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PAPER

EVALUATION OF TECHNOLOGICAL EFFECTS OF YEAST-BACTERIAL CO-INOCULATION IN RED TABLE WINE PRODUCTION

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ABSTRACT

The practice of inoculating selected bacteria before alcoholic fermentation (AF) (co-inoculation) to induce malolactic fermentation (MLF) was used to produce red table wines from several grape varieties. In most of the microvinification trials, MLF was completed before or within one week after de-vatting. The acetic acid content remained within an acceptable level (<0.60 g/L). In an industrial vinification (200 hL), MLF induced by co-inoculation was completed before de-vatting, while in the wines inoculated after AF, the total malic acid degradation was obtained 33 days later. The potential risk of increasing the volatile acidity was also evaluated by inducing MLF in partially fermented wines. The co-inoculation practice was compatible with the production of red wines. The decrease in vinification time with respect to traditional MLF management could offer a significant advantage for industrial wineries.

- Key words: malolactic fermentation, red wine, volatile acidity, yeast-bacterial co-inoculation -

INTRODUCTION

Malolactic fermentation (MLF) is a naturally occurring biological reaction in wine caused by indigenous lactic acid bacteria or induced by inoculation with selected bacterial starters. MLF is recommended in red wine production because of its positive impact on sensorial quality and on the microbial stabilization of wine (BAUER and DICKS, 2004).

The inoculation with selected starters of *Oenococcus oeni* in wine is a common winemaking practice. MLF starters are usually inoculated in wine at the end of alcoholic fermentation (AF). Previous studies have been reported on the timing of bacterial inoculation in order to have good control of the fermentation process in winemaking (BEELMAN and KUNKEE, 1985, HENICK-KLING and PARK, 1994, ROSI *et al.*, 2003). The inoculation of bacterial starters in the must before AF (yeast-bacterial co-inoculation) has been proposed as an alternative technique that offers some advantages with respect to the traditional one (BEELMAN and KUNKEE, 1985; KRIEGER, 2005). It has been demonstrated that bacteria inoculated in must performed better than those inoculated after AF, especially when cell growth conditions are not favourable (SIECKOWSKI 2004; JUSSIER *et al.,* 2006; KRIEGER *et al.,* 2007). ZAPPAROLI *et al.,* (2009) recently reported successful MLF in a high alcohol wine, such as Amarone, by inoculating *O. oeni* cultures in grape must (co-inoculation). In contrast a sluggish or stuck fermentation occurred in the same wine when the bacterial starters were inoculated after AF. Bacteria inoculated in wine at the end of AF often face difficult conditions, such as high ethanol content combined with low pH, that threaten their survival. In contrast, inoculation in grape must allows the bacteria to adapt gradually to the ethanol before it becomes toxic and the malolactic performance is enhanced.

MLF induced concurrently with AF can reduce the vinification time. The use of co-inoculation to terminate MLF faster with respect to traditional winemaking can help protect the wine from spoilage and save time. GERBAUX et al. (2009) observed that early inoculation of wine with malolactic bacteria may be a way to reduce the risk of volatile phenol production by *Brettanomyces*.

Nevertheless, there is still some scepticism about the application of co-inoculation due to the risk of an undesired increase in volatile acidity as well as the negative effects on AF due to possible interferences in yeast performance due to the MLF bacteria (ALEXANDRE *et al.,* 2004). Recently, MASSERA *et al.* (2009) studied the risk of early bacteria co-inoculation under Argentinean winemaking conditions. This technique is used very little by wineries because oenologists fear an excessive production of acetic acid as a consequence of sugar metabolism by LAB.

To obtain reliable data on the application of yeast-bacterial co-inoculation based on standard red winemaking procedures, MLF was induced in red wines produced by microvinifications of several grape varieties characterized by different physical-chemical parameters. The co-inoculation and traditional post AF inoculation methods were compared in an industrial vinification of the dry red table wine, Valpolicella. The potential threat to the wine quality was ascertained by inducing MLF into partially fermented wines, as a simulation of stuck wine. Acetic acid production and the time required for MLF were considered important qualitative and technological parameters for evaluating the suitability of using this bacterial inoculation technique in winemaking.

MATERIAL AND METHODS

Microorganisms

The yeast *Saccharomyces cerevisiae* VRB strain (Lallemand Inc., Montreal, Canada, Usa) was used to induce AF and the bacterial starters of *Oenococcus oeni*, Elios 1 and VP41 (Lallemand), were used to induce MLF. The pairing of the VRB-Elios 1 yeast-bacterium was used in microvinification trials and the VRB-VP41 was used in industrial vinifications. Commercial preparations of these microorganisms (active dry yeast for yeast and freeze-dried culture for bacteria) were used according to the manufacture's instructions.

Microvinifications

A total of 100 microvinifications were carried out with different local and international of red grape varieties (Table 1). Each grape variety was represented by one or more samples of 2008 vintage that differed according to vineyard area and the genetic line.

A traditional red vinification was carried out. The grape solids were extracted by means of maceration during AF. Trials were carried out in stainless steel vats (100 L) using 40 L of must and 15 kg of grape pomace. The yeast VRB strains (Lallemand) and bacterium Elios1, Lallemand) were inoculated at concentration of 5-8 and 2-4 10^6 x cfu/mL, respectively.

The fermenting must was mixed manually twice a day by soaking the pomace cap. A 2 L aliquot from each trial was not inoculated with the bacteria and was used as the control. Fifty mg/L of SO_2 were added to all the musts. Bacteria were inoculated 16 h after the yeast inoculation, when the free SO_2 concentration was less than 10 mg/L. AF was monitored by measuring the ethanol production and sugar con-

Table 1 - Composition of 100 musts of international and local varieties used for the microvinification trial. Values are the average of n. samples; the minimum and maximum values are in brackets.

Grape variety	Origin	n.[1]	pH	Reducing sugars °brix	Titratable acidity[2] (g/L)	L-malic acid (g/L)
Sangiovese	Italy	9	3.27 (3.10-3.44)	19.4 (17.9-21.5)	6.13 (5.12-6.92)	1.40 (0.98-2.08)
Corvina	Italy	36	3.25 (2.89-3.64)	21.0 (18.8-23.8)	6.28 (4.10-8.94)	1.50 (0.66-3.46)
Rondinella	Italy	4	3.19 (3.12-3.23)	19.4 (19.0-19.7)	4.61 (4.52-4.65)	0.96 (0.57-1.51)
Teroldego	Italy	2	3.18 (3.03-3.33)	18.0 (17.6-19.1)	8.49 (5.80-11.17)	1.43 (1.41-1.45)
Refosco	Italy	1	3.28	19.5	7.00	1.32
Longanesi	Italy	1	3.24	20.8	6.92	2.08
other local varieties[3]	Italy	22	3.28 (3.01-3.48)	20.1 (17.7-24.5)	7.05 (3.90-12.85)	2.60 (1.38-5.80)
Alfrocheiro Preto	Portugal	1	3.25	17.5	6.30	1.19
Castelão	Portugal	1	3.19	16.4	4.95	1.29
Tinta Aragonés	Portugal	1	3.20	18.0	5.05	1.45
Tinto Cão	Portugal	1	3.30	18.9	5.88	2.69
Touriga francesa	Portugal	1	3.39	17.8	5.04	1.29
Touriga national	Portugal	1	3.16	18.6	6.38	1.28
Trincadeira	Portugal	1	3.25	16.9	5.84	1.78
Tinta barrica	Portugal	1	3.58	19.1	4.44	1.24
Mencia	Spain	1	3.39	16.2	4.64	1.19
Pietro picudo	Spain	1	3.28	19.5	7.54	1.35
Tempranillo	Spain	1	3.31	19.2	4.99	1.68
Tinta de toro	Spain	1	3.42	20.5	5.03	1.71
Cabernet Sauvignon	France	9	3.36 (3.28-3.48)	21.4 (19.9-23.2)	5.54 (4.90-6.72)	2.06 (1.33-3.19)
Merlot	France	2	3.24 (3.20-3.27)	19.5 (18.8-20.2)	6.73 (6.38-7.07)	1.24 (1.23-1.25)
Syrah	France	1	3.38	19.9	6.69	3.11
Blaufränkisch	Austria	1	3.19	20.9	6.17	1.17

[1]number of samples; [2]as tartaric acid; [3]Recantina, Cabrusina, Cavrara, Corbina, Denela, Dindarella, Marzemina nera, Molinara, Pattaresca, Rossetta di montagna and Saccola.

sumption; AF was considered completed when the residual sugar level was less than 3.0 g/L. Wine devatting was carried out at the end of AF and the wines were left for two days to allow the clarification by natural sedimentation. MLF was monitored by measuring the L-malic acid depletion; it was considered terminated when the L-malic acid content in the wine was 0.1 g/L. The wines were analyzed at the end of AF and MLF. All trial wines were kept in a cellar at 18°-23°C; the temperature inside the vats did not exceed 27°C.

Industrial vinification

An industrial vinification of Valpolicella wine was carried out in steel tanks containing 200 hL of must and grape pomaces of the Corvina and Rondinella varieties. The must composition was as follows: pH 3.33, fermentable sugars 206 g/L, titratable acidity 6.80 g/L measured as tartaric acid, acetic acid 0.02 g/L, L-malic acid 2.59 g/L, L-lactic acid 0.07 g/L, D-lactic acid 0.05 g/L and citric acid 0.25 g/L. A traditional red vinification was carried out in duplicate. Fifty mg/L of SO_2 were added and three tanks were inoculated with the yeast VRB to induce AF. About 12 h after yeast inoculation, MLF starter VP41 was added to two of the three tanks (co-inoculation trial).

Must aeration and cap management were carried out by a pumping-over operation. At the end of AF, the wine was devatted and clarified by natural sedimentation for two days. Wine from the tank that was not inoculated with bacteria was divided into three fractions of 65 hL each, two were used for the inoculation of the MLF starter VP41 (inoculation post AF). The third was not inoculated with any MLF starters and was left to carry out a spontaneous MLF. AF and MLF were considered finished when the residual sugars and L-malic acid content was 3.0 and 0.1 g/L, respectively. At the end of AF and MLF, the wines were analysed. The temperature in the winery was held constant at about 18°C, while the temperature inside the vats did not exceed 28°C.

Bacteria in partially fermented wine

Wines obtained by fermenting the must of the Corvina grape variety at pH 3.20 and 3.60 (the original pH 3.34 was corrected with HCl and NaOH, respectively) were used as a natural growth substrate of *O. oeni*. The must composition was: fermentable sugars 230 g/L, titratable acidity 6.32 (pH 3.20) and 4.61 (pH 3.60) g/L measured as tartaric acid, acetic acid 0.01 g/L, L-malic acid 4.19 g/L, L-lactic acid 0.04 g/L, D-lactic acid 0.02 g/L and citric acid 0.25 g/L. The grape musts were fermented by *S. cerevisiae* VRB in a volume of 2.7 L. AF was monitored by measuring the consumption of reducing sugars and ethanol production. When the content of reducing sugars was approximately 80 g/L, the partially fermented wines (containing about 8.5-9.0 % v/v of ethanol) were divided into two equal fractions of 1.3 L, one for the bacterial inoculation, the other for the completion of AF as control. This latter, after the total consumption of reducing sugars (< 3.0 g/L) was devatted and inoculated with bacteria. Before the inoculation of a commercial culture of *O. oeni* VP41 (Lallemand), the partially fermented wines were pasteurised in order to eliminate the living yeast cells. The size of inoculation was about 2 x 10^6 cfu/mL. MLF trials were performed in triplicate and the fermentation was monitored by L-malic acid depletion and L-lactic acid production. The acetic acid, D-lactic acid and citric acid contents were measured during the fermentation.

Must and wine analysis

Titratable acidity was measured as tartaric acid by titration with 1 M NaOH. Total and free SO_2 were measured by iodometric titration. Ethanol levels were analyzed by NIR spectroscopy using an Alcolyzer Wine apparatus (Anton Paar GmbH, Graz, Austria). Sugar content during fermentation was determined by the Fehling method with automatic titration (Crison, Barcelona, Spain) using 0.5 M $Na_2S_2O_3$, 25% w/v H_2SO_4, 30% w/v KI and Fehling solutions. Organic acids were quantified using enzyme kits according to the manufacture's instructions (La Roche, Basel, Switzerland).

Lactic acid bacteria counts were carried out during must fermentations by plating on MRS (Fluka, Seelze, Germany) supplemented with 10% w/v tomato juice and 0.01% w/v actidione (Fluka). The plates were incubated anaerobically at 28°C using an Anaerocult A kit (Merck, KgaA, Darmstadt, Germany) and after 7 days the colony counts were carried out as colony forming units per mL.

The L-malic acid consumption rate was calculated as the average daily consumption (g/L per day) during the time between bacterial inoculation (or from wine devatting in the case of spontaneous MLF) and the end of MLF (< 0.1 mg/L L-malic acid).

RESULTS AND DISCUSSION

The performance of the MLF bacteria was measured during the vinification of grapes with different must compositions (Table 1). Among the main parameters that influence bacterial activity, pH, temperature, ethanol and SO_2 concentration (RIBÉREAU-GAYON *et al.,* 2006), pH was the most important variable in these grape musts. The SO_2 concentration did not constitute a limiting factor for bacteria (less than 10 mg/L in all trials at the time of bacterial inoculation) since free SO_2 rapidly binds to various must components, such as sugars, ketonic acids and polyphenols. The dose of 50 mg/L SO_2 is considered standard for red vinification and is compatible with co-inoculation, provided that bacteria are inoculated at least 6-8 h after SO_2 addition in order to allow the SO_2 to combine completely (RIBÉREAU-GAYON *et al.,* 2006).

The AF rates were not affected by the presence of bacteria. In all the trials, the fermentable sugars were completely consumed between 7 and 13 days after yeast inoculation. Moreover, the MLF was completed in all the wines. This fermentation was imputable to the starter strain because in control wines the L-malic acid concentration at the end of AF did not substantially change with respect to that contained in grape must before AF (data not shown).

Fig. 1 shows the pH and ethanol content of the 100 wines determined after MLF. The content of L-lactic acid, produced by degradation of L-malic acid, in all the wines is shown in Fig. 2. In all wines the content of D-lactic acid was less than 0.27 g/L (data not shown). The rate of consumption of L-malic acid was not correlated with the wine pH (Fig. 3). The management of MLF in wines with low pH (< 3.20) can be very difficult because of the negative impact on bacterial growth (BAUER and DICKS, 2004). In this

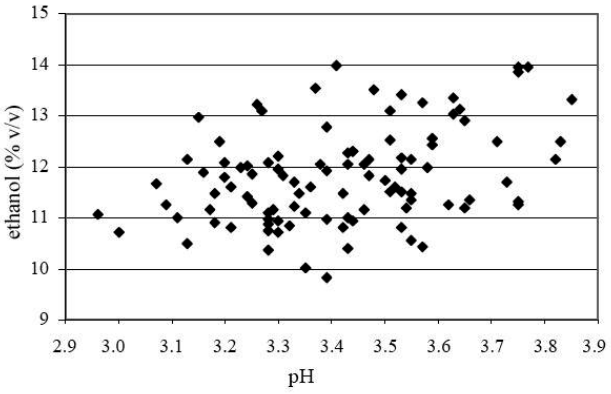


Fig. 1 - Plot of pH and ethanol levels in 100 wines that underwent MLF.

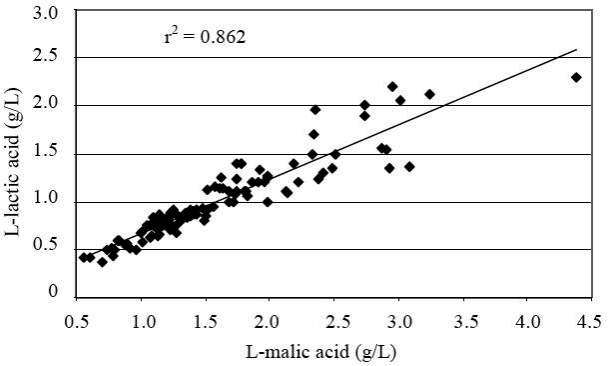


Fig. 2 - Relationship between L-lactic acid produced and L-malic acid consumed in 100 wines that underwent MLF with linear regression line and correlation coefficient (r^2).

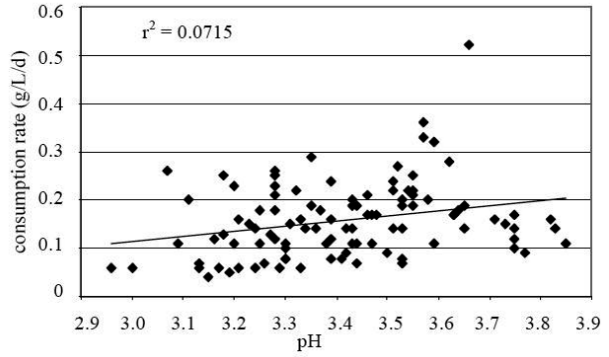


Fig. 3 - Relationship between pH and the L-malic acid consumption rate in 100 wines that underwent MLF with linear regression line and correlation coefficient (r^2).

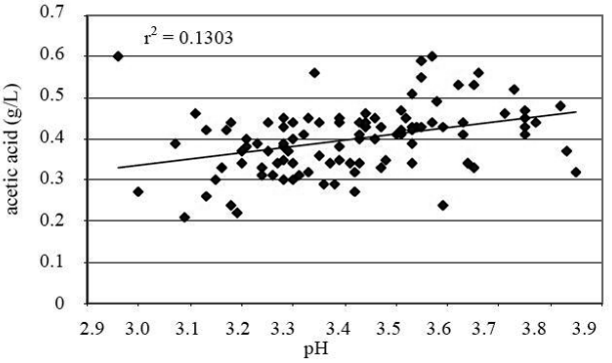


Fig. 4 - Relationship between the pH and acetic acid contents in 100 wines that underwent MLF with linear regression line and correlation coefficient (r^2).

study, yeast-bacterial co-inoculation permitted successful MLF under conditions that could have been limiting in post AF inoculation.

The maximum acetic acid concentration was 0.60 g/L and no relationship was observed between this acid and the pH of the wine (Fig. 4). These concentrations are considered to conform to the standard quality parameter for volatile acidity in red table wine. The development of spoilage bacteria often causes the acetic acid to increase in wine, especially when the pH is high (> 3.6). This risk did not occur in 18 wines with pH > 3.60.

In all the wines, L-malic acid consumption started before AF and lasted up to 12 days after the devatting (Fig. 5). A total of 39 wines out of 100 finished MLF within the devatting; in only 10 microvinifications, did MLF last more than one week after devatting.

From a technological point of view, these results are significant because of the short time needed to conclude the vinification processes. The vinification time is generally longer in sequential AF/MLF since it is necessary to complete MLF after devatting. JUSSIER *et al.* (2006) reported that fermentation times were greatly reduced by inoculating bacteria and yeast concurrently into Chardonnay must with respect to post AF inoculation. In Amarone wine, L-malic acid consumption was completed in 32 days in co-inoculated trials, compared to 72 days when the bacterial starter was inoculated after AF (KRIEGER *et al.*, 2007). The reduction in total time of fermentation and a better control of MLF inoculating starter bacteria in the must were also reported by MASSERA *et al.* (2009). Hence, the use of yeast-bacterial co-inoculation could favour the completion of L-malic depletion in wine before winter, when the cellar temperatures decrease which delays MLF.

The size of the tank in which fermentation is carried out is one of the technological parameters that can influence MLF. This is probably due to its interference on aeration, temperature, sulfitation and nutritive conditions for bacterial activity (RIBÉREAU-GAYON *et al.*, 2006). Therefore, the effects of yeast-bacterial co-inoculation on industrial wine production were evaluated. MLF induced by co-inoculation was fast and the L-malic acid was consumed completely during AF (L-malic acid consumption rate was 0.25 g/L per day) (Fig. 6). In the tank inoculated after AF, MLF lasted 33 days longer than the former (L-malic acid consumption rate was 0.05 g/L per day). Spontaneous MLF occurred with kinetics similar to MLF induced after AF. When the enological conditions are favourable, the growth of the bacterial cells starts just before the end of

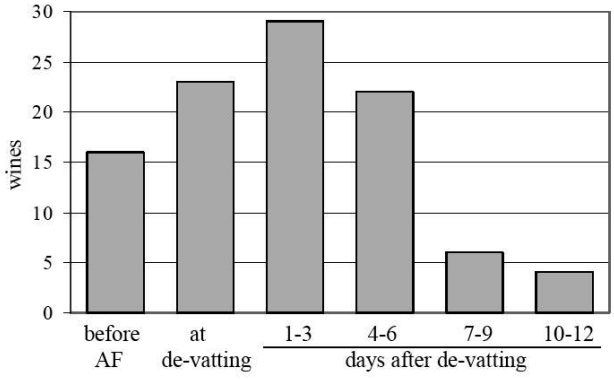


Fig. 5 - Frequencies of wines that terminated MLF before the end of alcoholic fermentation (before AF), at the de-vatting and 1-3, 4-6, 7-9, 10-12 days after the de-vatting.

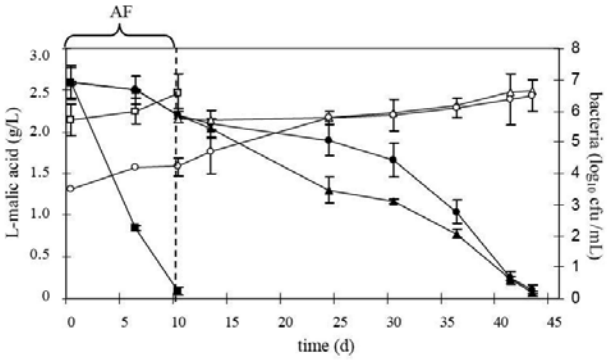


Fig. 6 - L-malic acid consumption (g/L, filled symbols) and lactic acid bacteria population (log_{10} cfu/mL, empty) measured during the industrial vinification of Valpolicella wine in tanks inoculated with bacteria before AF (square, simultaneous AF/MLF), after AF (triangle, sequential AF/MLF) and in a non-inoculated tank (circle, spontaneous MLF).

AF, therefore, when the wine was devatted, the onset of spontaneous MLF was rapid. Bacterial inoculation in grape must can promote a rapid colonization of the selected starter, while avoiding possible interference from the indigenous microflora. MLF induced by bacterial inoculation in Chardonnay must at pH 3.18 terminated rapidly because of the full adaptation of the *O. oeni* strain that allowed a fast colonization in the wine (ZAPPAROLI *et al.,* 2006). The acetic acid content in the co-inoculated Valpolicella wine was slightly lower than that measured in the same wine that underwent post AF and spontaneous MLF (Table 2). This study confirmed the findings in previous investigations where simultaneous AF/MLF did not cause an increase in acetic acid due to bacterial sugar catabolism (SIECKOWSKI, 2004; JUISSER *et al.,* 2006; ZAPPAROLI *et al.,* 2009; MASSERA *et al.*, 2009).

To ascertain the potential risks for wine quality due to the presence of growing malolactic bacteria during a sluggish or stuck AF, *O. oeni* cells were inoculated in partially fermented wines containing a high residual sugar content, to simulate stuck wine. All wines completed MLF, 9 and 7 days after bacterial inoculation in the pH 3.20 and pH 3.60 trials, respectively (Fig. 7). During MLF the glucose and fructose contents did not vary significantly ($p = 0.899$ and 0.921 at pH 3.20 and 3.60, respectively) in either wine, indicating that LAB growing at enological pH prefer to degrade L-malic acid rather than sugars when energy is needed to establish the intracellular pH (HENICK-KLING, 1995). The increased acetic acid and D-lactic acid contents were derived mainly from citrate metabolism, since most of the citric acid degradation occurred after MLF (Fig. 7). No significant differences were observed in the content of these organic acids in wines at the end of MLF between partially fermented wine and the controls (data not shown). In the case of a stuck AF, the winemaker can let MLF continue until it is complete before inhibiting the bacteria. Nevertheless, the permanence of bacteria in a prolonged stationary phase should be avoided due to the risk of wine spoilage, especially at high pH (RIBÉREAU-GAYON *et al.* 2006).

Since the incompatibility between yeast and bacteria can affect both AF and MLF, the choice of the yeast-bacterium couple is crucial for achieving a simultaneous AF/MLF (NEHME *et al.,* 2008). The bacterial starters used in this study were compatible with the yeast-bacterial co-inoculation strategy. Concerns for the negative impact on wine quality due to volatile acidity production can be minimized by inoculating bacteria early in the presence of sugars only when standard enological conditions and compatible yeast-bacteria pairings are done as in the current study.

In conclusion, yeast-bacterial co-inoculation is compatible with the production of red table wines. The different typology and large number of grape varieties are significant for testing this technique of bacterial inoculation for the red vinification. Further investigations are needed to evaluate the effects of yeast-bacterial co-inoculation under other different winemaking conditions before it can be accepted definitively as a recommended enological practice.

Table 2 - Composition of Valpolicella wine at the end of MLF obtained by industrial vinification in tanks inoculated with bacteria before AF (simultaneous AF/MLF), after AF (sequential AF/MLF) and in non-inoculated tank (spontaneous MLF).

		Simultaneous AF/MLF	Sequential AF/MLF	Spontaneous MLF
pH		3.38±0.03	3.37±0.02	3.40±0.03
ethanol	% v/v	12.1±0.1	12.1±0.0	12.1±0.2
residual sugars	g/L	2.3±0.5	2.4±0.3	2.4±0.3
titratable acidity[1]	g/L	5.8±0.2	6.0±0.1	6.0±0.1
acetic acid	g/L	0.21±0.04	0.25±0.01	0.26±0.02
L-malic acid	g/L	0.07±0.02	0.08±0.01	0.09±0.00
L-lactic acid	g/L	1.39±0.06	1.54±0.12	1.49±0.05
D-lactic acid	g/L	0.24±0.02	0.28±0.03	0.27±0.02
citric acid	g/L	0.22±0.01	0.13±0.02	0.13±0.03

[1] as tartaric acid.

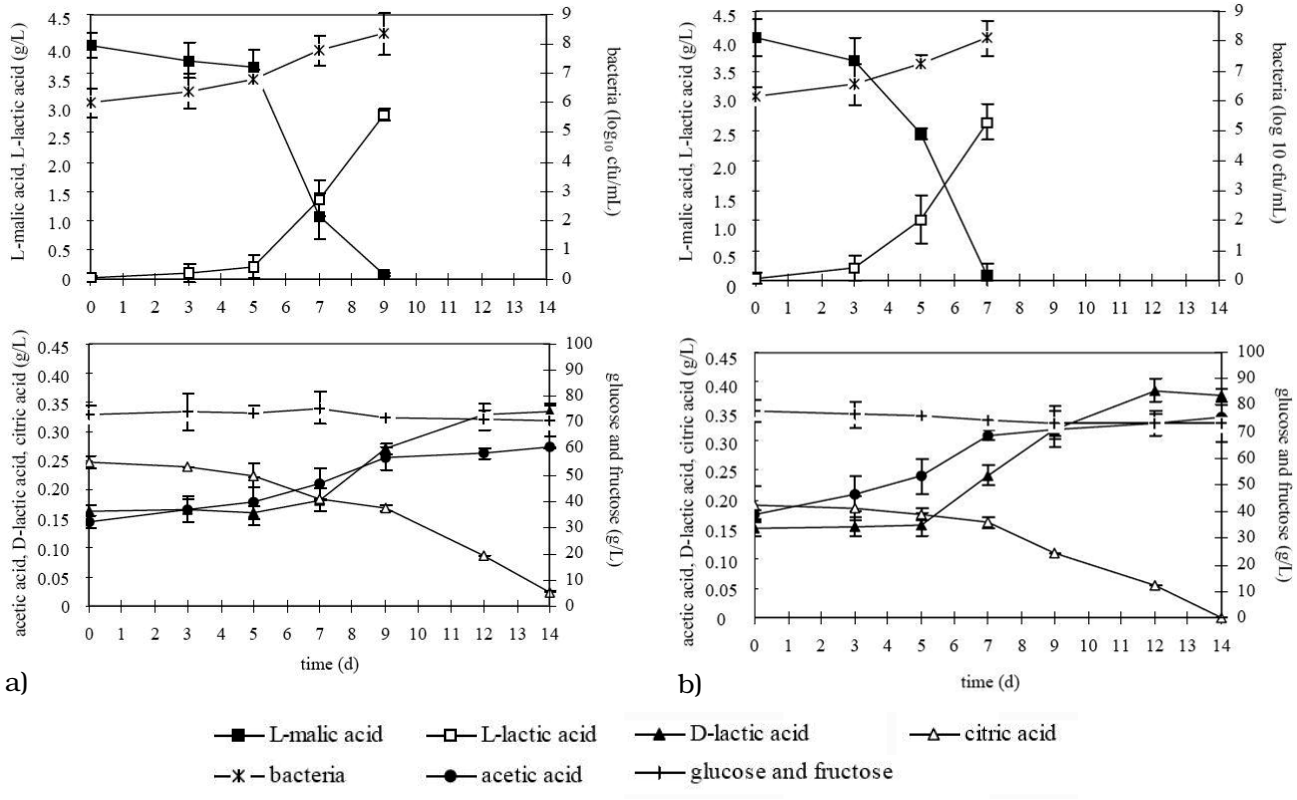


Fig. 7 - Concentration of organic acids and sugars (glucose and fructose) during and after MLF obtained by bacterial inoculation of wine obtained by partial alcoholic fermentation of grape must with pH 3.20 (a) and 3.60 (b).

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PAPER

RISK PERCEPTION ABOUT GMOS AND FOOD CHOICES AMONG ADOLESCENTS ATTENDING SECONDARY SCHOOLS: A TUSCAN CASE

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ABSTRACT

Young consumers' (students attending secondary schools) risk perception of biotechnologies, in general, and of GMOs, in particular, were investigated in Valdinievole (Italy), using a questionnaire adapted from Eurobarometer 64.3. The relationship between the social status and cultural capital of the students' families and their food choices were explored. A total of 502 questionnaires were completed. The adolescents' knowledge seemed rather confused and the answers were related more to the type of school attended rather than the cultural capital and/or social class of the family. In order to make informed decisions, educational training is needed. The schools should serve as a channel through which students gain information.

- Key words: adolescents, biotechnologies, GMOs, risk perception -

INTRODUCTION

According to the 90/220 EEC Directive, the term Genetically Modified Organism indicates an organism whose genetic material has been modified differently than would have naturally occurred by inbreeding or natural genetic recombination. The International Service for the Acquisition of Agri-biotech Applications reports that the global area of biotech crops increased from 2 to 59 million hectares from 1996 to 2002, and reached about 300 million hectares in 2008. In 2008, 25 countries were growing biotech crops, including 11 industrialized countries and 11 developing countries. The United States, Argentina, Brazil, Canada, India and China are the major producers (ISAAA, 2009).

From 1998 to 2004, the European Union banned importation and cultivation of GMOs in the 15 member States, despite the fact that the entire scientific community unanimously expressed its firm belief that there were no risks to human health or the environment. Presently, Spain is the leading European producer of GMOs; in 2008, 100,000 hectares were planted with GMOs (ISAAA, 2009).

The current state of knowledge about GM foods is that they are "substantially equivalent" to non-genetically modified foods. Although there are hypothesized health risks caused by their consumption, there is currently no evidence of harm. Some scientific studies maintain that GMOs can have potential benefits both for human health and for the environment (BONACCORSI, 2001; WU, 2004). On the other hand, negative consequences for the environment have been hypothesized and it has been suggested that there could be possible harm to humans, due to toxins, allergens or infectious agents that are produced, or to the environment, caused by increased susceptibility to biotic stresses (WHO, 2006).

Finding out what people think about GMOs is needed in order to decide how to regulate the GM food market (NAPIER *et al.*, 2004), as well as to understand and evaluate the effects that mass media can have on consumers' capacity to make informed choices (VERBEKE, 2008). Consumer policy is one of the strategic objectives of the European Union to improve its citizens' quality of life. Besides direct protection of consumer rights, the EU considers their interests in EC legislation in every political sector, in line with the "Gaining Health" political strategy (WHO, 2006).

An appropriate evaluation of both the benefits and risks related to new biotechnologies must consider not only the technical and scientific aspects but also the cultural and social context in which they are to be introduced. When experts underestimate public concern and deem it irrational, there is a lack of appropriate consideration of public demand, which leads to distrust (FREWER *et al.*, 1995; 2003; 2004).

The literature shows that the risk perception of biotechnologies is affected by many factors: level of knowledge, awareness of benefits, level of trust in the sources of information and the history, culture and socioeconomic conditions of the country in which the people live and demographic factors (BREDAHL *et al.*, 1998; COPPENS *et al.*, 2006; FINUCANE *et al.*, 2002; FINUCANE and HOLUP, 2005; FREWER *et al.*, 1995; 1998; 2004; LYNN *et al.*, 2002; SLOVIC *et al.*, 2002; SJÖBERG, 2004; TOWNSEND *et al.*, 2004; ZECHENDORF, 1994). Generally speaking, consumer evaluation of GMOs, as well as risk perception, varies greatly among countries and regions (DANNENBERG, 2009; SJÖBERG, 1999).

In 2005, a study conducted by the Pew Initiative on Food and Biotechnology showed that the public knowledge in the USA about GMOs is still quite low and that USA consumers, despite growing fears about possible risks connected with the consumption of GMOs, feel a ban on new technologies would not be useful. They would rather be sure that legislators do all they can to assure that new foods introduced in the marketplace are totally safe (PEW INITIATIVE ON FOOD AND BIOTECHNOLOGY, 2003).

In contrast, the general public opinion in Europe is strongly against GMOs which are perceived as "risky" and "useless". In Europe, the fiercest opposition to GMOs comes from Greece, Ireland and France. However, in Austria, Finland, Great Britain and Holland only one third of the population think that foods containing GMOs could turn out to be "dangerous" (FREWER *et al.*, 1997; GASKELL *et al.*, 1999; 2000; SAVADORI *et al.*, 2004). The European aversion to genetic modification of foods is increasing markedly (DANNENBERG, 2009).

Since 1991, a survey is carried out every three years on behalf of the European Commission, to study the opinions of EU citizens on scientific issues and the use of new biotechnologies. The most recent data available are based on answers given by 25,000 EU citizens, about 1000 from each Member State (EC, 2005). Generally, there is widespread support for medical (red) and industrial (white) biotechnologies, but as far as agricultural (green) biotechnologies are concerned, there is strong opposition in practically every country. The only countries where there is more support than opposition are Spain, Portugal, Italy, Malta, the Czech Republic and Lithuania.

The EU report is based on the overall results of the citizens surveyed, and does not give a specific picture of what the younger generation – the so-called stakeholders of tomorrow – thinks about the issue. Therefore, we carried out an investigation in a limited area of Tuscany, Italy, namely Valdinievole, to explore the level of knowledge and opinions of young people on biotechnology issues. The main objective was to focus on what factors affect the students' perception of risk

about food in general and GMOs in particular, and to determine if there are any links between the choices made by the adolescents and the social status of the students' families and/or the type of school attended. The students were also asked what sources of information they considered to be the most reliable.

MATERIALS AND METHODS

Rationale of the questionnaire

A questionnaire adapted from Eurobarometer 64.3, 2005 (EC, 2005) was used to investigate young consumers' risk perception on genetically modified foods and biotechnologies. Each question had three (yes/no/don't know) or five (very/quite/little not at all concerned/don't know) closed responses to which the subjects could answer in a relatively short time (around fifteen minutes to compile the questionnaire).

The questions in the first part concerned personal data, which, though anonymous, helped place the subject in a socio-cultural, geographic and educational context. The parents' professions and educational qualifications were obtained from the 2001 ISTAT (National Institute of Statistics) classification (ISTAT, 2001).

To combine the professions of both parents into a single variable, which could summarize the socio-economic condition of the family, a family social class variable was devised. Based on the model theorized by SCHIZZEROTTO (1988), the social classes of the father and mother, were determined by assigning: factory workers, housewives, unemployed people to the "low" social class; craftsmen, shopkeepers, members of the military forces and retired people to the "mid-low" social class; public or private employees and technical professionals to the "mid-high" social class, and entrepreneurs and scientific professionals to the "high" social class. Then, according to the Erikson dominance criterion, the family was assigned to the higher social class assigned to the mother and the father. In cases in which the profession of one parent was not reported, the reported parent's profession was used (CARDANO and MARINACCI, 2004).

To evaluate if there was a relationship between the educational level of the family and the perception of risks with respect to GMOs, the family cultural capital variable was created (CARDANO and MARINACCI, 2004). The educational qualification of each parent was classified as follows:

- "low", person with no education or up to primary school level;
- "mid-low", education up to the first degree of secondary school;
- "mid-high", education up to the second degree of secondary school;
- "high", education at university or higher.

The family cultural capital assigned was the higher of the two reported. When no information was provided about one of the parents, the one reported was used.

The questions investigated the following issues:

- which factors affect the perception of food risk in general and of GMOs in particular;
- which subjects are more or less worried about the potentially harmful effects of food;
- is there any relationship between the social status of the students' family and the choices they make;
- which sources of information do students perceive to be the most reliable.

In order to achieve these objectives, other questions were included to investigate the knowledge of European laws and risk perception on different issues, such as tobacco, food and chemical pollutants, in addition to specific questions on biotechnologies and/or biotechnologies applied to food and food safety.

The data were analysed with the SPSS (Statistical Package for Social Science) 16.0 software.

A descriptive analysis was made for each variable in the questionnaire. The null hypothesis of no difference in the frequency of distribution of responses with respect to cultural capital, social class and/or type of institution was tested by means of χ^2 test.

The interactions between and among the independent variables were assessed by correlation analysis and by multivariate logistic regression analysis.

The correlation between the type of school attended, the family cultural capital and social class was expressed by the Pearson correlation coefficient.

In the multivariate logistic regression analysis, the answers to each question were the dependent variables and the covariates were the type of school attended, the family cultural capital and the social class. The results of the regression analysis are expressed by the Odds Ratio (the probability that the event of interest occurs in relation to the probability that it does not) (BLAND and ALTMAN, 2000), i.e. the probability of a "no" response instead of a "yes" or "little/not at all concerned" rather than "very/quite concerned". In multivariate regression analyses each OR value is calculated considering the influence of the other variables and if multicollinearity occurs, the predictors that make a very small contribution are removed. Only the results of the analysis for those questions with statistically significant regression models ($p<0.05$ with Model χ^2 statistic) and with at least one variable showing at least one OR statistically different from 1 are reported. For all the analyses the alpha level considered was 0.05.

Area studied and choice of sample

Valdinievole is an area of 266 km^2 in the province of Pistoia (Tuscany, Italy), with a population of 116,337 (437.36 inhabitants/ km^2), of which 2,873 are between 15 and 17 years of age and 7,723 between 18 and 24 (PROVINCIA DI PISTOIA, 2006). The economy of Valdinievole is primarily based on tourism, trade and services; the territory does not have heavy industries, but there are numerous small-to-medium businesses, including paper, shoe and floral industries.

Carrying out a survey that is limited to a local situation is important in order to understand how, and to what extent, the socio-cultural context can influence and determine the risk perception.

For this study, the sample was composed of students attending the fourth year of secondary school (average age 17-18) in 2007, who should have acquired elements of knowledge and be independent to exert their own purchasing power. As such, the students can be easily classified as stakeholders who influence the marketplace and food sale trends. Furthermore, they can be a key for measuring public opinion about some food commodities and, therefore, for orienting EU policy regarding the protection of consumer rights. Seven secondary schools were involved in the study including vocational, technical, and *licei* (academically-oriented secondary schools).

RESULTS

A total of 502 questionnaires were completed (M 47%, F 53%); the average age of the students was 17.8 years. For the survey 41.2% of the questionnaires were compiled by students from vocational, 32.7% from *licei* and 26.2% from technical schools.

With respect to the parents 91.4% of the fathers and 80.4% of the mothers were born in Italy; Albania was the second most frequent birthplace of the parents (1.8% fathers, 1.6% mothers). Most of the subjects (65.9%) live in the boroughs within the province of Pistoia, the majority (61.1% of total students) live in Valdinievole; 31.7% live in the neighbouring provinces and 2.4% in more distant boroughs. Most of the families belong to the middle-upper class (28.9%), followed by the upper-class (27.7%), middle-lower class (20.9%) and lower class (16.1%) (6.4% missing data).

The most frequent cultural capital was the middle-upper class (46.4%), followed by middle-lower (28.9%), upper (15.3%) and lower cultural capital (3.6%) (5.8% missing data). Families tend to have a cultural capital level that is equal to or lower than their social class.

The distribution of subjects according to type of school differed significantly from the family cultural capital and social class: students who attend *licei* usually came from a family which belongs to a high social class or with a high cultural capital while those who attend vocational schools generally came from lower social classes with a lower cultural capital (Fig. 1).

The distribution of family cultural capital and social class was significantly non-independent: the frequency was much higher than that expected of subjects with the same family cultural capital and family social class (χ^2= 153.800; p<0.001).

The type of school, the family cultural capital and social class were significantly and positively correlated. In fact, the higher the cultural capital the higher was the family social class (r = 0.446, p<0.001) as well as the theoretical orientation of the school attended (from vocational to technical to *licei*, the latter being more academically-oriented) (r = 0.277, p<0.001). The higher the social class, the higher was the theoretical content of the school (r = 0.195, p<0.001). The Pearson coefficients in the last two cases were lower indicating a lower marked correlation than the first case.

Regarding the approach to food, "pleasure" was the first positive word that food evokes and occurred in 25.7% of the respondents, "taste" (25.1%) and "hunger" (17.9%), followed with no statistically significant differences with respect to family social class and cultural capital, or type of institution attended.

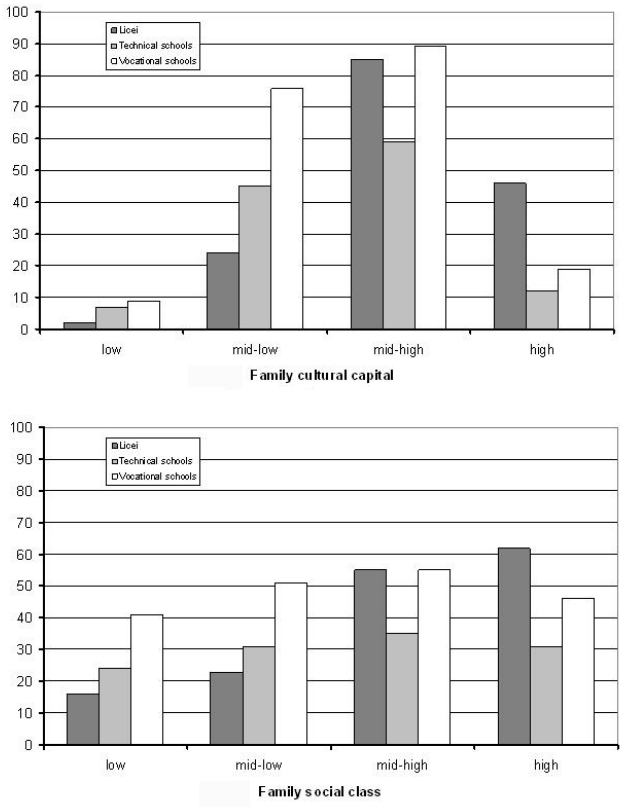


Fig. 1 - Distribution of subjects with respect to type of school, cultural capital (top, χ^2= 48.126; p<0.001) and family social class (below, χ^2=22.454; p=0.001). In both distributions, there are statistically significant differences (p <0.05).

Table 1 - Assessment of students' level of knowledge about biotechnologies and opinions about their use.

	Yes		No		Missing	
	N	%	N	%	N	%
Express your opinion by ticking "yes" or "no" to the following questions and statements						
Standard tomatoes do not contain genes that genetically modified tomatoes do	219	43.6	271	54.0	12	2.4
Would you eat longer lasting GM peaches?	108	21.5	392	78.1	2	0.4
Would you eat GM rice with a high content of vitamin D3?	207	41.2	286	57	9	1.8
Do biotechnologies applied to agriculture help to preserve and renew natural resources?	309	61.6	170	33.9	23	4.6
Could biotechnology increase the amount of food produced in developing countries?	385	76.7	108	21.5	9	1.8
Are GM foods a danger to future generations?	319	63.5	170	33.9	13	2.6
As a consumer, are you sure that the food you eat is without any risk to health?	63	12.5	434	86.5	5	1
Whatever risks may be related to GM foods, can we avoid them if we really want to?	329	65.5	163	32.5	10	2
Could GM food bring benefits to many people?	178	35.5	310	61.8	14	2.8
Of all the risks we take, GMOs are the least important for now	239	47.6	250	49.8	13	2.6
You would support the sale of genetically modified foods only if ...						
it were absolutely certain that there were no risks to consumers	402	80.1	70	13.9	30	6
possible risks were equal to or fewer than those taken with the consumption of non GM foods	218	43.4	210	41.8	74	14.7
risks were fewer than benefits in terms of lower costs and increased shelf life of products	117	23.3	298	59.4	87	17.3
I would never allow GM foods to be sold	174	34.7	258	51.4	70	13.9
Do you know if there is specific legislation regarding the following issues in the European Union?						
Food safety	243	48.4	40	8	216	43.6
Maximum food prices	97	19.3	152	30.3	253	50.4
Consumer rights	287	57.2	44	8.8	171	34.1
Health warnings on cigarette packets	394	78.5	28	5.6	80	15.9

Table 2 - Students' level of concern regarding biotechnology issues.

Specify your level of concern regarding the following issues	Very/quite concerned		Little/not at all concerned		Missing	
	N	%	N	%	N	%
Genetic testing for the diagnosis of hereditary diseases or cancer	298	59.4	165	32.9	39	7.8
Genetic engineering to cure diseases	183	36.5	278	55.4	41	8.2
Human cell cloning or therapeutic cloning	275	54.8	198	39.4	29	5.8
Animal cloning	293	58.4	178	35.5	31	6.2
Using microorganisms to return the natural environment, altered by contaminants, to its original condition (bioremediation)	187	37.3	198	39.4	117	23.3
Allergies to certain foods or beverages	271	54	215	42.8	16	3.2
Presence of additives in foods and beverages	278	55.4	201	40.0	23	4.6
Mad cow disease	359	71.5	129	25.7	14	2.8
Presence of pollutants such as mercury and dioxin	426	84.9	57	11.4	19	3.8
Presence of residues of antibiotics and hormones in meat	392	78.1	84	16.7	26	5.2
Residues of pesticides in fruit, egetables and grains	390	77.7	94	18.7	18	3.6
Presence of GMOs in food and beverages	327	65.1	151	30.1	24	4.8

Tables 1, 2 and 3 show the students' degree of knowledge of biotechnologies and their opinion about their use, the students' level of concern regarding biotechnological issues and the distribution of responses related to the type of school attended by the students, respectively.

The level of knowledge of biotechnologies of male and female students and their opinion on GMO use, as well as the potential associated risks were examined (Table 1). The general level of knowledge did not seem to be very high and the general opinion seemed to be confused, as evidenced by the correct answer to the question "Do common tomatoes contain genes that GM tomatoes do not?". Slightly more than half of the students (54%) gave the correct answer ("No"). This specific item showed a statistically significant difference with respect to family cultural capital and social class, as well as type of school. Regarding family social class, there was a downward trend of wrong answers among the subjects belonging to the lower class (59.5%) and the middle-upper class (34.5%), followed by a subsequent increase in the higher class (46.6%) (χ^2= 13.418; p=0.004).

A similar trend was observed in distribution according to family cultural capital: the percentage of wrong answers was similar in the lower and middle-lower categories (55.6% and 54.2%, respectively), and in the middle-upper and upper (38.9% and 39.7%, respectively) (χ^2= 9.950; p=0.019).

When the questionnaire asked if "biotechnologies applied to agriculture help to preserve and renew natural resources", 61.6% of the students answered "yes" with statistically significant dif-

Table 3 - Response distribution related to the type of school attended by the students (p<0.05).

	Type of school						
	Licei		Technical schools		Vocational schools		χ^2 (p)
	N	%	N	%	N	%	
Express your opinion by ticking "yes" or "no" to the following questions and statements[1]							
Standard tomatoes do not contain genes that genetically modified tomatoes do	46	29.1	55	43.0	118	57.8	29.939 (<0.001)
GM foods are a danger to future generations	88	56.1	90	70.9	141	68.8	8.751 (0.013)
As a consumer, are you sure that the food you eat is without any risk to health?	14	8.6	13	10.0	36	17.6	7.642 (0.022)
Whatever risks may be related to GM foods, can we avoid them if we really want to?	94	59.1	97	75.2	138	67.7	8.401 (0.081)
You would support the sale of genetically modified foods only if ...[1]							
I would never allow GM foods to be Sold	39	28.5	41	37.6	94	50.5	16.405 (<0.001)
Do you know if there is specific legislation in the European Union? [1]							
Health warnings on cigarette packets	131	80.4	115	87.8	148	71.8	14.316 (0.001)
Specify your level of concern regarding the following issues[2]							
Genetic testing for the diagnosis of hereditary diseases or cancer	74	48.7	82	70.7	142	72.8	24.396 (<0.001)
Mad cow disease	105	64.8	99	76.2	155	79.1	9.894 (0.007)
Presence of GMOs in food and beverages	95	60.1	98	77.8	134	69.1	10.172 (0.006)

[1]Number and percentage of affirmative answers are indicated
[2]Number and percentage of "Very/quite concerned" answers are indicated

ferences according to family cultural capital: 33.3% for lower, 68.4% for middle-lower, 62.9% for middle-upper and 71.6% for upper (p<0.05) family cultural capital.

GM food was considered a danger to future generations by 63.5% of respondents, with statistically significant differences according to the three above-mentioned items (family social class and cultural capital, type of school). The distribution by family social class showed a decreasing trend of affirmative answers: 73.8% for lower, 71.2% for middle-lower, 66.4% for middle-upper and 56.6% for upper family social class (χ^2= 8.699; p=0.034); a similar trend was observed by cultural capital (77.8% for lower, 66.7% for middle-lower, 68.8% for middle-upper and 52.0% for upper family cultural capital) (χ^2= 8.399; p=0.038). As regards the type of school, 56.1% of students attending *licei*, 70.9% from technical schools and 68.8% from vocational schools believed that GM foods are a threat to future generations.

The food they eat was considered to not be a risk for human health by a total of 13.5% of the students; with statistically significant differences by type of school (8.6% *licei*, 10.0% technical schools, 17.6% vocational schools).

When asked "Would you welcome the sale of genetically modified foods only if the risks are lower than the benefits that they could give in terms of lower cost and better storage capacity?", 23.3% of respondents answered positively, with statistically significant differences according to social class (27.2% for lower, 27.9% for middle-lower, 39.3% for middle-upper and 45.9% for upper) (χ^2= 7.482; p=0.05).

A total of 34.7% of the respondents stated that

they would never allow the sale of GM foods, with statistically significant differences among the type of school (28.5% from *licei*, 37.6% technical schools, 50.5% vocational schools).

The level of knowledge about EU legislation concerning food safety, sale of food and consumer rights was quite low (high percentage of no-responses) (Table 1).

The students' level of concern about specific health topics was also investigated (Table 2): 55.4% of the students answered that they have little or no concern at all about the use of genetic engineering to cure diseases. The students reported that they were very or fairly concerned about the presence of additives in foods and beverages (55.4%), pollutants such as mercury and dioxin (84.9%), antibiotic and hormone residues in meat (78.1%) and pesticide residues in fruit, vegetables and cereals (77.7%). A total of 65.1% respondents reported being very or fairly concerned about the presence of GMOs in food and beverages with statistically significant differences with regard to the family social class (χ^2= 8.689; p=0.034) and type of school attended. Students belonging to the lower social class (78.2%), to middle-lower (75.5%), to middle-upper (62.7%), and to upper family social class (64.9%), and students of *licei* (60.1%), of technical schools (77.8%), of vocational schools (69.1%) responded being very or fairly worriA total of 59.4% of respondents reported being very or quite concerned about genetic testing for the diagnosis of hereditary diseases or cancer, with a significant decreasing trend in the level of concern with increase of family cultural capital (very or fairly worried: 81.3% for lower, 70.6% for middle-lower, 63.3% for middle-upper and 47.8% for upper family cultural capital; χ^2= 12.468; p=0.006). The answers to this question showed statistically significant differences with respect to type of school with a percentage of very or fairly worried of 48.7% in *licei*, 70.7% in technical schools and 72.8% in vocational schools.

The answers for which a statistically significant difference was reported by type of school are shown in Table 3. There was an increasing trend in wrong answers, with fewer from *licei* students (29.1%), where there should be better theoretical teaching, than in technical schools (43.0%) and finally vocational schools (57.8%), in which there is a stronger focus on technical and/or vocational education.

The results of the multivariate logistic regression analysis are reported (Table 4). No multicollinearity was observed. A few questions show statistically significant models, and in these cases the type of school is the variable that is most frequently linked to the type of response. This is true for the following questions:

- "Standard tomatoes do not contain genes that genetically modified tomatoes do" the likelihood of negative response (instead of positive) was significantly lower for students attending vocational (OR = 0.331) or technical schools (OR = 0.554) compared to *licei* (reference category, OR = 1) and higher for students belonging to middle-upper family social class (OR = 1,788) than high (OR = 1);
- "Could GM food bring benefits to many people?" the likelihood of negative response was significantly higher for students belonging to lower (OR = 2.190) or middle-lower (OR = 2.086) family social class (OR = 2.190) than upper (OR = 1);
- "You would support the sale of genetically modified foods only if possible risks were equal or fewer than those taken with the consumption of non-GM foods" the likelihood of negative response was significantly higher for students attending vocational (OR = 1.868) or technical schools (OR = 1.444) compared to *licei* (OR = 1);
- "I would never allow GM foods to be sold" the likelihood of negative response is significantly lower for students attending vocational schools (OR = 0.430) compared to *licei* (OR = 1);
- "Specify your level of concern regarding genetic testing for the diagnosis of hereditary diseases or cancer" the likelihood of "little/not at all concerned" response (instead of "very/quite concerned") was significantly lower for students attending vocational (OR = 0.407) or technical schools (OR = 0.427) compared to *licei* (OR = 1), as well as for "regarding mad cow disease" (vocational school OR=0.478; technical schools OR=0.464) while for "presence of GMOs in foods and beverages" the likelihood of "little/not at all concerned" response was significantly lower only for students attending technical school (OR=0.438).

Students were asked to express their opinion on the credibility of various sources of information. The results showed that doctors are recognized as the best source in 44.8% of questionnaires, followed by universities (14.5%), consumer organizations (11.6%) and the Internet (9.4%). The answers were different in the vocational schools: physicians 42.2%, university 11.8%, consumer organizations, TV/newspapers and Internet 10.3%.

DISCUSSION AND CONCLUSIONS

The aim of this study was to investigate the opinions and the perception of risk that adolescents have regarding biotechnologies and genetically-modified foods; a topic of great interest that has generated intense debate on a global scale (FINUCANE and HOLUP, 2005; KUIPER *et al.*, 2001; SCHMIDT and WEI., 2006; SPENCE and TOWNSEND, 2006; VAN KLEEF *et al.*, 2007; WILSON, 2004).

The distribution of responses was more close-

Table 4 - Multivariate logistic regression - OR (Standard Error) for models with statistical significance (p<0.05).

	Express your opinion by ticking "yes" or "no" to the following questions and statements°		*You would support the sale of genetically modified foods only if ...* °		*Specify your level of concern regarding the following issues*#		
	Standard tomatoes do not contain genes that genetically modified tomatoes do[1]	*Could GM food bring benefits to many people?*[2]	*Possible risks were equal or fewer than those taken with the consumption of non GM foods*[3]	*I would never allow GM foods to be sold*[4]	*Genetic testing for the diagnosis of hereditary diseases or cancer*[5]	*Mad cow disease*[6]	*Presence of GMOs in foods and beverages*[7]
Type of schools							
Vocational	0.311 (0.247)*	1.435 (0.241)	1.868 (0.348)*	0.430 (0.259)*	0.407 (0.249)*	0.478 (0.264)*	0.786 (0.247)
Technical	0.554 (0.276)*	1.559 (0.270)	1.444 (0.283)	0.725 (0.295)	0.427 (0.281)*	0.464 (0.295)*	0.438 (0.296)*
Licei	Reference	Reference	Reference	Reference	Reference	Reference	Reference
Family cultural capital							
Low	0.830 (0.580)	0.557 (0.575)	0.747 (0.641)	0.756 (0.618)	0.396 (0.724)	2.274 (0.668)	1.542 (0.664)
Mid-low	0.830 (0.339)	1.159 (0.336)	0.867 (0.356)	1.238 (0.374)	0.687 (0.354)	1.688 (0.388)	0.802 (0.359)
Mid-high	1.070 (0.305)	1.408 (0.297)	1.335 (0.314)	0.963 (0.333)	0.681 (0.312)	1.017 (0.348)	0.723 (0.311)
High	Reference	Reference	Reference	Reference	Reference	Reference	Reference
Family social class							
Low	0.873 (0.329)	2.190 (0.342)*	1.790 (0.348)	0.592 (0.352)	0.661 (0.357)	0.745 (0.395)	0.560 (0.374)
Mid-low	1.276 (0.288)	2.086 (0.300)*	0.837 (0.307)	0.781 (0.315)	0.879 (0.314)	1.343 (0.332)	0.667 (0.325)
Mid-high	1.788 (0.271)*	1.136 (0.363)	1.355 (0.280)	1.049 (0.219)	1.085 (0.277)	1.556 (0.300)	1.235 (0.274)
High	Reference	Reference	Reference	Reference	Reference	Reference	Reference

°OR (Standard Error) of "no"; # OR (Standard Error) of "Little/not at all concerned"; * p<0,05

[1] χ^2: 40.284 (8 d.f.); p<0,01; R^2: 0,114; [2] χ^2: 19,162 (8 d.f.); p=0,015; R^2: 0,057 ; [3] χ^2: 16,232 (8 d.f.); p=0,039; R^2: 0,054; [4] χ^2: 18,306 (8 d.f.); p=0,019; R^2: 0,061; [5] χ^2: 29,384 (8 d.f.); p<0,01; R^2: 0,091; [6] χ^2: 16,205 (8 d.f.); p=0,04; R^2: 0,052; [7] χ^2: 20,322 (8 d.f.); p=0,01; R^2: 0,063

ly associated with the type of school attended by the students rather than the cultural capital or social class of their families. Other studies have shown a moderate relationship between risk perception and socio-economic status (SJÖBERG, 2004).

The study confirms a sense of hostility towards the application of biotechnology in food by the students in the Valdinievole secondary schools, even if there were statistically significant differences between the different types of schools.

Students attending *licei* seemed to be more open to and less anxious about the use of genetic testing for the diagnosis of diseases; the entire sample was not concerned about the use of genetic engineering to treat diseases (*licei* students are "not worried at all").

As far as the level of knowledge of the biotechnology issue is concerned, it seems that the Valdinievole students are rather confused, and this is particularly true for those attending the vocational and technical schools: *licei* students have more knowledge about these issues, which may be related to their average higher socio-economic and cultural background, although some authors have stated that the direct relationship between the resources and educational outcome is partially mediated through investments made by families and schools (ROSCIGNO *et al.*, 2006). This consideration could in part explain the stronger association observed between the type of school and risk perception.

In general, the level of knowledge in this study seems to be lower than that described by CAVANAGH *et al.* (2005), probably due to the particular context of the area that was analysed (rural area) (ROSCIGNO *et al.*, 2006).

For the group of adolescents surveyed, the type of school attended is the main factor that influenced their views and opinions about various social issues: the school is the place where they develop a critical sense of the world around them. Therefore it is important that they receive information on "new" issues (MAEKAWA and MACER, 2004) such as biotechnologies and genetically-modified foods, regardless of the type of school attended.

In several international educational systems, courses concerning bio-

technology, particularly genetic engineering applied to foods and drugs, have been taught (CAVANAGH *et al.*, 2005; HARMS, 2002; KITTO *et al.*, 2003).

In response to the results of the Eurobarometer, "biotechnology" has been integrated into the school curricula in the European Union in several countries as a subject. Multidisciplinary courses have been introduced as part of the EIBE project (European Initiative for Biotechnology Education), founded by the European Commission in 1996: informational materials are distributed to teachers and students. Ethical, social, legal and economic issues of the topic are discussed, in addition to the scientific perspective. This helps student develop a moral sense that enables them to make choices keeping in mind all possible implications for both the individual and the society in general. In order for students to make reasonable, informed decisions, there must be adequate educational training: schools should serve as a channel through which physicians and other professionals who gain students' trust can convey information.

It is also important to note that, apart from the type of school attended, every student eats every day and periodically buys food items: so, it does not seem ethical to link the possibility of making informed choices about food items based mainly on the type of school attended. Every single person should have the right to correct information on topics/issues which pertain to everyday life such as food; this information can be derived from school, family, friends or public sources, but it is the responsibility of the society as a whole.

The school can play a major role, but society – namely politicians, scientists and the media – must have the right to correct information, even in the field of genetic engineering.

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PAPER

FREE FATTY ACIDS AND LIPID OXIDATION IN VENISON CHORIZO SAUSAGES MADE AT DIFFERENT STAGES OF THE HUNTING SEASON AND UNDER DIFFERENT RIPENING CONDITIONS

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ABSTRACT

Changes in the free fatty acid profiles and lipid oxidation products, measured as thiobarbituric-reactive substances (TBARS), were examined during the ripening and subsequent vacuum-packed storage of venison chorizo sausage made from animals killed at the beginning and at the end of the hunting season and processed using both natural and controlled drying systems. Total free fatty acid (FFA) content generally increased over the first 7 days of ripening; after which, considerable changes were also noted in the FFA profiles. Oleic, linoleic, palmitic, stearic and palmitoleic acids predominated in all types of chorizo sausages and at all stages of ripening. Monounsaturated FFA were predominant in all cases and polyunsaturated FFA levels declined during ripening. The TBARS values were higher in chorizo sausages processed under controlled conditions and lipid oxidation increased progressively in all batches during the 21-day ripening period and after 45 days of storage in vacuum packaging.

- Key words: dry sausages, free fatty acid, lipid oxidation, TBARS, venison -

INTRODUCTION

Consumer interest in game meat has increased in the last years. Game meat can be distinguished from that of poultry and farmyard animals because it is generally darker, stronger tasting and often tougher, depending on the type and age of the animal. In addition, game meat has a low muscle fat content and a desirable polyunsaturated:saturated fatty acid ratio (HOFFMAN and WIKLUND, 2006).

Hunting is a major economic activity in various rural areas of Spain including Castilla-La Mancha, where it generates an average annual income of 240 m. euros. Hunters kill 40,000 large game animals (e.g. wild boar, red venison, roe venison, fallow venison) and 3,000,000 fowl and small animals (e.g. partridge, rabbit, hare) every year. This area is considered throughout Europe to be a paradise for game-based cuisine.

Although Castilla-La Mancha is a traditional producer of cured, fermented and dried products derived from various game species, marketing of these products is very restricted and highly season-dependent. Moreover, because the cottage-processing system is widely used in this area, product characteristics and types tend to be poorly-defined.

Ripening of fermented sausages causes considerable changes in protein and lipid profiles. Lipolysis plays an essential role in the development of dry-sausage flavour. Lipids in muscle and adipose tissue are hydrolyzed by enzymes generating free fatty acids, which are substrates for the oxidative changes responsible for flavour compounds. Lipolysis in dry sausages has been attributed to the activity of both microbial and meat-endogenous lipases (MOLLY *et al.,* 1997; HIERRO *et al.,* 1997; ORDÓÑEZ *et al.,* 1999). By the end of processing, free fatty acid levels range from 1 to 7%, depending on the product (NAVARRO *et al.,* 1998; SELGAS *et al.,* 1999; BOLUMAR *et al.,* 2001). Diet has a marked influence on the fatty-acid composition of game-meat. However, since game animals are herbivorous and eat a wide variety of indigenous plants, grains and fruit, it is not easy to quantify this factor with any precision.

Lipid oxidation takes place in the presence of oxygen, and is catalyzed by light, the presence of metal cations, moisture, heat and certain oxidative enzymes. The intensity of lipid oxidation is governed by a number of factors, including fatty acid unsaturation, and has a considerable influence on dry sausage flavour and taste. At the same time, lipid oxidation is a major cause of deterioration in the quality of stored meat products.

Very few studies have been found in the literature on the free fatty acid composition and lipid oxidation of dry venison sausages (VIOQUE *et al.,* 2003; SORIANO *et al.,* 2006). Hence, the present study was undertaken to evaluate differences in the free fatty acid composition and lipid oxidation, measured by the thiobarbituric-reactive substances (TBARS), between dry sausages made with venison meat from animals hunted at the beginning and at the end of the hunting season in 2001-2002, and ripened under controlled or natural conditions. Two protocols commonly used in the Castilla-La Mancha region (Spain) to produce the sausages were followed. Changes in free fatty acid and TBARS concentrations were determined during ripening and after 45 days of vacuum packed storage.

MATERIALS AND METHODS

Chorizo sausages and curing process

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

Two treatments were obtained from each company: the first was made at the beginning of the hunting season (November 2001) and the second at the end of the same season (March 2002). The four treatments were coded as follows: A: made at the beginning of the hunting season and ripened under controlled conditions; B: made at the end of the hunting season and ripened under controlled conditions; C: made at the beginning of the hunting season and ripened under natural conditions; D: made at the end of the hunting season and ripened under natural conditions.

The venison meat used in the manufacture of each treatment came from several animals killed on the same day in the same area. Venison meat obtained from hunters was stored at -18°C until processed. Venison and pork back fat (1:1) were ground in a grinder (Mainca, Granollers, Spain) fitted with a plate containing 12 mm-diameter holes. Ground meat and pork back fat were mixed in a mixer (Fuerpla, Valencia, Spain), with the following ingredients: a Ceylamix CH-L (Ceylan, S.L., Valencia, Spain) (90 g/kg) prepared formula containing salt, lactose, dextrose, dextrin, spices, polyphosphates, sodium ascorbate, potassium nitrate (E-252) and paprika; garlic (1.7 g/kg) and sodium nitrite (1 g/kg). The mixture was kneaded for four min and then stuffed into 30 mm diameter artificial casings using a stuffing machine (Fluerpla, Valencia, Spain). Casings were hydrated prior to being immersed in brine for 15-20 min.

Chorizo sausages of the A and B treatments were ripened in a drying room (relative humidity 76-80%, 10°-12°C) for 3 weeks; total weight loss was 41.0 and 45.2%, respectively. Chorizo sau-

sages of the C and D treatments were ripened in a natural drying room for 3 weeks in which the relative humidity and temperature varied according to outdoor weather conditions. In the natural drying room of treatment C, the temperature fluctuated between 3°-14°C and the relative humidity between 41-97%. Finally, sausages of the D treatment were ripened at a temperature of 7°-15°C and a relative humidity of 60-84%. Sausages of the C and the D treatments had a weight loss of 40.3 and 38.8%, respectively.

Sample preparation

Venison, pork back fat and deskinned chorizo samples were ground in a household blender, placed in hermetically-sealed containers and stored at -20°C for future analysis. Samples were analyzed in duplicate.

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

Physicochemical composition

Water activity (a_w) was measured directly using a Decagon CX-2 dew point hygrometer (Decagon Devices Inc., Pullman, WA, USA); pH was measured directly with a Crison 2001 pH meter (Crison Instruments S.A., Barcelona, Spain). Three measurements were made; the electrode was inserted in three different positions. Moisture, sodium chloride and fat content were analyzed according to AOAC methods (1990).

Free fatty acid analysis

The extraction of total lipids was performed according to KALLIO *et al.* (1998). Free fatty acids were separated from the rest of the neutral and polar lipids by extraction in solid phase using aminopropylsilica minicolumns (500 mg; Supelco). Free fatty acids of the samples and the calibration solutions were methylated with 10% borum trifluoride in methanol (GARCÍA-REGUEIRO *et al.,* 1994).

Analysis of fatty acid methyl esters was carried out in a Perkin Elmer gas chromatograph model 8600 equipped with a flame ionization detector at 250°C. The procedures were controlled with a ChemStation programme (Hewlett-Packard). The capillary column used was a SGL-1000 (50 m × 0.25 mm × 25 μm) (Sugelabor). The oven temperature was held for 35 min at 200°C.

Lipid oxidation

Oxidative stability was evaluated by changes in thiobarbituric acid-reactive substances (TBARS) during ripening. The thiobarbituric-reactive substances were measured following the method describe by SERRANO *et al.* (2006). The standard curve was constructed using 1,1,3,3-tetraethoxypropane (Sigma Chemical Co., St. Louis, MO, USA) as described by BOTSOGLOU *et al.* (1994). The determination was performed in triplicate and the TBARS value was expressed as mg of malonaldehyde (MA)/kg of dry matter (DM).

Statistical analysis

Data were subjected to analysis of variance using the SPSS program version 15.0 for Windows XP (SPSS, Inc, Chicago, Illinois). The Student-Newman-Keuls test was used to separate the means.

RESULTS AND DISCUSSION

Physicochemical composition of raw venison

The pH, a_w, dry matter (DM) and fat content of the venison meat used to make the four types of venison sausages are shown in Table 1. The four meats displayed similar values for all parameters except for fat content. The fat content was lower ($P < 0.05$) in meat obtained at the beginning of the hunting season (A and C). The animals were still in the mating period or just finishing it, and therefore their fat reserves were fairly depleted.

Physicochemical composition of venison chorizo sausages

The values for the physicochemical characteristics and composition of the four types of venison chorizo sausages during ripening and after vacuum-packed storage for 45 days are reported (Table 2). There was a general decline in a_w values and an increase in DM values throughout the curing process and after 45 days of storage. The changes after storage indicated that the material used by the two companies for the vacuum package did not maintain the properties of the sausages. During the first week of ripening, pH values decreased in all four treatments; thereafter, values remained constant or increased, possibly due to enzyme formation of non-protein nitrogen and ammonium ions. Concerning differences found among treatments, the two naturally-ripened treatments (C and D) displayed higher values of sodium chloride than the two treatments ripened under controlled conditions (A and B). The combination of the acidic pH values and low a_w was considered adequate for inhibiting the growth of pathogenic microorganisms (BACUS and BROWN, 1981) and correct for allowing the growth of the sodium chloride tolerance strains used in the industry to produce fer-

Table 1 - Mean values and standard deviations for physicochemical characteristics and composition of venison meat used to make four types of chorizo sausages.

	A	B	C	D
pH	5.77±0.37	5.48±0.01	5.83±0.11	5.47±0.01
a_w	0.991±0.002	0.988±0.001	0.994±0.003	0.980±0.007
DM (g/100 g sample)	21.97±0.33	22.87±0.19	22.31±0.39	22.36±1.54
Fat (g/100 g DM)	1.04±0.43[a]	8.26±0.02[b]	2.55±0.63[a]	11.57±1.43[c]

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

mentation in the sausages. In general, DM was slightly higher in sausages ripened under controlled conditions; higher weight losses were observed during ripening. The average fat content values expressed as dry matter were: 35.2% for treatment A, 34.1% for treatment B, 30.0% in treatment C and 34.6% for treatment D. Physicochemical values at the end of ripening were similar to those found by other authors in venison chorizo sausages (VIOQUE *et al.*, 2003; SORIANO *et al.*, 2006) and pork chorizo sausages (ASTIASARÁN *et al.*, 1990).

Table 2 - Mean values and standard deviations for physicochemical characteristics and composition of deer chorizo sausages during ripening and after 45 days of vacuum packed storage.

	Ripening time (days)				Storage
	0	7	14	21	
			A		
pH	5.32±0.061[a]	4.91±0.02[b]	4.81±0.01[c]	4.96±0.05[b]	4.99±0.05[b]
a_w	0.973±0.003[a]	0.950±0.005[b]	0.924±0.001[c]	0.903±0.013[d]	0.849±0.010[e]
DM (g/100 g sample)	35.53±0.62[a]	44.81±0.21[b]	54.88±1.20[c]	59.91±2.96[d]	67.27±0.57[e]
Fat (g/100 g DM)	37.30±1.19	34.55±2.06	36.96±1.19	36.19±3.90	34.12±0.92
NaCl (g/100 g DM)	5.95±0.16	5.71±0.04	5.61±0.18	5.83±0.23	5.55±0.12
			B		
pH	5.20±0.04[a]	4.95±0.04[b]	4.94±0.02[b]	4.96±0.04[b]	5.11±0.06[c]
a_w	0.966±0.001[a]	0.940±0.003[b]	0.915±0.007[c]	0.877±0.008[d]	0.827±0.004[e]
DM (g/100 g sample)	38.61±0.26[a]	48.53±1.63[b]	56.79±2.02[c]	64.28±1.54[d]	69.46±1.04[e]
Fat (g/100 g DM)	37.56±2.80	36.35±2.32	32.98±2.23	30.93±2.40	32.49±2.92
NaCl (g/100 g DM)	5.18±0.15	5.95±0.46	5.56±0.15	5.78±0.06	5.86±0.12
			C		
pH	5.72±0.03[a]	5.09±0.17[b]	5.23±0.07[b,c]	5.13±0.22[b,c]	5.32±0.01[c]
a_w	0.972±0.001[a]	0.946±0.005[b]	0.911±0.004[c]	0.887±0.025[d]	0.879±0.003[d]
DM (g/100 g sample)	34.18±1.12[a]	42.76±2.66[b]	52.71±0.91[c]	59.23±4.01[d]	59.58±0.18[d]
Fat (g/100 g DM)	32.07±4.46	27.36±3.61	30.37±0.38	30.78±1.56	29.18±3.77
NaCl (g/100 g DM)	7.55±0.44	7.23±0.08	7.40±0.24	7.12±0.14	7.15±0.28
			D		
pH	5.68±0.01[a]	5.07±0.02[b]	5.32±0.06[c]	5.40±0.11[d]	5.76±0.03[e]
a_w	0.969±0.002[a]	0.952±0.002[b]	0.944±0.004[c]	0.902±0.004[d]	0.849±0.003[e]
DM (g/100 g sample)	35.10±0.19[a]	41.46±0.74[b]	43.15±0.88[c]	56.26±2.20[d]	65.82±0.86[e]
Fat (g/100 g DM)	33.29±2.94	35.05±1.11	36.57±1.25	34.45±0.63	33.54±1.62
NaCl (g/100 g DM)	6.86±0.06	6.65±0.21	7.18±0.09	7.38±0.04	7.01±0.19

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

Free fatty acid content of raw material and chorizo sausages

The free fatty acid (FFA) levels in the four venison meats, pork back fats and chorizo sausages during ripening and after 45 days of vacuum-packed storage at room temperature are shown in Fig. 1. Venison used for sausages ripened under natural conditions showed higher concentrations of FFA than venison used for sausages ripened under controlled conditions. The FFA content found in pork back fats was similar except in treatment A, which had a higher FFA content than the other three treatments. The total FFA content in chorizo sausages displayed a marked increase over the first 7 days of ripening. After 14 and 21 days of ripening, the total FFA levels showed no significant changes, in any of the treatments, with respect to the values recorded after 7 days. Many authors have reported a progressive increase in FFA levels during ripening in dry-cured pork sausages (LIZARRAGA *et al.*, 1989; DOMÍNGUEZ and ZUMALACÁRREGUI, 1991; HIERRO *et al.*, 1997; NAVARRO *et al.*, 1998; BOLUMAR *et al.*, 2001).

The mean values after 21 days of ripening were similar to those reported by other authors. HIERRO *et al.* (1997) reported values of 0.7-2 g/100 g DM in dry-cured pork sausages.

During vacuum-packed storage, there were no changes in the mean FFA levels with respect to the end of ripening, except in treatment B, where a significant increase was noted ($P < 0.05$).

Free fatty acid profile of raw venison and pork back fat

The relative percentage (%) of each FFA found in the raw venison used to make chorizo sausages is given in Fig. 2. All four meats had higher amounts of saturated free fatty acids (SFFA), from 36.1 to 52.4%, than monounsaturated and polyunsaturated free fatty acids (MFFA and PUFFA). Venison obtained at the beginning of the hunting season had the lowest (17.4% in treatment A) and the highest percentage (37.2% in treatment C) of PUFFA. These meats had a high percentage of $C_{18:0}$, followed by $C_{16:0}$, $C_{18:1}$ and $C_{18:2}$. However, venison obtained at the end of the hunting season (treatments B and D) had higher percentages of $C_{18:1}$, $C_{16:0}$ and $C_{18:2}$ than of $C_{18:0}$. Meats obtained at the beginning of the hunting season showed higher percentages of $C_{20:1}$ than meat obtained at the end of the hunting season. The percentage of $C_{20:4}$ was markedly higher in all four meats compared with meat from other farmyard animals. The variation among the profiles of the four treatments could be attributable to the different feed of wild deer that is based on grass, as well as other parameters (sex, age).

The relative percentages (%) of each FFA in the pork back fat used to make chorizo sausages are given in Fig. 3. The profiles were similar for all four types of chorizo sausages. The amount of MFFA was the highest (41.6-52.3%), followed by SFFA (27.1-32.4%) and PUFFA (20.6-26.7%). $C_{18:1}$ had the highest relative percentage, followed by $C_{18:2}$, $C_{16:0}$ and $C_{18:0}$.

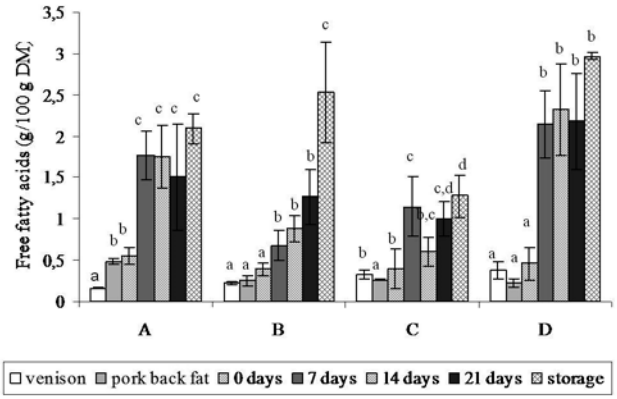


Fig. 1 - Free fatty acid concentrations of raw venison, pork back fat and chorizo sausages during ripening and after 45 days of vacuum packed storage. A: venison obtained at the beginning of the hunting season and used for sausages ripened under controlled conditions; B: venison obtained at the end of the hunting season and used for sausages ripened under controlled conditions; C: venison obtained at the beginning of the hunting season and used for sausages ripened under natural conditions; D: venison obtained at the end of the hunting season and used for sausages ripened under natural conditions. Different letters within a treatment indicate significant differences ($P < 0.05$).

Free fatty acid profile of chorizo sausages

The relative percentage (%) values for each FFA throughout ripening and storage of the treatments A, B, C and D are shown in Tables

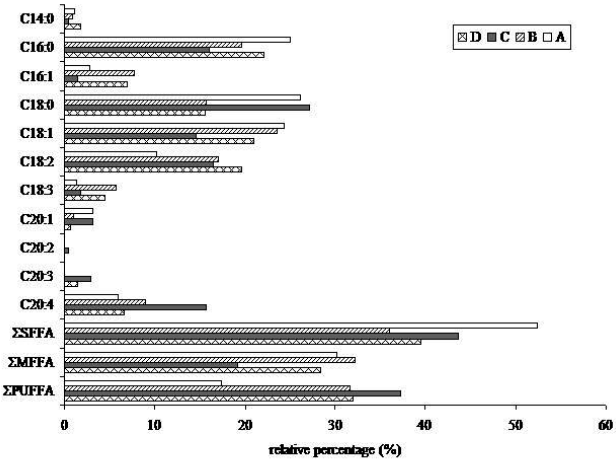


Fig. 2 - Free fatty acid profile of raw venison. A: venison obtained at the beginning of the hunting season and used for sausages ripened under controlled conditions; B: venison obtained at the end of the hunting season and used for sausages ripened under controlled conditions; C: venison obtained at the beginning of the hunting season and used for sausages ripened under natural conditions; D: venison obtained at the end of the hunting season and used for sausages ripened under natural conditions.

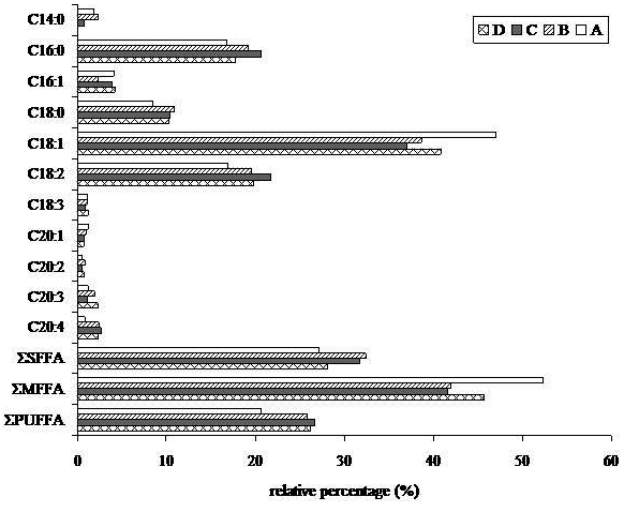


Fig. 3 - Free fatty acid profile of pork back fat. A: pork back fat used at the beginning of the hunting season for sausages ripened under controlled conditions; B: pork back fat used at the end of the hunting season for sausages ripened under controlled conditions; C: pork back fat used at the beginning of the hunting season for sausages ripened under natural conditions; D: pork back fat used at the end of the hunting season for sausages ripened under natural conditions.

3, 4, 5 and 6, respectively. It is difficult to compare the results obtained here with those reported by other authors because the degree of lipolysis and oxidation responsible for FFA levels varies considerably depending on processing temperature, the proportions of different ingredients (lean meat, fat, spices, salt), the amount of enzymes in the raw material and the degree of mincing. Moreover, few studies have addressed FFA levels in cured venison sausages (VIOQUE *et al.,* 2003; SORIANO *et al.,* 2006). The results obtained here are therefore compared mainly with those reported for dry-cured sausages made from lean and fat pork or beef.

The predominant FFAs in all treatments and at all stages were: $C_{18:1}$, $C_{16:0}$, $C_{18:2}$, $C_{18:0}$ and $C_{16:1}$. By the end of ripening, the relative percentages were: 41.7-48.1% for $C_{18:1}$, 17.1-18.5% for $C_{16:0}$, 15.0-19.9% for $C_{18:2}$, 8.2-10.5% for $C_{18:0}$, and 3.8-4.8% for $C_{16:1}$. Other FFAs accounted for less than 3% of the total. Similar findings have been reported by other authors for different dry-cured pork products (DOMÍNGUEZ and ZUMALACÁRREGUI, 1991; SAMELIS *et al.,* 1993; ZALACAIN *et al.,* 1997; NAVARRO *et al.,* 1998; BOLUMAR *et al.,* 2001) and also for venison sausages (VIOQUE *et al.,* 2003; SORIANO *et al.,* 2006).

The FFA profiles changed considerably after the first 7 days in all types of chorizo sausages. Changes in treatment D continued into the second and third weeks of ripening, possibly due to higher moisture content during this period (Table 2), which could influence the lipase activities. In general, no changes were found in FFA profiles exclusively due to ripening conditions or hunting season stages. Perhaps the lipase content could explain some of the results.

No changes in the relative proportions of $C_{14:0}$, $C_{16:0}$ and $C_{16:1}$ were recorded in the A, B or C treatments. In the D treatment, however, there was a significant decline in the relative percentages of $C_{14:0}$ and $C_{16:0}$ after the first 7 days, and an increase in $C_{16:1}$ after 14 days of ripening. Thereafter, values for $C_{14:0}$ and $C_{16:1}$ remained unchanged until the end of ripening, while $C_{16:0}$ increased progressively, approaching the initial values after 21 days. Several authors (LIZARRAGA *et*

Table 3 - Mean values and standard deviations of relative percentage (%) of free fatty acid extracted from chorizo sausages made at the beginning of the hunting season and ripened under controlled conditions (A treatment), throughout ripening and vacuum packed storage.

	Ripening time (days)				Storage
	0	7	14	21	
$C_{14:0}$	1.28±0.39^{a,b}	1.18±0.15^{a}	1.32±0.02^{a}	1.38±0.23^{a,b}	1.52±0.20^{b}
$C_{16:0}$	16.80±1.70	14.95±0.50	16.28±0.97	17.07±1.69	19.46±0.91
$C_{16:1}$	3.59±0.41	4.06±0.12	3.90±0.18	3.90±0.15	4.07±0.26
$C_{18:0}$	6.94±1.10^{a}	8.87±0.21^{b}	8.34±0.95^{a,b}	8.16±0.22^{a,b}	8.58±0.30^{a,b}
$C_{18:1}$	41.76±2.65^{a}	50.91±1.57^{b}	47.46±3.45^{b}	47.70±1.26^{b}	47.46±1.33^{b}
$C_{18:2}$	26.66±4.78^{a}	17.17±0.82^{b}	19.26±1.57^{b}	18.57±1.30^{b}	15.99±1.95^{b}
$C_{18:3}$	1.28±0.43^{a}	0.75±0.06^{a,b}	0.97±0.17^{a,b}	0.94±0.04^{a,b}	0.67±0.17^{b}
$C_{20:1}$	0.77±0.06^{a}	1.23±0.09^{b}	1.03±0.10^{b}	1.00±0.14^{b}	1.13±0.05^{b}
$C_{20:2}$	0.15±0.13^{a}	0.37±0.03^{b}	0.43±0.06^{b}	0.40±0.04^{b}	0.38±0.04^{b}
$C_{20:3}$	n.d.	0.08±0.14	0.19±0.17	0.17±0.15	0.20±0.03
$C_{20:4}$	0.75±0.65	0.42±0.15	0.81±0.28	0.69±0.13	0.54±0.19
ΣSFFA	25.02±3.11	25.01±0.74	25.94±1.89	26.61±2.04	29.55±0.80
ΣMFFA	46.12±2.88^{a}	56.20±1.43^{b}	52.38±3.63^{b}	52.61±1.26^{b}	52.66±1.60^{b}
ΣPUFFA	28.86±5.98^{a}	18.79±0.88^{b}	21.67±2.04^{b}	20.78±1.59^{b}	17.79±2.38^{b}

Different superscripts in the same row indicate significant differences ($P < 0.05$).
n.d.: non detected.
SFFA: saturated free fatty acids; MUFFA: monounsaturated free fatty acids; PUFFA: polyunsaturated free fatty acids.

Table 4 - Mean values and standard deviations of relative percentage (%) of free fatty acid extracted from chorizo sausages made at the end of the hunting season and ripened under controlled conditions (B treatment), throughout ripening and vacuum packed storage.

	Ripening time (days)				Storage
	0	7	14	21	
$C_{14:0}$	1.44±0.06	1.29±0.19	1.34±0.09	1.32±0.13	1.39±0.49
$C_{16:0}$	15.95±0.94	18.82±1.86	19.87±1.27	18.54±1.11	18.71±2.75
$C_{16:1}$	4.44±0.19	4.07±0.19	4.50±0.34	4.18±0.22	4.19±0.67
$C_{18:0}$	7.66±0.70^{a}	9.80±0.45^{b}	9.84±0.42^{b}	9.78±0.78^{b}	8.53±0.27^{a,b}
$C_{18:1}$	49.83±1.19^{a}	43.94±2.26^{b}	46.92±1.37^{a}	48.08±0.82^{a}	48.90±1.52^{a}
$C_{18:2}$	17.89±1.29	17.83±1.87	14.51±0.35	14.98±1.64	14.99±2.12
$C_{18:3}$	1.09±0.14	1.35±0.38	0.94±0.08	0.98±0.06	0.87±0.23
$C_{20:1}$	1.23±0.06	1.14±0.30	1.10±0.13	1.13±0.05	1.06±0.11
$C_{20:2}$	0.11±0.19	0.28±0.05	0.18±0.18	0.27±0.08	0.34±0.10
$C_{20:3}$	n.d.	0.47±0.43	0.15±0.27	n.d.	0.14±0.12
$C_{20:4}$	0.37±0.32^{a}	1.01±0.10^{b}	0.62±0.05^{a,b}	0.74±0.12^{a,b}	0.88±0.30^{a,b}
ΣSFFA	25.05±1.57^{a}	29.90±2.38^{a,b}	31.06±1.15^{b}	29.64±1.92^{a,b}	28.63±3.29^{a,b}
ΣMFFA	55.50±0.98^{a}	49.16±2.39^{b}	52.53±1.11^{a}	53.39±0.60^{a}	54.15±1.09^{a}
ΣPUFFA	19.45±0.96^{a}	20.94±1.93^{a}	16.42±0.19^{b}	16.97±1.56^{b}	17.22±2.83^{a,b}

Different superscripts in the same row indicate significant differences ($P < 0.05$).
n.d.: non detected.
SFFA: saturated free fatty acids; MUFFA: monounsaturated free fatty acids; PUFFA: polyunsaturated free fatty acids.

al., 1989; HIERRO *et al.,* 1997; BOLUMAR *et al.,* 2001) reported a progressive drop in the relative percentage of free $C_{14:0}$ during sausage ripening. LIZARRAGA *et al.* (1989) recorded a decrease in $C_{16:0}$ after the oven phase, followed by an increase, with values nearly returning to the initial levels by the end of ripening. Other authors (HIERRO *et al.,* 1997; NAVARRO *et al.,* 1998; BOLUMAR *et al.,* 2001) reported a gradual decrease in the relative percentage of $C_{16:0}$. LIZARRAGA *et al.* (1989) and NAVARRO *et al.* (1998) noted unchanging values of between 2.5 and 3.4% for $C_{16:1}$ during ripening. MELGAR *et al.* (1990) recorded a drop in the relative percentage of $C_{16:1}$ after the oven phase, followed by an increase during the remainder of the drying period, finally returning to the initial values (3.1%). SAMELIS *et al.* (1993) also found an increase in $C_{16:1}$ during ripening, between 3.6 and 4.7%, in dry-cured pork and beef products.

Table 5 - Mean values and standard deviations of relative percentage (%) of free fatty acid extracted from chorizo sausages made at the beginning of the hunting season and ripened under natural conditions (C treatment), throughout ripening and vacuum packed storage.

	Ripening time (days)				Storage
	0	7	14	21	
$C_{14:0}$	2.15±0.19	1.83±0.19	2.23±0.21	2.07±0.24	2.27±0.20
$C_{16:0}$	17.01±2.74	17.54±2.15	18.39±1.78	18.17±1.19	18.36±0.19
$C_{16:1}$	3.25±0.73^{a}	4.07±0.20^{a,b}	3.93±0.24^{a,b}	3.75±0.04^{a,b}	4.29±0.08^{b}
$C_{18:0}$	8.44±0.31	9.90±1.35	10.33±0.92	10.45±0.43	9.96±0.49
$C_{18:1}$	32.69±3.82^{a}	42.25±2.91^{b}	42.95±2.86^{b}	41.66±1.67^{b}	39.47±1.00^{b}
$C_{18:2}$	31.18±5.14^{a}	20.49±1.15^{b}	18.85±0.39^{b}	19.85±0.78^{b}	20.81±0.77^{b}
$C_{18:3}$	2.17±0.61^{a}	1.22±0.12^{b}	1.03±0.02^{b}	1.17±0.02^{b}	1.28±0.09^{b}
$C_{20:1}$	0.30±0.53^{a}	1.05±0.13^{b}	1.09±0.15^{b}	1.02±0.05^{b}	0.84±0.07^{b}
$C_{20:2}$	0.07±0.13^{a}	0.31±0.09^{b}	0.30±0.09^{b}	0.29±0.07^{b}	0.41±0.06^{b}
$C_{20:3}$	n.d.	0.13±0.22^{a}	n.d.	0.39±0.07^{b}	0.42±0.10^{b}
$C_{20:4}$	2.73±1.50	1.19±0.28	0.91±0.06	1.18±0.05	1.89±0.46
ΣSFFA	27.60±2.71	29.28±3.68	30.95±2.89	30.69±1.78	30.59±0.26
ΣMFFA	36.25±4.98^{a}	47.37±2.99^{b}	47.98±3.11^{b}	46.43±1.70^{b}	44.60±1.06^{b}
ΣPUFFA	36.15±6.93^{a}	23.35±1.36^{b}	21.08±0.32^{b}	22.88±0.90^{b}	24.81±1.32^{b}

Different superscripts in the same row indicate significant differences ($P < 0.05$).
n.d.: non detected.
SFFA: saturated free fatty acids; MUFFA: monounsaturated free fatty acids; PUFFA: polyunsaturated free fatty acids.

The relative percentage of $C_{18:0}$ in sausages ripened under controlled conditions (A and B) increased after the first 7 days and remained unchanged thereafter. There was no change in the relative percentage of this acid in treatment C, whereas in treatment D the percentage of $C_{18:0}$ declined after 14 days of ripening, nearly returning to the initial values. Several authors (SAMELIS *et al.*, 1993; HIERRO *et al.*, 1997; NAVARRO *et al.*, 1998; BOLUMAR *et al.*, 2001) have reported a gradual decline in the relative percentage of free stearic acid during the ripening of dry-cured products. In contrast, LIZARRAGA *et al.* (1989) and MELGAR *et al.* (1990) noted an increase in free stearic acid after the oven phase in pork and beef sausages, with a subsequent decrease at the end of the process. LIZASO *et al.* (1999) reported an increase until the end of the drying period, with a relative percentage of 12.7% at the end of ripening.

The relative percentage of $C_{18:1}$ in the A, C and D treatments increased during the first 7 days of ripening, and remained steady until the end of the process; similar findings were reported by LIZASO *et al.* (1999) for *salchichón*. In the B treatment, values fell during the first 7 days, increasing thereafter, to return to the initial levels, and then remained unchanged until the end of the process. LIZARRAGA *et al.* (1989) and MELGAR *et al.* (1990) also noted a decrease in the relative percentage of oleic acid during the first few days of ripening, followed by an increase, with values reaching 43.5 and 34.4% by the end of the process.

The relative percentage of $C_{18:2}$ decreased after the first 7 days of ripening in the A, C and D treatments and remained unchanged thereafter in the A and C treatments (also reported by LIZASO *et al.*, 1999) but decreased again in the D treatment after 14 days. In the B treatment, there was no change in the relative percentage of this acid throughout the ripening process. Other authors reported contrasting results: LIZARRAGA *et al.* (1989), SAMELIS *et al.* (1993), HIERRO *et al.* (1997) and NAVARRO *et al.* (1998) noted a progressive increase, while ZALACAIN *et al.* (1997) and SELGAS *et al.* (1999) recorded a decrease throughout the ripening period. DOMÍNGUEZ and ZUMALACÁRREGUI (1991) and BOLUMAR *et al.* (2001) reported an initial increase during the first few days of ripening, followed by a decline that continued until the end of the period, whereas MELGAR *et al.* (1990) noted that the relative percentage declined during the oven phase, and then increased until the end of processing in dry-cured pork products. Polyunsaturated fatty acids are much more susceptible to oxidation than monounsaturated acids: linoleic acid, for example, oxidises 10 to 15 times faster than oleic acid, which may account both for the decrease noted in the A, C and D treatments here, as well as for the disparate results reported by other authors.

The relative percentage of $C_{18:3}$ in sausages ripened under controlled conditions (A and B) remained steady over the 21 days of ripening; similar findings were reported by MELGAR *et al.* (1990) and BOLUMAR *et al.* (2001). However, in the naturally-ripened treatments (C and D) values declined after 7 days (treatment C) and 14 days (treatment D), thereafter remaining unchanged until the end of ripening. Several au-

Table 6 - Mean values and standard deviations of relative percentage (%) of free fatty acid extracted from chorizo sausages made at the end of the hunting season and ripened under natural conditions (D treatment), throughout ripening and vacuum packed storage.

	Ripening time (days)				Storage
	0	7	14	21	
$C_{14:0}$	2.52±0.57^{a}	1.18±0.21^{b}	1.68±0.38^{a,b}	1.90±0.19^{a,b}	2.02±0.27^{a,b}
$C_{16:0}$	19.33±0.40^{a}	11.25±1.08^{b}	14.02±2.15^{c}	18.36±0.90^{a}	22.15±1.28^{d}
$C_{16:1}$	3.74±0.23^{a}	3.98±0.33^{a,b}	4.85±0.46^{c}	4.83±0.09^{c}	4.50±0.26^{b,c}
$C_{18:0}$	8.98±0.18^{a,c}	8.09±0.67^{a}	7.25±0.39^{b}	8.27±0.20^{a,c}	9.18±0.48^{c}
$C_{18:1}$	33.68±1.24^{a}	45.71±1.02^{b,c}	50.01±1.61^{c}	45.94±3.51^{b,c}	44.90±1.36^{b}
$C_{18:2}$	26.84±1.80^{a}	23.69±2.26^{b}	18.11±0.65^{c}	16.98±2.36^{c,d}	14.12±0.44^{d}
$C_{18:3}$	2.19±0.27^{a}	2.09±0.31^{a}	1.31±0.07^{b}	1.22±0.34^{b}	0.87±0.02^{b}
$C_{20:1}$	0.86±0.13	0.94±0.11	0.97±0.09	0.95±0.13	1.00±0.05
$C_{20:2}$	0.13±0.23^{a}	0.56±0.04^{b}	0.48±0.07^{b}	0.35±0.01^{a,b}	0.35±0.04^{a,b}
$C_{20:3}$	0.28±0.49	0.24±0.22	0.20±0.18	0.23±0.04	0.11±0.09
$C_{20:4}$	1.45±0.47^{a}	2.27±0.44^{b}	1.11±0.11^{a}	0.97±0.40^{a}	0.81±0.06^{a}
ΣSFFA	30.83±0.97^{a,c}	20.52±1.91^{b}	22.96±2.27^{b}	28.53±0.83^{a}	33.36±1.59^{c}
ΣMFFA	38.28±1.59^{a}	50.63±1.38^{b}	55.82±1.22^{c}	51.72±3.68^{b}	50.40±1.20^{b}
ΣPUFFA	30.89±0.99^{a}	28.85±3.25^{a}	21.23±1.06^{b}	19.75±3.12^{b,c}	16.24±0.62^{c}

Different superscripts in the same row indicate significant differences ($P < 0.05$).
SFFA: saturated free fatty acids; MUFFA: monounsaturated free fatty acids; PUFFA: polyunsaturated free fatty acids.

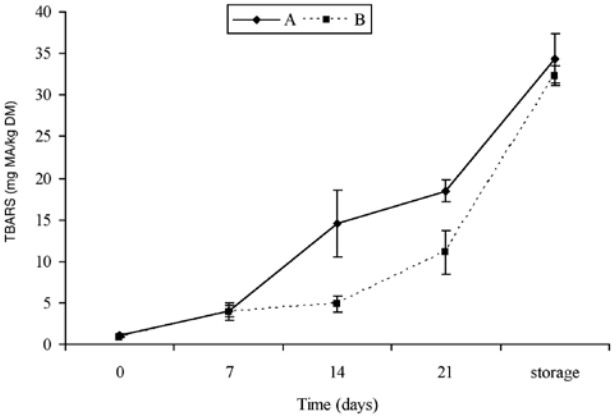


Fig. 4 - Thiobarbituric acid-reactive substances (Tbars) in venison chorizo sausages over ripening and after 45 days of vacuum packed storage of chorizo sausages ripened under controlled conditions. A: chorizo sausages made at the beginning of the hunting season and ripened under controlled conditions; B: chorizo sausages made at the end of the hunting season and ripened under controlled conditions. Different letters within a treatment indicate significant differences ($P < 0.05$).

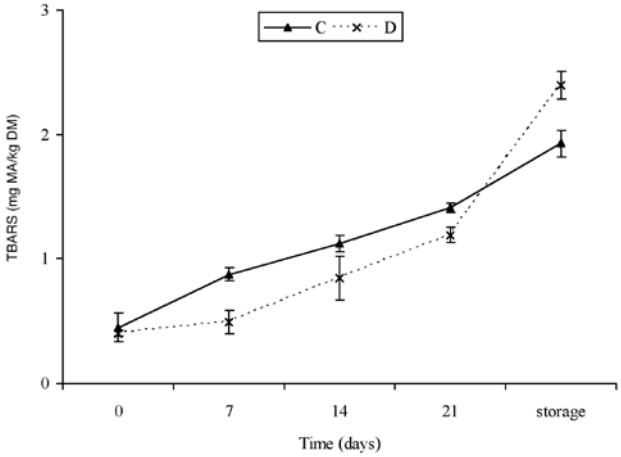


Fig. 5 - Thiobarbituric acid-reactive substances (TBARS) in venison chorizo sausages during ripening and after 45 days of vacuum packed storage of chorizo sausages ripened under natural conditions. C: chorizo sausages made at the beginning of the hunting season and ripened under natural conditions; D: chorizo sausages made at the end of the hunting season and ripened under natural conditions. Different letters within a treatment indicate significant differences ($P < 0.05$).

thors (LIZARRAGA *et al.,* 1989; NAVARRO *et al.,* 1998; SELGAS *et al.,* 1999) have reported an increase in the relative percentage of free linolenic acid during ripening, while others (ZALACAIN *et al.,* 1997; LIZASO *et al.,* 1999) have noted a progressive decrease.

Regarding the twenty carbon atoms, the relative percentage of $C_{20:1}$ rose after 7 days of ripening in sausages made at the beginning of the hunting season (A and C), while that of $C_{20:2}$ increased over the same period in all the treatments except treatment B. $C_{20:3}$ was detected after 7 days in the A, B and C treatments, and its relative percentage remained stable throughout the process in the A, B and D treatments; the values increased after 7 and 21 days of ripening in treatment C. Finally, the relative percentage of arachidonic acid ($C_{20:4}$) increased after 7 days in the end-of-season treatments (B and D), and remained stable in treatment B, but decreased to the initial values after 14 days in treatment D. NAVARRO *et al.* (1998) and BOLUMAR *et al.* (2001) reported a relative increase in $C_{20:1}$, $C_{20:2}$ and $C_{20:3}$ during *salchichón* ripening. MELGAR *et al.* (1990) and NAVARRO *et al.* (1998) noted an increase in the relative percentage of $C_{20:4}$ during ripening in chorizo and *salchichón*, whereas BOLUMAR *et al.* (2001) recorded fairly constant values for this acid during *salchichón* ripening.

In all cases, monounsaturated FFA (MUFFA) predominated, with an overall relative percentage between 36.3 and 56.2%. This fraction increased after 7 days of ripening in the A, C and D treatments, but decreased after the same period in treatment B, thereafter the value increased to near initial values. There was no significant change in total saturated FFA (SFFA) in any of the four types of chorizo sausages; however, total polyunsaturated FFA (PUFFA) levels decreased mainly after 7 days (A and C) and after 14 days (B and D). BOLUMAR *et al.* (2001) reported a decline in the saturated fraction and an increase in the monounsaturated fraction; the polyunsaturated fraction showed an initial increase but then declined throughout the ripening period.

After 45 days of vacuum-packed storage, no significant changes were noted in the relative percentages of FFA, except for an increase in palmitic acid in the D treatment.

Lipid oxidation

Determination of thiobarbituric-reactive substances (TBARS) can be used to measure products of lipid oxidation. Lipid oxidation influences flavour development in dry-cured sausages, and is influenced by several factors, including the presence of oxygen, moisture levels, temperature, and high unsaturation levels. TBARS behaviour was monitored during ripening under controlled conditions (A and B; Fig. 4) and natural conditions (C and D; Fig. 5). In all four types of chorizo sausages, there was a progressive increase in lipid oxidation throughout the 21-day ripening period; the difference was significant after 7 days for end-of-season treatments (B and D) and after 14 days for beginning-of-season treatments (A and C). After 45 days of vacuum-packed storage at room temperature, a significant increase ($P<0.05$) in lipid oxidation was observed in all treatments. SUMMO *et al.* (2006) also reported a significant increase in TBARS as a consequence of 40 days of vacuum-packed storage of dry pork sausages. Chorizo sausages made under controlled conditions displayed higher TBARS levels regardless of hunting-season stage, values rang-

ing from 0.9-1.1 mg MA/kg DM (day 0) to 11.1-18.2 mg MA/kg DM (after 21 days of ripening); values for treatments made in natural conditions ranged from 0.4-0.5 mg MA/kg (day 0) to 1.2-1.4 mg MA/kg DM (after 21 days of ripening). This difference may be due to the absence of marked variations in temperature and humidity in the A and B treatments, which could enhance enzyme activity and oxidative reactions. No significant correlation was found between the FFA content and the TBARS of the different types of chorizo sausages. The values found for chorizo sausages made under natural conditions at the end of ripening were similar to those noted by other authors in dry pork and beef sausages, which ranged from 0.8 to 2.8 mg MA/kg DM (ANSORENA *et al.,* 1998; BRUNA *et al.,* 2001).

CONCLUSIONS

Venison obtained at the beginning of the hunting season had a lower fat content than venison obtained at the end of the hunting season. The FFA profile of the intramuscular fat in venison always showed that the amount of SFFA was higher than MFFA and PUFFA, independent of the season. FFA profiles of venison chorizo sausages was more similar to the FFA profiles of pork back fat than to FFA profiles of venison fat. MFFA predominated in venison chorizo sausages. Concerning ripening, the FFA profiles changed considerably after the first 7 days in all types of chorizo sausages. The total FFA content increased markedly during this time (the MFFA increased and the PUFFA decreased), except in the treatment made at the end of the hunting season and ripened under controlled conditions. Chorizo sausages processed under controlled conditions had higher TBARS values and showed a progressive increase in lipid oxidation during the 21-day ripening period and after 45 days of storage in vacuum packaging in all four treatments.

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PAPER

CITRININ RISK IN BLACK AND GREEN TABLE OLIVES: SIMULTANEOUS DETERMINATION WITH OCHRATOXIN-A BY OPTIMIZED EXTRACTION AND IAC-HPLC-FD

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ABSTRACT

Mycotoxin citrinin (CIT) and ochratoxin A (OTA) were simultaneously identified using immunoaffinity column-high performance liquid chromatography with fluorescence detection (IAC-HPLC-FD) (Ex.333 nm; Em:495 nm) after an optimized extraction procedure. Both mycotoxins were eluted on a C_{18} RP support (250 × 4.6 mm I.D., ODS2, 5 μm particles) using an isocratic eluent consisting of acetonitrile/water/formic acid (60/38/2, v/v/v), acidified to pH 2.5 and pumped at a flow rate of 1.0 mL min^{-1}. The four categories of citrinin levels [0-0.55; 1.56-2.0; 0.66-2.64; 5.76-14.55 μg kg^{-1} of CIT] and three categories of ochratoxin levels [0 - < 0.1; 0.1-0.25; 0.30- 0.46 μg kg^{-1} of OTA] were found in 88 groups of olive samples. Recovery studies [y= 21416x - 7919.4 (R^2=0.9998) for citrinin and y= 0.0001x + 0.0074 (R^2=0.9999) for ochratoxin A] were performed and the mean analytical recoveries detected in CIT and OTA in table olives ranged from 92.65 - 96.83% and 88.92 - 95.58%, respectively. Limit of detection (LOD) was equivalent to 0.05 μg/kg for both CIT and OTA. With the proposed method, CIT and OTA were both quickly determined in table olives and could be used to detect of mycotoxinic risks in a HACCP quality system of olive and olive-based food products.

- Keywords: citrinin, HPLC, mycotoxin, ochratoxin-A, olive -

INTRODUCTION

The mycotoxin citrinin (CIT) (Fig. 1) and ochratoxin-A (OTA) are formed as a secondary metabolic product of the mould fungi *Penicillium*, *Aspergillus*, *Pythium* and *Monascus* on agricultural products (BRAGULAT *et al.*, 2008; BLANC *et al.*,1995). CIT and OTA have nephrotoxic (kidney damaging) and genotoxic potential (FÖLLMANN, 1999) based on *in vitro* tests and may have a carcinogenic effect in humans (ARAI and HIBINO, 1983; NTP, 1989; FRANK, 1992). OTA has been classified as a possible human carcinogen (Group 2B) by the International Agency for Research on Cancer based on animal data and epidemiological studies on humans (O'BRIEN and DIETRICH, 2005). It is reported that CIT enhances the carcinogenicity of OTA (KANISAWA, 1984; JESWAL, 1995). Recent studies confirm the impact of OTA and CIT *in vivo* (PFOHL-LESZKOWIZ *et al.*, 2008; 2007a,b).

A limited number of chromatographic studies to determine citrinin and ochratoxin determination in various foods including cereal grains, breakfast cereals and animal foodstuffs have been published (BRAGULAT *et al.*, 2008; SCUDAMORE and HETMANSKI, 1995; LEPOM, 1986; REINHARD and ZIMMERLI, 1999; SCUDAMORE *et al.*, 1998; VRABCHEVA *et al.*, 2000; MEISTER, 2003; MOLINIÉ *et al.*, 2005; SETSUKO *et al.*, 2008; TRI NGUYEN *et al.*, 2007). Some enzyme immunoassays (EIA) in the ppb-range have also been performed to determine citrinin and ochratoxin levels (VRABCHEVA *et al.*, 2000). As a result of cross reactions, EIAs frequently give high analytical results, therefore, the results of EIAs must be confirmed by using a sensitive analytical method such as HPLC.

Table olives (*Olea europaea* L.) are valuable commodities worldwide. Black and green table olives are frequently consumed for breakfast throughout the Mediterranean area, so citrinin and ochratoxin contaminated olives could be a potential risk to consumers. Only two studies were found in the literature that dealt with CIT in table olives. Using HPLC, 0.45-0.52 μg CIT/kg were found in black table olives sold on Moroccan markets (EL ADLOUNI *et al.*, 2006), 77% of the 69 black table olives contained CIT in Turkey, Marmara and the Aegean areas in 2000-2001. Pre-coated silica gel TLC plates and UV spectrometry were used in this analysis (HEPERKAN *et al.*, 2006).

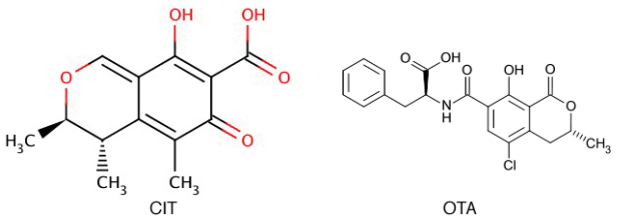


Fig. 1 - Chemical structure of mycotoxin citrinin (CIT) and ochratoxin-A (OTA).

No studies were found that reported an easily applicable, validated, reliable and sensitive IAC-HPLC method for the lower ppb range regarding simultaneous detection of CIT and OTA in olive and olive-based products. In this study, CIT and OTA were determined simultaneously in black and green table olives by immunoaffinity column-high performance liquid chromatography with fluorescence detection (IAC-HPLC-FD) after optimized extraction.

MATERIAL AND METHODS

Chemicals

Citrinin [Cas number: 518-75-2] and ochratoxin-A [CAS Number: 303-47-9] were purchased from Sigma Aldrich (St. Louis, MO, USA). All other chemicals and solvents (HPLC grade) were obtained from Merck (Darmstadt, Germany).

Table olive material

Black and green table olives were obtained from companies and local retail outlets (Table 1) in İzmir, Akhisar/Manisa, Havran/Balıkesir and Gemlik/Bursa in 2007/2008. For the professionally controlled treatments, HACCP points were carried out in companies. All samples were transferred at 4°±1°C to the quality laboratory and were immediately prepared for analysis.

Preparation of table olives

Fifty g of each sample were placed in a sterile stomacher bag (Fisher Scientific, Pittsburgh, PA, USA) and homogenized via stomacher (Interscience, BagMixer, Cole-Parmer, London, UK) after pre-homogenization in an aseptically sterilized stainless-steel blender (Waring, Torrington, CT, USA).

Analytical method of simultaneous extraction of citrinin and ochratoxin

Citrinin and ochratoxin were extracted using a modified method of EL ADLOUNI *et al.* (2006). Twenty five g of fresh pitted black and green table olives of each group were homogenized in a blender (Waring, Torrington, CT, USA). The homogenized samples were then mixed with acetonitrile (180 mL) + 4% KCl (20 mL) + 20% H_2SO_4 (2 mL) for 4 min (at 3500×*g*) and filtered through Whatman No. 4. Fifty mL of hexane were added to the filtrate and shaken in a separatory funnel for 10 min. Three phases, lipid (1), phenolic (2) and mycotoxin (citrinin + ochratoxin) (3) were separated as shown in Fig. 2. Ten mL of chloroform were added to 30 mL of the separated bottom phase (citrinin phase).

The mixture (40 mL) for each sample was then passed through an immunoaffinity column (IAC)

Table 1 - Number of samples based on the sampling region.

Black table olive samples (*n*=63)				**Green table olive samples** (*n*=25)			
Aegean region		Marmara region		Aegean region		Marmara region	
İzmir and Akhisar/Manisa markets	İzmir and Akhisar/Manisa companies	Balıkesir and Bursa markets	Havran/Balıkesir and Gemlik/ Bursa companies	İzmir and Akhisar/Manisa markets	İzmir and Akhisar/Manisa companies	Balıkesir and Bursa markets	Havran/Balıkesir and Gemlik/ Bursa companies
20	10	20	13	7	6	6	6

Table 2 - The presence and concentrations of citrinin and ochratoxin mycotoxins in black and green table olives obtained from two areas in Turkey[ab].

Citrinin (CIT) levels (µg kg^{-1})	**Mean[a] (µg kg^{-1})**	**Black table olives** (*n*=63)				**Green table olives** (*n*=25)			
		Aegean		Marmara		Aegean		Marmara	
		Number of samples (*n*=30)	Frequency (%)	Number of samples (*n*=33)	Frequency (%)	Number of samples (*n*=13)	Frequency (%)	Number of samples (*n*=12)	Frequency (%)
0 - 0.55	0.21±0.09	6	20%	3	9%	4	30%	3	30%
1.56 - 2.0	1.82±0.13	3	10%	2	6%	1	8%	ND	ND
0.66 - 2.64	2.05± 0.42	2	7%	7	21%	2	15%	2	17%
5.76 - 14.55	7.58±0.60	3	10%	9	27%	1	8%	2	17%
		14	**47%**	**21**	**63%**	**8**	**61%**	**7**	**64%**
Ochratoxin (OTA) levels (µg kg^{-1})	**Mean[a] (µg kg^{-1})**	**Black table olives** (*n*=63)				**Green table olives** (*n*=25)			
		Aegean		Marmara		Aegean		Marmara	
		Number of samples (*n*=30)	Frequency (%)	Number of samples (*n*=33)	Frequency (%)	Number of samples (*n*=13)	Frequency (%)	Number of samples (*n*=12)	Frequency (%)
0 - <0.1	0.02±0.00	17	56%	19	58%	9	70%	8	67
0.1 - 0.25	0.20±0.03	10	33%	9	27%	ND	ND	2	17
0.30 - 0.46	0.33±0.02	1	3%	3	9%	ND	ND	ND	ND
		28	**92%**	**31**	**94%**	**9**	**70%**	**12**	**84%**

[a]mean concentrations with ± standard deviations (n=3);
not detected = ND.

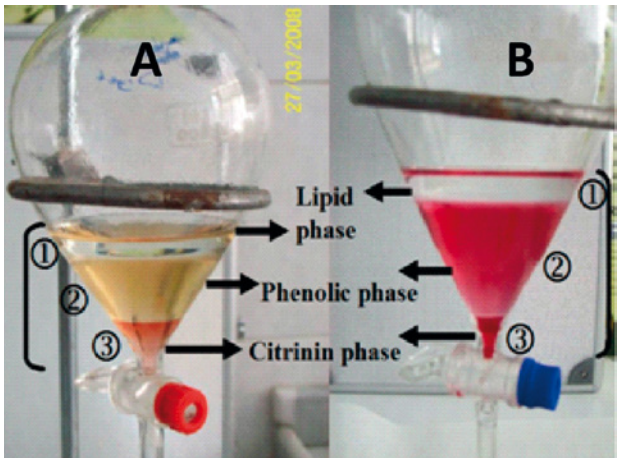


Fig. 2 - Three phases in black table olive solutions (A; light black table olive extract; B; normal fatted and salted black table olive extract).

(R-Biopharm Rhône Ltd., UK) at a flow rate of 1-2 drops/s. The eluate was evaporated to dryness in a water bath at 40°C (rotary evaporator, Heidolph, Cole-Parmer, London, UK) and was dried under N_2 atmosphere. The mobile phase (500 µL) acetonitrile/water/formic acid (60/38/2, v/v/v) (pH 2.5) was added to the aseptically dried extract, and 10 µL of this final solution was injected into the HPLC for citrinin and ochratoxin analysis. The HPLC citrinin data were confirmed by ELISA kits (RIDASCREEN FAST Citrinin, R-Biopharm AG, Darmstadt, Germany). Phases 1 and 2 of the olive extracts were confirmed by fatty acid profiles and phenolic substance analysis, respectively according to our previous data (TOKUŞOĞLU and BOZOĞLU, 2009; TOKUŞOĞLU *et al.*, 2008, 2009). Individual phenolics were confirmed by retention time and quantified on a dry basis against standard plots of known phenolic standards (0.10-15 g/g) [y= 3.142 x + 0.026 (R = 0.9996)]. The GC method of fatty acid profiles also validated the table olive lipid content within the 95% confidence limits [y= 2.784 x + 0.096 (R = 0.9999)] (TOKUŞOĞLU *et al.*, 2009).

Simultaneous HPLC analysis of citrinin and ochratoxin

Table olive extracts were analyzed by isocratic high performance liquid chromatography (HPLC) using fluorescence detection (Ex.333 nm; Em:495 nm). The HPLC equipment included a Shimadzu Class-VP and C18 column (Waters Spherisorb 5 µm, ODS2, 4.6 mm × 250 mm, Milford, MA, USA), a mobil phase mixture of acetonitrile/water/formic acid (60/38/2, v/v/v) (pH 2.5) was used and the flow rate was 1.0 mL min^{-1}. Detector sensitivity was 0.05 A.U.F.S and column oven was set at 30°C. Ten µL were injected and total elution time was 12 min. All determinations were performed on three separate extractions for each sample and each was injected in duplicate (n=6).

Method validation and recovery of CIT and OTA

The presence of CIT and OTA was confirmed by retention time and was quantified on a dry basis against standard plots of the mycotoxin standards using the same HPLC system under the same conditions. The mean retention times (R.T.) of CIT and OTA were 6.92 ± 0.1 min and 8.23 ± 0.2 min, respectively.

From the stock standard mixture, the working calibration solutions were prepared in methanol resulting in concentration levels of 0.5, 1.0, 5.0, 10.0, 25.0, 75.0, 150.0 µg/kg for citrinin [y= 21416x - 7919.4 (R^2=0.9998)] (Fig. 3a) and 0.125, 0.25, 0.5, 1.0, 2.5, 5.0 µg/kg for ochratoxin-A [y= 0.0001x + 0.0074 (R^2=0.9999)] (Fig. 3b), CIT and OTA standards had linear calibration curves through the origin. The HPLC procedure was validated at the 95% confidence limits for the CIT and OTA determination in black and green olives.

Recoveries were determined by analyzing spiked extracts at 0.5, 2.5, 5.0, 10.0, 25.0 µg/kg of CIT and 0.5, 1.0, 2.5, 5.0, 10.0 µg/kg of OTA. The mean analytical recoveries for spiked samples were detected in CIT and OTA in table olive extracts altered from 92.65 - 96.83% and 88.92 - 95.58%, respectively. The mean analytical recoveries for spiking ranged from 92.96 to 96.83% (CIT) and 90.76 to 95.58% (OTA) for black olives and from 92.65 to 96.71% (CIT) and 88.92 to 93.46% (OTA) for green table olives. The

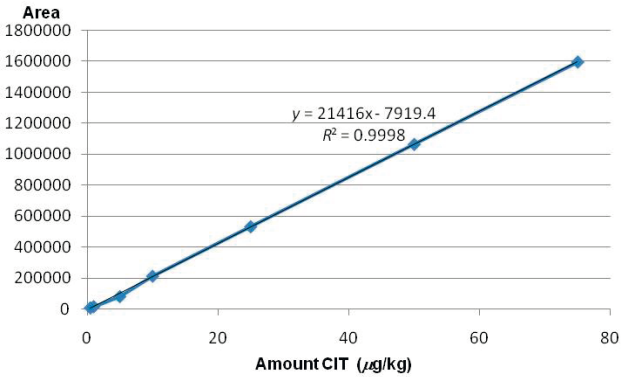


Fig. 3a - The calibration graph of citrinin (CIT).

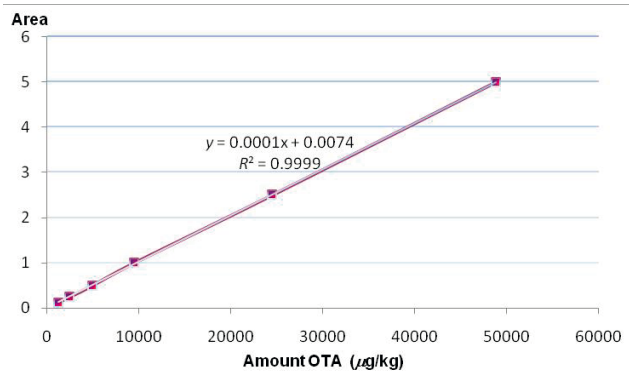


Fig. 3b - The calibration graph of ochratoxin (OTA).

limit of detection (LOD) was equivalent to 0.05 μg/kg for both CIT and OTA.

Statistical analysis

The statistical analysis was performed with the Microsoft version of SPSS 10.0 and significant differences between groups were analysed by ANOVA test.

RESULTS

The occurrence of citrinin (CIT) and ochratoxin A (OTA) was investigated in 63 groups of black table olives and 25 groups of green table olives collected in olive-producing areas in Turkey after the harvest in 2007-2008.

The distribution of the number of samples based on the sampling region is shown in Table 1 along with a map (Fig. 4) which shows the areas of olive harvest in Aegean and Marmara, Turkey.

The black (*n*=30) and green (*n*=13) olive samples from the Aegean Region were collected from İzmir markets in Edremit, Ayvalık, Akhisar, Gemlik and Urla and Akhisar/Manisa markets, including Akhisar; olives were also obtained from İzmir and Akhisar/Manisa olive collecting-manufacturing KOBIs. The Marmara Region black (*n*=33) and green (*n*=12) olive samples were collected from the Balıkesir and Bursa markets including Havran and Gemlik; olives were also obtained from the Fora Company/ Havran/Balıkesir and the Uludağ Company/Gemlik/Bursa (Table 1, Fig. 4).

Citrinin and ochratoxin A were perfectly separated under the chromatographic conditions used (Fig. 5 and 6) and this IAC-HPLC-FD procedure provided the perfect baseline simultaneous separation of CIT and OTA in table olives. As shown in Figs. 5 and 6, the CIT peak in our procedure had a shorter retention time than the value reported by EL ADLOUNI *et al.* (2006), which was more than 7 min (EL ADLOUNI *et al.*, 2006). CIT and OTA were simultaneously and quantitatively determined in this separation and the limit of detection (LOD) for both CIT and OTA was equivalent to 0.05 μg kg^{-1}.

The presence and concentrations of citrinin and ochratoxin in black and green table olives from the Aegean and Marmara areas were an important problem in 2007-2008. The four categories of citrinin (CIT) levels [0-0.55 μg kg^{-1}; 1.56 - 2.0 μg kg^{-1}; 0.66 - 2.64 μg kg^{-1}; 5.76 -14.55 μg kg^{-1}] were found in 88 groups of olive samples (Table 2). In the black table olives (*n*=63) obtained from the Aegean and Marmara areas, 47 and 63% of the samples, respectively, were found to contain CIT whereas in the green table olives (*n*=25) from the Aegean and Marmara areas, 61 and 64% of the samples, respectively, contained CIT (Table 2).

Nine of the thirty-three Marmara black olive samples analyzed had CIT concentrations ranging from 5.76 to 14.55 μg kg^{-1} (avg.7.58 ± 0.60 μg kg^{-1}) and seven of thirty-three samples ranged from 0.66 to 2.64 μg kg^{-1} (avg. 2.05 ± 0.42 μg kg^{-1}). It is shown that 35 of 63 (56%) black table olives and 15 of 25 (60%) green table olives were contaminated with citrinin (Table 2). The presence of CIT was similar in the two types of table

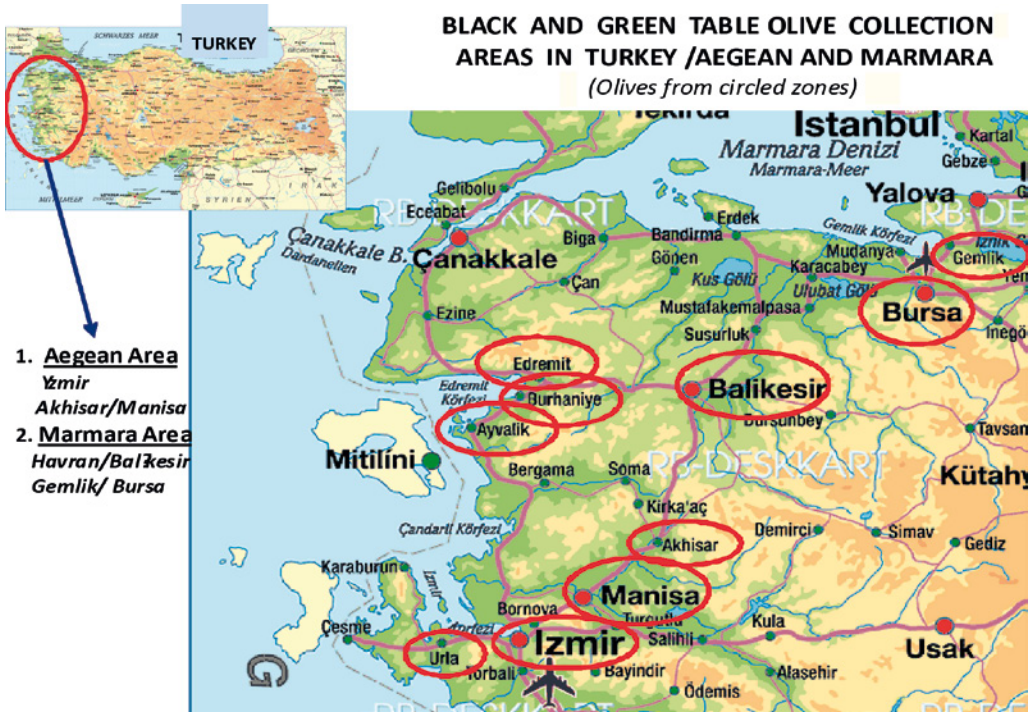


Fig. 4 - Olive collection points in Aegean and Marmara/Turkey.

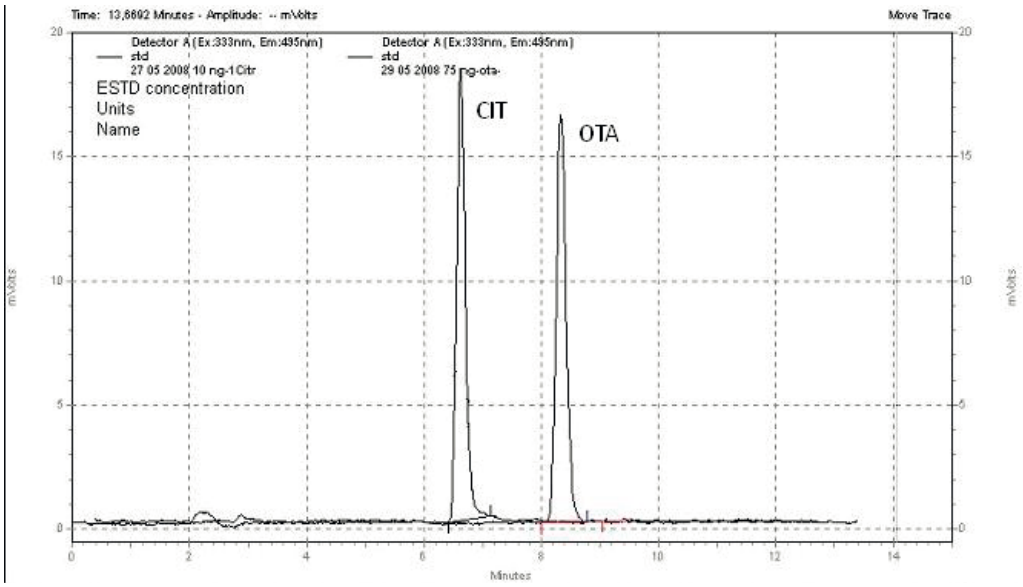


Fig. 5 -The chromatogram of simultaneous determination of citrinin (CIT) and ochratoxin (OTA).

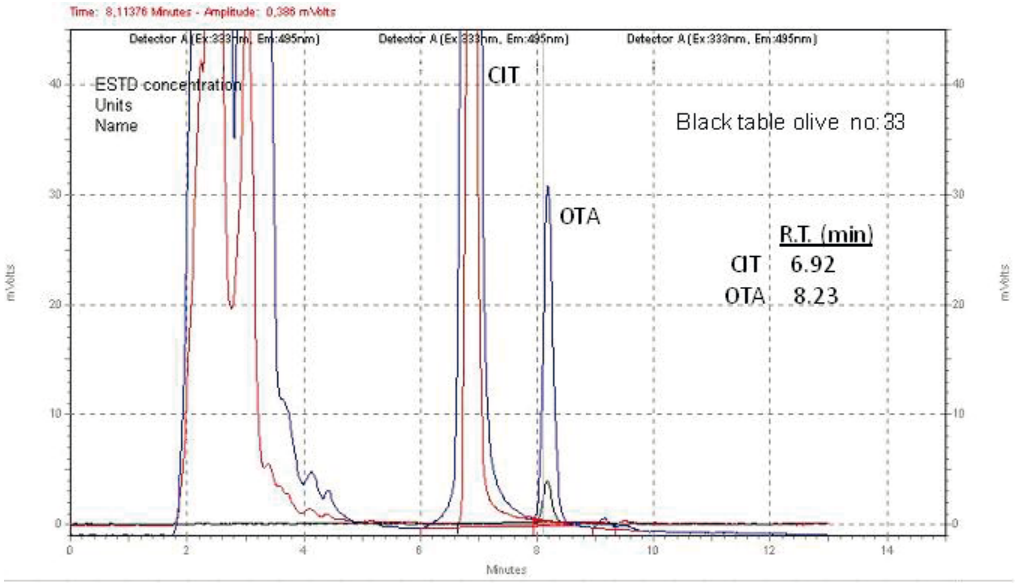


Fig. 6 - Citrinin (CIT) and ochratoxin (OTA) in black table olive extract from two different locations.

olives, the occurrence in black olives was higher than in green olives (Table 2).

Based on an HPLC study, EL ADLOUNI *et al.* (2006) reported that 0.45-0.52 μg CIT kg^{-1} were found in Moroccan black table olives. The results in our study (Table 1) are in accordance with the study described by EL ADLOUNI *et al.* (2006), CIT contamination of our samples from uncontrolled areas was higher than that in the study of EL ADLOUNI *et al.* (2006) (Table 2).

The highest CIT contamination levels in our study (CIT amount > 0.55 μg/kg) were found in uncontrolled packaged samples from markets and unpackaged olives from retailers. This was probably due to contamination during the manufacturing process while under storage conditions (Table 2).

Mycotoxin in table olives may be formed depending on the olive microflora, olive genetic variety, agronomic, climatological and ecological effects, the storage conditions of harvested olives, brine and packaging technologies used in small- and medium-sized enterprises (KOBIs) (TOKUŞOĞLU, 2008). Citrinin especially occurs in the white scum on the surfaces of olive docks or olive vats. It is not perceived by organoleptical tests and can not be removed by the washing process used by the olive company (TOKUŞOĞLU *et al.*, 2009; TOKUŞOĞLU and BOZOĞLU, 2009). After the harvest, olives are steeped on the olive docks or in olive vats for the traditional manufacturing of table olives. Olive microorganisms may form causing a deterioration of the soft tissue and a malodorous fermentation which also depends on the fermentation conditions in the docks and vats. The brine surface in the docks

and vats can be covered by a dense moldy layer. Citrinin mycotoxin can form in these layers as a secondary metabolic product of the mould fungi especially *Penicillium* and *Aspergillus*. At the marketing stage, mould growth may be visible in transparent packages of table olives. Consumer acceptance, quality and shelf life of the olives could diminish. These are critical control points (CCP) for the olive manufacturer and should be inspected (TOKUŞOĞLU *et al.*, 2009; TOKUŞOĞLU and BOZOĞLU, 2009; KABAHASAN, 2008).

Citrinin is produced by some *Penicillium*, *Aspergillus*, and *Monascus* species (BRAGULAT *et al.*, 2008). It is reported that the *Penicillium* spp. was the dominant flora in table olives and the mould species *P. crustosum, P. roqueforti* and *P. viridicatum* were encountered most frequently in the preliminary studies on black and green table olives by TOKUŞOĞLU *et al.* (2008). Mould growth was observed in 14 out of 30 (47%) samples obtained from the Aegean Area control samples and in 21 out of 33 samples (64%) obtained from the Marmara Area samples in Turkey (TOKUŞOĞLU *et al.*, 2009).

In a survey conducted by HEPERKAN *et al.* (2006), in markets from Istanbul and Aegean areas in 2000-2001, 77% of the 69 black table olives were found to contain CIT. Pre-coated silica gel TLC plates and UV spectrometry were used and according to their survey, CIT levels in black table olives were very high (25-375 $\mu g kg^{-1}$) (HEPERKAN *et al.*, 2006). The amount of CIT contamination found in our research was much lower (Table 2) than that reported by HEPERKAN *et al.* (2006).

In our study, three ochratoxin levels [0 - < 0.1 $\mu g\ kg^{-1}$; 0.1-0.25 $\mu g\ kg^{-1}$; 0.30 - 0.46 $\mu g\ kg^{-1}$ of OTA] were found in 88 groups of olive samples (Table 2). Based on our data in the black table olives (*n*=63) obtained from the Aegean and Marmara areas, a total of 92 and 94% of the samples, respectively, contained OTA, whereas in the green table olives (*n*=25) from the Aegean and Marmara areas, 70 and 84% of the samples, respectively, were contaminated with OTA (Table 2). The OTA occurrence in black olives was higher than by in green olives. Thirty-one samples out of 33 (94%) were contaminated by OTA in the black olive samples from Marmara (Table 2).

The black table olive samples from the Aegean (56%) and Marmara (58%) areas and the green table olives from the Aegean (70%) and Marmara (67%) areas had OTA concentrations above the LOD value of OTA (0.05 μg/kg) (Table 2).

Based on the Joint Expert Committee on Food and Additives (JECFA), the provisional tolerable weekly intake (TWI) of OTA is 100 ng/kg bodyweight/week for a pig. This nephrotoxicity is equivalent to 6 μg/week for a person weighing 60 kg (CASTEGNARO *et al.*, 2006).

According to our data, the Turkish daily intake of CIT and OTA through the consumption of table olives at breakfast [avg.five olives (4-5 g/day)] could contribute 1.56-2.0 $\mu g kg^{-1}$ of CIT intake and 0.1-0.25 $\mu g\ kg^{-1}$ of OTA intake, daily.

Our results suggest that human exposure to CIT and OTA through the consumption of black and green olives could contribute significantly to the total daily intake of these mycotoxins and consequently could be a public health hazard. In addition, consideration must be given to the possibility of synergism or additive effects with other mycotoxins, like AFB_1, present in the same food commodities contaminated even at low levels (ARAI and HIBINO,1983) (Commission of the European Community, Scientific committee on Food Opinion on Ochratoxin A. CS/CNTM/MTC/14 Final, Annex II to Document XXI/2210/98, Brussels: CEC, 1998).

Occurrence of mycotoxins in black and green table olives is related to the mycotoxigenic mold flora that forms in olives. Olive tanks and vats should be checked, regularly. There is a close association between geographic area and the presence of mycotoxin citrinin. Western Turkey is a geographical area where the climatic conditions are quite temperate, and the mountains run parallel to the coasts, so there are prominent discrepancies in climatic conditions from one region to another. The coastal regions of Aegean and Marmara receive rain and have milder climates, while the inland plateau areas (Inland Aegean and Marmara) have hot summers and cold winters with limited rainfall. The olive fly (olive insect) is also an important factor and contaminated olives develop these mycotoxins.

Mandatory, regularly scheduled controls and observations must be applied. Good handling procedures, application of GAP (Good Agricultural Practices) after harvesting in olive orchards and GMP (Good Manufacturing Practices) for manufacturing and storage in companies are required to prevent possible CIT and OTA contamination in olives and to reduce the risk of the mycotoxin-producing moulds. Currently, no limits have been set for CIT by the Turkish government or by the European Union, some countries may set stringent levels based on the dietary intake of their populations (PARK and TROXELL, 2002).

CONCLUSIONS

Table olives are mainly consumed as breakfast foodstuffs throughout the Mediterrenean area. The results of our research indicate that black and green table olives could be a considerable source of CIT in the human diet. Black and green table olives contained 0.21-7.58 $\mu g\ kg^{-1}$ of citrinin and 0.02- 0.33 $\mu g\ kg^{-1}$ of ochratoxin. The optimized extraction and IAC-HPLC-FD method is an easily applicable, reliable and sensitive method for determining the lower ppb range of CIT and OTA. This analytical method could be used for rapid detection of citrinin and ochratoxin in black and green olives for GMP and HACCP (Hazard Analysis and Critical Control Point) analysis in the olive and olive-based sec-

tor. Other phases of black and green table olive solution obtained in the extraction stage of the study can also be used for lipid (fatty acid) analysis and phenolic substance analysis in olives.

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PAPER

NEW CEREAL FOOD PRODUCTS WITH DRIED SHIITAKE MUSHROOM (*LENTINULA EDODES)* ADDED AS A SOURCE OF SELECTED NUTRIENTS

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ABSTRACT

Shiitake mushrooms (*Lentinulla edodes*) have documented health benefits and a relatively high nutritive value and are recommended as a daily dietary supplement. However before recommending that products with the addition of shiitake be included in the diet, especially as a source of minerals, the contents of these elements should be determined and the sorption of micro-elements from these products must be assessed. The aim of this study was to assess the chemical composition of cookies and breads with 10 and 20% dried shiitake added to the flour and to estimate the availability of Fe, Cu and Zn in such products under pH conditions similar to those found in the human digestive tract. The products were characterized by high contents of fiber, Fe, Cu, Mg and K, and low amounts of Na and Ca. The percentage of dried mushrooms added to the products had a significant effect on contents of insoluble fiber, Fe, Cu, Zn, K and Na. The highest amounts of Fe, Cu and Zn were recorded in bread with a 20% addition of dried mushrooms. A low sorption of Fe, Zn and Cu was observed in the material analyzed at pH=1.8, while at pH=8.7 the sorption values did not exceed 18%. The test results show that the products may be recommended as a dietary supplement.

- Key words: cereal food, nutritional value, shiitake, sorption of minerals -

INTRODUCTION

Mushrooms have traditionally been used in the human diet and in recent years the cultivation has increased. From a nutritional point of view, mushrooms are low in calories and fat, have higher protein content than most vegetables, and are rich in vitamins (B, D) and minerals (K, P, Mg, Ca, Zn, Cu, Mn, Se) (ISILOGLU *et al.*, 2001; BERNAS *et al.*, 2006). Cultivated mushrooms, especially shiitake (*Lentinula edodes),* have prebiotic properties, relatively high nutritive value and high contents of minerals. In many Countries they are used as a supplement to the daily diet. Shiitake has biologically active components. Studies have shown that these mushrooms contain effective substances which decrease the LDL fraction of cholesterol in blood. They also prevent the accumulation of serum triacylglycerols, thus decreasing the risk of developing cardiovascular disease (BOBEK *et al.*, 1993; STAMETS, 1993; WASSER and WEIS, 1999; MANZI and PIZZOFERRATO, 2000; MAU and SONG, 2002; ROWAN and SULLIVAN, 2002).

However before recommending the introduction of dried shiitake to the diet, particularly as a source of minerals, the mineral content should be determined as well as the capacity of these products to bind micro-elements. Trace minerals have specific biological functions that are essential for the absorption and utilization of many nutrients; they aid enzymes and hormones in vital activities. Iron plays a major role in oxygen transport and storage and is a component of hemoglobin in red blood cells and myoglobin in muscle cells. Cellular energy production requires many trace minerals, including iron, copper and zinc, which act as enzyme cofactors in the synthesis of many proteins, hormones, neurotransmitters and genetic material. Zinc is also essential for many other bodily functions, such as growth, development of sexual organs and reproduction. Zinc and copper prevent oxidative damage to cells. In addition to clinical deficiency diseases such as anemia and goiter, research indicates that trace minerals play a role in the development, prevention, and treatment of chronic diseases (LEE *et al.*, 2001; ZECCA *et al.*, 2004; VALKO *et al.*, 2005).

Balanced mineral levels in the human body depend on many factors including their content in the total daily food ration, as well as on their release and absorption in the digestive system (SANDSTROM, 2001).

Many Authors have reported the possibility of lowering mineral substance levels as a result of a high dietary fiber diet. On the basis of the literature data, it can be hypotheses that metal binding capacity of fiber preparations depends on the dietary fiber source, adsorbent type, processing method, pH and temperature of the medium, as well as on the metal (THOMPSON and WEBER, 1979; STACHOWIAK and GAWECKI, 1989; STACHOWIAK and KUBIAK, 1990; KORCZAK *et al.*, 1995; GORECKA and STACHOWIAK, 2002; STACHOWIAK and SMIGIELSKA, 2004; REGUŁA and SIWULSKI, 2007).

The aim of this study was to assess the chemical composition of cereal products with dried shiitake added and estimate the availability of Fe, Cu and Zn from these products under pH conditions similar to those found in the human digestive tract.

MATERIALS AND METHODS

Material

Cookies were made from maize flour, margarine, milk and water. While wheat flour, water, yeast and sugar were used to make bread. In order to obtain the test products with the addition of dried shiitake, 10 and 20% dried shiitake were substituted for the maize and wheat flour, respectively (Table 1).

The shiitake mushrooms were grown on a beech sawdust substrate enriched with 20% ground wheat, inoculated with granular mycelium of shiitake *cv* 'Sylvan 4080'. Incubation was carried out at the temperature of 25°C and relative humidity of 80-85%, and after incubation, mushrooms were grown at 17°-18°C and relative humidity of 85-90%. Shiitake mushroom fruiting bodies were dried at 40°C for 8 h, force dried at 70°C to constant weight and then ground into powder.

Nutritive value

Protein, fat and ash contents were determined using standard analytical methods (nitrogen according to Kjeldahl, fat according to Soxhlet, ash according to RUTKOWSKA, 1979). The protein content was calculated with a conversion factor of 4.38, taking into account the non-protein nitrogen in mushroom (AOAC, 1995). In contrast, all nitrogen compounds, including those of non-protein nature, considered when calculating the carbohydrate contents and energy values. Carbohydrates constituted the difference of 100 and the sum of water, ash, protein and fat. The energy value was calculated using the mean energy coefficients: 4 kcal/g protein, 9 kcal/g fat and 4 kcal/g carbohydrates.

Soluble dietary fiber (SDF) and insoluble dietary fiber (IDF) were determined enzymatically according to ASP *et al.* (1983). Total dietary fiber (TDF) was the sum of SDF and IDF.

The mineral and toxic metal contents were analyzed using atomic absorption spectrophotometry in a SOLAR 939 apparatus from UNICAM LIMITED (Cambridge, UK). The sample preparation for the analyses of Fe, Zn, Cd and Pb were carried out according to Polish Standard (PN-EN 14082, 2004). The Mg, Ca, K, Na and Hg contents

Table 1 - Composition of products with 10 and 20% addition of dried shiitake mushrooms (g/100 g).

Contents of	Cookies		Bread	
products (g/100 g)	10% addition	20% addition	10% addition	20% addition
Maize flour	45.5	41.7	-	-
Wheat flour	-	-	50	45.8
Shiitake powder	4.5	8.3	5	9.2
Margarine	16.6	16.6	-	-
Milk	12	12	-	-
Water	21	21	37	37
Yeast	-	-	5	5
Sugar	-	-	2.6	2.6
Salt	0.4	0.4	0.4	0.4
Baking time (min)	20		45	
Baking temperature °C	130		190	

were analyzed using methods described by LUDWICKI (1990). Analytical assays were performed in three simultaneous replications and the results are presented as grams per 100 g solids.

Sorption of minerals

To determine the capacity of bread to bind selected minerals (Cu, Zn and Fe) a methodology based on a study by STACHOWIAK and KUBIAK (1990) was applied. Product samples were shaken at the temperature of 37°C. The products were ground before being exposed to the solutions. The sorbates used were solutions containing $CuCl_2$, $ZnCl_2$ and $FeCl_3$ at the following concentrations, Cu 5 μg/mL, Zn 25 μg/mL and Fe 30 μg/mL. The dispersing solutions were buffer solutions with pH = 1.8 and pH = 8.7. Experimental conditions were set up to mirror the human digestive tract: the oral cavity and the stomach (pH = 1.8, shaking time 135 min) and the duodenum (pH = 8.7, shaking time 60 min). The solid fraction was then separated from the liquid one by centrifuging at 6,000 rpm. The Fe, Cu and Zn contents in both fractions were assayed using the AAS method. The volume of sorption was defined as the percentage ratio of the element bound by the product to the total amount of this element introduced into the system. Analytical assays were performed in three simultaneous replications.

Table 2 - Chemical composition of dried shiitake mushrooms (*Lentinula edodes).*

The energy value (kcal) and contents of macro-components (g/100 g dry matter)	
Energy value	361±1.4
Water	7.04±0.05
Protein	14.9±0.6
Fat	2.53±0.03
Carbohydrates	69.6±0.4
Ash	5.94±0.31
Soluble dietary fiber	1.95±0.32
Insoluble dietary fiber	42.0±0.3
Mineral content (mg/100 g dry matter)	
Fe	39.5±0.3
Cu	13.7±0.5
Zn	126±12
Mg	1620±4
Ca	158±0.3
K	31600±310
Na	138±1
Heavy metal content (mg/kg dry matter)	
Pb	nd*
Cd	0.232±0.012
Hg	0.005±0.0002
*nd: not detected.	

Statistical analysis

Statistical analysis was performed using ANOVA for factorial designs and the differences between individual groups were analyzed using the Tukey's HSD test. The statistical software package Statistica 7.0 by StatSoft was used.

RESULTS

The energy value, macro-components, fiber, minerals and toxic metal contents in dried shiitake powder and products with 10 and 20% dried shiitake added to the flour are presented in Tables 2, 3 and 4.

Dried shiitake (*Lentinula edodes)* was high in nutrients, particularly ash, as well as Fe, K, Mg, P, Zn and Cu. The dried mushrooms exhibited high contents of total dietary fiber (TDF). The mean level of dried shiitake mushrooms was 43.9 g/100 g dry matter. The soluble fraction was less than the insoluble fraction.

The nutritive value differed significantly in the analyzed products. The cookies had a higher energy value and fat content, the bread had a higher carbohydrate content. The added dried mush-

Table 3 - Chemical composition of cookies with 10 and 20% dried shiitake (*Lentinula edodes)* added.

The energy value (kcal) and contents of macro-components (g/100 g dry matter)		
	10% dried shiitake	20% dried shiitake
Energy value	513±1	508±4
Water	4.23±0.05[a]	6.08±0.13[b]
Protein	9.23±0.12	9.68±0.41
Fat	27.3±0.3	27.9±0.9
Carbohydrates	57.6±0.3	54.4±0.6
Ash	1.59±0.03	1.69±0.01
Soluble dietary fiber	5.36±0.51	4.89±0.15
Insoluble dietary fiber	11.6±0.1[a]	16.7±0.3[b]
Mineral content (mg/100 g dry matter)		
Fe	16.2±0.4	17.3±0.7
Cu	1.83±0.02[a]	2.72±0.04[b]
Zn	14.8±0.1[a]	19.7±0.4[b]
Mg	459±13	483±4
Ca	276±1a	309±12[b]
K	3450±110[a]	5560±90[b]
Na	717±26[a]	910±130[b]
Heavy metal content (mg/kg dry matter)		
Pb	nd*	nd*
Cd	0.166±0.01[a]	0.326±0.01[b]
Hg	0.00300±0.001	0.00200±0.0006

*nd: not detected.
Mean values with different superscript letters (a, b) in each column are significantly different (Tukey's HSD test, P<0.001).

Table 4 - Chemical composition of bread with 10% and 20% dried shiitake (*Lentinula edodes)* added.

The energy value (kcal) and contents of macro-components (g/100 g dry matter)		
	10% dried shiitake	20% dried shiitake
Energy value	425±1	427±1
Water	38.7±0.2[a]	42.7±0.1[b]
Protein	18.4±0.1	22.7±2.1
Fat	1.95±0.02	1.64±0.03
Carbohydrates	83.6±1.0	80.5±1.7
Ash	1.53±0.03	1.93±0.27
Soluble dietary fiber	1.55±0.45	1.99±0.66
Insoluble dietary fiber	10.8±0.4[a]	14.4±0.6[b]
Mineral content (mg/100 g dry matter)		
Fe	16.0±0.1[a]	23.4±1[b]
Cu	2.60±0.15[a]	3.45±0.2[b]
Zn	20.6±0.4[a]	26.1±0.6[b]
Mg	416±5[a]	488±19[b]
Ca	222±3	205±3
K	3880±200[a]	5684±80[b]
Na	665±5[a]	973±2[b]
Heavy metal content (mg/kg dry matter)		
Pb	0.0123±0.011[a]	0.0140±0.001[b]
Cd	0.382±0.021[a]	0.523±0.004[b]
Hg	0.0080±0.0006[a]	0.015±0.001[b]

Mean values with different superscript letters (a, b) in each column are significantly different (Tukey's HSD test, P<0.001).

rooms had no significant effect on the changes in the macro-component contents in either cookies or bread.

Both tested products were characterized by a high, varying content of total dietary fiber (TDF). The cookies had a mean of 18.8 g/100 g dry matter, while the bread value was 14.3 g/100 g dry matter. The soluble fraction was less then the insoluble fraction. The SDF content ranged from 1.55 g/100 g dry matter (bread with a 10% addition of dried mushrooms) to 5.36 g/100 g dry matter (cookies with a 10% addition of dried shiitake).

The percentage of dried mushrooms added to the products had a significant (P<0.001) effect on the insoluble fiber content, that increased by approx. 40% in both cookies and bread.

A significant (P<0.001) increase was also observed (from 15 to 60%) in the Fe, Cu, Zn, K and Na contents when the amount of dried shiitake added to the products changed. The highest Fe, Cu and Zn values were recorded in bread with a 20% addition of dried mushrooms. Compared to other products this one contained an average of 40% more iron and copper and 50% more zinc. All products were characterized by high Mg and K contents. An advantageous Na/K was observed both in cookies and bread. The heavy metal content never exceeded the admissible limits.

The statistical analysis of the results of the sorption of Cu, Zn and Fe by dried mushrooms showed that the level of binding for these metals was dependent (p<0.001) on the pH of the medium and the species from which the dried product was obtained.

Metal sorption values of were lowest at pH = 1.8, whereas Cu and Zn binding by dried shiitake at pH = 1.8 did not exceed 6% (Fig. 1). As the pH of the medium increased the metal binding capacity of the dried products increased. The strongest metal sorption capacity was found for dried shiitake in a medium at pH = 8.7; the highest value was reported for Fe.

The statistical analysis of the results of the availability of Cu, Zn and Fe from cookies and bread with dried shiitake added showed that the level of binding of these metals was dependent (p<0.001) on the ambient pH level and the type of product analyzed. The metal sorption values were the lowest at pH = 1.8; binding of Fe, Zn and Cu by products at this pH did not exceed 3% (Figs. 2 and 3). The addition of dried mushroom powder caused the sorption of Fe to increase from 4.5 to 10.2% in the cookies at pH = 8.7 (p<0.001)

DISCUSSION

For several years there have been reports on the prebiotic properties of shiitake (BREENE, 1990; BOBEK *et al.*, 1993; WASSER and WEIS, 1999; KALAC and SVOBODA, 2000; ISILOGLU *at al.*, 2001; SHIMADA *et al.*, 2002; BERNAS *et al.*, 2006). It is also believed that these mushrooms are a good source of micro- and macro-elements, such as K, Mg, P, Zn, Fe, and Cu. (ISILOGLU *et al.* 2001; RAJEWSKA and BALASINSKA, 2004; STACHOWIAK and SMIGIELSKA, 2004; REGUŁA and SIWULSKI 2007). These mushrooms are easy to grow and not costly to produce. While information on the nutritive value of shiitake mushrooms and their dried products is abundant in the literature, there are practically no comprehensive studies on foodstuffs prepared with these mushrooms, especially information on the range of metal binding capacity of the products analyzed, which may considerably reduce the availability of these micro-elements for the body.

This study attempted to address this problem, by adding 10 - 20% dried shiitake to cookies and bread. The products tested were characterized by a relatively high nutritive value. The protein content was similar to that recorded in cereal products; numerous Authors have reported that shiitake protein is relatively available and methionine is the amino acid that limits their nutritive value (KALAC and SVOBODA, 2000; RAJEWSKA and BALASINSKA, 2004; BERNAS *et al.*, 2006).

The high dietary fiber content, in both cookies and bread, exceeds the values recorded in cereals. High contents of ash as well as Fe, K, Mg, P, Zn and Cu were recorded in cookies and bread; the mineral content was significantly affected by the addition of dried shiitake to those products. Some Authors have suggested that foods that are rich in dietary fiber may disturb the mineral balance in the human body due to their adsorption (STACHOWIAK and KUBIAK, 1990; HARLAND and MORRIS, 1995; GORECKA and STACHOWIAK, 2002; STACHOWIAK and SMIGIELSKA, 2004, GORECKA *et al.*, 2009). Excessive adsorption of minerals is caused by the presence of phytates. To be a good source of minerals, a product cannot exhibit strong sorptive properties.

The sorption value was defined as the percentage ratio of the content of the element bound by the product with respect to the total amount of

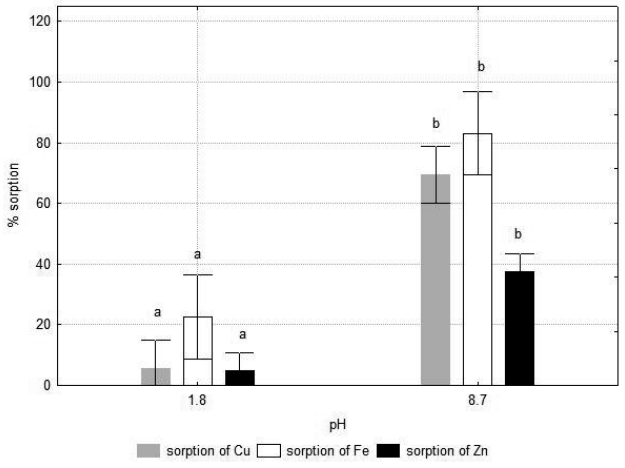


Fig. 1 - Mean sorption value of Cu, Fe and Zn ions by dry shiitake (*Lentinula edodes)* at pH=1.8 and pH=8.7. The volume of sorption was defined as the percentage ratio of the content of the element bound by the product to the total amount of this element introduced into the system.
Significant difference between pH values of each mineral is indicated by different superscript letters (Tukey's HSD test, P<0.001).

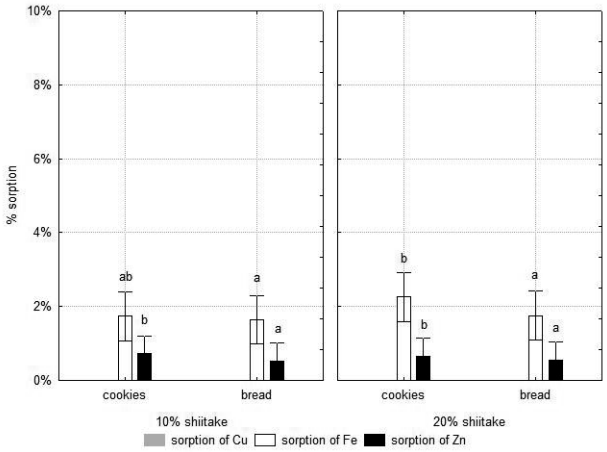


Fig. 2 - Mean sorption value of Cu, Fe and Zn ions by products at pH=1.8. The volume of sorption was defined as the percentage ratio of the content of the element bound by the product to the total amount of this element introduced into the system.
Significant difference between products for each mineral is indicated by different superscript letters (Tukey's HSD test, P<0.001).

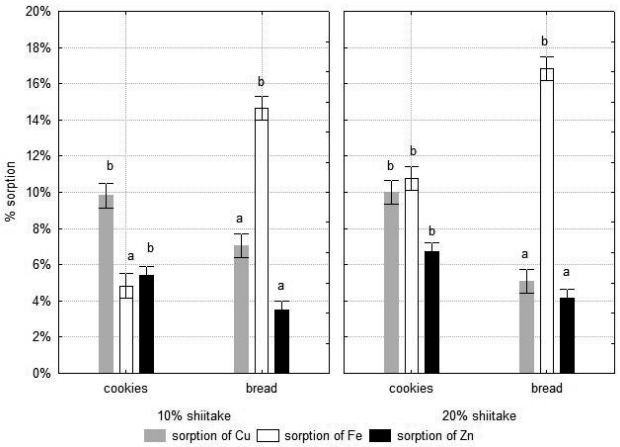


Fig. 3 - Mean sorption value of Fe, Cu and Zn ions by products at pH=8.7. The volume of sorption was defined as the percentage ratio of the content of the element bound by the product to the total amount of this element introduced into the system.
Significant difference between products for each mineral is indicated by different superscript letters (Tukey's HSD test, P<0.001).

this element introduced into the system. The sorption of Fe, Zn and Cu at pH 1.8 did not exceed 3%.

In this study the sorption of Zn, Cu and Fe was relatively high at pH = 8.7. This result is consistent with observations by other Authors that most sorbents of different origin (dried apples; dried carrot; wheat, maize, soy and rice bran; oat glumes and cellulose) bind metals at higher pH values. As acidity increased in the solution, the sorption decreased; at pH = 0.65 Cu, Zn and Fe were released (THOMPSON and WEBER, 1979; STACHOWIAK and KUBIAK, 1990; KORCZAK *et al.*, 1995; GORECKA and STACHOWIAK, 2002; REGUŁA and SIWULSKI, 2007).

KORCZAK *et al.* (1995) reported the highest cation exchange in dried carrot and dried apples at pH = 6.6 at 20°C and at pH=8.7 at 37°C, while the exchange was lower in a medium at pH = 1.8. The maximum sorption values were recorded at pH = 8.7 for Fe in bread with 10 and 20% dried mushrooms added (15 and 17%, respectively). This value is lower than those reported by other Authors (THOMPSON and WEBER, 1979; STACHOWIAK and KUBIAK, 1990; GORECKA and STACHOWIAK, 2002).

In vivo studies may be necessary to clarify the sorption capacity of these products and the mineral availability. Considering the relatively high nutritive value manifested in the protein, dietary fiber, Mg, K, Fe, Cu and Zn contents, as well as prebiotic properties reported by other Authors, the cookies and bread produced with dried mushrooms may be recommended as a dietary supplement. However, before promoting them as a good source of micro-elements, it is necessary to determine the sorption capacity and the availability of the minerals must be determined *in vivo* studies.

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PAPER

NON-DESTRUCTIVE EVALUATION OF SWEET CHERRY (*PRUNUS AVIUM* L.) RIPENESS USING AN ELECTRONIC NOSE

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ABSTRACT

The work is a preliminary study to verify the reliability of a commercial and portable electronic nose (PEN 2, Win Muster Airsense) as a tool to assess the differences among four sweet cherry cultivars and their ripening stages. Cluster Analysis was applied to maturity indices such as colour, titratable acidity, and total soluble solids in order to categorize sweet cherries into three clusters (ripening stages) referred to as "commercial ripe", "ripe" and "over-ripe". Principal Component Analysis applied to the electronic nose data highlighted the ability of the instrument to distinguish among cultivars and to assess the ripening stages of sweet cherries. From the Linear Discriminant Analysis, the electronic nose could effectively categorize fruit into the three ripening stages with the correct classification percentage of 95.0%. The predictive ability of Linear Discriminant Analysis classification model was confirmed by considering eight sweet cherries (two for each cultivar) analysed from postharvest to senescence.

- Key words: Cluster Analysis, electronic nose, fruit ripeness, Linear Discriminant Analysis, MOS sensors, Principal Component Analysis, sweet cherries -

INTRODUCTION

Quality evaluation of agricultural products is an important issue for both growers and consumers. The assessment of ripeness is an important part of quality evaluation since maturity at harvest can have a negative effect on sensory and storage properties of fruit. Many methods to monitor fruit ripeness have been proposed, but the majority of these are not practical and require the destruction of the samples (CHRISTENSEN, 1983; WANG *et al.*, 2004). This is why, nowadays, prediction of ripening stage is mainly based on practical experience or visual parameters such as colour changes, even if colour is, in many cases, only slightly correlated with fruit ripeness.

An alternative strategy for evaluating the state of ripeness consists of determining the volatiles emitted by fruit during ripening. There have been several reports on the changes in volatile composition during ripening, investigated by gas chromatography (GC) with headspace sampling, and GC combined with mass spectrometry (GC-MS) (BOUDHRIOUA *et al.*, 2003; IBANEZ *et al.*, 1998; LAVILLA *et al.*, 2002; PLUTOWSKA and WARDENCKI, 2007). These methods are not normally used to assess maturity since they are difficult to undertake and require expensive instrumentation. Furthermore, the high number of volatiles prevents a simple interpretation of results and, even if only the major volatile compounds are considered, a skilled analyst is still required.

The electronic nose (EN) overcomes some of the difficulties associated with traditional methods since it is easy to use, portable, non-destructive and rapid. The EN system is sensor-based technology which considers the total headspace volatiles and creates a smell print (SHALLER *et al.*, 1998). Unlike gas chromatography, EN does not identify all the individual components of the volatiles, but rather, responds to the whole set of volatiles in a unique digital pattern.

The EN has been successfully used in monitoring volatiles of climacteric fruit such as apples, peaches, pears, bananas and tomatoes (BREZMES *et al.*, 2000, 2001; DI NATALE *et al.*, 2001a; GOMEZ *et al.*, 2006a; GOMEZ *et al.*, 2008; LLOBET *et al.*, 1999; SAEVELS *et al.*, 2003, 2004, ZHANG *et al.*, 2008a, b). However, there is little information about using EN to detect the volatile compounds of non-climacteric fruit and monitoring their aroma changes during ripening (DI NATALE *et al.*, 2001b; GOMEZ *et al.*, 2006b).

The aim of this research was to assess the potential of a commercial and portable EN, based on 10 Metal Oxide Semiconductor (MOS) sensors to detect changes in the volatile profile of a non-climacteric fruit during ripeness and post-harvest life. The EN was tested on sweet cherries (*Prunus avium* L.) of different cultivars and ripening stages. The performance of the EN was compared with the results of classical analytical methods such as colour evaluation, titratable acidity, and total soluble solids. The data were analysed by multivariate statistical techniques including Principal Component Analysis (PCA), Cluster Analysis (CA) and Linear Discriminant Analysis (LDA).

MATERIALS AND METHODS

Four sweet cherries (*Prunus avium* L.) cultivars with the following features were chosen for the study:

-'Mora di Cazzano': medium ripening, bright and spotted red colour, medium size, juicy and excellent flavour;

-'Stella': late ripening, very dark red colour, large size, excellent sweet flavour;

-'Adriana': early ripening, dark red colour, medium-large size, sweet and low acid flavour;

-'Van': medium size, bright spotted red colour, excellent flavour, firm texture.

Sweet cherries (*Prunus avium* L.) were obtained from 22-year-old trees grafted on seedling rootstock of a commercial orchard in northern Italy in 2008. The fruit was randomly picked over three weeks from mid June to the first week of July in order to obtain three batches of fruit at different ripening stages referred to as "commercial ripe" (batch 1), "ripe" (batch 2) and "over-ripe" (batch 3). Each batch was composed of 10-12 cherries for each cultivar, selected for their homogeneous pericarp colour, uniform size and weight, absence of injuries and healthy greenish stems. In total 120 cherries from four different cultivars were considered, all of which were harvested and then left at 20°±1°C overnight and then used first for the EN analysis and then for chemical analysis (total soluble solids and titratable acidity). Furthermore, for each cultivar, two cherries of "commercial ripe" class (batch 1) were monitored with the EN at a constant temperature (20°±1°C) from the day after harvest until the fruit became senescent. Each monitoring cycle lasted about 10 days.

Electronic nose analysis

A Portable Electronic Nose (PEN2) from Win Muster Airsense (WMA) Analytics Inc. (Schwerin, Germany) was used. It consists of a sampling apparatus, a detector unit containing the array of 10 Metal Oxide Semiconductor (MOS) type chemical sensors, and a pattern recognition software (Win Muster v.1.6) for data recording and elaboration (BENEDETTI *et al.*, 2008). Table 1 lists the 10 sensors used and their main applications.

Each fruit was placed in a 50 mL airtight glass jar fitted with a pierceable Silicon/Teflon disk in the cap. After a two-hour equilibration at 20°C ± 1, the measurements began. The sweet cherry headspace was pumped over the sensor surfaces

Table 1 - Sensors and their applications in PEN2.

Number in array	Sensor name	General description	Reference
1	W1S	Aromatic compounds	Toluene, 10 mg kg^{-1}
2	W5S	Very sensitive, broad range sensitivity, react on nitrogen oxides, very sensitive with negative signal	NO_2, 1 mg kg^{-1}
3	W3C	Ammonia, used as sensor for aromatic compounds	Benzene, 10 mg kg^{-1}
4	W6S	Mainly hydrogen, selectively (breath gases)	H_2, 100 µg kg^{-1}
5	W5C	Alkanes, aromatic compounds, less polar compounds	Propane, 1 mg kg^{-1}
6	W1S	Sensitive to methane (environment) ca. 10 mg kg^{-1}. Broad range, similar to No. 8	CH_3, 100 mg kg^{-1}
7	W1W	Reacts on sulphur compounds, H_2S 0.1 mg kg^{-1}. Otherwise sensitive to many terpenes and sulphur organic compounds, which are important for smell, limonene, pyrazine	H_2S, 1 mg kg^{-1}
8	W2S	Detect alcohols, partially aromatic compounds, broad range	CO, 100 mg kg^{-1}
9	W2W	Aromatic compounds, sulphur organic compounds	H_2S, 1 mg kg^{-1}
10	W3S	Reacts on high concentrations > 100 mg kg^{-1}, sometime very selective (methane)	CH_3, 10 CH_3, 100 mg kg^{-1}

for 60 s (injection time) at a flow rate of 300 mL min^{-1}, during this time the sensor signals were recorded. After sample analysis the system was purged for 180 s with filtered air to allow re-establishment of the instrument base line. At the end of the analysis the glass jar was immediately opened in order to provide enough oxygen and avoid fruit fermentation. In each measurement cycle, the sensor drift was evaluated by including a standard solution of 5% ethanol in distilled water. For the entire experimental period (4 weeks max), no sensor drift was experienced. Each sweet cherry was evaluated three times and the average of the results was used for the statistical analysis.

Maturity indices

Skin colour was measured with a Minolta Chromameter CR- 200 reflectance colorimeter (Minolta Camera Co. Ltd., Osaka, Japan), which provided CIE L*, a* and b* values. These values were used to calculate chroma ($C^*=\sqrt{a^{*2}+b^{*2}}$), a measure of colour saturation. Juice was obtained by hand crushing 10 sweet cherries from each cultivar and ripening stage in plastic bags. This juice was used to determine the titratable acidity and soluble solids concentration. Total soluble solids were determined at 20°C with a hand refractometer (N1 Atago, Tokyo, Japan). Titratable acidity was evaluated by using a Crison compact-D titrator (Crison Strumenti S.p.a., Carpi, Italy). The titration was performed with 0.1 N NaOH to pH 8.1 and results are expressed as percent of malic acid.

Data analysis

The multivariate statistical techniques used were: Cluster Analysis (CA), Principal Component Analysis (PCA) and Linear Discriminant Analysis (LDA) were performed using Statistica software (v. 6.0, StatSoft Inc., Tulsa, Oklahoma, Usa).

PCA is a linear and unsupervised procedure that permits useful information to be extracted from the data. The data structure can be explored as well as the relationship between objects and the overall correlation of the variables (BEEBE *et al.*, 1998).

Cluster Analysis (CA) is an exploratory data analysis tool used to solve classification problems. Its object is to sort cases (people, things, events, etc) into groups, or clusters, so that the degree of association is strong between members of the same cluster and weak between members of different clusters. The hierarchy of clusters can be represented by a binary tree, called a "dendrogram". A final partition, i.e. the cluster assignment of each object, is obtained by cutting the tree at a specified level. There are many subjective choices to make in performing a cluster analysis. The linkage method, the distance measure, the level of resolution or the number of clusters have to be established on the basis of need and circumstance (GARDNER and BARTLETT, 1992).

LDA is one of the most widely used classification techniques (LACHLAN, 1992; MELOUN *et al.*, 1992). The method is a probabilistic parametric classification technique that maximizes the variance between categories and minimizes the variance within categories, by means of a data projection from a high dimensional space to a low dimensional space. In this way, a number of orthogonal linear discriminant functions equal to the number of categories minus one is obtained. The classification model was validated using a leave-one-out procedure. Each sample is removed from the data set, one at a time. The classification model is rebuilt and the sample removed is classified in this new model. All the samples are sequentially removed, reclassified and finally the percentage of correct classification is calculated. The quality of the LDA classification model was also evaluated by external validation applied to a new data set.

RESULTS AND DISCUSSION

Electronic nose response to sweet cherry aroma

Fig. 1 shows a typical response of MOS sensors. Each curve represents the conductivity variation of the sensors during the injection time (60 s) when the sweet cherry headspace is pumped over the sensor surfaces. The data are expressed as the ratio between G and G_0 where G is the conductivity of the sensors during sampling and G_0 is the conductivity of the sensors measured immediately before sampling. As can be seen, the G/G_0 increased rapidly, and stabilized or decreased slowly after 20-30 seconds. For each sensor the signal that is recorded and used for data analysis corresponds to the maximum value of G/G_0.

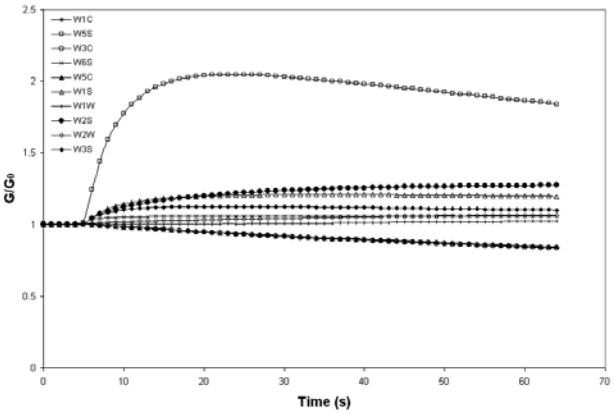


Fig. 1 - MOS sensor responses to sweet cherry aroma.

Characterization of sweet cherry cultivars by electronic nose

In order to evaluate whether EN was able to detect differences among cultivars on the basis of their aroma, PCA was carried out on the EN data collected on commercially ripe sweet cherries. Since EN variables were expressed in the same unit and the variances did not differ widely, PCA was performed in a covariance matrix. Examining the score plot (Fig. 2) in the area defined by the first two principal components (97.9% of the total variance) a clear separation of sweet cherries into four clusters according to the four cultivars is evident. In particular, on the first principal component there is a discrimination between the 'Mora di Cazzano' and 'Van' cultivars placed at the left of the plot and the 'Stella' and 'Adriana' cultivars placed at the right. On the second principal component the 'Stella' and 'Van' cultivars, placed in the upper part of the plot, are discriminated respectively from 'Adriana' and 'Mora di Cazzano' cultivars.

Statistical analysis of maturity indices

The three maturity indices, colour (L, a, b, chroma), titratable acidity and total soluble solids, were used to confirm the homogeneity of the fruit batches and also to correctly categorize fruit into the three ripening stages. For each cultivar the Hierarchical Cluster Analysis was applied to the maturity indices collected on 30 sweet cherries: fruit numbered from 1 to 10 were from batch 1, fruit from 11 to 20 from batch 2 and fruit from 21 to 30 from batch 3. Fig. 3 reports the dendrograms obtained using the Ward method and the Euclidian distance. The number of desired classes (clusters) determined where the trees were cut: cluster 1 corresponded to "commercial ripe", cluster 2 to "ripe" and cluster 3 to "over-ripe". The CA confirmed the homogeneity of fruit batches since only five samples (encircled in the figure) were clustered in different groups.

Statistical analysis of electronic nose data

To determine whether EN was able to evaluate the ripening stage of sweet cherries, sensor responses were collected and elaborated. The evolution of the signals generated by the sensor array is reported in Fig. 4. Each line represents the average signal for one sensor of the array. Considering the three ripening stages, the relative conductivity (G/G_0) of the W5S sensor has the highest increase, which implies that it is relevant to the discrimination of fruit ripeness, especially from commercial ripe and ripe to over-ripe. This result is in accordance with those reported in other studies concerning the applicability of EN for evaluating maturity in apple, peach, mandarin and tomato. In all these works it was demonstrated that W5S sensor was particularly relevant in monitoring changes in the volatile profile during fruit ripening (BENEDETTI *et al.*, 2008; BURATTI *et al.*, 2007; GOMEZ *et al.*, 2006a,b; 2007).

EN data were elaborated by PCA and LDA. PCA was performed on centered and normalized data. For each cultivar, the first two principal compo-

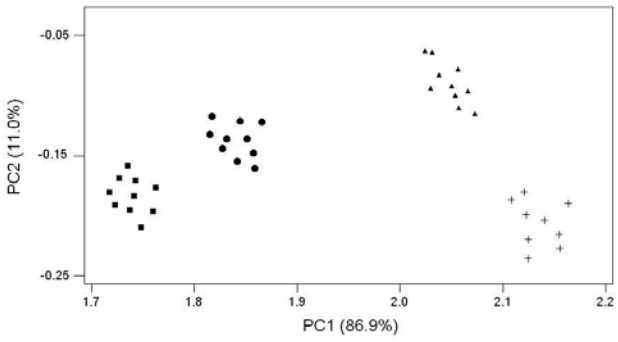


Fig. 2 - Principal component analysis score plot of four sweet cherry cultivars in the plane defined by the first two principal components (97.9% of the total variance). (▲) 'Stella', (+) 'Adriana, (●) 'Van', and (■) 'Mora di Cazzano'.

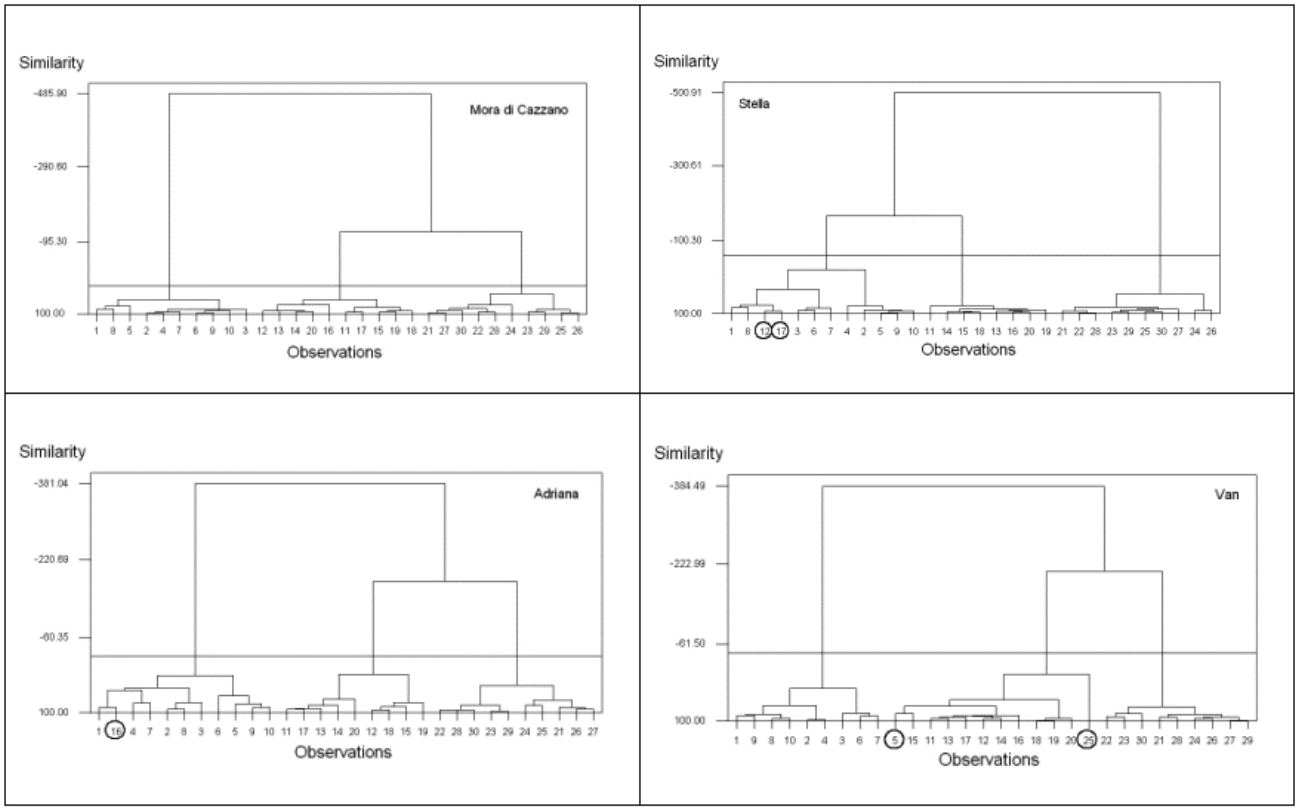


Fig. 3 - Classification dendrograms (Cluster Analysis) of the sweet cherries of different cultivars and ripening stages.

nents represented about 98% of the total variance but almost all of the variance (more than 97%) was in the first principal component. The exception was in cultivar 'Adriana' where the first principal component explained 71.8% of the total variance and the second explained 27.5%. The score plots in Fig. 5 show a clear separation of the samples according to the ripening stage. In particular, the first principal component discriminated mostly commercial ripe (1) and ripe (2) fruit from over-ripe fruit (3); in cultivar 'Adriana' the second principal component accounted for a significant discrimination between commercial ripe (1) and ripe (2) fruit.

The LDA analysis was applied to the whole data set (all cherry samples from all cultivars). The training set was composed of 120 samples and ten parameters were chosen (EN variables). Fig. 6 shows the first two LDA discrimination functions. Function 1 discriminates mostly over-ripe fruit from commercial ripe and ripe fruit. In the vertical direction (Function 2) there is a partial discrimination between commercial ripe and ripe fruit. The LDA results give 95.0% correct classification for the three classes (error rate 5%) and a leave-one-out cross validation error rate of 8.3%, due to the fact that commercial ripe and ripe fruit cannot be completely discriminated.

Since external validation is the best way to evaluate the robustness of a multivariate model, the classification model was applied to a new set of EN data. Eight commercial ripe sweet cherries (two for each cultivar) were followed during post-harvest life at 20°±1°C and analysed with the EN at the beginning of the storage (time 0) and after 5, 7, and 9 days. The new data set was used to validate the LDA model and the predictive ability of LDA model is reported (Table 2). The eight samples analysed at the beginning of storage were grouped into "commercial ripe" class, the eight samples stored for 5 days were classified in the "ripe" class, while the eight samples stored for 7 and 9 days were classified as "over-ripe".

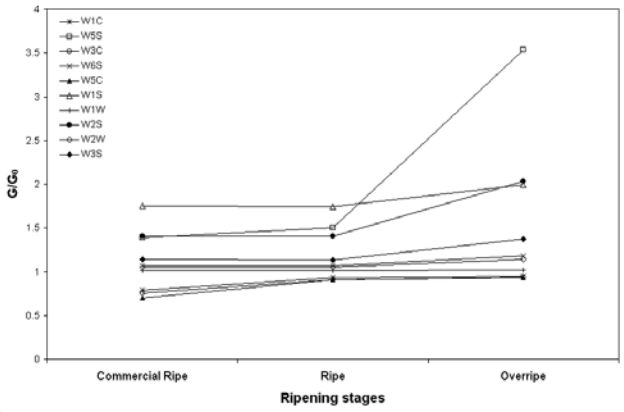


Fig. 4 - Relative conductivity of MOS sensors *vs* sweet cherry ripeness classes.

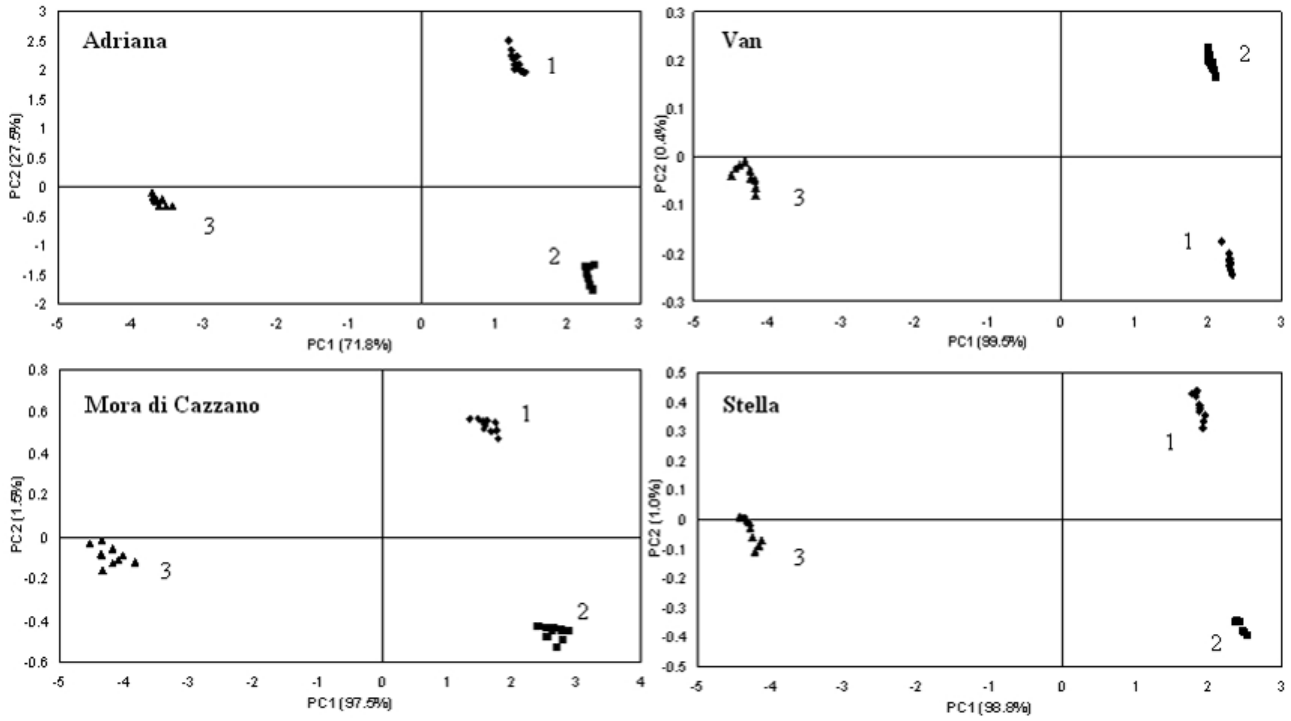


Fig. 5 - Principal component analysis score plots in the plane defined by the first two principal components of the four sweet cherry cultivars at three different ripening stages: 1 'commercial ripe', 2 'ripe', 3 'over-ripe'.

CONCLUSIONS

Based on three maturity indices (colour, soluble solid content and titratable acidity) an objective evaluation of the ripening stages of sweet cherries can be accomplished. PCA confirmed that EN was able to categorize sweet cherries into four different cultivars and three different ripening stages. As in the case of climacteric fruit, the W5S sensor seems to be relevant in the discrimination of sweet cherries on the basis of their ripeness.

LDA was applied in order to create a model for the classification of sweet cherries into three classes referred to as "commercial ripe", "ripe" and "over-ripe", and to predict the ripening stage of a new set of samples analysed during their postharvest life from the day after harvest until the fruit was senescent.

In this perspective EN could be a useful tool to characterize cultivars, by analyzing the complex evolution of the aroma profile of sweet cherries during ripening in a rapid, objective and non-destructive way. However, the practical applica-

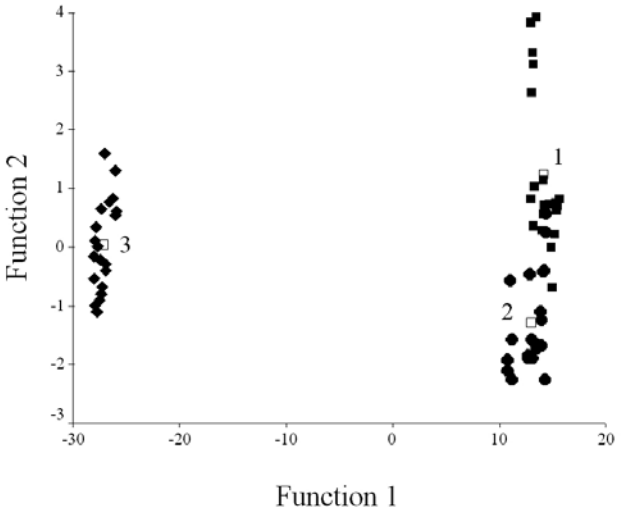


Fig. 6 - Projections of sweet cherry samples in the space defined by the LDA discriminant functions at three different ripening stages: 1 'commercial ripe', 2 'ripe' and 3 'over-ripe'.

Table 2 - Classification of sweet cherry samples by Linear Discriminant Analysis.

		Predicted Group			Total
	CLASS	1	2	3	
Original Count	1	38	2	0	40
	2	4	36	0	40
	3	0	0	40	40
	Ungrouped cases	10	6	16	32
Original (%)	1	95	5	0	100
	2	10	90	0	100
	3	0	0	100	100
	Ungrouped cases	31.25	18.75	50	100
Cross-validated Count	1	38	2	0	40
	2	8	32	0	40
	3	0	0	40	40
Cross-validated %	1	95	5	0	100
	2	20	80	0	100
	3	0	0	100	100

bility of EN requires robust training and testing over more harvest seasons and with a greater number of external validations.

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PAPER

CHANGES IN NUTRITIONAL PROPERTIES OF MINIMALLY PROCESSED FRESH FRUIT DURING STORAGE

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ABSTRACT

The aim of this work was to monitor the nutritional quality changes of a mixed fruit salad stored for 6 days at two different temperatures: 4° and 10°C. A commercial 200 g fruit salad (25% apple, 25% pineapple, 25% grape, 25% orange or nectarine) was tested. During cold storage the following parameters were evaluated: carotenoids, vitamin C, anthocyanin content, total phenolic content, and antioxidant capacity. Changes in carbon dioxide and oxygen concentrations were also monitored. The shelf life of the fruit salad kept at 4°C was longer than that kept at 10°C; the latter had an accelerated vitamin C degradation, lower phenolic content and lower total antioxidant capacity at the end of the storage period. The higher the storage temperature, the greater was the quality loss.

- Key words: antioxidant compounds, carotenoids, fresh-cut fruit, nutraceutical content, vitamin C -

INTRODUCTION

Food quality and safety are the main demands of consumers and the media, as great attention is paid to the effects of proper nutrition on health. The health benefits of eating fruit and vegetables are fully recognized. Eating minimally processed fruit could be important, due to the freshness and health benefits of their functional nutrients (MANFREDINI *et al.*, 2003; ROJAS-GRAU *et al.*, 2009). Fresh fruit contains sugars, soluble and insoluble fibers, vitamins, mineral salts, organic acids and antioxidant phytochemicals (MOULY *et al.*, 1999; CARRATU' and SANZINI 2005; FRAZZOLI, 2005, SHAHIDI, 2009). These nutrients help prevent several diseases (JOSEPH *et al.*, 2000; RAO and BALACHANDRAN, 2002).

Ready-to-eat or minimally processed fresh fruit and vegetables are becoming increasingly popular in Europe and the USA. These products have only recently appeared in Italy (WATADA *et al.*, 1996). The raw fruit or vegetables are washed, peeled, sliced or shredded. They are ready for use, generally packed in plastic bags, stored below 6°C, and sold within 5-10 days (MCKELLAR *et al.,* 2004).

Ready-to-eat salads are a suitable meal for today's lifestyles because they need no preparation and provide a great variety of vitamins, minerals and other phytochemicals. About 80% of the ready-to-eat products are vegetables, while only 20% are fruit (FRAZZOLI, 2005; REDIERS *et al.*, 2009). The demand for such products has increased significantly, although there are some hurdles in the production of these commodities, due to the difficulty of preserving their freshness during storage. The short shelf-life of fresh-cut fruit is mainly due to the cutting operations that damage and wound the fruit cell membrane. As a result, the plant tissues increase the respiration rate, ethylene production and tissue softening, which decreases the quality (MASSANTINI and SALCINI, 2003). In addition, the fruit becomes more susceptible to microbial attack due to the leakage of vesicular juice, and the absence of the protective peel (PRETEL *et al.*, 1998). Therefore refrigeration systems are required throughout the supply chain (DONATI, 2003). For these reasons, fresh-cut fruit is still under study because of the difficulties in preserving their fresh quality over prolonged periods (ABADIAS *et al.*, 2008; CONTE *et al.,* 2009; GUERRA and CASQUERO, 2008). Temperature, atmosphere, relative humidity and hygiene must be regulated to maintain fresh-cut quality (AGUAYO *et al.*, 2008; KOUKOUNARAS *et al.*, 2008; MARRERO and KADER, 2006; MONTERO-CALDERON *et al.*, 2008; OMS-OLIU *et al.,* 2008; WANG and BUTA, 2003).

The objective of this study was to investigate the qualitative and nutritional parameter trends of a minimally processed fruit preparation that is commercially available in Italy, stored at two different temperatures (4° and 10°C).

MATERIAL AND METHODS

Fruit salad

The study was carried out on commercial mixed fruit salad packs. The composition varied as a result of seasonal availability; they contained 25% apple, 25% pineapple, 25% grape, 25% orange or nectarine. The fruit was sorted, cleaned, cut and rinsed for 1 min in potable water with 50 gL^{-1} ascorbic acid (commercial practice). Excess water was completely drained, the fruit was mixed together, placed in plastic boxes (200 g per box) and wrapped in polypropylene film without gas injection. Two assays were conducted: the first in June, the second in July. Each pack was considered a replicate and all data points are means of three replicates.

Storage conditions

The samples were stored for seven days at two different temperatures: 4°C, the temperature recommended in the commercialization of these products and 10°C, to simulate commercial distribution and retail temperatures.

Chemical analysis

Each kind of fruit was analyzed separately as described below.

Vitamin C was determined by enzymatic analysis using a Boehringer Mannheim/R-Biopharm (Germany) kit number 10409677035. Colorimetric measurements were performed on a UV-vis Model DU530 Spectrophotometer (Beckman, Fullerton CA, USA). Ascorbic acid was determined by using a commercially available test kit which uses the capability of ascorbate to reduce 3-(4,5-dimethylthiazoyl-2)-2,5- diphenyltetrazolium bromide (MTT) in the presence of the electron carrier 5-methylphenazinium methosulfate (PMS) at pH 3.5 to the corresponding formazan molecule. This can be spectrophotometrically quantified by means of its light absorbance in the visible range at 578 nm. For the specific determination of ascorbic acid in a blank sample assay, ascorbic acid is the only fraction of the reducing substances present in the sample to be oxidatively removed by ascorbate oxidase in the presence of oxygen from the air. Dehydroascorbic acid does not react with MTT/PMS. In this method, the absorbance differences for both the sample and blank were determined. The absorbance differences for the blank sample were then subtracted from the absorbance differences for the sample ((*A*2 –*A*1) sample–(*A*2 –*A*1) blank sample). Finally, ascorbic acid con-

centrations were calculated with the aid of the extinction coefficient of MTT-formazan, according to the general equation:

$$C = V \times MW/\varepsilon \times d \times v \times 1{,}000\ (\times \Delta A)$$

where C is the concentration g ascorbic/L; V, the final volume (2,700 mL); MW, the molecular weight of the substance to be assayed (176.13 g/mol); ε, the extinction coefficient for MTT-formazan at 578 nm (16.9 l $mmol^{-1}$ cm^{-1}); d, the light path (1.00 cm); v, the sample volume (0.100 mL); and ΔA, the absorbance differences ($A2$–$A1$) sample–($A2$ –$A1$) blank sample.

Vitamin C was monitored just after packaging (time zero) and after three and six storage days.

Total carotenoids were extracted according to OKE *et al.* (2005); organic extracts were saponified according to LEE *et al.* (2001). The quantitative analysis of each carotenoid was performed by HPLC according to HART and SCOTT (1995) and the results are expressed as μg/100 g fresh weight. The analysis was performed using a HPLC Jasco Series LC2000 Plus equipped with a UV/Vis detector and a Supelco LC-18 column, 150 mm x 4,6 mm i.d., 5 μm particle size. Total extracted carotenoids were also determined spectrophotometrically (UV/Vis Hitachi U-2000 spectrophotometer) at λ 450 nm. Carotenoid content was monitored after 1, 2, 5, and 6 days of storage.

Anthocyanin content, phenolic content and total antioxidant capacity. Extracts were prepared by weighing 10 g of fresh fruit in a centrifuge tube, methanol was added (25 mL) and the sample was homogenized for 1 min. Extractions were performed under reduced light conditions. Tubes were centrifuged (3,000 rpm for 15 min) and the clear supernatant fluid was collected and stored at -26°C for further work.

The anthocyanin content was quantified according to the pH differential method of CHENG and BREEN (1991). Anthocyanins were estimated spectrophotometrically by their absorbance at 510 and 700 nm in a buffer at pH 1.0 and pH 4.5, where $A = (A_{515}\text{-}A_{700})_{pH1.0} - (A_{515}\text{-}A_{700})_{pH4.5}$. Results are expressed as mg of cyanidin-3-glucoside (C3G) per 100 g of fresh fruits weight.

Total phenolics were determined with Folin-Ciocalteu reagent by the method of SLINKARD and SINGLETON (1977) using gallic acid as a standard. Absorption was measured spectrophotometrically at 765 nm. Results are expressed as mg gallic acid equivalents (GAE) per 100 g of fresh weight.

Antioxidant activity was determined using ferric reducing antioxidant power (FRAP) assay, following the method of PELLEGRINI *et al.* (2003) with some modifications. The antioxidant capacity of a dilute fruit extract is determined by its ability to reduce ferric iron to ferrous iron in a solution of 2,4,6-tripidil-s-triazina (TPTZ) prepared in sodium acetate at pH 3.6. The reduction of iron in the TPTZ-ferric chloride solution (FRAP reagent) results in the formation of a blue-colored product (ferrous tripyridyltriazine complex), the absorbance of which is read spectrophotometrically at 595 nm 4 min after the addition of appropriately diluted fruit extract to the FRAP reagent. Results were calibrated against a standard curve produced by adding freshly prepared $FeSO_4 \times 7H_2O$ 100-1,000 μM solutions. Results are expressed as mmol Fe2+/kg of fresh weight. Anthocyanin content, phenolic content and total antioxidant capacity were monitored just after packaging (time zero) and after 3 and 6 days of storage.

Gas monitoring

The concentration of oxygen and carbon dioxide inside the packages was monitored daily by using a CANAL 121 (Vizag, Gas Analysis, France). A sample of 0.5 mL was automatically withdrawn from the headspace atmosphere with a pin-needle connected to the injection system. The CO_2 level was measured with an infrared sensor. The O_2 level was measured with an electrochemical sensor. The instrument was calibrated for air. The gas composition was determined daily.

Statistical analyses

The data were subjected to one-way analysis of variance (ANOVA), the sources of variance being days of storage and storage temperature. A Tukey's test HSP (honestly significant differences) was used to determine significant differences among the treatments. Mean values were evaluated for significant differences ($P \leq 0.05$).

Data were analyzed using the program package STATISTICA ver. 7.1 (Statsoft Inc., Tulsa, OK, USA).

RESULTS AND DISCUSSION

Vitamin C

Vitamin C content was determined in pineapple (June and July) and in orange (June) as shown in Table 1. In pineapple, in both assays and at both storage temperatures, the vitamin C content was significantly reduced after 6 days of storage. This finding is in accord with the data of ODRIOZOLA-SERRANO *et al.* (2009) for fresh-cut strawberries. In the first assay the vitamin C content decreased substantially (about 50%); the higher the storage temperature, the more substantial was the variation in ascorbic acid content. The same trend was found in the second assay after 3 days of storage; a significantly greater decrease was obtained in samples stored at 10°C. The decrease of vitamin C at high temperatures might be the consequence of low O_2

Table 1 - Changes in Vitamin C (mg/100 g fruit fresh weight) in minimally processed pineapple and orange stored at 4° and 10°C[a].
Mean values followed by the same letter are not significantly different at P≤0.05 level. Latin letters (a, b, c) in the same line are used to compare the storage time influence. Greek letters (α, β) in the same column are used to compare the temperature influence.

			Storage (days)		
	Fruit	Storage temperature	0	3	6
First assay (June)	Pineapple	4°C	43.94 a,α		21.22a,α
		10°C	43.94a,α		19.72b,α
	Orange	4°C	64.61a,α		43.57b,α
		10°C	64.61a,α		51.82a,α
Second assay (July)	Pineapple	4°C	50.61a,α	45.94 a,α	26.6a,α
		10°C	50.61a,α	32.42b,β	28.61a,α

[a]Data are means of 3 replications.

and high CO_2 accumulation inside the packages. In addition, it has been reported that vitamin C is a typical heat-sensitive nutrient (REYES *et al.*, 2007). A different trend was observed at the end of the storage period; the vitamin C content was similar at the two storage temperatures.

Orange samples stored at higher temperature (10°C) showed the highest values, and the decrease (from 64.61 to 51.82 mg/100 g fw) was much less compared to that of pineapple (Table 1).

Total carotenoids

Total carotenoid content, β-carotene and cryptoxsanthin were monitored in orange and in nectarine (Table 2).

The total carotenoid content in orange was between 20 μg/100 g fw and 21 μg/100 g fw during the storage period. The total carotenoid content decreased by the second day and increased again from the third day to the end of the storage period, but there were no significant differences either over time of storage or among treatments (4° and 10°C). β-carotene and cryptoxanthin content decreased from the first to the second day and then the concentration gradually increased to a value about equal that at the start.

Nectarine slices showed a different trend compared to orange samples as reported in Table 2. Total carotenoids increased slowly during storage at both storage temperatures. Significant changes in total carotenoids throughout storage was detected at 4°C. Nectarines had a higher carotenoid content after 6 days at 4°C, compared to the carotenoid content at the beginning of the storage period. Significant changes throughout the storage period were observed in nectarines stored at 10°C.

These trends could be attributed to an in-

Table 2 - Changes in carotenoid (μg/100 g fresh weight), β-carotene (mg/kg) cryptoxanthin (mg/kg) of nectarines and orange after 1, 3, 6 days stored at 4° and 10°C.
Mean values followed by the same letter are not significantly different at P≤0.05 level. Latin letters (a, b, c) in the same line are used to compare the storage time influence. Greek letters (α, β) in the same column are used to compare the temperature influence.

			Storage (days)			
Parameters	Fruit	Storage temperature	0	1	3	6
Carotenoid (μg/100 g fresh weight)	Orange	4°C	21.01a,α	20.08a,α	20.35a	20.68a
		10°C	21.01a,α	20.06a,α		
β-carotene (mg/kg fresh weight)		4°C	6.62c,α	3.76d,β	7.55a	6.74b
		10°C	6.62a,α	4.19b,α		
Cryptoxsanthin (mg/kg fresh weight)		4°C	11.78b,α	7.23d,α	9.2c	12.92a
		10°C	11.78a,α	7.99b,α		
Carotenoid (μg/100 g fresh weight)	Nectarine	4°C	3.36b,α	3.29b,α	3.77ab	4.75a
		10°C	3.36b,α	3.71a,α		
β-carotene (mg/kg fresh weight)		4°C	2.66a,α	1.29c,α	1.16d	1.74b
		10°C	2.66a,α	1.18b,β		
Cryptoxsanthin (mg/kg fresh weight)		4°C	1.02a,α	0.72b,α	0.51d	0.63c
		10°C	1.02a,α	0.51b,β		

Table 3 - Changes in anthocyanin content (mg/100 g of cyanidin-3-glucoside), phenolic content (mg/100g gallic acid equivalents) and total antioxidant capacity (mmol Fe2+/kg) of grape, apple, nectarines and pineapple after 1, 3, 6 days stored at 4° and 10°C[a].
Mean values followed by the same letter are not significantly different at P≤0.05 level. Latin letters (a, b, c) in the same line are used to compare the storage time influence. Greek letters (α, β) in the same column are used to compare the temperature influence.

Parameters	Fruit	Storage temperature	Storage (days) 0	3	6
Antocyanin content (mg/100 g of cyanidin-3-glucoside)	Grape	4°C	37.44 a,α	35.17 a,β	33.13a,α
		10°C	37.44a,α	55.75a,α	38.96a,α
Phenolic content (mg/100 g gallic acid equivalents)		4°C	132.68a,α	91.39a,α	130.96a,α
		10°C	132.68a,α	128.41a,α	139.79a,α
Total antioxidant capacity (mmolFe2+/kg)		4°C	13.6a,α	11.05a,α	12.95a,α
		10°C	13.6a,α	12.17a,α	11.61a,α
Phenolic content (mg/100 g gallic acid equivalents)	Apple	4°C	64.42c,α	75.13b,α	81.48a
		10°C	64.42a,α	55.66a,α	
Total antioxidant capacity (mmol Fe2+/kg)		4°C	10.37a,α	10.86a,α	11.6a
		10°C	10.37a,α	11.21a,α	
Phenolic content (mg/100 g gallic acid equivalents)	Nectarine	4°C	49.73ab,α	43.55b,α	59.09a
		10°C	49.73a,α	29.44b,β	
Total antioxidant capacity (mmol Fe2+/kg)		4°C	7.39a,α	6.96a,α	6.75a
		10°C	7.39a,α	4.73b,α	
Phenolic content (mg/100 g gallic acid equivalents)	Pineapple	4°C	40.2a,α	37.53a,α	48.06a
		10°C	40.2a,α	30.61a,α	
Total antioxidant capacity (mmol Fe2+/kg)		4°C	8.92a,α	7.91ab,α	7.21b
		10°C	8.92a,α	6.96b,β	

[a]Data are means of 3 replications.

creased bioavailability due to tissue decay, as previously reported in the literature (DING *et al.*, 2002; MARRERO and KADER, 2006; PAIVA and RUSSELL, 1999; RATHORE *et al.*, 2007). However, in nectarine samples β-carotene and cryptoxanthine decreased compared to the initial values.

Anthocyanin content

Total anthocyanins were quantified in a grape sample as reported in Table 3. The initial anthocyanin content of grape samples was 37.44 mg/100 g fw cyanidin 3-glucoside. Significant differences during storage were found after 3 days: 35.17 mg/100 g fw cyanidin 3-glucoside for the fruit stored at 4°C and 55.75 mg/100 g fw cyanidin 3-glucoside for the samples stored at the higher temperature.

Total phenolic content

In grape the trend of total polyphenols was similar to that for anthocyanins content (Table 3); there were no significant differences over time of storage or among treatments (4° and 10°C).

In the other fruit samples, the phenolic content increased slowly during storage at 4°C, and decreased during storage at 10°C. In apple this trend is probably related to an increase in ethylene, because the ethylene stimulates phenylalanine ammonia lyase activity (PAL), a key enzyme in the biosynthesis of phenol compounds (LEJA *et al.*, 2003).

In nectarine slices stored at 4°C a slight decrease was found and was followed by an increase on the sixth day, until reaching values higher than the start of the assay. Total polyphenols increased at the end of storage as previously reported (CONNOR *et al.*, 2002; KALT *et al.*, 1999; GIL *et al.*, 2006).

The total phenolic levels in fruit stored at 10°C decreased by more than 40% just after 3 days of storage. A similar trend was observed in pineapple samples because phenolics are degraded when exposed to oxygen, light and high temperatures (ROBLES-SANCHEZ *et al.*, 2009)

Total antioxidant capacity

The antioxidant activity in grape and apple slices was not significantly affected by storage time and temperature (Table 3).

However, significant reduction of total antioxidant capacity values during storage were found in fresh-cut pineapple at both temperatures and in nectarine at 10°C, due to a more intense met-

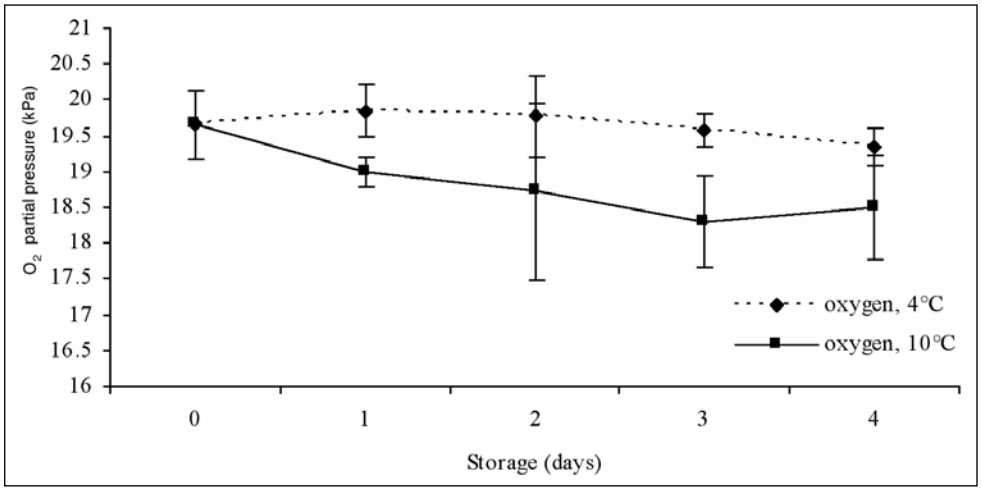


Fig. 1 - Range of O_2 partial pressure (kPa) (mean values and standard deviation) in the package headspace during 5 days of storage at indicated temperatures (4° and 10°C).

abolic activity. Operations such as peeling, cutting, and slicing are thought to induce a rapid enzymatic depletion of several natural antioxidants (NICOLI *et al.*, 1999; ROBLES-SANCHEZ *et al.*, 2009).

Gas monitoring

As shown in Figs. 1 and 2, the composition of the atmosphere inside the packs changed during the storage period. CO_2 partial pressure gradually increased while O_2 levels progressively decreased throughout storage. The packs stored at different temperatures showed different atmospheric compositions from the second day of storage on. During storage at 10°C, O_2 decreased and CO_2 drastically increased, suggesting an active metabolism.

At the lower temperature (4°C), O_2 partial pressure gradually decreased and CO_2 gradually increased during storage (Figs. 1 and 2). However, the atmosphere in these samples had lower O_2 and CO_2 levels than at 10°C. The increase of CO_2 inside the packs was not high enough to repress metabolic activity.

CONCLUSIONS

This study investigated the effects of different storage temperatures on the nutritional quality of commercial fresh-cut fruit salad. Carotenoids, vitamin C, anthocyanin content, total phenolic content and antioxidant capacity were monitored for 6 days. The nutritional value of the minimally processed fruit decreased during storage and

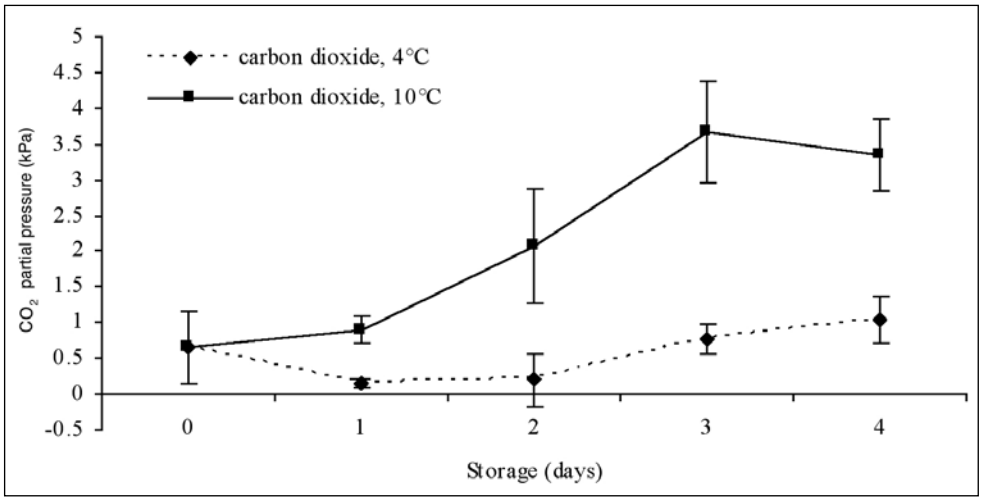


Fig. 2 - Range of CO_2 partial pressure (kPa) (mean values and standard deviation) in the package headspace during 5 days of storage at indicated temperatures (4° and 10°C).

the shelf life of packaged fruit was significantly affected by temperature. The results show that the shelf life of fruit stored at 4°C was longer compared to the fruit salad stored at 10°C. At low temperatures some compounds such as vitamin C and antioxidant compounds that are important to the quality of minimally processed fresh fruit are more stable. Fruit stored at 10°C had accelerated vitamin C degradation, lower phenolic content and lower total antioxidant capacity compared to the samples stored at the lower temperature.

For commercial purposes, nutrients and quality attributes of the fruit are maintained at 4°C. The recommended six-day storage appears to be excessive. The fruit salad was unsuitable for consumption when stored for more than three days at 10° C and over 5 days when stored at 4°C.

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PAPER

EVALUATION OF ANTIOXIDANT CAPACITY OF ANTIOXIDANT-DECLARED BEVERAGES MARKETED IN ITALY

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ABSTRACT

Clinical literature suggests a negative correlation between fruit juice consumption and the occurrence of various diseases. Consequently, many commercially available beverages are based on fruit juices or green tea extracts with specific additives that increase their antioxidant power. In order to fully estimate their potential antioxidant capacity, several products marketed in Italy were analyzed for total phenolics and flavonoids, DPPH· scavenging activity, TEAC, FRAP and ORAC-PYR. On average, fruit-based samples had more antioxidants than green teas, but specific additives significantly improved total antioxidant power. Differences between these samples and plain fruit juices were also evaluated. Total antioxidant supply remained almost constant during the entire shelf life of the products.

- Key words: antioxidant capacity, commercial juices, DPPH, ORAC-PYR, refrigerated storage, TEAC -

INTRODUCTION

Reactive oxygen species (ROS) are unavoidable products of aerobic metabolism (HALLIWELL, 2006; 2007) which cause significant tissue damage and human diseases (SIES, 1985). A huge number of molecules called antioxidants that can break radical chain reactions and prevent cellular damage are widespread in nature (SANJUST *et al.*, 2008). Since the high antioxidant content of some foods and beverages (particularly fruit-based ones) has been clearly demonstrated (PELLEGRINI *et al.*, 2003; WU *et al.*, 2004), consuming such foods and beverages has been suggested to significantly reduce the risk of various diseases (GEY *et al.*, 1991; LA VECCHIA *et al.*, 2001). In particular, due to the high antioxidant content and "ready-to-drink" availability of fruit- and vegetable-based beverages, their antioxidant properties have been investigated with promising results (MULLEN *et al.*, 2007; ZULUETA *et al.*, 2007; SEERAM *et al.*, 2008; PISOSCHI *et al.*, 2009). Moreover, various researchers have also suggested their possible role in preventing oxidative damage (PANZA *et al.*, 2008) and diseases like cancer (HIRVONEN *et al.*, 2006), and in delaying the onset of Alzheimer's disease (DAI *et al.*, 2006).

Nowadays, in addition to traditional fruit juices and extracts, fruit-based beverages with declared antioxidant additives (such as vitamins, phenolics, flavonoids, etc.) and alleged anti-aging/antioxidant/free radical scavenger abilities are commercially available. The aim of the present study was to measure the total antioxidant power of these ready-to-drink beverages. Accordingly, several antioxidant fruit-based beverages commercially available in Italy were analyzed, both fresh and during storage, in order to quantify their antioxidant supply throughout shelf life. Some green-tea-based beverages were also included in this study because their composition differs only slightly from most antioxidant-declared beverages; they differ in the amount and variety of additives. To the best of our knowledge, there have not been any similar screenings of such products.

Many methods involving both electron transfer (ET) and hydrogen atom transfer (HAT) (HUANG *et al.*, 2005; PRIOR *et al.*, 2005) have been proposed to assess the antioxidant capacity of food samples. Differences between the results arising from these methods have been found (HUANG *et al.*, 2005; TABART *et al.*, 2009; ZULUETA *et al.*, 2009); therefore, in order to make this study as comprehensive as possible, many methods were used for each sample, both ET (FRAP, TEAC, DPPH· scavenging assays) and HAT (ORAC-PYR assay). In addition, the amounts of the main compounds usually related to antioxidant power (phenolics and flavonoids) were determined for each sample.

METHODS AND MATERIALS

Samples

All samples were purchased from local stores from September 2008 through February 2009. For each sample, information regarding the composition was taken from the packaging. Samples were stored in strict accordance with vendors' instructions (room temperature for all samples, except A stored at 4°C). After the packages were opened, samples were immediately analyzed or were kept at 4°±2°C as suggested by vendors for the storage experiments.

Three natural and pasteurized citrus juices were analyzed for comparison; they were purchased from local stores in the same time period. They were exclusively red orange, orange and grapefruit juices; nothing else was added (including water or sugar). Samples were stored at 4°C.

Chemicals

All of the reagents were of reagent grade, and were used without further purification. In particular, Trolox (cat. No. 56510), DPPH free radical (cat. No. D9132), Pyrogallol Red (cat. No. 223239), Rutin (cat. No. 84082), Folin-Ciocalteu's reagent (cat. No. 47641), APH (cat. No. 44,091-4), Gallic acid (cat. No. 48630) and ABTS (cat. No. 11557) were all purchased from Sigma-Aldrich-Fluka (Milan, Italy). Ascorbic acid Baker Teststrips (cat. No. 4409-01) were purchased from Baker (Phillipsburg, NJ, Usa).

Total phenolic determination

The total soluble phenolic content was determined with Folin-Ciocalteu reagent according to a previously described method (SLINKARD and SINGLETON, 1977). Briefly, 1 mL of each sample was diluted with 2.5 mL Na_2CO_3 2% w/v. Following vortexing and one min incubation at 25°C, 0.25 mL 1 N Folin-Ciocalteu reagent was added. After vortexing again, the mixture was incubated at 25°C in the dark for 45 min. Absorbance at 760 nm was measured using an UltroSpec 2100 spectrophotometer (Amersham Bioscience, Milan, Italy). Gallic acid was used as the standard (linearity range 0.05-0.6 mM), and the results were calculated as gallic acid equivalent (GAE) using a standard curve.

Total flavonoid determination

The total flavonoid content was measured by using a previously described method (ZHISHEN *et al.*, 1999). Briefly, 0.25 mL of sample were diluted with 1.25 mL H_2O and 0.075 mL $NaNO_2$ (5% w/v). After 5 min incubation at 25°C, 0.15 mL $AlCl_3$ (10% w/v) was added. After 6 min 0.5 mL of 1 M NaOH and 0.275 mL H_2O were added. Mixtures were vortexed and absorbance at

510 nm was measured. Rutin was used as the standard (linearity range 0.1-0.6 mM) and the results are expressed as Rutin Equivalent (RE).

DPPH· (1,1-Diphenyl-2-picrylhydrazyl radical) scavenging assay

DPPH assay was performed as already described (HUANG *et al.*, 2005). Briefly, 0.3 mL of sample were added to 0.7 mL of DPPH solution 25 mg/L in ethanol. Decrease in absorbance at 515 nm was followed for 30 min at 25°C. The remaining DPPH (%$DPPH_{rem}$) was measured as follows: %$DPPH_{rem}$ = 100 x $[DPPH]_{rem}$ / $[DPPH]_{T=0}$. Trolox was used for the calibration curve (linearity range 5- 50 μM), and results are expressed as Trolox Equivalents (TE).

Ferric reducing antioxidant power (FRAP)

FRAP was measured as described by HUANG *et al.*, (2005). Briefly, 2.5 mL of 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) in 40 mM HCl were diluted in 25 mL of 0.1 M sodium acetate buffer (pH 3.6) and 2.5 mL of 20 mM $FeCl_3$. After warming to 37°C, 0.2 mL of this solution were added to 0.77 mL H_2O and 0.03 mL of sample. Mixtures were incubated for 6 min at 25°C and centrifuged at 8,000 *g* for 10 min. Absorbance at 593 nm was then measured. Trolox was used for the calibration curve (0.1-0.6 mM linearity range), and the results are expressed as Trolox Equivalents (TE).

Trolox Equivalent Antioxidant Capacity (TEAC) assay

The TEAC assay was performed according to RE *et al.*, (1999). Aqueous 2,2'-azinobis(3-ethylbenzothiazoline 6-sulphonate) (ABTS, 7 mmol) was diluted with 2.45 mmol of aqueous $K_2S_2O_8$. The mixture was incubated at 25°C for 16 h to give ABTS radical. This solution was then diluted with sodium phosphate buffer 75 mM (pH 7.4) until the absorbance reached 0.70±0.01 at 734 nm. 0.01 mL of sample was then diluted with 1 mL of this ABTS radical solution, and the differences in absorbance at 734 nm were measured after 6 min incubation at 25°C. Trolox was used for the calibration curve (linearity range 0.1-0.8 mM).

ORAC-PYR determination

ORAC-PYR was determined using the pyrogallol red method where APH was the radical releaser (ALARCON *et al.*, 2008). Briefly, 0.75 mL of 6.6 mM pyrogallol red solution in 75 mM potassium phosphate buffer (pH 7.4) and 0.125 mL of the sample were incubated at 25°C for 10 min. Then 0.125 mL of 0.153 mM APH solution in 75 mM potassium phosphate buffer (pH 7.4) were added and the decrease in absorbance at 540 nm was followed for 35 min at 25°C. For each sample, the area under the kinetic curve was calculated (AUC_{net}) by subtracting the area of the blank (AUC_{blank}) from the area of the sample (AUC_{sample}): $AUC_{net} = AUC_{sample} - AUC_{blank}$. Trolox was used for the calibration curve (linearity range 0.1-0.8 mM), and the results are expressed as Trolox Equivalents (TE).

Statistical analysis

The results are the averages of at least six independent determinations, and the data are reported as the mean value±standard deviation (SD). The Pearson correlation coefficient (*r*) and the associated *p*-value were used to show correlation and significance. Probability values of $p < 0.05$ were considered statistically significant. All of the statistical analyses were performed using R 2.5.1 software (R Foundation for Statistical Computing, Vienna, Austria).

RESULTS AND DISCUSSION

Total phenolic and flavonoid content

In order to fully evaluate the free radical scavenging power of commercial beverages that advertise their antioxidant content, the total phenolic and total flavonoid contents were determined, since these compounds have been positively correlated with antioxidant capacity (FANG *et al.*, 2009; FUKUSHIMA *et al.*, 2009). Vitamin C has also been correlated with the antioxidant power of commercial products. This information is usually reported on the packages and data are summarized in Table 1. The determinations of vitamin C content showed that the measured and declared vitamin C values were not significantly different, confirming the accuracy of the package-reported information (data not shown). This substance has already been extensively studied, and the present work focused on less investigated chemical compounds.

The results of total phenolic and flavonoid contents are reported in Table 2. This table shows that the concentration of total phenolics varied widely among samples. More than one order of magnitude difference was observed between the poorest (R, 0.63 mM GAE) and the richest (B, 17.97 mM GAE) beverage. In particular, green tea-based samples had significantly lower average total phenolic values. The richer beverages (B, C, and D) all came from the same producer, but had quite different compositions (complex mixtures of several fruit juices and tea extracts).

The results of the total flavonoid analyses were very similar; these data are quite well correlated with total phenolic data ($r = 0.95$). Flavonoids, on average, made up 15-20% of the total phenolic composition, except in Q in which the flavonoid content was less than 10%.

Table 1 - Composition of the beverages analyzed. All samples were stored at room temperature, and refrigerated storage was suggested after package opening. Only A was stored at 4°C.

Sample	Additives	Juice type
A	Vit. A (4 mg/L), C (0.3 g/L) and E (15 mg/L), coenzyme Q (0.015%)	Orange and mango juice
B	Vit. C (0.3 g/L) and E (25 mg/L)	Apple, orange, pomegranate, cherry juices. Apple, elderberry, and red grape extracts (0.15%)
C	Vit. C (25 mg/L), and E (25 mg/L), β-carotene	Peach, mango, apple, grape, orange, Maracuja juices. Apple, green, and white tea extracts (0.11%)
D	Vit. C (0.3 g/L) and E (25 mg/L)	Red grape, apple, and blueberry puree. Elderberry juice. Green tea, red grape leaves, elderberry, and apple extracts (0.28%)
E	Vit. C (0.24 g/L)	Red orange (18%), pomegranate (16.2%), and elderberry (0.1%) juices
F	Vit. C (0.15 g/L)	Red grape (13.5%), apple (12.5%), plum (12%), elderberry (1%), cherry (0.5%), and blueberry (0.5%) juices. Grape peel extract
G	Vit. C (0.18 g/L), fibers, quercetin (0.013%)	Pear (15%), white grape (14%), apple (7%) juices. Food fiber (0.6%)
H	Vit. C (0.3 g/L), β-carotene	White grape (11%), apple (10%) juices. Apricot (5%), mango (4%), and papaya (1%) purees
I	Vit. C (0.15 g/L), folic acid, lutein (0.005%)	Kiwi (15%), apple (12%), white grape (10%), and lime (3%) juices and purees
J	Vit. C (0.15 g/L) and E	Pink grapefruit (15%), white grape (2%) juices. Matè (0.05%) and ginseng (0.04%) extracts
K	Lipoic acid (58 mg/L), Vit. C (0.18 g/L)	*Paullinia sorbilis* and *Vitis vinifera* extracts
L	Vit. C (0.36 g/L) and Vit. E (48 mg/L), catechins (0.14 g/L)	Green tea extract (0.12%)
M	Vit. C (40 mg/L)	Green tea extract (0.2%)
N	Vit. C (0.4 g/L)	Green tea extract (0.168%)
O	Vit. C (0.14 g/L)	Green tea extract
P	Vit. C (1 g/L)	Green tea extract, polyphenols (200 mg/L). Peach and lemon juice
Q	Epigallocatechin gallate (0.18%), Vit. E (0.015%)	Green tea extract (0.2%)
R	Lemon juice	Green tea infusion (1%) and jasmine extract (0.1%)

Three samples (G, L, and Q) were declared to have flavonoid additives (quercetin, catechins and epigallocatechin-3-gallate, respectively). However, these additions were not always with a significantly higher total flavonoid or phenolic content in the final product. In fact, while L was the green tea-based sample with the highest amount of total flavonoids, Q had the lowest percentage of flavonoids among the total phenolics of all the samples.

Table 2 - Total phenolic and flavonoid values in the beverages. Values are means±SD ($n = 6$). Samples refer to table 1.

Sample	Total phenolics GAE (mM)	Total flavonoids RE (mM)
A	2.90±0.07	0.52±0.06
B	17.97±0.06	3.51±0.03
C	17.64±0.51	3.39±0.07
D	14.09±0.84	2.48±0.15
E	5.05±0.03	0.49±0.01
F	13.56±0.04	2.17±0.06
G	4.63±0.66	1.19±0.02
H	4.26±0.04	0.54±0.01
I	4.14±0.38	0.56±0.01
J	2.57±0.01	0.34±0.01
K	2.76±0.24	0.93±0.04
L	6.39±0.59	1.12±0.09
M	0.87±0.02	0.09±0.01
N	3.11±0.07	0.42±0.01
O	3.18±0.26	0.63±0.13
P	5.83±0.52	0.67±0.07
Q	3.21±0.35	0.19±0.01
R	0.63±0.03	0.08±0.01

In this context, the importance of low amounts of additives should be taken into account. For instance, G declared only 0.04 mM of quercetin added, one order of magnitude less than Q (0.4 mM of epigallocatechin-3-gallate); the total flavonoid content of G, however, was one order of magnitude higher than that of Q. This finding suggests that additives had only a marginal effect on the flavonoid composition of the beverages studied. This is probably due to the low amounts added. Conversely, additive-containing samples showed the highest DPPH, TEAC, FRAP and ORAC (*vide ultra*) values, suggesting that the antioxidant activity is not the same in all flavonoids. These data however do suggest that additives are crucial for total antioxidant power.

Total antioxidant capacity

Since notable differences have been reported between ET-based and HAT-based methods for evaluating antioxidant capacity (in particular, ET methods could underestimate antioxidant content) (HUANG *et al.*, 2005; ZULUETA

et al., 2009), both types of assays were included in this screening. Accordingly, the antioxidant power for each beverage was determined with DPPH, FRAP, TEAC (ET-based), and ORAC-PYR (HAT-based) assays. The results are reported in Table 3.

There was more than one order of magnitude of difference between the highest (L = 12.2 mM TE) and lowest (R = 0.2 mM TE) FRAP values. Similar results were observed for the TEAC and DPPH assays.

The differences were even greater for the ORAC-PYR results. There were more than two orders of magnitude of difference between the highest (P = 104.9 mM TE) and lowest sample values (R = 0.6 mM TE).

Due to the quite complex compositions of the beverages studied, it was not possible to associate specific composition patterns with high or low antioxidant capacity. However, the beverages that were almost completely composed of green tea extracts had quite low antioxidant power. Excluding L, P, and Q (which contain specific additives), the green teas had a mean TEAC value of 3.3 mM TE, while the mean for all the beverages was 7.5 mM TE. Even larger differences were obtained with ORAC-PYR: 4.3 mM TE was the mean for green tea, while 45.9 mM TE was the overall ORAC-PYR mean. The mean for green tea was therefore one order of magnitude lower than that for all beverages.

It is worth noting that most of the green teas contained vitamin C as a flavouring agent (only Q and R did not contain it); samples L, P, and Q are green teas that also have specific phenolics added. The total antioxidant power of the latter beverages was higher than in the simple green teas, and also higher than the overall mean values. The ORAC-PYR values were 44.5, 104.9, and 76.7 mM TE, respectively. There results suggest that, unless the commercial green tea extracts contain specific additives, they are less effective antioxidant beverages than common fruit juices. This is probably due to their high water dilution.

Among the fruit juice-based beverages, there were marked differences in reducing power. Samples B, C, D, and F had FRAP, TEAC, and DPPH values that were about twice as high as those of A, E, G, H, I, J, and K. Excluding J and K (the poorest fruit juice samples), all of the beverages had comparable ORAC-PYR values. This is in good agreement with the fact that this method evaluates overall antioxidant capacity, while FRAP, TEAC, and DPPH evaluate the presence of reducing compounds.

The FRAP, TEAC, and DPPH assays are all reduction-based. In light of this fact, it is not surprising that they all gave quite similar results for the same sample, according to previously reported studies (THAIPONG *et al.*, 2006; SEERAM *et al.*, 2008; FANG *et al.*, 2009). In contrast, ORAC-PYR results were always clearly higher than the mean values obtained with the ET-methods. This confirms that ORAC-PYR reports the total antioxidant power of samples, and not just the part due to the reducing compounds such as phenolics. These findings are in quite good agreement with previously reported studies that reported the risk that ET assays may underestimate total antioxidant capacity (TABART *et al.*, 2009; ZULUETA *et al.*, 2009).

The features of the methods used were further corroborated by their correlation coefficients versus total phenolic and total flavonoids contents (Table 4). The statistical analysis of the *p*-values associated with the Pearson test revealed that the ET assays were correlated with both the phenolics and flavonoids ($p < 0.005$ for DPPH and FRAP versus total flavonoids and $p < 0.001$ all other cases), according to published works with similar samples (THAIPONG *et al.*, 2006; FANG *et al.*, 2009; FUKUSHIMA *et al.*, 2009). In contrast, the HAT methods (like ORAC-PYR) are

Table 3 - Total antioxidant power (TE mM) of the samples. Values are means±SD ($n = 6$). Samples refer to table 1.

Sample	DPPH	TEAC	FRAP	ORAC-PYR
	TE (mM)			
A	0.45±0.03	2.30±0.03	1.24±0.08	29.0±2.50
B	5.3±0.20	16.7±0.40	8.8±0.20	58.3±1.9
C	11.2±0.10	14.3±0.50	9.9±0.50	77.3±14.70
D	8.2±0.20	12.3±0.30	9.7±0.2	68.9±1.90
E	3.7±0.10	5.7±0.10	4.0 ±0.10	65.8±2.80
F	13.3±0.30	16.6±0.90	7.9 ±0.20	61.3±1.50
G	2.6±0.10	4.7±0.20	3.8±0.10	91.8±22.20
H	3.3±0.30	3.2±0.10	3.0 ±0.10	79.6±20.30
I	2.3±0.40	2.6±0.20	2.2 ±0.20	40.6±1.30
J	1.03±0.02	2.99±0.15	1.58±0.02	5.3±0.10
K	0.85±0.09	2.0±0.10	0.51±0.01	5.4 ±0.20
L	8.2±0.30	16.4±0.30	12.2±0.30	44.5±2.50
M	0.52±0.07	1.2±0.10	0.42±0.01	1.7±0.20
N	3.8±0.20	6.9±0.30	4.6±0.10	8.7±1.10
O	1.9±0.10	4.7±0.10	2.7±0.20	6.1 ±0.20
P	8.3±0.20	13.0±0.50	10.0±0.10	104.9±8.90
Q	5.6±0.40	9.7±0.70	6.4±0.10	76.7±1.50
R	0.14±0.02	0.49±0.07	0.19±0.01	0.60 ±0.04

Table 4 - Pearson correlation coefficient of the total phenolic, total flavonoid, and declared vitamin C content in DPPH, TEAC, FRAP and ORAC-PYR assays.

*** $p < 0.001$ (two-tailed); ** $p < 0.005$ (two-tailed); * $p < 0.05$ (two-tailed); † $p < 0.1$ (two-tailed); § $p > 0.1$ (two-tailed)

Method	r		
	Total phenolics	Total flavonoids	Declared vitamin C
DPPH	0.76***	0.66**	0.21§
TEAC	0.81***	0.73***	0.30§
FRAP	0.73***	0.65**	0.41†
ORAC-PYR	0.49***	0.40†	0.44†

not directly related to reducing substances such as phenolics. They take into account the whole antioxidant capacity, which depends on chemicals that cannot always be regarded as reductants. In this perspective, lower correlation coefficients reported in the literature between ORAC-PYR and the total phenolics are not surprising (THAIPONG *et al.*, 2006; ALARCON *et al.*, 2008). In the present study the *p*-values for ORAC-PYR are much higher than those for the ET methods ($p < 0.05$ versus total phenolics and $p < 0.1$ versus total flavonoids). The correlation is weaker, but it is still good (Pearson *r* coefficient always > 0.4). These data suggest that phenolics make up a significant portion of the total antioxidant content of the beverages analyzed.

Conversely, both ET and HAT methods showed a weaker correlation with the vitamin C content ($p < 0.1$ for FRAP and ORAC-PYR, but $p > 0.1$ for the other assays), suggesting that the addition of vitamin C (usually done as flavouring or acidifying agent) has less effect on the antioxidant properties. This is confirmed by the widespread use of vitamin C as a flavouring additive in many foods and beverages that have no antioxidant declaration (like salads, dairy and egg-based products or alcoholic beverages).

These results emphasize the importance of using both ET and HAT-based methods to obtain a complete estimate of the total antioxidant capacity of beverages. While the ET methods offer an easier approach, to obtain quick and quite good estimates of antioxidant power, a full antioxidant capacity assessment is possible in combination with HAT methods.

Comparison with natural samples

To better understand the values reported, some commercial citrus juices were also analyzed. The values for red orange, orange and grapefruit juices were 54.8, 21.1, and 9.1 mM TE ORAC-PYR, respectively. The first showed antioxidant activity comparable to the mean of fruit-based antioxidant beverages (53.1 mM TE ORAC-PYR), but the others were considerably lower. They were about one order of magnitude higher than the values for most of the green teas analyzed; excluding L, P, and Q (samples with phenolic additives), the ORAC-PYR mean for green teas was 4.3 mM TE. This result could be partially attributed to the high dilution of commercial antioxidant samples. Simple fruit juices do not usually contain added water. Comparable results were obtained with ET-based methods.

These facts were corroborated by other studies. ZULUETA *et al.* (2007) analyzed fruit and milk-based beverages marketed in Spain. Their samples were quite similar in composition to samples in this study, but did not contain specific antioxidant additives (except vitamins A, C, and E). The samples had a mean TEAC value of 2.5 mM TE. This value is considerably lower than that of the antioxidant beverages in the present study (mean values 7.5 mM TE), indicating that specific antioxidant additives increased the antioxidant capacity. While, the order of magnitude remained the same, the differences were not substantial. PELLEGRINI *et al.* (2003) measured the antioxidant capacity of common beverages and reported their best samples (some red wines, coffee, lemon and orange juices). The TEAC values were similar to values for the antioxidant beverages (or even higher for very concentrated samples like espresso coffee). Only a few antioxidant samples (such as B, C, D, F, L, Q, and P) showed an antioxidant power that was clearly higher than that of plain natural juices.

All of this information suggests that antioxidant power can be improved through the use of additives. Samples containing additives, however, did not always have a significantly higher antioxidant capacity than plain natural juices.

Refrigerated storage effect

The beverages analyzed are usually marketed in 0.75 liter or 1 liter packages, so consumers do not usually drink the entire contents immediately after opening. Producers of these beverages typically indicate a shelf-life of 3-4 days after the package is opened. Accordingly, samples were stored at 4°C as suggested by producers, simulating domestic storage, and the total antioxidant capacity was evaluated for a few days.

The results of the experiments revealed that no significant loss of activity was detected during the shelf-life (data not shown); ET (FRAP, TEAC, DPPH· scavenging assays) and HAT (ORAC-PYR assay) methods gave similar TE results for at least 3-4 days. The decrease in antioxidant power was negligible for at least 10 days after opening the package. Such a time span is substantially longer than the expiry date suggested by the producer, as microbial degradation usually takes place before. This definitely allows consumers to obtain a full benefit of the antioxidant power of the beverages.

CONCLUSIONS

The results of this study show that, on average, fruit-based beverages had higher antioxidant power than green teas. The importance of additives has been outlined, because they can noticeably improve total antioxidant power. Simple fruit juices had a lower antioxidant content (justifying the typical higher price of antioxidant-declared beverages), but this difference was not always significant. Accordingly, consumers should exert extreme care when choosing beverages, since only specific and targeted additives (like polyphenols and catechins) in non-negligible amounts can significantly improve the antioxidant content of beverages when

compared to traditional fruit juices or tea extracts. In all cases, total antioxidant power was retained almost completely throughout the shelf life of the beverage. Clear differences were also reported between ET and HAT methods, confirming the importance of both approaches in order to fully estimate the antioxidant capacity of food samples.

ABBREVIATIONS

DPPH	1,1-Diphenyl-2-picrylhydrazyl
TEAC	Trolox Equivalent Antioxidant Capacity
FRAP	Ferric Reducing Antioxidant Power
APH	2,2'-Azo-*bis*(2-amidopropane)dihydrochloride
ORAC	Oxygen Radical Absorbance Capacity
GAE	Gallic Acid Equivalents
TE	Trolox Equivalents
ET	Electron Transfer
HAT	Hydrogen Atom Transfer

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PAPER

CHEMICAL COMPOSITION, ANTIOXIDANT AND ANTIBACTERIAL ACTIVITIES OF THE ESSENTIAL OILS OF FIVE TUNISIAN AROMATIC PLANTS

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ABSTRACT

The oil composition of five aromatic plants with commercial importance in Tunisia were analysed by GC-FID and GC-MS. *Mentha longifolia* L. and *Mentha pulegium* L., *Rosmarinus officinalis* L., *Eugenia caryophyllata* (*Syzgium aromaticum* L.) and *Thymus vulgaris* L. exhibited high levels of antimicrobial activity against thirteen *Vibrio alginolyticus* strains (100% for thyme and clove oil and 92.3% for the other oils). These oils reduced the DPPH· radical more effectively than the synthetic antioxidant (tert-butylated hydroxytoluene: BHT IC_{50} = 11.5 μg mL^{-1}). The results show that these plants, especially rosemary, thyme and clove, can be practical in cooking to protect against contamination by marine *V. alginolyticus*.

- Key words: antioxidant activity, anti-*Vibrio* activity, essential oils, GC-MS, MBC, MIC -

INTRODUCTION

Vibrio alginolyticus are motile, non-sporeforming, slightly curved Gram-negative bacteria with a single polar flagellum. They are both aerobic and facultatively anaerobic. Being halophilic, *Vibrio alginolyticus* is the most common species isolated from the Mediterranean seawaters and from the fish and shellfish in that area (BAKHROUF *et al.*, 1995; BALEBONA *et al.*, 1998; DERIU *et al.*, 2005). Currently, 12 *Vibrio* species are known to cause or to be associated with human infections: *V. alginolyticus*, *V. carchariae*, *V. cholerae*, *V. cincinnatiensis*, *V. damsela*, *V. fluvialis*, *V. furnissii*, *V. hollisae*, *V. metschnikovii*, *V. mimicus*, *V. parahaemolyticus* and *V. vulnificus* (KELLY *et al.*, 1991).

High concentrations of *V. alginolyticus* during the summer are related to an increase in the seawater temperature and are often the cause of human illness: severe gastroenteritis, wounds; intracranial infection, otitis (PIERSIMONI *et al.*, 1991; BRAVO FARINAS *et al.*, 1992) and extra-intestinal diseases (REINA *et al.*, 1995; GOMEZ *et al.*, 2003) which occur frequently when people swim in contaminated seawater and eat seafood that is contaminated by this bacterium (RIPABELLI *et al.*, 2003; OTTAVIANI *et al.*, 2003). Other *Vibrio* species that are pathogenic to humans (e.g. *V. parahaemolyticus* and *V. vulnificus*) have been isolated occasionally from mollusks, and fish harvested along the Mediterranean sea coasts. These were not included in this study.

Several spices and herbs have been used for centuries in the Mediterranean countries to enhance the flavour and aroma of foods. The Tunisian culture also recognizes the value of using spices and herbs when preserving foods and for medicinal purposes. For these reasons, many plants are used in Tunisian cooking including: rosemary (*Rosmarinus officinalis*), cloves (*Eugenia caryophyllata*), thyme (*Thymus vulgaris*), and mint (*Mentha longifolia* and *Mentha pulegium*). Monoterpenes, sesquiterpenes and their oxygenated derivatives (alcohols, aldehydes, esters, ethers, ketones, phenols and oxides) are the major components found in the essential oils of several spices and medicinal plants used around the world. Generally, the oil composition is a mixture of various compounds, although in many species one constituent may prevail over all the others (COWAN, 1999).

The aim of this study was to determine the chemical composition of some essential oils extracted from five aromatic plants commonly used in Tunisian cooking and to evaluate their antioxidant and antibacterial effects against *V. alginolyticus* isolated from seawater and edible fish.

MATERIAL AND METHODS

Plant material and oil extraction procedures

The plant materiel of *M. longifolia*, *T. vulgaris* and *R. officinalis* used in this study was collected from Sidi Bouzid (Centre of Tunisia) and *M. pulegium* from Ksour Essef (Mahdia, Tunisia). Cloves (*E. caryophyllata*) were obtained from a local market in Mahdia. One hundred grams of the aerial parts of these plants were dried for seven days at room temperature and the commercial clove buds were subjected to hydrodistillation for 3 h with 500 mL distilled water using a Clevenger-type apparatus according to the European Pharmacopoeia (1975). The oil obtained was collected and dried over anhydrous sodium sulfate and stored in sealed glass vials in a refrigerator at 4°-5°C prior to analysis.

GC and GC/MS

The gas chromatographic analysis of the five volatile oils was performed using a HP 5890-series II (Hewlett Packard, Ontario, Canada) equipped with flame ionization detectors (FID), HP-5 (BP-1) (5% phenyl+95% dimethylpolysiloxane) 30 m x 0.25 mm ID, 0.25 μm film thickness fused capillary column. The carrier gas was nitrogen (1.2 mL/min). The oven temperature program was 1 min isothermal at 50°C, then 50°-280°C (BP-1) and 50°-220°C (BP-20) at a rate of 5°C/min and held isothermal for 1 min. The injection port temperature was 250°C, detector 280°C. Volume injected: 1 μL of 1% solution (diluted in hexane). Percentages of the constituents were calculated by electronic integration of the FID peak areas.

The analyses of the volatile constituents were run on a Hewlett-Packard GC-MS (Ontario, Canada) system (GC: 5890-series II; MSD 5972). The fused-silica HP-5 MS capillary column (30 m x 0.25 mm ID, film thickness of 0.25 μm) was directly coupled to the MS. The carrier gas was helium, with a flow rate of 1.2 mL/min. Oven temperature was programmed (50°C for 1 min, then 50°-280°C at 5°C/min) and subsequently, held isothermal for 2 min. Injector port: 250°C, detector: 280°C, split ratio 1:50. Volume injected: 1 μL of 1% solution (diluted in hexane): HP 5972 recording at 70 e Volts; scan time 1.5 sec; mass Range 40-300 amu. Chem Station software was used to handle the mass spectra and chromatograms. The components of the oils were identified by comparing the mass spectra with those in the Wiley 275 GC-MS library and those in the literature (ADAMS, 2001), as well as by comparing the retention indices with the literature data (ADAMS, 2001, SIBANDA *et al.*, 2004). Retention indices (RI) of the components were determined relative to the retention times of a series

of *n*-alkanes (relative to C_9-C_{28} on the HP5 and HP-20M columns) (KOVATS, 1958):

$$RI = 100^* [n + [(T_{éch} - T_n)/(T_{n+1} - T_n)]]$$

n: number of *n*-alkane
$T_{éch}$: retention time of the sample
T_n: retention time of the *n*-alkane
T_{n+1}: retention time of the *n+1*-alkane

Antioxidant activity of the essential oil

Antiradical activity was evaluated by measuring the scavenging activity of the examined essential oil on the 2,2-diphenyl-l-1-picrylhydrazil ($DPPH^{\cdot}$) radical. The DPPH assay was performed as described by ESPIN *et al.* (2000) with minor modifications. The samples (from 0.10 to 10 μg mL^{-1}) were mixed with 1 mL of 90 μM $DPPH^{\cdot}$ solution and filled up with 95% methanol to a final volume of 4 mL. The absorbance of the resulting solutions and the blank (with the same chemicals, except for the sample) were recorded after 30 min at room temperature, against tert-butylated hydroxytoluene (BHT) as a positive control. The disappearance of $DPPH^{\cdot}$ was measured spectrophotometrically at 515 nm. The IC_{50} value is the concentration of the essential oil that caused 50% inhibition. A low IC_{50} value corresponds to a high antioxidant activity of the essential oil. The percentage of the radical scavenging activity was calculated using the following equation: $DPPH^{\cdot}$ scavenging effect (%) = $[(A_0 - A_1)/ A_0] \times 100$ where A_0 is the absorbance of the control at 30 min, and A_1 is the absorbance of the sample at 30 min.

Antibacterial activity assays

Bacterial microorganisms

Seven *V. alginolyticus* strains isolated from the Bay of Khenis (Centre of Tunisia) during the summer of 2006 were used in this study. All these strains were β-haemolytic, protease positive and adhered strongly to two cell lines used in a previous study: Caco-2 (cells from human colon adenocarcinoma) and Hep-2 (cells from human laryngeal carcinoma). The list of strains tested in this study also included two American Type Collection Culture (ATCC) reference strains (33787 and 17749) and two other bacteria isolated from the Port of Alghero (Sardegna, Italy). The last two strains were isolated from diseased *Dicentrarchus labrax* reared in a Tunisian fish farm located along the Mediterranean sea.

Screening for disk diffusion assay

Anti-*Vibrio alginolyticus* activity was achieved by the agar-well diffusion method (VUDDHAKUL *et al.*, 2007). For the experiments, a loopful of the bacterial working stocks was enriched in a tube containing 9 mL of alkaline peptone water (1% NaCl, pH: 8.4) then incubated at 37°C for 18 to 24 h. The overnight cultures were used to study the antimicrobial activity of the essential oils used and the optical density was adjusted to 0.5 McFarland turbidity standards with a DENSIMAT (Biomérieux l´Etoile, France). The inocula of the respective bacteria were streaked onto MHI agar plates using a sterile swab. A sterile filter disk (diameter 6 mm, Whatman paper No.3) was placed on the agar and was impregnated with the tested essential oils (10 μL /disk). The dishes were incubated at 37°C for 18-24 h.

The diameter of the zones of inhibition around each of the disks was taken as the measure of antimicrobial activity. Each experiment was carried out in triplicate and the mean diameter of the inhibition zone was recorded. The ATCC strains were used as a quality control. In a previous study, the results against *V. alginolyticus* showed that these strains were susceptible to tetracycline (30 μg/disk) and trimethoprim-sulfamethoxazole (1, 25/23, 75 μg/disk) which were used in this study as the positive controls. Tetracycline was also used by VUDDHAKUL *et al.* (2007) as a positive control in a study on the anti-*Vibrio parahaemolyticus* activity of 13 condiments used in Thai cooking.

The effect of clove, rosemary and thyme essential oils on the kinetic growth of *V. alginolyticus* type strain (ATCC 33787) was tested according to the protocol described by ACEVEDO *et al.* (2005). Cultures were grown on Luria Bertani broth supplemented with 1% NaCl for 18 to 24 h at 37°C. From the enrichment culture, 0.5 mL were used to inoculate sterile glass bottles containing 50 mL of new LB medium (amended with 0.5% Tween 80 and 1% NaCl) prepared with different concentrations of essential oils (1/2 MIC, MIC and BMC). Bottles were incubated on a rotary shaker (120 rpm) at 37°C. At regular time intervals, bacterial growth was evaluated by measuring the absorbance at 560 nm using a spectrophotometer after 0, 3, 6, 9, 12, 18, 24, 30 and 40 h of incubation.

Micro-well determination of MIC and MBC

The minimal inhibition concentration (MIC) and the minimal bactericidal concentration (MBC) values were determined for all bacterial strains used in this study as described by GULLUCE *et al.* (2007). The inocula of the bacterial strains were prepared from 12 h broth cultures (alkaline peptone water; 1% NaCl, pH: 8.4) and the suspensions were adjusted to 0.5 McFarland standard turbidity. Each essential oil, dissolved in 10% dimethylsulfoxide (DMSO), was first diluted to the highest concentration (20 mg/mL for the thyme, clove and rosemary oils and; 50 mg/mL for the two mint oils) to be tested, and then serial two-fold dilutions were made in a concentration range from 0.0195-20 mg/mL for thyme, clove and rosemary oils and 0.0488-50 mg/mL for peppermint and spearmint oils in 5 mL sterile test tubes containing nutrient broth (1% NaCl).

The 96-well plates were prepared by dispens-

ing 95 µL of nutrient broth (1% NaCl) and 5 µL of the inoculum. Into each well a 100 µL aliquot from the stock solutions of each essential oil was added into the first wells. Then, 100 µL from the serial dilutions were transferred into eleven consecutive wells. The last well containing 195 µL of nutrient broth (1% NaCl) without essential oil and 5 µL of the inoculum on each strip was used as the negative control. The final volume in each well was 200 µL. The plates were incubated at 37°C for 18-24 h. The results were interpreted with the naked eye or by using a mirror with reflection to observe turbidity at the bottom of the wells (COS *et al.*; 2006). The MIC was defined as the lowest concentration of the compounds that inhibited the growth of the microorganisms. The MBC values were interpreted as the highest dilution (lowest concentration) of the sample, which showed clear fluid with no development of turbidity and without any visible growth.

Statistical analysis

All the experiments were conducted in triplicate and average values were calculated using the SPSS 13.0 statistics package for Windows. The differences in means were calculated using the Duncan's multiple-range tests for means with 95% confidence limit ($P \leq 0.05$).

RESULTS

Essential oil composition

Eighty-two compounds were identified in the five oils used in this study. The qualitative and quantitative compositions of the oils are given in Table 1, where the components are listed according to their elution on the polar column. The different constituents of the samples were identified and quantified by GC and GC-MS. The volatile components found in both *M. longifolia* and *M. pulegium* were similar to those observed in the same plants in Jordan, Morocco, and Iran (GHOULAMI *et al.*, 2001; JAIMAND and REZAEE, 2002 and MONFARED *et al.*, 2002). The oil of *M. longifolia* was particularly rich in α-pinene (4.2%), 1.8-cineole (10.88%), menthone (12.38%), isomenthone (2.50%), pulegone (48.04%), α-terpineol (2.46%) and d-cadinene (2.74%). *M. pulegium* oil contains menthone (9.34%), isomenthone (19.05%), pulegone (44.27%), α-humulene (2.24%), piperitone (10.44%) and α-calacorene (2.17%).

Eugenol and eugenyl acetate (88.58% and 5.62%, respectively) were the major constituents of *E. caryophyllata*. *R. officinalis* oil was found to be rich in 1.8-cineole (43.49%), α-pinene (13.45%), camphre (8.95%), β-pinene (8.36%), camphene (5.86%), borneol (6.14%) and bornyl acetate (4.13%). In Turkey, ORHAN *et al.* (2008) characterized 37 compounds from the essential oil of *R. officinalis* which was particularly rich in 1.8-cineole (44.42%) followed by α-pinene (12.57%) and borneol (8.52%). However, ANGIONI *et al.* (2004) reported that Sardinian *R. officinalis* plants were rich in α-pinene, borneol and camphene. In Iran, piperitone, α-pinene, 1.8-cineole and camphor were the components found in the same essential oil (GACHKAR *et al.*, 2007).

The *T. vulgaris* oil (carvacrol chemotype) was found to be rich in carvacrol (60.27%), γ-terpinene (11.20%), p-cymene (7.58%) and bornyl acetate (4.93%). In fact, PICCAGLIA and MAROTTU (1993) reported that six chemotypes were identified in the *T. vulgaris* plants grown in France and other countries (geranial, linalool, gamma-terpinol, carvacrol, thymol and tran-thujan-4-ol/ terpinen-4-ol). The divergence in chemical composition of the essential oils tested in the present work can be attributed to the interaction between abiotic factors (soil, climate, and altitude) and a difference in biosynthetic enzymatic activity (YOUNIS and BESHIR SHADIA, 2004 and ZAOUALI and BOUSSID, 2008).

Antioxidant activity of the essential oils

The antioxidant activity may act in various ways by scavenging the radicals, decomposing peroxides and chelating metal ions. The ability of the essential oil to act as a donor for hydrogen atoms or electrons in the transformation of the DPPH radical to its reduced form DPPH-H was measured spectrophotometrically. DPPH is one of the compounds that has a proton free radical with a characteristic absorption, which decreases significantly when exposed to proton radical scavengers.

The concentration corresponding to 50% inhibition of radical DPPH shows that the antiradical activity of these five oils is very significant compared to the synthetic antioxidant used in this study (Table 2). Indeed, all the IC_{50} values were lower than the BHT values. The magnitude of the DPPH· radical quenching activity depended on the nature of the essential oil. *T. vulgaris*, *E. caryophyllata* and *R. officinalis* oils had a greater capacity to eliminate this radical than that from the mint oils. The essential oils were able to reduce the stable radical DPPH to the yellow DPPH-H, reaching 50% of reduction with an IC_{50} of 0.1, 0.2, 0.4, 9 and 10 µg mL^{-1} of the essential oils of *T. vulgaris*, *E. caryophyllata*, *R. officinalis*, *M. longifolia* and *M. pulegium*, respectively.

Anti-*Vibrio alginolyticus* activity

The antimicrobial activity of the essential oils was determined by the broth microdilution susceptibility method. The results show that the essential oils tested presented different degrees of

Table 1 - Percentage composition of the essential oils extracted from *M. longifolia* (1), *M. pulegium* (2), *E. caryophyllata* (3), *R. officianalis* (4) and *T. vulgaris* (5).

No.	Compounds	RI	1	2	3	4	5
1	Tricyclène	1015	-	-	-	0.2	-
2	**α-Pinene**	**1020**	**4.2**	tr	-	**13.45**	**2.83**
3	α-Thujene	1027	0.57	tr	-	-	-
4	α-Fenchene	1066	tr	tr	-	-	-
5	**Camphene**	1071	0.40	tr	-	**5.86**	0.29
6	**β-Pinene**	1113	1.06	tr	-	**8.36**	0.23
7	Sabinene	1124	0.55	tr	-	-	-
8	δ-3-Carene	1144	-	-	-	0.22	0.11
9	**Myrcene**	1161	0.39	tr	-	1.48	**2.48**
10	2-Heptanone	1172	-	-	0.93	-	-
11	**α-**Terpinene	1186	-	-	-	0.196	0.38
12	Limonene	1196	0.72	tr	-	0.55	2.24
13	**1,8-Cineole**	**1213**	**10.88**	tr	-	**43.49**	0.48
14	Ethyl hexanoate	1232	-	-	0.66	-	-
15	Z(β)Ocimene	1235	tr	tr	-	-	-
16	3-Octanone	1236	0.10	-	-	-	-
17	**γ-Terpinene**	1245	tr	tr	-	1.0	**11.20**
18	**p-Cymene**	1269	tr	0.34	-	1.39	**7.58**
19	Terpinolene	1286	tr	tr	-	0.36	0.24
20	2-Heptanol	1304	-	-	tr	-	-
21	Menthyl octanoate	1384	-	-	tr	-	-
22	3-Octanol	1388	tr	tr	-	-	0.45
23	2-Nonanone	1392	-	-	tr	-	-
24	Ethyl octanoate	1429	-	-	tr	-	-
25	1-Octenol-3	1441	tr	tr	-	-	0.16
26	α-Cubebene	1459	-	-	tr	-	-
27	Trans-Sabinene hydrate	1465	-	-	-	0.12	-
28	**Menthone**	**1473**	**12.38**	**9.34**	-	-	-
29	Menthofurane	1488	tr	tr	-	-	-
30	Copaene	1491	-	-	tr	-	-
31	**Isomenthone**	**1494**	**2.50**	**19.05**	-	-	-
32	2-Nonanol	1499	-	-	tr	-	-
33	**Camphor**	1517	-	-	-	**8.95**	**2.05**
34	B-bourbonene	1544	0.14	tr	-	-	-
35	Linalool	1548	tr	tr	tr	1.38	0.11
36	Linalyl acetate	1554	-	-	-	0.89	1.12
37	Methyl acetate	1565	0.59	0.41	-	-	-
38	**Bornyl acetate**	1586	-	-	-	**4.13**	**4.93**
39	2-Undecanone	1588	-	-	tr	-	-
40	Isopulegone	1590	0.81	0.78	-	-	-
41	Neoisomenthol	1591	0.37	tr	-	-	-
42	β-Caryophyllene	1595	-	-	1.38	-	-
43	Terpinen-4-ol	1600	0.27	tr	-	0.41	0.09
44	Menthol	1613	0.12	0.37	-	-	-
45	Menthyl benzoate	1619	-	-	tr	-	-
46	Ethyl benzoate	1647	-	-	tr	-	-
47	**Pulegone**	**1654**	**48.04**	**44.27**	-	-	-
48	**α-Humulene**	1668	0.38	**2.24**	0.19	-	-
49	trans -Piperitol	1669	1.65	-	-	-	-
50	Menthyl chavicol	1670	-	-	tr	-	-
51	α-Amorphene	1675	-	-	tr	-	-
52	**α-Terpineol**	**1693**	**2.46**	0.80	-	-	-
53	α-Terpinyl acetate	1695	-	-	tr	-	-
54	**Borneol**	1702	-	-	-	**6.14**	0.77
55	Germacrene -D	1703	0.63	0.81	-	tr	tr
56	α-Muurolene	1711	-	-	tr	-	-
57	Benzyl acetate	1714	-	-	tr	-	-
58	Carvone	1731	0.37	1.25	tr	-	-
59	**Piperitone**	1732	0.94	**10.44**	-	-	-
60	**δ-Cadinene**	**1755**	**2.74**	1.56	-	0.19	tr
61	γ-Cadinene	1756	0.12	-	tr	0.14	0.10
62	Myrtenol	1791	0.10	-	-	-	-
63	Carveol	1804	0.63	-	-	-	-
64	Germacrene-B	1813	0.66	-	-	-	-
65	2-Phenylethyl acetate	1826	-	-	tr	-	-

(continued)

(continued table1)

No.	Compounds	RI	1	2	3	4	5
66	(E)-Anethole	1827	-	-	tr	-	-
67	Calamenene	1828	-	-	0.10	-	-
68	Benzyl alcohol	1861	-	-	tr	-	-
69	Calacorene	1918	-	-	0.11	-	-
70	Caryophyllene oxide	1976	0.58	tr	tr	0.26	0.62
71	**α-Calacorene**	1912	1.09	**2.17**	-	-	-
72	Methyl eugenol	1985	-	-	tr	-	-
73	Humulene oxide	1986	-	-	tr	-	-
74	Cinnamic aldehyde	2018	-	-	tr	-	-
75	Ethyl cinnamate	2072	-	-	tr	-	-
76	Benzyl tiglate	2103	-	-	tr	-	-
77	**Eugenol**	**2151**	-	-	**88.58**	-	-
78	T-cadinol	2164	-	-	-	0.21	-
79	Thymol	2166	tr	1.16	-	-	0.28
80	**Carvacrol**	**2203**	-	-	-	-	**60.27**
81	**Eugenyl acetate**	**2263**	-	-	**5.62**	-	-
82	Humulenol	2265	-	-	0.27	-	-
	% of Identification		**96.69**	**95.04**	**98.27**	**99.57**	**99.1**

The compounds and their percentages are listed in order of their elution on polar column (HP-20M); RI: Retention indices relative to n-alkanes on the polar HP-20M; tr: trace (< 0.1%); -: not detected.

anti-*Vibrio alginolyticus* activity (Table 3). The MIC and MBC values (Table 4) indicate that the *T. vulgaris* essential oil was the most efficient essential oil tested against the selected bacteria followed by clove and rosemary oils. The antimicrobial activity of the volatile components of thyme, clove, and rosemary can be attributed to the presence of high concentrations of carvacrol (60%), eugenol (88%) and 1-8 cineole (43%), respectively. These components exhibit a high antibacterial property against several Gram-negative and Gram-positive bacteria (ROTA *et al.*, 2007; CHANGA *et al.*, 2008). The lowest MIC value was observed with thyme and clove essential oils (0.078 mg/mL and 0.156 mg/mL, respectively).

The zones of growth inhibition (mm) scored in Mueller-Hinton agar supplemented with 1% NaCl are summarized in Table 3. The zones of greatest growth inhibition were obtained when *T. vulgaris* essential oil was tested against *V. alginolyticus* strains, the diameter of inhibition ranged from 14 to 25 mm. Compared to the two antimicrobial agents used in this study as the most active antibiotics on these bacteria (SNOUSSI *et al.*, 2006), thyme oil showed the highest level of antibacterial activity followed by clove oil, the growth inhibition diameter ranged from 11 to 23 mm.

The effect of rosemary, thyme and clove essential oils on the reference strain of *V. alginolyticus* 33787 are shown (Fig. 1). The high efficiency of the rosemary, thyme and clove essential oils against *V. alginolyticus* type strains was confirmed on LB broth medium. A concentration as low as 0.078 mg/mL (thyme), 0.15 mg/mL (clove) and 0.31 mg/mL (rosemary) inhibited the growth of *V. alginolyticus* ATCC 33787. At high concentrations (MBC) (0.62; 1.25 and 5 mg/mL of thyme, clove and rosemary oils, respectively), the growth of ATCC 33787 strain was inhibited, signalling the bactericidal effect of these oils within the first hours of the experiment. A comparison of the curves obtained for the reference strain with the different oils tested showed that clove and thyme oils were very effective against these bacteria in liquid medium.

Table 2 - Antiradical activity of five Tunisian aromatic plant essential oils and positive control BHT. Activity is expressed as IC_{50} values for DPPH· radicals.

Essential oil	Inhibiting activity of radical DPPH· (IC_{50}: µg mL^{-1})
M. longifolia	9 b
M. pulegium	10 b
E. caryophyllata	0.2 c
R. officinalis	0.4 c
T. vulgaris	0.1 c
BHT	11.5 a

Means of three replicates. Values followed by the same letter are not significantly different at $P < 0.05$.

DISCUSSION

Several studies have been performed worldwide to study the antimicrobial (Gram-negative and Gram-positive bacteria), antifungal and antioxidant power of a large number of plants (BAKKALI *et al.*, 2008; BUSATTAA *et al.*, 2008; AL-BAYATI *et al.*, 2008). Few data are available regarding the antibacterial activity of several plants and spices traditionally used in cooking against *Vibrio* spp. (BEUCHAT *et al.*, 1976;

Table 3 - Zones of growth inhibition (mm) showing antibacterial activity for a number of selected plant volatile oil components against *V. alginolyticus* strains.

Strains	Inhibition zone in diameter (mm±SD) around the discs impregnated with 10 µL of essential oil					Tetracycline (30 µg/disc)	Trimethoprim-Sulfamethoxazole (1.25/3.75 µg/disc)
	R. officinalis	*M. longifolia*	*E. caryophyllata*	*T. vulgaris*	*M. pulegium*		
ATCC 33787	(-) dF	9.33±1.15aE	11.33±0.57defD	13.33±0.57fC	10±1abcE	20±0cB	22±1aA
ATCC 17749	7±0cC	7.66±0.57cdC	10.66±0.57fB	14±1efA	10.33±0.57abB	7±0eC	11.33±1.15fB
1	11±1aBC	7±0dD	11.66±1.15defB	22.66±2.08bcA	9.33±0.57bcdC	7±0eD	22±0aA
2	7±0cD	7±0dD	12±1cdefB	19.66±1.52dA	10±0abcC	7±0eBC	19.33±1.15bA
3	7.33±0.57cD	7.33±0.57cdD	20.33±0.57bB	26±1.73aA	8.66±0.57defD	19.66±0.57cB	13.66±0.57eC
4	9.66±0.57bD	(-) eF	11±1efC	25.33±0.57aA	(-) gF	7±0eE	14.66±0.57eB
5	7.66±0.57cD	8±0bcD	11±0efC	21.33±2.3cdA	8±0efD	21±0bA	16.66±0.57dB
6	7.66±0.57cDE	8±0bcD	11.66±0.57defC	24.33±0.57abA	7.66±0.57fDE	7±0eE	19.33±0.57bB
7	11±1aC	9±0aD	12.66±1.15cdB	21±1cdA	8±0efDE	20.66±0.57bA	17.66±0.57cdE
8	10.66±1.15abC	8.66±0.57abD	13.33±0.57cB	20.66±1.15cdA	9.33±0.57bcdCD	7±0eE	19.66±0.57bA
9	10.66±0.57abD	7±0dF	12.33±0.57cdeC	14±0efB	9±0cdeE	20±0cA	12±0fC
10	7±0cF	9±0aE	23.66±0.57aA	15.66±0.57eB	10.66±1.15aD	23±0aA	14±0eC
11	9.66±0.57bD	8±0bcF	11.66±0.57defC	20±0dA	8±0fD	9±0dEE	18±0cF

(-) Note: No activity; (a ,b, c, d, e, f, g, A, B, C, D, E, F): Means followed by the same letters are not significantly different at P = 0.05 based on Duncan's multiple range test. Small letters are used to compare means between strains for each oil while capital letters are used to compare means between oils and antibiotic for each strain.

LLEWELLYN *et al.*, 1981; ALBADO *et al.*, 2001; LIN *et al.*, 2005).

In fact, KARAPINAR and AKTUG (1987) tested the effect of four spice components (eugenol, thymol, menthol and anethole) against *S. typhimurium*, *S. aureus* and *V. parahaemolyticus* and found that eugenol and thymol were the most effective components, respectively, followed by anethole and menthol. MATA *et al.* (1994) demonstrated that lime juice protected cabbage, lettuce and fish when they were experimentally contaminated by *Vibrio cholerae*. With the globalization of food, *Vibrio* spp. strains may become a human health risk when associated with the Asian habits of consuming fish and shellfish raw or slightly cooked. *Wasabi japonica*, a Japanese spice, is commonly used to protect sushi fish from the risk of *V. parahaemolyticus* when they are eaten raw (HASEGAWA *et al.*, 1999).

DERIU *et al.* (2004) noted that rosemary oil offers a natural alternative to the use of antibiotics. This plant inhibits the growth of a wide range of bacteria including: *S. aureus*, *E. feacalis*, *S. typhimurium*, *P. aeruginosa* and clinical strains of A. *hydrophila*, *A. sorbia*, *C. albicans*, *C. glabrata* and *K. pneumonia*. The MIC of rosemary oil was about 2.5% (v/v) against environmental *V. alginolyticus* strain and only 0.01% (v/v) against *V. vulnificus*. The same oil inhibits the growth of the selected bacteria with a zone inhibition diameter about 20-25 mm. The leaves and inflorescence of *Lepechinia caulescens* (Labiatae), locally used in México to treat hypoglycemia, as a

Table 4 - The MIC and MBC values (mg/mL) of the five essential oils against *V. alginolyticus* strains tested in microdilution assay.

Strains	Origin	*R. officinalis*		*M. longifolia*		*E. caryophyllata*		*T. vulgaris*		*M. pulegium*	
		MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
ATCC 33787	Type strain	0.31	5	0.78	>0.31	0.15	1.25	0.078	0.62	0.097	3.12
ATCC 17749	Type strain	0.19	>5	0.78	6 .25	0.31	2.5	0.15	1.25	0.78	6.25
1	Seawater	0.62	>5	0.19	6.25	0.31	2.5	0.15	0.62	0.39	6.25
2	Seawater	1.25	>5	3.12	12.5	0.31	2.5	0.15	0.62	3.12	>12.5
3	Seawater	1.25	>5	3.12	12.5	0.31	2.5	0.31	0.62	3.12	12.5
4	Seawater	1.25	>5	3.12	12.5	0.31	2.5	0.31	0.62	3.12	12.5
5	Seawater	1.25	>5	3.12	12.5	0.31	2.5	0.31	0.62	1.56	12.5
6	Seawater	1.25	>5	1.56	12.5	0.31	2.5	0.15	>0.31	1.56	12.5
7	Seawater	1.25	>5	3.12	12.5	0.31	2.5	0.31	1.25	1.56	12.5
8	Alghero port	0.31	0.62	0.19	3.12	0.15	1.25	0.078	0.61	0.39	3.12
9	Alghero port	0.62	5	0.78	>6.25	0.75	>0.62	0.15	0.62	0.78	3.12
10	*Sparus aurata*	0.62	2.5	0.78	3.12	0.75	>0.62	0.15	>0.31	0.78	>6.25
11	*Sparus aurata*	0.31	2.5	0.78	>0.31	0.15	1.25	0.15	3.12	0.048	1.56

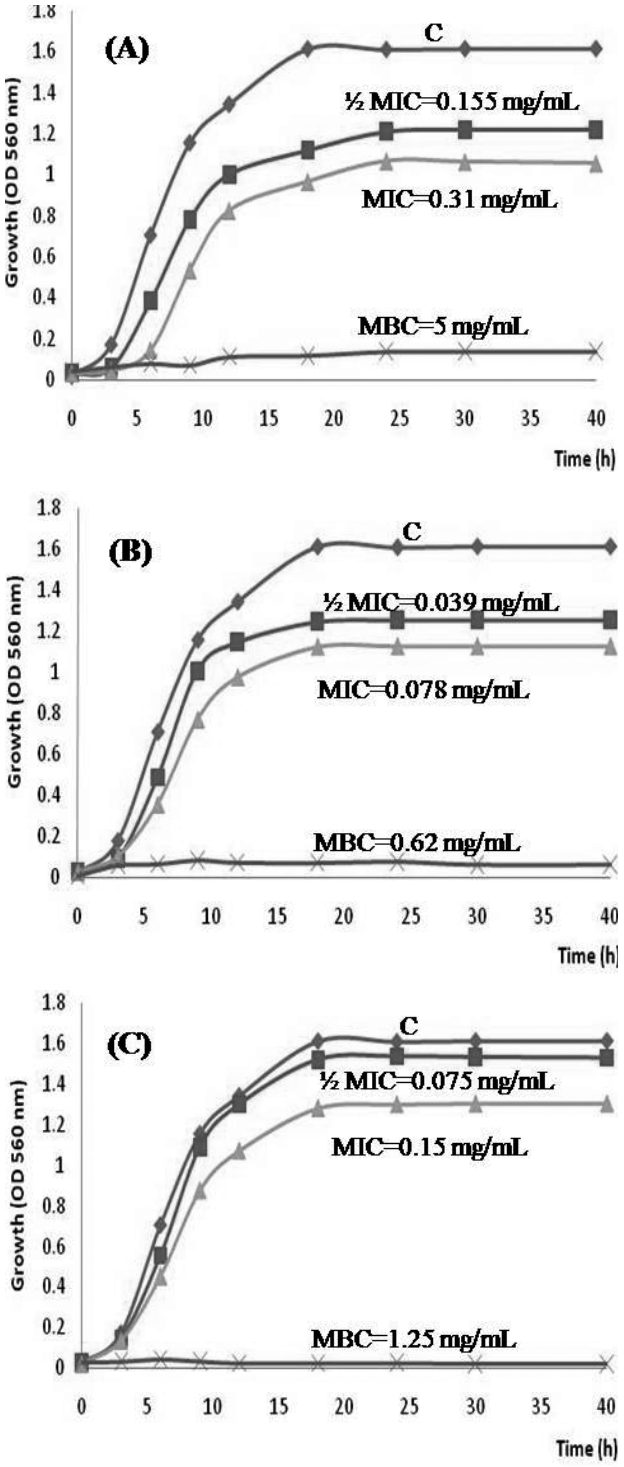


Fig. 1 - Growth kinetics of *V. alginolyticus* type strain ATCC 33787 (A, B, C) on LB medium (1% NaCl; 0.5% Tween 80) in absence C and presence of, respectively, ½ MIC, MIC and MBC of the essential oils (mg mL^{-1}) of *R. officinalis* (A), *T. vulgaris* (B) and *E. caryophyllata* (C). The essential oil was added to each experimental culture at time zero. Data represent the mean value of three measurements of the optical density at 560 nm.

gastrointestinal agent and for diarrhea, exhibits an anti-*V. cholerae* activity with MIC and MBC values of (3±1.4) and (4±0.5) μL/mL, respectively (ACEVEDO *et al.* 2005). In MH broth, MIC has a bacteriostatic effect, while MBC has a lethal effect (bactericidal activity) within the first 6 h. This antibacterial activity can be correlated with the action of borneol, camphor and *trans*-caryophyllene as the major components identified by CG/MS.

YANO *et al.* (2006) demonstrated that basil, clove, garlic, horseradish, majoram, oregano, rosemary and thyme were found to exhibit inhibitory activities against *V. parahaemolyticus* strains (serotype O3:K6). HASEGAWA *et al.* (1999) showed a similar sensitivity of *V. parahaemolyticus* strains to *R. officinalis* oil including serotype O4:K8. TOMOTAKE *et al.* (2006) noted that lemon (*Citrus limon* Burm.f.), lime (*Citrus aurantifolia* swingle) and *Citrus sudashi* are popular citrus fruit used in the Japanese cuisine when preparing raw or grilled fish. These citrus fruits are known for their anti-*V. cholerae* activity (DE CASTILLO *et al.*, 2000) and these juices exhibit high levels of inhibition against *Vibrio* spp. strains, especially *V. alginolyticus* and *V. anguillarum*.

PINTORE *et al.* (2006) reported that the essential oil of Sardinian *Stachys glutinosa* L. plants showed good bacteriostatic activities against *V. cholerae* (MIC 0.6% and MBC 2.5%). The same essential oil inhibits the growth of the only *V. alginolyticus* strain tested with MIC and MBC values >2.5%. In a recent work, VUDDHAKUL *et al.* (2007) reported that only three out of 13 condiments tested (garlic, galangal and lemon) were able to inhibit the growth of both pandemic (O3: K6 and O1: K25) and non-pandemic strains of *V. parahaemolyticus*.

In the present study, the essential oil of *T. vulgaris* exhibited a strong anti-*V. alginolyticus* and antioxidant activities which can be attributed to the high percentage of carvacrol in the *Thymus* genus (COSENTINO *et al.*, 1999). This phenolic compound extracted from *T. hyemalis* (carvacrol chemotype, > 40%) showed bacteriostatic and bactericidal activities at MIC and MBC under 0.2 μL/mL against several Gram-positive and Gram-negative bacteria, including *V. parahaemolyticus* strains (VUDDHAKUL *et al.*, 2007).

The antioxidant power of thyme, clove and rosemary oils was also confirmed (GÜLÇIN *et al.*, 2004; TOMAINO *et al.*, 2005; CHAIEB *et al.*, 2007). Several studies have demonstrated that essential oils contain antioxidant molecules including terpenoids and phenols which explain their use as natural food preservatives (AESCHBACH *et al.*, 1994). Indeed, LALAS *et al.* (2007) tested the effect of rosemary extract on two traditional Greek foods (Tarama salad and Deples). Their result showed that the rosemary extract at 400 ppm gave the highest protection against lipid oxidation of Tarama salad during 40 days of storage. It was superior to BHA (200 ppm). The extract at a concentration of 150 ppm protected Deples from oxidation during 35 days of storage. These natural antioxidants reduce fat oxidation; BHT and BHA (synthetic antioxidants) may be dangerous for living organisms (BARRATA *et al.*, 1998). The antioxidant activity found in clove oil

can be attributed to the high amount of eugenol (88.58%) as reported by OGATA *et al.* (2000). At low concentrations, eugenol acts as an antioxidant and anti-inflammatory agent, whereas at high concentrations it acts as a pro-oxidant (CHOGO and CRANK 1981; ASHA *et al.*, 2001).

CONCLUSIONS

The data obtained in the present work indicate the efficiency of the essential oils of thyme and clove against *V. alginolyticus* isolated from seawater and diseased *Sparus aurata* reared in a Tunisian fish farm located along the Mediterranean coast. These results confirm the potential use of thyme oil in the food industry and buds of clove used to prepare traditional Tunisian seafood dishes may offer a good alternative for protecting against possible contamination by environmental *V. alginolyticus* strains.

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PAPER

A NOVEL METHOD TO CALCULATE THE ANTIOXIDANT PARAMETERS OF THE REDOX REACTION BETWEEN POLYPHENOLIC COMPOUNDS AND THE STABLE DPPH RADICAL

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ABSTRACT

The antioxidant activities of five synthetic phenols (gallic acid, tannic acid, caffeic acid, hydroquinone and quercetin) and four natural plant extracts (*Vitis vinifera* variety Burgund Mare, *Calluna vulgaris*, *Camellia sinensis* and *Epilobium hirsutum*) were measured by stable free radical DPPH assay. Substrate concentrations that lower the initial DPPH· concentration by 50% (EC_{50}) were calculated by non-linear regression analysis (sigmoidal four-parameter curve) at different time intervals. Final state efficient concentration (EC_{50fs}) and the time required to reach final state (T_{EC50fs}) were obtained from an exponential decay equation. Redox reaction stoichiometry *n* was also calculated. EC_{50fs} varied in the order: tannic acid > quercetin ≥ gallic acid > hydroquinone > caffeic acid for the pure compounds and *C. sinensis* > *V. vinifera* > *E. hirsutum* > *C. vulgaris* for the plant extracts. T_{EC50fs} values had different rankings, hydroquinone and caffeic acid had the fastest kinetics followed by gallic acid, tannic acid and quercetin. In the series of natural products, the reaction kinetics decreased as follows: *V. vinifera* > *C. vulgaris* > *E. hirsutum* > *C. sinensis*; the *n* values followed the same order as EC_{50fs}. The procedure described is fast, reproducible and the parameters scored could be useful tools for characterizing the radical-scavenging properties of antioxidants.

- Key words: antioxidant activity, DPPH· assay, plant extracts, polyphenols, stoichiometry -

INTRODUCTION

Reactive oxygen species (ROS) are constantly generated during aerobic metabolism in living organisms, whereupon they can be detoxified by a wide array of antioxidant defence mechanisms (MATES and SANCHEZ-JIMENEZ, 2000; KIM *et al.*, 2005). A disruption in the balance between endogenous cellular antioxidant systems and ROS generation produces oxidative stress and the oxidative damage thus accumulated in biological macromolecules contributes to various pathologies such as cancer, neurodegenerative and cardiovascular diseases (KIM *et al.*, 2005).

Plant-derived antioxidants, which contain phenolic compounds, could be considered as potential candidates for reducing the levels of oxidative stress (BEHREND *et al.*, 2003; ROUSSIS *et al.*, 2005; NICHENAMETLA *et al.*, 2006).

The main characteristic of an antioxidant is its ability to scavenge free radicals and ROS. Therefore, it is important to evaluate the antioxidant properties, particularly of dietary polyphenols. There are numerous methods to do this, most of which quantify the antioxidant activity of pure (poly)phenols (BRAND-WILLIAMS *et al.*, 1995; SANCHEZ-MORENO *et al.*, 1998; VILLANO *et al.*, 2007).

The stable free radical, 2,2-diphenyl-1-picrylhydrazyl (DPPH·) assay is widely used to measure the free radical scavenging ability of various antioxidants (SANCHEZ-MORENO *et al.*, 1998; ROUSSIS *et al.*, 2005) and different procedures have been applied to determine the EC_{50} values, which represent the concentration of the antioxidant that causes 50% loss of the initial DPPH· concentration.

In earlier studies the time course variations of the DPPH· concentration were measured individually after adding various amounts of antioxidant. EC_{50} was obtained by plotting the antioxidant concentration vs. the remaining DPPH· concentration calculated from a calibration curve (SANCHEZ-MORENO *et al.*, 1998; VILLANO *et al.*, 2007).

In the present study the antioxidant properties of five synthetic phenols and four natural plant extracts were measured in reactions with the DPPH radical, in a methanol solution and are expressed as the efficient concentration (EC_{50}), final state EC_{50} (EC_{50fs}) and stoichiometric parameter (n). The procedure was based on the observation that variations in DPPH· absorbance decay vs. the variable concentrations of added antioxidant follows a sigmoidal-shaped curve when the antioxidant concentrations are expressed as logarithms. Thus, EC_{50} was calculated by non-linear regression analysis (sigmoidal four-parameter curve) at different time intervals. Final state efficient concentration (EC_{50fs}) and T_{EC50fs} were obtained from an exponential decay curve plotting the EC_{50} vs. the time measurements. The stoichiometric values, n, were derived from the EC_{50fs} values.

MATERIALS AND METHODS

Chemicals

Gallic acid and hydroquinone were obtained from Fluka, Buchs, Switzerland. Tannic acid (Mw=1701.2), caffeic acid, quercetin and DPPH· were from Sigma-Aldrich, Steinheim, Germany. Folin-Ciocalteu reagent was from Merck, Darmstadt, Germany.

Plant materials

The aerial parts of *Calluna vulgaris* L. Hull. (known as Common Heather, fam. Ericaceae) and *Epilobium hirsutum* L. (known as the Great Willowherb, fam. Onagraceae), leaves of *Camellia sinensis* L. (known as Green tea, fam. Theaceae) and red grape seeds of *Vitis vinifera* L. (variety Burgund Mare, Romania) were used.

Extract preparation

- *V. vinifera*: 1 g of ground dried seeds were extracted with 20 mL of an ethanol/water, (50/50, v/v) mixture, under reflux, for 30 min (POSTESCU *et al.*, 2007).

- *E. hirsutum*: 1 g ground dried plant was treated with 20 mL methanol and the mixture was kept under refluxing for 15 min.

- *C. sinensis*: 1.5 g of tea (Lipton) were added to 100 mL boiled water (95°C), brewed for 5 min, cooled and decanted.

After cooling, all extracts were filtered and adjusted with appropriate solvents to the initial volumes.

- *C. vulgaris*: 1 g plant material and 20 mL ethanol (70%) were macerated, at room temperature, for 7 days. The extract was concentrated 10-fold, under reduced pressure, at 35^{0}C.

Total polyphenol content (TPC)

TPC of the plant extracts was determined according to the Folin-Ciocalteu procedure (SINGLETON *et al.*, 1999). All extracts were diluted at a ratio of 1:40 or 1:80 before measuring. Each test sample (1 mL) was treated with 5 mL of Folin-Ciocalteu reagent. After 3 min of stirring, 4 mL of Na_2CO_3 (75 g/L) were added. After standing for 2 h in a dark room, the absorbance was measured at 740 nm (UV-Vis Spectrophotometer, Jasco V-530, Japan). The results are expressed as gallic acid equivalents (GA Eq).

Antioxidant activity (AO)

AO was measured by using the free radical DPPH· scavenging assay following the Brand-Williams method (BRAND-WILLIAMS *et al.*, 1995) with slight modifications. This method is based on the ability of the DPPH· to scavenge the an-

tioxidants and decrease the DPPH· absorbance at 515 nm. Eight successive dilutions of each sample were prepared and 5 μL of each dilution were transferred into plastic cuvettes (l = 1 cm) containing 3 mL of DPPH· (0.0025 mg/mL, 65.4 μM) in methanol. The absorbance at 515 nm was recorded after 1, 2, 5 and 60 min at 25°±1°C. The antioxidant concentrations in the reaction medium are expressed as mmol antioxidant / mmoles DPPH· for pure polyphenols and as mmol gallic acid equivalents / mmol DPPH· for natural extracts.

Statistical analysis

All measurements were made in triplicate (n=3). The fit of the sigmoidal curves, exponential decay curves and coefficient of determination (R^2) were performed with the GraphPad Prism program, version 5.0 (GraphPad, San Diego, CA, Usa). Results are expressed as the mean value±SEM or 95% confidence intervals (95% CI).

RESULTS AND DISCUSSION

Calculation of effective concentration EC_{50}

The method is based on the reduction of the highly colored DPPH radical in the presence of a phenolic antioxidant (PheOH), in a methanol solution:

$$\text{PheOH} + \text{nDPPH}\cdot \rightarrow \text{oxidation products} + \text{nDPPH–H}$$

Upon reduction, the color of the solution fades so the progress of the reaction can be easily followed by monitoring the decrease in free radical absorbtion at 515 nm (BRAND-WILLIAMS *et al.*, 1995; SANCHEZ-MORENO *et al.*, 1998; DANGLES *et al.*, 1999; VILLANO *et al.*, 2007).

The DPPH· concentration was held constant (0.0025 mg/mL, 65.4 μM) and the plot of the absorbance values vs. radical concentrations was linear, whereas the concentration values of the pure phenols covered a wide range (3-0.003 mg/mL). In this way, both redox reaction partners could be in excess at different times.

It was observed that the variation of DPPH· concentration with the concentration of added antioxidants followed a sigmoidal shape curve if the antioxidant doses are expressed as logarithms. Therefore, the EC_{50} values were calculated using a sigmoidal dose-response (variable slope) equation (GraphPad Prism, version 5):

$$y = y_{min} + \frac{(y_{max} - y_{min})}{1 + 10^{(\log EC_{50} - x)*slope}} \quad (1)$$

where y is the remaining DPPH·(%), y_{max} the top plateau, y_{min} bottom plateau, x the logarithm of the antioxidant concentration and the slope describes the steepness of the curve (Fig. 1). Each curve in the graph was fitted individually according to its corresponding measured data sets for the four parameters (top and bottom plateaus, EC_{50} value and slope).

The $EC_{50,}$ its 95% CI (95% confidence intervals of EC_{50}) and the coefficient of determination (R^2) values obtained for the antioxidants investigated at different time intervals (1 to 60 min) are listed in Table 1. EC_{50} was inversely related to the antioxidant capacity, i.e. the lower the EC_{50} values, the greater the antioxidant activities. The 95% confidence intervals, rather than standard errors, are reported in order to explain the quality of the curve-fitting to the data points.

Determination of EC_{50} and time at final state ($EC_{50fs,}$ T_{EC50fs})

Data from the plot showing the decay of EC_{50} over time, were used in Equation (2); an exponential curve (Fig. 2) was obtained. The EC_{50} at the final state (EC_{50fs}), and the time needed to reach the final state at a concentration of antioxidant corresponding to EC_{50fs} (T_{EC50fs}) were then determined (Fig. 2a).

$$y = a*e^{-bx}+c \quad (2)$$

where y is EC_{50}; a the amplitude; b a rate constant; x time and c EC_{50fs}.

For both synthetic and natural antioxidants the redox reaction displayed a biphasic course with a fast decay in absorbance in the first minutes, followed by a slower step which followed an asymptotic trend as it progressed toward the final values. The fast step essentially refers to the most labile H atoms, whereas the slow step reflects the remaining activity in the oxidation degradation-products (DANGLES *et al.*, 2000).

Table 2 shows the values for EC_{50fs}, R^2 for the exponential curves obtained for different antioxidants and the T_{EC50fs}. For the pure chemicals, the highest antioxidant capacity was exhibited by tannic acid (0.010), followed in order by quercetin (0.082), gallic acid (0.083), hydroquinone (0.176) and caffeic acid (0.196). These EC_{50fs} values obtained for the pure chemicals are in agreement with data reported by other authors for the steady state (Table 3).

In the plant extracts, known to be rich sources of polyphenols, *C. sinensis* and *V. vinifera,* in order, had higher antioxidant capacities than *E. hirsutum* or *C. vulgaris*. The time needed to reach the final state (T_{EC50fs}) had different rankings which indicated that the antioxidant redox kinetics differs from their antioxidant activities.

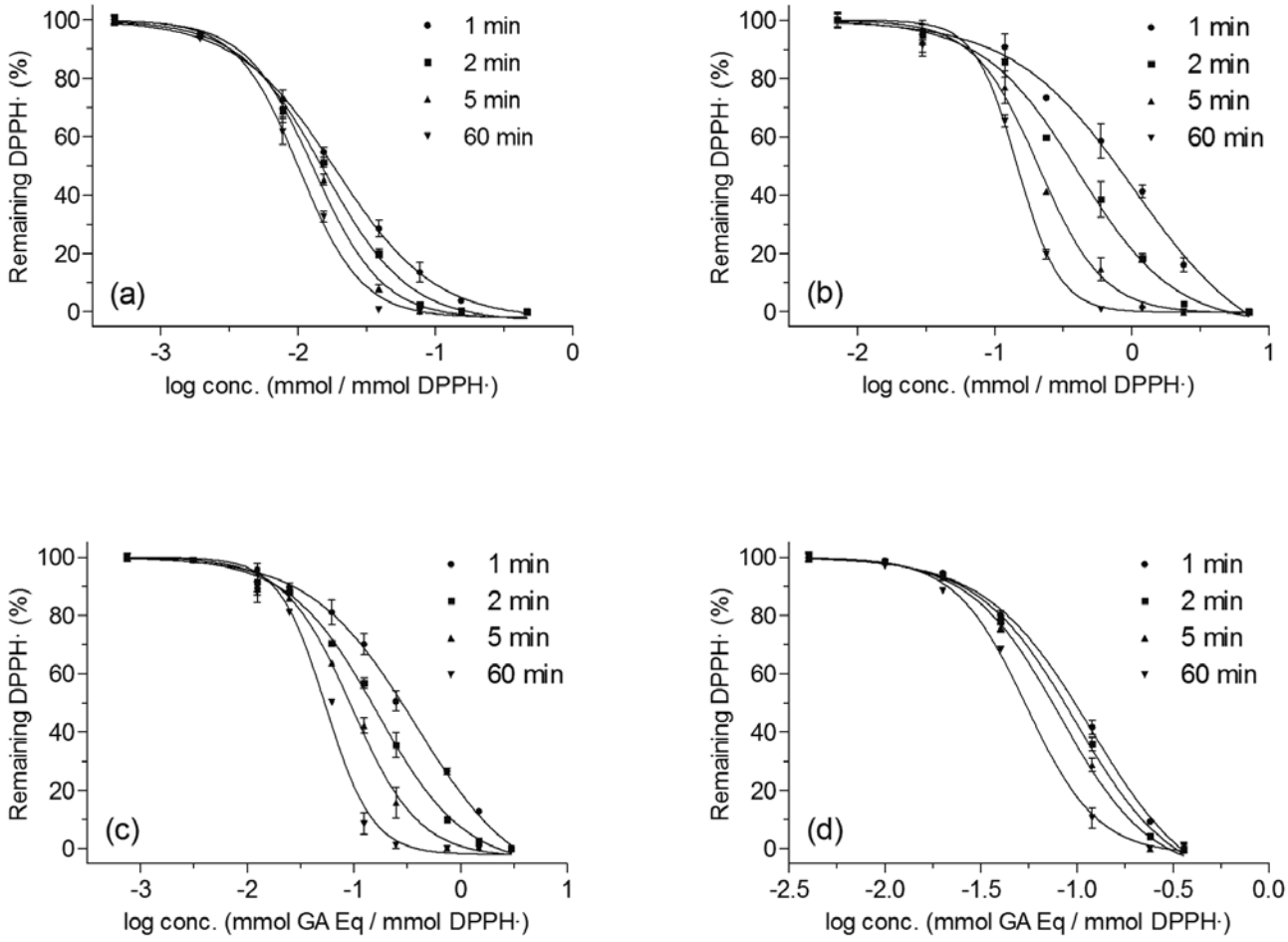


Fig. 1 - Some representative sigmoidal curves, at different time intervals, in the DPPH· scavenging process by: (a) tannic acid, (b) hydroquinone, (c) *V. vinifera* (var. Burgund Mare) and (d) C. sinensis. Data points are the means±S.E.M. (n = 3).

For example, tannic acid and *C. sinensis* extract, which were the most active antioxidants, both had long reaction times, indicating a slow kinetic behaviour.

Stoichiometry of reaction

Antioxidants are sources of transferable H atoms and can also be characterized by their stoichiometry. The antioxidant stoichiometry is determined by the antioxidant structure and its mechanisms of oxidation (VILLANO *et al.*, 2007). The stoichiometric values, *n*, indicate the number of DPPH· moles reduced by 1 mol of antioxidant; this can be calculated by Equation (3) (VILLANO *et al.*, 2007):

$$n = 1/2*EC_{50fs} \quad (3)$$

This equation estimates the number of hydrogen atoms involved in the process; values higher than the theoretical ones indicate that not only the parent phenol(s) but also their oxidation-degradation products can transfer H to DPPH· (DANGLES *et al.*, 1999; GOUPY *et al.*, 2003; VILLANO *et al.*, 2007).

The stoichiometry values given in Table 2 show that tannic acid is the strongest antioxidant among the phenolics examined, one mole of product was able to reduce 50 moles of DPPH·, while caffeic acid is able to reduce 2.6 moles. All pure phenols, with known chemical structures, had higher *n* values than the number of their hydroxyl groups. This demonstrates that the reaction is continued by the oxidation-degradation products. The enhanced antioxidant stoichiometry of tannic acid originates in its 10 residues of gallic acid born by a D-glucose core. Theoretically, its EC_{50fs} is expected to be 8 times lower and *n* should be 8 times higher than those of gallic acid. This fact was experimentally confirmed (Table 2). Thus, the proportionality between the DPPH· values and the number of OH groups in compounds with similar basic structure (PEREZ-JIMENEZ and SAURA-CALIXTO, 2008) was validated for tannic and gallic acids. In the series of plant extracts, the order of variation of *n* was: *C. sinensis* > *V. vinifera* > *E. hirsutum* > *C. vulgaris*.

Table 1 - EC_{50}, 95% CI values and R^2 for pure phenols and plant extracts at different time intervals.

Sample	Time (min)	R^2	EC_{50} (mmol / mmol DPPH·)	95% CI[a] (mmol/mmol DPPH·)
Gallic acid	1	0.995	0.118	0.111 to 0.126
	2	0.995	0.102	0.096 to 0.108
	5	0.995	0.094	0.089 to 0.099
	60	0.997	0.082	0.078 to 0.086
Tannic acid	1	0.993	0.0188	0.0165 to 0.0213
	2	0.994	0.0155	0.0141 to 0.0169
	5	0.993	0.0130	0.0120 to 0.0142
	60	0.992	0.0102	0.0094 to 0.011
Caffeic acid	1	0.993	0.389	0.336 to 0.451
	2	0.993	0.268	0.242 to 0.297
	5	0.992	0.212	0.193 to 0.233
	60	0.993	0.186	0.168 to 0.206
Hydroquinone	1	0.978	1.041	0.700 to 1.548
	2	0.980	0.409	0.327 to 0.510
	5	0.986	0.210	0.187 to 0.236
	60	0.991	0.147	0.137 to 0.157
Quercetin	1	0.990	0.127	0.115 to 0.140
	2	0.995	0.113	0.106 to 0.120
	5	0.996	0.107	0.101 to 0.113
	60	0.994	0.082	0.074 to 0.090
V. vinifera [b]	1	0.990	0.374	0.274 to 0.511
	2	0.994	0.164	0.142 to 0.189
	5	0.991	0.095	0.085 to 0.106
	60	0.987	0.054	0.048 to 0.062
C. vulgaris[b]	1	0.992	1.270	0.805 to 2.003
	2	0.994	0.674	0.537 to 0.846
	5	0.993	0.344	0.299 to 0.396
	60	0.990	0.154	0.136 to 0.175
C. sinensis[b]	1	0.996	0.118	0.103 to 0.135
	2	0.996	0.098	0.087 to 0.110
	5	0.996	0.081	0.073 to 0.090
	60	0.997	0.054	0.049 to 0.059
E. hirsutum[b]	1	0.995	0.164	0.148 to 0.181
	2	0.992	0.136	0.123 to 0.151
	5	0.991	0.121	0.108 to 0.134
	60	0.989	0.095	0.082 to 0.110

[a]95% CI: 95% confidence intervals of EC_{50};
[b]EC_{50} for the natural extracts are expressed as mmol GA Eq/mmol DPPH.

Table 2 - EC_{50} and T_{EC50} values at the final state obtained with the exponential curves for the different antioxidants. Stoichiometric values *n* of phenolic compounds are also reported.

Sample	EC_{50fs} (mmol/mmol DPPH·)	R^2	T_{EC50fs} (min)	*n*
Gallic acid	0.083±0.004	0.954	13.8	6.0
Tannic acid	0.010±0.001	0.979	21.8	50.0
Caffeic acid	0.196±0.011	0.990	7.7	2.6
Hydroquinone	0.176±0.03	0.996	6.6	2.9
Quercetin	0.082±0.006	0.962	31.4	6.1
V. vinifera[a]	0.072±0.002	0.987	7.5	6.9
C. vulgaris[a]	0.211±0.08	0.984	11.3	2.4
C. sinensis[a]	0.054±0.006	0.984	22.2	9.3
E. hirsutum[a]	0.096±0.01	0.958	18.3	5.2

[a]EC_{50fs} for the natural extracts are expressed as mmol GA Eq/mmol DPPH·.

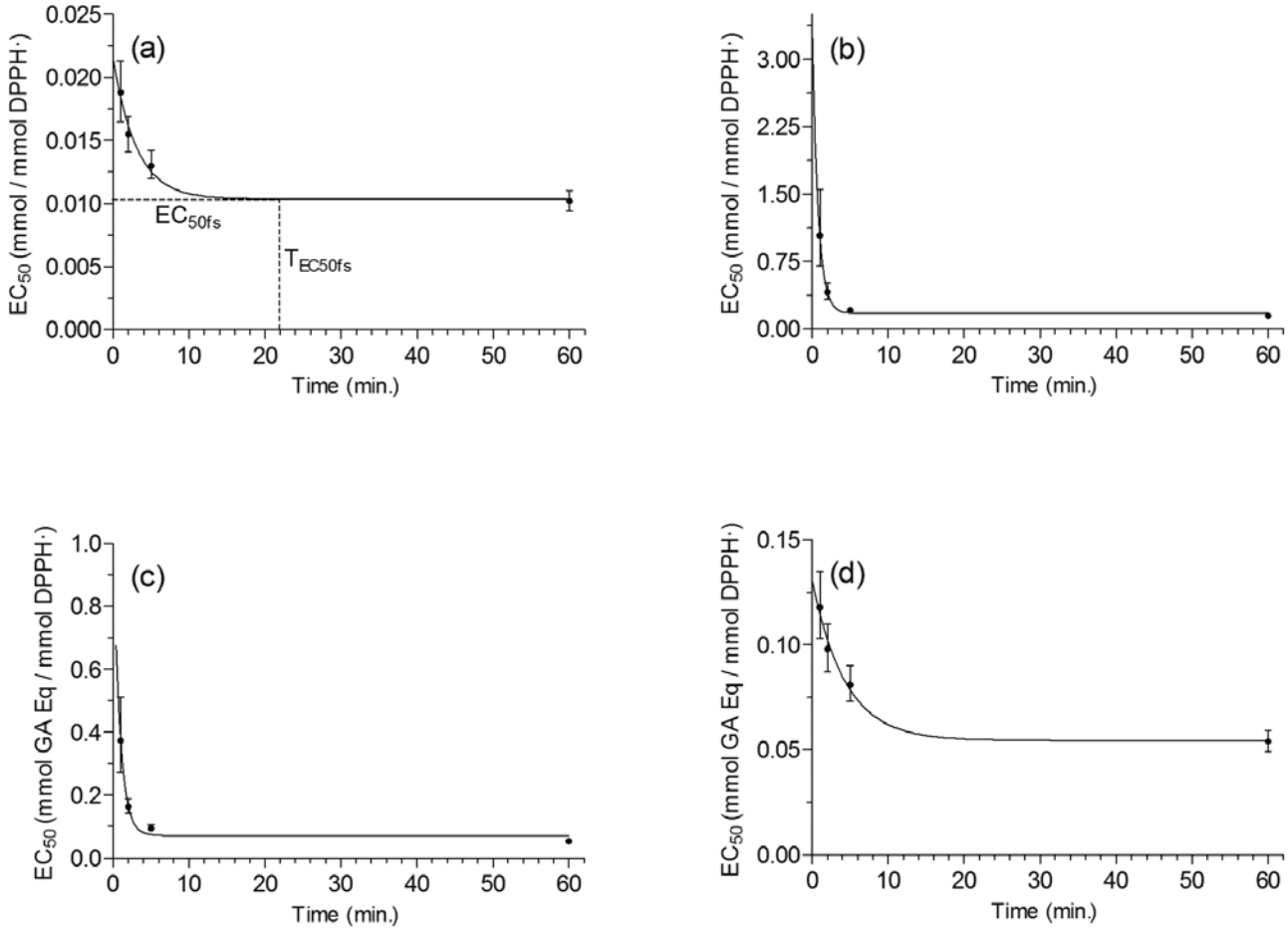


Fig. 2 - Kinetic exponential curves for the determination of the EC_{50fs} and T_{EC50fs} for: (a) tannic acid, (b) hydroquinone, (c) V. vinifera (var. Burgund Mare) and (d) C. sinensis. Data points are the means±95% CI for data shown in Table 1.

Table 3 - Comparison of EC_{50fs} with previous measurements (EC_{50} at the steady state).

Gallic acid (mmol/mmol DPPH·)	Tannic acid (mmol/mmol DPPH·)	Caffeic acid (mmol/mmol DPPH·)	Quercetin (mmol/mmol DPPH·)	References
0.083	0.010	0.196	0.082	Present study
0.082	-	0.192	0.087	VILLANO *et al.*, 2007
0.060	0.013	0.151	0.097	SÁNCHEZ-MORENO *et al.*, 1998
0.080	-	0.110	-	BRAND-WILLIAMS *et al.*, 1995

CONCLUSIONS

A quick and easy procedure for evaluating the antioxidant activity, kinetics and stoichiometric behavior of both pure phenols and natural phenolic extracts was presented. The method is based on the Brand-Williams principle (BRAND-WILLIAMS *et al.*, 1995) i.e. antioxidants in reaction with DPPH· produce a decay in stable free radical absorbance at 515 nm. This original approach is based on two mathematical models that are applied to calculate the antioxidant parameters, the versatility of measurements (wide range of antioxidant / DPPH· molar ratios) and finding the EC_{50} values at any time before the redox reaction is terminated. The results reported are in accord with the data given in the literature and calculated by other methods.

These parameters that can characterise antioxidants according to their reactivity and stoichiometry in their reactions as radical scavangers could be useful tools in choosing the doses and exposure times in experiments carried out particularly with natural extract samples of unknown and complex composition.

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SHORT COMMUNICATION

ANTIMICROBIAL ACTIVITY PRODUCED BY *BACILLUS* SPP. ISOLATED FROM FOOD OF PLANT ORIGIN

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ABSTRACT

A total of 109 bacterial strains isolated from Argentinean food of plant origin were screened for the production of bacteriocin-like substances. Antimicrobial activity of extracts from isolated cultures was evaluated according to the agar diffusion plate method. Twenty strains belonged to *Bacillus* spp. and *Paenibacillus* spp. were selected based on their activity against *Bacillus cereus*, *Bacillus pumilus*, *Bacillus subtilis*, *Paenibacillus larvae*, *Paenibacillus polymyxa* and *Staphylococcus aureus*. The antimicrobial activity of the extracts was sensitive to the proteolytic action of trypsin. This suggests that the antimicrobial substances produced would be bacteriocin-like substances. The identification of bacteriocin-like substances active against pathogenic and food-spoilage bacteria is an important aspect of food protection.

- Key words: *Bacillus* spp., bacteriocin-like substances, *Paenibacillus* spp. plant foods -

INTRODUCTION

Bacteriocins are ribosomally synthesized antimicrobial peptides or proteins produced by bacteria that display antimicrobial properties against other bacteria; they are often closely related to the producer strain (KLAENHAMMER, 1988; JACK *et al.*, 1995). These substances, which are often metabolites produced by various Gram-positive and Gram-negative microorganisms (RILEY and WERTZ, 2002) are a heterogeneous group of antibacterial peptides that vary in spectrum of activity, mode of action, molecular weight, genetic origin and biochemical properties (ABEE *et al.*, 1995).

Bacillus spp. produce a large number of peptide antibiotics, include a variety of industrially important species, and have a history of being safe for use in both food and industry (PAIK *et al.*, 1997). Several bacteriocins or bacteriocin-like substances (BLS) have already has been described for many *Bacillus* species such as *Bacillus amyloliquefaciens* (LISBOA *et al.*, 2006) *Bacillus cereus* (OSCÁRIZ and PISABARRO, 2000; BIZANI *et al.*, 2005), *Bacillus megaterium* (VON TERSCH and CARLTON, 1983), *Bacillus licheniformis* (CLADERA-OLIVERA *et al.*, 2004) *Bacillus subtilis* (SHELBURNE *et al.*, 2007; HAMMAMI *et al.*, 2009) and *Bacillus thuringiensis* (AHERN *et al.*, 2003).

Several bacterial strains produce bacteriocins that are active against important pathogens present in food such as *Listeria monocytogenes*, *Clostridium botulinum*, *Yersinia enterocolítica*, *Staphylococus aureus*, *Escherichia coli*, *Salmonella typhimurium* and *Bacillus cereus* (PAIK *et al.*, 1997; ESCUDERO-ABARCA and SÁNCHEZ ESQUIVEL, 2002; SIMON *et al.*, 2002; CHERIF *et al.*, 2003; MATTHEWES, 2004).

In spite of modern advances in technology, the preservation of foods is still a debated issue, not only for developing countries (where implementation of food preservation technologies are clearly needed) but also for the industrialized world (GÁLVEZ *et al.*, 2007). Nowadays, artificial chemical preservatives are used to limit the number of microorganisms capable of growing within food, but the increasing consumer demand for more natural food and an awareness of the health risks of some chemical preservatives has led researchers to examine the possibility of using bacteriocins as biopreservatives in addition to the application of bacteriocinogenic strains (ABEE *et al.* 1995; OMAR *et al.*, 2006).

The aim of the present study was to screen a number of *Bacillus* spp. and other related genera strains isolated from Argentinean foods of plant origin for antagonistic activity, in order to identify bacteriocin-like substances with broad inhibition spectra.

MATERIALS AND METHODS

Isolation of microorganisms

Samples of butternut squash (*Curcubita moschata*), potatoes (*Solanum tuberosum* subsp. *tuberosum*), raw rice (*Oryza L* genus), and wheat flour (*Triticum vulgare*) were kept at 4°C. All foods were analyzed within 24 h of purchase. Ten grams of each sample were mixed with 90 mL sterile Butterfield's phosphate-buffered dilution water (BUTTERFIELD, 1932), homogenized using a Stomacher 400 Circulator Homogenizer and heated for 10 min at 80°C to destroy vegetative bacterial forms. Ten-fold serial dilutions of the liquid supernatant were prepared with the same diluent. For pour plating, 1 mL of the dilutions was mixed with molten (45°C) mannitol-egg yolk-polymyxin (MYP) agar (FDA, 1998) and poured into the plates. After incubation at 37°C for 24-48 h, single colonies were isolated, identified and screened for antimicrobial activity.

Bacterial cultures

Indicator strains listed in Table 1 were laboratory stocks obtained from different sources. The strains were kept frozen in 20% (v/v) glycerol at -20°C.

Preparation of crude extracts

The strains were grown in brain-heart infusion (BHI) (Britania, Buenos Aires, Argentina) at 37°C for 24 h. After incubation, the bacterial

Table 1 - Media, temperature and time conditions of growth of indicator organisms.

Indicator organism	Medium*	Temperature (ºC)	Time (h)
Bacillus cereus (food isolated)	MH	37	24
Bacillus pumilus (food isolated)	MH	37	24
Bacillus subtilis (food isolated)	MH	37	24
Paenibacillus polymyxa (food isolated)	MH	37	24
Paenibacillus larvae (isolated from apiary)	MYPGP	37	48
Staphylococcus aureus ATCC 25923	MH	37	24
Escherichia coli ATCC 25922	MH	37	24

*Medium: MH: Mueller-Hinton agar; MYPGP: Mueller-Hinton, yeast extract, glucose and sodium pyruvate agar.

cells were removed by centrifugation at 10,000 rpm for 15 min and the supernatant was adjusted to pH 6.8 with 0.1 mol/L NaOH to eliminate the inhibitory activity from the acid. After neutralization, the supernatant was filtered through a 0.22 μm membrane and stored at -20°C until analysed.

Antimicrobial activity assay

The assessment of the antimicrobial activity was performed by the modified well diffusion agar assay method, according to PAIK *et al.* (1997). To prepare the plates for the dissemination of test wells, 20 mL of Mueller-Hinton Agar (Britania, Buenos Aires, Argentina) or Mueller-Hinton, yeast extract, glucose and sodium pyruvate agar (MYPGP) prepared according to the composition reported by DINGMANN and STAHLY (1983), previously inoculated with a suspension of corresponding indicator strain (Table 1) were plated onto Petri dishes. Wells were made in the solidified agar. Twenty μL of pretreated extracts of isolated strains were transferred to the wells in preseeded agar plates. After diffusion of the extract into the agar (2 hours at room temperature), the plates were incubated under conditions that correspond to each testing strain (Table 1). Finally, the plates were examined for the presence of inhibition zones and antimicrobial activity is expressed as average size (mm) of inhibition zones. The cross-sensitivity spectrum of the isolates was determined with the method described above in Mueller-Hinton agar.

Effect of trypsin on antimicrobial activity

To analyze the relationship between the effect of proteolytic and antimicrobial activity, samples of cell-free extract were incubated at 37°C for 1 h with trypsin (Difco; Detroit, MI, USA) at a concentration of 10 mg/mL. After treatment, the samples were tested for antimicrobial activity with the agar-disk diffusion assay against sensitive testing strains.

Identification tests
and bacterial characterization

Bacterial identification was based on biochemical and morphological tests and 16S rRNA gene sequencing analysis. Selected strains were examined microscopically for the presence of spores after growth on nutrient agar at 37°C for 2-5 days. The identification of *Bacillus* spp. was made following: Gram reaction; test for protein toxin crystals (FDA, 1998); catalase test (FDA, 1998); oxidase test (FDA, 1998); nitrate reduction test (AOAC, 1995); Voges Proskauer reaction (AOAC, 1995); indole production test (CLAUS and BERKELEY, 1986); growth on Simmons citrate agar (AOAC, 1995); growth with lysozyme (AOAC, 1995); sugar-fermentation test (CLAUS and BERKELEY, 1986); motility (AOAC, 1995); hydrolysis of starch, gelatin (CLAUS and BERKELEY, 1986) and casein (CLAUS and BERKELEY, 1986); anaerobic growth (CLAUS and BERKELEY, 1986). In addition, the isolates were characterized by biochemical reactions in the API 50 CH kits at 30°C according to the manufacturer's instructions (API System, BioMérieux S.A., Marcy l'Etoile, France). Bacterial 16S rRNA genes were amplified by PCR using the universal primer pair 27f (5'-AGA GTT TGA TCC TGG CTC AG- 3'), and 1492r (5'-GGT TAC CTT GTT ACG ACT T-3') (GUO *et al.*, 2007). A colony was added to a mix of PCR reaction composed of 2 μL of 10X Taq DNA polymerase buffer, 0.12 U/μL of Taq polymerase, 5 mmol L^{-1} $MgCl_2$, 0.2 mmol L^{-1} dNTPs and 5 pmol L^{-1} of each primer. The reaction conditions consisted of one initial denaturation cycle at 95°C for 3 min, 35 cycles of 95°C for 30 sec, 50°C for 1 min and 72°C for 3 min, and a final extension step at 72°C for 10 min. Amplification was carried out using an Eppendorf Mastercycler gradient (Eppendorf, Hamburg, Germany) with a reaction volume of 20 μL. The products of the PCR amplification were examined by electrophoresis in 1% agarose gel and displayed in a uv transilluminator. The partial sequence of the 16S rDNA (about 1,500-bp) was carried out using a BigDye DNA sequencing kit (PE Biosystems) according to the manufacturer's instructions. Every sequence type was compared with those available in GenBank by performing BLASTn program (http://www.ncbi.nlm.nih.gov).

Statistical analysis

The antimicrobial activity of the strains against each indicator strain was compared by Analysis of Variance (ANOVA) with Tukey tests with SPSS 15.0 of Windows.

RESULTS AND DISCUSSION

In the present study bacterial strains isolated from foods of plant origin were screened in order to detect antimicrobial effects. A total of 109 bacterial isolates obtained from starchy food were screened for antimicrobial activity against bacterial strains used as indicators (Table 1): *B. cereus* (16 isolates), *B. pumilus*, (2 isolates), *B. subtilis* (14 isolates), *P. larvae* (10 isolates), *P. polymyxa* (7 isolates), and *S. aureus* (3 isolates). In many cases, for a given indicator strain, some isolates showed a significantly higher antimicrobial activity ($P<0.05$) than others (Table 2). Antimicrobial activity of the crude extract analyzed by agar diffusion showed that 20 of the isolates produced antimicrobial substances that were active against at least one of the bacterial indicator strains.

Some bacteriocins produced by Gram-positive

Table 2 - Antimicrobial spectrum of selected bacteriocinogenics isolated from raw plant material.

Bacteriocin producer strains		Indicator strains*						
		B. cereus (food isolate)	*B. pumilus*	*B. subtilis* (food isolate)	*P. larvae*	*P. polymyxa*	*S. aureus* ATCC 25923	*E. coli* ATCC 25922
B. amyloliquefaciens	1	7.3±0.6^{a,b,c}	0	9.7±1.5^{b,c,d}	21.3±0.6^{e}	7±0^{a}	7.3±0.57^{a}	0
B. amyloliquefaciens	2	7^{a,b}	0	7±0^{d}	0	0	0	0
B. amyloliquefaciens	3	0	0	0	12.7±0.6^{a,b}	0	0	0
B. cereus	4	0	0	0	10.3±0.6^{a}	0	8.3±0.58^{b}	0
B. cereus	5	7.7±0.6^{a,,b}	0	10.7±1.2^{a,b}	0	0	0	0
B. licheniformis	6	6.7±0.3^{a}	0	8±1.7^{b,c,d}	0	0	0	0
B. licheniformis	7	6.7±0.3^{a}	9.3±0.6^{a}	0	0	0	0	0
B. licheniformis	8	9.7±1.5^{d}	0	8± 1.4^{b,c,d}	0	7.3±0.6^{a}	0	0
B. licheniformis	9	7±0^{a,b}	9.7±0.6^{a}	7.7±0.6^{b,c,d}	12.7±2.1^{a,b}	0	0	0
B. pumilus	10	6.7±0.28^{a}	0	0	0	0	0	0
B. pumilus	11	0	0	0	16.7±1.5^{d}	0	0	0
B. subtilis	12	0	0	7.7±0.6^{b,c,d}	14±1^{b,c,d}	7.7±0.6^{a}	0	0
B. subtilis	13	6.7±0.28^{a}	0	0	0	0	0	0
B. subtilis	14	8.7±0.6^{c,d}	0	9.7±1.5^{b,c,d}	20.7±3.5^{e}	7.7±0.6^{a}	7±0^{a}	0
B. subtilis	15	7±0^{a,b}	0	8.3±0.6^{b,c,d}	14±1.7^{b,c,d}	7.7±0.6^{a}	0	0
B. subtilis	16	8.3±0.6^{b,c}	0	8±1^{b,c,d}	15.7±0.6^{c,d}	7.3±0.6^{a}	0	0
B. subtilis	17	8±0^{a,b,c}	0	8.7±1.53^{b,c,d}	0	0	0	0
P. polymyxa	18	7.3± 0.6^{a,b,c}	0	13± 2.6^{a,b,c}	0	0	0	0
P. polymyxa	19	10±1^{d}	0	10.33±0.6^{a}	20.7±0.6^{e}	0	0	0
P. polymyxa	20	7±0^{a,b}	0	7.3 ± 0.6^{c,d}	0	7.7±0.6^{a}	0	0

*Values are the diameter of the inhibition zone in mm around the hole and correspond to the mean of three independent experiments ± standard deviation. Values against the same indictor strains (vertical columns) with different superscripts ($^{a, b, c, d}$ and ^{e}) differ significantly ($P < 0.05$) compared to other isolates.

bacteria have shown an antimicrobial activity against Gram-negative strains, like some type A lantibiotics such as nisin A and mutacin B-Ny266 that have been reported to be active against a number of medically important Gram-negative bacteria including *Campylobacter*, *Haemophilus*, *Helicobacter*, and *Neisseria* (RILEY and WERTZ, 2002). However, most of the Gram-positives bacteriocins are ineffective against Gram-negatives strains due to the barrier properties of the outer membrane (ABEE *et al.*, 1995; JACK *et al.*, 1995). In this study, *E. coli* ATCC 25922 was resistant to the activity of all 20 antimicrobial strains isolated.

B. cereus, B. licheniformis and *B. subtilis* were inhibited by bacteriocinogenic *Bacillus* spp strains (CHERIF *et al.*, 2001; TORKAR and MATIJAŠIC, 2003; LISBOA *et al.*, 2006; SIRTORI *et al.*, 2006). Almost all the strains showed an antimicrobial activity against *B. subtilis* and *B. cereus,* while *B. pumilus* showed more resistance to the antimicrobial strain activity.

Paenibacillus larvae is the causative agent of American foulbrood (AFB) which affects the larval and pupal stages of honeybees (*Apis mellifera* L.). In the present study, the inhibitory activity against *P. larvae* produced by the antimicrobial activity of the strains, was significantly higher than that produced against the other indicator strains.

Based on 16S rRNA gene sequencing analysis, morphological and biochemical examinations and the API 50 CHB results, the strains were identified as the *Bacillus* and *Paenibacillus* species.

B. amyloliquefaciens strains producers of antimicrobial activity, were identified in three of the 109 strains analyzed. One of these, strain 1, produced antimicrobial activity against *S. aureus*, *B. cereus*, *B. subtilis*, *P. larvae* and *P. polymyxa*. LISBOA *et al.* (2006) isolated a *B. amyloliquefaciens* strain that produced a bacteriocin-like substance that was active against *B. cereus* and *B. subtilis,* but was ineffective against *S. aureus and E. coli.*

B. cereus strain 4 produced antimicrobial activity against *S. aureus*, but showed little action against the other strains tested. On the other hand, *B. cereus* strain 5 showed antimicrobial activity against the indicator strains *B. cereus* and *B. subtilis*, also showing activity against *P. polymyxa* (strain 20) in the cross-sensitivity spectrum of bacteriocinogenic-like strains. OSCÁRIZ and PISABARRO (2000) identified a bacteriocin (cerein 7), a peptidic antibiotic produced by *Bacillus cereus* Bc7 (CECT 5148) which has a broad spectrum of antibacterial activity against Gram-positive bacteria but is inactive against Gram-negative bacteria. Alternatively, BIZANI *et al.* (2005) reported that cerein 8A produced by *Bacillus cereus* 8A, was effective for Gram-positive and Gram-negative bacteria, showing bactericidal action against *Bacillus cereus,* and in-

hibition of the growth of *E. coli* and *Salmonella enteritidis*.

None of the four *B. licheniformis* strains, 6, 7, 8 and 9 formed antimicrobial substances that were active against *S. aureus* or *E. coli;* they had low activity against some of the other testing strains such as *B. cereus* and *B. subtilis*. In addition, *B. licheniformis* strain 9 showed antimicrobial activity against the indicator strain *P. polymyxa* (strain 20) in the cross-sensitivity spectrum of the bacteriocinogenic-like strains. MARTIRANI *et al.* (2002) isolated a *B. licheniformis* bacteriocin, bacillocin 409, which showed low activity against *B. cereus* and *B. subtilis* and no activity against *E. coli* and *S. aureus*. The same result was found by CLADERA-OLIVERA *et al.* (2004) with *Bacillus licheniformis* strain P40.

A low antimicrobial activity was shown by the two bacteriocinogenic-like *B. pumilus* strains, 10 and 11. ASLIM *et al.* (2002) reported a *B. pumilus* strain with antimicrobial activity against *S. aureus,* but without activity against *E. coli.* Pumilicin 4 presented antimicrobial activity against *S. aureus* and several Gram-positive bacteria (AUNPAD and NA-BANGCHANG, 2007).

The *B. subtilis* strain 14 showed the highest activity against *P. larvae,* and was also active against *S. aureus.* In addition, it showed a significantly higher activity against some of the other given indicator strains ($P<0.05$) compared to other isolates. A bacteriocinogenic *Bacillus* spp strain P45, a *B. subtilis* group strain produced an antimicrobial substance that also showed activity against *Bacillus* spp. and some *S. aureus* strains (SIRTORI *et al.*, 2006).

Three *P. polymyxa* strains also produced antimicrobial activity against some indicador strains. Paenibacilin (HE *et al.*, 2007) is a bacteriocin isolated from *P. polymyxa* OSY-DF and active against a broad range of food-borne pathogenic and spoilage bacteria, including *B. cereus, B. subtilis, S. aureus*, but it was inactive against *E. coli.*

For all the producer strains, antimicrobial activity disappeared after treatment with trypsin (results not shown), which indicates the proteinaceous nature of the substance responsible for the antimicrobial activity; it is probably a bacteriocin.

Fourteen isolates were selected for further study due to their broad antimicrobial spectra. The antimicrobial activity of these strains was measured against other isolates with antimicrobial activity. All of the isolates were resistant to their own inhibitors with the exception of isolates 5, 8, 14 and 20 which produced inhibition on their own strain. These results are in agreement with those reported by OMAR *et al.* (2006).

The results are promising and bacteriocin-like activity should be analyzed further against other pathogens. Isolation of bacteriocinogenic *Bacillus* spp. strains from different sources could be an important source of natural antimicrobial substances with potential relevance for the biological control of pathogenic strains, spoilage microorganisms and food safety.

Some of these isolates could have a broad application potential in food fermentation. Due to the fact that strains 1 and 14 showed an antagonistic effect on five of the indicator bacteria, it could be recommended that these strains be used in certain biotechnological studies to obtain better understanding of their action. These substances could be novel antimicrobial agents with potential applications in food safety, with important implications for the biological control of pathogenic and spoilage microorganisms.

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SHORT COMMUNICATION

THE EFFECT OF STEAM STERILIZATION ON ANTIOXIDANT ACTIVITIES OF SAGE, OREGANO AND BASIL

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ABSTRACT

Sage, oregano and basil are aromatic herbs showing strong antioxidant activity. Total phenolic contents (279.1, 258.3 and 84.4 mg GAE/g), DPPH* radical-scavenging activity (EC_{50} values 2.07, 2.64 and 9.26), metal chelating activities (82.6, 63.9 and 92.9%) and hydrogen peroxide scavenging capacity (89.7, 97.2 and 7.4%) were determined for sage, oregano and basil, respectively. Total phenolic content and antioxidant activity of sage samples were not significantly influenced by the steam sterilization process, but basil was. Steam sterilization significantly reduced the total phenolic content, and hydrogen peroxide scavenging capacity of oregano.

- Key words: antioxidant activity, basil, oregano, sage, steam sterilization -

INTRODUCTION

Sage, oregano and basil (Family *Labiatae)* have strong antioxidant activities (KAUR and KAPOOR, 2001). Antioxidants are compounds that can delay, reduce or prevent oxidation processes by reacting with free radicals and chelating metals. They can also scavenge oxygen, the triplet as well as the singlet form, and transfer hydrogen atoms to the free radical structure (TORRES *et al.*, 2002; GULCIN *et al.*, 2004).

Spices and herbs are natural products that can be contaminated by microorganisms that survive the harvest, primary processing and/or drying process. Production of spices implicates a number of hygienic problems which can pose tremendous risks for producers and consumers (SCHWEIGGERT *et al.*, 2007). Several methods including fumigation with ethylene oxide, irradiation, steam treatments, high hydrostatic pressure, etc. have been developed to reduce the microbial loads on spices (SCHWEIGGERT *et al.*, 2007; CALUCCI *et al.*, 2003; WEBER, 2003; LEISTRITZ, 1997).

Steam sterilization systems have been working wonders for the sterilization of herbs, seeds, spices, teas, medicinal plants, etc. The temperature and time of processing depends on the microbiological load in the raw material. Steam sterilization systems are based on rapid heating to the required temperatures with saturated steam, holding the temperature for the required period, and rapid vacuum cooling/drying. The rapid heating and cooling minimize the loss of colour and of essential oils (ANON, 1993; ANON, 2009). Compared to conventional spices, steam-sterilized products are generally characterized by significantly lower microbial loads and brighter color (SCHWEIGGERT *et al.*, 2005). Steam is also the most ideal gas because it is non-toxic. BARBIERI *et al.* (2004) concluded that the use of a low pressure superheated steam method rendered a better product in terms of the content of aroma compounds compared with other methods.

The objective of the present study was to determine the effect of a steam sterilization process on commercial samples of sage, oregano and basil. Total phenolic content and antioxidant activity analysis (DPPH* radical-scavenging activity, metal chelating activity and hydrogen peroxide scavenging capacity) were measured in control and sterilized samples.

MATERIALS AND METHODS

Materials

Samples of sage (*Salvia officinalis L.*), oregano (*Origanum vulgare L.*) and basil (*Ocimum basilicum L.*) were provided by Safe Spice Co. (Izmir, Turkey). Each sample was divided into two lots of ~500 g, one of which was steam sterilized and the other was kept as control. Samples were enclosed in cotton bags. The steam sterilization process was performed in a commercial sterilization chamber. Thermal treatments of sage, oregano, and basil were accomplished by sterilization at 120°C, by means of steam (2 bar) for 2-4.5 min (internal temperature at sterilization was 92°-98°C). The sterilization process was carried out in triplicate.

Extraction

Herb samples, 2.5 g of each (ground), were homogenized with 20 mL methanol for 5 min in an Ultra Turrax (Ika-Werke GmbH&Co., Staufen, Germany). The extraction was repeated four times and all extracts were combined. The total extract was centrifuged at 4,500xg for 15 min and then the supernatants were filtered. The volume was made up to 50 mL with methanol.

Analysis

Total phenolic compounds were measured using the Folin-Ciocalteu method (SINGLETON and ROSSI, 1965) and results are expressed as gallic acid equivalents. DPPH* radical-scavenging activity of methanol extracts of samples were determined using the stable radical DPPH* (2,2 diphenyl-1-picryhydrazyl) (BRAND-WILLIAMS *et al.*, 1995). Chelating activity (YEN and WU, 1999) and hydrogen peroxide scavenging capacity (RUCH *et al.*, 1989) were also determined. All analyses were carried out in triplicate.

Statistical analysis

Results were analyzed statistically using a completely randomized design. When the means were significant they were separated by using Fisher's LSD procedure (SAS, 1999).

RESULTS

Several phenolic compounds with antioxidant activity were identified in sage, oregano and basil (AREIAS *et al.*, 2000; SUHAJ, 2006). The total phenolic contents of sage, oregano and basil extracts in control samples were 279.1 mg GAE/g, 258.3 mg GAE/g, 84.4 mg GAE/g, respectively (Table 1). There was a statistically significant difference among the total phenolic contents of the species. BERTELLI *et al.* (2004) reported the total phenolics in oregano (112.1 mg/g), sage (52.12 mg/g) and basil (40.05 mg/g). The total phenolics in the samples were decreased by the steam sterilization process, but the differences between the control and sterilized samples were not significant ($p<0.05$) in the sage samples.

Table 1 - Total phenolic contents, DPPH scavenging activity (EC_{50}), chelating activity and H_2O_2 scavenging capacity of control and sterilized sage, oregano and basil.

		Total phenolics (mg GAE / g)	DPPH scavenging activity (EC_{50} value)	Chelating activity, %	H_2O_2 scavenging capacity, %
Sage	control	279.1±2.1[a]	2.07±0.06[a]	82.6±1.9[a]	89.7±1.6[a]
	sterilized	271.3±1.3[a]	2.12±0.01[a]	79.2±1.3[a]	86.3±0.8[a]
Oregano	control	258.3±0.2[b]	2.64±0.04[b]	63.9±2.3[b]	97.2±3.5[b]
	sterilized	249.4±0.6[c]	2.73±0.06[b]	50.9±1.7[b]	71.1±4.2[c]
Basil	control	84.4±3.8[d]	9.26±0.01[c]	92.9±0.1[c]	7.4±0.4[d]
	sterilized	76.6±3.2[e]	10.79±0.75[d]	79.2±1.3[d]	4.7±0.2[e]

Results are expressed as means ± SD of three parallel replicates.
Different letters in a column indicate significant differences (p>0.05).

The antioxidant activity of the various spice extracts was measured in terms of hydrogen donating or radical-scavenging ability using the stable DPPH* radical (BRAND-WILLIAMS *et al.*, 1995). The amount of sample needed to increase the initial DPPH* concentration (EC_{50}) by 50% is a parameter that is widely used to measure the antioxidant activity. The lower the EC_{50}, the higher the antioxidant power. Basil, oregano and some other spices showed a good radical-scavenger property in the DPPH assay (TOMAINO *et al.*, 2005). The EC_{50} values of the samples are given in Table 1. The EC_{50} values of the control samples were 2.07 mg for sage, 2.64 mg for oregano and 9.26 for basil. Sterilization treatment caused an increase in the EC_{50} value. However the difference was only statistically significant for basil (p>0.05), the increase in EC_{50} values of sterilized sage and oregano samples were not significant (p>0.05).

In the chelating of ferrous ions by the extracts, ferrozine can quantitatively form complexes with Fe^{+2}. Measuring the rate of colour reduction allows the chelating activity of the coexisting chelator to be estimated (YAMAGUCHI *et al.*, 2000). The chelating activity was analyzed in 50 mg/mL extracts. As can be seen in Table 1, the chelating activities (%) of basil, sage and oregano extracts were 92.9%, 82.6% and 63.9%, respectively. Chelating activity was decreased by the sterilization process, but the differences between the control and the sterilized samples were not significant (p>0.05) in the sage and oregano samples, but it was significant (p<0.05) for the basil samples.

The scavenging ability of the extracts on hydrogen peroxide is shown in Table 1. These results show that both the sage and oregano extracts had strong hydrogen peroxide scavenging capacity; the capacities decreased in the order of oregano>sage>basil. CALUCCI *et al.*, (2003) reported that basil had the highest free radical content with slightly smaller contents found in oregano, sage, parsley and rosemary. This proves that the basil samples had less hydrogen peroxide scavenging capacity. After sterilization, a general decrease of H_2O_2 scavenging capacity was observed in all the samples, but the differences between the control and the sterilized samples were not significant for the sage samples (p>0.05).

DISCUSSION

In the present study, non-sterilized (control) sage, oregano and basil extracts showed higher antioxidant activity (DPPH* radical-scavenging activity, metal chelating activity and hydrogen peroxide scavenging capacity) than the sterilized samples. The decreases in total phenolic content and antioxidant activity were not significant for sage (p>0.05), but were significant (p<0.05) for basil. DPPH* radical-scavenging activity and metal chelating activity of oregano samples were not significantly affected by sterilization (p>0.05). Thermal treatment may be responsible for a significant loss of natural antioxidants because most of the compounds are relatively unstable.

TOMAINO *et al.* (2005) studied the influence of heating on antioxidant activity and the chemical composition of some spice essential oils. They reported that the heating (up to 180°C) of basil and oregano oils did not influence either their antioxidant activities or their chemical composition. SHOBANA and NAIDU (2000) reported that the antioxidant activity of spice extracts was retained even after boiling for 30 min at 100°C, indicating that the spice constituents were resistant to thermal denaturation.

In conclusion, the results of this research

suggest that sage, oregano and basil are strong antioxidant herbs and that steam sterilization is an effective, safe, and consumer-friendly method for sterilizing.

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SHORT COMMUNICATION

BEHAVIOUR OF SOME COMPOUNDS WITH λ_{MAX} AT 280 NM IN THE DETERMINATION OF TOTAL FLAVONOIDS OF GRAPE SKIN EXTRACTS MADE FROM A HYDROALCOHOLIC SO_2-RICH SOLVENT

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ABSTRACT

This paper reports the results of a study on the ambiguous behaviour of substances with λ_{max} at 280 nm in the determination of total flavonoids in whole grape skin extracts made from a hydroalcoholic SO_2-rich solvent, apparently not eluted with methanol after absorption on a C_{18} cartridge employed for sample pre-concentration and SO_2 removal. After rejecting the hypothesis that they were irreversibly absorbed on the C_{18} cartridge, it seemed that they reacted with other phenolics during passage through the C_{18} cartridge and were totally eluted with methanol in this new combined form. This new hypothesis seems very probable and is in accord with the more recent acquisition about the synthesis of anthocyanins and flavanols.

- Key words: anthocyanins, λ_{max}, polyphenols, proanthocyanidins, total flavonoids -

INTRODUCTION

Polyphenols are very important UV-absorbing compounds because of the contribution they make to the flavour of foods and beverages (GLORIES, 1988; THORNGATE, 1997; GAWEL *et al.*, 2000) and due to the fact that they can protect against some animal and human pathologies (DIXON *et al.*, 2005).

Among the polyphenols identified in grapes and wines, anthocyanins and monomeric, oligomeric and polymeric flavanols, belonging to the class of flavonoids, display a clear-cut absorption maximum at about 280 nm. As such, these compounds are the most important among the UV-absorbing substances of grapes and wines. Therefore measuring the absorbance at 280 nm was proposed by SOMERS and ZIEMELIS (1985) as a method for determining the amount of total flavonoids.

In contrast to the results obtained for wines in previous experiments, the total flavonoid values determined in whole grape skin extracts made from a hydroalcoholic SO_2-rich solvent, were abnormally higher than the total flavonoid and total phenolic values determined after pre-concentration on a C_{18} cartridge to remove SO_2 (DI STEFANO *et al.*, 2002). Assuming that this was due to interfering substances, experiments were performed to acquire information about their nature and presence in grape skin, seed extracts and wines.

MATERIALS AND METHODS

Experiments were performed on Barbera grapes grown in Piedmont (Italy) in 2004, Nero d'Avola, Syrah, Cabernet Sauvignon and Merlot grapes, harvested during ripening in western Sicily in 2006, Barbera, Nebbiolo, Refosco and Syrah grapes, harvested at technological maturity in Piedmont in 2006, and a group of Sicilian red wines of 2007 vintage.

Extraction of phenolics from grape skins and seeds

Fifty berries with petioles were selected from a sample of 400 berries collected from each vineyard. After weighing, berries were skinned, and the skins and seeds were placed in two different flasks, each containing 50 mL of pH 3.2 tartaric buffer (5 g/L tartaric acid, 22.2 mL/L NaOH 1M, 2 g/L $Na_2S_2O_5$, 125 mL/L 96% ethanol, to 1 L with distilled H_2O in a volumetric flask) for 4 h. The samples were then homogenised and centrifuged, and the liquors were collected in 100-mL volumetric flasks. Pellets were re-suspended in 40 mL of the previously mentioned buffer and after 1 h were centrifuged. The second extracts were added to the first and brought to 100 mL with the same buffer.

Spectrophotometric analysis of polyphenols

Proanthocyanidins in wines and in whole skin and seed extracts and after passage through a C_{18} cartridge were determined following the method of DI STEFANO and GENTILINI (2002). In brief: 0.1-0.2 mL of each sample and 24.9-24.8 mL ethanol:HCl 1:1, respectively, were placed in a 50 mL round-bottomed flask wrapped in tinfoil that was heated in a boiling water bath for 50 min with refrigerant, and then cooled with running water. The spectra of this solution from 340 to 700 nm were recorded before and after heating and the absorbances E'_{532} nm were calculated. The same determinations were performed after pre-concentration and cleaning of the samples by passage through a C_{18} cartridge. The proanthocyanidins of grape extracts and wines were calculated as cyanidin according to the following equation:

$$\text{Proanthocyanidins (mg/L)} = = \Delta E'_{532} \times 1162.5/v$$

where the volume of the sample was v; the length as a.u. (absorbance units) of the segments joining the peaks at 532 nm of spectra of the sample before and after heating, with the intersection point between the perpendicular drawn from the peaks at 532 nm to the λ axis and the tangent to the spectrum, were E'_1 and E'_2 (the difference between them was $\Delta E'_{532}$); 1162.5 was a coefficient deduced from ε of cyanidin and the yield of conversion of procyanidins in cyanidin in the above-described conditions. In the seed extracts, vanillin assay was performed following the method of BROADHURST and JONES (1978).

To determine total anthocyanins and total flavonoids, 0.5 mL of each sample of grape skin extracts and wines were diluted to 25 mL with ethanol:H_2O:HCl conc. 70:30:1 (ethanol-HCl), the spectra from 230 to 700 nm and the absorbances at 540 or 536 nm (for cultivars in which dioxygenated or trioxygenated anthocyanins, respectively, prevailed) were recorded and the E'280 was calculated. Total anthocyanins and total flavonoids of grape extracts and wines were calculated according to the following equations:

$$\text{Tot. Ant. mg/L} = 16.17 \times E_{540} \times 50$$
$$\text{Tot. Flav. mg/L} = 82.4 \times E'_{280} \times 50$$

16.17 was calculated from ε of malvidin-3-glucoside in ethanol-HCl deduced from ε = 33700 in methanol-HCl conc. (WULF and NAGEL, 1979); 82.4 was concentration/E'_{280} determined on a 10 mg/L solution of (+)-catechin; the dilution coefficient of the extracts was 50; the length as absorbance units (a.u.) of the segment joining the peak at 280 nm of the spectrum of the skin extract diluted in ethanol-HCl, with the intersection point between the perpendicular drawn from the peak at 280 nm to the λ axis and the tangent to the spectrum in the UV region, was E'_{280}.

For skin extracts, data in mg/L were converted to mg/kg.

Total flavonoids, anthocyanins and proanthocyanidins were also determined after absorption of phenolics of grape skin extracts and wine on a C_{18} cartridge, and elution with methanol.

Pre-concentration
of phenolics on a C18 cartridge

A sample of 0.5 mL, diluted to 5 mL with 5×10^{-3} M H_2SO_4, was passed through a 400 mg C_{18} cartridge (Sep Pak, Waters, Milan, Italy, part n° WAT036810) previously activated by sequential elution of 2 mL methanol and 3 mL 5×10^{-3} M H_2SO_4. After removal of interfering hydrophilic substances by washing with 3 mL of 5×10^{-3} M H_2SO_4, phenolics were recovered with 2-3 mL of methanol in a 25-mL volumetric flask and brought to 25 mL with ethanol-HCl.

To determine total flavonoids in seeds, the 230-400 nm spectra of the extracts diluted 1:10 with water were recorded, and the absorbance at 280 nm (E'_{280}) was calculated as for skin extracts. All measurements were performed in triplicate.

RESULTS AND CONCLUSIONS

During ripening of Barbera grapes in 2004, the anthocyanin contents determined at all samplings in whole skin extracts and after passage through a C_{18} cartridge were almost identical. In contrast, the total flavonoids showed a remarkable decrease after absorption on the cartridge (Table 1). Like Barbera 2004, anthocyanins from skin extracts of Barbera, Nebbiolo, Refosco and Syrah grapes (2006) at technological maturity were absorbed and quantitatively eluted with methanol through the C_{18} cartridge, while some compounds determined as flavonoids in whole extracts were apparently retained in the cartridge. Unexpectedly, the amount of proanthocyanidins did not decrease after passage of skin extracts through the C_{18} cartridges (Table 2). The differences in the amounts of flavonoids determined on whole extracts and after absorption through the C_{18} cartridge were more evident in grapes before maturity than at maturity (Table 3).

Since extracts were obtained by skin homogenisation in pH 3.2 tartaric buffer containing ethanol and SO_2, the possibility that this phenomenon was due to artefacts induced by the presence of such a remarkable amount of SO_2 was evaluated. Thus, total anthocyanins and total flavonoids were determined in whole skin extracts of Syrah grapes made from the SO_2-rich tartaric buffer as well as 1% HCl-methanol, and after passage through a C_{18} cartridge. The absorbance at 280 nm was also much higher in whole skin extracts made from 1% HCl-methanol than after passage through a C_{18} cartridge (results not reported). These discrepancies were not detected in wines passed through a C_{18} cartridges whose E'_{280} value was very similar to that of wines as a whole (Table 4). The results obtained for wines were also obtained with seed extracts, as shown in the data from total flavonoids (Table 5) determined on whole extracts and after passage through a C_{18} cartridge and elution with methanol. In this case the proanthocyanidin contents as well as total flavanols determined by vanillin assay did not vary.

The initial hypothesis was that substances with λ_{max} at 280 nm that apparently did not elute with methanol from the C_{18} cartridge were phenolic compounds linked to polysaccharide chains that were released from the cell wall by the hydrolytic action of the SO_3H^- or strong acids. Their phenolic component was supposed to be held by the C_{18} cartridge and not eluted with methanol due to the insolubility of the polysaccharide moiety in this solvent. Such a hypothesis was rejected after having ascertained the absence of turbidity or precipitate due to the dilution of the skin extract with ethanol (ethanol index, GLORIES, 1978). Under these conditions the phenolic compounds linked to the polysaccharide chain or to other polymers (proteins, glycoproteins) should have precipitated. Furthermore, the hypothesis that those substances were phenolics linked to the cutin structure was rejected, after having assessed that elution with chloroform after methanol did not allow the recovery of compounds with λ_{max} at 280 nm from the cartridge. Due to these unexpected results, it was hypothesized that all the substances with λ_{max} at 280 nm present in the skin extracts were eluted with methanol and that a part of them had not been determined by the measurement of $E'_{280,}$ because they were transformed during the process. The decrease in their content during ripening and their presence in skins, but not in seeds or wines, suggested that they were intermediates in the biosynthesis of anthocyanin. The existence of hypothetical intermediates derived from leucoanthocyanidins, before cyanidin and delphinidin, appears to be consistent with the data reported in this work. These intermediates would accumulate during the previous phase or at the beginning of anthocyanin synthesis, and would be converted into cyanidin and delphinidin by ANS (anthocyanidin synthase) when FGT (UDP-Glucose:Flavonoid 3-O-glucosyltransferase) is expressed. In the slightly acidic skin tissue they would be sufficiently stable (PFEIFFER *et al.*, 2006). During extraction with solvents containing strong acids or high amounts of SO_3H^- they would be converted into suitable forms in order to be absorbed, on the C_{18} cartridge along with proanthocyanidins. As noted for anthocyanins, washing of the cartridge with 5×10^{3} M H_2SO_4 to remove the interfering substances, would entail the separation of the intermediates from the SO_3H^- ion, the formation of a carbocation with the positive charge located at position 4 of the C ring, as well as the reaction

of this with proanthocyanidins adsorbed by the cartridge. In the new compounds the reactivity to vanillin (vanillin assay) remained almost unchanged, because of the increasing degree of polymerization, while the content in proanthocyanidins, determined by heating in a strong acid milieu was higher than the one reported in the whole skin extract. The intermediates present in the skin extracts obtained from the hydroalcoholic SO_2-rich buffer used in this work did not react with vanillin nor were they converted into anthocyanidins by heating in strong acidic milieu. The more marked formation of brown pigments, by heating the whole skin extract in strong acid milieu, during the determination of the proanthocyanidins, with respect to the sample obtained by passage through the C_{18} cartridge and elution with methanol suggests the presence of

Table 1 - Evolution of total anthocyanins and total flavonoids during ripening in skins of Barbera grapes grown in Piedmont (2004).

Harvest-period	Aug-12		Aug-19		Sept-16		Sept-28	
	whole	C_{18}	whole	C_{18}	whole	C_{18}	whole	C18
Non-absorbed Flavonoids*		71 ± 3		97 ± 9		174 ± 8		185 ± 6
Total Flavonoids*	806 ± 4	474 ± 9	924 ± 8	746 ± 10	3096 ± 15	2375 ± 28	2596 ± 14	1963 ± 17
Total Anthocyanins **	183 ± 8	183 ± 4	410 ± 5	400 ± 4	1456 ± 4	1450 ± 5	1153 ± 5	1164 ± 6

*Expressed in mg kg^{-1} as (+)-catechin equivalent
**Expressed in mg kg^{-1} as malvidin-3-glucoside equivalent
n = 3 samples; mean value ± standard deviation

Table 2 - Influence of passage through C18 cartridges of skin extracts of Barbera, Nebbiolo, Refosco and Syrah grapes from Piedmont at technological maturity (2006) in determining the total phenolics, total anthocyanins and proanthocyanidins.

Cultivar	Barbera		Nebbiolo		Refosco		Syrah	
	whole	C_{18}	whole	C_{18}	whole	C_{18}	whole	C_{18}
Total Flavonoids*	1728 ± 13	1330 ± 23	3030 ± 25	2278 ± 16	4192 ± 47	3464 ± 38	3172 ± 18	2472 ± 117
Total Anthocyanins**	755 ± 4	744 ± 1	820 ± 2	810 ± 1	1976 ± 4	1926 ± 7	1243 ± 14	1221 ± 18
Proanthocyanidins***	661 ± 16	686 ± 7	2861 ± 51	3815±165	2460 ± 10	2622 ± 91	1447 ± 121	1887 ± 2

*Expressed in mg kg^{-1} as (+)-catechin equivalent
**Expressed in mg kg^{-1} as malvidin-3-glucoside equivalent
***Expressed in mg kg^{-1} as cyanidin equivalent
n = 3 samples; mean value ± standard deviation

Table 3 - Total flavonoids and total anthocyanins during ripening in grape skins of Nero d'Avola, Syrah, Merlot and Cabernet Sauvignon grown in western Sicily (2006).

Total Flavonoids*										
Harvest-period	Aug-4		Aug-11		Aug-18		Aug-26		Sep-01	
	whole	C_{18}	whole	C_{18}	whole	C_{18}	whole	C_{18}	whole	C_{18}
Nero d'Avola	3847 ± 13	2415 ± 15	2619 ± 16	1300 ± 10	1974 ± 16	1600 ± 20	2242 ± 15	2047 ± 15	2677 ± 17	2465 ± 15
Syrah			2700 ± 20	1866 ± 16	1777 ± 17	1662 ± 15	2625 ± 20	2307 ± 25	2209 ± 11	1908 ± 12
Merlot	4046 ± 13	2213 ± 16	4594 ± 16	2571 ± 9	5196 ± 14	2780 ± 15	3185 ± 15	2501 ± 21		
Cabernet Sauv.	4606 ± 25	2549 ± 11	6625 ± 25	3708 ± 11	5633 ± 15	2312 ± 18	3207 ± 13	2859 ± 19		
Total Anthocyanins**										
Harvest-period	Aug-4		Aug-11		Aug-18		Aug-26		Sep-01	
	whole	C_{18}	whole	C_{18}	whole	C_{18}	whole	C_{18}	whole	C_{18}
Nero d'Avola	576 ± 4	567 ± 4	413 ± 3	412 ± 3	543 ± 3	543 ± 3	1106 ± 4	1097 ± 3	867 ± 3	855 ± 5
Syrah			585 ± 3	585 ± 3	571 ± 3	540 ± 6	864 ± 2	853 ± 2	745 ± 3	720 ± 3
Merlot	843 ± 7	811 ± 4	1056 ± 6	1009 ± 4	1322 ± 4	1278 ± 2	1256 ± 5	1197 ± 3		
Cabernet Sauv.	730 ± 5	710 ± 5	948 ± 2	953 ± 3	1343 ± 3	1214 ± 13	1087 ± 3	1097 ± 3		

*Expressed in mg kg^{-1} as (+)-catechin equivalent
**Expressed in mg kg^{-1} as malvidin-3-glucoside equivalent
n = 3 samples; mean value ± standard deviation

Table 4 - Total anthocyanins and total flavonoids in Sicilian red wines (2007) before and after passage through C18 cartridges.

Cultivar	Syrah		Cabernet S.		Cabernet S.		Cabernet S.		Merlot		Merlot	
	whole	C_{18}	whole	C_{18}	whole	C_{18}	whole	C_{18}	whole	C_{18}	whole	C_{18}
Total Flavonoids*	1787 ± 13	2055 ± 15	2873 ± 17	2853 ± 17	2219 ± 11	2044 ± 16	2477 ± 13	2450 ± 10	2280 ± 20	2182 ± 8	2704 ± 16	2894 ± 16
Total Anthocyanins**	199 ± 1	203 ± 3	542 ± 2	536 ± 4	618 ± 2	611 ± 4	416 ± 3	403 ± 3	182 ± 2	182 ± 3	410 ± 3	416 ± 4
Cultivar	**Merlot**		**Merlot**		**Merlot**		**Petit Verdot**		**Nero d'Avola**		**Nero d'Avola**	
	whole	C_{18}	whole	C_{18}	whole	C_{18}	whole	C_{18}	whole	C_{18}	whole	C_{18}
Total Flavonoids*	3115 ± 15	3113 ± 17	2251 ± 14	2208 ± 12	2149 ± 9	1963 ± 12	2877 ± 13	2658 ± 12	1765 ± 15	1763 ± 10	2153 ± 17	1975 ± 10
Total Anthocyanins**	396 ± 3	379 ± 3	764 ± 2	773 ± 4	555 ± 1	541 ± 2	1227 ± 3	1204 ± 3	274 ± 2	271 ± 2	885 ± 3	872 ± 3

*Expressed in mgL^{-1} as (+)-catechin equivalent
**Expressed in mgL^{-1} as malvidin-3-glucoside equivalent
n = 3 samples; mean value ± standard deviation

Table 5 - Evolution of total flavonoids, total flavanols (vanillin assay) and procyanidins during ripening in seeds of Barbera grapes from Piedmont (2004).

Harvest-period	Aug-12		Aug-19		Sept-16		Sept-28		Oct-20	
	whole	C_{18}	whole	C_{18}	whole	C_{18}	whole	C_{18}	whole	C_{18}
Total Flavonoids*	1456 ± 8	1446 ± 10	1366 ± 6	1384 ± 5	1294 ± 10	1291 ± 7	1033 ± 8	1034 ± 2	1168 ± 6	1151 ± 6
Total Flavanols*	1450 ± 10	1420 ± 5	1309 ± 6	1276 ± 4	1194 ± 4	1166 ± 7	1000 ± 5	976 ± 7	976 ± 6	947 ± 8
Procyanidins**	1168 ± 4	1225 ± 5	1252 ± 4	1265 ± 5	1175 ± 9	1217 ± 6	959 ± 5	1010 ± 5	1108 ± 5	1124 ± 6

*Expressed in $mgkg^{-1}$ as (+)-catechin equivalent
**Expressed in $mgkg^{-1}$ as cyanidin equivalent
n = 3 samples; mean value ± standard deviation

substances in all the extracts from which these brown pigments were produced. After incorporating the intermediates into proanthocyanidins, their conversion into brown pigments decreased. It can also be hypothesized that, since the anthocyanins absorbed onto the C_{18} cartridge were in the flavilium form, the reaction with the carbocations generated from the intermediate ones did not occur. The absorbance at 280 nm (E'_{280}) of molecules that originated from the reaction between the hypothetical intermediates and the proanthocyanidins was evidently less than that of the sum of the intermediates and proanthocyanidins found in the whole extract. These reactions cannot be observed in seed extracts and in wines, due to the lack of substrata (i.e. intermediates derived from the leucoanthocyanidins). As far as seeds are concerned, this is due to the fact that the proanthocyanidin synthesis precedes veraison and that anthocyanins are not synthesized. In wines the reason is probably that, at the moment of crushing, the reactions occur between the intermediates and proanthocyanidins, catechins, or other molecules. Experiments to identify the substances responsible for the discrepancies in determining the total flavonoids in whole skin extracts with SO_2-rich tartaric buffer and after passage on C_{18} cartridge are in progress. Results obtained with grape skin extracts made from neutral solvents, would confirm the consistency of the hypothetical model proposed.

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FOOD CONSUMPTION AND DIET COST: A NORTHERN ITALIAN SURVEY

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ABSTRACT

The aim of the present study was to estimate the cost of the Italian diet, analyzed for individual age groups and nine different family units, purchased in five different supermarkets located in Pavia, Italy. Foodstuff prices at discount stores, as well as the average daily and monthly diet costs for each age group, were always lower (ANOVA, $p<0.05$) than those in the other supermarkets. In addition, the comparison among the five supermarkets was significantly different ($p<0.05$) when the maximum daily and monthly cost of the diet purchased by the nine family units was considered. Depending on the family unit and the different supermarkets, food expenditure ranged from 19% to 53% of the income. For low-income families, the amount spent for food expenditures had a heavy impact on their income; they consumed high-energy-dense diets that have a relatively low cost, but are also of lower dietary quality.

- Key words: foodstuff prices, northern Italian survey, supermarkets, target specific diet cost -

INTRODUCTION

Since 2002, when the Euro was adopted in the European Union, in Italy the prices have continued to rise excessively, with the result that Italian families have begun to consume less, with decreased food purchasing towards the end of the month when the family wages have almost run out. This trend has also been observed by industries and supermarkets.

The cause underlying the reduction in the income of low-income Italian families, particularly employees and retired people, is inflation (ANFOSSI, 2004). The Euro could be the cause of this excessive price rise; indeed, since the Euro was adopted, prices have increased by about 50% for foodstuffs, especially fruit and vegetables. In 2003 the Italian family was spending about € 77 more per month than in 2001. In 2002 food expenditure was € 116 billion, € 468 per family and € 168 per consumer each month (CIA, 2003). Food expenditure increased, but, in the meanwhile, the purchase of selected foods, such as fruit and vegetables, decreased because of their price escalation. The mean increase was about 15%, with the maximum increase of 50% for a few foods, such as zucchini, French beans, tomatoes, chicory, apricots and peaches (CIA, 2003). Consequently, Italian consumers reduced fruit and vegetable consumption from 18% of the food expenditure in 2001 to 10% in 2003 (CIA, 2003).

Nevertheless, daily expenditure of the Italian family increased, so the adoption of the Euro is not the only reason for this escalation in food prices, especially for fruit and vegetables. Dry weather, frost, floods, cut-offs in national agricultural production, speculation and a mainly unwarranted rise in prices impacted the final cost of food. In addition, industrial processing and trade transport prices involving all widely consumed products must be considered.

In 2005 it was estimated that, due to increases in food prices, Italian families would have spent € 216 more on food (CILLIS, 2005). In recent years, the decrease in food consumption, caused mainly by increased prices, can be summarized as follows: in 1973 food expenditure was 34.4% of the family's total daily expenditure; in 2003 this percentage was 19.4% and in 2005 it was 19.0% (www.regione.piemonte.it).

According to ISTAT data (2005), in southern Italy, the percentage of food expenditure was higher than in the other areas and the highest percentages were those for bread and cereals and meat. In general, the expenditure for meat, especially beef and pork, is the highest in all the geographical areas.

In addition food expenditure depends on the number of family members, but the average expenditure does not increase in proportion to the number of components. For example, the average monthly food expenditure for a single person is about two thirds that for two members. In Italy, in 2005 the average expenditure for a single person was € 1571 and € 3072 for a four-member household. On average, families with five members spend less per person than those with three or four people. Larger families, mostly found in southern Italy where consumption expenditure is lower, have a lower income than families with fewer persons living in northern Italy.

The amount for food expenditure in the case of low-income families requires a higher proportion of their income compared with medium-high income families: it is about 30% of the total daily expenditure. Low-income families are less likely to consume healthy, less-energy-dense diets, such as diets rich in fruit and vegetables, because they are more expensive. Instead they choose high-energy-dense diets, which are less expensive but are also of a lower dietary quality, such as foods that are rich in fats, oil and sugar (AGRA, 2007; CASSADY *et al.*, 2007; NI MHURCHU and OGRA, 2007).

The aim of the present study was to estimate the costs of Italian diets, analyzed for individual age groups and family units with different numbers of members, purchased in different supermarkets located in various urban areas in Pavia, a northern Italian town. In addition, the impact of diet costs on the daily and monthly income of low socioeconomic groups was evaluated.

MATERIAL AND METHODS

Dietary consumption data

The dietary consumption data used to formulate the Italian diets according to age groups were drawn from the National Italian Household Survey carried out in 1994-96 by the Italian National Institute for Food and Nutrition Research (INRAN) (TURRINI *et al.*, 2001). This survey is the most representative Italian food consumption study since it is based on 1,978 subjects randomly selected according to age groups and is representative of the four main geographical areas in Italy (Northwest, Northeast, Centre and South). Although these data are not very recent, they were used because they are the most complete dietary consumption data of the Italian people.

Table 1 shows the consumption of the following food categories by various age groups (TURRINI *et al.*, 2001): cereals and tubers; meat and meat products, fish and fish products, eggs, legumes; milk and dairy products; vegetables and fruit; oil and fats; sweet foods; special foods and mixed dishes and beverages.

Table 1- Food consumption in Italy by age groups. Values reported in grams (TURRINI *et al.*, 2001).

Foodstuff	Children (1-9 yrs) mean	Adolescents (10-17 yrs) mean	Adults (18-64 yrs) mean	Elderly (> 64 yrs) mean
(1) Cereals and tubers				
Bread and pizza	87.4	177.8	155.4	130.6
Crackers, bread sticks, rusk, etc.	4.8	8.4	8.5	16.7
Pasta	73.7	112.2	93.9	98.7
Pasta made with eggs and filled	3.6	6.0	4.5	4.1
Rice	6.5	8.3	11.3	13.5
Grains and flour	5.3	8.4	8.7	6.2
Breakfast cereals	1.7	2.6	0.9	0.3
Potatoes, raw and cooked (including crisps)	30.2	48.4	40.9	43.2
(2) Meat and meat products, fish and fish products, eggs, legumes				
Beef	39.9	50.8	46.1	38.6
Pork	3.8	8.7	8.4	7.2
Sheep meat	2.1	6.5	3.0	3.1
Horse meat	1.1	2.6	2.7	0.3
Poultry	26.2	31.8	30.0	31.5
Rabbit and other meat	3.5	7.3	6.9	7.4
Ham, salami, etc	16.7	33.0	27.6	17.0
Meat, preserved	0.5	0.7	0.8	0.8
Offal and unspecified meat	5.0	5.3	6.7	7.4
Fish and seafood, fresh and frozen	20.7	29.8	33.5	28.6
Fish and seafood, preserved	3.4	6.2	8.0	6.6
Eggs	10.1	15.6	14.1	12.8
Legumes, dry and tinned	4.0	7.2	6.4	7.6
(3) Milk and dairy products				
Milk	198.0	142.1	119.3	143.1
Yoghurt	33.2	16.9	15.9	10.5
Cream	0.9	1.8	1.7	0.8
Cheese	29.4	36.8	45.4	42.1
(4) Vegetables and fruit				
Tomatoes for salad	187.0	32.8	44.4	41.0
Tomatoes, ripe	26.4	41.6	34.3	36.0
Vegetables, fresh and frozen	51.5	89.7	129.2	131.3
Vegetables, preserved	0.9	2.2	3.3	2.4
Legumes, fresh and frozen	6.5	11.7	15.4	17.6
Citrus fruit	28.0	33.1	38.5	34.4
Fruit, fresh	113.0	126.0	160.9	189.2
Fruit, preserved (including olives)	1.0	1.9	1.9	1.9
Nuts and dried fruit	1.5	1.6	2.4	1.3
(5) Oil and fats				
Olive oil	12.3	19.3	20.4	21.3
Seeds oil	1.4	3.3	3.2	2.2
Margarine	0.3	0.3	0.3	0.4
Butter	2.5	3.4	3.2	3.0
Other animal fats	0.0	0.0	0.1	0.1
(6) Sweet foods				
Cakes, biscuits, pastries, etc	52.1	60.2	43.1	21.1
Ice cream	17.8	27.4	14.4	4.8
Sugar	7.5	9.4	12.3	9.7
Honey, candies, jam, chocolate, etc.	16.6	14.6	7.1	4.6
(7) Special foods and mixed dishes				
Sandwiches, etc	4.3	6.8	4.3	2.7
Vinegar and salt	5.1	7.8	10.0	12.0
Sauces and dressing	1.7	2.7	2.5	2.2
Babyfoods	13.6	0.7	0.1	0.6
(8) Beverages				
Mineral water	194.3	272.2	329.5	286.9
Soft drinks	34.6	75.3	34.6	7.9
Fruit juices	59.3	40.7	16.4	13.2
Wine and sparkling wine	1.0	10.3	94.0	130.2
Beer	1.9	9.7	33.0	12.4
Coffee, powder	0.3	1.5	8.4	7.4

Selection of supermarkets

Five supermarkets located in Pavia, Italy were randomly selected:
- a large supermarket located a few kilometres outside of town, in a shopping centre, where many people from the surrounding area shop during the weekend (supermarket A);
- a large supermarket located in the suburbs (supermarket B), belonging to a supermarket chain with many outlets throughout northern Italy;
a large supermarket located in the town centre (supermarket C), belonging to a supermarket chain with many outlets throughout northern Italy;
- a small supermarket located in the suburbs (supermarket D);
- a small discount store located in the suburbs (supermarket E).

Recording of food prices

The research was carried out from March to May 2008 by two dieticians. The prices of typical foods and beverages of the Italian diet were recorded in the five supermarkets. In each supermarket the prices of five different brands were recorded for each food and beverage. The prices of the cheapest and the most expensive products were recorded, as well as those in the range in between. Foods on promotional sale were excluded from the investigation, since their prices change during the year. For each unpackaged product, the price/kilogram was recorded, while for each packaged food, the price per package was recorded along with its weight and then the price per kilogram or per litre of product was calculated.

Average price of each foodstuff

The average price/kilogram of each foodstuff was calculated from the five values recorded in each supermarket; in addition, the minimum and maximum prices were recorded.

Table 2 - Minimum and maximum prices/kg of foodstuff (€/kg) for all stores combined (n=5) (mean ± SD).

Food items	Minimum mean±SD	Maximum mean±SD
Cereals and tubers	2.2±1.8[a]	4.2±2.7
Meat and meat products, fish and fish products, eggs, legumes	8.6±6.0[a]	11.0±7.6
Milk and dairy products	2.6±1.9[a]	4.7±4.0
Vegetables and fruit	3.2±2.0[a]	4.8±3.8
Oil and fats	2.6±1.7[a]	4.4±2.4
Sweet foods	3.4±1.8[a]	6.6±3.4
Special foods and mixed dishes	3.7±2.8[a]	10.5±5.1
Beverages	0.4±0.3[a]	0.9±0.7

[a]ANOVA $p < 0.05$ across stores.

Calculation of daily diet cost for age groups

Based on the Italian food consumption data (TURRINI *et al.*, 2001) and on the food prices recorded, the minimum, mean and maximum cost of the daily diet purchased in each supermarket were calculated for each age group.

Calculation of daily and monthly diet cost for different family units

The minimum, mean and maximum cost of the daily and monthly diet purchased in every supermarket were also calculated for different family units, by summing the diet cost for each member of the family.
The following family units were considered:
- Couples without children;
- Parents with one child;
- Parents with one adolescent;
- Parents with two children;
- Parents with two adolescents;
- Parents with two children and two elderly;
- Parents with two adolescents and two elderly;
- A single adult;
- Two elderly.

Statistical analyses

Analysis of variance (ANOVA) was performed to identify differences between the five supermarkets in terms of foodstuff prices and the cost of the diet purchased for the various age groups and family units. Descriptive and statistical analyses were performed using the Statistical Package for Social Sciences /PC version 10, Chicago- SPSS Inc.

RESULTS

A total of 985 prices were recorded, 197 in each of the five supermarkets selected: 160 for cereals and tubers; 244 for meat and meat products, fish and fish products, eggs, legumes; 80 for milk and dairy products; 160 for vegetables and fruit; 80 for oil and fats; 80 for sweet foods; 76 for special foods and mixed dishes and 105 for beverages. The minimum and maximum prices/kg (€/kg) of the food items grouped according to the eight food categories (mean ± standard deviation) for all the stores combined are reported in Table 2.

ANOVA showed that there were no significant differences in prices among the five supermarkets, except for supermarket E, with significantly lower ($p < 0.05$) prices. In all the supermarkets, meat and meat products, fish and fish products, eggs and legumes were the most expensive items, while beverages were the cheapest.

Fig. 1 shows the minimum and maximum mean costs of the daily diet for the different age groups spent at the five supermarkets. The max-

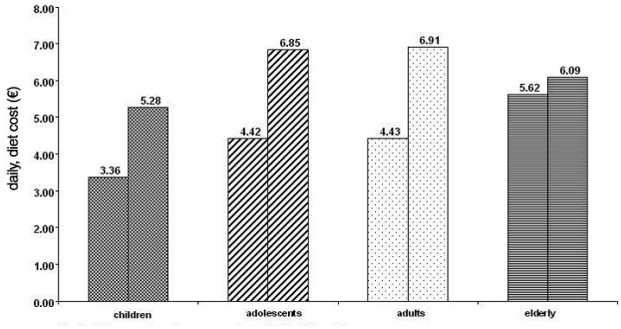


Fig. 1 - Minimum and maximum mean values of daily diet cost for age groups.

imum price for all age groups was in supermarket A located in the shopping centre, while the lowest was in the discount store (supermarket E). The mean price of the daily diet was the highest for adolescents, adults and the elderly in supermarket A, while it was the highest for children in supermarket B. It was the lowest in the discount store for all age groups. The minimum cost was higher for all age groups in supermarket D, while it was the lowest in the discount store.

Table 3 reports daily diet costs in the cheapest (discount store) and the most expensive (supermarket A) stores for different family units. For couples, the daily diet cost ranged from € 7-12 at the discount store and from € 9-21 at supermarket A. For two parents with one child, the daily diet cost ranged from € 10-16 at the discount store and from € 13-29 at supermarket A. Similar costs were recorded for two parents with one adolescent: € 10-18 at the discount store and from € 14-31 at supermarket A.

As the number of children per family increased, the daily diet cost range rose from € 12-21 at the discount store to € 16-37 at supermarket A, while when two adolescents were present in the family it went up from € 14-24 at the discount store to € 19-41 at supermarket A. The cost range increased even further when the above-mentioned family units also included two elderly, ranging from € 18-31 at the discount store to € 24-56 at supermarket A in the first family unit, and from € 20-34 at the discount store to € 27-60 at supermarket A in the second family unit.

Obviously, a single adult spent less: from €

Table 3 - Daily diet costs (minimum, mean, maximum values) for different family units in the cheapest and the most expensive store (values in Euro).

Family unit	Cheapest store			Most expensive store		
	min[a]	mean	max[b]	min	mean	max
Couples without children	7	9[c]	12	9	14	21
Parents with one child	10	12[c]	16	13	19	29
Parents with one adolescent	11	13[c]	18	14	21	31
Parents with two children	12	16[c]	21	16	24	37
Parents with two adolescents	14	18[c]	24	19	28	41
Parents with two children and two elderly	18	23[c]	31	24	36	56
Parents with two adolescents and two elderly	20	25[c]	34	27	40	60
A single adult	3	4[c]	6	5	7	11
Two elderly	6	8[c]	11	8	12	19

[a]minimum cost; [b]maximum cost; [c]ANOVA p<0.05 across stores.

Table 4 - Monthly diet costs (minimum, mean, maximum values) for different family units in the cheapest and the most expensive store (values in Euro).

Family unit	Cheapest store			Most expensive store		
	min[a]	mean	max[b]	min	mean	max
Couples without children	217	279[c]	372	279	434	651
Parents with one child	310	372[c]	496	403	589	899
Parents with one adolescent	341	403[c]	558	434	651	961
Parents with two children	372	496[c]	651	496	744	1,147
Parents with two adolescents	434	558[c]	744	589	868	1,271
Parents with two children and two elderly	558	713[c]	961	744	1,116	1,736
Parents with two adolescents and two elderly	620	775[c]	1,054	837	1,240	1,860
A single adult	93	124[c]	186	155	217	341
Two elderly	186	248[c]	341	248	372	589

[a]minimum cost; [b]maximum cost; [c]ANOVA p<0.05 across stores.

3-6 at the discount store to € 5-11 at supermarket A. Table 4 reports the monthly costs of diets in the cheapest (discount store) and the most expensive (supermarket A) stores for different family units.

Food prices in the discount store were always lower ($p<0.05$) when compared with those of the other supermarkets, which was also true for the average daily and monthly costs of diets purchased at the discount store for each age group compared with those purchased in supermarkets A-D ($p<0.05$). In addition, when the maximum daily and monthly costs of diets purchased in the five supermarkets by the nine family units are considered, the differences were always statistically significant ($p<0.05$). On the other hand, no statistically significant differences (p=NS) were observed when the minimum daily and monthly diet costs were considered. Finally, supermarket A had the highest costs, both for food items and the diet, without any significant difference (p=NS) compared with the other supermarkets, except for the discount store ($p<0.05$).

DISCUSSION

The food price data are not statistically different among the supermarkets, except for supermarket E (discount) that had the lowest prices ($p<0.05$) for all of the food items selected. The discount store is a supermarket that offers the consumer cheaper foods than other supermarkets, but the food choice is more limited. In addition, well-known brand products are not usually sold in discount stores, since their prices are higher due to advertising policy.

Even though food prices were not statistically different among the five supermarkets considered, except for the discount store, it was unexpected that prices of supermarket A would be more expensive than those of supermarket C, since the former was located outside of town, in a shopping centre, visited by many people coming from the surrounding area, while the latter was in the town centre. This trend was found for the daily diet of all age groups: the minimum, average and maximum prices were always higher in supermarket A than in supermarket C.

Average daily costs of diets purchased at the five supermarkets were not statistically different, except for the discount store. The mean daily diet cost for a single adult was € 4, if the diet was purchased at the discount store, € 6 if purchased at supermarkets C and D, and € 7 if purchased at supermarkets A and B. The difference of € 2-3 in the cost of the daily diet was not negligible for the consumer.

As expected, the daily diet cost differed among the age groups: it was higher for adolescents and adults, while it was lower for children and the elderly; the nutritional requirements for each group were different as well as the amount food that they consumed.

As far as family units are concerned, no linear relationship was evident between price increase and the number of family members. A family with two parents and one child spent on average € 12 at the discount store and € 19 at supermarket A; a family with two parents and one adolescent € 13 Euro at the discount store and € 21 at supermarket A; two parents and two children spent € 16 at the discount store and € 24 at supermarket A, while for two parents and two adolescents daily diet cost rose to € 18 at the discount store and € 28 at supermarket A. Therefore, the increase in family daily diet cost does not depend on the number of family members, but on the varying composition of the family unit: a family with two parents and two adolescents spent more than a family with two children, even though the number of members was the same. Indeed, if the family unit included two elderly persons, it spent even more: € 25 at the discount store and € 40 at supermarket A. The monthly diet cost was higher for a family unit with more members and had a greater impact on its income especially when only one parent was employed. For example, in a family with two parents and one child, if only one parent was employed and his/her monthly income was about € 2000, 19 and 29% of it was used for food expenditure purchased at the discount store and supermarket A, respectively. These percentages increased slightly if there was one adolescent present in the family unit (20% at the discount store and 33% at supermarket A). When two children or two adolescents were present in the family, these percentages increased even further: 25% at the discount store and 37% at the supermarket A, and 28% at the discount store and 43% at supermarket A, respectively. For families with lower incomes, these percentages increased even more.

If two elderly live on a minimum pension of € 700 each, not uncommon in Italy, 18% of the pension is used for food expenditure at the discount store and 27% at supermarket A.

If they purchased their food at the discount store it cost 35% of their pension, 53% at supermarkets A and B, and 49% at supermarkets C and D. Except when the food was purchased at discount stores, about half of the pension was used for food expenditure. This could result in cutbacks on the food purchased by the elderly, with a consequent effect on the status of malnutrition. Another important factor is that discount stores are not very widespread within towns. Elderly people, especially those who do not have a car, have to purchase their food in the more expensive supermarkets. In a society that is becoming increasingly more elderly, health services as well as politicians should consider adopting specific public policy measures.

For low-income families, a higher proportion

of their income is spent on food compared with medium-high income families. Low income families tend to consume unhealthy diets that are often less expensive than healthy ones (DARMON *et al.*, 2006; IRALA-ESTEVEZ *et al.*, 2000; GALOBARDES *et al.*, 2001; PERRIN *et al.*, 2002). Unhealthy eating patterns, particularly high-energy-dense diets, which cost less have a lower dietary quality, such as foods that are rich in fats, oil and sugar, with low fruit and vegetable consumption, which are more expensive (CASSADY *et al.*, 2007; DREWNOWSKI *et al.,* 2004, 2007; MAILLOT *et al.,* 2007; DARMOND *et al.,* 2004; DREWNOWSKI *et al.*,; CLARO *et al.*, 2007). High prices are likely to be a barrier to healthy eating, which can compromise the nutritional and health status among low-income people and other vulnerable groups, such as the elderly. As the World Health Organization (WHO, 2003) pointed out, diet quality varies with social status, and this contributes to health inequalities. Intervention to make basic healthy foods affordable and accessible to all people would help reduce the high burden of malnutrition in old people as well as that of chronic disease, including overweight and obesity, in low-income consumers (DREWNOWSKI and DARMON, 2005a; b;). A comprehensive policy approach is needed that takes into consideration the nutrition behaviour and the economics of food choices of all social and economic classes.

Supermarkets B and C belong to a supermarket chain that is very widespread in northern Italy and therefore the data may be considered representative of this entire area.

The Italian Household National Survey was carried out in 1994-96, so the data based on food consumption reported in this survey may not be recent, but these data are the most complete and representative of the Italian population that are available.

CONCLUSIONS

The discount store is the cheapest supermarket, but the food choices and number of different brands for each foodstuff are limited. The lack of availability of some foods in the discount store and the higher cost of healthy foodstuff in other supermarkets may be a deterrent to eating healthier among very low-income consumers. In addition, discount stores are not very widespread and are generally located in the suburbs, where public transport may be limited, especially for the elderly. Public policies should examine ways to make healthy foods more affordable to low-income families and the elderly.

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GUIDE FOR AUTHORS

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

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