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PAPER

THE EVOLUTION OF APPLE VOLATILES DURING STORAGE AS INFLUENCED BY FRUIT MATURITY

EVOLUZIONE DEL CONTENUTO IN SOSTANZE VOLATILI DURANTE LA CONSERVAZIONE IN MELE RACCOLTE A DIVERSI STADI DI MATURAZIONE

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ABSTRACT

The quality and methylene chloride-extractable volatiles were measured on multiple-harvested "Golden Delicious" apples at picking and after storage. These compounds have been rarely or never identified in apples but are presumed to be present in trace amounts. Esters, aldehydes, aliphatic and aromatic alcohols, other aliphatic compounds (overall acids), norisoprenoids, phenols, benzene derivatives and linalool were detected. At picking time no quantitative differences were detected

RIASSUNTO

Si sono valutati alcuni aspetti qualitativi ed il contenuto in sostanze volatili in mele "Golden Delicious"; le sostanze volatili sono state estratte con cloruro di metilene al fine di valutare composti che raramente sono stati identificati in mele e che si suppone siano presenti in tracce. Si sono identificate classi di composti quali esteri, alcoli, aldeidi, norisoprenoidi, fenoli ed altri derivati benzenici e linalolo. Alla raccolta non si sono verificate differenze quantitative nella dotazione in aromi totali e nel-

- Key words: apple quality, controlled atmosphere, flavour, *Malus x domestica* Borkh., regular atmosphere -

in the volatile concentration in multiple-harvested apples except for the alcohol concentration which was higher in late-harvested fruit. CA storage reduced the ester, norisoprenoid, linalool, alcohol and aldehyde contents. The return to RA storage conditions highly favored late-harvested fruit ester content and commercially-harvested fruit norisoprenoid content.

le singole classi di composti esaminate; solamente la concentrazione in alcoli è risultata superiore nei frutti raccolti tardivamente. La conservazione in AC ha ridotto il tenore in esteri, alcoli, aldeidi e norisoprenoidi. Il ritorno alle condizioni di AR ha nettamente favorito il contenuto in esteri dei frutti raccolti tardivamente ed il contenuto in norisoprenoidi dei frutti raccolti in epoca commerciale.

INTRODUCTION

Several studies have focused attention on the identification of volatile compounds of apples and many compounds have been reported to be important contributors to apple flavor, such as aliphatic esters, especially the C5 to C10 acid esters, ethyl, propyl and hexyl butanoates, butyl, isoamyl and hexyl hexanoates, aldehydes, especially hexanal and (E)-2-hexenal, ketones, aliphatic and aromatic alcohols, phenols, terpenes, estragol and β-damascenone (CUNNINGHAM *et al.*, 1986; FLATH *et al.,* 1967). The characteristic "apple aroma" is associated with ethyl 2-methyl butanoate, hexanal and (E)-2-hexenal (FLATH *et al.,* 1967; PLOTTO *et al.,* 1998). Golden Delicious is an acetate-ester prevalent cultivar, rich in butyl acetate, hexyl acetate, 2-methylbutyl acetate and ethyl-2-methyl butanoate (DIXON and HEWETT, 2001). Three hundred and seventy volatile compounds have been identified in apple (NIJSSEN *et al.,* 1997). The wide panorama is not only due to the large number of aromatic substances but also to the different analytical techniques used to isolate the volatiles responsible for aroma production, such as headspace (HS) or solid phase micro-extraction (SPME) techniques or steam distillation/solvent extraction (SDE). The extraction methods are sensitive enough to identify trace compounds (μg L^{-1}) (MOIO *et al.,* 1995; FUHRMANN and GROSCH, 2002). For most solvent extraction protocols, large amounts of plant material and high temperatures are required, which can cause chemical changes during extraction. It has been determined that extraction methods, together with very high efficiency condensation and concentration using Vigreux columns, are highly reproducible for extracting volatiles from small amounts of plant material (XU *et al.,* 2005). Analysis with n-pentane as a solvent indicated that no volatile degradation products or chemical derivatives were formed (XU *et al.,* 2005).

Other authors have used methylene chloride to extract free and bound volatiles from Chardonnay (SEFTON *et al.,* 1993) and muscadine grape (BAEK *et al.,* 1997) juice, grapefruit (JELLA *et al.,* 1998) and Red Delicious apples (KOLLMANNSBERGER and BERGER, 1992). In the methylene-chloride extraction method, all volatile compounds (low, medium and high volatility), with a high partitioning coefficient to the organic solvent, could be isolated, even if the required solvent evaporation could cause the loss or degradation of some volatiles or the formation of artefacts (SEFTON *et al.,* 1993). Volatile loss or artefact formation could be minimised by using a minimum solvent/sample ratio, which de-

creases the appearance of solvent impurities and improves reproducibility (ORTEGA-HERAS *et al.,* 2002).

Artefact formation in Chardonnay juice has been investigated and among the benzene derivatives, 4-vinylguaiacol and 4-vinylphenol may be derived from the corresponding cinnamic acid decarboxylation during gas chromatography, whereas the detection of 3-oxo-α-ionol, that was found only in the acid hydrolysates, has not been fully investigated (SEFTON *et al.,* 1993). The main disadvantage of using methylene chloride is its toxicity; however, the risk of methylene chloride loss can be minimized by using very small volumes of solvent and carrying out the extractions at low temperature in a closed container.

Apple flavor accumulation and fruit quality depend on a number of attributes including internal and external factors. Internal factors influencing the accumulation of volatiles are associated with aromatic precursor availability, particularly, amino acids and fatty acids. Linoleic and linolenic acid accumulation during ripening is associated with an increase in aldehydes (DEFILIPPI *et al.,* 2005). External factors that influence fruit volatile accumulation are linked to climatic conditions (ECHEVERRÍA *et al.,* 2004; FERRANDINO *et al.,* 1999; RIZZOLO and VISAI, 1990), harvest time (DIRINCK and SCHAMP, 1989; FELLMAN *et al.*, 2000; HANSEN *et al.,* 1992) and storage conditions (BRACKMANN, 1990). In "Delicious", "Gala", "Rome" and "Fuji" apples, the acetate ester concentration increases as ripening progresses, especially if fruit is left at room temperature for one week (FELLMAN *et al.,* 2000). The concentration of aroma-characterizing compounds in "Fuji" apples is higher in early-harvested fruit (ECHEVERRÍA *et al.,* 2004).

Controlled-atmosphere (CA) storage with low or ultra-low oxygen concentration has been largely used to extend the length of time of apple storage and to maintain fruit firmness, acidity and sugar content (LÓPEZ *et al.,* 1998; STREIF and BANGERTH, 1988). CA can suppress volatile emission (LÓPEZ *et al.,* 1998; MATTHEIS *et al.,* 1998; STREIF and BANGERTH, 1988). The reduction of lipid-derived esters under CA conditions is mainly due to the low oxygen concentration, while the high carbon dioxide concentration limits the formation of esters that are derived from amino acid degradation (BRACKMANN *et al.,* 1993). CA conditions can influence the capacity of the apple to produce volatiles, particularly esters, even after their removal from CA (MATTHEIS *et al.,* 1998; WILLAERT *et al.,* 1983). In multiple-harvest experiments it has been demonstrated that, as harvest maturity advances, the time required to regenerate volatiles after removal from CA storage decreases (FELLMAN *et al.,* 2003). CA storage can cause a decrease in the concentrations of free- and esterified fatty acids which are the dominant precursors of volatile aroma substances in apples (SAQUET *et al.,* 2003).

In order to understand the effects of time of harvest and storage conditions on Golden Delicious apples, the qualitative characteristics and volatile compounds responsible for aroma production in the fruit were evaluated. A liquid-liquid extraction method with methylene chloride as a solvent was used to extract volatiles (KOLLMANNSBERGER and BERGER, 1992; SEFTON *et al.,* 1993; JELLA *et al.,* 1998; BAEK *et al.,* 1997). The reason for choosing this method was to investigate those compounds that have been rarely or never identified in apples and that are supposed to be present in trace amounts.

Another aim was to evaluate the effect of time of harvest on fruit quality and volatiles after CA storage and CA followed by regular atmosphere (RA) storage on Golden Delicious apples cultivated in a mountainous zone at picking and after storage.

MATERIALS AND METHODS

Plant material

The trial was carried out in northwestern Italy (Aosta Valley) in an orchard with a southern exposure located at 750 m a.s.l. with east-west oriented rows. Seventeen-year-old "Golden Delicious" clone B trees (4 m between rows, 1.2 m within rows), trained to the spindle system and grown on M9 rootstock were divided into three blocks of fifteen trees. In each block, 5 trees were labelled and 15-18 apples were harvested from each of the 5 trees on September 13 (early harvest) and 23 (commercial harvest), and on October 3 (late harvest). Approximately 200 apples per picking were stored in RA at 1°C for ten days and then were kept in CA (1.8 kPa O_2, 2.8 kPa CO_2) at 1°C (ISOLCELL system, Laives, Bolzano, Italy). After 248 days of CA storage (postharvest 1, PH1) a batch of stored fruit was analysed for fresh weight (FW), soluble solid content (SSC), titratable acidity (TA), firmness (FI) and volatiles. Another batch of fruit was left in RA at room temperature (approximately 18°C) for 10 and 20 days to simulate the fruit shelf-life. The determinations were then carried out after 10 days of RA (PH2) and 20 days of RA (PH3). Qualitative determinations and the sample preparation for volatile analysis were carried out immediately after picking at harvest or immediately at the end of storage at PH1.

Qualitative determinations

Three replications of 20 apples each were used to measure qualitative characteristics. Each apple was weighed to assess FW (g). FI was measured with an Effegi type (Effegi, Milan, Italy) penetrometer on two peeled surfaces in the equatorial zone of the apple using a plug diameter of 11 mm; data are expressed in N. A quarter from each of the 20 apples was removed; all quarters from the 20 apples were peeled, ground and centrifuged to obtain juice on which the SSC and the TA were measured. The SSC was measured with a hand-refractometer (Atago, Tokyo, Japan) and the data are expressed as °Brix. Ten milliliters of juice were neutralized to pH 8.0 with 0.1 N NaOH, using phenolphthalein as indicator, and the data are expressed as g L^{-1} of malic acid. The pulp starch content was enzymatically determined (Boehringer-Mannheim starch kit n. 207748) on a second pool of apple quarters after a preliminary starch hydrolysis carried out by means of a thermostable α-amylase (Termamyl, Novo Industri A/S, Copenhagen) as described by SEAGER and HASLEMORE (1993). Ethylene was extracted from the core of 10 apples dipped in water, using a syringe that was hermetically sealed with a caoutchouc stopper; 500 µL of air from the caoutchouc stopper were drawn up with a GC syringe and injected into a GC (Perkin-Elmer 8500, FID detector), equipped with a Poropak QS column (2 m, 1/8, 80/100, Porapak_QS, Supelco Co., Bellefonte, PA). Operating conditions were as follows: oven temperature 110°C, injector temperature 120°C, detector temperature 180°C, N_2 was used as carrier gas at a flow of 40 mL min^{-1} (LARA and VENDRELL, 1998). Ten other apples were cut on the equatorial surface and dipped in the Lugol solution to measure the starch index (SI; Test Amidomètre AM93, Copa Informatique, S.A. 1-10 scale).

Extraction of volatiles

Three replications of 20 apples each were sliced, seeds were removed and then ground and homogenized, adding 100 mg of ascorbic acid as an antioxidant. The homogenate was clarified with a purified concentrated pectinase (without secondary glycosidase activity, Vinozym FCE G, Novo Nordisk, http://www.novonordisk.com) to obtain a clear juice. Samples were kept at fixed temperature (12°C) overnight to reduce the possibility of thermal fla-

vor degradation and chemical reactions, (MOIO *et al.*, 1995). The homogenate was then centrifuged twice and the supernatant was recovered and brought to a known final volume. One-hundred milliliters of the juice were added to 200 μL of a solution of 77.12 μg L^{-1} 2-nonan-1-ol (Fluka, Buchs, Switzerland) as internal standard and 30 mL of methylene chloride and stirred for 10 min. The emulsion was separated in a separator funnel; the methylene chloride fraction was dried (a half teaspoon of anhydrous sodium carbonate was added) and concentrated by fractional distillation through a Vigreux column (600 mm height) at 37°C. One microliter of the concentrated extract was immediately injected into the GC and into the GC/MS.

Gas Chromatographic and GC/MS analysis

The volatiles were separated using a Carlo Erba HRGC 5300 GC equipped with an Innovax capillary column (30 mx0.25 mm, 0.25 μm film thickness) and a FID detector that was kept at 250°C. The GC was programmed to start at 40°C, rise to 60° at 30°C/min, to 190° at 2°C/min, and then to 230° at 3°C/min and held this temperature for 10 min. The injector temperature was maintained at 250°C. Injection was splitless (2 min). Semi-quantitative evaluation was made by the internal standard method, using 2-nonan-1-ol as reference; the coefficient of variability (CV) of 2-nonan-1-ol was calculated on 32 extracts to evaluate the method reproducibility. The same sample was injected into a quadrupole GC/Mass Spectrometer Hewlett-Packard (GC5890 II Series, MSD5970) equipped with the same capillary column and with the same temperature program. Ionization energy was set at 70 eV. Helium was used as the carrier gas (flow rate of 1.2 mL/min).

The peaks were identified by comparing mass spectra and retention time of the sample compounds with those of pure compounds, when available, with those of mass spectra and retention times published in the literature or from our laboratory. Pure compounds were purchased from Fluka (Buchs, Switzerland). Results are expressed as μg kg^{-1} of apples.

Statistical analysis

Data were subjected to analysis of variance (ANOVA) and means were separated by the Tukey's test ($P \leq 0.05$ and 0.01) using SAS 8.2 for Windows (SAS Institute, Cary, USA).

RESULTS AND CONCLUSIONS

Fruit quality

The starch index was significantly lower in early-harvested than in late-harvested fruit, even if no significant differences were detected for starch concentration (Table 1). The ethylene concentration in early-harvested fruit was significantly lower than in commercial and late harvests; the ethylene threshold of 0.2 ppm indicated that the optimum time to begin harvest (DUVERNEY *et al.*, 1992) was reached between the early and commercial harvest.

Early-harvested apples weighed significantly less than those from commercial and late harvests but during storage no significant differences were detected among harvest dates (Table 2).

Time of harvest significantly affected the soluble solid content; late-harvested fruit had a higher SSC than early-harvested ones. The SSC of early- and commercially-harvested apples did not significantly vary during storage, while a significant reduction (for $P \leq 0.05$) was detected in late-harvested fruit after PH1 (Table 2).

Picking the fruit from early and commercial harvests showed significantly higher ($P \leq 0.05$) titratable acidity levels

in comparison with late-harvested fruit. During storage, the TA of early-harvested fruit was always significantly higher than that of late-harvested fruit except at PH2. The TA of commercially and late-harvested fruit decreased significantly after PH1, whereas in early-harvested fruit the TA only decreased after the return to RA conditions, i.e. PH2 (Table 2).

At harvest firmness was significantly higher in early-harvested fruit than in late-harvested ones; at PH3, FI was still significantly higher in fruit from early harvest. CA storage caused a general reduction in FI in fruit from all harvests (Table 2).

In this study early-harvested fruit showed good qualitative characteristics after storage; the SSC levels were similar to commercially-harvested fruit and had higher TA and FI values. Time in storage affected apple fruit quality. The TA and FI decreased notably when fruit was kept in RA for 20 days after CA storage, independent of the harvest date. In contrast the SSC, which was significantly influ-

Table 1 - Starch index and starch and ethylene concentrations of "Golden Delicious" apples picked at different harvest dates. Data were analysed with ANOVA and means were separated by Tukey's test. Means in rows followed by the same letters are not significantly different at P≤0.05 (small letters) and for P≤0.01 (capital letters).

	Time of harvest		
	Early	Commercial	Late
Starch index	6.6 bB	7.8 abAB	9.0 aA
Starch content (g/100g)	0.98 ns	0.80 ns	0.74 ns
Ethylene (ppm)	0.11 bB	0.28 aA	0.36 aA

Table 2 - Fresh weight, soluble solid content, titratable acidity and firmness of "Golden Delicious" apples picked at different harvest dates as influenced by storage (PH1 = 248 days of CA storage; PH2 = 248 days of CA followed by 10 days of regular atmosphere - RA - storage; PH3 = 248 days of CA followed by 20 days of RA).
Means were separated by Tukey's test; means followed by the same letters are not significantly different at P≤0.05 (small letters) and at P≤0.01 (capital letters).
+ comparison within each columns (among harvest dates);
++ comparison within each rows (between picking time and storage periods).

	Time of harvest	At picking time	+	++	PH1	+	++	PH2	+	++	PH3	+	++
Fresh weight (g)	early	166.2	b	ns	162.2	ns	ns	164.3	ns	ns	168.8	ns	ns
	commercial	183.8	a	ns	179.3	ns	ns	170.8	ns	ns	169.8	ns	ns
	late	196.5	a	ns	183.9	ns	ns	173.9	ns	ns	166.3	ns	ns
Soluble solids content (°Brix)	early	11.8	b	ns	12.2	ns	ns	12.4	a	ns	12.4	ns	ns
	commercial	12.5	ab	ns	12.6	ns	ns	11.7	ab	ns	12.1	ns	ns
	late	13.9	a	aA	12.5	ns	bAB	12.6	b	bAB	12.2	ns	bB
Titratable acidity (g L^{-1} malic acid)	early	4.5	aA	aA	3.3	a	abAB	2.5	ns	bcB	1.9	a	cB
	commercial	4.1	aAB	aA	2.5	ab	bcB	2.2	ns	bcB	1.6	ab	cB
	late	3.4	bB	aA	1.8	b	bcBC	2.3	ns	bB	1.2	b	cC
Firmness (N)	early	71.3	a	aA	56.7	a	bB	50.5	a	cC	48.3	a	cC
	commercial	68.6	ab	aA	49.6	b	bB	45.6	b	bcB	41.6	b	cB
	late	64.2	b	aA	52.2	ab	bB	46.9	ab	bcBC	42.1	b	cC

enced by time of harvest, was not influenced by postharvest treatments in early- or commercially-harvested fruit. Storage of late-harvested fruit significantly reduced the SSC compared to the levels at harvest.

Volatiles

The methylene chloride extraction method made it possible to identify flavor compounds that have rarely been identified in apples. This technique, however, was not well suited for high volatility compounds, particularly esters. The initial sample preparation required that the apples webe sliced, homogenized and then left overnight to let the pectolytic enzyme act, which probably resulted in the loss of esters. Nevertheless, the method was reproducible as seen by the 15.88% calculated internal standard coefficient of variation. The free volatile extracts of methylene chloride of "Golden Delicious" apples can be classified in the following categories: esters, aliphatic and aromatic alcohols, aldehydes, phenols and other benzene derivatives, norisoprenoids, monoterpenes (in trace amounts) and other aliphatic compounds (overall acids, Table 3).

The portion of esters that could be detected with the extraction method used, did not vary among harvests at picking and at PH1 (Table 4). The effect of the return to atmospheric O_2 concentration after CA (PH2 and PH3) was evident in late-harvested fruit in which the concentration of esters was significantly higher than in early- and commercially-harvest-

Table 3 - Free volatile compounds identified in methylene chloride extracts of "Golden Delicious" apples. R^z = reliability of identification: y = mass spectrum in agreement with spectra of the Wiley library and retention time are the same as those of pure substances available in the laboratory and evaluated on the same column and with the same GC program; x = the pure substance was not available; the mass spectrum was in agreement with those found in the literature. Compound identification was done according to: (1) DI STEFANO ***et al.***, 1998; (2) FLATH ***et al.***, 1967; (3) DI STEFANO, p.c. RT = retention times (minutes) of detected compounds according to the GC program and conditions reported in the text.

Esters	R^z	RT	Aliphatic and aromatic alcohols	R^z	RT	Aldehydes	R^z	RT	Phenols and other benzene derivatives	R^z	RT	Norisprenoids	R^z	RT	Aliphatic compounds	R^z	RT
ethyl butanoate	y(2)	3.2	butan-1-ol	y(2)	4.7	hexanal	y(2)	3.8	benzaldehyde	y(1)	17.0	β-damascenone	x(1)	35.5	acetoin	y(3)	8.4
isobutyl acetate	y(2)	3.6	3-penten-2-ol	y(2)	5.0	(E)-2-hexenal	y(2)	6.4	benzyl alcohol	y(1)	37.8	3-hydroxy-β-damascone	x(1)	68.3	acetic ac.	y(3)	15.3
isoamyl acetate	y(2)	4.3	isoamyl alcohol	y(3)	6.1				eugenol	y(1)	52.2	3-oxo-α-ionol	x(1)	69.2	unidentified		
2-phenylethyl ac.	y(2)	33.0	pentan-1-ol	y(3)	7.2				4-vinylguaiacol	x(1)	53.3				butyric ac.	y(3)	25.0
ethyl hexadecanoate	x(3)	57.0	(Z)-penten-1-ol	y(3)	9.7				ethyl benzaldehyde	x(1)	57.2				2-methyl-butanoic ac.	x(3)	27.9
linoleic acid ester	x(3)	68.1	hexan-1-ol	y(1)	11.2				4-vinylphenol	x(1)	62.2				hexanoic ac.	y(3)	37.0
linolenic acid ester	x(3)	71.2	(Z)-3-hexen-1-ol	y(1)	12.2				benzoic acid	y(3)	63.2	Monoterpenes			(E)-2-hexenoic ac.	y(3)	43.0
			2-butoxyethanol	x(1)	12.9				dihydroconiferyl alcohol	x(1)	77.2	linalool	x(1)	20.9	octanoic ac.	y(3)	47.8
			(E)-2-hexen-1-ol	y(1)	13.2										hexadecanoic ac.	y(3)	75.3
			2-phenylethanol	y(1)	39.4												

Table 4 - Volatiles (μg/kg) of "Golden Delicious" apples picked at different harvest dates as influenced by storage (PH1 = 248 days of CA storage; PH2 = 248 days of CA followed by 10 days of regular atmosphere - RA - storage; PH3 = 248 days of CA followed by 20 days of RA).
Means were separated by Tukey's test; means followed by the same letters are not significantly different at P≤0.05 (small letters) and at P≤0.01 (capital letters).
+ comparison within each columns (among harvest dates);
++ comparison within each rows (between picking time and storage periods).

	Time of harvest	At picking time	+	++	PH1	+	++	PH2	+	++	PH3	+	++
Esters	early	1,301.14	ns	a	69.40	ns	b	77.73	bB	b	149.09	bB	b
	commercial	1,031.94	ns	aA	90.80	ns	bB	80.49	bB	bB	89.64	bB	bB
	late	879.90	ns	a	76.55	ns	b	821.90	aA	a	712.62	aA	a
Aliphatic and	early	821.86	bB	b	1,142.43	ns	b	6,015.45	a	a	7,930.88	a	a
aromatic	commercial	1,260.58	bB	b	1,158.26	ns	b	3,678.10	ab	a	3,196.53	ab	ns
alcohols	late	3,228.18	aA	ns	1,380.40	ns	ns	1,776.60	b	ns	2,818.48	b	ns
Aldehydes	early	573.35	ns	ns	611.48	ns	ns	1,190.89	a	ns	1,005.93	a	ns
	commercial	719.51	ns	ns	582.16	ns	ns	714.64	ab	ns	336.18	b	ns
	late	1,042.13	ns	aA	471.27	ns	bAB	341.50	b	cAB	135.35	b	cB
Phenols and other	early	348.41	ns	a	210.87	ns	ab	166.35	ns	ab	118.51	ns	b
benzene derivatives	commercial	383.02	ns	a	160.91	ns	b	210.44	ns	ab	168.87	ns	b
	late	500.59	ns	aA	227.09	ns	bB	104.21	ns	bB	92.97	ns	bB
Norisprenoids	early	89.75	ns	ns	57.12	ns	ns	72.83	ns	ns	86.85	ns	ns
	commercial	67.75	ns	ns	61.34	ns	ns	50.15	ns	ns	127.10	ns	ns
	late	75.85	ns	ns	47.31	ns	ns	43.26	ns	ns	62.61	ns	ns
Linalool	early	26.31	ns	aA	5.52	ns	bB	10.05	aAB	bAB	3.63	ns	bB
	commercial	37.27	ns	aA	3.62	ns	cB	10.45	aA	bB	11.04	ns	bB
	late	31.16	ns	aA	2.75	ns	bB	2.39	bB	bB	9.16	ns	bB
Aliphatic	early	1,202.80	ns	a	322.22	ns	b	661.25	aA	ab	469.38	ns	b
compounds	commercial	718.03	ns	a	268.42	ns	b	546.87	aA	ab	264.20	ns	b
	late	775.50	ns	aA	204.33	ns	bB	318.34	bB	bAB	273.50	ns	bB
Total aromatic	early	4,363.62	ns	ab	2,419,05	ns	b	8,194.54	a	ab	9,764.27	a	a
compounds	commercial	4,218.10	ns	ns	2,328,16	ns	ns	5,291.14	ab	ns	4,848.63	ab	ns
	late	6,533.32	ns	a	2,409.70	ns	b	3,408.20	b	b	4,104.69	b	ab

ed fruit. At PH2 this increase was due to a very high linoleic acid ester concentration, whereas at PH3 it was associated with the high concentrations of ethyl butyrate and isobutyl acetate (Table 5). An obvious decrease in the total amount of esters was detected in early- and commercially-harvested fruit during CA stor-

Table 5 - Individual volatiles (μg/kg) of "Golden Delicious" apples picked at different harvest dates as influenced by storage (means ± standard errors; PH1 = 248 days of CA storage; PH2 = 248 days of CA followed by 10 days of regular atmosphere - RA - storage; PH3 = 248 days of CA followed by 20 days of RA; nd = not determined).

	Time of harvest	Individual volatiles	At picking time	PH1	PH2	PH3
Esters	early	ethyl butyrate	nd	8.4±0.9	5.1±0.0	5.1±0.7
		isobutyl acetate	19.3±2.9	7.3±2.2	4.0±1.4	5.6±0.6
		isoamyl acetate	15.7 ±0.9	9.7±2.3	14.6±5.2	9.2±3.8
		ethyl phenyl acetate	2.9±0.1	2.1±0.3	1.1±0.1	1.7±0.2
		ethyl esadecanoate	163.3±72.3	3.9±1.1	5.5±0.8	17.9±2.1
		linoleic acid ester	678.6±35.4	33.8±3.0	34.3±5.2	26.4±2.6
		linolenic acid ester	421.4±190.5	4.3±1.6	13.1±0.8	83.2±7.4
	commercial	ethyl butyrate	nd	5.3±2.7	4.3±1.4	8.5±2.0
		isobutyl acetate	14.3±2.3	5.5±2.2	4.7±2.9	7.0±0.9
		isoamyl acetate	43.15±11.0	26.2±7.7	17.7±7.0	16.0±0.7
		ethyl phenyl acetate	2.85±0.1	2.2±1.7	1.3±0.2	18.2±0.3
		ethyl esadecanoate	143.9 ±72.7	4.1±0.4	7.2±1.8	8.5±1.1
		linoleic acid ester	712.7±7.1	28.3±2.2	40.1±16.3	15.2±1.1
		linolenic acid ester	114.9±20.0	19.2±12.9	5.2±3.0	15.9±3.8
	late	ethyl butyrate	19.9±5.7	4.4±2.6	7.2±0.0	198.4±37.8
		isobutyl acetate	83.0±11.1	6.0±0.6	16.8±0.0	462.0±39.9
		isoamyl acetate	85.8±2.6	19.2±7.0	8.3±0.0	15.7±0.5
		ethyl phenyl acetate	4.3±1.0	3.3±0.5	nd	11.6±3.5
		ethyl esadecanoate	122.7±21.3	5.8±1.2	3.6±0.5	3.5±0.5
		linoleic acid ester	481.6±39.4	30.2±0.2	776.0±0.8	10.3±2.4
		linolenic acid ester	82.5±1.2	7.7±5.7	10.0±0.0	11.2±4.7
Aliphatic and aromatic alcohols	early	1-butanol	47.0±9.9	100.0±2.6	1901.0±48.2	3522±568.6
		3-penten-2-ol	27.0±2..2	21.8±0.0	13.0±5.4	130.0±0.6
		isoamyl alcohol	76.0±19.4	326.2±13.0	1700.0±88.4	2411.0±538.5
		1-pentanol	30.2±2.1	55.5±15.6	118.0±10.0	108.0±21.2
		cis-penten-1-ol	71.1±13.7	37.1±5.5	53.0±11.3	31.0 ±4.6
		hexanol	241.6±18.2	52.3±18.8	2075±108.4	1641.0±355.9
		cis-3-hexenol	132.2±15.7	7.1±0.9	3.0±0.5	4.0±0.3
		2-butoxyethanol	31.5±1.1	27.5±2.3	54.0±7.5	105.0±38.9
		trans-2-hexenol	145.7±21.2	31.2±0.2	53.0±15.6	49.0±13.9
		2-phenylethanol	23.9±0.8	14.6±0.59	46.0±0.5	47.0±9.4
	commercial	1-butanol	141.3±42.0	42.2±17.9	1055.3±288.0	1215.6±357.9
		3-penten-2-ol	27.4±3.4	10.6±3.3	12.1±2.1	9.0±1.0
		isoamyl alcohol	205.7±58.6	540.8±74.1	1127.2±166.5	968.1±148.2
		1-pentanol	41.2±9.4	45.0±11.0	77.2±12.2	55.9±8.7
		cis-penten-1-ol	75.2±7.0	31.5±9.2	48.5±8.6	18.2±2.4
		hexanol	459.2±77.8	446.9±53.5	1255.9±126.4	820.5±129.4
		cis-3-hexenol	52.3±7.8	3.9±0.5	2.8±0.8	2.9±0.1
		2-butoxyethanol	80.1±53.3	26.1±2.1	44.1±11.0	59.9±5.2
		trans-2-hexenol	150.5±5.8	24.1±7.1	18.9±2.6	17.0±5.0
		2-phenylethanol	27.7±2.5	13.2±1.3	36.0±5.9	29.4±0.1

(continua)

Table 5

	Time of harvest	Individual volatiles	At picking time	PH1	PH2	PH3
	late	1-butanol	943.1±181.5	88.5±29.1	435.5±0.3	1598.6±105.4
		3-penten-2-ol	30.9±5.4	4.8±0.6	8.2±0.0	8.9±2.3
		isoamyl alcohol	489.1±3.5	599.1±101.5	585.8±0.3	85.2±11.5
		1-pentanol	77.1±4.4	33.9±0.5	32.1±0.0	68.9±4.5
		cis-penten-1-ol	72.1±6.9	23.1±3.3	25.9±0.0	24.9±0.0
		hexanol	1257.2±13.9	556.5±7.9	570.0±0.2	901.3±103.9
		cis-3-hexenol	31.2±3.3	3.4±0.3	0.7±0.0	2.7±0.0
		2-butoxyethanol	38.3±12.4	21.3±5.6	71.3±0.1	78.2±6.3
		trans-2-hexenol	258.6±20.4	40.7±27.8	21.2±0.5	28.0±1.2
		2-phenylethanol	30.4±2.0	9.2±4.0	25.6±2.6	21.8±4.2
Aldehydes	early	hexanal	390.8±32.4	484.1±4.2	1021.2±120.7	997.3±274.4
		trans-2-hexenal	182.5±50.8	127.3±25.4	169.7±12.4	8.6±0.1
	commercial	hexanal	494.0±83.7	331.2±150.3	603.2±49.8	198.2±9.6
		trans-2-hexenal	225.5±40.6	202.4±17.5	111.4±11.2	135.6±10.2
	late	hexanal	686.4±61.3	392.5±83.1	267.5±0.1	125.0±6.7
		trans-2-hexenal	355.7±14.8	78.8±16.6	74.0±25.0	10.32±0.1
Phenols and other benzene derivatives	early	benzaldehyde	9.1±1.4	2.6±0.6	3.8±0.2	4.3±1.4
		benzyl alcohol	17.8±0.7	11.1±1.2	13.5±6.4	6.5±3.6
		eugenol	16.1±0.2	18.8±5.8	40.9±12.8	7.7±1.0
		4-vinylguaiacol	6.8±1.2	7.9±1.9	6.3±2.3	5.4±3.8
		ethyl benzaldehyde	18.4±2.2	68.4±26.7	22.9±4.1	33.9±21.2
		4-vinylphenol	7.8±1.4	8.8±2.2	7.9±1.0	9.7±2.8
		benzoic acid	174.6±6.7	14.1±7.7	8.9±5.6	12.4±6.9
		dihydroconiferyl alcohol	97.8±31.2	79.2±3.2	62.2±17.0	38.6±7.4
	commercial	benzaldehyde	8.4± 0.4	5.0±3.0	3.4±0.9	2.8±0.0
		benzyl alcohol	15.8±0.6	6.7±0.1	7.9±0.7	11.0±1.9
		eugenol	72.1±15.4	29.4±7.0	101.1±33.2	15.2±5.1
		4-vinylguaiacol	4.8±0.8	8.3±1.7	5.6±2.2	6.2±0.7
		ethyl benzaldehyde	16.4±0.4	34.0±10.8	21.9±8.0	17.2±2.8
		4-vinylphenol	5.2±0.4	4.5±1.1	7.8±1.2	21.8±0.1
		benzoic acid	147.7±4.2	13.0±1.9	13.4±3.6	40.9±5.5
		dihydroconiferyl alcohol	112.7±22.4	60.1±10.9	49.3±7.6	53.3±24.5
	late	benzaldehyde	7.2±0.1	3.3±0.9	1.4±0.0	3.0±0.3
		benzyl alcohol	12.4±2.0	7.7±1.5	7.1±0.0	5.9±0.7
		eugenol	177.52±2.2	53.6±22.0	24.8±0.5	14.0±2.1
		4-vinylguaiacol	4.8±0.9	3.0±1.0	13.1±5.5	4.3±1.3
		ethyl benzaldehyde	9.9±0.3	36.7±22.9	23.4±0.2	12.1±3.7
		4-vinylphenol	4.7±0.8	9.8±6.4	10.5±2.5	7.4±0.7
		benzoic acid	162.4±3.8	10.9±2.9	11.0±3.9	7.1±3.5
		dihydroconiferyl alcohol	121.6±6.0	102.1±1.2	12.9±6.3	39.2±5.2
Norisprenoids	early	b-damascenone	10.8±3.7	2.7±0.2	7.2±3.8	17.0±14.1
		3-hydroxy-b-damascone	79.0±4.1	46.4±1.3	44.7±2.3	46.4±6.0
		3-oxo-a-ionol	0,0	8.1±1.2	20.9±11.4	23.4±9.1
	commercial	b-damascenone	4.9±2.1	0.8 ±0.4	9.7±0.7	36.1±0.1
		3-hydroxy-b-damascone	62.9±0.6	39.9±1.1	30.3±18.9	51.9±15.6
		3-oxo-a-ionol	nd	24.9±9.0	10.1±1.8	39.1±12.0
	late	b-damascenone	3.0±1.4	1.8±0.3	8.0±2.4	4.2±0.4
		3-hydroxy-b-damascone	72.9±10.3	27.5±0.4	20.1±6.3	32.6±2.1
		3-oxo-a-ionol	0,0	18.0±9.6	15.9±5.8	25.8±3.8

(continua)

Table 5

	Time of harvest	Individual volatiles	At picking time	PH1	PH2	PH3
Linalool	early	linalool	26.3±2.0	5.5±2.7	10.0±0.4	3.6±1.7
	commercial	linalool	37.27±2.8	3.6±2.9	10.45±0.8	11.03±0.1
	late	linalool	31.2±0.3	2.8±1.1	2.4±2.3	9.2±3.8
Aliphatic compounds	early	acetoin	63.0±0.6	89.3±9.3	134.1±8.0	88.5±30.8
		acetic acid	201.8±52.6	39.5±0.2	204.4±48.5	109.3±77.5
		unidentified	212.7±19.3	4.4±0.0	40.8±29.2	5.3±3.3
		butyric acid	4.4±0.8	4.4±2.7	6.7±3.0	10.3±3.0
		2-methyl-butanoic acid	16.0±1.0	4.4±2.7	21.5±1.7	2.5±0.4
		hexanoic acid	42.7±3.4	74.5±1.6	115.4±10.6	85.4±6.8
		trans-2-hexenoic acid	12.2±0.4	17.4±9.3	21.7±1.3	14.6±1.3
		octanoic acid	50.6±4.3	42.9±5.2	33.0±0.3	19.0±4.0
		hexadecanoic acid	599.4±202.2	45.3±30.9	45.3±29.9	134.6±26.8
	commercial	acetoin	53.2±1.5	55.4±9.4	153.5±6.2	27.0±9.3
		acetic acid	131.7±3.9	25.8±5.8	61.7±7.6	13.0±0.2
		unidentified	190.8±26.2	nd	20.2±7.9	12.6±0.1
		butyric acid	6.2±1.1	3.9±2.2	9.9±0.7	6.9±0.0
		2-methyl-butanoic acid	16.6±0.3	4.1±0.5	19.9±0.7	1.5±0.0
		hexanoic acid	43.5±1.8	61.6±5.7	109.8±5.0	63.4±8.6
		trans-2-hexenoic acid	30.7±6.8	nd	22.0±4.5	nd
		octanoic acid	30.9±4.6	31.6±3.6	33.8±5.6	38.8±11.3
		hexadecanoic acid	214.2±63.5	86.0±49.2	116.1±4.1	100.9±22.4
	late	acetoin	51.2±1.7	58.8±16.3	61.7±12.5	31.3±7.8
		acetic acid	162.9±60.2	27.7±0.5	23.4±4.6	5.4±1.2
		unidentified	211.4±39.7	0.5±0.0	nd	12.7±0.9
		butyric acid	7.5±2.6	2.6±1.7	6.2 ±.2.8	3.1±0.7
		2-methyl-butanoic acid	25.3±0.6	6.5±3.5	10.6 ±.1.7	3.5±0.3
		hexanoic acid	44.3±2.8	57.1±4.2	85.4 ±.25.7	66.4±3.8
		trans-2-hexenoic acid	35.6±2.7	4.3±0.9	7.2±2.4	20.2±3.6
		octanoic acid	27.6±0.9	27.1±7.0	25.3±14.8	35.7±23.7
		hexadecanoic acid	209.7±15.8	20.9±14.7	98.6±23.4	92.2±9.9

(continua)

age. This confirms that CA storage reduces the capacity of apple to produce esters and/or maintain the existing concentrations (LÓPEZ *et al.*, 1998; MATTHEIS *et al.*, 1998; STREIF and BANGERTH, 1988). Prolonged storage in low-oxygen conditions is detrimental to ester production (FELLMAN *et al.*, 2000). The late-harvested apples were the only ones in which the concentration of esters increased after the return to RA (Table 4).

Early- and commercially-harvested fruit had lower alcohol concentrations than the late-harvested ones; this confirms what was reported previously by other authors (FLATH *et al.*, 1967; MEHINAGIC *et al.*, 2006). The return to RA conditions strongly influenced the alcohol concentration in the fruit. At PH3 it was almost 10 times greater than in early-harvested fruit and 3 times greater in commercially-harvested ones with respect to picking time. In late-harvested fruit, where alcohol concentration was already high at harvest, a decreasing trend during storage was detected (Table 4). CA balanced the total alcohol amount. No differences were detected among harvest dates at PH1, whereas after the return to RA it was possible to distinguish the fruit based on their picking time. Under RA storage conditions higher quantities of

alcohols were accumulated in fruit from the early harvest than in fruit from the late harvest (Table 4). Given that the increase in esters is facilitated by the availability of alcohol precursors (ECHEVERRÍA *et al.,* 2004), it was hypothesized that in the early- and commercially-harvested fruit the esterification of alcohols with acyl-CoA to produce esters had not yet happened, whereas the process of ester formation had already taken place in the late-harvested apples, resulting in the higher ester concentration detected after RA storage. This is in agreement with recent studies that reported that as harvest maturity progressed, the time required to regenerate volatiles decreases after removal from CA storage, particularly esters (FELLMAN *et al.,* 2003). After homogenization the linoleic and linolenic acids can serve as the substrate of lipoxygenase (LOX) enzymes which produce aldehydes. This event could be attributed to enzymes that became active after cell breakage that occurred during sample preparation, or to the post-climateric ripening that increased cell membrane permeability which made the activation of LOX possible (SEFTON *et al.*, 1993). In this method the effect of enzymes activated after cell breakage can be excluded, due to temperature control during homogenization and the use of ascorbic acid as an antioxidant. Hexanal is produced from linoleic acid lipooxygenation that is then reduced to hexanol which has a green-apple odor. (Z)-3-hexenal is produced from linolenic acid lipooxygenation and traces of it are transformed into the corresponding (Z)-3-hexenol, whereas most of it is isomerised into (E)-2-hexenal which is transformed into (E)-2-hexenol. This could explain the significantly higher aliphatic and aromatic alcohol concentration of late-harvested fruit at harvest (DEPOOTER *et al.,* 1987) which was essentially due to a particularly high hexanol concentration (Table 5).

The aldehydes, hexanal and (E)-2-hexenal, which have green apple-like odors (FLATH *et al.,* 1967; FUHRMANN and GROSCH, 2002), were not significantly different among pickings until PH1. The length of time that fruit was in RA after CA storage increased the aldehyde concentration of early-harvested fruit with respect to commercially- and late-harvested ones (Table 4). The aldehyde concentration in late-harvested fruit was significantly higher at harvest and at PH1 than afterwards (Table 4).

Time of harvest did not affect the phenol or other benzene derivative concentrations at harvest or during storage (Table 4). Phenols and the other benzene derivatives were influenced by the ageing of fruit during storage, particularly in fruit from the late harvest. Their concentrations decreased significantly after PH2 (Table 4), essentially due to the decrease in the eugenol concentration (Table 5). The return to RA storage conditions had no effect on the accumulation of these compounds. Eugenol, is a potent odorant and was the only compound that had already been identified in cv. Cox Orange apples (GALLIARD, 1968). To our knowledge, the other phenols and benzene derivative compounds have never been identified in apple, whereas they have been reported in other species such as benzaldehyde in tomatoes (BALDWIN *et al.*, 2004), sour cherries (GIRARD and KOPP, 1998; MATTHEIS *et al.*, 1997; POLL *et al.*, 2003), kiwifruit (YOUNG and PATERSON, 1995) and peaches (CHAPMAN *et al.*, 1991). Benzaldehyde, benzyl alcohol, 4-vinylguaiacol, 4-vinylphenol, benzoic acid, dihydroconiferyl alcohol and 2-phenylethanol were identified as free forms in the berries of Chardonnay and Sauvignon blanc grapevine varieties (SEFTON *et al.*, 1993, 1994). 4-Vinylguaiacol and 4-vinylphenol could be attributed to artefact formation, derived from the corresponding cinnamic acid decarboxylation during gas chromatography. The other benzene derivatives have never been identified as artefacts but as free constituents of the volatile profile (SEFTON *et al.*, 1993).

Among the norisoprenoids, we identified 3-hydroxy-β-damascone, a β-damascenone precursor (ROBERTS and ACREE, 1995), 3-oxo-α-ionol and β-damascenone, The latter is one of the highest odor potent volatiles in apples (CUNNINGHAM *et al.*, 1986; FURHMANN and GROSCH, 2002). These norisoprenoids were always in trace amounts (Table 5) and no differences were detected between harvest dates at picking and during storage (Table 4). 3-Hydroxy-β-damascone and β-damascenone have already been identified in apples (GALLIARD, 1968; FUHRMANN and GROSCH, 2002). 3-oxo-α-ionol, whose sensory impact is still unknown, could be derived from acid hydrolysis of the glyco-conjugated forms at the time of sample preparation. It was detected in fruit at harvest, independent of harvest time (Table 5). This compound has been isolated in Chardonnay juice (SEFTON *et al.*, 1993), in other grapevine varieties (WILLIAMS et al., 1982) and in apple leaves in the glyco-conjugated form (STINGL et al., 2002). Norisoprenoids seemed to take advantage of the return to RA storage conditions, particularly in the commercially harvested fruit (Table 4).

Linalool, already reported as a potent odorant in apples in the free-form (GALLIARD, 1968), was detected in trace amounts (Table 4). Time of harvest did not affect the linalool content at picking or at PH1, whereas at PH2 the linalool concentration was significantly higher in fruit from the early and commercial harvests than in fruit picked later. Storage greatly affected the linalool concentration; it was much lower after CA storage than at picking (Table 4). The return to 10 days of RA conditions significantly increased the linalool content of commercially-harvested fruit with respect to PH1 (Table 4). The partial recovery of linalool after CA storage was also evident in commercially harvested apples at PH2 (Table 4). The linalool concentration was generally higher at harvest than after storage.

The concentration of the other aliphatic compounds, mainly organic acids (Table 2), decreased during storage and time of harvest did not significantly affect their concentrations, except at PH2 (Table 4).

No differences were detected in the total aromatic compound contents at harvest and at PH1; the total volatile content was significantly higher in early-harvested fruit than in late-harvested apples at PH2 and PH3 and this was attributable to the higher concentrations of alcohols, aldehydes and miscellaneous compounds (Table 4).

As previously stated, the low ester concentrations detected were probably attributable to the analytical method used to extract the volatiles; the ester content, however, accounted for 29.8 and 24.5% of total volatile amount, in early- and commercially-harvested fruit, respectively, whereas, it accounted for just 13.5% in late-harvested fruit. In contrast, the alcohol content in late-harvested apples accounted for 49% of the total volatile amount versus 19 and 30% in early- and commercially-harvested fruit, respectively. The aldehyde percentage was very similar among the three pickings (13, 17, 16%, respectively, in early, commercial and late harvest) at harvest. This suggests a certain stability of these compounds that have already been indicated as stable, since they were not influenced by the fruit/leaf ratio (POLL *et al.*, 1996). Phenols and other benzene derivatives accounted for 8% of the total volatile content in early- and late-harvest and 9% at commercial harvest. During storage, the evolution of various classes of compounds evolved differently: CA markedly reduced the percentage of esters and it only increased in late-harvested fruit that was returned to atmospheric O_2 concentrations (24% at PH2 and 17% at PH3).

In conclusion, even if the general quality parameters of early-harvested fruit were satisfactory, the volatile content after storage was characterized by the highest alcohol concentration which could in-

dicate a delay in their conversion into esters; in this case, their qualitative characteristics could still improve. In contrast there was a regeneration of esters in late-harvested fruit after the return to RA conditions. In these apples, as harvest maturity advanced, the ester regeneration after the return to O_2 was greater than in earlier-picked fruit. Further studies are needed to establish the real degree of fruit maturation, i.e. the potential of alcohols to be transformed into esters in order to correlate the volatile concentration to routine quality indexes and study the sensory impact of volatiles, such as phenols, norisoprenoids and linalool on the sensorial features of apples.

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PAPER

OXIDATIVE STABILITY AND HEALTH ASSESSMENT INDEX OF RAINBOW TROUT (*ONCORHYNCHUS MYKISS* L.) REARED IN VALNERINA

STABILITÀ OSSIDATIVA E QUALITÀ NUTRIZIONALE DI TROTE IRIDEE (*ONCORHYNCHUS MYKISS* L.) ALLEVATE IN VALNERINA

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ABSTRACT

Forty rainbow trout (*Oncorhynchus mykiss L.*) reared in Valnerina (Umbria, Italy) were studied to determine the oxidative stability of fillets during a 5-day storage period at 2°C under continuous cool white fluorescent illumination. Chemical, physical and microbiological analyses were carried out. High quality fresh trout fillets were characterized by a low lipid and cholesterol content (4.2% and 37 mg/100 g, respectively), a high hypo-cholesterolemic/hyper-cholesterolemic fatty acid

RIASSUNTO

Per la sperimentazione sono state utilizzate 40 trote iridee (*Oncorhynchus mykiss* L.) allevate in Valnerina (Umbria); sui filetti conservati a 2°C per 5 giorni sotto luce fluorescente (simulazione banco vendita) è stata valutata la stabilità ossidativa oltre alle normali analisi chimiche, fisiche e microbiologiche. I risultati ottenuti dimostrano che le trote della Valnerina sono caratterizzate da un basso contenuto di lipidi e di colesterolo (4,2% and 37 mg/100 g, rispettivamente) da un ottimo rapporto acidi gras-

- Keywords: atherogenic index, oxidative stability, PUFA, rainbow trout, thrombogenic index -

ratio (2.9) and low atherogenic and thrombogenic index values (0.45 and 0.21, respectively). After three days of display, the oxidative stability of the fillets decreased (2.7 *vs* 0.8 mg MDA/kg, P<0.05) compromising the quality of the product. The lipid profile of the fillets also changed: the SFA and MUFA increased due to PUFA oxidation and/or drip loss, while EPA and DHA tended to decrease. Nevertheless the levels of both long-chain PUFA were higher with respect to the baseline values, indicating that drip loss, more than oxidation, affected the amount of these compounds in fillets at the end of the experimental storage. Hence, to preserve the nutritional value of this product, better packaging and storage conditions must be used.

si ipocolesterolemici/ipercolesterolemici (2,9) e da bassi valori degli indici aterogenico e trombogenico (0,45 and 0,21, rispettivamente). Dopo il terzo giorno di conservazione, i filetti hanno mostrato delle variazioni significative, pregiudizievoli per la *shelf-life* del prodotto. Infatti, oltre alla riduzione della stabilità ossidativa (2,7 vs 0,8 mg MDA/kg, P<0,05), si è notata una modificazione del profilo acidico con aumento percentuale dei saturi e dei monoinsaturi con riduzione dei polinsaturi, dell'EPA e del DHA con differenze significative al 4° e 5° giorno di conservazione. Tuttavia il contenuto in termini quantitativi dei polinsaturi a lunga catena è risultato superiore al livello di partenza, indicando un effetto della perdita di liquidi superiore a quello dell'ossidazione. Lo studio di adeguate metodologie di conservazione potrebbe comunque ulteriormente migliorare le caratteristiche qualitative di questo prodotto.

INTRODUCTION

Fish products come from either fishing or from aquaculture. Since 1980, aquaculture has increased considerably and this trend is expected to continue given the widespread collapse of the wild fisheries (DE SILVA, 2001).

In Europe, trout (*Oncorhynchus mykiss*) rearing is the most important fish-producing activity and Italy is one of the main European producers along with Norway, Denmark, Spain and France (FAO Fishery Statistics, 2002). About 10% of the trout fillets produced in Italy are reared in Valnerina (Umbria, Italy), an area in south-eastern Umbria, delimited by the hydrographic basin of the Nera River.

In recent years, the demand in Italy for processed aquaculture products (mainly fillets and eviscerated fish) has increased and the goals of the fish processing industry have been to: attain good sensory characteristics that correspond to the eating habits of consumers, improve the hygienic quality and preserve the intrinsic nutritional value.

Processed food products may undergo substantial negative alterations as a consequence of variations in pH and oxidative status and fish products, in particular, are known to deteriorate easily during processing and/or storage mainly due to microbial growth, endogenous enzyme activity and non-enzymatic lipid oxidation (HSIEH and KINSELLA, 1989).

To date, research on the alterations in fish fillets during storage has focused on variations in the sensory attributes (WHITTLE and HOBBS, 1990), volatile amine and hypoxanthine production and protein changes with subsequent changes in the physical properties (ASHIE *et al.*, 1996). The lipid fraction of fish

products has also received a great deal of attention since polyunsaturated fatty acids (PUFA) are highly prone to oxidation (HARRIS and TAL, 1994; RICHARDS and HULTIN, 2002). Studies on various fish species have shown that lipid damage may reduce the sensory quality and nutritional value of fish (KHALIL and MANSOUR, 1998; AUBOURG *et al.*, 1998).

The aim of this study was to evaluate the effect of retail display on the oxidative status and nutritional characteristics of fillets from rainbow trout (*Oncorhynchus mykiss*) reared in Valnerina.

MATERIALS AND METHODS

Rearing conditions and diets

The study was carried out on a quality certified (UNI EN ISO 9001-2000) fish farm (Tranquilli Fishery S.r.L., Preci, Perugia, Italy), that buys fertilized eggs and rears them in an open, 4-step production cycle: hatching and first feeding, fryer production, pre-fattening, fattening.

The water temperature in this habitat ranges from 6° to 15°C. The water is changed frequently and the farmer performs regular controls. The fish are reared at a low density and the feed is produced by a certified UNI EN ISO 9001-2000 farm. The main ingredients of the diet are: fish meal, herring meal, fish oil, soybean meal, wheat meal, colza oil and minerals. The same amounts of three standard feeds (first period: live fish weight 12-100 g; second period: 80-200 g; third period 170-500 g) were given to the fish; the quantity was 10% less than the feed manufacturer table values to ensure that all the feed was eaten. The chemical composition of the diets used during the three growth phases of rainbow trout is summarized in Table 1.

Under the above-mentioned conditions, it takes twenty-four months for a rainbow trout to reach a commercial weight of about 500 g. During this time, careful hygienic-sanitary and good management practices that respect the welfare of the animal are followed.

Fish and fillet handling procedures

After 12 hours of fasting, forty trout of mean weight 500±45 g were captured and immediately slaughtered by continuous electrical stunning. After washing with running water, the fish were mechanically eviscerated, the tails and heads were removed. Eighty dorsal and ventral fillets (mean weight of 130±2 g), were cut and transferred to the laboratory (Department of Plant Biology, Agro-environmental and Animal Biotechnologies, Animal Sciences Section, University of Perugia) for analyses.

Half of the fillet samples were analyzed in duplicate to determine the proximate composition, energetic value and cholesterol level. The remaining samples were placed on plastic foam trays covered with PVC film (600 cm^2) and displayed at +2°C under continuous cool white fluorescent illumination (intensity 2,300 lux) (MITSUMOTO *et al.*, 1991) for five days. Each day, during the storage period, the pH, color parameters, water loss, total mesophil bacterial flora, fatty acid profile and oxidative stability values were determined.

Table 1 - Chemical composition and digestible energy of diets used during the three growing phases of rainbow trout.

		Phase		
		First	Second	Third
Pellet diameter	mm	2.5	4.0	6.0
Crude protein	%	46.0	44.0	41.0
Crude lipid	%	22.0	24.0	26.0
Ash	%	10.0	9.3	8.8
Fiber	%	1.2	1.5	1.6
Phosphorus	%	1.3	1.2	1.1
Digestible energy	MJ/kg	19.8	20.2	20.4

Analyses

Moisture, ash and total nitrogen were assessed using the AOAC methods (N. 950.46B, 920.153, and 928.08, respectively). Total protein was calculated by Kjeldahl nitrogen using a 6.25 conversion factor. Total lipids were extracted in duplicate from 5 g of each homogenised sample and calculated gravimetrically (FOLCH *et al.*, 1957).

Cholesterol was quantified colorimetrically (wavelength 405 nm) using a Biochemia enzymatic kit n. 139050 (Boehringer, Mannheim, Germany – Manual for Food Analysis, 1995).

The pH was measured with a Knick digital pH-meter (Broadly Corp., Santa Anna, CA, USA) after homogenizing the fillet with iodoacetate (KORKEALA *et al.*, 1986).

The color parameters were evaluated by means of a tristimulus analyzer (Minolta Chroma Meter CR-200, Azuchi-Machi Higashi-Ku, Osaka 541, Japan) with a CIELAB Color System (1976). The *L a* b** color system consists of a luminosity or lightness component (*L**) and two chromatic components: the *a** component varies from green (-*a*) to red (+*a*) and the *b** component varies from blue (-*b*) to yellow (+*b*). The colorimeter was calibrated by using a standard white plate. The standard values were: L = 97.10, a = +0.13, and b = +1.88. Trout fillet color was measured at three different points: next to head portion, middle portion and caudal portion and values are expressed as the mean of the three positional measurements.

Enumeration of aerobic mesophiles was carried out on Plate Count Agar (PCA, Merck 5463, Darmstadt, Germany), after incubation for 48 h at 30°C and are reported as the number of colony forming units/g (CFU/g).

Fatty acids were determined by gas-chromatography after lipid extraction according to the FOLCH *et al.* method (1957). One mL of lipid extract was evaporated under a stream of nitrogen and the residue was derivatized by adding 3 mL of sulfuric acid (3% in methanol). After incubating at 80°C for one h, methyl esters were extracted with petroleum ether and 1 μL was injected into the gas chromatograph (Fisions Mega 2 Carlo Erba Gas Chromatograph, model HRGC Milano, Italy), equipped with a flame ionization detector. Separation of fatty acid methyl esters (FAME) was carried out on an Agilent (J&W) capillary column (30 m x 0.25 mm I.D, CPS Analitica, Milan, Italy) coated with a DB-Wax stationary phase (film thickness of 0.25 μm). The operating conditions on column injection of a sample volume of 1 μL were: the temperatures of the injector and detector were set at 270° and 280°C, respectively; the detector gas flows were: H_2 50 mL/min and air 100 mL/min. The oven temperature was programmed to give good peak separation: the initial oven temperature was set at 130°C; this temperature increased at a rate of 4.0°C/min to 180°C and was held for 5 min; the temperature was then increased at a rate of 5.0°C/min to 230°C; the oven was held at the final temperature for 5 min. Helium was used as carrier gas at a constant flow rate of 1.1 mL/min. Individual fatty acid methyl esters were identified by reference to the retention time of FAME authentic standards. The relative proportion of each fatty acid in the fatty acid pattern of the fillet is expressed as a percentage.

The mean value of each fatty acid was used to calculate the sum of the saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids and to calculate the peroxidability index (PI) according to the equation proposed by ARAKAWA and SAGAI (1986):

PI = (% monoenoic x 0.025) + (% dienoic x 1) + (% trienoic x 2) + (% tetraenoic x 4) + (% pentaenoic x 6) + (% hexaenoic x 8).

Based on the current knowledge regarding the effect of specific fatty acids

on cholesterol metabolism, the ratio between hypocholesterolaemic and hypercholesterolemic fatty acids (HH) was calculated using the following mathematical equation (SANTOS-SILVA *et al.*, 2002):

HH = (C18:1n-9 + C18:2n-6 + C20:4n-6 + C18:3n-3 + C20:5n-3 + C22:5n-3 + C22:6n-3)/(C14:0 + C16:0).

The concentration of each fatty acid (mg/100 g of fish) was calculated from the lipid content of the fish and the conversion factor 0.91 according to JOHANSSON *et al.* (2000). The lipid content of the stored fillets was quantified on the day of analysis to avoid overestimation of fatty acids due to drip loss.

The amount of each fatty acid was also used to calculate the indexes of atherogenicity and thrombogenicity as proposed by ULBRICHT and SOUTHGATE (1991):

- atherogenic index = (C12:0 + 4xC14:0 + C16:0)/[(ΣMUFA + Σ(n-6) + Σ(n-3)];
- thrombogenic index = (C14:0 + C16:0 + C18:0)/[(0.5 x ΣMUFA + 0.5 x (n-6) + 3 x (n-3) + (n-3)/(n-6)].

The index of nutritional quality (INQ) was calculated on the basis of the eicosapentaonic (EPA) + docosahexanoic (DHA) acid level using the formula suggested by GODBE (1994).

The extent of lipid oxidation was evaluated in fresh and stored fillets as thio-barbituric acid reactive substances (TBARS) according to the modified method of KE *et al.* (1977). Oxidation products were quantified as malondialdehyde (MDA) equivalents (mg MDA/kg muscle).

Data of the qualitative traits were analysed with a linear model (STATA, 2005) that also takes into account the effect of storage. Significant differences were evaluated as least significant differences (LSD).

Table 2 - Proximate composition, cholesterol and energy level of rainbow trout fillets at slaughter (n=40).

		Mean ± SD
Moisture	%	74.4±2.5
Protein	%	20.10±1.2
Lipid	%	4.2±0.6
Ash	%	1.3±0.2
Cholesterol	mg/100 g	37.00±3.6
Energy	Kcal/100 g	119.80±12.3

RESULTS AND DISCUSSION

The proximate composition, cholesterol and energy levels of the trout fillets are summarized in Table 2. These data are in agreement with the standard data for this species (TURCHETTO *et al.*, 1994). Compared to the findings of CARNOVALE and MIUCCIO (1984) and DE FRANCESCO *et al.* (2004) the lipid and cholesterol levels observed in this study were lower (4.20% *vs.* 6.98 and 5.50%; 37.00 mg/100 g *vs.* 51.04 and 47.74 mg/100 g). TURCHINI *et al.* (2003) found a low lipid content in brown trout fillets but a higher cholesterol level (2.8 *vs.* 4.2%; 107.06 *vs.* 37.00 mg/100 g, respectively). These findings are probably due to the particular management practices followed (feed composition and length of cycle) and the environmental conditions (water temperature).

The variations in the main traits of rainbow trout fillets during storage are reported in Table 3. The pH values decreased during the first three days and then increased to a value of 6.3. Nutritional status, stress and exercise of the fish before death are known to play a key role in determining the total amount of glycogen stored in fish and expressed in the *post mortem* pH, (HUSS, 1995). Once glycogen-related energy sources are exhausted, fat lipolysis (generates free acids) and protein oxidation (generates ammonia) take place which affect the fillet pH values, the pH decreases during the former biochemical reaction and increases during ammonia production. Notwithstanding the numerous *ante-mortem* fac-

tors that affect the pH after death, fish storage at 4°C for more than a day under the proposed conditions did not seem to inhibit biochemical degradation of lipids and proteins, as indicated by the variations in pH of the fillets. PEREZ-ALONZO *et al.* (2003) showed that Atlantic pomfret, stored on ice in an isothermal room at 4°C, underwent lipid damage by hydrolysis and oxidation after 19 days of storage. However, the storage conditions used in the present study were very different. Fluorescent illumination may also have contributed to the earlier lipid oxidative degradation.

The pH variation during storage may account for the water loss and changes in the color parameters of the fillets (Table 3). The pH is known to affect protein conformation and thus, protein water-binding properties. Water loss due to evaporation from the surface cannot be excluded. Furthermore, pH can affect the light reflectance properties, as well as the conformation of myoglobin. In particular, the muscle fiber structure is open at low pH and reflects light (OFFER *et al.*, 1989). Accordingly, high light values at low pH and low values at higher pH values were found (Table 3). Such a condition is also known to enhance enzymatic activity in the mitochondria, thereby increasing oxygen availability for heme reactivity (> oxyhemoglobin) which explains the redness of trout fillets. Pigment oxidation may also account for the color variation. Dietary keto-carotenoids (astaxanthin and canthaxanthin) are known to influence the color of the flesh of rainbow trout (FRANCIS, 1995). These carotenoids contain many conjugated double bonds, which are responsible for their color, as well as their higher susceptibility to oxidation in air (LIAAEN-JENSEN, 1971).

In contrast, greater oxygen availability could promote lipid peroxidation and consequently the production of TBARS. While the TBARS concentration was initially low, in accordance with previous reports (CHAIYPECHARA *et al.*, 2003), there was a 3.7-fold increase by the end of the storage period. In particular, the greatest increase of TBARS value was observed on the third day of display. This finding agrees with that of CHOUBERT *et al.* (2005), who studied the evolution of oxidative processes in cold smoked rainbow trout slices. The TBARS level is very important for the sensory appraisal of a product and can affect consumer choice (AHN *et al.*, 1996; LEI and VAN BEEK, 1997; SHAHIDI, 1998; GOMES *et al.*, 2003). In poultry, CASTELLINI *et al.* (2006) found a significant negative corre-

Table 3 - Changes in pH, drip loss, color, oxidative stability (TBARS) and mesophilic bacteria charge (MBC) of trout fillets during storage display at 2°C under continuous fluorescent illumination.

		Days					
		1	2	3	4	5	LSD
pH		6.62^{e}	6.45^{d}	5.92^{a}	6.20^{b}	6.31^{c}	0.14
Drip loss	%	0.84^{a}	1.76^{a}	5.46^{b}	0.04^{a}	1.25^{a}	0.29
Fillet Color							
L*		47.41^{b}	48.89^{c}	49.91^{c}	46.58^{a}	46.61^{a}	2.18
a*		0.77^{a}	1.96^{b}	2.41^{c}	2.59^{c}	2.61^{c}	1.16
b*		2.94^{a}	3.31^{a}	5.03^{b}	4.96^{b}	4.81^{b}	2.29
TBARS	mg/kg	0.79^{a}	0.97^{a}	2.67^{b}	2.79^{b}	2.93^{b}	0.34
MBC	10^{5} CFU/g	0.85^{a}	1.60^{a}	1.70^{a}	7.50^{b}	7.00^{b}	2.20

n=40. Level of significance: a..e: P<0.05.

lation (-0.86; P<0.01) between meat oxidative status and the general acceptability of the product when evaluated by a sensorial test panel. It was concluded that a TBARS level in meat greater than 2.5 mg MDA/kg can negatively affect consumer choice, based on a sensory appraisal of the product.

A significant increase in bacterial growth during storage was observed after the fourth day of display accompanied by a variation in pH, presence of water on the surface of the fillets (see drip loss) and oxidative processes. The results are shown in Table 3. These factors, together with temperature, oxygen availability, redox potential, competition with other bacteria and moisture availability of the substrate are known to play a key role in bacterial growth (BODDY and WIMPENNY, 1992).

The fatty acid profile of the trout fillet fat is reported in Table 4. Palmitic acid was the predominant saturated fatty acid, whereas oleic acid was the major monounsaturated fatty acid. Among the PUFAs, EPA and DHA were the most abundant long-chain fatty acids. The levels of these fatty acids were higher than those found by other authors in the same fish products (JOHANSSON *et al.*, 2000; HALILOGLU *et al.*, 2004; DE FRANCESCO *et al.*, 2004; KIESSLING *et al.*, 2001). This peculiar fatty acid profile was probably due to the age of the Valnerina trout that reached commercial weight at 24 months. KIESLING *et al.* (2001) showed that the length of productive cycle affects the fatty acid profile; SFA concentration decreases as the age and body weight of the fish increases.

Feed composition also, directly or indi-

Table 4 - Changes in fatty acid profile (%) and peroxidability index of rainbow trout fillets during display at 2°C under continuous fluorescent illumination.

		Days					
		1	2	3	4	5	LSD
C14:0		3.66	3.55	3.48	3.73	3.86	0.54
C16:0		17.84	18.15	18.24	18.44	18.32	2.13
C18:0		3.96	4.15	3.94	4.09	3.90	0.47
Others		1.38	1.44	1.37	1.83	2.39	0.19
SFA		26.84^{a}	27.29^{ab}	27.03^{ab}	28.09^{b}	28.47^{b}	3.05
C14:1n-6		0.03	0.04	0.05	0.05	0.04	0.01
C16:1n-7		4.13	3.89	4.15	4.30	4.46	0.47
C18:1n-9		13.78^{a}	14.74^{b}	14.44^{ab}	14.78^{b}	14.64^{b}	2.03
Others		3.97	3.69	3.96	4.04	4.24	0.86
MUFA		21.91^{a}	22.36^{ab}	22.60^{ab}	23.17^{b}	23.38^{b}	3.68
C18:2n-6	LA	17.81^{b}	16.50^{ab}	15.79^{a}	16.95^{ab}	16.22^{ab}	2.58
C20:4n-6		0.80	0.79	0.75	0.77	0.80	0.14
C18:3n-3	LNA	2.16	2.12	2.15	2.27	2.17	0.47
C20:3n-3		0.18	0.20	0.19	0.20	0.16	0.08
C20:5n-3	EPA	5.58^{b}	5.27^{b}	5.50^{b}	4.47^{a}	4.46^{a}	0.76
C21:5n-3		0.40	0.39	0.38	0.36	0.35	0.13
C22:5n-3		1.97^{b}	2.06^{b}	2.02^{b}	1.15^{a}	1.04^{a}	0.25
C22:6n-3	DHA	20.95^{b}	20.16^{b}	19.82^{ab}	18.75^{a}	18.72^{a}	2.14
Others		1.39^{a}	2.85^{a}	3.75^{b}	3.21^{ab}	4.22^{c}	1.06
PUFA		51.24^{b}	50.34^{b}	50.35^{b}	48.13^{a}	48.14^{a}	3.21
Peroxidability index		239.04^{b}	230.03^{b}	227.62^{ab}	209.17^{a}	207.32^{a}	21.36

n=40. Level of significance: a..e: P<0.05.

rectly, affects the fatty acid profile of lipids in fish. Fish meal and fish oil, rich in EPA and DHA, are commonly used in the diet of rainbow trout as protein and lipid sources (SARGENT and TACON, 1999). The fatty acid composition of trout grown on this diet was very similar to that of wild fish, with high levels of n-3 highly unsaturated fatty acids (n-3 HUFA) (ACKMAN, 1980; HENDERSON and TOCHER, 1987; BELL *et al.*, 2001, 2002). Moreover, the fatty acyl desaturase enzyme activities involved in the HUFA biosynthesis pathway are known to be under nutritional regulation in mammals (BRENNER, 1981) and have also been demonstrated in fish (SARGENT *et al.*, 2002). Taken together, these considerations may partially explain the high EPA and DHA contents found in the trout fillets.

The temperature of the rearing water regulates the desaturation-elongation and β-oxidation of fatty acids and therefore plays an important role in lipid fatty acid composition (NINNO *et al.*, 1974, HAZEL 1984, TOCHER *et al.*, 2004). Temperatures higher than 15°C reduce the efficiency of such reactions in both the hepatocytes and erythrocytes, while a temperature around 7°C is suitable for the maximum enzymatic activity. A study on the metabolic adjustments responsible for the "homeoviscous adaptation" of membrane lipids in rainbow trout, showed that desaturation of fatty acids, an important process for PUFA formation, proceeds more rapidly in trout reared in cold water than in those reared in warm water (HAZEL, 1984). Furthermore, certain fatty acids, particularly those formed by the Δ5-desaturase activity (n-3 and n-6 series), are preferentially incorporated into phospholipids at a cold temperature in cold-acclimated trout. This is probably due to the direct effect of temperature on the substrate preferences of the phospho- and acyltransferase enzymes for the *de-novo* phospholipid biosynthesis. Finally, NINNO *et al.* (1974) found that in *Pimelotus maculate* there was a 2-4-fold increase in the Δ6- and Δ5-desaturase activity when the temperature decreased from 30° to 16°C. Taken together, these findings may explain the high PUFA content, especially n-3 fatty acids, found in the trout reared in Valnerina where the water temperature ranges from 6° to 15°C. Investigating the adaptive changes in fatty acid metabolism of rainbow trout in relation to variations in the water temperature, CALABRETTI *et al.* (2003) reported an increase in the PUFA/SFA and PUFA/MUFA ratios in the fish meat as a consequence of the cold adaptation. RADY (1993) found that the C20:5n-3/C20:4n-6 ratio decreased from 2.7 to 0.5 in carp (*Cyprinus carpio*) in relation to an increase in water temperature from 5° to 25°C. A similar trend was also found in *Platichthys flesus* (SORENSEN, 1993).

During storage, the fatty acid profile of trout fillets varied greatly. The SFA and MUFA (mainly oleic acid) increased, whereas the total PUFA, EPA and DHA decreased; the differences were statistically significant on the 4th and 5th days of storage. The percent reduction of these fatty acids at the end of the trial was 7.33, 20.1 and 10.6%, respectively. OZDEN (2005) also reported a significant increase of SFA and a concomitant decrease of PUFA in trout fillets stored under different conditions.

The processing and retail conditions (temperature and light, pro-oxidant factors), as well as a high level of unsaturated fatty acids were probably responsible for the high lipid oxidation of the trout fillets during storage. In fact, the peroxidability index (PI) that measures the relationship between the fatty acid composition of a tissue and its susceptibility to oxidation, was very high (239.04) as a consequence of the abundance of long-chain fatty acids. This value is in agreement with that found by TESTI *et al.* (2006). With respect to mammalian species (e.g. the PI of rabbit is 42.70;

CASTELLINI *et al.*, 2001), the peroxidability index of trout fillets was noticeably higher.

The nutritional properties of rainbow trout fillets are reported in Table 5. On the first day of storage, the PUFA, DHA and EPA contents were higher (2,055.53, 840.42 and 223.85 mg/100 g, respectively) than those reported by DE FRANCESCO *et al.* (2004) in the same fish species (DHA and EPA were 479 and 182 mg/100 g, respectively). Given these high values, a 448 g serving of Valnerina trout fillet is enough to satisfy the weekly human PUFA requirement (4.55 g/2,000 kcal diet, SIMOPOULOS, 2003). Furthermore, the DHA level in fresh fillets was considerably higher than that recommended by the Life Sciences Research Office (1998). The values were 668.37 mg DHA/100 kcal compared to 8 mg/100 kcal, making it an ideal food.

During display, the absolute amount of fatty acids increased significantly from the third day onwards (Table 5). This was probably due to the storage-induced drip loss and the consequent concentration of total lipids in the fillets. PUFA oxidation may also contribute to the increase of SFA and MUFA during storage. Unexpectedly, PUFA values did not decrease, which suggested that drip loss had a stronger effect on PUFA concentration than oxidation when the fillets were stored for 5-days at 2°C under continuous cool white fluorescent illumination.

The indexes for human health in relation to fatty acid composition and content (the n-6/n-3 ratio, the hypocholesterolaemic and hypercholesterolaemic fatty acid ratio, HH, the atherogenic and thrombogenic indexes and the index of nutritional quality, INQ) are considered when product quality is being assessed.

The n-6/n-3 ratio of fresh trout fillets was 0.62, which is higher than that found in other studies. TESTI *et al.* (2006) found a ratio of 0.22 and 0.24 in dorsal

Table 5 - Changes in nutritional characteristics of rainbow trout fillets during display at 2°C under continuous fluorescent illumination.

		Days					
		1	2	3	4	5	LSD
SFA	mg/100 g fillet	1076.71^{a}	1202.24^{a}	1515.24^{b}	1577.00^{b}	1672.27^{b}	170.24
MUFA	"	878.94^{a}	985.05^{a}	1266.91^{b}	1300.79^{b}	1373.29^{b}	115.69
∑ n-3	"	1271.80^{a}	1372.29^{a}	1754.24^{b}	1587.11^{ab}	1662.68^{b}	112.42
∑ n-6	"	783.73^{a}	845.40^{a}	1065.47^{b}	1114.96^{b}	1164.97^{b}	89.18
n-6/n-3		0.62	0.62	0.61	0.70	0.70	0.20
EPA	"	223.85^{a}	232.17^{a}	308.32^{b}	250.39^{ab}	261.97^{ab}	35.98
DHA	"	840.42^{a}	888.13^{a}	1111.07^{b}	1052.64^{b}	1099.58^{b}	96.12
PUFA	"	2055.53^{a}	2217.69^{a}	2822.51^{b}	2702.07^{b}	2827.65^{b}	225.41
EPA	mg/100 kcal	178.02^{b}	168.13^{b}	175.46^{b}	142.60^{a}	142.28^{a}	12.54
DHA	"	668.37^{b}	643.23^{b}	632.32^{ab}	598.18^{a}	597.22^{a}	28.41
PUFA	"	1634.71^{b}	1606.02^{b}	1606.33^{b}	1535.50^{a}	1535.81^{a}	124.26
HH*		2.93^{b}	2.84^{b}	2.78^{ab}	2.66^{a}	2.61^{a}	0.55
Atherogenic index		0.45	0.45	0.44	0.47	0.47	0.05
Trombogenic index		0.21	0.21	0.21	0.23	0.23	0.02
Index Nutritional Quality		29^{b}	25^{ab}	25^{ab}	23^{a}	23^{a}	1.20

*Ratio hypocholesterolaemic/ hypercholesterolemic fatty acids.
n=40. Level of significance: a..e: P<0.05.

and ventral fillets of rainbow trout, respectively. JOHANSSON *et al.* (2000), investigated the effect of changes in ration levels on the sensory characteristics, lipid content and fatty acid composition of rainbow trout and found a n-6/n-3 ratio that ranged from 0.20 to 0.21. The high values observed in Valnerina trout are probably imputable to feeding factors (Soya bean extract) responsible for the marked increase in linoleic acid. TURCHINI *et al.* (2003) reported different values for the n-6/n-3 ratio in brown trout fed with different lipid sources. In particular, they reported values of 0.27 for fish oil, 0.62 for canola oil, 0.64 for poultry fat, 0.59 for pork lard and 0.46 for oleine oil. When the authors used diets rich in C18:2n-6 (canola oil, 14.3% and poultry fat, 14.2%) they observed a detrimental effect on the above-mentioned index.

The HH ratio was slightly higher than that found by TESTI *et al.* (2006) in the dorsal (2.93 *vs.* 2.40) and ventral rainbow trout fillets (2.93 *vs.* 2.46). The atherogenic and thrombogenic indexes were both lower than those reported by TURCHINI *et al.* (2003) for brown trout (0.45 *vs.* 0.50 and 0.21 *vs.* 0.25, respectively) and DAL BOSCO *et al.* (2004) for rabbit meat (0.45 and 0.21 *vs.* 0.70 and 0.99). Finally, the INQ index was similar to that found in dorsal rainbow trout fillets (26 *vs.* 24) by TESTI *et al.* (2006), but was lower than that found in ventral fillets (26 *vs.* 33). Only HH and INQ were significantly affected during storage.

CONCLUSIONS

The environmental (cold water) and rearing conditions (24-month production cycle), as well as diet are responsible for the low fat and cholesterol levels and for the particular lipid fatty acid composition of Valnerina rainbow trout fillets. The nutritional characteristics of this fish species make it suitable for a healthy human diet.

All the analyzed fillet parameters were stable for the first 3 days under the storage conditions, after which they began to vary, resulting in a general decline in product quality.

Drip loss and PUFA oxidation (as revealed by the high levels of MDA) were both responsible for the high SFA and MUFA levels found at the end of the experimental period. Regarding long-chain PUFA, the highest concentration of EPA and DHA were reached on the third day of storage (due to drip loss) and tended to decrease afterwards (due to oxidation). These results, taken together, demonstrate that rainbow trout fillet should not be stored under the proposed conditions for more than three days if nutritional characteristics are to be preserved. Further studies on packaging (modified atmosphere or vacuum) and storage conditions (light, temperature, time) are needed in order to better preserve the nutritional quality of this fish species.

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PAPER

ARTISAN STRAWBERRY ICE CREAM MADE WITH SUPPLEMENTATION OF LACTOCOCCI OR *LACTOBACILLUS ACIDOPHILUS*

GELATO ALLA FRAGOLA DI PRODUZIONE ARTIGIANALE CON AGGIUNTA DI LATTOCOCCHI O *LACTOBACILLUS ACIDOPHILUS*

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ABSTRACT

The main aim of this research was to determine whether strawberry, a highly acidic fruit, can mask the sour taste of ice cream made with the addition of lactic acid bacteria (LAB). The effect of different LAB on strawberry ice cream quality was assessed. Six strawberry ice creams containing single strains of commercial LAB were produced. Two lactic acid supplementing methods were used. In one of them, pasteurised ice cream mix was inoculated with a single starter of *Lactobacillus ac-*

RIASSUNTO

Lo scopo principale di questo studio era quello di valutare se la fragola, un frutto altamente acido, può mascherare il sapore amaro del gelato prodotto con l'aggiunta di batteri lattici. Sono stati prodotti sei gelati alla fragola contenenti un singolo ceppo commerciale di batteri lattici. Sono stati utilizzati due metodi di aggiunta di acido lattico. In uno di questi il gelato pastorizzato è stato inoculato con un singolo starter di *Lactobacillus acidophilus*, *Lactococcus lactis* subsp. *lactis* e *Lactococcus lac-*

- Key words: highly acidic fruits, lactic acid bacteria, probiotic bacteria, strawberry ice cream -

idophilus, Lactococcus lactis subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris* and incubated until the pH dropped to 5.6. In the second method, the milk part of the ice cream mix was fermented with *Lactobacillus acidophilus, Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris* separately. Sufficient live bacterial counts were obtained in both LAB supplementation methods. In addition, fermenting the mix to pH 5.6 resulted in better taste and flavour scores. The sour taste resulting from the addition of LAB can be masked by highly acidic fruit. The sensorial properties of strawberry ice cream made with LAB supplementation were preferred to the control. The addition of the LAB to the highly acidic fruity ice cream is beneficial for both sensorial and physico-chemical properties.

tis subsp. *cremoris* ed incubato fino al raggiungimento di pH 5.6. Nel secondo metodo la porzione di latte della miscela per il gelato è stata fermentata con *Lactobacillus acidophilus, Lactococcus lactis* subsp. *lactis* e *Lactococcus lactis* subsp. *cremoris* separatamente. È stata rilevata un conta sufficiente di batteri lattici vivi in entrambi i metodi. La miscela fermentata a pH 5,6 è risultata di sapore migliore e di aroma marcato. Il sapore acido risultante dall'aggiunta dei batteri lattici può essere mascherato con frutta altamente acida. Le proprietà sensoriali del gelato alla fragola prodotto con aggiunta di batteri lattici sono state preferite al controllo organolettico. L'aggiunta di batteri lattici al gelato di frutta altamente acida ha un effetto benefico sia per le proprietà chimico fisiche sia per le sensoriali.

INTRODUCTION

Consumer interest in functional foods is increasing due to a desire to live healthier life styles. People are not only consuming food for their essential needs but they are also demanding a positive effect on their health. Probiotic dairy products are a response to this demand. The consumption of these microorganisms may affect the composition of the indigenous microbiota and may have several beneficial effects on human health such as maintaining a balanced flora, alleviating lactose intolerance symptoms and providing resistance to enteric pathogens (ROY, 2001).

Non-viable microorganisms can have probiotic properties, but in most cases, viable microorganisms have better effects (OUWEHAND and SALMINEN, 1998). Several authors (VINDEROLA *et al.*, 2000; OLIVEIRA *et al.*, 2001) have suggested that ingestion of 10^6-10^9 viable cells per day is needed to develop beneficial effects in humans. Therefore, the survival rate of the probiotic bacteria in food must be kept as high as possible prior to consumption.

Various lactic acid bacteria are used in the production of fermented dairy products (*Lactococcus lactis*, *Streptococcus thermophilus*, and *Leuconostoc mesenteroides*), but they do not grow in the gastrointestinal tract and, thus, are considered to have lower probiotic potential (CHAMPAGNE and GARDNER, 2005). Some dairy strains, *L. bulgaricus* and *L. lactis* ssp. *cremoris*, show a relatively high incidence of binding to intestinal mucus, even though they are not considered colonising strains (OUWEHAND *et al.*, 1999). Recent studies have focused on the wide-scale use of probiotic bacteria which has threatened to undervalue the benefit of other LAB. It should be remembered, however, that LAB reach high numbers in fermented milks and may produce health-promoting metabolites, such as exopolysaccharides, peptides etc. (CHAMPAGNE and GARDNER,

2005). KIMOTO *et al.* (2002) showed that some strains of lactococci had the ability to remove cholesterol from laboratory media during growth. In addition, bread baked with lactic acid improved glucose metabolism in obese and hyperinsulinaemic Zucker rats (ÖSTMAN *et al.*, 2005). Lactic acid is an important fuel for the body during rest and exercise. It is used to synthesize liver glycogen and is one of the most important energy sources. Lactate is the preferred fuel source in highly oxidative tissues, such as heart muscle and slow-twitch skeletal muscle fibers. It is used rapidly by the body and is a valuable component in fluid replacement beverages for athletes (FAHEY, 2006).

Research over the years has indicated that the desired benefits of probiotic microorganism consumption are obtained not only by their presence in sufficient numbers but also by the presence of live bacteria (e.g. yoghurt bacteria). Ice cream could serve as a suitable vehicle for delivering and maintaining a sufficiently high number of living probiotic and LAB cells. The bacteria can increase to high numbers in ice cream mix and remain viable during frozen storage. However, the perception of an acidic taste produced by lactic acid in ice cream can have a negative effect on the consumer. A pleasant taste and attractive texture are essential for all dairy products, regardless of their health status (SAXELIN *et al.,* 1999). Concerning consumer preferences, HEKMAT and MCMAHON (1992) suggested that a pH of 5.5 is suitable for probiotic ice cream.

It is well known that many fruits have an acidic taste and are readily consumed. We have hypothesized that the addition of LAB to ice cream prepared with a highly acidic fruit could result in a minimum acidic flavour perception. In this study, strawberry, with a pH<4 in the ripe stage, was selected as the fruit additive (MOING *et al.,* 2001; KAFKAS *et al.*, 2007). *Lactobacillus acidophilus* was selected as the probiotic bacteria because it is used extensively in dairy products. *Lactococcus lactis* subsp. *lactis* was chosen because of its widespread use and low acid producing capacity compared to the *Lactobacillus* genus. Since ice cream is a fatty product, *Lactococcus lactis* subsp. *cremoris* was selected because it is used in butter manufacturing.

The aims of this research were to determine a) the effect of probiotics and LAB on the quality of artisanal strawberry ice cream and consumer preferences, b) the optimum fermentation procedure (fermentation of the whole mix versus fermentation of only the milk of the mix), c) whether the microbiological quality is improved and d) at what level the microorganisms survive.

MATERIALS AND METHODS

Ice cream mixes were prepared by adding water, skim milk-powder (Pinar Dairy Company, İzmir, Turkey), cream, sucrose, stabilizer/emulsifier (Cremodan SE 38 VEG, Danisco A/S, Denmark) and fresh strawberries. The ice cream formulas were calculated by conventional methods (ARBUCKLE, 1986) and two LAB-adding methods (fermentation of whole mix and fermentation of milk only at 10% of mix weight) were used. The production method of HAGEN and NARVHUS (1999) was followed. The fat content of the ice cream was adjusted to 6% based on the results of panel acceptance after sensorial analyses (taste and flavour).

After blending the ice cream mix ingredients (6% fat, 12% sucrose, 11.2% skim milk dry matter, 0.8% stabilizer/emulsifier) at 40°C, the mixture was then pasteurized by pouring it into a bain-marie-type stainless steel vessel and heating it to 75°C for 5 min, cooling it immediately to 68°C, and then maintaining it for 30 min before cooling it at 4°C (Fig. 1).

Six strawberry ice creams containing single strains of commercial LAB were produced. Two lactic acid supplement-

ing methods were used. In one method, the pasteurised mix was inoculated with a single starter of LAB culture (*Lactobacillus acidophilus* Lyofast SLH 41107 at 40°C (LAF), *Lactococcus lactis* subsp. *lactis* Lyofast CMS 19131 at 30°C (LLF), *Lactococcus lactis* subsp. *cremoris* Lyofast CMS 19132 at 30°C (LCF)) at 0.2% of mix weight and incubated at pH 5.6. In the second method, the milk part of the mix was fermented with single strains of LAB cultures (*Lactobacillus acidophilus* Lyofast SLH 41107 at 40°C (LAA), *Lactococcus lactis* subsp. *lactis* Lyofast CMS 19131 at 30°C (LLA), *Lactococcus lactis* subsp. *cremoris* Lyofast CMS 19132 at 30°C (LCA)) until the milk coagulated from the other components of the mix. The coagulated milk was then combined with the remaining parts of the ice cream mix.

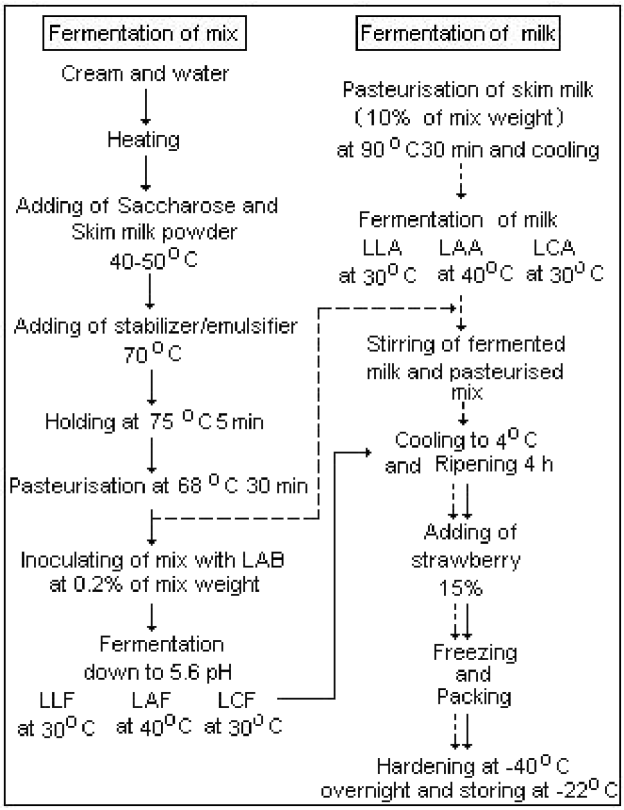

Fig. 1 - Flowsheet of the main steps in ice cream production.

Following an ageing step at 4°C for 4 h, all mixes were frozen separately in a batch freezer of 5 kg capacity (Ugur, Nazilli, Turkey). Strawberries, at 15% (w/w) were added in the last stage of the freezing process. As a control (WLAB), one batch of strawberry ice cream was manufactured without any LAB supplementation.

At the end of the production process, the ice creams were packaged separately in sanitized ice cream cases (0.5 L) with lids and hardened in a deep freezer at -40°C for 12 h, and then stored at -22°C.

Mixes and ice cream analyses

The microbiological quality of the ice creams was determined by incubating the cultures in media as follows; for yeasts and molds, acidified Potato Dextrose Agar (Oxoid, CM-139, UK) at 25°C for 5 days (FRANK *et al.*, 1992); for coliform bacteria, Violet Red Bile Agar (Oxoid, CM-107, UK) at 32±1°C for 24±2 h (CHRISTEN *et al.*, 1992); for *Staphylococcus aureus,* Baird-Parker Agar (Oxoid-CM-275, UK) at 35°C for 45-48 h (FLOWERS *et al.*, 1992). Injured bacteria cannot grow on media that are selective for them, even though they are metabolically active. However, if given adequate time and proper conditions they can repair their injuries (ARANY *et al.,* 1995). Because of this, Standard Plate Count Agar (Oxoid, CM-465, UK) was used for the enumeration of *Lactobacillus acidophilus* (at 37°C, 3 days), *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris* (at 35°C, 2 days).

To determine the effects of LAB on

strawberry ice cream characteristics, some quality indexes (overrun, viscosity, dry matter, melting time, titratable acidity, pH and sensory properties) were evaluated. Viscosity and overrun were evaluated as described by ARBUCKLE (1986). To determine the viscosity, the ice cream was heated in a waterbath to 20°C. The viscosity was then checked by observing the time it took for the meniscus to fall from 0 to 50 in a 50 mL burette with a 4.0-mm-diameter exit. The viscosity (η) values of the ice cream samples were calculated as follows:

$$\eta = \rho \times t$$

η is expressed as centipoises (cP).
ρ is the density of ice cream
t is the flow time of ice cream

For overrun; 250 mL of ice cream at 4°C were weighed and the density was calculated. The volume of the whole ice cream was calculated by dividing its weight by the previously calculated ice cream density value. The volume of mashed strawberry and mix were computed similarly. Overrun was calculated by the following equation:

$$\text{Overrun \%} = \frac{A-(B+C)}{B}$$

A = volume of ice cream;
B = volume of mix;
C = volume of strawberries.

The fat ratio of samples was determined by the Rose-Gottlieb method (ARBUCKLE, 1986) and expressed as fat content. The quantities of dry matter and protein were assigned according to the AOAC method (1995). To determine the melting characteristics of the ice creams, 20 g of sample were weighed and put into a Petri dish (10 cm diameter) and allowed to melt at 20°C until it covered the whole base of the Petri dish, and the characteristic was expressed as partial melting time (ÖZTÜRK, 1963). The titratable acidity and pH values (using a WTW 330 pH meter) were set as reported by BRADLEY *et al.* (1992). Sensory evaluations (taste and flavour, structure and consistency) were rated on a 5-point scale [best (5 points), good (4 points), slight defect (3 points), strong defect (2-1 points)] by eight experienced panel members according to Turkish Ice Cream Standard TS 4265 (ANONYMOUS, 1992). The ice cream analyses were carried out on days 1, 30, 60, 90 and 120 after manufacturing. The experimental treatments of production and analyses were replicated twice.

A completely randomized block design was used for all tests and the data were analysed by ANOVA using a SPSS statistical packet. Differences among means were compared by Duncan's Multiple Range Test. To normalize data, LAB counts were transformed to $\log_{10}$ before analysis.

RESULTS AND DISCUSSION

Microbiological properties

The determination of a viable population of microorganisms in functional foods is important in order to assure the consumer that a product corresponds to certain norms (CHAMPAGNE and GARDNER, 2005). Table 1 indicates that the number of all LAB used were within the established limit values (10^6 cfu/g). This indicated that both methods of LAB supplementation were effective.

The *L. acidophilus* sp. was more sensitive i.e. fewer bacteria survived the freezing process than the other bacterial species. Similarly, CALCOTT (1986) and JOHNSON *et al.* (1984) reported that the sensitivity of *L. acidophilus* to cryogenic treatments made its preservation difficult. The sensitivity of this bacterial species is due to their cylindrical shape. Because the surface area exceeds the vol-

ume (DUMONT *et al.*, 2004) more water comes out of the cell which leads to dehydration of the cytoplasm and an increase in solute density. Highly concentrated solutes can denature intracellular enzyme systems (THUNELL, 1996).

When the first day counts of LAB of strawberry ice cream were compared with the LAB counts on the 120th day, the value decreased at an approximate rate of 0.5 log cfu/g. This rate of decline however, was not uniform over the 120 days. It is thought that the alteration of LAB counts in different days was due to injured cells or inherent errors within the microbiological methods. Different results have been obtained by other researchers. LOPEZ *et al.* (1998) observed only a slight decline in LAB in three batches (pH = 4.32, 5.09, and 5.53) of commercial frozen yogurt stored at -23°C for 1 year. CHRISTIANSEN *et al.* (1996) reported that the number of viable bacteria decreased by 0.1-0.7 log units during storage at -20°C for 16 weeks. In contrast, DAVIDSON *et al.* (2000) reported that frozen storage of their product had little or no effect on culture survival, and bacterial counts remained at levels sufficient to offer the suggested therapeutic effects. These data corroborate with our results.

Coliform bacteria and *Staphylococcus aureus* were not detected in any of the samples. Yeast was determined at a level of 10^2cfu/g on the first day for LLF and on the 30th day for LCA. This was probably due to a re-contamination of the ice creams during the packing process (Table 1).

Physico-chemical properties

Air in ice cream provides a light texture and influences the physical properties of melt down and hardness (SOFJANA and HARTEL, 2004). Our study indicates that overrun increases as pH increases (Table 2). When overrun and pH values of ice creams produced with the same species but different inoculation methods were compared, there was a significant linear correlation ($p<0.01$) between the overrun and pH values. In both LAB addition methods, *Lactococcus cremoris* supplementation provided the highest overrun. In addition, the greatest overrun was obtained with fermentation of the entire mix when the pH was lowered to 5.6. When WLAB was compared with

Table 1 - LAB counts (log_{10} cfu/g) throughout storage of ice cream samples.

	Storage time (days)				
Samples	1	30	60	90	120
LCFd	7.756±1.15	7.176±1.52	7.568±1.30	7.114±1.48	7.176±1.35
LAWb	6.491±1.25	6.447±1.28	6.380±1.32	6.362±1.21	6.398±1.40
LLFd	7.556±1.21	7.380±1.19	7.491±1.25	7.544±1.40	7.477±1.23
LCAe	8.041±1.33	7.875±0.92	7.763±1.23	7.708±1.30	7.462±1.27
LAAc	7.146±1.20	7.114±1.22	7.079±1.32	7.079±1.37	7.000±1.28
LLAe	8.079±1.43	7.857±1.10	7.839±1.36	7.716±1.27	7.447±1.43
WLABa	-	-	-	-	-

Different lower cases indicate significant differences ($p<0.01$) among samples.
LCF: mix was inoculated with *Lactococcus lactis* subsp. *cremoris*, LAF: mix was inoculated with *Lactobacillus acidophilus*, LLF: mix was inoculated with *Lactococcus lactis* subsp. *lactis*, LCA: mils part of the mix was fermented with *Lactococcus lactis* subs. *cremoris*, LAA: milk part of the mix was fermented with *Lactobacillus acidophilus*, LLA: milk part of the mix was fermented with *Lactococcus lactis* subsp. *lactis*, WLAB: control.

the others, except LCF, *Lactococcus lactis* subsp. *lactis* and *Lactobacillus acidophilus* did not contribute to an increase in overrun. In this investigation, commercial starters were used and it was not determined whether these starters produce exopolysaccharides (EPS). However, it is well known that many lactic acid bacteria are capable of synthesizing EPS. When the stabilizer quantity is increased, the overrun decreases (STANLEY *et al.*, 1996). The decrease of overrun in ice cream made with *Lactobacillus acidophilus* may have been caused by their EPS synthesis property. This opinion is supported by the viscosity results. As seen in Table 2, the viscosity value of *Lactobacillus acidophilus* indicates that this bacterium had a stabiliser effect. According to the viscosity and overrun values, *Lactobacillus acidophilus* had a greater stabiliser effect when supplemented in the milk rather than in the mix. In other words, milk components are more suitable for *L. acidophilus*-mediated stabiliser effect than the mix. This means that fewer stabilisers could be used in ice cream manufacturing.

When ice cream is consumed it should not melt or lose its shape. The partial melting time test is performed to determine the stability of the ice cream at the time of its consumption, in a real sense. First, the partial melting time was significantly ($p<0.01$) and positively affected by the use of LAB and the different LAB addition methods. In control samples produced without LAB, the partial melting time was shorter, resulting in a loss of ice cream shape in the shortest time. Despite the fact that *Lactobacillus acidophilus* supplementation showed the longest partial melting time in both culture addition methods, the best result was seen with the fermentation of milk with the above-mentioned bacteria. This result could also be due to EPS synthesis. The lower pH used could also explain the melting time. Longer partial melting time could also be due to a greater stretching of milk proteins at this pH value which could provide a firmer and more stable structure.

As seen in Table 2, there are differences among the dry matter and fat content values. These dissimilarities appeared to be due to variations in batch process conditions such as evaporation of water phase especially at the pasteurisation step.

Table 2 - Physico-chemical properties of the ice cream samples.

Samples	Overrun	Viscosity (Cp)	Dry matter (%)	Fat (%)	% acidity	pH	PMT
LCF	42.3±7.1f	15.9±1.4a	28.59±0.23d	5.88±0.12c	0.34±0.03a	5.68±0.06d	59.6±9b
LAF	35.9±4.9c	21.3±2.1c	29.29±0.10f	5.90±0.07ef	0.33±0.03a	5.74±0.11d	83.6±11e
LLF	37.3±5.8d	16.3±1.5b	28.43±0.11c	5.89±0.02de	0.35±0.03a	5.66±0.06d	74.4±10c
LCA	32.2±4.0b	22.2±2.2d	28.39±0.07c	5.89±0.04cd	0.75±0.04b	4.57±0.22b	87.7±12f
LAA	31.4±4.4a	26.3±0.7e	27.91±0.07a	5.77±0.07a	0.71±0.01b	4.72±0.15c	93.1±1.5g
LLA	31.6±4.9a	15.7±1.6a	28.24±0.14b	5.90±0.9f	0.90±0.01c	4.33±0.17a	79.6±11d
WLAB	39.8±6.8e	16.4±1.1b	28.96±0.15e	5.85±0.1b	0.28±0.02a	5.89±0.03e	51.3±4a

Different lower cases indicate significant differences ($p<0.01$) among samples.
LCF: mix was inoculated with *Lactococcus lactis* subsp. *cremoris*, LAF: mix was inoculated with *Lactobacillus acidophilus*, LLF: mix was inoculated with *Lactococcus lactis* subsp. *lactis*, LCA: mils part of the mix was fermented with *Lactococcus lactis* subs. *cremoris*, LAA: milk part of the mix was fermented with *Lactobacillus acidophilus*, LLA: milk part of the mix was fermented with *Lactococcus lactis* subsp. *lactis*, WLAB: control, PMT: partial melting time (mm).

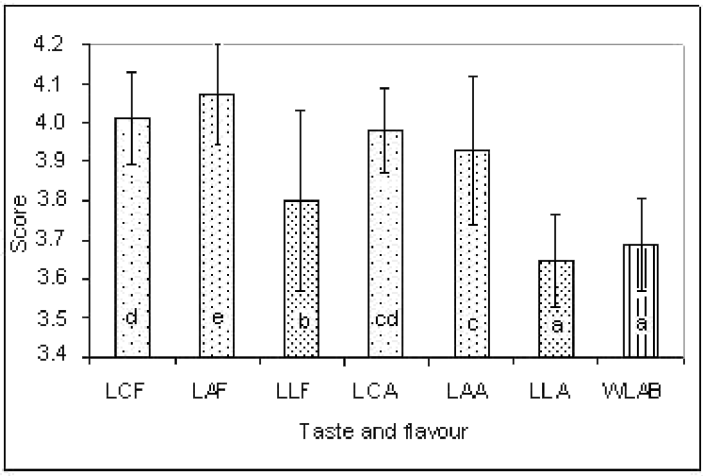

Fig. 2 - Taste and flavour scores (max 5 points).
Different lower case letters indicate significant differences ($p<0.01$) among samples. LCF: mix was inoculated with *Lactococcus lactis* subsp. *cremoris*, LAF: mix was inoculated with *Lactobacillus acidophilus*, LLF: mix was inoculated with *Lactococcus lactis* subsp. *lactis*, LCA: milk part of the mix was fermented with *Lactococcus lactis* subsp. *cremoris*, LAA: milk part of the mix was fermented with *Lactobacillus acidophilus*, LLA: milk part of the mix was fermented with *Lactococcus lactis* subsp. *lactis*, WLAB: control.

The highest score for structure and consistency was obtained in ice creams prepared by the addition of fermented milk at a pH value of approximately 4.5 (Fig. 3). The addition of LAB was positively correlated with these sensorial criteria and the best result was obtained with LCA supplementation.

CONCLUSIONS

Supplementation of strawberry ice cream with *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris* had some positive effects on the sensorial properties. With the fermentation of milk at 10% of mix weight

Sensorial properties

The results of the taste and flavour tests to evaluate the effects of LAB supplementation in strawberry ice cream are shown in Fig. 2. The incubation of the mix at pH 5.6 gave better results in terms of taste and flavour. With the exception of LLA, the use of LAB to make strawberry ice cream had a positive effect on the sensorial attributes. The best taste and flavour scores were obtained with LAF, at 0.328% acidity. SPECK (1983) stated that frozen yoghurts with the lowest titratable acidity (0.28 to 0.38%) received the highest overall quality scores.

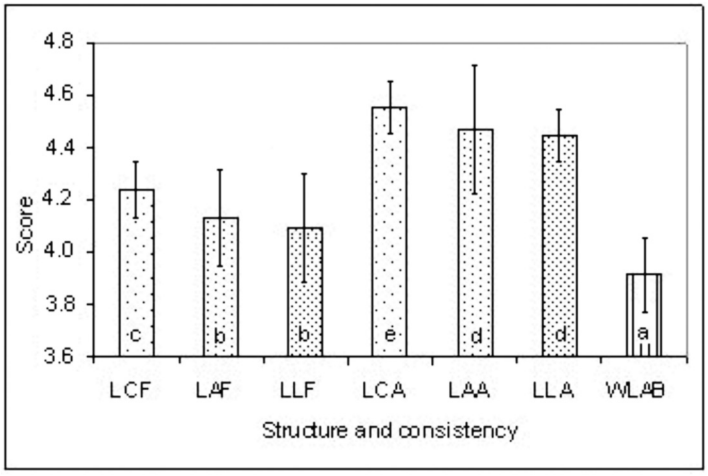

Fig. 3 - Structure and consistency scores.
Different lower case letters indicate significant differences ($p<0.01$) among samples. LCF: mix was inoculated with *Lactococcus lactis* subsp. *cremoris*, LAF: mix was inoculated with *Lactobacillus acidophilus*, LLF: mix was inoculated with *Lactococcus lactis* subsp. *lactis*, LCA: milk part of the mix was fermented with *Lactococcus lactis* subsp. *cremoris*, LAA: milk part of the mix was fermented with *Lactobacillus acidophilus*, LLA: milk part of the mix was fermented with *Lactococcus lactis* subsp. *lactis*, WLAB: control.

or fermentation of the entire mix at pH 5.6 an adequate number of viable organisms were retained. Since ice cream is usually consumed as a dessert, a sour taste is often unappealing. The taste and flavour properties obtained by fermentation of the ice cream mix at pH 5.6 were accepted better than those from samples where the pH values were below 5.

Our results showed that the sour taste resulting from probiotic LAB supplementation can be hidden by adding highly acidic fruit to ice cream. In addition, strawberry ice creams made with LAB had better sensorial properties than the control ice creams prepared without LAB supplementation. Fermenting the mix at pH 5.6 successfully masked the sour taste. Supplementing ice cream with LAB also had a positive effect on the quality criteria such as overrun, viscosity etc. The stabiliser effect, which may have been due to the production of EPS, may reduce the need for the addition of extraneous stabilisers. The low overrun values also had a positive effect on the structure and consistency.

Lactic acid bacteria, used in ice cream manufacturing, should have a low acid-producing capacity and attain a minimum viable cell count of 10^8 cfu/g before freezing. The different quality results obtained by using LAB indicate that further studies are needed to determine which lactic acid bacteria would be must suitable for fruity ice creams.

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PAPER

"VINO COTTO" COMPOSITION AND ANTIOXIDANT ACTIVITY AS AFFECTED BY NON ENZYMATIC BROWNING

INFLUENZA DELL'IMBRUNIMENTO NON ENZIMATICO SULLA COMPOSIZIONE E L'ATTIVITÀ ANTIOSSIDANTE DEL "VINO COTTO"

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ABSTRACT

"Vino cotto", a typical product mainly produced in central Italy, is obtained by fermenting cooked must. Nine "vino cotto" samples from the Abruzzo region were analysed for their chemical composition. Alcohol content ranged from 8 to 16%, reducing sugars content from 83 to 350 g L^{-1} and total acidity from 9 to 19 g L^{-1} of tartaric acid. On the basis of compositional data and literature data, "vino cotto" can be classified as either a dry or sweet dessert wine depending on the chemical composition.

RIASSUNTO

Il "vino cotto" è un prodotto tipico dell'Italia centrale ottenuto dalla fermentazione dei mosti cotti. La composizione chimica di nove campioni di "vino cotto" prodotti in Abruzzo è stata analizzata: il contenuto in alcool è risultato variabile tra 8 e 16%, il contenuto in zuccheri riducenti tra 85 e 350 g L^{-1} e l'acidità totale da 9 a 19 g L^{-1} di acido tartarico. Sulla base dei risultati analitici ottenuti in questo studio e di alcuni dati di letteratura, il "vino cotto" può essere classificato come vino da dessert

- Key words: composition, cooked must, melanoidins, non-enzymatic browning, radical scavenging activity, "vino cotto" -

The must cooking process caused the activation of non-enzymatic browning reactions and the formation of Maillard reaction products (MRPs). The radical scavenging activity (RSA) of "vino cotto" was tested by the DPPH· and $ABTS^{+\cdot}$ decolouration assays. The RSA is highly correlated with both the polyphenol and MRP content; these two classes of compounds showed an additive combined effect on RSA. Phenolic (PE) and non-phenolic (NPE) extracts were obtained from "vino cotto" and the MRPs were mainly eluted with the NPE. The sum of the antioxidant activities of the two extracts were equal to the total RSA of "vino cotto" samples. In highly browned "vino cotto" samples the antioxidant activity of NPE was higher than that of PE and accounted for 56% of the overall antioxidant activity.

dolce o secco a seconda della sua composizione chimica. Il processo di cottura del mosto durante la produzione di "vino cotto" ha determinato l'attivazione della reazione di imbrunimento non-enzimatico e la formazione di composti della reazione di Maillard (MRPs). L'attività antiradicalica del "vino cotto" valutata mediante i saggi di decolorazione dei radicali DPPH· e $ABTS^{+\cdot}$ è risultata altamente correlata con la concentrazione sia di polifenoli che di MRPs, e queste due classi di antiossidanti hanno mostrato un effetto combinato additivo sulla capacità antiradicalica del "vino cotto". Estratti fenolici (PE) e non fenolici (NPE) sono stati ottenuti dal "vino cotto" e i prodotti della reazione di Maillard sono risultati eluiti con la frazione non fenolica. La somma delle attività antiradicaliche degli estratti fenolici e non fenolici è risultata uguale alla capacità antiradicalica totale e nei campioni maggiormente imbruniti l'attività antiossidante della frazione non fenolica risulta maggiore dell'attività antiossidante della frazione fenolica, rappresentando fino al 56% dell'attività antiossidante totale.

INTRODUCTION

"Vino cotto" (namely cooked wine) is a dessert wine produced from the fermentation of cooked must, with or without the addition of fresh must. This is a practice in a specific area of the eastern regions of central Italy, mostly in the Marches and Abruzzo (FORLANI, 1904; DE ROSA, 1987).

"Vino cotto" is of historical importance in Italy (FORLANI, 1904, DE ROSA, 1987) but very few data are available in the literature about its composition (DE PISIS, 1898) because, until 2000, it was not possible to market a product obtained from fermented cooked musts (except Marsala) according to Italian law. Even if it was not marketed, "vino cotto" retained its traditional importance in the regions of production, being produced for self-consumption (DE ROSA, 1987). "Vino cotto" was inserted in the "national list of traditional food products" for Marches and Abruzzo regions in 2000 and 2003, respectively, and can be marketed as a traditional product (REPUBBLICA ITALIANA, 2000; 2003).

"Vino cotto" is traditionally produced from white grapes, mainly of Trebbiano, Passerina, Montonico and Moscato cultivars, even if red grapes such as Montepulciano and Sangiovese can be used (FORLANI, 1904; DE ROSA, 1987). The must is heated directly in copper boilers until it is concentrated to 40-90% and it becomes

darker and denser. Once cooled, the must is decanted and fermented in chestnut barrels of 3-5 hL (FORLANI, 1904; DE ROSA, 1987). The must fermentation is very slow due to the high sugar content reached upon concentration; thus actively fermenting must (mosto fiore) is sometimes added to the cooked must in order to fasten the rate of fermentation (FORLANI, 1904; DE ROSA, 1987). The product remains in the barrel for a long time and is periodically filled up with fresh cooked must (DE ROSA, 1987). "Vino cotto" can also be obtained by adding 10-15% sapa (must concentrated to 30% of its initial volume) to actively fermenting must; this wine requires at least three years of ageing and is characterized by a sweet taste (FORLANI, 1904).

The wine-making practice of adding cooked must to actively fermenting and fermented must is also used for other sweet wines: the Spanish "colour wine" and Marsala, respectively (DE ROSA, 1987; ARENA, 1981; RIVERO-PEREZ *et al.*, 2002).

When the must is heated, non-enzymatic browning reactions (NEB) such as the Maillard reaction and caramelisation occur (RIVERO-PEREZ *et al.*, 2002). The former shows an Arrhenius-type dependence on temperature and is limited at low pH (LABUZA *et al.*, 1994; MARTINS *et al.*, 2001). The latter requires temperatures higher than 120°C and a pH 3-9 (KROH, 1994), so it is expected to play a minor role during must heating at atmospheric pressure.

The Maillard reaction could take part in the formation of coloured brown compounds such as melanoidins (RIVERO-PEREZ *et al.*, 2002) and of volatile aroma compounds that contribute to the aroma of sweet wines during ageing (CUTZACH *et al.*, 1998a; b).

The generation of colour and flavour by heating also have a secondary use in foods: one is the enhancement of antioxidant activity which is associated with the formation of brown coloured melanoidins (ELIZALDE *et al.*, 1992; NICOLI *et al.*, 1999; MORALES and JIMENEZ-PEREZ, 2001) that positively affect the antioxidant activity of sweet wines (RIVERO-PEREZ *et al.*, 2002; MANZOCCO *et al.*, 1999; MORALES and JIMENEZ-PEREZ, 2004).

In previous studies (MANZOCCO *et al.*, 1999, MASTROCOLA *et al.*, 2001), a "vino cotto" sample was shown to have a higher antioxidant activity than the white wine produced from the same grapes. The purpose of this study was to investigate the composition and the antioxidant activity of nine "vino cotto" samples as affected by product browning.

MATERIALS AND METHODS

Samples

Nine samples of "vino cotto" from the province of Teramo (Italy) were selected from the private collection of Prof. Leonardo Seghetti (Ascoli Piceno, Italy). A Trebbiano white wine, vintage 2003, from the Abruzzo region was obtained from Trebbiano grapes which are the most common raw material for "vino cotto". Three replicates were used for each sample. A model solution of MRPs was obtained by heating a solution of L-glutamine (280 mg L^{-1}), glucose (90 g L^{-1}) and fructose (90 g L^{-1}) for 20 h at 100°C. Chemicals were from Sigma (Steinheim, Germany).

Solid phase extraction of the phenolic and non-phenolic fraction

Commercially available octadecyl C_{18} cartridges (1 g, 6 mL) (International Sorbent Technology, Tucson, AZ) were used for extraction of the phenolic fraction according to the following protocol: 1 mL of sample was loaded onto a column previously conditioned with 2 mL of methanol and 5 mL of water (DI STEFANO and GUIDONI, 1989). The column was eluted with 2x4 mL of 0.1 N sulphuric acid to eliminate all the water-soluble compounds and the

solution obtained accounted for the non-phenolic extract (NPE). The compounds retained by the column were recovered by eluting with 2x4 mL of 60% methanol solution and the solution collected accounted for the phenolic extract (PE). Trapping and release of the phenolic fraction from the C_{18} solid phase was performed according to DI STEFANO and GUIDONI (1989). Both fractions (NPE and PE) were made up to a final volume of 10 mL and the pH of the NPE fraction was adjusted to that of the original sample.

Analytical determinations

Chemical analyses were conducted using the official EU Community methods for the analysis of wines (EEC, 1990).

The total polyphenol content was determined by the colourimetric reaction with the Folin Ciocalteau reagent. Before analysis, the phenolic compounds were extracted by solid phase extraction as previously reported because substances such as reducing sugars, alcohol and tartaric acid, as well as Maillard reaction products, could interfere in the determination of the polyphenols with the Folin Ciocalteau reagent (DI STEFANO and GUIDONI, 1989).

Hydroxymethylfurfural (HMF) content was determined by the official EU Community methods for the analysis of wines (EEC, 1990). 5 - (Hydroxymethyl) - furfural (Sigma, Steinheim, Germany) was used for the calibration curves.

The melanoidin content was determined by spectrophotometric analysis reading the absorption value at a wavelength of 280 nm (RIVERO-PEREZ *et al.*, 2002). Before melanoidin analysis, the "vino cotto" samples were dialysed using cellulose dialysis tubing (Sigma) that retains most molecules ≥12.000 Da. Samples (15 mL) were put into dialysis tubing which were then placed in a glass vessel with 1 L of water. This solution was maintained at 5°C under stirring for 12 h. This procedure was repeated. The volume of sample which remained in the dialysis tubing was diluted to a known volume with double distilled water.

Colour determinations

Reflectance analyses were carried out on a Minolta (Osaka, Japan) CM-500 spectrophotometer using the CIE illuminant D65 and 10° standard observer conditions. Before analysis, the instrument was calibrated on a white standard (L*: 96.58; a*: -0.09; b*: -0.05 on SCE modality). Samples were poured into white 1 cm tall conical beakers (L*: 68.08; a*: -0.54; b*: -0.52 on SCE modality) that were then covered with a transparent glass in order to set the optical path constant. Transparent glass did not significantly influence colour determinations (data not reported). Colour measurements were carried out in SCE (Specular Component Excluded) modality because the glass causes a high specular reflection.

Antioxidant activity determination

The radical scavenging activity was determined using two radicals, DPPH· and $ABTS^{+\cdot}$, and defined: $RSA_{DPPH\cdot}$ and $RSA_{ABTS+\cdot}$.

$RSA_{DPPH\cdot}$ was measured by the methodology described by BRAND-WILLIAMS *et al.* (1995). The bleaching rate of a stable free radical, 2,2-diphenyl-1-picrilhydrazyl (DPPH·) in the presence of the sample was monitored at 515 nm using a Lambda Bio 20 spectrophotometer (Perkin Elmer, Boston, MA). A volume of 1.9 mL of 6.1 10^{-5} M DPPH· methanol solution was used. The reaction was started by adding 5-20 m μL of "vino cotto" samples. DPPH· bleaching was monitored at 25°C for at least 60 min and the decolouration after 5 min was used as the measure of antioxidant activity. In this dilution range, the DPPH· -bleaching was proportional to the concentration of sample added to the medium and the regression coefficient of the dose-response curve was

taken as a measurement of the antioxidant activity. Antioxidant activity was measured as mmoles DPPH· inhibited per mL of sample.

$RSA_{ABTS+\cdot}$ was measured by the methodology described by RE *et al.* (1999). The bleaching rate of $ABTS^{+}\cdot$ in the presence of the sample was monitored at 734 nm using a Perkin Elmer Lambda Bio 20 spectrophotometer. A volume of 2.97 mL of $ABTS^{+}\cdot$ solution (Abs = 0.7±0.02) was used. The reaction was started by adding 30 m µL of either "vino cotto" or extract samples diluted with double distilled water up to 1:100 and 1:10 respectively. $ABTS^{+}\cdot$-bleaching was monitored at 25°C for at least 60 min and the decolouration after 5 min was used as the measure of antioxidant activity. In this dilution range, the $ABTS^{+}\cdot$ bleaching was proportional to the concentration of the sample added to the medium and the dose-response curve was fitted by a linear model. Antioxidant activity was measured as µmoles of Trolox equivalents per mL of sample and was calculated by the ratio of the regression coefficient of the dose response curve of the sample and the regression coefficient of the dose response curve of Trolox (the hydrophilic homologue of tocopherol).

Statistical analysis

Principal components analysis (PCA) was applied to describe the data set and to detect the most important variables for determining the data structure. Final data processing by PCA was performed by subtracting non-significant variables (loading < 0.70) and redundant variables. Stepwise multiple regression analysis was applied to data and the response equations were calculated using a definitive model which considers only the influence of factors significant at a $p<0.01$ level. The goodness of fit of the models was checked by: the adjusted determination coefficient R^2_{adj}, and the *p* value associated with the Fisher test (PIKE, 1986). Data were analyzed using the STATISTICA for Windows (StatSoft™, Tulsa, OK) package.

RESULTS AND DISCUSSION

The composition of the nine "vino cotto" samples varied greatly (Table 1). The alcohol content ranged from 8 to 16%, reducing sugar content from 83 to 350 g L^{-1} and total acidity from 9 to 19 g L^{-1}. DE ROSA (1987) reported reducing sugar values from 130 to 170 g L^{-1}, alcohol content ranging from 13 to 17% and a total acidity of about 12 g L^{-1}. However, DE PISIS (1898) reported acidity values up to 24 g L^{-1}, reducing sugar values up to 450 g L^{-1} and alcohol contents from 8 to 13.5%; these data were more similar to those found in this study. The sulphur dioxide values of the "vino cotto" samples in this study were very low (from 6.4 to 15.6 mg L^{-1}) and all the samples except S3 were in the 10-15 mg L^{-1} range in agreement with DE ROSA (1987). Samples S3 and S8 had copper content values that were higher than the 1 mg L^{-1} limit fixed for wines by Italian law (REPUBBLICA ITALIANA, 1987). This could be due to the use of copper boilers. All the "vino cotto" samples had very high concentrations of iron that were due to the metal concentration during cooking and to the common practice of adding a piece of iron to the cooking must in order avoid the copper taste in the wine (DE ROSA, 1987). The data obtained in this study and in previous studies confirm the high variability in "vino cotto" composition.

To find the simplest model to describe the data set, the compositional data were processed by principal component analysis. The results showed an explained variance of 74.03% (58.07 and 15.95% along the first and second principal components, respectively Fig. 1). Based on the distribution of the samples, two groups could be identified, the first characterised by high sugar content, dry extract and acidity and the second characterised

Table 1 - Chemical composition of nine "vino cotto" samples and a white Trebbiano wine (TRB).

	TRB	S1	S2	S3	S4	S5	S6	S7	S8	S9
alcohol (% vol.)	11.56	8.47	12.61	12.48	12.30	13.80	16.04	10.26	7.87	11.79
reducing sugars (g L^{-1})	1.280	257.5	171.6	139.2	83.06	93.63	114.4	156.1	343.5	350.1
total dry extract (g L^{-1})	22.4	260	190	170	110	130	180	210	410	370
dry extract (g L^{-1})	21.1	2.50	18.4	30.8	26.9	36.4	65.6	53.9	66.5	19.9
pH	3.14	3.00	3.21	3.68	3.31	3.18	3.21	3.68	3.05	3.16
total acidity (g L^{-1})*	5.10	11.0	10.4	9.60	8.60	15.8	17.6	12.2	16.8	19.2
volatile acidity (g L^{-1})**	0.30	1.75	1.39	1.30	1.15	1.80	1.92	1.85	2.21	1.75
malic acid (g L^{-1})	0.28	3.50	2.75	2.12	2.10	3.04	2.84	3.28	2.16	2.7
tartaric acid (g L^{-1})	2.96	4.20	3.45	3.65	3.30	3.60	3.65	3.52	3.48	3.50
lactic acid (g L^{-1})	0.64	1.74	2.10	2.40	1.56	2.14	1.92	1.95	1.84	2.10
total sulphur dioxide (mg L^{-1})	76.8	12.8	9.60	6.40	9.60	12.8	9.60	15.6	12.8	9.60
ash content (g L^{-1})	1.24	2.94	2.74	2.36	2.56	2.82	2.64	2.56	3.12	3.26
alkalinity of ash (meq L^{-1})	14.8	32.0	29.6	27.2	28.4	28.6	29.2	28.4	32.4	34.0
iron (mg L^{-1})	1.80	16.5	9.42	20.2	9.70	5.65	8.40	26.4	19.2	22.1
copper (mg L^{-1})	0.15	1.72	1.04	2.14	0.96	0.86	0.72	1.18	2.30	0.84
TPP (mg L^{-1} GAE)	277	852	407	482	693	556	860	807	793	1214

Coefficient of variation < 5% for all the analyses.
*Expressed as tartaric acid; **expressed as acetic acid.

by low sugar and high alcohol content. Samples with characteristics similar to those of the first and second group were described as sweet and dry dessert wines, respectively, by other authors (FORLANI, 1904; DE ROSA, 1987).

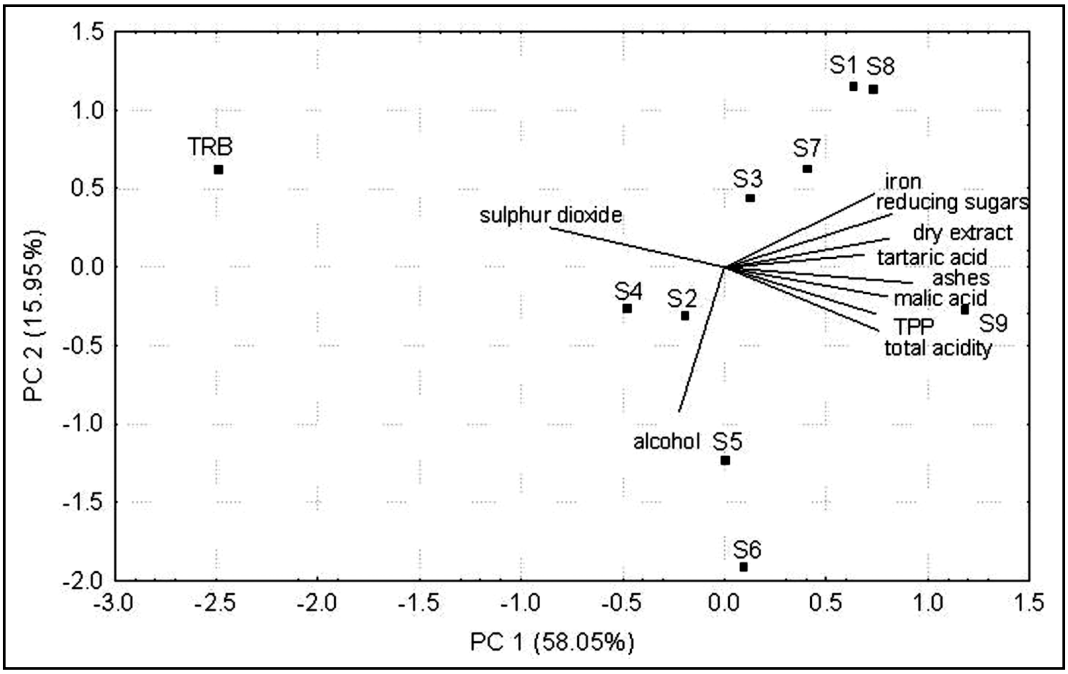

Fig. 1 - Loading plot and scores from compositional analysis of nine "vino cotto" samples (S1-S9) and a white Trebbiano wine sample.

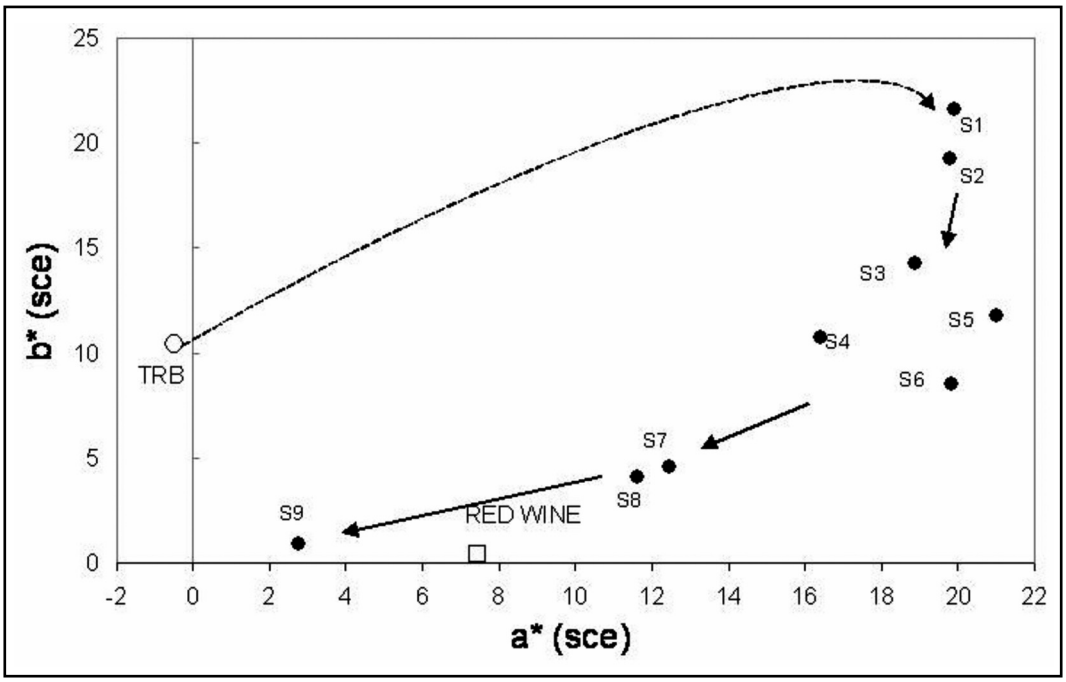

Fig. 2 - Colour of nine "vino cotto" samples, a commercial Trebbiano (white) and a Montepulciano (red) wine on the a*b* chromaticity plane.

In order to investigate the colourimetric characteristics of the nine "vino cotto" samples, colour was analyzed by reflectance spectrophotometry because most samples did not allow light transmittance in the 300 to 450 nm range and sample dilution could influence the determination of chromatic characteristics.

From S1 to S9 it was possible to observe a reduction in both lightness (L*) and hue angle (h°) which describe a darkening effect and a shift of colour toward the red zone (h° = 0°); this colour change can be described as browning (MASTROCOLA and LERICI, 1991). The cooking process of musts, in fact, activates non-enzymatic browning (NEB) reactions (RIVERO-PEREZ *et al.*, 2002; MANZOCCO *et al.*, 1999; MORALES and JIMENEZ-PEREZ, 2004) that could affect the colour (BARBANTI *et al.*, 1990; MASTROCOLA and LERICI, 1991).

The h° values show a positive linear correlation with lightness (L*) values (r = 0.936, $p<0.01$), thus both these parameters could be used as browning indices.

In order to better describe the "vino cotto" colour, the chromaticity values of the different samples were reported on the a*b* chromaticity plane (Fig. 2) together with those of a white Trebbiano wine and a red Montepulciano d'Abruzzo wine.

The white Trebbiano wine had a bright yellow color. S1 sample had a darker colour than white Trebbiano wine with a typical orange hue (h° = 45°) due to the thermal treatment of the musts. From S1 to S9, the hue shifted towards red (h° = 0°) until the chroma values were similar to that of a red wine but the redder samples had a darker colour than that of red wine (Table 2). The colour shift on the a*b* chromaticity plane described the classical loop observed during a Maillard reaction development (MORALES and JIMENEZ-PEREZ, 2001; MASTROCOLA and LERICI, 1991; MAC DOUGALL and GRANOV, 1998).

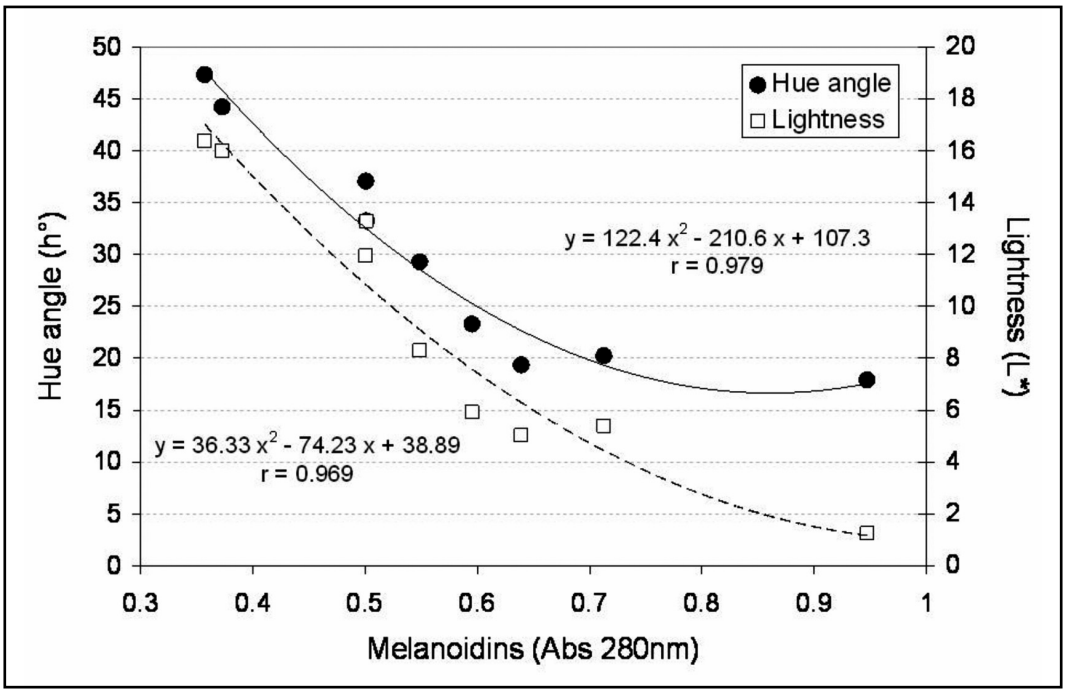

Fig. 3 - Regression between colour parameters L* and h° and melanoidins.

The occurrence of the Maillard reaction was also confirmed by absorbance at 420 nm and chemical indices (Table 2); the HMF and melanoidins increased from S1 to S9.

The colour parameters L* and h° were correlated with the concentration of MRPs and showed a linear correlation with HMF (r = -0.576 and -0.641, $p<0.05$, respectively) and a quadratic correlation with melanoidins (Fig. 3); in both cases, L* values decreased as the MRP content increased.

Colour parameters were better corre-

Table 2 - Colour and MRP concentration of "vino cotto" samples and commercial white Trebbiano (TRB) and red Montepulciano (MTP) wines.

Sample	L*	h°	Abs 420 nm	HMF (mg L^{-1})	Melanoidins (Abs 280 nm)
TRB	38.96±0.39	92.68±1.29	0.013	-	-
S1	16.37±0.78	47.34±0.82	0.450	657.0±18.2	0.357±0.010
S2	15.99±1.17	44.21±0.61	0.473	767.0±27.9	0.373±0.050
S3	11.96±0.49	37.06±0.67	0.469	480.1±23.1	0.501±0.022
S4	13.30±1.53	33.25±1.33	0.792	901.0±2.4	0.501±0.018
S5	8.31±0.80	29.30±0.18	0.952	763.6±13.4	0.549±0.050
S6	5.93±0.89	23.29±0.76	0.880	1044±16	0.595±0.028
S7	5.40±0.09	20.25±0.28	0.677	541.9±0.1	0.713±0.052
S8	5.03±1.71	19.36±0.38	1.180	1154±58	0.639±0.040
S9	1.25±0.08	17.93±0.07	1.744	1781±23	0.947±0.064
MTP	6.55±0.89	3.10±0.77	-	-	-

lated with melanoidins than HMF. The former compounds are formed in the final stages of the Maillard reaction, when product browning becomes visible, while the latter is mainly formed in the advanced stage of Maillard reaction (MARTINS *et al.*, 2001) and, once formed, could either decrease or increase depending on the relative rate of condensation and degradation reactions.

Most of the browned products had generally high values of dry extract, total acidity and TPP (Table 1) because the naturally occurring compounds are concentrated during the cooking process along with the development of the Maillard reaction (FORLANI, 1904; DE PISIS, 1898).

The radical scavenging activity of the "vino cotto" samples was evaluated by two different methods: the ABTS and the DPPH assay (Table 3). The former allows the antioxidant activity to be measured in aqueous solutions, while the latter is measured in alcoholic or amphiphilic solutions. These two methods were used because melanoidins have been reported to be effective antioxidants in alcoholic solutions and lipids (LABUZA *et al.*, 1994; ELIZALDE *et al.*, 1992; MANZOCCO *et al.*, 2001; DALLA ROSA, 1996) and weak antioxidants in watery fluids (MORALES and BABBEL, 2002).

The antioxidant activity of the "vino cotto" samples was up to 9-times higher than that of the white Trebbiano wine (Table 3) and the most browned and concentrated products showed antioxidant activities that were similar to those of red wines as reported by FERNÁNDEZ-PACHÓN *et al.* (2004). However, even if the total RSA is affected by the concentration due to cooking, the RSA of "vino cotto" expressed on dry matter was always higher than that of the Trebbiano wine.

The antioxidant activity increased from S1 to S9, as the product browning increased. The relationship between antioxidant activity and browning phenomena in plant foods has been largely reviewed and attributed to either the oxidation of natural antioxidants or to the formation of compounds with antioxidant activity due to the occurrence of NEB (MANZOCCO *et al.*, 2001).

Table 3 - Antioxidant activity of nine "vino cotto" samples and a white Trebbiano wine (TRB).

Sample	$RSA_{ABTS\cdot+}$ (μmoles Trolox eq. mL^{-1})	$RSA_{DPPH\cdot}$ (mmoles $DPPH^{\cdot}mL^{-1}$)
TRB	0.914±0.107	1.184±0.059
S1	1.445±0.017	3.178±0.105
S2	1.364±0.007	2.258±0.090
S3	2.160±0.05	2.054±0.109
S4	1.880±0.077	3.003±0.153
S5	2.178±0.241	3.587±0.251
S6	4.361±0.263	4.527±0.272
S7	3.343±0.177	5.105±0.276
S8	3.546±0.155	5.318±0.271
S9	8.216±0.235	10.67±0.267

In the case of "vino cotto", the increase of antioxidant activity during thermal processing could be due to changes in the naturally-occurring antioxidant compounds, such as polyphenols, as well as to the formation of compounds with antioxidant activity (DALLA ROSA, 1996; NICOLI *et al.*, 1999) such as melanoidins, which have been proven to be effective antioxidants (ELIZALDE *et al.*, 1992; NICOLI *et al.*, 1999; MANZOCCO *et al.*, 2001).

Antioxidant activity values obtained by the DPPH and ABTS assays showed a positive quadratic correlation with melanoidins and TPP and as the content of these antioxidant compounds increased, the antioxidant activity increased.

Simple regression analysis of RSA as a function of the two classes of antioxidants (TPP and melanoidins) did not take into account the correlation between the two independent variables. This correlation is due to the fact that samples with higher melanoidin content also showed a higher TPP content (Tables 1 and 2) because the concentration of naturally-occurring compounds and the formation of MRPs were both determined by the cooking process.

Multiple regression analysis was thus carried out to evaluate the individual

and interactive effect of the melanoidins and TPP on the total antioxidant activity of "vino cotto". The total RSA was positively correlated with the sum of the individual effects of the TPP and melanoidin content (Table 4) and no interactive effect was observed.

Both melanoidins and polyphenols showed a lower regression coefficient in aqueous solution than in methanolic solution (Table 4) but, on the basis of the statistical data, it is not possible to assert that melanoidins are weak antioxidants in aqueous solutions.

The statistical analysis showed that both TPP and MRPs contributed to the antioxidant activity of "vino cotto" but the relative contribution of these two classes of compounds to the overall antioxidant activity could not be quantified. In order to investigate this aspect, a tentative separation of the phenolic (PE) and non-phenolic (NPE) fraction of "vino cotto" samples was carried out. Only the $RSA_{ABTS+\cdot}$ was tested on each fraction because it was deemed important to test the RSA in aqueous solution at biological pH.

The two fractions were separated by SPF, which has been reported to be a suitable technique of separation for phenolic compounds (ANTOLOVICH *et al.*, 2000; NACKZK and SHAIDI, 2004). The NPE, which contained MRPs, was recovered after washing with an acidic aqueous solution. MRP elution from C_{18} with acidified aqueous solutions has been reported in other studies (RESMINI *et al.*, 1990; DELGADO *et al.*, 1992; RUFIÁN-HENARES

Table 4 - Multiple regression analysis of antioxidant activity as a function of TPP and melanoidin concentration.

Antioxidant activity	Equation
$RSA_{DPPH\cdot}$	-4.0265 + 0.0047 [TPP] + 8.624 [Melanoidins] R^2_{adj} = 0.910; $F_{(2.6)}$ = 41.34; p<0.001; S.E. = 0.787
$RSA_{ABTS+\cdot}$	-3.635 + 0.0028 [TPP] + 8.212 [Melanoidins] R^2_{adj} = 0.874; $F_{(2.6)}$ = 28.90; p<0.001; S.E. = 0.761

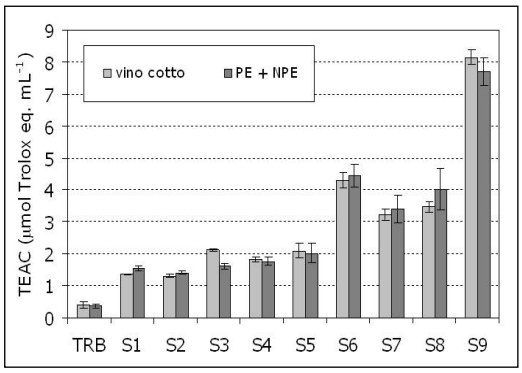

Fig. 4 - Antioxidant activity of "vino cotto samples" and sum of the antioxidant activities of their phenolic (PE) and non-phenolic (NPE) extracts.

et al., 2004). RSA recovery after washing with sulphuric acid was tested on a MRP model system and the recovery was 72%. The model system was obtained by heating an aqueous solution with a total sugar and amino acid content similar to that of a Trebbiano must. Glutamine was chosen because it is the most abundant amino acid in Trebbiano (CHINNICI *et al.*, 1999) and it has a medium tendency to browning (SHINODA *et al.*, 2005).

The sum of the TEAC values of the PE and NPE was not significantly different from the overall antioxidant capacity $TEAC_{TOT}$ in all samples except S1 and S3 (Fig. 4).

The recovery of antioxidant compounds from the solid phase of the C_{18} column was almost complete even if SPE did not allow a perfect separation of the MRPs and polyphenols. MRPs that were not eluted in the aqueous solution were further eluted by the 60% methanol solution since some MRPs are methanol-soluble (BORRELLI *et al.*, 2003), and this was also confirmed by the SPE of the MRP model solution. Thus the theoretical PE also contained small quantities of Maillard products which eluted together with phenolics and caused an overestimation of the RSA of PE and an underestimation of that of NPE. In contrast, the SPE could not separate the phenolic compounds that were incorporated in

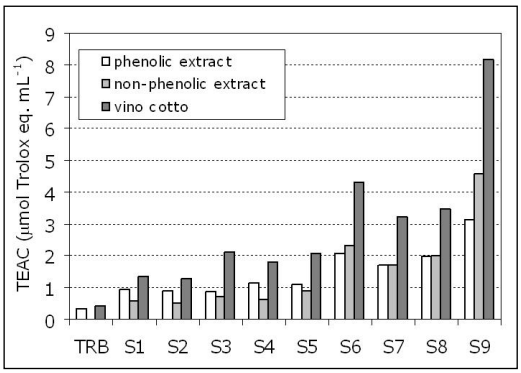

Fig. 5 - Antioxidant activity of "vino cotto" samples and their phenolic (PE) and non-phenolic extracts (NPE).

the melanoidin structures and cause the opposite effect.

The overall antioxidant activity of "vino cotto" samples and those of the PE and NPE are reported in Fig. 5.

The antioxidant activity of NPE showed a positive and significant correlation with the browning and Maillard reaction indices and the antioxidant activity of the phenolic fraction showed a positive and significant correlation with TPP (Table 5).

The contribution of the antioxidant activity of NPE to the total antioxidant activity was significantly correlated to product browning as described by L* ($r = -0.857$; $p < 0.01$). Thus, although it has been reported that melanoidins show a low antioxidant activity in aqueous solutions (MORALES and BABBEL, 2002), in most of the browned "vino cotto" samples the contribution of $TEAC_{NPE}$ to $TEAC_{TOT}$ was higher than that of the $TEAC_{PE}$.

This result is important because the bioavailability of polyphenols in human metabolism has been proven (MANACH *et al.*, 2005; WILLIAMSON & MANACH, 2005) and their bioefficacy is generally accepted. The metabolism of food-borne advanced MRPs has not been completely elucidated and it is still an open question whether isolated melanoidin structures undergo metabolic biotransformation and subsequently cause physiological effects *in vivo*. Moreover MRPs are known to act either as carcinogenic or as anticarcinogenic compounds (MANZOCCO *et al.*, 2001; LEE and SHIBAMOTO, 2002), so a more in-depth understanding of the toxicology of these functional compounds is needed.

Table 5 - Correlation between the antioxidant activity of the phenolic RSA_{PE} and the non-phenolic extracts RSA_{NPE}, the total polyphenol content and the Maillard reaction indices.

	RSA_{PE}	RSA_{NPE}
TPP	0.864	n.d.
HMF	n.d.	0.868
Melanoidins	n.d.	0.892
L*	n.d.	-0.861
Abs 420 nm	n.d.	0.901

n.d. = not determined.

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PAPER

PASTEURISATION OF GRAPE MUST AND TOMATO PASTE BY DENSE-PHASE CO_2

PASTORIZZAZIONE DI MOSTO D'UVA E PASSATA DI POMODORO CON CO_2 IN PRESSIONE

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ABSTRACT

Microorganism inactivation by high pressure CO_2 treatment was determined in fresh grape must and tomato paste under both natural and inoculated conditions. The microorganisms present in these two substrates were isolated and identified. A number of samples were then subjected to dense phase CO_2, and the reduction of microorganism viability was measured. Results are reported and discussed for natural grape must processed in a multi-batch contactor under conditions be-

RIASSUNTO

Sono state determinate le cinetiche di inattivazione microbica attraverso CO_2 in pressione in mosto d'uva e passata di pomodoro naturali, sia tal quali che inoculati. Dapprima, sono stati isolati ed identificati i microrganismi presenti in questi due substrati. Successivamente, sono stati trattati numerosi campioni con CO_2 in pressione, misurando poi la sopravvivenza dei microrganismi. Vengono presentati e discussi i risultati ottenuti in mosto d'uva allo stato naturale trattato tra 80 e 120

- Keywords: dense-phase CO_2, food pasteurisation, grape must, microbial inactivation, tomato paste -

tween 80-120 bar, 30°-40°C, for 5 to 50 min, and for natural tomato paste between 75-110 bar, 32°-50°C, up to 24 h. Different combinations of temperature and pressure were also applied to fresh grape must inoculated with wild strains of *Pichia awry* and *Bacillus subtilis*. The dense phase CO_2 treatment sterilized the grape must but only reduced the microbial load of tomato paste by less than 1 log.

bar, 30°-40°C, per un tempo di trattamento da 5 a 50 minuti, e in passata di pomodoro allo stato naturale tra 75 e 110 bar, 32°-50°C, fino a 24 ore. Vengono applicate differenti combinazioni di temperatura e pressione in mosto d'uva naturale inoculato con ceppi selvaggi di *Pichia awry* e *Bacillus subtilis*. Nel loro insieme i risultati ottenuti dimostrano che il trattamento con CO_2 in pressione è in grado di sterilizzare il mosto d'uva e di ridurre la carica microbica della passata di pomodoro.

INTRODUCTION

In a safe food, the spoilage and pathogenic microorganisms are eliminated by pasteurisation, and the growth of possible residual microbial load is suppressed in order to obtain a product with a long, stable shelf-life. If harmful spores are present in the foodstuff, a more severe treatment, sterilization, is normally required to eliminate them. This treatment is also required to reduce enzymatic activity, which is a further aim in the industrial preservation of food.

Thermal treatment is the most widely used technology for food preservation. In some cases, it induces positive effects, such as the elimination of some anti-nutritional components, but in most cases, it causes an undesirable deterioration of food quality. This is particularly negative since the freshness of the food is of utmost importance. Loss of vitamins, denaturation of proteins, loss of fresh taste, colour, aroma and texture are the most frequent drawbacks of thermal treatment because of the high temperature applied to the natural substrate. Thus, efforts are currently being made to optimize the heat exchange mechanism to overcome the inevitable physical limits of thermal treatment (LEE and COATES, 1999; ZANONI *et al.*, 2003). At the same time, there is increasing consumer demand for minimally processed, fresh-tasting foods. The food industry is therefore looking for advances in technology that cause the least deterioration in product quality. Investigations are intensifying in alternative, non-thermal technologies, including the Pulsed Electric Field process (PEF), the application of ionizing radiation, UV light, ultrasound, the use of membrane filtration and oscillating magnetic fields, microwave and radio frequency processes (POTHAKAMURY *et al.*, 1995; KNORR *et al.*, 2004; TAJCHAKAVIT *et al.*, 1998) and high hydrostatic pressure (PORRETTA *et al.*, 1995; BUTZ *et al.*, 2002). Combinations of these methods have also been proposed, sometimes in association with classical thermal treatment (RASO *et al.*, 1998). Except for high hydrostatic pressure, no industrial application using new technologies has been successfully implemented at the industrial scale.

Dense-phase CO_2 processing is a treatment that seems promising, for reducing both microbial and enzymatic activity. Using this technique foodstuffs are pasteurised at moderate temperature and pressure, and the CO_2 significantly inactivates vegetative bacterial cells, moulds and yeasts and, under certain conditions, intracellular and pectolytic

enzyme activity can be reduced (KAMIHIRA *et a*l., 1987;. BALABAN *et al.*, 1991). Numerous studies have confirmed the antimicrobial action of CO_2 under pressure on food substrates (HAAS *et al.*, 1989; WEI *et al.*, 1991) as well as on polymeric materials for pharmaceutical and cosmetic use (DILLOW *et al.*, 1999). However, despite the large quantity of data collected, the mechanism of microbial inactivation induced by high pressure CO_2 remains unknown. Some researchers have hypothesized that the internal pH of the microbes is decreased to a lethal level due to the easy penetration of CO_2 into the cells. It has been demonstrated that the amount of dissolved carbon dioxide is responsible for cell death, which is not necessarily accompanied by cell lysis (ISENSCHMID *et al.*, 1995; HAAS *et al.*, 1989). BERTOLONI *et al.* (2006) showed that CO_2 treatment induced the release of cellular enzymes.

Other studies have demonstrated that dense-phase CO_2 can only inactivate spores at drastic conditions (60°-95°C, 120 min, 300 bar). Therefore, the sterilisation process by dense phase CO_2 is not practical for industrial applications (ISHIKAWA *et al.*, 1997; WATANABE *et al.*, 2003).

Most of the high-pressure CO_2 inactivation studies currently available in the scientific literature have been performed in inoculated or spoiled foods, in inoculated simple model systems and liquid substrates, such as physiological saline solution or culture media. To date less attention has been paid to the treatment of foodstuffs in their natural conditions. The lack of studies using real foodstuffs, instead of model systems, and the fact that the structural and microbiological properties of food are modified due to CO_2 treatment, are major obstacles to the development of this technology for industrial applications.

High pressure CO_2 pasteurisation of grape must and tomato paste has been given little attention. For example, to improve processing plants to obtain better quality products by thermal processes, LEE and COATES (1999) studied the changes in colour due to thermal pasteurisation of red grapefruit juice from two cultivars treated at 91°C using a plate heat exchanger. Thermal pasteurisation caused a change in the colour of the juice, with a slight shift towards a lighter, brighter juice. TALCOTT *et al.* (2003) applied the pasteurisation method by high hydrostatic pressure to muscadine grape juice, but this processing was detrimental to juice quality, probably due to the action of residual oxidase enzymes. In contrast, WU *et al.* (2005) reported that PEF treatment to inactivate microorganisms in red and white grape juice only gave satisfactory results when combined with temperatures above 50°C.

The effects of time-temperature sterilisation conditions (90°, 95° and 100°C) on the overall quality of tomato puree were studied by ZANONI *et al.* (2003); heat and oxidative damage was observed in the samples. During sterilisation, the ascorbic acid content and antioxidant activity of the hydrophilic fraction decreased, and a colour change occurred, but no significant variations were found in the lycopene content. PORRETTA *et al.* (1995) subjected tomato juice to ultra-high-hydrostatic-pressure; changes in the structure of the product were detected, and the physical-chemical properties were altered when the tomato juice was treated at 5,000, 7,000 and 9,000 bar, the viscosity and total pectin content increased and protein coagulation and high free glutamic acid levels were observed. In particular, the treated samples were inedible because of a strong rancid taste. This was due to a marked increase in n-hexane content, the same substance that, at a concentration less than 1-1.2 mg/kg, imparts the typical fresh tomato flavour.

In this work, a semi-continuous and multi-batch apparatus was designed to

investigate real foodstuff treatment by dense-phase CO_2. Experiments were performed with fresh red grape must and tomato paste at different CO_2 pressures, temperatures and treatment times; the foodstuffs were used without any previous technological treatments. Different combinations of process parameters were also applied to fresh grape must that had been inoculated with wild strains of yeast (*Pichia awry*) and bacteria (*Bacillus subtilis*), in order to investigate the pasteurisation efficiency of pressurised CO_2 in these cases, and to determine the level of survival of their typical microbial population.

MATERIALS AND METHODS

Pressure treatment: apparatus and procedure

The experiments were carried out in both a multi-batch and semi-continuous apparatus, specifically designed and constructed to investigate high-pressure CO_2 food treatment and determine the related kinetics. The design of the apparatus is shown in Fig. 1. The system has a high pressure CO_2 storage tank primarily to reduce the pressurisation time of the reactors and to prevent pressure fluctuations. The CO_2 reservoir is temperature-controlled by using circulating water. Details of this apparatus are reported elsewhere (PARTON *et al.*, 2007).

During an experimental run, each batch reactor was loaded with about 1.8 mL of well-mixed sample under sterile conditions, and then connected to the high-pressure apparatus. After temperature equilibration, the six samples were subjected to the same CO_2 pressure for different treatment times.

If larger sample volumes have to be treated, the system can be equipped with a single tubular reactor of 70 mL internal volume (SCR in Fig. 1), which can be used to carry out experiments in a semi-continuous mode in order to improve the mass transport of CO_2 to the substrate.

The experimental runs carried out in this study are listed in Table 1. Different combinations of temperature and pressure were investigated. Each experiment was done at least twice, and the average value is reported. Values of process parameters were selected on the basis of previous experience with the pasteurisation of other fruit juices; the operating temperature should be less than 40°C to prevent any changes in organoleptic properties.

Microbial characterisation of food

Red grape must and tomato paste were supplied by the Sapio S.r.l. Company (Monza, Italy); the grape must came from mountains of Valtellina (Sondrio, Italy), whereas the tomato paste came from an Italian farm. These substrates were used as delivered, without any oth-

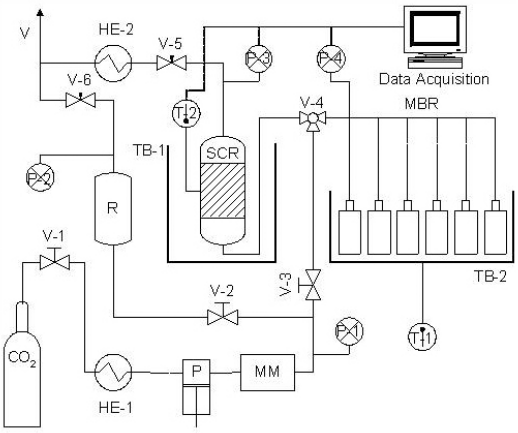

Fig. 1 - Sketch of the multi-batch system for determination of inactivation kinetics of enzymes and microorganisms subjected to high pressure CO_2. V-1, V-2, V-3, V-4: on-off valves; V-5, V-6: micrometering valves; HE-1, HE-2: heat exchanges; P: pump; MM: pressure controller; R: high pressure CO_2 reservoir; SCR: single cell reactor; MBR: multi-batch reactor; TB-1, TB-2: thermostatic bath; V: vent; P-1, P-2, P-3, P-4: pressure indicators; T-1, T-2: temperature indicators.

Table 1 - Experimental runs carried out for microbial inactivation in fresh grape must and tomato paste by dense CO_2. * Thermal shock; ** Semi-continuous; *** Inoculated.

	Temperature (°C)	Pressure (bar)	Time (min)
Grape must	30	80	5, 10, 20, 30, 40, 50
	30	100	5, 10, 20, 30, 40, 50
	30	120	5, 10, 20, 30, 40, 50
	35	80	5, 10, 20, 30, 40, 50
	32	85	5, 10, 20, 30, 40, 50 *
	40	85	60 **
	32	85	5, 10, 20, 30, 40, 60 ***
	32	110	5, 10, 20, 30, 40, 60 ***
	40	85	5, 10, 20, 30, 40, 60 ***
	40	110	5, 10, 20, 30, 40, 60 ***
Tomato paste	40	110	10, 20, 30, 45, 60, 75
	35	75	15, 30, 45, 60, 75, 90 *
	50	100	20, 30, 40, 60, 80, 100
	32	110	60 **
	40	110	Up to 24 h

er treatment. Both foods were wholesome and very aromatic. The pH value of the red grape must was 3.10 and the tomato paste was 4.03 (values provided by the suppliers).

Samples of the substrates (about 1 L) were maintained at -12°C until analysis. Freezing and thawing did not significantly affect the microbial population. The same microbial population was identified by viable plate count in both a fresh sample and a frozen/thawed sample. This assessment was carried out before all the sample was frozen. Samples were slowly thawed at 4°C; after shaking, aliquots of 20 mL were taken for seeding in both solid and liquid growth medium for microbial culturing. Qualitative and quantitative microbiological analyses were carried out in both substrates, in order to determine the typology and quantity of the microorganisms present in natural substrates.

For the quantitative analyses, the samples were serially 10-fold diluted in: i) liquid media (Brain Heart Infusion Broth, BHI, and thioglycolate) and incubated in aerobic and anaerobic conditions, respectively; ii) physiological saline solution (0.9% NaCl) and, in order to determine colony forming units per mL (cfu/mL), each dilution was plated in two sets of plates for aerobic (28°C) and anaerobic (37°C) incubation conditions, respectively. For the qualitative analyses, aliquots of sample were centrifuged at 3,000 g for 30 min, and the sediment was spread on suitable growth media (Difco and Becton Dickinson, USA): Brain Heart Infusion Agar (BHA) for bacteria, Sabouraud Dextrose Agar (SAB) for yeasts and moulds, Agar Rogosa (AR) for lactic bacteria, Mannitol Salt Agar (MSA) for *Staphylococcus* sp., Mac Conkey Agar (McC) for coliforms and intestinal pathogens, Columbia for Gram positive *cocci*, growth medium for *Acetobacter* and *Gluconobacter* (only for grape must), Glucose Yeast Peptone (GYP) for yeast in grape must and Tomato Juice Agar (TJA) for *Lactobacillus* sp. (only for tomato paste). To avoid the colony masking due to the sediment, each pellet was distributed in five plates of each medium.

The isolated strains were identified based on morphological characteristics, the biochemical profile according to the manufacturer's instructions (API system,

BioMerieux, France), and by sequencing 16S (bacteria) or 26S (fungi) rDNA as described below.

DNA extraction, amplification and sequencing

Two or three colonies of each microbial strain were suspended in 100 µL of sterile double-distilled water. Cells were lysed by heating for 5 min at 100°C, centrifuged at 13,000 g for 15 min at 4°C and the supernatant (containing DNA) was transferred to a microfuge tube. Two µL of each DNA samples were used as a template in the PCR (Polymerase Chain Reaction) assay after extraction. The primers used for prokaryotic cells were 355F (5'- CCT ACG GGA GGC AGC AG -3') and 910R (5' –CCC GTC AAT TCC GAG TT – 3'), while those used for eukaryotic cells were NL1 forward (5' GCA TAT CAA TAA GCG GAG GAA AAG 3') and NL4 reverse (5' GGT CCG TGT AAG ACG G 3'). In a final 50 µL volume, 5 µL of forward primer 10 µM (Sigma), 5 µL of reverse primer 10 µM (Sigma), 2 µL of template DNA, 5 µL of 10X Taq DNA polymerase buffer (Sigma), 4 µL MgCl2 (25 mM) and 0.5 µL Taq DNA polymerase (5 U/µL, Sigma) were used. The PCR conditions for bacteria were: 95°C for 1 min, followed by 30 cycles at 95°C for 30 sec, 50°C for 1 min, and 72°C for 1 min, followed by one final extension at 72°C for 6 min. The PCR conditions for fungi were: 95°C for 5 min, followed by 30 cycles at 95°C for 45 sec, 53°C for 45 sec., and 72°C for 45 sec, followed by one final extension at 72°C for 10 min.

The amplicons were purified using the Microcon PCR columns (Millipore, CA, USA) and the purified products were eluted with 35 µL of MilliQ sterile water. Eighty ng of this DNA were used for a PCR sequencing reaction performed as follows: 3.2 µL of forward primer (1 µM), 6 µL of Big Dye Buffer (Applied Biosystems), 2 µL of Big Dye Mix (Terminator RR Mix, Applied Biosystems), milliQ sterile water up to 20 µL. The PCR conditions were the same as previously described. The DNA obtained was treated with 45 µL of pure ethanol (4°C) and 3.75 µL of EDTA (125 µM), incubated for 15 min in the dark and centrifuged at 15,000 g for 15 min at 4°C. The pellets were washed with 150 µL of 70% ethanol and centrifuged again at 15,000 g for 15 min at 4°C. The supernatants were discharged and the pellets dried for 15 min at 37°C. The samples were suspended in 15 µL formammide and sequenced in an ABI PRISM 310 Genetic Analyzer (Perkin Elmer). The sequences obtained were compared to those present in the National Center for Biotechnology Information (NCBI) genome bank in order to identify the microorganisms with a percentage of similarity greater than 98.5%.

Microbiological procedures

Experiments were carried out on fresh foodstuffs both with their natural microbial load and after inoculation with a yeast (*Pichia awry*) and a bacterium (*Bacillus subtilis*). These microorganisms were isolated from original grape must micro-flora.

Treatment of fresh foodstuffs in their natural state did not require special procedures for sample preparation; microorganisms were cultured according to the identification procedure presented above.

The yeast cultures were prepared by inoculating 2-3 colonies in 15 mL of Sabouraud broth and incubating the resulting suspension at 32°C for 48 h. Under these conditions, the yeast population reached the stationary phase and a concentration of about 10^6 cfu/mL.

The bacterial cultures were prepared by inoculating 2-3 colonies in 15 mL of BHI and incubating the resulting suspension at 37°C for 18 h. Under these conditions, the vegetative bacterial population reached the stationary phase and a concentration of about 10^7 cfu/mL.

Cell survival assay

The number of viable cells in treated and untreated substrates was determined by the viable plate count method. A well-mixed volume of 100 μL of diluted 1:10 or non-diluted cell suspension was plated on suitable solid media. After incubation at 32°C for 48 h (yeasts) and at 37°C for 24 h (bacteria) the colonies were counted. The final concentration was obtained by taking the arithmetic mean of the counts determined in the plates of the same sample; the total microbial load was measured as colony forming units per mL (cfu/mL).

Each run was carried out in duplicate and the results are reported as the average of the separate counts; the calculated standard errors are also reported. Inactivation was expressed as $Log(N_0/N)$, where N_0 is the number of microorganisms initially contained in the sample at time 0 (control sample), and N is the number of microorganisms counted after treatment at any time t. The total inactivation (N=0) was conventionally expressed as $>Log(N_0)$.

RESULTS

Microbial isolation and identification

The results obtained after the liquid media was incubated in aerobic conditions showed microbial growth in grape must until a 10^{-3} dilution, indicating a total microbial load between 1,000 and 9,000 cfu/mL; in tomato paste growth was observed until a 10^{-4} dilution, indicating a total microbial load between 10,000 and 90,000 cfu/mL. The same samples, incubated in anaerobic conditions, showed microbial growth until a 10^{-4} dilution in both cases, indicating that the total anaerobic microbial load was between 10,000 and 90,000 cfu/mL. These data were confirmed by cfu/mL determinations (Table 2).

The isolated and identified microbial strains in grape must and tomato paste are shown in Tables 2 and 3. The majority of bacterial strains identified in the grape must belonged to the genus *Bacillus* followed by *Acetobacter* and *Gluconobacter* and *Lactobacillus* in the tomato paste. The genus *Clostridium* was scarcely present in both samples.

Table 2 - Isolated and identified bacteria in natural grape must and tomato paste.

Grape must		Tomato paste	
Microorganisms	Total load cfu/mL	Microorganisms	Total load cfu/mL
Acetobacter sp.	1,000	*Bacillus pumilus*	16,000
Gluconobacter oxydans	1,000	*Bacillus sphaericus*	2,000
Paenibacillus sp.	100	*Bacillus clausii*	1,000
Bacillus macroides	1,500	*Bacillus licheniformis*	100
Bacillus pumilus	2,000	*Bacillus magaterium*	100
Bacillus licheniformis	100	*Bacillus subtilis*	60
Bacillus subtilis	180	*Bacillus mojavensis*	30
Bacillus cereus	10	*Bacillus cereus*	20
Clostridium perfrigens	<10	*Pediococcus acidilactici*	2,000
Clostridium barati	<10	*Acetobacter sp.*	<10
		Lactobacillus delbrueckii	2,500
		Lactobacillus reuteri	8,000
		Clostridium beijerinckii	<10
		Clostridium xylanolyticum	<10

Table 3 - Isolated and identified yeasts in grape must.

Microorganisms	Total microbial load (cfu/mL)
Issatchenkia terricola (Pichia)	1,000
Pichia sp. AWRI 1272	4,000
Pichia kluyveri	1,500
Candida valida	130
Candida vinaria	200
Zygoascus hellenicus (Candida hellenica)	3,000
Candida stellata	200
Candida vini	200
Saccharomyces elongisporus	2,000
Saccharomyces crataegensis	160

While yeasts were absent in the tomato paste, different strains belonging to the genera *Candida*, *Pichia* and *Saccharomyces* were present in the grape must (Table 3).

The mould load was very low in both foodstuffs (~10 cfu/mL), and they were environmental microorganisms (*Alternaria* and *Penicillium)*.

Based on the results, the growth media chosen to determine the inactivation by dense-phase CO_2 were BHA for most of the detected bacteria, SAB for the yeasts and moulds, and TJA for lactobacilli (only for tomato paste).

Microbial inactivation in fresh grape must

The results (Figs. 2 and 3) from the pasteurisation of fresh grape must showed that yeasts, moulds and bacteria were almost completely inactivated by 30 min of treatment with dense-phase CO_2 (Fig. 2). Moreover, it was observed that the two groups of microorganisms showed the greatest sensitivity to the treatment at different pressures: 100 bar and 80 bar for bacteria and fungi, respectively. In all cases the inactivation trends were initially fast and then reached a plateau after 10-20 min of treatment.

Under the same treatment conditions, with the exception of the data at 10-20 min for yeast (Fig. 2) and 5-10 min for bacteria (Fig. 3), the inactivation of microorganisms in fresh grape must was comparable with good repeatability.

Another experimental run was performed at 35°C and 80 bar of CO_2; the results are shown in Figs. 4 and 5, and are compared to those obtained at 30°C. The 5°C increase in the process temperature did not substantially improve the extent of yeast inactivation (p=0.6); on the contrary, it was statis-

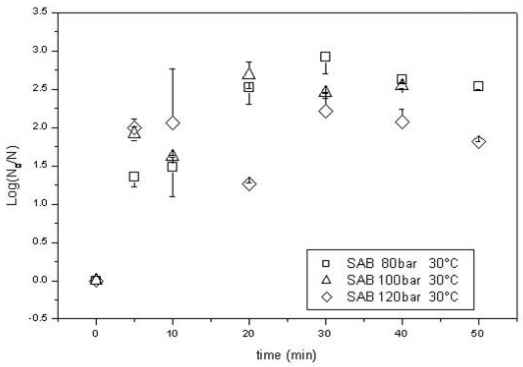

Fig. 2 - Inactivation curves for yeasts naturally contained in fresh grape must treated at 30°C at different values of CO_2 pressure, 80, 100 and 120 bar, for up to 50 min. (Error bars, ± standard error).

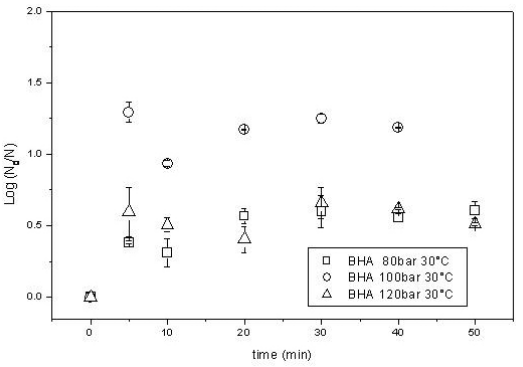

Fig. 3 - Inactivation curves for bacteria naturally contained in fresh grape must treated at 30°C at different values of CO_2 pressure, 80, 100 and 120 bar, for up to 50 min. (Error bars, ± standard error).

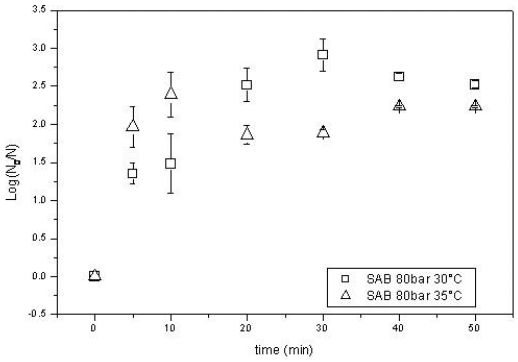

Fig. 4 - Temperature effect on inactivation curves for wild yeasts in fresh grape must treated at 80 bar at two different temperatures, 30° and 35°C, for up to 50 min. (Error bars, ± standard error).

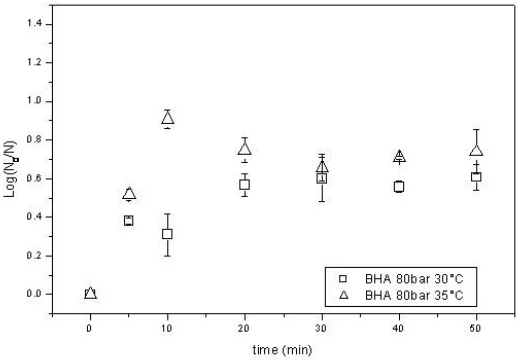

Fig. 5 - Temperature effect on inactivation curves for wild bacteria in fresh grape must treated at 80 bar at two different temperatures, 30° and 35°C, for up to 50 min. (Error bars, ± standard error).

tically significant (p=0.01) for bacteria (Fig. 5).

To determine the pasteurisation efficiency of dense-phase CO_2 in fresh grape must in a different contact system, a run was carried out in the semi-continuous apparatus with a single cell reactor, loaded with 20 mL of sample. A continuous flow of high pressure CO_2 (5 mL/min at 85 bar) was maintained for 60 min through grape must at a constant temperature of 40°C. Under these conditions, there should be better contact between phases; results are given in Table 4. A total inactivation of yeast and mould cells was confirmed, while a residual load of bacteria persisted, as already seen.

Table 4 - Yeast (SAB) and bacteria (BHA) inactivation in fresh natural grape must treated by continuous flow of high pressure CO_2 at 85 bar and 40°C for 60 min. Samples were then kept in a refrigerator (4°C) for one month (last row) under atmospheric conditions.

Treatment time (min)	N (cfu/mL)		$Log(N_0/N)$	
	SAB	BHA	SAB	BHA
0	4,630	1,228	0	0
60	0	920	>3.67	0.13
after 1 month	0	653	>3.67	0.28

After continuous exposure of dense-phase CO_2, the treated and control samples were maintained in a refrigerator at 4°C in sterile tubes. After one month, a plate count was executed on treated and control grape must. No growth was observed for the treated sample, whereas the untreated sample showed an enormous microbial load due to grape must fermentation (~10^7 cfu/mL). In this case the results suggest that the cells injured by high pressure CO_2 were not able to repair their damage and restore the initial biological state necessary for cellular reproduction. The induced damage was probably irreversible and the microbial inactivation corresponded to real cell death rather than a transitory inhibiting effect.

The inoculated strains, *Bacillus subtilis* and *Pichia awry*, were isolated from the grape must itself; they were chosen as representative wild-type microorganisms normally contained in natural must.

Fig. 6 shows the results obtained for inoculated *Bacillus subtilis* (in vegetative form). It can be seen that 85 bar and 40°C, and 110 bar and 32°C, were the most effective for the inactivation of *B. subtilis*. In addition, most bacteria were eliminated within the first 5 min of treat-

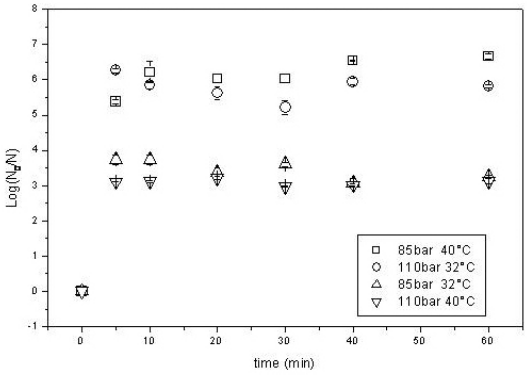

Fig. 6 - Inactivation trends of vegetative form of *Bacillus subtilis* wild type inoculated in fresh grape must treated under the conditions listed in Table 1. (Error bars, ± standard error).

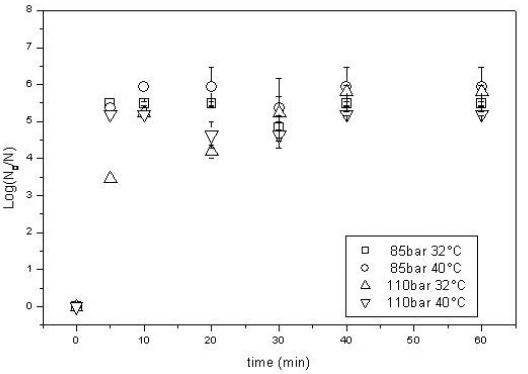

Fig. 7 - Inactivation trends of *Pichia awry* yeast wild type inoculated in fresh grape must treated under the conditions listed in Table 1. (Error bars, ± standard error).

ment. The final inactivation values after 60 min are almost identical to the results after 5 min; this confirms a rather fast kinetics with respect to the time scale considered.

Fig. 7 shows the results obtained for inoculated *Pichia awry* yeast. As already observed, when treating fresh natural grape must, the yeast cells were very sensitive to the dense-phase CO_2 treatment. The best inactivation conditions are similar (40°C and 85 bar) to those observed for *Bacillus subtilis*, but the degree of inactivation is much higher for the yeasts cells under all the conditions considered.

Microbial inactivation in fresh tomato paste

Microbial culturing in fresh tomato paste was carried out in BHA for bacteria and TJA for lactobacilli; however, some problems with sample culturing, such as rapid and irregular growth of the bacterial colonies, were always noted.

A preliminary experimental run carried out at 40°C and 110 bar gave satisfactory results (Table 5); some inactivation was achieved, but tomato paste pasteurisation was quite modest.

The inactivation under conditions

Table 5 - Microbial inactivation in natural fresh tomato paste treated at 40°C and 110 bar of CO_2 up to 75 min. TJA indicates the growth of lactobacillus, BHA indicates the growth of bacteria. (SE, standard error)

Time (min)	N (cfu/mL)		Log (N_0/N)	
	TJA (±SE)	BHA (±SE)	TJA (±SE)	BHA (±SE)
0	32,000 (±1,000)	25,000 (±720)	0	0
10	1,110 (±577)	8,640 (±120)	1.70 (±0.39)	0.45 (±0.05)
20	1,140 (±611)	6,965 (±903)	1.74 (±0.44)	0.56 (±0.10)
30	2,250 (±150)	7,980 (±952)	1.15 (±0.04)	0.50 (±0.00)
45	985 (±516)	8,330 (±912)	1.76 (±0.39)	0.48 (±0.09)
60	1,100 (±565)	7,365 (±730)	1.69 (±0.36)	0.53 (±0.01)
75	767 (±288)	7,130 (±479)	1.73 (±0.24)	0.54 (±0.08)

close to the critical point of CO_2 (35°C and 75 bar) was studied, supposing that its special properties could lead to higher microbial inactivation, as observed in previous cases (SIMS and ESTIGARRIBIA, 2002). In addition, the treated and control samples were subjected to thermal shock to detect the presence of spores; in fact, in tomato paste, a large microbial population is composed of spore-forming bacteria. However, the number of spores detected was much lower than expected and the survival level obtained in previous experiments was confirmed. Reasons for the moderate inactivation in tomato paste must be sought.

In an attempt to improve the microbial destruction, temperature and treatment time were further increased (50°C and 100 min, respectively), but the microbial load in treated tomato paste remained quite high, as is evident in Table 6.

Tomato paste is a highly viscous and complex substrate, with many organic suspensions and fibers. The resistance to high pressure CO_2 treatment may not be due to the strains of microorganisms, but rather to the properties of the substrate. To test this hypothesis, the tomato paste (previously sterilised by heat) inoculated with the same microorganisms isolated from the paste itself was treated and there was complete inactivation (data not reported). It may be that the particular texture of natural fresh tomato paste creates some resistance to the mass transfer and diffusion of CO_2 within tomato pulp particles, thus reducing the treatment effectiveness; alternatively, some heat labile components in the tomato paste may protect the naturally present microorganisms.

In an attempt to increase the degree of microbial inactivation taking into account the mass transfer limitation, experimental runs under continuous CO_2 flow were carried out with an improved phase contact between tomato paste and CO_2. The single cell reactor was used, operating at 32°C and 110 bar with a CO_2 flow equal to 5 mL/min for 60 min. No significant improvement with respect to previous data was found ($Log(N_0/N)$ = 0.35 in TJA, $Log(N_0/N)$ = 0.12 in BHA). The results obtained for a very long treatment time (up to 24 h) are presented in Fig. 8. Under these conditions, the mass transfer should be very effective, but once again microbial inactivation was modest, with a slight trend towards increased values. After 24 h of treatment, bacteria were reduced to about one quarter of the initial population, and the lactobacilli were about halved.

Table 6 - Microbial inactivation in natural fresh tomato paste treated at 50°C and 100 bar of CO_2 up to 100 min. TJA indicates the growth of lactobacillus, BHA indicates the growth of bacteria. (SE, standard error).

Time (min)	N (cfu/mL)		$Log(N_0/N)$	
	TJA (±SE)	BHA (±SE)	TJA (±SE)	BHA (±SE)
0	12,300 (±750)	19,800 (±606)	0	0
20	9,500 (±565)	14,400 (±294)	0.11 (±0.05)	0.14 (±0.00)
30	5,800 (±115)	13,050 (±418)	0.33 (±0.01)	0.18 (±0.03)
40	5,450 (±375)	11,450 (±288)	0.35 (±0.00)	0.24 (±0.01)
60	7,150 (±790)	11,800 (±577)	0.24 (±0.02)	0.22 (±0.02)
80	5,600 (±496)	10,450 (±721)	0.34 (±0.01)	0.28 (±0.01)
100	5,400 (±565)	9,000 (±317)	0.36 (±0.01)	0.34 (±0.02)

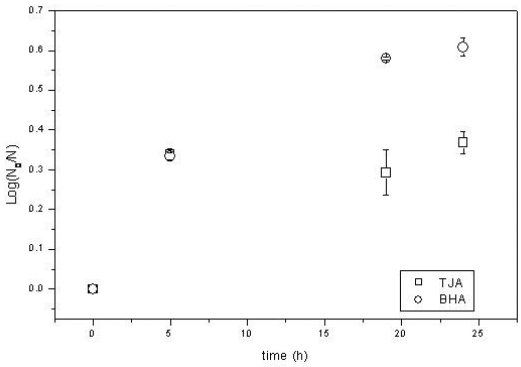

Fig. 8 - Inactivation of lactobacilli in TJA and bacteria in BHA, for 24 h of treatment under 110 bar of CO_2 at 40°C in natural fresh tomato paste. (Error bars, ± standard error).

CONCLUSIONS

An accurate and repeatable experimental procedure was set up to investigate dense-phase CO_2 food pasteurisation in a new multi-batch apparatus, expressly designed and developed to evaluate inactivation kinetics.

The effectiveness of high pressure CO_2 treatment was determined on natural fresh grape must with respect to both its natural microbial population and inoculated strains. The yeasts contained in the must were sensitive to the treatment at 30°C and 80 bar; complete inactivation was achieved at temperatures and pressures slightly higher than these. Bacterial strains were more difficult to eliminate: after 40 min of treatment at the above-cited conditions about 20% of the initial microbial population survived in terms of resistant microorganisms and probably spores.

In the inoculated grape must, the best inactivation, both for *Pichia awry* yeast and *Bacillus subtilis* bacterium, was obtained at 40°C and 85 bar. In these cases the inactivation kinetics was always fast with regard to the time scale considered (60 min); most microorganisms were inactivated after only 5 min.

The effectiveness of dense-phase CO_2 treatment was determined on natural fresh tomato paste. Low microbial inactivation was obtained at the conditions investigated, except in the case of a very long treatment time (around 24 h).

No physical or chemical changes were qualitatively detected in natural fresh grape must or tomato paste after dense-phase CO_2 treatment. Normally, the foodstuffs maintained their typical colour and fragrance; thus, the dense-phase CO_2 treatment is a promising alternative technology for the mild preservation of food, in order to obtain a valuable product with an extended shelf-life.

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PAPER

RHEOLOGICAL PROPERTIES OF DOUGH AND SENSORIAL QUALITY OF BREAD MADE FROM A WHOLEMEAL RYE-WHEAT BLEND WITH THE ADDITION OF GLUTEN

PROPRIETÀ REOLOGICHE DELL'IMPASTO E QUALITÀ SENSORIALI DEL PANE PREPARATO CON UNA MISCELA DI FARINA INTEGRALE DI SEGALE E FRUMENTO CON L'AGGIUNTA DI GLUTINE

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ABSTRACT

Baking assays under standardized conditions were carried out in order to compare the efficacy of ascorbic acid, cysteine and gluten as improvers of 50-50% wholemeal rye-wheat flour doughs. A fermentation time of 240 min was chosen taking into account the specific loaf volume without additives; dough yeast profile, pH and bread crumb structure. Compos-

RIASSUNTO

Sono state condotte prove di cottura al forno, in condizioni standardizzate, allo scopo di confrontare l'efficacia dell'acido ascorbico, cisteina e glutine nel migliorare gli impasti integrali di farina di segale e grano tenero (50-50%). È stato scelto un periodo di fermentazione di 240 minuti tenendo conto del volume della forma di pane ottenuto senza additivi, del tipo di lievito per l'impasto, del pH e

- Key words: ascorbic acid, cysteine, gluten, wholemeal rye-wheat dough -

ite doughs baked with different gluten levels were studied in regard to rheological, sensorial and microbiological changes. No significant changes in bread volume were observed when either ascorbic acid or cysteine were used at 50 and 100 ppm. Specific loaf volume was significantly increased ($p<0.05$) with 8% gluten addition. Strength value of the dough was significantly increased with gluten addition; furthermore, texture and overall acceptance were improved in comparison with bread without gluten. No significant differences ($p<0.05$) were observed in other attributes like crumb color, smell and taste, in agreement with *Lactobacillus* spp. and yeast counts.

della struttura della mollica. Sono stati studiati i cambiamenti delle proprietà reologiche, sensoriali e microbiologiche degli impasti, con differenti livelli di glutine, cotti al forno. Non sono state osservate variazioni significative nel volume del pane in seguito all'utilizzo sia di acido ascorbico sia di cisteina a concentrazioni di 50 e 100 ppm. Si è osservato un significativo aumento del volume del pane ($p<0,05$) con l'aggiunta dell'8% di glutine. La resistenza dell'impasto è aumentata significativamente con l'aggiunta di glutine; sono, inoltre, migliorate la tessitura e l'accettabilità generale del prodotto dopo confronto con il pane senza glutine. Non sono state osservate differenze significative ($p<0.05$) in altri attributi come il colore della mollica, l'odore ed il sapore, in accordo con i cambiamenti nelle conte microbiche dei *Lactobacillus* spp. e dei lieviti.

INTRODUCTION

Rye is an important source of fiber and other nutrients. Rye products are traditionally consumed in many European countries. For example, in Finland rye products comprise 20-30% of the fiber intake. Epidemiological studies have shown that consumption of foods rich in whole grains and cereal fiber reduce the risk of chronic diseases such as diabetes, cardiovascular disease and certain types of cancer, as reviewed by MURTAUGH *et al.* (2003) and JACOBS and GALLAHER (2004), and shown by LARSSON *et al.* (2005). Bakery products, like whole grain breads, are an important source of fiber in the diet. However, the addition of fiber has a negative effect on the final bread quality. The most evident effects are reduced loaf volume, increase of crumb firmness and dark crumb appearance (POMERANZ *et al.*, 1977; LAI *et al.*, 1989; KNUCKLES *et al.*, 1997). In particular, rye flour has a lower baking quality than wheat flour, often due to the lower gas-holding capacity of rye flour dough (WARNERBERGUER *et al.*, 1997). Because rye proteins are deficient both in polymerizing and in forming high-molecular-weight aggregates, rye doughs release CO_2 during fermentation and proofing (HE and HOSENEY, 1991). Moreover, the fiber-rich dough exhibits high water absorption, becomes shorter and has a reduced fermentation tolerance (GAN *et al.*, 1992; LAURIKAINEN *et al.*, 1998).

The addition of dietary fibers to baked products requires changes in the processing techniques in order to obtain products that have good consumer acceptability (LAURIKAINEN et al, 1998). Another option is to use appropriate additives. L-threo ascorbic acid (Vitamin C) is widely used as a bread improver in wheat breads; it oxidizes into dehydro-ascorbic acid (DAA) which becomes the oxidiz-

ing agent (ELKASSABANY and HOSENEY, 1980; GROSCH and WIESER, 1999).

When L-threo-ascorbic acid (AA) is added to flour, it reduces dough stickiness, increases loaf volume and improves the crumb structure. The concentrations commonly used in bread-making are in the range of 20-150 mg/kg of flour. The amount of AA added depends on the wheat cultivars, the type and storage time of the flour, and the processing technology and type of bread (KOEHLER, 2003). The fact that L-ascorbic acid is a permitted food ingredient that is readily available, and does not leave toxic compounds in the final product makes it superior to other oxidizing agents (MAFORIMBO *et al.*, 2005).

Rheological studies have shown that the addition of either sulfhydryl compounds or sulfhydryl-blocking reagents allows for the formation of disulfide bonds that are vital to dough stability. A small amount of cysteine increases the extensibility of dough (DONG and HOSENEY, 1995). It has been reported that the addition of cysteine to wheat flour accelerates stress relaxation and structural relaxation (FRATER *et al.*, 1961). POMERANZ (1988) reported that cysteine reduces the mixing time of the dough; it reacts with wheat proteins by splitting the disulphide bonds rapidly which favors faster dough development.

SAIZ *et al.* (1997) found that the addition of gluten improved the baking performance of wheat dough. The wheat flour dough was easier to handle, the loaf volume increased and sensory properties of the breads improved.

Lactic acid bacteria (LAB) and yeasts contribute to several organoleptic features: homofermentative LAB are responsible for the development of a good grain and elastic crumb in the final bread, whereas heterofermentative LAB improve the taste and promote the leavening process (DIMIANI *et al.*, 1996).

The aim of the present work was to evaluate the effects of gluten on the sensorial quality and rheological and microbiological characteristics of doughs made from a 50-50% wholemeal rye-wheat blend. Since the specific volume of bread is one of the most important characteristics that determines consumer acceptability, this parameter was used both to select the optimum fermentation time and to verify that gluten is an appropriate conditioner compared with ascorbic acid and cysteine.

MATERIALS AND METHODS

Materials

A commercial 000-quality wheat flour without additives and wholemeal rye flour (Lagomarsino S. A. mills, Buenos Aires province) were used. For the baking test, composite flours were made up of 50% wholemeal rye flour and 50% wheat flour.

The L-ascorbic acid and L-cysteine standards used were analytical grade and were purchased from Sigma Chemical Co (St. Louis. MO, USA). Gluten flour was obtained from a local market. Commercial gluten flours are obtained by washing the wheat flour and separating the protein from the starch, followed by freeze-drying and grinding (WADHAWAN and BUSHUK, 1989). The composition of the flours and gluten is shown in Table 1.

L-ascorbic acid or L-cysteine was added at concentrations of 50 and 100 ppm. Gluten substituted 6, 8 and 10% of the 50-50% composite flour. The composite flours were mixed thoroughly to ensure uniform distribution of the flour and conditioner particles.

Experimental design

The baking process was performed according to the experimental method suggested by the SECRETARÍA DE AGRICULTURA Y GANADERÍA (1952).

Table 1 - Chemical composition (g/100 g of dry matter) of the flours and gluten (harvest year 2003-2004).

Flour	Moisture content	Protein	Dietary fiber	Ash
Wheat flour	12.37±0.10	12.01±0.10	2.4±0.40	0.66±0.18
Wholemeal rye flour	10.73±0.33	10.60±0.40	15±0.40	2.15±0.80
Gluten	6.68±0.06	71.78±4.57	0.90±0.40	0.77±0.11
Values represent means ± standard deviation of three replicates.				

In order to determine the optimum water absorption, mixing and fermentation times, alveograms of wheat flour (control) were carried out according to the method of the AACC (2000).

Dough samples were prepared by mixing 300 g composite flour (without additives) with 189 mL water, 9 g of pressed Baker´s yeast, 7.5 g of sugar and 3 g of salt. Samples were incubated at 30°C for the initial rising and proofing time.

Fermentation times of 100, 120, 180, 240, 300 and 360 min were evaluated for the 50-50% wholemeal rye-wheat blends. The 100 min fermentation time corresponded to that of the baking process for wheat flour. Each fermentation time tested was divided as follows: 44% of the total fermentation time spent for the initial rising, which was sub-divided into two steps, 66 and 34%. After punching, the dough was divided into three equal pieces and each one was rolled and fitted into a mold. The dough was then proofed for the remaining 56% of the total time prior to baking. The loaves were then baked at 230°C for 30 min. After baking the breads were taken out of the moulds and left for 1 h at room temperature before physical measurements and subjective determinations were made. The baking assays were repeated three times.

The same baking procedure was carried out using the composite blend and conditioners (L-ascorbic acid, L-cysteine, gluten). The optimum fermentation time, as described above, was taken into account.

Microbiological analyses

Ten g of composite dough were homogenized in 90 mL of sterile Butterfield`s phosphate-buffered dilution water (0.25 M KH_2PO_4, pH 7.2) for 3 min using a Stomacher 400 Circulator. Microbiological analyses were carried out in triplicate. *Lactobacillus* spp. and yeasts were analyzed using the following culture media and culture conditions: *Lactobacillus* spp. on MRS agar (*Lactobacillus* selective agar) supplemented with ketoconazol (Janssen-Cilag Farmacéutica S.R.L., Buenos Aires, Argentina) at 50 ppm, incubated in anaerobic jars with Anaerocult C (Merck, Darmstadt, Germany) at 30°C for 5 days. Yeasts were analyzed on standard yeast extract-glucose-chloramphenicol (YGC) agar incubated at 22°-25°C for 3-5 days (ICMSF, 1983). Counts obtained are expressed as colony forming units (cfu g^{-1}). The *Lactobacillus* genus was confirmed by: Gram reaction, catalase activity, motility, oxidase test, growth on MRS agar with oxygen-depleted atmosphere and CO_2-enriched at 15°, 37° and 45°C (CLAUS and BERKELEY, 1986).

Sensory evaluation

A sensory panel was made up of 30 untrained judges (graduate and undergraduate students from the Universidad Nacional de Mar del Plata (20-50 years old). They were introduced to the principles of sensory evaluation and descriptive analysis by a panel leader. The organoleptic characteristics of the breads

evaluated by the judges were: crumb color, smell, taste, texture and overall acceptability. A five-point Hedonic Rating Scale ranging from like a lot (2) to dislike a lot (-2) was used (ANZALDUA-MORALES, 1994).

Physicochemical analyses

Loaf volume was measured by the rapeseed displacement method. The specific loaf volume of the breads was calculated as mL/100 g bread. The crust-crumb ratio was calculated as the weight of crust per weight of crumb (BARBER *et al.*, 1987). The pH values were determined on 10 g of dough blended with 90 mL of distilled water using a HANNA pH-meter (model HI 9321, Portugal).

Alveograph characteristics

The alveograms of wheat flour (control) and wholemeal rye-wheat blends with and without gluten were determined in order to evaluate extensibility, elasticity and strength of the different doughs according to the method of AACC (2000).

The blends used for the alveographic measures contained 10, 25 and 50% wholemeal rye flour. In addition, 8% gluten was added to each blend. All determinations were carried out in triplicate.

Statistical analysis

Physical measurements and sensory scores of breads were subjected to analysis of variance (ANOVA). When there were significant differences in ANOVA, means were compared using the Duncan's Multiple Range test. The level of significance was 0.05. Sensory and analytical data were analyzed using the Statistical Analysis System (SAS) program package for analysis of variance (ANOVA) and Duncan's test. The bread scores were subjected to ANOVA using a completely randomized block design.

RESULTS AND DISCUSSION

Fermentation time selection

Bread volume was used as a quality parameter to choose the optimal fermentation time of composite breads baked from 50-50% wholemeal rye-wheat blends. The dough yeast profile, pH and bread crumb structure were also taken into account.

Fig. 1 shows the specific loaf volume, pH and yeast population in the dough at different fermentation times. No significant differences ($p<0.05$) were found for specific loaf volumes between 240 and 300 min of fermentation, therefore the time selected as optimal was 240 min. The bread volume increased 17% from 217 mL at 100 min of fermentation time to 252 mL at 240 min of fermentation time, and the pH did not show any significant changes.

At 240 min of fermentation the yeast population increased 3-log cycles resulting in dough rising activity. When dough gets saturated with CO_2, the lactic acid bacteria begin to grow and consequently the pH decreases (FAID *et al.*, 1993). At the fermentation time selected, the lactic acid bacteria population was as low as 6.5×10^3 cfu g^{-1}; consequently the dough pH decrease from 6.5 to 5.8 was not significant which allowed an acceptable spread of the loaf. This is in agreement with POMERANZ *et al.* (1984), who reported that gluten can exert its unique viscoelastic properties better in the relatively neutral wheat dough than in the acidic rye dough. Indeed the optimum pH of carbohydrate-degrading enzymes such as amylase, pentosanase or cellulase varies widely (3.6-5.6) depending on the wheat cultivar and germination status (FOX and MULVIHILL, 1982).

In addition, the crumb texture showed a homogeneous distribution of alveolus, and the sensorial characteristics were appealing (data not shown).

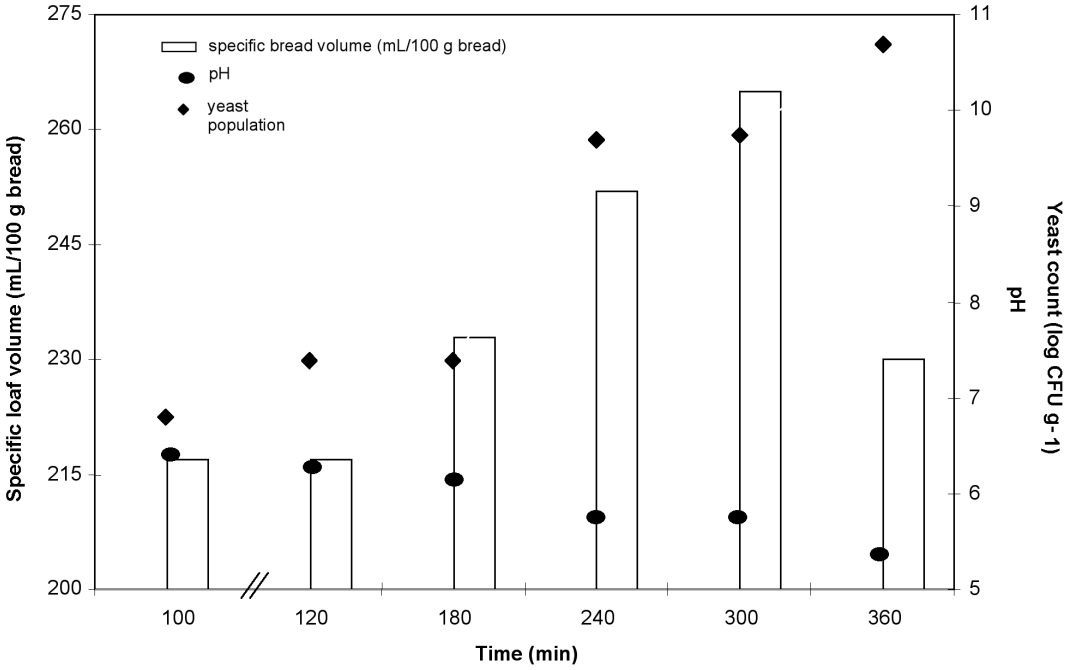

Fig 1 - Specific loaf volume, pH and yeast population at different fermentation times for a 50-50% wholemeal rye-wheat blend.

Effect of conditioners on the quality of bread baked from 50-50% wholemeal rye-wheat blends

Table 2 shows the results of the effect of L-ascorbic acid, L-cysteine and gluten on the specific loaf volume and crust-crumb ratio for the wholemeal rye-wheat breads baked at 240 min fermentation time.

No significant modifications ($p<0.05$) in specific loaf volume were observed when L-ascorbic acid was added at concentrations of 50 and 100 ppm. These results are in agreement with ANDREWS *et al.* (1995) who reported that dough mixing causes gluten to stretch points on the disulfide bonds causing its breakage and resulting in thiol formation. This was detected in farinograph experiments where L-ascorbic acid failed to improve the resistance to mixing soy-wheat dough but improved dough strength during resting. These findings, together with those of JONES *et al.* (1974) suggest that the stretching of disulfide bonds during dough mixing can exceed the effect of the oxidizing agents. The addition of 100 ppm ascorbic acid produced a more acceptable crust crumb ratio compared to that obtained with the addition of 50 ppm.

Slight changes in bread volume occurred with the addition of cysteine at concentrations of 50 and 100 ppm. Pentosans play an especially important role in rye baking. They influence the viscosity and gas retaining ability of doughs (MEUSER and SUKOW, 1986) and retain gas in wheat dough (HOSENEY, 1984). Arabinoxylans, that constitute the major cell-wall polysaccharides of wheat and rye endosperm can form gels by oxidative coupling of their feruloyl groups (GEISSMANN and NEUKON, 1973). Cysteine has been shown to hinder the

Table 2 - Effect of L-ascorbic acid, cysteine and gluten on the specific volume and crust-crumb ratio for wholemeal rye-wheat bread (50-50%) baked after 240 min fermentation time.

Flour blend and conditioners	Specific loaf volume (mL/100 g bread)	Crust/crumb ratio
Wholemeal rye-wheat flour 50-50%	252^{d}	0.20^{d}
Wholemeal rye-wheat flour 50-50% + 50 ppm L- ascorbic acid	262^{c}	0.17^{d}
Wholemeal rye-wheat flour 50-50% + 100 ppm L- ascorbic acid	263^{c}	0.33^{a}
Wholemeal rye-wheat flour 50-50% + 50 ppm L-cysteine	247^{d}	0.18^{d}
Wholemeal rye-wheat flour 50-50% + 100 ppm L-cysteine	253^{d}	0.27^{b}
Wholemeal rye-wheat flour 50-50% + 6% gluten	305^{b}	0.18^{b}
Wholemeal rye-wheat flour 50-50% + 8% gluten	371^{a}	0.25^{bc}
Wholemeal rye-wheat flour 50-50% + 10% gluten	369^{a}	0.23^{cd}

Values are the means of triplicate analyses.
Values with different letters in the same column are significantly different at p=0.05.

oxidative gelation of pentosans in the presence of the H_2O_2/wheat peroxidase (POD) system (HOSENEY and FAUBION, 1981; FIGUEROA-ESPINOZA *et al.*, 1998). Wheat POD has been studied and purified by different authors (JEANJEAN *et al.*, 1975; ZMRHAL and MACHACKOVA, 1978; BILLAUD *et al.*, 1999). In addition LIAO *et al.* (1998) showed that H_2O_2 can be formed by baker´s yeast during fermentation. These findings could explain why no significant modifications were observed in composite bread volume when different amounts of cysteine were added.

Composite breads made from 50-50 wholemeal-wheat blend with 6, 8 and 10% gluten added had specific volumes that were greater than the control, 21, 47 and 46%, respectively. The crust-crumb ratios were acceptable for each addition. The 8 and 10% gluten levels rendered the specific loaf volume increases similar to those obtained for bread made from wheat flour with a 6% gluten addition (PEREZ BORLA *et al.*, 2004). Breads with 10% gluten flour added had dark crusts and a gummy mouth feel, this was also reported by FINNEY (1984). The addition of 8% gluten enhanced the volume without having a detrimental effect on bread quality (Tables 2 and 3).

Bread quality, microbiological and physicochemical analysis on wholemeal rye-wheat composite dough with 8% gluten added

As is shown in Table 3, composite breads without additives were assessed as acceptable by the judges, scoring > 0 for crumb color, smell and taste. The scores for texture and overall acceptance were 0, neither liked nor disliked. Gluten addition at 8% improved the composite bread scores for all the characteristics. Differences between breads with and without gluten were significant at the 95% level for texture and overall acceptability. These findings are in line with the results reported for breads made wheat with 6% gluten added (PEREZ BORLA *et al.*, 2004). However, in sensory scores for crumb color, smell and taste, no significant differences ($p<0.05$) were observed between composite breads made from wholemeal rye-wheat flour (without gluten) and wholemeal rye-wheat flour with 8% gluten added. This last result is supported by microbiological analysis. Dough made with and without 8% gluten added did not show significant differences for the microbial population tested. The counts were $6.5x10^3$ and $4.5x10^3$ cfu g^{-1} for *Lactobacillus* spp., and $8x10^9$

Table 3 - Sensory characteristics of bread prepared from wholemeal rye-wheat flour (50-50%) with and without gluten addition.

Composite bread	Crumb color	Smell	Taste	Texture	Overall acceptability
Wholemeal rye-wheat Flour 50-50% (0% gluten)	0.8[a]	1.1[a]	0.1[a]	0[b]	0[b]
Wholemeal rye-wheat Flour 50-50% + 8% gluten	1.2[a]	1.3[a]	0.4[a]	0.8[a]	1[a]

Organoleptic characteristics were determined by using a five point Hedonic Rating Scale ranging from like a lot (2) to dislike a lot (-2).
Means within each sensory attribute followed by different letters are significantly different (p=0.05).

and $7.5x10^9$ cfu g^{-1} for yeast, respectively, for both doughs. FAID *et al.*, (1993) reported that both lactic acid bacteria and yeasts take part in the bread fermentation process. Many of these microorganisms have metabolic activities that can be associated with the development of typical bread flavor. Similar scores obtained for taste, smell and crumb color are in agreement with the slight changes observed in the microbial counts.

On the other hand, the alveographic technique was used to evaluate and compare the effect of gluten on the extensibility, elasticity and strength of the wholemeal rye-wheat dough (Table 4).

As expected, addition of rye flour had a negative effect on the properties of the wheat flour dough. As the concentration of wholemeal rye increased from 10 to 50% the elasticity of the dough increased slightly, while the extensibility decreased significantly ($p<0.05$). The dough elasticity is related to the consistency, plasticity and water-absorptiveness and the dough extensibility, depending on the elasticity of gluten and the ability of dough to hold gases. The ratio of elasticity to extensibility (P/L) needs to be balanced for leavened bread. A bread dough has to be extensible to allow it to expand during fermentation. However, as expansion occurs, elastic forces produce contraction. The 50% wholemeal rye flour addition to wheat flour increased the dough elasticity ($p<0.05$) from 122.54 to 152.17 mm, but the extensibility decreased markedly. Bran particles disrupted the co-

Table 4 - Alveographic characteristics of wholemeal rye-wheat flour blends with and without gluten.

Flour	0% gluten Elasticity (P) (mm)	8% gluten Extensibility (L) (mm)	Strength (W) ($x10^{-4}$ J)	Elasticity (P) (mm)	Extensibility (L) (mm)	Strength (W) ($x10^{-4}$ J)
Wheat 100%	122 [bd]	76 [bc]	344 [bc]	135 [ad]	88 [ac]	412 [ac]
10% wholemeal rye	142 [bc]	54 [bd]	291 [bd]	170 [ae]	67 [ad]	438 [ac]
25% wholemeal rye	145 [bc]	29 [be]	172 [be]	190 [ac]	36 [ae]	298 [ad]
50% wholemeal rye	152 [c]	23 [f]	162 [f]	Nr	Nr	Nr

Values represent means of triplicate analyses. Nr: not recorded.
Means within a row followed by different letters (a or b) are significantly different (p=0.05).
Means within a column followed by different letters (c, d, e or f) are significantly different (p=0.05).

hesive gluten matrix and weakened the dough (POMERANZ *et al.*, 1984). When 8% gluten was added the strength of the dough increased due to more cross-linked dough and greater resistance to dough breakdown. Stronger dough has more potential to hold the expanding air cells during fermentation, which expands the gluten network to give a porous structure and high loaf volume (BUSUGU *et al.*, 2001)

The addition of 8% gluten increased the strength of the composite dough ($p<0.05$) from $291x10^{-4}$ to $438x10^{-4}$ J (64%) with 10% wholemeal rye and from $172x10^{-4}$ to $298x10^{-4}$ J (70%) with 25% wholemeal rye.

At 50% wholemeal rye substitution with 8% gluten, the alveographic data could not be technically recorded. However, the organoleptic characteristics (Table 3) and loaf volume values (Table 2) improved the rheological properties of the dough, which was supported by results on composite dough with 10 and 25% wholemeal rye substitution (Table 4).

CONCLUSIONS

Gluten was an appropriate conditioner for bread made from 50-50% wholemeal rye-wheat flour. At 8% addition, the scores improved for all the sensory characteristics judged. Texture and overall acceptance increased significantly compared with the control composite bread. This result was in agreement with the improved strength value; therefore dough development characteristics and baking performance were also enhanced. In contrast, no significant differences were observed in the sensory characteristics such as crumb color, smell and taste between breads with and without the addition of gluten. This is in agreement with the slight changes observed in the *Lactobacillus* spp. and yeast counts.

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PAPER

TEXTURAL CHARACTERISTICS OF TYPICAL ITALIAN "GRISSINO STIRATO" AND "RUBATÀ" BREAD-STICKS

CARATTERISTICHE STRUTTURALI DEI GRISSINI TIPICI ITALIANI "GRISSINO STIRATO" E "RUBATÀ"

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ABSTRACT

Stretched ("Grissino stirato") and rolled ("Rubatà") bread-sticks are typical Italian bakery products that are also widely produced in other EU countries. Despite their wide distribution there are no studies available on these products. Therefore, the aim of this study was to distinguish them according to their chemical composition and textural characteristics determined by a compression test using a Texture Analyser. Fifty samples (25 of each kind of bread-stick), produced by different

RIASSUNTO

Il "Grissino stirato" ed il "Rubatà" sono grissini tipici italiani ampiamente prodotti però anche in altri Paesi europei, ma sui quali non sono mai stati svolti studi o ricerche. Lo scopo di questo lavoro è stato quindi quello di caratterizzare dal punto di vista compositivo, ma soprattutto strutturale questi prodotti. La ricerca ha interessato 50 campioni (25 per ciascuna tipologia) e le misure di texture sono state eseguite mediante un Texture Analyser in modalità di compressione. I dati raccolti

- Key words: artificial-neural-network, bread-sticks, grissino, texture analysis -

bakers, were examined. Variance analysis showed that the two bread-sticks only differed with respect to texture parameters. There were no differences in chemical parameters. The higher values of the maximum force and of the average puncturing force indicate that the rolled "Rubatà" bread-stick has a firmer character than the stretched "Grissino stirato" bread-stick. On the contrary, the number of spatial ruptures and the ruggedness index were higher for the stretched "Grissino stirato" bread-stick. For product authentication an Artificial Neural Networks (ANN) with a three-layer, fully interconnected, feed-forward architecture was used. ANN is an effective and powerful tool for distinguishing these products with a very high average learning (94% for rolled bread-stick and 93% for the stretched bread-stick). The model obtained can be easily used for product authentication.

sono stati elaborati mediante l'analisi della varianza ed i campioni sono stati discriminati mediante una Rete Neurale Artificiale a tre livelli con connessioni complete. I maggiori valori di forza di rottura sono stati riscontrati per il grissino "Rubatà" mentre il numero di rotture nell'unità di spazio e l'indice adimensionale di frastagliatura sono risultati maggiori per il "Grissino stirato". La Rete Neurale ha confermato queste differenze evidenziando una capacità di riconoscimento del 94% per il grissino "Rubatà" e del 93% per il "Grissino stirato". Il modello così costruito potrà essere vantaggiosamente utilizzato dagli Organi di Controllo per la definizione dell'origine del prodotto.

INTRODUCTION

Traditional bakery products such as "pizza" from Naples, "panettone" from Milan, "pandoro" from Verona and "bread-sticks" from Turin-Piedmont have a ubiquitous presence on the Italian food market. Some of these items, like pizza and panettone, have been become important symbols of Italian cuisine worldwide. Bread-sticks are also well known and appreciated and rank high among Italian food exports. Bread-sticks are native to the Piedmont region (north-western Italy), where they have been produced since 1600. There are currently two commercial types, "Grissino stirato" and "Rubatà" on the market (SCARLINO and MAINA, 1989). The production technology and raw materials are well-established and the products are very similar. There are no noticeable differences between the two types with respect to chemical composition or appearance but they differ in production technologies and they differ in structure (QUAGLIA, 1984; BARBERIS, 2000) and commercial price. Bread-sticks are crispy products and it is crispness that appears to be the single most versatile characteristic that has determined their success and is also the factor that distinguishes one from the other. Even if crispness has not yet been satisfactorily defined, it is agreed that it is a textural characteristic that has many positive connotations.

Until the 1960s crispness was defined as the quality of fracturing into many small pieces under compressive pressure. It is associated with brittleness. Later, progress was made in optimising

testing methods and refining the vocabulary of crispness, though interchangeable terms describing the texture status are still in use. Most published studies investigating the importance of different sensory perceptions on consumer acceptability conclude that texture, flavour and appearance are the most important sensory modalities (VALLES PAMIES *et al.*, 2000; GÁMBARO *et al.*, 2002; SCHER and HARDY, 2002; BOOTH *et al.*, 2003; KARADZHOV and ISERLIYSKA, 2003; MEULLENET *et al.*, 2003).

In addition to its direct contribution to consumer acceptance, texture has important secondary effects, through modulation of flavour release and its influence on appearance. Texture and food structure are inextricably linked: the micro- and macro-structural composition of foods will determine sensory perception and any change in the structure carries the risk of changing perceived texture and violating consumer expectation.

Based on recently published literature (BASMAN and KÖKSEL, 1999; PIAZZA *et al.*, 2001; BOURNE, 2002; CROWLEY *et al.*, 2002; MURRAY *et al.*, 2002; ZEPPA *et al.*, 2002; RAFFO *et al.*, 2003; SHOGREN *et al.*, 2003; DEMIREKLER *et al.*, 2004; PASQUALONE *et al.*, 2004) the aim of this study was to define the textural characteristics of the two commercial types of typical Italian bread-sticks "Grissino stirato" and "Rubatà" and to develop a model for distinguishing them by using the Artificial Neural Networks (ANNs) approach. As their product aspect is similar they can be easily mistaken by consumers but the "Rubatà" bread-stick purchase price is higher than that of "Grissino stirato" bread-stick. So a technique to distinguish them is very important for product authentication and consumer safeguard. Among the mathematical techniques for prediction, classification or control, the Neural networks provide a powerful tool for characterising, distinguishing and authenticating food products.

ANNs is an information processing paradigm that is inspired by the way biological nervous systems process information and they have more recently been applied in many scientific fields: physics, medicine, botany and food science (BOS *et al.*, 1992; DAWN, 1994; VALLEJO-CORDOBA *et al.*, 1995; HORIMOTO *et al.*, 1997; GERBI *et al.*, 1998; NI and GUNASEKARAN, 1998; MANCUSO *et al.*, 1998; CICCHELLI *et al.*, 2000; MITTAL and ZHANG, 2000; BALESTRIERI *et al.*, 2001). An ANN is a computational system which processes numerical input (training data) through a learning process and converts it into a final output. It is typically organised into "layers" which are made up of a number of interconnected "nodes". The data (in this case the texture values) are usually transferred to ANN via the "input layer" and with a system of "transfer functions" and "node weights". These weights correspond to synaptic efficacy in a biological neuron. Each neuron also has a single threshold value. When the weighted sum of the inputs is formed the threshold is subtracted to compose the activation of the neuron. This activation signal is passed through an activation function and the network computes a response at the output layer. These values are then compared to the actual values of the response variable (in this case the bread-stick classifications) and an error is computed. With a back-propagation algorithm this error is used to adjust the "node weights" and the network computes a new response. The iterative procedure of processing inputs, determining the errors and adjusting the weights is the "learning process". Iteration continues until the network response errors are kept to a minimum. Very important in this architecture is the weight of input neurons that are changed by an amount proportional to the difference between the desired output and the actual output and are a proportional index of importance of

these input neurons for product classification.

Neural networks, with their remarkable ability to derive meaning from complicate or imprecise data, can be used to extract patterns and detect trends that are too complex to be noticed by either humans or other computer techniques. Unlike other mathematical systems, such as Discriminant Analysis or Cluster Analysis, for the classification of different samples, the mathematical model developed in the ANNs can be used independently from the value used for its construction and subsequently applied to product control. In this work ANNs were then applied to the textural characteristics of "Rubatà" and "Grissino stirato" bread-sticks to define a mathematical model that can be used for product characterisation and authentication and consequently for market control.

MATERIALS AND METHODS

Samples

Bread-sticks were produced by fifty Piedmont (north-western Italy) bakers: twenty-five produce stretched bread-sticks or "Grissino stirato" and the other twenty-five produce rolled bread-sticks or "Rubatà".

Two production batches were examined for each baker and a sample of 1 kg of bread-sticks was collected for each production batch.

In the stretched "Grissino stirato" bread-stick technology, flour (type 00), water (40-60%), yeast (0.5-4%), pork fat (0.8-8%), olive oil (0.6-7%), salt (1.3-2.5%) and malt (0.5-3%) are typically used. Ingredients are mixed (about 20 min) and then the dough is divided into small portions. After the fermentation step (about 1 h), cylindrical strips are hand-rolled into bread-sticks. These cylinders are gently lengthened by hand-stretching to about 100 cm. Baking follows at 205°-300°C for 10-30 min. The attribute "stretched" ("stirato") is therefore really due to this unique hand stretching mode that serves to give the dough the peculiar elongated properties characteristic of the final baked structure.

In the rolled "Rubatà" bread-stick technology, flour (type 00), water (50-60%), yeast (0.1-2.6%), pork fat (2-7%), salt (0.8-2.5%) and malt (2-4%) are used. Ingredients are mixed (about 20 min) and then the dough is divided into small portions. After the fermentation step (about 1 h), the dough is formed into cylindrical strips from which the bread-sticks are made. These cylinders are gently rolled by hand to about 50 cm length and then baked at 190°-270°C for 5-20 min. Shaping dough by means of a rolling action promotes relevant biaxial extension stresses in the structure and the consequent viscoelastic properties differ from those of the stretched bread-stick dough where only elongational stress induced in the shaping technology results in more elastic attributes. The rolled "Rubatà" bread-stick can be compared to a special elongated baked crumble bread.

Chemical analysis

Chemical analyses were performed immediately after each production. Bread-stick moisture, ash and protein (nitrogenx5.7) contents were determined according to the AACC methods 44-15A, 08-01 and 46-11A, respectively (AACC, 2000). Fat and starch were determined according to an official Italian method (ITALIAN GOVERNMENT, 1994). Glucose, fructose, lactose and maltose were determined according to ZEPPA *et al.* (2001) with an HPLC system (Thermo Electron Corporation, Waltham, MA, USA) equipped with an isocratic pump (P1000), a multiple autosampler (AS3000) fitted with a 20 μL loop and a refractive index RI-150 detector. Data were collected on a EZChrom ver. 6.6

system (Thermo Electron Corporation, Waltham, MA, USA).

The analyses were performed isocratically at 1 mL/min and 65°C with a 300x7.8 mm i.d. ion exclusion column Aminex HPX-87H equipped with a Cation H^+ Microguard cartridge (Bio-Rad Laboratories, Hercules, CA, USA). The mobile phase was 0.013 N H_2SO_4 prepared by diluting reagent grade sulphuric acid with distilled water, filtered through a 0.20 μm membrane filter (Sartorius AG, Göttingen, Germany) and degassed under vacuum. Five grams of bread-stick were added to 25 mL of 0.013 N H_2SO_4 (mobile phase), the resulting suspension was then extracted for 10 min with a Stomacher (PBI, Milan, Italy). The use of a Stomacher laboratory blender allowed a time reduction because the extraction is considerably more effective than when using a magnetic stirrer. The extract was subsequently centrifuged for 5 min (7,000 g) and the supernatant was filtered through a 0.20 μm disposable syringe membrane filter (Sartorius AG, Göttingen, Germany).

Sugars were represented as the sum of glucose, fructose, lactose and maltose while carbohydrates are treated as the sum of sugars and starch.

Cholesterol was determined by HRGC according to the AOAC 976.26 Method (2003). Analytical grade reagents were used as standards (Sigma-Aldrich Corporation, Milan, Italy).

Image and texture analyses

Bread-sticks used for texture analysis were stored at 20°C and 20% relative humidity and analysed no more than 24 h after production.

The moisture content of each sample was determined before texture analysis according to the AACC methods 44-15A (AACC, 2000).

Five cut specimens (10 mm long) were obtained from each sample to give a total of 250 replicates for each class. Each specimen was cut from the centre of different bread-sticks with an electric hacksaw.

Geometrical measurements of the samples to be tested were taken by means of image analysis techniques. The cross-section image of each side of a specimen was captured with an Epson Perfection 1650 scanner (Seiko-Epson Corporation, Nagano, Japan) at 12,800 dpi in a black and white photo with a 16-bit resolution. Digitalized pictures were analysed by means of the commercial software Sigma Scan Pro rel. 5.0 (Systat Software, Richmond, CA, USA). The cross-section area of the two sides was quantified and the mean value for each specimen was calculated.

For texture analysis, each specimen was analysed in the compression mode using a TA.XT2i Texture Analysis (Stable Micro Systems, Godalming, UK) fitted with a plate-plate geometry (Flat probe P/75; 75 mm diameter). The crosshead speed was 0.9 mm/s and data were acquired with a resolution of 500 Hz. All samples were analysed for 50% deformation.

For the acquisition of the compression stress-strain relationships, Texture Export Exceed software (Stable Micro Systems, Godalming, UK; release. 2.54) was used.

Following VALLES PAMIES *et al.* (2000) and PIAZZA *et al.* (2001), the following parameters were taken from the force/deformation curve:

* Number of spatial ruptures (mm^{-1}): $N_{sr} = N_0 / d$
* Average Puncturing Force (N): $F_m = A/d$
* Crispness work (N mm): $W_c = F_m / N_{sr}$
* Average drop-off (N): $F_s = \Sigma \Delta F / N_0$
* Maximum Force (N): F_{max}
* Fracture work (N mm): W_f
* Ruggedness adimensional index: $RI = l_r / d_r$

where N_0 is the total number of peaks,

d is the compression distance, F_{max} is the value of the higher compression peak, ΔF is the individual force drops for each peak from F_{max} to the defined final compression point, A is the area under the force deformation curve, W_f is the area under the first breaking point; l_r is the length of the line on the force/displacement plot encompassing from F_{max} to the end of the curve and calculated using the Pythagorian theorem ($L_r=\Sigma[(\Delta force)^2+(\Delta length)^2]^{0.5}$) and d_r is the real displacement of the dynamometer cross-head encompassing from F_{max} to the end of the compression curve.

Since the results of the texture analysis are directly correlated to sample humidity, all texture data taken or calculated from the stress/strain curves were normalised for sample humidity, i.e. the values were divided by the sample humidity. Instead F_m, W_c, F_s, F_{max} and W_f indexes were divided by the mean area value of each specimen.

Statistical analysis

Conventional statistical methods were used to obtain a basic statistical evaluation of the chemical and mechanical data. Analysis of Variance (ANOVA) was applied using STATISTICA for Windows Release 7.1 (StatSoft Inc., Tulsa, OK, USA) to determine differences between bread-sticks.

For the Artificial Neural Network (ANN), the software used was NeuroShell 2, Rel. 2 (Ward System Groups Inc., Frederick, MD, USA). The network architecture used was a three-layer, fully interconnected, feed-forward type with 7 nodes in the input, 16 in the hidden and 2 in the output layer. A learning rate of 0.1 and a momentum of 0.1 were used. For the input slab the linear activation function was used while the logistic functions were used for hidden and output layer slabs. Data set inputs were normalised in the -1/+1 range before use in training and testing of the ANN. To avoid network overtraining, NET-PERFECT™ was used. This is an implemented procedure of NeuroShell 2 that creates an entirely separate set of data, called test-set, and is used to evaluate how well the network is predicting. NET-PERFECT™ was used to compute the optimum point for saving the network when it is able to generalise new data well. Testing data were fed into test trained ANN after 200 training epochs. In particular, the network learning was carried out with a limit of 200,000 events after the minimum mean value of re-classification error of the test set was reached. All samples were used for ANNs. These samples were randomly subdivided into a training set (70%) and a validation set (30%). There were approximately 175 samples in the training set and 75 samples in the validation set. The percentage distribution of the samples between the two data sets was chosen empirically and was a compromise between the need to have the maximum number of samples in the training set, while at the same time having all product categories represented in the validation set. The ANN construction process, from the two data set extractions through learning, was repeated five times.

RESULTS AND DISCUSSION

Chemical evaluation

The composition of the typically-produced bread-sticks (Table 1) was similar to that of bread and crackers with low water and fat contents. The standard deviation of data was very high as expected for an artisanal production, particularly due to the variability in the percentage and type of raw materials used by each producer. Hence, there was a statistically significant difference ($p<0.01$) between the stretched bread-sticks and the rolled bread-sticks only with respect to the moisture and protein contents. In

Table 1 - Chemical composition of stretched "Grissino stirato" bread-sticks and rolled "Rubatà" bread-sticks and results of the Analysis of Variance performed between the two products.

	Stretched bread-sticks		Rolled bread-sticks		Significance
	X	σ	X	σ	
Moisture (%)	6.63	1.51	8.62	2.32	**
Ash (without sodium chloride) (% dm)	0.44	0.30	0.62	0.40	*ns*
Energy (kcal/100 g)	369	28	349	28	**
Protein (%)	12.17	0.98	11.02	1.49	**
Carbohydrates (%)	64.90	3.92	64.35	4.46	*ns*
Sugars (%)	3.95	1.76	4.12	1.30	*ns*
Glucose (%)	0.46	0.52	0.73	1.14	*ns*
Fructose (%)	0.22	0.08	0.26	0.12	*ns*
Lactose (%)	0.12	0.03	0.14	0.04	*ns*
Maltose (%)	3.42	1.60	3.31	0.98	*ns*
Starch (%)	60.95	4.67	60.23	4.91	*ns*
Fat (%)	6.70	3.62	5.25	2.68	*ns*
Cholesterol (mg/100 g)	4.07	4.26	3.55	2.49	*ns*

(X mean; σ standard deviation; dm dry matter; ** $p<0.01$; *ns* not significant).

particular, the stretched bread-sticks had the highest protein content, while the rolled bread-sticks had the highest moisture content.

Mechanical evaluation

Bread-sticks have a rigid, slightly-deformable, stiff structure that suddenly collapses with a brittle fracture and a peculiar decay of the forces after the starting fracture point [i.e. the maximum stress (F_{max}) applied in the compression mode test]. Fig. 1 shows typical compression curves for the two types of bread-sticks which are characterised by three zones: 1) a steep ascent of stress with strain culminating in a fracture; 2) a jagged oscillating stress-strain function for a considerable strain range that expresses the crack propagation in crisp structures and involves cell wall break-

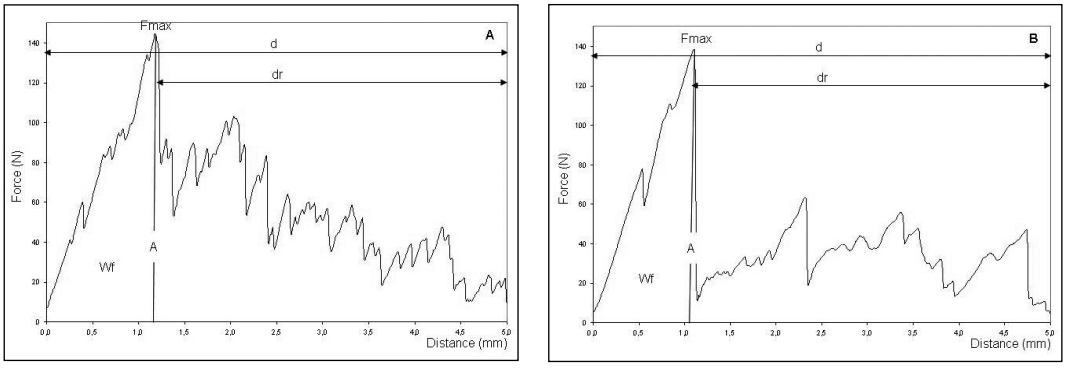


Fig. 1 - Typical force-deformation curves for "Grissino stirato" (A) and "Rubatà" (B) bread-sticks. For symbols see text.

age and 3) a second steep stress with ascent trend (PIAZZA *et al.*, 2001). High values of jaggedness, typical of low moisture foods, are present for both stretched and rolled bread-sticks (BARRETT *et al.*, 1992; ROHDE *et al.*, 1993). Instrumental parameters of crispness are summarised in Table 2. Differences in the shape and in the amplitudes of the force-deformation curves are reflected in the calculated parameters that were tentatively chosen to quantify the brittle fracture behaviour.

The well-established influence of water on all these parameters was taken into consideration by normalising the data on water content. The final moisture content of bread-sticks accounts for both compositional differences, as well as baking time-temperature conditions. This artefact allows the quantified mechanical parameters to be related to the intrinsic and typical macro-structure of the products. The extremely high level of the standard deviation of the mean values for each parameter within the same product typology is due to variation in the composition of bread-sticks from the various manufacturers and the different traditions in the territory. So, the aim of this work was to try to classify these traditional products on the basis of objective measurements, notwithstanding the variability in the particular traditional recipe and processing.

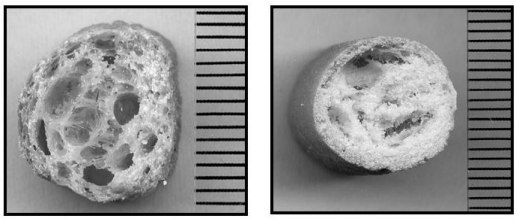
The number of spatial ruptures (N_{sr}) and the ruggedness index (RI) were higher for the stretched "Grissino stirato" bread-stick. This product is characterised by higher porosity compared to the rolled "Rubatà" bread-stick, due to larger cells with thin walls (Fig. 2). On the other hand, the rolling mode applied in the shaping step of the production technology results, from a macro-structure point of view, in a more regular sponge-like network.

The higher values of the maximum force (Fmax), the average drop-off (F_s) and the average puncturing force (F_m) indicate that the porous structure of the rolled "Rubatà" bread-stick is firmer than that of the stretched "Grissino stirato" one. Work (W_c) is a derived parameter of crispness ($W_c = F_m/N_{sr}$). Its significance might be doubtful because of the extremely high level of the statistical standard deviation, but its mean values differentiate the rolled bread-sticks and classify them as the less crisp product.

Table 2 - Values of the mechanical parameters for stretched "Grissino stirato" bread-sticks and rolled "Rubatà" bread-sticks and results of the Analysis of Variance performed between the two products.

	Stretched bread-sticks		Rolled bread-sticks		Significance
	X	σ	X	σ	
N_{sr}	1.172	0.467	0.716	0.316	**
F_m (× 10E5)	4.788	2.519	7.697	4.988	**
W_c (× 10E3)	5.062	0.478	1.099	2.032	**
F_s	0.011	0.006	0.015	0.013	**
RI	28.208	11.785	21.613	10.98	**
F_{max}	0.151	0.081	0.229	0.117	**
W_f (× 10E5)	8.767	5.413	14.75	13.45	**

(X-mean; σ-standard deviation; ** $p<0.01$).
Legend (see text for definitions): N_{sr}: Number of spatial ruptures (mm^{-1}); F_m: Average Puncturing Force (N); W_c: Crispness work (N mm); F_s: Average drop-off (N); RI: Ruggedness adimensional index; F_{max}: Maximum Force (N); W_f: Fracture work (N mm).



A B

Fig. 2 - Digitalized images of stretched "Grissino stirato" bread-stick (A) and rolled "Rubatà" bread-stick (B) sections.

The degree of jaggedness of the force-deformation curves for numerous cooked crisp cereal foods (crackers, snack foods, puffed extrudates and bread-sticks) has been quantified by using various approaches, in particular, Fractal Analysis and Fast Fourier Transform Analysis (BARRETT *et al.*, 1992; SCHER *et al.*, 2004) or peak analysis (SRISAWAS *et al.*, 2003). In this work the peak analysis approach was used and the results were then examined with ANNs.

ANNs constructed with the experimental texture analysis parameters show a very high average learning (94% for the rolled "Rubatà" bread-stick and 93% for the stretched "Grissino stirato" bread-stick) with small problems in the ANNs self-reconfiguration and only 6-7% reclassification errors. To evaluate the importance of each textural parameter on ANNs activities their "node weights" for input layers were extracted for each cycle and mean values for the five learning process were calculated (Table 3). As the total weight for each input neuron is directly correlated to the weight of the connection and the network structure the most important parameters for the ANNs construction are N_{sr}, F_m, F_{max} and W_c, while F_s, RI and W_f are less important. This confirms the texture differences between the two classes of products; in particular the rolled "Rubatà" bread-stick has a higher hardness attribute and the stretched "Grissino stirato" bread-stick has a higher friability.

CONCLUSIONS

Food authenticity and the determination of the geographic origin are two aspects that are receiving increased attention in food technology. In the bakery product sector, due to the absence of a definite production protocol for traditional, regional and home-made bread-sticks, many imitations have been produced to the detriment of the Piedmont producers and the image of this product.

Results obtained in this work do not definitively solve authenticity problems, but they clearly show that the traditional stretched "Grissino stirato" and traditional rolled "Rubatà" bread-sticks are two well-defined, distinguishable bakery products even though the artisan manufacturers' production modes are responsible for high variability.

Texture analysis performed by means of an objective instrumental method is a consolidated approach for quantifying the crispness attributes of these types of low-moisture bakery products and can define their characteristics, above all when combined with advanced ANN models. With this approach a mathematical model can be defined that can also be applied by food regulation authorities for product authentication. In particular rolled bread-sticks are stiffer

Table 3 - Values of weight coefficient calculated for the mechanical parameters used in the ANNs.

N_{sr}	0.458
F_m	0.323
W_c	0.318
F_s	0.194
RI	0.217
F_{max}	0.279
W_f	0.215

Legend (see text for definitions): N_{sr}: Number of spatial ruptures (mm^{-1}); F_m: Average Puncturing Force (N); W_c: Crispness work (N mm); F_s: Average drop-off (N); RI: Ruggedness adimensional index; F_{max}: Maximum Force (N); W_f: Fracture work (N mm).

than stretched bread-sticks which, on the other hand, are characterised by a higher crumbliness.

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SHORT COMMUNICATION

ANTIMICROBIAL ACTIVITY OF THE LICHENS *ASPICILIA CINEREA*, *COLLEMA CRISTATUM*, *OCHROLECHIA ANDROGYNA*, *PHYSCIA AIPOLIA* AND *PHYSCIA CAESIA*

ATTIVITÀ ANTIMICROBICA DEI LICHENI *ASPICILIA CINEREA*, *COLLEMA CRISTATUM*, *OCHROLECHIA ANDROGYNA*, *PHYSCIA AIPOLIA* E *PHYSCIA CAESIA*

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ABSTRACT

The antibacterial and antifungal activities of acetone, methanol and aqueous extracts of the lichens *Aspicilia cinerea*, *Collema cristatum*, *Ochrolechia androgyna*, *Physcia aipolia* and *Physcia caesia* were tested *in vitro* against six bacterial and 11 fungal species. Antimicrobial activity was checked by the disk diffusion test and by determining the

RIASSUNTO

Sono state testate in vitro le attività antibatteriche e antifungine dell'acetone, del metanolo e degli estratti acquosi dei Licheni *Aspicilia cinerea*, *Collema cristatum*, *Ochrolechia androgyna*, *Physcia aipolia* e *Physcia caesia*, nei confronti di sei specie batteriche e undici fungine. Le attività antimicrobiche sono state determinate tramite il metodo di

- Key words: antibacterial and antifungal activity, lichen extracts -

minimal inhibitory concentration (MIC) by the broth tube dilution method. In general, methanol extracts inhibited the greatest number of microorganisms tested. The methanol extract of *Aspicilia cinerea*, in relation to *Klebsiella pneumoniae* and its acetone extract against *Bacillus mucoides* had the lowest MIC (0.09 mg/mL of extract). Aqueous extracts of all of the lichens had the lowest activity. They were inactive against all of the tested organisms, with the exception of the aqueous extract of *Physcia aipolia* (which only inhibited some of the bacteria tested) and that of *Collema cristatum* (which inhibited all of them). Aqueous extracts of the lichens did not have antifungal activity.

diffusione su piastra e determinando la concentrazione inibitoria minima (MIC) tramite il metodo di diluizione in brodo di coltura (in provetta). In generale, gli estratti metanolici mostravano capacità inibitoria più alta nei confronti dei microrganismi testati. L'estratto metanolico di *Aspicilia cinerea* (nei confronti di *Klebsiella pneumoniae*) e il suo estratto acetonico (nei confronti del *Bacillus mucoides*) presentavano la MIC più bassa (0,09 mg/mL di estratto). Gli estratti acquosi di tutti i licheni presentavano l'attività più bassa. Essi risultavano inattivi contro tutti gli organismi testati, ad eccezione dell'estratto acquoso di *Physcia aipolia* (che inibiva solo alcuni dei batteri saggiati) e quelli di *Collema cristatum* (che li inibiva tutti). Gli estratti acquosi dei licheni non presentavano attività antifungina.

INTRODUCTION

Lichens are symbiotic organisms composed of a fungus (the mycobiont) and one or more algae (photobionts). About 17,000 species have been recorded worldwide (HUNECK, 1999). They commonly grow on rock surfaces, on poorly developed soils such as those found in arid lands and boreal-arctic regions, and as epiphytes on trees and shrubs (TAYLOR *et al.*, 1995).

Lichens are an important food for many animals, including man (RICHARDSON, 1991). The topical use of lichen extracts goes back to ancient Egyptian times (VARTIA, 1973). Many different bioactive secondary metabolites have also been isolated from lichen species (CULBERSON, 1969; DEMBITSKY *et al.*, 1992; HUNECK and YOSHIMURA, 1996; HUNECK 1999). They have also been used in pharmaceutical sciences (MÜLLER, 2001).

Certain metabolites of lichens are presumed to protect these organisms from herbivores and pathogenic microorganisms (LAWREY, 1986; 1989). It has been demonstrated that some lichen metabolites are toxic and repellent to insect larvae (EMMERICH *et al.*, 1993), while others exert nematocidal action (AHAD *et al.*, 1991). It has also been suggested that sequestered lichen compounds in insects may be utilized for chemical defense (HESBACHER *et al.*,1995). As certain phenolic compounds produced by lichens strongly absorb UVB light, these agents are used as photoprotectors (FERNANDEZ *et al.*, 1996), and their antioxidant capacity (HIDALGO *et al.*, 1994) warrants their use as preservatives in cosmetic creams.

They are used in alcohol production, in the perfume and drug industries, and in folk medicine (VARTIA, 1973; RICHARDSON, 1988). Several lichen extracts have been used for various remedies in folk medicine, and screening tests with

lichens have indicated the frequent occurrence of metabolites with antibiotic, antimycobacterial, antiviral, antitumor, analgesic and antipyretic properties (VARTIA, 1973; LAWREY, 1986; 1989; INGOLFSDOTTIR *et al.*, 1995; 1997 and 1998; HUNECK, 1999; MÜLLER, 2001).

Owing to the pronounced antimicrobial activity of their secondary metabolites, lichens together with algae, macrofungi, and higher plants, are attracting considerable attention as significant new sources of bioactive substances (MITSCHER *et al.*, 1987; HOSTETTMAN *et al.*, 1997; INGOLFSDOTTIR *et al.*, 1997).

The purpose of the present study was to investigate the antimicrobial activity of methanol, acetone and aqueous extracts of five different species of lichens, *Aspicilia cinerea*, *Collema cristatum*, *Ochrolechia androgyna*, *Physcia aipolia* and *Physcia caesia*, in relation to a number of microorganisms, including agents of human, animal and plant diseases, mycotoxin producers, and food spoilage agents.

MATERIALS AND METHODS

Lichen samples

Samples of the lichens *Aspicilia cinerea* (L.) Körb., *Collema cristatum* (L.) FH Wigg, *Ochrolechia androgyna* (Hoff.) Arnold, *Physcia aipolia* (Ehrh. ex Humb.) and *Physcia caesia* (Hofffm.) Fürnr. were collected on Mt. Zabljak (Montenegro) in August of 2005. The samples were dried at room temperature for 24 h. Documentation samples were deposited in the mycological herbarium of the Department of Biology, Faculty of Science, University of Kragujevac (MHDB): *Aspicilia cinerea* (L.) Körb. 189; *Collema cristatum* (L.) FH Wigg 204; *Ochrolechia androgyna* (Hoff.) Arnold 177; *Physcia aipolia* (Ehrh. ex Humb.) 291; and *Physcia caesia* (Hofffm.) Fürnr. 312.

Aspicilia cinerea contains the "lichen substances" norstictic acid (CULBERSON, 1969), and stictic acid (BRODO *et al.*, 2001). "Lichen substances" were not extracted from the lichen *Collema cristatum*, although it is believed that the photobiont can synthesize some metabolites (TORRES *et al.*, 2003). Extract of the lichens *Ochrolechia androgyna* contains norstictic acid (CULBERSON, 1969) and stictic acid (BRODO *et al.*, 2001). *Physcia aipolia* contains the "lichen substances" atranorin and zeorin (CULBERSON,1969), while *Physcia caesia* contains atranorin, leucotylin and zeorin (WALKER and LINTOTT, 1997).

Preparation of lichen extracts

Three solvents (water, methyl alcohol and acetone) were used to extract the lichens. To obtain aqueous extracts, dried lichen thalli (50 g of material from each species, separately) were ground to a particle size of <2.5 mm and flushed with distilled water. Extraction was performed in a Soxhlet extractor at 80°C for 7 h. The extract obtained was filtered through Whatman No. 1 filter paper and evaporated in a water bath at 80°C until dry material was obtained. Dry extract was obtained in a quantity of 2.8 g (*Aspicilia cinerea*); 3.8 g (*Collema cristatum*); 2.1 g (*Ochrolechia androgyna*); 3.1 g (*Physcia aipolia*) and 4.32 g (*Physcia caesia*).

Acetone extracts. Powdered lichens (50 g) were extracted with 250 mL acetone using the Soxhlet extractor for 7 h at a temperature less than the boiling point (56.2°C). The acetone extracts were filtered using Whatman No. 1 filter paper. The extracts obtained were evaporated on a rotary vacuum evaporator until a solid concentrate was recovered. Dry extract was obtained in a quantity of 0.73 g (*Aspicilia cinerea*); 2 g (*Collema cristatum*); 2.2 g (*Ochrolechia androgyna*); 2.27 g (*Physcia aipolia*) and 2 g (*Physcia caesia*).

Methanol extracts. Powdered lichens (50 g) were extracted with 250 mL me-

thanol using the Soxhlet extractor for 7 h at a temperature less than the boiling point (LIN *et al.*, 1999). The methanolic extracts were filtered using Whatman No. 1 filter paper and then concentrated in vacuum at 40°C using a Rotary Evaporator. Dry extract was obtained in a quantity of 4.64 g (*Aspicilia cinerea*); 3.2 g (*Collema cristatum*); 2.3 g (*Ochrolechia androgyna*); 7.36 g (*Physcia aipolia*) and 7.00 g (*Physcia caesia*).

The extracts were further dissolved in dimethyl sulfoxide (DMSO) for the disk diffusion test, and minimal inhibitory concentrations (MIC) were determined by making a series of dilutions in an appropriate substrate ranging of from 0.05 to 50 mg/mL. The final concentration of DMSO did not exceed 2%.

Microorganisms and media

The test organisms used in this study were the following: *Bacillus mycoides* (IPH), *Bacillus subtilis* (IPH), and *Staphylococcus aureus* (IPH) (Gram-positive bacteria); and *Enterobacter cloaceae* (IPH), *Escherichia coli* (IPH), and *Klebsiella pneumoniae* (IPH) (Gram-negative bacteria). All of the bacteria used were isolates from the National Institute for Protection of Health (IPH) in Kragujevac (Serbia). Their identification was confirmed in the Microbiological Laboratory, Department of Biology, University of Kragujevac. The following fungi were used in the study: *Aspergillus flavus* (ATCC 9170), *Aspergillus fumigatus* (DBFS 310), *Botrytis cinerea* (DBFS 133), *Candida albicans* (IPH 1316), *Fusarium oxysporum* (DBFS 192), *Mucor mucedo* (ATCC 52568), *Paecilomyces variotii* (ATCC 22319), *Penicillium purpurescens* (DBFS 418), *Penicillium verrucosum* (DBFS 262), *Saccharomyces cerevisiae* (DBFS 234) and *Trichoderma harsianum* (DBFS 379). The fungi came from the mycological collection of the Mycological Laboratory, Department of Biology, Faculty of Science, University of Kragujevac (DBFS). Bacterial cultures were maintained on Mueller-Hinton agar from Torlak (Belgrade, Serbia). Fungal cultures were maintained on potato dextrose agar and Sabourad dextrose agar from Torlak. All cultures were preserved at 4°C and subcultured every 15 days.

Antimicrobial activity

The sensitivity of microorganisms to extracts of different species of lichens was ascertained by the standard disk diffusion method approved by the National Committee for Clinical Laboratory Standards (BAUER *et al.*, 1966 or NCCLS, 1993). The minimal inhibitory concentrations (MIC) of the extracts were also determined.

Bacterial inocula were obtained from bacterial cultures incubated for 24 h at 37°C on Meuller-Hinton substrate and diluted to approximately 10^8 CFU/mL in relation to the 0.5 McFarland standard. Suspensions of fungal spores were prepared from fresh mature (3- to 7-day-old) cultures reared at 30°C on a PDA substrate. Spores were rinsed with sterile distilled water. Turbidity of spore suspensions was determined spectrophotometrically at 530 nm, after which they were further diluted to approximately 10^6 CFU/mL according to the recommended procedure (NCCLS, 1998).

Disk diffusion was conducted as follows: suspensions of microorganisms were poured over solid nutrient substrates, viz., Mueller-Hinton agar (for bacteria) and Sabourad dextrose agar (for fungi), in Petri dishes, 10 cm in diameter.

Filter paper disks (7 mm in diameter) were soaked with 15 μL of a lichen extract (50 mg/mL) and placed on the inoculated substrates. After incubation at 37°C for 24 h in the case of bacteria and at 27°C for 2-14 days in the case of fungi, the diameter of the zone of inhibition of the tested microorganisms by the given extract was measured. Disks soaked

with 15 μL of DMSO in the same concentration as that of the lichen extract solution were used as a negative control. Streptomycin was used as a positive control of growth in the case of bacteria, while ketoconazole was used as a positive control of growth in the case of fungi. All experiments were performed in duplicate. Diameters of the inhibition zones were measured in millimeters and are expressed as mean values.

The minimal inhibitory concentration (MIC) was determined by the broth tube dilution method. The concentration of lichen extracts for MIC determination ranged from 50 mg/mL (starting concentration) to 0.05 mg/mL (final concentration). A series of 12 sterile test tubes with stoppers (1 to 12) was used for each extract against each microorganism tested. Two mL of a solution of a certain lichen extract (50 mg/mL) was put in the test tube, after which 1.0 mL of serial Mueller-Hinton broth (for bacteria) or Sabourad dextrose broth (for fungi) was added to each of the other test tubes. A measured volume (mL) of the dissolved lichen extract was then transferred from the first tube to the second one. The contents of the second tube were stirred with a special sterile pipette, after which 1 mL was transferred to the third tube. Dilution was carried out in this manner up to tube 11; the pipette was changed each time. After this, 1 mL of contents of tube 11 was removed and discarded. The 12th tube served as a control with no lichen extract (it only contained 1 mL of the substrate). Twenty μL of a bacterial or fungal culture were then added to each tube (1-12). The tubes were subsequently incubated at 37°C for 24 h in the case of bacteria or at 27°C for 2-12 days in the case of fungi. The minimal inhibitory concentration was determined by evaluating the visible growth of microorganisms in the tubes. The boundary dilution without any visible growth was defined as the minimal inhibitory concentration (MIC) for the tested microorganism at the given concentration of a certain lichen extract. The starting concentration in studying the antimicrobial activity of lichen extracts was 1 mg/mL. All experiments were performed in duplicate.

RESULTS

The antimicrobial activity of acetone, methanol and aqueous extracts of the lichens *Aspicilia cinerea*, *Collema cristatum*, *Ochrolechia androgyna*, *Physcia aipolia* and *Physcia caesia* against the tested microorganisms was determined on the basis of the presence or absence of inhibitory zones, and their diameter (Table 1) and MIC values (Table 2).

The acetone and methanol extracts of *Aspicilia cinerea* manifested antibacterial activity. They inhibited all of the tested bacterial species except *Staphylococcus aureus*, which was resistant. The largest zones of inhibition (24 mm in diameter) were obtained with the acetone extract against the species *Bacillus subtilis*. Minimal inhibitory concentrations (MIC) ranged from 1.56 to 6.25 mg/mL of extract in relation to the tested bacteria. Extracts of this lichen acted selectively on the tested fungi. Antifungal activity was manifested against five of the 11 fungal species tested. The diameter of inhibition zones ranged from 12-18 mm for the acetone extract and 11-16 mm for the methanol extract. The MIC values varied from 0.78 to 25 mg/mL of extract. The aqueous extract of this lichen had no inhibitory activity in relation to any of the tested microorganisms. Our results are similar to those reported by LAND and LUNDSTROM (1998), MADAMOMBE and AFOLAJAN (2003) and RANKOVIĆ and MIŠIĆ (2007), who likewise recorded weak antimicrobial activity for aqueous extracts of different lichen species against the tested species of bacteria and fungi.

The *Collema cristatum* extracts manifested antibacterial activity against the

Table 1 - Antimicrobial activities of different lichen extracts using the disk diffusion method.

Organisms	Lichen species															Antibiot.	
	A. cinerea			*C. cristatum*			*O. androgyna*			*Ph. aipolia*			*Ph. caesia*				
	A[a]	B	C	A	B	C	A	B	C	A	B	C	A	B	C	S	K
Bacillus mycoides	18[b]	13	-	15	16	21	35	28	-	16	15	14	23	18	-	28	-
Bacillus subtilis	24	13	-	13	15	22	29	20	-	16	15	13	18	22	-	26	-
Enterobacter cloacae	19	16	-	13	16	18	36	27	-	12	14	13	21	17	-	25	-
Escherichia coli	22	20	-	14	13	20	13	11	-	20	20	-	-	-	-	15	-
Klebsiella pneumoniae	18	21	-	14	13	30	35	31	-	17	30	13	25	20	-	40	-
Staphylococcus aureus	-	-	-	14	14	23	18	16	-	16	14	-	24	18	-	20	-
Aspergillus flavus	-	-	-	-	-	-	-	-	-	12	12	-	-	20	-	-	27
Aspergillus fumigatus	-	-	-	-	-	-	-	-	-	12	11	-	20	23	-	-	34
Botrytis cinerea	-	-	-	-	-	-	-	-	-	14	12	-	-	16	-	-	39
Candida albicans	14	12	-	-	-	-	20	14	-	17	12	-	-	15	-	-	40
Fusarium oxysporum	12	11	-	-	-	-	20	9	-	14	12	-	16	18	-	-	35
Mucor mucedo	16	14	-	-	-	-	-	-	-	-	-	-	-	-	-	-	17
Paecilomyces variotii	-	-	-	-	-	-	-	-	-	14	13	-	22	20	-	-	40
Penicillium purpurescens	-	-	-	-	-	-	-	-	-	11	9	-	-	-	-	-	38
Penicillium verrucosum	-	-	-	-	-	-	12	11		18	16	-	20	20	-	-	36
Saccharomyces cerevisiae	18	16	-	-	-	-	-	-	-	10	10	-	17	15	-	-	30
Trichoderma harsianum	14	11	-	-	-	-	-	-	-	-	-	-	14	14	-	-	18

[a] A - acetone extract; B - methanol extract; C - aqueous extract - all 50 mg/mL.
[b] Diameter of inhibition zone (mm) including disk diameter of 7 mm. Values are the means of three replicates.
Antibiotics: K - ketokonazole, S - streptomycin, 1 mg/mL.

Table 2 - Minimum inhibitory concentration (MIC) of the lichen extracts against the test organisms expressed as mg/mL.

Organisms	Lichen species															Antibiot.	
	A. cinerea			*C. cristatum*			*O. androgyna*			*Ph. aipolia*			*Ph. caesia*				
	A[a]	B	C	A	B	C	A	B	C	A	B	C	A	B	C	S	K
B. mycoides	3.12[b]	3.12	-	25	12.5	6.25	0.09	0.39	-	0.39	0.78	1.56	0.78	1.56	-	7.81	-
B. subtilis	3.12	1.56	-	25	12.5	6.25	0.19	1.56	-	0.78	0.19	1.56	0.19	0.39	-	7.81	-
E. cloacae	3.12	1.56	-	25	6.25	6.25	0.39	0.19	-	0.39	0.39	0.19	0.78	0.78	-	1.95	-
E. coli	1.56	1.56	-	25	12.5	12.5	12.5	25	-	25	25	-	-	-	-	31.25	-
K. pneumoniae	6.25	6.25	-	12.5	6.25	6.25	0.31	0.09	-	0.19	0.19	0.19	0.39	0.78	-	1.95	-
S. aureus	-	-	-	25	12.5	6.25	1.56	1.56	-	1.25	12.5	-	0.62	0.39	-	31.25	-
A. flavus	-	-	-	-	-	-	-	-	-	6.25	25	-	-	3.12	-	-	3.9
A. fumigatus	-	-	-	-	-	-	-	-	-	6.25	12.5	-	-	3.12	-	-	3.9
B. cinerea	-	-	-	-	-	-	-	-	-	1.56	3.12	-	-	6.25	-	-	1.95
C. albicans	0.78	1.56	-	-	-	-	6.25	25	-	1.56	6.25	-	-	1.56	-	-	1.95
F. oxysporum	25	25	-	-	-	-	3.12	50	-	6.25	25	-	6.25	3.12	-	-	3.9
M. mucedo	0.78	3.12	-	-	-	-	-	-	-	-	-	-	-	-	-	-	31.25
P. variotii	-	-	-	-	-	-	-	-	-	1.56	3.12	-	1.56	3.12	-	-	1.95
P. purpurescens	-	-	-	-	-	-	-	-	-	6.25	25	-	-	-	-	-	3.9
P. verrucosum	-	-	-	-	-	-	25	12.5	-	3.12	12.5	-	3.12	6.25	-	-	3.9
S. cerevisiae	0.78	0.78	-	-	-	-	-	-	-	12.5	12.5	-	3.12	3.12	-	-	1.95
T. harsianum	12.5	25	-	-	-	-	-	-	-	-	-	-	6.25	12.5	-	-	7.81

[a] A - acetone extract; B - methanol extract; C - aqueous extract.
[b] Minimum inhibitory concentration (MIC); values given as mg/mL for lichen extract and as µg/mL for antibiotics.
Antibiotics: K - ketokonazole, S - streptomycin, µg/mL.

tested bacterial species. The strongest antibacterial activity was manifested by the aqueous extract, and the most sensitive bacterial species was *Klebsiella pneumoniae* (whose diameter of inhibition zone measured 30 mm). The MIC values ranged from 6.26-25 mg/mL of extract. Extracts of this lichen did not inhibit the tested fungal species. In agreement with our results, TURK *et al.* (2003) and YILMAZ *et al.* (2005) reported antibacterial activity for the lichen extracts, but no inhibitory action on filamentous fungi. Contrary to these results, SHAHI *et al.* (2003) found an aqueous extract of the lichen *Parmelia cirrhatum* to be very active against certain pathogenic fungi and recommended it as a medicine against external fungal infections in humans.

The acetone and methanol extracts of *Ochrolechia androgyna* exerted strong inhibitory action on all of the tested bacteria. The largest zones of inhibition were recorded with the acetone extract, which were especially large in the case of *Enterobacter cloaceae* (36 mm). The MIC values for the acetone and methanol extracts of this lichen in relation to the tested bacteria ranged from 0.09 to 25 mg/mL of extract. With MIC values of 0.09 mg/mL, the acetone extract manifested maximal activity against the species *Bacillus mucoides* and *Klebsiella pneumoniae*. The minimal inhibitory concentration in relation to the tested fungal species ranged from of 3.12-50 mg/mL. Each of these extracts acted on only three of the fungal species tested. The aqueous extract was inactive in relation to all of the tested organisms. GULLUCE *et al.* (2006) reported that ethanol extracts of five different lichen species manifested varying antimicrobial activity against the tested bacteria and fungi, which is in agreement with these results.

Extracts of the lichen *Physcia aipolia* had selective, but relatively strong antimicrobial activity. The acetone and methanol extracts acted on all of the tested bacteria, the largest zones of inhibition (with diameter of 30 mm) were recorded for the methanol extract in relation to the bacterial species *Klebsiella pneumoniae*. The MIC values ranged from 0.19-25 mg/mL of extract. The aqueous extract did not act on the bacteria *Escherichia coli* and *Staphylococcus aureus*, nor did it exert any antifungal action. The acetone and methanol extracts of this lichen manifested significant antifungal activity toward nine of the 11 fungal species tested. The MIC values ranged from 1.56-25 mg/mL of extract.

The acetone and methanol extracts of *Physcia caesia* exerted strong antibacterial action on all of the tested bacteria except *Escherichia coli*, which was resistant. The antifungal activity of these extracts was selective, the acetone extract inhibited six of the tested fungal species, and the methanol extract nine. The aqueous extract of this lichen did not manifest antimicrobial activity. BRODO (2001) reported that crude extracts of lichens of the genus *Physcia* have antibiotic properties and inhibit the bacterial species *Staphylococcus aureus* and *Bacillus subtilis*, which was confirmed by our investigations.

The results obtained in these investigations indicate that the antimicrobial activity of lichen extracts differs as a function of both the species of lichen in question and the extracting solvent used. Similar differences were also noticed by other investigators (INGOLFSDOTTIR *et al.* 1997; TURK *et al.*, 2003; YILMAZ *et al.*, 2004 and 2005; RANKOVIĆ and MIŠIĆ , 2007). Except in the case of *Collema cristatum*, aqueous extracts from all of the investigated lichen species exerted the weakest inhibitory action on the tested microorganisms. The reason for the weak activity of aqueous extracts is that active substances present in lichen thalli are insoluble or poorly soluble in water (KINOSHITA *et al.*, 1994).

CONCLUSIONS

Different crude extracts of the lichens studied moderately and, in some cases, significantly inhibited the tested microorganisms, the majority of which are pathogens of man, animals or plants. The results indicate which kinds of extracts of the lichen species possess potentially isolable components with antibacterial and antifungal properties. Naturally, if the active ingredients of different extracts of these lichens prove to be isolable, it can then be asserted that the lichens in question are an interesting new source of bioactive substances. They could be used to treat bacterial and fungal infections and diseases ad an alternative to synthetic antimicrobial medicines.

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SHORT COMMUNICATION

ANTIBACTERIAL ACTIVITY OF A STABLE STANDARDIZED *IN VITRO* CULTURE OF *RUBUS ULMIFOLIUS* SCHOTT AGAINST FOOD-BORNE PATHOGENIC BACTERIA

ATTIVITÀ ANTIBATTERICA DI UNA COLTURA *IN VITRO* STANDARDIZZATA DI *RUBUS ULMIFOLIUS* SCHOTT SU MICRORGANISMI PATOGENI ALIMENTARI

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ABSTRACT

Methanolic extracts of micropropagated *Rubus ulmifolius* plantlets were tested for antimicrobial activity against different strains of food-borne pathogenic bacteria. Crude methanolic extract effectively reduced the growth (up to 70 and 90%) of seven *Escherichia coli* and five *Salmonella* strains, and caused a 40% and

RIASSUNTO

È stata saggiata l'attività antimicrobica di un estratto metanolico ottenuto da plantule di *R. ulmifolius* micropropagate in vitro, su diversi ceppi di batteri patogeni contaminanti gli alimenti. L'estratto riduceva efficacemente (dal 70 al 90%) la crescita di sette ceppi di *Escherichia coli* e cinque di *Salmonella*, mentre inibiva ri-

- Key words: antibacterial activity; micropropagated plantlets; *Rubus ulmifolius* -

10% growth inhibition on one strain of *Pseudomonas* spp. and *Listeria monocytogenes*, respectively. Tannin-rich fractions, obtained by crude extract fractionation, were active against *E. coli* (about 65% inhibition), while fractions containing chlorogenic acid or caftaric acid and caffeoyl derivatives were almost ineffective (about 10% inhibition). The results suggest that *in vitro* cultures of *R. ulmifolius* could be a source of secondary metabolites to be used as preservatives in food products and as antimicrobial agents.

spettivamente del 40 e del 10% la crescita di un ceppo di *Pseudomonas* spp. e *Listeria monocytogenes*. Frazioni dell'estratto ricche in tannini sono risultate attive contro *E. coli* (circa il 60% di inibizione), mentre quelle contenenti acido clorogenico o acido caftarico e caffeoil-derivati, risultavano inefficaci (circa 10% di inibizione). I risultati ottenuti suggeriscono un possibile impiego delle colture *in vitro* di *R. ulmifolius* per l'estrazione di composti dotati di attività conservante ed antimicrobica per uso alimentare.

INTRODUCTION

In recent years, particular attention has been given to antimicrobial compounds of plant origin. Several of these molecules are synthesized by the plant in response to microbial attack (ZABEL and MORREL, 1992). Many studies have been conducted on plant extracts (HEISEY and GORMAN, 1992; HERALD and DAVIDSON, 1983; MASOOD *et al.*, 1994; SATO *et al.*, 1994) to evaluate their antimicrobial activity and their potential use in various applications, including food preservation (HERRMANN, 1989; MASOOD *et al.*, 1994). In particular, it has been reported that extracts of *Rubus* (blackberry) exhibit antimicrobial activity on bacteria and fungi (McCUTCHEON *et al.*, 1994; RAUHA *et al.*, 2000). *Rubus ulmifolius* (Rosaceae) is a perennial shrub with deciduous leaves, common in shady and uncultivated areas in the northern hemisphere and present in Italy from 0 to 1,100 m a.s.l. (PIGNATTI, 1982). Antimicrobial activity of extracts with increasing polarity of *Rubus ulmifolius* Schott was observed by PANIZZI *et al.* (2002); crude methanolic extract showed a wide range of activity against Gram+ and Gram- bacteria, and yeasts, while n-hexane-, chloroform-, and chloroform/methanol 9:1- extracts were less active. The highest activity was exhibited by the phenolic and tannin fractions (McCUTCHEON *et al.*, 1994; PANIZZI *et al.*, 2002). The presence of different flavonoids and flavonoid glycosides in leaves, branches and flowering tops of *Rubus* species, associated with a marked inhibitory activity against various Gram+ and Gram- bacteria has also been demonstrated (PANIZZI *et al.*, 2002).

Despite the widespread distribution of *R. ulmifolius*, the quantity of plant (such as leaves, young branches, and flowering tops) material suitable for the extraction of secondary metabolites is strictly dependent on the season. Moreover, it is well-known that the secondary metabolite contents of medicinal plants fluctuates with changing environmental and geophysical conditions (FONSECA *et al.* 2006). Therefore, the conditions required for a useful application are: the availability of large amounts of plant material, year-round, that is homogeneous in secondary metabolite content and free of phytopathogens and pesticides. For this reason, we have developed micropropagation protocols for *R. ulmifolius* to obtain uniform material, that is easy to handle and monitor, and has a polyphenol content and an antioxidant activity similar to those in *in vivo* grown plants (FRATERNALE *et al.*, 2006). The activity of the methanolic extract (crude and frac-

tionated) obtained from micropropagated *R. ulmifolius* plantlets against several human food-borne pathogenic bacteria is reported.

MATERIAL AND METHODS

In vitro plant culture

Plantlets were induced from nodal explants taken from young branches of *Rubus ulmifolius* Schott according to FRATERNALE *et al.* (2006). Explants were cultured in MS medium (MURASHIGE and SKOOG, 1962) supplemented with 3 mg L^{-1} N^6-benzyladenine (BA) and 0.2 mg L^{-1} naphthaleneacetic acid (NAA) and incubated in a growth chamber at 25°C ± 2 in the light (cool white fluorescent light at 50 μmol m^{-2} s^{-1}) under a 16-h photoperiod. The newly formed shoots were dissected and transferred to the same medium supplemented with 0.1 mg L^{-1} BA to promote growth. Finally, elongated shoots were transferred to half-strength hormone-free MS medium for root initiation (rooting phase). Shoots and plantlets were maintained in a growth chamber under the same conditions reported above.

Methanolic extract and fractions

Thirty-day-old *R. ulmifolius* plantlets in the rooting phase were collected, deprived of roots and dried at room temperature. The dried pulverized material was successively subjected to extraction under the same conditions reported by McCUTCHEON *et al.* (1994). Briefly, 40 g of powder were extracted in 100 mL of methanol with 3 washes of 100 mL. The crude methanolic extract (300 mL) was first filtered through a Buchner funnel using N. 4 filter paper. The filtrate was evaporated to dryness on a rotary evaporator and then reconstituted with 10 mL of methanol. Therefore, each millilitre of the concentrated methanolic extract corresponded to 4 g of extracted dry material.

The crude methanolic extract was fractionated by Sephadex LH-20 column chromatography with the resolution of 5 peaks (F1-F5) corresponding to phenolic fractions differentiated on the basis of their hydrophobic characteristics. Total phenolics were determined with Folin-Ciocalteu reagent according to the method of SINGLETON *et al.* (1999). Each chromatographic fraction was analyzed with an HPLC equipped with an UV/diode array detector as reported by SISTI *et al.* (2005); the main phenolic compounds were identified on the basis of the wavelength on which the maximum UV-Vis absorption was observed and by the use of commercial standard molecules. Each fraction (F1-F5) was adjusted to 35 mg mL^{-1} of phenolics which was the concentration of the working solution of the whole methanolic extract.

Microbial cultures

Fourteen bacterial strains were tested: five clinical isolates of *Salmonella* genus (*S. newport*, *S. meleagridis*, *S. derby*, *S. london*, and *S. bredeney*); seven clinical isolates of *Escherichia coli* (three verocytotoxigenic, VTEC, strains: 1952, F146 and ATCC 35150; two heat labile toxin-producing strains: 100B and 68B; two strains without associated virulence factors: EC1, EC2, used as control). One strain of *Pseudomonas* spp. and one strain of *L. monocytogenes* (ATCC 9525).

The strains collected from clinical samples and food poisoning cases were supplied by the Istituto Zooprofilattico Umbria e Marche (all the toxin-producing *E. coli*, *L. monocytogenes* and *Salmonella* strains) and by the Urbino Hospital (EC1, EC2 and *Pseudomonas* spp.). All the microorganisms were routinely grown in our laboratory on Tryptone Soya Agar (TSA, Oxoid).

Evaluation of antimicrobial activity of methanolic extract and fractions

Bacterial cultures ($5x10^5$ CFU mL^{-1})

were grown in K medium [1% glucose, 1% casamino acids, 1 μg mL^{-1} thiamine hydrochloride, 25 μg mL^{-1} $MgSO_4 \cdot 7H_2O$ and 2 μg mL^{-1} $CaCl_2$ in M9 salts; M9 salts: 34 mM Na_2HPO_4, 22 mM KH_2PO_4, 8.5 mM NaCl, 7.5 mM $(NH_4)_2SO_4$] in the presence of different concentrations of the extract (0.25-8 μg μL^{-1}) for 1-24 h at 37°C with agitation. Growth control (GC) with a corresponding volume of methanol was included. After different contact times, cultures were serially diluted in M9 salts and plated in tri- and quadruplicate onto TSA. After 24 h incubation at 37°C, colony-forming units (CFU) were counted and the percentages of growth inhibition were calculated compared to the GC.

Antimicrobial activity of fractions F1-F5 was evaluated on the EC1 strain of *E. coli* with the same procedure and at the same concentrations of phenolics used for the crude methanolic extract.

Statistical analysis

Analysis of variance was performed by 1-way ANOVA following the Bonferroni post-hoc test. Differences in the data were considered statistically significant at $P<0.05$.

RESULTS

The antibacterial effect of different concentrations of the crude methanolic extract was initially tested on *S. Newport*, *L. monocytogenes*, *Pseudomonas* spp. and on the EC1 strain of *E. coli*.

Dose-response curves are reported in Fig. 1. A marked inhibitory effect of the extract was observed on S. Newport and *E. coli* (Fig. 1A and B), in particular, at concentrations higher than 1 mg mL^{-1} and within 5 h. This effect significantly diminished after long-term exposure (24 h), after which bacterial cells started to grow again (data not shown). The growth of *Pseudomonas* spp. was moderately affected by the extract (Fig. 1C), whereas *L. monocytogenes* was mildly susceptible (data not shown).

In subsequent experiments, the methanolic extract was tested on a panel of different strains of the genus *Salmonella* and pathogenic *E. coli*. Fig. 2 shows that the growth of all strains tested was inhibited by the extract, with a reduction greater than 98% at the highest dose (8 mg mL^{-1}) after 5 h.

The analysis of the phenolic fractions obtained by chromatographic separations

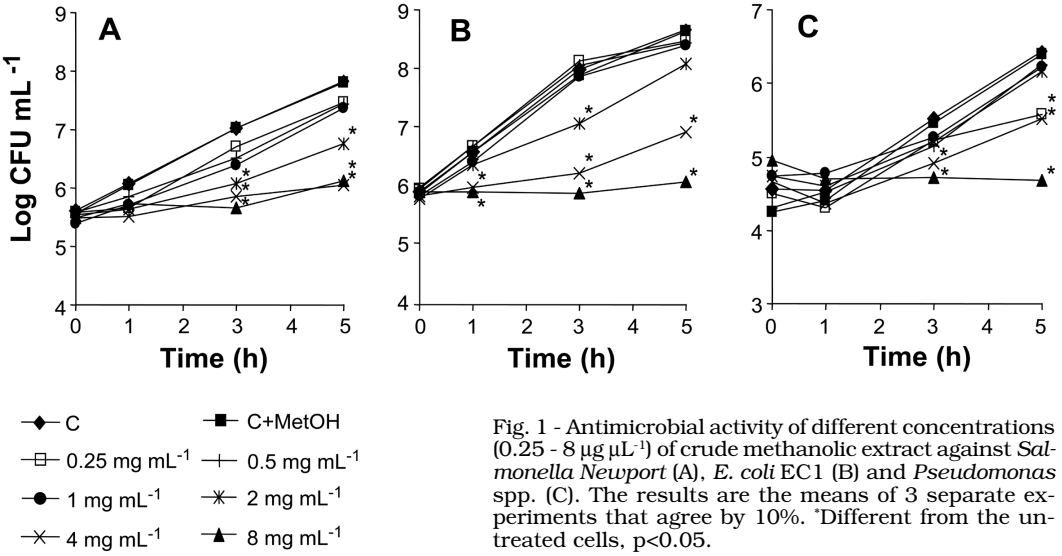

Fig. 1 - Antimicrobial activity of different concentrations (0.25 - 8 μg μL^{-1}) of crude methanolic extract against *Salmonella Newport* (A), *E. coli* EC1 (B) and *Pseudomonas* spp. (C). The results are the means of 3 separate experiments that agree by 10%. *Different from the untreated cells, $p<0.05$.

from methanolic extract showed that the main components of F1-F2 were chlorogenic and caftaric acid, respectively; caffeoyl derivatives were found in F3, while fractions F4-F5 were rich in tannins. F5 was the most active against *E. coli* EC1, in partial accordance with PANIZZI *et al.* (2002), with a 65% inhibition after 3 h. Finally, fractions rich in caffeoyl derivatives and caftaric acid were ineffective, while F4 slightly inhibited (about 10%) the growth of the EC1 *E. coli* strain.

DISCUSSION

R. ulmifolius Schott contains substances that have antimicrobial properties against bacteria and fungi (McCUTCHEON *et al.*, 1994; RAUHA *et al.*, 2000). However, the concentrations of the active ingredients can be markedly influenced by environmental and intrinsic factors, resulting in a pronounced variability in the antimicrobial activity.

In this study the antibacterial properties of a methanolic extract obtained from *in vitro* micropropagated *R. ulmifolius* plantlets was evaluated. This extract effectively reduced the growth of all the *E. coli* and *Salmonella* strains tested with a maximum effect after 5 h of treatment when an inhibition of at least 2 log was observed. However, the antimicrobial effect seemed to be bacteriostatic; indeed the bacterial growth resumed after long-term exposure (24 h).

According to the results obtained by McCUTCHEON *et al.* (1994) and PANIZZI *et al.* (2002), tannin-rich fractions are the most active against *E. coli*. In the present study, fractions containing chlorogenic acid, caftaric acid and caffeoyl derivatives had only a slight activity or none at all.

Previous studies have shown that the activation of the phenolic pathway is critically involved in plant defense responses against phytopathogenic microorganisms (BROWN and MORRA, 1997; NITA-LAZAR *et al.*, 2004). The present work

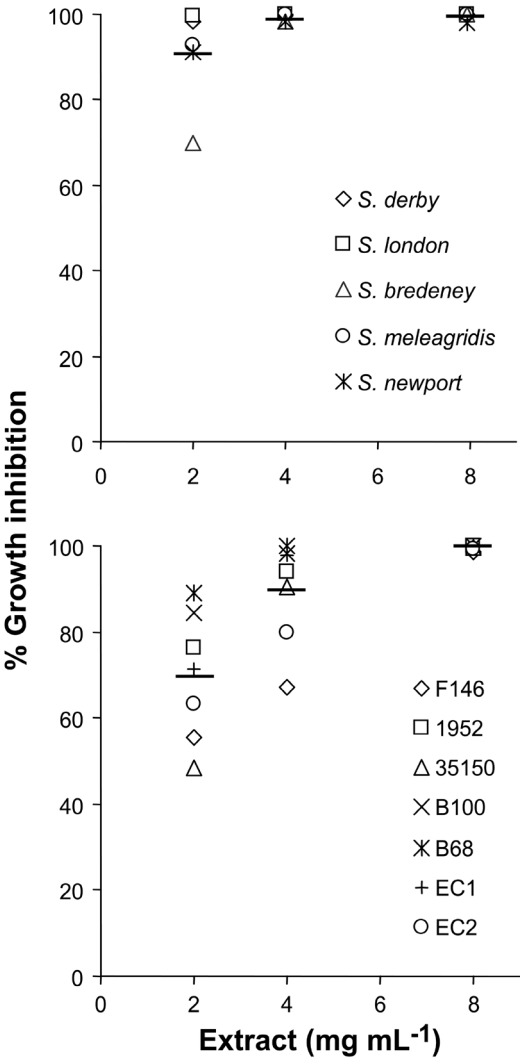

Fig. 2 - Antimicrobial activity of crude methanolic extract (2-8 mg mL^{-1}) on different strains of *E. coli* and *Salmonella* spp. Each point represents the mean of a triplicate determination.

highlights the effect of phenolic agents of plant origin against microorganisms that are pathogenic for humans and animals. The mechanisms thought to be responsible for phenolic toxicity to microorganisms include enzyme inhibition by the oxidized compounds, possibly through reaction with sulfhydryl groups, and the subsequent impairment of processes of energy production and structural component

synthesis, by weakening or destroying the permeability barrier of the cell membrane or by altering the physiological status of the cells and affecting nucleic acid synthesis (CUTTER, 2000).

The availability of a stable *in vitro* culture of *R. ulmifolius* plantlets, would therefore provide molecules, most probably in the form of phytocomplexes, that can inhibit the growth of some of the most common Gram-bacteria implicated in foodborne diseases. Thus, the main object of future work will be to evaluate the antimicrobial activity of *R. ulmifolius* extract and its fractions in different food systems. The activity of the substances could be modified by an interaction with food components, such as fats, carbohydrates, proteins, and salts as well as by pH value, as already reported by some authors for other plant extracts (CUTTER, 2000; HOLLEY and PATEL, 2005; DUPONT *et al.*, 2006). Furthermore, the absence of negative effects on the organoleptic properties of *R. ulmifolius* extracts should be investigated.

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FATTY ACID AND CONJUGATED LINOLEIC ACID PROFILES OF INFANT FORMULAS THROUGH DIRECT TRANSESTERIFICATION OF ACYL LIPIDS

PROFILI DEGLI ACIDI GRASSI E DELL'ACIDO LINOLEICO CONIUGATO IN FORMULATI PER L'INFANZIA MEDIANTE TRANSESTERIFICAZIONE DIRETTA DI GRASSI ACILICI

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ABSTRACT

Fatty acid (FA) values, including C8:0, C10:0, C12:0, C13:0, C14:0, C16:0, C18:0, C18:1*t*, C18:1*c*, C18:2, C18:2 *CLA*, C20:0, C18:3 and C22:0, in sixteen infant formulas were determined through direct transesterification of acyl lipids with sodium methoxide by capillary gas chromatography. Total FA values ranged from 250.25-256.06 mg/g sample and the conjugated linoleic acid (CLA) (*cis*-9, *trans*-

RIASSUNTO

Sono stati determinati per transesterificazione diretta dei lipidi acilici con metossido di sodio e gas cromatografia capillare, i valori degli acidi grassi, inclusi il C8:0, C10:0, C12:0, C13:0, C14:0, C16:0, C18:0, C18:1*t*, C18:1*c*, C18:2, C18:2 *CLA*, C20:0, C18:3 e C22:0, in sedici formulati per l'infanzia. Nei formulati per l'infanzia i valori degli acidi grassi totali variavano da 250,25-256,06 mg/g e i valo-

- Key words: Conjugated linoleic acids, fatty acids, infant formulas -

11 octadecadienoic acid) values were 1.41-2.02 mg/g sample (p<0.01) in the infant formulas. The total saturated fatty acid, monounsaturated fatty acid and polyunsaturated fatty acid values were also obtained. The C18:3 (linolenic acid (n-3)) values were 0.63-0.88 mg/g sample, whereas the C18:2 (linoleic acid (n-6)) values ranged from 35.64-35.82 mg/g sample. FA standard mix including CLA had linear calibration curves through the origin (R^2 = 0.9999). The precision of the analytical method was (using C13:0, methyl tridecanoate, as internal standard) within the 95% confidence limits and the mean recoveries determined for individual fatty acids in infant formulas varied from 99.8 to 100%.

ri dell'acido linoleico coniugato (CLA) (acid *cis*-9, *trans*-11 octadecadienoico) erano 1,41-2,02 mg/g (p<0.01). Sono stati inoltre determinati i valori totali di acidi grassi saturi, monoinsaturi e poliinsaturi. I valori di C18:3 (acido linolenico (n-3)) erano 0,63-0,88 mg/g, mentre i valori di C18:2 (acid linoleico (n-6)) erano 35,64-35,82 mg/g. La miscela di acidi grassi standard, incluso il CLA, presentava una retta di calibrazione lineare passante per l'origine (R^2 = 0,9999). La precisione del metodo analitico (utilizzando il C13:0, metiltridecanoato, come standard interno) era all'interno del limite di confidenza per il 95% e la media dei recuperi per ogni acido grasso nelle formule per l'infanzia variava fra il 99,8 e il 100%.

INTRODUCTION

Infant milk formulas are designed to provide infants with the required nutrients for optimal growth and development. Follow-on infant milks are formulated to be included as part of a mixed feeding diet for term infants from the age of 4 months upwards (EC COMMISSION DIRECTIVE, 1996). These milks are usually marketed as spray-dried powders to be reconstituted with water, or as ready-to-feed infant formulas. Many fatty acids are known to be very important in early childhood development. Follow-on infant milks can be considered as one of the principal sources of essential fatty acids (FAs) during this stage of life (AKALIN and TOKUSOGLU, 2004; TOKUSOGLU *et al.*, 2002). Therefore, their identification and quantification using a quick analytical method is crucial. Total nutritional fat analysis has generally included extraction of the crude fat and gravimetric analysis, but this concept changed with the implementation of the Nutritional Labeling and Education Act (NLEA) of 1990 (CANTELLOPS *et al.*, 1999).

Several methods have been established to determine fatty acids in foods. According to these methods, fats are extracted from the matrix with organic solvents, and the extracted fats are saponified with acid or base hydrolysis to free FAs, that are then methylated to form fatty acid methyl esters (FAMEs) (FOLCH *et al.*, 1957; LANZA *et al.*, 1980; HOUSE *et al.*, 1994). In the methods that use either acid hydrolysis or base hydrolysis or a combination of both depending upon sample matrix, the lipid extract is hydrolyzed by refluxing for 30 min or longer. The sample obtained is extracted with ether and the free FAs are then methylated 30 min or longer with boron trifluoride (BF_3) or other sillilated reagents; the

FAMEs obtained are then analyzed by gas chromatography (GC). The various stages are time-consuming, labor intensive and require laboratory resources. In addition, hazardous materials are used in these methods which are dangerous for the environment. Therefore, it is necessary to develop new procedures for effective analysis.

The objective of this study was to determine the FAMEs and conjugated linoleic acids (CLA) of term follow-on infant formulas including spray-dried powders and ready-to-feed formulas consumed in Turkey. Direct transesterification of acyl lipids was used to optimize the extraction and capillary GC procedure.

MATERIALS AND METHODS

Material

Sixteen term follow-on infant formulas including different brands (*Nestle, Milupa, Hero, Ülker)* were obtained from Turkish, German and Swiss companies which manufacture infant foods. These samples included spray-dried powders to be used in feeding bottles and teats, ready-to-feed infant formulas such as spoon-fed baby foods and complementary formulas. Production and expiration dates for all infant formulas were similar. FA analyses were performed in triplicate.

Standards and equipment

n-Hexane (analytical grade), methanol (99%) (CH_3OH) (analytical grade), sodium hydrogen sulfate monohydrate ($NaHSO_4.H_2O$) and sodium chloride (NaCl) were purchased from E. Merck Co. (Darmstadt, Germany), transesterification solution (sodium methoxide) ($NaOCH_3$) from Sigma Chemical Company (Poole, Dorset, UK), Supelcowax-10 capillary GC column (30 m x 0.25 mm ID x 0.25 μm df) from Phenomenex, Zebron (Los Angeles CA, USA), FAMEs standard solution and triglyceride internal standard solution C13:0, methyl tridecanoate ($C_{42}H_{80}O_6$) from Sigma Chemical Company (Poole, Dorset UK).

Direct extraction of lipids and methylation from a dry matrix of sample

The direct extraction methylation procedure was modified from previously published methods with infant formula powder (CANTELLOPS *et al.*, 1999), dry grain products (LONG *et al.*, 1988) and milk and milk product standard (ISO/DIS 1987). According to our procedure, the internal std. C13:0 (methyl tridecanoate) (300 μL) was transferred to a screw-capped reaction vial and evaporated to dryness with N_2. The dry infant food matrix (0.5 g) was put into this vial. Two mL of 0.05 N sodium methoxide ($NaOCH_3$) in methanol+hexane (4/2 v/v) mixture were prepared and 2 mL of this mixture were added to infant formula+ internal std. matrix. The reaction mixture in the test tube was heated at 80°C for 10 min with shaking. The mixture was then equilibrated to room temperature (25±1°C). The methyl ester phase was perfectly separated and the upper organic phase (including FAMEs in hexane) was transferred (1 mL) into a GC vial. Subsequently, FAMEs containing CLA were analyzed (1 μL) by GC. With this procedure, the hexane is responsible for extracting the lipids from the dry matrix of the infant foods. Simultaneously, the $NaOCH_3$ transesterified the extracted acyl lipids to form FAMEs. Care was taken to prevent the liquid from evaporating from the reaction media.

Chromatographic GC conditions

The capillary GC procedure for subsequent FA profiles and CLA was described by TOKUSOGLU *et al.* (2003).

Column	:	SGE (BP70X fatty acid column), 60 m capillary; 0.25 μm; 0.25 mm WCOT fused-silica
Model	:	Perkin Elmer (Auto System) model gas chromatograph
Column temp	:	
Initial temp.	:	150°C (0 min isotherm)
Ramp 1	:	175°C (2 min isotherm) (15°C/min)
Ramp 2	:	220°C (15 min isotherm) (2.2°C/min)
Split rate	:	50:1
Col.pressure	:	23 psi
Flow rate	:	1.5 mL/min
Carrier gas	:	Helium (He)
Inject.temp.	:	250°C
Detect. temp.	:	250°C (Flame Ionization Detector) (FID)
Inject.volume	:	1 μL

Analytical quality control

The retention times (RT) of the peaks were compared to the retention times of pure standards. Peak identity was confirmed when peak retention times were identical to those of the pure standard FA mixture. Analytical precision (using C13:0, methyl tridecanoate, as internal standard) was within the 95% confidence limits. Linearities of FA standard solutions were determined in triplicate (in the range 5, 10, 50, 100, 200, 400, 600 μL) ($p<0.01$). Recovery studies of FAs including CLA were performed in triplicate by spiking the extraction solution of the sample (two different samples of Ülker Hero Baby) with varying quantities of FA standard mixture ranging from 5 to 20 μg/mL (50% of the measured content) prior to analysis.

Statistical analysis

Data were analyzed with Statistica for Windows using a one-way analysis of variance (ANOVA) (STATISTICA, 1998).

RESULTS AND DISCUSSION

The analytical parameters of the method are given in Table 1. The FA standard mixture including CLA had linear calibration curves through the origin (R^2 = 0.9998-0.9999). Mean recoveries determined from the individual FAs in infant formulas varied from 99.8 to 100%. This is a rapid, interference-free, reproducible method for quantitatively identifying FAMEs in infant food.

FAMEs of 16 infant formulas consumed in Turkey were determined by using a simultaneous extraction-derivatization procedure that included base-media transesterification by capillary gas chromatographic separation. The GC chromatogram of FAMEs obtained from a follow-on infant milk (*Milupa*) is shown in Fig. 1.

Table 2 shows the fatty acid composition of the sixteen infant formulas. In general, the infant formulas contained similar amounts of individual fatty acids. Total FA values ranged from 250.25 to 256.06 mg/g sample. (All calculations were performed with an internal standard $C_{13:0}$).

Total saturated fatty acid (SFA) content values ranged from 127.55 to 131.31 mg/g sample and constituted 50.76-51.42% of the total FAs in the infant formulas. These fatty acids are considered to be energy sources or substrates for the synthesis of intermediate compounds (GIOVANNINI *et al.*, 1991).

Table 1 - The analytical parameters of FAs and CLA in the infant formulas.

		FAs	CLA
Linearity	R^2	0.9998-0.9999	0.9999
Interday precision *(n=9)*	μg/100 g infant formula	1.33±0.00 -1.35±0.01	1.35±0.01
Relative Std. Deviation	RSD (%)	1.08-1.19	1.18
Recovery *(n=6)*	(%)	99.8%-100.0%	100.0%
Detection limit	μg/100 g infant formula μg 100 mL^{-1} assay	0.090-0.100 0.050-0.055	0.100 0.050

KOLETZO and BREMER (1989) and HAYAT *et al.* (1999) reported similar values for the percent of SFA in the total FA content (51 and 51.35%, respectively) of infant formulas. In the present study, palmitic acid ($C_{16:0}$) did not constitute the highest proportion of SFA in infant formula samples (between 20.03 and 22.02%), in contrast to the findings of HAYAT *et al.* (1999) who found that half the source of the SFA (50.8%) was palmitic acid. Most of the SFA came from lauric acid ($C_{12:0}$) in all samples. However the intermediate fatty acids, lauric acid ($C_{12:0}$) and myristic acid ($C_{14:0}$), did not exceed the recommended value (15% of FA) (EC COMMISSION DIRECTIVE, 1991) in any of the infant formulas.

Monosaturated fatty acids (MUFA) are known to supply energy and serve as structural membrane components; this explains the high proportion of these fatty acids (> 30%) in both human and formula milk fat. Elaidic $C_{18:1t}$ and oleic $C_{18:1c}$ acids were determined as MUFA in infant milk formulas. Although oleic acid was the most abundant fatty acid in the samples, its proportion of the total FAs (ranging from 18.50 to 18.97%) were lower than those reported by other researchers (HAYAT *et al.*, 1999; LOPEZ-LOPEZ *et al.*, 2002). The same researchers reported similar values for the proportion of MUFA in the total FA content of infant formulas.

Polyunsaturated fatty acids (PUFA) were determined as linoleic acid - $C_{18:2}$ (n-6), linolenic acid - $C_{18:3}$ (n-3) and CLA. The content of the essential fat-

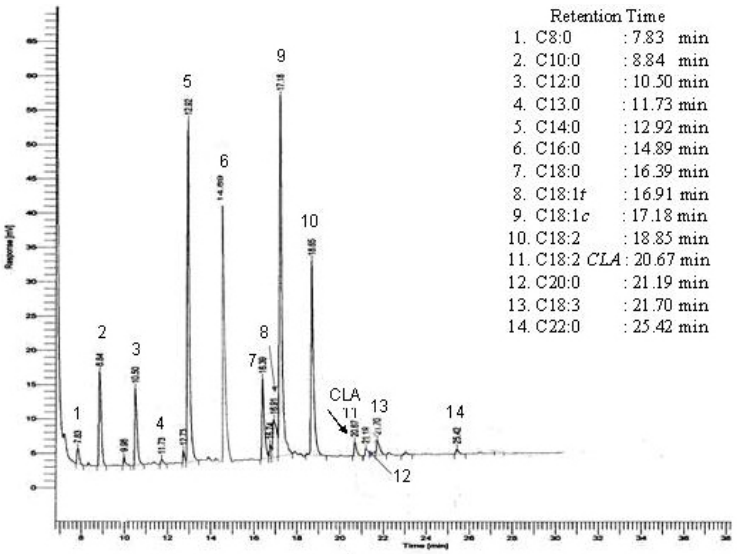

Fig. 1 - GC chromatogram of FAMEs from a follow-on infant milk (Milupa).

ty acid, linoleic acid ranged from 35.64 to 35.82 mg/g sample and made up 13.97-14.29% of the total FA content. The ratios meet the criteria recommended by the EC COMMISSION DIRECTIVE (1991) and LSRO (1998). In contrast, the proportion of linolenic acid in the total FA content was less than 1% which is less than the criteria determined by FAO/WHO (1994), LSRO (1998) and the HEALTH COUNCIL of the NETHERLANDS (2001).

HAYAT *et al.* (1999) and LOPEZ-LOPEZ *et al.* (2002) found similar linoleic acid levels in infant formulas, while the linolenic acid levels were higher.

The 18-carbon fatty acids, α-linolenic and linoleic acids, are converted into long-chain PUFAs by a series of elongation and desaturation steps. The dietary ratio of linoleic acid to α-linolenic acid is more important than the absolute intake of either of these essential fatty acids because they compete for the same enzyme for further desaturation and chain-elongation to form biologically active LCPUFAs. The current study shows that the linoleic acid/α-linolenic acid ratio ($C_{18:2}$ (n-6)/ $C_{18:3}$ (n-3)) ranged from 56.59 to 40.68; the ratios were not within the 1:5 and 1:15 range suggested by the ESPGHAN COMMITTEE ON NUTRITION (1991). On the other hand, these linoleic acid to α-linolenic acid ratios are extremely high compared to other studies that reported a mean value of 13.49±5.50 and in the range of 4-13, resp. (HAYAT *et al.*, 1999; LOPEZ-LOPEZ *et al.*, 2002). HAYAT *et al.* (1999) also reported high linoleic acid to α-linolenic acid ratios in three infant milk formulas.

Table 2 - Fatty acid methyl esters (FAMEs) in infant formulas as mg/g sample.

Infant Formula	C8:0 (Caprylic)	C10:0 (Capric)	C12:0 (Lauric)	C13.0 *Methyl tridecanoate*	C14:0 (Myristic)	C16:0 (Palmitic)	C18:0 (Stearic)	C18:1*t* (Elaidic)	C18:1*c* (Oleic)	C18:2 (Linoleic)	C18:2 (CLA)	C20:0 (Arach)	C18:3 (Linolenic)	C22:0 (Behenic)
No:1	8.13	6.17	36.97	5.50	15.62	26.76	27.90	38.43	47.61	35.65	1.70	0.42	0.63	0.50
No:2	8.28	6.26	37.62	5.75	14.98	27.55	27.87	37.92	47.80	35.79	1.68	0.36	0.76	0.48
No:3	8.08	6.35	37.71	5.51	15.07	28.98	27.96	38.54	47.38	35.80	2.00	0.38	0.88	0.46
No:4	8.36	6.17	36.99	5.56	15.26	26.68	27.86	37.76	46.85	35.75	1.41	0.40	0.70	0.50
No:5	8.17	6.22	37.28	5.82	15.83	28.16	27.89	39.00	47.23	35.64	1.56	0.38	0.82	0.51
No:6	8.42	6.45	38.66	5.68	14.89	27.11	27.95	37.67	47.54	35.69	1.95	0.37	0.69	0.47
No:7	8.40	6.51	37.85	5.57	15.62	26.68	27.93	39.14	46.92	35.73	1.62	0.40	0.81	0.46
No:8	8.33	6.13	38.09	5.84	15.94	25.93	27.91	38.36	47.65	35.82	1.90	0.36	0.73	0.49
No:9	8.12	6.19	37.80	5.56	14.92	26.09	27.97	38.68	46.97	35.77	1.52	0.43	0.77	0.47
No:10	8.10	6.25	39.00	5.87	15.75	25.98	27.86	39.03	47.98	35.68	1.73	0.39	0.69	0.48
No:11	8.15	6.53	38.54	5.79	15.37	27.75	27.95	38.56	47.65	35.76	2.02	0.37	0.80	0.46
No:12	8.11	6.05	37.95	5.53	14.96	28.62	27.87	39.23	46.99	35.74	1.85	0.42	0.85	0.48
No:13	8.10	6.20	39.05	5.66	15.57	27.64	27.94	37.80	47.75	35.81	1.68	0.38	0.73	0.47
No:14	8.39	6.47	38.49	5.60	15.65	26.17	27.88	38.15	47.63	35.69	1.93	0.41	0.66	0.47
No:15	8.37	6.28	37.72	5.83	14.97	26.43	27.95	37.95	47.84	35.67	1.53	0.37	0.80	0.46
No:16	8.41	6.42	38.50	5.55	15.73	27.90	27.94	39.05	47.70	35.78	1.77	0.39	0.75	0.47

CLA is an important fatty acid for human health. This physiological fatty acid was detected in all samples and the levels varied between 1.41 and 2.02 mg/g sample (Table 2). McGUIRE *et al.* (1997) found lower CLA values in infant formula preparations.

Long-chain derivatives of linoleic acid (n-6) LCP and α-linolenic acid (n-3)LCP were not detected in our follow-on infant formulas. LCPUFAs (arachidonic acid and docosahexaenoic acid) make up a major proportion of the phospholipid content in the cell membranes of the retina and brain. Their supplementation in infant formula is under debate. Many committees of experts and professional organizations have recommended that arachidonic acid and docosahexaenoic acid should be added to infant formulas (ALLES *et al.*, 2002), while WROBLE *et al.* (2002) concluded that LCPUFA supplements should not be added to formulas for term, typically-developing infants. HAYAT *et al.* (1999) and LOPEZ-LOPEZ *et al.* (2002) detected arachidonic acid in only one out of fourteen infant formulas and docosahexaenoic acid in four out of eleven infant formulas.

Part of this study was presented at the 2002 AOCS "World Conference and Exhibition on Oilseed and Edible, Industrial and Specialty Oils", August 12-15, Military Museum, Istanbul, Turkey.

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