ISSN 1120-1770

ITALIAN JOURNAL OF FOOD SCIENCE

Rivista italiana di scienza degli alimenti

IJFS

Volume XIX
Number 3
2007

Spedizione in ab. post. comma 26 - art. 2 - legge 549/95 n. 3/2007 - Torino

CHIRIOTTI EDITORI

ITALIAN JOURNAL OF FOOD SCIENCE

(RIVISTA ITALIANA DI SCIENZA DEGLI ALIMENTI)

Property of the University of Perugia
Official Journal of the Italian Society of Food Science and Technology
Società Italiana di Scienze e Tecnologie Alimentari (S.I.S.T.Al)
Initially supported in part by the Italian Research Council (CNR) - Rome - Italy
Recognised as a "Journal of High Cultural Level"
by the Ministry of Cultural Heritage - Rome - Italy

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Publisher:
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E-mail: info@chiriottieditori.it - URL: www.chiriottieditori.it

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Review Policy:
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Frequency: Quarterly - One volume in four issues. Guide for Authors is published in each number and annual indices are published in number 4 of each volume.

Impact Factor: 0.506 published in the 2006 Journal of Citation Reports, Institute for Scientific Information

Subscription Rate: 2007: Volume XIX

Ordinary	€ 150.00
Supporting	€ 1,000.00

IJFS is abstracted/indexed in: Chemical Abstracts Service (USA); Foods Adlibra Publ. (USA); Gialine - Ensia (F); Institut Information Sci. Acad. Sciences (Russia); Institute for Scientific Information; CurrentContents®/AB&ES; SciSearch® (USA-GB); Int. Food Information Service - IFIS (D); Int. Food Information Service - IFIS (UK); EBSCO Publishing.

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PAPER

PHOSPHATES AS INHIBITORS OF YEASTS ISOLATED FROM FOOD SOURCES

L´USO DEI FOSFATI COME INIBITORI DI LIEVITI ISOLATI DA FONTI ALIMENTARI

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ABSTRACT

The inhibition of twenty yeast strains isolated from food sources by six commercial phosphates (polyphosphates A and B; sodium tripolyphosphate – high solubility, TAS; sodium tripolyphosphate, TRI; sodium neutral pyrophosphate, N; sodium acid pyrophosphate, PAS) was investigated. The assays were performed at neutral (all phosphates), acid (2.5, 3.5 and 4.5) (polyphosphates A and B) and natural (without adjustment) (phosphates TAS, TRI, N and PAS) pH values, inoculating the yeasts

RIASSUNTO

È stata studiata l'inibizione di venti ceppi di lievito isolati da fonti alimentari da parte di sei fosfati commercializzati (polifosfati A e B; sodio tripolifosfato, ad alta solubilità, TAS; sodio tripolifosfato, TRI; sodio pirofosfato neutro, N; sodio pirofosfato acido, PAS). I saggi sono stati eseguiti inoculando i lieviti nel brodo di conta su piastra con fosfati a concentrazioni di 0,1, 0,3, 0,5, 1,0 e 1,5% (p/v), a valori di pH neutro (tutti i fosfati), acido (2,5, 3,5 e 4,5) (polifosfati A e B) e naturale (senza modifi-

- Key words: antimicrobial activity, phosphates, yeast spoilage -

in plate count broth with phosphates at concentrations of 0.1, 0.3, 0.5, 1.0 and 1.5% (w/v). The minimal inhibitory concentration (MIC) was determined. The phosphates with chain lengths greater than 15 phosphate units were more inhibitory and had the highest sequestering power. At neutral pH values, these polyphosphates showed MIC values of 0.3% for some yeasts and all the strains were inhibited completely with 1.5% phosphates. At natural pH values (pH obtained after phosphate addition), alkaline phosphates (TAS, TRI and N) were more inhibitory than at a neutral pH. Phosphate TAS (0.5%) increased its inhibitory capacity (percentage of strains inhibited) from 5% (pH= 7.0) to 70% (pH= 8.15). When phosphates A and B (chain lengths between 15 and 20 phosphate units) were assayed at acidic pH values, there was a clear inhibitory action that was exclusively due to the addition of these phosphates. These results demonstrate that some phosphates could be used in the food industry to inhibit yeasts associated with food spoilage.

che) (fosfati TAS, TRI, N e PAS). È stata determinata la Concentrazione Inibitoria Minima (CIM). I fosfati con capacità inibitoria più elevata erano quelli con catene più lunghe di 15 unità di fosfato e presentavano il più alto potere sequestrante. A valori di pH neutro, questi polifosfati mostravano valori di MIC dello 0,3% per alcuni lieviti e tutti i ceppi venivano completamente inibiti con l'1,5% di fosfati. A valori di pH naturali (pH ottenuto dopo aggiunta di fosfato), i fosfati alcalini (TAS, TRI ed N) risultavano con capacità inibitoria più elevata che a pH neutro. Il fosfato TAS (0,5%) aumentava la sua capacità inibitoria (percentuale di ceppi inibiti) dal 5% (pH = 7,0) al 70% (pH = 8,15). Quando i fosfati A e B (lunghezze di catena tra 15 e 20 unità di fosfato) venivano saggiati a pH acido, si osservava una chiara azione inibitoria esclusivamente dovuta all'aggiunta degli stessi. I risultati ottenuti dimostrano che alcuni fosfati potrebbero essere utilizzati nelle industrie alimentari per inibire i lieviti associati al deterioramento degli alimenti.

INTRODUCTION

Phosphates are considered GRAS (Generally Recognized As Safe) compounds. They are widely used in the food industry (mainly in the meat and dairy industries) because of their functional properties. In meat processing, for example, they are used to increase water binding, enhance emulsification, retard oxidative rancidity and color deterioration, and enhance cured-color development (MOLINS, 1991). Although the antimicrobial effect of some (poly) phosphates is well known, these compounds have not yet been added to food as antimicrobial agents. Many studies have demonstrated that the inhibitory activity of phosphates on bacteria is linked to the chelation of essential metal ions (JEN and SHELEF, 1986; KNABEL *et al.*, 1991; LEE *et al.*, 1994a, 1994b, 1994c). In general, Gram-positive bacteria appear to be more sensitive than Gram negative bacteria (MOLINS *et al.*, 1984; SHELEF and SEITER 1993; RAJKOWSKI *et al.*, 1994; LOESSNER *et al.*, 1997; SUÁREZ *et al.*, 2005a). However, some studies have demonstrated that Trisodium Phosphate (TSP) has an important antimicrobial effect on Gram-negative bacteria, which are natural contaminants of poultry meat (SLAVIK *et al.*, 1994; DEL RIO *et al.*, 2005; KANELLOS and BURRIEL, 2005; MEHYAR *et al.*, 2005). Furthermore, several studies have reported the

antibotulinal activities of phosphates (SOFOS and BUSTA, 1980; NELSON *et al.*, 1983; SOFOS, 1986; ECKNER *et al.*, 1994; LOESSNER *et al.*, 1997).

Yeasts are normally present in raw food material and processed foods and are a main cause of product spoilage in food with a high sugar concentration, low pH and low water activity (RAY, 2001a). Spoilage due to these microorganisms depends exclusively on the initial concentration and the characteristics of the food process. To date, there have been no systematic studies on the effect of phosphate addition against yeasts. The aim of this work was to obtain data on the inhibitory capacity of phosphates commonly used in foods on different yeasts isolated from food sources.

MATERIALS AND METHODS

Microorganisms and culture conditions

A total of 20 yeasts were used (Table 1). They were isolated from fruit juices, cheese, artisanal cultures for cheeses and the cheese plant environment. YGCB (Merck, Darmstadt, Germany) plates incubated for 4 d at 25°C were used for isolation. Purification was made using YM agar (0.3 g yeast extract (Merck), 0.3 g malt extract (Biokar, Beauvois, France), 0.5 g casein peptone (Merck), 1 g glucose (Anedra, Buenos Aires, Argentina), 1.5 g agar (Britania, Buenos Aires, Argentina), in 100 mL distilled water). Yeasts were stored in YM broth with 15% glycerol (-20°C) (CARRASCO *et al.*, 2006).

Table 1 - Phosphate inhibitory activity on yeasts isolated from food sources (pH = 7.0±0.3).

Organism/ Strain	Origin	Minimal Inhibitory Concentration (MIC, % w/v)*					
		A	B	TAS	TRI	N	PAS
Candida humicola 108	hard cheese	1.5	1.5	>1.5	>1.5	>1.5	>1.5
Geotrichum penicillatum 105	citric fruit juice	1.5	1.5	>1.5	>1.5	>1.5	>1.5
Cryptococcus laurentii 67	soft cheese	1.5	1.5	>1.5	>1.5	>1.5	>1.5
Brettanomyces claussenii 15	citric fruit juice	1.5	1.5	>1.5	>1.5	>1.5	>1.5
Candida famata 19	citric fruit juice	1.5	1.0	>1.5	>1.5	>1.5	>1.5
Cryptococcus heveanensis 17	soft cheese	1.5	1.0	>1.5	>1.5	>1.5	>1.5
Cryptococcus albidus 14	soft cheese	1.5	1.0	>1.5	>1.5	>1.5	>1.5
Candida parapsilosis 36	soft cheese	1.5	1.0	>1.5	>1.5	>1.5	>1.5
Candida famata 11	soft cheese	1.0	1.0	>1.5	>1.5	>1.5	>1.5
Cryptococcus laurentii 65	natural milk starter	0.5	1.0	>1.5	>1.5	>1.5	>1.5
Trichosporon sp. 44	natural milk starter	1.0	0.5	>1.5	>1.5	>1.5	>1.5
Candida famata 24	soft cheese	1.0	0.5	>1.5	>1.5	>1.5	>1.5
Candida famata 22	soft cheese	1.0	0.5	>1.5	>1.5	>1.5	>1.5
Candida famata 16	citric fruit juice	1.0	0.5	>1.5	>1.5	>1.5	>1.5
Geotrichum sp. 57	citric fruit juice	0.5	0.5	1.5	1.0	1.5	>1.5
Brettanomyces custersii 13	citric fruit juice	0.5	0.5	>1.5	>1.5	>1.5	>1.5
Candida famata 32	soft cheese	0.5	0.3	1.5	1.5	>1.5	>1.5
Brettanomyces intermedius 12	soft cheese	0.5	0.3	>1.5	>1.5	>1.5	>1.5
Cryptococcus albidus 8	citric fruit juice	0.5	0.3	>1.5	>1.5	>1.5	>1.5
Trichosporon cutaneum 10	dairy environment	0.3	0.3	0.5	0.5	1.0	0.5

A and B: polyphosphates (15 to 20 phosphate units); TAS: sodium tripolyphosphate - high solubility; TRI: sodium tripolyphosphate; N: sodium neutral pyrophosphate; PAS: sodium acid pyrophosphate.
* Values obtained from three determinations.
Phosphate concentrations assayed: 0.1, 0.3, 0.5, 1.0 and 1.5% (w/v).

Table 2 - pH values of plate count broth with phosphates added (% w/v).

Phosphate	0.1	0.3	0.5	1.0	1.5
A	6.95	6.91	6.87	6.82	6.75
B	6.92	6.88	6.84	6.79	6.72
TAS	7.89	8.05	8.15	8.39	8.44
TRI	8.04	8.13	8.23	8.47	8.55
N	7.92	8.41	8.53	8.76	8.80
PAS	5.85	5.70	5.55	5.17	5.04

Phosphates

Six available food-grade phosphates (SUDAMFOS S.A., Buenos Aires, Argentina) with different chain lengths were used: polyphosphates A and B (15 to 20 phosphate units), sodium tripolyphosphate-high solubility (TAS), sodium tripolyphosphate (TRI) ($Na_5P_3O_{10}$), sodium acid pyrophosphate (PAS) ($Na_2H_2P_2O_7$) and sodium neutral pyrophosphate (N) ($Na_4P_2O_7$).

The sequestering power values of phosphates were provided by the supplier (SUDAMFOS S.A.) and determined by titration with a calcium chloride solution until turbidity in a solution of 1.2% phosphate was observed.

Inhibition assays

Plate count broth (PC) was used as the basal medium (LOESSNER *et al.*, 1997) to which different concentrations (0.1, 0.3, 0.5, 1.0 and 1.5% w/v) of phosphates were added. For this purpose, 5% (w/v) phosphate stock solutions were prepared in distilled water and subsequently sterilized by filtration (Millipore membranes, 0.45 μm pore diameter) (LEE *et al.*, 1994b). PC-phosphate media were prepared using distilled water and the necessary volumes of stock solutions to reach the final phosphate concentrations cited. Media were used at two pH conditions: neutral (pH = 7.0±0.3) and natural (without pH adjustment) pH values. Neutral pH values were obtained by adding 6N solutions of NaOH or HCl, according to the phosphate used. Natural pH values obtained are shown in Table 2. Yeasts were inoculated in tubes with 5 mL of medium and incubated for 24 h at 30°C. The assays were performed three times and the minimal inhibitory concentration (MIC) values were calculated. This parameter (MIC) was defined as the lowest phosphate concentration that gives an absorbance (600 nm) value (as yeast growth measurement) lower than 0.2 units (LOESSNER *et al.*, 1997).

Inhibition assays at acid pH

The ability of yeasts to grow at acidic pH values (2.5, 3.5 and 4.5), with and without phosphates, was determined. The results are expressed as growth rates (% of growth, measured as A_{600nm}) with respect to a control (growth at pH 7.0 in broth without phosphate). A growth rate value lower than 25% was considered as inhibition. For these assays, polyphosphates A and B were used (at concentrations of 0.5 and 1.0%) since they showed the highest inhibitory activity at neutral pH.

RESULTS

At neutral pH, the more inhibitory phosphates for all strains studied were those with chain lengths greater than 15 phosphate units (phosphates A and B) (Table 1). The resistance of yeasts to phosphates was strain-dependent. A group of strains (strains 11, 14, 15, 17, 19, 36, 67, 105 and 108) was inhibited by concentrations ≥1.0%, while another group (strains 8, 10, 12, 13, 32 and 57) was inhibited by ≤0.5% phosphates. The other phosphates (TAS, TRI, N and PAS) showed a remarkably lower inhibitory capacity since MIC values ≤1.5% were only found for strains 10, 32 and 57 (phosphates TAS and TRI), strains 10 and 57 (phosphate N) and strain 10 (phosphate PAS).

Table 3 - Phosphate inhibitory activity on yeasts isolated from food sources (natural pH - See Table 1).

Organism/ Strain	Origin	Minimal Inhibitory Concentration (MIC, % w/v)*			
		TAS	TRI	N	PAS
Candida humicola 108	hard cheese	0.5	0.5	0.5	>1.5
Geotrichum penicillatum 105	citric fruit juice	>1.5	1.0	>1.5	>1.5
Cryptococcus laurentii 67	soft cheese	0.5	1.0	1.0	>1.5
Brettanomyces claussenii 15	citric fruit juice	0.5	0.5	0.5	>1.5
Candida famata 19	citric fruit juice	0.5	0.5	1.0	>1.5
Cryptococcus heveanensis 17	soft cheese	1.0	0.5	1.0	>1.5
Cryptococcus albidus 14	soft cheese	0.5	1.5	0.5	>1.5
Candida parapsilosis 36	soft cheese	0.5	0.5	0.5	>1.5
Candida famata 11	soft cheese	0.5	1.0	0.5	>1.5
Cryptococcus laurentii 65	natural milk starter	0.5	0.5	0.5	>1.5
Trichosporon sp. 44	natural milk starter	0.5	1.0	1.0	>1.5
Candida famata 24	soft cheese	1.0	0.5	1.0	>1.5
Candida famata 22	soft cheese	0.5	0.5	0.5	>1.5
Candida famata 16	citric fruit juice	1.0	0.5	1.0	>1.5
Geotrichum sp. 57	citric fruit juice	0.5	0.5	0.5	>1.5
Brettanomyces custersii 13	citric fruit juice	1.0	1.5	1.5	>1.5
Candida famata 32	soft cheese	0.5	0.5	0.5	>1.5
Brettanomyces intermedius 12	soft cheese	0.5	1.0	0.5	>1.5
Cryptococcus albidus 8	citric fruit juice	1.5	1.0	1.5	>1.5
Trichosporon cutaneum 10	dairy environment	0.5	0.5	0.5	>1.5

A and B: polyphosphates (15 to 20 phosphate units); TAS: sodium tripolyphosphate - high solubility; TRI: sodium tripolyphosphate; N: sodium neutral pyrophosphate; PAS: sodium acid pyrophosphate.
* Values obtained from three determinations.
Phosphate concentrations assayed: 0.1, 0.3, 0.5, 1.0 and 1.5% (w/v).

At natural pH, phosphates that had alkaline pH values when they were added to the medium (TAS, TRI and N) dramatically increased their inhibitory capacity (Table 3). For 0.5% TAS, the percentage of strains inhibited increased from 5% (pH = 7.0) to 70% (pH = 8.15). On the other hand, 0.5% of the other phosphates increased their inhibitory activity from 5% (pH = 7.0) to 60% (pH = 8.23) of the phosphate TRI strains and from 0% (pH = 7.0) to 55% (pH = 8.53) of the phosphate N strains. All yeasts were capable of growing in PC broth adjusted to pH values from 5.0 to 8.5, corresponding to the addition of the different phosphates at the concentrations assayed (data not shown). Phosphate PAS was acidic in solution and lost its scarce inhibitory activity shown at pH 7.0 on only one (*Trichosporon cutaneum* 10) (MIC = 0.5%) when it was tested at natural pH.

The sequestering powers of phosphates TAS, TRI and N at natural pH (13, 13 and 8 g Ca/100 g phosphate, respectively) were higher than at neutral pH (12, 12 and 5 g Ca/100 g phosphate, respectively). Phosphate TAS showed a poor sequestering power (3 g Ca/100 g phosphate), while phosphates A and B had similar sequestering power (15 g Ca/100 g phosphate) at pH values near 7.0.

Table 4 shows the growth rates (% of growth) of yeasts at acidic pH values (without phosphates added) using the growth at neutral pH as a control (100% of growth). As can be observed,

Table 4 - Yeast growth* in PC broth at different pH values.

Organism / Strain	pH		
	2.5	3.5	4.5
Cryptococcus albidus 8	89.9	100	100
Trichosporon cutaneum 10	88.5	100	100
Brettanomyces intermedius 12	76.9	100	100
Geotrichum penicillatum 105	100	100	100
Candida parapsilosis 36	11.8	100	100
Geotrichum sp. 57	7.6	96.6	100
Candida humicola 108	13.0	78.3	100
Candida famata 16	11.1	27.0	87.3
Candida famata 11	13.0	25.5	98.0
Brettanomyces custersii 13	8.2	15.4	100
Brettanomyces claussenii 15	10.0	13.0	100
Cryptococcus laurentii 65	10.0	10.9	100
Candida famata 19	7.4	13.9	100
Candida famata 32	14.7	13.3	96.0
Candida famata 22	10.0	13.7	90.0
Candida famata 24	15.4	12.3	41.5
Cryptococcus heveanensis 17	12.9	16.5	48.2
Trichosporon sp. 44	16.3	16.3	25.0
Cryptococcus laurentii 67	11.7	11.7	20.2
Cryptococcus albidus 14	15.0	13.3	13.3

* Rate (%) values of cell growth (measured at A_{600nm}, after 24 h at 30°C), using growth at pH 7.0 as control. The values are the means of three determinations.

80% of the total strains were inhibited (rate growth < 25%) in broth acidified to pH = 2.5 and this percentage reached 55 and 10% at pH 3.5 and 4.5, respectively. The highest resistance (rate growth from 76.9 to 100%, at pH 2.5) was exhibited by strains *Cryptococcus albidus* 8, *Trichosporon cutaneum* 10, *Brettanomyces intermedius* 12 and *Geotrichum penicillatum* 105. At this pH value, the other strains showed rate growth values ≤16.3%.

The inhibitory activity of phosphates at acidic pH values was determined by using phosphates A and B since these had shown the lowest MIC values at a neutral pH. A clear inhibitory effect due exclusively to phosphate addition was observed (Table 5). The percentage of inhibited strains increased up to 100% (pH 2.5 and 3.5) and 85% (pH 4.5) with 0.5% phosphate A. At this pH value, 100% of the tested strains were inhibited with phosphate A at 1.0%. For phosphate B, 0.5% inhibited all the strains at the three pH values assayed.

DISCUSSION

Yeasts are responsible for spoilage mainly of dairy products, fresh fruit, fruit juices and fruit products, honey, sugar syrups, molasses and fermented vegetables (RAY, 2001a). The yeast genera used in this study (*Candida*, *Geotrichum*, *Trichosporon*, *Brettanomyces* and *Cryptococcus*) are frequently linked to spoilage of these foods (ICMSF, 1980). In this study, yeast inhibition, at neutral

Table 5 - Percentage of inhibited* strains (n=20) at different pH values, with and without addition of phosphates (%w/v).

pH	Without phosphate addition	Phosphate A		Phosphate B	
		0.5	1.0	0.5	1.0
7.0 (control)	0	35	60	50	80
4.5	10	85	100	100	100
3.5	55	100	100	100	100
2.5	80	100	100	100	100

* Cell growth was measured at A_{600nm} in plate count broth after 24 h at 30°C using growth at pH 7.0 as control. A rate (%) value of cell growth <25% was considered as inhibition.

pH, was mainly observed when polyphosphates A and B (with the longest chain lengths) at concentrations from 0.3 to 1.5%, depending on the strain, were used. MAIER *et al.* (1999) have demonstrated that some yeasts such as *Candida albicans* and *Saccharomyces cerevisiae* were inhibited with 0.3% of the commercial polyphosphate JOHA® HBS (sodium polyphosphate glassy, 69±1% P_2O_5). These results could be related to the sequestering capacity of divalent cations by polyphosphates, and are in agreement with those obtained in a previous study on food spoilage molds (SUÁREZ *et al.*, 2005b). This theory is essentially supported by the report of reversal of phosphate-induced inhibition on bacteria when an excess of these ions is added back to the growth medium (JEN and SHELEF, 1986; KNABEL *et al.*, 1991; LEE *et al.,* 1994b; ZAIKA *et al.*, 1997). Long-chain phosphates show a greater inhibitory effect on bacteria than shorter chains phosphates (LEE *et al.*, 1994a; SUÁREZ *et al.*, 2005a); this could be due to the fact that the sequestering activity of divalent cations increases with chain length (MOLINS, 1991).

On the other hand, our results showed that the pH had a notable influence on the inhibitory capacity of phosphates. For phosphates that showed an alkaline reaction in the culture medium (phosphates TAS, TRI and N), this activity was higher at natural pH values. This fact can be linked to the increased sequestering capacity of metal cations for alkaline phosphates (MOLINS, 1991). These results were similar to those obtained for molds when the same phosphates were used (SUÁREZ *et al.*, 2005b). JEN and SHELEF (1986) studied the effect of pH on *Staphylococcus aureus* 196E, by using 0.5% sodium tripolyphosphate (STPP) and sodium hexametaphosphate (SHMP). They also observed an increased inhibition by phosphates at pH values higher than 7.0 (from 7.4 to 9) and hypothesized that chelators became increasingly dissociated and the quantity of complexed cations increased. As a consequence, essential divalent cations were not available for cellular growth and, therefore inhibition was enhanced.

However, there is no data about the inhibitory action of polyphosphates at acid pH values. Results obtained in our study demonstrated that polyphosphates A and B increased their inhibitory activity against yeasts under acidic conditions. Yeast strains capable of growing in acidified broth were inhibited when phosphates A and B were added at concentrations from 0.5 to 1.0%. These concentrations were similar to or lower than those used when phosphates are used as additives (MOLINS, 1991). These results should be evaluated to determine their possible use for the preservation of acidic foods such as acid fruits or fruit juices since, for example, the pH values of citric fruit juices range from 2.5 (concentrated juices) to 4.5 (fresh juices) (SOBRERO, 2002).

REFERENCES

Carrasco M.S., Moragues L.G., Vignatti C.I., Scarinci H.E. and. Simonetta A.C. 2006. Characterization and technological aspects of yeasts isolated from raw milk and different types of cheeses produced in Argentina. Austr. J. Dairy Technol. 61:21.

Del Rio E., Alonso-Calleja C. and Capita R. 2005. Effectiveness of trisodium phosphate treatment against pathogenic and spoilage bacteria on poultry during refrigerated storage. J. Food Prot. 68: 866.

Eckner K.F., Dustman W.A. and Rys-Rodriguez A.A. 1994. Contribution of composition, physicochemical characteristics and polyphosphates to the microbial safety of pasteurized cheese spreads. J. Food Prot. 57: 295.

ICMSF (International Commission on Microbiological Specifications). 1980. Ecología microbiana de los alimentos 2 - Productos alimenticios. Acribia S.A., Zaragoza, España.

Jen C.M. and Shelef L.A. 1986. Factors affecting sensitivity of *Staphylococcus aureus* 196E to polyphosphates. Appl. Environ. Microbiol. 52: 842.

Kanellos T.S. and Burriel A.R. 2005. The bacteri-

cidal effects of lactic acid and trisodium phosphate on *Salmonella enteritidis* serotype pt4, total viable counts and counts of *Enteriobacteriaceae*. Food Prot. Trends 25: 346.

Knabel S.J., Walker H.W. and Hartman P.A. 1991. Inhibition of *Aspergillus flavus* and selected Gram-positive bacteria by chelation of essential metal cations by polyphosphates. J. Food Prot. 54: 360.

Lee R.M., Hartman P.A., Olson D.G and Williams F.D. 1994a. Bactericidal and bacteriolytic effects of selected food-grade phosphates, using *Staphylococcus aureus* as a model system. J. Food Prot. 57: 276.

Lee R.M., Hartman P.A., Olson D.G. and Williams F.D. 1994b. Metal ions reverse the inhibitory effects of selected food-grade phosphates in *Staphylococcus aureus*. J. Food Prot. 57: 284.

Lee R.M., Hartman P.A., Stahr H.M., Olson D.G. and Williams F.D. 1994c. Antibacterial mechanism of long-chain polyphosphates in *Staphylococcus aureus*. J. Food Prot. 57: 289.

Loessner M.J., Maier S.K., Schiwek P. and Scherer S. 1997. Long-chain phosphates inhibit growth of *Clostridium tyrobutyricum* in processed cheese spreads. J. Food Prot. 60: 493.

Maier S.K., Scherer S. and Loessner M. 1999. Long-chain polyphosphate causes cell lysis and inhibits *Bacillus cereus* septum formation, which is dependent on divalent cations. Appl. Environ. Microbiol. 65: 3942.

Mehyar G., Blank G., Han J.H., Hydamaya A. and Holley R.A. 2005. Effectiveness of trisodium phosphate, lactic acid and commercial antimicrobials against pathogenic bacteria on chicken skin. Food Prot. Trends 25: 351.

Molins R.A. 1991. "Phosphates in Food". Boca Raton, FL: CRC Press.

Molins R.A., Kraft A.A., Olson D.G. and Hotchkiss D.G. 1984. Recovery of selected bacteria in media containing 0.5% food grade poly- and pyrophosphates. J. Food Sci. 49: 948.

Nelson K.A., Busta F.F., Sofos J.N. and Wagner W.K. 1983. Effect of polyphosphates in combination with nitrite-sorbate or sorbate on *Clostridium botulinun* growth and toxin production in chicken frankfurter emulsions. J. Food Prot. 46: 846.

Rajkowski K.T., Calderone S.M. and Jones E. 1994. Effect of polyphosphate and sodium chloride on the growth of *Listeria monocytogenes* and *Staphylococcus aureus* in ultra-high temperature milk. J. Dairy Sci. 77: 1503.

Ray B. 2001a. Indicators of microbial food spoilage. In "Fundamental Food Microbiology", p. 287. Boca Ratón, FL: CRC Press.

Ray B. 2001b. Spoilage of specific food groups. In "Fundamental Food Microbiology", p. 239. Boca Ratón, FL: CRC Press.

Shelef L.A. and Seiter J.A. 1993. Indirect antimicrobials. In "Antimicrobials in Foods" P.M. Davidson (Ed), p. 539. Marcel Dekker, Inc., New York.

Slavik M.F., Kim J.W., Pharr M.D., Raben D.P., Tsai S. and Lobsinger C.M. 1994. Effect of trisodium phosphate on *Campylobacter* attached to post-chill chicken carcasses. J. Food Prot. 57: 324.

Sobrero M.S. 2002. Identificación de mohos en el proceso de elaboración de jugos cítricos. Tesis para acceder al grado académico de Magíster en Ciencia y Tecnología de Alimentos, Facultad de Ingeniería Química, Universidad Nacional del Litoral, Santa Fe, Argentina.

Sofos J.N. 1986. Use of phosphates in low-sodium meat products. Food Technol. 40: 52.

Sofos J.N. and Busta F.F. 1980. Alternatives to the use of nitrite as an antibotulinal agent. Food Technol. 34: 244.

Suárez V.B., Carrasco M., Frisón L., Basilico M., Simonetta A., Rivera M., Reinheimer J. 2005a. Potencial de los fosfatos como nuevos antimicrobianos en la industria de alimentos. X Congreso CYTAL (AATA), 18-20 de Mayo, Mar del Plata, Argentina.

Suárez V.B., Frisón L., Basílico M.Z. de, Rivera M. and Reinheimer J.A. 2005b. Inhibitory activity of phosphates on molds isolated from foods and food processing plants. J. Food Prot. 68: 2475.

Zaika L.L., Scullen O. and Fanelli J. 1997. Growth inhibition of *Listeria monocytogenes* by sodium polyphosphate as affected by polyvalent metal ions. J. Food Sci. 62: 867.

Revised paper received December 4, 2006 Accepted January 22, 2007

PAPER

COMPARATIVE STUDY ON CACIOCAVALLO CAMPANO CHEESE

STUDIO COMPARATIVO CONDOTTO SUL CACIOCAVALLO CAMPANO

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ABSTRACT

Caciocavallo is a hard *pasta filata* cheese produced by artisanal methods in southern Italy. This study focused on Caciocavallo cheese made in the Campania region (Caciocavallo Campano). Ten 8-month-old and three 4-month-old cheeses were obtained over a two-year period from five dairies and subjected to compositional and biochemical analysis. Large differences between and within dairies were recorded. Six of these cheeses were selected for descriptive sensory and con-

RIASSUNTO

Il caciocavallo è un formaggio duro a pasta filata prodotto in Italia meridionale. Il presente lavoro è stato condotto su caciocavalli prodotti in Campania (Caciocavallo Campano). Dieci formaggi a 8 mesi di maturazione e tre a 4 mesi di maturazione, acquistati da cinque caseifici, sono stati caratterizzati dal punto di vista chimico-biochimico, evidenziando grandi differenze inter- e intracaseificio. Tra i suddetti formaggi, sei sono stati sottoposti ad analisi descrittive sensoriali e di preferenza che han-

- Key word: Caciocavallo Campano Cheese, chemical-biochemical and sensory characteristics -

sumer preference analysis that highlighted a preference for cheese with piccante, mouldy and rancid flavour. More production control is needed in order to improve product quality and meet consumer demands.

no mostrato un gradimento per i caciocavalli caratterizzati da un "flavour" piccante, rancido e di muffa. Per rispondere alle richieste del consumatore sarebbe opportuno introdurre un maggiore controllo nella produzione per migliorare la qualità del prodotto.

INTRODUCTION

Pasta filata cheeses, characteristically produced in Mediterranean countries, are characterised by a unique stretching process in which the acidified curd is kneaded in hot water, drawn into long strands and formed into various shapes (CORSETTI *et al.*, 2001). They include soft or semi-soft varieties, usually consumed fresh or after a brief period of ripening, e.g., Mozzarella and semi-hard or hard varieties which are consumed after considerable ageing, e.g., Provolone and Caciocavallo (KINDSTEDT, 1995; GOBBETTI *et al.*, 2002). Caciocavallo is very popular in southern Italy, but to date is produced only by artisanal methods in several small dairies. Many types of Caciocavallo cheese are produced in all regions of southern Italy (e.g., Caciocavallo Campano, Caciocavallo Silano and Caciocavallo Pugliese) and the cheese is generally defined as a hard *pasta filata* variety made from whole cows' milk (DECRETO DEL PRESIDENTE DELLA REPUBBLICA, 1955).

Studies on the microbial and biochemical characteristics of 2-month-old Caciocavallo Pugliese and Caciocavallo Silano have been published (GOBBETTI *et al.*, 2002; CORSETTI *et al.*, 2001), however to our knowledge no studies have been reported on the compositional and biochemical characteristics of Caciocavallo Campano cheese. Moreover, no comparative studies have been published on the biochemical characteristics of Caciocavallo cheese at different stages of ripening and produced by different dairies. In view of the increasing market popularity of Caciocavallo Campano cheese, chemical-biochemical and sensory data and consumer preference for a selection of cheeses were analysed to identify the distinctive characteristics of this type of cheese. It is hoped that information obtained may lead to more established production practices, greater consistency and better quality cheese than presently available.

MATERIALS AND METHODS

Samples

Cheeses were purchased from five dairies in the Campania region of Italy, all located in the province of Avellino. The characteristic manufacturing protocol followed is shown in Fig. 1. Caciocavallo Campano may be sold after 2-4 months, as a "sweet" variety, or after approximately 8 months when the flavour is more piquant. In the present study, all manufacturers used kid rennet paste. In order to respect the confidentiality of the suppliers and their products, each product was assigned a code.

Five 8-month-old cheeses were obtained in 2003 (3A8, 3B8, 3C8, 3D8 and 3E8) and five 8-month-old cheeses in 2004 (4A8, 4B8, 4C8, 4D8 and 4E8) from five dairies (A, B, C, D and E). Three 4-month-old cheeses were obtained in 2004 from dairies A, C and D (4A4, 4C4, 4D4). Each of these chees-

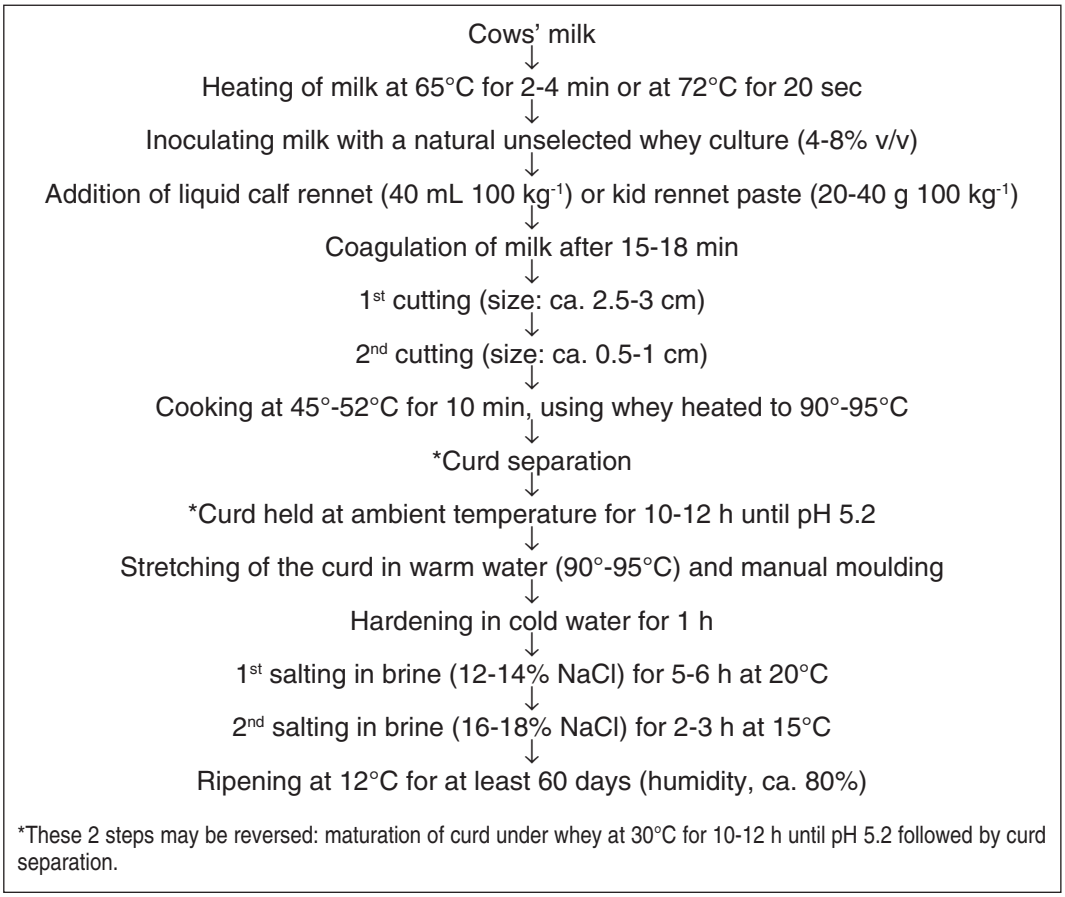

Fig. 1 - Manufacturing protocol for Caciocavallo cheese in the Campania region (adapted from CNR, 1996).

es was analysed for compositional and biochemical properties. Based on the compositional and biochemical properties, ripening time and dairy source, six of the cheeses purchased in 2004 were selected for descriptive sensory analysis and consumer preference analysis (4A4, 4C4, 4D4, 4C8, 4D8 and 4E8) in order to determine the most preferred sensory characteristics of Caciocavallo Campano cheese.

Each cheese was cut in half. One-half was used for compositional and biochemical analyses. After the rind (3 mm) was removed, the piece was cut into two roughly 3-4 cm thick sections, an outer section (OS) and the core or inner section (IS). Samples were grated and stored at -20°C prior to analysis. The second half, to be used for sensory evaluation and consumer preference studies, was vacuum-packed and stored at 4°C.

Compositional analysis

Cheese samples were analysed in triplicate for moisture (IDF, 1982), salt (FOX, 1963), protein (IDF, 1986) and fat (Gerber method: IIRS, 1955). pH was measured in triplicate as described by the International Dairy Federation (IDF, 1989).

Biochemical analysis

Proteolysis

Proteolysis was monitored by measuring the percentage of total N soluble in water at pH 4.6 (pH 4.6-WSN) by the method of KUCHROO and FOX (1982). Nitrogen was determined by the macro-Kjeldahl method (IDF, 1986). Cheeses were analysed in triplicate.

The insoluble fraction of the cheeses was analysed by urea polyacrylamide gel electrophoresis (Urea-PAGE) using a Protean II vertical slab gel unit (Bio-Rad) and the stacking gel system described by ANDREWS (1983). The gels were stained directly with Comassie Brilliant blue G250 by the method of BLAKESLEY and BOEZI (1977).

The peptide profile of the 70% ethanol-soluble fraction (ESF) of the IS of the cheeses, prepared according to REVILLE and FOX (1978), was determined by reverse-phase HPLC (RP-HPLC) using a Nucleocil C8 column according to the method of ROHM *et al.* (1996), with the following modifications: samples were diluted with mobile phase A (0.1% TFA in water) to a peptide concentration of 10 mg mL^{-1}. A Varian ProStar 230 solvent delivery system was used with a Varian ProStar 410 autosampler (Varian Associates Inc., Walnut Creek, CA, USA). Column eluates were monitored at 214 nm using a Varian ProStar 310 UV-Vis detector, interfaced with a PC running on Varian Star Chromatography Workstation Software, version 5.51.

Lipolysis

Free fatty acids (FFA; $C_{4:0}$, $C_{6:0}$, $C_{8:0}$ $C_{10:0}$, $C_{12:0}$, $C_{14:0}$, $C_{16:0}$, $C_{18:0}$, $C_{18:1}$, $C_{18:2}$, $C_{18:3}$) in the inner sections of the cheese were quantified by GC-FID (gas chromatography-flame ionisation detection), according to the method of DE JONG and BADINGS (1990), with the following modifications: solid-phase extraction was carried out using NH_2 cartridges (Waters Corporation Milford, MA 01757, USA) with a capacity of 500 mg and neutral lipids were extracted with hexane/2-propanol (3:2).

Cheeses were analysed on a Varian CP-3800 gas chromatograph with a Varian 8410 CX Autosampler, a Varian 1079 injector, a flame ionisation detector and associated Star Workstation software (JVA Analytical Ltd, Dublin, Ireland). An internal standard solution containing $C_{5:0}$, $C_{9:0}$ and $C_{17:0}$ was used to determine the recovery of each FFA. Separation of FFAs was achieved using a Varian/Chromopack WCOT fused silica column, 25 m x 0.32 mm ID, coated with FFAP-CB (DF 0.3). The column was attached to a fused silica guard column (2.5 m x 0.53 mm) with a quick-seal connector. The sample (3 µL) was injected directly into the column at 65°C which was ramped to 250° at 200°C min^{-1}, held for 1 min, then cooled to 65°C and held for 20 min. The oven temperature was 65°C, ramped to 240° at 10°C min^{-1} and held for 20 min. The detector temperature was 240°C. Flow pressure was maintained at 55 kPa over 44 min.

Analyses were performed in triplicate and results are expressed as mg kg^{-1} of cheese.

Sensory analysis

Descriptive Sensory Analysis

Three 4-month-old and three 8-month-old Caciocavallo Campano cheeses (4A4, 4C4, 4D4, 4C8, 4D8 and 4E8) were assessed by seven trained assessors, in duplicate. All assessments were conducted in individual booths, under red light, at the sensory laboratory at University College, Cork, Ireland, which complies with international standards for the design of test rooms (ISO, 1988). Prior to analysis, assessors participated in a number of group discussions of 2 h duration, during which, using a previously-agreed vocabulary (LAWLOR and DELAHUNTY, 2000), all attributes of Caciocavallo Campano

cheese were discussed and the vocabulary revised. The final vocabulary included nine odour and fifteen flavour attributes (Table 1). Prior to assessment, the rind (3 mm) of each cheese was removed and the samples were cut into slices (5 g) from the outer edge to the centre and served at room temperature (21°C) on coded plates. The order of tasting was balanced to account for order and carry-over effects (MACFIE *et al.*, 1989). Descriptive sensory analysis was carried out following the method of LAWLOR *et al.* (2002). Data were recorded using the *Compusense five* version 4.0 sensory data acquisition programme (Compusense Inc., Guelph, ON, Canada).

Consumer preference testing

A consumer preference test on the same cheeses used for Descriptive Sensory Analysis was conducted in Avellino, Italy. All participants (107) were famil-

Table 1 - Vocabulary and definitions developed for descriptive analysis of Caciocavallo Campano cheese.

ATTRIBUTE	DEFINITION
ODOUR	
PUNGENT	Physically penetrating sensation in the nasal cavity; sharp-smelling irritant.
SWEATY	The aromatics reminiscent of perspiration-generated foot odour, i.e., sour and stale.
RANCID	The aroma associated with sour milk.
FRUITY	The aromatic blend of different fruity characteristics.
CARAMEL	The aromatics associated with butter and sugar that has been cooked (condensed milk, burnt sugar or syrup; toffee made with sugar that has been melted further).
BUTTERY	Fatty, buttery taste, of the nature of, or containing, butter.
MOULDY	The aromatics associated with moulds; usually earthy, dirty, stale, cardboard, musty.
NUTTY	The non-specific nut-like aromatics characteristic of several different nuts, e.g., peanuts, hazelnuts, pecans, almonds.
COWY-PHENOLIC	Aroma associated with barns and stock trailers, indicative of abnomal sweat and waste.
FLAVOUR	
PUNGENT	Physically penetrating sensation in the nasal cavity; sharp-smelling irritant.
RANCID	The taste associated with sour milk.
MOULDY	The taste associated with moulds; usually earthy, dirty, stale, cardboard, musty and slightly sour.
CARAMEL	The taste associated with butter and sugar that has been cooked (condensed milk, burnt sugar or syrup; toffee made with sugar that has been melted further).
FRUITY	The taste of a blend of different fruity characteristics.
BUTTERY	Fatty, buttery tasting, of the nature of, or containing, butter.
NUTTY	The non-specific nut-like taste characteristic of several different nuts, e.g., peanuts, hazelnuts, pecans, almonds.
SOAPY	A detergent-like taste; similar to that of a food tainted with a cleansing agent.
SWEET	Fundamental taste sensation, of which sucrose is typical.
SALTY	Fundamental taste sensation, of which sodium chloride is typical.
ACIDIC	A sour tangy, citrus-like taste; a fundamental taste sensation, of which lactic and citric acids are typical.
BITTER	A chemical-like taste; a fundamental taste sensation, of which caffeine and quinine are typical.
ASTRINGENT	A mouth-drying and harsh sensation; the complex of drying, puckering and shrinking sensations in the oral cavity causing contractions of the body tissue.
ALCOHOL	Flavour associated with alcohol, particularly Sherry.
PICCANTE	A slightly burning, prickling and/or numbness of the tongue and/or mouth surface.

iar with Caciocavallo Campano cheese and expressed their preference for each of the six cheeses sampled on a nine-point hedonic scale (PERYAM and PYLGRIM, 1957). Cheese samples were prepared as described for Descriptive Sensory Analysis and randomly coded. The order of tasting was balanced to account for order and carry-over effects (MACFIE *et al.*, 1989). Consumers were provided with water and unsalted crackers and encouraged to cleanse their palate between cheeses.

Statistical analysis

Data treatment of compositional and biochemical properties

A randomised complete block design, with incorporated treatments, was used to analyse the response variables, related to moisture, pH, NaCl and pH 4.6 WSN. ANOVA was carried out using GLM procedure of SAS (2003) where the effects of treatment and replicates were estimated for all response variables. Duncan's multiple-comparison test was used as a guide for pair comparison of the treatment means. The level of significance was determined at $P<0.05$.

Data treatment of sensory analysis

The mean panel scores of the quantitative descriptive data were subjected to one-way ANOVA using SPSS version 10.0 (SPSS Inc. Chicago, IL 60611, USA) to determine which terms effectively discriminated amongst the cheeses. Descriptive terms which did not significantly discriminate ($P<0.05$) between the cheeses were removed from subsequent analyses. The data were then standardised (1/Standard Deviation) and Principal Component Analysis (PCA) was performed using The Unscrambler® version 8.0 (CAMO PROCESS AS, Nedre Vollgate 8, N-0158 Oslo, Norway). PCA was used to identify the factors responsible for the similarities and differences between the sensory characteristics of the cheeses. How each PC discriminated between the sensory characteristics of the cheeses was tested using one-way ANOVA (SPSS version 10.0).

The consumer preference ratings were subjected to PCA (Internal Preference Mapping (SCHLICH, 1995; MCEWAN, 1996), using the Unscrambler® version 8.0 (CAMO AS). This identified the direction and intensity of preferences of the individual consumers.

RESULTS

Compositional analysis

The composition of the OS and IS of all the cheeses is shown in Tables 2a and 2b, respectively. Significant differences were found between all cheeses for moisture, pH, NaCl and fat content ($P<0.0001$). In addition, significant differences were also evident between cheeses from the same dairy in 2003 and 2004, and for the 4- and 8-month-old cheeses from the same dairy.

The moisture content was highest in the IS of all 8-month-old cheeses, in agreement with the data reported for Caciocavallo Pugliese cheese (GOBBETTI *et al.*, 2002). Very large differences in moisture and NaCl content were found. The NaCl content was highest in the IS, in agreement with 60-day-old Caciocavallo Pugliese cheese (GOBBETTI *et al.*, 2002). pH values were usually higher than those reported for Caciocavallo Silano (CORSETTI *et al.*, 2001) and Caciocavallo Pugliese (GOBBETTI *et al.*, 2002) cheeses, but this was not unexpected because the cheeses in this study were ripened for a longer time and the pH is known to increase with ripening time. Large variations in fat content were also evident, ranging from 25.5 to 36.5% in the OS and 21.3 to 32.0% in the IS. Most cheeses in this study had higher levels of fat than those reported for Caciocavallo Pugliese cheese (GOBBETTI *et al.*, 2002),

but lower than those reported for Caciocavallo Silano cheese (CORSETTI *et al.*, 2001). The protein content varied extensively for the OS, 28.5 to 36.9%, but less for the IS, 24.4 to 29.6%. The protein level was generally higher than that found in Caciocavallo Silano cheese by CORSETTI *et al.* (2001). In general, differences in composition between cheeses may have been due to the quality of the raw milk, the breed and feeding regime of the cows and period of lactation. This information could not be obtained from the manufacturers.

Proteolysis

The level of pH 4.6-WSN, expressed as a percentage of total nitrogen, is shown in Table 2a and 2b for OS and IS, respectively. The data showed that the level of pH 4.6-WSN was greater in the IS than in the OS of all cheeses, in agreement with the results of GOBBETTI *et al.* (2002) for

Table 2 - Compositional and proteolytic parameters of the outer section (OS) (a) and of the inner section (IS) (b) of Caciocavallo Campano cheese.

SAMPLE	Moisture (%)	Fat (%)	Protein (%)	NaCl (%)	pH	% pH 4.6 SN/TN
(a)						
3A8	33.6^{cd} (2.1)	31.5^{cde}(0.5)	29.9^{g}(0.1)	1.88^{g}(0.01)	5.39^{e}(0.05)	10.5^{g}(0.0)
3B8	26.8^{gh}(0.1)	28.5^{g}(0.6)	35.7^{b}(0.1)	3.14^{a}(0.06)	5.22^{h}(0.03)	13.8^{e}(0.1)
3C8	36.5^{ab}(0.6)	25.5^{h}(0.6)	30.8^{f}(0.2)	1.76^{h}(0.05)	5.44^{d}(0.02)	14.0^{d}(0.2)
3D8	31.8^{de}(1.3)	32.0^{cd}(0.6)	29.5^{g}(1.0)	2.07^{e}(0.01)	5.29^{fg}(0.03)	16.2^{c}(0.2)
3E8	25.9^{h}(0.1)	31.0^{de}(0.6)	35.8^{b}(0.1)	2.18^{d}(0.01)	5.63^{b}(0.04)	16.0^{c}(0.1)
4A8	29.8^{ef}(1.2)	32.5^{c}(0.6)	33.7^{c}(0.1)	2.73^{b}(0.06)	5.59^{bc}(0.03)	13.7^{e}(0.1)
4B8	33.0^{cd}(1.7)	30.5^{ef}(0.6)	32.1^{e}(0.4)	2.45^{c}(0.02)	5.57^{bc}(0.01)	10.5^{g}(0.1)
4C8	28.3^{fg}(1.8)	30.0^{f}(0.3)	36.9^{a}(0.7)	1.90^{fg}(0.08)	5.69^{a}(0.02)	16.8^{b}(0.7)
4D8	29.9^{ef}(1.4)	36.5^{a}(0.6)	28.6^{h}(0.2)	1.89^{g}(0.11)	5.28^{g}(0.04)	12.8^{f}(0.0)
4E8	28.2^{fg}(0.6)	34.5^{b}(0.5)	32.6^{de}(0.1)	2.40^{c}(0.02)	5.56^{c}(0.02)	24.6^{a}(0.0)
4A4	37.5^{a}(0.7)	28.5^{g}(0.5)	29.5^{g}(0.5)	3.10^{a}(0.0)	5.45^{d}(0.03)	8.43^{h}(0.2)
4C4	34.8^{bc}(0.9)	28.5^{g}(0.5)	33.0^{d}(0.6)	1.99^{ef}(0.08)	5.55^{c}(0.05)	16.4^{c}(0.3)
4D4	30.2^{ef}(0.0)	34.0^{b}(0.0)	28.5^{h}(0.5)	1.99^{ef}(0.05)	5.34^{ef}(0.03)	13.8^{e}(0.1)
(b)						
3A8	38.4^{c}(0.2)	27.0^{e}(0.0)	24.4^{abcde}(0.1)	1.94^{i}(0.06)	5.32^{gh}(0.03)	14.6^{i}(0.2)
3B8	35.6^{e}(0.5)	24.7^{fg}(0.6)	29.6^{a}(0.2)	3.92^{a}(0.08)	5.31^{h}(0.01)	18.7^{g}(0.1)
3C8	43.3^{a}(0.2)	21.3^{i}(0.6)	28.3^{abcd}(0.4)	1.96^{i}(0.03)	5.51^{de}(0.03)	20.2^{f}(0.2)
3D8	39.3^{c}(0.3)	28.0^{d}(0.0)	27.1^{bcde}(3.6)	2.44^{fg}(0.03)	5.25^{i}(0.02)	21.8^{e}(0.6)
3E8	38.7^{c}(0.2)	25.3^{f}(0.6)	28.9^{ab}(0.2)	3.02^{d}(0.05)	5.71^{b}(0.01)	24.8^{c}(0.3)
4A8	37.3^{d}(1.7)	29.0^{c}(0.0)	26.6^{cde}(0.2)	3.48^{b}(0.01)	5.86^{a}(0.05)	23.6^{d}(0.1)
4B8	42.2^{b}(0.7)	23.5^{h}(0.5)	26.1^{de}(0.2)	3.15^{c}(0.04)	5.50^{e}(0.01)	13.5^{i}(0.0)
4C8	37.1^{d}(0.3)	25.0^{f}(0.0)	29.3^{ab}(2.3)	2.50^{f}(0.01)	5.71^{b}(0.04)	29.1^{b}(2.4)
4D8	39.3^{c}(0.6)	26.5^{e}(0.5)	28.8^{abc}(0.5)	2.28^{h}(0.04)	5.36^{gf}(0.03)	17.6^{gh}(0.4)
4E8	34.0^{f}(0.1)	32.0^{a}(1.0)	28.4^{abc}(0.1)	2.89^{e}(0.02)	5.55^{cd}(0.01)	32.4^{a}(0.1)
4A4	42.4^{ab}(0.2)	24.5^{fg}(0.5)	25.9^{e}(0.2)	3.14^{c}(0.05)	5.28^{hi}(0.04)	11.7^{l}(0.0)
4C4	41.9^{b}(0.4)	24.0^{gh}(0.0)	28.6^{abc}(0.1)	2.14^{i}(0.01)	5.56^{c}(0.02)	20.5^{f}(0.2)
4D4	39.3^{c}(0.3)	30.0^{b}(0.0)	25.5^{e}(0.1)	2.38^{g}(0.05)	5.40^{f}(0.02)	17.4^{h}(0.1)

Data presented are the means of triplicate analysis.
$^{a-h}$ Values in columns not sharing a common superscript differ significantly (P<0.05).
Values in brackets are standard deviations.
$^{a-j}$ Values in columns not sharing a common superscript differ significantly (P<0.05).
Values in brackets are standard deviations.

Caciocavallo Pugliese cheese. Significant differences ($P<0.05$) were found between cheeses for the level of pH 4.6-WSN. The level of pH 4.6-WSN ranged from 10.5 to 24.6% for the OS of 8 month-old cheese and 13.5 to 32.4% for the IS of 8-month old cheese. In general, 4-month-old cheeses had lower levels of pH 4.6-WSN than the 8-month-old cheeses, as expected. Very little difference was noted in the cheeses from dairy D at 4 and 8 months, probably due to comparable moisture and pH values which influence enzymatic activity and microbial growth. Fig. 2 shows urea-PAGE electrophoretograms of the pH 4.6-insoluble fraction of the IS of all cheeses studied. Urea-PAGE electrophoretograms of the pH 4.6-insoluble

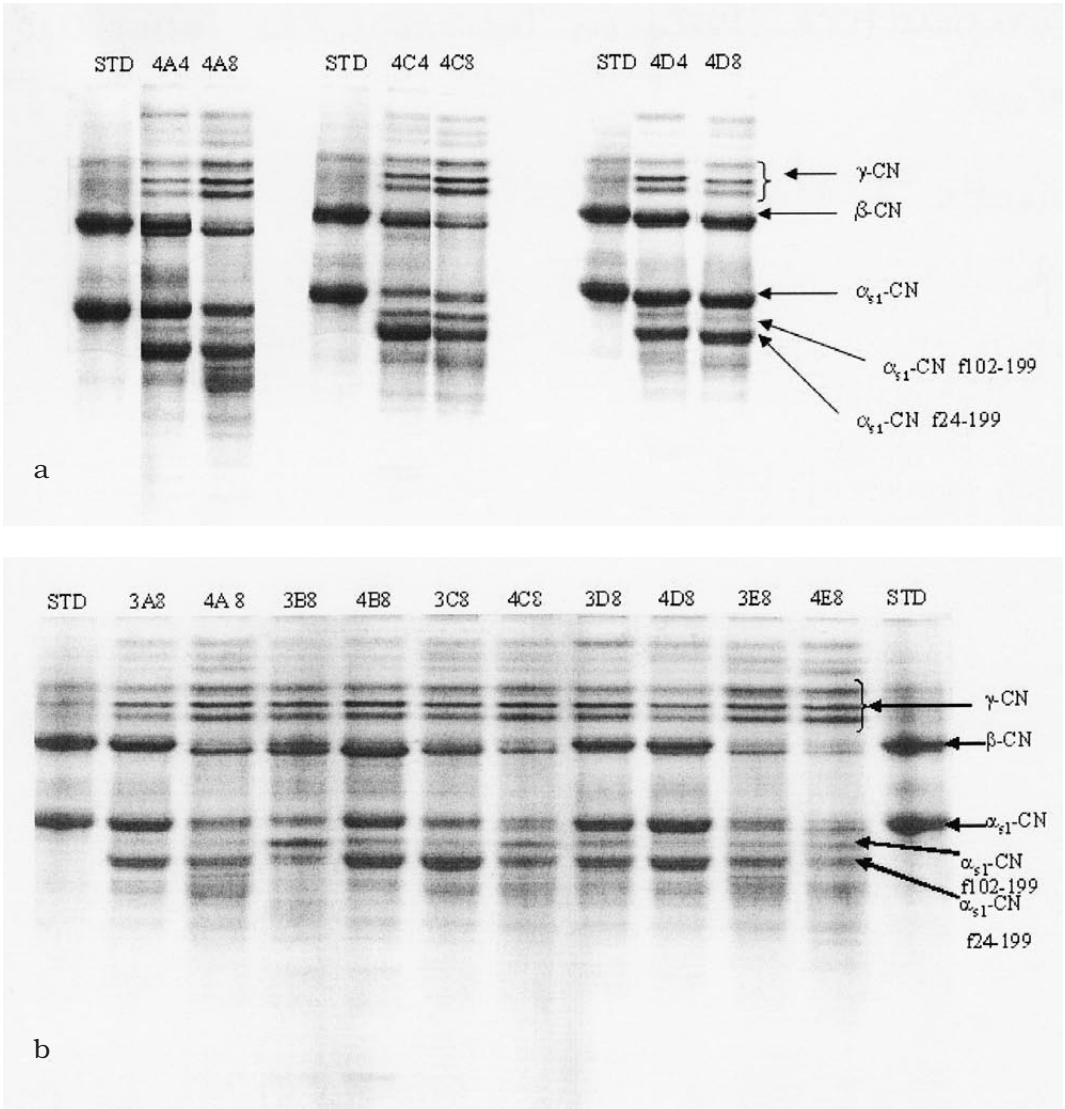

Fig. 2 - Urea-PAGE of pH 4.6-insoluble fraction of the inner section (IS) of 4- and 8-month-old Caciocavallo Campano cheeses obtained in 2004 (a) and of 8-month-old Caciocavallo Campano cheeses obtained in 2003 and 2004 (b). Sodium caseinate (STD) was used as a control.

fraction of the OS were similar to those of the IS (data not shown). All cheeses showed greater degradation of α_{s1}-casein (CN) than β-CN, which is in agreement with previous studies (MILANOVIĆ *et al.*, 1998; GOBBETTI *et al.*, 2002). In the 4-month-old cheeses the main degradation product of α_{s1}-CN was α_{s1}-CN f24-199. In the 8-month-old cheeses, the degradation of α_{s1}-CN increased, e.g., in cheese 4A8, peptides of high electrophoretic mobility were present (Fig. 2a). In the 8-month-old cheeses, α_{s1}-CN was hydrolysed mainly to α_{s1}-CN f24-199 and to a lesser extent to α_{s1}-CN f102-199, with the exception of cheese 3B8, where the intensity of α_{s1}-CN f102-199 was the greatest (Fig. 2b). The limited hydrolysis of β-CN may have been due to a fairly high NaCl concentration which inhibits the hydrolysis of β-CN by chymosin, as shown by the absence of the peptide β-CN f1-189/92, and stimulates the hydrolysis of α_{s1}-CN (MULVIHILL and FOX, 1978; CARLES and RIBADEAU-DUMAS, 1985). The limited hydrolysis of β-CN to γ-CNs was probably due to plasmin activity (EIGEL *et al.*, 1984; FOX *et al.*, 1994).

RP-HPLC profiles of the ESF of all cheeses showed a large number of peaks, indicating a heterogeneous mixture of peptides (Fig. 3). The profiles of the ESF

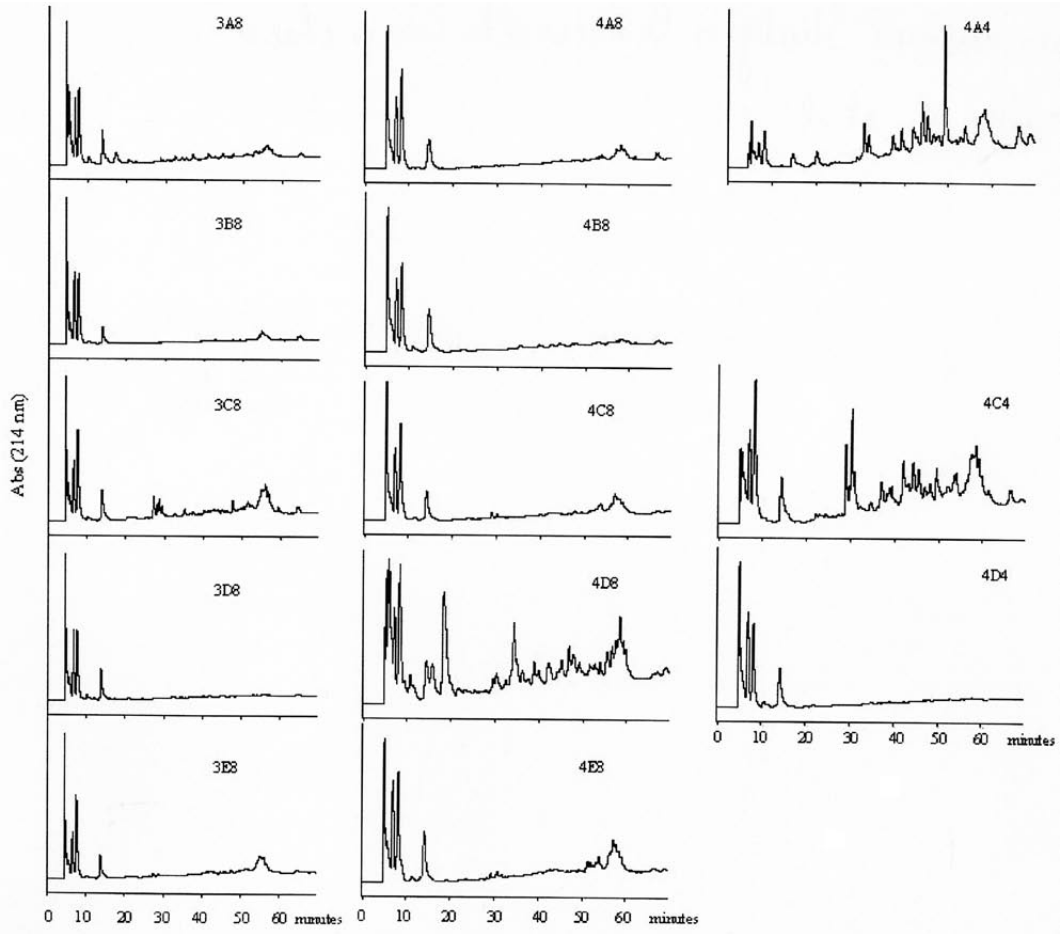

Fig. 3 - RP-HPLC profiles of the ethanol-soluble fraction of the inner section (IS) of all Caciocavallo Campano cheeses.

of the 8-month-old cheeses obtained in 2003 and 2004 were similar to each other and were characterised by small peptides and amino acids (peaks with a short elution time), the only exception being the cheese 4D8 which contained hydrophobic peptides. The profiles of cheeses 4A4 and 4C4 showed a high level of hydrophobic peptides, which were not present in cheeses 4A8 and 4C8. This difference in peptide profile may have been due to the hydrolysis of larger peptides to smaller peptides, as ripening progressed. The profile of 4D8 showed hydrophobic peptides, which were not evident in cheese 4D4, which contained mainly hydrophilic and small peptides and amino acids. These unusual profiles of samples 4D4 and 4D8 were probably due to an uncontrolled manufacturing process or possibly to high levels of non-starter lactic acid bacteria or different contaminating advantageous microflora.

Lipolysis

The concentration of total FFAs and the percentage of individual FFAs in the IS of all cheeses is shown in Table 3. The percentages of individual FFAs in the OS were similar to those of the IS of the same cheeses (data not shown). Significant differences were found for FFA levels in cheeses from the same and different dairies in both years. Age appeared to have no influence on FFA content. The extent of lipolysis in all cheeses in this study was higher than that found in Caciocavallo Pugliese cheese (GOBBETTI *et al.*, 2002), but lower than that for Caciocavallo Silano cheese (CORSETTI *et al.*, 2001). Caciocavallo cheeses appear to be characterised by a high level of $C_{4:0}$ as noted in the results of GOBBETTI *et al.* (2002) and CORSETTI *et al.* (2001) and in many of the cheeses in this study (3A8, 3B8, 3D8, 4A8, 4C8, 4D8, 4E8 & 4D4). However, a low level of $C_{4:0}$ was found in some of the cheeses (3C8, 3E8, 4B8, 4A4 & 4C4), despite having quite a high level of lipolysis. A high concentration of $C_{4:0}$ reflects the use of kid rennet paste, as claimed by manufacturers, which contains pregastric esterase which is specific for the FA at the Sn 3 position, and which is predominantly a short chain FA, such as $C_{4:0}$ (FOX *et al.*, 1993; COLLINS *et al.*, 2004). High concentrations of $C_{16:0}$, $C_{18:1}$ and $C_{14:0}$ predominated, reflecting their abundance in milk fat (DE JONG and BADINGS, 1990; IDF, 1991). A contribution by lipases secreted by the cheese microflora may also be supposed. It was postulated that the activity of lipases from several lactobacilli was stimulated by concentrations of NaCl similar to those observed in the present study (c. 2%) (COLLINS *et al.*, 2004).

Descriptive Sensory Analysis

Descriptive sensory analysis was carried out using a final vocabulary of nine odour and fifteen flavour terms (Table 1). The attributes "pungent", "sweaty", "fruity", "caramel", "mouldy" and "nutty" odour and "pungent", "caramel", "acidic" and "astringent" flavour did not significantly ($P<0.05$) distinguish between cheeses and were consequently not included in subsequent analysis (ELMORE *et al.*, 1999). The data matrix of the discriminating attributes was analysed by PCA which showed that the first four PCs indicated significant differences between the samples ($P<0.05$) and accounted for 98% of the explained variance (Figs. 4a and 4b). PC 1 and 2 described 67% of the variation in the data. PC1 distinguished the sensory character of cheeses 4C8 and 4E8 from 4A4. Cheese 4C8 scored the highest for the intensity of "piccante" and "mouldy" flavours and had a "cowey/phenolic" odour. This cheese had a reasonably high level of lipolysis and a level of $C_{4:0}$ which would have contributed to the "piccante" flavour. Cheese 4E8 had the most "rancid" odour and intense "soapy" and "rancid" flavour, in addition

Table 3 - Free fatty acids as a percentage (%) on total free fatty acids and total free fatty acid concentration in the inner section (IS) of Caciocavallo Campano cheese.

SAMPLE	$C_{4:0}$	$C_{6:0}$	$C_{8:0}$	$C_{10:0}$	$C_{12:0}$	$C_{14:0}$	$C_{16:0}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$	Total (mg kg^{-1})
3A8	21	9	2	5	5	9	18	8	20	3	0	4,605^{e}(463)
3B8	27	6	3	4	4	9	18	11	18	0	0	6,777^{c}(452)
3C8	9	3	1	5	5	12	28	11	23	3	0	7,923^{b}(237)
3D8	39	13	3	6	5	6	10	3	12	3	0	7,216^{c}(495)
3E8	7	3	2	5	5	14	28	10	24	1	1	11,869^{a}(34)
4A8	21	3	2	4	4	10	30	7	15	4	0	5,502^{d}(251)
4B8	11	4	3	4	4	10	24	16	24	0	0	1,062^{h}(109)
4C8	20	4	2	4	4	10	28	8	17	3	0	4,605^{e}(274)
4D8	27	6	2	4	3	7	21	8	16	6	0	1,895^{g}(127)
4E8	15	4	3	4	4	9	26	7	22	4	2	7,860^{b}(440)
4A4	10	3	2	4	5	11	35	7	17	4	2	3,302^{i}(79)
4C4	8	2	2	4	4	11	35	9	20	5	0	3,195^{i}(138)
4D4	50	12	3	6	4	5	10	4	5	1	0	3,671^{f}(25)

Data presented are the means of triplicate analysis.
$^{a-h}$ Values in columns not sharing a common superscript differ significantly ($P<0.05$).
Values in brackets are standard deviations.

to a "piccante" and "mouldy" flavour. This cheese had the highest level of lipolysis of the six cheeses which would have explained its rancid, soapy character. In addition, $C_{4:0}$ was one of the most abundant FFAs in cheese 4E8 which would have contributed to its piccante flavour. In comparison, 4A4 was found to have the most "buttery" odour and the most "buttery" and "salty" flavour. This cheese had the highest level of NaCl of the six cheeses studied and had a relatively low level of lipolysis, which would explain its buttery, salty characteristics. PC2 showed the differences between the sensory character of 4C4 from 4E8 and 4A4. Cheese 4C4 had the most "fruity" and "alcohol" flavour and intense "sweet" flavour. PC3 described the differences between 4D8 and 4C8, 4E8, 4C4 and 4A4. Cheese 4D8 scored highest for "bitter" flavour amongst all the cheese samples, which may have been due to a relatively low level of primary proteolysis. This cheese was also characterised by an intense "nutty" and "mouldy" flavour. PC4 distinguished the sensory characteristics of cheese 4D4 from those of cheeses 4D8, 4C8, 4E8 and 4C4. Cheese 4D4 was described as having the most "rancid" and "cowey/phenolic" odour and the most "nutty" and "sweet" flavour. This cheese was very unusual in that 50% of the total FFA was $C_{4:0}$ which would have contributed strongly to its flavour characteristics. There appears to be some correlation between the descriptive sensory results and the biochemical data; however more detailed analysis of volatile compounds is required to better discern such differences.

Consumer preference testing

The consumer preference map (Fig. 5) shows the preference of individual consumers for the six cheeses (4A4, 4C4, 4D4, 4C8, 4D8 and 4E8) sampled. The position of the cheeses was determined by the strength and direction of the consumers' preference and illustrated the most important differences between the cheeses. Cheeses 4C8, 4E8 and 4D4 were grouped on the right side of the map

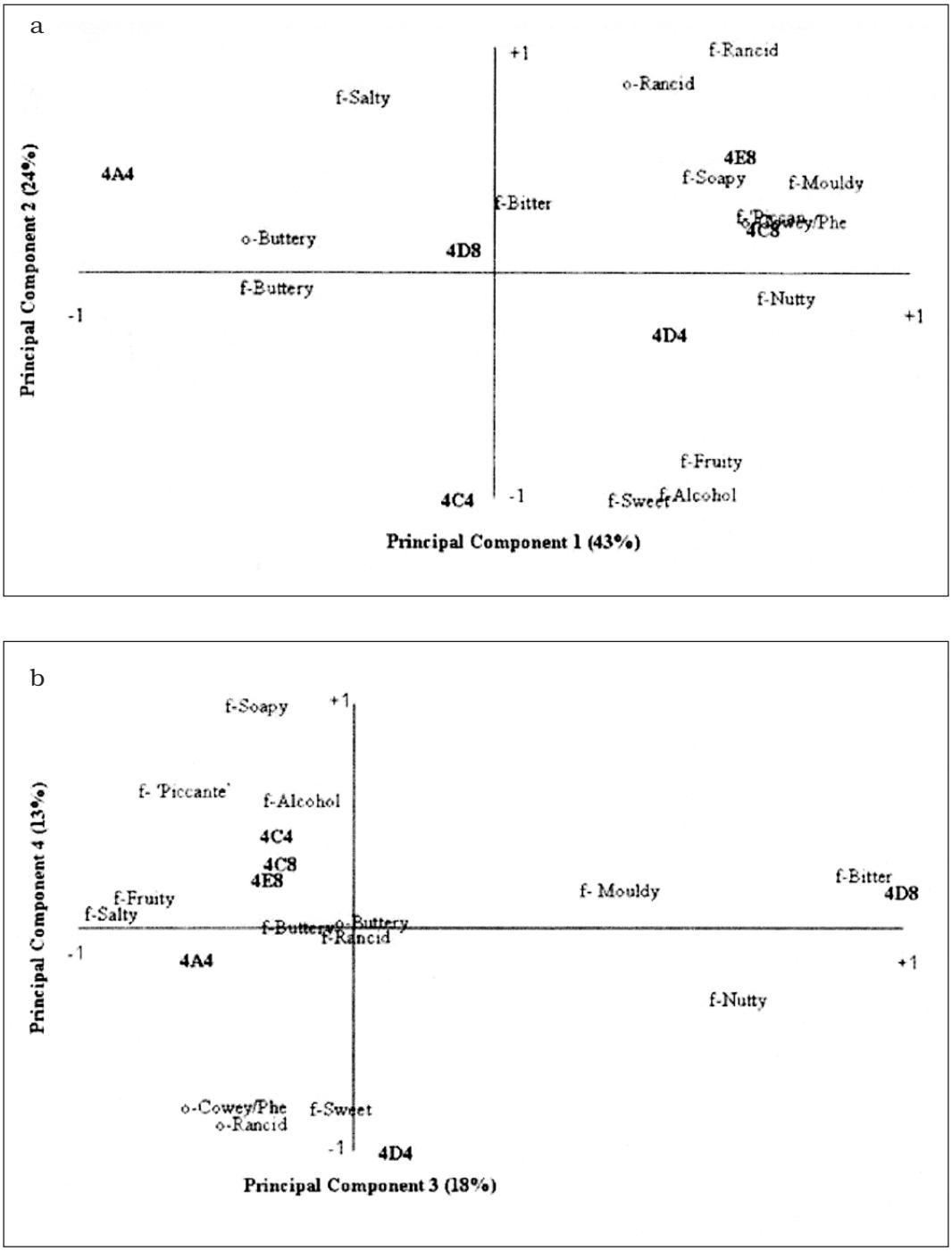

Fig. 4 - Descriptive sensory analysis of the 6 cheeses tested. Principal Component Analysis (PCA): biplot of scores and loadings for PC1 and PC2 (a) and biplot of scores and loadings for PC3 and PC4 (b).

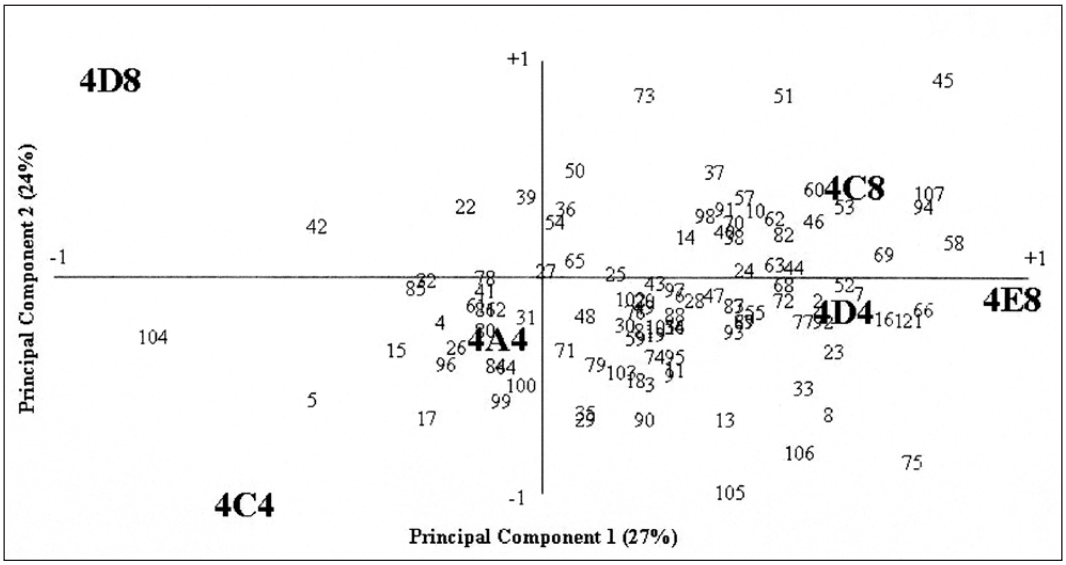

Fig. 5 - The Internal Preference Map obtained from Principal Component Analysis of consumer preference data, showing the individual preference of 107 consumers for the 6 cheeses tested.

and were the most preferred cheeses as most consumers were positioned in this area of the sensory map. These cheeses had the highest level of lipolysis of the six cheeses tested. It also appears that consumers prefer Caciocavallo Campano cheese to have a mouldy, rancid flavour with a cowey/phenolic odour as each of these cheeses was characterised by these attributes. In addition, cheeses 4C8 and 4E8 had a piccante flavour. Cheeses 4D8, 4C4 and 4A4 were situated on the left side of the map and were differentiated from each other and from the other cheeses. Cheeses 4D8, characterised by bitter flavour, and 4C4, characterised by alcohol flavour, were the least preferred cheeses as very few consumers were positioned in these areas of the sensory space. These results may indicate that consumers disliked cheeses that had a bitter or alcohol flavour. Cheese 4A4 was positioned near the centre of the map, suggesting that consumers neither strongly liked nor disliked this cheese. It was characterised as having a buttery odour and a buttery and salty flavour and therefore appears to have no desirable or disagreeable characteristics.

CONCLUSIONS

The aim of the present study was to establish chemical-biochemical and sensory characteristics and consumer preference for a selection of Caciocavallo Campano cheeses to identify the best representative attributes of this cheese. Because of its increasing market popularity, it is important to introduce greater production controls in order to improve product quality.

Although artisanal production did not allow compilation of detailed data about the manufacturing protocol followed by each dairy and limited production did not allow for a wider and more complete sampling protocol, the present study has provided useful information on the chemical-biochemical and sensory characteristics of Caciocavallo Campano cheese. In general, the large differences in compo-

sitional, proteolytic and lipolytic indices between and within dairies indicate poor manufacturing methods, which are often associated with such artisanal production. Such differences may have been due to the quality of the milk, the standard of hygiene and/or poorly controlled methods of production, such as milk standardisation, salt concentration, pH, cooking and stretching temperatures, rennet concentration and microflora composition. However, some common characteristics were observed such as, a) high levels of hydrophobic peptides in the 4-month-old cheeses which were absent in the 8-month-old cheeses which were characterised by small peptides and amino acids, b) a high level of lipolysis and in particular a high concentration of $C_{4:0}$ which reflects the use of kid rennet paste and c) a consumer preference for Caciocavallo Campano cheese with piccante, rancid, mouldy flavours, which correlate with high level of lipolysis. The manufacture of Caciocavallo Campano cheese needs to be improved in order to produce cheese with a desirable piccante, rancid, mouldy flavour which would best meet consumer preference.

ACKNOWLEDGMENT

This work was supported by Biotech Millennium. The assessors and the consumers are thanked for their enthusiastic participation in this study.

REFERENCES

Andrews A.T. 1983. Proteinases in normal bovine milk and their action on the caseins. J. Dairy Res. 50: 45.

Blakesley R.W. and Boezi J.A. 1977. A new staining technique for proteins in polyacrylamide gels using Comassie Blue G250. Anal. Biochem. 82: 580.

Carles C. and Ribadeau-Dumas B. 1985. Kinetics of action of chymosin (rennin) on some peptide bonds of bovine β-casein. Biochemi. 23: 6839.

CNR. 1996. "I Prodotti Caseari del Mezzogiorno". Consiglio Nazionale delle Ricerche, Roma.

Collins Y.F., McSweeney P.H.L. and Wilkinson M.G. 2004. Lipolysis and catabolism of fatty acids in cheese. In "Cheese: Chemistry, Physics and Microbiology", Fox P.F., McSweeney P.H.L., Cogan T.M. and Guinee T.P. (Ed.) Vol. 1, 3rd ed. p. 373 Elsevier Applied Science London.

Corsetti A., Corbo M.R., Albenzio M., Di Cagno R., Gobbetti M. and Fox P.F. 2001. Microbiology and biochemistry of Caciocavallo Silano cheese. Ital. J. Food Sci. 13: 297.

Decreto del Presidente della Repubblica 1955. D.P.R. 30th October 1955, n. 1269 Rome.

de Jong C. and Badings H.T. 1990. Determination of free fatty acids in milk and cheese, procedures for extraction, clean up and capillary gas chromatographic analysis. J. High Res. Chromatograph. 13: 94.

Eigel W.N., Butler J.E., Ernstrom C.A., Farrell H.M., Harwalkar V.R., Jennes R. and Whitney R.McL. 1984. Nomenclature of proteins of cow's milk: fifth revision. J. Dairy Sci. 67: 1599.

Elmore J.R., Heymann H., Johnson J. and Hewett J.E. 1999. Preference mapping: relating acceptance of "creaminess" to a descriptive sensory map of semi-solid. Food Qual. Pref. 10: 465.

Fox P.F. 1963. Potentiometric determination of salt in cheese. J. Dairy Sci. 46: 744.

Fox P.F., Singh T.K. and McSweeney P.L.H. 1994. Biogenesis of flavour compounds in cheese. In "Chemistry of Structure-Function Relationships in Cheese" Malin E.L. and Tunick M.H. (Ed.) p. 59 Plenum Press. New York.

Gobbetti M., Morea M., Baruzzi F., Corbo M.R., Maturante A., Considine T., Di Cagno R., Guinee T. and Fox P.F. 2002. Microbiological, compositional, biochemical and textural characterization of Caciocavallo Pugliese cheese during ripening. Int. Dairy J. 12: 511.

IDF (International Dairy Federation). 1982. Determination of the total solid content. Standard 4A. In: Cheese and processed cheese. International Dairy Federation. Brussels.

IDF (International Dairy Federation). 1986. Determination of nitrogen content (Kjeldahl method) and calculation of crude protein content. Standard 20A. International Dairy Federation. Brussels.

IDF (International Dairy Federation). 1989. Determination of pH. Standard 115A. International Dairy Federation. Brussels.

IDF (International Dairy Federation). 1991. Determination of free fatty acids in milk and milk products. Bulletin 265. International Dairy Federation. Brussels.

Institute for Industrial Research and Standards. 1955. Determination of the percentage of fat in cheese. Irish Standard 69. Institute for Industrial Research and Standards Dublin.

International Organization for Standardization. 1988. Sensory analysis. General guidance for the design of test rooms. Standard 8589. In-

ternational Organization for Standardization Paris.

Kindstedt P.S. 1995. Factors affecting the functional characteristics of unmelted and melted Mozzarella cheese. In "Chemistry of Structure-Function Relationships in Cheese" Malin E.L. and Tunick M.H. (Ed.) p. 27 Plenum Press. New York.

Kuchroo C.N. and Fox P.F. 1982. Soluble nitrogen in Cheddar cheese: comparison of extraction procedures. Milchwissenschaft 37: 331.

Lawlor J.B. and Delahunty C.M. 2000. The sensory profile and consumer preference for ten speciality cheeses. International J. Dairy Technol. 53: 28.

Lawlor J.B., Delahunty C.M., Wilkinson M.G. and Sheehan J. 2002. Relationships between the gross, non-volatile and volatile compositions and the sensory attributes of eight hard-type cheeses. Int. Dairy J. 12: 493.

MacFie H.J., Bratchell N., Greenhoff K. and Vallis I.V. 1989. Designs to balance the effect of order of presentation and first-order carry-over effects in hall tests. J. Sens. Stud. 4: 129.

McEwan J.A. 1996. Preference mapping for product optimisation. In "Multivariate Analysis of Data in Sensory Science" Naes T. and Risvik E. (Ed.) p. 71 Elsevier Applied Science. London.

Milanović S., Kaláb M. and Carić M. 1998. Structure of Kashkaval curd manufactured from milk of UF retentate using enzymes of various origin. Lebensm.-Wiss. U.-Technol. 31: 377.

Mulvihill D.M. and Fox P.F. 1978. Proteolysis of bovine β-casein by chymosin: influence of pH, urea and sodium chloride. Irish J. Food Sci. Technol. 2: 135.

Peryam D.R. and Pilgrim F.J. 1957. Hedonic scale method of measuring food preferences. Food Technol. 11: 9.

Reville W.J. and Fox P.F. 1978. Soluble protein in Cheddar cheese: a comparison of analytical methods. Irish J. Food Sci. Technol. 2: 67.

Rohm H., Jaros D., Rockenbauer C., Riedler-Hellrigl M., Uniacke-Lowe T. and Fox P.F. 1996. Comparison of ethanol and trichloracetic acid fractionation for measurement of proteolysis in Emmental cheese. Int. Dairy J. 6: 1069.

SAS. 2003. User's Guide: Statistics, Version 9.1.3 Edition. SAS Institute, Cary NC.

Schlich P. 1995. "Preference Mapping: Relating Consumer Preferences to Sensory or Instrumental Measurements" Étiévant P. and Schreier P. (Ed.) p. 135 Bioflavour 95. INRA. Paris.

Revised paper received December 19, 2006 Accepted January 22, 2007

PAPER

PROTECTION OF TRADITIONAL GREEK FOODS USING A PLANT EXTRACT

SALVAGUARDIA DEGLI ALIMENTI TRADIZIONALI GRECI UTILIZZANDO UN ESTRATTO VEGETALE

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ABSTRACT

The effect of rosemary extract on two traditional Greek foods (Tarama salad and Deples) was evaluated using the Rancimat method and sensory evaluation. The rosemary extract at 400 ppm gave the highest protection against lipid oxidation of Tarama salad during 40 days of storage. It was superior to BHA (200 ppm). The extract at a concentration of 150 ppm protected Deples from oxidation during 35 days of storage. Panelists clearly preferred products with the rosemary extract.

RIASSUNTO

È stato valutato l'effetto di un estratto di rosmarino su due tradizionali alimenti greci (l'insalata Tarama e il Deples) utilizzando il metodo Rancimat e la valutazione sensoriale. L'estratto di rosmarino a 400 ppm esplicava la migliore protezione contro l'ossidazione lipidica dell'insalata Tarama durante 40 giorni di conservazione e risultava superiore al BHA (200 ppm). L'estratto ad una concentrazione di 150 ppm proteggeva il Deples dall'ossidazione durante 35 giorni conservazione. Gli assag-

- Key words: deples, induction period, rosemary extract, sensory evaluation, Tarama salad -

The shelf-life of products was calculated using the linear adjustment equation. Foods with rosemary had 17-102% longer shelf-life.

giatori preferivano chiaramente prodotti contenenti l'estratto di rosmarino. La shelf life dei prodotti veniva calcolata utilizzando un'equazione lineare. Gli alimenti con estratto di rosmarino presentavano una shelf life più lunga del 17-102%.

INTRODUCTION

Traditional foods contribute significantly to the human diet in many countries around the world since they are closely related to the local culture. Some of them are produced by simply combining the ingredients and then mixing them (e.g. Tzatziki salad), while others, after mixing, undergo further processing such as frying (e.g. pan cakes) or baking (e.g. baklava). Tarama salad and deples are traditional Greek foods. Tarama salad is a traditional delicacy that looks like a pink or red paste; it is consumed mainly during the forty-day period of fasting before Easter. It is made of oil, fish roe, bread crumbs or bread and water, and is stored at 6°-8°C. It is a traditional home-made food, but it can also be purchased in supermarkets. The lipid phase accounts for more than 50% of the salad mass. Within two weeks, oxidation makes the product unacceptable, especially when the salad is stored unsealed in the fridge.

Deples is another traditional Greek delicacy (which comes in the form of a swirled or twisted crispy pastry); it is mainly consumed during the Christmas holidays. It is made of flour, orange juice and whole egg to produce a dough which is then fried in vegetable oil and stored at room temperature for up to two weeks. It contains a high amount of absorbed oil, which oxidises easily making it commercially unacceptable. The use of highly unsaturated vegetable oil (e.g. soybean oil) increases the susceptibility to oxidation (RUDNIK *et al.*, 2001).

Lipid peroxidation, which involves a series of free radical-mediated chain reaction processes, is also associated with several types of biological damage. The role that free radicals and active oxygen plays in causing atherogenesis in humans is becoming clearer (KISHK and EL-SAYED, 2007). Antioxidants are used to reduce or retard deterioration of the lipid phase. In addition, the use of antioxidants minimises discoloration, and off-flavours and contributes to emulsion stability which can be negatively affected by the degradation of lipids in foods (MITSUMOTO *et al.*, 2005; KISHK and EL-SAYED, 2007). ESTEVEZ *et al.* (2007) reported that artificial antioxidants (usually butylated hydroxyanisole-BHA) are added to food to inhibit lipid oxidation and obtain a longer shelf-life. Synthetic antioxidants are not popular among consumers, since it has been shown that they act in promoting carcinogenesis and mutagenesis (FARAG *et al.*, 1989). On the other hand, plant extracts especially rosemary (*Rosemarinus officinallis* L.) and sage (*Salvia officinallis* L.) provide varying levels of antioxidant activity, depending on the method of assessment in different fats and oils (CHE MAN and TAN, 1999; KIM *et al.*, 1994; ZHANG *et al.*, 1990). In an earlier study the effect of the natural extract from rosemary, dissolved in frying oil, was evaluated against the oxidation of oil, during intermittent frying of potato chips (LALAS and DOURTOGLOU, 2003). In the present work, rosemary extract was used to protect the lipid phase of the two tradition-

al Greek foods mentioned, prepared under different temperature conditions and storage. This time, the antioxidant was added to the food ingredients and not to the frying oil.

The main objectives of this study were: first, to assess the oxidative susceptibility of two traditional foods (Tarama salad and Deples) prepared under different conditions (high and low temperature) and second to evaluate the possibility of slowing this oxidation by addition of a natural extract obtained from rosemary. Extension of the shelf-life was also predicted. The potential substitution of the synthetic antioxidant BHA (normally used in commercial preparations) in Tarama salad was also examined.

MATERIALS AND METHODS

Materials

Rosemary extract (Rosemax®) is a commercial product (Vioryl Chemical and Agricultural Industry Research S.A., Afidnes, Greece) with the following composition: 43% caffeic acid, 7% ferulic acid, 10% carnosic acid, 20% rosmarinic acid, 5% vanillic acid, 5% carnosol, 3% p-hydroxy benzoic acid and 7% ursolic acid (LALAS and DOURTOGLOU, 2003). Butylated hydroxyanisole (BHA) was obtained from Sigma Chemicals Company Co. (St. Louis, USA). Soybean oil (Kope, Athens, Greece), bread flour (Jotis S.A., Athens, Greece), fresh oranges and eggs were purchased from the local market. Salted roe of *Gadidae* spp. was provided by Olympus Foods S.A. (Nea Efessos, Pieria, Greece).

Tarama salad recipe

The salad recipe used was: 65% soybean oil, 8% salted roe, 26% water, 1% breadcrumbs and a selected amount of antioxidant (extract or BHA). The ingredients were mixed using a Vorwerk Thermomix 3300 (Vorwerk France s.a., Paris). The salad was poured into plastic containers (200 g per container) and stored unsealed at 5°C for 40 days. In total, 10.0 kilograms of salad were divided into five different batches (produced by adding: 0, 100, 200, 400 ppm rosemary extract, and 200 ppm BHA, respectively) and stored at 6°C in the dark. Two containers of each batch were removed from cold storage each day. The first container was subjected to sensory evaluation (day 0 and 40). The lipid phase from the salad of the other container was extracted with n-hexane (to determine the oxidative condition) and centrifuged for 10 min at 6,500 rpm using a Sorvall General-Purpose RC-3 Automatic Refrigerated Centrifuge (Ivan Sorvall Inc., Newtown, CT, USA). Its oxidative condition was then determined using the Rancimat method. Induction period was evaluated at day 0, 10, 20, 30 and 40.

In total three replicate experiments were carried out (using the same recipe and experimental conditions for all determinations).

Deples recipe

Deples were prepared using the following recipe: 63% bread flour, 17% orange juice and 20% whole egg (about 9 eggs). The ingredients were mixed with mixer (Vorwerk Thermomix 3300) and the dough was divided into four batches. Rosemary extract at concentration levels of 0, 50, 100 and 150 ppm were added. Each batch was then passed through a pasta machine (Roma/Weston Pasta Machine 6in - Model 150P, Pragotrade USA, Inc. Strongsville, Ohio) and cut into parallelogram pieces of uniform thickness (15x5 cm). Each piece was then properly swirled to form a helical shaped piece before frying.

In total three replicate experiments were carried (using the same recipe and experimental conditions for all determinations).

Frying method of Deples

Frying was performed in four different 22 cm diameter pans using soybean oil. Temperature was monitored with an IKA Labortechnik ETS-D4 fuzzy (IKA Labortechnik, Germany) digital thermometer. Each batch of Deples was deep-fried independently in 1 L of soybean oil. The batches of Deples were put in the oil when the temperature reached 170°C and fried for 2 min. After frying they were placed on absorbent paper to remove the excess oil. Sample batches were packed in unsealed plastic bags and kept in the dark at 4°C for 35 days.

Deples were subjected to sensory evaluation (day 0 and 35) and determination of the oxidative condition of the lipid phase was determined over a six-week period (day 0, 7, 14, 21, 28 and 35). The lipid phase was extracted from ground Deples (using a Krups GVA2 Mini-chopper, Zentralkundendienst Krups GmbH, Solingen, Germany) with 250 mL of n-hexane in conical flasks for 30 min, in the dark. The oil was collected after the solvent was evaporated, under vacuum using a rotary evaporator (Büchi Rotavapor R-215 and B-160 Vacobox pump-BÜCHI Labortechnik AG, Flawil, Switzerland). The efficiency of oil extraction was 98-100%. Induction period was measured after extraction.

Determination of the oxidative state of foods

The determination of the induction period was determined by the Rancimat method (100°C and 20 L/h) adapted from LALAS and DOURTOGLOU (2003).

Sensory evaluation of products

Deples and Tarama salad were placed in plastic dishes to evaluate their sensory quality using the method described by TSAKNIS and LALAS (2002). Twenty panellists were selected from laboratory staff. Each sample was coded with a three-digit code number. Panellists evaluated the overall acceptability of each sample (taking into account any flavour of rancidity and undesirable taste) using a numerical scale of 1 to 9 (with 1 = not acceptable, 9 = extremely good) as well as the colour by using a numerical scale of 1 to 4 (with 4 = white-yellow, 1 = brown for Deples and 4 = red, 1 = light pink for Tarama salad). In addition, panellists had to state their overall preference taking into account any flavour of rancidity and unpleasant taste or colour.

Statistical analysis

The results are the mean values of triplicate analyses of three different experiments (triplicate analysis per experiment). The statistical significance of the differences between the mean values was assessed by ANOVA. Changes in the induction period of the lipid phase of Tarama salad and Deples were correlated with the time past since the production date by a linear regression equation ($Y = a + bX$), where Y = induction period and X = number of days since the production date. By applying the developed linear equations in which shelf-life corresponded to a value for an induction period of 10 hours (Tarama Salad) or 1 hour (Deples), the effective shelf-life of each product was expressed in days before the product became unacceptable.

RESULTS AND CONCLUSIONS

Tarama salad

Oxidation of the lipid phase of the Tarama salad was inhibited by both rosemary extract and BHA. The results showed that, the longer the salads were kept in storage, the shorter was the induction period (Table 1). The reduction of induction period for all batches was significant ($P<0.05$) in all cases. The rose-

Table 1 - Changes in induction period* of the lipid phase Tarama salad samples (0, 100, 200, 400 ppm of rosemary extract and 200 ppm BHA) during 40 days of storage.

Day	No antioxidant	Extract 100 ppm	Extract 200 ppm	Extract 400 ppm	BHA 200 ppm
0	$37.4\ (1.2)^{a}_{a}$	$38.0\ (1.7)^{b}_{a}$	$41.9\ (1.7)^{c}_{a}$	$42.0\ (1.2)^{c}_{a}$	$39.0\ (1.2)^{d}_{a}$
10	$33.2\ (1.5)^{a}_{b}$	$34.6\ (1.2)^{b}_{b}$	$37.0\ (1.0)^{c}_{b}$	$37.9\ (1.3)^{c}_{b}$	$35.6\ (1.4)^{b}_{b}$
20	$30.5\ (1.2)^{a}_{c}$	$33.3\ (1.0)^{b}_{c}$	$35.1\ (1.3)^{c}_{c}$	$36.7\ (0.6)^{d}_{c}$	$34.0\ (1.6)^{b}_{c}$
30	$25.3\ (1.2)^{a}_{d}$	$31.0\ (1.9)^{b}_{d}$	$33.8\ (1.0)^{c}_{d}$	$35.4\ (0.7)^{d}_{d}$	$31.9\ (1.5)^{b}_{d}$
40	$17.5\ (1.3)^{a}_{e}$	$25.2\ (1.0)^{b}_{e}$	$31.9\ (0.9)^{c}_{e}$	$33.0\ (1.3)^{d}_{e}$	$26.4\ (0.7)^{b}_{e}$

* Values are the mean and standard deviation (in parentheses) of triplicate determinations of three replicate experiments carried out simultaneously (nine determinations in total). Means within each row with different superscripts are significantly (P<0.05) different. Means within each column with different subscripts are significantly (P<0.05) different.

mary extract at a concentration of 400 ppm was the most effective antioxidant followed by the extract at 200 ppm after 40 days of storage. BHA (200 ppm) showed antioxidant activity equal to the extract at 100 ppm (no significant difference at P<0.05).

These results were also reflected in the sensory evaluation of Tarama salad after 40 days of storage (Table 2). On the day of preparation (day 0), the overall acceptability was good for all samples (no significant difference at P<0.05). After 40 days of storage the batches containing 200 and 400 ppm of extract had the highest (significant at P<0.05) overall acceptability among all batches and no significant change (P<0.05) in colour. The other batches showed a significant (P<0.05) reduction in their overall acceptability. In addition, no discolouration was observed in the Tarama salad with rosemary extract at 200 or 400 ppm. At day 40, the colour of the batches of salad with rosemary extract at 100 ppm, BHA or with no antioxidant showed significant (P<0.05) changes from red to pink. As determined by the overall preference, on the last day of the experiment the panellists showed a clear pref-

Table 2 - Sensory scores of Tarama salad after 40 day of storage.

Sensory attributes	Day	No antioxidant	Extract 100 ppm	Extract 200 ppm	Extract 400 ppm	BHA 200 ppm	
Overall acceptability*	0	$9\ (0.0)^{a}_{a}$	$9\ (0.0)^{a}_{a}$	$9\ (0.0)^{a}_{a}$	$9\ (0.0)^{a}_{a}$	$9\ (0.0)^{a}_{a}$	
	40	$3\ (1.0)^{a}_{b}$	$5\ (1.0)^{a}_{b}$	$8\ (1.0)^{b}_{b}$	$8\ (0.9)^{b}_{b}$	$7\ (0.9)^{c}_{b}$	
Colour**	0	$4\ (0.0)^{a}_{a}$	$4\ (0.0)^{a}_{a}$	$4\ (0.0)^{a}_{a}$	$4\ (0.0)^{a}_{a}$	$4\ (0.0)^{a}_{a}$	
	40	$1\ (0.1)^{a}_{b}$	$2\ (0.8)^{b}_{b}$	$4\ (1.0)^{c}_{a}$	$4\ (1.0)^{c}_{a}$	$2\ (0.9)^{d}_{b}$	
Overall preference***	0	1	0	1	1	1	16^{****}
	40	1	3	4	9	3	0^{****}

* Values represent the scale of overall acceptability (9 to 1) and the mean and standard deviation (in parentheses) of 20 observations. Means within each row with different superscripts are significantly (P<0.05) different. Means within each column with different subscripts are significantly (P<0.05) different.
** Values represent the scale of colour (4 to 1). Values are the mean and standard deviation (in parentheses) of 20 observations. Means within each row with different superscripts are significantly (P<0.05) different. Means within each column with different subscripts are significantly (P<0.05) different.
*** Number of panellists out of 20 who expressed their preference for the particular sample.
**** Number of panellists out of 20 who did not distinguish any differences.

erence for salad with rosemary at a concentration of 400 ppm. The salad without antioxidant had developed a rancid off-flavour. Caffeic acid, carnosol, carnosic and rosmarinic acid and other polyphenols present in extract have been reported to have good antioxidant action (alone or synergistically) (SHAHIDI, 2000; CUVELIER *et al.*, 1996) and, obviously, protected the product from oxidative deterioration.

Setting 10 hours as the end limit of the induction period, after which the product had unacceptable sensory properties, an estimation of the shelf-life of Tarama salad could be made using the linear equation. The theoretical decrease in the induction period after the 40th day until the induction period became 10 hours was calculated using the linear equation Y = a + bX (where Y = induction period and X = number of days) taking into account the intercepts and slopes of each batch. The calculated values were Y=38.797-0.477X (salad without antioxidant), Y=37.73-0.218X (salad with 100 ppm of rosemary extract), Y=40.402-0.222X (salad with 200 ppm of extract), Y=41.325-0.205X (salad with 400 ppm of extract) and Y=38.679-0.219X (salad with 200 ppm BHA). According to the above equations, the number of days before the induction period of each batch of salad reached the value of 10 hours (Y=10) was calculated to be 102.3 (without antioxidant), 127.2 (100 ppm of extract), 139.9 (200 ppm of extract), 152.8 (400 ppm of extract) and 131.1 (200 ppm of BHA). All antioxidants increased the shelf-life of the product. The rosemary extract at 400 ppm (increase 49%) was the most effective followed by the same extract at 200 ppm (34%) and BHA (28%), while the rosemary extract at 100 ppm increased the shelf-life the least (24%).

The rosemary extract at 400 ppm gave the highest protection against oxidation during the 40 days of storage and the longest shelf-life to the salad (as calculated by the linear equation) followed by the 200 ppm concentration. However, the same extract at 100 ppm showed the least protection (significant at $P<0.05$) during the 40 days of storage. The artificial antioxidant (BHA) showed moderate protection and extended the shelf-life of the product less than the rosemary extracts at 200 and 400 ppm but more than the extract at 100 ppm. Therefore, the natural antioxidant appears to be a good substitute for the synthetic one (BHA) at concentrations of 400 and 200 ppm. The results reported by ESTEVEZ *et al.* (2007) and SEBRANEK *et al.* (2005), regarding use of rosemary essential oils or extract to protect against the oxidative deterioration of liver paté and pork sausage, are in agreement with our results. In most cases, the natural antioxidant activity was proved superior to that of the artificial ones (BHT and BHA). However, these authors used substantially higher concentrations (1,000 to 2,500 ppm) of rosemary extract or essential oil. Other authors (CHE MAN and TAN, 1999) have also reported that rosemary extract had a higher antioxidant activity than BHA and BHT. Since Tarama salad is kept in the fridge, the addition of an antioxidant delays the oxidation of the lipid phase and prolongs the shelf-life of the product.

Deples

Table 3 illustrates the changes in the induction period of the oil extracted from Deples. The antioxidant improved the resistance to oxidative rancidity. The decrease in the induction period was significant ($P<0.05$) in all cases. The rosemary extract at a concentration of 150 ppm gave the highest protection (significant at $P<0.05$) to the product followed by the extract at a concentration of 100 ppm. The polyphenolic composition is responsible for the higher protection of the extract.

Setting 1 hour as the end limit of the induction period after which the product

Table 3 - Changes in induction period* of the lipid phase extracted from Depleses samples with rosemary extract during 35 days of storage.

Day	No antioxidant	Extract 50 ppm	Extract 100 ppm	Extract 150 ppm
0	$26.1\ (1.1)^{a}_{a}$	$31.9\ (1.8)^{b}_{a}$	$34.3\ (1.7)^{c}_{a}$	$37.3\ (1.3)^{d}_{a}$
7	$22.2\ (0.8)^{a}_{b}$	$27.0\ (1.1)^{b}_{b}$	$30.2\ (1.2)^{c}_{b}$	$35.2\ (1.5)^{d}_{b}$
14	$15.2\ (0.7)^{a}_{c}$	$20.7\ (1.3)^{b}_{c}$	$25.1\ (0.8)^{c}_{c}$	$30.4\ (0.9)^{d}_{c}$
21	$10.1\ (0.9)^{a}_{d}$	$15.2\ (0.8)^{b}_{d}$	$20.8\ (1.1)^{c}_{d}$	$25.6\ (1.1)^{d}_{d}$
28	$4.5\ (0.7)^{a}_{e}$	$10.3\ (0.9)^{b}_{e}$	$15.8\ (1.0)^{c}_{e}$	$22.5\ (1.1)^{d}_{e}$
35	$1.5\ (0.7)^{a}_{f}$	$5.1\ (0.6)^{b}_{f}$	$10.9\ (1.3)^{c}_{f}$	$20.2\ (0.7)^{d}_{f}$

* Values are the mean and standard deviation (in parentheses) of triplicate determinations of three replicate experiments carried out simultaneously (nine determinations in total). Means within each row with different superscripts are significantly ($P<0.05$) different. Means within each column with different subscripts are significantly ($P<0.05$) different.

has unacceptable sensory properties, an estimation of the shelf-life of Depleses can be made using the linear equation. The equations were Y=26.949-0.7396X (no antioxidant), Y=32.683-0.7739X (50 ppm of extract), Y=35.271-0.6714X (100 ppm of extract) and Y=38.229-0.5241X (150 ppm of extract). According to the above equations, the number of days before the induction period of each batch reached the value of 1 hour (Y=1) were calculated as 35.1 (no antioxidant), 40.9 (50 ppm), 51.0 (100 ppm) and 71.0 (150 ppm). The greatest increase was achieved with the rosemary extract at 150 ppm (102.3% increase) followed by 100 ppm (45.3%) and 50 ppm (16.5%).

The inhibition of oxidation of the frying oil absorbed by Depleses is very important. The oil continues to oxidize during storage which results in an unacceptable and unhealthy product. These results are in accordance with those of the sensory evaluation after 35 days of storage (Table 4). The overall acceptability as expressed by the score 9 to 1 was equal in all cases (not significant at $P<0.05$) at Day 0. According to Table 4 Depleses were generally acceptable with or without antioxidant since there was no

Table 4 - Sensory scores of Depleses.

Sensory attributes	Day	No antioxidant	Extract 50 ppm	Extract 100 ppm	Extract 150 ppm	
Overall acceptability*	0	$9\ (0.0)^{a}_{a}$	$9\ (0.0)^{a}_{a}$	$9\ (0.0)^{a}_{a}$	$9\ (0.0)^{a}_{a}$	
	35	$4\ (1.0)^{a}_{b}$	$6\ (2.0)^{b}_{b}$	$8\ (1.1)^{c}_{b}$	$8\ (1.0)^{c}_{b}$	
Colour**	0	$1\ (0.0)^{a}_{a}$	$4\ (0.0)^{b}_{a}$	$4\ (0.0)^{b}_{a}$	$4\ (0.0)^{b}_{a}$	
	35	$1\ (0.0)^{a}_{b}$	$4\ (0.1)^{b}_{a}$	$4\ (0.2)^{b}_{a}$	$4\ (0.1)^{b}_{a}$	
Overall preference***	0	2	2	1	1	14^{****}
	35	0	2	5	12	1^{****}

* Values represent the scale of overall acceptability (9 to 1) and are the mean and standard deviation (in parentheses) of 20 observations. Means within each row with different superscripts are significantly ($P<0.05$) different. Means within each column with different subscripts are significantly ($P<0.05$) different.
** Values represent scale of colour (4 to 1) and the mean and standard deviation (in parentheses) of 20 observations. Means within each row with different superscripts are significantly ($P<0.05$) different. Means within each column with different subscripts are significantly ($P<0.05$) different.
*** Number of panellists out of 20 who expressed their preference on the particular sample.
**** Number of panellists out of 20 who did not distinguish any differences.

statistically significant difference in total acceptability at Day 0. However, after 35 days of storage Deples containing 100 and 150 ppm of rosemary extract were the most acceptable (significant at $P<0.05$) among the whole lot of products. In evaluating the overall preference, the panellists showed a preference for Deples containing antioxidant after 35 days of storage compared to Deples without antioxidant. Rosemary extract, at all concentrations, significantly ($P<0.05$) reduced the darkening of Deples while the product without antioxidant turned brown. According to LALAS and DOURTOGLOU (2003), the colour of fried products (potato chips) is produced mainly by Maillard reactions. Antioxidants not only reduce oxidation but also, indirectly block the Maillard reaction by inhibiting of the final pyrazine formation phase (PORTER *et al.*, 2006). This reduces the formation of the dark colour that occurs as a result of reactions between free sugars and amino acids during frying, especially in the present case where orange juice (high sugar content) was used in the recipe. Finally, a pungent taste (due to the oxidation of the absorbed oil in Deples), made the product unacceptable by the panellists, especially after 35 days of storage (0 in overall preference) (Table 4).

In conclusion, rosemary extract protected Tarama Salad and Deples against oxidation and extended their shelf-life. The use of rosemary extract has a positive effect on consumer acceptance (as indicated by the sensory evaluation) of all the products that contain a considerable amount of oil (absorbed during frying or added during preparation).

ACKNOWLEDGMENT

We are grateful to VIORYL S.A. for providing Rosemax®.

REFERENCES

Che Man Y.B. and Tan C.P. 1999. Effects of natural and synthetic antioxidants on changes in refined, bleached, and deodorised palm olein during deep-fat frying of potato chips. J. Am. Oil Chem. Soc. 76: 331.

Cuvelier M.E., Richard H. and Berset C. 1996. Antioxidative activity and phenolic composition of pilot-plant and commercial extracts of sage and rosemary. J. Am. Oil Chem. Soc. 73: 645.

Estevez M., Ramirez R., Ventanas S. and Cava R. 2007. Sage and rosemary essential oils versus BHT for the inhibition of lipid oxidative reactions in liver pate. Lebensm. Wiss. Technol. 40: 58.

Farag R.S., Badel A.Z.M.A., Hewedl F.M. and El-Baroty G.S.A. 1989. Antioxidant activity of some spice essential oils on linoleic acid oxidation in aqueous media. J. Am. Oil Chem. Soc. 66: 792.

Kim S.Y., Kim J.H., Kim S.K., Oh M.J. and Jung M.Y. 1994. Antioxidant activities of selected oriental herb extracts. J. Am. Oil Chem. Soc. 71: 633.

Kishk Y.F.M. and Al-Sayed H.M.A. 2007. Free-radical scavenging and antioxidative activities of some polysaccharides in emulsions. Lebensm. Wiss. Technol. 40: 270.

Lalas S. and Dourtoglou V. 2003. Use of rosemary extract in preventing oxidation during deep fat frying of potato chips. J. Am. Oil Chem. Soc. 80: 579.

Mitsumoto M., O'Grady M.N., Kerry J.P. and Buckley D.J. 2005. Addition of tea catechins and vitamin C on sensory evaluation, colour and lipid stability during chilled storage in cooked or raw beef and chicken patties. Meat Sci. 69:773.

Porter W.L., Conca K.R., Yeomans W.G., Diotte S., Lynch A. and Tate J. 2006. Modification of Maillard browning in a microwaved glucose/glycine model system by water-soluble natural antioxidants and foods containing them. J. Am. Oil Chem. Soc. 83: 697.

Rudnik E., Szczucinska A., Gwardiak H., Szulc A. and Winiarska A. 2001. Comparative studies of oxidative stability of linseed oil. Thermochim. Acta 370: 135.

Sebranek J.G., Sewalt V.J.H., Robbins K.L. and Houser T.A. 2005. Comparison of a natural rosemary extract and BHA/BHT for relative antioxidant effectiveness in pork sausage. Meat Sci. 69: 289.

Shahidi F. 2000. Antioxidants in food and food antioxidants. Nahrung 3: 158.

Tsaknis J. and Lalas S. 2002. Stability during frying of *Moringa oleifera* seed oil variety "Periyakulam 1". J. Food Compos. Anal. 15: 79.

Zhang K.Q., Bao Y.D., Wu P., Rosen R.T. and Ho C.T. 1990. Antioxidative components of Tanshen (*Salvia miltiorrhiza* Bung). J. Am. Oil Chem. Soc. 38: 1194.

Revised paper recieved February 2, 2007 Accepted March 27, 2007

PAPER

ANTIOXIDANT ACTIVITIES OF SOME EDIBLE WILD MEDITERRANEAN PLANTS

ATTIVITÀ ANTIOSSIDANTE DI ALCUNE PIANTE ALIMENTARI MEDITERRANEE NON COLTIVATE

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ABSTRACT

The antioxidant activities of ethanol extracts of eleven edible plants grown in Jordan (eminium, Judean sage, Spanish thistle (stem), pepperwort, coriander, golden thistle, cyclamen, Roman nettle, silver nailroot, santolina milfoil, and musky archangel) were investigated using reducing power and DPPH radical tests. Santolina milfoil, Judean sage and silver nailroot showed the highest antioxidant activities, while pepperwort and Roman nettle showed the lowest activities. A significant, pos-

RIASSUNTO

Utilizzando i saggi del potere riducente e del radicale DPPH, sono state determinate, le attività antiossidanti di estratti etanolici di 11 piante commestibili cresciute in Giordania: l'Eminium, la salvia di Giudea, il cardo spagnolo (gambo), il Lepidio latifoglio, il coriandolo, il cardo dorato, il ciclamino, l'ortica romana, la Paronichia argentea, la Santolina e l'arcangelo muschiato. La Santolina, la salvia di Giudea e la Paronichia argentea mostravano le migliori attività antiossidanti, mentre il Lepi-

- Key words: antioxidant, oil, oil-water emulsions, plant extracts -

itive correlation was found between the antioxidant activity measured by reducing power or DPPH scavenging activity and the total phenolic content of the extracts. The antioxidant activity of the extracts (at 2,000 μg g^{-1}) of santolina milfoil, eminium and pepperwort that showed high, moderate and low antioxidant activity, respectively, were evaluated in corn oil and corn oil-water emulsion. The results were compared with the standard antioxidants Trolox (the carboxyl analog of α-tocopherol) and α-tocopherol (at 100 ppm). The antioxidant activity of these extracts was evaluated by determining the percentage of conjugated dienes. Trolox was more effective than α-tocopherol in corn oil and the opposite was found in the emulsified oil. Similarly, the plant ethanol extracts were more effective as antioxidants in corn oil than in the oil-water emulsion. The order of the antioxidant activity of these antioxidants in corn oil was santolina = Trolox > eminium > pepperwort = α-tocopherol, while the order in corn oil-water emulsions was α-tocopherol > santolina > eminium > pepperwort = Trolox.

dio latifoglio e l'ortica romana mostravano le più basse attività antiossidanti. È stata rilevata una correlazione significativa e positiva fra l'attività antiossidante misurata attraverso il potere riducente o l'attività di scavenging del DPPH e il contenuto in polifenoli totali degli estratti. I valori delle attività antiossidanti degli estratti (2.000 μg g^{-1}) di Santolina, Eminium e Lepidio latifoglio (che mostravano, rispettivamente, alta, moderata e bassa attività antiossidante) sono stati determinati nell'olio di mais e in un'emulsione di olio di mais ed acqua. I risultati sono stati confrontati con gli standards antiossidanti Trolox (l'analogo carbossilico dell'α-tocoferolo) e l'α-tocoferolo (100 ppm). L'attività antiossidante di questi estratti era stata valutata determinando la percentuale di dieni coniugati. Il Trolox risultava più efficace dell'α-tocoferolo nell'olio di mais mentre il comportamento opposto veniva rilevato nell'emulsione dell'olio di mais con acqua. Analogamente, gli estratti etanolici delle piante risultavano più efficaci come antiossidanti nell'olio di mais rispetto all'emulsione olio-acqua. L'ordine del potere antiossidante di questi antiossidanti nell'olio di mais era: santolina = Trolox > eminium > Lepidio latifoglio = α-tocoferolo, mentre l'ordine nelle emulsioni olio-acqua era α-tocoferolo > santolina > eminium > Lepidio latifoglio = Trolox.

INTRODUCTION

Lipid oxidation during food processing and storage significantly decreases the nutritional value and overall quality of food. It is also associated with aging, membrane damage, heart disease, stroke, and cancer in living organisms (ADDIS and WARNER, 1991). The addition of antioxidants to food is an effective way to limit the degree of oxidation of fat. While some of these antioxidants occur as natural dietary constituents (DUH and YEN, 1997), others are synthetic and some of these such as BHA (butylated hydroxyanisole) and BHT (butylated hydroxytoluene) are believed to be carcinogenic (IMADIA *et al.*, 1983). This finding together with consumer preference for natural products

Table 1 - Ethnobotanical data of the plants studied [1].

Family name	Scientific name	English name
Araceae	*Eminium spiculatum*	Eminium
Labiatae (Lamiaceae)	*Salvia juduica*	Judean sage
Compositae (Asteraceae)	*Centaurea iberica*	Spanish thistle
Cruciferae	*Lepidium latifolium*	Pepperwort
Umbelliferae (Apiaceae)	*Coriandrum sativum*	Coriander
Compositae (Asteraceae)	*Scolymus maculatus*	Golden thistle
Primulaceae	*Cyclamen persicum*	Cyclamen
Urticaceae	*Urtica pilulifera*	Roman nettle
Caryophyllaceae	*Paronychia argentea*	Silver nailroot
Compositae (Asteraceae)	*Achillea santolina*	Santolina milfoil

[1]Adopted from: AL-EISAWI (1998).

has resulted in an increased effort to find natural antioxidants. It has been demonstrated that the extracts of many plants have potent antioxidant activity (AMR, 1991; ECONOMOU *et al.*, 1991; KARIN *et al.*, 1992; AL-ISMAIL and ABURJA, 2003; VEKIARI *et al.*, 1993; MARIE-ELISABITH *et al.*, 1996).

It has been reported that Trolox (a carboxylic acid analog of α-tocopherol) plus ascorbic acid, and rosemary extract, which are hydrophilic, are more active as antioxidants in bulk oil than their lipophilic analogues (α-tocopherol and ascorbyl palmitate), but they are less active in oil/water emulsions (FRANKEL *et al.*, 1994; 1996). These results indicated that the differences in antioxidant efficiency could be related to their affinities toward the air-oil interfaces in oils and to the oil-water interfaces in emulsions (HUANG *et al.*, 1994).

Many natural antioxidants have been found in plant leaves, seeds and nuts and various solvents have been used to extract these antioxidants. They will therefore have different polarities and complex interfacial affinities between air-oil and oil-water interfaces. The information related to the behavior of these antioxidants in pure oils or in oil-water emulsions becomes very important when these antioxidants are used in food products.

The aims of this study were to screen the antioxidant activities of ethanol extracts of ten edible plants grown in Jordan and compare the antioxidant activities of some of these extracts in corn oil and oil-water emulsion.

MATERIALS AND METHODS

Plant samples

The plants used in this study were collected in the spring of 2003 from different locations in northern Jordan (Table 1).

The edible parts of the plants were removed by hand, dried at 50°C in a circulated air oven. They were then milled using a Brown domestic coffee mill (Brown GmbH, Germany) for 3-5 min and then passed through a 0.5 mm sieve. The particles that did not pass through the sieve were remilled to pass through the sieve. Fifty grams of the milled plant material were used to obtain the extract under reflux conditions with 95% ethanol for 2 h each according to VEKIARI (1993). The extracts were filtered and the solvents were completely evaporated by rotary evaporator

at 35°C. The extract yields are reported in Table 2.

Determination of total phenolic compounds

The phenolic compounds present in the ethanol extracts of the selected plants were determined by using the Folin-Denis reagent (PIN-DER and GOWCHIN, 1997). Twenty-five μL of ethanol extract solutions (1 mg/mL) were diluted with 20 mL of ethanol in a volumetric flask (50 mL). Folin-Denis reagent (2.5 mL) was added and the solution was mixed thoroughly. After 3 min, 2.5 mL of sodium carbonate solution (10% w/v) was added and the solution was made up to the mark with ethanol. The solution was left to stand for 30 min and the absorbance of the sample was then measured at 760 nm using a spectrophotometer (Biotech Engineering Management Co. Ltd, Cambridge UK). The results are expressed as milligrams gallic acid per gram of dried extract (mg g^{-1}).

Table 2 - The yield and total phenolic compounds (TPC) expressed as gallic acid equivalent of the ethanol extracts of the plants.

Plant extract	Yield (mg g^{-1} dried plant) [1, 2]	TPC (mg g^{-1} extract)
Judean sage (leaves)	33.2±1.7[e]	380.5±10.5[a]
Santolina milfoil (whole)	54.0±1.1[c]	328.2±12.4[b]
Golden thistle (stem)	13.7±1.7[f]	300.1±13.4[c]
Eminium (leaves)	67.0±2.9[b]	272.2±6.5[d]
Silvery nailroot (whole)	58.8±4.6[c]	221.1±6.4[e]
Coriander (whole)	73.3±5.4[a]	170.4±10.3[f]
Cyclamen (leaves)	67.4±2.1[b]	166.9±8.5[f]
Spanish thistle (stem)	65.3±2.4[b]	135.4±5.1[g]
Roman nettle (stem)	75.0±4.5[a]	127.0±5.2[h]
Pepperwort (whole)	40.0±1.9[d]	111.6±4.2[i]

[1]values are means of three replicates ± standard deviation.
[2]Means with different letters within a column are significantly different (P≤0.05) according to Duncan multiple range test.

Antioxidant activity

Determination of reducing power

The reducing power of the extracts was determined according to the method of OYAIZU (1986). One mL of plant extract solution (100 g L^{-1}) was placed in a capped test tube and the solvent was evaporated under a flow of nitrogen. The residue was mixed with 5 mL of phosphate buffer (2 M, pH 6.6) and 5 mL of potassium ferricyanide solution (10 g L^{-1}) and the mixture was heated at 50°C for 20 min. Five mL of trichloroacetic acid solution (100 g L^{-1}) was added and then centrifuged at 5,000 rpm for 10 min. Five mL of the upper layer of the solution was mixed with 5 mL distilled water and 1 mL of ferric chloride solution (1 g L^{-1}). The absorbance of this solution was measured at 700 nm using a spectrophotometer (Biotech Engineering Management Co. Ltd, Cambridge, UK). Increased absorbance of the reaction mixture indicated increased reducing power. Ascorbic acid at three concentrations was used as standard.

2,2-Diphenyl-1-picrylhydazyl (DPPH) free radical scavenging activity of the extracts

The DPPH radical scavenging effect was determined according to the method of MATTHAUS (2002). A 0.2 mL ethanol solution of DPPH (0.5 g L^{-1}) was mixed with different amounts of ethanol and the mixture was brought to a total volume of 4.0 mL with the corresponding extracting solvent. The mixture was mixed thoroughly and allowed to stand for 45 min in a dark place. The absorbance was then measured at 515 nm using a spectrophotometer (Biotech Engineering Management Co. Ltd, Cambridge, UK). A control sample of ethanol extract was also run. The scaveng-

ing activity of the extracts was calculated as:

$$\text{Inhibition (\%)} = \frac{\text{Abs control - Abs sample}}{\text{Abs control}}$$

IC_{50} (the concentration of the extracts in mg/mL required to scavenge 50% of the added DPPH radical) was calculated from the dose-response curves.

Evaluation of the antioxidant activity of three ethanol extracts in corn oil and corn oil-water emulsions

The ethanol extracts of santolina milfoil, eminium and pepperwort that showed strong, moderate and weak antioxidant activity as measured by the reducing power and DPPH radical tests were used to determine their antioxidant activities in corn oil and corn oil-water emulsion. Samples of oil and oil-water emulsions were prepared according to FRANKEL *et al.* (1994) as follows: refined corn oil sample with no antioxidant and samples that contained α-tocopherol, Trolox, santolin milfoil, eminium and pepperwort. The concentration of the two standard antioxidants was 100 μg g^{-1} and the concentration of the 3 plant extracts was 2,000 μg g^{-1}. Ten percent (w/v) corn oil/water emulsions were prepared with no antioxidants. The oils containing the antioxidants and extracts were prepared using 1 g of Tween 20 as emulsifier. The emulsification was carried out in an ice bath using a sonicator (Elma Transonic T310m, Singen, Germany) at high power for 8 min. These emulsions were physically stable during oxidation at 60°C.

Oxidation

Twenty-five g of the prepared corn oil samples were put into a screw-capped 100 mL bottle and 100 g of the 10% emulsion samples (75 mL) were put into 200 mL flasks and oxidized at 60°C in an electric oven. Samples were removed after 0. 1, 2, 4, 6 and 8 days of heating and the conjugated dienes were determined.

The percentage of conjugated dienes in the samples was determined according to the A.O.C.S methods (1973). One-hundred mg of the oil samples were dissolved in 100 mL of isooctane. For the oil-water emulsion samples, 10 mL was extracted twice with 5 mL of a methanol/hexane mixture (1:1). The methanol layer was washed three times with 3 mL of hexane. The hexane extracts were then combined and evaporated to dryness under nitrogen. One-hundred μL of each prepared solution was diluted with 5 mL of isooctane and the absorbance was measured at 232 nm using a spectrophotometer (Biotech Engineering Management Co. Ltd, Cambridge, UK). The percentage of conjugated dienes was calculated according to the following formula:

$$\text{\% conjugated dienes} = 0.91\ ((A/bc)-k)$$

where:
A = absorbance at 232 nm;
b = cell length in cm;
c = concentration of the sample (g L^{-1});
k = ester absorptivity = 0.07.

The percentage inhibition of diene formation was calculated according to the following formula:

$$\text{\% Inhibition} = [(\text{control-sample/control})] \times 100$$

Statistical analysis

Statistical analysis was carried out using the Statistical Analysis Systems (SAS, 1985) software package. Analysis of variance was performed by ANOVA procedures. Significant differences between means were determined by Duncan's multiple range tests.

RESULTS AND DISCUSSION

Total phenolic compounds

Most natural plant antioxidants are believed to be phenolic compounds (KIM *et al.*, 1997). YEN *et al.* (1993) reported that peanut hulls showed antioxidative activity in oil which was attributed to their phenolic compound content. YEN and CHEN (1995) found that polyphenols were the most abundant compounds in plant leaves which showed antioxidant activity. These findings indicate that phenolic compounds are responsible for antioxidant activity. The total phenolic compounds (TPC) expressed as gallic acid equivalent of the ethanol used are reported in Table 2. The data show that the total phenolic compound content of the ethanol extracts was in the range of 111-380 mg g^{-1}. The ethanol extract of Judean sage had the highest total phenolic compounds, while pepperwort had the lowest.

Antioxidant activity

While many methods can be used to measure the antioxidant activity of natural extracts, in this study, the scavenging of the DPPH radical and reducing power methods were used.

Reducing power

Table 3 shows the reducing power of the ethanol extracts of the selected plants. The ethanol extract of santolina milfoil had the strongest reducing power with an absorbance value of 1.614, while pepperwort had the weakest reducing power with an absorbance value of 0.148. The ethanol extracts were divided into three groups according to whether they showed strong, moderate or weak reducing power. The strong group included santolina milfoil, Judean sage, silvery nailroot and cyclamen with absorbance value ranging from 0.9 to 1.6. Eminium, golden thistle, coriander, Spanish this-

Table 3 - Reducing power and DPPH radical scavenging activity expressed as IC_{50} of the ethanol extract of the selected plants.

Plant extract	Reducing power[1,2]	Plant extract	DPPH (IC_{50})
Santolina milfoil	1.614 [a]	Santolina milfoil	0.17
Silver nailroot	1.113[c]	Silver nailroot	0.48
Judean sage	1.392[b]	Judean sage	0.23
Cyclamen	0.960[d]	Eminium	0.64
Eminium	0.525[h]	Cyclamen	1.33
Golden thistle	0.515[hi]	Spanish thistle (stem)	2.25
Coriander	0.449[ji]	Coriander	2.6
Spanish thistle (stem)	0.390[jk]	Golden thistle	2.67
Roman nettle	0.383[jk]	Pepperwort	3.1
Pepperwort	0.148[L]	Roman nettle	5.09
Ascorbic acid (0.05 mg)	0.323[k]		
Ascorbic acid (0.1 mg)	0.594[g]		
Ascorbic acid (0.15 mg)	0.885[e]		

[1]Values are means of three replicates.
[2]Means with different letters within a column are significantly different ($P \leq 0.05$) according to Duncan multiple range test.

tle and Roman nettle had moderate reducing power with absorbance values in the range of 0.38 to 0.5 and pepperwort has weak reducing power.

Ascorbic acid, which is a reducing agent as well as a reductone (SHIMADA *et al.*, 1992), was used at three different concentrations as a reference to compare its antioxidant activity, measured by the reducing power test, with that of the extracts used in this study at 2,000 mg kg^{-1}. It is evident that the ethanol extracts of santolina milfoil, Judean sage, silvery nailroot and cyclamen were significantly more effective as antioxidants than 0.10 mg ascorbic acid. Eminium, golden thistle, coriander, Spanish thistle and Roman nettle were more effective than 0.05 mg of ascorbic acid, while pepperwort was the least effective (Table 3).

The results indicate that these extracts could react with free radicals (as electron donors) to convert them into stable products and terminate the radical chain reaction (PIN-DER and GOW-CHIN, 1997). TANAKA *et al.* (1988) reported that the antioxidant activity exponentially increases as a function of the reducing power, indicating that antioxidant properties are concomitant with the development of reducing power.

2, 2-Diphenyl-1-picrylhydazyl (DPPH) free radical scavenging effect

It is well known that lipids containing polyunsaturated fatty acids and their esters are readily oxidized by molecular oxygen. Such oxidation, called autoxidation, proceeds by a free radical chain reaction mechanism (FRANKEL *et al.*, 1987). On the other hand, antioxidants are believed to scavenge free radicals and suppress free radical chain oxidation by molecular oxygen (MAGDALENA *et al.*, 2001). DPPH is a stable free radical that accepts an electron or hydrogen radical to become a stable molecule (GOUPY *et al.*, 1999). It has been used to evaluate the antioxidant action of various natural materials (ALEJANDRO *et al.*, 2000). Table 3 shows the DPPH radical scavenging activity of the extracts expressed as IC_{50} (the concentration mg/mL of the extract required to scavenge 50% of the added radical). The IC_{50} of the extracts varied from 0.17 mg/mL for santolina milfoil to 5.09 mg/mL for Roman nettle. The extracts were then grouped according to their strong, moderate or low radical scavenging activity. Three out of the 11 extracts, santolina milfoil, Judean sage and silver nailroot showed strong radical scavenging capacity ($IC_{50} < 1$ mg/mL). The group that showed moderate activity ($IC_{50} \geq 1$ and ≤ 2 mg/mL) included the eminium and cyclamen extracts. The Spanish thistle, coriander, golden thistle, pepperwort and Roman nettle extracts showed weak radical-scavenging capacity ($IC_{50} > 2$ mg/mL).

The statistical analysis showed a highly significant relationship between the total phenolic compounds (TPC) content and the reducing power ($r = 0.799$) and between the TPC content and radical-scavenging activity (expressed as the reciprocal of IC_{50} ($r = 0.927$). This finding agrees with the results of other workers who found a linear relationship between the antioxidant activity of plant extracts and the TPC content (TSUSHIDA *et al.*, 1994; VELIOGLU *et al.*, 1998; KAUR and KAPOOR, 2002). In contrast, some other workers did not find any correlation between phenolic compounds and antioxidant activity (CUVELIER *et al.*, 1992; HEINONEN *et al.*, 1998; STATUE-GRACIA *et al.* 1997). They reported that their finding could be attributed to the different phenolics which had different response for Folin-Ciocalteu, to molecular antioxidant response of phenolics, which depends on their chemical structure or to the different systems used to evaluate the antioxidant activity. On the other hand, a positive and highly significant relationship ($r = 0.886$) was found between the reducing power and radical-scavenging activity (expressed as the reciprocal of IC_{50}),

indicating that these two tests could be used to evaluate the antioxidant activity of the phenolic compounds.

Antioxidant activity of the extracts of santolina milfoil, eminium and pepperwort in corn oil and corn oil-water emulsion

PORTER (1993) postulated that polar antioxidants such as trolox and propyl gallate are more effective in bulk oils than in emulsified oils, while non-polar antioxidants such as BHA and BHT are more effective in emulsified oil than in bulk oils. To determine if this hypothesis can be applied to natural extracts of plants, the antioxidant activities of the ethanol extracts (as lyophobic antioxidants) of santolina milfoil, eminium and pepperwort (strong, medium or low antioxidant activities, respectively) were evaluated in bulk oil or corn oil-water emulsion incubated at 60°C for different times. The results were then compared with those observed for Trolox (hydrophobic) and α-tocopherol (lyophilic) standard antioxidants. The results are reported in Fig. 1 and Table 4. Trolox was more effective in depressing the formation of conjugated dienes in corn oil than in the emulsified oil, while the opposite was found for α-tocopherol (Fig. 1 & Table 4). In corn oil, Trolox showed an antioxidant activity comparable to that of santolina; it had an activity that was significantly ($P<0.05$) higher than those of the other two extracts. In corn oil-water emulsion, α-tocopherol was the most effective antioxidant followed by santlolina, while Trolox and pepperwort showed the lowest activities. The order of the antioxidant activities of the three extracts in the oil and emulsified oil systems was santolina > eminium > pepperwort. These results might be attributed to their TPC content (Table 2). Similar to the lyophobic antioxidant (Trolox), the antioxidant activity of the three ethanol extracts in corn oil was significantly higher than that observed in corn oil-water emulsion. These results support the hypothesis of PORTER (1993) and are also in agree-

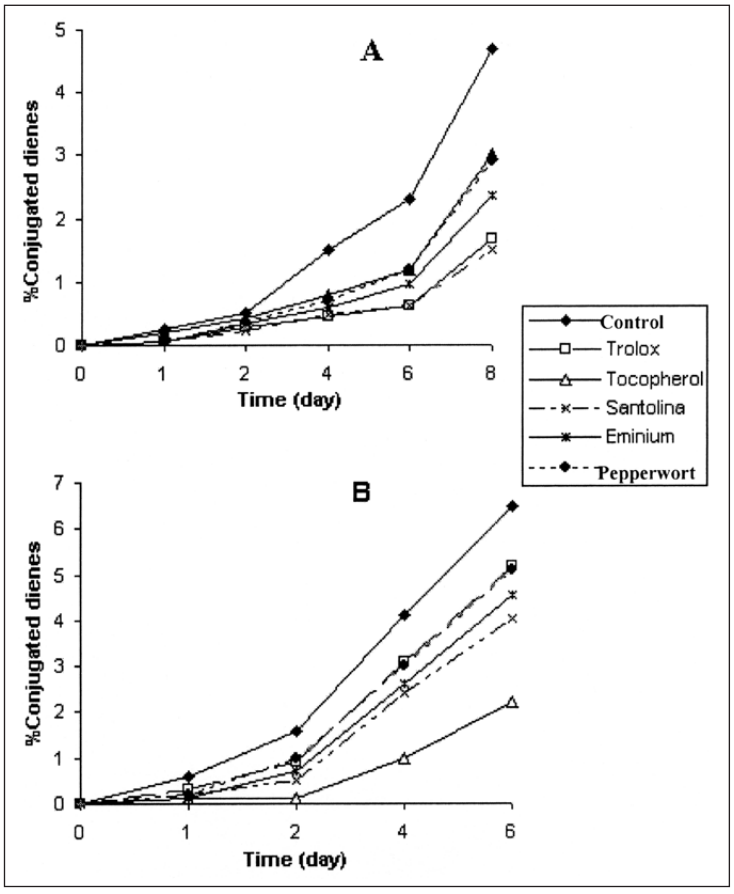

Fig. 1 - The antioxidative activity of trolox, tocopherol and three selected extracts in (A) corn oil and (B) corn oil-water emulsion.

Table 4 - Inhibition of conjugated diene formation by two standard antioxidants and 3 plant extracts.

Sample	Corn oil [1] Oxidation Time (days)		Corn oil-water emulsion Oxidation Time (days)	
	6	8	4	6
Trolox	73.0 [2a]	64.0[b]	24.4[d]	20.0[d]
α-tocopherol	47.8[c]	44.3[d]	75.6[a]	66.2[a]
Santolina milfoil	72.6[a]	67.9[a]	41.5[b]	38.2[b]
Eminium	57.8[b]	52.6[c]	36.6[c]	30.0[c]
Pepperwort	47.8[c]	42.6[d]	26.8[d]	21.5[d]

[1]Values are means of three replicates.
[2]Means with different letters within a column are significantly different (P≤0.05) according to Duncan multiple range test.

ment with the results of other workers (FRANKEL *et al.*, 1994; 1996) who reported that lipophilic antioxidants such as α-tocopherol and ascorbyl acetate were more effective in emulsified oil systems than in bulk oil, while the hydrophilic antioxidants such as Tolox, ascorbic acid, rosmerinic acid, and the water extract of rosemary were more effective in bulk oil than in the emulsified oil system. They explained these findings on the basis of their interfacial properties. In the emulsified oil system, the molecules of the lipophilic antioxidants which orient themselves on the oil-water surface, provide more protection against oil oxidation than the hydrophilic antioxidants. In the bulk oil, the molecules of the hydrophilic antioxidants, which orient themselves on the air-oil surface, provide more protection against oil oxidation than the lipophilic antioxidants.

CONCLUSION

The results indicate that the reducing power and DPPH radical test can be used to evaluate the antioxidant activity of plant extracts. The results demonstrate that the lipophobic antioxidant Trolox and the three selected ethanol extracts showed greater antioxidant activities in corn oil than in the corn oil-water emulsion. In contrast, the lipophilic antioxidant α-tocopherol was more effective in the corn oil-water emulsion than in corn oil. A linear, positive correlation was found between the TPC content and the antioxidant activity measured by the DPPH radical and reducing power tests.

REFERENCES

Addis P.B. and Warner G.J. 1991. The potential health aspects of lipid oxidation products in food. In "Free Radical and Food Additives", p. 77. Taylor & Francis (Ed), London.

Al-Eiswi D. M. 1998. "Wild Flowers of Jordan and Neighbouring Countries" National Library, Amman, Jordan.

Al-Ismail K. and Aburjai T. 2003. Antioxidant activity of water and alcohol extracts of chamomile flowers, anise seeds, and dill seeds. J. Sci. Food- Agric., 84:173.

Alejandro T., Gabriela E. F., Daniel B., Luis A., Cristina, T. and Guillermo S. H. 2000. Biological active alkaloids and free radical scavenger from prosopis species. J. Ethnopharmacol. 71:241.

Amr A. 1991. Effectiveness of synthetic and potential natural antioxidants in improving the stability of sheep's anhydrous butter fat during long-term storage. J. Sci. Food Agric., 55:75.

AOCS. 1973. "Official Methods and Recommended Practices of the American Oil Chemists' Society", 3rd ed, R.O. Walker (Ed.), American Oil Chemists' Society, Champaign.

Cuvelier M.E., Richard H. and Berset C. 1992. Comparison of antioxidative activity of some acid-phenols: structure-activity relationship. Biosci. Biotech. Biochem., 56:324.

Duh P. and Yen G. 1997. Antioxidative activity of three herbal water extracts. J. Agric. Food Chem., 60:639.

Economou K.D., Oreopoulou V. and Thomopoulos C.D. 1991. Antioxidant activity of some plant extracts of the family Labiatae. J. Am. Oil Chem. Soc., 68:109.

Frankel E.N., Nash A.M. and Snyder J.M. 1987. A methodology study to evaluate quality of soybeans stored at different moisture levels. J. Am. Oil Chem. Soc., 74:387.

Frankel E.N., Huang S., Kanner J. and German B. 1994. Interfacial phenomena in the evaluation of antioxidants: bulk oils *vs* emulsions. J. Agric. Food Chem., 42:1054.

Frankel E.N., Huang S., Aeschbach R. and Prior E. 1996. Antioxidant activity of a rosemary extract and its constituents, carnosic acid, carnosol, and rosmaric acid, in bulk oil and oil-in-water emulsion. J. Agric. Food Chem. 44:131.

Goupy P., Hugues M., Boivin P. and Amiot. 1999. Antioxidant composition and activity of barley (*Hordeum vulgare*) and malt extracts and of isolated phenolic compounds. J. Sci. Food Agric. 79:1625.

Heinonen M., Lehtonen P.J. and Hopia A. 1998. Antioxdative activity of berry and fruit wines and liquor. J. Agric. Food Chem., 46:25.

Huang S.W., Frankel E.N. and German J.B. 1994. Antioxidant properties of α- and γ-tocopherols in bulk oil and in oil-in-water emulsions. J. Agric. Food Chem., 42:2108.

Imadia K., Fukushima S., Shirai T., Ohtani M., Nakanish K. and Ito, N. 1983. Promoting activities of butylated hydroxytoluene on 2-stage urinary bladder carcinogenesis and inhibition of γ-glutamyl transpeptidase-positive foci development in the liver of rats. Carcinogenesis, 4:895.

Karin S., Waldemer T., and Eberhard S. 1992. Antioxidative constituents of *Rosmarinus officinalis* and *Salvia officinalis*. III. Stability of phenolic diterpens of rosemary extracts under thermal stress as required for technological process. Z. Lebensm. Unters. Forsch., 195:104.

Kaur C. and Kapoor C. 2002. Anti-oxidant activity and total phenolic content of some Asian vegetables, Inter. J. Food Sci. Tech., 37:153.

Kim B.J., Kim J.H., Kim H.P. and Heo M.I. 1997. Biological screening of 100 plants for cosmetic use (II): Antioxidative activity and free radical scavenging activity. Int. J. Cosmet. Sci., 19:299.

Magdalena M.T., Antonia M.J., Silverio R., Natale F. Rosanna S. and Antonia M.M. 2001. Antioxidant properties of Mediterranean spices compared with common food additives. J. Food Prot., 64:1412.

Marie-Elisabeth C., Hubert R. and Claudette B. 1996. Antioxidant activity composition of pilot-plant and commercial extracts of sage and rosemary. J. Am. Oil Chem. Soc., 73:645.

Matthaus B. 2002. Antioxidant activity of extracts obtained from residues of different oilseeds. J. Agric. Food Chem., 50:3444.

Oyaizu M. 1986. Studies on Products of browning reaction: antioxidative activity of products of browning reaction prepared from glucosamine. Jpn. J. Nutr., 44:307.

Pin-Der Duh and Gow-Chin Yen. 1997. Antioxidatant efficacy of methanolic extracts of peanut hulls in soybean and peanut oil. J. Am. Oil Chem., 74:745.

Porter W.L. 1993. Paradoxical behavior of antioxidants in food and biological systems. In "Antioxidants: Chemical, Physiological, Nutritional and Toxicological Aspects" Williams, G.M. (Ed.), p. 93. Princeton Scientific: Princeton, N.J.

SAS. 1985. Statistical Analysis System, 5th ed. SAS Institute, Cary, NC, USA.

Shimada K., Fujikawa K., Yahara K. and Nakamura T. 1992. Antioxidative properties of xanthan on the autoxidation of soybean oil in cyclodextrin. J. Agric. Food Chem., 40:945.

Statue-Gracia M.T., Heinonen M. and Frankel E.N. 1997. Antioxidant activity of anthocyanin in LDL and lecithin liposome systems. J. Agric. Food Chem., 5:362.

Tanaka M., Kuei W., Nagashima Y. and Taguchi T. 1988. Application of antioxidative Maillard reaction products from histidine and glucose to sardine products. Nippon Suisan Gakkaishi, 54:1409.

Tsushida T., Suzuki T.M. and Kurogi M. 1994. Evaluation of antioxdative activity extracts and determination of some active compounds. Nippon Sokuuhin Kogyo Gakhaishi. 41:611.

Vekiari S.A., Tzia C., Oreopoulou V. and Thomopoulos C.P. 1993. Isolation of natural antioxidant from oregano. Rivista Italiana delle Sostanze Grasse, 70:25.

Yen G. and Chen H. 1995. Antioxidant activity of various tea extracts in relation to their antimutagenicity. J. Agric. Food Chem., 43:27.

Yen G., Duh P. and Tsai C. 1993. Relationship between antioxidant ability and maturity of peanut hulls. J. Agric. Food Chem., 41:67.

Velioglu S., Mazza G., Gao L. and Oomah B.D. 1998. Antioxidant activity and total phenolics in selected fruits, vegetables and grain products. J. Agric. Food Chem., 46:4113.

Revised paper received February 5, 2007 Accepted April 10, 2007

PAPER

HOME-MADE DRY SAUSAGES PRODUCED IN SARDINIA: AN INVESTIGATION ON THE MICROFLORA

SALSICCE ARTIGIANALI PRODOTTE IN SARDEGNA: UN'INDAGINE SULLA MICROFLORA

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ABSTRACT

An investigation on the microflora of home-made Sardinian sausage was carried out. Two batches of sausages produced according to traditional methods by two artisanal producers were analysed during ripening. A total of 91 lactic acid bacteria (LAB) and 60 coagulase-negative staphylococci (CNS) were isolated and identified by species–specific PCR or 16S rDNA sequencing. The lactic microflora was mainly constituted by *Lactobacillus sakei* (80% of isolates) and *Lactobacillus curvatus*

RIASSUNTO

Allo scopo di condurre uno studio sulla composizione della microflora della salsiccia sarda artigianale, sono stati analizzati campioni di carne condita prima dell'insacco e di salsiccia a diversi stadi di maturazione, provenienti da due lavorazioni tradizionali. Sono stati isolati e identificati tramite PCR specie-specifica o sequenziamento del 16S rDNA, 91 batteri lattici (LAB) e 60 stafilococchi coagulasi negativi (CNS). Fra i LAB l'80% apparteneva alla specie *Lactobacillus*

- Key words: CNS, home-made Sardinian sausage, LAB, molecular identification -

(15% of isolates), *Leuconostoc mesenteroides* and *Lactobacillus reuteri* were also found. Among CNS, *Staphylococcus xylosus* was the main species isolated. The incidence of *Enterobacteriaceae* can be considered safe. The pH values were relatively high in both batches. This investigation indicates that home-made Sardinian sausages can be classified as a typical Mediterranean-type of dry, naturally-fermented sausages, characterised by a low acidification rate and high final pH.

sakei, il 15% alla specie *Lactobacillus curvatus*. Altre specie meno rappresentate erano *Lactobacillus reuteri* e *Leuconostoc mesenteroides*. Fra i CNS, lo *Staphylococcus xylosus* era la specie più frequente. Questo studio indica che le salsicce sarde artigianali analizzate possono essere ricondotte al tipo mediterraneo di salumi insaccati stagionati fermentati naturalmente, caratterizzato da una bassa acidificazione.

INTRODUCTION

Dry sausages are cured fermented meat. Two types of cured meat have been characterised in Europe: the Northern European products and the Mediterranean ones. Traditional manufacturing processes and association with a given geographical region of production confer cured fermented meats specific properties (BLAIOTTA *et al.*, 2004). Therefore, the nature of the meat, production technology, specific composition and metabolic activity of the indigenous microflora are critical factors in the characterisation of a typical product in terms of its microbiological quality and organoleptic properties (AMATO *et al.*, 1999; SAMELIS *et al.*, 1998; AYMERICH *et al.*, 2003).

In the Mediterranean regions cured meat products are submitted to long-term ripening during which biochemical, lipolytic and proteolytic transformations take place; these modifications give distinctive aromas and flavours to the cured meats (FLORES, 1997).

Compared to the Northern European sausages the Mediterranean-type is characterised by a lower acidification rate, with higher pH values that are often not lower than 5.2, and by a great variety within the different regions (SAMELIS *et al.*, 1998; PARENTE *et al.*, 2001b; AYMERICH *et al.*, 2003; BLAIOTTA *et al.*, 2004). These products are ripened at 10°-16°C and their pH values increase at the end of the ripening period when proteolysis produces compounds that give them a less acidic flavour (FLORES, 1997). Different authors have reported final pH values varying from 5.4 (COCOLIN *et al.*, 2001a) to 6.0 (COPPOLA *et al.*, 1995a; FLORES, 1997).

Lactic acid bacteria and coagulase-negative staphylococci are the main bacterial groups involved in the fermentation and ripening of cured sausages (NYCHAS and ARKOUDELOS, 1990; AYMERICH *et al.*, 2003). The indigenous microbiota which characterises Mediterranean dry sausages is also constituted by some species of halotolerant moulds and yeasts. Lactic acid bacteria come from raw meat and environmental contamination and are responsible for the production of lactic acid. The decrease in pH causes the coagulation of the muscle protein that is important for sliceability, firmness and cohesiveness of the product (HUGAS and MONFORT, 1997). LAB also produce small amounts of acetic acid, ethanol, acetoin, pyruvic acid and

carbon dioxide, some of which contribute to the dry sausage aroma (BEDARGUÉ *et al.*, 1993). In additon, the production of bacteriocins inhibits the growth of pathogenic and spoilage bacteria (HUGAS and MONFORT, 1997; HUGAS, 1998). The release of intracellular enzymes after cell-lysis also contributes to the development of flavour in the sausage (ZAMBONELLI *et al.*, 2002).

Coagulase-negative staphylococci play a major role in the development of sensory characteristics of fermented Mediterranean sausages (BLAIOTTA *et al.*, 2004). They reduce nitrates to nitrites which contribute to colour stability, and decompose peroxides preventing rancid taints from the fat. Their proteolytic and lypolytic activities produce compounds that are important for the aroma and flavour of the products (SAMELIS *et al.*, 1993; MIRALLES *et al.*, 1996; HUGAS and MONFORT, 1997; TALON and MONTEL, 1997; OLESEN *et al.*, 2004). The catabolism of branched-chain amino acids produces important compounds for the aroma such as methyl-branched aldehydes and their corresponding alcohols and acids (MONTEL *et al.*, 1998). The content of esters in dry sausage is affected by the capacity of *Staphylococcus* to synthesize or hydrolyze esters (TALON and MONTEL, 1997; MONTEL *et al.*, 1998).

The species of LAB mainly found in dry sausages are *Lactobacillus sakei*, *Lb. curvatus*, *Lb. plantarum* (HUGAS *et al.*, 1993, SAMELIS *et al.*, 1998). Other lactobacilli found in naturally-fermented sausages include *Lb. alimentarius*, *Lb. casei*, *Lb. paracasei*, *Lb. paraplantarum*, *Lb. pentosus, Lb. buchneri, Lb. rhamnosus, Lb. sanfranciscensis* and *Lb. maltaromicus* (COPPOLA *et al.*, 1995b; 2000; PAPAMANOLI *et al.*, 2003; RANTSIOU *et al.*, 2005).

Among CNS, *Staphylococcus xylosus* is the species most frequently isolated from dry fermented sausages (MIRALLES *et al.*, 1996; COPPOLA *et al.*, 1997; GARCÍA-VARONA *et al.*, 2000; AYMERICH *et al.*, 2003). *Staph. saprophyticus* is also frequently isolated from Greek sausages (SAMELIS *et al.*, 1998; PAPAMANOLI *et al.*, 2002; DROSINOS *et al.*, 2005). Other species reported in the literature are *Staph. carnosus, Staph. haemolityycus, Staph. warneri, Staph. equorum, Staph. cohnii, Staph. epidermidis, Staph. hominis, Staph. capitis, Staph. intermedius, Staph. chromogenes, Staph. pulvereri/vitulus, Staph. succinus, Staph. lentus* and *Staph. pasteuri* (BLAIOTTA *et al.*, 2004; SAMELIS *et al.*, 1998; PAPAMANOLI *et al.*, 2002; COPPOLA *et al.*, 2000; MAURIELLO *et al.*, 2004).

Species of *Micrococcus* and *Kokuria* genera constitute a minority of the microbiota of fermented sausages.

Although numerous studies concerning typical Mediterranean sausage technology and microbiota are reported in the literature (SAMELIS *et al.*, 1994; 1998; COPPOLA *et al.*, 1997; 1998; 2000; PARENTE *et al.*, 2001a; 2001b; PAPAMANOLI *et al.*, 2002; BLAIOTTA *et al.*, 2004; DROSINOS *et al.*, 2005; RANTSIOU *et al.*, 2005), to date, few studies are available regarding Sardinian homemade sausages. Despite the extent of pig farming and a thousand-year-old tradition of pork processing in Sardinia, in-depth studies have never highlighted the quality and tradition of the island's cured meat products.

The artisanal production and consumption of traditional, dry, fermented sausages is widespread on the island. Most rural families produce sausages only for their own consumption. Meat is processed during the coldest period of the year from November to March-April.

Pork meat, pork fat, salt, spices (black pepper, garlic, fennel seeds) and wine are used as ingredients; sometimes potassium nitrate is added. Some of the abovementioned components may be absent. The proportion of ingredients varies depending on the production region and family tradition.

Coarse minced meat is manually mixed with fat and the other ingredients. The mixture is left to stand, for a variable period, usually a few hours or overnight, after which it is stuffed in natural pork or calf casings. Then the sausages are hung and allowed to drip for 2-3 days without any control of the environmental conditions. After this, the sausages may be smoked using wood of the Mediterranean flora (e.g. oak, lentisk, myrtle, cistus). The smoking usually takes place for 2-3 h per day for 3 days without controlled temperature conditions.

The sausages are ripened for about 30 days in a cellar or 'farmhouse kitchen' where temperature and relative humidity are not controlled. The fermentation occurs through the natural growth of the microbiota present in the meat and in the environment, without the addition of any starter culture.

The aim of this work was to investigate the microflora of home-made Sardinian sausage in order to obtain more information about this traditional product. The study of the natural microbial population present in traditional Sardinian meat products is an important step for preserving the sensory characteristics of these products, improving their organoleptic quality and assuring their safety.

MATERIAL AND METHODS

Sausage manufacture and sampling

Two batches of sausages were produced according to traditional methods without the use of starter cultures by two different artisan producers (hereinafter called producers A and B, respectively) located in different areas of Sardinia.

Producer A used a formulation composed of pork meat (haunch and lean meat) and pork fat at a ratio of 70:30, NaCl (26 g/kg), ground black pepper (1 g/kg), garlic (2 g/kg) and KNO_3 (0.5 g/kg). No carbohydrates were added.

The meat and fat were minced with an electric mincer with holes of 22 mm diameter, then mixed with NaCl and left to stand overnight at about 5°-8°C. The following morning the meat was mixed again after the addition of garlic, pepper and potassium nitrate and left to stand for about 90 min at about 10°-12°C before stuffing. Natural beef casings (4 cm diameter, 50-60 cm in length) were filled with the mixture using a manual sausage stuffer. The stuffed casings (50-60 cm) were tied with string by hand.

The sausages were smoked for 2-3 h per day for 3 d without controlling the temperature using wood of Mediterranean flora (e.g. oak, lentisk, myrtle, cistus). Then they were ripened for 42 days in a room without any environmental conditioning (average temperature 9.7°C; average relative humidity 68%).

Producer B used the following formulation: pork meat (all meat cuts except bacon) and pork fat (65:35), NaCl (30.7 g/kg), ground black pepper (3.8 g/kg), garlic (1 g/kg) and fennel seeds (2.5 g/kg). No carbohydrates were added. The meat and fat were minced in an electric mincer with 18 mm diameter holes, then the minced meat was left on the table to drip for 3 h. The meat was mixed manually with the other ingredients. The mixture was stuffed into natural beef casings (4 cm diameter) that were tied with string and perforated with a needle to remove any airpockets. The sausages were smoked for 2 days after dripping (3 days) and ripened up to 42 days (average temperature 9°C; average relative humidity 76%).

Two samples of the meat mixture (300 g) before stuffing, and three sausage samples for each batch at 3, 7, 14, 28, 42 days of ripening were analysed.

pH and a_w measurements

The pH was determined potentiometrically by mixing 10 g of sample with 90

mL of distilled water. A digital pH meter was used (Crison Instruments, S.A., Barcelona, Spain).

Water activity was determined at 25°C using Novasina AW Sprint (Axair Ltd., Pfäffikon, Switzerland). Two independent measurements were made on each sample.

Microbiological analysis

The sampling of the sausages was performed starting with the aseptic removal of the casings. An aliquot of 20 g of each sample was taken aseptically and homogenised with 180 mL of sterile diluent containing peptone (1 g/L), NaCl (0.85 g/L) and Tween 80 (1 mL/L) in a Stomacher blender (Seward Medical, London, UK).

Ten-fold dilutions of the homogenates were made in sterile peptone water (1 g/L). Aliquots of dilutions were then plated in duplicate on: Plate Count Agar (PCA, Oxoid, Unipath Ltd. Basingstoke, UK) incubated at 30°C for 72 h, for mesophilic aerobic total count; FH agar (ISOLINI *et al.*, 1990) for mesophilic facultative heterofermentative lactobacilli and OH agar (ISOLINI *et al.*, 1990) for mesophilic obligate heterofermentative lactobacilli, incubated anaerobically (Gas Generating Kit, Oxoid, Unipath Ltd., Basingstoke, UK) at 37°C for 72 h; Mannitol Salt Agar (MSA, Oxoid, Unipath Ltd., Basingstoke, UK) incubated at 30°C for 72 h, for *Micrococcaceae*; Slanetz and Bartley agar (SBA, Oxoid, Unipath Ltd., Basingstoke, UK) incubated at 42°C for 48 h, for enterococci; Violet Red Bile Glucose agar (VRBG Oxoid, Unipath Ltd., Basingstoke, UK) incubated at 37°C for 24 h, for *Enterobacteriaceae*; Oxytetracycline-glucose-yeast extract (OGYE, Oxoid, Unipath Ltd., Basingstoke, UK) agar incubated at 25°C for 5 days, for yeasts and moulds.

Results are expressed as number of colony forming units/gram (CFU g^{-1}).

Isolation of LAB and CNS

Ninety-one colonies (about 5 at each sampling time for each batch) were isolated from FH (25 from batch A and 26 from batch B) and OH (17 from batch A and 23 from batch B) agar plates. Sixty colonies (5 at each sampling time for each batch) were isolated from MSA. The colonies were randomly picked up from plates with the lowest countable number. The isolates coming from FH agar and OH agar were purified to pure culture by streaking a drop of the broth culture three times on MRS agar. The MSA isolates were purified in the same way on MSA. The isolates were stored at -80°C in MRS broth (Oxoid, Unipath Ltd. Basingstoke, UK) (FH and OH isolates) or BHI broth (Oxoid, Unipath Ltd. Basingstoke, UK) (MSA isolates) containing 50% sterile glycerol, before being subjected to molecular identification.

Molecular identification of isolates

After checking for morphology, Gram staining, and catalase reaction, the pure isolates were submitted to species-specific PCR for identification. The PCR-species-specific primers (BERTHIER and EHRLICH, 1998; AYMERICH *et al.*, 2003) used in this study are indicated in Table 1. The oligonucleotide primers were synthesized by Invitrogen (Milan, Italy).

About 1 mL of log-phase cells in MRS broth or BHI broth was centrifuged (Centrifuge 5402, Eppendorf, Hamburg, Germany) at 5,000 rpm for 5 min at 4°C and the pellet was washed twice with sterile distilled water. Finally, it was re-suspended in ca. 0.5 mL of sterile distilled water. Cell lysis was performed by microwave oven treatment of the suspension for 15 min at 700W (in order to avoid evaporation and dryness problems, a beaker containing 200 mL of distilled water was put into the microwave oven). An aliquot of 5 μL of lysed cell suspension was used to perform PCR (MANNU *et al.*, 2000).

Table 1 - Primers used in this study.

Species or gene	Primer pair	Product size (bp)	Reference
Lactobacillus curvatus	Lc/16S	222	BERTHIER and EHRLICH (1998)
Lactobacillus sakei	Ls/16S	226	BERTHIER and EHRLICH (1998)
Staphylococcus xylosus	Fb/Rb	417/317/217	AYMERICH *et al*. (2003)
16S rDNA	P0/P6	1468	GRIFONI *et al*. (1995)

PCRs were perfomed in a total volume of 25 μL using the protocols reported by BERTHIER and EHRLICH (1998) and AYMERICH *et al.* (2003) in a model 2400 GeneAmp PCR System thermal cycler (Perkin-Elmer Corp., Wellesley, USA). The PCR products were resolved by electrophoresis on 2% agarose gel in Tris-acetate buffer at 100V and then stained in ethidium bromide solution (0.5 μg mL^{-1}).

The isolates which were not identified by species-specific PCR were submitted to 16S rDNA amplification and sequencing.

Colonies from a pure culture of each isolate were picked up from agar plates, suspended in 5 μL of sterile distilled water and treated in the microwave oven as described above.

The synthetic oligonucleotide primers P0 (5'-GAGAGTTTGATCCTGGCTCAG) and P6 (5'-CTACGGCTACCTTGTTACGA), *Escherichia coli* position 27f and 1495r, respectively, (GRIFONI *et al.*, 1995), were used to amplify the 16S rDNA.

After purification (Microcone, Millipore, Billerica, USA) PCR products were sent to a commercial service for sequencing (BMR-sequencing service, Padova, Italy). The primer P0 was used for sequencing.

The partial 16S rDNA sequences obtained were aligned with those of GenBank with the BLAST (ALTSCHUL *et al.*, 1997) search program.

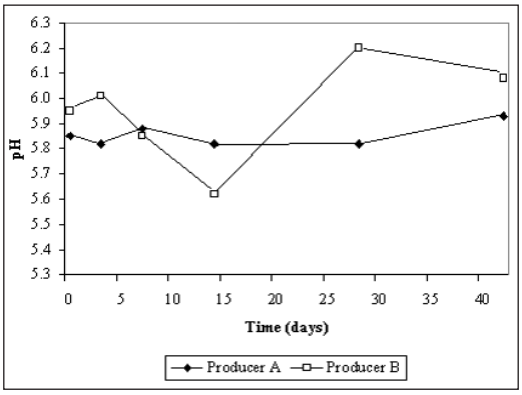

Fig. 1 - Evolution of pH in sausages from two different producers during ripening.

RESULTS AND DISCUSSION

The pH evolution during ripening of the sausages from the two producers (A and B) is shown in Fig. 1. The pH of batch A did not vary greatly during ripening. Its value was 5.85 at the beginning and 5.93 at the end and never reached a value below 5.82. Batch B had a greater variation in pH values. It varied from 5.95 at the start of ripening to the minimum value of 5.62 on the 14th day of ripening and then reached a maximum of 6.02 on the 28th day. However, the pH of the samples from batch B was higher than that of batch A, except for the pH values measured on the 7th and 14th days of ripening.

The relatively high pH values detected in the products of the two batches indicate a weak acidification as found in other typical naturally-fermented Mediterranean dry sausages, like Spanish chorizo and fuet (GONZÁLEZ-FANDOS *et al.*, 1999; AYMERICH *et al.*, 2003) and Southern Italian sausages (*salsiccia sotto sugna* salami, *soppressata* salami, *sal-*

siccia salami) (COPPOLA *et al.*, 1995a; FORASTIERO *et al.*, 1999; AMATO *et al.*, 1999; PARENTE *et al.*, 2001b; BLAIOTTA *et al.*, 2004; RANTSIOU *et al.*, 2005). The high pH values may be due either to the fact that no fermentable sugars were added to the meat or to a limited acidifying activity of the indigenous microbiota. A high pH may positively affect the activity of lipolytic enzymes produced by some *Staphylococcus* spp. (MONTEL *et al.*, 1998) with the consequent release of free fatty acids that may affect the aroma and taste of the product.

The high pH value at the end of the ripening process in the samples from batch B could be due to the release of amino acids and ammonia caused by the proteolytic activity of yeasts and moulds (DURÁ *et al.*, 2004; HERRANZ *et al.*, 2004).

The a_w during ripening is shown in Fig. 2. The a_w decreased from 0.95 to 0.82 in the samples from batch A and to 0.81 in those from batch B at the end of ripening. The values determined during ripening are similar to those found in other typical, naturally-fermented dry sausages of the Mediterranean regions (AYMERICH *et al.*, 2003; BLAIOTTA *et al.*, 2004).

Table 2 reports the evolution of the viable counts in the different media of the sausages of the two producers throughout ripening.

The aerobic mesophilic count in PCA increased by about 2 log from a value of $6x10^5$ to $5x10^7$ CFU g^{-1} in the samples from batch A. Although the aerobic mesophilic count was very high at the beginning of ripening in samples from batch B, 10^7 CFU g^{-1}, it did not increase a lot during ripening (1.2 log). These counts are comparable with those determined in other home-made Italian dry sausages like 'soppressata' and 'salsiccia' salami produced in Southern Italy (FORASTIERO *et al.*, 1999).

The selectivity of FH and OH media was very low. In fact, bacterial counts in these media were almost the same after the 3rd day of ripening for batch A and after the 7th day for batch B. The trend of the presumptive mesophilic lactobacilli (FH counts) (Table 2) was almost the same for the two batches, even though batch B had a higher number of these organisms (1 log) than batch A throughout the whole ripening period. As reported in other home-made Mediterranean dry sausages (COPPOLA *et al.*, 1995a; FORASTIERO *et al.*, 1999; AMATO *et al.*, 1999; PARENTE *et al.*, 2001b), the lack of added fermentable carbohydrates to the meat did not prevent the growth of LAB. After three days a rapid increase of mesophilic lactobacilli, from 10^3 to $3x10^6$ CFU g^{-1} for batch A, and from $2x10^4$ to 10^8 CFU g^{-1} for batch B, was observed. After a small increase between the 7th and 28th days, the number of mesophilic lactobacilli remained almost constant.

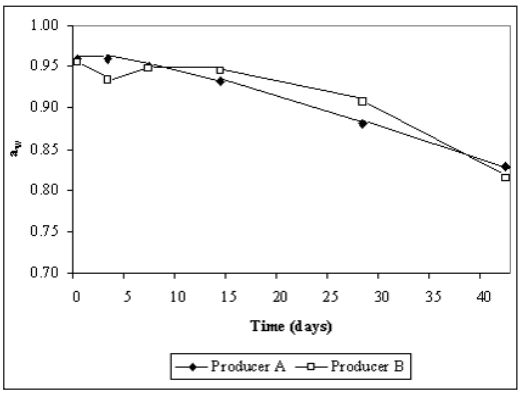

Fig. 2 - Evolution of a_w in sausages from two different producers during ripening.

The number of presumptive *Micrococcaceae* was high from the first stage of ripening: 10^4 CFU g^{-1} and $5x10^5$ for batch A and B, respectively. The growth of *Micrococcaceae* was not inhibited by the indigenous lactobacilli. This was probably due to the low acidification, and therefore the development of the two microbial groups was independent in both products. The number of colonies observed in the meat from batch B was about 2 log units higher than in the sample from batch A, with a value of $2x10^8$ CFU g^{-1}

Table 2 - Microbial counts: CFU g^{-1} during ripening in sausages from two different producers.

Microbial group	Time of ripening					
	Meat	3 days	7 days	14 days	28 days	42 days
	Producer A					
Total aerobic count	$6x10^5$	$2x10^4$	$4x10^6$	$6x10^7$	-[a]	$5x10^7$
Mesophilic FHL[b]	3x10	$1x10^3$	$3x10^6$	$5x10^7$	$7x10^7$	$2x10^7$
Mesophilic OHL[c]	-[a]	$4x10^3$	$3x10^6$	$5x10^7$	$1x10^8$	$3x10^7$
Micrococcaceae	$1x10^4$	$1x10^4$	$1x10^6$	$4x10^6$	$3x10^6$	$5x10^6$
Enterococci	$1x10^2$	$3x10^2$	$4x10^2$	$3x10^3$	$1x10^2$	n.d.[d]
Moulds and Yeasts	$3x10^2$	$6x10^2$	$6x10^4$	$1x10^5$	$5x10^4$	$6x10^4$
Enterobacteriaceae	2x10	9x10	n.d.[d]	n.d.[d]	n.d.[d]	n.d.[d]
	Producer B					
Total aerobic count	$1x10^7$	$1x10^7$	$4x10^8$	$2x10^8$	-[a]	$1x10^8$
Mesophilic FHL[b]	$3x10^2$	$2x10^4$	$1x10^8$	$4x10^8$	$5x10^8$	$5x10^7$
Mesophilic OHL[c]	7x10	3x10	$9x10^7$	$3x10^7$	$6x10^8$	$5x10^7$
Micrococcaceae	$5x10^5$	$2x10^7$	$3x10^8$	$2x10^8$	$2x10^8$	$7x10^7$
Enterococci	$7x10^2$	$2x10^2$	$2x10^2$	$5x10^3$	$1x10^3$	8x10
Moulds and Yeasts	-[a]	-[a]	$2x10^7$	$5x10^6$	$1x10^7$	$3x10^6$
Enterobacteriaceae	$2x10^3$	$3x10^3$	$6x10^3$	$3x10^3$	$8x10^2$	4x10

-[a]: no data available; [b]: facultative heterofermentative lactobacilli; [c]: obligate heterofermentative lactobacilli; [d]: no bacteria detected in 1 g.

on the 28th day of ripening (Table 2). The number of *Micrococcaceae* in these two products was similar to that described for other fermented Italian dry sausages (MAURIELLO *et al.*, 2004; PARENTE *et al.*, 2001b; AMATO *et al.*, 1999; FORASTIERO *et al.*, 1999; COCOLIN *et al.*, 2001a).

The sausages of batch B had more moulds and yeasts counted in OGYE (Table 2). The values reached about 10^7 CFU g^{-1} *vs* 10^5 CFU g^{-1}. This microbial group was stable after the 7th day of ripening.

The number of presumptive enterococci during the first 14 days of ripening was almost the same in the two batches. It reached the maximum of $3x10^3$ CFU g^{-1} (A) and $5x10^3$ CFU g^{-1} (B) on the 14th day and then decreased during ripening. The decrease was greater in the sample from batch A than for that from batch B: enterococci were absent in 1 g of product after 42 days of ripening in batch A. This microbial group is always present in fermented sausages (HUGAS *et al.*, 2003) and some authors consider enterococci important for ripening and for the development of aroma in traditional sausages (FRANZ *et al.*, 2003). Although they could be opportunistic pathogens and not considered "generally recognised as safe" (GRAS) (GIRAFFA *et al.*, 1997), recent studies indicate that enterocci isolated in food have a lower pathogenicity potential than clinical strains (HUGAS *et al.*, 2003; MANNU *et al.*, 2003). Their ability to produce bacteriocins could have an antimicrobial effect against pathogens and microorganisms responsible for meat spoilage (HUGAS *et al.*, 2003). The number of enterococci in the products analysed in the present study was slightly lower than that detected in different Italian dry sausages by other authors (COCOLIN *et al.*, 2001a; LUONGO *et al.*, 2001) and was much lower than in traditional Greek sausages (SAMELIS *et al.*, 1998; DROSINOS *et al.*, 2005).

We estimated the number of *Enterobacteriaceae* in order to evaluate the hygienic quality of the sausages analyzed, although the EU regulation on microbiological criteria for foodstuffs, Reg. EC N. 2073/2005, (EUROPEAN COMMISSION, 2005), does not provide for this microbial group as regards this meat product.

Enterobacteriaceae counts were different in the two batches. The initial number

of Enterobacteria was very low in batch A and they were absent in 1 g of product after the 7th day of ripening. In batch B the initial count was $2x10^3$ CFU g^{-1} and remained relatively constant during the first 14 days of ripening, after which it decreased to 4x10 CFU g^{-1} after 42 days of ripening. This is probably due to the decrease of a_w that had a value of 0.90 on the 28th day. VILAR *et al.* (2000) reported that *Enterobacteriaceae* are sensitive to salt and normally grow at a_w values higher than 0.93. The number of Enterobacteria detected in batch A and at the first stages of ripening did not differ greatly compared to those detected in other traditional dry sausages (SAMELIS *et al.*, 1998; LUONGO *et al.*, 2001; COCOLIN *et al.*, 2001b; PARENTE *et al.*, 2001b). The *Enterobacteriaceae* counts at the time of consumption (28-42 day) (Table 2) can be considered "acceptable" for the microbial quality and safety of fermented meat as reported by the "Guidelines for the interpretation of results of microbiological analysis of some ready-to-eat foods sampled at point of sale" (http://www.fsai.ie/publications/guidance_notes/gn3.pdf).

A total of 91 colonies of LAB isolated from FH and OH agar and 60 colonies of CNS isolated from MSA were identified by molecular methods. The number of isolates identified by species-specific PCR and 16S rDNA sequencing is reported in Table 3. All the isolates identified by sequencing had an identity of 99-100% with sequences in GenBank.

As regards LAB, 80% of the isolates were identified as *Lb. sakei*; the second main species isolated was *Lb. curvatus* (15.4% of the isolates). These results are in agreement with the results reported by SAMELIS *et al.* (1998) and PAPAMANOLI *et al.* (2003), who stated that *Lb. sakei* is normally more competitive then other LAB species in fermented sausage niches and is the dominant species within the lactic microflora of these products. The above-mentioned species were isolated from both FH and OH medium in almost the same proportion. Other minor species found were *Leuc. mesenteroides* (1 isolate from FH, 2 isolates from OH) and *Lb. reuteri* (1 isolate from OH).

As shown in Table 3, in the samples of producer A almost all the LAB isolates belonged to *Lb. sakei* (95%); only two were identified as *Lb. curvatus*. A higher presence of *Lb. curvatus* (24%) was observed in the samples of producer B. These two dominant species were found throughout the ripening of the products, while *Leuc. mesenteroides* was represented by only 3 isolates and was only found in the mixture of batch B before stuffing. Only one strain of *Lb. reuteri* was isolated and it came from product B after 3 days of ripening. These results are in agreement with the results reported for other similar products, where heterofermentative mesophilic lactobacilli were rarely isolated and were only found in the earliest stages of ripening (PARENTE *et al.*, 2001a). The presence of *Leuconostoc* spp. only during the first stages of ripening of dry sausages was reported by PAPAMANOLI *et al.* (2003). The prevalence of *Lb. sakei* and *Lb. curvatus* in naturally-fermented sausages is well documented in the literature (HUGAS *et al.*, 1993; SAMELIS *et al.*, 1994; 1998; COPPOLA *et al.*, 1998; 2000; PARENTE *et al.*, 2001a; PAPAMANOLI *et al.*, 2003; RANTSIOU *et al.*, 2005).

Among CNS, *Staph. xylosus* was the main species isolated (70% of the isolates); in the batch from producer B it had a higher frequency (80% of the isolates) than in the batch from producer A (60% of the isolates). This species was present at all the ripening times analysed for both producers (Table 3). The high prevalence of *Staph. xylosus* in the microflora of naturally-fermented dry sausages (both Spanish and Italian) (COPPOLA *et al.*, 1997; 2000; GARCÍA-VARONA *et al.*, 2000; BLAIOTTA *et al.*, 2004; COCOLIN *et al.*, 2001a) was also found in the samples analysed in this study. A higher diversity of CNS species (6 *vs* 4) was found in the batch from producer A compared

Table 3 - Distribution of species of LAB and CNS at different stages of ripening of traditional Sardinian sausages.

Species	Meat	N. of isolates					
		3 days	7 days	14 days	28 days	42 days	Total
	Producer A						
Lactobacillus sakei	-	5	9	8	8	10	40
Lactobacillus curvatus	-	-	1	-	1	-	2
Staphylococcus xylosus	1	2	4	4	3	4	18
Staphylococcus epidermidis	1	1	-	-	-	-	2
Staphylococcus equorum subsp.*linens*	-	-	-	-	1	1	2
Staphylococcus pasteuri	2	1	-	-	-	-	3
Staphylococcus succinus	-	1	1	1	1	-	4
Staphylococcus warneri	1	-	-	-	-	-	1
	Producer B						
Lactobacillus sakei	-	3	8	10	5	7	33
Lactobacillus curvatus	1	2	2	-	5	2	12
Lactobacillus reuteri	-	1	-	-	-	-	1
Leuconostoc mesenteroides	3	-	-	-	-	-	3
Staphylococcus xylosus	3	3	5	5	4	4	24
Staphylococcus equorum subsp.*linens*	1	-	-	-	1	1	3
Staphylococcus succinus	-	2	-	-	-	-	2
Staphylococcus pulvereri/vitulus	1	-	-	-	-	-	1

-: non detected.

to that of producer B. *Staph. equorum* subsp. *linens* and *Staph. succinus* were present in the sausages of both producers at different sampling times. *Staph. epidermidis, Staph. pasteuri* and *Staph. warneri* only occurred in the sausage of producer A and were limited to the earliest stages of ripening. *Staph. pulvereri/vitulus* was only isolated from the mixture of producer B. BLAIOTTA *et al.* (2004) detected this species almost exclusively in artisan products and it was always more abundant at the beginning of ripening. No isolates of *Staph. saprophyticus* were found in this study, although this species was shown to be the dominant one in dry Greek sausages (SAMELIS *et al.*, 1998; PAPAMANOLI *et al.*, 2002; DROSINOS *et al.*, 2005) and was highly represented in other fermented sausages of Southern Italy (MAURIELLO *et al.*, 2004). *Staph. carnosus*, widely used as micrococcal starter culture (HAMMES and HERTEL, 1998), was not found in the samples analysed in the present study, nor in other naturally-fermented Mediterranean sausages (COPPOLA *et al.*, 1997; SAMELIS *et al.*, 1998; GARCÍA-VARONA *et al.*, 2000; BLAIOTTA *et al.*, 2004; MAURIELLO *et al.*, 2004; DROSINOS *et al.*, 2005).

CONCLUSIONS

This preliminary study dealing with the characterisation of home-made Sardinian sausages indicates that this product is a typical Mediterranean type of dry, naturally-fermented sausage, characterised by low acidification rates and high final pH. The microflora is mainly represented by LAB and CNS that reach high counts from the 7th day of ripening. The main species isolated were *Lb. sakei, Lb. curvatus* and *Staph. xylosus*. The incidence of *Enterobacteriaceae* was similar to that of other analogous home-made products and can be considered safe. Further studies are needed to better characterise the microbial population of this product. It could also be important to investigate the genetic diversity

at the strain level and the technological features of the isolates in order to evaluate their role in the ripening process and their potential use as starters.

ACKNOWLEDGEMENTS

This work was supported by a grant of the European Union Interreg Project IIIA.

The authors sincerely thank the technician Maria Carmen Fozzi for her precious contribution, the Lei and Ligios families and Mr. Nino Lei for their availability during the manufacturing and collection of the samples.

REFERENCES

Altschul S.F., Madden T.L., Schäffer A.A., Zhang J., Zhang Z., Miller W. and Lipman D.J. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucl. Acids Res. 25: 3389.

Amato D., Di Gennaro P., Fiore C., Lanorte M.T., Caruso M., Suzzi G. and Laurita C. 1999. Caratterizzazione dei prodotti tipici lucani: la "salsiccia sotto sugna". Industrie Alimentari. 38: 683.

Aymerich T., Martín B., Garriga M. and Hugas M. 2003. Microbial quality and direct PCR identification of Lactic Acid Bacteria and nonpathogenic staphylococci from artisanal low-acid sausages. Appl. Environ. Microbiol. 69: 4583.

Bedargué J.L., Monteil P., Montel M.C. and Talon R. 1993. Effects of starter cultures on the formation of flavour compounds in dry sausage. Meat Sci. 35: 275.

Berthier F. and Ehrlich S.D. 1998. Rapid species identification within two groups of closely related lactobacilli using PCR primers that target the 16S/23SrRNA spacer region. FEMS Microbiol. Lett. 161: 97.

Blaiotta G., Pennacchia C., Villani F., Ricciardi A., Tofalo R. and Parente E. 2004. Diversity and dynamics of communities of coagulase-negative staphylococci in traditional fermented sausages. J. Appl. Microbiol. 97: 271.

Cocolin L., Manzano M., Aggio D., Cantoni C. and Comi G. 2001a. A novel polymerase chain reaction (PCR) – denaturing gradient gel electrophoresis (DGGE) for the identification of *Micrococcaceae* strains involved in meat fermentations. Its application to naturally fermented Italian sausages. Meat Sci. 57: 59.

Cocolin L., Manzano M., Cantoni C. and Comi G. 2001b. Denaturing Gradient Gel Electrophoresis analysis of the 16S rRNA gene V1 region to monitor dynamic changes in the bacterial population during fermentation of Italian sausages. Appl. Environ. Microbiol. 67: 5113.

Coppola R., Iorizzo M., Giagnacovo B., Sorrentino A., Sorrentino E., Grazia L. and Corino C. 1995a. La soppressata molisana: caratteristiche microbiologiche e tecnologiche. Industrie Alimentari. 34: 851.

Coppola R., Giagnacovo B., Iorizzo M. and Grazia L. 1998. Characterization of lactobacilli involved in the ripening of soppressata molisana, a typical southern Italy fermented sausages. Food Microbiol. 15: 347.

Coppola R., Iorizzo M., Saotta R., Sorrentino E. and Grazia L. 1997. Characterization of micrococci and staphylococci isolated from soppressata molisana, a Southern Italy fermented sausage. Food Microbiol. 14: 47.

Coppola R., Marconi E., Rossi F. and Dellaglio F. 1995b. Artisanal production of Naples-type salami: chemical and microbiological aspects. Ital. J. Food Sci. 7: 57.

Coppola S., Mauriello G., Aponte M., Moschetti G. and Villani F. 2000. Microbial succession during ripening of Naples-type salami, a southern Italian fermented sausage. Meat Sci. 56: 321.

Drosinos E.H., Mataragas M., Xiraphi N., Moschonas G., Gaitis F. and Metaxopoulos J. 2005. Characterization of the microbial flora from a traditional Greek fermented sausage. Meat Sci. 69: 307.

Durá M.A., Flores M. and Toldrá F. 2004. Effect of *Debaryomyces* spp. on the proteolysis of dry-fermented sausages. Meat Sci. 68: 319.

European Commission 2005. Commission Regulation (EC) N. 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs (Text with EEA relevance). Official Journal of the European Union L 338: 1.

Flores J. 1997. Mediterranean vs northern European meat products. Processing technologies and main differences. Food Chem. 59: 505.

Food Safety Authority of Ireland. 2001. Guidelines for the interpretation of results of microbiological analysis of some ready-to-eat foods sampled at point of sale. Dublin. Website: http://www.fsai.ie.

Forastiero V., Vergara A., Tantillo G. and Ceci E. 1999. Valutazione di requisiti microbiologici e chimico-fisici di prodotti di nicchia. La soppressata e la salsiccia dell'area del Pollino. Industrie Alimentari. 38: 692.

Franz C.M.A.P., Stiles M.E., Schleifer K.H. and Holzapfel W.H. 2003. Enterococci in foods- a conundrum for food safety. Int. J. Food Microbiol. 88: 105.

García-Varona M., Santos E.M., Jaime I. and Rovira J. 2000. Characterisation of *Micrococcaceae* isolated from different varieties of chorizo. Int. J. Food Microbiol. 54: 189.

Giraffa G., Carminati D. and Neviani E. 1997. Enterococci isolated from dairy products: a review of risks and potential technological use. J. Food Prot. 60: 732.

González-Fandos M.E., Sierra M., García-Lopez M.L, García-Fernández M.C. and Otero A. 1999. The influence of manufacturing and drying conditions on the survival and toxinogenesis of *Sta-*

phylococcus aureus in two Spanish dry sausages (chorizo and salchichón). Meat Sci. 52: 411.

Grifoni A., Bazzicalupo M., Di Serio C., Fancelli S. and Fani R. 1995. Identification of *Azospirillum* strains by restriction fragment length polymorphism of 16S rDNA and of histidine operon. FEMS Microbiol. Lett. 127: 85.

Hammes W.P. and Hertel C. 1998. New developments in meat starter cultures. Meat Sci. 49: S125.

Herranz B., De la Hoz L., Hierro E. and Ordóñez J.A. 2004. Microbiota de los embutidos fermentados (embutidos crudos curados). La Industria Carnica Latinoamericana. 133: 30.

Hugas M., Garriga M., Aymerich T. and Monfort J.M. 1993. Biochemical characterization of lactobacilli from dry fermented sausages. Int. J. Food Microbiol. 18: 107.

Hugas M. and Monfort J.M. 1997. Bacterial starter cultures for meat fermentation. Food Chem. 59: 547.

Hugas M. 1998. Bacteriocinogenic Lactic Acid Bacteria for biopreservation of meat and meat products. Meat Sci. 49: S139.

Hugas M., Garriga M. and Aymerich M.T. 2003. Functionalty of enterococci in meat products. Int. J. Food Microbiol. 88: 223.

Isolini D., Grand M. and Glattli H. 1990. Selective media for the enumeration of obligately and facultatively heterofermentative lactobacilli. Schweiz Milchwirtschaft Forschung. 19: 57.

Luongo D., Giagnacovo B., Fiume I., Iorizzo M. and Coppola R. 2001. Volatile compounds in "Soppressata molisana" style salami fermented by *Lactobacillus sakei*. Ital. J. Food Sci. 13: 19.

Mannu L, Comunian R. and Scintu M.F. 2000. Mesophilic lactobacilli in Fiore Sardo Cheese: PCR-identification and evolution during cheese ripening. Int. Dairy J. 10: 383.

Mannu L., Paba A., Daga E., Comunian R., Zanetti S., Duprè I. and Sechi L.A. 2003. Comparison of the incidence of virulence determinants and antibiotic resistance between *Enterococcus faecium* strains of dairy, animal and clinical origin. Int. J. Food Microbiol. 88: 291.

Mauriello G., Casaburi A., Blaiotta G. and Villani F. 2004. Isolation and technological properties of coagulase negative staphylococci from fermented sausages of Southern Italy. Meat Sci. 67: 149.

Miralles M.C., Flores J. and Perez-Martinez G. 1996. Biochemical tests for the selection of *Staphylococcus* strains as potential meat starter cultures. Food Microbiol. 13: 227.

Montel M.C., Masson F. and Talon R. 1998. Bacterial role in flavour development. Meat Sci. 49: S111.

Nychas G.J.E. and Arkoudelos J.S. 1990. Staphylococci: their role in fermented sausages. J. of Appl. Bacteriol. Symposium Supplement 69: 167S.

Olesen P.T., Meyer A.S. and Stahnke L.H. 2004. Generation of flavour compounds in fermented sausages - the influence of curing ingredients, *Staphylococcus* starter culture and ripening time. Meat Sci. 66: 675.

Papamanoli E., Kotzekidou P., Tzanetakis N. and Litopoulou-Tzanetaki E. 2002. Characterization of *Micrococcaceae* isolated from dry fermented sausage. Food Microbiol. 19: 441.

Papamanoli E., Tzanetakis N., Litopoulou-Tzanetaki E. and Kotzekidou P. 2003. Characterization of lactic acid bacteria isolated from a Greek dry-fermented sausage in respect of their technological and probiotic properties. Meat Sci. 65: 859.

Parente E., Grieco S. and Crudele M.A. 2001a. Phenotypic diversity of lactic acid bacteria isolated from fermented sausages produced in Basilicata (Southern Italy). J. Appl. Microbiol. 90: 943.

Parente E., Martuscelli M., Gardini F., Grieco S., Crudele M.A. and Suzzi G. 2001b. Evolution of microbial populations and biogenic amine production in dry sausages produced in Southern Italy. J. Appl. Microbiol. 90: 882.

Rantsiou K., Drosinos E.H., Gialitaki M., Urso R., Krommer J., Gasparik-Reichardt J., Toth S., Metaxopoulos I., Comi G. and Cocolin L. 2005. Molecular characterization of *Lactobacillus* species isolated from naturally fermented sausages produced in Greece, Hungary and Italy. Food Microbiol. 22: 19.

Samelis J., Aggelis G. and Metaxopoulos J. 1993. Lipolytic and microbial changes during the natural fermentation and ripening of Greek dry sausages. Meat Sci. 35: 371.

Samelis J., Maurogenakis F. and Metaxopoulos J. 1994. Characterization of lactic acid bacteria isolated from naturally fermented Greek dry salami. Int. J. Food Microbiol. 23: 179.

Samelis J., Metaxopoulos J., Vlassi M. and Pappa A. 1998. Stability and safety of traditional Greek salami - a microbiological ecology study. Int. J. Food Microbiol. 44: 69.

Talon R. and Montel M.C. 1997. Hydrolysis of esters by staphylococci. Int. J. Food Microbiol. 36: 207.

Vilar I., Garcìa Fontán M.C., Prieto B., Tornadijo M.E. and Carballo J. 2000. A survey on the microbiological changes during the manufacture of dry-cured lacón, a Spanish traditional meat product. J. Appl. Microbiol. 89: 1018.

Zambonelli C., Chiavari C., Benevelli M. and Coloretti F. 2002. Effects of Lactic Acid Bacteria autolysis on sensorial characteristics of fermented foods. Food Technol. Biotechnol. 40: 347.

Revised paper received November 10, 2006 Accepted January 30, 2007

PAPER

CHEMICAL COMPOSITION OF 15 SPINACH (*SPINACEA OLERACEA* L.) CULTIVARS GROWN IN IRAN

COMPOSTI A VALENZA NUTRIZIONALE PRESENTI IN 15 CULTIVARS DI SPINACIO (*SPINACEA OLERACEA* L.) COLTIVATE IN IRAN

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ABSTRACT

The chemical composition of 15 spinach (*Spinacea oleracea* L.) cultivars (cvs) grown in different areas of Iran was investigated. The ranges of the moisture, ash, total fat, crude fiber, oxalate, total phenolic and protein contents were 88.2-91.1 (g/100 gfw), 1.63-2.46 (g/100 gfw), 0.14-0.36 (g/100 gfw), 1.80-2.22 (g/100 gfw), 0.53-1.17 (g/100 gfw), 55.2-103.9 (mg TAE/100 gfw) and 1.62-3.19 (g/100 gfw), respectively. The mineral (K, Ca, Mg, Na, Fe, Zn, Cu and P) contents

RIASSUNTO

È stata analizzata la composizione chimica di 15 cultivars di spinaci (*Spinacea oleracea* L.) cresciute in diverse aree dell'Iran. I range di umidità, ceneri, grassi totali, fibra grezza, ossalato, composti fenolici totali e proteine erano, rispettivamente, 88,2-91,1 (g/100 gpf), 1,63-2,46 (g/100 gpf), 0,14-0,36 (g/100 gpf), 1,80-2,22 (g/100 gpf), 0,53-1,17 (g/100 gpf), 55,2-103,9 (mg TAE/100 gpf) e 1,62-3,19 (g/100gpf). I contenuti in minerali (K, Ca, Mg, Na, Fe, Zn, Cu and P) erano rispettivamente (595,8-

- Key words: anti-nutritional factors, chemical composition, fatty acids, mineral elements -

were (595.8-1,106.6), (61.6-112.8), (67.4-108.3), (34.0-117.7), (2.61-8.01), (0.963-2.606), (0.074-0.443) and (40.4-90.9) mg/100 gfw, respectively. The main fatty acids were palmitic (17.27-24.58%), palmitoleic (0.85-11.00%), hexadecatrienoic (1.85-6.10%), stearic (0.81-3.17%), oleic (6.68-16.51%), linoleic (13.59-22.50%), linolenic (31.60-44.04%) and erucic acid (0.55-5.43%). The differences in the nutrient composition correspond to the high genetic diversity found within the 15 cultivars. This variability in nutrients could be useful for selecting specific cultivars for special purposes.

1.106,6), (61,6-112,8), (67,4-108,3), (34,0-117,7), (2,61-8,01), (0,963-2,606), (0,074-0,443) e (40,4-90,9) mg/100 gpf. I principali acidi grassi erano il palmitico (17,27-24,58%), il palmitoleico (0,85-11,00%), l'esadecatrienoico (1,85-6,10%), lo stearico (0,81-3,17%), l'oleico (6,68-16,51%), il linoleico (13,59-22,50%), il linolenico (31,60-44,04%) e l'erucico (0,55-5,43%). Le differenze in composizione in nutrienti corrispondono all'alta diversità genetica trovata fra le 15 cultivars. Questa variabilità in nutrienti potrebbe essere utile per selezionare specifiche cultivars per scopi particolari.

INTRODUCTION

Spinach (*Spinacea oleracea* L.) was first cultivated in southwestern Asia, perhaps in Persia. The word itself is derived from the Persian word *Esfenaj*. The Chinese referred to it in 647 as the herb of Persia. It is an important leafy vegetable, now grown throughout the temperate regions of the world. It is most productive in cool seasons and climates, since heat causes spinach to go to seed too early.

Spinach is rich in iron, but the bioavilability of iron is dependent on its absorption. All of the iron in grains and vegetables is nonheme iron (WILLIAMS, 1993); therefore, its absorption may vary widely depending on the persence of a binder such as fiber or an enhancer such as vitamin C. Spinach contains high levels of oxalate that bind to iron or calcium to form insoluble calcium or ferrous oxalates, which cannot be absorbed by the body (WU *et al.*, 1999). Therefore, analysis of oxalate is of great importance in food. Spinach still has a large nutritional value, especially when fresh, steamed, or boiled quickly. It is low in calories and is a good source of vitamin A, C, E, folic acid, β-carotene as well as minerals and dietary fiber (USDA, 2006; KAWAZU OKIMURA *et al.*, 2003; TOLEDO *et al.*, 2003). Spinach has notable flavonoid content (more than 1,000 mg/kg) comparable to other flavonoid-rich vegetables such as broccoli and red onion (HOWARD *et al.*, 2002). The phenolic compounds in spinach exhibit a wide range of biological effects including antioxidative, antihypertensive, antiproliferative, anticarcinogenic and antimutagenic properties (AEHLE *et al.*, 2004; YANG *et al.*, 2004; CHU *et al.*, 2002).

Finally, spinach contains about 5% lipids on a dry basis, and the lipid content consists of palmitic (15.04%), hexadecatrienoic (7.67%), linoleic (11.84%), and linolenic (58.46%) acids (LEE *et al.*, 2000). Because of the presence of highly unsaturated fatty acids and other components, such as chlorophyll and β-carotene, the addition of spinach as an ingredient to foods may affect food processing properties and the quality of the final products. Significant variations in the chemical composition of different varieties of spinach have been reported over

the years by various researchers (JAWORSKA, 2005; GÜLSER, 2005; USDA, 2006). Due to the influence of environmental and cultivar differences on nutritional values of spinach, more work is required in this area.

Due to the lack of information about the chemical composition of spinach in Iran, the present study was conducted to compare the contents of moisture, ash, total fat, crude fiber, oxalate, total phenolic, protein, some mineral elements (Na, K, Ca, Mg, Fe, Cu, Zn and P), and fatty acid profiles of fifteen potential spinach cultivars for fresh consumption and further industrial use.

MATERIALS AND METHODS

Plant material and chemicals

Fifteen spinach cultivars (cvs), (Mobarakeh (N. 1), Dorood (N. 2), Saleh-abad (N. 3), Borougerd (N. 4), Ardestan (N. 5), Karaj 2 (N. 6), Yazd (N. 7), Ghoochan (N. 8), Shiraz (N. 9), Lahijan 1 (N. 10), Rahim-abad (N. 11), Qom (N. 12), Saveh (N. 13), Hamedan (N. 14), and Rahnan (N. 15)) were planted on 1 November 2005 at the experimental field of Tarbiat Modares University. The field plot plan was a randomized complete block design with three replicates. The plants were grown under identical growing conditions and received uniform fertilization, irrigation and herbicide treatments. The EC and pH of the soil were 1.2 ds/m and 7.5, respectively. Total nitrogen, available phosphorus and potassium were 0.15%, 8.2 and 3.4 ppm, respectively. One hundred kg of N (as urea), 50 kg of P (as P_2O_5), and 100 kg of K (as K_2O) were used. The leaves were collected within 2h on the same day (3 March 2006) from 15 randomly selected plants of each cultivar. For each cultivar, 30 leaves were collected, placed in polyethylene bags in refrigerated coolers, and transported to the Food Science Department within 30 min. After measuring the fresh weight of the samples, they were stored at -20°C until total phenolic analysis (for a maximum of 8h). The leaf samples of each cultivar were then placed in an electric oven at 70°C for 3 days (for other experiments, e.g. elemental analysis). All samples were analyzed in triplicate.

Folin-Ciocalteu reagent and 14% BF_3-methanol were obtained from Fluka (Taufkirchen, Germany). Methanol, tannic acid, hexane, and sodium hydroxide were purchased from Merck (Darmstadt, Germany). Standards of pentadecanoic, palmitic, palmitoleic, hexadecatrienoic, stearic, oleic, linoleic, linolenic and erucic acids were obtained from Sigma (Missouri, USA). All reagents used were of analytical grade.

Moisture, ash, protein, crude fiber, total fat, oxalate and total phenolic determination

The recommended methods of the Association of Official Analytical Chemists (AOAC, 1984) were adopted to determine the levels of moisture, ash, crude fiber, and total oxalate. Protein contents in the ground leaf samples were determined by the Kjeldahl method. Results were calculated using the general factor 6.25 (AOAC, 1984). For total lipid determination, three grams of dried milled leaves were placed in an extraction thimble and the extraction was carried out for 8h using 100 mL petroleum benzene (b. p. 40°-60°C). The solvent was then evaporated by a rotary evaporator at 40°C and then cooled for 30 min in a desiccator and weighed.

An aliquot of the material from each spinach cultivar was freeze-dried, ground into a fine powder and stored in a desiccator until the extraction of the phenolic compounds was carried out. Samples were extracted in triplicate. Each sample consisting of 2 g of freeze-dried material was extracted with 15 mL distilled water. The samples were shaken for 10

h at 4°C and 150 rpm and then centrifuged at 6,500 g at 4°C for 10 min. The total soluble phenolics in the water extracts were determined with Folin-Ciocalteu reagent according to the method of SINGH *et al.* (2002) (spectrophotometric method) using tannic acid as a standard. Results are expressed as mg of tannic acid equivalents per 100 g of fresh weight (mg TAE/100 gfw).

Mineral element analysis

The minerals sodium (Na), potassium (K), calcium (Ca), magnesium (Mg), iron (Fe), zinc (Zn) and copper (Cu) were determined by atomic absorption spectrophotometry. Ten grams of fresh spinach leaf samples were washed with double distilled water and oven-dried at 65°C for 3 days and then ground. The samples were then dry-ashed in a muffle furnace at 550°C for 6 h until a white residue of constant weight was obtained. The minerals were extracted from the ash by adding 5.0 mL of 37% HCl, heated in a steam bath and after filtration (Whatman N. 42), the liquid was transferred quantitatively to a 25 mL volumetric flask. It was diluted to volume with deionised water, stored in clean polyethylene bottles and the mineral contents were determined using an atomic absorption spectrophotometer (Shimadzu, Kyoto, Japan) on a fresh weight (fw) basis.

Phosphorous (P) was determined by the phosphovanadomolybdate method. The phosphovanadomolybdate complex formed between the phosphate, ammonium vanadate and ammonium molybdate is bright yellow and its absorbance is measured at 460 nm (JEFFERY *et al.*, 1989). The P was extracted from ash by adding 5.0 mL of 37% HCl, heated in a steam bath and after filtration (Whatman N. 42), was transferred quantitatively to a 25 mL volumetric flask. Four mL of dissolved ash, 10 mL of ammonium vanadate (2.5 g NH_4VO_3 dissolved in 500 mL hot water, with the addition of 20 mL conc. HNO_3 and diluted with water to 1L), and 10 mL of ammonium molybdate (50 g $(NH_4)_6Mo_7O_{24}$, $6H_2O$ dissolved in warm water and diluted with water to 1L), were then transferred to a 100 mL graduated flask, respectively, and diluted to the mark. The absorbance of this solution (after 10 min) at 460 nm was measured by using a Scinco 2100 spectrophotometer (Seoul, South Korea).

Fatty acid analysis

The fatty acid methyl esters (FAMEs) were prepared according to the procedure described by METCALF *et al.*, (1966). Fifty mg of extracted oil was saponified with 5 mL of methanolic NaOH (2%) solution by refluxing for 10 min at 90°C. After addition of 2.2 mL BF_3-methanol, the sample was boiled for 5 min. The FAMEs were extracted from a saturated salt mixture with hexane. The FAMEs were then analyzed using a gas chromatograph (UNICAM model 4600, Cambridge, UK) coupled with a FID detector. The column used for fatty acid separation was a fused silica BPX70 column, 30 m x 0.22 mm i. d. x 25 μm film thickness (from SGE, Griesheim, Germany). The oven temperature was held at 180°C during separation; the injector and detector temperatures were 240° and 280°C, respectively. The carrier gas (helium) flow rate was 1 mL/min. One μL of methyl esters of free fatty acids was injected into the split injector. The split ratio was adjusted to 1:10. The compounds were identified by comparison of their retention times with authentic compounds. The internal standard C15:0 was used in the quantitative analysis of the separated fatty acids (data were calculated as percentage total fatty acids).

Statistical analysis

The analysis of variance was done using SPSS software and significant differences among means from a triplicate

analysis at (P≤0.05) were determined by Duncan`s multiple range test (DMRT).

RESULTS AND DISCUSSION

The proximate composition of the 15 spinach cultivars analyzed is presented in Table 1. The mean moisture content of the cultivars was 90.0 g/100 gfw, which is slightly lower than the values reported by the USDA (91.4%) (2006), CHAKRABORTI *et al.*, (91.5%) (1989) and RUBATZKY and YAMAGUCHI (91.5%) (1997) but, in agreement with the mean value reported by KAWAZU OKIMURA *et al.*, (90.7%) (2003). Cultivars with low moisture content are suitable for processing.

The ash content ranged from around 1.63 g/100 gfw in cv N. 1 to 2.46 g/100 gfw in cv N. 8. This average was slightly higher than the values reported by other researchers (USDA 2006; RUBATZKY and YAMAGUCHI 1997).

The total fat (lipid) content ranged from 0.14 to 0.36 g/100 gfw for cvs N. 12 and N. 14. The mean value of total fat was lower than the values reported by the USDA (2006) and SALUNKHE and KADAM (1998).

There were significant differences in crude fiber of the cultivars. Values varied from 1.70 (g/100 gfw, cv N. 1) to 2.22 (g/100 gfw, cv N. 7). Similar results were reported by the USDA (2006), but RUBATZKY and YAMAGUCHI (1997) reported higher values.

The crude protein contents of the 15 cultivars ranged from 1.62 (cv N. 13) to 3.19 g/100 gfw (cv N. 10) with an average of about 2.41 g/100 gfw. Some cultivars (N. 3, 5, and 11) had protein contents similar to that reported by the USDA (2006).

In the present study the minimum and maximum of the total oxalate contents were 0.53±0.05 g/100 gfw (cv N. 12) and 1.17±0.07 g/100 gfw (cv N. 5). Statistically significant differences were found among all the samples. The average oxalate content of the cvs was 0.77 g/100 gfw, which is lower than the value of 8.7%

Table 1 - Some chemical composition parameters of the 15 cultivars (a).

cv N.	Total phenolic (mg TAE/100 gfw)	Oxalate (g/100 gfw)	Protein (g/100 gfw)	Crude fiber (g/100 gfw)	Total fat (g/100 gfw)	Ash (g/100 gfw)	Moisture (g/100 gfw)
1	66.5±0.4e	0.85±0.08d	2.46±0.01e	1.70±0.05g	0.27±0.01b	1.63±0.06g	89.4±0.3ab
2	67.4±0.1e	0.88±0.07d	2.11±0.02h	1.80±0.06f	0.20±0.03d	1.90±0.03de	89.4±0.2ab
3	103.9±0.3a	0.80±0.06d	2.69±0.05c	2.17±0.09abc	0.35±0.02a	1.87±0.27def	88.2±0.1b
4	75.3±0.6d	0.92±0.08c	2.39±0.00f	2.05±0.04de	0.23±0.02c	1.88±0.14de	90.4±0.2ab
5	64.0±0.7f	1.17±0.07a	2.75±0.01b	2.10±0.01cd	0.17±0.05g	1.93±0.07d	91.1±0.4a
6	80.8±1.9c	0.56±0.06f	2.27±0.02g	2.10±0.04cd	0.17±0.01g	2.08±0.04c	90.9±0.2ab
7	87.5±0.5b	1.08±0.08b	2.60±0.01d	2.22±0.08a	0.16±0.04h	1.78±0.04f	89.6±0.1ab
8	74.7±0.8d	0.62±0.07e	2.39±0.02f	1.99±0.03e	0.17±0.01g	2.46±0.05a	90.3±0.3ab
9	80.2±1.8c	0.60±0.06e	2.59±0.03d	2.21±0.05ab	0.19±0.03e	1.80±0.03ef	89.7±0.2ab
10	55.2±1.8h	0.55±0.07f	3.19±0.01a	1.99±0.02e	0.27±0.02b	2.14±0.07c	89.7±0.1ab
11	57.6±0.4g	0.81±0.09d	2.78±0.05b	2.09±0.06cde	0.19±0.03e	1.90±0.05de	90.3±0.1ab
12	87.1±1.2b	0.53±0.05f	2.14±0.02h	1.84±0.05f	0.14±0.00i	1.90±0.04de	90.5±0.5ab
13	63.8±0.9f	0.70±0.06e	1.62±0.01j	2.06±0.08de	0.26±0.01b	1.78±0.06f	90.5±0.2ab
14	68.1±0.6e	0.67±0.05e	2.25±0.02g	1.88±0.01f	0.36±0.04a	2.08±0.06c	89.5±0.3ab
15	89.0±0.5b	0.82±0.09d	1.91±0.02i	2.12±0.02bcd	0.18±0.02f	2.31±0.04b	90.1±0.2ab
Mean	74.7	0.77	2.41	2.02	0.22	1.96	90.0

(a) Values are mean ± standard deviation of triplicate determinations. Values in the same column having the same letters are not significantly different (Duncan's multiple range test, at P≤0.05).

reported by JAWORSKA (2005), 10.2% dry weight reported by KAWAZU OKIMURA *et al.* (2003) and 8.7% dry weight reported by JAWORSKA and KMIECIK (1999). An excessive dietary oxalate intake may lead to renal insufficiency. A high intake of oxalate plays a key role in secondary hyperoxaluria, a major risk factor for calcium oxalate stone formation (HODGKINSON, 1978) and also decreases calcium absorption (WEAVER *et al.*, 1987). Treatment of diet-dependent hyperoxaluria begins with the restriction of oxalate-rich foods. Without adequate knowledge of the oxalate content in food, dietary guidelines cannot be established.

The total phenolic content of the 15 spinach cultivars presented in Table 1 ranged from 55.2±1.8 (cv N. 10) to 103.9±0.3 (cv N. 3) mg TAE/gfw. These results are higher than those reported by PANDIAITAN *et al.* (2005).

Minerals

The mineral concentrations in the Iranian spinach cultivars are shown in Table 2. Significant variations occurred in the mineral concentrations among the samples. Spinach contains some important minerals. The potassium concentration was the highest, followed in descending order by calcium, magnesium, sodium, phosphorous, iron, zinc and copper. This order was reported earlier by the USDA (K> Ca> Mg=Na> P> Fe> Zn> Cu) (USDA 2006).

The mean value of the K content in the 15 spinach cvs was 864.5 mg/100 gfw. This value can be compared with the value of 558 mg/100 gfw given by the USDA (2006), 570 mg/100 gfw by WILLS *et al.* (1986), and 870-1,300 mg/100 g determined by OGUCHI *et al.* (1996). The content of calcium in spinach is high in fresh weight, but unfortunately, a significant part of this element occurs in the form of oxalates that limit its utilization in the diet. In general, the calcium content reported in this study was near or exceeded the values published in the literature. The USDA (2006) reported 99.0 mg/100 gfw,

Table 2 - Mineral elements of the 15 spinach cultivars (mg 100/gfw) (a).

cv N.	P	Cu	Zn	Fe	Na	Mg	Ca	K
1	65.1±0.4d	0.134±0.004c	1.486±0.000g	4.03±0.02j	117.7±1.0a	108.3±0.5a	90.4±0.5f	1027.6±5.0c
2	90.3±0.3a	0.208±0.008b	1.868±0.004c	6.60±0.02c	85.7±0.4e	91.3±0.8bc	106.4±0.4c	1106.6±2.7a
3	90.9±0.4a	0.123±0.001d	2.208±0.002b	8.01±0.01a	74.5±1.6g	90.7±0.3c	110.6±0.6b	958.8±10.0e
4	66.1±0.4c	0.443±0.004a	1.696±0.004e	3.57±0.02m	68.2±1.1i	77.5±1.0g	75.0±0.6m	942.5±12.6f
5	63.6±0.4e	0.104±0.004f	1.212±0.002m	4.02±0.02k	59.9±0.3j	85.6±0.3e	61.6±0.8o	735.8±4.70j
6	55.5±0.3h	0.083±0.002g	1.376±0.001k	6.31±0.02d	70.4±1.5h	67.9±0.3i	80.5±0.5i	663.7±12.2k
7	40.4±0.3n	0.102±0.003f	1.458±0.001j	5.93±0.03e	94.9±0.6d	81.3±0.4f	79.9±0.4k	997.8±8.1d
8	45.9±0.3k	0.102±0.002f	1.767±0.003d	7.88±0.04b	61.4±1.2j	92.0±0.5b	112.8±1.5a	1063.5±4.0b
9	41.9±0.4m	0.104±0.003f	1.570±0.001f	3.98±0.02l	108.7±1.0b	76.4±0.1h	105.2±1.7d	775.7±12.1h
10	44.2±0.3l	0.104±0.004f	0.963±0.002o	5.09±0.01f	99.4±0.2c	88.7±0.1d	83.7±0.6h	784.8±1.2h
11	56.4±0.4g	0.112±0.003e	1.473±0.001h	3.14±0.01n	79.2±1.5f	81.6±0.4f	79.4±0.8l	806.0±2.2g
12	48.9±0.5j	0.074±0.002h	1.188±0.003n	4.26±0.00i	34.0±0.4l	91.5±0.4bc	84.4±0.3g	595.8±3.6l
13	51.3±0.41i	0.103±0.001f	1.307±0.000l	2.61±0.02o	41.3±1.7k	67.4±0.5i	66.5±0.8n	738.7±1.3ij
14	74.1±0.6b	0.084±0.001g	2.606±0.003a	4.27±0.00h	67.9±0.9i	87.8±1.0d	95.9±1.0e	750.5±5.6i
15	62.5±0.5f	0.123±0.003d	1.465±0.002i	4.34±0.01g	72.8±0.7g	87.9±0.5d	80.3±2.27j	1018.9±4.5c
Mean	59.8	0.134	1.576	4.94	75.7	85.1	87.5	864.5

(a) Values are mean ± standard deviation of triplicate determinations. Values in the same column having the same letters are not significantly different (Duncan's multiple range test, at P≤0.05).

OGUCHI *et al.* (1996) 80-180 mg/100 gfw and JAWORSKA and KMIECIK (1999) 109 mg/100 gfw. Magnesium is generally among the deficient elements in the human diet; daily magnesium consumption by adults should amount to 300-380 mg (JAWORSKA and KMIECIK 1999). The mean value of Mg determined in this work was higher than the values reported by SALUNKE and KADAM (1998), USDA (2006) and JAWORSKA and KMIECIK (1999) and but was significantly lower than those found by OGUCHI *et al.* (1996) (110-180 mg/100 gfw).

Currently, no deficiency of sodium has been observed in the human diet. Our results were similar to the value reported by the USDA (2006), but higher than the amount reported by JAWORSKA and KMIECIK (1999) (30 mg/100 gfw) and lower than the amount published by RUBATZKY and YAMAGUCHI (1997).

The mean P value of the cvs was similar to the value reported by JAWORSKA and KMIECIK (1999), RUBATZKY and YAMAGUCHI (1997), and CHAKRABORTI *et al.* (1989) and higher than that obtained by the USDA (2006) (49.0 mg/100 g). Spinach is an excellent source of iron. In comparison to red meat, it provides iron for fewer calories and is totally fat-free. Iron is an integral part of hemoglobin, which transports oxygen from the lungs to all body cells. Growing children and pregnant women need iron. The estimated daily requirement of iron in adults is 15-26 mg. In the 15 cvs, the iron content was high; these results were higher than the values obtained by the USDA (2006), KIMURA and ITOKAWA (1990), WILLS *et al.* (1986) and RUBATZKY and YAMAGUCHI (1997).

Zinc is widespread throughout living organisms due to its biological significance. The minimum and maximum values of Zn were found in cvs N. 10 and 14, respectively. Statistically significant differences were observed between all of the cultivars ($P \leq 0.05$). The zinc content of spinach samples in the literature have been reported to be 0.53 mg/100 gfw USDA (2006) and 0.70 mg/100 gfw CHARKRABORTI *et al.* (1989). The zinc content reported in this study is higher than those reported in the literature.

The highest copper content was seen in cv N. 4 and the lowest in cv N. 12. The average value obtained in this study was in agreement with that reported by the USDA (2006) and was higher than the amount found by CHARKRABORTI *et al.* (1989). These differences are probably related to the spinach cultivars, and the agro-climatic and environmental conditions.

Fatty acid composition

The fatty acid composition of the 15 Iranian spinach cultivars was determined. The results (Table 3) showed that the oil contained eight fatty acids: palmitic, palmitoleic, hexadecatrienoic, stearic, oleic, linoleic, linolenic, and erucic acid. Linolenic acid was the predominant fatty acid. Significant differences were observed between the fatty acids of the different cvs. The fatty acid composition had the following average profile: C18:3> C16:0> C18:2> C18:1> C16:1> C16:3> C22:1> C18:0. The highest amounts of palmitic and palmitoleic acids were found in cv N. 14 (24.58 and 11.00%). These results are quite similar to those obtained by other authors. The USDA (2006) and LEE *et al.* (2000) reported the following profiles: C18:3 (52.2%)> C16:0 (18.60%)> C18:2 (10.00%)> C18:1=C16:1 (1.9%)> C18:0 (1.52%), and C18:3 (58.46%)> C16:0 (15.04%)> C18:2 (11.84%)> C16:3 (7.69%)> C14:0 (3.24%)> C16:1 (2.26%)> C18:1 (1.16%), respectively. In another study, CHOE *et al.* (2001) obtained the following order: C18:3 (57.11%)> C16:0 (17.63%)> C18:2 (9.56%)> C16:3 (8.01%)> C14:0 (2.57%)> C18:1 (2.48%)> C16:1 (1.92%)> C18:0 (0.65%). In con-

Table 3 - Fatty acid profiles of the 15 cultivars (%, percentage of total fatty acids) (a).

cv N.	USFA/SFA	C22:1	C18:3	C18:2	C18:1	C18:0	C16:3	C16:1	C16:0
1	3.14	4.18±0.11c	35.03±0.67f	17.13±0.18g	15.69±0.19b	2.84±0.32a	2.96±0.36e	0.85±0.24h	21.30±0.52c
2	4.22	1.98±0.10e	44.04±0.86a	19.34±0.32e	6.68±0.21g	1.55±0.11cde	4.36±0.16c	4.44±0.21f	17.61±0.28f
3	3.87	0.78±0.14h	40.47±0.32c	21.84±0.19ab	7.41±0.47f	1.19±0.36ef	4.35±0.04c	4.62±0.04f	19.34±0.24de
4	4.40	1.05±0.04g	43.54±0.39a	20.55±0.28cd	7.71±0.16f	1.27±0.19def	4.10±0.27cd	4.51±0.58f	17.27±0.79f
5	3.65	1.11±0.08g	37.65±0.25d	21.11±0.41bc	7.45±0.12ef	1.60±0.17cd	4.86±0.21b	6.30±0.40d	19.92±0.56d
6	2.94	2.14±0.12e	33.72±0.54g	17.50±0.76g	7.97±0.20f	1.38±0.09def	3.74±0.21d	9.58±0.39b	23.97±0.72a
7	3.70	1.01±0.08g	41.53±0.17b	21.42±0.29b	7.67±0.16e	1.39±0.35def	4.27±0.12c	2.82±0.12g	19.94±0.36d
8	3.08	1.97±0.07e	31.67±0.64h	18.33±0.35f	16.51±0.72a	3.17±0.13a	1.85±0.07f	5.14±0.18ef	21.36±0.09c
9	3.13	1.98±0.03e	33.52±0.61g	20.18±0.82d	8.69±0.34d	2.10±0.14b	5.16±0.23b	6.28±0.09d	22.10±0.31bc
10	3.87	0.55±0.14i	40.08±0.15c	21.80±0.08ab	8.67±0.41d	1.05±0.07fg	3.71±0.32d	4.70±0.21f	19.45±0.39de
11	3.76	2.14±0.09e	39.71±0.28c	14.23±0.49hi	8.84±0.15d	1.46±0.10cde	6.10±0.33a	8.01±0.25c	19.52±0.87de
12	3.31	5.43±0.11a	36.05±0.47e	14.86±0.63h	7.31±0.25f	1.24±0.08def	4.17±0.09cd	8.99±0.60b	21.95±0.16c
13	4.16	1.50±0.08f	39.68±0.08c	22.50±0.47a	8.60±0.26de	0.81±0.04g	2.85±0.14e	5.49±0.29e	18.56±0.46e
14	2.78	3.32±0.25d	31.60±1.52h	15.01±0.32h	8.45±0.71de	1.82±0.35bc	4.22±0.60c	11.00±0.78a	24.58±1.07a
15	2.88	4.65±0.30b	32.22±0.37h	13.59±0.82i	14.59±0.27c	2.81±0.19a	2.83±0.21e	6.36±0.51d	22.95±0.37b
Mean	3.53	2.25	37.37	18.63	9.48	1.71	3.97	5.94	20.65

(a) Values are mean ± standard deviation of triplicate determinations. Values in the same column having the same letters are not significantly different (Duncan's multiple range test, at $P \leq 0.05$).

trast to MURCIA *et al.* (1992), in this study C16:2 was not found. It was confirmed that the unsaturated fatty acids were predominant in all the cultivars and linolenic acid was the main fatty acid. The unsaturated/saturated fatty acid (USFA/SFA) ratio was generally high. The highest and lowest ratios were 4.22 (cv N. 2) and 2.78 (cv N. 14). These ratios were lower than the values reported by LEE *et al.* (2000) and quite similar with the value of 4.12 reported by the USDA (2006). Because of the presence of highly unsaturated fatty acids, the addition of spinach as an ingredient to foods may affect food processing properties as well as the quality of the final products (LEE *et al.*, 2002).

CONCLUSIONS

Practically no literature data are available concerning Iranian spinach and the present work reports the chemical composition of some of them. Spinach, which is cultivated throughout the year, provides many nutrients and is not expensive. It contains lipids (with high amounts of unsaturated fatty acids) and other components, such as chlorophyll and β-carotene. The addition of spinach as an added ingredient to food may affect food processing properties and the quality of the final product. In conclusion, spinach is a good source of minerals with K, Ca, Mg, Na and P being the major inorganic matrix of spinach followed by Fe, Zn, and Cu. The spinach cultivars studied showed a great variability in the chemical composition mainly due to genetic factors. Considering the increased interest in recent years to have foods with specific properties that prevent diseases, it would be useful to develop a selection program that would be extended to other spinach cvs in an attempt to identify cvs with high contents of nutrients and phenolic compounds that

are known for their numerous biological activities.

ACKNOWLEDGMENT

This research was supported by the Research Councils of Tarbiat Modares and the K. N. Toosi Technology Universities.

REFERENCES

Aehle E., Grandic S.R., Ralainirina R., Baltora-Rosset S., Mesnard F., Prouillet C., Maziere J.C. and Fliniaux M.A. 2004. Development and evaluation of an enriched natural antioxidant preparation obtained from aqueous spinach (*Spinacia oleracea*) extracts by an adsorption procedure. Food Chem. 86: 579.

AOAC (1984). "Official Methods of Analysis" 14th ed. Association of Official Analytical Chemists, Arlington, USA.

Chakraborti N., Mandal I. and Bannerjee G.C. 1989. Chemical composition of some common herbs and shrubs, aquatic plants and kitchen greens. Indian J. Dairy Sci. 42: 375.

Choe E., Lee J., Park K. and Lee S. 2001. Effects of heat pretreatment on lipid and pigments of freeze-dried spinach. J. Food Sci. 66: 1074.

Chu Y.F., Sun J., Wu X. and Liu R.H. 2002. Antioxidant and antiproliferative activities of common vegetables. J. Agric. Food Chem. 50: 6910.

Gülser F. 2005. Effects of ammonium sulphate and urea on NO_3- and NO_2- accumulation, nutrient contents and yield criteria in spinach. Sci. Hortic. 106: 330.

Hodgkinson A. 1978. Evidence of increased oxalate absorption in patients with calcium-containing renal stones. Clin. Sci. Mol. Med. 54: 291.

Howard L.R., Pandjaitan N., Morelock T. and Gil M.I. 2002. Antioxidant capacity and phenolic content of spinach as affected by genetics and growing season. J. Agric. Food Chem. 50: 5891.

Jaworska G. 2005. Nitrates, nitrites, and oxalates in products of spinach and New Zealand spinach – Effect of technological measures and storage time on the level of nitrates, nitrites, and oxalates in frozen and canned products of spinach and New Zealand spinach. Food Chem. 93: 395.

Jaworska G. and Kmiecik W. 1999. Content of selected mineral compounds, nitrates III and V, and oxalates in spinach (*Spinacia oleracea* L.) and New Zealand spinach (*Tetragonia expansa Murr.*) from spring and autumn growing seasons. Electron. J. Pol. Agric. Univ., Food Sci. Technol. Vol. 2, Issue 2.

Jeffery G.H., Basset J., Mendham J. and Denney R.C. 1989. "Vögels Textbook of Quantitative Chemical Analysis" 5th ed., p. 702, Longman Scientific and Technical Publisher, London.

Kawazu Okimura Y.M., Ishii T. and Yui S. 2003. Varietal and seasonal differences in oxalate content of spinach. Sci. Hortic. 97: 203.

Kimura M. and Itokawa Y. 1990. Cooking losses of minerals in foods and its nutritional significance. J. Nutr. Sci. Vitaminol. 36: S25.

Lee J., Lee S., Lee H., Park K. and Choe E. 2002. Spinach (*Spinacia oleracea*) powder as a natural food-grade antioxidant in deep-fat-fried products. J. Agric. Food Chem. 50: 5664.

Lee J., Park K., Lee S. and Choe E. 2000. Lipid changes of freeze dried spinach by various kinds of oxidation. J. Food Sci. 65: 1290.

Metcalf L.D., Schmitz A.A. and Pelka J.R. 1966. Rapid preparation of fatty acid esters from lipids for gas chromatographic analysis. Anal. Chem. 38: 514.

Murcia M.A., Vera A. and Garcia- Carmona F. 1992. Effects of processing methods on spinach: proximate composition in fatty acids and soluble protein. J. Sci. Food Agric. 59: 473.

Oguchi Y., Weerakkody W.A.P., Tanaka A., Nakazawa S. and Ando T. 1996. Varietal differences of quality-related compounds in leaves and petioles of spinach grown at two locations. Bull. Hiroshima Prefectural Agric. Res. Center. 64: 1.

Pandjaitan N., Howard L.R., Morelock T. and Gil M.I. 2005. Antioxidant capacity and phenolic content of spinach as affected by genetics and maturation. J. Agric. Food Chem. 53: 8618.

Rubatzky E. and Yamaguchi M. 1997. "World Vegetables: Principles, Production and Nutritive Values" p. 458, Chapman and Hall, New York, USA.

Salunkhe D.K. and Kadam S.S. 1998. "Handbook of Vegatable Science and Technology: Production, Composition, Storage and Processing" p. 721, Marcel Dekker Inc., New York.

Singh R.P., Murthy K.N.C. and Jayaprakasha G.K. 2002. Studies on the antioxidant activity of pomegranate (*Punica granatum*) peel and seed extracts using *in vitro* models. J. Agric. Food Chem. 50: 81.

Toledo M.E.A., Ueda Y., Imahari Y. and Ayaki M. 2003. L-ascorbic acid metabolism in spinach (*Spinacia oleracea* L.) during postharvest storage in light and dark. Postharv. Biol. Technol. 28: 47.

USDA. 2006. United State Department of Agriculture. http://www.nal.usda.gov/fnic/foodcomp.

Weaver C.M., Martin B.R., Ebner J.S. and Krueger C.A. 1987. Oxalic acid decreases calcium absorption in rats. J. Nutr. 117: 1903.

Williams S.R. 1993. "Nutrition and Diet Therapy" 7th ed., Mosby, St. Louis, MO.

Wills R.B.H., Lim J.S.K. and Greenfield H. 1986. Composition of Australian foods. Leafy, stem and other vegetables. Food Technol. Australia 38: 416.

Wu F., He Z., Luo Q. and Zeng Y. 1999. HPLC determination of oxalic acid using tris (1, 10-phenanthroline) ruthenium (II) chemiluminescence-application to the analysis of spinach. Food Chem. 65: 543.

Yang Y., Marczak E.D., Usui H., Kawamura Y. and Yoshikawa M. 2004. Antihypertensive properties of spinach leaf protein digests. J. Agric. Food Chem. 52: 2223.

Revised paper received December 23, 2006 Accepted January 19, 2007

PAPER

ANALYSIS OF CARBOHYDRATES IN SICILIAN DOC WINES BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH EVAPORATIVE LIGHT SCATTERING DETECTION

ANALISI DI ZUCCHERI IN VINI DOC SICILIANI MEDIANTE HPLC-ELSD

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ABSTRACT

A high performance liquid chromatographic method with low temperature evaporative light scattering detection (HPLC-LT ELSD) was developed for the direct analysis of some pentoses, hexoses and disaccharides in Sicilian Controlled Denomination of Origin (DOC) wines. The chromatographic separation was performed using a Prevail Carbohydrate ES column under isocratic conditions with a mixture of water-acetonitrile. The detection limits of ELSD (S/N=3) were between 7 and 30 mg/L

RIASSUNTO

Nel presente studio è stata utilizzata una metodica HPLC in fase normale, con un rivelatore Evaporative Light Scattering a bassa temperatura (LT-ELSD) per l'analisi di ramnosio, xilosio, fruttosio, glucosio, saccarosio, lattosio e maltosio in vini siciliani DOC bianchi, rossi e Marsala. La separazione è stata effettuata utilizzando una colonna di Prevail Carbohydrate applicando eluizione isocratica con una miscela di acqua e acetonitrile. Ottimizzando le condizioni cromatografiche sono

- Key words: carbohydrates, HPLC/ELSD, Marsala, Sicilian wines -

for different carbohydrates, whereas precision and accuracy were 4.2 and 96.5%, respectively. Glucose and fructose were the most abundant sugars in all the wines. Residual levels of xylose, rhamnose and lactose were detected only in Marsalas; maltose was present in all the Sicilian wines.

stati ottenuti limiti di rivelabilità compresi tra 7 e 30 mg/L, mentre la precisione e l'accuratezza sono entro 4,2 e 96%, rispettivamente. Glucosio e fruttosio sono gli zuccheri più abbondanti in tutti i campioni, residui di xilosio, ramnosio e lattosio sono state rilevate solo in alcuni vini Marsala, il maltosio è invece presente in tutti i campioni esaminati in concentrazioni.

INTRODUCTION

Without sugar and yeast there could be no wine. Yeast converts sugar into CO_2 and alcohol, through enzymatic action. In the process, there is a little sugar left over that the yeast converts into energy for its own metabolic needs.

D-glucose and D-fructose are fermentable and make up about 99% of the total sugar content of wine; residual sugars including D-glucose isomers (D-mannose and D-galactose), pentoses (D-xylose, D-ribose, L-rhamnose) and disaccharides (lactose, saccharose and maltose) may also be present. Almost all of these residual sugars are usually unfermentable and are believed to have either desirable or undesirable effects on the quality of wine during aging. Wine is usually fermented to dryness because residual sugars would make the wine microbially unstable. Residual sugars in a wine which contains less than 16% alcohol create a substrate for possible yeast growth, making the wine potentially unstable or liable to re-ferment in the bottle. Most enologists consider primary fermentation as complete when residual sugars are in the range of 0.1-0.2%. Using the reducing sugar measurement, wines below 0.2% are generally considered stable with regard to the possibility of re-fermentation in the bottle. At this point most of the residual sugars present are pentoses which are unfermentable by Saccharomyces yeast (RIBÉRAU-GAYON *et al.*, 2003). Furthermore, sugar analysis in wine is of great concern since, unlike what is allowed by the European Union and by the regulations of other countries, in Italy the addition of substances extraneous to the grape (e.g. saccharose) to wine is not permitted.

A considerable amount of data on residual sugars in wines has been published (AYESTARÁN *et al.*, 2004; ESAU and AMERINE, 1967; NAKASHINI and YOKOTSUKA, 1989), but no such data have been reported for Sicilian Controlled Denomination of Origin (DOC) wines. In the last years the products of Sicily have been revalued; Sicily produces at least 17% (8.7 million hL) of all the wine produced in Italy, and most of these wines (about 90%) are now protected by the DOC regulation. Four per cent of the Sicilian wine and must production is exported abroad, particularly to France (75%), Spain (12%) and the UK (5%), with some overseas consumers such as the USA, Canada and Japan.

In recent years, wine has been one of the few Sicilian agricultural products that have seen a rise in export levels, both in quantity and average unit value compared to previous years (CRESCIMANNO *et al.*, 2002). Among the Sicilian white wines, the most distinctive ones are Insolia, Damaschino or Grillo, sometimes blended with Chardonnay. Among the red wines, the most diffused native va-

rieties are Nero d'Avola and Nerello Mascalese that are vinified in their purest varietal form or blended with indigenous varieties such as Cabernet Sauvignon, Merlot and Syrah. Until recently, Sicily was best known for fortified aged wines such as Marsala. These wines are characterized by a high sugar content that is generally added during the vinification process through a fortification with must and wine. The sweetest Marsalas are called "Dolce" (total sugars > 100 g L^{-1}), followed by "Semi-secco" (total sugars from 40 to 100 g L^{-1}) and "Secco" (total sugars < 40 g L^{-1}) which are the driest; further information on Marsala wine classification is reported in the Italian Official Gazette (ITALIAN REPUBLIC, DPR 17/11/1986).

Various techniques for sugar analysis in wine have been developed including paper chromatography, capillary electrophoresis, spectrometry and high-performance liquid chromatography (HPLC) using refractometric detection (RID) (ARAUJO *et al.*, 2000; LIMA *et al.*, 1994; NAKASHINI and YOKOTSUKA, 1989; O'FLAHERTY *et al.*, 2001). Refractive index (RI) measurement is the most popular detection method for carbohydrates (BERTHOD *et al.*, 1998; GLYAD, 2002). However, RI has many disadvantages in LC analysis, such as a lack of sensitivity, dependence on temperature and flow-rate, and incompatibility with gradient elution. The use of evaporative light scattering detection (ELSD) in LC has increased during the last decade as a universal technique capable of detecting any semi- and non-volatile analyte. The ELSD measures the photons scattered from particles after nebulization and evaporation of the mobile phase. Because its response is independent of the optical absorption characteristics of the target substance and of the solvent, it can reveal sample components that are transparent to other detectors. WEI and DING (2000) developed a HPLC-ELSD method with a dynamically modified amino column to detect fructose, glucose, sucrose, maltose, lactose and raffinose in beverages with detection limits ranging from 10 to 60 mg/L. NOGUEIRA *et al.* (2005) described a HPLC method with evaporative light scattering detector optimized and validated for quantification of carbohydrates in beer; the chromatographic separation was achieved using a Spherisorb NH_2 chromatographic column and gradient elution with acetonitrile/water.

This paper describes the use of a HPLC-ELSD LT method to analyze rhamnose, xylose, fructose, glucose, saccharose, lactose and maltose, in Sicilian DOC wines. Sugar separation was achieved with a column packed with a rugged hydrophilic polymeric gel which provides a performance that is superior to traditional amino columns and ion exclusion columns. Because of its rugged bonding technology, the column delivers quiet, stable baselines and excellent peak shapes. Detection limits ranging from 10 to 30 mg/L were achieved.

MATERIALS AND METHOD

Chemicals

All the reagents used were of analytical grade. The standard sugars, D-fructose, D-glucose, D-(+) lactose, D-maltose, L-(+)-rhamnose, D-(+) saccharose, and D-(+) xylose, were purchased from Sigma Aldrich (Milan, Italy). Acetonitrile and water (HPLC-grade) were obtained from Carlo Erba Reagents (Milan, Italy).

Stock solutions of each carbohydrate were prepared as an aqueous solution at a concentration of 5,000 mg/L. The standard mixture of sugars was prepared using these stock solutions.

Apparatus

Carbohydrate analysis was performed using a liquid chromatographic system

equipped with two 10 Avp pumps, a vacuum degasser, a 20 μL manual injector and an ELSD LT detector (Shimadzu, Milan, Italy). N_2 generated from a Chromgas generator (Parker-Balston Corp., Haverhill, MA) was used as the carrier gas to transport the analyte substance from the drift tube into the detection chamber of the ELSD. The overall system operated under the control of the CLASS VP software package (Shimadzu, Milan, Italy).

HPLC-ELSD LT conditions

A separation Prevail Carbohydrate ES column (250x4.6 mm, 5 μm particle size) (Alltech Italia, Milan, Italy) packed with a rugged hydrophilic polymeric gel and a guard column of the same material was used. The separation was achieved at ambient temperature. The mobile phase for isocratic elution was a mixture of water-acetonitrile (20:80, v/v) at the flow-rate of 1.0 mL/min; the total run time was 20 min. The temperature of the ELSD drift tube was 40°C, the carrier gas pressure was 250 KPa at a flow-rate of 2.0 mL/min.

Samples

Fifteen samples of five different types of Marsala wines were studied; three samples of each wine type produced in the DOC zone of Marsala (Trapani, Sicily) were analysed. The vines grew on a dry calcareous soil near the coast. In all the crop years considered (1999, 2002, 2003), the grapes were harvested in the period between 25 August and 5 October. The newly harvested grapes were crushed, destemmed, and subjected to soft pressing in contact with the skins in order to extract the aromatic compounds. After fermentation by selected yeasts, at a controlled temperature (15°C) in stainless-steel containers, all the wines, except Marsala Vergine Soleras, were spiked with cooked must, wine (13% alcoholic degree) and alcohol. Marsala Vergine wines were fortified with only wine and alcohol. After the fortifications, the wines were left to mature in oak barrels. Three months after the end of the aging period, the wines were bottled and allowed to refine in the bottles for two months before uncorking and consuming. Table 1 reports all the information concerning the samples studied. Fifteen samples of red wines and nine samples of white wines from different autochthonous and allocthonous grape varieties were also analysed (Table 2). All the studied wine samples were bottled in dark bottles and stored at 4°C for the duration of the analysis.

Quantitative analysis and analytical performance

Calibration was carried out using the external standard method by preparing four aqueous solutions of carbohydrates of different concentrations in the range of 10-5,000 mg/L. The performance parameters, precision, accuracy (expressed as recovery factor), reproducibility, and detection limits, were evaluated using model wine solutions (14% v.v. aqueous ethanol, 1 g/L gallic acid, saturated with potassium hydrogen tartrate and adjusted to pH 3.5 with tartaric acid) spiked with different levels of D-fructose, D-glucose, D-(+) lactose, D-maltose, L-(+)-rhamnose, saccharose, and D-(+) xylose. Fig. 1 shows the chromatogram of the model wine solution (a) and of a red wine (b). Table 3 reports the retention times, calibration curves, detection limits (S/N=3) and quantification limits (S/N=10) (WINEFORDNER and LONG, 1983). Table 4 shows the average mean precision, given as the relative standard deviation of nine runs at different concentration levels (100, 500 and 1,000 mg/L), the recovery factors and the reproducibility, expressed as long-term stability of the method, evaluated by analysing the model wine solution contain-

Table 1 - Description of the Marsala wine samples produced in the DOC zone of Marsala (n=3).

	Grape variety	Production year	Ageing (years)	Alcohol %
Superiore Ambra Secco (SAS)	Grillo	2002	2	18
Fine Ambra Secco (FAS)	Grillo	2003	1	19
Vergine Soleras (VS)	Grillo-Inzolia-Cataratto	1999	5	18
Fine Oro Dolce (FOD)	Grillo-Cataratto	2003	1	18
Superiore Riserva (SR)	Grillo-Cataratto	1999	5	18

Table 2 - Description of the red and white wine samples produced in Sicily from autochthonous and allocthonous grape varieties (n=3).

Grape variety	Production year	Alcohol %
	RED WINES	
Nero d'Avola (NA)	2002	14
Nero d'Avola-Cabernet S. (NA-CS)	2002	14.5
Nero d'Avola-Cabernet S.-Merlot (NA-CS-M)	2001	15
Frappato-Syrah(F-S)	2002	14.5
Nerello Mascalese-Merlot (NM-M)	2002	14
	WHITE WINES	
Grillo (G)	2003	12
Inzolia (I)	2003	12.5
Inzolia-Chardonnay (I-C)	2003	12

ing 1,000 mg/L of each assayed sugar over five consecutive days.

Analysis of wines

The wines were analysed directly after being filtered through a 0.45 μm nylon membrane. Filtered red and white wines were diluted 2-fold, whereas sweet Marsalas and dry Marsalas were diluted with water 100-fold and 10-fold, respectively, prior the analysis.

RESULTS AND DISCUSSION

Analytical method

A sensitive, rapid and accurate HPLC-ELSD method for analysing monosaccharides and disaccharides in wines is described. Most of the HPLC methods reported in the literature for the determination of mono- and oligosaccharides use an amino-bonded silica column and acetonitrile-water as the mobile phase. This method can cause problems for the ELSD because the column exhibits hydrolytic instability in the presence of water, especially at concentrations greater than 10% (WEI and DING, 2000).

In this study the separation of sugars was achieved with a column packed with a rugged hydrophilic polymeric gel which has a performance that is superior to traditional amino columns, diol or polyol bonded silica gel columns and ion exclusion columns (BERTHOD 1998; TORTO *et al.*, 1995). This stable gel provides an efficient separation of some pentoses (rhamnose and xylose), hexoses (fructose and glucose) and disaccharides (saccharose, lactose and maltose) in wines in a single chromatographic profile, without any sample pre-treatment. ELSD measurement allows HPLC separations by gradient program elution. In this study many gradient pro-

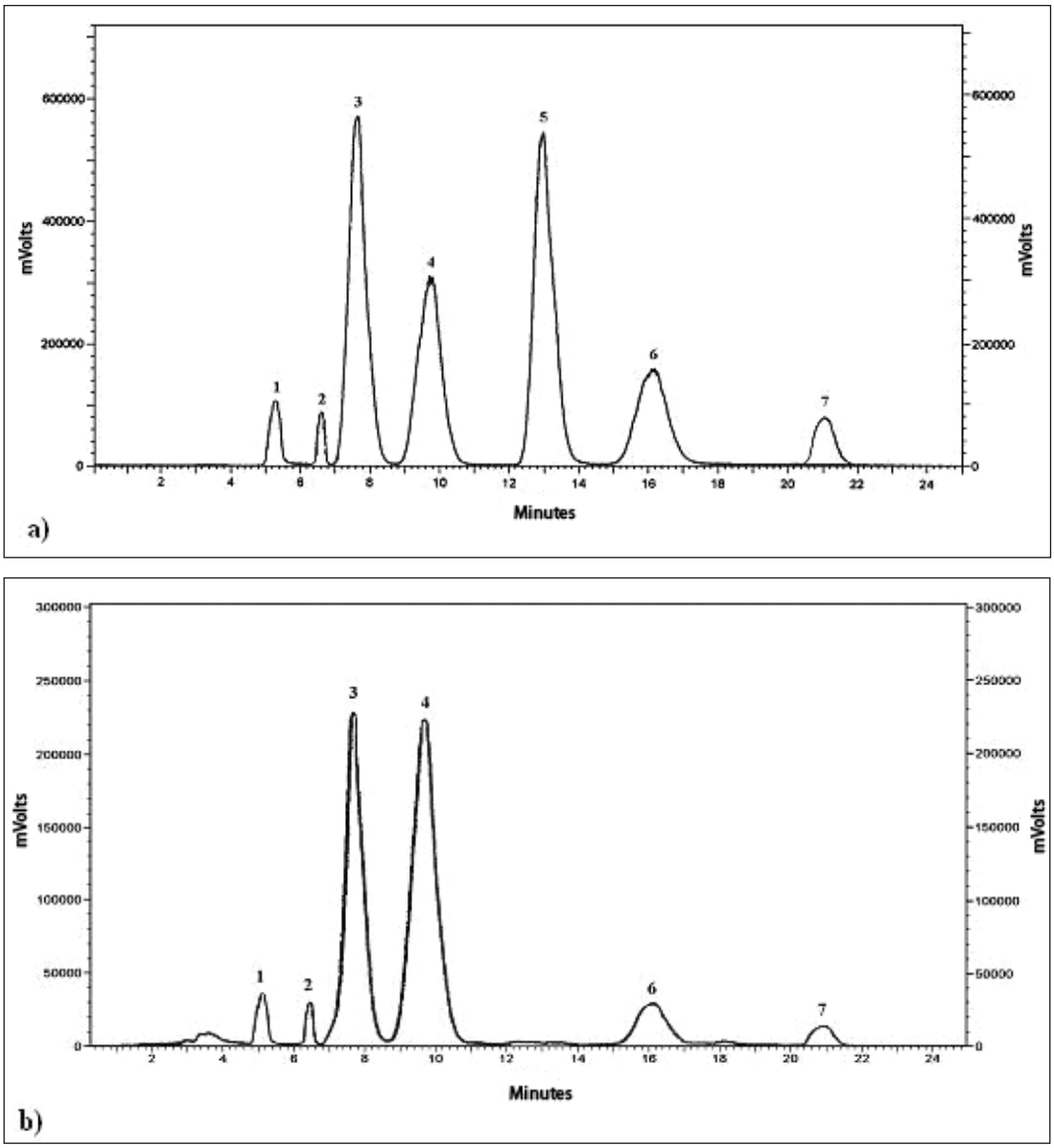

Fig. 1 - HPLC-ELSD chromatogram of a) model wine solution containing 1,000 mg/L of each assayed sugar; b)Sicilian red wine. Column: Prevail Carbohydrate ES (250x4.6 mm I.D., 5 μm); mobile phase: water-acetonitrile (25: 75 v/v); flow-rate: 1.0 mL/min; drift tube temperature of ELSD 40°C; gas flow-rate of ELSD: 2.0 mL/min. Peak identification: 1) rhamnose, 2) xylose, 3) fructose, 4) glucose, 5) saccharose, 6) lactose, 7) maltose.

grams were tested using water and acetonitrile to separate wine carbohydrates. The analysis times were shorter than those of isocratic elution but pentose separation was not achieved by any of the gradient programs used. Nevertheless isocratic elution using different ratios of water and acetonitrile as the mobile phase were studied. The content of water had a great effect on the retention

of the carbohydrates; as the water content increased, the carbohydrates were eluted more quickly. The chromatograms of a standard mixture and a red wine sample (Fig. 1a, b) indicate that the best separation, particularly between xylose, fructose and glucose was achieved using water-acetonitrile in a ratio 20:80 v/v.

The sensitivity of this method is affected by three instrumental parameters: detector temperature, nebulizing gas pressure and photomultiplier gain. The effect of temperature was evaluated by injecting a known amount of sample at different detector temperatures (30°, 40°, 50° and 60°C). The optimum temperature appeared to be 40°C. This temperature allowed complete solvent evaporation and negligible baseline noise. The airflow rate (pressure) of the air compressor was set at 250 KPa to reduce the baseline noise. A photomultiplier gain of 6 provided an average sensitivity for carbohydrate detection giving an adequate signal-to-noise ratio. Several authors have established that the ELSD response is linear for a wide range of concentrations (PONS *et al.*, 1998; WEI and DING, 2000). However, some workers have demonstrated that ELSD provides a non-linear response, that is the result of several light scattering mechanisms and particle size distribution (HIERRO *et al.*, 1992). The non-linear ELSD response is described by the widely used empirical model ($y = a/m^b$) where *m* is the amount injected and *a* and *b* are numerical coefficients. In this study the linearity of

Table 3 - Retention times, calibration curves, detection limits (S/N=3) and quantification limits (S/N=10) for HPLC-ELSD LT analysis of sugars in wine.

Sugar	t_R±SD	y = kx	R^2	LOD (mg/L)*	LOQ (mg/L)**
L-rhamnose	5.76±0.121	9,239.8 m	0.9987	20	67
D-xylose	6.35±0.183	11,044 m	0.9924	22	73
D-fructose	8.34±0.061	7,284.6 m	0.9915	9	30
D-glucose	10.47±0.115	10,244 m	0.9973	10	33
saccharose	13.70±0.093	16,550 m	0.9903	7	23
D-lactose	16.87±0.144	8,239.8 m	0.9925	30	100
D-maltose	21.25±0.183	5,866 m	0.9946	10	33

*defined as the signal height at a signal/noise ratio S/N=3;
**defined as the signal height at a signal/noise ratio S/N=10.

Table 4 - Performance of the HPLC-ELSD method for sugar analysis in wine.

Sugar	Precision * %	Recovery %	Reproducibility** %
L-rhamnose	4.2±0.21	95.8	3.3±0.25
D-xylose	4.0±0.32	96.0	3.6±0.37
D-fructose	4.0±0.38	96.0	3.6±0.59
D-glucose	3.5±0.20	96.5	3.7±0.38
saccharose	4.0±0.33	96.0	2.1±0.21
D-lactose	3.7±0.27	96.3	2.4±0.33
D-maltose	3.5±0.21	96.5	2.7±0.50

*Precision is expressed as the average of the relative standard deviations of nine runs at different concentration levels (100, 500 and 1,000 mg/L).
**Reproducibility is expressed as long term stability of the method, evaluated by analysing the model wine solution containing 1,000 mg L^{-1} of each assayed sugar over five consecutive days.

the method was determined by analysing of increasing amounts of aqueous standard solutions of seven wine carbohydrates. From our experimental results it was found that calibration curves between peak areas and the mass m of the analyte injected were linear for these seven carbohydrates following the equation y=a*m*, with coefficients of determination ranging from 0.9903-0.9987, in the concentration range studied (10-5,000 mg/L) (Table 3).

Of all the sugars studied, rhamnose, xylose, saccharose and lactose were present in the wine in very small amounts. Therefore a very sensitive analytical technique is required for their detection. The method described allows very low concentrations of sugars to be detected: the mass limits of detection (mLOD) ranged from 0.14 μg (saccharose) to 0.6 μg (lactose), corresponding to a concentration limit of detection range (cLOD) of 7-30 mgL^{-1} with an injection volume of 20 μL (Table 3). These values are lower than those reported by WEI and DING (2000) that were obtained with a dynamically modified amino column. Furthermore this method offers good precision (3.5-4.2%) and reproducibility (2.1-3.7%); the accuracy, expressed as recovery factor, was within 96% (Table 4).

Sugars in Sicilian wines

Sicilian wines have finally made it onto the international stage; more modern viticulture and winemaking techniques coupled with the unique blending of local Sicilian grape varieties and international ones, have helped improve the quality of this unique product. For these reasons it is interesting to study in detail the presence of chemical compounds, such as sugars, that mainly contribute to wine quality. In this study the concentrations of D-fructose, D-glucose, saccharose, D-lactose, D-maltose, L-rhamnose, D-saccharose, and D-xylose were determined in samples of Sicilian DOC wines. In particular fifteen samples of five different types of Marsala wines, fifteen samples of red wines and nine samples of white wines from different autochthonous and allocthonous grape varieties were studied.

The amount of sugar in the grapes determines what the final alcohol level will be. In cool climates, where it is a struggle for the grapes to ripen, sugar levels will be minimal, and consequently such wines often only reach 8 or 10% alcohol (WAHL, 1988). In very warm climates, such as in Sicily, the final alcohol level will be determined not so much by the amount of sugar but rather by the yeasts themselves. Once the alcohol level reaches about 14% the yeasts can no longer function and rapidly die off. For this reason, wines with a strength of more than 15% are almost certainly fortified. Marsala is a fortified wine with an alcohol content of around 18%; it is usually made from native Grillo, Catarratto or Inzolia grapes. As Table 5 shows, glucose and fructose were the most abundant sugars and their ratio was about 1 in all the Marsalas; their concentrations decrease in the following order: FOD>SR>SAS>FAS>VS. Among the pentoses studied, rhamnose was present in all the Marsalas in similar amounts, whereas xylose was detected only in the driest wines, SAS, FAS and VS. Among the disaccharides studied, the saccharose levels were lower than the LOD in all the Marsalas, maltose was detected in all the samples in concentrations ranging from 0.17 to 0.32 g/L, whereas lactose was found in all wines except VS, at a concentration lower than 0.32 g/L.

To have further information about the presence of non-fermentable residual sugars in Marsalas, a statistical elaboration of the data was performed to correlate the concentration of rhamnose, xhylose, lactose and maltose both with the ageing time of the wines (Pearson Correlation Coefficient, $p>0.01$) and with

Table 5 - Sugar concentrations (g/L) in Sicilian DOC wines determined by HPLC-ELSD (n=3).

Wines	Rh	Xy	Fr	Gl	Sac	Lac	Mal	*TOT %*
				MARSALA				
SAS	0.33±0.10	0.37±0.08	18.57±3.65	19.60±4.00	<0.007	0.31±0.10	0.16±0.03	*3.9*
FAS	0.33±0.09	0.41±0.14	4.39±1.25	3.92±0.99	<0.007	0.26±0.06	0.20±0.05	*0.9*
VS	0.25±0.08	0.34±0.09	1.21±0.95	1.23±0.08	<0.007	<0.03	0.32±0.11	*0.3*
FOD	0.37±0.10	<0.022	65.00±8.21	71.46±7.15	<0.007	0.32±0.07	0.17±0.09	*13.7*
SR	0.34±0.12	<0.022	55.43±6.55	56.10±4.96	<0.007	<0.03	0.25±0.10	*11.2*
				RED WINES				
NA	<0.02	<0.022	1.31±0.20	0.55±0.07	<0.007	<0.03	0.19±0.09	*0.2*
NA-CS	<0.02	<0.022	3.1±0.98	0.66±0.04	<0.007	<0.03	0.34±0.18	*0.4*
NA-CS-M	<0.02	<0.022	2.23±0.77	0.54±0.04	<0.007	<0.03	0.31±0.18	*0.3*
F-S	<0.02	<0.022	1.35±0.35	1.24±0.35	<0.007	<0.03	0.27±0.14	*0.3*
NM-M	<0.02	<0.022	3.1±1.05	0.53±0.06	<0.007	<0.03	0.13±0.11	*0.4*
				WHITE WINES				
G	<0.02	<0.022	2.73±0.55	0.65±0.03	<0.007	<0.03	0.29±0.11	*0.4*
I	<0.02	<0.022	6.6±1.67	3.3±0.80	<0.007	<0.03	0.27±0.16	*1.1*
I-C	<0.02	<0.022	1.66±0.91	0.6±0.08	<0.007	<0.03	0.25±0.09	*0.3*

the grape varieties used in wine making (Linear Discriminant Analysis): Grillo, Grillo-Inzolia, Grillo-Inzolia-Catarratto. Since no statistically significant results were obtained, the presence of residual sugars may be a result of the fortification process during winemaking. The red "table wines" had an alcohol content of about 14.5%, the white ones 12%. All of these can be defined as "dry bone" wines since their total sugar content was less than 0.5%, with the exception of samples from the Inzolia variety that showed a total sugar content of about 1%. While fructose was the most abundant compound, glucose is the preferred fermentable substrate for the majority of wine yeast (RIBÉRAU-GAYON *et al.*, 2003). The glucose-to-fructose ratio ranged from 0.2 to 0.4 in all the studied red and white wines except in the samples obtained from a blend of the Frappato and Syrah varieties where this ratio was about 1. White wines had a higher total sugar content with respect to the red wines; pentoses and lactose were not detected in any of the red and white wines. This confirms that their presence in Marsalas is the result of the fortification process with cooked must during winemaking. Maltose concentrations in table wines ranged from 0.13 to 0.34 g/L accounting at most for 11% of the total sugar fraction. Among the white wines, the sample obtained from the Inzolia variety had the highest levels of fructose and glucose, whereas samples obtained from a blend of Inzolia and Chardonnay varieties had the lowest. Among the red wines, the samples obtained by mixing Nerello Mascalese and Merlot varieties had the highest fructose and glucose levels while the wines from Nero d'Avola had the lowest.

CONCLUSION

A high performance liquid chromatographic method with low temperature evaporative light scattering detection (HPLC-LT ELSD) was developed for the direct analysis of some pentoses, hexoses and disaccharides in wine. Under the optimised analytical conditions, high sensitivity and accuracy coupled with a

short analysis time were achieved. The method described was able to provide some new information about the carbohydrate composition of Sicilian DOC wines. The results showed that glucose and fructose were the most abundant sugars in all the wines studied. Residual levels of xylose, rhamnose and lactose were only detected in Marsalas, while maltose was present in all the Sicilian wines studied. The saccharose levels were lower than the LODs in all the studied samples.

REFERENCES

Araujo A.N., Lima Jose´ L.F.C., Rangel A.O.S.S. and Segundo M.A. 2000. Sequential injection system for the spectrophotometric determination of reducing sugars in wines. Talanta 52: 59.

Ayestarán B., Guadalupe Z. and León D. 2004. Quantification of major grape polysaccharides (*Tempranillo* v.) released by maceration enzymes during the fermentation process. Analytica Chimica Acta 513: 29.

Berthod A., Chang S.S.C., Kullman J.P.S . and Armstrong D.W. 1998. Practice and mechanism of HPLC oligosaccharide separation with a cyclodextrin bonded phase. Talanta 47: 1001.

Crescimanno M., Ficani G.B. and Guccione G. 2002. The production and marketing of organic wine in Sicily. Br. Food J. 104: 274.

Esau P. and Amerine M.A. 1967. Quantitative estimation of residual sugars in wine. Am. J. Enol. Vitic. 17: 265.

Glyad V.M. 2002. Determination of monosaccharides, disaccharides, and oligosaccharides in the same plant sample by high-performance liquid chromatography. Russian J. Plant Physiol. 49: 277.

Hierro M.T.G., Tomas M.C., Fernandez-Martın F. and Santa Maria G. 1992. Determination of the triglyceride composition of avocado oil by high-performance liquid chromatography using a light-scattering detector. J. Chromatogr. B 607:329.

Italian Republic, DPR 17 November 1986. Published in the Italian Official Gazette n. 163, 15 July 1987.

Lima J.L.F.C., Neves O.B.A.O. and Rangel A.O.S.S. 1994. Flow injection determination of reducing sugar in wine in a wide concentration range using a dialysis unit and a stream splitting. In "Automatic Control of Food and Biological Processes" Bimbenet J.J., Dumoulin E. and Trystram G. (Eds.). p. 67. Elsevier.

Nakashini K. and Yokotsuka K. 1989. Isolation of two glucodisaccharides from Japanese wines. J. Ferment. Bioengin. 67: 148.

Nogueira L.C., Silva F., Ferreira I.M.P.L.V.O. and Trugo L.C. 2005. Separation and quantification of beer carbohydrates by high performance liquid chromatography with evaporative light scattering detection. J. Chromatogr. A 1065: 207.

O'Flaherty B., Yang W.P., Sengupta S. and Cholli A.L. 2001. Fast detection of anionic components in sugar and wine samples using a novel device based on capillary zone electrophoresis. Food Chem. 74: 111.

Pons S.M., Bargalló A.I.C. and Sabater M.C.L. 1998. Evaluation by high-performance liquid chromatography of the hydrolysis of human milk triacylglycerides during storage at low temperatures, J. Chromatogr. A 823:475.

Ribérau-Gayon P., Glories Y., Maujen A. and Dubourdieu D. 2003. "Trattato di Enologia II" Ch. 3, p. 59. Ed. Agricole, Bologna, Italy.

Torto N., Buttler T, Gorton L, Marko-Varga, G. and Stålbrand H. 1995. Monitoring of enzymatic hydrolysis of ivory nut mannan using on-line microdialysis sampling and anion-exchange chromatography with integrated pulsed electrochemical detection. Anal. Chimica Acta, 313: 15.

Wahl K. 1988. Climate and soil effects on grapevine and wine: Situation on the northern border of viticulture: the example of Franconia. In "Proceedings of 2nd International Symposium of Cool Climate Viticulture and Oenology", Eds. R.E. Smart, R. Thornton, S.B. Rodriquez and Young J. p. 1. Auckland, New Zealand.

Wei Y. and Ding M.Y. 2000. Analysis of carbohydrates in drinks by high-performance liquid chromatography with a dynamically modified amino column and evaporative light scattering detection J. Chromatogr. A 904:113.

Winefordner J.D. and Long. G.L. 1983. Limits of detection. A closer look at the IUPAC definition. Anal. Chem. 7: 712.

Revised paper received January 9, 2007 Accepted March 26, 2007

PAPER

THE RELATIONSHIP BETWEEN THE COMPOSITION OF DIFFERENT TABLE GRAPE (*VITIS VINIFERA* L.) EXTRACTS AND THREE METHODS OF MEASURING THEIR FREE RADICAL SCAVENGING PROPERTIES

RELAZIONE TRA LA COMPOSIZIONE DI DIFFERENTI ESTRATTI DI UVA DA TAVOLA (*VITIS VINIFERA* L.) E TRE METODI DI MISURA DELLE LORO PROPRIETÀ ANTIRADICALICHE

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ABSTRACT

Grape (*Vitis vinifera* L.) polyphenols are known to be beneficial as free radical scavengers. Previous experiments demonstrated that molecules other than polyphenols, such as simple carbohydrates and organic acids, can act as free radical inhibitors. In this study these hypotheses were verified by comparing the composition and the free radical scavenging of table grapes. Fremy's salt and DPPH quenching after 1 minute at 25°C were higher in skin (44

RIASSUNTO

I polifenoli dell'uva (*Vitis vinifera* L.) sono noti per le loro proprietà antiradicaliche. Precedenti esperimenti hanno dimostrato che altre molecole rispetto ai polifenoli, come gli zuccheri semplici e gli acidi organici, possono agire come inibitori dei radicali liberi. Questo studio ha suffragato questa ipotesi, confrontando la composizione e le proprietà antiradicaliche di uva da tavola. L'abbattimento del sale di Fremy e del DPPH dopo 1 minuto a 25°C è maggiore

- Key words Antioxidant properties, 1,1 diphenyl-2-picrylhydrazyl, free radical scavenging activity, Fremy's salt, grape polyphenols, hydroxyl radical -

and 89%) than in pulp (3 and 11%), while hydroxyl radical data gave similar values for skin (48%) and pulp (46%). Whole grape extracts were fractionated, separating sugars and organic acids from polyphenols. The scavenging activity was significant in polyphenol-enriched fractions for Fremy's salt and DPPH assays, resulting more active (2-fold and 25-fold, respectively). Hydroxyl radical was mainly quenched by fractions rich in sugars and organic acids, 5-fold more active than polyphenols-enriched fractions.

nelle bucce (44 e 89%) rispetto alle polpe (3 e 11%), mentre il radicale idrossile dà valori simili per buccia (48%) e polpa (46%). Estratti totali di uva sono stati frazionati separando gli zuccheri e gli acidi organici dai polifenoli. L'attività antiradicalica è significativa nelle frazioni ricche di polifenoli per il sale di Fremy e il DPPH, risultando rispettivamente più attive (2 e 25 volte). Il radicale idrossile è stato principalmente inattivato dalle frazioni ricche in zuccheri e in acidi organici, 5 volte più attive delle frazioni ricche in polifenoli.

INTRODUCTION

Grapes (*Vitis vinifera* L.) contain bioactive compounds (e.g., antioxidants). Health benefits seem to be related to the presence of phenolic compounds which act as antioxidants or free radical scavengers (BURNS *et al.*, 2000; SANCHEZ-MORENO *et al.*, 1999). A high intake of phenolics in the diet has been correlated with a low incidence of degenerative disorders which are probably caused by an excess of free radicals (BAZZANO *et al.*, 2002; FLOOD *et al.,* 2002).

The mechanism by which polyphenols exert these beneficial effects is not fully understood. Most experiments are performed on *in vitro* systems or *ex vivo* and there is evidence that free radical scavenging activity does not sufficiently explain some of the observed health effects.

The three main phenolic groups in grapes can be classified as flavonoids (defined by a C_6-C_3-C_6 carbon skeleton) and non-flavonoids, (cinnamic acid derivatives, C_6-C_3 system and phenolic acids, C_6-C_1) (MACHEIX *et al.,* 1996; BURNS *et al.,* 2001; SOUQUET *et al.,* 1996). Red grape cultivars are characterized by the presence of anthocyanins, a particular type of flavonoid.

Many studies have been carried out on the identity of the active compounds involved in the antioxidant and antiradical action of grapes (SANCHEZ-MORENO *et al.,* 1999; REVILLA and RYAN-JOSÈ, 2000) and it is widely affirmed that the most important anatomical parts of grapes are the skin and seeds which are rich in antioxidant polyphenolic compounds (YILMAZ and TOLEDO, 2004).

In fact, the "red wine making process" includes the maceration of skins in the fermenting must, to enrich the wine with polyphenols and anthocyanins which account for the colour of red wines.

Surprisingly, relatively little was found in the literature about the direct comparison of the antioxidant potential of grape pulp and skin. Most articles have been focused on the skin components and the wine.

Attention was focused on the scavenging activity of •OH because it is the most powerful, reactive and harmful free radical from oxygen catabolism (ARUOMA, 1998; CZAPSKY, 1984; VALKO *et al.,* 2005). It is implicated in the onset of many diseases; in fact, a bibliographic check on PubMed database

resulted in more than 600 references (about 130 reviews) for "hydroxyl radical disease".

Some preliminary trials of separate extractions of the pulp and skin of grape have already been performed on some grape varieties suitable for wine production (LO SCALZO *et al.,* 2006) and it was found that hydroxyl radical was equally quenched by the pulp and skin extracts, despite the well known fact that the most active compounds in the antioxidant action are in the skin. Some biological trials have also been carried out to confirm this hypothesis: rats were fed grape skin and pulp, and, surprisingly, the parameters related to the cardioprotective action were similar for both trial groups (FALCHI *et al.,* 2006).

Hence, the action of molecules other than polyphenols could be hypothesized: other experiments have demonstrated a significant action of simple sugars (GRAY and MOWER, 1991; WEHMEIER and MOORADIAN, 1994; MORELLI *et al.,* 2003) and organic acids (KAYASHIMA and KATAYAMA, 2002; CHANG *et al.,* 2001) on the hydroxyl radical (•OH). The aim of this study was to further confirm these findings by performing a separate ethanol extraction of skin and pulp from some commercial table grape varieties considering the main grape components (soluble carbohydrates, organic acids and polyphenols). The chemical composition and free radical scavenging properties of the grape extracts were evaluated by comparing three methods: spin-trapping of the Fenton-generated hydroxyl radical (•OH), Fremy's salt inhibition and 1,1 diphenyl-2-picrylhydrazyl (DPPH•) quenching. The separate extraction of skin and pulp was coupled with a simple fractionation of whole grape extracts on reversed phase in a fraction enriched with hydrophilic compounds (e.g. simple carbohydrates and organic acids) and in a fraction with relatively hydrophobic ones (e.g. polyphenols). Single fractions were analysed for their chemical composition and for their free radical scavenging properties.

MATERIALS AND METHODS

Sampling collection and extraction

Approximately 10 kg of four grape varieties were sampled in 2004 at commercial maturity. Two were collected in central Italy in the experimental vineyard of the Istituto Sperimentale per l'Enologia, Velletri (Rome): a red variety, Incrocio Prosperi (PRO) and a white one, Italia, harvested in October (ITAOTT). The other two table grape varieties came from commercial vineyards in southern Italy: a red variety, Palieri (PAL) and a white, Italia, harvested in September (ITASET). In the laboratory, 175 berries of each variety were randomly selected for their uniform size, absence of defects and apparent maturity level. Each lot was further divided into seven aliquots of 25 berries each (Fig. 1). One aliquot was squeezed, homogenated with a Waring blender, centrifuged at 20,000xg for 15 min and the juice was analysed for its refractive index and titratable acidity. Four aliquots (20 g each) were extracted with 40 mL of EtOH/CH_3COOH (9:1, v/v) then homogenised with a Waring blender, shaken for 15 min at room temperature and centrifuged at 2°C at 126,000xg. The supernatant was decanted and filtered through glass wool (RAW) and stored at -20°C until analysis.

The skins (SK) and pulp (PL) of two berry aliquots were carefully separated with a lancet, excluding the seeds, and extracted as described above.

Five mL of each extract were dried in a rotary evaporator, redissolved with the same volume of water and re-dried, in order to eliminate residual EtOH and CH_3COOH. All dried samples were stored at -20°C until analysis and further fractionations.

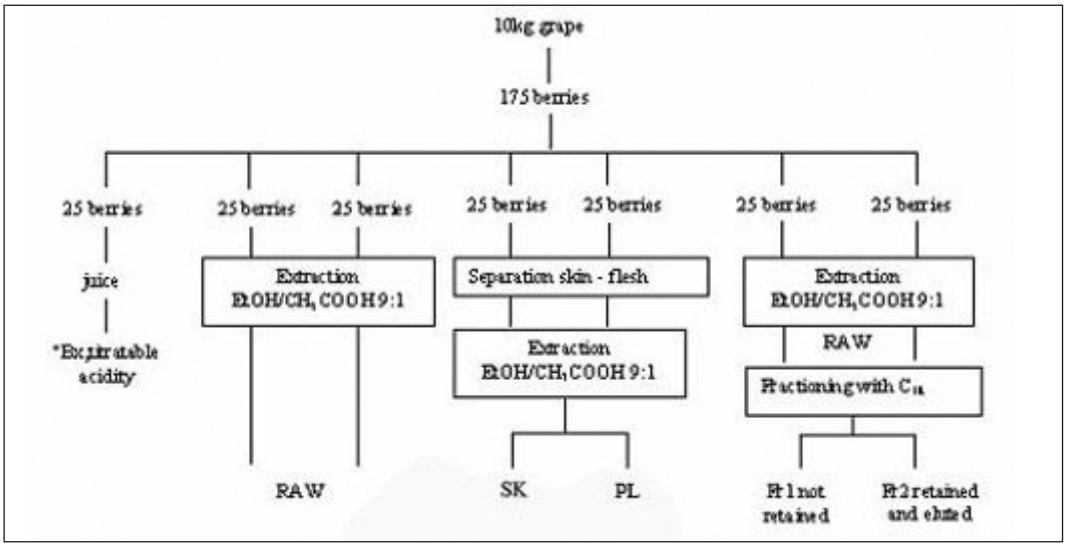

Fig. 1 - Scheme of the table grape sample processing for RAW, SK, PL, Fr1 and Fr2. Each extract was obtained in duplicate.

Solid phase fractionation on C_{18}

Two aliquots of the dried RAW samples were brought to the original extract volume by dissolving them in 5 mL of 1% aqueous CH_3COOH v/v: 2 mL of each solution were placed on a C_{18} column (Silica RP 18, 32-63 mesh 60A, 5x1 cm, ICN Biomedicals, Germany) previously conditioned twice with 3 mL of 1% CH_3COOH. The directly eluted fraction was obtained by collecting the direct eluate and rinsing with 2 mL of 1% CH_3COOH (Fr1). The elution was performed twice with 2 mL of EtOH 50% acidified with 1% CH_3COOH (Fr2). Fr1 and Fr2 samples were dried and stored at -20°C.

Sugars, organic acids, polyphenols, anthocyanin and cinnamic analysis

Each dried grape extract sample (RAW, SK, PL, Fr1 and Fr2) was dissolved in the same volume of double-distilled water as the original extract (sample 3-fold diluted). In the simple sugar analysis, an aliquot of each sample (0.2 mL) was diluted with 0.8 mL of water, filtered (0.45 μm) and injected into an analytical HPLC unit (5 μL). The HPLC system consisted of a Jasco (Tokyo, Japan) model 880-PU pump equipped with a Jasco AS-1555 intelligent sampler, a Jasco RI-930 detector. The chromatograms were recorded on a Shimadzu (Kyoto, Japan) C-R6A recorder. A 300x7.8 mm CARBOsep COREGEL-87C (CPS Analitica, Milano, Italy) column was used and the mobile phase consisted of double-distilled deaerated water at 0.6 mL/min; elution was carried out at 85°C. In these conditions, the peaks of the tested compounds showed the following retention times: sucrose, 7.82 min; glucose, 9.70 min; fructose, 12.24 min.

The concentration of sugar is expressed as g/100 g of fresh tissue (skin, pulp or raw grape) weight, after interpolation of the peak areas in each sample with those of commercial standards of known concentration.

Organic acid determination was performed on an aliquot of each sample (0.2 mL) diluted with 0.8 mL of water and

injected into an analytical HPLC Jasco (Tokyo, Japan) unit after filtration (0.45 μm). The analytical HPLC column was a 250x6 mm i.d., Intersil ODS-3 (CPS Analitica, Milano, Italy), maintained at 20°C and the mobile phase consisted of orthophosphoric acid 0.02 mol/L at a flow rate of 0.5 mL/min. Samples of 30 μL were injected and monitored at 214 nm. The concentration of tartaric and malic acids were calculated from the experimental peak area by analytical interpolation in the commercial standard calibration curve, and are expressed as mg/100 g fresh tissue (skin, pulp or raw grape) weight.

The total polyphenolic index was evaluated by a modified version of the Folin-Ciocalteu method (SINGLETON and ROSSI, 1965; MATAIX and LUQUE DE CASTRO, 2001): grape extracts (0.5 mL) were mixed with 3 mL of water, 1.5 mL of Folin-Ciocalteu reagent (Sigma-Aldrich, US), re-mixed and further treated with 3.0 mL of a 20% Na_2CO_3 water solution. The samples were kept in the dark for 2 h at room temperature. The standard curve was obtained with water solutions of gallic acid at 20, 10, 5 mg/100 mL. The absorbances were measured at 730 nm in a 1 cm cuvette. Data are reported as mg/100 g fresh tissue (skin, pulp or raw grape) expressed as gallic acid content.

The total anthocyanin index was measured following the pH differential method of RAPISARDA *et al.* (2000): grape extracts were diluted 25 times with pH 1.0 and pH 4.5 buffers, respectively, their absorbances at 510 nm were read and the total amount of anthocyanins was evaluated as equivalents of cyanidin-3-glucoside according to the following formula:

$$C=(A_{pH1.0}-A_{pH4.5}) \times 484.82 \times \\ \times 1{,}000/24{,}825 \times DF$$

where C represents the concentration (mg/100 g f. w. tissue), A the absorbance, 484.82 the molecular weight of cyanidin-3-glucoside, 24825 its molar extinction coefficient and DF the dilution factor.

Cinnamates were quantified by RP-HPLC Jasco (Tokyo, Japan) equipped with a diode-array system on a 250x6 mm Intersil ODS-3 (CPS Analitica Milano, Italy) column maintained at 40°C and the separation was isocratically performed (aqueous CH_3COOH 2%/MeOH acidified with CH_3COOH 2% 95:5), flow rate of 0.8 mL/min with a gradient program for cleaning the column, reaching the concentration of 70%. Samples of 50 μL were injected and monitored at 320 nm.

Trans-caftaric acid, *cis* and *trans*-coutaric acid and *trans*-fertaric acid were identified by their chromatographic properties (respective retention times were 9.9, 15.2, 16.0 and 19.5 min), by their spectroscopic characteristics and by matching the results with those of previous studies (MOZETIČ *et al.,* 2006). The quantification (mg/100 g f.w. tissue) was made with the response factor obtained from a solution of caffeic, *p*-coumaric and ferulic acids of known concentration analysed in the same conditions as the table grape extracts. The results showed the concentration of the main cinnamic ester of grape, *trans*-caftaric acid, and the sum of all the cinnamic esters found and quantified. All analyses were repeated three times.

Free radical scavenging properties

All measurements were made by electronic paramagnetic resonance (EPR) using a MiniScope MS 200 Magnettech (Berlin, Germany) and all solutions were previously deaerated by bubbling with pure N_2. Each dried grape extract was dissolved with deaerated double-distilled water to the original volume, consisting in a three-fold diluted fresh grape extract. Further dilutions are indicated in the single experiments. All experiments were performed in triplicate.

Hydroxyl radical scavenging activity

The main method of BUETTNER (1982) was followed for the measurement of •OH. Some modifications were made in a previous study to validate the method (MORELLI *et al.*, 2003). The •OH was generated by a process known as redox cycling or Fenton reaction, catalysed by a transition metal such as Fe^{2+}:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + \bullet OH + OH^-$$

The hydroxyl radical (2 mmol/L) in the reaction mixture was trapped with 5,5-dimethylpyrrolidine-N-oxide (DMPO). The resultant adduct DMPO-•OH consisted of a quartet resonance with 1:2:2:1 relative intensities composed of a doublet of triplet resonance. A blank solution contained 0.1 mL of phosphate buffer solution (PBS, 0.1 mol/L pH 7.4), 0.1 mL of water, 0.1 mL of Fe-EDTA (10 mmol/L), 0.1 mL of DMPO (50 mmol/L), and 0.1 mL of H_2O_2 (10 mmol/L) was run in each assay.

The assayed grape extracts (RAW, SK, PL, Fr1, Fr2) were diluted 5-fold with water and 0.1 mL of the resulting solution was mixed with the previously described Fenton reagent. The solution with the test scavenging compound was exactly composed as the blank, by replacing 0.1 mL of water with 0.1 mL of diluted grape extract.

These solutions were carefully mixed in an eppendorf with vortex, put into a 50 μL capillary and subsequently placed in the EPR. EPR spectra were recorded after exactly 1 min at 25°C.

The experimental conditions were: field set, 3,350 G; scan range, 150 G; scan time, 1 min; modulation amplitude, 3,000 mG; microwave attenuation 7 dB; receiver gain, $8x10^2$.

The percent scavenger activity of the test sample for •OH was expressed by the following formula:

$$I = 100 - [(h_x / h_o)] \times 100$$

where I is scavenger activity, h_o and h_x are the relative heights of the most abundant signal at 3,339.64 G in a reaction mixture with and without the scavenger solution, respectively.

Fremy's salt assay

The potential free radical scavenging activity of grape samples was measured by EPR with the inhibition of grape extracts of a solution of a stable N-oxide free radical, potassium nitrosodisulfonate, better known as Fremy's salt (GARDNER *et al.*, 2000).

The control reaction was prepared with a 2 mmol/L solution of Fremy's salt dissolved in PBS (0.05 mL), mixed with 0.45 mL of PBS and 0.5 mL of H_2O. The scavenger reaction consisted of 0.05 mL Fremy's salt, 0.45 mL of PBS, 0.45 mL of H_2O and 0.05 mL of grape extract.

EPR instrumental conditions were set at 3,350 G as field set, scan range, 100 G; scan time, 45 s; modulation amplitude, 600 mG; microwave attenuation 4 dB; receiver gain, 20.

The EPR spectrum consisted of a symmetric triplet, and the measure of intensity in the absence and in the presence of grape extract was made on the signal at 3,336.01 G after exactly 1 min of reaction at 25°C. The scavenging activity is expressed in percent activity compared to the control, as previously shown for •OH.

Scavenging effect on 1,1-diphenyl-2-picrylhydrazyl (DPPH•) radical

The scavenging activity of grape samples was measured by DPPH• radical quenching (BRAND-WILLIAMS *et al.*, 1995), with modifications. DPPH• is a commercially available free radical that is soluble and stable in ethanol and the free-radical scavenging activity is determined by measuring the decrease in EPR signal amplitude.

The experimental conditions of the spectrometer were: field set, 3,350 G; scan range, 50 G; scan time, 45 s; modulation amplitude, 2,000 mG; microwave attenuation 7 dB; receiver gain, 7x10.

Two-fold diluted grape extract (0.1 mL) was used for analysis, added to 0.1 mL H_2O, 0.5 mL EtOH and 0.1 mL of 0.8 mmol/L DPPH• ethanolic solution. The control sample was prepared using 0.2 mL of water, 0.5 mL EtOH and 0.1 mL of 0.8 mmol/L DPPH• ethanolic solution.

The radical scavenging activity against DPPH• of each extract is expressed in percentage by the same formula previously used, monitoring the signal at 3,350.16 G after exactly 1 min of reaction at 25°C.

Statistical analysis

The data obtained were submitted to analysis of variance and the averages were compared by Tukey's multiple range test. Correlation by simple linear regression analysis was carried out between the chemical parameters and free radical scavenging properties. A Statgraphics software (Version 7 for DOS) package was used.

RESULTS AND DISCUSSION

Sugars, organic acids, polyphenols, anthocyanin and cinnamates

The sugar content of table grapes measured on grape juices by °Bx averaged 16 g/100 g fresh weight (Table 1), with the highest value in PAL (18.1) and the lowest in PRO (12.0). The amount of glucose and fructose in the RAW grape extract, using EtOH/CH_3COOH 9:1, was closely related to the °Bx of grape juice (Table 1).

A significant difference in sugar content was found between skin and pulp extracts. The pulp had higher amounts of glucose and fructose than the skin in all the samples, similar to the data of COOMBE (1987).

The organic acid content, measured on grape juice as titratable acidity, ranged from 281 to 590 mg/100 g f.w., expressed as malic acid. Two red cultivars (PAL and PRO) had a lower acid content than white ITASETT and ITAOTT.

The HPLC measurements of organic acids extracted by EtOH/CH_3COOH 9:1 on RAW grape and the respective skin and pulp showed the same trend as in raw grape juice. There was a slight decrease in extraction yield, averaging 75%, probably due to the low solubility of tartaric acid in the extraction solution compared to pure water. This hypothesis is confirmed by the fact that there was less tartaric acid found in the extracts compared to malic acid.

All skin extracts showed a significantly higher amount of malic acid. This fact confirms the findings of COOMBE (1987).

The total polyphenol concentration, was measured on EtOH/CH_3COOH 9:1 extracts (Table 2). RAW grape showed significant amounts of polyphenols in the red varieties: the highest amount was in PAL grapes, followed by PRO. As expected the two samples of "Italia" grape had a significantly lower polyphenol concentration with respect to the red pigmented varieties.

The polyphenol concentration was clearly higher in skin than in pulp, with different average skin/pulp ratios for red varieties (19.4) compared to white (9.2). The anthocyanins were obviously present only in RAW and skin extracts of the red varieties, with an average content of 93.5 and 235.5 mg/100g f.w. of grape and skin tissue, respectively.

The cinnamate content was relevant in the ITAOTT grapes. In all samples, the skin extracts had the highest presence of both *trans*-caftaric acid and total cinnamates.

Table 1 - Refractive index (°Bx), titratable acidity, simple carbohydrates and organic acids of table grape extracts. The identity of the samples is given in the Materials and Methods section. In each column different letters stand for significant differences ($p<0.05$).

	°Bx	Glucose (g/100 g f.w.)	Fructose (g/100 g f.w.)	Titratable acidity (mg/100 g malic acid)	Tartaric acid (mg/100 g f.w.)	Malic acid (mg/100 g f.w.)
PAL RAW	18.1 a	8.7 a	8.9 a	281 b	93 c	89 g
SK		5.5 d	5.9 c		45 e	149 e
PL		7.6 b	7.3 b		70 d	96 f
Fr1		7.7 b	7.5 b		74 d	68 g
Fr2		0.4 g	0.4 f		10 g	7 h
PRO RAW	12.0 c	4.5 e	4.8 d	322 b	55 e	182 d
SK		3.9 e	4.5 d		59 de	274 c
PL		5.5 d	5.2 cd		60 de	107 f
Fr1		4.3 e	4.4 d		51 e	175 d
Fr2		0.1 h	0.2 f		17 g	7 h
ITASETT RAW	16.5 b	7.1 c	7.5 b	545 a	210 a	248 c
SK		6.2 d	6.5 c		36 f	322 bc
PL		7.0 c	7.0 b		63 d	211 d
Fr1		6.1 d	6.3 c		177 b	169 d
Fr2		1.0 f	1.2 e		37 f	53 g
ITAOTT RAW	17.3 ab	6.9 c	7.5 b	590 a	63 d	378 b
SK		6.2 d	6.4 c		50 e	522 a
PL		7.9 b	8.0 ab		77 d	300 c
Fr1		7.0 c	7.4 b		50 e	340 b
Fr2		0.5 fg	0.5 f		16 g	47 g

Fractionation was performed in order to separate hydrophilic compounds (directly eluted fraction, Fr1) such as simple sugars and organic acids, from relatively hydrophobic products (C_{18} retained and EtOH 50% eluted fraction, Fr2) such as polyphenols.

Simple sugars and organic acids were mainly present in the Fr1 samples (glucose and fructose averaged 93% and 90% presence with respect to RAW, Table 1), while the average presence of organic acid was 84% for tartaric acid and 83% for malic acid. To summarize, Fr2 samples were significantly poorer in simple carbohydrates and organic acids compared to Fr1.

On the other hand, polyphenols and anthocyanins for the red varieties and cinnamates were almost completely retained by C_{18} and were easily eluted in Fr2 by EtOH 50% acidified with CH_3COOH. Thus, the amounts of total polyphenols, anthocyanins and cinnamates in Fr2 were very similar to the RAW samples (Table 2) and significantly different from the Fr1 values in all the samples.

Free radical scavenging activity

RAW grape extracts and the respective partitioned SK and PL were assayed for their free radical scavenging activity by three methods: quenching of stabilized free radicals (DPPH• and Fremy's salt) and quenching of spin-trapped •OH, generated by the Fenton reaction.

All grape extracts were effective as free radical scavengers in all the tests. It is interesting to compare the tests and the correlation between the composition and the free radical scavenging potential.

Table 2 - Total polyphenols, anthocyanins and cinnamates of table grape extracts. The identity of samples is given in the Materials and Methods section. In each column different letters stand for significant differences ($p<0.05$).

	Total polyphenols (mg/100 g f.w.)	Total anthocyanins (mg/100 g f.w.)	*trans*-caftaric acid (mg/100 g f.w.)	Total cinnamates (mg/100 g f.w.)
PAL RAW	274 b	63 c	4.2 cd	4.8 d
SK	444 a	236 a	5.3 c	7.2 c
PL	24 e	0 d	1.1 f	1.3 f
Fr1	8 f	0 d	0.2 g	0.2 g
Fr2	188 c	42 c	3.8 d	4.4 d
PRO RAW	257 b	124 b	1.8 e	2.5 e
SK	448 a	235 a	3.4 d	4.8 d
PL	22 e	0 d	1.1 f	1.2 f
Fr1	1 g	0 d	0.1 g	0.1 g
Fr2	254 b	117 b	2.1 e	2.7 e
ITASETT RAW	98 d		2.5 e	2.6 e
SK	211 c		6.8 c	7.2 c
PL	15 f		0.8 f	0.9 f
Fr1	17 f		0.3 g	0.3 g
Fr2	85 d		2.4 e	2.9 e
ITAOTT RAW	132 d		11.9 b	13.6 b
SK	238 b		23.4 a	27.3 a
PL	34 e		6.8 c	7.2 c
Fr1	34 e		1.9 e	2.0 e
Fr2	119 d		11.7 b	13.6 b

Compared to pulp extracts, RAW and skin extracts were particularly effective in scavenging Fremy's salt (Fig. 2a). Fremy's salt assay did not give a significant difference between red and white cultivars except for skin of PAL and ITAOTT. RAW and skin extracts of red cultivars were more active in DPPH• quenching than the corresponding samples from white grapes (Fig. 2b), while, as previously seen for Fremy's salt, skin extracts were much more active than pulp.

As for •OH quenching (Fig 2c), the difference between RAW, skin and pulp samples was less pronounced. PAL showed a significantly higher value in RAW with respect to its skin and pulp, and PRO showed significantly low quenching in pulp extract. Instead, the quenching values in white grape cultivar extracts did not change, except for ITAOTT pulp, that showed an increase. The highest activity was found in PAL RAW samples, while pulp of the white varieties showed significantly higher activity than the pulp of red grape.

As previously reported (MUÑOZ-ESPADA *et al.,* 2004), grape skin polyphenols have a definite role compared to the pulp components for Fremy's salt and DPPH• quenching (Figs. 2a and 2b). This was demonstrated by the marked difference in total polyphenols and anthocyanin content between the SK and PL extracts (Table 2). On the other hand, there is clear evidence that there is little difference in the •OH inhibition of SK and PL compared to that in Fremy's salt and DPPH• tests. Therefore it is possible that the activity towards •OH is not only linked to the polyphenols, but also to some other active compounds.

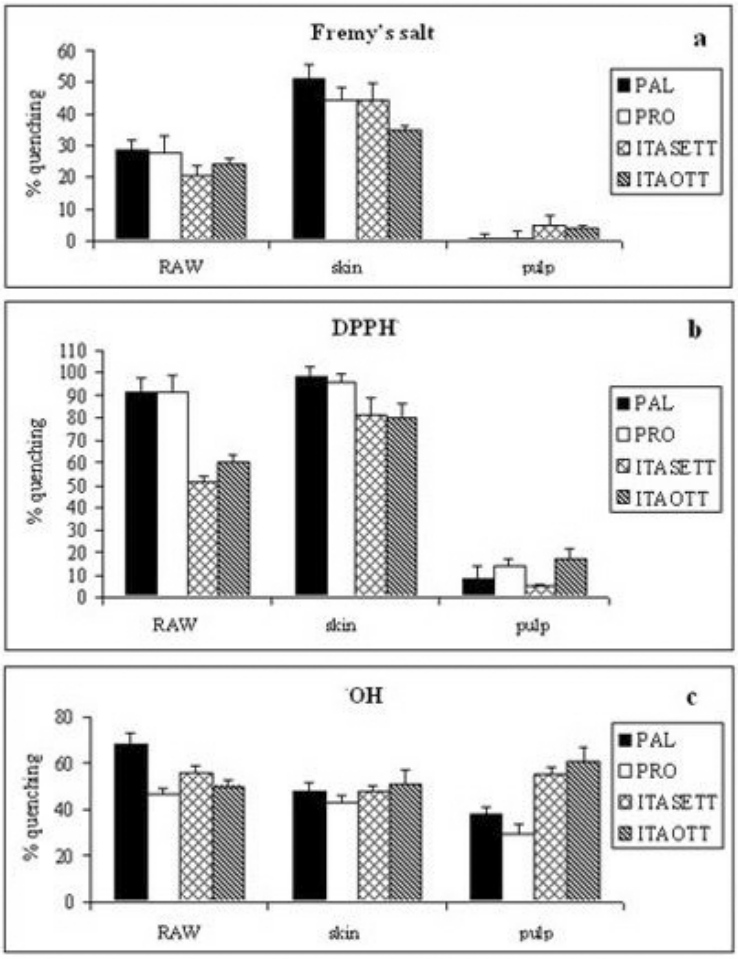

Fig. 2 - Free radical percent quenching after 1 minute reaction at 25°C of RAW, SK and PL extracts of table grape. Free radical final concentrations used were: a) Fremy's salt 0.1 mmol/L, b) DPPH· 0.1 mmol/L and c) ·OH 2 mmol/L. The identity of samples is given in the Materials and Methods section. Bars indicate standard errors.

Despite the low amount of total polyphenols it is possible that the significant activity of PL extracts, could be compensated by the presence of some very active components. This fact was verified by the C_{18} fractionation of the RAW grape extract.

In this experiment, Fr2 samples were significantly more effective than Fr1 in scavenging Fremy's salt and the DPPH•. This was very evident for DPPH• assay (Fig. 3b), which was significantly more quenched by Fr2 extracts from red cultivars, which highlights the important role of anthocyanins in its scavenging activity. The Fr2 extracts of white and red grape cultivars did not inhibit Fremy's salt differently (Fig. 3a), but the activity of Fr2 was significantly higher than Fr1 in all the samples.

As for the •OH quenching test (Fig. 3c), Fr1 extracts from all cultivars were surprisingly more active than Fr2, despite their poor content of total polyphenols and lack of anthocyanins in the red cultivars. The activity indices of Fr1 were very close to the value of the RAW extracts; the highest activity index was in the PAL variety.

The Fr1 sample contained more polar compounds than Fr2: the chemical analysis profile indicates that, com-

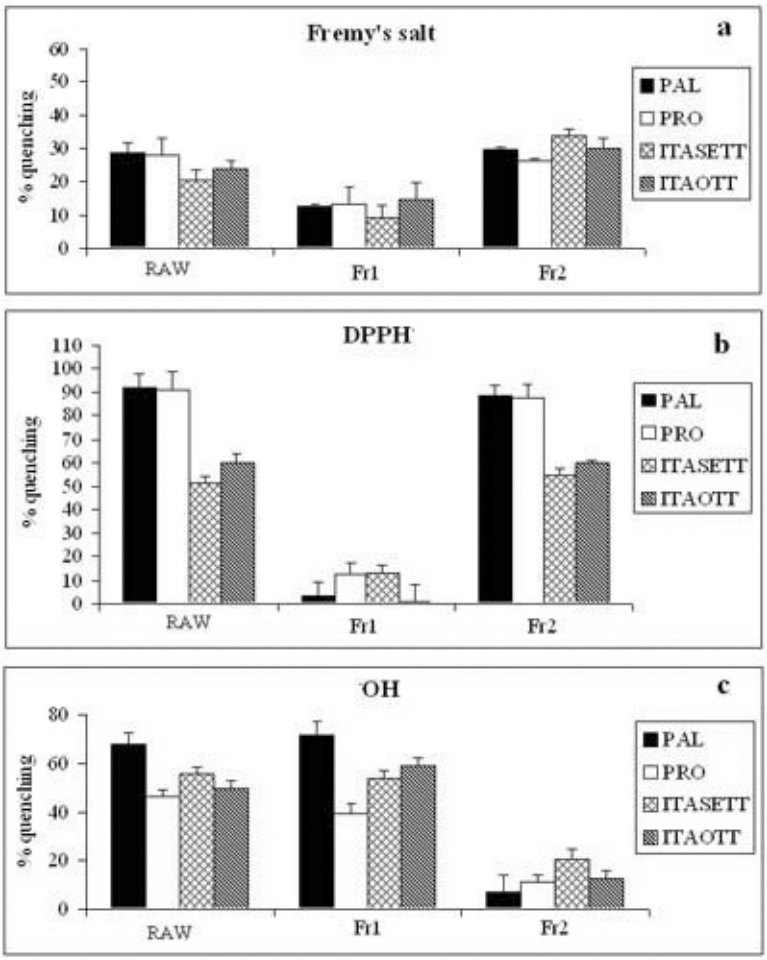

Fig. 3 - Free radical percent quenching after 1 minute reaction at 25°C of RAW, Fr1 and Fr2 extracts of table grape. Free radical final concentrations used were: a) Fremy's salt 0.1 mmol/L, b) DPPH· 0.1 mmol/L and c) ·OH 2 mmol/L. The identity of samples is given in the Materials and Methods section. Bars indicate standard errors.

pared to Fr2, Fr1 is rich in carbohydrates and organic acids (Table 1) and poor in polyphenols (Table 2). However, the presence of undetected polar substances present in Fr1 very active toward •OH and not against Fremy's salt and DPPH• cannot be excluded. The last results seem to confirm the hypothesis that other compounds than polyphenols play a role in scavenging •OH radical in these experimental conditions.

A statistical correlation (n=60) was made between the chemical data of table grape extracts and the respective index of free radical quenching (Table 3).

Fremy's salt and DPPH• quenching indices had a nearly linear correlation for total polyphenols (0.85 and 0.90, respectively) and anthocyanins (0.91 and 0.78). No other parameters were statistically significant.

There was a very good correlation between the quenching value of •OH and the amount of sugar (0.92 and 0.93 for glucose and fructose, respectively), while no other parameters were significant. It should be noted that there was no correlation for polyphenols (-0.12) and anthocyanins (0.01). Organic acids had slightly positive values (0.55 for tartaric and 0.52 for malic acid).

Table 3 - Correlation coefficients by simple linear regression between chemical parameters and free radical scavenging potential in different extracts of table grape (n=60). The values with one asterisk are significant for $p<0.05$, two asterisks for $p<0.01$.

	Glucose	Fructose	Tartaric acid	Malic acid	Total polyphenols	Total anthocyanins	trans-caftaric	Total cinnamates
Fremy's Salt	-0.37	-0.31	-0.34	0.11	0.85**	0.91**	0.40	0.43
DPPH·	-0.41	-0.36	-0.32	-0.03	0.90**	0.78*	0.38	0.42
·OH	0.92**	0.93**	0.55	0.52	-0.12	0.01	-0.02	-0.03

CONCLUSIONS

The use of different antioxidant tests on the same samples did not give the same response; in fact, it has become very useful to use different tests to ascertain the antioxidant activity of a plant extract. The antiradical action against Fremy's salt and DPPH• was mainly due to grape skin extracts, while it is clear that the antiradical action against hydroxyl radical was equally distributed between skin and pulp extracts, confirming previous results.

This fact was confirmed by C_{18} solid phase fractionation, where Fremy's salt and DPPH• were strongly quenched by Fr2 extracts rich in polyphenols. On the other hand, Fr1 fractions poor in polyphenols were found to be very active against •OH.

In recent years, there has been scientific debate about the antioxidant action of compounds other than those commonly considered, such as ascorbic acid (LEONARD *et al.,* 2002); the present study enforces this hypothesis: the quenching of •OH generated by a Fenton reaction and trapped by DMPO shows a very significant correlation with the amount glucose and fructose in table grape extracts, while no correlation was found for the concentration values related to polyphenol content.

The scavenging activity towards •OH by simple sugars was demonstrated in a study carried out on model solutions (MORELLI *et al.,* 2003). Disaccharides (maltose and sucrose) were more active than monosaccharides (glucose, fructose, deoxyribose and sorbitol), and their activity was strongly correlated to the number of free hydroxyl residues. An HPLC assay showed that less active sugars were more damaged by •OH respect to more active ones.

Furthermore, the role of organic acids needs to be explored because the already-cited literature and some of our recent unpublished laboratory trials demonstrate that organic acids scavenge Fenton-generated •OH in a significant manner. Finally, it is feasible that the antioxidant action of grape could be the result of a complex synergy of phenomena between well known and unusual antioxidant compounds, such as polyphenols, simple carbohydrates and organic acids. This needs to be elucidated.

ACKNOWLEDGEMENTS

The present work was conducted with the funding of Italian Ministry of Agricultural Policy, "Progetto Qualità" directed by the Italian Institute of Research on Nutrition, Rome.

REFERENCES

Aruoma O.I. 1998. Free radicals, oxidative stress and antioxidant in human health and disease. J. of Am. Oil Chem. Soc.. 75: 199.

Bazzano L.A., He J., Ogden L.G., Loria C.M., Vupputuri S., Myers L. and Whelton P. K. 2002. Fruit and vegetable intake and risk of cardiovascular disease in US adults: the first National Health Examination Survey Epidemiologic Follow-up Study. Am. J. Clin. Nutr. 76: 93.

Brand-Williams W., Cuvelier M.E. and Berset C. 1995. Use of a free radical method to evaluate antioxidant activity. Lebensm. Wiss. Technol. 28: 25.

Buettner G.R. 1982. The spin trapping of superoxide and hydroxyl radicals. Vol. 2. In "Superoxide Dismutase" L.W. Oberley (Ed.), p. 64. CRC Press, Boca Raton, FL.

Burns J., Gardner P.T., O'Neil J., Crawford S., Morecroft I., McPhail D.B., Lister C., Matthews D., MacLean M.R., Lean M.E., Duthie G.G. and Crozier A. 2000. Relationship among antioxidant activity, vasodilatation capacity, and phenolic content of red wines. J. Agric. Food Chem. 48: 220.

Burns J., Gardner P.T., Matthews D., Duthie G.G., Lean M.E. and Crozier A. 2001. Extraction of phenolics and changes in antioxidant activity of red wines during vinification. J. Agric. Food Chem. 49: 5797.

Chang C., Chen J. and Wong H. 2001. Protective effects of organic acids on human vascular endothelial cells. Zhonghua Yu Fang Yi Xue Za Zhi, 35: 79.

Coombe B.G. 1987. Distribution of solutes within the developing grape berry in relation to its morphology. Am. J. Enol. Vitic. 38: 120.

Czapsky G. 1984. Reaction of ·OH. Methods in Enzymology. 105: 209.

Falchi M., Bertelli A., Lo Scalzo R., Morassut M., Morelli R., Das S., Cui J. and Das D.K. (2006). Comparison of cardioprotective abilities between the pulp and skin of grapes. J. Agric. Food Chem. 54: 6613.

Flood A., Velie E.M., Chaterjee N., Subar A.F., Thompson F.E., Lacey J.V. Jr, Schairer C., Troisi R. and Schatzkin A. 2002. Fruit and vegetable intakes and the risk of colorectal cancer in the Breast Cancer Detection Demonstration Project follow-up cohort. Am. J. Clin. Nutr. 75: 936.

Gardner P.T., White T.A.C., McPhail D.B. and Duthie G.G. 2000. The relative contribution of vitamin C, carotenoids and phenolics to the antioxidant potential of fruit juices. Food Chem. 68: 471.

Gray J. and Mower H.F. 1991. The role of simple carbohydrates in the suppression of hydroxyl free radicals in γ-irradiated papaya juice. Food Chem. 41: 293.

Kayashima T. and Katayama T. 2002. Oxalic acid is available as a natural antioxidant in some systems. Biochim. Biophys. Acta, 1573: 1.

Leonard S.S., Cutler D., Ding M., Vallyathan V., Castranova V. and Shi X. 2002. Antioxidant properties of fruit and vegetable juices: more to the story than ascorbic acid. Annals of Clin. & Lab. Science. 32: 193.

Lo Scalzo R., Morassut M., Bianchi G. and Morelli R. 2006. Free radical scavenging activity of grape's (*Vitis vinifera* L.) skin and pulp. Vol. 17. Ch. 15. In "Chronic and Common Disease I". J.N. Govil (Ed.), p. 321. Studium Press LLC, Houston, Tx, USA.

Macheix J.J., Pulpeuriet A. and Billot J. 1996. "Fruit Phenolics". CRC Press Inc., Boca Raton FL.

Mataix E. and Luque de Castro M.D. 2001. Simultaneous determination of the total polyphenols index and density in wines by flow injection. Analyst. 126: 251.

Morelli R., Russo-Volpe S., Bruno N. and Lo Scalzo R. 2003. Fenton-dependent damage to carbohydrates: free radical scavenging activity of some simple sugars. J. Agric. Food Chem. 51: 7418.

Mozetić B., Tomažić I., Škvarć A. and Trebše P. 2006. Determination of polyphenols in white grape berries cv. Rebula. Acta Chemica Slovenica 53: 58.

Muñoz-Espada A.C., Wood K.V., Bordelon B. and Watkins B.A. 2004. Anthocyanin quantification and radical scavenging capacity of Concord, Norton, and Marechal Foch grapes and wines. J. Agric. Food Chem. 52: 6779.

Rapisarda P., Fanella F. and Maccarone E. 2000. Reliability of analytical methods for determining anthocyanins in blood orange juice. J. Agric. Food Chem. 48: 2249.

Revilla E. and Ryan-Josè M. 2000. Analysis of several phenolic compounds with potential antioxidant properties in grape extracts and wines by high-performance liquid chromatography-photodiode array detection without sample preparation. J. Chrom. A 881: 461.

Sànchez-Moreno C., Larrauri J.A. and Saura-Calixto F. 1999. Free radical scavenging capacity and inhibition of lipid oxidant of wines, grape juices and related polyphenolic compounds constituents. Food Res. Int. 32: 407.

Singleton V.L. and Rossi J.A. 1965. Colorimetry of Total phenolic with phosphomolybdic-phosphotungstic acid reagents. Am. J. Enol. Vitic. 16: 144.

Souquet J., Cheynier V., Brossaud F. and Moutounet M. 1996. Polymeric proanthocyanidins from grape skin. Phytochemistry. 43: 506.

Valko M., Morris H. and Cronin M.T. 2005. Metals, toxicity and oxidative stress. Curr. Med. Chem. 12: 1161.

Wehmeier K.R. and Mooradian A.D. 1994. Autoxidative and antioxidative potential of simple carbohydrates. Free Radic. Biol. Med. 17: 83.

Yilmaz Y. and Toledo R.T. 2004. Major flavonoids in grape seeds and skins: antioxidant capacity of catechin, epicatechin, and gallic acid. J. Agric. Food Chem. 52: 255.

Revised paper received January 15, 2007 Accepted March 23, 2007

SHORT COMMUNICATION

HYDROPHILIC AND LIPOPHILIC ANTIOXIDANT CAPACITIES OF COMMERCIAL DIETARY ANTIOXIDANT SUPPLEMENTS

CAPACITÀ ANTIOSSIDANTE LIPOFILA E IDROFILA
DI ALCUNI ANTIOSSIDANTI COMMERCIALI PER USI DIETETICI

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ABSTRACT

The ORAC (oxygen radical absorbance capacity) assay was applied to the hydrophilic (H) and lipophilic (L) fractions of 17 commercial dietary antioxidant supplements. The total ORAC (H-ORAC + L-ORAC) values varied from 0.22 to 8.26 μmol of Trolox equivalents per mg of supplement, demonstrating a great variability among them. Hydrophilic antioxidants are the main portion (86.3-99.3% of the total ORAC value) of the supplements, in comparison to the lipophilic fraction (0.7-13.7%). The daily total antioxidant intake provided by the supplements is reported.

RIASSUNTO

Il saggio ORAC (capacità inibente i radicali dell'ossigeno) è stato applicato alle frazioni idrofile e lipofile di 17 integratori alimentari commerciali di antiossidanti. I valori totali ORAC (H-ORAC + L-ORAC) variavano fra 0,22 e 8,26 μmol di equivalenti di Trolox per mg di integratore, mostrando una grande variabilità fra gli stessi. Gli antiossidanti idrofili sono la porzione fondamentale (86,3-99,3% del valore totale ORAC) degli integratori, in confronto alla frazione lipofila (0,7-13,7%). È riportato l'apporto giornaliero di antiossidanti fornito.

- Key words: dietary supplements, lipophilic and hydrophilic antioxidants, ORAC, radical scavenging activity -

INTRODUCTION

Consumption of dietary supplements has increased markedly in developed countries in recent years. A survey in the UK showed that 35.4% of adults usually take at least one dietary supplement (HARRISON *et al.*, 2004), and those containing vitamins, minerals and/or antioxidants are probably the most in demand. Dietary antioxidant supplements are complex formulations containing both hydrophilic and lipophilic ingredients. Vitamins (E and C), minerals (Se and Zn), grape extract, soy isoflavones, tomato concentrate, rosemary extract and citrus flavonoids are, among others, the main ingredients of dietary antioxidant supplements (DÁVALOS *et al.,* 2003). Due to the variability of the raw plant material and of the production process, antioxidant supplements are normally standardised by examining the contents of active compounds, which is also used as a parameter to establish the commercial lifetime of the products. In any case, antioxidant tests are not extensively used by the nutraceutical industries to characterize and standardize dietary supplements, probably due to insufficient information about the applicability of current antioxidant methodologies to the supplements (PRIOR *et al.*, 2005).

Among the methods proposed to evaluate the *in vitro* antioxidant capacity of food products, measurement of the oxygen radical absorbance capacity (ORAC) is perhaps one of the most suitable (PRIOR *et al.*, 2005). In this assay, peroxyl radicals generated by thermal decomposition of 2,2'-azobis(2-methylpropionamidine) dihydrochloride (AAPH) react with a fluorescent probe (fluorescein, FL) to form nonfluorescent products. In the presence of an antioxidant, peroxyl radicals are scavenged and decay of the fluorescence curve is delayed. The protective effect of an antioxidant is quantified by assessing the "area under the fluorescence decay curve" (AUC) of the sample compared to that of the blank in which no antioxidant is present (OU *et al.*, 2001). This methodology is not only applied to hydrophilic (H-ORAC$_{FL}$) compounds/fractions but also to lipophilic ones (L-ORAC$_{FL}$) by using randomly methylated β-cyclodextrin as a solubility enhancer (HUANG *et al.*, 2002). The L-ORAC$_{FL}$ method has been successfully applied to commercial vitamin E formulations (NAGUIB *et al.*, 2003) and the H-ORAC$_{FL}$ method to commercial multi-component antioxidant supplements (DÁVALOS *et al.*, 2004).

The objective of this paper was to determine both the hydrophilic and lipophilic antioxidant capacities (H-ORAC$_{FL}$ and L-ORAC$_{FL}$ values) of commercial dietary supplements containing hydrophilic and lipophilic antioxidants. An additional objective was to establish the variability of the antioxidant capacity of commercial products from different batches. For this purpose, a total of the 17 most consumed dietary antioxidant supplements on the European market was selected, and several production batches were supplied for one of the brands.

MATERIALS AND METHODS

Chemicals

Fluorescein disodium salt was purchased from Sigma Chemicals (St. Louis, MO, USA). 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and AAPH were purchased from Aldrich (Milwaukee, WI, USA). Randomly methylated β-cyclodextrin (RMCD) was purchased from Cyclolab R&D Ltd. (Budapest, Hungary). An FL stock solution (1.17 mM) was made in 75 mM phosphate buffer (pH 7.4) and stored at 4°C for 4 weeks. A 7% RMCD solution was prepared in acetone/water (50:50, v/v). AAPH was dissolved in 75 mM phos-

phate buffer (pH 7.4). Trolox (100 μM) was prepared in 75 mM phosphate buffer (pH 7.4) and further diluted with the same phosphate buffer for the H-ORAC$_{FL}$ assay, or with the 7% RMCD solution for the L-ORAC$_{FL}$ assay. The AAPH and Trolox solutions were prepared daily.

Dietary antioxidant supplements

Seventeen commercial dietary antioxidant supplements (named #1-17) were obtained from local markets. Table 1 lists the composition and daily dose of the supplements as indicated by the manufacturers. Supplements #2, 3, 4, 5, 6, 7, 12, 14, 15, 16 and 17 are multi-ingredient formulations, whereas supplements #1, 8, 9, 10, 11 and 13 only contain one active ingredient: wine marc (#1), grape seed (#8, 9 and 13), soy (#10) and *Pinus Maritima* bark (#11, Pycnogenol®; DEVARAJ *et al.*, 2002). For supplement #17, six different production batches were studied: batch #1 (expiration date 10/07), #2 (expiration date 10/07), #3 (expiration date 10/07), #4 (expiration date 12/07), #5 (expiration date 12/07) and #6 (expiration date 1/08). Three different bottles/boxes were analysed for each supplement and batch.

Sample preparation

Supplements were ground using a coffee-bean miller to a particle size of less than 50 μm. For extraction of the lipophilic antioxidants (HUANG *et al.*, 2002), 0.02 g were extracted with 10 mL of hexane in a sonicate bath for 5 min. After this time, the mixture was kept at room temperature for 15 min, centrifuged (5,000 rpm, 5°C, 10 min), and the hexane phase collected. The extraction was repeated once, and the solid residue was kept for hydrophilic antioxidant extraction. The two hexane extracts were combined and brought to dryness under vacuum. The residue was dissolved in 250 μL of acetone and 750 μL of 7% RMCD in acetone/water (50:50, v/v), and was centrifuged (5,000 rpm, 5°C, 10 min). The supernatant was ready for L-ORAC$_{FL}$ analysis after further dilution with the 7% RMCD solution.

To extract the hydrophilic antioxidants after lipophilic extraction (DÁVALOS *et al.*, 2004), the residue was mixed with 10 mL of methanol/HCl (1,000:1, v/v) and sonicated for 5 min. The mixture was kept at room temperature for 15 min, and was centrifuged (5,000 rpm, 5°C, 10 min). After centrifugation, the supernatant was ready for H-ORAC$_{FL}$ analysis after further dilution with 75 mM phosphate buffer (pH 7.4).

Determination of antioxidant activity

Antioxidant activity was determined by the ORAC-fluorescein (ORAC$_{FL}$) assay of OU *et al.* (2001) and modified by DAVALOS *et al.* (2004). The reaction was carried out in 75 mM phosphate buffer (pH 7.4) and there were 200 μL of the final reaction mixture. Antioxidant (20 μL) and FL (120 μL; 70 nM, final concentration) solutions were preincubated for 10 min at 37°C. The AAPH solution (60 μL; 12 mM, final concentration for the H-ORAC$_{FL}$ analysis, and 27.6 mM final concentration for the L-ORAC$_{FL}$ analysis) was added and, after shaking before the first reading, the fluorescence was recorded every 56 seconds for 96 min. Trolox was used as standard (1-8 μM, final concentration) and the sample was measured at different concentrations. A Polarstar Galaxy plate reader (BMG Labtechnologies GmbH, Offenburg, Germany) with 485-P excitation and 520-P emission filters was used. The equipment was controlled by the Fluostar Galaxy software version (4.11-0) for fluorescence measurement. Black 96-well microplates (96F untreated, Nunc™, Denmark) were used. All reaction mixtures were prepared in duplicate.

Fluorescence measurements were normalized to the curve of the blank

(no antioxidant). From the normalized curves, the area under the fluorescence decay curve (AUC) was calculated as:

$$AUC = 1 + \sum_{i=1}^{i=80} f_i / f_0 \quad (1)$$

where f_0 is the initial fluorescence reading at 0 min and f_i is the fluorescence reading at time *i*.

The net AUC for a sample was calculated as follows:

$$\text{net AUC} = AUC_{antioxidant} - AUC_{blank} \quad (2)$$

The regression equation between net AUC and antioxidant concentration was estimated. To calculate the $ORAC_{FL}$ value of the supplements, the slope of the curve (net AUC/mg supplement) was divided by the slope of the Trolox calibration curve (net AUC/μmol of Trolox). $ORAC_{FL}$ values were then expressed as μmol of Trolox equivalent (TE)/mg of supplement.

Statistical analysis

To investigate the existence of differences among supplements and batches, one-way ANOVA (3 replicates per measurement) was performed using the STATGRAPHICS Plus program for Windows (2.1).

RESULTS AND DISCUSSION

Fig. 1 depicts the kinetics of fluorescein degradation by peroxyl radicals generated from AAPH, in the absence and in the presence of the hydrophilic and lipophilic antioxidant fractions from dietary supplements. Both hydrophilic and lipophilic antioxidants neutralize the radicals, which delays the decay in the fluorescence curve until a certain time proportional to the antioxidant concentration. In the range of concentration studied, all the supplements showed a linear response between the net area under the curve (net AUC) and the concentration in the assay. The $ORAC_{FL}$ value was calculated by dividing the slope of the supplement curve by the slope of the Trolox calibration curve. On average, the Trolox calibration curve was: net AUC = 25,787 * μmol Trolox +2.19 for the hydrophilic ORAC assay (H-$ORAC_{FL}$), and net AUC = 10,742 * μmol Trolox + 0.96 for the lipophilic ORAC assay (L-$ORAC_{FL}$).

Table 1 reports the H-$ORAC_{FL}$ and L-$ORAC_{FL}$ values of the supplements assayed. The total antioxidant capacity (TOTAL-$ORAC_{FL}$) was calculated by summing H-$ORAC_{FL}$ and L-$ORAC_{FL}$ (Table 1). The antioxidant hydrophilic capacity of the supplements ranged from 0.190 to 8.00 μmol of TE/mg of supplement, which represented between 86.3 and 99.3% of the total antioxidant capacity. The lipophilic fraction represented from 0.7 to 13.7% of the total antioxidant capacity of the supplements, with (L-$ORAC_{FL}$) values ranging from 0.0130 to 0.450 μmol of TE/mg of supplement. The TOTAL-$ORAC_{FL}$ of the supplements varied from 0.220 to 8.26 μmol of TE/mg of supplement (a 37.5-fold maximum-minimum difference). The variability in the antioxidant capacity of the supplements (expressed as the variation coefficient with respect to the mean) was lower for the hydrophilic fraction (76%) than for the lipophilic fraction (147%). Our results are in agreement with previous data reported on the percentage of lipophilic to the total antioxidant capacity of foods of plant origin (WU *et al*, 2004a). In this study of 28 common foods, these authors found that the percentage of L-$ORAC_{FL}$ to TOTAL-$ORAC_{FL}$ was highly variable; <5% for most fruit (fresh and dried), from 3.9 to 18.6% for vegetables, and from 0.8 to 2.1% for nuts. In relation to previous reports on dietary supplements, the total antioxidant capacity of the supplements studied (mean value = 2.51 μmol of TE/mg of supplement) was similar to the antioxidant ca-

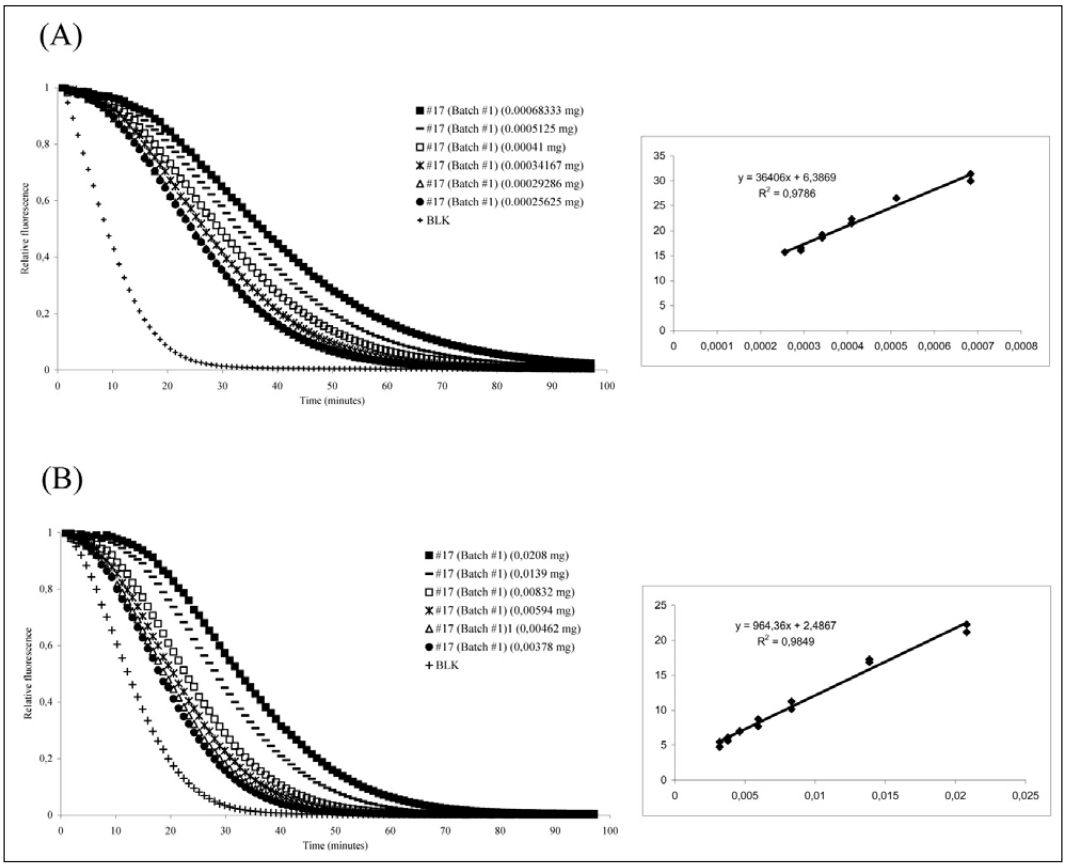

Fig. 1 - Time course of the reaction of fluorescein with AAPH in the absence (BLK) and in the presence of the hydrophilic antioxidant fraction (A) and the lipophilic antioxidant fraction (B) of dietary supplement #17. Regression analysis of net AUC (net area under the curve) against antioxidant concentration is included in both graphs.

pacity found for vitamin E formulations (1.30-2.03 μmol of TE/mg of supplement) (NAGUIB *et al.*, 2003).

The analysis of variance (ANOVA) showed significant differences ($P<0.05$) in the hydrophilic and lipophilic antioxidant capacity of the supplements studied. In general, the 95% confidence intervals overlapped among supplements, although supplement #9 for H-ORAC$_{FL}$ and supplements #16, 1, 9 and 13 for L-ORAC$_{FL}$ clearly differed from the rest (data not shown). All these supplements were composed of *Vitis vinifera* L. by-products (Table 1). However, other supplements made from these materials showed a low antioxidant efficiency (i.e., #8; Table 1), indicating that, not only the ingredient, but also the manufacturing procedure (procedure to obtain the ingredient, excipients, etc.) influences the final antioxidant properties of the supplements. Curiously, the supplements with the lowest (#8) and the highest (#9) H-ORAC$_{FL}$ values contained grape seed extracts as active ingredients. The main phenolic antioxidants found in commercial ingredients derived from *V. vinifera* L. by-products are: gallic acid, monomeric flavan-3-ols ((+)-catechin, (-)-epicatechin and epicatechin-3-*O*-gallate) and procyanidins in grape seeds, anthocyanins

Table 1 - Composition, and hydrophilic (H-ORAC$_{FL}$) and lipophilic (L-ORAC$_{FL}$) antioxidant capacity of the dietary antioxidant supplements studied.

#	Composition	Recommended daily dose (mg)	H-ORAC$_{FL}$ (μmol of TE/mg)[a]	L-ORAC$_{FL}$ (μmol of TE/mg)[a]	TOTAL-ORAC$_{FL}$ (μmol of TE/mg)	H-ORAC$_{FL}$ (%)	L-ORAC$_{FL}$ (%)	TOTAL-ORAC$_{FL}$ intake (μmol of TE)
1	Red wine marc extract	227	3.27±0.18	0.208±0.010	3.48	94.0	6.0	790
2	Vitamins (C, D, E, B1, B2, B3, B5, B6 and B12), β-carotene, folic acid, metals (Fe, Zn, Mn, Se and Cr), glutathione, and grape seed and tomato extracts	1588	2.03±0.02	0.0224±0.0021	2.08	98.9	1.1	3255
3	Grape seed extract, *trans*-resveratrol, and citrus flavonoids	948	2.00±0.09	0.0784±0.0081	2.08	96.2	3.8	1972
4	Green tea, olive leaf, grape seed, natural carotenoids, vitamin E, lutein, Se, Zn, and coenzyme Q_{10}	1396	1.55±0.11	0.0253±0.0024	1.58	98.4	1.6	2205
5	Green tea, olive leaf, grape seed, vitamin E, quercetin, Se, Zn, and coenzymeQ_{10}	1212	3.66±0.03	0.0283±0.0033	3.69	99.3	0.7	4472
6	Green tea, olive leaf, grape seed, wine polyphenols, vitamin E, lycopene, Se, Zn, and coenzyme Q_{10}	1396	2.07±0.04	0.0335±0.0029	2.10	98.4	1.6	2932
7	Skins and grape seed extracts	1192	3.05±0.20	0.0273±0.0027	3.07	99.1	0.9	3659
8	*Vitis vinifera* L. grape seed extract	763	0.190±0.008	0.0302±0.0010	0.220	86.3	13.7	168
9	Grape seed extract	809	8.00±0.92	0.257±0.047	8.26	96.9	3.1	6682
10	Soy extract (isoflavones)	1178	0.943±0.056	0.0130±0.0012	0.956	98.6	1.4	1126
11	Extract from *Pinus Maritima* bark (Pycnogenol®)	487	3.15±0.15	0.137±0.016	3.29	95.8	4.2	1602
12	Lacto-lycopene™, soy isoflavones, and vitamin C	2158	1.09±0.02	0.0636±0.0032	1.15	94.5	5.5	2482
13	Grape seed concentrate	1125	2.02±0.14	0.254±0.026	2.27	88.8	11.2	2554
14	Citrus flavonoids, *Ruscus aculeatus* L., *Ginkgo biloba*, *Equisetum arvense*, *Hamamelis virginiana* L., and vitamins C and B_1	2623	2.02±0.23	0.110±0.009	2.13	94.8	5.2	5587
15	Cranberry, red grape and pineapple extracts	926	0.312±0.025	0.0307±0.0025	0.343	91.0	9.0	318
16	*Vitis vinifera* L grape seed and leaf extracts	1834	3.71±0.17	0.450±0.021	4.16	89.2	10.8	7629
17	Grape seed, tomato and soy extracts, and vitamin C	1229	1.18±0.06	0.0535±0.0038	1.23	95.7	4.3	1512

[a] Results are the mean (n=3) ± SD.

in grape skins, and anthocyanins and flavonols in leaves (MONAGAS *et al.*, 2006).

The variability among production batches was studied for supplement #17. The ORAC$_{FL}$ values varied from 1.18 to 1.77 μmol of TE/mg of supplement for the hydrophilic antioxidant capacity, and from 0.0485 to 0.0878 μmol of TE/mg of supplement for the lipophilic antioxidant capacity (Table 2). The variation coefficient with respect to the mean was 14% for the hydrophilic fraction and 27% for the lipophilic fraction. In both cases, the ANOVA showed significant differences (P<0.05) among production batches, but homogenous groups were arranged in different patterns for the hydro and lipophilic capacity (Fig. 2). No relationship was found either between hydro or lipophilic capacity and the expiration date of the batches. The variability in the antioxidant capacity of the supplement among production batches

Table 2 - Hydrophilic (H-ORAC$_{FL}$) and lipophilic (L-ORAC$_{FL}$) antioxidant capacity of different batches of the dietary supplement #17.

Batch (#)	H-ORAC$_{FL}$ (μmol of TE / mg)[a]	L-ORAC$_{FL}$ (μmol of TE / mg)[a]	TOTAL-ORAC$_{FL}$ (μmol of TE / mg)	H-ORAC$_{FL}$ (%)	L-ORAC$_{FL}$ (%)
1	1.18±0.06[a]	0.0535±0.0038	1.235	95.7	4.3
2	1.56±0.13[a]	0.0485±0.0053	1.606	97.0	3.0
3	1.77±0.04[a]	0.0509±0.0083	1.820	97.2	2.8
4	1.34±0.08[a]	0.0850± 0.0046	1.423	94.0	6.0
5	1.37±0.04[a]	0.0567±0.0058	1.427	96.0	4.0
6	1.34±0.02[a]	0.0878±0.0038	1.425	93.8	6.2

[a] Results are the mean (n=3) ± SD.

was attributed to differences in the richness of the hydro- and lipophilic ingredients employed in each batch, and to differences in the production process that may affect the hydro/lipophilic ingredients differently.

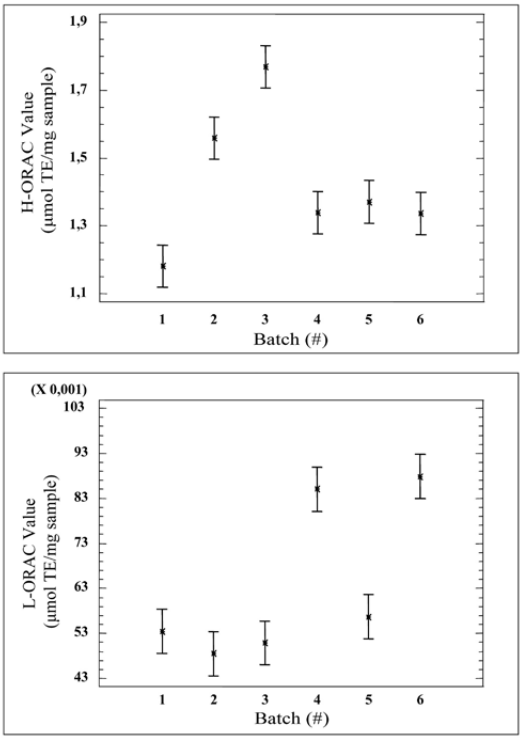

Fig. 2 - ANOVA of the hydrophilic (H-ORAC) and lipophilic (L-ORAC) antioxidant capacity of different batches of the dietary supplement #17. Error bars represent the 95% confidence intervals for mean.

In an attempt to compare the potential health benefits of the dietary supplements studied with those provided by food, the daily antioxidant intake provided by these supplements was calculated according to the dose recommended by the manufacturers (Table 1). The daily total antioxidant intake provided by the supplements varied from 168 (supplement #8) to 7,629 μmol of TE (supplement #16) (a 45.5-fold maximum-minimum difference). Using the same methodology, WU *et al.* (2004b) determined the total antioxidant capacity of 100 different kinds of foods (fruits, vegetables, nuts, dried fruit, spices, cereals, and infant and other foods) and estimated that the daily antioxidant intake of the USA population from consumption of vegetables, fruit and fruit juices was around 5,724 μmol of TE. However, this value would be lower (~2,200 μmol of TE) if the average number of servings of fruit and vegetables consumed daily in the USA is assumed to be 2.5 (WU *et al.*, 2004b). In any case, supplementation with most of the antioxidant formulations studied in this work could provide an antioxidant intake similar to that estimated for an average diet. Although the ORAC assay – which uses peroxyl free radicals, the most common radicals in human biology – can provide a good approximation of the potential benefits of antioxidant supplementation, other important factors such as bioavailability and nutrient

interactions have to be taken into consideration. Thus, the bioavailability of polyphenols is largely dependent on their structure (MANACH *et al.*, 2005). Comparatively, the isoflavones present in soy extracts (supplement #10) will be better absorbed than the flavonoid glucosides from citrus (supplement #3), and the latter will be better absorbed than the procyanidins present in grape extracts (supplements #8, 9 and 13), which are poorly absorbed (RASMUSSEN *et al.*, 2005). Finally, a positive response of a compound/preparation in an i*n vitro* antioxidant test is indicative but not a guarantee of having beneficial health effects in humans. Further *in vivo* studies are required to verify this.

In conclusion, the antioxidant capacity of dietary supplements assayed by the $ORAC_{FL}$ methodology, was variable among the commercial supplements studied. The hydrophilic ingredients represent around 86.3-99.2% of the total antioxidant capacity in contrast to the lesser relevance of the lipophilic constituents (0.8-13.7%); the variability among supplements and batches was greater for the lipophilic than for the hydrophilic antioxidant capacity. In order to accurately characterize the antioxidant capacity of dietary supplements, both lipophilic and hydrophilic fractions must be measured.

ACKNOWLEDGEMENTS

I. Garrido was the recipient of a fellowship from the I3P Program funding by the European Social Fund. This work has been supported by the Spanish Comision Interministerial de Ciencia y Tecnología (CYCIT) (Project AGL03-01088).

REFERENCES

Dávalos A., Gómez-Cordovés C. and Bartolomé B. 2003. Commercial dietary antioxidant supplements assayed for their antioxidant activity by different methodologies. J. Agric. Food Chem. 50: 2512.

Dávalos A., Gómez-Cordovés C. and Bartolomé B. 2004. Extending applicability of the oxygen radical absorbance capacity (ORAC-Fluorescein) assay. J. Agric. Food Chem. 52: 48.

Devaraj S., Vega-López S., Kaul N., Schönlau F., Rohdewald P. and Jialal I. 2002. Supplementation with a pine bark extract rich in polyphenols increases plasma antioxidant capacity and alters the plasma lipoprotein profile. Lipids 37: 931.

Harrison R.A., Holt D., Pattison D.J. and Elton P.J. 2004. Are those in need taking dietary supplements? A survey of 21923 adults. Brit. J. Nutr. 91: 617.

Huang D., Ou B., Hampsch-Woodill M., Flanagan J.A. and Deemer E.K. 2002. Development and validation of oxygen radical absorbance capacity assay for lipophilic antioxidants using randomly methylated β-cyclodextrin as the solubility enhancer. J. Agric. Food Chem. 50: 1815.

Manach C., Williamson G., Morand C., Scalbert A. and Rémésy C. 2005. Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. Am. J. Clin. Nutr. 81: 230S.

Monagas M., Garrido I., Bartolomé B. and Gómez-Cordovés C. 2006. Chemical characterization of commercial dietary ingredients from *Vitis vinifera* L. Anal. Chim. Acta 563: 401.

Naguib Y.M.A., Hari S.P., Passwater R. and Huang D. 2003. Antioxidant activities of natural vitamin E formulations. J. Nutr. Sci. Vitaminol. 49: 217.

Ou B., Hampsch-Woodill M. and Prior R. 2001. Development and validation of an improved oxygen radical absorbance capacity assay using fluorescein as the fluorescent probe. J. Agric. Food Chem. 49: 4619.

Prior R.L., Wu X. and Schaich K. 2005. Standardized methods for the determination of antioxidant capacity of phenolics in foods and dietary supplements. J. Agric. Food Chem. 53: 4290.

Rasmussen S.E., Frederiksen H., Krogholm K.S. and Poulsen L. 2005. Dietary proanthocyanidins: occurrence, dietary intake, bioavailability, and protection against cardiovascular disease. Mol. Nutr. Food Res. 49: 159.

Wu X., Gu L., Holden J.M., Haytowitz D.B., Gebhardt S.E., Beecher G.R. and Prior R.L. 2004a. Development of a database for total antioxidant capacity in foods: a preliminary study. J. Food Comp. Anal. 52: 4026.

Wu X., Beecher G.R., Holden J.M., Haytowitz D.B., Gebhardt S.E., Prior R.L. 2004b. Lipophilic and hydrophilic antioxidant capacities of common foods in the United States. J. Agric. Food Chem. 52: 4026.

Paper received July 7, 2006 Accepted December 15, 2006

SHORT COMMUNICATION

AN INITIAL COMPARISON OF THE CONTENT OF BITTER SUBSTANCES IN *CYNARA SCOLYMUS* L. PLANTS OBTAINED FROM ROOTED OFFSHOOTS AND MICROPROPAGATION

UN PRIMO CONFRONTO DEL CONTENUTO DI SOSTANZE AMARE IN PIANTE DI CARCIOFO (C*YNARA SCOLYMUS* L.) OTTENUTE MEDIANTE CARDUCCI E MICROPROPAGAZIONE

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ABSTRACT

Cynaropicrin, a sesquiterpene, is the main bitter compound in globe artichoke and is important for the organolectic and nutritional quality of the fresh product and leaf extracts. Artichoke plants of the late variety "Grato 1" obtained from rooted offshoots and by micropropagation were compared with respect to the bitter content in the young leaves and heads. The bitter content, expressed as cynaropicrin (% of dry weight), was determined

RIASSUNTO

La cinaropicrina, un sesquiterpene importante per la qualità organolettica e nutrizionale sia del prodotto fresco che degli estratti fogliari, è il principale composto amaro del carciofo. Nel presente lavoro è stato messo a confronto il contenuto di sostanze amare in foglie e capolini di piante di carciofo della varietà tardiva "Grato 1" ottenute per carducci e per micropropagazione. Il contenuto di sostanze amare espresse come cinaropicrina (% di sostanza sec-

- Key words: artichoke, bitter compounds, cynaropicrin, micropropagation -

by an alkalimetric method. The results show a bitter content ranging from 6.82 to 9.63%; the leaves of the micropropagated plants had 29% less bitter content than the plants traditionally propagated by rooted offshoots.

ca) è stato determinato tramite un metodo alcalimetrico. I risultati indicano un contenuto di sostanze amare variabile da 6,82 a 9,63%; nelle foglie delle piante ottenute in vitro è stata inoltre osservata una riduzione di circa il 29% rispetto alle piante propagate mediante carducci.

INTRODUCTION

Artichoke (*Cynara scolymus* L.) is a perennial Asteracea that is widely cultivated in the Mediterranean Basin and Italy is the main producer in the world (FAOSTAT data, 2006). Artichoke bloom heads have been a part of the Mediterranean diet since ancient times. Artichoke leaf extracts, which have been used in traditional folk medicine, are nowadays being used for the industrial preparation of standardised phytomedical products and for the extraction of bitter compounds. Artichoke extracts possess choleretic and hypocholesterolemic properties due to the high content of polyphenols with antioxidant activity (GEBHARDT and FAUSEL, 1997; GEBHARDT, 1997; KRAFT, 1997; WANG *et al.*, 2003; ALAMANNI and COSSU, 2003; CURADI *et al.*, 2005).

The characteristic bitter taste of artichoke is due to the endogenous content of sesquiterpene lactones, a group of colourless, bitter C_{15} terpenoids that are common in most genera of the Asteraceae (RODRIGUEZ *et al.*, 1976). Artichoke sesquiterpene lactones are cynaropicrin, dehydrocynaropicrin, cynaratriol and grosheimin (BERNHARD *et al.*, 1979). Among these, cynaropicrin (Fig. 1) is the quantitatively predominant bitter substance, being responsible for about 80% of the total bitterness of artichoke leaf extracts (SCHNEIDER and THIELE, 1974; FRITSCHE *et al.*, 2002). Consumers usually have an aversion to bitter compounds in plant foods so they are removed by selective breeding or during industrial processing (DREWNOWSKI and GOMEZ-CARNEROS, 2000). However a certain degree of bitterness is expected in artichoke.

Cynaropicrin is important for the organolectic quality of the edible fresh product and for the technological quality of the leaf extracts used for the industrial preparation of bitter liqueurs. This sesquiterpene exerts intense bitterness, more than alkaloids like caffeine and quinine (CRAVOTTO *et al.*, 2005). Recently it has been studied for its antihyperlipidemic and antitumoral properties (SHIMODA *et al.*, 2003; CHO *et al.*, 2004; CHOI *et al.*, 2005). Cynaropicrin may also act as an insect feeding deterrent during plant growth (BHATTACHARYYA *et al.*, 1995), as observed for other sesquiterpenes that function as protective agents against herbivorous insects (NAHRSTEDT, 1989).

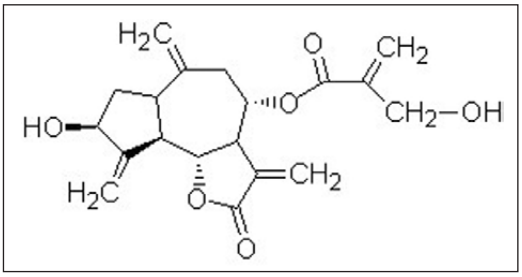

Fig. 1- Chemical structure of cynaropicrin.

The cynaropicrin content in artichokes is subject to a wide range of variation that depends on the plant genotype, the stage of the biological cycle, the organ considered, the environmental growth conditions and the cultivation site (SCHNEIDER and THIELE, 1974; FRITSCHE *et al.*, 2002), as found for bitter sesquiterpenes in *Cichorium intybus* L. (FOSTER *et al.*, 2006). Rooted offshoots (commonly known as "carducci") have traditionally been used for vegetative reproduction. In recent years the *in vitro* propagation of artichoke has been studied and further developed for large-scale multiplication, which offers genetic homogeneity and very healthy plant material. The low percentage of rooting may be improved by choosing suitable late artichoke cultivars, that show a greater potential for *in vitro* rooting than early cultivars (ANCORA *et al.*, 1981; TAVAZZA *et al.*, 2004). The artificial environment and different growth conditions during micropropagation phases may interfere with the secondary metabolism of the plantlets which could cause variations in the levels of bitter compounds during plant development.

Considering the lack of information available in the literature about Italian artichoke varieties, the aim of the present study was to investigate the content of bitter substances expressed as cynaropicrin in the young leaves and heads (edible parts and external bracts) of the Italian late artichoke variety "Grato 1" in both traditionally propagated plants obtained from rooted offshoots (RO) and in micropropagated plants (M).

MATERIALS AND METHODS

Chemicals

Toluene and petroleum ether (40°-70°) were all analytical grade quality and were purchased from Carlo Erba (Milan, Italy); dried $MgSO_4$ was obtained from Alfa Alesar (Karlsruhe, Germany); 0.01N NaOH, 0.01N HCl and phenolphtalein were purchased from Carlo Erba (Milan, Italy).

Plant material

Artichoke plants of the late variety "Grato1" were obtained from rooted offshoots of mother plants and cultivated in the open field of the Dipartimento di Biologia delle Piante Agrarie, located at San Piero a Grado, Pisa, Italy. Micropropagated plantlets of the same variety were obtained from offshoot apices in the micropropagation laboratory of the Dipartimento di Biologia delle Piante Agrarie and transplanted after acclimatization in a single row for open field growth on 5 October 2004, with a distance between the plantlets of about 90 cm. The soil had a medium depth and fertility.

Sample preparation

Plant material was collected on 27 May 2005. Nine leaves and nine heads of artichoke plants var. "Grato 1" obtained in vitro were harvested, and the same was done for artichoke plants of the same variety obtained from rooted offshoots. After harvesting, the plant material was cleaned, weighed fresh, and the heads were subsequently divided into external bracts and edible parts (inner bracts). Samples (leaves, inner and external bracts) were made up of 3 replications (n=3), each one formed by 3 young leaves or 3 heads collected from different plants. Plant material was then dry weighed after 8 days at 60°C in a thermoventilated oven, ground to a fine powder using an analytical mill and put into plastic flasks for quantitative analysis.

Quantitative analysis of bitter compounds

The artichoke bitter compounds were determined by means of a modified

form of the alkalimetric method previously described by SCHNEIDER and THIELE (1974). Each sample repetition was extracted as follows. Five grams dry weight of powdered sample were pre-extracted in Soxhlet for 8 h using 250 mL petroleum ether 40°-70° to remove lipophilic substances, and then extracted in Soxhlet for 30 h using 250 mL of toluene. The toluene extract containing the artichoke bitter compounds was then reduced to 80 mL with a Rotavapor and was partitioned 3 times with 30 mL of 2% NaOH aqueous solution in order to eliminate acids. The organic phase was then collected and the water removed with dried $MgSO_4$. The toluene extract was then filtered, the volume adjusted to 100 mL and transferred to a closed dark flask and stored at room temperature. For titration, 10 mL of the extract were mixed with 15 mL of 0.02N NaOH and stirred for 5-6 min at 45°C. Under these conditions, cynaropicrin is transformed into a water-soluble salt, and after about 10 min it remains stable without hydrolysis of the ester group. After cooling, the two phases were separated, and 10 mL of aqueous phase were titrated with 0.02N HCl using phenolphtalein as indicator. Given that 1 mL NaOH=6.92 mg of cynaropicrin (SCHNEIDER and THIELE, 1974), the content of bitter substances expressed as cynaropicrin was calculated and the results were then transformed into percentage dry weight (% d.w.).

Statistical analysis

Data are the mean of 3 sample replications (±S.E.M.; n=3); the values for each replication were obtained as the mean of 3 quantitative analyses of the same toluene extract. Data were subjected to ANOVA analysis using a Costat statistical program and the means were compared with the Duncan multiple range test (P≤0.05).

RESULTS AND CONCLUSIONS

The content of bitter substances expressed as cynaropicrin in the Italian late variety "Grato 1" showed a wide variability in the young leaves (Table 1). The highest value was found in the young leaves of the "Grato 1" plants obtained by vegetative propagation (9.63% d.w.). In all the tissues analysed, the bitter content was less in plants obtained *in vitro*. The percentage of reduction in the leaves, inner and external bracts of the heads of micropropagated plants (M), in comparison to the same organs of plants obtained from rooted offshoots (RO), were 29.13, 7.41 and 6.28%, respectively. The differences in the bitter content between M and RO plants were most evident in the leaves.

The statistical significance of the within-sample differences was determined using the analysis of variance and Duncan's multiple range test at the 5% level of significance (P≤0.05). Duncan's multiple range test indicated that there were no significant differences between and among the external bracts and the inner bracts of the heads (both in M and RO plants). However, a significant difference was noted between the leaves of the RO plants and the other samplings,

Table 1 - Content of bitter substance expressed as cynaropicrin in young leaves and heads (inner and external bracts) of artichoke plants var. "Grato 1" obtained from rooted offshoots (RO) and by micropropagation (M).

	Cynaropicrin (% d.w.) *
Grato 1 RO – Leaves	9.63±0.238 a
Grato 1 M – Leaves	6.82±0.047 c
Grato 1 RO – Inner bracts	7.60±0.213 b
Grato 1 M – Inner bracts	7.04±0.131 bc
Grato 1 RO – External bracts	7.48±0.220 b
Grato 1 M – External bracts	7.01±0.217 bc

* Row means ± S.E.M.; (n=3).
The means followed by the same letters are not significantly different at P≤0.05.

and among the leaves of the RO and M plants (Table 1).

Growth conditions during micropropagation phases may interfere with the secondary metabolism of the plantlets which could cause variations in the levels of endogenous sesquiterpenes during plant development. Data obtained in the present study indicate that, independent of the propagation method used, the young leaves of "Grato 1" had higher values of bitter content in comparison with those previously reported in the literature for different artichoke varieties (SCHNEIDER and THIELE, 1974) and commercial leaf extracts (FRITSCHE *et al.*, 2002). This result could be due to varietal differences and/or environmental conditions at the different cultivation sites during plant growth (FOSTER *et al.*, 2006; GIORGI *et al.*, 2005). SCHNEIDER and THIELE (1974) reported the absence of bitter compounds in completely developed artichoke heads and a content ranging from 0.5% d.w. to 6.4% d.w. in leaf laminas, with higher values occurring in the younger leaves. The data in the present study showed a different distribution, with a consistent quantity of bitter compounds in both the edible parts of the head (inner bracts) and the external bracts. While the distribution of cynaropicrin within the tissues of the head showed no significant differences between the inner and external bracts in both M and RO plants, higher values were found in the heads of plants obtained from rooted offshoots in comparison with those obtained *in vitro* (mean content in the whole head of 7.54% d.w. and 7.02% d.w., respectively).

The present data indicate that the extraction of bitter sesquiterpenes from an organic phase with the opening of the lactone ring and formation of a water-soluble saline form could be used for the quantitative determination of the artichoke bitter compounds. The results obtained with the alkalimetric method of SCHNEIDER and THIELE (1974) for artichoke leaf extracts were recently confirmed by means of HPLC-DAD using an external standard calibration with acetylgrosheimin as described by FRITSCHE *et al.* (2002). They found a maximum cynaropicrin content of about 2.2% d.w. in commercial artichoke leaf extracts. This value is comparable to the average bitter compound content of 2.5% d.w. reported by SCHNEIDER and THIELE (1974) for artichoke leaf extracts and calculated as cynaropicrin by titration after lactone cleavage. However, it should be noted that the alkalimetric method is not totally specific for cynaropicrin (BLOSZYK *et al.*, 1978). It allows all the artichoke bitter compounds, expressed as cynaropicrin to be quantified, given that this is the quantitatively predominant bitter substance in artichoke extracts. Further research is needed to define alternative less time-consuming methodologies that have greater sensitivity. Quantitative differences between micropropagated and non-micropropagated plants were found during the present preliminary study on the young leaves. The relationships between the cynaropicrin content and its potential insect-feeding deterrent activity in different Italian artichoke varieties should be investigated in order to identify more resistant varieties that have higher qualitative profiles.

ACKNOWLEDGEMENTS

The present research was carried out with the financial support of MIPAF. Thanks are due to Dr. Michele Leonardi for technical assistance.

REFERENCES

Alamanni M.C. and Cossu M. 2003. Antioxidant activity of the extracts of the edible part of artichoke (*Cynara scolymus* L.) var. Spinoso Sardo. Ital. J. Food Sci. 15: 187.

Ancora G., Belli-Donini M.L. and Cozzo L. 1981. Globe artichoke plants obtained from shoot apices through rapid *in vitro* propagation. Sci. Hort. 14: 207.

Bhattacharyya P.R., Barua N.C. and Ghosh A.C. 1995. Cynaropicrin from *Tricholepis glaberrima*: a potential insect feeding deterrent compound. Ind. Crop. Prod. 4: 291.

Bernhard H.O., Thiele K. and Pretsch E. 1979. Cynaratriol, ein neues Guajanolid aus der Kardone *Cynara carduncul*us L. und der Artischoke *C. scolymus* L. *(Compositae)*. Helv. Chim. Acta. 62: 1288.

Bloszyk E., Geppert B. and Drozdz B. 1978. Quantitative determination of sesquiterpene lactones in plant material by infrared spectroscopy. Planta Med. 34: 79.

Cho J.Y., Kim A.R., Jung J.H., Chun T., Rhee M.H. and Yoo E.S. 2004. Cytotoxic and pro-apoptotic activities of cynaropicrin, a sesquiterpene lactone, on the viability of leukocyte cancer cell lines. Eur. J. Pharmacol. 492: 94.

Choi S.Z., Choi S.U. and Lee K.R. 2005. Cytotoxic sesquiterpene lactones from *Saussurea calcicola*. Arch. Pharm. Res. 28: 1142.

Cravotto G., Nano G.M., Binello A., Spagliardi P. and Seu G. 2005. Chemical and biological modification of cynaropicrin and grosheimin: a structure-bitterness relationship study. J. Sci. Food. Agric. 85: 1757.

Curadi M., Picciarelli P., Lorenzi R., Graifenberg A. and Ceccarelli N. 2005. Antioxidant activity and phenolic compounds in the edible parts of early and late Italian artichoke (*Cynara scolymus* L.) varieties. Ital. J. Food Sci. 17: 33.

Drewnowski A. and Gomez-Carneros C. 2000. Bitter taste, phytonutrients, and the consumer: a review. Am. J. Clin. Nutr. 72: 1424.

FAOSTAT data, 2006. URL http://faostat.data.org. Last updated February 2005.

Foster J.G., Clapham W.M., Belesky D.P., Labreveux M., Hall M.H. and Sanderson M.A. 2006. Influence of cultivation site on sesquiterpene lactone composition of forage chicory (*Cichorium intybus* L.). J. Agr. Food Chem. 54: 1772.

Fritsche J., Beindorff C.M., Dachtler M., Zhang H. and Lammers J.G. 2002. Isolation, characterisation and determination of minor artichoke (*Cynara scolymus* L.) leaf extract compounds. Eur. Food Res. Technol. 215: 149.

Gebhardt R. 1997. Antioxidative and protective properties of extracts from leaves of the artichoke (*Cynara scolymus* L.) against hydroperoxide-induced oxidative stress in cultured rat hepatocytes. Toxicol. Appl. Pharmacol. 144: 279.

Gebhardt R. and Fausel M. 1997. Antioxidant and hepatoprotective effects of artichoke extracts and constituents in cultured rat hepatocytes. Toxicol. In Vitro. 11: 669.

Giorgi A., Bononi M., Tateo F. and Cocucci M. 2005. Yarrow (*Achillea millefolium* L.) growth at different altitudes in the central Italian Alps: biomass yield, oil content and quality. J. Her. Spi. Med. Pl. 11: 47.

Kraft K. 1997. Artichoke leaf extracts – Recent findings reflecting effects on lipid metabolism, liver and gastrointestinal tracts. Phytomed. 4: 369.

Nahrstedt A. 1989. The significance of secondary metabolites for interactions between plants and insects. Planta Med. 55: 333.

Rodriguez E., Towers G.H.N. and Mitchell J.C. 1976. Biological activities of sesquiterpene lactones. Phytochem. 15: 1573.

Schneider G. and Thiele K. 1974. Eigenschaften und Bestimmung des Artischocken-Bitterstoffes Cynaropicrin. Planta Med. 25: 150.

Schneider G. and Thiele K. 1974. Die Verteilung des Bitterstoffes Cynaropicrin in der Artischocke. Planta Med. 26: 174.

Shimoda H., Ninomiya K., Nishida N., Yoshino T., Morikawa T., Matsuda H. and Yoshikawa M. 2003. Anti-hyperlipidemic sesquiterpenes and new sesquiterpene glycosides from the leaves of artichoke (*Cynara scolymus* L.): structure requirement and mode of action. Bioorg. Med. Chem. Letters. 13: 223.

Tavazza R., Papacchioli V. and Ancora G. 2004. An improved medium for *in vitro* propagation of globe artichoke (*Cynara scolymus* L.) cv. "Spinoso sardo". Acta Hort. 660: 91.

Wang M., Simon J.E., Aviles I.F., He K., Zheng Q. and Tadmor Y. 2003. Analysis of antioxidative phenolic compounds in artichoke (*Cynara scolymus* L.). J. Sci. Food. Agric. 51: 601.

Paper received September 11, 2006 Accepted February 13, 2007

SHORT COMMUNICATION

RESIDUE ANALYSIS AND EFFECTS OF 17α-METHYLTESTOSTERONE ON BODY COMPOSITION OF RAINBOW TROUT, *ONCORHYNCHUS MYKISS*

EFFETTO DEL 17α-METILTESTOSTERONE SULLA COMPOSIZIONE DELLA TROTA ARCOBALENO, *ONCORHYNCHUS MYKISS*

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ABSTRACT

The effects of oral administration of 3 mg of 17α-methyltestosterone per kg of administered diet were studied in 35-day-old rainbow trout. The protein, fat, ash and moisture of meat, carcass efficiency, fillet efficiency and the hepatosomatic and gonadosomatic indexes were measured as well as the amount of residual methyl testosterone that might have remained in the meat. Compared to controls, there were no changes in crude protein and ash of the fish meat and in the fillet efficiency, carcass effi-

RIASSUNTO

Sono stati studiati gli effetti della somministrazione orale di 3 mg di 17α-metiltestosterone per kg di dieta somministrata a trote arcobaleno di 35 giorni. Sono stati misurati il contenuto in proteine, grassi, ceneri ed umidità della carne, il rendimento della carcassa, gli indici epatosomatico e gonadosomatico, insieme ai residui di testosterone potenzialmente rimasti nella carne. Dal confronto con i controlli, non si evidenziavano variazioni né contenuto di proteine e ceneri nella carne del pesce, nel

- Key words: body composition, methyltestosterone testosterone, *Oncorhynchus mykiss*, residue, trout -

ciency and gonodosomatic index of the trout. Crude fat of the fish meat and the hepatosomatic index decreased while the moisture increased. No residual 17α-methyltestosterone was found in the meat of treated trout.

filetto, nel rendimento della carcassa e nell'indice ganadosomatico della trota. I grassi totali della carne e l'indice epatosomatico diminuivano, mentre l'umidità aumentava. Non sono stati trovati residui di 17α-metiltestosterone nella carne delle trote trattate.

INTRODUCTION

Genetic manipulation has recently gained much attention for improving body composition and fish growth. Growth-promoting agents have also been widely used to improve the quality of trout (VANDENBERG and MOCCIA, 1998), encouraging the use of steroids and their synthetic analogues in fish culture (MATTY, 1985; GANNAM and LOVELL, 1991).

Protein accretion is the net difference between the rates of protein synthesis and degradation. These processes are harmonized by complex interactions between various endogenous growth regulators, the actions of which are influenced by a broad spectrum of intrinsic-and extrinsic factors (BELL *et al.*, 1998; BREIER, 1999; RONSHOLDT and MCLEAN, 2004). Disturbances to the growth-regulating hormonal milieu can negatively impact protein status. Conversely, enhanced presence of growth regulators can promote protein accretion and thus influence body composition (BREIER, 1999).

Some steroid compounds can cause different results in the body development and composition of various kinds of fish (YU *et al.*, 1979). It has been reported that increased concentrations of 17α-methyltestosterone (MT) resulted in increased moisture, crude protein and ash content but a decrease in crude fat of the fish meat (DEGANI, 1985).

MT did not change the fat and protein content in European eel, *Anguilla anguilla,* (DEGANI, 1986) or in red sea bream, *Chrysophrys major,* (WOO *et al.*, 1993). It has also been reported that MT given to coho salmon, *Oncorhynchus kisutch,* did not change the protein and ash content, but decreased the fat content (YU *et al.*, 1979). The hepatosomatic index (HSI) value increased when MT was given to red sea bream (WOO *et al.*, 1993), but decreased in sea bass, *Dicentrarchus labrax* (PIFERRER *et al.*, 1986).

A recent trend in salmon farming has been the development and application of high-energy diets, the manufacture of which relies upon extrusion and vacuum-coating technologies that permit incorporation of high levels of lipids. Such aqua-feed formulations offer several advantages (MAYER and MCLEAN, 1995), but also lead to increased fat deposition in both whole body and filet fractions. While such lipid buildup may be beneficial with respect to weight gain, it decreases slaughter yield and sometimes impairs end-product quality due to negative impacts on texture, taste and appearance (ALSTED, 1991; REGOST *et al.*, 2001). These negative effects can be reduced by using steroids which decrease fat storage in fish (YU *et al.*, 1979; MIWA and INUI, 1986).

In order to comply with the recommendations of the United Nations Food and Drug Organization and of the Environmental Protection Organization on the type and quantity of chemicals used in fish farming (OSTROWSKI and GARLING, 1988) and with restrictions put into practice in Turkey by the General Directorate of Protection and Control of The Turkish Ministry of Agriculture (ANONYMOUS,

2000), the question whether MT remains in fish meat needs to be resolved.

The present study was conducted to determine the effects of MT on meat quality, hepatosomatic and gonadosomatic indexes, carcass efficiency and fillet efficiency of rainbow trout and to measure the amounts of residual MT in trout meat.

MATERIALS AND METHODS

The present study was conducted over a period of 355 days on 800 35-day-old rainbow trout fry. At the beginning of the experiments, the average weight of the specimens was 0.32 g. The fish were kept in fiberglass tanks (2.5x0.8x0.7 m (1.4 m^3) filled with aerated well water. The water temperature and dissolved oxygen were recorded throughout the study. The fish were fed a commercial feed in pellets and granules obtained from Abalıoglu Food Production Plant, Denizli, Turkey. The composition of the feed is given in Table 1.

In order to conduct duplicate runs of all experiments, the fish were divided into four groups of 200 specimens each; two control and two experimental (MT) groups. This was done so that the method of random coincidence plots (YILDIZ and BIRCAN, 1991) could be used.

17α-methyltestosterone purchased from Sigma Corporation (Istanbul, Turkey) was added to the diet of the experimental groups. MT (3 mg/kg feed) was dissolved in a few mL of butyl alcohol and then diluted with 95% ethanol so that it could be sprayed onto the feed while turning in a mixer. The feed used for the controls was sprayed with ethanol only. The feeding material was left to air-dry overnight to eliminate the alcohol and then stored at 4°C for use during the experiments (INGRAM, 1988; GANNAM and LOVELL, 1991).

The fish were fed 5-6 times per day in the fry period and 3 times a day, *ad-libitum*, from the fingerling period to market size. The experimental group received feed treated with MT for 56 days. After this period, they were fed a non-treated diet for the remaining 299 days. The fish in the control group were given feed without MT and after this period normal feed was given until the end of the experiment.

At the end of the experiment, eight fish were randomly taken from each group for chemical residue analysis and 25 fish (average weight 295 g) were taken at random for measurements of the internal organs and carcass, and to determine fillet efficiency, and the hepato- and gonadosomatic indexes. The chemical analyses were carried out in compliance with the Weende methods of analysis (AKKILIC and SURMEN, 1979).

Residue analysis in fish muscle

Residue analysis was conducted in fish muscle to determine the presence

Table 1 - The chemical composition of the granule and pellet feed used.

	Control group		Methyltestosterone group	
Parameters	Granule feed	Pellet feed	Granule feed	Pellet feed
Moisture (%)	9.85	10.48	9.85	10.48
Crude protein (%)	51.73	45.29	51.73	45.29
Crude fat (%)	12.65	13.12	12.65	13.12
Crude cellulose (%)	1.82	2.80	1.82	2.80
Ash (%)	9.20	11.22	9.20	11.22
M.Energy (kcal/kg)	4,000	3,800	4,000	3,800
Methyltestosterone (mg/kg)	-	-	3	3

of residual 17α-methyltestosterone. Eight fish were randomly selected from the control and study groups (duplicate runs), frozen and put in clean styrofoam boxes (ANONYMOUS, 2000) for transport to the pharmacology laboratory of Bornova Veterinary Institute for Control and Research, Izmir, Turkey.

The residue was determined by GC-MS, modifying the procedures reported by HEITZMAN (1994) and SAEED *et al.* (1999). Two sets of samples were prepared by homogenizing muscle from individual subjects separately and from pooled muscles from each group. The following steps were then carried out for residue analysis.

Enzymatic hydrolysis

The MT hormone was first hydrolyzed by mixing 1 g of homogenized muscle with 300 μL β-glucoronidase in pH 9 buffer followed by incubation at 60°C for 2 hours. The enzymes in the liquid phase were separated and 5 mL diethyl ether was added onto the hydrolyzed samples and evaporated to dryness at 40°C using a rotary evaporator. This procedure was repeated twice.

The residue was dissolved in 100 μL absolute ethanol and 4 mL distilled water. Sep-Pak chromatographic cartridges (Waters GmbH, Eschborn, Germany) were previously conditioned with 5 mL of methanol and then with 5 mL of distilled water. The residue was injected into these cartridges. In the residue, methyltestosterone was retained on the SPE-cartridge under these conditions. After washing the cartridge, methyltestosterone was eluted with 2 mL of absolute ethanol. The eluate was transferred to a small sealed.

Derivative formation

To obtain the trimethylsilyl derivative (TMS) needed for chromatographic analysis, the ethanol in the hydrolyzed residue was evaporated and treated with 100 μL bis(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS), mixed in a blender and incubated for one h at 60°C. After incubation, the mixture was evaporated to dryness under a stream of nitrogen at 50°C and the residue was dissolved in 25 μL iso-octane and injected into the GC-MS (SAEED *et al.*, 1999; MEINERTZ *et al.*, 1999).

GC-MS analysis

For residue analysis, a Hewlett-Packard (HP) model 6890 gas chromatograph (Agillent, Waldbronn, Germany) with a HP5973 Mass Selective Detector was used to identify and quantify the MT in 2 μL aliquots of the TMS-derived samples.

Chromatographic conditions

A 25 m, 0.25 ID fused silica capillary column coated with SE-52 was used. The initial temperature was 100°C, the final temperature 280°C was reached in increments of 20°C/min. Helium was used as carrier gas at a flow rate of 2 mL/min. The samples were injected at a temperature of 280°C. For detection, the ionization energy was 70 eV, full scanning from 40 to 700.

The chemical structure of fish, crude protein (CP), crude fat (CF), ash (A) and moisture, the hepatosomatic (HSI) and gonadosomatic (GSI) indexes, carcass efficiency (CE) and fillet efficiency (FE) were examined and the formulas suggested by AKYILDIZ (1984), HALVER (1989) and HEPHER (1990) were used for the calculations.

The results were statistically analyzed using a one-way dimensional analysis of variance (ANOVA) contained in the SPSS software package.

RESULTS AND DISCUSSION

The differences between the crude protein and ash contents of the fish meat in the experimental and control groups

were not significant (P>0.05), but the crude fat and moisture contents were significant (P<0.05) (Table 2). The differences between the values of internal organ, carcass, and fillet efficiency values, GSI and HSI of the groups were not significant (P>0.05).

It is known that there is a negative correlation between the moisture ratio and raw fat in fish (VARLIK, 2004). SHELBOURN *et al.* (1992) reported that applying 17α-methyltestosterone to salmon (*O. kisutch*) decreased the fat ratio by 30%. SIMONE (1990) reported that MT administration decreased moisture, fat and protein ratios in fish meat. In contrast, other authors have reported that there is no change in moisture, fat and protein ratios in fish meat with MT administration (MIWA and INUI, 1986; DEGANI, 1986; WOO, *et al.* 1993). Anabolic steroids like MT cause a decrease in fat and lower fat synthesis, thus causing a decrease in fat storage (KAYA, 1984; QUATTRUCCI, 1987).

While the application of MT decreased the HSI value in rainbow trout, the decrease was not significant (Table 2). PIFERRER *et al.* (1986) reported the same results. The decrease in the HSI value could be the result of MT preventing the storage of some food (such as fat) which was stored in the liver or preventing the development of cells by degenerating liver cells with a pathological effect (HERMAN and KINCAID, 1988).

MT administration did not change the internal organ ratio, carcass, fillet yield and GSI parameters (Table 2). OSTROWSKI and GARLING (1988) reported similar results. Age, gender, internal organ composition and GSI values directly influence the carcass and fillet yields of fish (GJERDE and GJERDEM, 1984; HORSTGEN-SCHWARK *et al.*, 1986).

Unlike most other steroids, 17α-methyltestosterone did not leave any residue. In previous studies it has been reported that the amount of MT in fish tissues as a result of androgen and estrogen application was the same as that in wild fish. Therefore, there are no drawbacks to consuming these fish in terms of human health (GOUDIE *et al.*, 1986; STICKNEY, 1991; ARIMAN, 2000). The results of this study show that there is no MT residue in the fish and support the use of 17α-methyltestosterone administration in trout culture.

Table 2 - Influence of experimental diets on body composition of rainbow trout in the methyltestosterone (MT) and control groups. Mean±SE n=25.

	Control	MT
Moisture*	73.30±0.42	73.98±0.16
Crude protein	19.17±0.17	19.43±0.13
Crude fat*	4.32±0.01	3.43±0.19
Ash	1.37±0.02	1.40±0.07
Internal organs	11.44±3.47	11.43±3.51
Carcass efficiency	83.70±1.17	82.88±1.18
Fillet efficiency	68.14±1.25	67.81±1.18
Hepatosomatic index	0.94±.11	0.86±0.13
Gonadosomatic index	8.41±1.28	9.02±1.43

* Indicates a significant difference (p<0.05) between MT-treated and control groups.

17α-Methyltestosterone can be given to rainbow trout because of its fat decreasing effect and because there is no MT residue. Low fat content in fish meat gives it a dietetic property that affects consumer preference in a positive way. However, further studies are needed to determine the effects of 17α-methyltestosterone on the liver and other organs.

REFERENCES

Akkilic M. and Surmen S. 1979. "Yem Maddeleri ve Hayvan Besleme Laboratuarı El Kitabı". AU. Veteriner Fak. Yayın no: 357, Ankara.

Akyildiz A.R. 1992. "Balık Yemleri ve Teknolojisi". p. 192. AU. Ziraat Fak. Yayınları no: 1280, Ankara.

Alsted N.S. 1991. Studies on the reduction of discharges from fish farms by modification of the diet. In "Nutritional Strategies and Aquaculture Waste". Cowey, C.B., Cho, C. Y. (Eds.), Univ. Guelph, Ontario, Canada.

Anonymous 2000. "Su Ürünleri Kalite Kontrol El Kitabı". p. 229. Tarım ve Köy İşleri Bakanlığı Koruma ve Kontrol Genel Müdürlüğü Yayini, Ankara.

Ariman H. 2000. Effect of the different water temperatures and various levels of growth agent on the growth features and meat composition of rainbow trout *(Oncorhynchus mykiss)* fingerlings. Atatürk Üniv., Fen Bilim. Enst., Doktora Tezi, Erzurum. Turkiye.

Bell A.W., Bauman D.E., Beermann D.H. and Harrell R.J. 1998. Nutrition, development and efficacy of growth modifiers in livestock species. J. Nutr. 128: 360.

Breier B.H. 1999. Regulation of protein and energy metabolism by the somatotropic axis. Domest. Anim. Endocrinol. 17: 209.

Degani G. 1985. The influence of 17α-methyltestosterone on body composition of eels (*Anguilla anguilla* L.). Aquaculture 50: 23.

Degani G. 1986. Effect of combined dietary 17β-estradiol and 17α-methyltestosterone on growth and body composition of European eels (*Anguilla anguilla*). Aquaculture 59: 169.

Gannam A.L. and Lovell R.T. 1991. Growth and bone development in channel catfish fed 17α-methyltestosterone in production ponds. J. World Aquaculture Soc. 22: 95.

Gjerde B. and Gjerdem T. 1984. Estimates of phenotypic and genetic parameters for carcass traits in Atlantic salmon and rainbow trout. Aquaculture 36: 97.

Goudie C.A., Shelton W.L. and Parker N.C. 1986. Tissue distribution and elimination of radiolabeled, methyltestosterone fed to sexually undifferentiated blue tilapia. Aquaculture 58: 153.

Halver J.E. 1989. "Fish Nutrition". p. 785. Academic Press, New York, London.

Heitzman R.J. 1994. "Veterinary Drug Residues". Residues in food producing animals and their products: Reference materials and method, Published on behalf of commission of the European Communities, Luxembourg.

Hepher B. 1990. "Nutrition of Pond Fishes". p. 386. Cambridge University Press, Cambridge, New York.

Herman R.L. and Kincaid H.L. 1988. Pathological effects of orally administered estradiol to rainbow trout. Aquaculture 72: 165.

Hörstgen-Schwark G., H. Fricke. and H.J. Langholz. 1986. The effect of strain crossing on the production performance in rainbow trout. Aquaculture 57: 141.

Ingram M. 1988. Farming rainbow trout in fresh water tanks and ponds. In "Salmon and Trout Farming". L. Laird and T. Needham (Ed.) Ellis Horwood Limited, England.

Kaya S. 1984. Hayvansal üretimde gelişmeyi hızlandırıcı maddeler ve sakıncaları. Ankara Üni. Veteriner Fakültesi Dergisi 3: 410. Ankara.

Mayer I. and McLean E. 1995. Biotechnological and bioengineering strategies for reduced waste aquaculture. Water Sci. Technol. 31: 85.

Matty A.J. 1985. "Fish Endocrinology". p. 267. Timber Press Ltd. Portland, USA.

Meinertz J.R., Stehly G.R., Hubert T.D and Bernardy J.A. 1999. Liquid chromatographic determination of benzocaine and N-acetylbenzocaine in the edible fillet tissue from rainbow trout. J. Chromatog. 855: 255.

Miwa S. and Inui Y. 1986. Inhibitory effects of 17α-methyltestosterone and 17β-estradiol on smoltification of sterilized amago salmon (*Oncorhynchus rhodurus*). Aquaculture 53: 21.

Ostrowski A.C. and Garling D.L. 1988. Influences of anabolic hormone treatment and dietary protein; energy ratio on condition and muscle deposition of rainbow trout. The Progressive Fish-Culturist 50: 136.

Piferrer F., Carrillo M. and Zanuy S. 1986. Effects of injections of 17α-methyltestosterone on the external features and growth of juvenile Sea Bass (*Dicentrarchus labrax L.*). Inv. Pesq. 50: 213.

Quattrucci E. 1987. Natural sex hormones and their residues in food of animal origin. Hormones in food: Occurence and hazards. "Toxicological Aspects of Food". Elsevier Applied Science Publishers Ltd., England.

Regost C., Arzel J., Cardinal M., Laroche M. and Kaushik S.J. 2001. Fat deposition and flesh quality in sea water reared, triploid brown trout (*Salmo trutta*) as affected by dietary fat levels and starvation. Aquaculture 193: 325.

Ronsholdt B. and McLean E. 2004. Effect of growth hormone and salbutamol on growth performance, filet proximate composition and pigmentation of rainbow trout (*Oncorhynchus mykiss*). Aquaculture 229: 225.

Saeed T., Naimi I., Ahmad N. and Sawaya W.N. 1999. Assessment of the levels of anabolic compounds in Kuwait meat industry: optimization of a multiresidue method and the results of a preliminary survey. Food Control 10: 169.

Shelbourn J.E., Clarke W.C., McBride J.R., Fagerlund U.H.M. and E.M. Donaldson. 1992. The use of 17α-methyltestosterone and 3,5,3-triiodo-L-thyronine for sterilizing and accelerating the growth of zero-age coho salmon smolts (*Oncorhynchus kisutch*). Aquaculture 103: 85.

Simone D.A. 1990. The effects of the synthetic steroid 17α-methyltestosterone on the growth and organ morphology of channel catfish (*Ictalurus punctatus*). Aquaculture 84: 81.

Stickney R.R. 1991. "Culture of Salmonid Fishes". p. 189. CRC Press, Florida.

Vandenberg G.W. and Moccia R.D. 1998. Growth performance and carcass composition of rainbow trout, *Oncorhynchus mykiss*, fed the β-agonist ractopamine. Aquac. Res. 29: 469.

Varlık C. 2004. "Su Urunleri İsleme Teknolojisi". İstanbul Uni. Su Urunleri Fakultesi Yayınları. İstanbul.

Woo N.Y.S., Chung A.S.B. and Ng T.B. 1993. Influence of oral administration of estradiol-17 beta and testosterone on growth, digestion, food conversion and metabolism in the under yearling red sea bream (*Chrysophrys major*). Fish Physiol. Biochem. 10: 377.

Yildiz N. and Bircan H. 1991. "Arastirma ve Deneme Metodlari". p. 227. A.Ü. Zir. Fak. Ders Kitabı. Erzurum. Turkiye.

Yu T.C., Sinnhuber R.O. and Hendricks J.D. 1979. Effect of steroid hormones on the growth of juvenile Coho salmon, *Oncorhynchus kisutch.* Aquaculture 16: 351.

Revised paper received December 4, 2006 Accepted February 12, 2007

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256 pages, hardback
January 2007
ISBN: 9780813828558
Price: £ 105,00
Publisher: Blackwell Publishing

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March 2007

ISBN: 9780813829685

Price: £ 105,00

Publisher: Blackwell Publishing

"Advances in Thermal and Non-Thermal Food Preservation" provides current, definitive and factual material written by experts on different thermal and non-thermal food preservation technologies. Emphasizing inactivation of microorganisms through the application of traditional as well as newer and novel techniques and their combinations, the book's chapters cover:

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4th INTERNATIONAL MILK GENOMICS & HUMAN HEALTH SYMPOSIUM SPEAKER PROGRAM ANNOUNCED

Brussels, 6 August 2007 - The speaker program for the 4th International Symposium on Milk Genomics & Human Health, to be held November 7-9 at the COPIA Center for Wine, Food & the Arts in Napa, California has been released.

This two-and-a-half-day event, organized by the California Dairy Research Foundation (CDRF) and sponsored by IDF and other partners, will gather international experts in nutrition, genomics, bioinformatics and milk to address the status of milk-specific genomic research. Members of the International Milk Genomics Consortium (IMGC) also will meet to set future agendas for the group.

Day one sessions will focus on presentations of recent research breakthroughs in the areas of evolutionary genetics; bioinformatics, tools and applications; the bovine genome; and genetic diversity in milk and lactation.

Day two will focus on the activities of milk and genomics centers from around the world with the goal to increase coordination among researchers. Topics include the targets of milk bioactives;

mammary epithelia and milk production; the milk fat globule proteome; and bacteria the other consumers of milk.

The Symposium will wrap up with highlights from researchers throughout the world followed by an in-depth discussion of the International Milk Genomics Consortium, including knowledge management tools developed for the Consortium, led by Matthew Lange and Danielle Lemay of UC Davis and the IMGC Web Portal Group.

For registration information, visit www.milkgenomics.org or register online at www.acteva.com/go/cd rf

DAIRY INDUSTRY TO EXPLORE DEVELOPMENTS IN ICE CREAM PRODUCTS
3rd IDF International Symposium on Ice Cream
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Many representatives of major research institutes, universities, dairy industry, owners of companies, managing directors, heads of development and production, quality and technical managers will gather at "INTER-ICE 2007", the Third IDF International Symposium on Ice Cream, 4-6 December 2007 in Cologne, to provide new insights into current and future research, technology and industrial application in the field of ice cream.

Thirty-four top international professionals from eleven countries from academia and industry will present latest developments in ice cream. Presentations will cover areas such as processing, technology, analysis, new markets and raw materials.

IDF ANNOUNCEMENT INTERNATIONAL SYMPOSIUM ON REVOLUTION IN FOOD SAFETY MANAGEMENT
Nusa Dua, Bali - Indonesia
13-15 February, 2008

The International Dairy Federation has announced that an International Symposium on Revolution in Food Safety Management will be organized with the technical cooperation of the Food and Agriculture Organization of the United Nations (FAO) and co-sponsored by the World Health Organization (WHO) in Nusa Dua, Bali/Indonesia, 13-15 February 2008.

The symposium aims at improving food safety and dairy development worldwide by providing practical guidance for the implementation of new concepts and tools of a risk-based approach in the dairy production chain. Various elements in effective management of microbiological and chemical hazards relevant to the dairy chain will be covered. This will enable the dairy sector, including dairy businesses in emerging countries, to better understand these new approaches and consider the feasibility of their application in their sector. It will therefore focus on practical experiences, challenges, planning and feasibility by means of presentations on the various concepts. Case studies and practical examples will also be presented.

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The paper must also be submitted by e-mail or on a digital support (cd-rom or floppy disk). Indicate which word processor was used to generate the file and save the file also in format "Text only", DCA-RTF or ASCII, if you do not have programs for Macintosh; **graphics, pictures and diagrams must be saved at 300 dpi in TIF, JPEG, EPS or PICT formats** (not included in MsWord documents).

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

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(Anonymous)
Anonymous. 1982. Tomato product invention merits CTRI Award. Food Technol. 36(9): 23.

(Book)
AOAC. 1980. "Official Methods of Analysis" 13th ed. Association of Official Analytical Chemists, Washington, DC.
Weast, R.C. (Ed.). 1981 "Handbook of Chemistry and Physics" 62nd ed. The Chemical Ruber Co. Cleveland, OH.

(Bulletin, circular)
Willets C.O. and Hill, C.H. 1976. Maple syrup producers manual Agric. Handbook No. 134, U.S. Dept. of Agriculture, Washington, DC.

(Chapter of book)
Hood L.F. 1982. Current concepts of starch structure. Ch. 13. In "Food Carbohydrates". D.R. Lineback and G.E. Inglett (Ed.), p. 217. AVI Publishing Co., Westport, CT.

(Journal)
Cardello A.V. and Maller O. 1982. Acceptability of water, selected beverages and foods as a function of serving temperature. J. Food Sci. 47: 1549.
IFT Sensory Evaluation Div. 1981a. Sensory evaluation guide for testing food and beverage products. Food Technol. 35 (11): 50.
IFT Sensory Evaluation Div. 1981b. Guidelines for the preparation and review of papers reporting sensory evaluation data. Food Technol. 35(4): 16.

(Non-English reference)
Minguez-Mosquera M.I., Franquelo Camacho A, and Fernandez Diez M.J. 1981. Pastas de pimiento. I. Normalizacion de la medida del color. Grasas y Aceites 33 (1): 1.

(Paper accepted)
Bhowmik S.R. and Hayakawa, K. 1983. Influence of selected thermal processing conditions on steam consumption and on mass average sterilizing values. J. Food Sci. In press.

(Paper presented)
Takeguchi C.A. 1982. Regulatory aspects of food irradiation. Paper No. 8, presented at 42nd Annual Meeting of Inst. of Food Technologists, Las Vegas, NV, June 22-25.

(Patent)
Nezbed R.I. 1974. Amorphous beta lactose for tableting U.S. patent 3,802,911, April 9.

(Secondary source)
Sakata R., Ohso M. and Nagata Y. 1981. Effect of porcine muscle conditions on the color of cooked cured meat. Agric. & Biol. Chem. 45 (9): 2077. (In Food Sci. Technol. Abstr. (1982) 14 (5): 5S877).
Wehrmann K.H. 1961. Apple flavor. Ph. D. thesis. Michigan State Univ., East Lansing. Quoted in Wehrmann, K.H. (1966). "Newer Knowledge of Apple Constitution", p. 141, Academic Press, New York.

(Thesis)
Gejl-Hansen F. 1977. Microstructure and stability of freeze-dried solute containing oil-in-water emulsions. Sc. D. Thesis, Massachusetts Inst. of Technology, Cambridge.

(Unpublished data/letter)
Peleg M. 1982. Unpublished data. Dept. of Food Engineering., Univ. of Massachusetts, Amherst.
Bills D.D. 1982. Private communication. USDA-ARS. Eastern Regional Research Center, Philadelphia, PA.

CONTRIBUTORS

Gratitude is expressed to the following entities for contributing to the realization of the Journal by being supporting subscribers for 2007.

Si ringraziano i seguenti Enti, Ditte ed Istituti per aver voluto contribuire fattivamente alla realizzazione della Rivista, sottoscrivendo un abbonamento sostenitore per il 2007.

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ITALIAN JOURNAL OF FOOD SCIENCE
Rivista Italiana di Scienza degli Alimenti
DIRETTORE RESPONSABILE: Giovanni Chiriotti
AUTORIZZAZIONE: n. 3/89 in data 31/1/1989
del Tribunale di Perugia
Proprietà dell'Università di Perugia
TIPOGRAFIA Giuseppini - Pinerolo
Una copia € 6.00

ISSN 1120-1770 © 2007

CHIRIOTTI EDITORI sas - 10064 Pinerolo - Italy

publishes the technical magazines:

VOLUME XIX IJFS No. 3, 2007

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