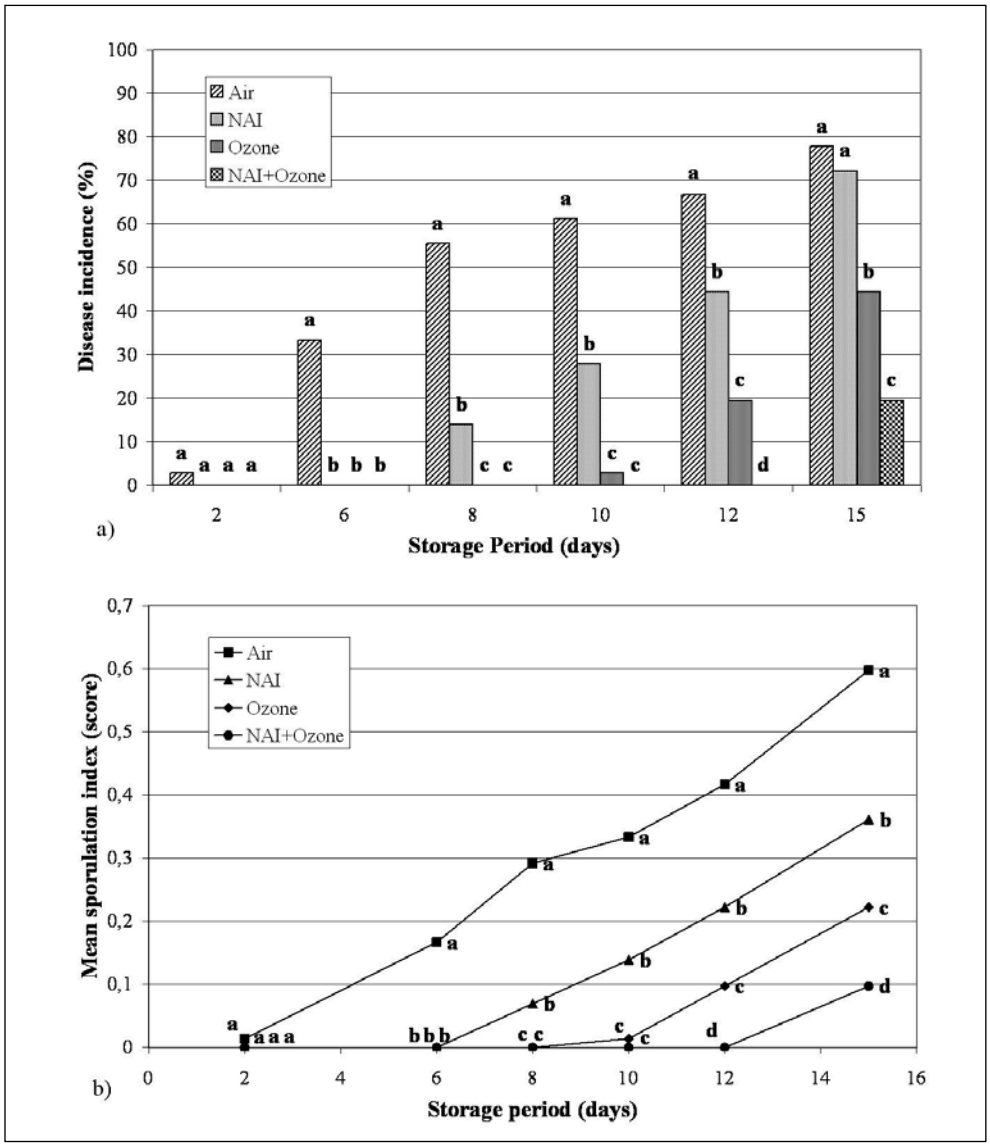


Fig. 3 - Disease incidence (a) and mean sporulation index (b) of strawberries inoculated with *B. cinerea* stored at 2°C under 95-98% RH and subjected to the following treatments: Air, NAI, Ozone and NAI+Ozone. Values that contain different letters indicate a statistically significant difference (P≤ 0.05) between the different treatments according to Duncan's test.

was significantly lower under the NAI, Ozone and NAI+Ozone treatments (Fig. 3b). In addition, by analyzing the single values constituting the mean index, no fruit sample showed any trace of conidia under any oxidizing treatment over the entire test period. The beneficial effect of NAI, Ozone and NAI+Ozone is much more remarkable because sporulation phenomena occur under the Air treatment; index values of 1 and 2 were achieved for nearly 30% of the samples. No treatment completely inhibited disease incidence, but each treatment significantly reduced growth and thoroughly inhibited fungal sporulation. In conclusion, fungal growth inhibition was observed to last only for the duration of the treatment period; i.e., no fungistatic effect was detected at the end of the treatments (after 15 days), even for the NAI+Ozone treated fruit (data not shown).

The incidence and severity of the blue mold disease caused by *P. expansum* are illustrated in Figs. 4a and 4b, respectively. When examining these graphs, it is noteworthy that the developmental temperature limit for *P. expansum* is 0°C, while the limit for *B. cinerea* is -4°C. Therefore, cold storage at 2°C will presumably enhance the sensitivity of *P. expansum* to fungistats. This hypothesis is apparently confirmed by the results presented in Fig. 4 because these values appear to be lower than those reported in Fig. 3. In the Air treatment, mycelia only occurred on the 8th day. The percentage of infected fruits increased with time; after 15 days, a number of spores were detected on approximately 10% of the fruits that contained mycelium. Additionally, a greater number of infected fruits were observed in comparison with *B. cinerea*, although they displayed less devel-

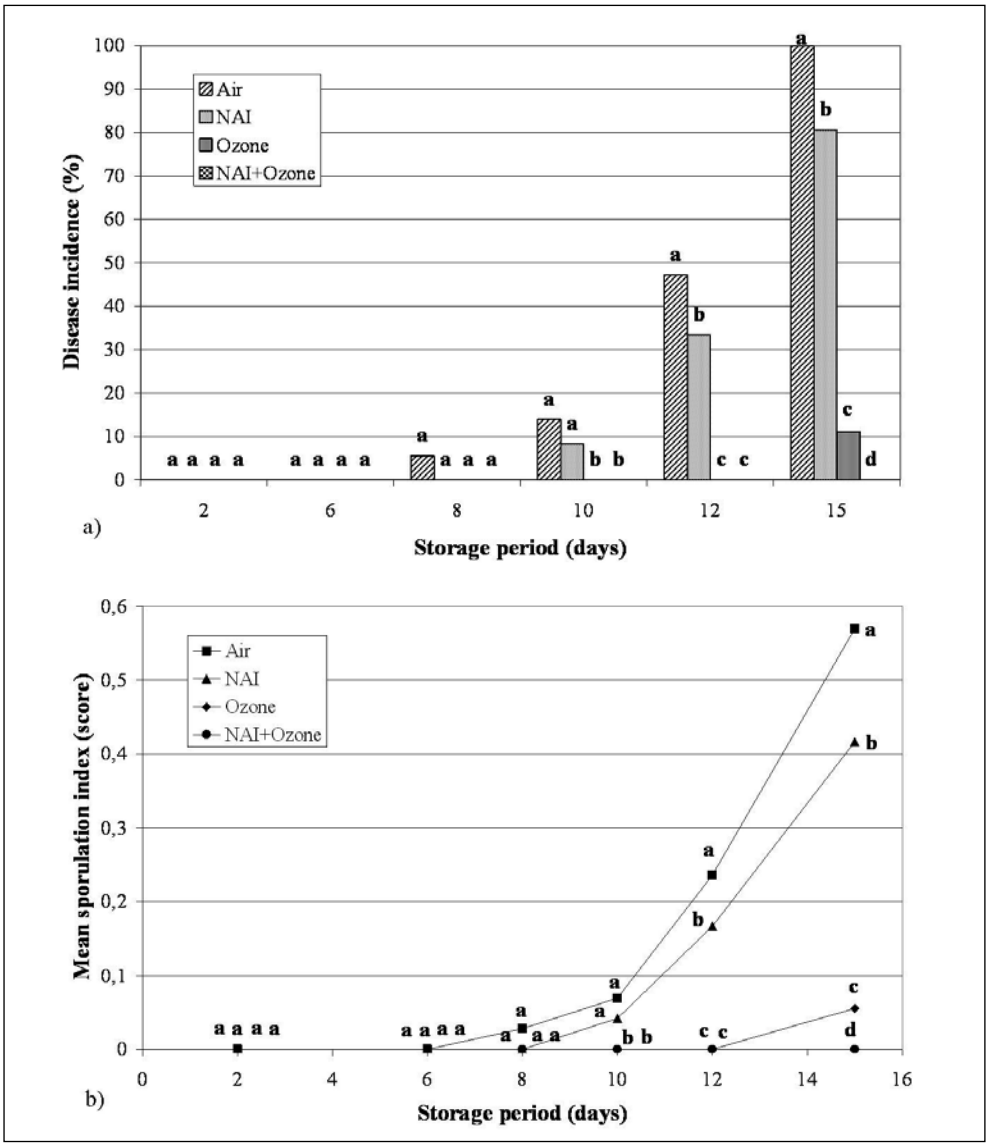


Fig. 4 - Disease incidence (a) and mean sporulation index (b) of the strawberries inoculated with *P. expansum* stored at 2°C under 95-98% RH and subjected to the following treatments: Air, NAI, Ozone and NAI+Ozone. Values that contain different letters indicate a statistically significant difference (P≤ 0.05) between the different treatments according to Duncan's test.

opment and less spore production. In the NAI treatment, mycelia formed on the 10th day, and sporulation was apparent in a small portion of the strawberries on the 15th day. Clearer improvement was observed for the Ozone and NAI+Ozone treatments. During the 15-day storage period, the NAI+Ozone mixture completely inhibited the germination and growth of blue mold. However, this inhibition is reversible, even for *P. expansum* (data not shown).

Finally, for both of the molds, the gap between the fungistatic treatments and the control tended to increase with time because the different growth rates overcame the differences in mold development over time. For example, for *P. expansum*, no remarkable difference was noted until the third sampling. As the days passed, this gap became even more statistically significant.

Decay development experiments – Tomatoes

The incidence and development of gray mold on the inoculated tomatoes increased much more rapidly than on the strawberries (Figs. 5a and 5b), which probably occurred due to the higher storage temperature (12°±1°C). In fact, more than 30% of the Air-exposed tomatoes were already infected after 2 days, and more than 90% were infected at the last sampling. The NAI treatment did not affect the disease incidence during the 15-day exposure period (Fig. 5a). However, delayed fungal development and sporulation were observed during long exposure periods (Fig. 5b). Significant improvements were noted for the Ozone and NAI+Ozone treatments ever since the first sampling. In the Ozone treatment, formation of mycelia was detected on the sixth day. Subsequently, the

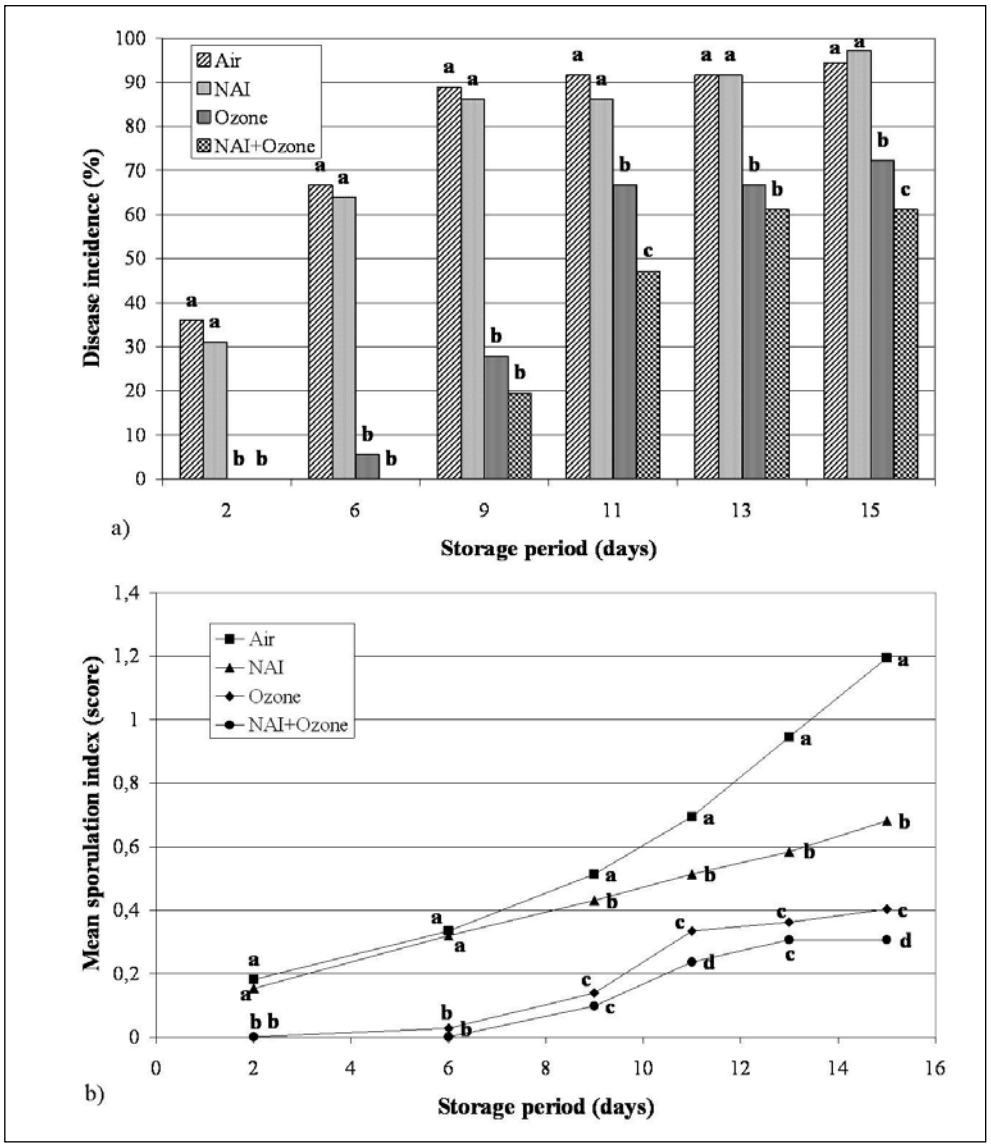


Fig. 5 - Disease incidence (a) and mean sporulation index (b) of the tomatoes inoculated with *B. cinerea* stored at 2°C under 95-98% RH and subjected to the following treatments: Air, NAI, Ozone and NAI+Ozone. The values that contain different letters indicate a statistically significant difference (P≤ 0.05) between the different treatments according to Duncan's test.

number of infected berries increased progressively, but sporulation was observed on few tomatoes at the fifth sampling, and this number did not increase at the last sampling. Finally, in the NAI+Ozone treatment, formation of mycelia was observed on several tomatoes on the ninth day (with a delay of 5 days in comparison with Air), and the disease incidence was greater than 60% at the last sampling without achieving sporulation. NAI+Ozone acted synergistically only for disease incidence, which is similar to the results obtained for the strawberries. In the last test, an abnormal *B. cinerea* conformation characterized by a short, compact, dark mycelium was observed (Fig. 6b). PALOU (2002) noted a similar conformation of *B. cinerea* hyphae growth on inoculated peaches during cold storage under 0.3 ppm ozone. Once they had formed, the mycelia did not expand on the surface of the berries; instead, they maintained their initial dimensions for the duration of the test.

Fig. 7 displays the results for the tomatoes inoculated with *P. expansum*. A higher storage temperature favored the germination and development of blue mold of this fungus. In fact, the formation of mycelia had already occurred in the first sampling under Air exposure (Fig. 7a). In the second sampling, the number of infected tomatoes surpassed the number of healthy tomatoes, and formation of the first spores was observed. The number of infected berries and the dimensions of the infection increased steadily, and on the last day, more than 60% of the inoculated tomatoes exhibited a sporulation index of 2. In keeping with the results for *B. cinerea*, the NAI treatment did not produce successful results with respect to

controlling disease incidence. Improvement of the sporulation index was only observed for long exposure times (Fig. 7b); NAI halted the expansion of superficial mold, and sporulation was greatly inhibited.

The Ozone and NAI+Ozone treatments produced better results against *P. expansum* than *B. cinerea*. In the Ozone test, formation of mycelia was only observed on the ninth day (less than 5%), which is shown in Fig. 7a. This level increased continually at a slow rate, but no sporulation occurred (Fig. 7b). In the NAI+Ozone test, further improvement and a synergistic effect for disease incidence were observed (Fig. 7a); mycelia were observed to be growing on a small percentage of berries in the fourth sampling, with no significant increase being detected until the end of the test. The mean sporulation index values were even lower than in the Ozone treatment (Fig. 7b).

For the NAI+Ozone and Ozone treatments and, to a lesser degree, the NAI treatment, the tomato samples infected with *B. cinerea* and *P. expansum* exhibited a smaller damaged surface area compared to the control samples (Fig. 6). This finding was probably due to the different activities of the fungal peptolytic enzymes. As for the strawberries, the difference in the sporulation index among the treatments for the tomatoes became progressively evident as time progressed. This result was particularly true for the NAI treatment, which was performed similarly to the Air treatment in the first two samplings for *B. cinerea* and in the first three samplings for *P. expansum*.

DISCUSSION

NAI and ozone reduced the epiphytic flora on tomatoes and strawberries. A strong synergism was observed between ozone and NAI for microbial cell death, and the degree of this effect probably varied based on the microbial species (FAN *et al.*, 2002). SOBSEY (1989) reviewed studies performed to inactivate health-related microorganisms in water using several disinfectants and concluded that gram-positive bacteria were more resistant to these treatments than gram-negative bacteria. When electrostatic filters were used, an improvement was observed because the incidence of dust particles and airborne microorganisms decreased, while the concentration of sanitizers increased. Researchers agree that a higher concentration of the gas results in greater effects (SINGH *et al.*, 2002; KOWALSKI *et al.* 1998). NAI and ozone can be efficiently used to reduce pathogens and food decaying microorganisms. However, it is critical to understand what sanitizer concentrations and exposure times can be used to avoid oxidizing food constituents.

Although NAI and ozone exhibited a microbicidal effect on vegetative bacterial and yeast

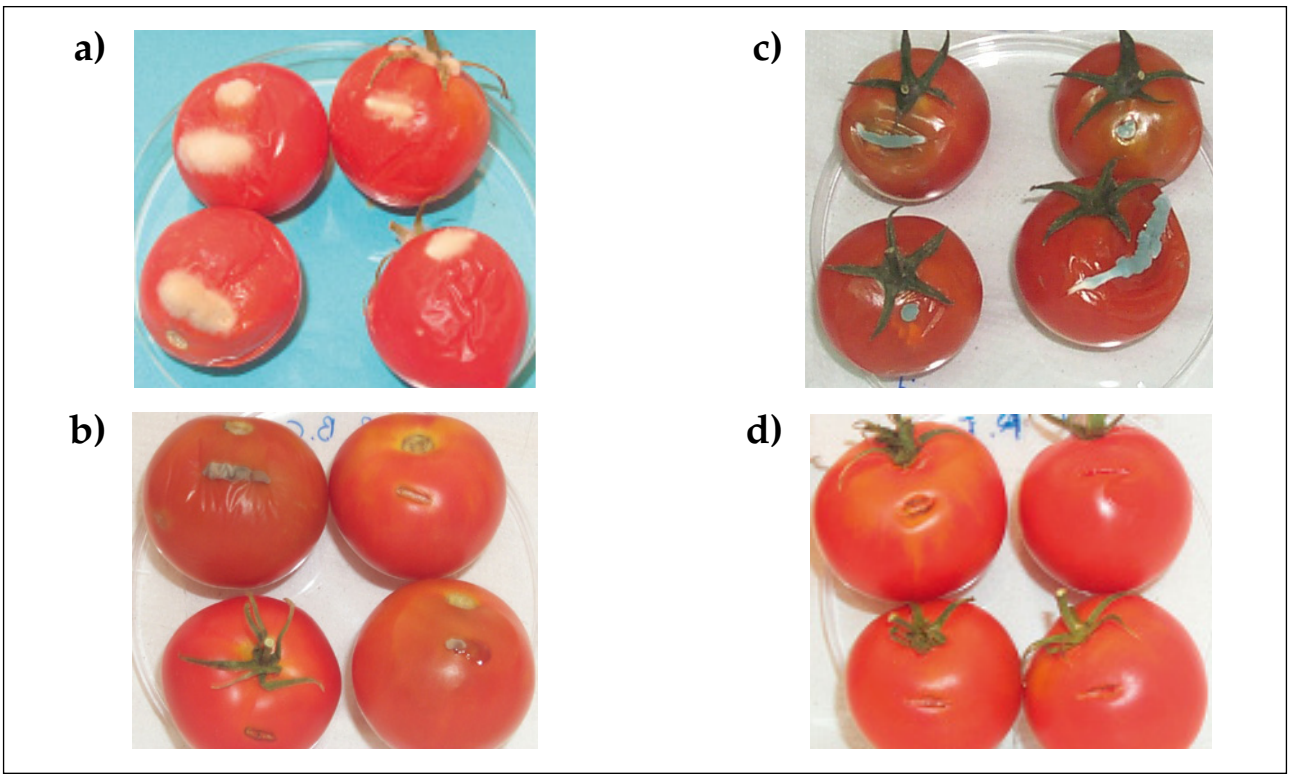


Fig. 6 - Table tomatoes inoculated with *B. cinerea* and exposed for 15 days to Air (a) or NAI+Ozone (b). Table tomatoes inoculated with *P. expansum* and exposed for 15 days to Air (c) or NAI+Ozone (d).

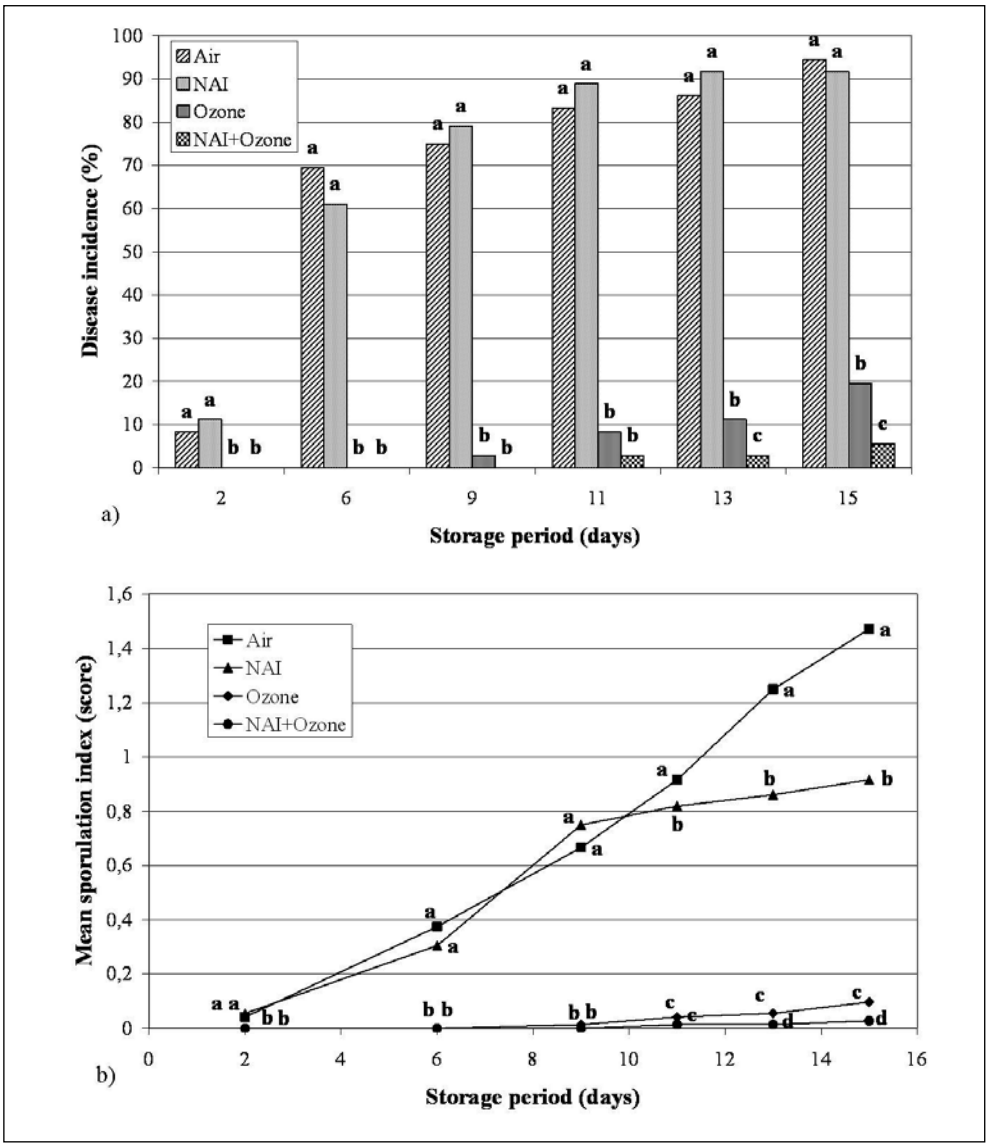


Fig. 7 - Disease incidence (a) and mean sporulation index (b) of tomatoes that were inoculated with *P. expansum* stored at 2°C under 95-98% RH and subjected to the following treatments: Air, NAI, Ozone and NAI+Ozone. The values that contain different letters indicate a statistically significant difference (P≤ 0.05) between the different treatments according to Duncan's test.

cells, a fungistatic effect was only observed for fungi. TUFFI *et al.* (2009), who reported similar results from *in vitro* experiments, concluded that these agents achieved reversible inhibition of the fungi. However, an important improvement was observed in the ability of NAI and ozone to inhibit fungal development based on comparing these *in vivo* results with previous *in vitro* results. In the current experiments, despite the inability to completely control decay, the tested sanitizing agents (primarily the NAI+Ozone mixture) inhibited germination and normal aerial growth of mycelia and greatly reduced sporulation from lesions among the infected fruits. In fact, the adopted *in vitro* experimental conditions were optimal for the growth of microorganisms. The fungal colonies were subjected to the treatments when they were already completely developed under the most favorable growth conditions. This finding was observed because in addition to nutrients, the culture media provided physical protection to the hyphae that prevented the agents from reaching them executing their action (PALOU *et al.*, 2001). Among other properties, the importance of the type of interactions between microorganisms and the substrate on which they grow is well known. TZORTZAKIS *et al.* (2007; 2008) suggested that the better results observed *in vivo* in comparison with those observed *in vitro* occurred due to ozone, which probably induced changes in fruit-pathogen interactions and suppressed mold development. Furthermore, when the pathogens penetrate deeply into a wound or remain under the surface of a fruit, gaseous ozone and ozone in water cannot effectively perform their oxidant activities. Ozone treatment did not control postharvest infection in wounds on table grapes (SHIMIZU *et*

al., 1982), apples (SCHOMER and MCCOLLOCH, 1948), pears (SPOTTS and CERVANTES, 1992), citrus (HOPKINS and LOUCKS, 1949; SMILANICK *et al.*, 1999), and other commodities (SPALDING, 1966, 1968; OGAWA *et al.*, 1990). The fungal structures inside a wound were apparently protected from the oxidant effects of ozone; this likely occurred due to a reduction in ozone penetration, an interaction of Ozone with the tissue or the components of the fruit and the presence of antioxidants that reduced the effective ozone concentration at the site of action. However, several of these factors are similar to those that explain the failure of other strong oxidant agents, such as chlorine and chlorine dioxide, in controlling infections inside wounds (SPOTTS and PETERS, 1980; ADASKAVAGE, 1995).

In all of the tests, NAI guaranteed a certain degree of inhibition, but ozone seemed to be primarily responsible for the observed fungistatic activity, which became more evident as the temperature increased. Only minimal differences were detectable in the disease incidence between Air and NAI exposure at 12°C, as well as for the mean sporulation index and disease incidence between Ozone and NAI+Ozone. This result indicates that the contribution of NAI to the fungistatic effect was notably low. NAI was probably more effective under conditions in which the fungi were already weakened by limited growth conditions (for example, low temperature). Conversely, when the temperature increased, the fungi were more resistant to the fungistatic effect of NAI. In a similar but less evident manner, ozone activity also decreased at higher temperatures; the improvement obtained for the tomatoes inoculated with *P. expansum* was clear. Therefore, temperature appears to be a decisive parameter. Although the fruit products were different, and the response may be affected by the fruit species tested, optimal performance was obtained at a low temperature. The NAI+Ozone treatment produced the best results with respect to disease incidence and growth, and it was the only treatment that completely inhibited the sporulation of *B. cinerea* and *P. expansum* on both strawberries and tomatoes. A synergistic effect was observed only on disease incidence when NAI alone did not have any effect. In addition, different microorganisms may display different susceptibilities to ozone and NAI due to their structure, conformation and general characteristics (KHADRE *et al.*, 2001). *P. expansum* was clearly more sensitive to NAI and/or ozone than *B. cinerea*, and this effect was also observed in an *in vitro* experiment (TUFFI *et al.*, 2009).

The ozone and NAI concentrations in a storage room are dependent on the environmental conditions and the amount of stored produce. Ozone and NAI concentrations decrease rapidly when the temperature, humidity or food load increases. The results obtained in this study demonstrated a synergistic effect between ozone and NAI at low temperatures. The best results were obtained for strawberries, which were stored at a lower temperature than the tomatoes. Humidity is also important for the activities of ozone and NAI. SMILANICK (2003) observed that killing spores of some pathogens (*P. digitatum, P. italicu*, and *Geotrichum citri-auranatii*) in dry air (35% RH) required a dose that was 5 to 10 times higher than the dose in humid air (95% RH). Ozone and NAI likely react with water vapor to form additional reactive compounds.

NAI and ozone greatly reduced the sporulation from lesions in infected fruits after development of the lesions. Growth and sporulation were inhibited only as long as the gaseous oxidants were present; this result is consistent with those of other authors (PALOU *et al.*, 2001; HARDING, 1968; KRAUSE and WEIDENSAUL, 1977). Fungi grew quickly and sporulated when infected fruits were removed from the fungistat atmosphere. Nevertheless, the lack of spores on fruits and vegetables that occurs while products are stored in the presence of ozone and NAI gases could be greatly advantageous because an infected fruit will be prevented from contaminating adjacent healthy fruits, packages and the walls and floors of rooms, which can cause the loss of entire production lots. Furthermore, a lack of sporulation on decayed, stored fruit could be particularly useful for reducing the use of fungicidal treatments and preventing the proliferation of fungicide-resistant pathogen strains. Cold-stored fruits have generally been previously treated with fungicides in the packing line. Therefore, many spores that are generated during storage are derived from resistant strains. Any practice that reduces inoculum production on decayed fruit following fungicide treatment can be effective for preventing or managing resistance in target pathogen populations (PALOU *et al.*, 2001). In addition, the use of ozone and NAI can decrease the load of airborne microorganisms in a storage facility and subsequently reduce the amount of inoculum available for the reinfection of stored produce. Finally, ozone and NAI slow the fruit and vegetable ripening process and oxidize ethylene gas, which is a growth hormone in many fruits and vegetables (RICE, 2002; PALOU *et al.*, 2001; DICKSON *et al.*, 1992).

Despite the favorable results obtained in these experiments, it is necessary to consider that the exposure of the berries to the sanitizers has been optimized. The efficacy of the gases could decrease when the fruits are stored under commercial conditions (stacked in pallets or packed in cardboard boxes and polyethylene bags) in which the exposure of fruit to ozone and/or NAI could be reduced. In industrial cold storage rooms, it will probably be necessary to use higher concentrations of oxidant agents and open boxes or bins that contain large vents to ensure good penetration of ozone and NAI at concentrations

that are sufficiently high to reach the stocked product in the internal portions of the packaging (HARDING, 1968; PALOU *et al.*, 2003). Furthermore, NAI has a low mobility (DANIELS, 2002). In fact, we found that ions concentration decreased rapidly away from the NAI source (TUFFI, 2007). This finding explains why only a portion of the treatment chamber was used such that the position of the plates inside the pilot plant did not affect the results. A large number of NAI generators will probably be required on an industrial scale to ensure a uniform ions distribution among the fruit products. In future studies, the impact of current commercial conditions on the efficacy of ozone and NAI during cold storage will be evaluated.

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PAPER

BIOCHEMICAL AND TECHNOLOGICAL PROPERTIES OF *PENICILLIUM ROQUEFORTI* AND *GEOTRICHUM CANDIDUM* STRAINS ISOLATED FROM CABRALES, A SPANISH TRADITIONAL, BLUE-VEINED, STARTER-FREE CHEESE

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ABSTRACT

Autochthonous starters are thought to increase the quality and safety of traditional cheeses while preserving their typicity. In order to select appropriate ripening cultures, 12 *Penicillium roqueforti* and 10 *Geotrichum candidum* strains isolated from a Spanish, traditional, blue-veined cheese were subjected to different biochemical and technological assays. All strains were weakly proteolytic, whereas all *P. roqueforti* and four *G. candidum* strains proved to be lipolytic. None of the strains produced biogenic amines in broth media containing the required amino acid precursors. Under simulated cheese conditions (10°C, pH=4.5, 5% NaCl), test strains showing technological performances superior to those of commercial strains currently in use were readily identified.

- Keywords: *Penicillium roqueforti*, *Geotrichum candidum*, cheese, starters, adjunct cultures, dairy moulds -

INTRODUCTION

In the dairy industry, moulds and yeasts are common contaminants that contribute to the spoilage of milk and milk-derived products (PITT and HOCKING, 1997). However, in some dairy products including kefir, Scandinavian fermented milks, cheeses of the Camembert and Brie types and blue-veined varieties (BERESFORD *et al.*, 2001; WOUTERS *et al.*, 2002), fungi are essential for the development of desirable rheological, organoleptic and visual characteristics (CANTOR *et al.*, 2004). Well-characterised fungal strains could be used as ripening cultures for the manufacture of such products (LARSEN *et al.*, 1998; FERREIRA and VILJOEN, 2003). These could also be contemplated as components of specific starters designed to ensure the growth of the correct types of microbe and their relative proportions in traditional fermentations (PARENTE and COGAN, 2004). Specific starters are particularly important for generating the complex sensory profiles of Protected Designation of Origin (PDO) cheeses (SMIT *et al.*, 2005). Lactic acid bacterial strains from many of these have been characterised in order to design such starters (AYAD *et al.*, 1999; RANDAZZO *et al.*, 2008; DE ANGELIS *et al.*, 2008; FRANCIOSI *et al.*, 2009). However, attention to the eukaryotic microorganisms that might also be of benefit has only rarely been paid (FARAHAT *et al.*, 1990; FERREIRA and VILLOEN, 2003).

Penicillium roqueforti is used as a starter for the production of blue-veined cheeses, such as Roquefort, Gorgonzola, Stilton and Danish-blue (CANTOR *et al.*, 2004). This mould is well adapted to the cheese environment, where it contributes to the formation of cheese texture and flavour through the action of its proteolytic and lipolytic systems. Wide variation in phenotypic properties among different *P. roqueforti* strains has been reported in several studies. Some of these variations, such as the colour of the mycelium, its proteolytic and lipolytic activities (LÓPEZ-DÍAZ *et al.*, 1996), growth at different temperature and NaCl concentrations (YOUSEF and MARTH, 1987; LÓPEZ-DÍAZ *et al.*, 1996), and mycotoxin production (BOYSEN *et al.*, 1996; GEISEN *et al.*, 2001), are of great technological interest.

Geotrichum candidum is a filamentous fungal species dominant in most smear and mould-ripened cheese varieties (BOUTROU and GUÉGUEN, 2005). It shows properties of both moulds and yeasts, although nowadays it is regarded as a true yeast (MARCELLINO *et al.*, 2001). The predominance of a yeast-like or filamentous morphology mostly depends on the cheese technology employed. *G. candidum* strains consume lactate and produce enzymes for the breakdown of proteins and fats, leading to the softening of the cheese matrix and the formation of aroma compounds such us alcohols, ketones, esters and different sulphide compounds (JOVILLET *et al.*, 1994; BERGER *et al.*, 1999). These strains may also interact with lactic acid bacteria and/or *P. roqueforti* strains to promote the growth of starter or adjunct cultures (ADDIS *et al.*, 2001; GADAGA *et al.*, 2001). Strain-related phenotypic variability has been reported (BERGER *et al.*, 1999; MARCELLINO *et al.*, 2001).

Cabrales is the most famous of the Spanish, traditional, blue-veined cheeses, and has enjoyed PDO status since 1981. The development of *P. roqueforti* in this cheese is responsible for its typical appearance. It also drives the biochemical changes that occur during ripening (ALONSO *et al.*, 1987). Traditionally, no fungal spores are added during Cabrales manufacture (FLÓREZ *et al.*, 2006). *P. roqueforti* is thought to enter into the cheese matrix from the environment of the natural caves in which the cheeses are ripened (minimum 60 days). During a recent microbial characterisation of Cabrales (FLÓREZ *et al.*, 2006), *G. candidum* and other yeast species were encountered in similar numbers to those of *P. roqueforti*, suggesting the former may play a significant role in ripening.

This paper reports the biochemical and technological characterisation of representative *P. roqueforti* and *G. candidum* strains isolated from several batches of Cabrales cheese made from raw milk using traditional technology. The aim of this work was to identify strains that might be used as adjunct cultures for making and ripening this cheese type. Enzyme activities with biotechnological potential were also identified.

1. MATERIAL AND METHODS

1.1. Fungal strains and growth conditions

The studied organisms were 12 *P. roqueforti* strains (1AM6, 1AM8, 1AM10, 1AM11, 1AM12, 1AM13, 1AM14, 3AM8, 3AM11, 3AM12, 3AM15 and 3AM16) and 10 *G. candidum* strains (1AM1, 1AM3, 3AM1, 3AM3, 3AM4, 3AM9, 3AM10, 3AM19, 3AM20 and 3AM20), previously isolated during the manufacture and ripening of two batches of traditional Cabrales cheese (FLÓREZ *et al.*, 2007). As a control, the commercial *P. roqueforti* PB6 (Chr. Hansen, Denmark) and *G. candidum* VTT1 (Valio Ltd., Finland) strains were also subjected to the same analyses. Stock cultures of the strains were maintained at 4°C on slants of Malt Extract Agar (MEA; Oxoid Ltd., Basingstoke, Hampshire, UK) over the analytical period. For long storage, strains were deep-frozen at -80°C in Malt Extract broth (ME; Oxoid) with 15% glycerol. Unless otherwise stated, strains were grown in ME or on MEA at 25°C for 72 h or 5 days respectively. Cellular suspensions were prepared from the cultures, enumerated and used as inocula in the different assays.

1.2. Plate assays for proteolytic and lipolytic activities

Proteolytic activity was first determined on Skimmed Milk Agar (SMA; Oxoid) and then repeated on Mycological Agar (MA; Oxoid) and MEA prepared with 20% (v/v) of 10% (w/v) skimmed milk added immediately before the pouring of the plates. Lipolytic activity was determined according to HARRIGAN and MCCANCE (1976) with Tributyrin Agar (TA; Oxoid). Plates were spot-inoculated in triplicate with 24 h cultures showing a cell density of around 10^7 colony forming units (cfu) mL^{-1}. These were then incubated at 25°C for 7 d, at which time the diameter of the clearance zone was measured.

1.3. Lipolytic activity in olive oil-based media

Strains producing lipolysis halos on TA plates were assayed on MEA plates supplemented with 1% (v/v) olive oil and 0.10 mg mL^{-1} Nile Blue (Sigma; Sigma-Aldrich Co., St. Louis, Mo., USA). The solution was emulsified in an Ultra-Turrax T-25 blender (IKA, Staufen, Germany) (three pulses of 2 min each at maximum speed). Strains were spot-inoculated onto the plate surface as above and incubated at 25°C for 7 d. Positive lipolytic strains produced a blue halo around the spots. Extracellular lipase activity was also determined in supernatants from cultures grown for 72 h in ME broth with olive oil via the titration of the fatty acids released with 50 mmol/L NaOH, as reported by PIGNÈDE *et al.* (2000) (performed in duplicate). One arbitrary unit of lipase was defined as the amount of enzyme catalysing the release of 1 μmol L^{-1} of fatty acid per min at 37°C.

1.4. Azocasein test

The intracellular and extracellular proteolytic activities of positive strains were analysed by the azocasein method after their growth in Yeast-extract Glucose Chloramphenicol medium (YGC; Oxoid) at 28°C for 48 h. Cultures were centrifuged at 15,000 x g at 4°C for 20 min and the supernatants filtered through a 45 μm membrane. Cells were suspended in 50 mmol/L phosphate buffer pH 6.5, disrupted by sonication in a Vibra Cell apparatus (Sonic and Materials Inc., Danbury, Ct., USA), and centrifuged as above for 20 min. Equal volumes of supernatants or cell-free extracts were mixed with a 1% azocasein solution in 0.1 mol/L phosphate buffer pH 6.5. Reactions proceeded for 1 h at 35°C and were then stopped by the addition of a volume of 10% trichloroacetic acid (TCA). The supernatants were centrifuged and absorbance measured at 336 nm in an Uvikon 930 spectrophotometer (Kontron Instruments, Milton Keynes, UK). An arbitrary unit of proteolytic activity was defined as an increase in absorbance of 0.01 over 1 h of incubation with respect to the value at time zero.

1.5. Enzyme profiles

Enzyme activities were assessed using the semi-quantitative API-ZYM method (bioMérieux, Montalieu-Vercieu, France). After cultivation in ME, the cells were washed and diluted in physiological saline to an optical density of 0.1 at 550 nm (approximate cell density of 10^6 cells mL^{-1}). Following the manufacturer's recommendations, the wells of the strips were inoculated with 65 μL of the strain suspensions and enzyme activities recorded after 4 h of incubation at 25°C.

1.6. Growth of *P. roqueforti* and *G. candidum* strains under different conditions

MEA plates (initial salt concentration 0%, pH 7.6), inoculated in triplicate in a central position, were incubated at 10° and 25°C and the diameter of the colonies produced by the different strains measured daily from day 2 to day 10. The rate of growth was calculated as the slope of the colony diameter/incubation time (mm d^{-1}) regression curve (Yousef and Marth, 1987). Similarly, the effect of NaCl on growth was determined by adding this compound to MEA plates at a final concentration of 1, 2, 3, 4, 5 or 6% (w/v). The influence of pH on growth was assessed in MEA plates acidified to pH 4.0, 4.5, 5.0 and 5.5; these pHs were achieved by adding sterile 10% lactic acid to the liquid medium before pouring the plates. The beginning of sporulation was visually determined by the appearance of a green colour in the colonies.

The optimum values for the different variables studied were combined to recreate the best possible cheese ripening conditions. The growth of the strains was thus evaluated on MEA plates at pH 4.5 with 5% NaCl, incubated at 10°C for up to 10 days.

1.7. Production of biogenic amines

All strains were examined for the production of biogenic amines using the agar plate assay described by GARDINI *et al.* (2006). Briefly, a solution containing 0.1 g of glucose, 0.06 g of bromocresol purple, 10 g of each amino acid under assay, 900 mL of water and 15 g of agar was sterilised and supplemented with 100 mL of filter-sterilised Yeast Nitrogen Base (Difco, Sparks, Maryland, USA). Histidine, phenylalanine, tyrosine, ornithine and lysine (all from Sigma) were the amino acids tested as precursors. Plates were spot-inoculated with 10^6 cfu mL^{-1} and incubated at 25°C for up to 7 d. An increase in pH during growth turns the yellow colour of the plates to purple, indicative of the presence of biogenic amines.

1.8. Statistical analysis

Statistical analysis was performed using SPSS 11.0 software for Windows (SPSS Inc., Chicago, IL, USA). Growth rates were subjected to one-way ANOVA. Means were compared by the least-significant difference test (LSD test). Significance was set at $p<0.05$.

2. RESULTS

Strains of *P. roqueforti* and *G. candidum* were assayed for proteolytic and lipolytic activities on SMA and TA plates respectively; the results are summarised in Table 1. Under the conditions of the assay, only one strain, *G. candidum* 3AM4, showed appreciable proteolytic activity on the SMA plates. To determine whether a lack of proteolytic activity was responsible for the poor growth of the other strains in this medium, all strains were further assayed on MA and in MEA plates supplemented with 2% skimmed milk. However, 3AM4 was the only strain to show a large proteolytic halo in all three media. A further method was used to assess whether the absence of proteolytic activity was due to a lack of production of extracellular enzymes by these fungal strains. Culture supernatants and cell free-extracts were independently measured for proteolytic activity using the azocasein test. The results indicated the cell-free extracts of both the *P. roqueforti* and *G. candidum* strains to retain most of their proteolytic activity. However, after normalisation to the protein content, the relative activity was always greater in the supernatants. As scored by the defined arbitrary units, the level of proteolysis was low; values ranged among species and strains from 8.2 to 26.3, with a single peak of 89.6 for strain 3AM4.

In contrast to the proteolytic results, all of the *P. roqueforti* strains were lipolytic on the TA plates, although small differences in the size of the halos were observed (Table 1). The *G. candidum* isolates seemed to be less lipolytic, with only two strains showing a halo comparable to that produced by the *P. roqueforti* strains. The results on the MEA plates with olive oil (a method frequently used to distinguish between lipase and esterase activities) agreed with those obtained on TA; a clear halo of lipolysis was observed for all *Penicillium* strains and for two *Geotrichum* strains. The extracellular lipase activity measured in the *P. roqueforti* strains varied between 12 and 28 arbitrary units (with strain 1AM13 showing the highest value). Most of the *G. candidum* strains, however, showed undetectable levels of extracellular lipase; the two positive strains 3AM4 and 3AM9 showed 16 and eight units respectively.

Table 2 shows the different enzyme activities recorded for all *P. roqueforti* and *G. candidum* isolates using the semi-quantitative API-ZYM system. None of the strains showed lipase C14, trypsin, α-chymotrypsin, β-glucuronidase or α-fucosidase activity. While different activity profiles were recorded for *P. roqueforti* and *G. candidum*, only small differences were recorded for some activities between isolates of the same species. The *P. roqueforti* isolates showed several glucosidase and galactosidase activities, whereas these activities were absent in *G. candidum*. Alkaline and acid phosphatase activities were also lower in the strains of *G. candidum* than in those of *P. roqueforti*, as was that of naphto-AS-BI-phosphohydrolase. In contrast, the *G. candidum* strains showed greater valine arylamidase activity than the *Penicillium* strains. Further, all the *G. candidum* strains showed low levels of cysteine arylamidase activity, while the *P.*

Table 1 - Proteolytic and lipolytic activities of *P. roqueforti* and *G. candidum* strains from Cabrales cheese.

Species	Strain	Proteolytic activity[a] Skim Milk Agar (SMA)[b]	Lipolytic activity[a] Tributyrine Agar (TA)[b]
P. roqueforti	1AM6, 1AM8, 1AM10, 1AM11, 1AM12, 1AM14, 3AM8, 3AM11, 3AM12, 3AM15, 3AM16	-	++
	1AM13	-	+++
	PB6[c]	-	++
G. candidum	1AM1, 1AM3, 3AM1, 3AM10, 3AM19	-	-
	3AM3, 3AM20	-	(+)
	3AM4	++	++
	3AM9	-	++
	VTT1[c]	-	-

[a]Key of activities: -, no halo of clearing; (+) weak halo, ++ halo between 2.5 and 4 mm around the colonies; +++, clearing halo >4 mm.
[b]Similar results were obtained in other media, such as MA and MEA plates with skim milk (proteolysis) and MEA plates with olive oil (lipolysis).
[c]PB6 is a *P. roqueforti* commercial starter strain (Chr. Hansen, Denmark) and VTT1 is a *G. candidum* strain isolated from commercial viili (Valio Ltd., Finland).

Table 2 - Enzymatic activities measured with the API ZYM system in *P. roqueforti* and *G. candidum* strains from Cabrales cheese.

Species	Strain	Enzymatic activities[a]													
		Alcaline phosphatase	Esterase (C4)	Esterase lipase (C8)	Leucine arylamidase	Valine arylamidase	Cysteine arylamidase	Acid phosphatase	Naphtol-AS-BI-phosphohydrolase	α-galactosidase	β-galactosidase	α-glucosidase	β-glucosidase	N-acetyl-β-glucosaminidase	α-mannosidase
Penicillium roqueforti	1AM6	30[b]	20	5	>40	0	0	30	20	10	0	20	20	>40	0
	1AM8	>40	10	5	>40	5	0	>40	10	20	10	10	20	>40	5
	1AM10	>40	20	5	>40	5	0	>40	20	20	5	20	20	>40	0
	1AM11	>40	20	0	>40	5	0	>40	10	10	5	5	5	>40	0
	1AM12	>40	20	10	>40	5	0	>40	20	30	20	20	20	>40	0
	1AM13	>40	20	10	>40	5	0	>40	30	30	5	20	30	>40	0
	1AM14	>40	30	10	>40	0	0	>40	30	30	5	20	30	>40	0
	3AM8	>40	20	5	>40	5	0	>40	>40	20	10	30	20	>40	0
	3AM11	>40	30	5	>40	5	0	>40	30	30	10	30	30	>40	0
	3AM12	>40	20	10	>40	5	0	>40	>40	30	30	30	>40	>40	0
	3AM15	>40	30	10	>40	5	0	>40	>40	30	10	>40	>40	>40	0
	3AM16	>40	30	5	>40	0	0	>40	30	30	5	20	>40	>40	0
	PB6[c]	30	20	5	>40	5	0	>40	10	20	5	20	20	>40	0
Geotrichum candidum	1AM1	10	20	10	>40	5	5	30	10	0	0	0	0	0	0
	1AM3	20	30	10	>40	5	5	10	5	0	0	0	0	0	0
	3AM1	20	20	20	>40	5	5	>40	10	0	0	0	0	0	0
	3AM2	5	20	20	>40	5	5	10	5	0	0	0	0	0	0
	3AM3	10	20	20	>40	5	5	20	5	0	0	0	0	0	0
	3AM4	>40	20	10	>40	>40	20	10	10	0	0	0	5	5	5
	3AM9	10	20	10	>40	5	5	30	5	0	0	0	0	0	0
	3AM10	20	20	10	>40	5	5	10	5	0	0	0	0	0	0
	3AM19	10	20	20	>40	10	5	20	5	0	0	0	0	0	0
	3AM20	10	20	20	>40	5	5	>40	5	0	0	0	0	0	0
	VTT1[c]	10	20	10	>40	5	5	10	5	0	0	0	0	0	0

[a]Lipase C14, trypsin, α-quimiotrypsin, β-glucuronidase, and α-fucosidase activities were never detected.
[b]Units of activity are expressed as nanomol of substrate hydrolyzed under the assay conditions.
[c]PB6 and VTT1 are commercial starter strains from Chr. Hansen and Valio Ltd., respectively, used as controls.

roqueforti strains were negative for this activity. The two control strains PB6 and VTT1 showed enzymatic profiles similar to those of the other strains of their respective species (Table 2).

Table 3 shows the growth rates (measured as the slope of the increase in size of the colony per day) of the *P. roqueforti* and *G. candidum* strains at different NaCl concentrations (from 0 to 6%) and at 10° and 25°C. Fig. 1 shows the effects of these variables on growth in *P. roqueforti* 1AM6. Low NaCl concentrations stimulated growth (control condition: MEA plates without added salt at 25°C; Table 3; open squares in Fig. 1), while concentrations of 4% or higher were clearly inhibitory. Low salt concentrations also promoted sporulation in *P. roqueforti*, which occurred 1-2 days earlier on plates with just 1-2% NaCl. In general, the *P. roqueforti* strains showed higher growth rates than did *G. candidum* under all conditions. Further, increasing the salt concentration or lowering the temperature was more inhibitory to the *G. candidum* than to the *P. roqueforti* strains. Lowering the temperature to 10°C dramatically affected the growth rate of both species' strains (Table 3), rendering it two to three times lower than that at 25°C (the optimum temperature for both species). The growth rates of *P. roqueforti* were, however, always higher than those of the *G. candidum* strains. Commercial strains, PB6 and VTT1 showed lower growth rates than the autochthonous strains under all conditions. In most cases, these differences were statistically significant (Table 3).

Although strain to strain variation in growth was recorded, the different pHs tested in the present work did not really affect the growth of either the *P. roqueforti* or *G. candidum* strains (data not shown).

The development of fungi in cheese depends on all the variables analysed acting synergistically and at the same time. Therefore, the growth of all strains was assessed on MEA plates containing 5% NaCl acidified to pH 4.5 and incubated at 10°C for up to 10 d. Colony growth was recorded daily and compared to that seen on MEA plates incubated under optimal con-

Table 3 - Average of growth rates (mm d^{-1}) from triplicate analysis of the *P. roqueforti* and *G. candidum* strains in MEA at several salt concentrations and at two distinct temperatures. Results of one way ANOVA and multiple means comparison by the LSD test are also indicated.

	Salt concentration (%) (at 25°C)						Temperature (°C)	
Strain	**1%**	**2%**	**3%**	**4%**	**5%**	**6%**	**25**	**10**
P. roqueforti								
1AM6	9.41 [a]	9.42 [a]	9.21 [a]	8.32 [a]	7.40 [a]	6.24 [a]	8.43 [a]	3.93 [a]
1AM8	9.39 [a]	9.81 [b]	9.41 [a]	8.29 [a]	7.49 [a]	6.46 [a]	8.19 [a]	4.12 [a]
1AM10	9.99 [b]	9.17 [c]	9.03 [b]	7.81 [b]	7.51 [a]	5.88 [b]	8.25 [a]	4.24 [b]
1AM11	9.83 [b]	9.64 [a]	9.09 [a]	8.24 [a]	7.47 [a]	6.19 [a]	8.27 [a]	3.83 [c]
1AM12	9.63 [a]	9.68 [b]	9.20 [a]	7.16 [c]	7.20 [b]	6.39 [a]	8.40 [a]	3.88 [a]
1AM13	9.52 [a]	9.21 [c]	9.03 [b]	8.04 [b]	7.29 [a]	6.18 [a]	8.02 [b]	3.47 [d]
3AM8	9.71 [b]	9.28 [c]	9.09 [a]	8.29 [a]	7.73 [c]	6.31 [a]	8.16 [b]	3.63 [d]
3AM11	9.60 [a]	9.41 [a]	9.33 [a]	8.41 [a]	7.56 [a]	6.40 [a]	8.24 [a]	3.58 [d]
3AM12	8.86 [c]	8.68 [d]	8.21 [c]	7.66 [d]	6.93 [d]	6.07 [b]	8.15 [b]	3.89 [a]
3AM15	9.64 [a]	9.43 [a]	8.87 [b]	8.13 [a]	7.32 [a]	6.07 [b]	8.31 [a]	4.11 [a]
3AM16	9.41 [a]	9.17 [c]	8.96 [b]	8.27 [a]	7.39 [a]	6.19 [a]	8.47 [a]	3.95 [a]
PB6[a]	9.41 [a]	9.02 [d]	8.14 [c]	7.17 [c]	6.14 [e]	5.09 [c]	8.16 [b]	3.36 [e]
G. candidum								
1AM1	8.44 [a]	8.45 [a]	8.36 [a]	8.24 [a]	7.13 [a]	5.44 [a]	8.41 [a]	3.55 [a]
1AM3	8.56 [a]	8.63 [a]	8.58 [a]	7.93 [a]	7.01 [a]	5.03 [b]	8.54 [a]	3.62 [a]
3AM1	8.77 [b]	8.72 [a]	8.39 [a]	7.79 [b]	7.32 [a]	5.29 [a]	7.99 [b]	3.77 [b]
3AM3	8.36 [a]	8.46 [a]	8.28 [a]	7.45 [c]	6.89 [b]	5.14 [b]	8.36 [a]	3.54 [a]
3AM4	9.02 [b]	9.07 [b]	8.93 [b]	8.15 [a]	7.35 [a]	5.67 [c]	8.89 [c]	3.88 [b]
3AM9	8.90 [b]	8.97 [b]	8.43 [a]	7.48 [c]	6.92 [b]	5.05 [b]	8.67 [c]	3.60 [a]
3AM10	8.48 [a]	8.72 [b]	8.32 [a]	7.99 [a]	6.88 [b]	4.98 [b]	8.52 [a]	3.35 [c]
3AM19	8.88 [b]	9.05 [b]	8.78 [b]	7.58 [c]	6.95 [a]	5.13 [b]	8.89 [c]	3.51 [a]
3AM20	8.56 [a]	8.68 [a]	8.68 [b]	7.95 [a]	6.45 [c]	4.87 [d]	8.62 [a]	3.49 [a]
3AM20	8.93 [b]	9.02 [b]	8.83 [b]	7.36 [d]	7.03 [a]	5.36 [a]	8.88 [c]	3.26 [d]
VTT1[a]	8.32 [a]	8.44 [a]	8.14 [c]	7.02 [e]	5.77 [c]	4.93 [b]	8.24 [a]	3.39 [c]

[a]PB6 is a *P. roqueforti* commercial starter strain (Chr. Hansen, Denmark) and VTT1 is a *G. candidum* strain isolated from commercial viili (Valio Ltd., Finland).

Independent statistical analysis was performed for strains of the two species; means within the same column not followed by the same letter differ significantly ($p<0.05$). For clarity, standard deviations were omitted.

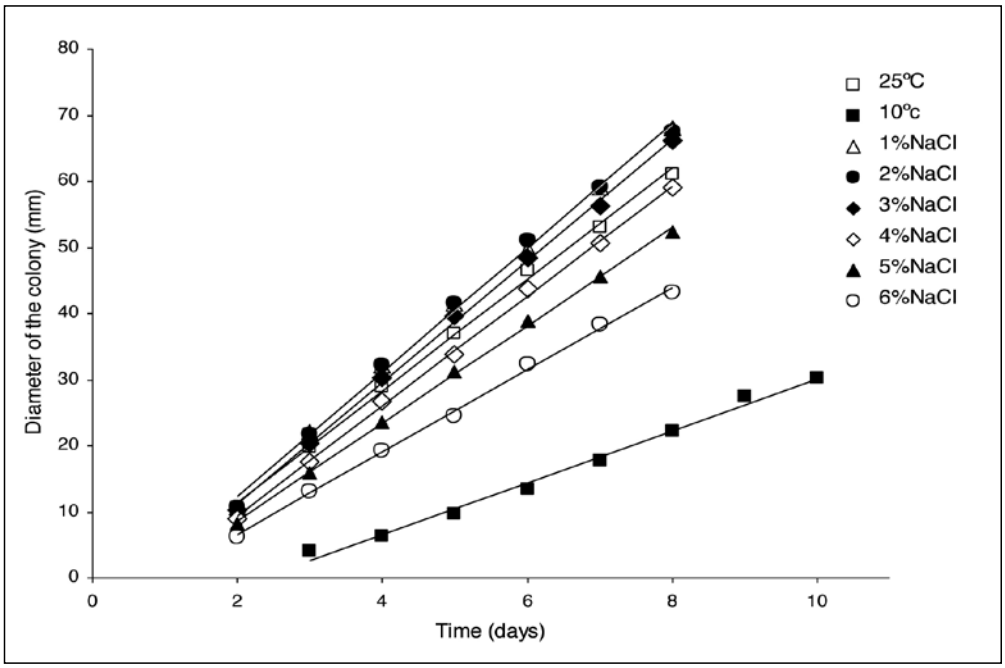


Fig. 1 - Evolution of the colony size in mm of *P. roqueforti* 1AM6 grown in MEA plates at 25° and 10°C and at different salt concentration at 25°C.

ditions (25°C, neutral pH, 0% NaCl). Strain to strain variations were observed with the majority of autochthonous strains growing faster and producing greater final cell counts than the commercial strain PB6. Fig. 2 shows the growth achieved by the *P. roqueforti* strains. Lower final cell counts were observed for the strains of *G. candidum* (Table 3).

Finally, the strains of both *P. roqueforti* and *G. candidum* proved to be negative for the production of the most common biogenic amines in cheese (tyramine, histamine, putrescine, cadaverine and 2-phenylethylamine) under the present conditions.

3. DISCUSSION

Filamentous *G. candidum* strains have been found in numbers similar to those of *P. roqueforti* during the manufacture and ripening of Cabrales cheese (FLÓREZ *et al.*, 2006), suggesting the former may play a significant role in ripening. *P. roqueforti* is responsible for the final appearance of this cheese, and for driving the biochemical changes that take place during ripening (ALONSO *et al.*, 1999). *G. candidum* consumes lactate and produces several enzymes that break down proteins and fats (BOUTROU and GUÉGUEN, 2005) - processes involved in the neutralisation and softening of the cheese matrix as well as in the formation of aroma components in the mature cheese (BONNARME *et al.*, 2001; ARFI *et al.*, 2001).

Variation in technological variables among strains has been repeatedly reported for isolates of both *P. roqueforti* (FARAHAT *et al.*, 1990; LÓPEZ-DÍAZ *et al.*, 1996; LARSEN *et al.*, 1998) and *G. candidum* (BERGER *et al.*, 1999; MARCELLINO *et al.*, 2001). These differences include all the technological variables examined in the present work (proteolytic and lipolytic activities and growth at different salt concentrations and temperatures). The manufacturers of blue cheeses acknowledge having fungal strains with different technological properties at their disposal in order to satisfy the visual and organoleptic preferences of consumers.

The proteolytic and lipolytic activities of fungi used in cheesemaking are extremely important. Their proteolytic activity is responsible for the degree of proteolysis obtained in Camembert, Brie and blue cheeses which, in turn, determines the development of texture and flavour (SABLÉ and COTTENCEAU, 1999). Alkaline and acid proteases have both been purified from *P. roqueforti* (CANTOR *et al.*, 2004), but less is known about the proteolytic abilities of *G. candidum* strains, for which proteolytic and non-proteolytic variants have been reported (MARCELLINO *et al.*, 2001). In the present study, only a single *G. candidum* strain, 3AM4, showed proteolytic activity in the plate assays. Highly proteolytic *G. candidum* strains have been associated with mouldy filamentous colonies with abundant hyphae; in contrast less proteolytic strains are more yeast-like colonies and have abundant arthrospores (MARCELLINO *et al.*, 2001). However, the heterogeneous nature of the potentially distinct taxa currently belonging to the poorly-defined *G. candidum* may contribute towards confusion when try-

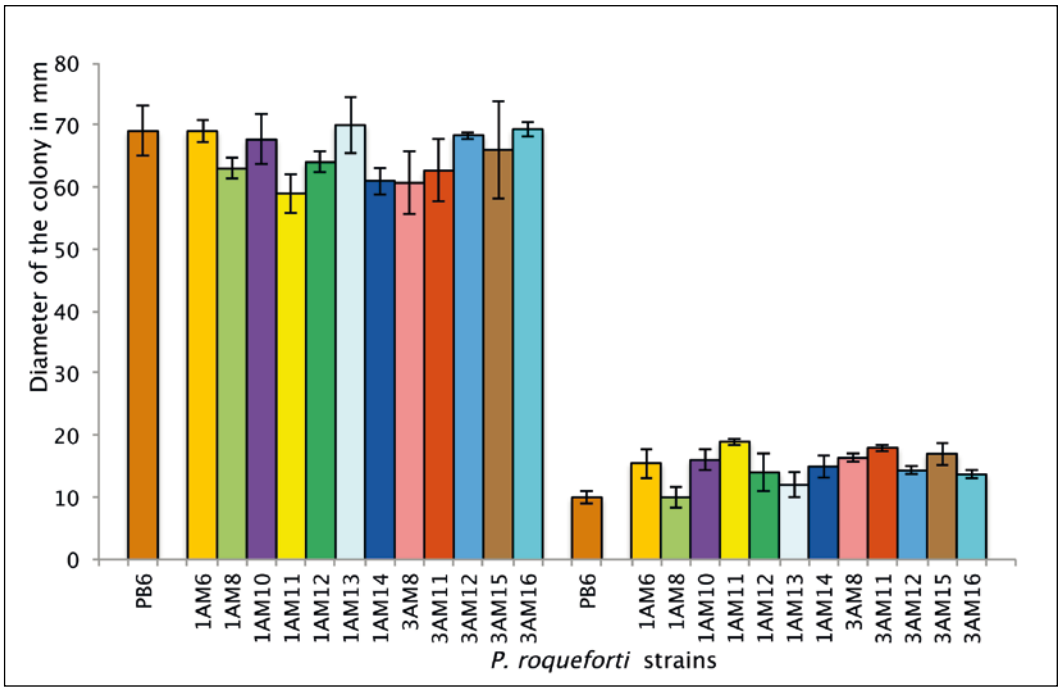


Fig. 2 - Final growth of the *P. roqueforti* strains in MEA plates at optimal conditions (25°C, pH 7.6, 0% NaCl) as compared with conditions simulating cheese ripening (10°C, pH 4.5, 5% NaCl).

ing to link morphology and biochemical properties (BOUTROU and GUÉGUEN, 2005). Certainly, *G. candidum* 3AM4 showed no distinct colony morphology; indeed, all the *G. candidum* strains showed the same fungal-like morphology. LARSEN *et al.* (1998) argue that agar diffusion tests may not have the required sensitivity for assaying low-level proteolytic activity and recommend more sensitive assays, such as the azocasein assay, be performed. However, the present work suggests this test to be insufficiently sensitive as well. Capillary electrophoresis may be able to show more clear differences among species and strains with similar levels proteolytic of activity (FERNÁNDEZ-BODEGA *et al.*, 2009).

The proteolysis performed by *G. candidum* may be affected by environmental conditions, as reported for *P. roqueforti* by LARSEN and JENSEN (1999). Moreover, under cheese-ripening conditions, proteolysis may be enhanced by calcium since divalent cations have been shown to increase the extracellular proteolytic activity of *G. candidum* strains (ÁLVAREZ-MARTÍN *et al.*, unpublished). The fact that the greatest proteolytic activity was associated with the supernatants may be of practical importance in cheese ripening; enzyme release would appear to be necessary for interactions with milk components to take place (SMIT *et al.*, 2005).

Lipolytic activity was detected in all the *P. roqueforti* and in two *G. candidum* strains. Real lipase activities were detected in positive strains by plate and liquid assays involving olive oil. The results did not agree, however, with those obtained by the API-ZYM system, which returned negative lipase C14 activity for all strains. This suggests that substrates might be in different biochemical states in the different tests. Alternatively, the incubation time in the API-ZYM assay may be too short to detect low activities.

The biochemical profiles measured with the API-ZYM system distinguished between the strains belonging to *G. candidum* and *P. roqueforti* quite well. Several glucosidases and galactosidases seemed to be exclusive to the *Penicillium* strains, whereas *G. candidum* showed more valine and cysteine arylamidases. The latter two enzymes are involved in the degradation of amino acids in the cheese preceding the synthesis of flavour compounds by these species (BONNARME *et al.*, 2001; ARFI *et al.*, 2003). These fungi may also be an excellent source of extracellular enzymes with potential biotechnological and industrial applications (BUZZINI and MARTINI, 2002).

During cheese ripening, *P. roqueforti* has to develop in the high salt concentration present in the aqueous phase of the cheese and at a rather low temperature (usually 8°-10°C). Growth rate variation among *P. roqueforti* strains at increasing salt concentrations and at optimal and low temperatures has been described elsewhere (GODINHO and FOX, 1981; YOUSEF and MARTH, 1987; LÓPEZ-DÍAZ *et al.*, 1996). In all these studies, the stimulatory effect on sporulation of a low concentration (1%) of salt has also been reported. The fact that the autochthonous isolates of both *P. roqueforti* and *G. candidum* species grew faster than the commercial controls suggests they may be more suited to industrial conditions.

They might therefore be appropriate components of secondary and/or ripening cultures. Indeed, a few of the *G. candidum* strains analysed in the present work have already been shown to grow to high cell densities in milk while being compatible with starter lactic acid bacteria species (ÁLVAREZ-MARTÍN *et al.*, 2008). Alongside lactic acid bacterial starters, the use of fungal starter cultures of for Cabrales might help improve its standardisation and food safety (FLÓREZ *et al.*, 2006), both of which are major issues for traditional cheeses.

As well as the need to be able to precisely identify the strains used in food systems, they must also be shown to be safe. None of the present strains were producers of biogenic amines according to a plate assay containing amino acid precursors. These results were confirmed using a sensitive HPLC method as reported by FERNÁNDEZ *et al.* (2007) (data not shown). In fact, *G. candidum* may degrade these compounds or inhibit their production by other microorganisms (BOUTROU and GUÉGUEN, 2005). Although not analysed in this work, the toxigenic effect of *P. roqueforti* strains in cheese does not seem to be a matter of concern; under ripening conditions only a low level of roquefortine is produced (ERDOGAN and SERT, 2004).

4. CONCLUSIONS

Twenty two strains of filamentous fungi belonging to *P. roqueforti* (12) and *G. candidum* (10) isolated from traditional batches of Cabrales cheese were characterised for their biochemical, technological and safety traits. Strains of both species showing properties that render them of interest as ripening and adjunct cultures were readily identified. In particular, *P. roqueforti* 1AM10 and 1AM13, which respectively showed the highest and lowest growth rates at low temperatures, will be used in controlled cheesemaking trials in the future. Further, *G. candidum* 1AM1, 3AM4, 3AM9, with their varying degrees of proteolytic, lipolytic and other enzyme activities, are currently being assayed as adjunct cultures for the manufacture of Cabrales cheese. The characterised autochthonous *P. roqueforti* and *G. candidum* strains might also complement currently available strains for the industrial manufacture of other dairy products. In addition, they could be used to valorise the PDO status of Cabrales cheese.

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PAPER

KINETIC CHARACTERIZATION OF BACTERIAL ISOLATES ABLE TO PROMOTE MALOLACTIC FERMENTATION AT REDUCED TEMPERATURES

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ABSTRACT

Eight malolactic bacterial strains were isolated through enrichment cultures kept at 10°C from different Tuscan wines. Six strains were identified as *Oenococcus oeni* by ARDRA and species-specific PCR. Two strains were tentatively assigned to *L. brevis*. A kinetic approach was used to assess their activity at 25° and 10°C. All strains induced a remarkable conversion of L-malate at 25°C. Four strains showed a measurable fermentative activity at 10°C, among which *L. brevis* LB2 gave a significant conversion of L-malate. The kinetic activity of this strain appears to be unaffected by the components dissolved in wine.

- Keywords: malolactic fermentation, low temperatures, cryophilic wild strains, kinetics -

INTRODUCTION

The malolactic fermentation (MLF) in wine is a microbiological process involving the decarboxylation of L(-) malic acid to produce α, D- and L-lactic acid (ARTHURS and LOYD, 1999; VOLSCHENK, 2006). The transformation is promoted by a group of Gram+ asporogenous Eubacteria (*Oenococcus*, *Leuconostoc*, *Pediococcus*, *Lactobacillus*) which prefer anaerobic conditions but can proliferate also in the presence of air (O_2 tolerant). These microorganisms are able to metabolize different carbohydrates (monosaccharides, disaccharides, and polyalcohols), giving rise to a large range of by-products, and to induce a remarkable modification of wine organoleptic traits. This occurs as a consequence of the reduction of fixed acidity and the production of a variety of metabolites (LONVAUD-FUNEL, 1999). Moreover, at the end of the MLF an increase of the wine biological stability is achieved (VERSARI *et al.*, 1999). As a function of the bacterial strains involved, different effects on the wine flavour and aroma are obtained, and it is generally accepted that MLF reduces herbaceous character and increases fruity and butter aromas (DAVIS *et al.*, 1985; VINCENZINI *et al.*, 2005).

Due to the effects induced on sensorial traits, this secondary fermentation is particularly desired for structured red wines while, on the contrary, it can produce deleterious effects on microbial stability and organoleptic properties of wines produced in warm climates, often characterised by very low malic acidity (HERVÉ *et al.*, 2004).

Wine represents a growth medium particularly harsh for malolactic bacteria (COUCHENEY *et al.*, 2005). Their replication rate appears to be affected by a number of physical-chemical factors (mainly pH, temperature, ethanol, and SO_2 concentration) which can act synergistically on bacterial population growth. Among these factors, the temperature seems to play a primary role, being the replication rate of many malolactic bacteria strains greatly reduced at low temperatures (< 10°C).

Usually MLF takes place at end of alcoholic fermentation in a period of the year located at end of October or at the beginnings of November when the temperatures of the cellars decrease, thus slowing or not allowing this microbial process to take place. Therefore it is necessary to reach the following spring and more favourable environmental conditions to have again significant metabolic activity (SAAYMAN and VILJOEN-BLOOM, 2006). To avoid long term storage of unstable wines not having completed their MLF, two approaches are possible: a) to heat wines at the end of alcoholic fermentation to increase their temperatures, thus allowing microbial populations to promote MLF; in this case, there are high energy demands to be met; b) to utilise selected strains of malolactic bacteria able to promote this transformation also at lower temperatures.

At the difference of alcoholic fermentation, where a large number of selected strains of yeasts are now commercially available, a reduced amount of malolactic bacteria can be found on the market. Moreover the selection of these strains has been often carried out under environmental conditions, temperatures, different from those at which ML bacteria are used. This encompasses usually a loss of activity, and makes it difficult to promote a significant conversion of L-malate to L- and D-lactate.

The possibility to select bacterial strains, isolated from must and wines maintained at reduced temperatures (~ 10°C), and able to carry on MLF in cooler conditions, could represent a progress in winemaking. These microbial starters, being linked to their production area, could provide a better characterization of the final wine possibly recognizable by consumers.

The kinetic behaviour of malolactic bacteria isolated from musts and wines maintained at reduced temperature (T ~ 10°C), and in particular their ability to promote MLF at reduced temperatures, has been investigated in this study.

In order to carry out this study, a kinetic model (ZINNAI *et al.*, 2011), enabling to evaluate the specific activity shown by a microbial population to promote MLF in different environmental conditions, has been utilised. The results obtained have been compared and integrated with data supplied by another more traditional technique (optical density evaluation).

MATERIALS AND METHODS

Enrichment cultures and bacteria isolation

Microbial populations, able to promote malic acid conversion to produce lactic acid, were isolated from white and red wines samples taken during different stage of winemaking of several grape varieties in different Tuscan wineries of Chianti area. The wine samples were inoculated in flasks containing 50 mL of a modified MRS medium (DE MAN *et al.*, 1960) (Fluka, Milan, Italy), namely MRS-TJC, containing 20 g L^{-1} Tomato Juice Broth (Fluka), 5 g L^{-1} L-malic acid (Fluka), 100 mg L^{-1} cycloheximide, adjusted to pH 5. Standing cultures in flasks were incubated without subculturing at 10°C for one month.

Microbiological analyses were performed using MRS-TJC supplemented with 20 g L^{-1} agar. The plates were incubated anaerobically in jars (Anaero*Gen*™, Oxoid, Milan, Italy) at 10°C for up to 28 days.

Colonies developed on the agar surface identified as bacterial cells by microscopic inspection, were confirmed as lactic acid bacteria (LAB) by positive Gram staining and negative

catalase test, and purified by repeated streaking on the same medium. Eight isolates were collected and stored at -80°C in 20% (v/v) of glycerol and subsequently identified through molecular methods.

DNA isolation and molecular techniques

The isolates were grown for more than 10 days on MRS-TJC agar plates at 10°C. For each strain, a single colony was picked up, resuspended in 10 µL of 0.005 mol L^{-1} Na phosphate buffer, 20% of sucrose (pH 7.0), lysozyme 2.5 mg mL^{-1} and incubated at 37°C for 45 min. At the end of the incubation period, 50 µL lysis buffer (0.05 mol L^{-1} NaOH, 0.25% SDS) were added to each sample which was vortexed for 2 min, heated to 95°C for 30 min and centrifuged at 10,000 g for 10 min. The lysate cell suspensions were diluted 1:10 with sterile distilled water for PCR amplification.

Extracted DNA was analysed by PCR amplification with 27F-1495R primers (DI CELLO *et al.*, 1997) and specific primers On1-On2 for *O. oeni* (ZAPPAROLI *et al.*, 1998) utilising a thermocycler (model 2400; Perkin-Elmer). *Oenococcus oeni* OE101 and *Lactobacillus brevis* LB110 (Collection of the Department of Crop Biology, Pisa) were used as reference strains during the characterization of the isolates. Amplification of 16S rDNA was performed with 1 µL of each diluted cell lysate in a 25 µL PCR mixture containing 1x Buffer Finnzyme (50 mmol L^{-1} KCl, 10 mmol L^{-1} TrisHCl pH 8.3, 0.1% Triton X-100, 1.5 mmol L^{-1} $MgCl_2$), 0.2 mmol L^{-1} each dNTPs, 0.2 µmol L^{-1} of each primer, 0.5 U DyNazyme II DNA polymerase (Finnzyme, Celbio, Milan, Italy). Cycler conditions were: initial denaturation at 95°C for 2 min followed by 35 cycles at 94°C for 1.20 min, 54°C for 60 s, 72°C for 1.30 min, with final extension at 72°C for 5 min. 5 µL of 16S rDNA amplified by PCR were digested with *Hinf*I, *Hae*III, *Rsa*I, *Cfo*I, and *Dpn*II endonucleases according to the instructions of the supplier (Roche Diagnostic, Germany, Mannheim). The ARDRA profiles were obtained in 2% (w/v) agarose gel electrophoresis in 1x TBE buffer containing 0.5 µg mL^{-1} of ethidium bromide. Molecular weight of the fragments was estimated by comparison with the DNA molecular mass marker VI (Boeringer, Mannheim, Germany). The profiles obtained were captured as TIFF format files by Liscap program for Image Master VDS system (Pharmacia Biotech, Milan, Italy).

Species-specific amplification using On1 and On2 primers was carried out as described by ZAPPAROLI *et al.* (1998). Cycler conditions were: initial denaturation at 94°C for 2 min followed by 30 cycles at 94°C for 45 sec, 64°C for 2 min, 72°C for 2 min, with final extension at 72°C for 10 min. Amplification products were analysed as described above.

Kinetic characterisation of the isolates

The kinetic characterization of isolates was carried out at two different temperatures (10° and 25°C) following the experimental procedure already reported (ANDRICH *et al.* 1988; ZINNAI *et al.*, 2011). To assess the performance of the isolates in working conditions, their metabolic activity was evaluated in white wine (*Trebbiano toscano*). L-malic acid was added to this wine up to a concentration of about 4 g L^{-1}.

The specific statistical program BURENL was used to identify the best values to be assigned to the constants of the kinetic model utilised to describe the kinetic behaviour shown by the microbial populations tested (ZINNAI *et al.*, 2011).

RESULTS AND DISCUSSION

Eight bacterial strains, isolated from Tuscan wines through enrichment cultures, possibly due to the selective (*i.e.* reduced temperature) conditions of isolation, were identified as malolactic bacteria based on their positive Gram reactions, non motility, absence of catalase activity, rod or coccal shape, and the ability to decarboxylate L-malic to L-lactic acid. These strains were further characterized by the restriction analysis of the amplified 16S-rDNA (PCR-ARDRA) with the *Hinf*I, *Hae*III, *Rsa*I, *Cfo*I, and *Dpn*II endonucleases. The resulting ARDRA profiles of 6 strains were identical to that obtained from a *O. oeni* culture collection strain. The same applies for the ARDRA profiles of the other two strains with respect to that obtained from the *L. brevis* culture collection strain (results not shown). The identification of the six strains as *O. oeni* was confirmed through a PCR method using species-specific primers which amplify a fragment (1,025 bp) of the malolactic enzyme gene of *O. oeni* (ZAPPAROLI *et al.*, 1998). Indeed, the six strains and the *O. oeni* culture collection strain generated a single product of about 1,025 bp (results not shown). No amplification product was observed for the negative control and for the other two strains. The latter were tentatively assigned to *L. brevis* on the basis of its ARDRA profile compared to that obtained using the *L. brevis* culture collection strain.

The activity of the isolates was characterized from a kinetic point of view for their ability to grow in wine at 25° and 10°C. Table 1 shows the origin of the isolated strains and their growth rate at 10° and 25°C determined by measuring the optical density of the medium.

In particular their ability to grow at the temperature analysed (T=T), was evaluated looking at the ratio ($R_{T=T}$) between the value of optical density measured at the end of microbial growth, when the turbidity of the growing solution appears to reach its asymptotic maximum

Table 1 - Isolation sources of the microbial populations (mp) analysed and the values assumed, at two different temperatures (10° and 25°C), by the parameter $R_{T=T}$ ($R_{T=T}$ = O.D.$_{max,T=T.}$/O.D.$_{init.,T=T}$) adopted to evaluate the ability shown by mp tested to increase their values of optical density (O.D.$_{.,T=T}$), while $Q_{25/10}$ gives a measure of the dependence of this parameter ($R_{T=T}$) from temperature.

mp	Isolates	Source	$R_{T=10°C}$	$R_{T=25°C}$	$Q_{25/10}=\left(\frac{R_{T=25°C}-R_{T=10°C}}{R_{T=10°C}}\right)\times 100$
a	*O. oeni OE1*	Wine Chardonnay	4.2	7.1	69.0
b	*L. brevis LB1*	Wine Chardonnay	12.7	15.0	18.1
c	*O. oeni OE2*	Wine Chardonnay	5.5	40.8	641.8
d	*O. oeni OE3*	Wine Chardonnay	7.2	7.1	- 1.4
e	*O. oeni OE4*	Must Sangiovese	2.7	3.7	37
f	*O. oeni OE5*	Wine Merlot	10.7	10.3	- 3.7
g	*O. oeni OE6*	Must Sangiovese	8.2	10.1	23.2
h	*L. brevis LB2*	Wine Carignano	15.7	19.2	22.3

value (O.D.$_{max,T=T}$), and that (O.D.$_{init.,T=T}$) determined immediately after the inoculation of the microbial population to the growing solution (Fig. 1). In order to elucidate how the change of the growth temperature (decreasing from 25° to 10°C) could affect the propagation of the different microbial species analysed, we introduced the $Q_{25/10}$ parameter:

$$Q_{25/10}=\left(\frac{R_{T=25°C}-R_{T=10°C}}{R_{T=10°C}}\right)\times 100$$

High $Q_{25/10}$ values are related to high levels of thermo-sensitivity, while low values would indicate that the growth rate of the microbial population is slightly affected by the temperature changes. Hence, the microbial strains presenting a growth ratio at 25°C which does not change significantly when temperature is reduced to 10°C (reduced value of per cent of variation = $Q_{25/10}$), could represent the microbial population to be utilised to promote malolactic fermentation also when the environmental temperature decreases (Table 1). In particular the two *Lactobacillus* strains (LB1 and LB2) show sufficiently high value of $R_{T=T}$ also at 10°C (Table 1), which do not seem to change significantly when the temperature is increased ($Q_{25/10}$ values). However, as the rapid fermentation of the substrate could require a higher density of microbial cells if the specific activity of the unit microbial cell is reduced, we used a previously kinetic model (ZINNAI *et al.*, 2011) to determine this kinetic parameter.

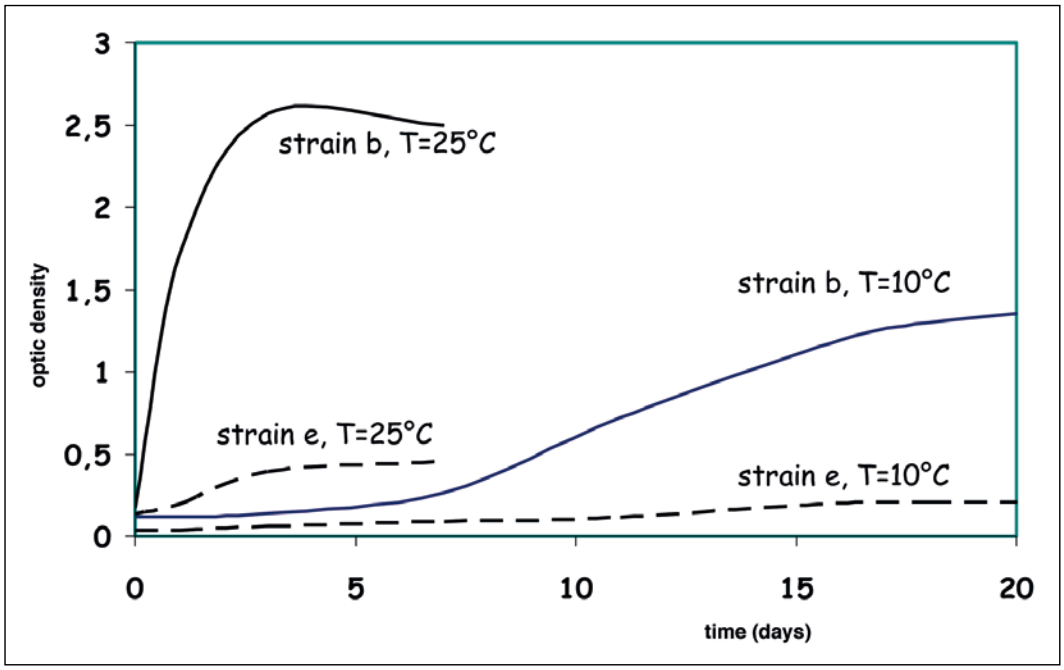


Fig. 1 - Time evolutions of optic density measured for two microbial strains (b and e) at the two temperatures tested (10° and 25°C).

According to this kinetic model, the evolution along time of the bacterial population ($[B]_{t=t}$), L-malate ($[M]_{t=t}$) decrease and D-/L- lactic acid ($[L]_{t=t}$ and $[D]_{t=t}$) accumulation could be effectively described by the following mathematical equations:

$$1)\ [B]_{t=t} = [B]_{t=0} \cdot e^{k_B \cdot t}$$

$$2)\ [M]_{t=t} = [M]_{t=0} \cdot e^{k_M/k_B \cdot B_{t=0} \cdot (1 - e^{k_B \cdot t})}$$

$$3)\ R_{L/,M} + R_{D/,M} = [L]_{t=t}/([M]_{t=0} - [M]_{t=t}) + + [D]_{t=t}/([M]_{t=0} - [M]_{t=t}) = 1$$

$$4)\ [L]_{t=t} = R_{L,M} \cdot ([M]_{t=0} - [M]_{t=t}) = R_{L,M} \cdot \cdot [M]_{t=0} \cdot (1 - e^{k_M/k_B \cdot B_{t=0} \cdot (1 - e^{k_B \cdot t})}$$

$$5)\ [D]_{t=t} = R_{D,M} \cdot ([M]_{t=0} - [M]_{t=t}) = (1-R_{L,M}) \cdot \cdot [M]_{t=0} \cdot (1 - e^{k_M/k_B \cdot B_{t=0} \cdot (1 - e^{k_B \cdot t})})$$

which involve the following five functional parameters:

- the initial number of active bacterial cells for unit volume ($[B]_{t=0}$; CFU/L);
- the concentration of L-malate initially dissolved in the reaction medium ($[M]_{t=0}$; mol/L);
- the kinetic constant related to time evolution of bacteria population (k_B; min^{-1});
- the kinetic constant connected to the specific metabolic activity shown by the unit microbial cell of the analysed bacterial population (k_M; $L \cdot CFU^{-1} \cdot min^{-1}$);
- the ratios ($R_{L/,M}$ and $R_{D/,M}$) occurring between the concentrations of the two stereo-isomers L- and D-lactate produced by MLF at a random time t=t ($[L]_{t=t}$ and $[D]_{t=t}$) and that of L-malate converted ($[M]_{t=0} - [M]_{t=t}$).

The identification of the best values, to be assigned to the five functional parameters involved in the kinetic model was carried out utilising the experimental data related to the time course of the bacterial population ($[B]_{t=t}$), the L-malate ($[M]_{t=t}$) consumption and those of L ($[L]_{t=t}$) and D ($[D]_{t=t}$)-lactate accumulation evaluated at two different temperatures, *i.e.* 25°C (Table 2) and 10°C (Table 3). The kinetic constant related to the evolution of the bacterial population (k_B) was affected by a high variability so that the related values of the square of correlation coefficient (r_B^2), showed a remarkable variation, namely from 0.01 to 0.89. As the number of bacterial cells did not change significantly during many experimental runs, a very low correlation between cells number and run time was found (Tables 2 and 3).

However, also positive values were obtained for k_B (Tables 2 and 3) and in some cases the bacterial population showed a slight increase.

While all the bacterial populations were able to induce the conversion of L-malate at 25°C, only a few of them succeed to promote a measurable fermentative activity when the working temperature was reduced to 10°C.

According to literature (LIU, 2003) the ability to produce together with L-lactate also the stereoisomer D- depends on the strain used. The accumulation of D-lactate during MLF could occur by hydrogenation of pyruvate coming directly from L-malate by a single reaction step (dehydrogenation and decarboxylation) (LONVAUD *et al.*, 1977) or by a previous dehydrogenation of L-malate to produce oxalacetate (LAFON-LAFOURCADE, 1975), which is then decarboxylated to give pyruvate. Indeed some strains (OE1, OE4, OE5, and OE6) are characterised by values of $R_{L,M}$ (fraction of L-lactate accumulated in the reaction medium per unit of L-malate converted) very close to the unit so that the L form appears quite the only stereoisomer produced, other strains (LB1, OE2, and LB2) show a more significant production also of D-lactate (Table 2). Moreo-

Table 2 - Parameters (mean ± CI) and corresponding square values of the correlation coefficients of the kinetic model adopted to describe the time evolution of the bacterial population and of L-malate (M), L-lactate (L) and D-lactate (D) concentrations during malolactic fermentation carried on at 25°C by the different populations of malolactic bacteria tested which were added to an acetate buffer (pH=3.4) aqueous solution of L-malate.

N.	$k_B\ 10^4$ (min^{-1})	$[B]_{t=0}\ 10^{-11}$ ($CFU\ L^{-1}$)*	$k_M\ 10^{16}$ ($L\ CFU^{-1}\ min^{-1}$)	$[M]_{t=0}$ ($mmol\ L^{-1}$)	$R_{L,M}$	r_B^2	r_M^2	r_L^2	r_D^2
a	-1.02±0.01**	0.68±0.01	10.5±13.1	63.6±0.1	0.97±0.01	0.01	0.97	0.97	0.48
b	-0.64±0.01	0.46±0.01	9.07±12.6	60.9±0.1	0.87±0.01	0.19	0.92	0.96	0.58
c	0.14±0.01	6.81±0.01	17.80±13.57	70.0±0.1	0.66±0.01	0.06	0.99	0.99	0.91
d	0.58±0.01	0.04±0.03	9.57±16.23	56.6±0.3	***	0.04	0.76	***	***
e	0.07±0.01	2.17±0.01	1.07±14.53	50.0±0.1	0.99±0.01	0.13	0.94	0.73	0.34
f	0.08±0.01	0.30±0.01	10.27±16.43	53.9±0.2	0.99±0.01	0.74	0.80	0.97	0.28
g	3.89±0.02	0.35±0.01	10.75±13.86	60.7±0.7	0.99±0.01	0.01	0.91	0.84	0.33
h	0.02±0.01	1.12±0.02	24.40±13.71	56.3±0.1	0.88±0.02	0.12	0.99	0.99	0.98
hw	-0.21±0.01	1.17±0.01	26.72±12.84	31.3±0.1	0.91±0.01	0.21	0.99	0.99	0.92

*CFU = colony forming units; k_B, kinetic constant of bacterial population evolution; $[B]_{t=0}$, concentration of bacteria at the initial time; k_M, kinetic constant of L-malate conversion; $[M]_{t=0}$, concentration of L-malate at the initial time; $R_{L,M}$, fraction of L-lactate accumulated in the reaction medium per unit of L-malate converted; **p = 0.05; *** the experimental values related to the time evolution of the two stereoisomers L/D of lactic acid were not determined.

ver when temperature decreases the $R_{L,M}$ value increased for strain OE2, remained substantially unchanged for two strains (OE1 and LB2), and decrease slightly for the strain LB1.

The specific kinetic constant k_M can be assumed as a reliable measure of the ability shown by the different microbial strains to promote malolactic fermentation in the adopted reaction conditions. High k_M values mean significant malolactic activities while reduced values of this constant are related to low conversion of L-malate. While at 25°C all the strains tested appeared to be able to promote a measurable conversion of L-malate (Table 2) only the half of them (OE1, LB1, OE2, and LB2) succeeded to determine a significant transformation of this substrate when temperature was reduced to 10°C (Table 3). At 25°C five (OE1, LB1, OE3, OE5, and OE6) of the eight strains tested, presented values of k_M constant very similar ($9 \div 11 \cdot 10^{-16}$ L CFU^{-1} min^{-1}) but significantly lower than those measured for strains OE2 and LB2 ($k_{M,c} = 17.8\ 10^{-16}$ and $k_{M,h} = 24.4$ L CFU^{-1} min^{-1}) and greater than strain OE4, the microbial population less active (~$1 \cdot 10^{-16}$ L CFU^{-1} min^{-1}). This distribution underwent a relevant change at 10°C. At this temperature some strains (OE3, OE4, OE5 and OE6) became practically inactive, a kinetic behaviour which could be easily forecasted for strain OE4, but not for the other three bacterial populations. In fact all these strains were characterised by a considerable optical density and also by a clearly detectable cell growth at 10°C (Table 1). Moreover strain OE4 shows a slight increase of microbial population as evidenced by the positive value assumed by the related constant k_B although the L-malate is the only available substrate in the reaction medium (Table 3).

When temperature is reduced to 10°C, a significant conversion of L-malate, brought about by an increased number of cells, could be insufficient if the corresponding value assumed by the specific kinetic constant k_M is too low. This underlines the role played by kinetic model to carry out a correct identification of a microbial strain effectively able to promote MLF also at reduced temperatures. In fact, while the strain OE2 lost a great part of its specific kinetic activity when temperature is reduced from 25° to 10°C ($k_{M,c,10°}/k_{M,c,25°} = 0.06$), the reduction shown by strain LB2 is lower ($k_{M,h,10°}/ k_{M,h,25°} = 0.41$). A similar situation is found also for the cell growth, as $Q_{25/10}$ of strain OE2 is 641.8 while that of strain LB2 is 22.3 (Table 1). The results therefore indicate that the strain LB2 is a potential candidate to promote MLF also when the temperature of the cellars and wine is drastically reduced. The kinetic activity of strain LB2 was then evaluated using "Trebbiano toscano" white wine (pH=3.18±0.01; titratable acidity=9.1±0.2 g L^{-1} tartaric acid; malic acid=4.12±0.02 g L^{-1}; lactic acid (D- + L-)= 0.66 ± 0.1 g L^{-1}; volatile acidity = 0.56 ± 0.03 g L^{-1} acetic acid; SO_2=not detectable; ethanol = 10.5± 0.1% v/v; residual sugars < 2 g L^{-1}) previously sterilised by filtration and added with malic acid to a final concentration of ca. 4 g L^{-1}.

The values obtained from the kinetic parameters when LB2 strain was inoculated in wine (hw) are reported in Table 2 in order to be directly compared (run h) with those determined adding the same strain to the model solution (acetate buffer pH= 3.4 aqueous solution of L-malate). As the values obtained from the specific kinetic constant (k_M) appear to be statistically equivalent, no significant differences were found as a function of the reaction medium. The kinetic activity shown by this bacterial strain appears to be almost unaffected by the components dissolved in wine.

Fig. 2 reports the time course of L-malate decrease along with that of L- and D-lactate accumulation. The good level of overlap occurring between experimental and calculated values, and

Table 3 - Parameters (mean ± CI) and corresponding square values of the correlation coefficients of the kinetic model adopted to describe the time evolution of the bacterial population and of L-malate (M), L-lactate (L) and D-lactate (D) concentrations during malolactic fermentation carried on at 10°C by the different populations of malolactic bacteria tested which were added to an acetate buffer (pH=3.4) aqueous solution of L-malate.

N.	$k_B\ 10^4$ (min^{-1})	$[B]_{t=0}\ 10^{-11}$ (CFU L^{-1})*	$k_M\ 10^{16}$ (L CFU^{-1} min^{-1})	$[M]_{t=0}$ (mmol L^{-1})	$R_{L,M}$	r_B^2	r_M^2	r_L^2	r_D^2
a	-0.29±0.01**	0.59±0.01	4.83±0.01	74.1±0.1	0.99±0.01	0.68	0.95	0.96	0.39
b	0.05±0.01	1.96±0.01	6.29±9.46	75.5±0.1	0.73±0.01	0.01	0.99	0.99	0.98
c	-0.32±0.01	8.62±0.01	0.97±5.57	70.0±0.1	0.84±0.01	0.02	0.99	0.99	0.95
d	-0.79±0.01	22.70±0.03	-	71.8±0.2	-	0.74	-	-	-
e	0.43±0.01	0.05±0.01	-	74.3±0.2	-	0.89	-	-	-
f	-0.05±0.01	0.07±0.01	-	70.3±0.4	-	0.52	-	-	-
g	-0.23±0.01	0.12±0.01	-	73.6±0.7	-	0.39	-	-	-
h	-2.63±0.02	1.48±0.02	9.94±11.1	36.7±0.1	0.93±0.02	0.20	0.93	0.97	0.74

*CFU = colony forming units; k_B, kinetic constant of bacterial population evolution; $[B]_{t=0}$, concentration of bacteria at the initial time; k_M, kinetic constant of L-malate conversion; $[M]_{t=0}$, concentration of L-malate at the initial time; $R_{L,M}$, fraction of L-lactate accumulated in the reaction medium per unit of L-malate converted; **p = 0.05; *** the experimental values related to the time evolution of the two stereoisomers L/D of lactic acid were not collected.

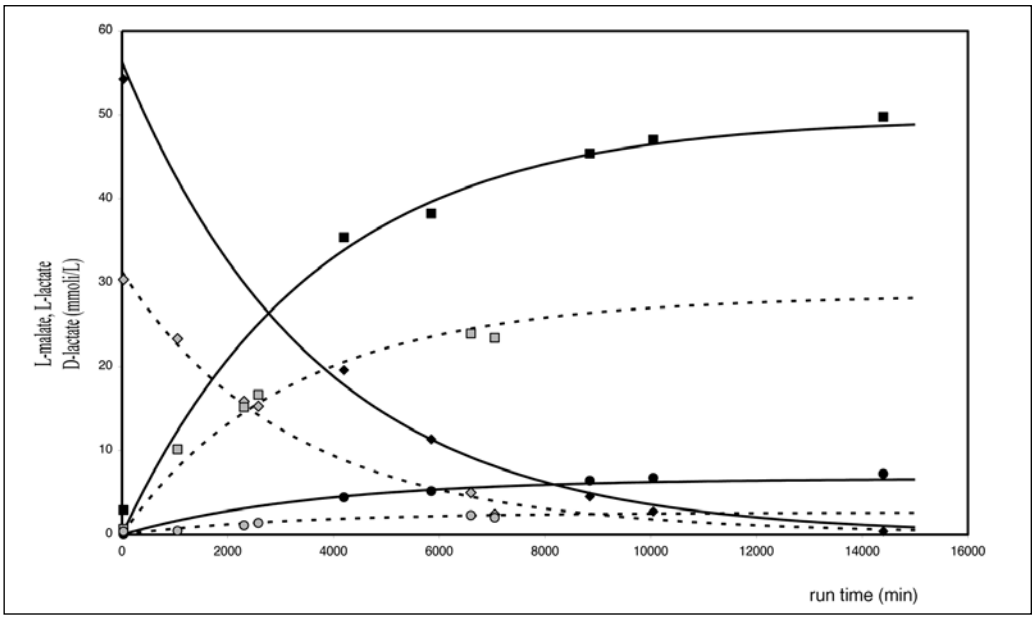


Fig. 2 - The time evolution of experimental and calculated values of L-malate ($[M]_{t=t}$; diamonds for experimental values), D- ($[D]_{t=t}$; circles for experimental values) and L-lactate ($[L]_{t=t}$; squares for experimental values) concentrations, when bacterial cells of strain 'h' were inoculated in a real wine added with L-malic acid (—; closed symbols for experimental values) or to an acetate buffer (pH=3.4) aqueous solution of L-malic acid (---; empty symbols for experimental values).

the high values obtained by the squares of correlation coefficients calculated for the substrate (L-malate; r_M^2) and end-products (D- and L-lactate; r_L^2 and r_D^2) of malolactic fermentation (Tables 2 and 3), provide a satisfactory level of reliability of the kinetic model utilised.

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VOLATILE COMPOSITION OF *"A LA PIEDRA" TURRÓN* ENRICHED WITH ROYAL JELLY

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ABSTRACT

"*A la piedra*" (ALP) *turrón* was used as model matrix to add a functional ingredient: royal jelly (RJ, 1.0%). The aroma profiles of control and RJ enriched ALP *turrón* were studied; 58 compounds were found (21 terpenes, 14 terpenoids, and 8 aldehydes), most of them coming from lemon peel. The attributes toasted, nutty and cinnamon were also of importance. The addition of RJ did not change the aroma profile and only slightly the sensory profile of this confection but will improve its health benefits by incorporating in the consumers' diet 500 mg RJ per 50 g of RJ enriched ALP *turrón*.

- Keywords: almond, descriptive sensory analysis, GC-MS, lemon, nougat, torrone -

INTRODUCTION

The main ingredients of "*a la piedra*" (ALP) *turrón* are toasted almonds, sugars, lemon peel and cinnamon; it is a typical Christmas confection. This product can be considered the simplest version of *turrón* because only grinding and refining operations are involved in its manufacture (VÁZQUEZ-ARAÚJO *et al.*, 2008). First, the ingredients are added into a stone grinder and are ground until the crystalline texture of sugars disappears. Next, the mass is placed in refining grinders in order to reduce the particle size below 50 μm. Later, the mixture is moulded and cinnamon is added on top of the product. Finally, the product is cut, packed, and stored until it is distributed in the market, mainly Spanish markets. Other types of Christmas confections, such as Spanish Jijona and Alicante *turrón*, Italian *torrone* or French *nougat* involve heating steps and the functional ingredients should be thermo-resistant (VÁZQUEZ-ARAÚJO *et al.*, 2006; VÁZQUEZ *et al.*, 2007; VERDÚ *et al.*, 2007). This simple manufacturing process and the fact that it does not involve any heating steps makes ALP *turrón* the perfect matrix to add functional ingredients. In this way, ALP *turrón* can be used as a model to imitate other similar confections, such as *turrón*, *torrone* or *nougat*.

Unusual ingredients in *turrón* manufacturing, such as cinnamon and lemon peel, are included in the formulation of ALP *turrón* because no honey is used and because the full flavor potential of toasted almonds is not built up due to the absence of thermal treatments (VÁZQUEZ-ARAÚJO *et al.*, 2008). The addition of a bee product, such as royal jelly, to ALP *turrón* could be useful to include this type of confection in the list of products under the Protected Geographical Indication (PGI) "*Jijona y Turrón de Alicante*" because turrón is defined as "confection made with toasted almonds and honey produced in a traditional way".

Bees are known to exist for more than 125 million years and they are able to live in virtually all habitats on Earth (BANKOVA, 2005). This evolutionary success is mainly due to specific products that bees manufacture and use for different purposes: honey, royal jelly, pollen, propolis, beeswax and venom. Royal jelly (RJ) is the main food for the honeybee queen; it is a yellowish, creamy, and acidic material with a slightly pungent odour and taste. RJ possesses several pharmacological activities, such as life-span-elongating, antifatigue, antiallergic, antitumor, antihypercholesterolemic, antihypertensive and anti-inflammatory effects (MELLIOU and CHINOU, 2005). These effects are related to its chemical composition, including proteins, sugars, vitamins and bioactive substances such as *trans*-10-hydroxy-2-decenoic acid. In fact, RJ has been used worldwide as commercial medical products, health foods and cosmetics.

The main objective of this study was to describe qualitatively and quantitatively the volatile composition of ALP *turrón* enriched with royal jelly. Aroma profiles of royal jelly, cinnamon and lemon peel were also studied. This study is included within a general project in which new products based on *turrón* are being designed and developed.

MATERIAL AND METHODS

Materials

Functional "*a la piedra*" (ALP) *turrón* was prepared at the facilities of the *turrón* company *Pablo Garrigós Ibáñez S.L.*, located in Jijona (Alicante, Spain). Royal jelly was purchased from the *Mieles Llovell S.L.* (Carcer, Valencia, Spain). Two hundred bars of ALP *turrón* enriched with RJ were prepared in four different batches (50 bars batch^{-1}). The ingredients composition of the functional ALP *turrón* was as follows: toasted almonds (56-60%), sugar, lemon peel, cinnamon, and propolis (1.00%). The control samples (50 bars) did not contain any RJ.

Threshold of royal jelly detection

The objective of the study was to determine the detection threshold (maximum amount needed for detecting the stimulus, in %) of RJ in ALP *turrón*. A consumer panel of 40 panellists (regular consumers of *turrón* during Christmas time) was used for this sensory test. ALP *turrón* with eight royal jelly concentrations was studied (0, 0.25, 0.50, 0.75, 1.00, 1.25, 1.50, 1.75 and 2.00%). The panellists were asked to say whether they noticed something different from the initial sample of ALP *turrón* (control sample with no RJ addition).

Simultaneous Steam-Distillation Extraction (SDE)

A suspension of 30 g of ALP *turrón* in 75 mL of ultrapure water (Milli-Q, Millipore Corp., Bedford, MA, Usa) was placed in flask A of the Likens-Nickerson distillator (Afora, Barcelona, Spain) with 10 g NaCl (Merck, Darmstadt, Germany). In flask B, 50 mL of Cl_2CH_2 (Labscan Ltd., Dublin, Ireland) was introduced. Boiling chips were added to flask A together with 10 drops of silicon as an antifoaming agent and the internal standard, 2-pentanol (100 μL of 1 g L^{-1}). Flask B was heated in a water bath at 55°C and flask A in an oil heater at 160°C. The vapours were condensed by means of a cold refrigerant maintained at -5°C by a cryostat, model Frigiterm (Selecta, Barcelona, Spain). After 120 min of extraction, the organic solvent contained the aroma compounds from ALP *turrón* samples (VÁZQUEZ *et al.*, 2007). The extract was dried over 5 g anhy-

drous Na_2SO_4 (Panreac Química S.A., Barcelona, Spain) and concentrated to about 1 mL in a Vigreaux column.

GC-MS analytical conditions

GC-MS analyses were performed with a GC-MS-QP5050A Shimadzu (Shimadzu Corporation, Kyoto, Japan) model gas chromatograph-mass spectrometer equipped with an AOC-20i autoinjector and a TRACSIL Meta.X5 column, 95% dimethyl-polysiloxane and 5% diphenyl-polysiloxane (Teknokroma S. Coop. C. Ltd, Barcelona, Spain; 30 m × 0.25 mm × 0.25 μm film thickness). The experimental conditions were those reported earlier by BOLECHOWSKI *et al.* (2011). Data handling was made through *GCMSolution 1.01* (Shimadzu).

The quantification of the volatile compounds was performed using a Shimadzu 2010 gas chromatograph with a flame ionization detector (FID). The column and chromatographic conditions were similar to those reported for the GC-MS analysis. The injector temperature was 250°C and nitrogen was used as a carrier gas (1 mL min^{-1}). Data handling was carried out by means of *GCsolution 2.3* (Shimadzu).

Tables 1 and 2 show the authentic chemicals used as standards (std), the system of identification of each compound and experimental and literature Kovats indices (KI) for ALP turrón and royal jelly, respectively. Identification was considered tentative when it was based exclusively on mass spectral data.

Sensory evaluation with trained panel

A panel of 10 judges, aged 20 to 60 (6 females and 4 males) was trained for the descriptive evaluation of *turrón* and its main raw materials, i.e. almonds and honey. The details about panel selection and training can be found in VÁZQUEZ-ARAÚJO *et al.* (2005).

Descriptive Sensory Analysis (DSA) has been successfully used for comparing odour and taste attributes in foods and their products (ALASALVAR *et al.*, 2003). *Turrón* samples were assessed using a flavour profile method (MEILGAARD *et al.*, 1999). Prior to DSA, the panellists discussed the flavour properties of ALP *turrón* during two preliminary orientation sessions, each lasting 90 min, until they agreed on their use of flavour attributes. During these orientation experiments, the panellists evaluated six different coded samples of ALP *turrón* from different manufacturing companies. Fourteen flavour attributes were identified and the standards were made available to the panellists. DSA was performed for 14 flavour attributes (Table 3).

The measurements were performed in individual booths with controlled illumination (70-90 fc) and temperature (23°±2°C) (MEILGAARD *et al.*, 1999). Individual *turrón* samples were scored for the intensity of the studied attributes using a 100 mm-long line with line anchors (0 = no intensity; 100 = very intense). The panellists relied on their training experience to score products. The samples were randomly presented to each panellist (coded with a three-digit random number). The samples were evaluated in triplicate.

Statistical analysis

For each of the above-mentioned analyses at least 3 replications were carried out. The ± values represent the standard errors of the measurements. The data from the DSA experiments were subject to the analysis of variance (ANOVA) and the Tukey's least significant difference multi-comparison test in order to determine significant differences between the samples. Statistical analyses were done using a Statgraphics Plus 5.1 software (Manugistics, Inc., Rockville, MD, Usa) and graphics using a Sigma Plot 9.0 (SPSS Science, Chicago, Usa).

RESULTS AND DISCUSSION

Detection threshold

Duo to the peculiar and not pleasant flavour of royal jelly (phenolic odour, not sweet, acidic and slightly pungent and rancid), it was of great importance to determine its detection threshold. Fig. 1 shows that the detection threshold of royal jelly (the lowest stimulus capable of producing a sensation) was approximately 1.03%; at this concentration, 50% of the consumers included in the panel were able to detect a significant difference from the control sample. Consequently, a final concentration of 1.0% of royal jelly was chosen as an ingredient to ALP *turrón*.

Biological activity

Royal jelly contains considerable amounts of proteins, free amino acids, lipids, vitamins, and sugars, and it is known to have several diverse physiological and pharmacological functions in humans. These include vasodilative, hypotensive, anti-hypercholesterolemic, and anti-tumor activities (NARITA *et al.*, 2006; MATEESCU and BARBULESCU, 1999). All these health-related benefits make RJ attractive as a natural preservative in new food applications. This meets the demand for natural antioxidants and antimicrobials, fuelled by the increasing consumer awareness of natural, minimally processed foods with traditional preservatives either absent or at very low concentrations (KALOGEROPOULOS *et al.*, 2009). Besides, royal jelly proved to have higher antioxidative activities than honey (NAGAI *et al.*, 2001).

The functional ALP *turrón* studied here will provide consumers with 1,000 mg of RJ per

Table 1 - Volatile compounds identified in alp *turrón* enriched with royal jelly, their identification methods and Kovats indices for the present study and literature values (NIST, 2011).

Compound	Peak	RT (min)	Kovats Index (KI)		Concentration (mg kg^{-1})	
			Exp.	Lit.	Control	Royal Jelly
Pyrrole	1	5.05	769	762	0.05±0.02	0.10±0.01
Dihydro-2-methyl-3(2H)-furanone†	2	6.83	808	804	0.06±0.02	0.06±0.02
2-Methylpyrazine†	3	7.58	829	825	0.05±0.02	0.04±0.01
Furfural†	4	8.03	842	833	0.22±0.05	0.09±0.01
Heptanal†	5	11.62	902	903	0.06±0.01	0.06±0.01
2,5-Dimethylpyrazine	6	12.05	910	926	0.08±0.05	0.04±0.01
α-Thujene	7	12.99	927	923	0.95±0.03	1.01±0.10
α-Pinene†	8	13.32	933	933	4.21±0.16	4.51±0.45
Camphene	9	14.13	947	945	0.16±0.02	0.17±0.02
cis-2-Heptenal	10	14.68	957	951	0.03±0.01	0.02±0.01
Benzaldehyde†	11	14.79	959	960	0.34 ±0.08	0.26±0.02
Sabinene	12	15.62	974	973	6.46±0.06	6.59±0.56
β-Pinene†	13	15.78	977	981	36.5±0.9	38.2±2.8
1-Octen-3-ol†	14	16.27	986	982	0.04±0.01	0.03±0.01
6-Methyl-5-hepten-2-one	15	16.41	988	994	0.09±0.01	0.09±0.01
Myrcene	16	16.70	994	991	3.72±0.05	3.81±0.28
Octanal†	17	17.34	1005	1006	0.72±0.05	0.82±0.09
α-Terpinene	18	18.06	1018	1014	0.59±0.05	0.65±0.22
p-Cymene	19	18.55	1027	1027	3.55±0.24	3.49±0.24
Limonene†	20	18.98	1035	1029	229±4	231±28
Benzyl alcohol†	21	19.34	1042	1036	0.67±0.10	0.62±0.05
Phenylacetaldehyde†	22	19.50	1045	1050	0.55±0.16	0.56±0.06
cis-β-Ocimene	23	19.92	1052	1051	0.22±0.01	0.23±0.02
γ-Terpinene	24	20.52	1063	1059	36.4±0.4	36.6±4.1
cis-Sabinene hydrate	25	21.07	1073	1073	0.30±0.03	0.28±0.04
1-Octanol†	26	21.38	1079	1072	0.23±0.01	0.22±0.02
Terpinolene	27	22.01	1090	1084	1.61±0.01	1.62±0.14
trans-Sabinene hydrate	28	22.76	1104	1080	0.33±0.01	0.32±0.03
Linalool	29	22.91	1107	1098	0.51±0.03	0.45±0.07
Nonanal†	30	22.99	1109	1103	2.11±0.08	2.16±0.22
cis-Limonene oxide	31	24.73	1142	1139	1.53±0.05	1.50±0.14
Citronellal	32	25.59	1159	1161	0.67±0.02	0.64±0.06
Terpinen-4-ol	33	26.98	1185	1178	0.28±0.01	0.26±0.03
α-Terpineol	34	27.78	1201	1193	2.07±0.04	2.02±0.21
Decanal†	35	28.38	1213	1209	0.43±0.01	0.42±0.05
cis-Cinnamaldehyde†	36	28.96	1224	1215	0.32±0.05	0.31±0.03
trans-Carveol	37	29.22	1230	1207	0.75±0.09	0.63±0.06
Nerol†	38	29.59	1237	1233	0.39±0.02	0.44±0.04
Neral	39	30.11	1248	1235	2.66±0.10	2.54±0.28
Geraniol	40	30.98	1265	1276	0.37±0.21	0.78±0.08
trans-2-Decenal	41	31.24	1271	1278	0.09±0.01	0.08±0.02
trans-Cinnamaldehyde†	42	31.72	1280	1283	19.3±1.1	18.1±1.9
(Z,Z)-2,4-Decadienal†	43	32.85	1304	1293	0.05±0.01	1.48±0.08
(E,E)-2,4-Decadienal†	44	33.93	1327	1316	0.34±0.09	0.18±0.01
Citronellyl acetate	45	35.59	1363	1357	0.21±0.01	0.20±0.02
Eugenol†	46	35.84	1368	1364	0.28±0.01	0.28±0.05
Neryl acetate	47	36.07	1373	1365	2.25±0.04	2.19±0.29
α-Copaene	48	36.76	1388	1377	0.10±0.02	0.07±0.02
Geranyl acetate	49	36.97	1392	1382	3.09±0.12	2.88±0.19
Tetradecane†	50	37.91	1413	1400	0.18±0.01	0.19±0.01
(*Z*)-β-Farnesene	51	38.53	1427	1431	0.14±0.01	0.11±0.02
trans-Caryophyllene	52	38.79	1433	1428	1.18±0.03	1.11±0.06
α-Bergamotene	53	39.47	1448	1446	2.27±0.12	2.14±0.11
(*E*)-Cinnamyl acetate	54	39.86	1457	1441	0.08±0.01	0.06±0.01
β-Santalene	55	40.60	1474	1476	0.10±0.06	0.04±0.01
(*E,E*)-α-Farnesene	56	41.68	1499	1500	0.07±0.01	0.05±0.01
(*E*)-β-Bisabolene‡	57	42.44	1517	1508	0.22±0.01	0.20±0.03
γ-Bisabolene‡	58	42.73	1524	1525	2.76±0.12	2.60±0.13
Total					**373±7**	**376±29**

† Both Kovats indexes and retention times of authentic standards were used for identification besides the general comparison of experimental mass spectra to spectra of authentic standards and Wiley library. ‡ Tentatively identified.

Table 2 - Volatile compounds identified in royal jelly with their identification method and Kovats indices for the present study and literature values (NIST, 2011).

Compound	Peak	RT (min)	Kovats Index (KI)		Concentration (mg kg^{-1})
			Experimental	Literature	
1-Pentanol	1	5.72	778	771	0.28±0.01
Benzaldehyde†	2	14.79	955	960	0.54±0.05
1-Heptanol	3	15.75	971	970	0.14±0.01
1-Octen-3-ol†	4	16.27	980	982	0.10±0.01
Benzyl alcohol†	5	19.34	1034	1036	0.60±0.06
Phenylacetaldehyde†	6	19.50	1039	1050	0.16±0.01
cis-Sabinene hydrate	7	21.07	1066	1068	0.38±0.02
1-Octanol†	8	21.38	1072	1072	0.20±0.01
trans-Sabinene hydrate	9	22.76	1097	1080	0.54±0.02
Linalool	10	22.91	1099	1098	0.30±0.05
Nonanal†	11	22.99	1103	1103	0.16±0.02
cis-Limonene oxide	12	24.73	1135	1139	0.33±0.03
Terpinen-4-ol	13	26.98	1177	1178	0.14±0.01
α-Terpineol	14	27.78	1191	1193	1.06±0.11
cis-Cinnamaldehyde†	15	28.96	1215	1215	0.90±0.09
trans-Carveol	16	29.22	1219	1207	0.12±0.01
Neral	17	30.11	1239	1235	0.72±0.07
trans-Cinnamaldehyde†	18	31.72	1269	1283	11.0±0.55
Total					**17.6±1.3**

† Both Kovats indexes and retention times of authentic standards were used for identification besides the general comparison of experimental mass spectra to spectra of authentic standards and Wiley library.

each 100 g of the product. The data in literature show that a daily intake of 200 mg of RJ is recommended (RDI), and needed to have positive a positive impact on health (ASÍS, 1996). A daily consumption of 20 g of the RJ enriched ALP *turrón* will cover 100% of the RDI. In fact, the normal daily consumption of *turrón* during Christmas is usually higher (about 50 g).

Summing up, ALP *turrón* enriched with RJ will be of superior quality (according to its biological activity) as compared to the traditional ALP product and other types of *turrón*, such as Jijo-

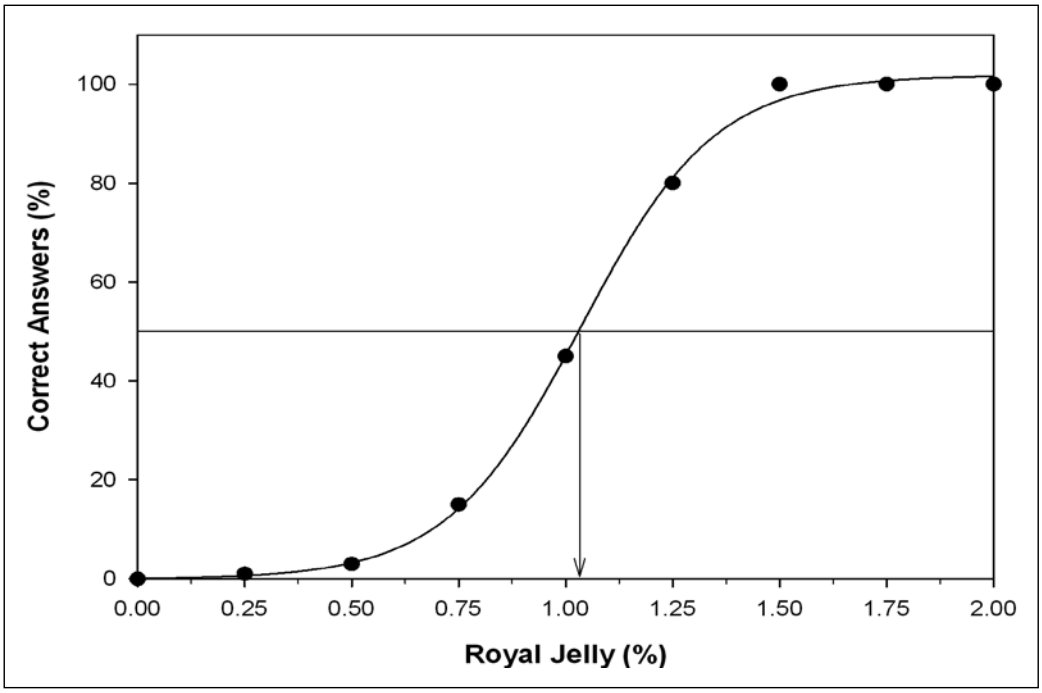


Fig. 1 - Determination of royal jelly detection threshold in *"a la piedra" turrón*.

Table 3 - Flavour attributes selected for DSA.

Attribute	Characteristics
Acid	Taste associated with citric acid
Aftertaste	Remaining desirable and delicate flavour after swallowing
Astringent	Causing contraction of mouth tissues
Bitter	Taste associated with caffeine
Burnt	Smell of grilled meat, burnt smell
Cinnamon	Aroma and flavour of cinnamon
Lemon	Aroma and flavour of lemon
Nutty	Delicate, characteristics flavour of tree nut products
Oily	Oily taste or mouthfeel
Pungent	Burning or stinging sensation
Rancid	Associated with old or oxidised fat
Sweet	Taste associated with sugar
Toasted	Flavour of toasted nuts
Woody	Odour of almond hard shell

na or Alicante due to higher biological activity of RJ as compared with honey.

Volatile compounds

Total concentrations of volatile chemicals in control and functional ALP *turrón* samples were 373 ± 7 mg kg^{-1} and 376 ± 29 mg kg^{-1}, respectively. Table 1 shows that the addition of RJ at a concentration of 1.00% did not increase the concentrations of volatile compounds.

According to the composition (percentage of each ingredient) of ALP *turrón*, its ingredients can be ordered as follows: toasted almonds > sugar > lemon peel > cinnamon ≈ royal jelly. However, the order of the concentration of total volatiles from different raw materials was quite different: lemon peel > cinnamon > toasted almonds ≈ sugars > royal jelly. Total concentrations of the raw ingredients not studied until this study were: (i) lemon peel (15,135 ± 176 mg kg^{-1}), (ii) cinnamon (8,220 ± 41 mg kg^{-1}), and (iii) RJ (17.6 ± 1.3 mg kg^{-1}). The concentrations of total volatiles in sugar matrix and toasted almonds were previously established by VÁZQUEZ *et al.* (2007) and VÁZQUEZ-ARAÚJO *et al.* (2008) and were 174 mg kg^{-1} (for sugars at the end of the concentration step of honey, sugars, and water) and 228 mg kg^{-1} (mean value of almonds from three different cultivars: *Marcona*, *Comuna*, and *California*), respectively.

Therefore, the first conclusion from this study was that ALP *turrón* was more aromatic than Alicante and Jijona *turrón* mainly due to the lemon peel and cinnamon used in its formulation. Besides, the role of RJ in the aromatic profile of ALP *turrón* is insignificant due to its low concentration of volatile compounds.

The main compounds identified in the lemon peel were: limonene (7,890 mg kg^{-1}, 52.1% of total concentration of volatiles), γ-terpinene (2,342 mg kg^{-1}, 15.5%), β-pinene (1,220 mg kg^{-1}, 8.1%), α-terpineol (464 mg kg^{-1}, 3.1%), geranial (408 mg kg^{-1}, 2.7%), neryl acetate (301 mg kg^{-1}, 2.0%), neral (291 mg kg^{-1}), and geranyl acetate (202 mg kg^{-1}, 1.3%). These compounds have already been reported for lemon products (ALLEGRONE *et al.*, 2006).

trans-Cinnamaldehyde was the main compound in cinnamon (7,456 mg kg^{-1}) and represented almost 90.7% of the total concentration of volatile compounds in this raw material. Other important compounds were: cinnamyl acetate (199 mg kg^{-1}, 2.4% of total concentration of volatiles), eugenol (152 mg kg^{-1}, 1.8%), linalool (74.8 mg kg^{-1}, 0.9%), *cis*-cinnamaldehyde (62.5 mg kg^{-1}, 0.8%), α-terpineol (60.6 mg kg^{-1}, 0.7%), and terpinen-4-ol (45.0 mg kg^{-1}, 0.5%). The same compounds but at different concentrations were found by KAUL *et al.* (2003) in their studies of essential oils isolated from different parts of cinnamon (*Cinnamomum zeylanicum* Blume) from India and Sri Lanka.

Eighteen volatile compounds were found and tentatively identified in the RJ extracts (Table 2). The main compound was *trans*-cinnamaldehyde, which represented 62.5% of the total concentration of volatiles; its main sensory descriptors are cinnamon, clove and spice. *trans*-Cinnamaldehyde seems to be responsible, at least in part, of the spice flavour of RJ. The most abundant compounds were α-terpineol, *cis*-cinnamaldehyde, neral, and benzyl alcohol, which represented 6.0, 5.1, 4.1 and 3.4% of the total concentration of volatiles.

Fifty-eight compounds were identified in both control and RJ enriched ALP *turrón* and can be grouped in eleven chemical families: monoterpenoids, monoterpenes, sesquiterpenes, aldehydes, aromatic hydrocarbons, furans, pyrazines, alcohols, pyrroles, ketones, and linear hydrocarbons. Monoterpenes, monoterpenoids, sesquiterpenes, aromatic hydrocarbons and aldehydes were the predominant chemical groups. The volatile compounds from the first three chemical groups are due to the addition of lemon peel to this confection, while aromatic hydrocarbons and aldehydes result from the addition of almond and/or cinnamon.

Taking into account low aromatic intensity of RJ (17.6 mg kg^{-1}) and its low percentage in the confection formula (1%), its on modification of the ALP *turrón* seems to be impossible.

The major group of chemicals found in control and RJ enriched ALP *turrón* extracts was that of monoterpenes (with 12 hydrocarbons present) comprising 86.8 and 87.3% of the concentration of total volatiles, respectively. A large amount of limonene (229 and 231 mg kg^{-1}) was composed of 61.4% of both the control and functional ALP *turrón*. γ-Terpinene, β-pinene, sabinene and α-pinene were the next most abundant monoterpenes found in ALP *turrón* samples. Even though limonene was more abundant than other monot-

erpenes and because of its high odour threshold (1 mg L^{-1}), the other compounds, such as α-pinene will play more important role in the final flavour of this product due to its very low odour threshold (0.006 mg L^{-1}). The odour units (concentration/threshold) of α-pinene and limonene were 727 (mean of both ALP *turrón* samples) and 230, respectively, showing a relatively low effect of limonene on ALP *turrón* flavour. In fact, β-pinene with a mean value of 267 also showed higher odour units than limonene.

On the other hand, the chemical group with the highest number of chemicals was monoterpenoids, 14. Within this group, the most abundant compounds were geranyl acetate, neral, neryl acetate, α-terpineol, and limonene oxide. However, the combination of relatively low concentrations and high odour thresholds reduced contribution of monoterpenoids to the odour and aroma of ALP *turrón*.

Finally, the less important chemical group present in the lemon peel was that of sesquiterpenes, representing only about 1.9% of the concentration of total volatiles. Eight compounds were included in this group, with γ-bisabolene and β-bergamotene being the most abundant ones.

Eight aldehydes (heptanal, 2-heptenal, Octanal, nonanal, etc.) were found in ALP *turrón*. Aldehydes increased with toasting of nuts and prolonged storage, due to the development of rancidity. They can enhance the aroma of foods both due to their high concentrations and low odour thresholds (VÁZQUEZ-ARAÚJO *et al.*, 2008; VÁZQUEZ *et al.*, 2007). This situation can also be true with ALP *turrón*, especially because of the low odour thresholds. The mean odour units of the most important aldehydes found in the two types of ALP *turrón* under investigation were: 4,300 for decanal, 3,714 for (E,E)-2,4-decadienal, 2,140 for nonanal, and 1,100 for octanal.

Seven aromatic hydrocarbons were detected in ALP *turrón* (benzaldehyde, benzyl alcohol, *cis*- and *trans*-cinnamaldehyde, etc.). The predominant aromatic hydrocarbon was *trans*-cinnamaldehyde, present both in cinnamon and RJ. However, due to its high odour threshold (20 mg kg^{-1}), its importance is insignificant for the final aroma of the ALP *turrón*.

Only one or two compounds from the following chemical families: pyrazines, furans, pyrroles, ketones, alcohols and linear hydrocarbons, were found in ALP turrón, and their concentrations were low enough to affect the final odour and aroma of this confection. The low concentrations of pyrazines, furans and pyrroles also proved that almonds used in the manufacture of this confection are only slightly toasted.

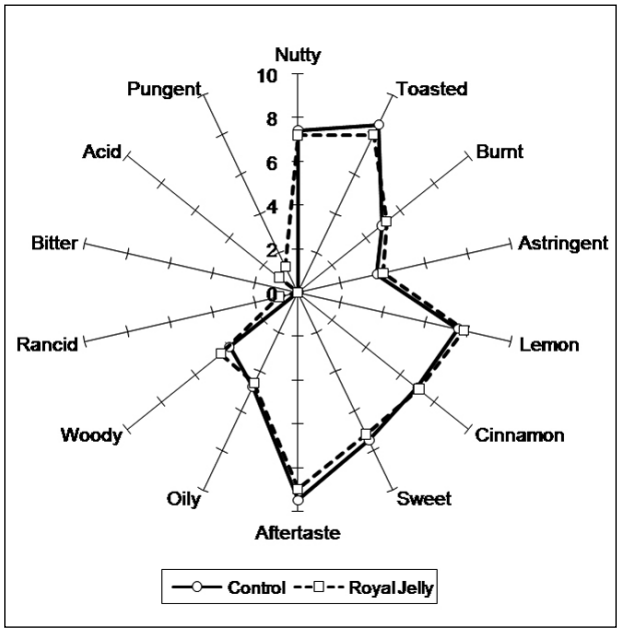


Fig. 2 - DSA of flavour attributes in control and functional (enriched with royal jelly) "*a la piedra*" *turrón*.

Sensory evaluation

The flavor of RJ could be defined as having low but significant impact on the following attributes of the product: astringent, acid, pungent, spicy and/or rancid. Besides, *trans*-cinnamaldehyde, the most abundant compound in RJ, has a sharp spicy and even pungent taste. The addition of RJ is expected to improve the taste rather than the odour or aroma of ALP *turrón*, due to its low aromatic intensity.

Intensities of a number of flavour attributes (nutty, toasted, burnt, astringent, lemon, cinnamon, sweet, aftertaste, oily, and woody) did not show significant differences ($p > 0.05$) (Fig. 2) between the control and RJ enriched ALP *turrón* samples. The contribution of rancidity, acidity, and pungency increased in about one unit (scale 0 to 10) in RJ enriched products as compared to the negligible intensities of these three attributes in the control samples.

CONCLUSIONS

Royal jelly was added at a maximum concentration possible without changing the main sensory properties of "*a la piedra*" (ALP) *turrón*, as perceived by a consumer panel; this concentration was determined at 1.0%. A total of 58 compounds was detected in both the control and the royal jelly (RJ) enriched ALP *turrón*, including monoterpenes, sesquiterpenes, monoterpenoids, pyrazines, furans, pyrroles, aldehydes, and aromatic hydrocarbons. The main chemical groups, terpenes and terpenoids, originated from the lemon peel, and their total concentration represented more than 90% of the total volatile compounds. However, the other compounds, *e.g.* aldehydes could also play an important role in the final aroma of the ALP *turrón*, due to their

low odour thresholds. Descriptive sensory analysis proved that the main attributes characterizing this confection were lemon, toasted, nutty, sweet, cinnamon, and aftertaste; the addition of RJ slightly increased the scores of rancidity, acidity and pungency (these attributes were noticed by the trained panel but not by the consumer panel). Both the sensory and instrumental analyses of ALP *turrón* demonstrated the predominant role of the compounds present in lemon in the overall aroma of the product. The addition of RJ to the ALP *turrón* did not change its instrumental aroma profile or sensory properties significantly, but it will contribute with 500 mg per 50 g of ALP *turrón* ingested.

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PAPER

MONITORING BACTERIOLOGICAL QUALITY OF PRECOOKED LASAGNA

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ABSTRACT

The effects of two preparation methods, two packaging materials, and modified atmosphere condition, on the bacteriological quality and safety of precooked lasagna, were studied. Microbiological analyses focused on *Salmonella* spp., *Listeria monocytogenes*, *Bacillus cereus*, *Escherichia coli*, *Staphylococcus aureus*, spores of sulphite-reducing clostridia, and on mesophilic and psychrotrophic aerobic bacteria, total coliforms and lactic acid bacteria. In total, 81 samples were examined, for up to 28 days. No pathogenic microorganisms were detected. After 28 days, spoilage-hygiene markers levels were non-compliant to reference standards. In conclusion, lasagna prepared with hot method in polyethylene packages were acceptable for up to 21 days.

- Keywords: precooking, modified atmosphere packaging, lasagne, catering -

1. INTRODUCTION

In Italy it is estimated that state school catering services serve more than 2,700,000 students daily. This represents a huge "social restaurant" in which the State, through local authorities, basically chooses the organization, raw materials, and the menus (Emilia-Romagna, Italy, 2009; Conferenza Permanente Stato-Regioni, 2010). Considering the complexity of the organizations involved, the vulnerability of the people who receive the foodservices, and the consequences of possible adverse events, it is clear that catering services are of great strategic importance in the program to develop the right nutrition and to ensure food safety and nutritional quality.

In Italy the traditional "cook-serve" foodservice system is the most common in school catering (CIAPPELLANO, 2009), in which ingredients are assembled and food is produced on-site, kept either heated or chilled for up to two hours, and served to consumers.

Nevertheless, in the school kitchens that prepare thousands of meals per day there are some dishes that cannot be prepared using the cook-serve method, due to the complexity of the recipe and the long period of time requested for assembly and cooking. In these cases the caterers often purchase already precooked, chilled or frozen dishes from outside suppliers, such as lasagna.

Lasagna is a classic Italian pasta casserole dish, which consists of alternate layers of pasta, cheese and often other ingredients such as minced beef with tomato sauce. It is one of the most well-liked recipes in Italian school foodservices (DRAGONI *et al.*, 2005).

Modified atmosphere packaging (MAP) in combination with chill storage (<4°C) is a well-known food preservation method, but in the Italian school catering is still not widespread in meals preparation systems (CIAPPELLANO, 2009). The right combination of gases (CO_2, N_2 and O_2) in the headspace of food packs suppresses the microbial flora of perishable foods that normally develop under aerobic conditions (PATSIAS *et al.*, 2003). A minimum CO_2 concentration of 20-30% is necessary to produce inhibitory effects (STILES, 1991). Chilling slows the deterioration of stored foods but if the atmosphere surrounding the product is also modified to reduce oxygen concentration, the shelf-life is increased considerably because of a further reduction in the rate of chemical oxidation by oxygen and in the growth of aerobic microorganisms (PHILLIPS, 1996). Nevertheless, concerns have been expressed that the increase in shelf-life of MAP products provides sufficient time for human pathogens to multiply to levels which render the food unsafe while still edible (JAY, 1992).

Pathogens that are able to multiply at chill temperatures (-1° to +7°C) (RAY, 2004) and that are able to growth in very low levels of oxygen as psychrotrophic *Clostridium botulinum* and *Listeria monocytogenes* (minimum growth temperature: 0° to 1°C) (RAY, 2004) are of a particular concern (PHILLIPS, 1996; RAY, 2004). Some of the major microbiological hazards associated with modified atmosphere packaging are: 1) *Clostridium botulinum* type E, which is capable of growth and toxin production at 3°C; 2) *Listeria monocytogenes*, enterotoxigenic *Escherichia coli* and spore-formers such as *Bacillus cereus* can survive inadequate heat and then grow during the chilled storage of the product; 3) any leaks in the seal and packaging material can lead to post-thermal processing contamination by pathogens (NYATI, 2000). However, the use of MAP with precooked meals that are subsequently cooked is considered less hazardous because cooking (if correctly carried out) kills vegetative pathogens such as *Listeria monocytogenes*, *Salmonella* spp., *Escherichia coli*, *Staphylococcus aureus*, *Bacillus cereus*, and *Clostridia* (HOTCHKISS, 1988).

The aim of this study was to evaluate and compare the bacteriological quality and safety of refrigerated precooked lasagna, experimentally prepared by a traditional cook-serve centralized school kitchen in Lombardy, Italy. This study was carried out testing the efficacy of two production processes and two types of packaging materials, under a modified atmosphere, and stored at a refrigerator temperature (cold room – Angelo Po Grandi Cucine S.p.A., Italy – set at 2°C) for up to 28 days. The ultimate goal was to determine the most suitable production method and type of packaging to ensure as high a level of microbial safety as possible. The microbiological analysis focused on pathogenic and potential-pathogenic microorganism markers (*Salmonella* spp., *Listeria monocytogenes*, *Bacillus cereus*, *Escherichia coli*, *Staphylococcus aureus*, and spores of sulphite-reducing clostridia); as well as spoilage-microorganisms and hygienic markers: mesophilic aerobic and psychrotrophic bacteria, total coliforms and lactic acid bacteria.

2. MATERIALS AND METHODS

This study was conducted from June 2010 to July 2010, involving a cook-serve kitchen of an Italian school catering establishment in Lombardy, which provides 3,000 meals a day for 3 to 14 year-old children.

2.1 Preparation of lasagne

The lasagna samples were prepared on 23 June 2010. The production was conducted in a separate area of the establishment. The ingredients consisted of: a) Bolognese sauce (40%) (Table 1), b) Béchamel sauce (40%) (Table 2) and c) lasagna sheets (20%) (durum wheat, wheat flour type 00, pasteurized whole egg – 20% – and water). Bolognese and Béchamel sauces

Table 1 - Bolognese sauce recipe.

Bolognese sauce	
Ingredients	**%**
Minced Italian beef	44.0
Double concentrated tomato	27.5
Frozen diced onion	3.5
Frozen diced carrot	3.5
Frozen diced celery	3.5
Extra virgin olive oil	2.3
Red wine	2.5
Salt	1.0
Water	2.9
Tomato pulp	2.9
Tapioca starch	1.0
Butter	2.7
Wheat flour	2.7

were cooked in the on-site, pasta sheets were provided precooked and frozen (Food Valley S.r.l., Florence, Italy). The lasagna consisted of five layers of pasta sheets interleaved with three layers of Bolognese sauce and three layers of Béchamel.

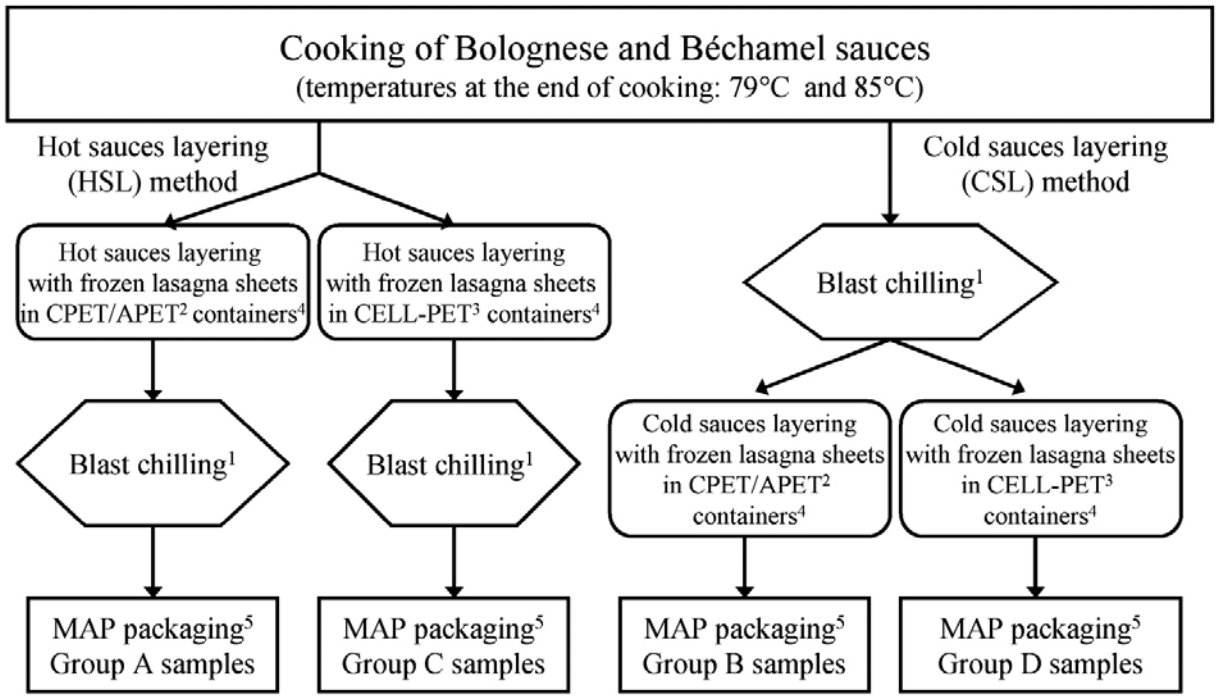
The two lasagna preparation methods and the two types of packaging materials are shown and described in Fig. 1.

2.2 Storage conditions and sampling

The lasagna (n = 81) was stored in a cold room set at 2°C for 28 days. The temperature of the cold room was monitored every 30 minutes by a temperature recorder (iLOG, Escort Data Loggers Inc., Usa). For the purpose of this study, precooked lasagna portions were divided into four groups according to their preparation and packaging methods. Group A (n = 15): HSL method and CPET/APET packaging; Group B (n = 15): CSL method and CPET/APET packaging; Group C (n = 15): HSL method and CELL-

Table 2 - Béchamel sauce recipe.

Béchamel sauce	
Ingredients	**%**
Water	75.0
Wheat flour	7.0
Butter	9.5
Screamed milk powder	6.2
Salt	0.9
Sugar	0.8
Tapioca starch	0.5
Nutmeg	0.1



[1] rapid chilling in a blast chiller (BCF 180A, Zanussi Professional, Italy), cooling time: 2 hours, lasagna temperature at the end of chilling: 3°C

[2] CPET/APET: crystalline polyethylene terephthalate with a top layer of amorphous polyethylene terephthalate (Arcoplastica S.r.l., Italy)

[3] CELL/PET: cellulose with PET (92%+8%) (S.I.S. S.p.A., Italy)

[4] Containers: half size gastronorm (dimensions: 320 x 260 mm, depth: 50 mm). Top film: polyethylene, 30 µm in thickness, with O_2 permeability of 56 ml/m^2/d/atm at 65% RH/23°C and a water vapour transmission rate of 13 g/m^2/d at 90% RH/38°C (Tecnofood Pack S.p.A., Italy)

[5] Gas mixture: CO_2/N_2 (50%/50%). Automatic Packaging machine Mondini, CV/PN-50VG, Italy

Fig. 1 - Flow-diagram of lasagna samples preparation.

PET packaging; Group D (n = 15): CSL method and CELL-PET packaging. During the storage, the analysis of the headspace gas concentration and sampling for the microbiological tests were performed on day 0 and after 7, 14, 21 and 28 days of storage. At time 0, the frozen precooked pasta sheets, and the Béchamel and Bolognese (n = 9) sauces after cooking were packaged and analysed separately. At time 28, three samples *per* group (n = 12) were analysed after cooking at 180°C for 30 minutes. The cooking was carried out using a convection oven (Zanussi S.p.A., Italy) set at "dry cooking". All samples were transported to the laboratory in containers with ice and were analysed the same day.

2.3 Headspace gas analysis

Gas concentrations in the headspace of packages (CO_2 and O_2) were determined using an OXYBABY®-M headspace analyzer (WITT GasetechniK GmbH & CO., England).

2.4 Microbiological analysis

An analytical unit (10 g) was aseptically taken from each sample unit, added to 90 mL of sterile diluent solution (0.85% NaCl and 0.1% peptone), and homogenized in a stomacher 400 (Colworth, UK) for 1 min at room temperature and then serial 10-fold dilutions were prepared in a sterile saline.

Psychrotrophic aerobic plate counts (PAPC) were determined using a pour plate technique on Plate Count Agar (Oxoid, Basingstoke, UK); plates were incubated at 10°C for 10 days. This medium was chosen since it is a well-known and frequently applied tool for the enumeration of bacteria in food quality control programs in the food industry (ESPE *et al.*, 2004; BERNARDI *et al.*, 2009), and it is the medium indicated by official methods (ISO 17410: 2001). Mesophilic aerobic plate counts (MAPC) were enumerated using a Petrifilm™ Aerobic Count (3M™, St. Paul, Minnesota, USA), following the AFAQ/AFNOR 3M 01/1-09/89 method. Petrifilm™ plates were also used to determine total coliforms (TC), *Escherichia coli* (EC), *Staphylococcus aureus* (SA), using the following methods AFNOR 3M 01/2-09/89A, AFAQ/AFNOR 3M 01/08-06/01 and AFNOR 3M 01/9-04/03 respectively. Lactic acid bacteria (LAB) were enumerated on de Man-Rogosa-Sharpe agar (Oxoid, Basingstoke, UK), at pH 5.5. Plates were incubated at 30°C for 48 h under anaerobic conditions in an anaerobic jar (Oxoid, Basingstoke, UK) with an AnaeroGen™ sachet oxygen absorber (Oxoid, Basingstoke, UK); *Salmonella* spp. detection was in accordance with ISO 6579:2002 Cor.1:2004. The detection of *Listeria monocytogenes* were performed in accordance with AFNOR BRD 07/4-09/98 and AOAC No. 060402 2006. For *Bacillus cereus* enumeration, 0.1 mL of each dilution sample was put onto polymixin-piruvate-egg yolk-mannitol-bromothymol blue agar (PEMBA, Oxoid, Basingstoke, UK) with 50,000 IU of polymixin per litre and egg yolk emulsion (Oxoid, Basingstoke, UK). The plates were air dried and incubated at 30°C for 24 to 48 h (TESSI *et al.*, 2002). For the isolation and enumeration of spores of sulphite-reducing clostridia (SSC), the first step was the heat treatment of the tubes containing the first decimal dilution in a thermostatically controlled water bath at 80°±0.1°C for exactly 10 min after the temperature reached 80°C in a control tube, in order to eliminate vegetative cells. In the second step, spores were enumerated by the pour plate method (SWANSON *et al.*, 1992) onto tryptose-sulfite-cycloserine (TSC) agar without egg yolk (CM587, Oxoid, Basingstoke, UK). The agar was supplemented with a 1% filter-sterilized D-cycloserine (Oxoid, Basingstoke, UK) solution (4% wt/vol.). An amount of 0.1 mL of heated dilution was put onto empty sterile Petri dishes, poured plates were overlaid with a thin layer (5 mL) of freshly prepared TSC agar (supplemented with D-cycloserine as previously described). Upon solidification of the TSC overlay, plates were placed in anaerobic jars (Oxoid, Basingstoke, UK) with an AnaeroGen™ sachet oxygen absorber (Oxoid, Basingstoke, UK) and incubated at 37.0°C for 48 h. Characteristic black colonies, if present, were enumerated. All analyses were performed in triplicate.

2.5 Evaluation of microbial safety

Codex Alimentarius Commission guidelines (1997) were followed for evaluating the microbial safety of samples: *L. monocytogenes* (not detected in 25 g), *Salmonella* spp. (not detected in 25 g), *E. coli* (satisfactory <1.00 $\log_{10}$ CFU/g, unsatisfactory ≥1.00 $\log_{10}$ CFU/g), *B. cereus* (satisfactory <2.00 $\log_{10}$ CFU/g, unsatisfactory ≥2.00 $\log_{10}$ CFU/g), *S. aureus* (satisfactory <2.00 $\log_{10}$ CFU/g, unsatisfactory ≥2.00 $\log_{10}$ CFU/g), spores of sulphite-reducing clostridia (satisfactory <1.00 $\log_{10}$ CFU/g, unsatisfactory ≥1.00 $\log_{10}$ CFU/g). Mesophiles and psychrotrophic aerobic plate counts, total coliforms and lactic acid bacteria provide a general estimation of the total number of microorganisms on the product, and are very helpful in estimating/comparing the microbial quality of each group of samples analysed (KOKKINAKIS *et al.*, 2007). In our study we established the following acceptance limits: MAPC/PAPC = 6.00 $\log_{10}$ CFU/g; TC = 3.00 $\log_{10}$ CFU/g and LAB = 6.00 $\log_{10}$ CFU/g.

3. RESULTS AND CONCLUSIONS

Salmonella spp. and *Listeria monocytogenes* were not detected in any of the samples analysed. *Escherichia coli* was <1.00 $\log_{10}$ CFU/g, *Bacillus cereus* was <2.00 $\log_{10}$ CFU/g, spores of sulphite-reducing clostridia were <1.00 $\log_{10}$

CFU/g in all the samples examined. In Groups A and D LAB counts were lower than the acceptance limit up to 28 days of storage (mean counts ranged from <2.00 at day zero to 5.66±0.02 $\log_{10}$ CFU/g at day 28), while in Group B and C LAB counts exceeded the acceptance limit on 28th day of storage (respectively with 6.98±0.02 and 6.11±0.07 $\log_{10}$ CFU/g).

Staphylococcus aureus was found with a frequency of contamination of 37% (mean counts varied from <1.00 to 2.88±0.08 $\log_{10}$ CFU/g). In half of the positive samples *S. aureus* counts were unsatisfactory (mean values were ≥ 2.00 $\log_{10}$ CFU/g), and the unsatisfactory counts were found in Groups B and D (cold sauce layering) from day 14 (Group B) and from day 28 (Group D), respectively. No difference in contamination levels was found between CELL-PET and CPET/APET packages.

It is well known that *S. aureus* lives on humans, food equipment, environmental surfaces, and animals, and exists in the nasal passages, throats, hair, and skin of 50% or more of healthy individuals. This bacterium is highly vulnerable to heat treatment and nearly all sanitizing agents (YOON *et al.*, 2008). For these reasons we can assume that the presence of *S. aureus* may be an indication of an environmental contamination during the filling process. In case of the hot fill system *S. aureus* has been eliminated by the high temperatures of the sauce, in the cold fill system *S. aureus* has not been eliminated.

The evolution of microbial populations (MAPC, PAPC, TC, LAB) in the four Groups are shown in Figs. 2-5. The trend in the percentage of carbon dioxide measured in the headspace of packages is shown in Fig. 6. During the period of 28 days, the mean temperature in the cold room recorded by the data logger was 2.1°C.

As regards the enumeration of mesophilic (MAPC) and psychrotrophic (PAPC) microorganisms, we found that the counts were the same order of logarithmic magnitude (Figs. 2 and 3). We can thus hypothesize that almost all the mesophilic bacteria detected had the capacity to grow at refrigeration temperatures.

At day 0, precooking seemed the most important factor to affect initial bacterial populations, as mean mesophilic and psychrotrophic counts ranged from 2.00±0.04 to 3.98±0.02 $\log_{10}$ CFU/g and total coliforms counts (Fig. 4) ranged from <1.00 to 2.87±0.03 $\log_{10}$ CFU/g.

The results of the tests performed separately on the three elements of lasagna (at day 0), carried out immediately after packaging, showed that mean MAPC and PAPC in frozen precooked sheets were respectively 4.00±0.03 $\log_{10}$ CFU/g and 4.04±0.06 $\log_{10}$ CFU/g, in Béchamel sauce MAPC and PAPC were respectively 2.60±0.06 $\log_{10}$ CFU/g and 2.00±0.05 $\log_{10}$ CFU/g. In the Bolognese sauce they were <1.00 $\log_{10}$ CFU/g, and all the other microbiological parameters (TC, SA, LAB) were lower than the detection limits.

At day 14, the inhibitory effect of CO_2 was particularly significant as the populations were

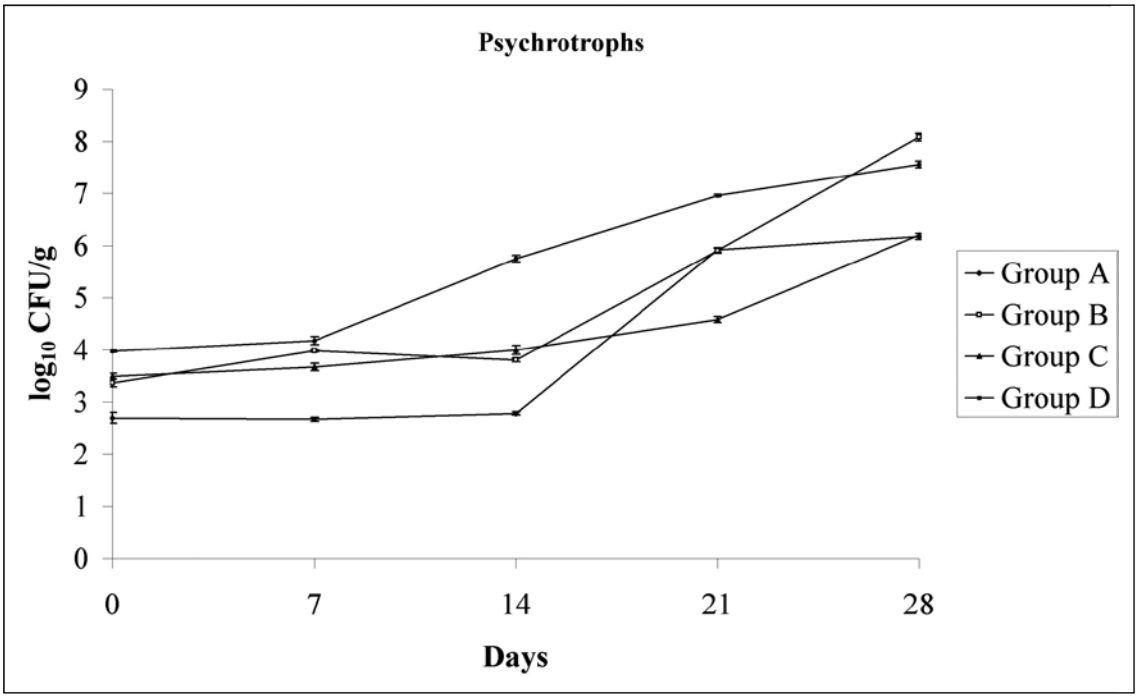


Fig. 2 - Evolution of Psychrotrophs during storage of Groups: Group A, hot sauce layering method and CPET/APET packaging; Group B, cold sauce layering method and CPET/APET packaging; Group C, hot sauce layering method and CELL-PET packaging; Group D, cold sauce layering method and CELL-PET packaging. CFU: colony-forming units. The data are the mean values of three experiments. Bars: standard deviation.

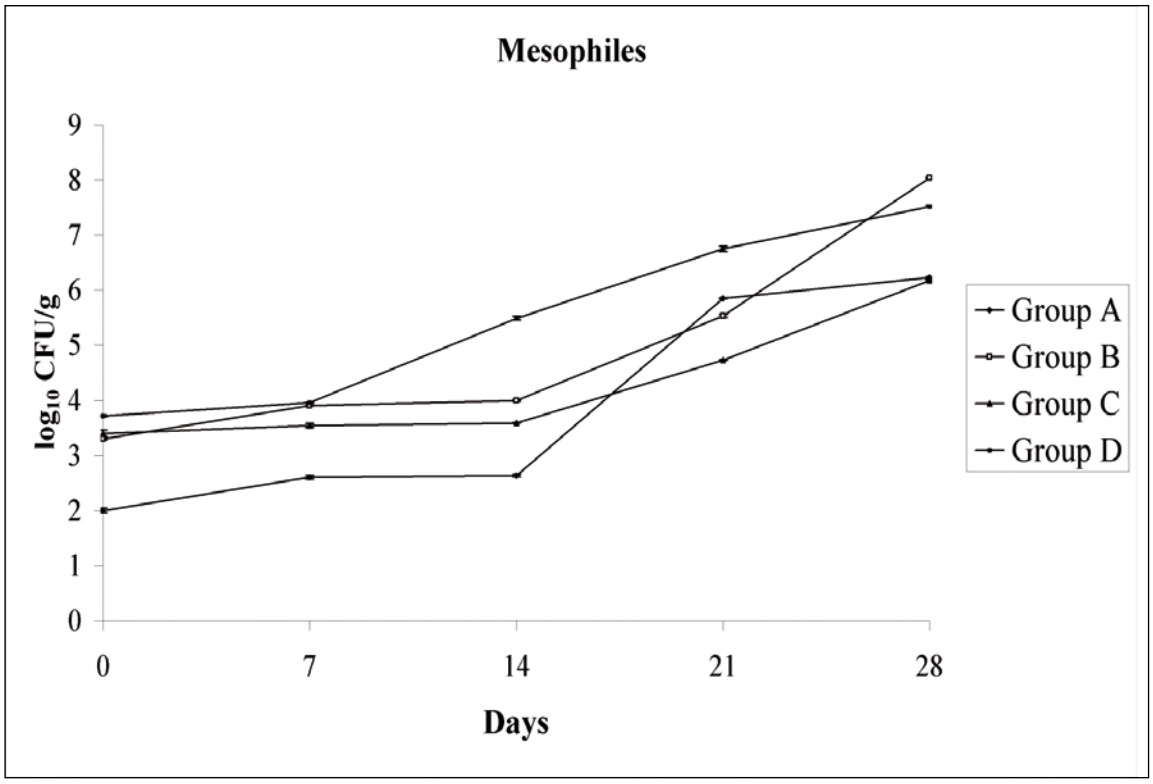


Fig. 3 - Evolution of Mesophiles during storage of samples: Group A, hot sauce layering method and CPET/APET packaging; Group B, cold sauce layering method and CPET/APET packaging; Group C, hot sauce layering method and CELL-PET packaging; Group D, cold sauce layering method and CELL-PET packaging. The data are the mean values of three experiments. Bars: standard deviation.

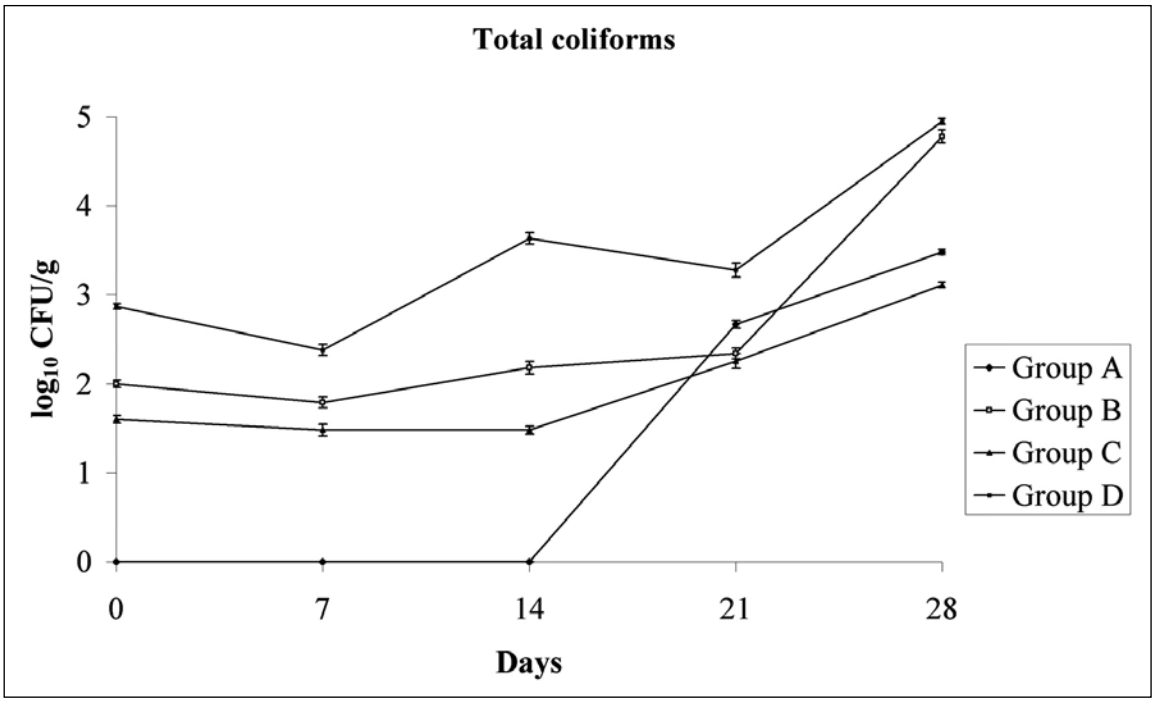


Fig. 4 - Evolution of total coliforms during storage of samples: Group A, hot sauce layering method and CPET/APET packaging; Group B, cold sauce layering method and CPET/APET packaging; Group C, hot sauce layering method and CELL-PET packaging; Group D, cold sauce layering method and CELL-PET packaging. CFU: colony-forming units. The data are the mean values of three experiments. Bacterial counts below the detection limit (<1.00 $\log_{10}$ CFU/g) are conventionally represented in this figure as 0 $\log_{10}$ CFU/g. Bars: standard deviation.

substantially unvaried in Groups A, B and C; only in Group D (CELL-PET packaging and CSL method) mean values of MAPC, PAPC and total coliforms counts increased by 1.25-1.57 $\log_{10}$ units, and TC mean counts were unsatisfactory according to the acceptance limits (mean value was 3.63±0.06 $\log_{10}$ CFU/g).

At day 21, MAPC, PAPC and TC mean counts exceeded the acceptance limits only in Group D.

At day 28, the populations detected were higher than the acceptance limits in all the four Groups analysed (mean MAPC and PAPC ranged from 6.18±0.02 to 8.08±0.07 $\log_{10}$ CFU/g, TC mean values varied from 3.11±0.03 $\log_{10}$ CFU/g in Group C, to 4.95±0.03 $\log_{10}$ CFU/g in Group D).

The results we obtained confirm that the CO_2 concentration inhibited the growth of microorganisms as long as a sufficient concentration of dissolved CO_2 was maintained on the surface of the lasagna. As shown in Figure 6, the carbon dioxide percentage decreased rapidly until day 7, then decreased more slowly up to day 28. C and D were the groups in which the CO_2 decreased to the lowest levels.

These data indicate that probably the heat sealing of CELL-PET containers with a PET top film could be less effective over time compared to the polyethylene containers. The inefficiency of heat-sealing was also demonstrated by the percentage of oxygen (Fig. 7) measured in the headspace. In fact, during the 28 days of storage, in Group C oxygen increased from 2.3 to 18.8%, in Group D it increased from 0.0 to 2.9%, while in Groups A and B it increased respectively from 0.2 to 2.2% and from 0.0 to 1.3%. The level of oxygen may have inhibited the surface growth of pathogenic anaerobic bacteria, which were not detected in this investigation. No difference in aerobic bacterial counts was found between higher and lower percentage oxygen packages, most probably because the bacterial populations were also able to grow also in an environment with an oxygen deficiency (<1%).

At day 28, the results on the samples analysed after cooking showed almost a complete destruction of microbial populations, in all the groups, thus demonstrating the efficacy of cooking timing and temperature.

In conclusion, this study investigated the possible extension of the shelf-life of refrigerated precooked lasagna prepared by using two different processes and packaged using two materials, under MAP. The microbiological results suggest that the potential risk of the growth of pathogenic microorganisms is minimal.

Both packaging methods (CPET-APET and CELL-PET) ensured a sufficiently long shelf-life for most catering purposes. The only difference worth noting was that cellulose had higher values of oxygen in the headspace, which could affect its future use in catering due to the greater probability of aerobic bacterial growth. In conclusion, the use of MAP, and both the HSL/CSL

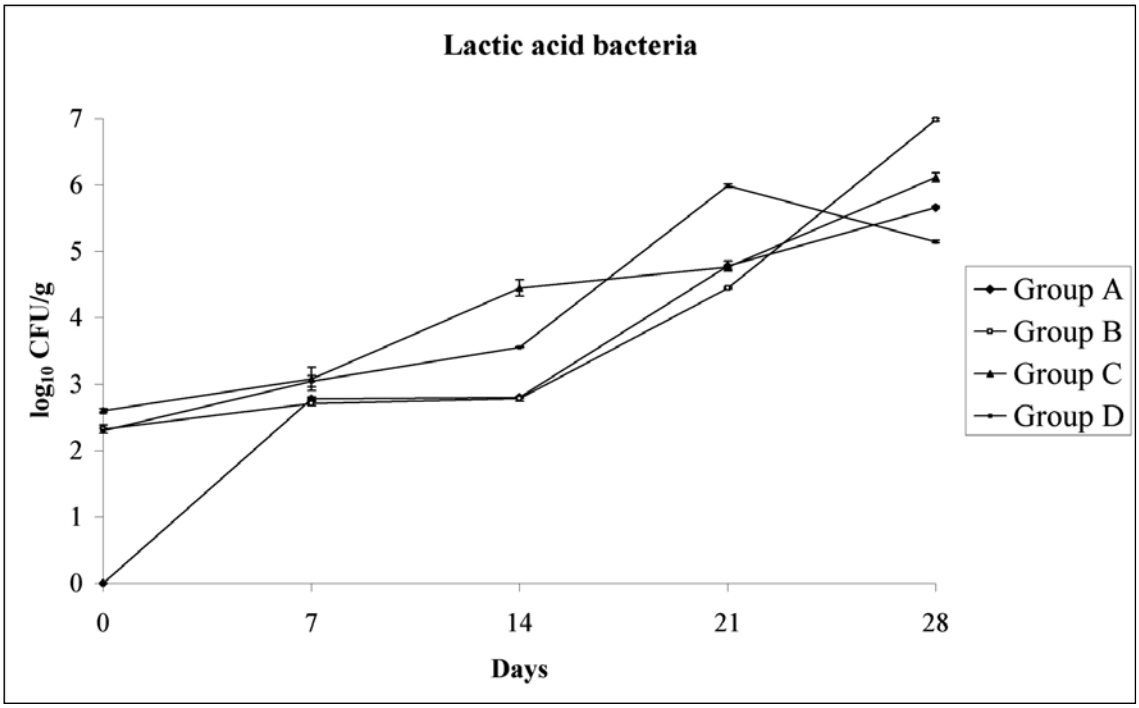


Fig. 5 - Evolution of lactic acid bacteria during storage of samples: Group A, hot sauce layering method and CPET/APET packaging; Group B, cold sauce layering method and CPET/APET packaging; Group C, hot sauce layering method and CELL-PET packaging; Group D, cold sauce layering method and CELL-PET packaging. CFU: colony-forming units. The data are the mean values of three experiments. Bacterial counts below the detection limit (<2.00 $\log_{10}$ CFU/g) are conventionally represented in this figure as 0 $\log_{10}$ CFU/g. Bars: standard deviation.

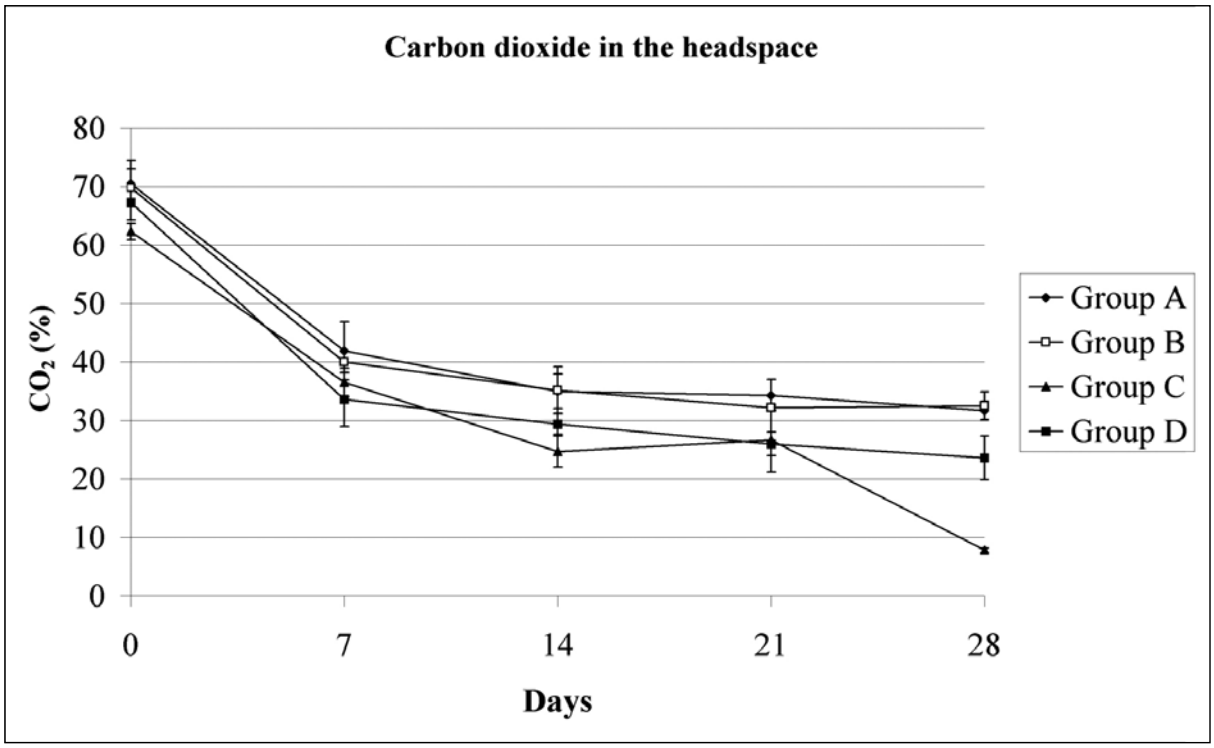


Fig. 6 - Concentration of CO_2 during storage of lasagna sample Groups: Group A, hot sauce layering method and CPET/APET packaging; Group B, cold sauce layering method and CPET/APET packaging; Group C, hot sauce layering method and CELL-PET packaging; Group D, cold sauce layering method and CELL-PET packaging. Bars: standard deviation.

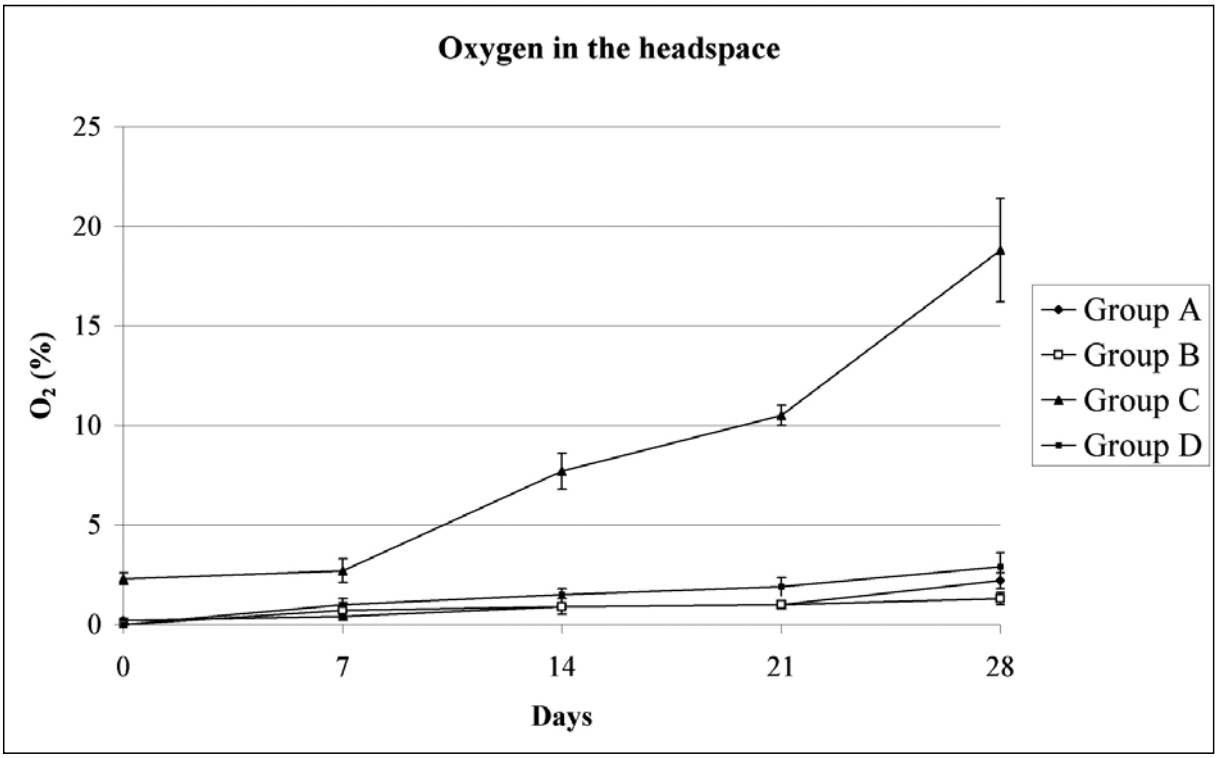


Fig. 7 - Concentration of O_2 during storage of lasagna sample Groups: Group A, hot sauce layering method and CPET/APET packaging; Group B, cold sauce layering method and CPET/APET packaging; Group C, hot sauce layering method and CELL-PET packaging; Group D, cold sauce layering method and CELL-PET packaging. Bars: standard deviation.

methods associated with CPET-APET packaging and the HSL method associated with CELL-PET containers appeared to be an optimal solution for increase the shelf-life up to 21 days.

The results obtained could be useful to implement new production processes within the widespread Italian cook-serve school foodservice establishments, which currently, due to the large number of consumers to be served, acquire ready-to-cook frozen lasagna from outside suppliers in order to optimize time and labor.

There are several direct and indirect advantages from the implementation of this new production system in the largest cook-serve catering establishments: a) greater flexibility in menus; b) possibility of customize the recipes for ethnic and religious minorities (for example using poultry meat instead of beef), and for children suffering from food allergies or intolerances; c) meet the contemporary trend for consumption of fresh rather than frozen products.

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PAPER

RELATIONSHIP BETWEEN THE INNER CELLULATION OF SYNTHETIC STOPPERS AND THE BROWNING OF A WHITE WINE OVER EIGHTEEN MONTHS OF STORAGE

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ABSTRACT

We attempted to predict the performance of cylindrical stoppers by evaluating their inner morphology. The same white wine lots were stored for eighteen months under controlled humidity and temperature conditions. Stopper cellulation was analysed using scanning electron microscopy. The results show that the wine-browning kinetics were significantly different for each stopper used and were related to the regularity of stopper cellulation. Combining our observations regarding the inner closure morphology with data on browning kinetics, we propose an arbitrary scale for classifying stopper quality. This scale can be practically applied during pre-bottling operations to choose the most efficient cylindrical closure.

- Keywords: bottle storage, browning kinetics, cylindrical closures, white wine -

INTRODUCTION

The production of white wine involves great efforts to avoid extensive contact with oxygen, which is deleterious in terms of colour alteration (browning) and the eventual deterioration of overall product quality and marketability (ESCUDERO *et al.,* 2000; SILVA FERREIRA *et al.,* 2002). It is well established that the relevant reactions leading to browning in white wines depend principally on their polyphenolic compositions (ROSSI and SINGLETON, 1966; GOZALES-CARTAGENA *et al.,* 1994), although factors affecting polyphenol oxidation, such as transition metal ions and the presence of sulphur dioxide and ascorbic acid (DANILEWICZ, 2007), are of equal importance. Among phenolic compounds, flavan-3-ols are the substances most directly related to the browning process in most white wines (CHEYNIER *et al.,* 1989). Acetaldehyde can promote the polymerisation of flavan-3-ols, especially during the biological aging of sherry wines (FULCRAND *et al.,* 1996) whereas browning is related more to cinnamates (FERNÀNDEZ-ZURBANO *et al.,* 1998) than to flavan-3-ols (FABIOS *et al.,* 2000; IBERN-GÒMEZ *et al.,* 2000). In general, caffeic and caftaric acids, as well as catechin, epicatechin and their derivatives, are recognised to produce a number of oxidation products that are regarded as potential browning agents (OSZMIANSKI *et al.,* 1996; PALMA and GARCIA BARROSO, 2002) that lead to changes in the antioxidant status of wine (SIOUMIS *et al.,* 2005). Conditions such as high storage temperatures (SOMMERS and POCKOCK, 1990), light and oxygen dissolution (SINGLETON, 1987) are conducive to wine browning (GÒMEZ *et al.,* 1995). The evolution of absorbance at 420 nm can be used to monitor browning analytically (GÒMEZ *et al.,* 1995; SIOUMIS *et al.,* 2006), although some authors hold that this method is not the best indicator of the oxidation status of white wine (SKOUROMOUNIS *et al.,* 2005).

Wine closure, packaging options, and storage conditions offer opportunities to alter a white wine's colour and flavour profile. The capacity for closure or packaging to directly remove volatile compounds through sorptive processes is termed scalping and has been shown to vary with closure type for several wine odorants (BRAJKOVICH *et al.,* 2005; PICKERING *et al.,* 2009; LEQUIN *et al.,* 2010). Because of its oxygen permeability, the closure chosen for sealing a bottle of wine plays a fundamental role in promoting or limiting wine oxidation (KEENAN *et al.,* 1999; LOPES *et al.,* 2005; LOPES *et al.,* 2006; LOPES *et al.,* 2009; BROTTO *et al.,* 2010; KARBOWIAK *et al.,* 2010; WIRTH *et al.,* 2010). Much prior research has demonstrated that white wines closed with synthetic stoppers, screw caps, or cork closures (CHATONNET *et al.,* 2000; GODDEN *et al.,* 2001; MAS *et al.,* 2002; SILVA *et al.,* 2003; MARIN *et al.,* 2007; LOPES *et al.,* 2009), or packaged in bags within boxes (FU *et al.,* 2009) exhibit different behaviours during secondary shelf-life.

In recent years, as a consequence of higher market prices for cork and of qualitative problems with natural stoppers (PENA-NEIRA *et al.,* 2000; VAREA *et al.,* 2001; MAZZOLENI and MAGGI, 2007), winemakers, mostly those in non-cork-producing countries, have begun to use alternative seals (CHATONNET *et al.,* 1999; JUNG and ZURN, 2000; VASSEROT *et al.,* 2001). To regulate quality and obturation, synthetic closures and screw caps require standardised dimensions (CHATONNET and LABADIE, 2001; GODDEN *et al.,* 2001) with respect to their shapes and mechanical properties (GIUNCHI *et al.*, 2008) and should be chemically and sensorially inert (CHATONNET *et al.,* 1999; CHATONNET *et al.,* 2000; CHATONNET *et al.,* 2001; SILVA *et al.,* 2003). Nevertheless, the first applications of synthetic stoppers revealed some problems, demonstrating the need to focus more research on the characterisation of the materials employed and on their interactions with the "wine matrix" (CHATONNET *et al.,* 2000; JUNG and ZURN, 2000). However, little information is available (DE FILIPPIS *et al.,* 2007; GIUNCHI *et al.,* 2008) on the characterisation of synthetic closures, especially regarding their inner structures (ROSA *et al.,* 2001) and their relationships with closure performance during wine post-bottling evolution.

In this work, we aimed to analyse stopper inner cellulation and its effect on the chemical evolution of a white wine over eighteen months of bottle storage. Nine types of cylindrical stoppers were used to seal the white wine Bianco di Custoza, and their performances were compared with those of a natural cork. The rate of browning was analysed to establish a relationship between stopper cellulation quality and the oxidative evolution of bottled wine.

MATERIALS AND METHODS

Cylindrical closures

Trials were performed by bottling the same white wine with the following stoppers: a natural cork stopper (N), a cork-based technical stopper (S1), and seven synthetic stoppers, denoted S2, S3, S4, S5, S6, S7, and S8. All closures made of synthetic materials were produced using moulding technology. The stopper surface treatment consisted of lubrication with 1 kg of silicone/500,000 stoppers. The sizes of the closures, their characteristics, provided in the manufacturer's technical sheets, and their countries of production are reported in Table 1.

Wine

The white wine used in this study was Bianco di Custoza, made from Trebbiano Toscano and Garganega grapes. This wine was made via a traditional white winemaking process in a wine

Table 1 - Stoppers tested in the study.

Stopper	Size: Diameter x Length (mm)	Density (g/cm^3)	Stopper typology and related description	Producer Country
N	24.0 x 44.0	0.18	Natural cork stopper	Italy
S1	24.5 x 44.0	0.22	Technical stopper composed of synthesis micro spheres and suberine.	USA
S2	23.0 x 39.0	0.40	Synthetic stopper obtained by moulding technology. No specifications about raw materials.	Italy
S3	23.0 x 37.0	0.40	Synthetic stopper obtained by moulding technology. No specifications about raw materials.	Italy
S4	21.5 x 38.0	0.54	Synthetic stopper obtained by moulding technology. It is composed of expanded ethylene copolymer with chemical agents and adjuvants.	Australia
S5	22.4 x 38.0	0.42	Synthetic stopper obtained by moulding technology. It is composed of polyethylene resin with expanding agents.	France
S6	21.5 x 37.0	0.52	Synthetic stopper obtained by moulding technology. It is composed of an expanded thermoplastic polymer.	Italy
S7	21.5 x 36.5	0.51	Synthetic stopper obtained by moulding technology. It is composed of a thermoplastic elasthomer coming from biomedical field.	USA
S8	22.2 x 38.0	0.48	Synthetic stopper obtained by moulding technology. It is composed of elasthomeric polyethylene with expanding agents.	Italy

cellar in Verona. The general characteristics of the wine at bottling were as follows: 12.10% v/v alcohol, 3.5 g/L reducing sugars, 17.6 g/L dry extract, pH 3.26, 5.0 g/L titratable acidity, 0.17 g/L volatile acidity, 89 mg/L total sulphur dioxide, 18 mg/L free sulphur dioxide, and 0.34 mg/L dissolved oxygen.

Bottling and storage

White glass Bordeaux-style bottles with a diameter of 17.5 mm at the bottle mouth were used. Bottling was performed at the winery. The bottles were filled with the wine to a level of 70 mm below the bottle mouth, nitrogen gas was then injected into the headspace of the bottle, and the bottles were finally corked. Nine bottling runs were performed with the same four-head corker: the first using an N stopper and the remaining eight in the order S1, S2, S3, S4, S5, S6, S7, and S8. After bottling, the 270 samples were maintained for eighteen months at a temperature of 16°C and a relative humidity of 60%.

Analysis of the stopper inner cellulation

Prior to bottling, the stoppers were submitted to cellular ultra-structural analysis using a Philips XL30 ESEM Scanning Electron Microscope (SEM) (Royal Philips Electronics, Holland). Using four samples of each closure, small slices, 1-2 mm thick, were cut with a surgical scalpel in the radial direction. The fragments were taken from six different points of the stopper inner body along its length and placed onto a stainless steel support. The whole surface of each fragment was observed using SEM in a chamber under low vacuum at a magnification of 30X. To determine the average diameter of the inner cells of a stopper label, measurements were made at ten points sampled on each of the six fragments taken from each of the four samples.

Wine analyses

At bottling, the dissolved oxygen content in the wine was analysed using an Orbisphere Micro Logger 3650-O_2 O_2 probe (Oxytech Instruments, Switzerland). Measurements were performed on five replicates for each bottle run (e.g., for each stopper type), revealing an average oxygen concentration of 0.34±0.06 mg/L At bottling and at six, nine, twelve, and eighteen months after storage, four samples of each closure were uncorked, and the alcohol content, the pH, the volatile acidity, the total and free sulphur dioxide (SO_2), and the absorbance at 420 nm ($A_{420\ nm}$) were determined according to the analytical methods described in European Regulation No. 2676/90. The total acetaldehyde content was analysed as described in Italian Regulation D.M. 12/3/1986.

Statistical analysis

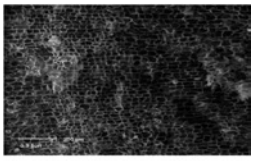
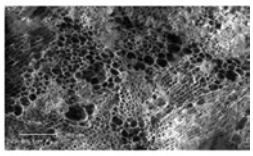
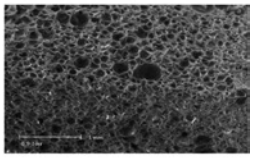
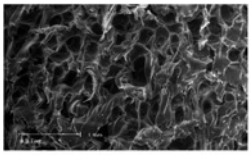
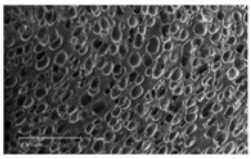
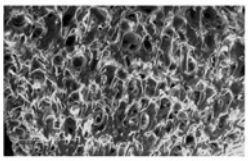
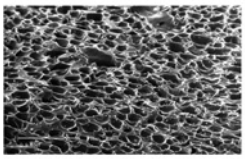
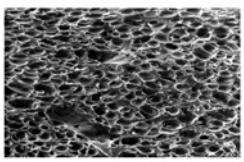
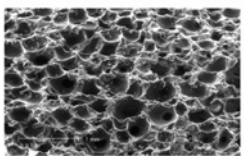
The experiments were performed on four replicates and the analyses were performed in triplicate. SPSS 13.0 software for Windows was used to perform the statistical analyses (SPSS, 2008). The differences in wines sealed with the different cylindrical closures were tested for significance with analysis of variance (ANOVA) techniques. Principal component analysis (PCA) was used as a descriptive method to examine the relationships among the variables and the grouping among samples (LEWI, 1992). The rotation of principal components was performed using the Varimax normalised method (MELOUN *et al.*, 1992). Correlations between parameters were evaluated by Pearson's test at a significance level of $p < 0.05$.

RESULTS AND CONCLUSIONS

Stopper inner cellulation

SEM analysis permitted the verification of the presence of holes and provided information regarding the regularity of internal stopper morphology. Through this SEM procedure, it was possible to evaluate the porosity of the internal stopper body by measuring, from each SEM image, the sizes of a large number of cells. Table 2 lists descriptions of the inner closure cellulation and the associated range of cell dimensions. Except for the natural cork stopper (N), in which cells appeared as small bricks along the radial section, the cells of the synthetic stoppers appeared to be shaped like small spheres. The natural cork stopper (N) showed a defined and uniform network of

Table 2 - Ultra-structure and morphology description of the stopper inner body observed by scanning electron microscope (SEM).

Stopper	SEM Image	Morphology description	Cell diameter range (µm)
N		Regular net of cells	37 ÷ 74
S1		Quite regular net of cells	51 ÷ 88
S2		Quite regular net of cells with some holes	100 ÷ 400
S3		Collapsed structure with many holes	300 ÷ 400
S4		Quite regular net of cells with some holes	100 ÷ 200
S5		Collapsed structure with some holes	100 ÷ 200
S6		Quite regular net of cells with many holes	200 ÷ 400
S7		Quite regular net of cells with many holes	100 ÷ 600
S8		Quite regular net of cells with some holes	300 ÷ 400

cells, the most restricted range of cell diameters and the lowest cell size. It was used as the reference for homogeneity of the inner structure. The cork-based technical stopper (S1; cork powder and synthetic microspheres) had a regular inner structure very similar in shape and size to that of the natural cork. The stoppers made of plastic materials revealed a large and variable cellulation, always with cell diameters of 100 μm or more. S4 and S5, which showed similar cell sizes, featured 100-200-μm cells and unique morphologies consisting of a regular network of cells and a collapsed structure, respectively. In both cases, holes were rarely observed. S2 had a heterogeneous inner structure with a wide range of cell diameters, in which holes were infrequently found. S8 had cells that were generally larger than those of the other samples and had few holes. S6, S7 and S3 displayed low-quality cellulation in that many holes were entrapped in the cell network; for S3, a collapsed structure was also observed. The differences in the inner structures of the moulded synthetic stoppers have been reported to be related to the particularities of the moulding technology process. In fact, they can depend on several aspects: the properties of the initial expanded-polymer material, the management of the heating temperatures, the injection and cooling times of the fused raw material into the stamp, and the type of the expansion additive used to create bubbles in the resin. These factors are known to affect the physical-mechanical properties of the final stopper, e.g., density, elasticity, and permeability (CHATONNET *et al.*, 1999; JUNG and ZURN, 2000; LOPES *et al.*, 2006).

Wine evolution during bottle storage

PCA was separately applied to wine parameters collected at six, twelve, and eighteen months after bottling using total and free SO_2 levels, volatile acidity, acetaldehyde content, and $A_{420\,nm}$ as a matrix for PCA analysis. The first two principal components specified, respectively, the following variations: 34.9 and 25.9% of the variation at six months; 36.9 and 26.6% of the variation at twelve months, and 43.8 and 24.1% of the variation at eighteen months.

A general conclusion concerning the evolution of the overall characteristics of the wine can be drawn by relating the loadings extracted from different PCA analyses (Fig. 1). A plot of the an-

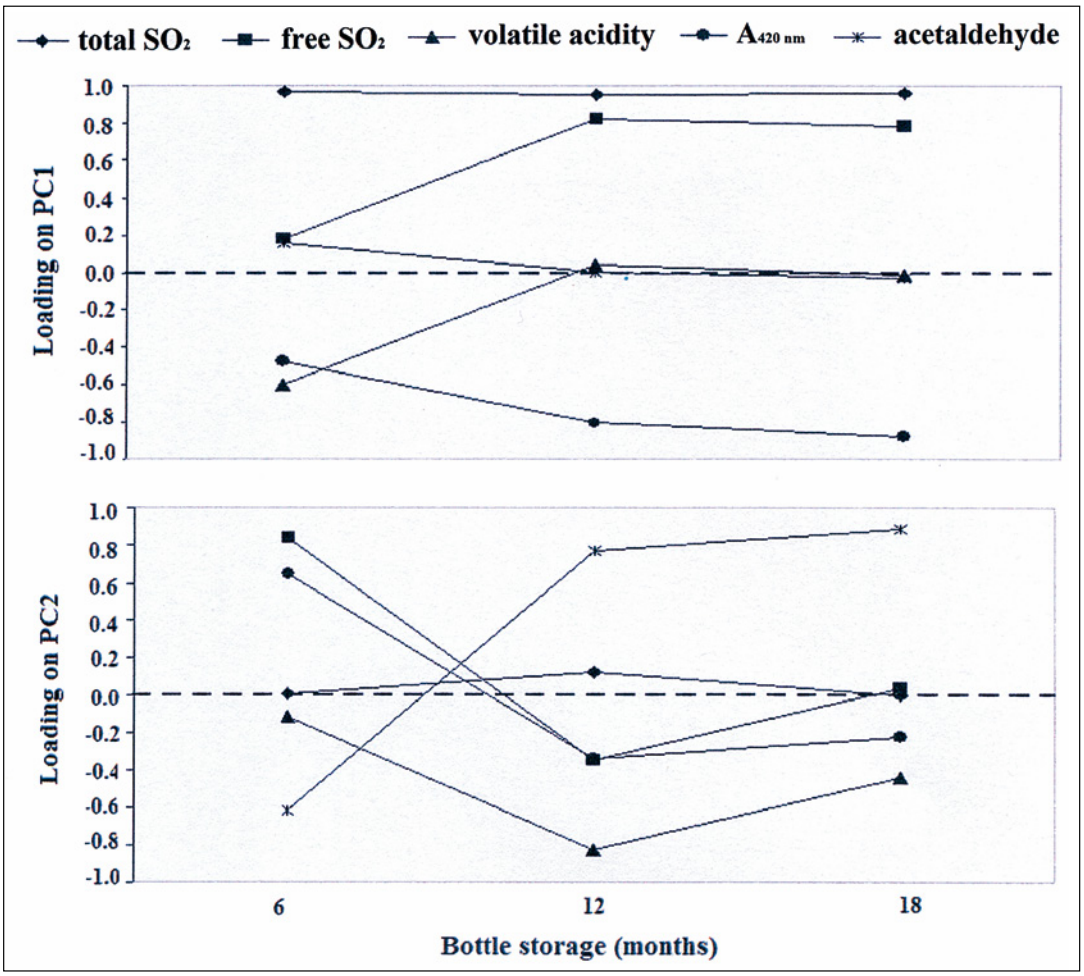


Fig. 1 - Wine parameter loadings on PC1 and PC2 plotted against storage time after PCA of data from wine samples at six, twelve, and eighteen months.

Table 3 - Probability values of analysis of variance (ANOVA) for the wine parameters (n.s., not significant).

Parameter	Time	Stopper	Time x Stopper
Total SO_2	$p < 0.01$	n.s.	n.s.
Free SO_2	$p < 0.01$	n.s.	n.s.
Volatile acidity	$p < 0.01$	n.s.	n.s.
Alcohol content	$p < 0.01$	n.s.	n.s.
$A_{420\ nm}$	$p < 0.01$	$p < 0.01$	$p < 0.01$
Acetaldehyde	$p < 0.01$	n.s.	n.s.

alytical parameter loadings on PC1 versus storage time shows that the total SO_2 had a strong positive correlation throughout the bottle storage period whereas free SO_2 was highly correlated after twelve months. $A_{420\ nm}$ was negatively linked to PC1 and the correlation increased with storage time. Analytical parameter loadings on PC2 versus storage time showed that free SO_2 and $A_{420\ nm}$ had positive correlations at six months and were then weakly negatively linked to PC2. Volatile acidity showed a strong negative correlation at twelve months. Acetaldehyde changed its PC2 loading from a negative value at six months to a positive correlation at twelve months after bottling. At eighteen months, it showed a high positive loading on PC2.

In agreement with other studies (EDWARDS *et al.,* 1985; ALDAVE *et al.,* 1993; GÒMEZ *et al.,* 1995; SILVA *et al.,* 2003), our data relating to wine evolution confirm that storage time significantly affects the studied variables, particularly total and free SO_2, acetaldehyde, volatile acidity, and browning at 420 nm (Table 3). Over time, the type of stopper significantly affected only $A_{420\ nm}$ – i.e., the onset of browning varied with the different stopper types.

Browning kinetics

An investigation of the kinetics of browning onset during storage for each stopper type was performed by plotting $A_{420\ nm}$ as a function of time. Browning was generally characterised by a three-phase course, with a fairly linear increase during the first six months followed by a rapid growth between six and twelve months, and a deceleration from twelve to eighteen months after bottling. The fairly linear increase in the first phase may be due to the consumption of oxygen present in the wine at bottling (SINGLETON, 1987). These reactions also involve SO_2, as suggested by the significant loss of total SO_2 observed between six and twelve months of storage. In fact, the initial oxidation of phenolic compounds in wine leads to the formation of quinones that can be directly reduced by SO_2 (SAUCIER and WATERHOUSE, 1999) or by coupled reactions with flavanols (CHEYNIER *et al.,* 1989; CHEYNIER and RICARDO DA SILVA, 1991). The subsequent polymerisation of flavanols and quinones (GUYOT *et al.,* 1996; SIOUMIS *et al.,* 2005), together with a low availability of SO_2, may produce the rapid increase in browning observed between six and twelve months of storage. In the third phase, the polymerisation process leads to a slowing of browning rates.

A combined model of zero-order and first-order kinetics correctly describes the browning reaction rate in white wines (PEREZ-ZUNIGA *et al.,* 2000). This model assumes that the colour evolution of white wines (as measured by the changing absorbance at 425 nm) results from a combination of the formation of coloured polymeric compounds (described by zero-order kinetics) and the concurrent loss of colour intensity due to polymeric dissociation (described by first-order kinetics) (KARBOWIAK *et al.,* 2010). Because polymeric dissociation was not considered in our study due the short storage time (PEREZ-ZUNIGA *et al.,* 2000), a zero-order reaction model was applied to fit the browning data:

$$A_{420\ nm} = A_{420\ nm[0]} + kt$$

where $A_{420\ nm}$ is the concentration of brown product(s), $A_{420\ nm[0]}$ is the initial concentration of brown product(s) in the wine and k is the rate constant ($A_{420\ nm}$/month). The browning kinetic parameters for each stopper type are listed in Table 4. The results show that natural cork produced an increase in browning of 0.0022 $A_{420\ nm}$/month. The browning rate for S1 was lower than that for the natural closure. The browning onsets with stoppers S4 and S5 were similar to that of the natural cork. The wines sealed with S2, S3, S6, S7 and S8 showed faster browning rates than that of wines sealed with N. The proposed kinetic model reveals different rates of evolution among white wines sealed with natural cork and synthetic closures and among samples closed with different types of moulded synthetic stoppers. These data confirm the results of recent studies (SANCHEZ and ARACIL, 1998; LOPES *et al.,* 2005; LOPES *et al.,* 2006; SIOUMIS *et al.,* 2006; SALACHA *et al.,* 2008). Under accelerated test conditions, the browning kinetics of white wines measured by $A_{420\ nm}$ changes obey zero-order kinetics, with reported kinetic constants ranging from 15 to 75 $\times 10^{-3}$ day^{-1} (SIOUMIS *et al.,* 2006) or from 8.8 to

Table 4 - Browning kinetic parameters ($A_{420\ nm[0]} = 0.095$) of wine samples sealed with the different stoppers.

Stopper	k ($A_{420\ nm}$/month)	r^2
N	0.0022	0.9234
S1	0.0012	0.9514
S2	0.0032	0.9030
S3	0.0030	0.9096
S4	0.0024	0.8772
S5	0.0026	0.8544
S6	0.0031	0.8853
S7	0.0032	0.9158
S8	0.0029	0.9176

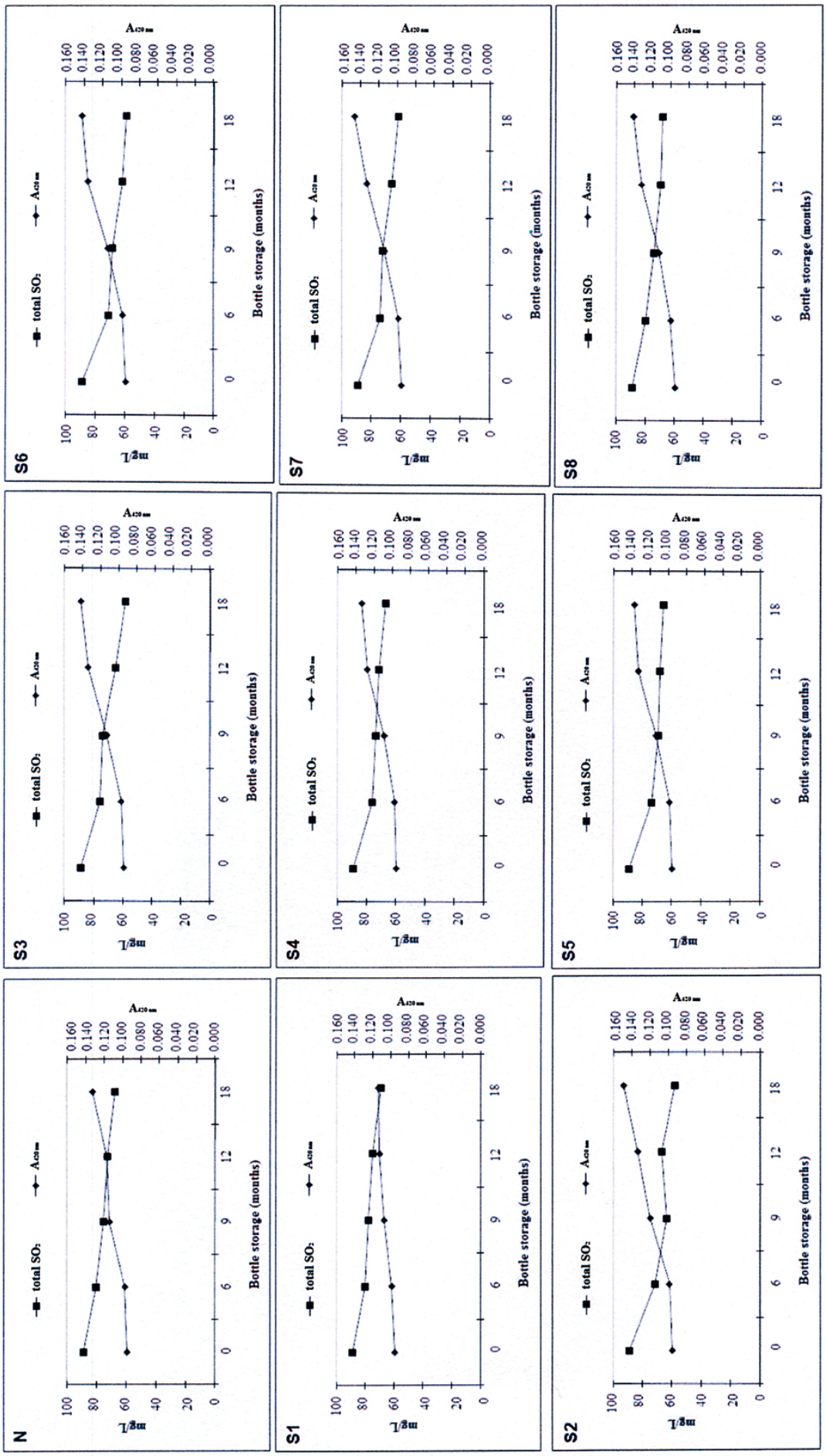


Fig. 2 - Mirror course of total SO_2 and of browning (measured by $A_{420\ nm}$) of wine samples *versus* storage time.

6.1 k × $A_{420\,nm}$ day^{-1} (SALACHA *et al.*, 2008). Moreover, wines sealed with some synthetics (S2, S3, S6, S7, and S8) showed high levels of browning, as reported in previous studies (MAS *et al.*, 2002; CHATONNET and LABADIE, 2003; SKOUROMOUNIS *et al.*, 2005).

Finally, a significant inverse linear correlation (r^2 = - 0.683 at $p < 0.01$) was observed between the browning rate constants and the total SO_2. By plotting both parameters against storage time, a "mirror" image was obtained (Fig. 2). This behaviour, which has also been observed when monitoring the oxidation of white wines closed with cork stoppers (WATERS *et al.*, 1996), revealed further differences among the stoppers evaluated in this study. The differences were particularly significant when comparing the natural (N) and cork-based technical (S1) stoppers with their synthetic counterparts. For natural cork and cork-based technical closures, the intersection of the line describing the increase in browning with that describing the drop in SO_2 content occurred at twelve months or later, although this intersection occurred earlier (at approximately nine months) with the synthetics. This information suggests that in the wines closed with the synthetic stoppers examined herein, the loss of SO_2 and the onset of browning occurred more quickly than in wines sealed with cork.

Relationship between stopper cellulation and wine browning

Several observations can be made regarding the homogeneity of the inner morphology of the stopper (Table 2) and the browning rates during wine storage (Table 4). The natural cork stopper, N, and the cork-based technical stopper, S1, displayed regular networks of cells with diameters smaller than 100 μm and the slowest browning kinetics. The stoppers made of plastic materials had wide cellulation ranges and gave rise to faster browning.

Comparing the homogeneity of the stopper inner morphology and wine browning rates during bottle storage revealed a positive relationship between the range of cell diameters of the stopper inner body and the browning rate (Fig. 3). Among the synthetics, S4 and S5 had cells of the same diameter (between 100 and 200 μm) and produced browning kinetics similar to those of the natural stopper. S3 and S8 showed similar ranges of cell diameters, but the cells were larger, i.e., between 300 and 400 μm. These two stoppers produced higher browning rates than S4 and S5. Stopper S6 had a more heterogeneous cellulation than all the previously listed stoppers, with sizes ranging from 200 to 400 μm, and yielded browning rates faster than those of S3 and S8. For the S2 and S7 closures, the range of cell diameters was the widest, from 100 to 400 μm and from 100 to 600 μm, respectively, but the relative browning rates were no higher than those observed for the S6 stopper.

These investigations of the stopper inner body show that a smaller cell size and a more restricted range of cell diameters (up to a value of 200 μm) result in slower browning rates during bottled wine storage. Consequently, an arbitrary scale used to evaluate stopper performance is suggested. The placement of the stopper type

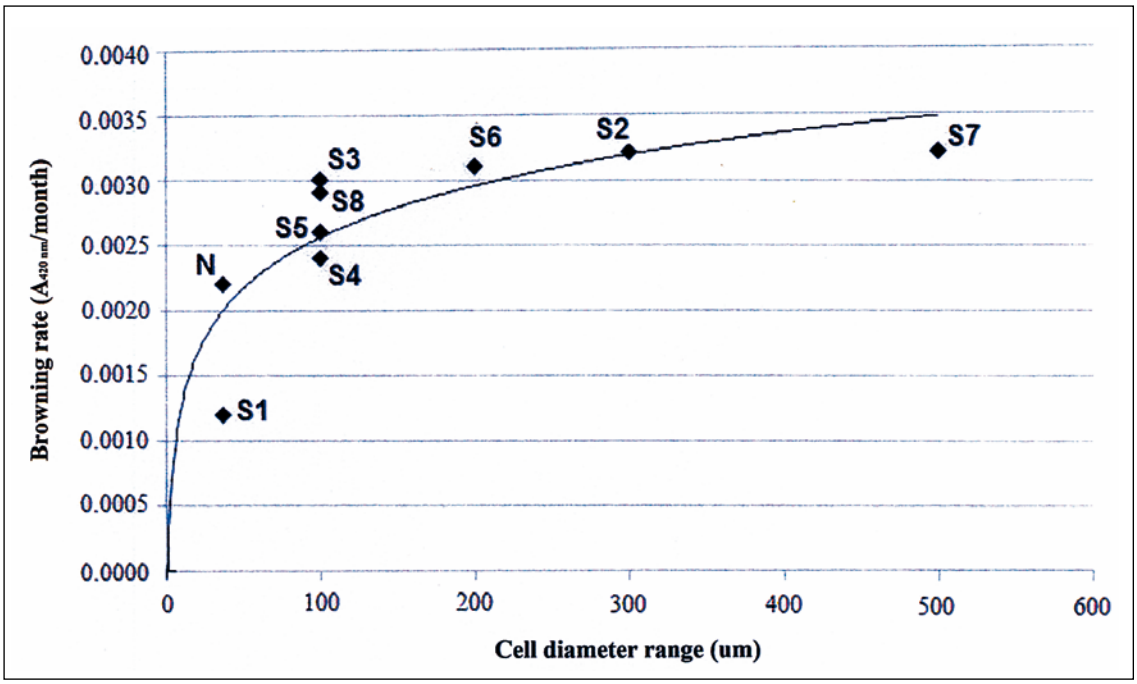


Fig. 3 - Relationship between wine browning rate ($A_{420\,nm}$ / month) and cell diameter range of stopper inner body.

on the quality scale should be based on the following concepts: a smaller inner cellular structure size and a more restricted range of diameters correspond to a higher quality level on the proposed scale. This scale, which is based on inner stopper cellulation quality, can be a useful tool for determining the performance of cylindrical closures with respect to the evolution of bottled white wine and can be practically applied during the pre-bottling steps of white winemaking to choose the most efficient cylindrical closure. Further studies should be conducted to consider other stopper typologies, e.g., co-extruded synthetics, to enhance the scale for stopper quality assessment proposed herein.

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PAPER

COMPARATIVE STUDY OF THE PHENOLIC COMPOSITION OF SEEDS FROM GRAPES *CV* CARDINAL AND ALPHONSE LAVALLEE DURING LAST MONTH OF RIPENING

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ABSTRACT

During the last month of ripening, the phenolic composition of seeds from two widely distributed table grapes, *cv* Cardinal and Alphonse Lavallee, was determined by HPLC/DAD/ESI/MS. Besides, the concentrations of nutrients in leaf blades of grapevine were measured. Generally, the most abundant phenolic in grape seed was gallic acid, followed by methyl gallate and monomeric flavan-3-ols. In comparison to Alphonse Lavallee, the amounts of phenolics were higher in grape seed of Cardinal, in which gallic acid glucoside was not detected. Among analyzed phenolics, the increase of gallic acid was evidenced in grape seed of Cardinal. The most of phenolics decreased during the last month of grape ripening, and some of them had no significantly different content. Results of bivariate correlations showed that the amounts of some phenolics in grape seed of Cardinal increased with increasing of the content of potassium and phosphorus in leaves.

- Keywords: correlation, grape, leaf, nutrient, phenolic, seed -

INTRODUCTION

The investigation of the changes of phytochemicals during ripening of fruit contributes to the understanding of the biochemical and physiological processes in the developing fruit (USENIK *et al.*, 2008; VEBERIC, 2010). The phenolic compounds, as secondary metabolites, in grapes have attracted much interest recently, because of potential beneficial effects of phenolics on health and their strong contribution to the organoleptic characteristics of grapes. Numerous studies showed that the contents of phenolic compounds in grape berries are genetically determined and also depends on climatic and geographical factors, agricultural practices, stage of ripeness, and vegetative vigor of the plant (CANTOS *et al.*, 2002; VIAN *et al.*, 2006; PEREIRA *et al.*, 2006; LOVINO *et al.*, 2006; GODJEVAC *et al.*, 2010; OBREQUE-SLIER *et al.*, 2010). During the first period of berry growth, phenolics are accumulated, while colouring occurring during the second period (ripening) is characterized by increasing of the content of anthocyanins. Changes in the phenolic composition can also be observed in the seeds during grape maturation (FERRER-GALLEGO *et al.*, 2010; KENNEDY *et al.*, 2000; BRAIDOT *et al.*, 2008; OBREQUE-SLIER *et al.*, 2010).

In grape berries, phenolic compounds are present mainly in skins and seeds (OBREQUE-SLIER *et al.*, 2010). Grape seeds are rich in flavan-3-ols: monomers such as (+)-catechin, (-)-epicatechin and (-)-epicatechin 3-*0*-gallate as well as proanthocyanidins. Proanthocyanidins (condensed tannins) are oligomers and polymers composed of flavan-3-ol units (including 3-*0*-gallates) linked mainly through C4→C8 bond, but the C4→C6 linkage also exits (both are called B-type) (FULEKI and RICARDO DA SILVA, 1997).

Almost all flavonoids of the seed are contained in the outer integument, between the cuticle and the hard seed coat, whereas tannins localize in the epidermis and in the last layers of the inner integument (ADAMS, 2006; CADOT *et al.*, 2006). The flavonoid composition changes throughout the overall process of seed maturation, together with macroscopic changes in the tissues, such as the color and hardness (BRAIDOT *et al.*, 2008).

These compounds affect the taste; produce a sensation of astringency arising from the precipitation of oral proteins and mucopolysaccharides. Beside flavan-3-ol monomers (BELL *et al.*, 2000), there is an evidence that proanthocyanidins are also absorbed into the bloodstream (HOLT *et al.*, 2002). The flavan-3-ols are powerful antioxidants that demonstrate antibacterial, antiviral, anticarcinogenic, anti-inflammatory, and vasodilatory activities (KALIN *et al.*, 2002; ENG *et al.*, 2003; JAYAPRAKASHA *et al.*, 2003; STANKOVIC *et al.*, 2008). Recognition of the health benefits of flavan-3-ols initiated the manufacture of grape seed extracts as dietary supplements. In addition, seed-containing grapes could be useful to make juice because during the crushing the juice is enriched with flavan-3-ols coming from the seeds (CANTOS *et al.*, 2002).

On the contrary to wine grape varieties, which were widely investigated, very little information is available on identification and quantification of phenolics in table grape varieties.

The objective of the present study was to determine and to compare the phenolic composition of grape seeds of two widely distributed table grape varieties, Cardinal and Alphonse Lavallee, during the last month of ripening. Besides, considering the important role of plant nutrients in photosynthesis (SALISBURY and ROSS, 1992) and consequently their potential effect on accumulation of phenolics, the relationship between macro- and microelements in grapevine leaf and phenolics in grape seed was investigated.

MATERIALS AND METHODS

Sampling site characteristics

The commercial vineyard (13 Jul - Plantaže a.d.) is located about 10 km southeast from the town Podgorica (N 42° 27', E 19° 28', 10-50 m AMSL), Montenegro, in the area with Mediterranean climate. In Podgorica, for period from the beginning of April to the end of August 2008, the sum of rainfall was 325.3 mm, the sum of the sunlight hours 1406.7 h and mean temperature 23.5°C. The soil type is euthric brown on a fluvioglacial deposit consisting of carbonates. *Vitis vinifera* L. *cv* Cardinal on SO4 rootstock (Selection Oppenheim Nr. 4) and Alphonse Lavallee on Paulsen rootstock were planted in 1997/98. Vine spacing was 1.2 m, with a row spacing of 2.6 m. Cardinal is a table grape cultivar with an early ripening period, and Alphonse Lavallee with medium to late ripening period. The studied vines of one cultivar were within the same row, whereas the distance between rows with vines of *cv* Cardinal and Alphonse Lavallee was about 200 m. All applied agrotechnical measures were the same (pruning, fertilization through soil, irrigation, plant protection, etc.).

Sampling of grapevine leaves

The leaves of grapevine were taken from the opposite grapes near the middle of the shoot, just before the first sampling of grapes (on 16th of July for Cardinal and 6th of August for Alphonse Lavallee). The leaf blades were immediately separated from petioles. Twenty leaf blades from five vines (within one replicate) represented one sample.

Leaf blade analyses

N and S were determined on CHNS/O elemental analyzer (Vario EL III, Elementar, Germany). For determination of the other nutrients, dried (for 24 h at 65°-70°C) and grinded (in porcelain mortar with pestle) plant material was digested by HNO_3 and $HClO_4$ (RYAN *et al.*, 2002). P was spectrophotometrically determined (Cary 100, Varian, Australia); K and Ca flame photometrically (PFP 7, Jenway, United Kingdom); Mg, Fe, Mn, Cu and Zn by flame atomic absorption spectrophotometry (AA – 6800, Shimadzu, Japan). Boron was determined after dissolving of ashed material in 20% HCl (MUNTER and GRANDE, 1984) by inductively coupled plasma optical emission spectrometry (iCAP 6500 Duo ICP, Thermo Fisher Scientific, United Kingdom). These results are given on dry basis at 105°C.

Sampling of grapes

The samples of grape were collected 3 times during last month of ripening (for Cardinal I: 16th of July; II: 30th of July and III: 13th of August; whereas for Alphonse Lavallee I: 6th of August, II: 19th of August and III: 1st of September). For each sampling date, about 2.5 kg of grape were taken on five vines (in average about 0.5 kg per vine). There were 8 replications (in total 40 vines per cultivar) with 3 buffer vines between replications.

Determination of general parameters

Total soluble solids – TSS (by manual refractometer) and titratable acidity – TA (with 0.1M NaOH) were determined in the grape juice (obtained by crushing and pressing of grape berries by hand through two layers of gauze).

Preparation of grape seed extracts

Grape seeds were manually separated from pulp and dried on filter paper. Air dried grape seeds (about 2.4 g) were extracted in 30 mL of 50% methanol. The mixtures were sonicated on ultrasonic bath (12 h), filtered over 0.45 μm syringe cellulose filter and transferred into HPLC vials.

HPLC/DAD and LC/MS analyses

Were described by GODJEVAC *et al.* (2010). HPLC analysis of extracts was performed using an Agilent 1200 equipped with DAD model G1315B, Bin pump model G1312A, autosampler model G1313A, and RR Zorbax Eclipse Plus C18 column (1.8 μm, 150 x 4.6 mm). Mobile phase A was 0.2% formic acid in water and mobile phase B was acetonitrile. The injection volume was 5 μL, and elution at 0.95 mL/min with gradient program (0-20 min 5-16% B, 20-28 min 16-40% B, 28-32 min 40-70% B, 32-36 min 70-99% B, 36-45 min 99% B, 45-46 min 99-5% B).

UV-VIS detection was carried out at 230, 280, and 320 nm. Quantification was based on the measured integration area applying the calibration equation of the corresponding standard. The concentrations used for the calibration were 0.1-1.0 and 0.02-0.2 mg/mL for catechin and gallic acid (Sigma, St. Louis, MO, Usa), respectively. Some compounds were quantified as equivalents of the most similar chemical structures: gallic acid for methyl gallate and gallic acid glucoside; but catechin for all other compounds.

LC/MS analysis was performed on an Agilent MSD TOF coupled to an Agilent 1200 series HPLC, using the same column and gradient program as those for HPLC–DAD analysis. Mass spectra were acquired using an Agilent ESI-MSD TOF. Drying gas (N_2) flow was 12 L/min; nebulizer pressure was 45 psig; drying gas temperature was 350°C. For ESI analysis, the parameters were: capillary voltage, 4,000 V; fragmentor, 140 V; skimmer, 60 V; Oct RF V 250 V, for positive and negative modes. The mass range was from 100 to 2,000 m/z. Processing of data was done with the software Molecular Feature Extractor. Characteristic ions were used to assign procyanidin oligomers, but in the absence of authentic standards, oligomers differing in combinations of epimeric catechin and epicatechin units cannot be distinguished.

Statistical analysis

Descriptive statistics (mean and standard error), Pearson correlation (2-tailed), one way ANOVA and multiple range test using Duncan's test at $p < 0.05$ were performed by SPSS 10.0 Program.

RESULTS AND DISCUSSION

Sugars are among the most important ingredients determining fruit quality, which are responsible for the sweet taste of fruit. The acidity affects not only the sour taste, but also sweetness by masking the taste of sugars (NELSON *et al.*, 1973). Towards common practice for determination of harvest time, the total soluble solids and titratable acidity in grape juice were measured during the last month of ripening. As expected, TSS increased and TA decreased during the last month of ripening (Fig. 1). A significant difference in these parameters was noticed between Cardinal and Alphonse Lavallee at the first sampling date. On the contrary to expectations, due to the relatively high variability of the data for second date, the means of total soluble solids were not statistically different (p = 0.192). The same was for titratable acidity (p = 0.080). At the end of sampling i.e. at the state of full maturity, the grapes of both cultivars were

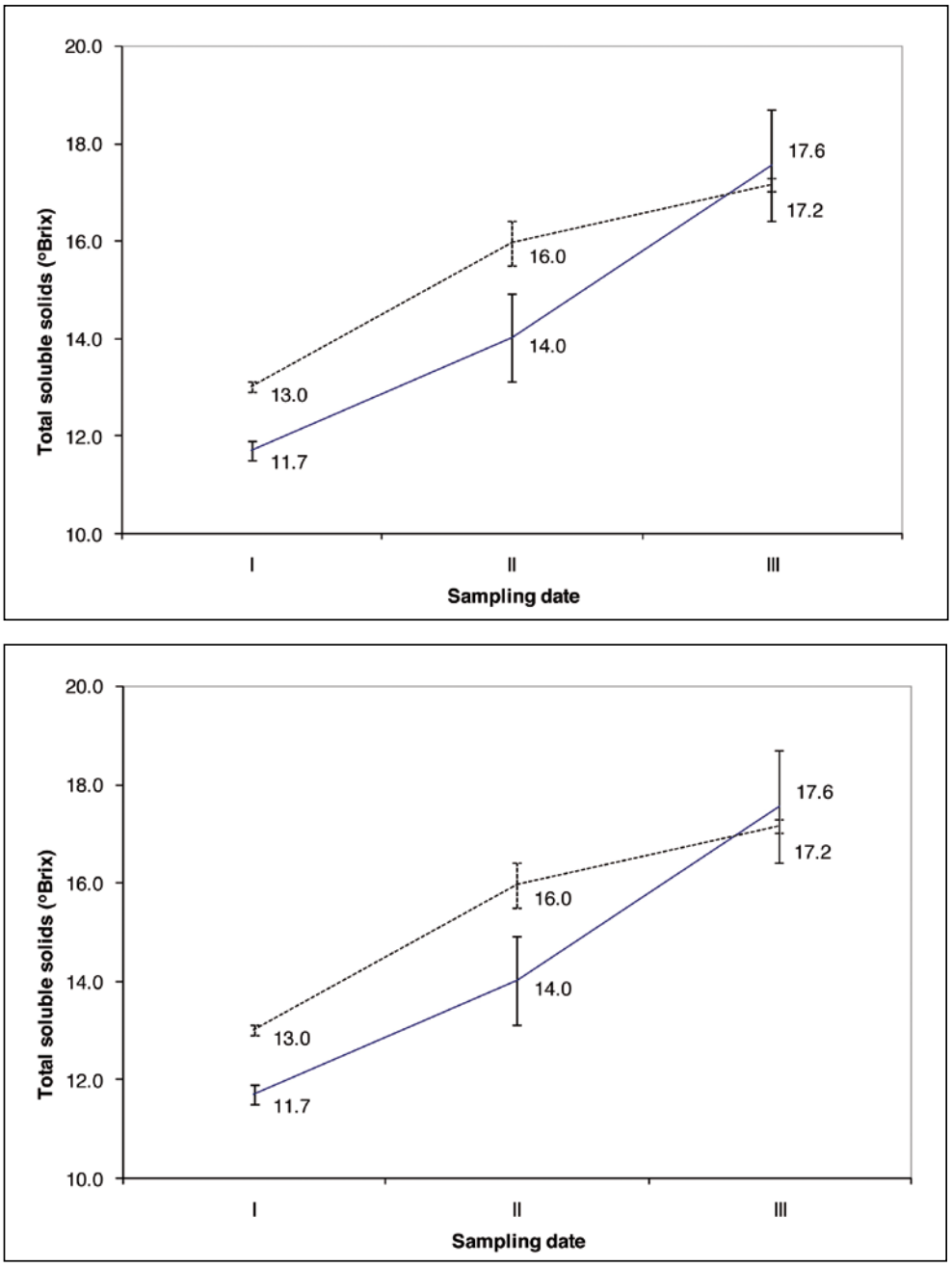


Fig. 1 - Total soluble solids (°Brix) and titratable acidity (g TAE/L) of grape juice (solid line: Cardinal; dashed line: Alphonse Lavallee) at three sampling dates (n = 8).

not different in the average of TSS and TA. The ratio between °Brix and titratable acids ranged from 16.9 at the first sampling date through 34.2 at second date to 53.2 at the end of ripening for Cardinal, and from 27.4 through 49.8 to 67.1 for Alphonse Lavallee, respectively. In study of the relation between Brix/acid ratio and favour preference, NELSON *et al.* (1973) stated that the rate of increase in consumer acceptability decreased at the higher Brix/acid ratios, which had been demonstrated with samples of Cardinal grapes with ratios as high as 45:1.

The amounts of analyzed phenolic compounds are presented in Table 1. The most abundant phenolic in grape seed of Cardinal and Alphonse Lavallee was gallic acid, followed by methyl gallate and monomeric flavan-3-ols. There were differences in the content of phenolics in grape seeds of both cultivars during the last month of ripening. The exception was gallic acid at the first sampling date as well as methyl gallate which amount was not significantly different at the first and second sampling date. Having in mind already mentioned difference in general parameters at the beginning, the difference in phenolic composition of seed was expected. However, at the end of ripening, when TSS and TA were similar for both cultivars, the evidenced difference

was mainly influenced by genetic factors. Gallic acid glucoside was not found in grape seed of Cardinal. For Alphonse Lavallee gallic acid glucoside was not detected at the first sampling date and was at almost constant value thereafter.

During the last month of grape ripening for both cultivars, the contents of catechin, proanthocyanidin trimer, epicatechin, proanthocyanidin dimer monogallate and epicatechin gallate decreased from the first to second sampling date and were not significantly different thereafter, while proanthocyanidin dimer (t_R = 13.2) was at similar level. In grape seed of Alphonse Lavallee the same trend as for epicatechin gallate was found for gallic acid and methyl gallate, while proanthocyanidin dimer (t_R = 8.9) was significantly lower at the end in comparison with the first sampling date. Differently to Alphonse Lavallee, in grape seed of Cardinal there were no significant differences in the content of methyl gallate and proanthocyanidin dimer (t_R = 8.9), the concentration of proanthocyanidin dimer (t_R = 9.8) decreased from the first to second sampling date and was similar thereafter, while the content of gallic acid significantly increased from the beginning to the end of the last month of ripening. The increasing of gallic acid in seeds of Cardinal could be partly caused by hydrolysis of some compounds such as methyl gal-

Table 1 - The content (mean ± standard error, n = 8) of the analysed compounds in grape seeds in mg/kg DW. Values with asterisk in the row are not different at $p > 0.05$ and without asterisk are different at $p < 0.05$. Values followed by different letters within column for each phenolic compound are significantly different according to Duncan's multiple range test at $p < 0.05$.

Compound	Molecular formula/Mass/Ion species	t_R (min)	Cardinal		Alphonse Lavallee	
Gallic acid	$C_7H_6O_5$ 170.0215 M-H	3.3	Jul 16 Jul 30 Aug 13	1532.5±90.8^{a*} 1842.5±180.2^{ab} 2080.0±139.6^{b}	Aug 6 Aug 19 Sep 1	1371.3±121.9^{b*} 1070.0±54.3^{a} 813.8±76.7^{a}
Gallic acid glucoside	$C_{13}H_{16}O_{10}$ 332.0744 M-H	4.8	Jul 16 Jul 30 Aug 13	nd nd nd	Aug 6 Aug 19 Sep 1	nd 240.0±97.5^{a} 236.3±51.0^{a}
Proantho-cyanidin dimer	$C_{30}H_{26}O_{12}$ 578.1424 M-H, 2M-H	8.9	Jul 16 Jul 30 Aug 13	203.8±13.5^{a} 153.8±19.5^{a} 138.8±32.3^{a}	Aug 6 Aug 19 Sep 1	81.3±14.4^{b} 53.8±6.8^{ab} 47.5±7.5^{a}
Proantho-cyanidin dimer	$C_{30}H_{26}O_{12}$ 578.1424 M-H, 2M-H	9.8	Jul 16 Jul 30 Aug 13	197.4±21.0^{b} 126.1±14.9^{a} 151.8±7.8^{ab}	Aug 6 Aug 19 Sep 1	53.8±15.5^{a} 37.5±4.5^{a} 30.0±3.8^{a}
Methyl gallate	$C_8H_8O_5$ 184.0372 M-H	10.2	Jul 16 Jul 30 Aug 13	930.0±79.3^{a*} 792.5±77.5^{a*} 902.5±48.5^{a}	Aug 6 Aug 19 Sep 1	1228.8±131.8^{b*} 821.3±120.9^{a*} 558.8±81.0^{a}
Catechin	$C_{15}H_{14}O_6$ 290.0790 M-H	10.6	Jul 16 Jul 30 Aug 13	1426.3±144.6^{b} 901.3±114.9^{a} 780.0±70.7^{a}	Aug 6 Aug 19 Sep 1	820.0±158.5^{b} 428.8±49.4^{a} 317.5±38.7^{a}
Proantho-cyanidin trimer	$C_{45}H_{38}O_{18}$ 866.2058 M-H, M-2H	11.4	Jul 16 Jul 30 Aug 13	147.5±11.0^{b} 102.5±17.5^{a} 120.0±12.8^{ab}	Aug 6 Aug 19 Sep 1	53.8±9.4^{b} 32.5±4.5^{a} 28.8±3.5^{a}
Proantho-cyanidin dimer	$C_{30}H_{26}O_{12}$ 578.1424 M-H, 2M-H	13.2	Jul 16 Jul 30 Aug 13	111.3±8.1^{a} 113.8±10.8^{a} 136.3±8.4^{a}	Aug 6 Aug 19 Sep 1	65.0±10.9^{a} 50.0±8.5^{a} 53.8±8.9^{a}
Epicatechin	$C_{15}H_{14}O_6$ 290.0790 M-H	14.9	Jul 16 Jul 30 Aug 13	801.3±59.8^{b} 593.8±76.5^{a} 632.5±33.7^{ab}	Aug 6 Aug 19 Sep 1	411.3±67.4^{b} 227.5±26.3^{a} 153.8±21.5^{a}
Proantho-cyanidin dimer monogallate	$C_{37}H_{30}O_{16}$ 730.1534 M-H, 2M-H	18.3	Jul 16 Jul 30 Aug 13	501.3±50.4^{b} 241.3±37.2^{a} 152.5±18.3^{a}	Aug 6 Aug 19 Sep 1	148.8±31.1^{b} 57.5±11.3^{a} 46.3±8.4^{a}
Epicatechin gallate	$C_{22}H_{18}O_{10}$ 442.0900 M-H, 2M-H	22.7	Jul 16 Jul 30 Aug 13	955.0±128.3^{b} 182.5±40.3^{a} 165.0±15.7^{a}	Aug 6 Aug 19 Sep 1	190.0±40.0^{b} 68.8±13.6^{a} 58.8±9.3^{a}

late, epicatechin gallate etc. Although PENA-NEIRA *et al.* (2004) found that with exception of seeds from low vigor vines of *cv* Cabernet Sauvignon, gallic acid concentration decreased during ripening. There is an evidence that extractable flavan-3-ol monomers (Mr: 290-442) and low molecular weight seed tannins (Mr: <900) decrease during grape ripening (KENNEDY *et al.*, 2000; BRAIDOT *et al.*, 2008; OBREQUE-SLIER *et al.*, 2010). The ratio between catechin and epicatechin for Cardinal decreased from 1.8 to 1.2 during last month of ripening, whereas for Alphonse Lavallee was relatively close 1.9-2.1. Similarly, GODJEVAC *et al.* (2010) found in grape seed of some cultivars this ratio between 1 and 2, e.g. 1.25 for Muscat Hamburg the most widespread table grape in Serbia. The relative proportion of flavanol monomers also changed in grape seed of Cardinal, while was almost similar for Alphonse Lavallee (Fig. 2). According to KENNEDY *et al.* (2000), differences in the relative proportions of flavan-3-ol monomers are consistent with a strong relation between the variety and the chemical evolution of monomeric composition in seeds during ripening.

The amount of phenolics was higher in grape seed of Cardinal than of Alphonse Lavallee. Thus, at third sampling date, when total soluble solids was above 17°Brix, proanthocyanidin dimer (t_R=9.8) was even 5-fold, proanthocyanidin trimer and epicatechin were more than 4-fold; proanthocyanidin dimer (t_R=8.9), proanthocyanidin dimer monogallate and epicatechin gallate about 3-fold; gallic acid 2.6-fold; catechin and proanthocyanidin dimer (t_R=13.2) 2.5 and methyl gallate 1.6-fold higher in grape seed of Cardinal. In this regard, literature data also differ. Comparing to our data for both cultivars at third sampling date, in grape seed of Muscat Hamburg (GODJEVAC *et al.*, 2010) much lower concentration of gallic acid, gallic acid glucoside and methyl gallate, but higher of catechin and epicatechin was reported. A concentration of epicatechin gallate was higher than in grape seed of Alphonse Lavallee, but lower than for Cardinal. FULEKI and RICARDO DA SILVA (1997) found concentrations more similar to our results for catechin in grape seeds of Merlot and Riesling as well as for epicatechin in Cabernet Sauvignon and Riesling.

Table 2 - The content (mean ± standard error, n = 8) of nutrients in leaf blade of grapevine. Values with asterisk in the row are not different at $p > 0.05$ and without asterisk are different at $p < 0.05$.

Cultivar	Cardinal	Alphonse Lavallee
Date	16 July	6 August
N (%)	2.52±0.05	1.99±0.04
S (%)	0.36±0.01	0.40±0.01
K (%)	0.73±0.03	0.57±0.04
P (%)	0.18±0.00*	0.19±0.01*
Mg (%)	0.27±0.01	0.46±0.01
Ca (%)	4.70±0.09	4.22±0.14
Fe (mg/kg)	99.95±3.67	114.09±3.27
Mn (mg/kg)	85.90±1.40	63.72±2.30
Zn (mg/kg)	13.87±0.39*	14.09±0.35*
Cu (mg/kg)	4.64±0.22*	4.58±0.21*
B (mg/kg)	36.23±0.57	27.74±0.85

A leaf tissue analysis is commonly used as a diagnostic tool to determine the nutritional program of grapevine. Having in mind that grape quality can be affected by the nutrient composition of leaf (FREGONI, 1998), an objective of this study was to investigate the relationship between macro- and micronutrients in the grapevine leaf and phenolics in the grape seed. The content of nutrients in grapevine leaves just before the last month of grape ripening (Table 2)

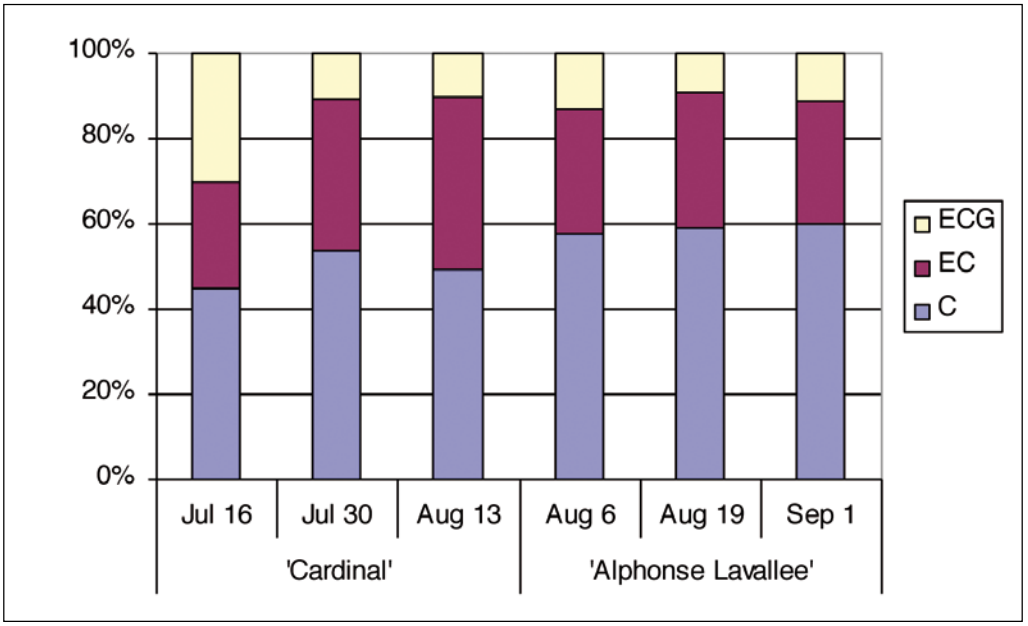


Fig. 2 - Relative proportion of flavanol monomers.

Table 3 - Correlation matrix for macro- and microelements in grapevine leaf blade and phenolics in grape seed of Cardinal (n=8, * significant at < 0.05, ** significant at < 0.01).

	GA	PCD 8.9	PCD 9.8	MG	C	PCT	PCD 13.2	EC	PCDG	ECG
N	0.620	-0.391	-0.678	0.392	0.277	0.157	-0.076	0.475	-0.002	0.248
S	0.204	0.267	0.173	0.128	0.453	0.437	0.375	0.429	0.526	0.500
K	0.913**	0.503	0.124	0.848**	0.771*	0.836**	0.711*	0.936**	0.686	0.647
P	0.681	0.053	-0.235	0.460	0.752*	0.545	0.193	0.598	0.472	0.759*
Mg	-0.378	-0.419	-0.049	-0.227	-0.311	-0.600	-0.215	-0.226	-0.175	-0.450
Ca	-0.782*	-0.312	-0.044	-0.794*	-0.730*	-0.600	-0.706*	-0.909**	-0.677	-0.526
Fe	0.455	-0.216	-0.374	0.208	0.128	0.229	0.053	0.436	0.076	0.156
Mn	-0.366	-0.288	-0.304	-0.484	-0.451	-0.244	-0.221	-0.323	-0.379	-0.279
Zn	-0.660	0.148	0.486	-0.575	-0.077	-0.195	0.019	-0.462	0.127	0.006
Cu	-0.388	0.133	0.457	-0.433	-0.152	-0.078	-0.405	-0.446	-0.049	0.010
B	-0.758*	-0.199	-0.017	-0.736*	-0.616	-0.461	-0.325	-0.674	-0.453	-0.441

GA: gallic acid; PCD 8.9: proanthocyanidin dimer t_R = 8.9; PCD 9.8: proanthocyanidin dimer t_R = 9.8; MG: methyl gallate; C: catechin; PCT: proanthocyanidin trimer; PCD 13.2: proanthocyanidin dimer t_R = 13.2; EC: epicatechin; PCDG: proanthocyanidin dimer monogallate; ECG: epicatechin gallate.

indicated significant differences between studied cultivars for the majority of nutrients, with the exception of P, Zn, and Cu. The leaf blade of Cardinal has higher concentration of N, K, Ca, Mn and B than one of Alphonse Lavallee, which was more abundant in S, Mg and Fe. It has been known that leaf contents of individual elements are not a constant, they keep changing during the growing period. Besides, element contents depend on the variety, soil chemical properties, weather conditions as well as on the anthropogenic impact (fertilization). Taking into account the same soil properties and the same fertilization of vines (similar values of soil agrochemical parameters) as well, these differences might be attributed to sampling date and cultivar characteristics.

For Alphonse Lavallee, there were no significant correlations between above mentioned nutrients in leaves and analyzed seed phenolics (data not shown). For Cardinal (Table 3), the content of gallic acid, methyl gallate, catechin, proanthocyanidin trimer, proanthocyanidin dimer (t_R=13.2) and epicatechin in grape seed increased with increasing of the content of potassium in leaf blade. Moreover, positive correlations were found between catechin, as well as epicatechin gallate and phosphorus. The importance of potassium for photosynthesis, translocation of photosynthates, activation of plant enzymes (among which ones important for pentose phosphate pathway and Krebs cycle when the precursors of secondary metabolites are originated), as well as of phosphorus in energy transfer, photosynthesis, transformation of sugars and starches (SALISBURY and ROSS, 1992) can explain obtained significant correlation of K and P with the content of phenolics.

The negative correlations between some phenolics in grape seed and calcium and boron were likely the consequence of negative correlations between these nutrients and K (data not shown). Namely, the content of gallic acid, methyl gallate, catechin and epicatechin were indirectly proportional with calcium, but gallic acid and methyl gallate showed negative relationships with boron in grapevine leaf.

The investigations indicate that potassium and calcium are antagonistic when the other element is available in higher concentrations (GARCIA *et al.*, 1999). Due to the fact that vineyard soil being highly calcareous, the negative correlation between Ca and K in leaf was expected. Although high potassium concentration can reduce boron, it is difficult to explain the negative correlation between K and B, because their contents in grapevine leaf blade were optimal.

CONCLUSION

The phenolic composition of seed from table grape depends directly on the cultivar and ripening time, as growing conditions being the same for Cardinal and Alphonse Lavallee. In general, the most abundant phenolic in grape seed was gallic acid, followed by methyl gallate and monomeric flavan-3-ols. The amounts of phenolics were higher in grape seed of Cardinal. The majority of phenolics decreased during last month of grape ripening for both cultivars.

Significant correlations between potassium as well as phosphorus in grapevine leaf and some phenolic compounds in grape seed of Cardinal indicate the possible positive effect of these elements on the content of seed phenolics.

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PAPER

FAST NON-DESTRUCTIVE DETERMINATION OF CHLOROPHYLLS IN APPLE SKIN

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ABSTRACT

The evaluation of the chlorophyll content in fruit peel is usually performed by means of complex destructive procedures that include extraction and separation. In the present work, a non-destructive procedure, based on UV-Vis spectroscopy in the diffuse reflectance mode, is proposed for the quantitative measurement of both chlorophylls *a* and *b*, utilising the spectral range in which molecules other than chlorophylls show low absorbance. Two methods are presented and compared, one based on multivariate analysis over the whole range, and the other based on a linear regression at two meaningful wavelengths.

- Keywords: calibration, non-destructive methodology, chlorophyll *a*, chlorophyll *b*, diffuse reflectance, multivariate analysis, UV-Vis analysis -

1. INTRODUCTION

The ripening of apples is usually assessed using indexes related to firmness, acidity, starch content, sugar content and ground colour (MCGLONE *et al.*, 2002; DESHPANDE *et al.*, 1984; PEIRS *et al.*, 2005). The latter parameter is related to the presence of pigment in the skin, such as chlorophyll, carotenoids and anthocyanins (MERZLYAK, 2006; LENK *et al.*, 2007). The most representative pigment is chlorophyll, of which the most common and abundant species are chlorophylls *a* and *b* (hereafter, Chp *a* and Chp *b*, respectively) (MCGLASSON *et al.*, 2007; GITELSON *et al.*, 2003). Degradation of both types of chlorophyll occurs during ripening in climacteric fruit, therefore, in principle, their content may be used as a fingerprint of fruit ripening (SOLOVCHENKO *et al.*, 2005; MERZLYAK *et al.*, 1999). In practice, the chlorophyll content is not commonly used as an indicator of ripening, because a complex destructive procedure, based on extraction and separation, is required for its determination (LICHTENTHALER and BUSCHMANN, 2005). These operations require well-equipped laboratories and skilled operators, and they are time-consuming, thus constituting a serious drawback for many sectors of the food supply chain. Moreover, artefacts may arise due to the addition of the stabilizing agents that are used to avoid degradation and pheophytization of the chlorophylls (SOLOVCHENKO *et al.*, 2001; ANTOLOVICH *et al.*, 2000; MERZLYAK *et al.*, 2003). As an alternative, UV-Vis spectroscopy is currently being proposed to characterize pigments in fruit. For instance, ZUDE-SASSE *et al.* (2002) have recently utilized a mixed reflectance-transmission method together with Partial Least Squares (PLS) modelling to non-destructively measure the total amount of chlorophyll in apples skins. The aim of the present work is to show that UV Vis spectroscopy can be satisfactorily used in the diffuse reflectance mode, which is traditionally adopted in the material science field to determine the concentrations of both Chp *a* and Chp *b* chlorophylls.

2. MATERIALS AND METHODS

2.1. Materials

Twenty-four Golden Delicious apple specimens, from different markets, were considered. UV-Vis measurements were conducted in the diffuse reflection mode by simply putting a selected zone of the equatorial region of the whole fruit in contact with the entrance port of the instrument. In order to obtain spectra calibration, the chlorophyll concentration corresponding to each spectrum was determined by extracting in the same area used for UV-Vis measurements, according to the method proposed by the Association of Official Analytical Chemists (AOAC, 2006). This involved two steps: the determination of the extinction coefficients of bands related to Chp *a* and Chp *b* in a water/acetone mixture, and the measurement of the UV-Vis spectra in apple extracts. The extraction procedure was carried out as described hereafter.

2.2. UV-Vis reflectance measurements

The fruit was rinsed with water, hand-dried and measured without any further treatment. A UV-Vis double-beam spectrophotometer Varian Cary 500 device, equipped with an integrating sphere, was used. The advantage of a double-beam instrument is that it automatically compensates for instrumental variations, such as the overriding of lamp effects due to random fluctuations or temperature. A white disk filled with compressed Spectralon powder was used as a reference. Spectra were collected in the 350-800 nm range at a resolution of 3 nm. The instrumental set-up allows diffuse radiation to be measured, therefore the specular component of the reflected light is excluded.

Destructive chlorophyll analyses of the Chp *a* and Chp *b* extinction coefficients were conducted in a water/acetone mixture (volume to volume ratio = 15:85) using standard solutions of either Chp *a* or Chp *b* from Fluka. Fifty solutions were prepared in a concentration range of between 1×10^{-4} M and 1×10^{-3} M for Chp *a* and of between 5×10^{-5} M and 2×10^{-4} M for Chp *b*, as this is the expected range for the concentrations in the extract. These solutions were measured by means of UV-Vis spectroscopy in transmission mode with the same instrument, using the same water/acetone mixture as the reference. The spectra were collected in the 400-750 nm interval at a resolution of 2 nm. Figure 1a reports the spectra for the water/acetone mixture in the 400-750 nm range, expressed as the molar extinction coefficients as a function of the wavelength: Chp *a* shows a main absorption peak at 663 nm and a Soret absorption band at 431 nm; Chp *b* instead shows a Soret band at 459 nm and an absorption band at 646 nm. For each apple, a peel area of 1.54 cm^2 was cut, sliced thinly and homogenized in the water-acetone mixture saturated with Na_2CO_3 using an Ultra Turrax T 25. After separation of the solid residues, by means of filtration (on a 45 μm PTFE filter), the extract solutions were measured using the same method described for the standard solutions. An example of the UV-Vis spectra of an apple extract is shown in Fig. 1b. Apart from Chp *a* and Chp *b*, other molecules were also extracted from the water/acetone mixture of the apple skin: carotenoids and anthocyanins. As a consequence, in the case of Golden Delicious apples, the spectrum in Fig. 1b is only amenable for the direct superposition of the two spectra in Fig. 1a in the 550-700 nm range.

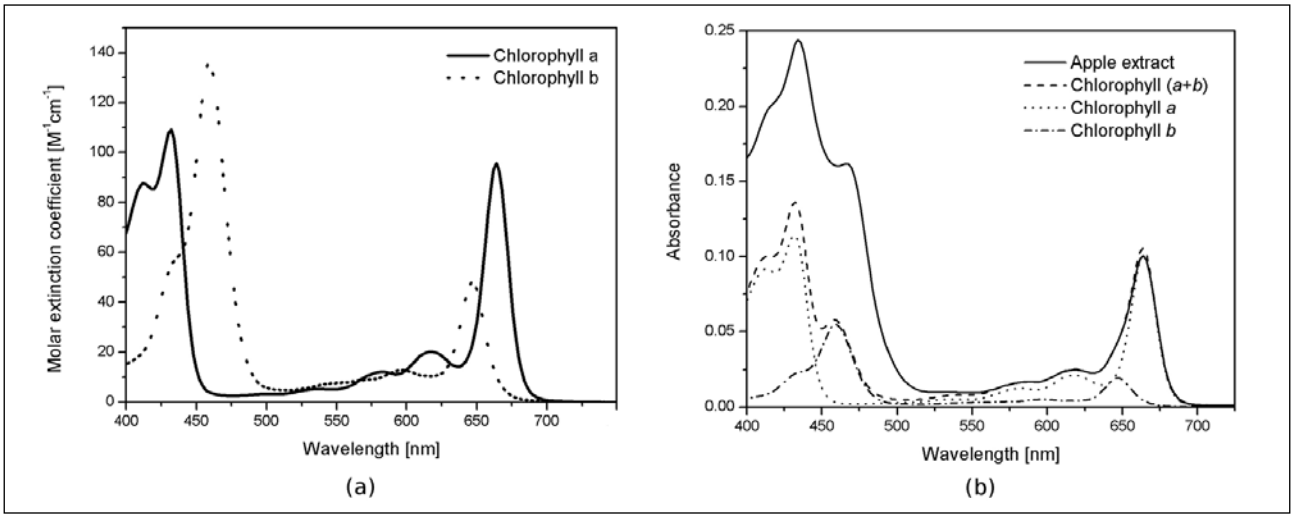


Fig. 1 - Section a: calculated extinction coefficients of Chp *a* (solid curve) and Chp *b* (dotted curve).
Section b: a typical transmission UV-Vis spectrum of apple extract, together with the calculated spectra of Chp *a*, Chp *b* and the sum of *a*+*b*.

2.3. Collection mode for UV-Vis spectra

The diffuse reflectance (DR) method offers a basic advantage over other measurement methods, because it allows quantitative estimates to be obtained. Denoting the reflectivity of a sample of infinite thickness at a given wavelength with R, and considering the case of only one substance being present, the Schuster-Kubelka-Munk function of R:

$$F(R) = \frac{(1-R)^2}{2R} \qquad \text{eqn. (1)}$$

can be written as (WENDLANDT and HECHT, 1966):

$$F(R) = \frac{k}{s} \cdot C = K' \cdot C \qquad \text{eqn. (2)}$$

where k is the absorptivity of the sample at the given wavelength, C is the sample concentration and s is the scattering coefficient. At a given wavelength, if the absorptivity and scattering coefficient are constant, there is direct proportionality between the intensity of the peaks expressed as F(R) through the coefficient K' and the concentration of the absorbing species in the matrix (WENDLANDT and HECHT, 1966).

Partial least squares regression modelling (PLS) was performed using the MATLAB software package. The 24 samples were divided into two groups, i.e a calibration set and a validation set, with a relative ratio population of about 5:1, in order to build a robust model. Five samples were randomly selected and included in the validation set for external validation purposes, and the remaining nineteen samples were used in the calibration set to build the PLS models. The performance of the PLS regression model was evaluated in terms of root mean square error of cross validation (RMSECV), which was determined by leave-one-out cross validation (LOO-CV) on the calibration set where:

$$RMSECV = \sqrt{\frac{\sum (y_i - \hat{y}_i)^2}{N}}$$

where $\hat{y}_1$ is the predicted response of the experiment, y_1 is the measured response and N is the number of experiments. The root mean square error of prediction (RMSEP) was used for the validation set to estimate the prediction of the developed model. Finally, correlation coefficients of the validation set were also calculated between the reference and prediction concentration.

3. RESULTS AND DISCUSSION

The DR spectra of the apples are shown in Fig. 2a at different ripening degrees. The chlorophylls in the apple matrix are subjected to a different environment from the acetone-water mixture, therefore the absorption peaks, in principle, can be found at different wavelengths: differences are also expected as far as the intensities are concerned. Recourse was made to the second derivatives of the curves in Fig. 2a (Fig. 2b) in order to obtain an estimate of the shifts undergone by the bands under consideration. It results that the characteristic band of Chp *a* is found at 678 nm in the apple skin (664 in acetone/water mixtures), thus showing a red-shift of about 14 nm, while that of Chp *b* appears as a shoulder at the same value of 645 nm, and no shift is apparently present. From a general point of view, if the entire DR spectra in the 400-700 nm range is considered, it is possible to write an appropriate ver-

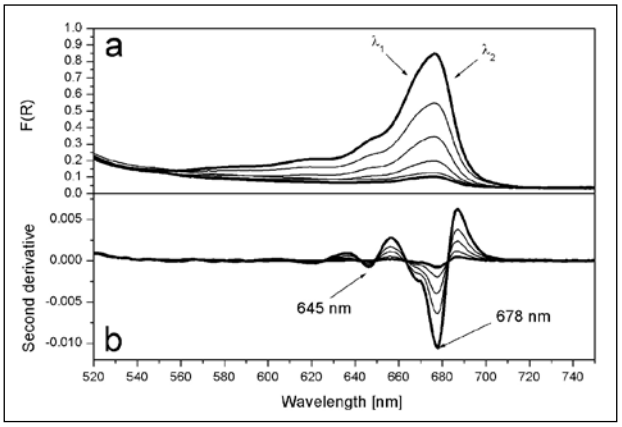


Fig. 2 - Section a: DR-Vis spectra of various apple skins with different chlorophyll concentrations.
Section b: second derivatives of the same spectra; the minima are used to locate the position of the absorption peaks of Chp *a* and Chp *b*. The meaning of the two arrows is given in the text.

sion of eqn. (2) for more than one chemical species. The spectral intensity at a given wavenumber, as expressed by F(R), is given by:

$$F(R) = K'_a X_a + K'_b X_b + \sum (K'_n X_n) \quad \text{eqn. (3)}$$

where X_a and X_b are the concentrations of Chp *a* and Chp *b*, respectively, K_a and K_b are basically the extinction coefficient of the two chlorophyll species, but for the scattering coefficient of the sample surface, and X_n and K_n represent the concentrations and the absorption coefficients of other molecules that absorb in the selected range. The Partial Least Square Method is in fact able to handle such a situation, even in the presence of foreign species. However, attempts in that direction have proved less satisfactory than the other methods our group has tried. We therefore limited ourselves to the 550 to 700 nm spectral range where, for Golden Delicious apples, only Chp *a* and Chp *b* show a significant absorbance. In this case, equation (3) reduces to the first two terms. Two methods have been attempted, one utilising two meaningful fixed wavelengths, the other using the PLS method over the 550-700 nm range.

3.1. Fixed wavelength calibration

The method is based on searching for two wavelengths, one, λ_1, which is representative of the absorption of both types of chlorophylls, and thus allows the total chlorophyll concentration X_{tot} to be determined, while the other, λ_2, is only representative of Chp *a*. At wavelength λ_1 when $K_a = K_b = K$:

$$F(R) = K'(X_a + X_b) = K' X_{tot} \quad \text{eqn. (4)}$$

In the case of acetone/water mixtures, λ_1 is at 652 nm. In the case of extracts, the values of K_a and K_b are different from those of the acetone-water mixtures, and cannot be evaluated separately. The value of λ_1 has therefore been estimated as the wavelength at which, by imposing that F(R) is proportional to the total chlorophyll content, the least error is found. This means that at each wavelength λ, K' has been evaluated for each spectrum as

$$K' = \frac{F(R)}{X_{tot}} \quad \text{eqn. (5)}$$

Figure 3a illustrates the trend of *K* with the wavelength. The relative error of *K* at each wavelength has been calculated as the standard deviation / average ratio of *K*. The results are plotted in Fig. 3b. A rather shallow minimum is observed, extending from 665 to 686 nm. The value chosen for the optimal wavelength is 668 nm, as this value represents the absolute minimum. On the other hand, it is possible to note that, in the case of acetone-water mixtures, λ_1 is 652 nm; the spectrum of Chp *a* has shifted with respect to Chp *b* by 14-16 nm, therefore the value of λ_1, in the present case, is expected to be no higher than 668 nm. The value of F(R) at 668

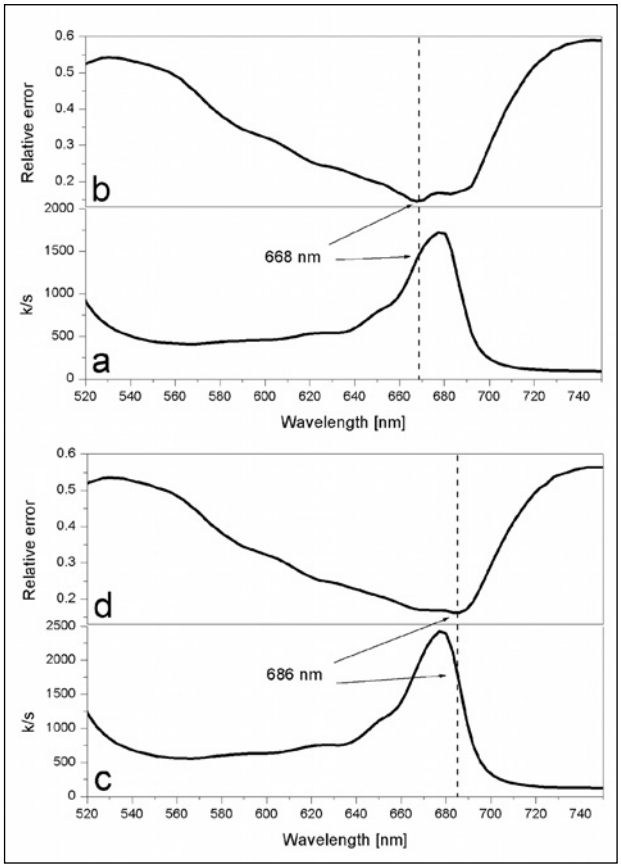


Fig. 3 - Average k/s value and its relative error at each wavelength calculated as σ(k/s)/μ(k/s) obtained for the DR-UV-Vis spectra of apples with different chlorophyll concentrations for the total chlorophyll concentration (left hand side) and for Chp *a* (right hand side).

nm is linearly correlated to the total concentration of chlorophyll, as shown in Fig. 4. The proportionality constant obtained by ordinary least squares fitting is 1.1 x 10^3. The model has an RMSD associate error of 2.3 x 10^{-7} mol/cm_2. It should be pointed out that Fig. 4 allows the total chlorophyll content to be evaluated with a single measurement at one wavelength. The value of λ_2 is obtained through a similar procedure, this time involving only Chp *a*. In this case, as shown in Figs. 3c and 3d, the optimal value of λ_2 is 686 nm, where the absorption of Chp *a* is still relatively high, and Chp *b* do not contribute to the absorption. A similar linear regression to that shown in Fig. 4 is obtained, with an RMSD associate error of 2.2 x 10^{-7} mol/cm^2. This procedure, in principle, allows the concentration of Chp *b* to be evaluated as the difference between the total chlorophyll content and that of Chp *a*. However, it should be noted that the error associated with this operation is influenced by both the error in the evaluation of the total chlorophyll and of Chp *a*. The partial least square (PLS) method allows a calibration to be obtained, even in the presence of correlated variables, and therefore offers better performances than the previously presented two-frequency method.

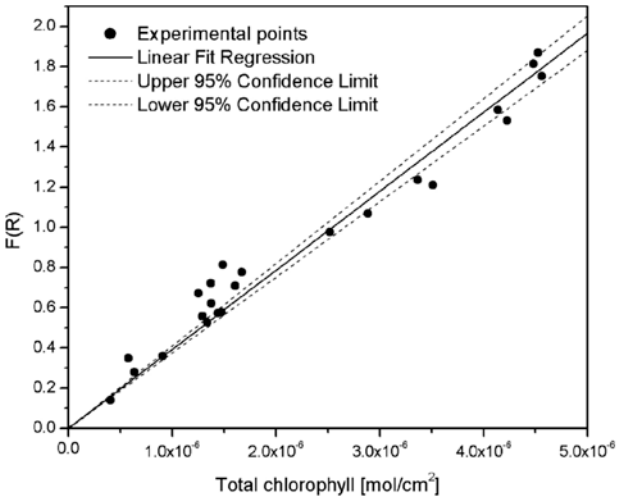


Fig. 4 - Linear correlation between F(R) at 668 nm and the total chlorophyll concentration.

3.2. Multivariate calibration

The quantitative PLS models were built by optimizing the principal component number, and applying the following spectral pre-processing methods: baseline correction, first derivative, standard normal variate correction (snv), first derivative + snv, but no data pre-processing. The results of both Chp *a* and Chp *b* are compared in Table 1. Better cross-validation results were obtained in the 560-740 nm range after a first derivative spectra pre-treatment. In this case, 2 PLS models were evaluated, one using 1 factor for Chp *a* and one using 2 factors for Chp *b*. These models allow the concentrations to be determined simultaneously with a reasonable error, calculated in cross validation RMSECV, of 1.5 x 10^{-7} mol/cm^2 for

Table 1 - PLS models results obtained from the spectral pre-processing methods: first derivative, baseline correction, standard normal variate correction (snv), first derivative + snv and no data pre-processing. The considered intervals are 740-560 nm and 700-640 nm.

Analyte	Pre-processing	Wavelength range nm	RMSECV	R^2
Chlorophyll *a*	First derivative	740-560	1.50*10^{-7}	97.8
		700-640	2.55*10^{-7}	93.8
	baseline correction	740-560	2.78*10^{-7}	93.3
		700-640	2.64*10^{-7}	93.6
	standard normal variate correction (snv)	740-560	4.88*10^{-7}	80.1
		700-640	3.58*10^{-7}	88.5
	first derivative + snv	740-560	5.20*10^{-7}	80.5
		700-640	5.85*10^{-7}	77.5
	no data pre-processing	740-560	2.78*10^{-7}	93.2
		700-640	2.72*10^{-7}	93.4
Chlorophyll *b*	First derivative	740-560	1.00*10^{-7}	96.6
		700-640	4.55*10^{-8}	99.2
	baseline correction	740-560	7.70*10^{-8}	97.6
		700-640	8.03*10^{-8}	97.5
	standard normal variate correction (snv)	740-560	1.67*10^{-7}	90.5
		700-640	9.33*10^{-8}	97
	first derivative + snv	740-560	2.07*10^{-7}	85.7
		700-640	2.28*10^{-7}	82.9
	no data pre-processing	740-560	7.77*10^{-8}	97.8
		700-640	9.17*10^{-8}	96.9

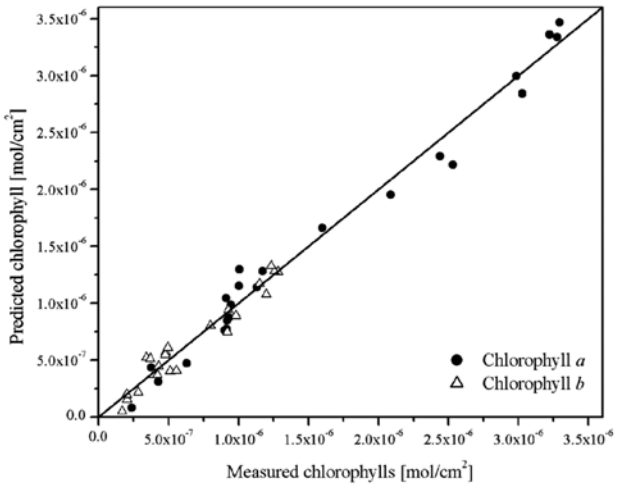


Fig. 5 - Measured *vs* predicted Chp *a* (•) and Chp *b* (Δ) contents in Golden Delicious apple skins, obtained with the PLS calibration model.

Chp *a* and 1.0 x 10^{-7} mol/cm^2 for Chp *b*, and an RMSEP of 2.13 x 10^{-7} mol/cm^2 for Chp *a* and 6.70 x 10^{-8} mol/cm^2 for Chp *b*. The predicted *vs* measured values are reported in Fig. 5. As expected, the use of PLS offers better results than the analysis at fixed wavelengths. The root mean square deviation for Chp *a*, using the PLS algorithm, is 1.5 x 10^{-7} mol/cm^2 compared to 3.8 x 10^{-7} mol/cm^2 obtained working at fixed wavelengths.

4. CONCLUSIONS

The reported results show that non-destructive UV-Vis analysis can be used in the DR mode to measure the concentrations of both types of chlorophylls in apple skins. Two methods have been presented, one working at two fixed wavelengths, the other (PLS) working over the 560-740 nm range. Both methods offer satisfactory results. However, the use of PLS is more convenient as the root mean square deviation for Chp *a* is 1.5 x 10^{-7} mol/cm^2, when using the PLS algorithm, compared to the value of 2.2 x 10^{-7} mol/cm^2 obtained with the method at fixed wavelengths. If a fast and simple method is required, the measurement of reflectance at 668 nm could be proposed, as it allows the total concentration of the chlorophylls to be determined, even with inexpensive instrumentation.

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PAPER

EFFECT OF MODIFIED ATMOSPHERE PACKAGING ON HALVAS FARSALON QUALITY DURING REFRIGERATED STORAGE

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ABSTRACT

Halvas Farsalon, a traditional confection of Greece, was packaged under modified atmosphere conditions (MAP) with four different gas mixtures (groups: A: 80%N_2 - 20%CO_2; B: 30%N_2 -70%CO_2; C: 100%N_2, and Control: atmospheric air) and stored at 4°C for 28 days. The sensory properties, color, textural performance, and oxidative stability of halvas were evaluated during MAP storage. Results showed that storage affected the fatty acid profile of the control group by decreasing the percentage proportion of MUFAs and PUFAs and increasing SFAs, while had no effect on the other groups among which group A presented better quality characteristics.

- Keywords: Halvas Farsalon, modified atmosphere packaging, lipid oxidation, fatty acids, sensory properties, refrigerated storage -

INTRODUCTION

Halvas, a traditional and popular confection in the Mediterranean and Middle East countries, is well known as a versatile and quality dessert. It usually consists of a sweetened sesame paste that contains sugar, glucose, vegetable oils (mainly palm oil) and tahina but there have been several variations based on the different ingredients used for halvas recipes. In Greece, one of the most popular and renowned traditional product is halvas Farsalon, which is manufactured in the town of Farsala, central Greece. This product consists mainly of sugar, corn starch, ovine butter, almond nuts and citric acid. Starch gelatinization, which is characterized by irreversible granule swelling, loss of birefringence and loss of crystallinity due to transition to the amorphous state is an important procedure that takes place during preparation of halvas Farsalon (BEMILLER and WHISTLER 1996; FELICIDAD and YRJO, 2008). This unique recipe gives to halvas Farsalon unique sensory and textural properties and renders the product very popular.

Halvas Farsalon is usually sold in local shops in the region of Farsala. It is stored in pans kept at ambient or refrigerated temperature and is sold to consumers after it is cut in smaller pieces. The product has usually a shelf life of only 5-6 days at ambient temperature but the shelf life is extended at refrigerated temperature. Since no packaging is applied, it is difficult to commercialize in other regions of Greece. The rapid loss of overall quality is mostly attributed to oxidative rancidity and textural changes that limit the retail value of the product, as well as the possibility to promote the product to other regions of Greece or even other countries.

Modified atmosphere packaging (MAP) is an attractive modern method of preservation of foods and has been extensively used for the refrigerated storage of various foods (MICALI *et al.*, 2009). MAP involves the use of O_2 concentrations below atmospheric levels, CO_2 concentrations at relatively high concentrations, usually higher than 20%, and N_2 as an inert filler gas, in order to preserve freshness and ensure/retain food safety. Some workers have pointed out the adverse effect of CO_2 on the physicochemical and sensory properties (eg. taste or odor) of certain foods packaged under MAP (OLARTE *et al.*, 2001; MICALI *et al.*, 2009).

Recent studies showed an elongation of shelf life and better quality characteristics for other confection products like marmalade candies (MUIZNIECE-BRASAVA *et al.*, 2011) or milk pomade sweets (VORMA *et al.*, 2010) stored under MAP conditions. To the best of our knowledge there are no publications on the effect of MAP on quality characteristics of this food product. Thus, the objective of this work was to investigate the effect of MAP on the quality characteristics of halvas Farsalon during refrigerated storage.

MATERIALS AND METHODS

Manufacture of halvas Farsalon

Sugar, water, corn starch, ovine butter, almond nuts and citric acid used for the manufacture of halvas Farsalon were purchased from local retail shops. HalvasFarsalon was manufactured in a confectionery shop at the town of Farsala, central Greece, following the traditional recipe. The preparation of halvas Farsalon is shown in the flow chart of Fig. 1. After preparation, halvas was left to cool at ambient temperature for 6 h, and then was cut into rectangular shape 500-g samples, sized (10 x 8 x 3 cm), which were used in the present study.

Modified atmosphere packaging (MAP)

Halvas samples were packaged in plastic trays of 16 x 20.5 x 4.8 cm (Cryovac UBRT®, Cryovac, Sealed Air S.r.l., Passirana di Rho, Italy), and

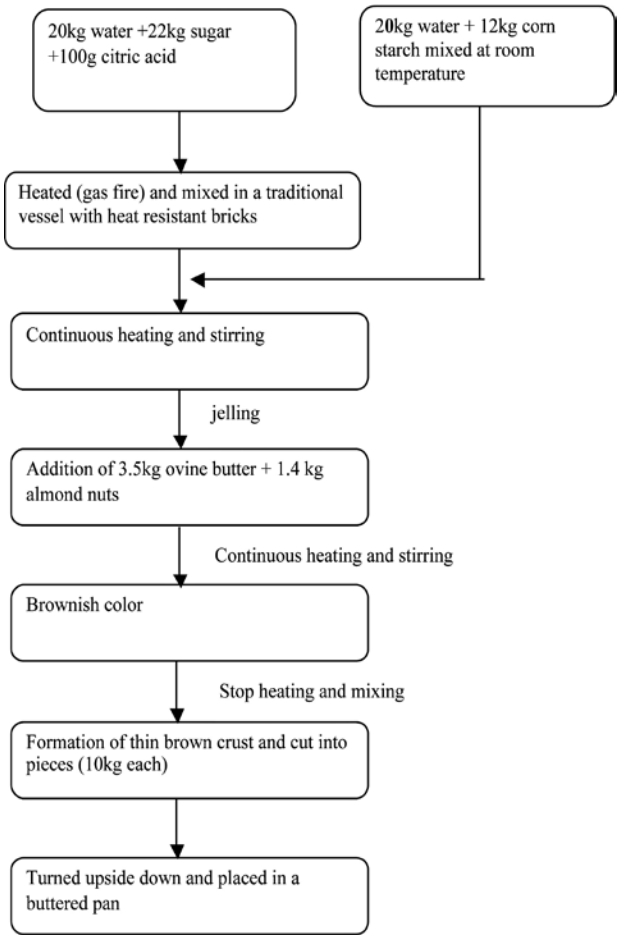


Fig. 1 - Flow chart of halvas Farsalon manufacture.

sealed by a sealing machine (model TSM105 Food tech, Minipack-torre S.p.A., Dalmine, Italy) with a film (OPET, Cryovac) of 39 μm thickness, under MAP conditions.

Four groups with four different gas mixtures (A group: 80%N_2 - 20% CO_2, B group: 30% N_2 - 70% CO_2, C group: 100% N_2, and Control group: atmospheric air) were used for the MAP evaluation tests of halvas Farsalon. All samples were stored at 4°C for 28 days. The gas mixtures were obtained from Air Liquid SA, Athens, Greece, and chosen in preliminary experiments, since there were no available literature data about the effect of MAP gases on halvas quality characteristics.

Physicochemical analysis

Total sugars were determined by a modification of the method of LANE and EYNON (AOAC, 1968). Corn starch was determined according to MCCLEARY *et al.* (1997). Moisture content was calculated by a gravimetric method using oven drying, fat content was quantified using the Soxhlet method, whereas protein concentration was calculated as total nitrogen content by the Kjeldahl method (AOAC, 2006). At each sampling time, pH values of halvas samples were determined with a pH meter (WTW, type 525, Wissennchaftlich-Technische Werkstatten, GmbH, D 82362 Weilheim, Germany).

Sensory evaluation

A 12-member trained descriptive attribute panel was used to evaluate sensory attributes such as of halvas Farsalon aroma intensity on a 9-point intensity scale (9=intense; 1=none) and rancid flavor intensity on a 9-point intensity scale (9=none; 1-intense). Panelists were selected among students and staff of the department and trained on the grading of halvas Farsalon sensory attributes. Each sample was served in dishes coded with three digits random numbers and presented in individual booths to each panelist for evaluation.

Headspace gas determination

Prior to opening, CO_2 and O_2 of the headspace of all packages were determined by using a gas analyzer Checkmate O_2/CO_2 (PBI-Dansensor A/S, Ringsted, Denmark) equipped with a needle for penetrating through the package. A rubber septum (Syntech Ltd, Glasgow, UK) was glued on the surface of the package and pierce with a 23 gauge needle connected to the gas analyzer.

Determination of lipid oxidation

Oxidative changes were monitored at 0 day and at 7-d intervals for up to 28 days of refrigerated storage on the basis of the malondialdehyde (MDA) formed. MDA, the compound used as an index of lipid peroxidation, was determined by a selective third-order derivative spectrophotometric method (BOTSOGLOU *et al.*, 1994). In brief, 2-g samples were homogenized in presence of 8 mL of a solution of 5 g trichloroacetic acid (Merck, Darmstadt, Germany)/100 mL water and 5 mL of a solution of 0.8 g butylated hydroxytoluene (Sigma Chemical Co, St. Louis, MO)/100 mL hexane, and the mixture was centrifuged. The top layer was discarded, and a 2.5-mL aliquot from the bottom layer was mixed with 1.5 mL of a solution of 0.8 g 2-thiobarbituric acid (Sigma Chemical Co, St. Louis, MO)/100 mL water to be further incubated at 70°C for 30 min. Following incubation, the mixture was cooled under tap water and submitted to conventional spectrophotometry (Shimadzu, Model UV-160A, Tokyo, Japan) in the range of 400-650 nm. Third-order derivative spectra were produced by digital differentiation of the normal spectra using a derivative wavelength difference setting of 21 nm. The concentration of MDA in samples was calculated on the basis of the height of the third-order derivative peak at 521.5 nm by referring to slope and intercept data of the computed least-squares fit of standard calibration curve prepared using 1,1,3,3-tetraethoxypropane (Sigma Chemical Co, St. Louis, MO).

Fatty acid analysis

The lipid fraction of samples was extracted according to FOLCH *et al.* (1957) with some modifications. The lipid extract was saponified and fatty acids were converted to methyl esters according to the one-step extraction-transesterification procedure described by SUKHIJA and PALMQUIST (1988). Separation and quantification of the fatty acid methyl esters were carried out using a capillary column gas chromatographic system (Shimadzu, Model GC-15A, Shimadzu Corp., Kyoto, Japan) equipped with a flame ionization detector, an automatic sampler, model AOC-17, and a fused silica capillary Supelco column model 24110-u SP-2380™, 30 m x 0.25 mm i.d., 2 μm film thickness. Column temperature was set initially at 70°C and maintained for 0.3 min; it was then increased to 180°C at a rate of 30°C/min and held there for 10 min; finally, column temperature was raised to 225°C at a rate of 5°C/min and held there for 3 min. The temperature of both the injection port and the flame ionization detector was set at 240°C. Helium carrier gas flow was set at 1 mL/min, hydrogen at 30 mL/min, and air at 300 mL/min. Injection of the 1-μL samples was performed with a split ratio of 20:1. Identification of individual fatty acids was based on comparisons of retention times of unknown peaks to authentic fatty acid methyl ester standards (Supelco FAME Mix 18919,

Bellefonte, PA, Usa) and by addition of standards when necessary. Peak areas were integrated and converted to fatty acid percentages (direct area normalization) by means of the Shimadzu, Model Class-VP chromatography data system program. The relative content of each fatty acid methyl ester was reported as percent area of total fatty acid methyl esters.

Color measurement

Color changes were assessed in the inner core (with no surface crust) of the halvas samples using a spectro-colorimeter (tristimulus color machine) with the CIE lab color scale (CHROMAmeter CR 410, Minolta, Japan). This color assessment system is based on the Hunter *L**, *a** and *b** coordinates, where *L** represents lightness, *a** redness and *b** yellowness in the reflection mode.

Texture measurements of halvas

The texture of halvas was determined using an AEGIS, Texture Analyzer machine (model: expert 5601 ADMET, Inc.) fitted with an SM-250 (1,000 N) load cell at room temperature (23°C). Halvas samples of cylindrical shape (height 30 mm and diameter 90 mm) were used for texture measurement. Each sample was compressed to 70% of its original height using a 20 mm diameter cylindrical probe, and a penetration speed of 100 mm/min. The texture was evaluated by estimating the peak force (N) required for the compression.

Statistical analysis

Data were subjected to analysis of variance in the general linear model using the SPSS 10.05 statistical program (SPSS Ltd. Woking, UK). When significant treatment effects were disclosed, Duncan's multiple range tests with examination for significant differences at each storage interval for individual treatment were employed. Sensory data were also analyzed by ANOVA and t-tests for separation of mean differences. A probability level of $P \leq 0.05$ was used in testing the statistical significance of all experimental data.

RESULTS AND DISCUSSION

Physicochemical analysis

Chemical analysis of halvas samples showed values of 60.5±0.2% for total sugars, 13.0±0.1% for starch, 2.7±0.1% for fat, 0.3±0.04% for protein, and 33.1±0.2% for moisture. Sugars content was rather high (60.5%), while protein content was low (0.30%). EISSA and ZOHAIR (2006) reported the composition of another type of halvas produced in Middle East (protein 24.7% and fat 58.9%), which is different than halvas Farsalon. However, the basic ingredient for the production of that type of halvas was tahina and not the corn starch used as the basic ingredient for the production of halvas Farsalon. Results showed that total sugars, starch, protein and moisture contents did not change significantly ($P>0.05$) among samples throughout storage at 4°C.

In A, C and control groups, halvas samples showed pH values in the range 4.95-5.15 without any significant changes ($P>0.05$) during storage at 4°C. However, in group B (30% N_2-70% CO_2) pH values were in the same range by the 21st day of storage whereas they reduced thereafter to values (4.87), which were significantly lower ($P<0.05$) than the pH of the other groups. Previous studies showed that a modified atmosphere packaging with a high CO_2 content could cause a pH decrease due to dissolution of CO_2 in the moisture and the formation of carbonic acid (OLARTE *et al.*, 2002; DERMIKI *et al.*, 2008). Determination of the headspace gases within packages also revealed that the decrease of CO_2 in the modified atmosphere of group B was higher than group A during the refrigerated storage due, possibly, to higher dissolution of CO_2 in the aqueous phase of halvas.

Sensory evaluation

The sensory evaluation scores of the A, B, C and the control groups during storage at 4°C for 28 days are shown in Fig. 2. Aroma and rancid flavor intensity scores were significantly higher ($P<0.05$) for group A compared to control, after the 7th day of storage. Aroma and rancid flavor intensity scores of group A were significantly higher ($P<0.05$) than those of groups B and C, after the 7th and 14th days of storage, respectively. Therefore, halvas Farsalon packaged under MAP with a mixture of 80%N_2 - 20% CO_2 showed better sensory properties than control and the rest gas mixtures during the 28 days of refrigerated storage. Other studies showed that MAP offered better sensory properties than atmospheric air packaging for various foods during refrigerated storage (SANTOS *et al.*, 2005; GOVARIS *et al.*, 2010; SINGH and GOYAL 2011).

Color assessment

*L**, *a** and *b** values of samples from all groups decreased throughout the refrigerated storage of 28 days (Fig. 3). Control group presented *L** and *a** or *b** values significantly lower ($P<0.05$) than the MAP groups (A, B and C) after the 7th and 14th days of storage, respectively. Group A presented *L** and *a** values significantly higher ($P<0.05$) than groups B and C after the 14th day of storage, while A, B and

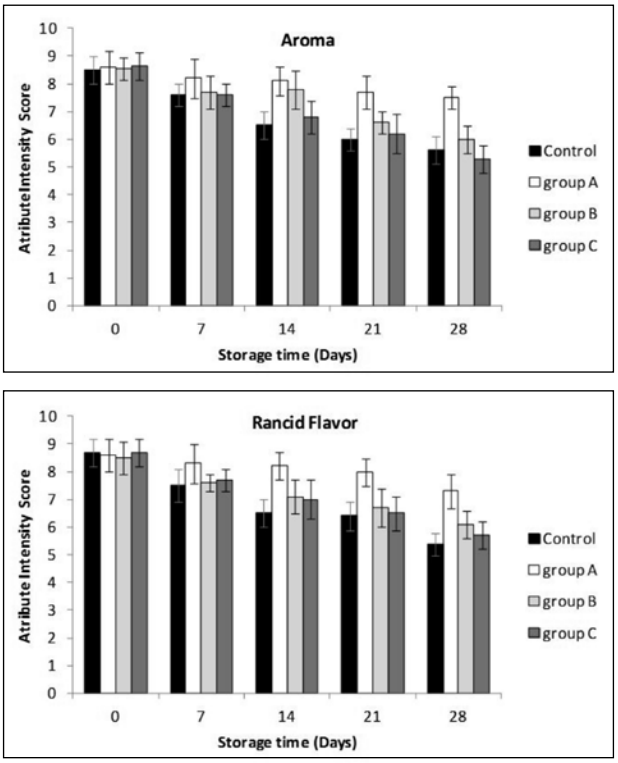


Fig. 2 - Sensory evaluation of halvas Farsalon of MAP groups A, B, C and control during storage at 4°C for 28 days. Groups: A: 80%N_2 - 20%CO_2; B: 30%N_2 -70%CO_2; C: 100%N_2, and Control: atmospheric air. Mean values from six samples analysed±standard deviation.

C groups presented *b** values not differing (P>0.05) among each other, throughout the refrigerated storage.

In contrast to the control, all other groups presented colors closer to the fresh product, and this might be due to the absence of oxygen. Group A (80% N_2 - 20% CO_2) presented the closer to the fresh product color, as compared to other groups throughout the 28 days refrigerated storage. Previous studies indicated that the absence of oxygen in modified atmosphere packaging kept the fresh color of several food products for a longer time, since the oxygen can cause discoloration of several color compounds of foods (GOULAS *et al.,* 2005; SINGH and GOYAL, 2011; ROJAS-GRAU *et al.,* 2009).

Determination of headspace gases

The O_2 content of the headspace of all packages was determined at 0.5% on 0 day and kept almost the same throughout the refrigerated storage. The initial CO_2 content was found 19.75 or 67.90% on 0 day and then decreased to reach 19.30 or 67.10% by the 28th day of storage, for the groups A and B, respectively. The decrease of CO_2 might be due to dissolution of CO_2 in the aqueous phase of halvas Farsalon or absorption of CO_2 in the halvas Farsalon and is in accordance with similar observations in previous studies for other foods (DERMIKI *et al.,* 2008; SIMPSON *et al.,* 2009).

Lipid oxidation

Fig. 4 shows that the extent of lipid oxidation in halvas Farsalon stored at 4°C for up to 28 days, varied with the storage time and the MAP treatment. A, B, and C groups presented MDA values that were not significantly different (P<0.05), as compared to each other, but significantly lower (P<0.05) than the control group, throughout the refrigerated storage. The absence of oxygen in groups A, B and C offered a better oxidative stability to halvas Farsalon as compared to the control group packaged under atmospheric conditions. In agreement to our study, previous studies also showed better oxidative stability of other foods stored under modified atmosphere conditions with absence of oxygen or lower concentration of oxygen than the atmospheric air composition (ARASHISAR *et al.,* 2004; TIAN *et al.,* 2004; GOULAS *et al.,* 2005).

Effect of MAP on fatty acid profile

Table 1 shows that total saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs) in halvas samples prior to storage (day 0) were 67.71, 25.54 and 4.81%, respectively. The predominant saturated fatty acid was palmitic acid (C16:0) with a proportion of 33.15%, whereas the predominant monounsaturated and polyunsaturated fatty acids were oleic acid (C18:1) and linoleic acid (C18:2) with proportions of 22.99 and 4.05%, respectively.

In the control group, storage at 4°C for 28 days resulted in decrease of the proportion of MUFAs and PUFAs and increase of SFAs. The proportions of palmitoleic acid (C16:1), oleic acid (C18:1), linoleic acid (C18:2) and linolenic acid (C18:3) were decreased (P<0.05), the proportions of palmitic acid (C16:0) and stearic acid (C18:0) were increased (P<0.05), while the other saturated fatty acids remained unchanged, after 28 days of storage at 4°C. Similar changes in the fatty acid composition of SFAs, MUFAs and PUFAs were also observed in other foods such as eggs (MAZALLI and BRAGAGNOLO, 2009), pork (MONIN *et al.,* 2003) or fish (STAMATIS and ARKOUDELOS, 2007) during refrigerated storage.

In A, B and C groups, no significant changes (P>0.05) were observed among SFAs, MUFAs and PUFAs during refrigerated storage at 4°C for 28 days. These results indicate that the absence of oxygen inhibits lipid oxidation and lend support to previous studies with other foods such as cheese (TEMIZ, 2010), chocolate (MEXIS *et al.,* 2010) or fish (STAMATIS and ARKOUDELOS, 2007) during refrigerated storage.

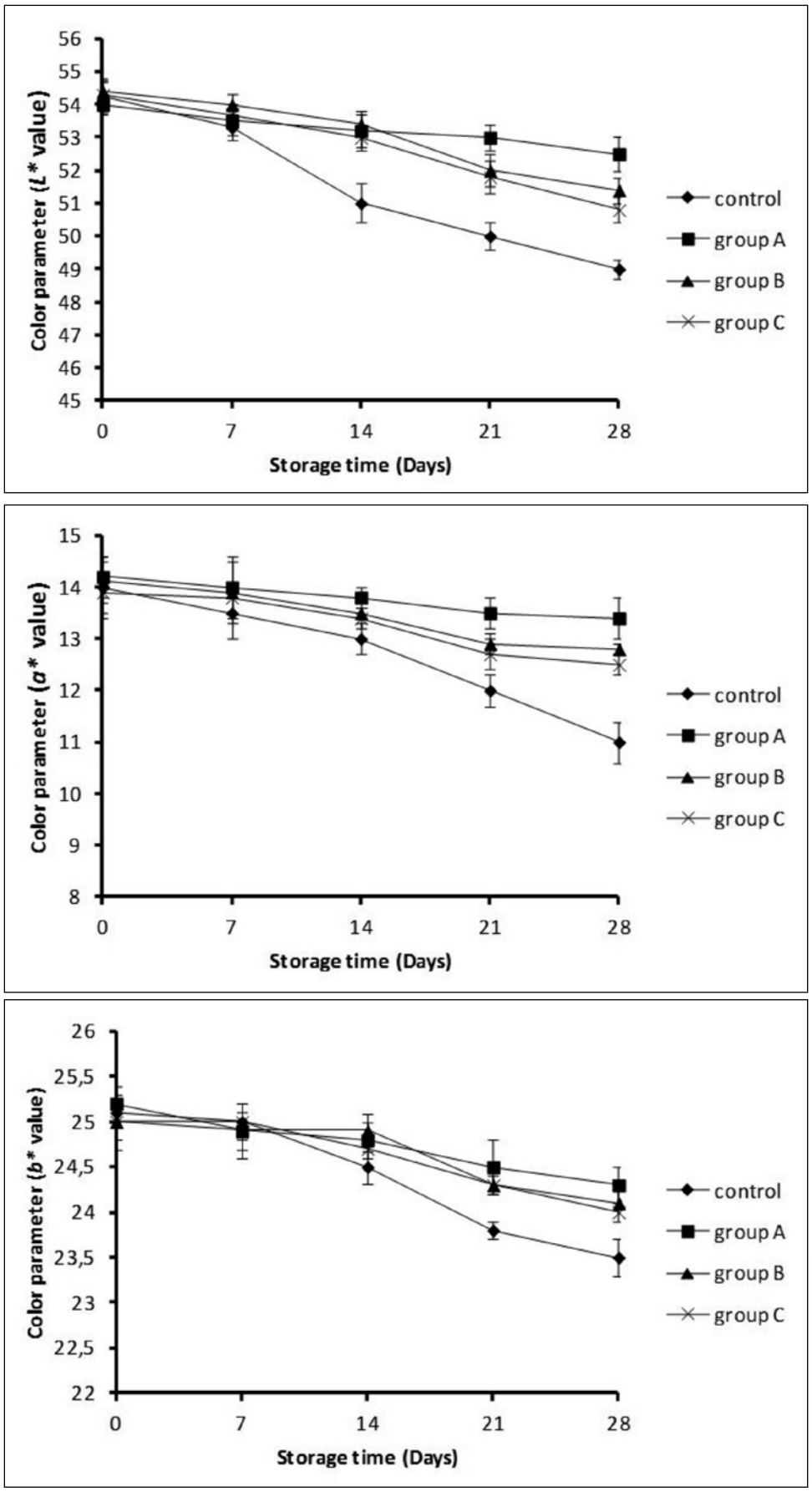


Fig. 3 - Color assessment with estimation of *L**, *a** and *b** values of halvas Farsalon of MAP groups A, B, C and control during storage at 4°C for 28 days. Groups: A: 80%N_2 - 20%CO_2; B: 30%N_2 -70%CO_2; C: 100%N_2, and Control: atmospheric air. Mean values from six samples analysed±standard deviation.

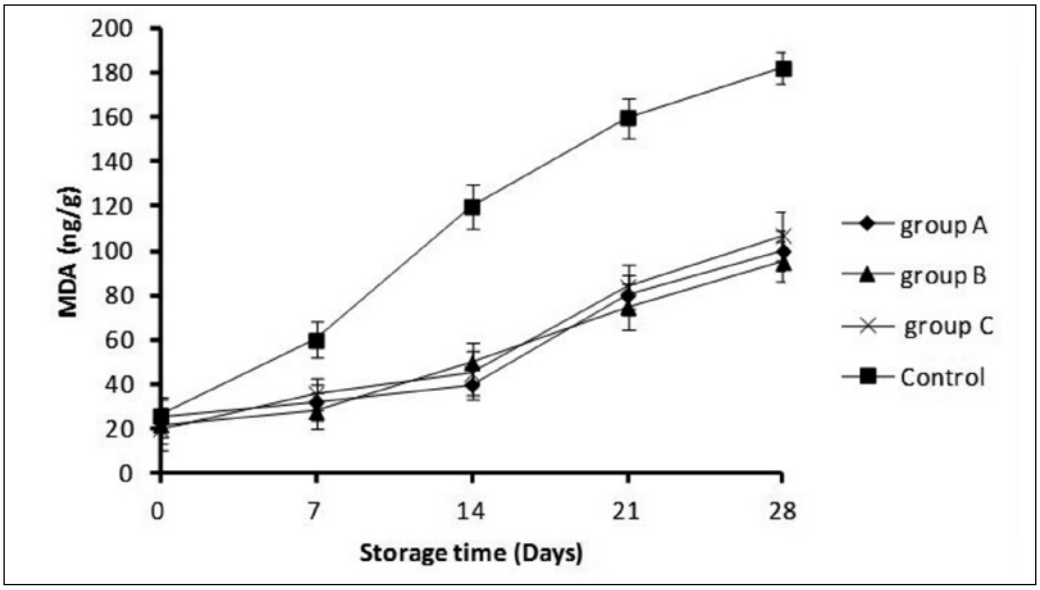


Fig. 4 - Effect of MAP groups A, B, C and control on lipid oxidation of halvas Farsalon during storage at 4°C for 28 days. Groups: A: 80%N_2 - 20%CO_2; B: 30%N_2 -70%CO_2; C: 100%N_2, and Control: atmospheric air. Data points are mean malondialdehyde (MDA) concentrations from six samples analyzed ± standard deviation.

Effect of MAP on texture

Results showed that the peak force changes of control group were significantly (P<0.05) higher than those of the A, B and C groups throughout the 28 days of refrigerated storage, indicating that storage under atmospheric conditions (control) caused a harder texture than under modified atmosphere conditions (Fig. 5). Among the MAP groups, the better textural performance of modified atmosphere treatments were shown by group A, since the peak force values of this group were significantly (P<0.05) lower than those of groups C

Table 1 - Fatty acid profile of halvas samples stored under MAP and ambient conditions at 4°C for 28 days.

	Peak area percentage of fatty acids*				
	0 day of storage	28th day of storage			
Fatty acids		Control**	Group A**	Group B**	Group C**
C10:0	5.98±0.27	6.12±0.28	6.09±0.40	6.14±0.19	5.92±0.44
C12:0	3.51±0.24	3.35±0.24	3.44±0.37	3.20±0.45	3.38±0.15
C14:0	11.86±0.45	12.08±0.36	11.68±0.37	11.98±0.24	12.02±0.29
C16:0	33.15±0.63a	34.66±0.39b	33.54±0.55a	33.72±0.40a	33.80±0.35a
C18:0	12.53±0.31a	13.25±0.22b	12.36±0.26a	12.23±0.28a	12.27±0.25a
C20:0	0.58±0.06	0.42±0.08	0.54±0.12	0.46±0.14	0.43±0.13
C24:0	0.10±0.04	0.08±0.03	0.12±0.02	0.11±0.03	0.13±0.02
Total SFA	67.71±0.38a	69.96±0.26b	67.77±0.44a	67.84±0.30a	67.95±0.28a
C16:1	2.55±0.14a	2.02±0.10b	2.49±0.12a	2.58±0.18a	2.46±0.08a
C18:1	22.99±0.55a	22.04±0.12b	22.82±0.42a	23.01±0.45a	22.90±0.30a
Total MUFA	25.54±0.38a	24.06±0.12b	25.31±0.34a	25.56±0.28a	25.36±0.22a
C18:2	4.05±0.15a	3.58±0.08b	4.12±0.08a	4.04±0.06a	4.10±0.10a
C18:3	0.76±0.08a	0.42±0.08b	0.84±0.12a	0.80±0.10a	0.82±0.10a
Total PUFA	4.81±0.10a	4.00±0.07b	4.96±0.09a	4.84±0.08a	4.92±0.10a

*Mean values from six samples analysed±standard deviation. Mean values in the same row followed by a different letter are significantly different (P<0.05).

**Groups: A: 80%N_2 - 20%CO_2; B: 30%N_2 -70%CO_2; C: 100%N_2, and Control: atmospheric air.

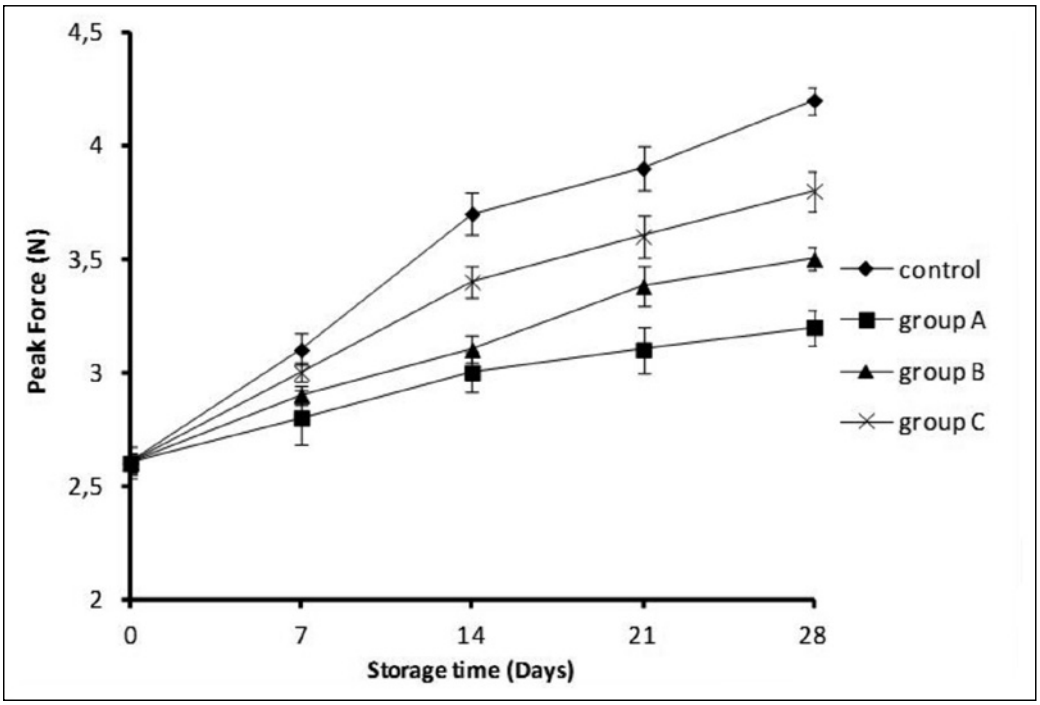


Fig. 5 - Effect of MAP groups A, B, C and control on texture of halvas Farsalon during storage at 4°C for 8 days. Groups: A: 80%N_2 - 20%CO_2; B: 30%N_2 -70%CO_2; C: 100%N_2, and Control: atmospheric air. Data points are peak force (N) values from six samples analyzed ± standard deviation.

and B, after the 7th and the 14th day of storage, respectively. These differences of textural performance of halvas samples stored under atmospheric or modified atmosphere packaging conditions might be attributed to different rates of starch retrogradation, where a gradual transition of amorphous starch to a partially crystalline state takes place (FELICIDAD and YRJO, 2008). The rate of this transition depends on many factors including product formulation, cooking method and storage conditions (FELICIDAD and YRJO, 2008). In halvas, where there is just enough moisture to gelatinize starch granules, amylose retrogradation may be largely complete by the time the product has cooled to ambient temperature. The presence of lipids such as MUFAs and PUFAs are protecting starch from retrogradation in the jells (BEMILLER and WHISTLER, 1996). However, exposure of starch to atmosphere conditions can accelerate lipid oxidation and starch cross-linking leading to faster texture deterioration (BEMILLER and WHISTLER, 1996). The better textural performance under modified atmosphere packaging conditions was also reported for other foods such as bread (CENCIC *et al.,* 1996), cheese tart (SANGUINETTI *et al.,* 2009), or chocolate (MEXIS *et al.,* 2010).

In conclusion, halvas packaged under modified atmosphere conditions (MAP) with a gas mixture of 80% N_2-20% CO_2 presented better quality characteristics than those of other tested groups.

ACKNOWLEDGEMENTS

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SHORT COMMUNICATION

QUALITY AND SAFETY OF ITALIAN WINE VINEGAR

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ABSTRACT

The aim of this work was to investigate the most common frauds in the production of wine vinegar in Italy. By means of isotopic analyses (SNIF-NMR and IRMS techniques), 100 samples of commercial wine vinegars and 100 samples of red and white wines used for vinegar production were analysed to detect any illegal practices such as watering, sugaring, addition of synthetic acetic acid or table grapes. For further investigations, samples were also submitted to chemical, physical, microbiological and sensorial analyses. The results show that the addition of synthetic acetic acid is still a very common fraud.

- Keywords: frauds, isotopic, quality, safety, vinegar, wine -

INTRODUCTION

Vinegar is an acidic liquid derived from the oxidation of ethanol (CH_3CH_2OH), which has previously been fermented from sugar. The result is the production of acetic acid (CH3COOH), the main component of vinegar. This process was first explained by PASTEUR. In 1864, he made an important contribution to the scientific study of this substance, by demonstrating that the oxidation of ethanol by means of acetic acid bacteria led to the formation of acetic acid.

Vinegar has long been known and appreciated. It was employed as a culinary ingredient and preservative as well as a medicament and disinfectant in ancient Greece, Roman times and the Middle Ages. The Italian term "aceto" derives from the Latin *acetum* (acid and sour liquid obtained from wine) whereas the English term "vinegar" comes from the Old French *vin aigre* meaning "sour wine". Both words link vinegar with sourness and wine, but this important by-product can be made also from malt, apple cider, fruit, rice, coconut, cane, beer, honey, etc. However, the most common type is wine vinegar. The aim of this study was to investigate the most common frauds relating to Italian wine vinegars, pointing out the infringements of legal limits and quality standards or the risks to human health. These are very important of food quality and safety issues, as vinegar is still widely used in cooking and food preservation.

In Italy, the 20 February 2006 No. 82 Law incorporated the EU Directive for the Common Market Organization (CMO) that regulates wine and also vinegar production. As for commercial wine vinegars, it fixes legal limits for the following parameters: total acidity, alcoholic degree, ashes, sulfur dioxide, zinc, copper, lead, boric acid, bromine and sorbitol. According to Italian legislation, wines used to produce wine vinegars can only be affected by sourness. The Italian Ministry of Agriculture controls food products in order to detect frauds or non-conformities to the law. The term "fraud" generally indicates an intentional deception made to secure unfair advantage or unlawful gain. Our laboratory is involved in this prevention effort, the aim of which is to secure quality and safety for consumers. We carry out analyses on irregular samples of wines, musts, spirits and wine vinegars, already examined by other agencies, in order to confirm or refute the previous results (Review Analysis).

In this work, 100 samples of commercial wine vinegars and 100 samples of red and white wines used in vinegar production were analysed. The results were compared with standards set out in current legislation in order to evaluate the incidence of the most common frauds.

With regard to vinegars, we first need to investigate the addition of synthetic acid, a very common illegal practice. Until the introduction of radiocarbon analytical method, this addition was very difficult to detect (RESMINI and VOLONTERIO, 1974; MATTA and GIANNESSI, 1987). Vinegar containing synthetic acetic acid can be assessed by the content of ^{14}C. The amounts of ^{14}C in natural wine vinegars are determined by the year of production. Other adulterations such as watering and sugaring are today easily detectable by means of isotopic analyses (SNIF-NMR and IRMS techniques).

Isotopic techniques were first introduced in France in the 1980s and soon became widely applied in oenology (MARTIN *et al.*, 1986; FORSTEL *et al.*, 1996; JAMIN *et al.*, 2004; THOMAS and JAMIN, 2009). They are based on the fact that a particular molecule may have different isotope contents, depending on the biochemical processes involved in its synthesis. These analyses also made it possible to control mislabelling, a practice concerning both wines and primary products like grapes. As for wines used for vinegar production, it is now easily to verify much of the data or statements reported on their labels. By means of isotopic analyses, we detected the use of table grapes, such as *Pergolone*, instead of wine grapes. Table grapes and wine grapes have similar isotopic parameters but a different potential alcohol degree. Unscrupulous producers take advantage of the availability of table grapes which cost less, and due to their use on a massive production scale they can make greater profits. As the potential amount of alcohol is lower in table grapes than in wine grapes, a solution of water and sugar is usually added to increase it.

MATERIALS AND METHODS

Materials

Red and white wine vinegars

In this work, 100 commercial samples of white and red wine vinegars originating from the most important Italian production areas were analysed anonymously and indicated by progressive numbers. The samples were collected in the following regions: Piemonte, Lombardia, Veneto, Emilia Romagna, Campania, Sicilia.

Red and white wines used in vinegar production

Other 100 red and white wines employed in vinegar production were also analysed anonymously and indicated by progressive numbers. The samples were collected in the following regions: Piemonte, Lombardia, Veneto, Friuli, Emilia Romagna, Lazio, Puglia, Sicilia, Campania.

Methods

Red and white wine vinegars

The chemical and physical analyses covered

the following legal parameters: total acidity, ethanol, copper, zinc and lead content, presence of synthetic acetic acid. They were carried out according to the standards established by the resolution of the International Organisation of Vine and Wine (OIV): "Oenology 2000 – Methods of analysis of vinegars".

- **Total acidity** was determined by titration with sodium hydroxide 0.1 N. (phenolphthalein indicator).

- **Ethanol** was determined after distillation by titration with the addition of an excess of potassium dichromate and then back titrated with a solution of ferrous sulfate and ammonium.

- **Heavy metals** were determined in vinegars using a Zeeman 5100 atomic absorption spectrometer (Perkin Elmer). Copper was determined by flame ionization spectroscopy. In the case of very low concentrations, we used the method of a standard addition. Zinc was also determined by flame ionization spectroscopy. Lead was determined by ashing.

- **Synthetic acetic acid** was quantified by the amount of ^{14}C detected. Natural wine vinegars have amounts of ^{14}C which relate to their year of production. The addition of synthetic acid causes a noticeable reduction in ^{14}C values. Measurements were carried out by means of a ^{14}C liquid scintillation spectrometer. As a reference, we used our data-base of radioactivity values for natural vinegar.

Red and white wines used
for vinegar production

All the samples of wines were submitted to chemical, physical and isotopic analyses, according to the resolutions established by the OIV methods. The chemical and physical analyses covered the following legal parameters: total sulfur dioxide, copper, zinc, lead, methanol, isotopic parameters. Organoleptic faults were ascertained through a series of sensory analyses. Italian laws states that all the values detected in wines used for vinegar production must be within the legal ranges. Only the volatile acidity can be over the legal limit.

- **Total sulfur dioxide**. It was oxidized by sulfuric acid with oxygenated water and measured by titration with 0.1 N sodium hydroxide using the mixed indicator Methyl Red-Methylene Blue – alcoholic solution.

- **Heavy metals**. These were directly determined in wines using a Zeeman 5100 atomic absorption spectrometer (Perkin Elmer). Lead was determined by furnace whereas copper was determined by flame ionization spectroscopy.

- **Methanol**. It was quantified according to the OIV gas chromatographic method and extracted from wine by distillation and then quantified using a Carlo Erba model 5300 gas chromatograph and an Innowax column equipped with a flame ionization detector, according to the OIV reference method.

- **Isotopic parameters**. These were determined by applying the following methods:

SNIF-NMR Isotopic Ratio (D/H)I determinations were performed on a Bruker Avance 300 (Cryomagnet 300/52) NMR spectroscope. The analysis was carried out on distilled wine (Fig. 1).

$^{13}C/^{12}C$ isotopic ratios were determined by isotopic ratio mass spectrometry, using a Fisons, Optima model. The variation from a standard value attributed to a reference substance by the International Atomic Energy Agency of Vienna (IAEA) was measured. The analyses were carried out on distilled wine (Fig. 2).

$^{18}O/^{16}O$ isotopic ratio measurements were per-

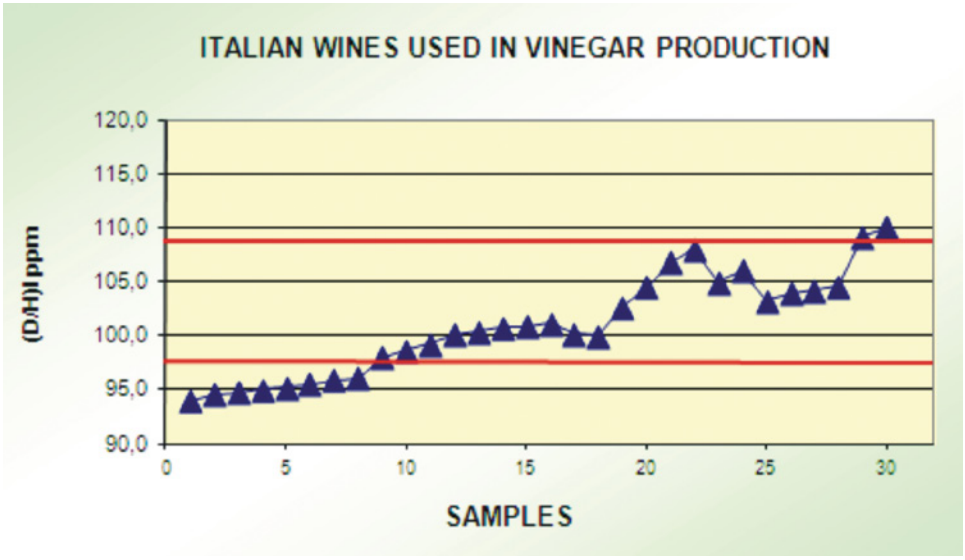


Fig. 1 - Isotopic parameter (D/H)I values compared with the reference values of the National Annual Data Bank on Authentic Wines.

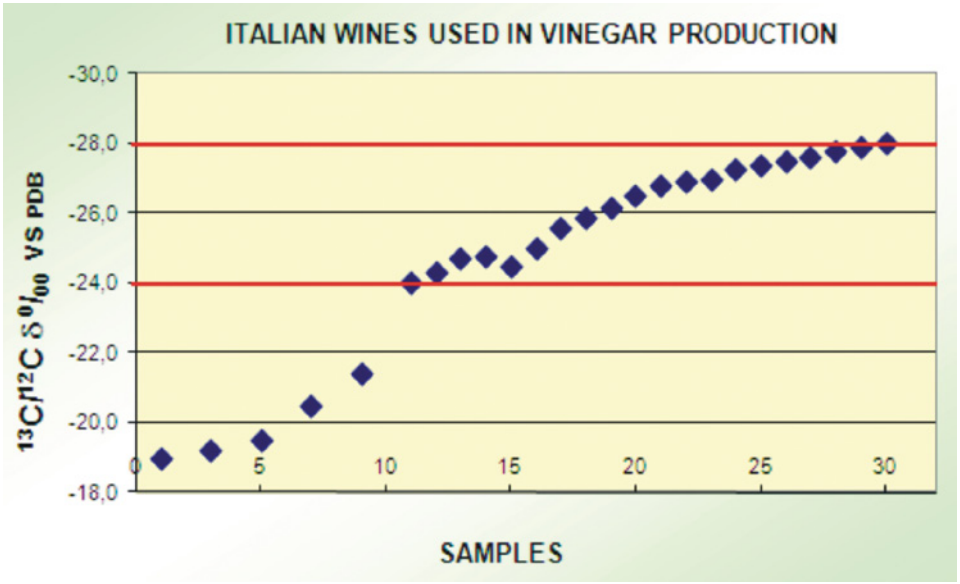


Fig. 2 - $^{13}C/^{12}C$ isotopic parameter values compared with the reference values of the National Annual Data Bank on Authentic Wines.

formed on the same mass spectrometer. These values represent the % variation from the standard value attributed to ocean water, the Vienna Standard Mean Ocean Water (V-SMOW). The analyses were carried out directly on wine without any distillation.

- **Organoleptic faults**. These were detected through a series of sensorial analyses carried out by the CRA-ENO wine tasting panel, formed by five wine tasters trained to detect wine faults.

RESULTS

Red and white wine vinegar

For red and white wine vinegars, we detected the addition of synthetic acid and dried currants.

Addition of synthetic acetic acid radiocarbon

In the past, the addition of pyroligneous acid or "wood vinegar" to vinegars was a very common practice. This synthetic acid was very difficult to detect, because it has the same chemical structure as vinegar. In the determination of ^{14}C in our vinegar samples, we recorded a minimum value of 11.29 dpm/g C and a maximum value of 14 dpm/g C. The reference value of 14 dpm/g for authentic vinegars was determined in our laboratory. Values that are 5% or more under the reference value are considered to be irregular because of the addition of acetic acid. In our investigation, 20 samples were found to be irregular. Although the incidence of this fraud has diminished, it still remains one of the most widespread.

Addition of dried currants

The use of dried currants in vinification is another common practice. Their presence in wine is exposed by the variation in isotopic parameters. In vinegars, this addition is very difficult to detect, if water is added to dilute the wines used for vinegar production. This is a legal oenological practice but it changes the isotopic parameters of the wines. We found 3 irregular samples.

Red and white wines used for vinegar production

For the red and white wines used for vinegar production, we detected the following isotopic parameters.

The determination of the isotopic parameter (D/H)I produced values ranging from a minimum of 94 ppm to a maximum of 108 ppm. Reference values were obtained from the National Annual Data Bank on Authentic Wines: the range established was from 98 to 108 ppm. We found 10 irregular samples because of sugaring. Values from 94 to 98 ppm are due to the addition of sugar beet to wine, whereas values over 110 ppm are due to the addition of sugar cane.

The determination of the $^{13}C/^{12}C$ isotopic parameter showed values ranging from a minimum of -19 δ% to a maximum of -28 δ%. The range established from the National Data Bank is from -24 to -28 δ%. Values below -24 δ% indicates the addition of exogenous sugars made from sugar cane or maize. We found 5 irregular samples.

The determination of the $^{18}O/^{16}O$ isotopic parameter $^{18}O/^{16}O$ showed values ranging from a minimum of -5 δ% (V-SMOW) to positive values

(Fig. 3). Reference values are determined yearly and were positive for the year concerning our samples. In general, negative values of this parameter indicate watering of the wine employed for vinegar production. This dilution increases the total volume of wines. We found 15 irregular samples.

We also found 5 samples for which all three isotopic parameters did not correspond to the reference values. This indicated the use of table grapes, e.g. *Pergolone*, instead of wine grapes (mislabelling). The two types of grapes have similar isotopic parameters but different potential alcohol degrees. When table grapes are illegally used in wine production, water and sugar are added to increase the alcohol content. This addition can be now identified by carrying out the isotopic analyses described.

Subsequently, we investigated the possible presence of heavy metals in our samples.

Copper

Copper sulfate pentahydrate is a fungicide. Mixed with calcium oxide it forms the so-called "Bordeaux Mixture" which is used to control fungus on grapes. In wines, this compound is usually below 1 mg/L, because it is partially eliminated during fermentation in the form of insoluble salts absorbed by lees. Thus the presence of copper in wines may be due to other causes: releases from the winery equipment or wine treatments with copper sulfphate to eliminate bad odours. In the analysed samples, we found a minimum content of 0,3 mg/L and a maximum of 3 mg/L. Wine legislation establishes a limit for copper of 1 mg/L. We found 12 samples with a content over the legal limit.

Zinc

Although zinc is an essential requirement for our health, an excess of zinc can be harmful. Its presence in wine may be due to its release from the storage containers. Zinc alloys have been used for thousands of years: brass is doubtless the most ancient. It was widely employed because of its resistance and the large availability of its component materials. The introduction of steel in wineries limited the use of zinc and its alloys. The content in zinc of our samples varied from a minimum of 2 mg/L and a maximum of 6 mg/L. The legal limit is 5 mg/L. We found 1 sample over the legal limit.

Lead

Lead is very dangerous to human health because it is highly toxic and may cause saturnism. The lead content in wines has been object of many studies carried out by OIV and E.U. experts. The legal limit of 0.2 μg/L is very low. The values determined for the analysed samples ranged from a minimum of 0.1 μg/mL to a maximum of 0.6 μg/mL. We found 2 irregular samples. A higher lead content may be due to traffic pollution near vineyards (fuel contains tetraethyl lead), vineyard treatments with copper salts contaminated by lead, releases by the storage container, winery machinery, or from the screw caps of wine bottles.

Methanol

This is a very noxious compound. Today its content in wines is generally low. Higher contents can be found in the so-called "hard-pressed wines" because of the natural degradation of pectins, during fermentation. This occurs when pomace is overpressed in order to extract the juice that it still contains. The values determined in our samples varied from a minimum of 0.1 mL % of alcohol anhydride (a.a.) to a maximum of 0.45 mL % a.a. The legal limit for alcohol anhydride is 0.20 mL % mL a.a., for white wines, and 0.25 mL % a.a. for red wines. We found 5 irregular samples.

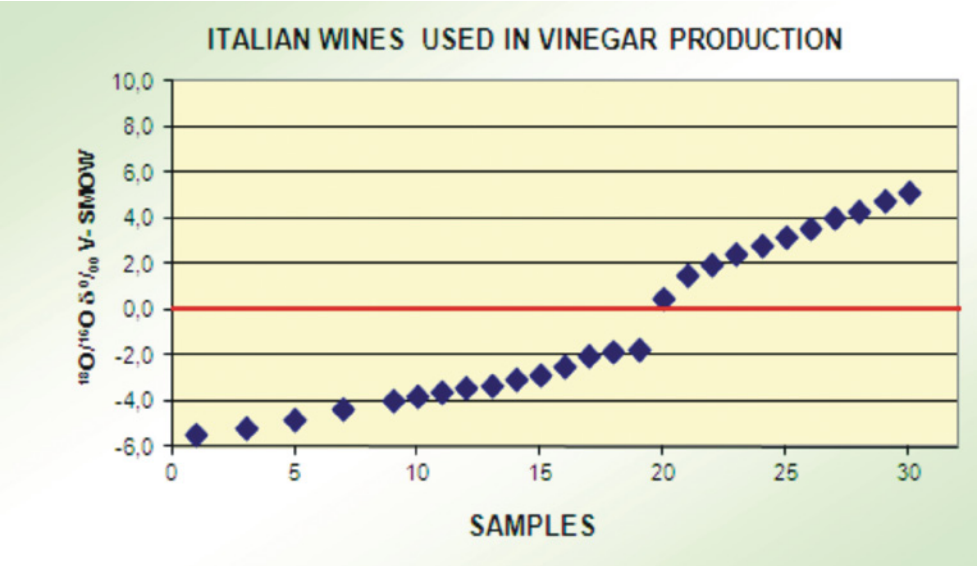


Fig. 3 - $^{18}O/^{16}O$ isotopic parameter values compared with the reference values of the National Annual Data Bank on Authentic Wines.

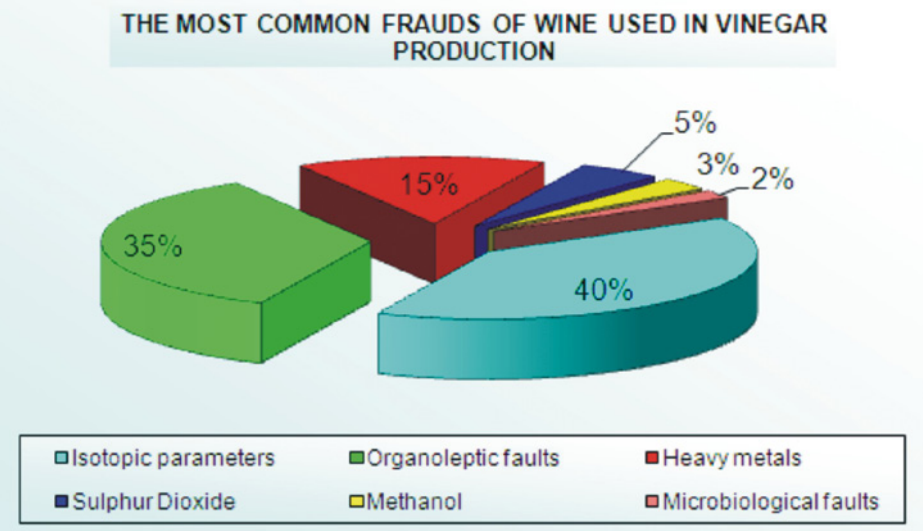


Fig 4 - The most common frauds and non-conformities to the law detected in red and white wines employed for vinegar production. The percentages were calculated on the total number of samples analysed.

Sulfur dioxide

High contents of sulfur dioxide are toxic. Nowadays wine quality is better than in the past, but current sulfur dioxide legal limits are still high, especially for sweet wines. The values determined in our samples varied from a minimum of 50 mg/L to a maximum of 360 mg/L. The legal limit established by E.U. laws is 200 mg/L for white wines and 150 mg/L for red wines. We found 3 irregular samples.

To complete our survey, we investigated the possible presence of organoleptic faults or microbiological contamination.

Organoleptic faults

Some organoleptic faults were detected in 35 samples. Well-known wine defects such as (oxidation, casse, flowers of wine or mould) were the most common ones. Organoleptic faults cannot be considered "frauds" in a strict sense and are generally related to wine storage problems.

Microbiological contamination

Lactic bacteria and re-fermentation were detected in 2 samples.

CONCLUSIONS

The quality of Italian wine vinegars has generally improved. Today, the availability of modern technologies and the use of good oenological products are more widespread. However, some infringements of the law are still committed (Fig. 4). To detect the slightest amount of an illegal or noxious compound, more reliable analytical methods, such as isotopic analyses, are now applied. The introduction of a restrictive legislation and effective measures to prevent frauds has produced good results. Therefore, it is very important to continue to assess the number and variety of these infringements in order to further improve food quality and safety.

ACKNOWLEDGEMENTS

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SHORT COMMUNICATION

EFFECTS OF ORGANIC FERTILIZATION ON QUALITY AND ANTIOXIDANT PROPERTIES OF HULLED WHEATS

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ABSTRACT

The results of an agronomic trial of two levels of nitrogen fertilization for organic farming in comparison with conventional farming are discussed. Two groups of wheat were compared; namely, durum and soft-wheat cultivars, including the emmer and spelta genotypes. Analyses were carried out to determine lignan, total phenolic content, antioxidant activity, the mineral elements and the dough rheological properties. Statistical analysis showed that under conditions of organic farming, the lignans and antioxidant activity reached the greater levels. For the qualitative parameters, the conditions of organic farming with N fertilization did not damage the indices analyzed. For the mineral content, the effects of the fertilization were not significant.

- Keywords: organic cultivation, fertilization, hulled wheat, lignans, phenolics, total antioxidant capacity -

INTRODUCTION

Epidemiological studies have indicated that consumption of whole-grain wheat is associated with a reduction in the incidence of coronary heart disease, diabetes and cancer (SLAVIN *et al.*, 2001). Whole-grain wheat flour contains significant levels of antioxidants (HEMERY *et al.*, 2007), although these are not evenly distributed throughout the kernel. Recent studies have demonstrated that the wheat-grain fractions (bran, flour, and shorts) have different antioxidant capacities, with the wheat bran displaying the highest levels (LIYANA-PATHIRANA and SHAHIDI, 2007). ZHOU *et al.* (2004) showed that the antioxidant properties of the outermost layers of the wheat kernel are due to the presence of the aleurone layer. These antioxidant properties are attributed to the relative abundance of phenolic compounds in the aleurone layer with respect to other bran tissues, primarily as ferulic acid (MATEO ANSON *et al.*, 2008). The aleurone also contains most of the lignans of the wheat kernel (BURI *et al.*, 2004), which are polyphenols that can produce metabolites that act as antioxidants, show antitumoral activities, and have been reported to modulate the effects of estrogens (QU *et al.*, 2005).

Recent studies have indicated that changes in agricultural practices have induced changes in the nutrient composition of fruits and vegetables (WORTHINGHTON, 2001; Del AMOR *et al.*, 2007). Conventional and organic agriculture differ significantly in their land-use methods, their use of pesticides, and their environmental impact (WINTER and DAVIS, 2006). In organic farming, nutrients can be supplied through crop rotation, application of animal manure, growing of cover crops, and biological preparations. Hulled wheat is a crop that is well adapted to low-input agricultural systems, and the present trends in organic agriculture and the consumption of healthy food products have led to a renewed interest in hulled wheat.

Hulled wheat comprises three cultivated species with different ploidy levels: einkorn (*Triticum monococcum* L., 2n = 2x = 14), emmer (*T. dicoccum*, 2n = 4x = 28), and spelt (*T. spelta* L., 2n = 6x = 42). To date, very few studies have addressed the effects of organic cultivation on the rheological indices and antioxidant properties of these hulled wheat species. The aim of the present study was to investigate the effects of two levels of organic fertilization on lignans and phenolics accumulation, and on the flour and semolina quality parameters of these hulled wheat species.

MATERIALS AND METHODS

Field material

The field trials were sown at the beginning of November 2007, under wet conditions, in the experimental fields of the CRA-Centro per la Cerealicoltura di Foggia in Southern Italy. A complete randomised block design (tree replicates) was applied with the application of two levels of organic nitrogen (N) fertilization for the organic system (Bio Ilsa N10): no N fertilization (0 org), and 100 units N/ha (100 org). The conventional farming comparator received 100 units N/ha (100 con). The seedling density was 300 seeds m^{-2}, and this wheat followed the previous crops of chick-pea and fallow for the organic and conventional farming, respectively. The N fertilizers were split into two applications: one third at sowing, and the remaining two-thirds at tillering. The soil type was clay loam with a pH of 7.8. The biological cycle of the plant materials extended from November 2007 to the end of June 2008. The size of the plot was 10.2 m^2 (eight 7-m-long rows, 0.17 m apart). The wheat genotypes belonged to four species: emmer (Molisano, Garfagnana, Leonessa, G. Paolo, Padre Pio, and Davide), spelt (Roquin, Altgold Rotkorn, Ostar, Giuseppe, Benedetto, and Pietro), durum wheat (Saragolla, a cultivar adapted for pasta-making use), and common wheat (Blasco, a cultivar well adapted for bread-making use). After the harvest, each emmer and spelt sample was hulled with a thresher equipped of aspiration apparatus (Colombini S.a.S. Novara, Italy) and stored at +4°C until analysis.

Milling

The clean grain of emmer and durum wheat samples (800 g) were tempered to 16.5% for 18 h prior milling, and then they were milled with a laboratory mill (Bona, Monza, Italy) that separates semolina from bran and shorts. The spelta and the common wheat flour samples were obtained after grain samples had been moistened for 18 h at 14% humidity, and then milling with a laboratory mill (Brabender TYPE 27900 for common wheat, Belotti Strumenti S.r.l., Milan, Italy) that separates flour from bran and shorts. The extraction rate ranged from 55 to 60% for both the semolina and the flour. The hull-free grain (50 g) was ground with a Tecator1093 Cyclotec (Foss Italia, Padova, Italy) sample mill (1 and 0.5 mm sieves) to obtain the wholemeal for antioxidant extractions and minerals determination.

Quality parameters

Protein content (%N × 5.70) was determined in triplicate samples by means of the UNI 10 274 method, and were expressed on a dry-matter basis. Dry gluten was determined in duplicate samples for the semolina and flour according to the UNI 10 689 method, using the Glutomatic 2020 system (Perten, Sweden). For the determination of dry gluten, the wet gluten was dried with a Glutorx 2020 at 150°C for 5 min, and left to cool for 3 min.

The rheological characteristics of the semoli-

na and flour samples were evaluated by alveographic indices: the deformation energy or flour strength (W), and the ratio P/L of the resistance to extension or tenacity (P) and the extensibility (L), (Chopin Alveograph, Chopin, Montpellier, Cedex France), according to standard UNI 10 453 method. The yellow index (YI) was used to determine the semolina and flour colours, using a chromameter (ChromaMeter CR-200 Minolta, Tokyo, Japan) equipped with a xenon arc lamp. The colorimeter was calibrated on a standard white tile of L* = 97.34, a* = –0.53 and b* = 2.39 (CIE 1976) with the CIE illuminant D65. Each measure was performed in triplicate

Extraction and determination of total phenolic content

The extraction for the total phenolic content (TPC) was carried out according to the method proposed by BETA *et al.* (2005). Extracts (0.2 mL) were added to 1.5 mL 10-fold diluted Folin-Ciocalteau reagent (SINGLETON and ROSSI, 1965). The mixture was allowed to equilibrate for 5 min and then neutralized with sodium carbonate (from a stock solution of 60 g/L). After incubation at room temperature for 90 min, the absorbance of the mixtures was measured at 725 nm. Acidified methanol was used as the blank. Ferulic acid was used as the standard, and the data are expressed in μg ferulic acid/g. Determinations were performed in triplicate for each extract and are reported on a dry-matter basis. The moisture content was determined by drying the samples at 130°C for 3 h in an oven.

Trolox equivalent antioxidant capacity

Trolox equivalent antioxidant capacity (TEAC) was determined according to the procedure of RE *et al.* (1999), with minor modifications. This present procedure was previously fully described by FARES *et al.* (2010). All of the tests were conducted in triplicate. The TEAC of the extracts was calculated using a Trolox standard curve, based on the percentage inhibition of absorbance at 734 nm, and it was expressed in μmol Trolox/g dry matter (dm).

Extraction and determination of lignan

Extraction and determination of the total lignan content (TLC) was performed according to LIGGINS *et al.* (2000), with minor modifications. The whole-grain samples were ground with a Cyclotec (Foss) laboratory mill (1-mm screen), cooled in liquid N_2, and then defatted (Soxhelet) for 6 h with diethyl ether and dried in an oven equipped with a vacuum pump (30°C; vacuum, 760 mmHg). Then 1 g wholemeal and 10 mL 1.5 M HCl were added together in a 15-mL screw-capped tube, and shaken in a waterbath for 60 min (100°C). Thereafter, the samples were allowed to cool for 30 min. The HCl was then neutralized by the addition of 10 M sodium hydroxide. The aglycone lignans from each sample were separately partitioned from the aqueous hydrolysis mixture into 2 mL of an equal mixture of ethyl acetate and methyl *tert*-butyl ether (1:1, v/v). The organic phases were separated from the aqueous phases by centrifugation, and then the organic phases were washed five times with organic solvent before they were combined. A total of 3 mL of the organic extract was evaporated using a centrifugal concentrator at room temperature. The dried samples were dissolved in a 1 mL mixture of pyridine and N,O-bis (trimethylsilyl) triflouroacetamide (4:1, v/v) with 1% trimethylchlorosilane, and 20 μL 0.5 mg/mL anthraflavic acid was added as an internal standard. The vials were sealed and heated to 60°C for 1 h to facilitate the formation of the trimethylsilyl derivatives of the lignans. Calibration standards of known quantities of secoisolariciresinol, shonanin, matairesinol and isolaricinresinol were derivatized in an identical manner. One microliter of the derivatized samples or standards was injected into a gas chromatography-mass spectrometer (GC-MS, Agilent 6890N). The injection was operated in the splitless mode at 280°C. The capillary column was an Equity (15 m × 0.25 mm × 1 μm) Supelco (Supelco, Park, Bellefonte, PA); the initial oven temperature was 180°C; after 10 min, the temperature was increased at 10°C per min, to 320°C for 5 min. The mass spectrometer range was from 50 to 650 Da. The lignans in the samples were identified and quantified by comparisons with known quantities of the reference standards analyzed in the same runs. The results were summed for TLC.

Mineral determination by inductively coupled plasma optical emission spectrometry

The mineral element concentrations (Ca, K, P, Mg, Mn, Na Cu, Fe, and Zn) of the whole-meal was determined using a Varian Vista-MPX simultaneous inductively coupled plasma optical emission spectrometer (ICP-OES). For the microwave digestion, 10 mL 10 M nitric acid was added to 500 mg whole-meal sample and dried for 3 h at 85°C, using 50 mL of digested sample; the determination was carried out with CCD (charge coupled device). The reference technique (internal standardization) and ionization buffering were used throughout the analysis.

Statistical analysis

Standard ANOVA procedures (one factor randomized complete block design with three replicates) were applied to the dataset using the statistical package STATISTICA (StatSoft Italia Srl, Vers.7.1, 2005). The means were separated by the last significant difference (LSD) test at a $P = 0.05$ probability level.

RESULTS AND DISCUSSION

Table 1 shows the variations in the technological indices for the two groups of wheat grown under organic and conventional farming with the different N fertilization. In general, the absence of N fertilization in the organic farming system resulted in a slight decrease in all of the indices assessed. Instead, if the effects of the same units of N fertilization (100 units) are compared across the organic and conventional farming, slight differences were found. In a long-term study conducted in Europe by MADER *et al.* (2007), the protein content of wheat grown in organic farming was lower than in samples from conventional management, by about 6%. This reduction is very close to that observed in the organic farming without N fertilization in our field trial; in contrast, with the same N units, the grains from the organic farming showed a protein content equal to that of the conventional cultivation system.

For the effects of farming and fertilization on the two groups of wheat, hexaploid wheat showed the highest values of PC, GI, and W. In particular, the alveographic indices obtained by the hexaploid group at the highest level of organic and conventional fertilization are typically of flours for bread-making use, for the extensibility (P/L) and force (W) showed. For tetraploid wheats instead, the GI and the PC are too low for pasta making, moreover the alveographic indices (W and P/L) showed a strong disequilibrium of semolina samples, more marked in the condition with no fertilization. The tetraploid wheat showed also low values of YI (b*) for obtaining pasta with the typical amber yellow colour. Even considering the two groups separately, the positive, although slight, effects of the N fertilization, regardless of the type of cultivation (organic or conventional), was confirmed. Although obtained from a one-year trial, our data confirm the observations of MADER *et al.* (2007), that the performance of wheat under organic farming is comparable with that achieved under conventional farming.

Figure 1 shows the concentrations of the mineral elements in the whole grain samples. In analogy with the above-mentioned study (MADER *et al.*, 2007), although not in full agreement with WORTHINGHTON (2001), we did not find significant differences between the minerals as a function of these different farming conditions. On the other hand DANGOUR *et al.* (2009) reviewing about organic and conventional food characteristics and arguing on the suggestion of a possible superiority of organic foods in relation to mineral content (Mg, Ca, K, Zn, and Cu) reported that no significant differences could be ascribed to production procedures.

In recent years, some studies have shown positive impacts of organic farming on some secondary metabolites, and particularly on organic acids and polyphenols, which are also considered as promoters of human health. The main hypothesis is that in the absence of synthetic products, the crops are stimulated more towards the production of secondary metabolites in response to environmental stress. Even if there are still controversies concerning the effectiveness of organic cultivation in enhancing the vitamins and minerals in foods, more agreement in the literature data is reported about the poliphenols (LIMA and VIANELLO, 2011). REN *et al.* (2001) in a studies conducted on vegetables of organic origin showed that the concentration of quercetin and caffeic acid in organic crop were 1.3- 10.4 times higher than that found in conventional plants.

As expected, the Total Lignan Content (TLC) was highest when the crops were grown under organic conditions in the absence of N fertilization (Table 2). Moreover, the antioxidant activity (TEAC) increased under organic farming when there was no N fertilization, and it is in agree-

Table 1 - Effects of the farming systems and fertilization levels on the quality indices of the two sets of wheat.

Wheat	Fertilization level	PC (% dm)	GC (% dm)	b*	GI	W ($x10^{-4}$ joule)	P/L
Tetraploid wheat	0 organic	12.4 b	10.1 d	14.6 b	37.3 d	78.8 d	3.32 a
	100 organic	12.8 b	10.0 e	15.2 a	33.7 e	94.1 c	2.92 b
	100 conventional	12.6 b	10.7 c	15.2 a	37.3 d	94.1 c	2.92 b
Hexaploid wheat	0 organic	12.6 b	10.1 d	-	61 c	147 b	1.11 c
	100 organic	14.5 a	13.4 a	-	69 b	176 a	0.95 c
	100 conventional	13.5 ab	12.2 b	-	76 a	176 a	0.95 c
Mean	0 organic	12.8 b	12.5 b	-	49 b	113 a	2.22 a
	100 organic	13.2 a	13.6 a	-	51 b	135 a	1.94 b
	100 conventional	13.2 a	13.1 ab	-	57 a	135 a	1.94 b

Different letters in the same column indicate statistical differences by LSD test ($p < 0.05$). Data are means of three determinations.
PC, protein content; GC, gluten content; b*= yellow index determined only on semolina samples; GI, gluten index; W, deformation energy or flour strength; P, resistance to extension or tenacity or elasticity; L, extensibility.

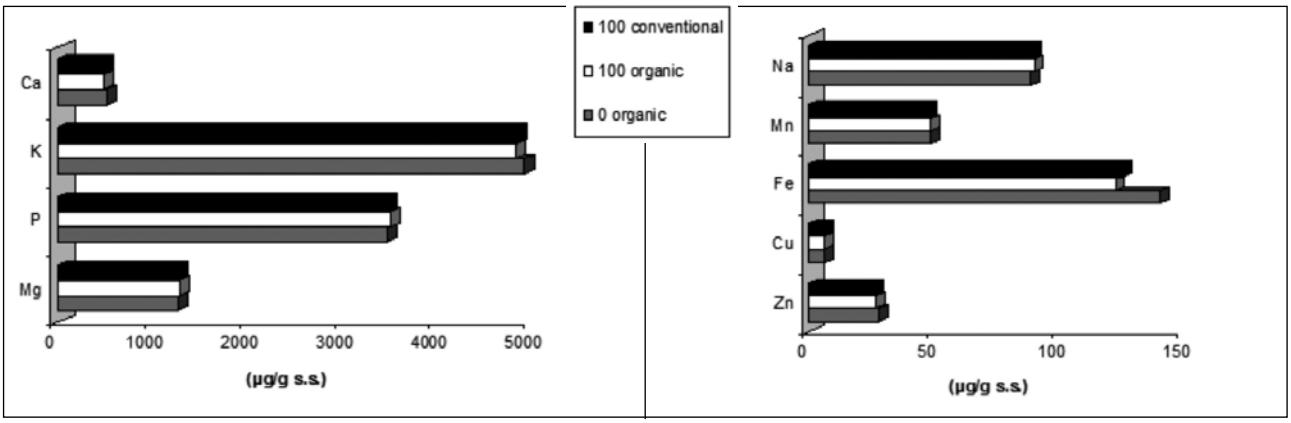


Fig. 1 - Effects of the farming systems and fertilization levels on the mineral element concentrations[a].
[a]No statistical differences (P<0.05) among fertilization levels and farming system were found.

ment with the quantitative response observed for lignans. It appeared that the antioxidant activity measured *in vitro* increased in response to the conditions of organic farming, and by more with limited organic N fertilization. For the Total Phenolic Content (TPC) instead, the trend was different, as the data showed the greatest accumulation under conventional farming, and a slightly lower TPC under organic farming without N fertilization. Regarding to the two groups of wheat, we have observed the highest TPC and the highest TEAC of the hexaploid wheats with respect to the tetraploid wheats, in accord with a previous study conducted by MENGA *et al.* (2010). The TLC of hexaploid wheat instead, showed middle and no different values among treatments that ranged from the maximum reached by tetraploid wheats in organic farming with no nitrogen and the minimum reached in conventional farming. Therefore, also in this set of wheats, there was a tendency to accumulate more secondary metabolites under organic farming conditions. Similar results were observed on the phenolics and peroxidase activities of red sweet peppers in organic farming (DEL AMOR *et al.*, 2007). Indeed, in organic farming, the N availability is lower, which leads to an increase in the biosynthesis of carbon-containing compounds, including secondary metabolites. The activation of the phenylpropanoid pathway by N deficiency results in more accumulation of phenolics like lignans and phenolic acids (ZUCHOWSKI *et al.*, 2011).

CONCLUSION

This study, although carried out for a one-year trial, has showed that the dough quality of the two groups of wheat grown under organic farming with N fertilization is comparable with that achieved under conventional farming. On the contrary, under conditions of organic farming with no N fertilization, the lignans and antioxidant activity showed the greatest levels. The mineral content instead seemed to be not influenced by treatments.

Table 2 - Effects of farming systems and fertilization levels on antioxidant properties of the two sets of wheat.

Wheat	Fertilization level	TPC (µg/g Ferulic acid d.m.)	TEAC (µmol Trolox/g d.m.)	TLC (µg/g d.m.)
Tetraploid wheat	0 organic	778.4 e	1.03 d	795.1 a
	100 organic	760.4 f	0.88 f	697.0 ab
	100 conventional	810.2 d	0.97 e	637.7 b
Hexaploid wheat	0 organic	917.7 b	1.43 a	772.3 ab
	100 organic	891.5 c	1.34 b	738.6 ab
	100 conventional	924.5 a	1.27 c	693.1 ab
Mean	0 organic	848.0 b	1.23 a	787.5 a
	100 organic	826.0 c	1.11 b	710.9 b
	100 conventional	865.2 a	1.12 b	656.2 b

Different letters in the same column indicate statistical differences by LSD test ($p < 0.05$). Values are means of three determinations.
TPC, Total phenolic content; TEAC, Trolox equivalent antioxidant capacity; TLC, Total Lignans Content.

The data reported in the present study are in agreement with those produced in other studies (D'EGIDIO *et al.*, 2007; MADER *et al.*, 2007) namely, that the organic farming can provide comparable results of technological properties obtained in conventional crop. At present, the really gains of farming cultivation seemed to be obtained in terms of antioxidants that even not directly related to food nutritional quality, have peculiar biological activity, beneficial for human health.

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REVIEW

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Compositional Analysis and Antioxidant Profile of Yellow, Orange and Purple Polignano Carrots
M. Cefola, B. Pace, M. Renna, P. Santamaria, A. Signore and F. Serio

SURVEY

Classification of Commercial Omani Halwa by Physico-Chemical Properties and Instrumental Texture Profile Analysis (TPA)
Mohammad Shafiur Rahman, Qassim Al-Shamsi, Aminah Abdullah, Michel R. Claereboudt, Buthaina Al-Belushi, Rabea Al-Maqbaly and Jamal Al-Sabahi

GUIDE FOR AUTHORS
ITALIAN JOURNAL OF FOOD SCIENCE -IJFS

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

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