ISSN 1120-1770

ITALIAN JOURNAL OF FOOD SCIENCE

Rivista italiana di scienza degli alimenti

IJFS

Volume XVIII
Number 3
2006

CHIRIOTTI EDITORI

Spedizione in ab. post. comma 26 - art. 2 - legge 549/95 n. 3/2006 - Torino

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

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Frequency: Quarterly - One volume in four issues. Guide for Authors and annual indices will be published only in number 4 of each volume.

Impact Factor: 0.400 published in the 2005 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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Reprints (100) will be sent free of charge.

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PAPER

AFLATOXIN M_1 DETERMINATION IN MILK: METHOD VALIDATION AND CONTAMINATION LEVELS IN SAMPLES FROM SOUTHERN ITALY

DETERMINAZIONE DELL'AFLATOSSINA M_1 NEL LATTE: VALIDAZIONE DEL METODO E LIVELLI DI CONTAMINAZIONE IN CAMPIONI DELL'ITALIA DEL SUD

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ABSTRACT

Aflatoxin M_1 in milk and dairy products represents a concern for food safety, and laboratories involved in its determination have to use reliable and robust test methods. To this purpose, both method validation and participation in proficiency studies are mandatory requirements for demonstrating adequate technical competence. In this work, the validation of a HPLC method for determination of aflatoxin M_1 (AFM_1) in milk, through both proficiency testing and intra-laboratory study,

RIASSUNTO

L'aflatossina M_1 nel latte e nei prodotti lattiero-caseari rappresenta un serio problema per la sicurezza alimentare. I laboratori preposti al controllo ufficiale degli alimenti devono utilizzare metodi di prova affidabili; sia la validazione del metodo di analisi che la partecipazione a studi inter-laboratorio sono requisiti indispensabili per dimostrare un'adeguata competenza tecnica del laboratorio. In questo lavoro è descritto il procedimento di validazione di un metodo HPLC per la determinazione del-

- Key words: aflatoxin M_1, heat-treated milk, raw milk, survey, validation -

according to the UNI CEI EN ISO/IEC 17025 standard is described. A survey about AFM_1 levels in both raw and heat-treated milk from Southern Italy over the years 2002-2005 is also reported. A total of 552 samples from cows, buffaloes, sheep and goats were analysed; AFM_1 was detected in 248 samples (44.9%), from all the species. Most of these samples contained AFM_1 concentrations that were well below the legal maximum residue limit (MRL); only 33 samples were non-compliant (6.0%); bovine milk was the most contaminated. Moreover, the first long-term survey regarding contamination of raw buffalo milk is described and discussed. The highest contamination levels were detected in 2003 and 2005.

l'aflatossina M_1 (AFM_1) nel latte di massa e nel latte trattato termicamente, sia mediante uno studio intra-laboratorio che la partecipazione a circuiti inter-laboratorio, come richiesto dalla norma UNI CEI EN ISO/IEC 17025. Inoltre, sono riportati i livelli di contaminazione da AFM_1 nel latte di massa e termicamente trattato proveniente dalle regioni Campania e Calabria nel periodo 2002-2005. Sono stati analizzati 552 campioni di latte bovino, bufalino ed ovi-caprino; la AFM_1 è risultata presente in 248 campioni (44,9%), ed in tutti i tipi di latte. Nella maggior parte dei campioni sono stati determinati livelli di AFM_1 inferiori al limite massimo di residuo (MRL); il latte bovino è risultato il più contaminato. Inoltre, per la prima volta sono descritti e discussi i dati di un monitoraggio a lungo termine circa la contaminazione nel latte di bufala. I campioni di latte non conformi sono stati solo 33 (6,0%); i livelli di contaminazione più elevati sono stati riscontrati nel 2003 e nel 2005.

In recent years, there has been growing attention given to the presence of organic contaminants in food in the European Union. Among the organic contaminants, mycotoxins represent a class of compounds that is of great interest, because, through animal feeding, they can enter the consumers' diet through derived foods. Aflatoxins are a group of structurally-related toxic compounds produced by some strains of the moulds *Aspergillus flavus* and *Aspergillus parasiticus*, under favourable conditions of temperature and humidity (SEMPLE *et al.,* 1989). Aflatoxin B_1 is the most toxic and widespread compound of this class, and is the only one for which legal limits have been set for feed (COMMISSION DIRECTIVE, 2003). Aflatoxin M_1 (AFM_1) is the major metabolite of aflatoxin B_1 in mammals, and is usually excreted in the milk and urine of animals that have consumed aflatoxin-contaminated feed (PRUCHASE and STEYN, 1967; APPLEBAUM *et al.,* 1982). AFM_1 is stable to thermal, chemical and physical treatment; it can be found in both raw and heat-treated milk, and in derived products, such as cheese (LOPEZ *et al.,* 2001). AFM_1 is hepatotoxic, and has been classified as a genotoxic, teratogenic and carcinogenic substance of class 2^B by IARC (IARC, 1987; WOGAN, 1992). It has been estimated that between 0.2% and 5% of aflatoxin B_1 ingested through feedstuff by dairy cows is excreted in the milk as the metabolite AFM_1. This carry-over rate increases proportionally with milk pro-

duction, and it is higher in early lactation, showing high individual variability (APPLEBAUM *et al.,* 1982). The European Commission has set a maximum residue limit (MRL) of 0.05 ng/g for AFM_1 in milk (COMMISSION REGULATION, 2001) and prohibits the use of products non-compliant with this limit as ingredients for manufacturing other foodstuffs. It is noteworthy that the MRL for AFM_1 in milk-based food for infant formulas is 0.025 ng/g (COMMISSION REGULATION, 2004). Since 1996, the European Commission has required that member States carry out national monitoring programmes on both aflatoxin B_1 levels in feed and AFM_1 in raw milk. Within this framework, several official controls are performed annually, both on raw milk produced in Italy and in other European Community countries, as well as on dairy products. In late 2003, the Italian Ministry of Health issued an alert for aflatoxin contamination in maize used as feed as a consequence of the dry, hot summer in Northern Italy, and pointed out the problem of high AFM_1 levels in milk and dairy products (ITALIAN MINISTRY OF HEALTH, 2004).

Routine laboratory analysis for AFM_1 is performed by screening methods, such as ELISA and lateral flow strips, and by confirmatory methods, such as HPLC with fluorescence detection. Recently, to guarantee both technical laboratory competence and time maintenance efficiency of analytical performance, the European Commission established that public official control laboratories have to be accredited according to the UNI CEI EN ISO/IEC 17025 (2000) international standard for quality management system and must participate in internationally recognised external quality control assessment and accreditation programmes (COMMISSION DECISION, 1998; 2002). In our laboratory, AFM_1 is determined by a confirmatory method, based on immuno-affinity chromatography purification from milk, prior to HPLC determination by fluorescence detection (DRAGACCI *et al.,* 1995). The method has been validated with periodic external quality controls through the participation in internationally recognised proficiency studies. The mean recovery, repeatability and within-laboratory reproducibility of the method at, below and above the MRL have been evaluated by intra-laboratory studies. Finally, the AFM_1 contamination levels determined between 2002 and 2005 in raw milk from different species, as well as in retail heat-treated milk from the regions of Campania and Calabria, are reported and discussed.

MATERIALS AND METHODS

Materials

A 1 mL vial at 1 mg/mL AFM_1 was obtained from Carlo Erba Reagents (Rodano, MI, Italy).

The standard stock solution of AFM_1 at 5 µg/mL in acetonitrile/methanol 3/2 v/v is stable for 1 year if properly stored at -18°C. A standard solution at 100 ng/mL prepared by dilution with acetonitrile/methanol 3/2 v/v is stable for 6 months at -18°C; standard working solutions at 5.0, 2.0, 1.0, 0.8, 0.4 and 0.1 ng/mL in acetonitrile/methanol 3/2 v/v were prepared daily by serial dilutions of the 100 ng/mL standard solution, and used during HPLC analysis.

HPLC grade acetonitrile and methanol were obtained from Carlo Erba Reagents (Rodano, MI, Italy) and HPLC grade water was obtained from a laboratory MilliQ system (Waters Corp., Milford, MA, USA).

VICAM Afla-M_1 Immuno-affinity disposable SPE columns (Safefood, S. Vitale Braganza, PR, Italy), were used for AFM_1 purification, according to manufacturer's instructions (SAFEFOOD, 1997). A SPE vacuum manifold system (Waters Corp., Milford, MA, USA) was used to perform SPE chromatography.

Sample purification

Fifty grams of milk were poured into a polypropylene tube and centrifuged at 3300 *g* for 5 min at 4°C, and the upper fat layer was removed by a spatula; this step was repeated twice; then the defatted sample was kept at -18°C for 30 min. The sample was filtered through filter paper (Millipore, Milford, MA, USA) and loaded onto an Afla-M_1 Immuno-affinity disposable SPE column, at about 1 mL/min flow rate; then, the column was washed with 3×3 mL of MilliQ water and AFM_1 was eluted with 2 mL of acetonitrile/methanol 3/2 v/v, and collected in a centrifuge tube. Then, the immuno-affinity column was dried under vacuum, to recover completely the elution solvent. After centrifugation at 3300 *g* for 5 minutes at 4°C the sample was analysed by HPLC.

HPLC analysis with fluorescence detection

HPLC analysis was performed with a Waters system, equipped with a 600E quaternary pump, a 717 Plus autosampler and a 2475 fluorescence detector (Waters Corp., Milford, MA, USA). Chromatographic separation was performed on a 4 μm particle 250 × 4.6 mm 80 Å Polar-RP Synergi stainless steel column (Phenomenex, Torrance, CA, USA), at 1 mL/min flow rate, with water/acetonitrile/methanol 52/22/26 v/v/v mobile phase, injecting 100 μL sample volume. Fluorescence detection was carried out setting 360 nm as the excitation and 440 nm as the emission wavelengths. Quantitative analysis was performed by interpolation of the external standard calibration curves, calculated daily by linear regression of peak area versus concentration of the standard solutions; the concentration of AFM_1 in contaminated samples was calculated, then was corrected for recovery. During each working session, a blank reagent, three samples of bovine, buffalo or goat milk spiked at 0.025, 0.050 and 0.075 ng/g, corresponding to 0.5, 1.0 and 1.5 MRL, respectively, were analysed.

Method validation by intra-laboratory study and proficiency testing

The method was validated both by intra-laboratory study and external quality control, according to the schemes suggested by the UNI CEI EN ISO/IEC 17025 standard. During the intra-laboratory study, uncontaminated milk samples from all species, spiked at 0.025, 0.050 and 0.075 ng/g (corresponding to 0.5-, 1- and 1.5-fold the MRL), were analysed over a period of nine months. Method repeatability was evaluated by analysing six replicates at each contamination level; within-laboratory reproducibility was calculated by different analysts testing six replicates at each spiking level, repeated during three different working sessions. The overall data were used to calculate the mean recoveries at all the spiking levels. Method specificity was tested by analysing 30 milk samples, raw, UHT and pasteurised, non-contaminated by AFM_1, and belonging to the different species (bovine, buffalo, goat, sheep). The limit of quantification (LOQ) and the linearity of detector response were also calculated. Moreover, laboratory performances were tested by taking part in several proficiency test rounds, organised by FAPAS (CSL, Sand Hutton, York, UK) and Progetto Trieste (Tecna, Science Park, Trieste, Italy), from June 2002 to December 2005. In each round the unknown sample was analysed at least in duplicate, and the results were evaluated in terms of the z-score, as calculated by the proficiency test organiser.

Milk field samples

AFM_1 levels in raw bovine, buffalo, goat and sheep milk, as well as in heat-

treated retail milk, both pasteurised and UHT, were determined. All samples were collected from the regions of Campania and Calabria, in southern Italy, by public control authorities, within the framework of official surveillance activities of local health services.

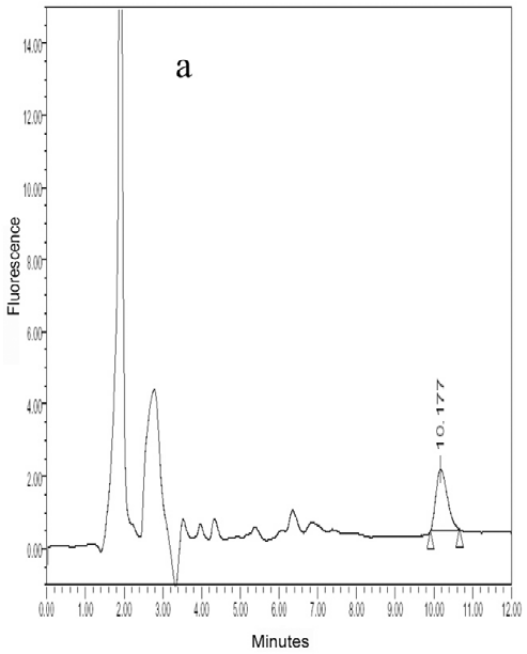
RESULTS AND DISCUSSION

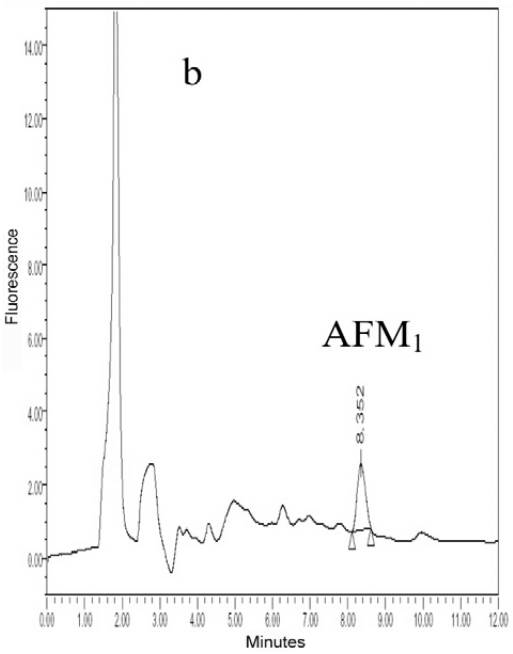
Test methods suitable for official control purposes have to minimise matrix interferences and improve specificity. Usually, for the reversed phase HPLC analysis of AFM_1, a C-18 stationary phase is used. Considering the AFM_1 molecular structure, the ether-linked phenyl phase with polar end-capping of the 80 Å Polar-RP Synergi from Phenomenex was tried. This polar stationary phase maximises retention and selectivity of aromatic compounds in the presence of mobile phases containing methanol. Very good peak sharpness and symmetry were observed with respect to the classical C-18 stationary phase previously employed, thus allowing the limit of quantification (LOQ) to be lowered. During method development, the LOQ was determined by analysing in triplicate several blank milk samples spiked at decreasing concentrations, 0.004 ng/g being the lowest concentration level determined with a 5/1 signal-to-noise ratio. The chromatograms of a blank milk sample, a sample spiked at 0.004 ng/g and a sample contaminated by AFM_1 are shown (Fig. 1). The analyte was identified on the basis of the retention time (t_r), with a tolerance range of ±2.5%; to confirm a positive sample, co-chromatography with an approximately equal amount of AFM_1 standard was performed, according to the Commission Decision 2002/657/EC requirements. The linearity of the fluorescence detector response was between 0.1 and 5.0 ng/mL of the AFM_1 standard, corresponding to a range of 0.004-0.200 ng/g in the sample. The absence of chromatographic peaks that interfered with the analyte identification in 30 uncontaminated milk samples proved the method specificity.

To verify method reliability, mean recoveries, repeatability and within-laboratory reproducibility were calculated at 0.025, 0.050 and 0.075 ng/g, corresponding to 0.5-, 1- and 1.5-times the legal MRL, respectively. The results (Table 1) were very satisfactory, in terms of mean percentage recoveries and RSD%, at all the spiking levels. No appreciable qualitative or quantitative differences were observed in recoveries from milk samples of different species, or between raw and heat-treated milk; the initial defatting step probably allows the most relevant matrix interferences to be removed from the samples.

The results obtained in several proficiency testing rounds between 2002 and 2005 were satisfactory ($-2 \leq z\text{-score} \leq +2$), proving the technical competence of the laboratory, both for positive and negative samples (Table 2).

This method was applied in the years between 2002 and 2005 to determine AFM_1 in milk samples of different origin from the regions of Campania and Calabria; some samples were unsuitable for the analysis, because of protein clotting due to unsuitable conditions during transportation to the laboratory (Table 3). Most of the 552 samples analysed were from bovine (235 samples, 42.6%) and buffalo (207, 37.5%) herds, while only a small portion of the samples (93, 16.8%) were pasteurised/UHT retail milk, and sheep and goat milk (17, 3.1%) (Table 4). Attention was mainly focused on bovine milk, even if only a few samples of heat-treated milk were collected. It should be noted that only a few sheep and goat milk samples were collected for official control. The surveillance on buffalo milk was quite constant during the study period, because it was carried out within the Italian Na-





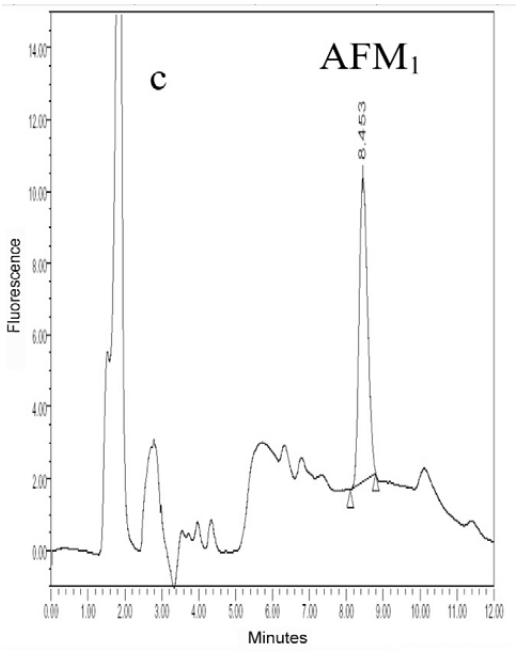


Fig. 1 - HPLC chromatograms of: a) blank raw bovine milk sample; b) raw bovine milk spiked at 0.004 ng/g AFM_1 (retention time 8.35 minutes); c) pasteurised milk sample containing 0.058 ng/g AFM_1 from FAPAS 2003 proficiency test (retention time 8.45 minutes).

tional Monitoring Programme. AFM_1 was detected in all of the kinds of milk (Table 4); bovine milk was the most contaminated (more than 50% of the samples), with similar percentages of contaminated raw and heat-treated milk. There were fewer contaminated samples of raw buffalo milk. A statistical evaluation of the goat and sheep milk is not possible, because of the few samples collected, even if AFM_1-contamination seems appreciable. An evaluation of overall concentration levels shows that most contaminated samples contained AFM_1 amounts quite below the legal MRL (Fig. 2). The concentration ranges of AFM_1 for each year and for the different milk samples are also reported (Table 4). Again, raw bovine milk had the highest contamination levels; heat-treated milk had lower AFM_1 levels as a consequence of combining raw milk from different farms.

A comparison with similar surveys reported in the literature, shows that until 2003 the incidence of AFM_1 contam-

Table 1 - Mean percentage recoveries, repeatability and within-laboratory reproducibility of the method, expressed as standard deviation (SD) and coefficient of variation (RSD%), at 0.025, 0.050 and 0.075 ng/g spiking levels, tested over nine months by different analysts. The number of replicates (n) is also reported.

AFM_1 spiking level (ng/g)	Mean percentage recovery (%)	Repeatability			Within-laboratory reproducibility		
		SD (ng/g)	RSD %	n	SD (ng/g)	RSD %	n
0.025	95.7	0.0016	7.0	6	0.0021	8.4	18
0.050	96.0	0.0026	5.6	6	0.0046	9.6	18
0.075	97.0	0.0028	3.8	6	0.0031	4.2	18

Table 2 - Results of the proficiency tests conducted between June 2002 and December 2005 to determine AFM_1 in milk, with the respective organizing body and laboratory z-score.

Year	Proficiency study organizing body	AFM_1 assigned value (ng/g)	Laboratory result (z-score)
2002	Progetto Trieste	Negative sample	Satisfactory
2002	Progetto Trieste	0.017	-1.19
2003	Progetto Trieste	Negative sample	Satisfactory
2003	FAPAS	0.058	-1.2
2004	FAPAS	0.039	-0.1
2005	FAPAS	0.034	-1.6

Table 3 - Results from milk samples analysed from 2002 to 2005.

Year	Total number of samples	Contaminated samples	Negative samples	Unsuitable for analysis
2002	82	12	61	9
2003	116	52	55	9
2004	161	69	80	12
2005	239	115	108	16

Table 4 - The distribution of contaminated milk samples; the total sample number, the contaminated samples and their relative percentages, the contamination ranges determined per year are reported for each kind of milk.

		2002	2003	2004	2005
Raw bovine milk samples	Total	20	42	59	114
	Contaminated (percentage)	3 (15.0%)	22 (52.4%)	33 (55.9%)	68 (59.6%)
	Range, ng/kg	7 - 14	6 - 244	6 - 770	4 - 1262
Raw buffalo milk samples	Total	43	47	59	58
	Contaminated (percentage)	4 (9.3%)	20 (42.6%)	18 (30.5%)	18 (31.0%)
	Range, ng/kg	4 - 39	4 - 43	5 - 23	4 - 676
Raw sheep/goat milk samples	Total	1	7	8	1
	Contaminated (percentage)	0 (0.0%)	4 (57.1%)	2 (25.0%)	1 (100%)
	Range, ng/kg	-	6 - 9	9 - 27	0 - 31*
Heat-treated bovine milk samples	Total	9	11	23	50
	Contaminated (percentage)	5 (55.6%)	6 (54.5%)	15 (65.2%)	28 (56.0%)
	Range, ng/kg	4 - 64	4 - 20	6 - 29	5 - 88

* 1 sample analysed.

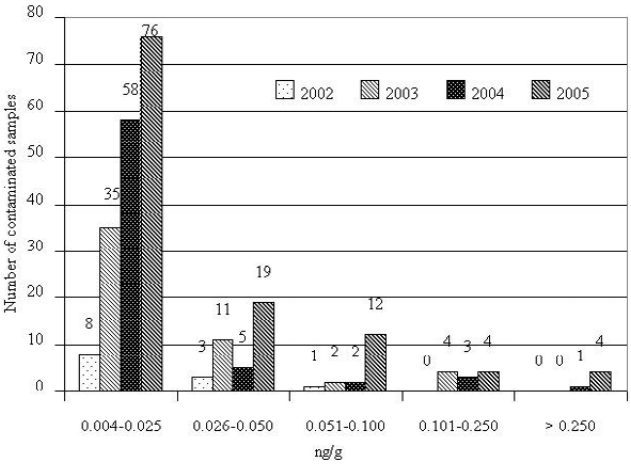


Fig. 2 - The overall contamination levels of AFM_1 in contaminated milk samples from all the species for each year.

ination in bovine milk from Campania and Calabria was lower than in other regions of Italy (CAPEI and NERI, 1996; GALVANO *et al.*, 1996; 1998; 2001; SERRAINO *et al.*, 2003; PAOLONI *et al.*, 2004; GHIDINI *et al.*, 2005), even if the concentration levels were quite similar. After 2003, the range became much wider for raw bovine milk. Regarding raw buffalo milk, since most buffaloes are raised in Campania, this survey appears to be the most representative and up-to-date for this species. The data indicate a significant contamination rate (between 9.3 and 42.6% samples) at relatively low concentration levels. On the whole, only 33 samples (6.0%) were non-compliant: 1 in 2002 (1.4%), 6 in 2003 (5.6%), 6 in 2004 (4.0%) and 20 in 2005 (9.0%). As reported in Table 5, all the non-compliant samples were raw and heat-treated bovine milk, except for 2 raw buffalo milk samples in 2005.

CONCLUSIONS

The method for AFM_1 determination was validated and accredited because these requirements are mandatory for an official control laboratory. Its reliability and specificity, as well as the satisfactory participation in proficiency testing, give evidence of technical competence that ensure reliable results and stimulate analysts to improve their own performance.

AFM_1 monitoring in milk has increased considerably in recent years. In the last 3 years the contamination ranges detected have become considerably wider, especially in 2005, when several non-compliant samples were found in all kinds of milk. It is interesting to note that in late 2005 the Italian Ministry of Health initiated a special national monitoring programme for controlling AFM_1 content in retail heat-treated milk, to evaluate real consumer exposure. This confirms that it was oppor-

Table 5 - The number of non-compliant milk samples and their relative percentages over the period 2002-2005, per year and per each kind of milk.

Year	Raw bovine milk Samples		Raw buffalo milk samples		Raw sheep/goat milk samples		Heat-treated bovine milk samples	
	Total	Non compliant (percentage)	Total	Non compliant (percentage)	Total	Non compliant (percentage)	Total	Non compliant (percentage)
2002	20	0	43	0	1	0	9	1 (11.1%)
2003	42	6 (14.3%)	47	0	7	0	11	0
2004	59	6 (10.2%)	59	0	8	0	23	0
2005	114	15 (13.2%)	58	2 (3.4%)	1	0	50	3 (6.0%)

tune to carry out a wider surveillance activity, in agreement with our data, showing that more than half of the bovine milk samples were contaminated, but very little testing on heat-treated milk has been conducted. Moreover, the number of samples of sheep/goat milk is insufficient, considering the relatively high number of contaminated samples. On the other hand, these data are the first to describe a long-term survey on AFM_1 contamination in buffalo milk. The data show a large distribution of this mycotoxin, but it is lower than in bovine samples. In our opinion, there should be more official control of buffalo and goat/sheep milk and the dairy products derived from them, because these kinds of milk are not usually consumed directly, but are widely distributed on the market in mozzarella and pecorino cheese.

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Revised paper received May 2, 2006 Accepted June 16, 2006

PAPER

MICROBIOLOGICAL DIVERSITY IN NATURAL WHEY STARTERS USED TO MAKE TRADITIONAL ROCAMADOUR GOAT CHEESE AND POSSIBLE RELATIONSHIPS WITH ITS BITTERNESS

DIVERSITÀ MICROBIOLOGICA IN SIERI NATURALI USATI COME STARTER PER LA FABBRICAZIONE DEL "ROCAMADOUR" (FORMAGGIO DI CAPRA TRADIZIONALE) E POSSIBILI RELAZIONI CON L'AMARO DEL PRODOTTO

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ABSTRACT

Rocamadour is a farmhouse-made cheese that is based on the use of a natural whey starter (NWS). Eight NWS from seven different cheese houses were analysed, mainly for their lactic acid bacteria population, because bitterness has become a problem. The isolates were identified on the basis of phenotypic and biochemical capacities. Lactococci were often the most abundant microflora followed by *Leuconos-*

RIASSUNTO

Il Rocamadour è un formaggio artigianale la cui produzione è basata sull'utilizzo di sieri naturali usati come starter (NWS "Natural whey starter"). Sono stati analizzati 8 NWS provenienti da 7 diversi caseifici, in particolare per quanto riguarda il loro contenuto in batteri lattici, a causa dell'insorgere del gusto amaro.Gli isolati batterici sono stati identificati sulla base delle caratteristiche fenotipiche e biochimiche. I

- Key words: Bitterness, goat cheese, lactic acid bacteria, microbiological diversity, natural whey starter, whole-cell protein patterns -

toc, with levels ranging from 10^8 to 10^9 cfu/mL. Citrate-positive bacteria levels were about the same. In particular, the *Lactococcus lactis* subsp. *cremoris* levels were always greater than those of *Lc lactis* subsp. *lactis* cases, except in the two NWS suspected of causing bitterness. *Leuconostoc mesenteroïdes* subsp. *mesenteroïdes* and *Ln mesenteroïdes* subsp. *cremoris* were the only two subspecies identified. The technological characterisation of 165 lactococci and leuconostocs and the analysis of whole-cell protein patterns allowed different bacterial behaviours to be observed. In some NWS, a few strains were dominant whereas in some others, the microflora was more diverse.

Lattococchi risultavano essere la microflora più abbondante, seguiti da *Leuconostoc*, con livelli da 10^8 e 10^9 cfu/ml. I livelli di batteri citrato-positivi risultavano più o meno identici. In particolare, i livelli di *Lactococcus lactis* subsp. *cremoris* spesso risultavano maggiori di quelli del *Lc lactis* subsp. l*actis*, con l'eccezione dei due NWS sospettati di essere implicati nello sviluppo del sapore amaro nel formaggio. Il *Leuconostoc mesenteroïdes* subsp. *mesenteroïdes* e il *Ln mesenteroïdes* subsp. c*remoris* erano le uniche 2 sottospecie identificate. La caratterizzazione tecnologica dei 165 lactococci e leuconostoc e l'analisi del pattern proteico cellulare, permettevano di osservare comportamenti batterici diversi. In alcuni NWS, pochi ceppi risultavano essere dominanti, mentre in altri la microflora risultava essere molto differenziata.

INTRODUCTION

In 2002, 98,000 tons of goat milk cheese were produced in France which represented 3.9% of the total national cheese production (CHAUSSON *et al.*, 2004). This quantity has been increasing yearly which indicates a renewed consumer interest in such products. Eleven goat cheeses, totalling 6,370 tons (6.5%), are of Controlled Denomination of Origin (CDO). Rocamadour cheese is one of these.

Rocamadour, a traditionally-made farmhouse goat cheese, is mainly produced in the Lot region in south-western France. This cheese is moulded in small cylinders (6 cm in diameter, 1.6 cm in height) and weighs about 35 g. The technology is based on clotting of the full fat raw milk due to the activity of a natural whey starter (NWS); this practice is widespread in traditional French goat milk cheese technology. During the first 10 hours, the pH of the milk drops from 6.6 to 4.8. Thereafter, it decreases slowly until it reaches 4.4 after 20 h of culture. The curd is not cut but drained after 12 hours. Salt is then mixed with the curd before moulding. Cheeses are ripened at 10°C for at least six days, which allows *Geotrichum candidum* to develop on the rind surface. Typical sensory characteristics include a creamy, buttery and nutty aroma, and a creamy and oily texture. For further information, readers are invited to look at an Internet site dedicated to this cheese (http://www.aoc-rocamadour.com).

The use of NWS is an ancient practice that is still frequently used to make cheese with cow (Parmigiano Reggiano, Comté), sheep milk (Pecorino Sardo) or goat milk (Cameros, Valdeteja) (OLARTE *et al.*, 2000; ALONSO-CALLEJA *et al.*, 2002, CANDIOTI *et al.*, 2002). NWS are

known to contribute to the development of the cheese flavour and provide resistance in case of phage attack (MAURIELLO *et al.*, 2001; CANDIOTI *et al.*, 2002). The whey of the cheese is removed and cultured for several hours. Depending on the technology, the acidity level may vary from 60-70 Dornic degrees (°D) to 130-140°D (1°D is equivalent to 0.1g of lactic acid). The bacteria that make up these starters come from three sources: (1) a previous cheese-making process, (2) the raw milk and (3) the cheese house environment (BOUTON *et al.*, 1998). The NWS of cheeses processed with a mesophilic technology have a majority of mesophilic lactic acid bacteria (LAB), *i.e.* lactococci, leuconostocs, enterococci and mesophilic lactobacilli (HATZIKAMARI *et al.*, 1999; ALLONSO-CALLEJA *et al.*, 2002; MANOLOPOULOU *et al.*, 2003). In contrast, in Italian and Argentinean hard cheeses, which are mainly produced using a thermophilic technology, the NWS are generally composed of thermophilic LAB (NEVIANI, 1993; REINHEIMER *et al.*, 1996). To our knowledge, except for one congress report (TORMO *et al.*, 2004), there is no published information about the microbial diversity of NWS used in goat cheese technology, including Rocamadour technology. This lack of data is probably due to the technical difficulty involved in studying such a complex microbial population which is in constant evolution (GIRAFFA, 2003).

Cheese makers currently using NWS often face some recurrent problems, including a decrease in the acidifying capacity of the strains and the development of strong bitter taste. This latter defect has occasionally been detected in Rocamadour. According to BERGÈRE and LENOIR (1997), bitterness is derived from three major sources: (1) the starter, (2) the rennet and (3) an excessive quantity of *Pseudomonas*. Since the amount of rennet added to Rocamadour is very slight, it seemed more important to determine if the starter plays a role in bitterness development.

Consequently, the aim of this research was to study the microbial diversity among eight different natural whey starters used to make Rocamadour cheese. One-hundred-and-sixty-five LAB isolates were biochemically and technologically characterised. Their whole–cell protein patterns were also studied. Since two NWS were suspected of being the cause of bitterness development, attempts were made to detect any differences which might have been related to this problem.

MATERIALS AND METHODS

Origins of the samples

Eight natural whey starters were analysed and one of these was analysed at two different times. They were identified as follows: V1, V2, V3a, V3b, V4, V5, V6 and V7.

In agreement with the technical adviser of the Rocamadour region, seven different cheese houses were selected which are located in areas in the region where two main edaphic conditions prevail: the "Causses" region, characterised by barren lands (V1, V4, V5 and V6), and the "Limargue" region which has more fertile soils (V2, V3 and V7).

The NWS were all prepared in a similar manner. After draining the curd, the whey was collected and cultured at dairy plant temperature (20 - 24°C). As a consequence of LAB metabolism, the acidity of the whey increased to approximately 65-70°D; this acidified NWS (1-3 L) was then added to the raw milk.

The natural whey starters V1 - V4 were all collected on February 24, 2004; V5 and V6 were collected two months later. V3 was re-sampled along with V7, on May 25, 2004. Samples V1 - V5 originated from cheese houses that produce typical products. Samples V6 and

V7 were suspected of producing bitterness in cheese.

Sampling

Thirty millilitres of NSW were collected in sterile screw-capped jars that also contained 30 mL of sterile reconstituted milk (10%, w/v, AES, Combourg, France). After gentle homogenisation, the jars were immediately frozen at -20°C. This procedure was carried out to limit problems associated with possible delays between sampling and microbiological analyses. The frozen samples were not stored for more than 7 days before thawing and further analyses. Preliminary studies were conducted to ensure that there were no differences between the levels of lactococci, lactobacilli, leuconostocs, enterococci and citrate-positive bacteria, before and after freezing. Compared with the addition of a 30% glycerol solution, sterile milk was the preferred cryoprotectant. Microbial analyses were carried out within 24 h after thawing.

Enumeration

After thawing, samples were diluted in sterile distilled water containing peptone (1%, w/v, AES, Combourg, France). The following bacterial populations were then investigated:

- Lactococci on M17 agar (Biokar diagnostics, Beauvais, France): aerobic incubation at 22°C for 48 h,
- Lactobacilli on MRS agar (AES, Combourg, France): anaerobic incubation at 37°C for 72 h,
- Leuconostocs on MSE agar (MAYEUX *et al.*, 1962): aerobic incubation at 22°C for 48 to 72 h,
- Citrate-positive microflora on KCA (NICKELS and LEESMENT, 1964): aerobic incubation at 30°C for 72 h,
- Enterococci on KF agar (Biokar diagnostics, Beauvais, France): aerobic incubation at 37°C for 48 to 72 h,
- *Pseudomonas* on CFC agar, (Biokar diagnostics, Beauvais, France): aerobic incubation at 22°C for 48 to 72 h. This flora was only counted in samples V6 and V7.
- Yeasts and moulds on chloramphenicol glucose agar (Biokar diagnostics, Beauvais, France): aerobic incubation at 25°C for 48 to 72h.

Strain isolation

One-hundred-and-sixty-five strains were picked from the M17, MSE and KCA media and 24 from MRS agar. The enterococci levels were 10^3 to 10^4 times lower than the other bacterial groups. The decision was then made to focus the study specifically on the main flora, *i.e.* lactococci and leuconostocs. Consequently, no isolates were taken from the KF agar. Seventeen, 19, 19, 15, 13, 19 and 21 colonies were isolated and characterised from the V2, V3a, V3b, V4, V5, V6 and V7 starters, respectively; and 42 colonies were isolated from the V1 starter. It was observed that 20 colonies isolated from MRS agar were coccus-shaped, while four were lactobacillus-shaped. These 24 MRS-isolates were not characterised thereafter.

All strains were cultivated at 22°C in M17 broth for 24 h. After growth, purity of the isolates was checked once on M17 agar before thawing. Each isolate was then stored at -80°C in the presence of M17 broth and 30% (w/v) glycerol in 2 mL cryotubes (Nunc cryotube™ Vials).

Isolate characterisation

Preparation of inocula

After thawing, bacteria were cultivated on M17 broth for 24 to 48 h at 22°C. Cells were then harvested and washed twice in 0.9% NaCl distilled water. Optical density at 600 nm was checked and adjusted between 0.2 and 0.3. This final suspension was used to inoculate the media to be tested at 2%. All the in-

cubations were made at 22°C, which is the natural whey starter culture temperature in the cheese house.

Biochemical characterisation

Cocci were tested for their ability to grow on KF agar. Salt resistance was evaluated in M17 broth at 22°C in the presence of 3%, 4% and 6.5% NaCl (w/v). Growth at 15°C and 37°C was performed in M17 broth. Sugar fermentation was tested using API 20A basic medium (BioMérieux, Marcy l'Etoile, France). Arginine dihydrolase capability was tested on BHI broth (Biokar diagnostics, Beauvais, France) supplemented with an arginine solution (0.3%, w/v). Citrate utilisation was checked on KCA agar. These tests were selected in order to differentiate between lactococci and leuconostoc, and more specifically between *Lactococcus lactis* subsp. *lactis*, *Lc lactis* subsp. *cremoris*, *Lc lactis* subsp. *lactis* biovar. *diacetylactis*, *Leuconostoc mesenteroïdes* subsp. *mesenteroïdes* and *Ln mesenteroïdes* subsp. *cremoris* (DELLAGLIO *et al.*, 1994).

Technological characterisation

The isolates were also analysed for the following characteristics that are of dairy interest:

- Acidification ability was performed on reconstituted milk (10%, w/v, AES, Combourg, France). Tubes were incubated for 16 h and cooled immediately. pH was then determined.

- Proteolysis was evaluated according to the procedure described by BOUTON *et al.* (1993).

- Autolysis was evaluated by inoculating cells in a tube containing 10 mL of 0.1 M NaH_2PO_4. Optical density at 600 nm (OD) was followed for 48 h. If the OD decreased, the strains were considered "strongly autolytic (Au)"; if a temporary increase was observed, strains were classified as "slightly autolytic (SAu)". A stabilisation of OD indicated "intermediate (In)" behaviour.

- Acidification, reduction and coagulation were evaluated on bactolithmus milk according to the methodology described by LIMSOWTIN *et al.* (1994). Depending on the strains, behaviour differs when cultured on bactolithmus milk. For instance, some strains first reduce the milk, then, the milk is acidified and finally coagulation occurs. This sequence, reduction, acidification, coagulation (RAC), is an indication of LAB metabolism. Some other behaviour that was observed included just reduction (R), reduction followed by acidification (RA), and reduction, acidification and slight coagulation (RA(C)).

Whole-cell protein pattern

Cells were washed in a sucrose buffer (sucrose, 103 g, 2M Tris (pH 8), 25 mL, Sigma, St Louis, MO, USA). After centrifugation, the supernatant was removed and replaced with a buffer containing lysozyme (100 mg, Sigma, St Louis, MO, USA), 1 mL of EDTA 0.5 M (Sigma, St Louis, MO, USA), 3 mL of the sucrose buffer and 2 mL of sterile distilled water. Incubation was carried out for 1 h at 37°C. After centrifugation (20 000 g, 2 min.), the supernatant was carefully removed. A solution containing 1.56 mL of 1 M Tris HCl (pH 6.8), 0.5 g of SDS (Sigma, St Louis, MO, USA), 2.5 g of glycerol, 3.78 mL of sterile distilled water and 100 µL of 0.5% bromophenol blue (w/v, Sigma, St Louis, MO, USA) was added. After gentle homogenisation, tubes were heated for 5 min in a boiling bath, cooled and frozen at -20°C.

Before gel electrophoresis, cell extracts were warmed to 20°C and 100 µL were sampled in a 0.5 mL Eppendorff tube that also contained 22.4 µL of β-mercaptoethanol (Sigma, St Louis, MO, USA).

Gel preparation and whole cell protein electrophoresis were performed according to the procedure of BARREAU and WAGE-

NER (1990). Gels were scanned using a HP 3770 ScanJet (Hewlett Packard, Evry, France). The band patterns were then normalised and processed by using the GelCompar 3.1 program (Applied Maths, Kortrijk, Belgium). The densitometric traces were analysed using Pearson's product moment correlation coefficient (VAUTERIN and VAUTERIN, 1992) and were clustered using the unweighted pair group method with arithmetic averages (UPGMA) (SOKAL and MICHENER, 1958).

Chromatography

Proteolysis products resulting from the growth of lactic acid bacteria in reconstituted milk (10%, w/v, 22°C, 24 h) were analysed by reversed phase high performance liquid chromatography (RP-HPLC) according to THOMAS and PRITCHARD (1987). Measurements were made on a Merck L4000 UV Detector apparatus linked to a Merck HSM 7000 PC integrator (Paris, France). Technical requirements were the same as those used by BOUTON *et al.* (1994) to study peptides in cheese.

Statistical analyses

Correlations, multiple correspondences factorial analyses (MCFA) between isolates and technological abilities (acidification, proteolysis and autolysis) and hierarchical classifications were performed using the STATITCF software (5th version, 1995, Institut Techniques des Céréales et des Fourrages, Paris, France).

RESULTS

Microbial composition of the natural whey starters

High levels of bacteria, irrespective of the populations enumerated and the origin of the sample can be observed in Table 1. In particular, presumptive lactococci always exceeded $10^{8.78}$ cfu/mL. Presumptive lactobacilli were also systematically found at levels ranging from $10^{5.69}$ to $10^{9.52}$ cfu/mL. However, microscopic observations, as well as biochemical characterisations, revealed that this population was only composed of cocci. Enterococci concentrations did not exceed 10^5 cfu/mL (results not shown). Presumptive leuconostoc and citrate-positive bacteria, which were frequently dominant, were not identified in the V6 starter. However, this is not necessarily correlated to the development of a bitter taste, since the V7 starter had even higher levels of leuconostoc and citrate-positive bacteria, $10^{9.07}$ and $10^{8.96}$ cfu/mL, respectively. Furthermore, no *Pseudomonas* colonies were obtained from these two NWS. Differences of less than 1 $\log_{10}$(cfu)/mL were recorded for V3a and V3b that were sampled at 0 and three months.

The yeast and mould levels did not exceed $10^{6.0}$ cfu/mL, irrespective of the NWS analysed (data not shown).

Consequently, these preliminary results indicate some variability in the microbial composition of the NWS, depending on the origin and time of the sampling.

Characterisation of the isolates

While the count of the MRS population was high, it was not representative of the lactobacilli population. Only four out of 24 isolates were identified as lactobacilli. The estimated level of this population was similar to that of the enterococci. Therefore, subsequent tests were focused on the dominant lactococci and leuconostoc populations.

One-hundred-four isolates were sampled from M17 agar, and all were identified as *Lactococcus* strains. As a consequence, the levels of lactic acid bacteria enumerated on M17 seemed representative of the *Lactococcus* population. Six of the 25 colonies sampled

Table 1 - Comparison of the four main microflora levels in the different natural whey starters according to their origin.

	V1	V2	V3a	V3b	V4	V5	V6*	V7*
Citrate positive microflora	9.30	9.30	9.30	8.53	9.30	5.70	Absent	9.07
Presumptive lactococci	8.90	8.78	8.86	9.54	8.78	8.92	8.88	9.77
Presumptive lactobacilli	8.78	8.78	8.78	9.11	7.93	7.67	5.69	9.52
Presumptive leuconostoc	9.30	9.30	9.30	9.11	9.30	6.64	Absent	8.96

Data expressed in log colony forming units (cfu) per millilitre, log(cfu/mL). Absent: detection limit: 1 log(cfu/mL).
*: Natural whey starters suspected of giving favour bitterness to cheeses.

on MSE belonged to the *Lactococcus* genus and 19 to the *Leuconostoc* genus. Thirty-six colonies were isolated from KCA agar. Twenty-four belonged to the *Lactococcus* genus and 12 to the *Leuconostoc* genus. The *Leuconostoc* levels reported in Table 1 were probably excessively high. The lactococci seem to dominate the leuconostocs in Rocamadour NWS.

The specific identification of the lactococci isolates revealed different compositions depending on the origin of the starters (Table 2). *Lactococcus lactis* ssp ranged from 80.8% (V1) to 87.5% (V7) and from 93.8% (V3b) to 100% (V2, V3a, V4, V5, V6) of the characterised strains. *Lc plantarum* was identified in V1, V3b and V7, and *Lc raffinolactis* was identified in V1 and V7. Since the lactococci levels in NWS are known to be high, it can be assumed that *Lc plantarum* and *Lc raffinolactis* were sub-dominant; their counts were estimated to be six to 20 times lower than that of *Lc lactis*.

Among the *Lc lactis* strains, the subspecies *hordniæ* was only identified in V3b. There were two isolates out of 19, which indicates a rather high concentration of this population in this NWS. *Lc lactis* subsp. *cremoris* was always the most important population except in V6 and V7 which gave bitter cheeses (in which *Lc lactis* subsp. *lactis* dominated), and V3a (in which *Lc lactis* subsp. *lactis* biovar. *diacetylactis* dominated).

Except for the V3a NWS, there is a

Table 2 - Proportions of species and subspecies among lactococci and leuconostocs isolates.
Each species or subspecies of lactococci is expressed as the proportion of isolates reported with respect to the total number of isolates identified as lactococci.

	V1	V2	V3a	V3b	V4	V5	V6*	V7*
Lactococcus lactis subsp. *cremoris*	42.3	53.8	40.0	50.0	69.2	90.9	26.3	0.0
Lactococcus lactis subsp. *lactis*	7.7	23.1	0.0	6.3	30.8	0.0	73.7	56.3
Lactococcus lactis subsp. *lactis biovar. diacetylactis*	30.8	23.1	60.0	25.0	0.0	9.1	0.0	31.2
Lactococcus lactis subsp. *hordniae*	0.0	0.0	0.0	12.5	0.0	0.0	0.0	0.0
Lactococcus plantarum	7.7	0.0	0.0	6.2	0.0	0.0	0.0	6.3
Lactococcus raffinolactis	11.5	0.0	0.0	0.0	0.0	0.0	0.0	6.2
Leuconostoc mesenteroïdes subsp. *mesenteroïdes*	12.5	100.0	100.0	0.0	34.0	100.0	0.0	0.0
Leuconostoc mesenteroïdes subsp. *cremoris*	87.5	0.0	0.0	100.0	66.0	0.0	0.0	100.0

Each subspecies of leuconostoc is expressed as the proportion of isolates reported to the total number of isolates identified as leuconostoc.
*: Natural whey starter suspected of giving favour bitterness to cheeses.

strong negative correlation between the levels of *Lc lactis* subsp. *cremoris* and *Lc lactis* subsp. *lactis* microflora (r = -0.70). Compared to this, the "r" values between *Lc lactis* subsp. *lactis* biovar. *diacetylactis* and *Lc lactis* subsp. *lactis* or *Lc lactis* subsp. *cremoris* were -0.43 and -0.31, respectively. *Lc lactis* subsp. *lactis* biovar. *diacetylactis* always seemed to be present at low, rather stable levels compared with the subspecies *cremoris* and *lactis*, which fluctuated according to the NWS.

Considering the leuconostoc isolates, *Leuconostoc mesenteroïdes* subsp. *cremoris* and *Ln mesenteroïdes* subsp. *mesenteroïdes* were the only subspecies identified. There was a low negative correlation between *Lc lactis* subsp. *lactis* and *Ln mesenteroïdes* subsp. *mesenteroïdes* (r = -0.46).

Technological characterisation of the isolates

One-hundred-and-thirty-four lactococci strains were isolated from the eight NWS and their technological aptitudes were characterised. The resulting data for this main bacterial group were analysed together by means of MCFA. Five axes were kept for the analysis. Based on the coordinates on these five axes, strains were grouped together by means of hierarchical classification. Twenty-three clusters were obtained (Table 3). It should be noted that clusters belonged to two different catego-

Table 3 - Technological characteristics of *Lactococcus* strains according to the origin of the natural whey starter (NWS) and with respect to the cluster after multiple correspondence factorial analysis and hierarchical classification.

Clusters	NWS	Identification	Growth on Bactolithmus Milk	Autolysis	Proteolysis	pH variation
1	V1	*Lc plantarum*	RAC	Au	3.04-3.90	1.47-1.61
	V1	*Lc lactis* subsp. *cremoris*	R(A)	Au	2.95-3.33	0.45-1.10
	V1	*Lc lactis* subsp. *lactis biovar. diacetylactis*	RAC	Au	3.51-4.13	0.61-1.48
2	V1	*Lc raffinolactis*	R(A)	In	1.68-1.96	2.03-2.10
3	V1	*Lc lactis* subsp. *cremoris*	RA	Au	2.72	1.15
4	V1	*Lc lactis* subsp. *lactis biovar. diacetylactis*	RAC	In	2.84-2.98	1.05-1.11
	V1	*Lc lactis* subsp. *cremoris*	RAC	SAu	2.70-3.03	0.58-1.50
5	V1	*Lc lactis* subsp. *lactis biovar. diacetylactis*	RAC	In	2.44	0.97
6	V1	*Lc lactis* subsp. *cremoris*	R(A)	Au	1.93-2.36	0.38-0.48
	V6*	*Lc lactis* subsp. *cremoris*	RAC	Au	1.21	0.37
7	V2	*Lc lactis* subsp. *lactis*	RAC	SAu	1.76	2.48
8	V3b	*Lc plantarum*	RAC	In	1.51	0.68
	V3b	*Lc lactis* subsp. *cremoris*	R(A)	In	1.47	0.71
	V3b	*Lc lactis* subsp. *lactis biovar. diacetylactis*	RAC	In	1.36	0.77
9	V7*	*Lc lactis* subsp. *lactis biovar. diacetylactis*	R	SAu	1.13	0.47
	V7*	*Lc lactis* subsp. *lactis*	R	SAu	1.13-1.20	0.14-0.47
10	V6*	*Lc lactis* subsp. *lactis*	RAC	SAu	1.59-1.63	1.70-1.84
	V6*	*Lc lactis* subsp. *cremoris*	RAC	SAu	1.54-1.62	1.75-1.87
11	V1	*Lc lactis* subsp. *cremoris*	RAC	In	2.4	2.81
	V2	*Lc lactis* subsp. *lactis*	RAC	In	1.73	2.50
	V4	*Lc lactis* subsp. *lactis*	RAC	In	1.4	2.62
	V3b	*Lc lactis* subsp. *lactis*	RAC	In	1.58	2.01
	V7*	*Lc lactis* subsp. *lactis*	RAC	In	1.49	2.89

Table 3 (continued).

Clusters	NWS	Identification	Growth on Bactolithmus Milk	Autolysis	Proteolysis	pH variation
12	V2	*Lc* lactis subsp. *lactis*	RA	SAu	2.04	2.31
	V4	*Lc* lactis subsp. *lactis*	RA	Au	1.23	2.12
13	V3a	*Lc* lactis subsp. *lactis biovar. diacetylactis*	RA	SAu	1.36	1.82
	V3a	*Lc* lactis subsp. *cremoris*	RA	SAu	1.22-1.24	1.03-1.95
	V4	*Lc* lactis subsp. *cremoris*	RA	SAu	1.16-1.42	0.87-1.16
	V4	*Lc* lactis subsp. *cremoris*	R(A)	Au	1.40-1.42	0.88-1.45
	V5	*Lc* lactis subsp. *cremoris*	R(A)	SAu	1.20-1.24	0.42-1.38
	V7*	*Lc lactis* subsp. *lactis*	R(A)	SAu	1.43	0.44
14	V5	*Lc* lactis subsp. *cremoris*	R	Au	1.1	0.30
15	V6*	*Lc* lactis subsp. *cremoris*	R(A)	In	0.88	0.78
	V3b	*Lc* lactis subsp. *cremoris*	R(A)	In	1.33	0.70
16	V3b	*Lc* lactis subsp. *cremoris*	R	In	1.16-1.30	0.72-0.80
	V7*	*Lc lactis* subsp. *lactis biovar. diacetylactis*	R	In	1.14-1.39	0.83-0.89
17	V3b	*Lc* lactis subsp. *hordniæ*	R	Au	1.18	1.22
18	V7*	*Lc plantarum*	R	SAu	0.42	0.18
19	V1	*Lc* lactis subsp. *lactis biovar. diacetylactis*	RAC	Au	2.23-2.39	1.29-2.54
	V1	*Lc* lactis subsp. *lactis*	RAC	Au	1.82-2.31	1.94-2.75
	V2	*Lc* lactis subsp. *lactis biovar. diacetylactis*	RAC	Au	1.55-1.65	1.51-2.35
	V3a	*Lc* lactis subsp. *lactis biovar. diacetylactis*	RAC	Au	1.45-1.76	1.38-2.68
	V4	*Lc* lactis subsp. *lactis*	RAC	Au	1.47	1.90
	V3b	*Lc* lactis subsp. *lactis biovar. diacetylactis*	RAC	Au	1.58-1.67	2.07-2.24
20	V5	*Lc* lactis subsp. *cremoris*	R(A)	SAu	1.21-1.34	0.57-0.82
	V6*	*Lc* lactis subsp. *cremoris*	R(A)	In	1.53	1.94
	V3b	*Lc* lactis subsp. *hordniæ*	RAC	In	1.95	1.04
	V3b	*Lc* lactis subsp. *lactis biovar. diacetylactis*	RAC	In	1.57	1.40
	V3b	*Lc* lactis subsp. *cremoris*	RAC	In	1.32	1.04
21	V3b	*Lc* lactis subsp. *cremoris*	RAC	In	1.53	0.85
22	V7*	*Lc* lactis subsp. *lactis biovar. diacetylactis*	R	In	1.20-1.31	0.79-1.05
23	V1	*Lc raffinolactis*	RA	In	1.87	1.94
	V1	*Lc* lactis subsp. *cremoris*	R	Au	1.99	0.48
	V2	*Lc* lactis subsp. *cremoris*	R - RAC	SAu	1.26-1.37	1.14-2.11
	V3a	*Lc* lactis subsp. *cremoris*	RAC	SAu	1.18-1.59	1.97-2.29
	V3a	*Lc* lactis subsp. *lactis biovar. diacetylactis*	R	SAu	1.62-1.64	1.58-1.82
	V4	*Lc* lactis subsp. *cremoris*	R et RAC	SAu	1.15-1.45	1.11-1.74
	V4	*Lc* lactis subsp. *lactis*	R	SAu	1.18	1.76
	V5	*Lc* lactis subsp. *lactis biovar. diacetylactis*	RAC	SAu	1.63	1.63
	V6*	*Lc* lactis subsp. *lactis*	RAC	SAu	1.49-1.79	1.54-2.17
	V7*	*Lc raffinolactis*	RAC	SAu	1.53	2.73
	V7*	*Lc* lactis subsp. *lactis*	RAC	SAu	1.20-1.56	1.68-2.75

Growth on bactolithmus milk: R, reduction, A, acidification, C, coagulation. Letters in brackets indicate that these activities were rather weak. Autolysis: strong, Au, intermediate, In, and slight, SAu, autolytic potential.
Proteolysis: expressed in µmoles of glycine per mL.
pH variation: difference between initial and final pH after culture in milk for 16 h at 22°C.
*: Natural whey starter suspected of giving favour bitterness to cheeses.

ries. Clusters 1, 2, 3, 4, 5, 7, 8, 9, 10, 14, 17, 18, 21 and 22 contained strains that originated from only one NWS. The other clusters contained strains from two to seven different NWS.

When the behaviour of the different strains was observed within each NWS, the mean activities of all the isolates from the same NWS were rather close. For instance, the mean acidifying and proteolytic activities were equal to 1.85±0.59 pH units and 2.52±0.64 μmoles of glycine/mL for the V1 NWS and to 0.82±0.44 pH units and 1.27±0.13 μmoles of glycine/mL for the V5 NWS. Moreover, strains from V1 NWS and V5 NWS were shared among nine clusters for the V1 NWS and four clusters for the V5 NWS. It was also observed that the strain composition of the V2, V3b and V7 NWS was rather heterogeneous whereas the *Lactococcus* population of V3a and V6 was dominated by only two or three technological profiles.

The "bitter" V6 and V7 NWS were not composed of isolates that showed any specific characteristics. For instance, the mean proteolytic activity of the lactococci strains was equal to 1.61 and 1.27 μmoles of glycine/mL and the mean acidifying activity was equal to 1.80 and 1.44 pH units. These two parameters ranged from 1.27 to 2.52 μmoles of glycine/mL and from 0.82 to 1.89 pH units for the strains of the other NWS.

Proteolytic products of the V1 and V6 strains, *i.e.* from non-bitter and bitter NWS, were analysed by RP-HPLC (Fig. 1). The "hydrophobic" part of the chromatogram was specifically focussed on, since hydrophobic peptides are known to be linked with bitterness. It is interesting to note that the V6 strains induced the appearance of many hydrophobic compounds, both quantitatively and qualitatively compared to the V1 strains. However, this observation was not confirmed after comparison of the V3b and V7 strains (results not shown). This means that NWS probably contribute to bitterness along with other factors. This analysis was not relevant to *Leuconostoc* because there were so few isolates.

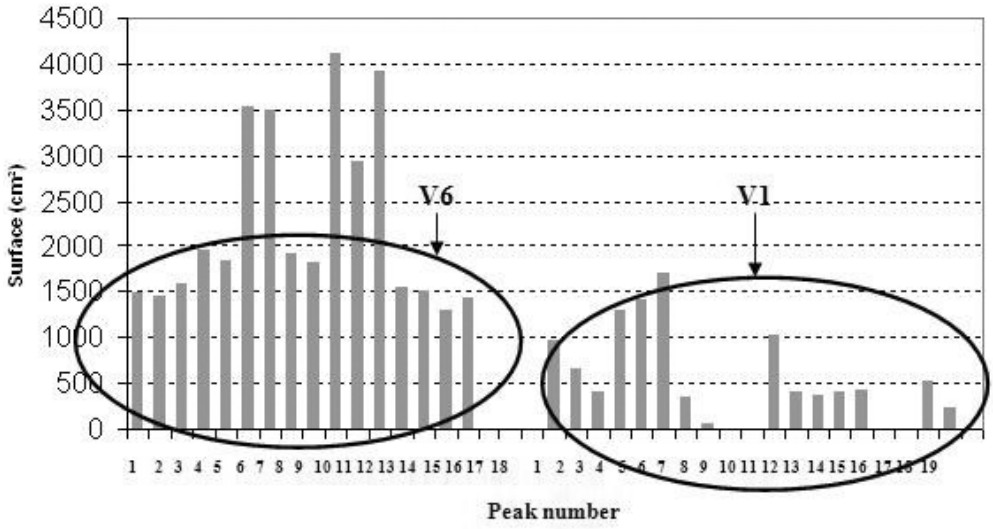

Fig. 1 - Comparison by RP-HPLC of the number and quantity of hydrophobic proteolytic compounds obtained after culture of V1 and V6 *Lactococcus* strains in milk for 24h at 22°C. V1 corresponds to a "normal" natural whey starter and V6 to a "bitter" NWS.

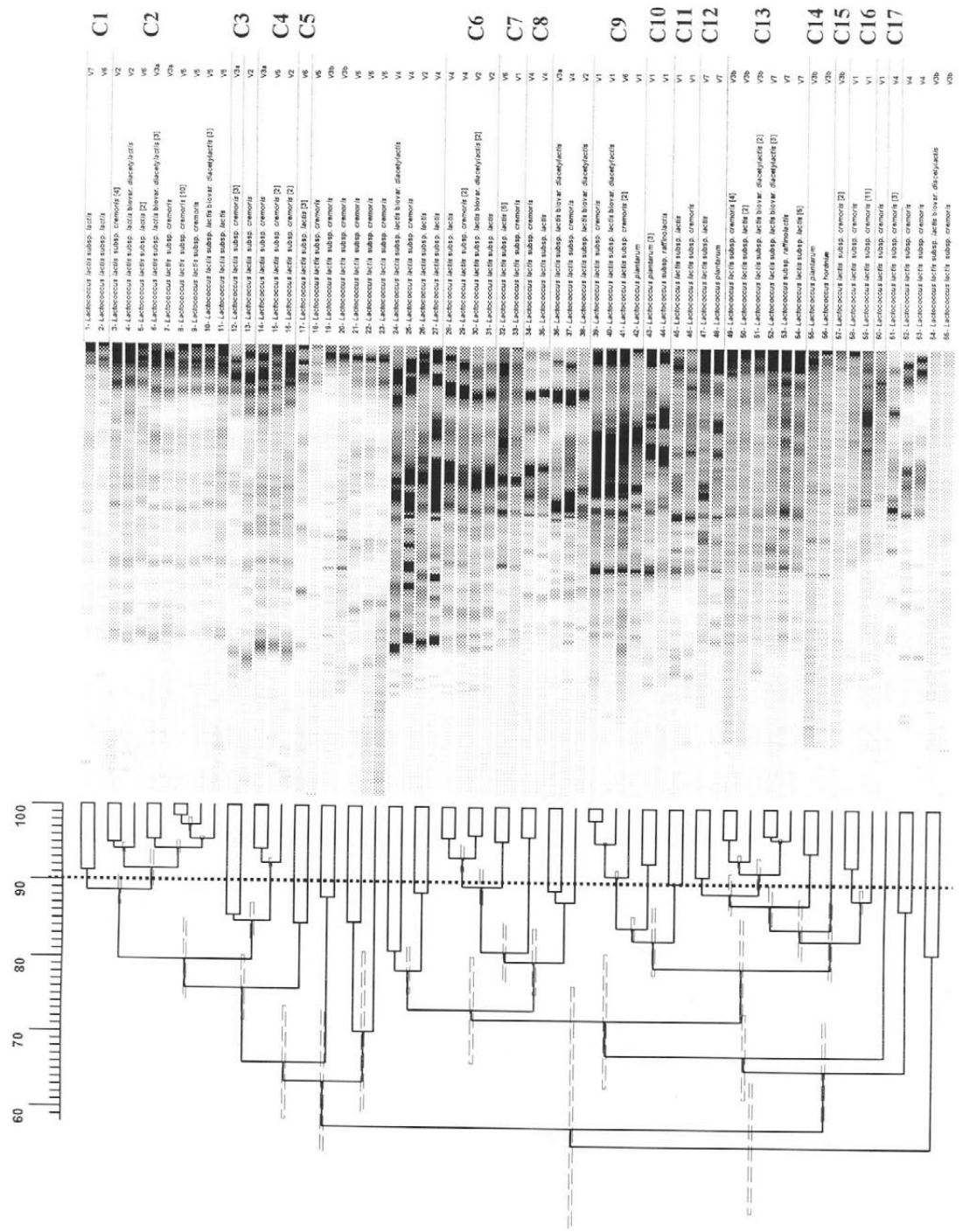

Fig. 2 - Dendrogram and whole-cell protein patterns clustering wild *Lactococcus* strains. Normalised patterns were analysed using Pearson's product moment correlation coefficient and were clustered by the UPGMA method. The coefficient of similarity (90%) is indicated on the figure by the dotted line. Values in brackets after the name of the bacteria refer to the total number of closely related patterns.

Whole-cell protein patterns

Whole-cell protein patterns were also used to characterise NWS and to compare strains. As noted by BARREAU and WAGENER (1990), *Leuconostoc* and *Lactococcus* strains are clearly discriminated at the genus, species and subspecies levels. However, the low number of *Leuconostoc* isolated did not allow interpretable results to be obtained. The analysis of the whole-cell protein patterns was therefore restricted to the *Lactococcus* isolates.

The *Lactococcus* strain group included 121 whole-cell protein patterns, distributed among the eight NWS, *i.e.* V1, 25, V2, 13, V3a, 10, V3b, 14, V4, 12, V5, 17, V6, 18 and V7, 13. Thirteen strains gave unusable profiles and were not kept. In the first analysis (results not shown) four main clusters were identified that contained more than nine strains and 11 smaller clusters that contained two to five strains. Based on a reproducibility test, the similarity coefficient was determined and fixed at 90%. A second analysis was made, keeping the most discriminating patterns. The final dendrogram, presented in Fig. 2, includes no more than 65 patterns divided into 17 strain clusters and 19 lone strains. The number of clusters reported in relation to the number of isolates is a rough indication of the tendency in strain diversity within each NWS.

The V1, V5 and V7 NWS seemed to be composed of a few dominant strains, the isolates shared among a few clusters, whereas the diversity of the V3b and V4 NWS was greater. The diversity of strains in the "bitter" NWS, V6 and V7 was not noteworthy in that the results were comparable with those of the V1 and V5 NWS. Consequently, the only difference which could partly explain the bitter defect is the ratio between the *lactis* and *cremoris* subspecies. Analysis of technological properties of strains gave different conclusions. The study of NWS diversity imposes the use of several different methods.

DISCUSSION

In this work, eight different natural whey starters used in the Rocamadour cheese-making technology from seven different cheese houses were analysed for their bacterial composition. It is difficult to compare these results with previous data because most of the published research deals with bacterial communities in the curd and during the ripening of goat cheeses, and many involve different technologies such as those used for hard cheeses.

Depending on the origin of the samples, differences were observed between the major populations as well as between genus, species, subspecies and strains. Such conclusions are not surprising. For instance, MAURIELLO *et al.* (2003) reported that the flavouring capability of different NWS was related to their geographical origin. GIRAFFA *et al.* (1998) and GATTI *et al.* (2003) indicated that *Lactobacillus helveticus* strains from NWS used to make Parmigiano Reggiano were dairy-linked, which means they are related to the source of the isolates. In a recent work, LAITHIER *et al.* (2004) also pointed out how feeding and milking practices influenced goat milk bacterial composition and resulting acidification curves. No attempt was made here to correlate edaphic or feeding conditions with microbial diversity because the number of NWS analysed was too low.

The lactococci, leuconostoc and citrate-positive bacteria were identified systematically in all the cheese houses except that of V6. Lactococci levels ranged from $10^{8.78}$ to $10^{9.77}$ cfu/mL. These results are in agreement with TORMO *et al.* (2004), although they observed greater variations, at least 2 log, between NWS. They also found significant levels of lactobacilli, from 10^{4} to $10^{7.5}$ cfu/mL,

after enumeration on MRS agar. However, it is possible that the colonies counted on MRS were not lactobacilli at all. ZARATE *et al.* (1997) and HATZIKAMARI *et al.* (1999) noted the predominance of lactococci in Tenerife and Anevato curd, but reported different results concerning lactobacilli.

We counted more leuconostocs than TORMO *et al.* (2004). However, the estimated ratio between lactococci and leuconostocs, ranging from 1.8 to 8.5, is congruent with previous research (ZARATE *et al.*, 1997; ALLONSO-CALLEJA *et al.*, 2002). The citrate-positive population, including lactococci, leuconostocs and lactobacilli strains, was counted because of the fundamental role played by citrate metabolism in flavour development during ripening (HUGENHOLZ, 1993). We observed high levels of citrate-positive bacteria in NWS. The importance of this population is probably linked to the typical creamy and buttery notes that characterise Rocamadour cheese.

TORMO *et al.* (2004) identified other microbial populations in NWS, although at lower levels: enterococci $\approx 10^2$ cfu/mL, yeasts and molds, $< 10^4$ cfu/mL, coliforms, $< 10^6$ cfu/mL and staphylococci, $< 10^4$ cfu/mL. In different goat milk curds, ALLONSO-CALLEJA *et al.* (2002) reported nearly the same populations, with varying levels. Although we actually detected enterococci, the levels were always lower than 10^5 cfu/mL and even as low as $10^{2.5}$ cfu/mL. The yeast and mould levels in two NWS were $10^{5.8}$ and $10^{6.0}$ cfu/mL, respectively. The other populations were not checked.

One-hundred-and-thirty-four isolates belonging to the genus *Lactococcus* and 31 to the genus *Leuconostoc* were characterised. Except for NWS V6 and V7, *Lc lactis* subsp. *cremoris* dominated *Lc lactis* subsp. *lactis*. *Lc lactis* subsp. *lactis* biovar *diacetylactis* was also identified which confirmed the prevalence of the citrate-positive population in the NWS. We only identified the species *Ln mesenteroïdes* with the two subspecies, *mesenteroïdes* and *cremoris*. These results are different from those reported in the literature on goat milk curds. TORNADIJO *et al.* (1995) ZARATE *et al.* (1997), OLARTE *et al.* (2000) and MAS *et al.* (2002) generally observed the dominance of *Lc lactis* subsp. *lactis* over *Lc lactis* subsp. *cremoris*, although the ratios could change radically from one cheese to another. Some articles also referred to the frequent presence of *Ln mesenteroïdes* subsp. *dextranicum*; this was never isolated during the course of the present work. For instance, CIBIK *et al.* (2000) isolated *Ln mesenteroïdes* subsp. *mesenteroïdes* and *Ln citreum* from traditional French cheeses. Such differences are not surprising considering the variety of cheese-making technologies. To be more precise, our study only used phenotypic behaviour to separate *Lc lactis* subsp. *cremoris* from *Lc lactis* subsp. *lactis*. Phenotypic characteristics may fail to properly discriminate *Lc lactis* subsp. *lactis* strains (CORROLER *et al.*, 1998). While *Lc lactis* subsp. *cremoris* is generally correctly identified irrespective of the technique used, *Lc lactis* subsp. *lactis* strains are sometimes misclassified when based only on phenotypic features. In earlier work (TORNADIJO *et al.*, 1995; ZARATE *et al.*, 1997; OLARTE *et al.*, 2000; MAS *et al.*, 2002), the frequency of *Lc lactis* subsp. *cremoris* may have been more important than indicated. On the other hand, it is unlikely to find any trace of other species or subspecies *i.e. Lc lactis* subsp. *hordniæ*, *Lc plantarum* and *Lc raffinolactis* in these articles. Although rarely characterised, these strains appeared to be subdominant. Nevertheless, it is necessary to use molecular methods, such as DNA/DNA hybridisation or species-PCR, to confirm the species identification of these isolates.

The presence of the subspecies *cremoris* may be surprising. The disappearance of this group of bacteria in raw milk has already been described by SALAMA *et al.* (1993).

The biodiversity of the eight NWS was studied by means of two different methods. The strains were characterised on the basis of their technological abilities and on their whole-cell protein patterns. The capability of whole-cell protein patterns to discriminate strains belonging to the genus *Lactococcus* and *Leuconostoc*, as described by BARREAU and WAGENER (1990) and ELLIOTT *et al.* (1991), was also studied. They noted that the technique could not be used to separate the different species and we confirm this observation.

Significant results were obtained for the *Lactococcus* group. As previously stated, we confirmed the observations of GIRAFFA *et al.* (1998), GATTI *et al.* (2003), MAURIELLO *et al.* (2003) and especially of FORTINA *et al.* (1998) who, in part, used a similar approach. The use of the technological abilities of the strains and the whole-cell protein patterns allowed the NWS to be divided into three groups. Some NWS were dominated by a few strains, while others showed greater diversity. The third group included NWS which could not be classified in the first or second groups. However, the use of two methods led to different conclusions; the NWS classifications were not identical. Each method gave its own information. The first one gave the technological identification of the strains which is of interest to cheese-makers.

It is important to note that the stain diversity of some NWS was less important irrespective of the technique used. The domination of one or two strains could then lead to a phage infection which would explain the appearance of acidification defects frequently observed in Rocamadour.

This work was undertaken because of recurrent problems linked to the use of NWS. In detail, the V6 and V7 NWS were reported to cause bitterness in cheese. In the case of Rocamadour cheese, bitterness is probably the result of an impaired activity of the starter (BERGÈRE and LENOIR, 1997). In the cheeses made with the two NWS, no *Pseudomonas*, a common agent frequently implicated in bitter peptide production, were found. Even though *Lactococcus* strains of V6 and V7 presented very low similarity levels, they did share one specific feature: in both cases, *Lc lactis* subsp. *lactis* strains dominated over *Lc lactis* subsp. *cremoris* strains. V6 also seemed to be characterised by the absence of *Leuconostoc*. V6 NWS strains tended to produce high quantities of rather hydrophobic peptides. BERGÈRE and LENOIR (1997) reported that there is a greater risk of bitterness if lactic acid bacteria grow and acidify quickly. However, the acidification abilities of the V6 and V7 strains did not differ from the other strains. These first results constitute initial guidelines and, as such, will have to be confirmed by analysing other "bitter" NWS.

CONCLUSION

The study of eight Rocamadour NWS showed that they are composed of different bacterial communities. In particular, bitterness, in the absence of *Pseudomonas* or other identified factors, could be associated with a change in the ratio between *Lc lactis* subsp. *lactis* and *Lc lactis* subsp. *cremoris*. The role of *Leuconostoc* is unclear. Populations of lactobacilli and enterococci, which are of far less importance, may interfere with the major flora. These hypotheses must be studied further by extending our observations to other "bitter" and non-bitter NWS as well as to other microbial populations and by integrating the dynamic component of these ecosystems.

ACKNOWLEDGEMENTS

A part of this work was recently presented at "Produits alimentaires fermiers" -farmhouse made products- Congress (DEMARIGNY *et al.*, 2004).

The authors would like to thank N. Ballot and G. Durand for re-reading the paper and for their kind advice concerning the writing of it. They also thank M. Chareyron for the English corrections and L. Dousseau for the Italian translation. This project was supported by the Massif Central CDO cheese association (Pôle Fromager AOC Massif Central), the Rocamadour cheese maker association (Syndicat des producteurs de fromages Rocamadour) and the regional council of Midi-Pyrénées (France).

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Revised paper received April 20, 2006 Accepted June 8, 2006

PAPER

FUNCTIONALITY OF BREAD MADE WITH PASTEURIZED WHEY AND/OR BUTTERMILK

LE PROPRIETÀ FUNZIONALI DEL PANE PRODOTTO CON SIERO PASTORIZZATO E/O LATTICELLO

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ABSTRACT

The dairy by-products whey and buttermilk were pasteurized (74°C 20 s) and used to replace water in white wheat bread-making at the following combinations: A: 100% water (control dough); B: 50% whey + 50% water; C: 100% whey; D: 50% buttermilk + 50% water; E: 100% buttermilk. The effects of whey and buttermilk on dough properties, bread characteristics and mineral content of white wheat bread were investigated. The water absorption, stability time, development time and

RIASSUNTO

Il siero ed il latticello, sottoprodotti dell'industria lattiero casearia, sono stati pastorizzati (74°C 20 s) ed utilizzati al posto dell'acqua nella produzione di pane con farina bianca nelle combinazioni seguenti: A: 100% di acqua (pasta di controllo); B: 50% di siero + 50% di acqua; C: 100% di siero; D: 50% di latticello + 50% di acqua; E: 100% di latticello. Sono stati studiati gli effetti del siero e del latticello sulle proprietà della pasta, sulle caratteristiche del pane e sul contenuto in minerali della fari-

- Key words: buttermilk, dough properties, mineral content, wheat bread, whey -

softening degree of the bread doughs in a Brabender farinograph improved (P<0.05) as the whey and buttermilk addition increased resistance to extension (Rmax) and energy values (P<0.05) in an extensograph compared to control dough. The greatest improvement in those values was obtained with 100% buttermilk replacement. Replacement of pasteurized whey and buttermilk at 50% and/or 100% resulted in a better sensory score than the control sample. No significant differences (P>0.05) were recorded in bread volume and crumb firmness values between fresh control bread and breads containing whey and buttermilk. However, use of whey and buttermilk had a retarding effect on crumb firmness scores after a 72 h storage period. Replacement of water with whey and buttermilk in the bread recipe significantly (P<0.05) increased macro (Na, K, Ca, Mg and P) and micro mineral (Zn, Mn, Cu and Fe) contents except for Na and Mn in sample B.

na bianca. L'assorbimento di acqua, la stabilità nel tempo, il tempo di lievitazione e il grado di sofficità degli impasti miglioravano quando analizzati in un farinografo Brabender (P<0,05), mentre l'aggiunta di siero e di latticello aumentava la resistenza all'allungamento (Rmax) e il valore di energia (P<0,05) quando misurati in un estensografo, in confronto all'impasto di controllo. Il miglioramento più rilevante in questi valori era stato ottenuto con la sostituzione di acqua con il 100% di latticello. La sostituzione di acqua con siero e con latticello al 50% e/o al 100% comportava un migliore impatto sensoriale rispetto al campione di controllo. Differenze non significative (P>0.05) erano state rilevate per quanto riguardava il volume del pane e il raffermamento della mollica fra il pane fresco di controllo ed i pani contenenti siero e latticello. Tuttavia, l'impiego di siero e latticello presentavano un effetto ritardante sul raffermimento della mollica dopo un periodo di 72 ore di stoccaggio. La sostituzione dell'acqua con siero e latticello nella ricetta del pane incrementava significativamente (P<0.05) i contenuti in macro (Na, K, Ca, Mg and P) e micro minerali (Zn, Mn, Cu and Fe) ad eccezione del Na e del Mn nel campione B.

INTRODUCTION

Dairy ingredients are added to bakery products in various forms (whole milk powder, skim milk powder, whey powder and/or whey protein concentrates) to increase nutritional and functional properties. Nutritional properties include increasing calcium and protein content, and supplementing the essential amino acids including lysine, methionine and tryptophan. Functional properties of dairy ingredient incorporation include improvement of crust color, bread flavour, crumb structure and crumb texture. These functional benefits are a direct result of the effects of milk fat and protein (ERDOGDU *et al.*, 1996; YOUSIF *et al.*, 1998; SANCHEZ *et al.*, 1998; HOLTON and HASP, 1999; KENNY *et al.*, 2000; GALLAGHER *et al.*, 2003).

Whey is a by-product of cheesemaking in which the butterfat, casein and some lactose and mineral components are separated from the milk in the form of curd. The remaining milk serum, or whey, retains practically all of the wa-

ter-soluble vitamins and minerals of the milk, and most of its lactose and non-casein proteins. Buttermilk is the principle by-product of butter production. When cream is churned, butter, representing the fat phase of the emulsion, and buttermilk, representing the aqueous phase, are obtained. In addition to the water-soluble constituents of the cream, buttermilk consists of some milk fat that did not separate with the butter, and the phospholipid-rich membrane material that originally surrounded each fat globule (PYLER, 1988).

The manufacture of cheese and butter constitute a major part of the Turkish dairy industry. Unfortunately, nearly all of the whey and buttermilk by-products are discarded. Bread is the principal food of Turkish people and daily average consumption is around 400 g per capita. Particularly in rural areas, bread consumption exceeds the national average and more than half of the daily caloric intake comes from bread (ANON., 1987). In this study, the effects of adding pasteurized whey and buttermilk on bread dough properties, bread characteristics and mineral content were investigated.

MATERIAL AND METHODS

Materials

The flour used in the present study was a commercial wheat flour (Eks Un Com., Tekirdag, Turkey) which contained 13.5% moisture, 11.8% protein (wet basis) and 0.58% ash (dry basis). Compressed yeast (Pakmaya Com., İzmit, Turkey) was purchased from a local market, and pasteurized whey and buttermilk (at 74 °C for 20 s) were provided by a local dairy (Unallar Dairy Com., Tekirdag, Turkey). Table 1 summarizes the composition of the pasteurized whey and buttermilk used in the breadmaking. The dry matter was determined by heating (5.0 g) at 105°C to constant weight and the fat content was estimated by the Gerber method. The protein content was determined using a Kjeldahl method (AOAC, 1990).

Dough properties and formulation

Rheological properties of dough samples were tested using a farinograph and extensograph according to the ICC standard methods no. 115 and 114, respectively (ICC, 1972). Each result is the average of three determinations.

A traditional formula was used in dough preparation, and ingredients included 3% yeast and 2% salt on a flour weight basis. Water addition was calculated as farinograph water absorption at 500 BU (Brabender Units). Five different dough samples were prepared according to the percentage of water replacement with pasteurized whey and buttermilk, and each sample was coded with a letter A, B, C, D and E. Combinations were as follows: A: 100% water (control dough), B: 50% whey + 50% water, C: 100% whey, D: 50% buttermilk + 50% water, and E: 100% buttermilk.

Table 1 - Composition (%) of pasteurized whey and buttermilk.[a]

Ingredient	Dry Matter	Fat	Non-fat dry matter	Protein
Whey	6.2	0.5	5.7	0.85
Buttermilk	9.5	0.6	8.9	0.75

[a]Mean values of three determinations.

Bread-making procedure and baking tests

A straight dough process was used to prepare the bread samples. Doughs containing 1000 g of flour were mixed for 15 min, and fermented in bulk for 90 min at 30°C and 85% relative humidity. Next, the dough was punched and moulded by hand. Dough pieces (250 g) were put into ½-kg pans and proofed under the above-mentioned conditions for 30 min, then baked in an electric oven (PFS-9 Ozkoseoglu, Istanbul-Turkey) for 35 min at 220°C. The loaves were cooled to room temperature and evaluated for quality properties. Loaves which were stored for crumb firmness were packed in polyethylene bags (5 g/m^2/ 24 h water vapour permeability) and stored at 18-20°C.

Bread quality

Weight, specific volume, crumb firmness, crumb and crust colour were determined and sensory evaluation was carried out. Bread volume was determined using rapeseed displacement. Crumb firmness was measured at 2 h, 24 h and 72 h after baking using a LFRA Texture Analyzer TA-1000 model (CNS Farnel, UK). A 25 mm diameter aluminium cylindrical probe was used in the compression test. The probe speed during the test was set at 2 mm/s and the compression distance was 10 mm. The resulting peak force was measured in Newtons. In the crumb firmness analysis, six 2.5-cm thick slices were measured and the average result is reported. Crumb and crust colour were evaluated using a Hunter Lab DP-9000 optical sensor D-25A, Sterling, Virginia, USA (Standard of 45° Tristimulus Color, Standard No C2-34548, References CIE Illuminant C. 2°, Observer Standard Perfect Reflecting Diffuser Pub. CIE No 15) and Hunter *L* values were recorded as the measure of lightness. Sensory evaluation of bread samples was carried out by 10 trained panelists (academic staff) using a one to five hedonic scale for the overall acceptability (1=very poor, 2=poor, 3=fair, 4=good, 5= very good).

Mineral content

Bread samples were cut into small cubes and homogenized. For the determination of elements, samples were ashed at 550°C. Ashed samples were extracted with dilute hydrochloric acid. Magnesium, iron, zinc, manganese and copper concentrations were determined with a Shimadzu AA-660 Model Atomic Absorption Spectrophotometer-Kyoto/ Japan (AOAC, 1990 and ANON., 1990). Sodium and potassium concentrations of the samples were determined with a Jenway PFP 7 Model Flame Photometer and phosphorus content with a Pye Unicam PU 8600 UV/Vis Model spectrophotometer. Calcium concentration of the samples was determined by the titrimetric method (AOAC, 1990). To avoid interference, the final solutions for the calcium and magnesium determinations contained about 1% lanthanum. Results are given as mean values of three replications on a dry basis.

Statistical analysis

The analytical data collected from three replications of each treatment were subjected to analyses of variance and multiple range tests. The analysis was carried out using the MSTAT software program, and the least square difference test was applied in the case of high significance. Significant differences were determined at the $P<0.05$ level.

RESULTS AND DISCUSSION

Dough properties

Farinograph data of the samples including the control dough (A) and those in which water was replaced with pas-

teurized whey and buttermilk are presented in Table 2. The water absorption increased significantly ($P<0.05$) in the samples in which whey and buttermilk were added. The dough sample with 100% buttermilk (E) had the highest water absorption (63.4%) compared to the other replacement levels. The higher water absorption of dough samples B, C, D and E may have been due to the increased hydration capacity of the two dairy by-products, particularly buttermilk. This increase could be attributed to the water-soluble components of whey and buttermilk such as lactose, albumins and globulins. It has been well established that milk solids influence dough absorption. It is a general rule that dough absorption increases by a percentage equal to that of the dairy product used. (PYLER, 1988). On the other hand, ERDOGDU *et al.* (1996) found that bread dough prepared with acid whey powder required 1% more water than did the control when heated at 80°C.

Dough development time and dough stability, which are indicators of dough strength, also increased significantly ($P<0.05$) in all the replaced doughs. As for water absorption, sample E had the longest development time (5.5 min) and stability time (12.2 min). The significant increase in development and stability time of the whey- and buttermilk-added samples may be due to the physico-chemical properties of whey and buttermilk which contain different protein sources, milk fat and lactose. According to Table 2, replacement with whey and buttermilk decreased the softening degree significantly ($P>0.05$) compared to the control and the lowest value (50 BU) was obtained for 100% buttermilk addition. There was no difference between 50% and 100% whey addition levels with respect to softening degree.

There is general agreement about the link between extension testing and baking performance. A good loaf volume is achieved if the gas bubbles within the fermented dough expand with minimal rupturing during proofing and baking. Therefore, baking performance is related to the interplay between resistance to extension (Rmax) and extensibility (ANDERSSEN *et al.*, 2003). Extensograph results for the samples are given in Table 3. Doughs replaced with whey and buttermilk had significantly higher Rmax values ($P<0.05$) compared to the control sample. The highest Rmax value occurred for sample E with a value of 440 BU. Energy value, on the other hand, is a good indicator of flour strength and bread properties. Whey and buttermilk addition increased the energy value significantly ($P<0.05$) and the highest value was measured in sample E (76 cm^2). However, the replacement of water with whey and buttermilk decreased the extensibility and the low-

Table 2 - Effects of replacing water with pasteurized whey and buttermilk on farinograph measurements.[1]

Doughs[2]	Water absorption (%)	Development time (min)	Stability time (min)	Softening degree (BU)
A	59.0d	1.4c	5.7e	90a
B	60.8c	4.4b	7.0c	80b
C	62.3b	4.2b	6.5d	80b
D	62.1b	5.1a	10.3b	60c
E	63.4a	5.5a	12.2a	50d

[1]Means in the same column with different letters as superscripts are significantly different ($P<0.05$).
[2]A: control, B: 50% whey+50% water, C: 100% whey, D: 50% buttermilk+50%water, E: 100% buttermilk.

Table 3 - Effects of replacing water with pasteurized whey and buttermilk on extensograph measurements[1] (values at 135 min, Rmax maximum resistance to extension).

Doughs[2]	Rmax (BU)	R (BU)	Extensibility (mm)	Energy (cm^2)
A	300e	210e	156a	61
B	360d	260d	153b	65
C	410b	300b	152b	72
D	380c	280c	152b	68
E	440a	320a	148c	76

[1]Means in the same column with different letters as superscripts are significantly different (P<0.05)
[2]A: control, B: 50% whey+50% water, C: 100% whey, D: 50% buttermilk+50% water, E: 100% buttermilk.

est extensibility value (148 mm) was obtained from the dough in which water was replaced with 100% buttermilk. In general, the increase in Rmax and energy values, and the decrease in extensibility must have been the result of the dough strengthening effect of the milk solids. They must have enhanced the molecular exothermic reaction associated with dough formation (GRAS *et al.*, 2000). The use of dairy ingredients improves the handling properties of bread dough, as well as the bread quality. However, the complexity of the bread-making system, including several stages of processing and interaction among the components, makes it difficult to predict the performance of a particular dairy product (ERDOGDU *et al.*, 1996). It is well known that the reduced mixing properties of bread dough are due to uncomplexed β-lactoglobulin (whey protein) and **k**-casein (casein subunit) in non-heated dairy ingredients. The b-lactoglobulin and **k**-casein, when heated together, interact through disulfide linkages; therefore, heat treatment of dairy ingredients increases dough stability (SAWYER, 1969; LING *et al.*, 1976; ERDOGDU *et al.*, 1996; KADHARMESTAN *et al.*, 1998). In our study, the increase in Rmax and energy values, and the decrease in extensibility may be attributed to the dough strengthening effect of pasteurized whey, particularly buttermilk, due to better disulfide linkages. Our results on dough properties are in agreement with the findings of YOUSIF *et al.* (1998).

Bread characteristics

The effect of pasteurized whey and buttermilk on the specific volume of bread is shown in Fig. 1. Replacement of water with pasteurized whey and buttermilk did not significantly decrease the specific volume of bread compared to the control. Addition of 50% and 100% whey or buttermilk produced breads with similar specific volumes. The slight decrease in specific volume could be related to the way that the proteins in whey and buttermilk interact with the flour and yeast to form a dough. KENNY *et al.* (2000) reported that bread made with whey protein concentrates generally had less volume compared to the control made without dairy ingredients. GELINAS *et al.* (1995) also found that incorporation of fermented dairy ingredients in a pan bread formulation decreased specific volume. However, the loss of loaf volume was reversed by heating the dairy ingredients at 80°C (ERDOGDU *et al.*, 1996). On the other hand, LING *et al.* (1976 and 1977) showed that the addition of nonfat dry milk increased loaf volume if the level of potassium bromate increased. This supports the idea that whey and butter-

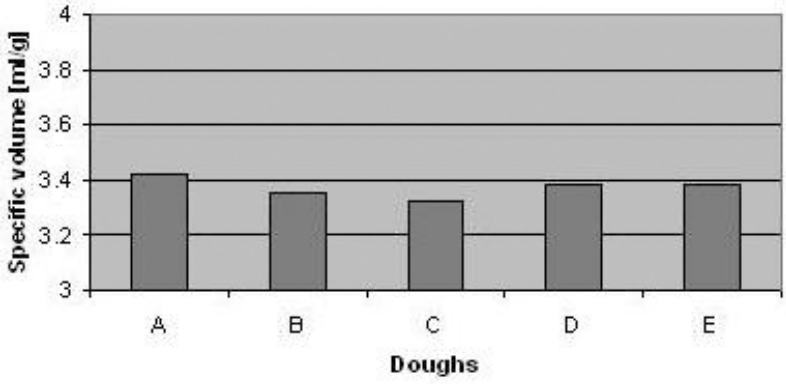

Fig. 1 - Effect of replacing water with pasteurized whey and buttermilk on specific volume of breads.
A: control, B: 50% whey+50% water, C: 100% whey, D: 50% buttermilk+50% water, E: 100% buttermilk.

milk proteins have a negative effect on loaf volume.

Fig. 2 shows Hunter *L* values determined for crumb and crust colours of bread samples. Crumb colour of sample B and D were slightly darker compared to the control bread. However, 100% whey (sample C) and 100% buttermilk addition (sample E), significantly changed ($P<0.05$) the Hunter L value associated with a crumb colour. An important change (darkening) in crust colour was observed when water was replaced with 100% buttermilk (E). This was attributed mainly to Maillard and caramelization reactions which occurred more intensively during baking due to the higher lactose and protein content of samples C and E compared to the other samples. The higher lactose content of whey and buttermilk, combined with the greater reactivity of albumin with reducing sugars, favours a more pronounced crust coloration by the Maillard reaction and caramelization (PYLER, 1988; YOUSIF *et al.*, 1998; KENNY *et al.*, 2000).

Fig. 3 shows the crumb firmness values of the bread samples measured 2 h, 24 h and 72 h after baking. The fresh

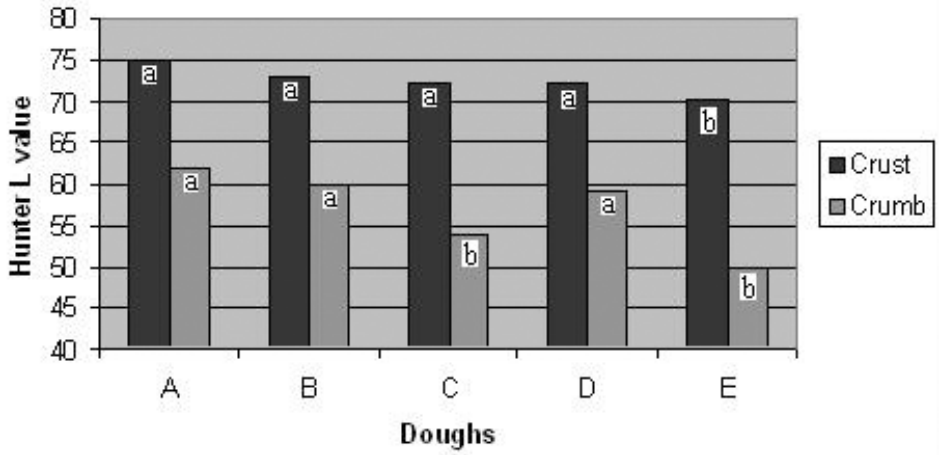

Fig. 2 - Effect of replacing water with pasteurized whey and buttermilk on crumb and crust colour of breads.[1]
[1]Means in the same bar with different letters as superscripts are significant1y different ($P<0.05$)
A: control, B: 50% whey+50% water, C: 100% whey, D: 50% buttermilk+50% water, E: 100% buttermilk.

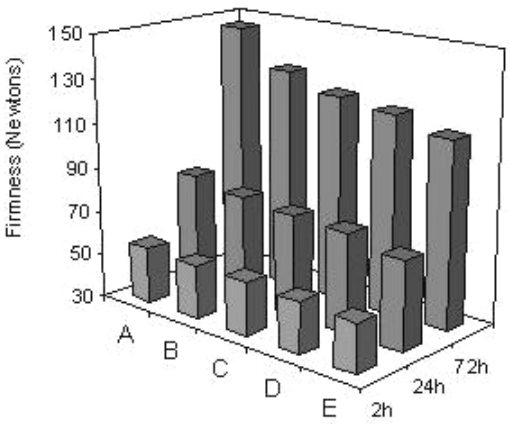

Fig. 3 - Effect of replacing water with pasteurized whey and buttermilk on crumb firmness of breads.
A: control, B: 50% whey+50% water, C: 100% whey, D: 50% buttermilk+50% water, E: 100% buttermilk.

whey and buttermilk breads had firmness values similar to the control and the differences in firmness among fresh breads were not significant. The crumb firmness values of the samples were lower than the control after 72 h of storage. Replacement of water with 100% pasteurized buttermilk gave the lowest firmness value after 72 h which indicates softer crumb. Previous research results indicated that the addition of heat-treated dairy ingredients slowed bread staling and extended its keeping quality. Some improvement in crumb softness was also obtained with lactose (VOLPE and ZABIK, 1975; YOUSIF *et al.*, 1998; ERDOGDU *et al.*, 1996).

The average sensory scores for the overall acceptability of bread samples are shown in Fig. 4. In general, panelists preferred breads with whey and buttermilk added. Their overall acceptability scores were slightly higher than those for the control sample. No significant differences ($P<0.05$) were found among the bread samples for the overall acceptability score, except for the 100% whey-added sample (C). Bread C was the most popular and the differences in the scores were significant ($P<0.05$) compared to the control. These results on bread acceptability agree with those reported by YOUSIF *et al.* (1998) and ERDOGDU *et al.* (1996) for heat-treated whey, and with KENNY *et al.* (2000) for whole and skim milk powders, but contrast with the results of SANCHEZ *et al.* (1998) for whey protein concentrate.

Mineral content

Conversion of wheat into white flour by milling reduces the original level of nutrients in the wheat. Therefore, the mineral content of bread varies considerably within and between different bread types depending on the flour processing and on the ingredients used in the recipe (POMERANZ, 1987; TAHVONEN and KUMPULAIEN, 1994). Table 4

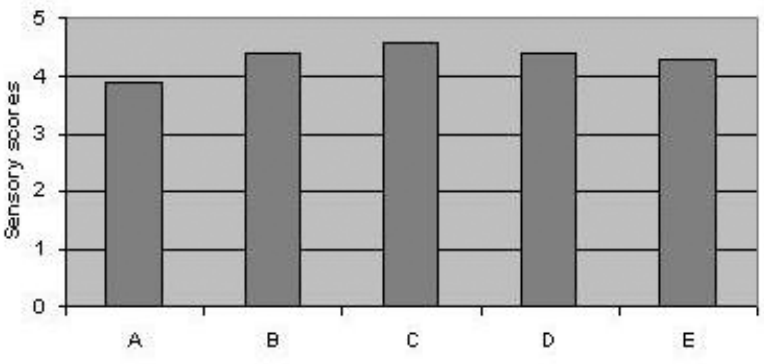

Fig. 4 - Effect of replacing water with pasteurized whey and buttermilk on sensory scores for overall acceptability of breads.
A: control, B: 50% whey+50% water, C: 100% whey, D: 50% buttermilk+50% water, E: 100% buttermilk.

Table 4 - Effects of replacing water with pasteurized whey and buttermilk on mineral content of breads (on dry basis).

	Macro minerals (mg/100 g)					Micro minerals (mg/kg)			
Bread[2]	Na	K	Ca	Mg	P	Zn	Mn	Cu	Fe
A	580d	180d	115d	240d	116d	7.1d	9.9d	3.3c	12.6d
B	590d	225c	178c	255c	137c	7.4c	10.0d	3.3c	13.l c
C	640c	263a	215b	265b	146b	7.8b	10.5c	3.4bc	14.4b
D	680b	229c	216b	267b	141c	7.7b	11.0b	3.5b	14.3b
E	750a	252b	292a	274a	162a	8.7a	11.3a	3.8a	14.9a

[1]Means in the same column with different letters as superscripts are significantly different (P<0.05).
[2]A: control, B: 50% whey+50% water, C: 100% whey, D: 50% buttermilk+50% water, E: 100% buttermilk.

presents the macro- and micro-mineral concentrations of the bread samples. The replacement of water with pasteurized whey and buttermilk increased the macro mineral concentrations, particularly the calcium and potassium levels (P<0.05) of the bread samples compared to the control sample. Breads in which water was replaced with 100% whey or buttermilk had higher levels of both macro and micro minerals (P<0.05) than those of other bread samples. However, the greatest increase was observed in the 100% buttermilk-added bread (E) which had 153% more calcium and 40% more potassium and phosphorous compared to the control sample. These results are due to the whey and buttermilk by-products which are a good source of these minerals. The calcium content in whey and buttermilk-added bread was higher than that reported by MOUSA *et al.* (1992), DAGLIOGLU and TUNCEL (1999) and ISSERLIYSKA *et al.* (2003). On the other hand, the micro mineral concentrations of the whey and buttermilk replaced breads increased at various levels compared to the control; the greatest increase was recorded for zinc (23%) in 100% buttermilk-added bread (E). Except for iron, the micro mineral contents of the samples were higher than those reported by ISSERLIYSKA *et al.* (2003) for white wheat bread, and similar to those of TAHVONEN and KUMPULAIEN (1994) for French bread and Finnish coffee bread.

A significant proportion of the human population appears to be at risk due to a deficiency in one or more micronutrients. Since bread is a major dietary source for the population, whey and buttermilk could be used as functional ingredients to improve the mineral and micronutrient contents. Moreover these dairy by-products improve bread quality, retard staling and promote the non-enzymatic browning, which has a positive effect on product appearance and flavour development.

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Paper received January 8, 2006 Accepted May 26, 2006

PAPER

ACID HYDROLYSIS OF POTATO TUBER SPROUT GLYCOALKALOIDS AND KINETICS OF SOLANIDINE EXTRACTION IN THREE-PHASE SYSTEMS

IDROLISI ACIDA DEI GLICOALCALOIDI DI GERMOGLIO DI TUBERO DI PATATA E CINETICHE DI ESTRAZIONE DI SOLANIDINA IN SISTEMI A TRE FASI

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ABSTRACT

A solid-liquid-liquid system was investigated in order to find the optimal conditions for a simpler, faster procedure for obtaining solanidine. The system consists of dried and milled tuber sprouts of potato (*Solanum tuberosum* L.) as the solid phase, solutions of hydrochloric acid in aqueous acetic acid, methanol or ethanol as the first liquid phase, and chloroform, trichlorothylene or carbon tetrachloride as the second liquid phase. The procedure combines the processes of glycoalkaloid extrac-

RIASSUNTO

È stato valutato un sistema solido-liquido-liquido al fine di determinare le condizioni ottimali per un procedimento di estrazione di solanidina semplice e veloce. Il sistema consiste in germogli di tubero di patata, essiccati e macinati, come fase solida, soluzioni di acido cloridrico in soluzione acquosa di acido acetico, metanolo od etanolo, come prima fase liquida, cloroformio, tricloroetilene, e tetracloruro di carbonio, come seconda fase liquida. Il procedimento combina i processi di estrazione di gli-

- Key words: glycoalkaloids, hydrolysis, potato, solanidine, tuber sprouts -

tion and hydrolysis and solanidine extraction into a single step. The degree of solanidine hydrolytic extraction was 65% and the corresponding solanidine yield was 1.02 g per 100 g of dried tuber sprouts. The method can be modified for analytical purposes.

coalcaloidi e l'estrazione di solanidina in un unico step. Il grado di estrazione idrolitica di solanidina era del 65% e la corrispondente resa in solanidina era 1,02 g per 100 g di germogli di tubero essiccati. Il metodo può essere modificato per scopi analitici.

INTRODUCTION

Potatoes are an excellent source of carbohydrates (starch and free sugars), amino acids, and good quality proteins (RODRIGUEZ-SAONA and WROLSTAD, 1997). They are consumed throughout the world and are an important part of the diet (BU-CONTRERAS and RAO, 2002), being prepared in different ways (SEVERINI *et al.*, 2004). They also contain biologically active compounds, including the inhibitors of digestive enzymes, polyphenols and steroidal glycoalkaloids (FRIEDMAN *et al.*, 1992; 1997). All parts of the potato plant contain steroidal glycoalkaloids (GAs). The GA content is the highest (6 - 50 g/kg) in dried tuber sprouts; it is almost ten times higher than in the dried haulm (3 - 6 g/kg) (POGORELOVA, 1968). STANKOVIC (1984) showed that the potato cultivar Désirée contained a higher GA content in the haulm (5.2 g/kg) than cvs Kenebec and Ostara (4.6 and 1.7 g/kg, respectively).

The procedure of GA hydrolysis, which exploits the difference in polarity between GAs and their aglycones by using a two phase liquid-liquid system, was first proposed by GELDER (1984). The hydrolysis medium consisted of pure GAs dissolved in a mixture of methanol and chloroform in ratio of 2:1 v/v, resulting in two immiscible phases. The polar GA is hydrolyzed in the acid phase and the nonpolar aglycone formed is continuously withdrawn from the acid by the organic liquid. Such a system has a reaction mixture temperature below 85°C compared to 97°C in a one-phase process. The lower reaction temperature has a favorable influence on hydrolysis and prevents the loss of aglycones which usually occurs during hydrolysis (GELDER, 1984). In preliminary experiments the partitioning of solanine and its hydrolytic products in a two-phase system consisting of hydrochloric acid (HCl) and chloroform, carbon tetrachloride or hexane, was investigated. By using chloroform, solanidine is quantitatively protected from dehydration to solanthrene (GELDER, 1984). A similar system consisting of aqueous HCl and toluene for the isolation of solanidine and other steroidal alkaloids was explored by WEISSENBERG (2001). It was described as a "one-pot" process, combining in a single step, direct acid hydrolysis of the glycosides in the plant material and extraction of released aglycones after alkali treatment. Different two-phase systems for GA extraction and hydrolysis, such as a liquid-liquid (STANKOVIC and NIKOLIC, 2002) and a solid-liquid system (NIKOLIC and STANKOVIC, 2003) have been explored. The liquid-liquid system consisted of GA extracts to which HCl was added as the first liquid phase followed by chloroform as the second liquid phase. The solid-liquid system consisted of dried and milled potato haulm as the solid and an ethanol solution of HCl mixed with chloroform as the liquid phase. In addition to acid hydrolysis, hydrolysis of GAs by enzymes present in plant material (GUSEVA and PASESHNICHENKO, 1957; STANKOVIC *et al.*, 1994) or by enzymes previously ob-

tained from plant material (NIKOLIC and STANKOVIC, 2005) is possible.

The research in this paper deals with the kinetics of solanidine extraction from tuber sprouts in different solid-liquid-liquid systems in an attempt to choose an optimized system for GA hydrolysis and solanidine isolation, because solanidine is an important intermediary in the chemical degradation to steroidal hormones (GASI *et al.*, 1996). In these systems, three different processes were combined into a single step: GA extraction from tuber sprouts, GA acid hydrolysis and solanidine extraction by organic liquid phase. Three-phase systems make the procedure for solanidine isolation simpler, faster and more economical. The term "hydrolytic extraction of solanidine" is used to indicate these processes. Using tuber sprouts in which GA content is the highest, higher solanidine yield can be obtained. Since tuber sprouts are a secondary product in potato production the starting material for obtaining solanidine is free. Further modifications of this method for analytical purposes are possible and could be the subject of a future investigation.

MATERIALS AND METHODS

Plant material

Tuber sprouts of potato (*Solanum tuberosum* L.) cv Désirée were grown at 20-22°C for 60 days in the dark. Then they were dried at room temperature to a moisture content of about 10 % and milled to an average particle size of 0.21 mm.

Quantification of glycoalkaloids

The glycoalkaloid (GA) content was determined spectrophotometrically by using methyl orange as the color reagent (BRINER, 1969), and by modifying the method of TUKALO and TSARIK (1970) as follows: the tuber sprouts (0.5 g) were treated with 10 mL of 100 g/L HCl in ethanol (95% vol.) at boiling temperature under reflux for 30 min and then filtered. The filtrate was evaporated until dry under vacuum and the dry residue was dissolved in 10 mL of 20 g/L aqueous solution of acetic acid at boiling temperature under reflux for 30 min. Then the pH of the cooled solution was adjusted to 4.0 by adding aqueous sodium hydroxide (first with a solution of 500 g/L and then by a solution of 10 g/L). The solutions were transferred to a separatory funnel and 2 mL of 0.5 g/L aqueous methyl orange was added for complex formation. The yellow solanidine complex was extracted with chloroform (5 times with 5 mL), the chloroform layer was dried with anhydrous sodium sulfate and put in a volumetric flask (25 mL). The flask was filled to the mark with chloroform and the absorbance (A) was read at 420 nm (UV-ViS Spectrophotometer, Lambda V, Perkin Elmer, Wellesley, MA, USA). The glycoalkaloid content of was determined on the standard curve as GAs (mg) = 1.05 x A.

Ratio of α-solanine to α-chaconine

The tuber sprouts (0.5 g) were treated with 10 mL of 100 g/L aqueous acetic acid at boiling temperature under reflux for 120 min and then filtered. An aliquot of 0.03 mL was applied on silica gel G60 TLC plate (20x20 cm, thickness 250 μm; Merck, Darmstadt, Germany). The plate was developed to a height of 16 cm, with a lower layer of a mixture of methanol:chloroform:10 g/L ammonium-hydroxide (50:50:25 v/v/v). The spots of α-solanine and α-chaconine were scraped from the plate, 10 mL of 20 g/L aqueous acetic acid was added and eluted under stirring for 30 min. Then, the solution was centrifuged and 1 mL of HCl was added to the supernatant and refluxed with stirring for 30 min. The pH of the solutions was adjusted to 4.0 and the α-solanine and α-chaconine contents were spectrophotometrically determined as above. The ratio of α-solanine to α-chaconine was then calculated.

Kinetics of solanidine hydrolytic extraction

Dried and milled tuber sprouts (40 g) were treated with various solutions: 50 and 150 g/L HCl in 20 g/L aqueous acetic acid in ratio of tuber sprouts to solution of 1:20 w/v; 20 g/L HCl in 100 g/L aqueous acetic acid in a ratio of 1:17 w/v; 100 g/L HCl in 50% aqueous methanol in ratio of 1:10 w/v; 50 g/L HCl in 50% aqueous ethanol in a ratio of 1:20 w/v. The organic phases, chloroform, trichloroethylene or carbon tetrachloride in a volume to acid phase ratio of 1:1 were added in three different flasks for each solution. The flasks were placed in the bath with boiling water and connected to a reflux condenser. The temperature of the lower layer, chloroform, trichloroethylene and carbon tetrachloride was approx. 65, 90 and 80°C, respectively, and the temperature of the upper layer was approx. 70, 95 and 85°C, respectively. Aliquots of 1 mL of organic liquid phase were taken from each flask at 10, 15, 30, 45, 60, 90 and 120 min intervals, in order to determine the solanidine content. A model of these processes is presented in Fig. 1.

The organic phase was evaporated under vacuum until dry. The dry residue was dissolved in 10 mL of 20 g/L aqueous acetic acid by using the water bath at boiling temperature and a reflux condenser. Then the pH of the cooled solutions was adjusted to 4.0 and the subsequent procedure was the same as that for determining the GA content. The solanidine content was calculated according to the GA content and the α-solanine and α-chaconine ratio in tuber sprouts, considering that one mole of α-solanine or α-chaconine yields one mole of solanidine.

Enzymatic hydrolysis

Fresh and milled haulm (20 g) was treated at 35°C and after a certain time of incubation (12 - 96 hours), 1 g of fresh incubated haulm was taken. Then, 10 mL of 20 g/L aqueous acetic acid solution was added and solanidine was extracted at room temperature under (200 min^{-1}) for 60 min. The pH of the extract was adjusted to 4.0 and solanidine content was determined as above.

For enzyme hydrolysis by enzyme preparation, 100 mg of the preparation (NIKOLIC and STANKOVIC, 2005) was added to 5 mL of the GA solution (2 g/L) in 20 g/L aqueous acetic acid solution. The pH of the solution was adjusted to 5.4 by adding 50 g/L and 10 g/L aqueous NaOH solution, and the mixture of the GA solution and the enzyme preparation was incubated at 35°C. After incubation (12 - 96 hours), the mixture was filtered and the solution of 20 g/L aqueous acetic acid was added to a volume of 10 mL. Then the pH of the solution was adjusted to 4.0 in order to determine the solanidine content.

Solanidine isolation

The dried and milled tuber sprouts (200 g) were treated with 2 L of 100 g/L HCl in 50% methanol and by 2 L chloroform, in boiling water bath under reflux for 1 hour. Then the system was allowed to cool and the chloroform layer was separated from the systems with a separatory funnel. Activated carbon was added (1 g) to the chloroform extract and heated in a water bath at boiling temperature under reflux. Then the extract was hot filtered through a 2 cm bed of celite and type 391 filter paper

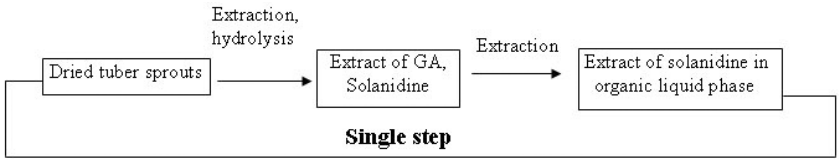

Fig. 1 - Model of solanidine hydrolytic extraction from potato tuber sprouts in a solid-liquid-liquid system.

(Filtrak, Bärenstein, Germany) and the filtrate was evaporated under vacuum. The dry residue was dissolved in a minimum amount of 96 % ethanol by heating in a water bath at boiling temperature under reflux. Solanidine was precipitated by adding concentrated aqueous ammonia to pH 10. The precipitate was separated by centrifuging for 15 min at 1700 *g*. The precipitate was washed twice with 30 mL of distilled water and centrifuged again. Tile apparatus was used for determination of melting point of solanidine.

GC-MS method

A Varian 3700 Gas Chromatograph-Mass spectrometer MAT 311A with Class 5000 software (Palo Alto, CA, USA) was used to obtain the electron impact mass spectrum. The MS ionization was set at 70 eV and helium was used as the carrier gas, at a linear velocity of 30 m/s. The instrument was equipped with an OV-101 capillary column (30 m), and the oven temperature was programmed from 150 to 290°C at 2°C/min. The volume of 0.1 µL split was injected by using split mode, at detector temperature of 250°C.

RESULTS AND DISCUSSION

Plant material

The content of glycoalkaloids (GAs) in tuber sprouts was 33.8 g/kg and the α-solanine/α-chaconine ratio was 1.12:1. The corresponding maximum yield of solanidine was 15.7 g per kg of dried potato sprouts.

TLC and GC/MS of isolated solanidine

About 80% of the possible solanidine yield was isolated from the chloroform phase. Solanidine ($S_{27}H_{43}ON$) has one basic tertiary nitrogen and one secondary hydroxyl, as well as a double bond. It is a white crystalline powder or looks like long needles and it melts at 219°C. In this study, the isolated solanidine had a melting point at 220.5°C. TLC gave an R_f - value of 0.98 for solanidine corresponding well with that (0.97) obtained earlier with a mobile phase system containing a mixture of chloroform-methanol-1 % w/w ammonia (FILADELFI and ZITNAK, 1983). GC/MS analysis, showing a parent (molecular) ion at m/z 397 with other diagnostic fragments at m/z 204 and m/z 150, largely agreed with literature data (LAWSON *et al.*, 1997).

Kinetics of solanidine hydrolytic extraction

The variations in the degree of solanidine hydrolytic extraction (DHE) and solanidine yield during the hydrolytic extraction with 100 g/L hydrochloric acid in 50% methanol as the first liquid phase and with chloroform, trichloroethylene or carbon tetrachloride as the second liquid phase, are shown in Fig. 2. The maximum DHE, hydrolytic extraction time, and possible solanidine yield (q_s) achieved with different systems are given in Table 1.

DHE is expressed as the ratio of solanidine content in the organic liquid phase after a certain hydrolytic extraction time to the maximum solanidine yield which should be achieved with the plant material used. The maximum solanidine yield which should be obtained from tuber sprouts was calculated according to the GA content and α-solanine and α-chaconine ratio in tuber sprouts, considering that one mole of α-solanine or α-chaconine yields one mole of solanidine. The maximum DHE (65.0 %) was achieved with 100 g/L HCl in 50% methanol as the first liquid phase and chloroform as the second liquid phase after 1 hour (Table 1, Fig. 2). The lowest DHE value was obtained with carbon tetrachloride as the organic phase, independent of the first liquid phase (Table 1). The maximum solanidine yield was 10.2 g solanidine per kg of dried and milled

Table 1 - Maximal achieved degree of solanidine hydrolytic extraction from tuber sprouts, time of solanidine hydrolytic extraction and calculated solanidine yield from dried tuber sprouts by using different solid-liquid-liquid systems.

First liquid phase		Second liquid phase		
		Chloroform	Trichloroethylene	Carbon tetrachloride
50 g/L HCl in 20 g/L acetic acid	DHE [1]	27.2	13.7	11.2
	T [2]	120	60	60
	YS [3]	4.3	2.2	1.8
100 g/L HCl in 20 g/L acetic acid	DHE [1]	54.4	41.5	24.6
	T [2]	120	120	120
	YS	8.5	4.2	3.9
20 g/L HCl in 100 g/L acetic acid	DHE [1]	42.2	20.0	13.8
	T [2]	120	120	60
	YS [3]	6.6	3.1	2.2
100 g/L HCl in 50% methanol	DHE [1]	65.0	57.6	40.2
	T [2]	60	60	60
	YS [3]	10.2	9.0	6.3
10% w/v HCl in 96% ethanol	DHE [1]	48.4	40.0	27.5
	T [2]	120	120	120
	YS [3]	7.6	6.3	4.3

[1] - degree of solanidine hydrolytic extraction in percent; [2] - time of solanidine hydrolytic extraction in minutes; [3] - solanidine yield in g/kg of dried sprouts.

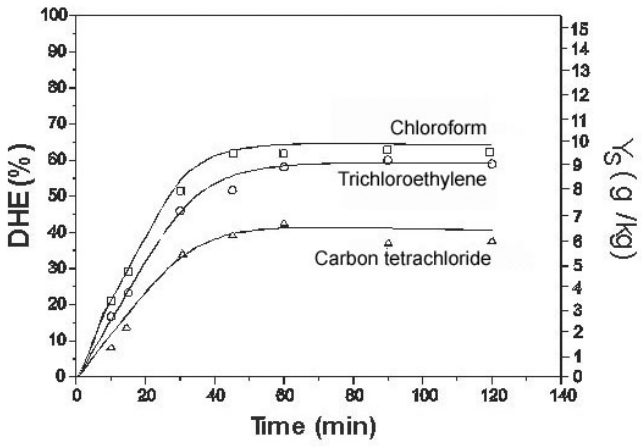

Fig. 2 - The variation of the degree of solanidine hydrolytic extraction (DHE) and solanidine yield (Y_s) from potato tuber sprouts during solanidine hydrolytic extraction at water bath boiling temperature with 100 g/L hydrochloric acid in 50% methanol as the first liquid phase and chloroform, trichloroethylene or carbon tetrachloride as the second liquid phase, in a volume ratio of 1:1.

tuber sprouts. The solanidine yield was calculated according to DHE and the maximum possible yield of solanidine which can be obtained from the tuber sprouts used.

Equations (1), (2) and (3) for calculating DHE expressed in %, solanidine yield (Y_S) expressed in g of solanidine per kg of dried and milled tuber sprouts, by using 100 g/L HCl in 50% methanol and chloroform as liquid phases and solanidine concentration in the extract and solanidine concentration in organic liquid phase (C_s), in g/L, depending on the hydrolytic extraction time in Maple V Release program, were:

$$DHE = 0.66 \cdot (1 - e^{-0.064t}) \quad (1)$$

$$Y_S = 1.03 \cdot (1 - e^{-0.064t}) \quad (2)$$

$$C_S = 0.502 \cdot (1 - e^{-0.064t}) \quad (3)$$

where t is the time of solanidine hydrolytic extraction, expressed in min.

The first two-phase system applied by GELDER (1984) was used to improve the methods of estimating GAs in *Solanum* spp. and was carried out with standard solutions made up of pure GAs and solanidine. The key data of solanidine hydrolytic extraction from plant material under the optimal operating conditions in different hydrolyzing systems is compared in Table 2. In a solid-liquid system the solanidine extract obtained has to be purified and another extraction with chloroform is needed, while in a liquid-liquid system first, the GAs have to be extracted from the plant material and then hydrolyzed to solanidine and solanidine extracted with chloroform. In both procedures, the degree of hydrolysis is greater than 80% but the process of obtaining solanidine consisted of two steps which are combined into one step in the solid-liquid-liquid system.

The process of enzymatic hydrolysis is long-term and the degree of hydrolysis is below 70%. In the case of hydrolysis by enzymatic preparation, a complicated preparation is needed. This makes the procedure especially complex if it has to be modified for analytical purposes. Even though a lower DHE was achieved by a solid-liquid-liquid system, with tuber sprouts as the source of GAs, instead of haulm, the solanidine yield was the best (4.25 times higher than the yield with haulm) and the time needed for hydrolysis only 90 min. Furthermore, the method of GA hydrolysis can be downscaled for example, to 1g of plant material with the appropriate downscaled amount of solvents and used for determination of GA content.

Table 2 - The key data of solanidine hydrolytic extraction from plant material under optimal operating conditions in different hydrolyzing systems.

System / Hydrolysis	DHE[1]	Time[2]	Y_s [3]
Solid-liquid system	85	120	0.21
Liquid-liquid system	93	120	0.23
Solid-liquid-liquid system			
a) haulm	98	60	0.24
b) sprouts	65	90	1.02
Enzymatic hydrolysis			
a) by enzymes in haulm	65	*50	0.15
b) by enzyme preparation	68	*20	0.17

[1] - degree of solanidine hydrolytic extraction in percent
[2] - time of solanidine hydrolytic extraction in minutes and *hours
[3] - solanidine yield in g/kg of dried sprouts.

In Fig. 3, the variations in the rate of solanidine hydrolytic extraction calculated as the moles of solanidine per cubic decimeter per second in solid-liquid-liquid systems (composed of HCl in methanol as the first liquid phase and chloroform as the second liquid phase), and in the corresponding solid-liquid system (system without chloroform) versus hydrolytic extraction time is presented. The maximum rate in both systems was achieved after 5 min of hydrolysis time; the values were 3.61×10^{-6} and 6.45×10^{-6} mole of solanidine dm^{-3} s^{-1}, respectively. It can be concluded, that the maximum rate of solanidine in a solid-liquid system is nearly double the maximum rate of solanidine hydrolytic extraction in a solid-liquid-liquid system, 2.84×10^{-6}. The reason is that in a solid-liquid system the reactions of GA extraction and their hydrolysis to solanidine are involved, while in a solid-liquid-liquid system in addition to these reactions, the reaction of solanidine extraction by chloroform is also included. The rates of solanidine hydrolytic extraction in these systems are different up to 30 minutes of hydrolytic extraction at which time they become almost equal as all of the solanidine obtained by GA hydrolysis is extracted by chloroform.

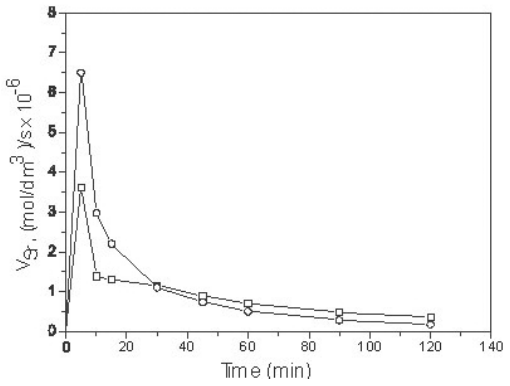

Fig. 3 - The rate of solanidine hydrolytic extraction (v_{sr}) in the optimal solid-liquid-liquid system composed of tuber sprouts, hydrochloric acid in methanol and chloroform (□) and in the optimal solid-liquid system composed of tuber sprouts and solution of hydrochloric acid in methanol (○).

CONCLUSIONS

A solid-liquid-liquid system was used to obtain solanidine. In the procedure, glycoalkaloid extraction and hydrolysis and solanidine extraction were combined into a single step. The degree of solanidine hydrolytic extraction was 65% and the corresponding solanidine yield was 1.02 g per 100 g of dried tuber sprouts. Compared to enzymatic hydrolysis, the new procedure is faster, while the other systems in which haulm was the source of glycoalkaloids, had the highest solanidine yield.

ACKNOWLEDGEMENT

This work was supported under the project TR-6708 B by the Ministry of Science and Environment Protection of the Republic of Serbia.

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Revised paper received April 20, 2006 Accepted May 31, 2006

PAPER

ANTIOXIDANT ACTIVITIES OF AQUEOUS EXTRACTS OF PURSLANE (*PORTULACA OLERACEA* SUBSP. *SATIVA* L.)

ATTIVITÀ ANTIOSSIDANTI DEGLI ESTRATTI ACQUOSI DI PORCELLANA COMUNE (*PORTULACA OLERACEA* SUBSP. *SATIVA* L.)

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ABSTRACT

The antioxidant activities of the infusion and aqueous extracts of purslane (*Portulaca oleracea* subsp. *sativa* L.) were investigated. Different antioxidant tests were employed, namely reducing power, total antioxidant activity, free radical scavenging, superoxide anion radical scavenging, hydrogen peroxide scavenging, hydroxyl radical scavenging, metal chelating activities and β-carotene bleaching effect. In addition, the results were compared with natural and synthetic antioxidants, e.g.

RIASSUNTO

Sono state studiate le attività antiossidanti del decotto e degli estratti acquosi di porcellana comune (*Portulaca oleracea* subsp. *sativa* L.). Sono stati utilizzati differenti test antiossidanti, per la precisione il potere riducente, l'attività antiossidante totale, l'inattivazione dei radicali liberi, l'inattivazione dei radicali anioni superossido, l'inattivazione del perossido di idrogeno, l'inattivazione dei radicali idrossilici, le attività chelanti i metalli ed la decolorazione del β-carotene. Inoltre, i risultati

- Key words: antioxidant activity, aqueous extract, infusion, purslane (*Portulaca oleracea* subsp. *sativa* L.) -

α-tocopherol, butylated hydroxytoluene and butylated hydroxyanisole. The levels of anthocyanins, total flavonoids and total phenolics of the extracts were also determined. Total phenolic and total flavonoid contents of the infusion were found to be higher than those of the aqueous extract. Infusion of purslane exhibited a strong Fe^{2+} chelating effect, free radical scavenging activity, hydrogen peroxide scavenging ability, and hydroxyl radical scavenging activity. Antioxidant activity of the aqueous extract increased with increased concentrations, in the range of 20-100 μg/mL. The aqueous extract also exhibited a strong superoxide radical scavenging activity. This study showed that purslane extracts exhibited antioxidant activity in all tests and the extracts could be considered as a source of natural antioxidants.

sono stati confrontati con quelli di antiossidanti naturali e sintetici ad esempio α-tocoferolo, l'idrossitoluene butilato e l'idrossianisolo butilato. Sono stati determinati inoltre i livelli di antocianidine, di flavonoidi totali e di composti fenolici totali degli estratti. Il contenuto di composti fenolici totali e di flavonoidi totali del decotto sono risultati più alti di quelli dell'estratto acquoso. Il decotto di porcellana comune presentava forte potere chelante il Fe^{2+}, attività di inattivazione dei radicali liberi, capacità di inattivazione del perossido di idrogeno ed attività di inattivazione dei radicali idrossilici. L'attività antiossidante dell'estratto acquoso aumentava con l'incremento delle concentrazioni, nel range da 20 a 100 μg/mL. L'estratto acquoso esibiva inoltre una potente attività di inattivazione dei radicali superossido. Questo studio mostrava che gli estratti di porcellana comune esibivano attività antiossidante in tutti i tests e che gli estratti potrebbero essere considerati come una fonte di antiossidanti naturali.

INTRODUCTION

Antioxidants are vital substances that protect the body from damage caused by free radical-induced oxidative stress. Free radicals can be generated from metabolic pathways within body tissues and can also be introduced from external sources such as drugs, food, UV radiation and environmental pollution (MILIAUSKAS *et al.*, 2004). Free radicals have been implicated in the cause of several diseases such as liver cirrhosis, atherosclerosis, cancer and diabetes and play an important role in ageing. Oxidative stress can also contribute to the development of neuro-degenerative disorders, such as Alzheimer's and Parkinson's as well as other diseases. Reactive oxygen species (ROS) have been known to cause tissue injury through covalent binding and lipid peroxidation. They have also been shown to damage proteins, carbohydrates and DNA. Such reactive species include hydroxyl radicals, superoxide anions, singlet oxygen and nitroso compounds. These free radicals attack unsaturated fatty acids of biomembranes, resulting in lipid peroxidation and desaturation of proteins and DNA, causing a series of deteriorative changes in the biological systems leading to cell inactivation (CHEN *et al.*, 2005). Thus, antioxidants are important inhibitors of lipid peroxidation, not only for food protection but also in defending living cells against oxidative damage. Lipid peroxidation is an important deteriorating reaction in

food during storage and processing that causes a loss in nutritional quality. The addition of antioxidants is required to preserve food quality. Antioxidant supplements or antioxidant-rich food are used to help the human body reduce oxidative damage from free radicals and active oxygen species (JUNTACHOTE and BERGHOFER, 2005). Synthetic antioxidants such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), tert-butylhydroquinone (TBHQ) and propyl gallate (PG) are widely used as antioxidants in the food industry. However, they have been shown to have toxic and/or mutagenic effects (CHUNG *et al.*, 2005). Because of their toxicity, the development and isolation of natural antioxidants from plant species, especially edible plants, are in progress.

Purslane (*Portulaca oleraceae* subsp. *sativa* L.), belonging to the Portulacaceae family, is an annual green herb with branched, succulent stems that are decumbent near the base and ascending near the top. In warm climates it grows to a height of 15-30 cm. It is fast growing, self-combatible and produces large numbers of seeds that have a long viability (RASHED *et al.*, 2003). In Turkey, it is well known and is commonly consumed raw in salad or cooked as a vegetable where it is known as "semizotu". Purslane has been described as a "power food" of the future because of its high nutritional and antioxidant properties. It has also been reported to be an excellent source of omega-3 fatty acids, antioxidant vitamins (SIMOPOULOS *et al.*, 1992) such as ascorbic acid, α-tocopherol (MILLER *et al.*, 1984) and β-carotene (SU *et al.*, 2002) as well as glutathione, and the amino acids isoleucine, leucine, lysine, methionine, cysteine, phenylalanine, tyrosine, threonine and valine (MILLER *et al.*, 1984). Portulaca is listed as a plant genus contains oxalates and has a reported oxalic acid content of up to 9% (OBIED *et al.*, 2003). It also contains alkaloids, coumarins, flavonoids, anthraquinone glycosides and betacyanin (PALANISWAMY *et al.*, 2004). Purslane is used as a diuretic, anti-scorbutic, antiulcerogenic, antispasmodic, antipyretic, anti-asthma, anti-inflammatory, anti-tussive, analgenic and muscle relaxant (CHAN *et al.*, 2000). In traditional medicine, this plant is used against vomiting, bleeding, hepatitis and in the treatment of gastric mucosal diseases. In some middle eastern countries, it is considered beneficial for small tumors, inflammation, urinary disorders, liver obstruction and ulcers of the mouth and stomach (KARIMI *et al.*, 2004). Recent research indicates that purslane offers better nourishment than the major cultivated vegetables (ARRUDA *et al.*, 2004). Purslane is not only a healthy food, but is also useful in preventing various degenerative diseases. The aim of this study was to investigate the antioxidant effects of aqueous extracts of purslane and to elucidate their antioxidative action.

MATERIALS AND METHODS

Plant material

The aerial part of fresh purslane was purchased from a local market in Istanbul, Turkey. The fresh plants were cleaned, washed with water, cut into small pieces and dried at 40°C in a hot air oven. The dried aerial parts were put in a plastic bag and stored at -20°C until used.

Preparation of infusions and aqueous extractions

Plant extracts were prepared as a 10% (w/v) infusion with distilled water or extracted with distilled water using a reflux method. For aqueous infusions, dried sample (20 g) was extracted with boiling water (200 mL) for 15 min while stirring. For the reflux method, 20 g of dried aerial parts of purslane were weighed

into a round-bottomed flask fitted with a condenser and a heating mantle. All of the contents were refluxed with 400 mL of distilled water for 2 h. After filtering through linen cloth, the filtrates were evaporated to dryness under vacuum at 35°C on a rotary evaporator. The yield for the infusion and aqueous extractions were 4.50 (22%) and 8.02 (40%) g, respectively. These crude extracts were stored at 4°C and dissolved in distilled water; they were used for the assessment of antioxidant activity.

λ_{max} values were obtained at 200 - 600 nm ultraviolet-visible (UV) for infusions and aqueous extracts of purslane.

Determination of total phenolic compounds

Samples were analyzed spectrophotometrically for total phenolic content with Folin-Ciocalteau reagent according to the method of SLINKARD and SINGLETON (1977) using pyrocatechol (20-200 µg/mL) as a standard phenolic compound. Briefly, 1 mL of the sample solution containing 1000 µg of extract was mixed with 45 mL distilled water. Folin-Ciocalteau reagent (1 mL) was added and the contents were mixed thoroughly. This mixture was allowed to stand for 3 min before adding 3 mL of 2% (w/v) Na_2CO_3. The solution was then allowed to stand for 2 h with occasional shaking before reading at 760 nm in a spectrophotometer. The total phenol content was expressed as µg equivalents to the standard used per mg of extract. The equation obtained for the standard curve was $y = 0.0025x - 0.0144$ for pyrocatechol.

Determination of total flavonoid content

Total flavonoid content was determined using a colorimetric method (ZHISHEN *et al.*, 1999). Briefly, 0.25 mL of the extract (1000 µg/mL) or (+)-catechin standard solution (10-100 µg/mL) was mixed with 1.25 mL of distilled water in a test tube, followed by the addition of 75 µL of a 5% (w/v) sodium nitrite solution. After 6 min, 150 µL of a 10% (w/v) aluminium chloride solution was added and the mixture was allowed to stand for a further 5 min before 0.5 mL of 1 M sodium hydroxide was added. The mixture was brought to 2.5 mL with distilled water and mixed well. The absorbance was measured immediately at 510 nm using a spectrophotometer. The results are expressed as the mean (±SD) mg of (+)-catechin equivalents per gram of extract.

Anthocyanin determination

The anthocyanin content of the dried purslane sample was determined according to the modified method of PADMAVATI *et al.* (1997). The dried purslane (25 mg/mL) was mixed with acidified methanol (1% HCl/methanol) for 24 h at 4 °C in the dark, and then centrifuged at 1000 x g for 15 min. The anthocyanin concentration in the supernatant was measured spectrophotometrically at 530 and 657 nm and the absorbance values are indicated as A_{530} and A_{657}. The extinction coefficient of 31.6 $M^{-1}cm^{-1}$ was used to convert the absorbance values into anthocyanin concentration. The concentration was calculated using the following equation: anthocyanin concentration (µmol/g) = $[(A_{530} - 0.33 \times A_{657})/31.6] \times$ [volume (mL)/weight (g)]. Results are expressed as the average of triplicates.

β-carotene bleaching test

Approximately 10 mg of trans-β-carotene was dissolved in 10 mL of chloroform and 0.2 mL of the solution was placed in a boiling flask containing 20 mg linoleic acid and 200 mg of Tween-40. After removal of chloroform, 50 mL of distilled water was added to the flask and vigorously shaken. Five mL of this emulsion were added to tubes containing

the 0.2 mL of antioxidants (2 mg/mL). The tubes were stoppered and placed in a water bath at 50°C. Spectrophotometric readings at 470 nm were taken after 60 and 120 min of incubation. Controls consisted of BHA (positive control; 2 mg/mL) and a linoleic acid and β-carotene emulsion (negative control). Relative antioxidant activities (RAA) were calculated with the following formula:

RAA = Absorbance of sample / Absorbance of BHA (BRUNI *et al.*, 2004).

Reducing power

The reducing power of the extracts was measured according to the method of OYAIZU (1986). Various concentrations of extracts (20-100 μg) in 1 mL of distilled water were mixed with 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and 2.5 mL potassium ferricyanide [$K_3Fe(CN)_6$] (1%, w/v), and then the mixture was incubated at 50°C for 30 min. Afterwards, 2.5 mL of trichloroacetic acid (10%, w/v) was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. Finally, 2.5 mL of upper-layer solution was mixed with 2.5 mL distilled water and 0.5 mL $FeCl_3$ (0.1%, w/v), and the absorbance was measured at 700 nm. α-tocopherol, BHA and BHT were used as standard antioxidants. Higher absorbance of the reaction mixture indicated greater reducing power.

Chelating activity on Fe^{2+}

The chelating activity of the infusion and aqueous extract on ferrous ions (Fe^{2+}) was measured according to the method of DECKER and WELCH (1990). Aliquots of 1 mL of different concentrations (0.25, 0.50, 0.75 and 1.0 mg/mL) of the samples were mixed with 3.7 mL of deionized water. The mixture was incubated with $FeCl_2$ (2 mM, 0.1 mL) for 5, 10, 30 and 60 min. After incubation the reaction was initiated by addition of ferrozine (5 mM and 0.2 mL) for 10 min at room temperature, and then the absorbance was measured at 562 nm in a spectrophotometer. A lower absorbance indicates a higher chelating power. The chelating activity of the infusion and aqueous extract on Fe^{2+} was compared with that of EDTA at a level of 0.037 mg/mL. Chelating activity was calculated using the following formula:

Chelating activity (%) = [1 – (Absorbance of sample/Absorbance of control)] x 100

Control test was performed without addition of the purslane extracts.

Free radical-scavenging activity

The free radical scavenging activity of the infusion and aqueous extract was measured with 1,1-diphenyl-2-picrylhydrazil (DPPH•) using the slightly modified methods of BRAND-WILLIAMS *et al.* (1995) and TAKASHIRA and OHTAKE (1998). Briefly, $6x10^{-5}$ mol/L DPPH• solution in ethanol was prepared and 3.9 mL of this solution was added to 0.1 mL of the infusion and aqueous extract (2-6 mg/mL) and the trolox solution (0.02-0.06 mg/mL). The mixture was shaken vigorously and the decrease in absorbance at 517 nm was measured at 5, 10, 30 and 60 min. Water (0.1 mL) in place of the plant extract was used as control. The percent inhibition activity was calculated using the following equation:

$$\text{Inhibition activity (\%)} = [(A_0 - A_1)/A_0 \times 100],$$

where A_0 is the absorbance of the control reaction and A_1 is the absorbance in the presence of the extract sample.

Total antioxidant activity determination

The antioxidant activity was determined according to the thiocyanate method with slight modifications (OSAWA and NAMIKI, 1981). For the stock solu-

tion, 10 mg of extracts were dissolved in 10 mL water. Then the solution containing different amounts of stock solution or standards samples (20, 40, 60, 80, 100 μg) in 2.5 mL of potassium phosphate buffer (0.04 M, pH 7.0) was added to 2.5 mL of linoleic acid emulsion. Fifty mL linoleic acid emulsion contained Tween-20 (175 μg), linoleic acid (155 μL) and potassium phosphate buffer (0.04 M, pH 7.0). On the other hand, 5.0 mL of control contained 2.5 mL of linoleic acid emulsion and 2.5 mL of potassium phosphate buffer (0.04 M, pH 7.0). Each solution was then incubated at 37°C in a glass flask in the dark. At 24 h intervals during incubation, 0.1 mL of this incubation solution was added to 4.7 mL of 75% (v/v) ethanol and 0.1 mL of 30% (w/v) ammonium thiocyanate. Precisely 3 min after 0.1 mL of 0.02 M $FeCl_2$ in 3.5% (w/v) HCl was added to the reaction mixture, the absorbance of the red colour was measured at 500 nm in a spectrophotometer. The solutions without added extracts or standards were used as the control. The inhibition of lipid peroxidation in percentage was calculated by the following equation:

Inhibition % = $[(A_0 - A_1)/A_0 \times 100]$, where A_0 is the absorbance of the control reaction and A_1 is the absorbance in the presence of the extract sample or standards.

Superoxide radical scavenging activity

Measurement of superoxide anion scavenging activity of infusions and aqueous extracts of purslane was based on the method described by LIU *et al.* (1997). Superoxide anions were generated in a non-enzymatic phenazine methosulfate-nicotinamide adenine dinucleotide (PMS-NADH) system by oxidation of NADH and assayed by reduction of nitroblue tetrazolium (NBT). In this experiment, the superoxide anion was generated in 3 mL of Tris-HCl buffer (16 mM, pH 8.0) containing 1 mL of NBT (50 μM) solution, 1 mL of NADH (78 μM) solution and different concentrations (0.1-1.0 mg/mL) of sample solution. The reaction was started by adding 1 mL of PMS solution (10 μM) to the mixture. The reaction mixture was incubated at 25°C for 5 min and absorbance at 560 nm was recorded against blank samples in a spectrophotometer. Epicatechin, trolox, BHA and ascorbic acid were used as standard samples (0.1-1.0 mg/mL).

Hydrogen peroxide scavenging activity

The extract (20-100 μg/mL) was dissolved in 3.4 mL of 0.1 M phosphate buffer (pH 7.4) and mixed with 0.6 mL of 43 mM hydrogen peroxide solution. The absorbance value (at 230 nm) of the reaction mixture was recorded after 40 min. For each concentration, a separate blank sample was used for background subtraction (RUCH *et al.*, 1989). α-tocopherol, BHA, and BHT (20-100 μg/mL) were used as standard antioxidants. The solutions without added extracts or standards were used as the control. The percentage of scavenged hydrogen peroxide of samples and standard compounds was calculated using the following equation:

Scavenged H_2O_2 % = $[(A_0\text{-}A_1)/A_0 \times 100]$

where A_0 is the absorbance of the control and A_1 is the absorbance in the presence of the extract samples and standards.

Hydroxyl radical scavenging activity

The effect of hydroxyl radical was assayed by using the 2-deoxyribose oxidation method (CHUNG *et al.*, 1997). 2-Deoxyribose is oxidized by the hydroxyl radical that is formed by the Fenton reaction and degraded to malondialdehyde. The reaction mixture contained 0.45 mL of

0.2 M sodium phosphate (pH 7.4), 0.15 mL of 10 mM 2-deoxyribose, 0.15 mL of 10 mM $FeSO_4$-EDTA, 0.15 mL of 10 mM hydrogen peroxide, 0.525 mL of distilled water and 0.075 mL (20-100 μg/mL) of sample solution in a tube. The reaction was started by the addition of hydrogen peroxide. After incubation at 37°C for 4 h, the reaction was stopped by adding 0.75 mL of 2.8% (w/v) trichloroacetic acid and 0.75 mL of 1.0% (w/v) of thiobarbituric acid. The mixture was boiled for 10 min, cooled in ice and then measured at 520 nm. The reaction mixture not containing test sample was used as the control. The scavenging activity on hydroxyl radicals (HRSA) was expressed as:

$$HRSA = [(A_0 - A_1)/ /A_0 \times 100]$$

where A_0 is the absorbance of the control reaction and A_1 is the absorbance in the presence of the extract sample.

RESULTS AND DISCUSSION

UV-visible absorption spectroscopy is one of the most useful techniques available for structural analysis of phenolic compounds. The UV-visible spectra of the diluted infusion and aqueous extracts of purslane is shown in Fig. 1. Absorption maxima at 223 and 265 nm may be due to the presence of flavone/flavonol derivatives indicating that purslane may contain phenolic compounds. Phenols are very important plant constituents as they are good scavengers due to their hydroxyl groups.

Determination of total phenolic compounds

Phenolic compounds are aromatic secondary plant metabolites that are widespread throughout the plant kingdom. Phenolics have been associated with colour, sensory qualities, and nutritional and antioxidant properties of food. Phenolic compounds are reported to have multiple biological effects, including antioxidant activity, antitumor,

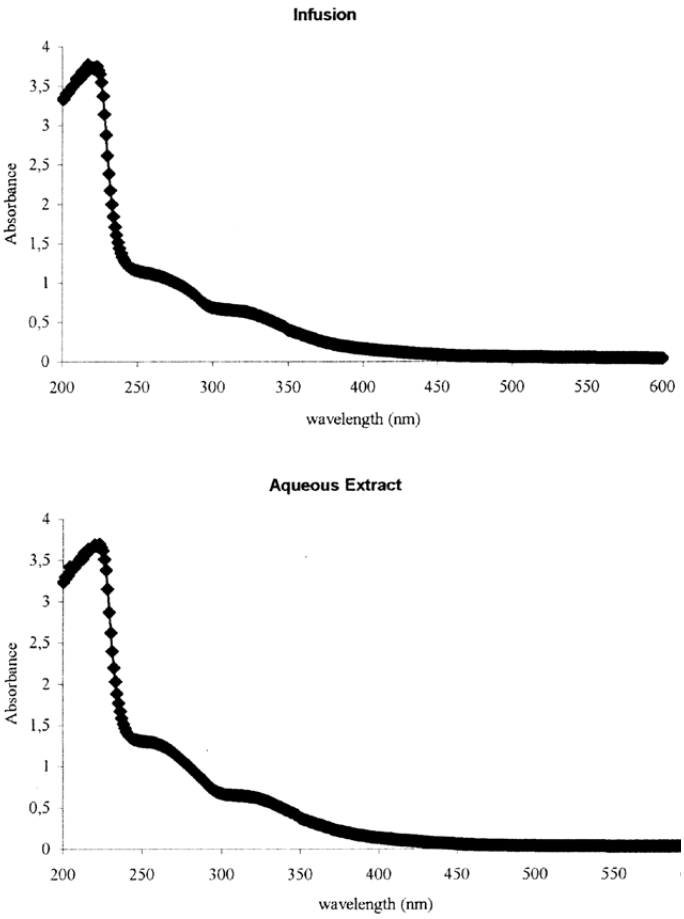

Fig. 1 - UV-vis spectra of an infusion and aqueous extract of purslane.

antimutagenic, and antibacterial properties. The antioxidant activity of phenolic compounds is mainly due to their redox properties, which can play an important role in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen and decomposing peroxides (BHANDARI and KAWABATA, 2004). The amount of total phenolics in purslane extracts was determined using the Folin-Ciocalteau method. Values are expressed as μg pyrocatechol per mg of extracts. It was determined that there were 21.80 ± 1.75 and 17.91 ± 2.42 μg pyrocatechol equivalent of phenolic compounds in the 1 mg of the infusion and aqueous extract of purslane, respectively. It is suggested that some polyphenolics have inhibitory effects on mutagenesis and carcinogenesis in humans, when up to 1.0 g from a diet rich in fruits and vegetables are ingested daily (YEN *et al.*, 2005).

Determination of total flavonoid content

Flavonoids are diverse secondary metabolites abundant in plant tissues. Flavonoids possess an ideal structural chemistry for free radical scavenging activity, and have been shown to be more effective antioxidants in vitro than tocopherols and ascorbate. It was determined that there were 19.51 ± 0.79 and 14.63 ± 0.49 μg (+)-catechin equivalent of flavonoids per mg of infusion and aqueous extract of purslane, respectively.

Anthocyanin determination

Plant anthocyanins are widely distributed in the human diet through vegetables, fruit, beans, cereals, tea, coffee, natural herbs and spice extracts, and have been found to possess significant antioxidant activities that are associated with lower incidences of and lower mortality rates from certain human diseases (IGARASHI *et al.*, 2000). In this study, the anthocyanin content of purslane was found to be 0.410 ± 0.014 μmol/g. The antioxidant activity of purslane may be attributed in great part to it.

β-carotene bleaching test

β-carotene is found in dark green and orange vegetables and fruit (PRADEEP and KUTTAN, 2003). It has a strong biological activity and is a physiologically important compound. Two important properties of β-carotene are its ability to trap certain organic free radicals and to deactivate the excited molecules, particularly excited or singlet oxygen (PRADEEP and KUTTAN, 2003). In the β-carotene bleaching test, the infusion and aqueous extracts of purslane were similar to the positive control, BHA, at 60 min and only slightly less after 120 min of incubation (Table 1). Thus, it is apparent that purslane extracts have strong effects against the discoloration of β-carotene.

Reducing power

The reducing power has been used as one of the antioxidant capability indicators of plants (BHANDARI and KAWABATA, 2004). In the reducing power assay, the presence of reductants (antioxidants) in the tested samples resulted in the reduction of the Fe^{3+}/ferricyanide complex to the ferrous form (Fe^{2+}). The amount of Fe^{2+} complex can therefore be monitored by measuring the for-

Table 1 - β-carotene bleaching test[a].

Sample	RAA[b] 60 min	RAA 120 min
Infusion	0.92±0.17	0.86±0.32
Aqueous extract	0.91±0.19	0.78±0.25
BHA	1	1
Negative control[c]	0.13±0.09	0.13±0.03

[a]Mean±SD; [b]Relative Antioxidant Activities; [c]Negative control: Linoleic acid and β-carotene emulsion.

mation of Perl's Prussian Blue at 700 nm (CHUNG *et al.*, 2005). Figure 2 shows the reducing power of infusion and aqueous extracts from purslane. The reducing power of all the extracts increased with increasing concentration. Based on a comparison of the absorbance at 700 nm, the reducing power of purslane at a concentration of 20 μg/mL was similar to that of α-tocopherol at 20 μg/mL. This indicates that the extracts of purslane were electron donors and could also react with free radicals, converting them to more stable products and terminate the radical chain reaction.

Chelating activity on Fe^{2+}

Iron is the most important lipid oxidation pro-oxidant due to its high reactivity. The ferrous state of iron can stimulate lipid peroxidation by the Fenton type reaction and also accelerates peroxidation by decomposing lipid hydroperoxides into peroxyl and alkoxyl radicals that can themselves abstract hydrogen and perpetuate the chain reaction of lipid peroxidation. The Fe^{3+} ion also produces radicals from peroxides, although the rate is ten times less than that of the Fe^{2+} ion. The Fe^{2+} ion is the most powerful pro-oxidant among the various species of metal ions (MILLER, 1996). Ferrozine, a chelating reagent, was used to indicate the presence of chelator in the reaction system. Ferrozine forms a complex with free Fe^{2+} but not with Fe^{2+} bound by extracts. In the presence of chelating agents, the complex formation of ferrous iron and ferrozine is disrupted, resulting in a decrease in the red color of the complex. Measurement of color reduction therefore allows the metal chelating activity of the coexisting chelator to be estimated (YAMAGUCHI *et al.*, 2000).

The purpose of the test of ferrous ion chelating activity was to determine the capacity of purslane to bind the ferrous ion catalyzing oxidation. The ferrous ion chelating effect of purslane extracts is presented in Fig. 3a-b. All samples at 1.0 mg/mL concentration showed more than 40% chelating effect on ferrous ions at an incubation time of 60 min. The chelating activity of samples increased with increasing incubation times with $FeCl_2$. At the same amounts, infusions exhibited higher chelating activity on Fe^{2+} than aqueous extracts. However, the chelating activity of infusions of 1.0 mg/mL was nearly equal to EDTA at 0.037 mg/mL (43.67%) for an incubation time of 30 min. This indicates that the chelation property of the samples on Fe^{2+} ions may afford protection against oxidative damage.

Free radical-scavenging activity

The proton radical scavenging action is known as an important mechanism of antioxidation. 1,1-Diphenyl-2-picrylhydrazil (DPPH•) is used as a free radical to evaluate the antioxidative activity of some natural sources (CHUNG *et al.*, 2005). The DPPH radical scavenging ef-

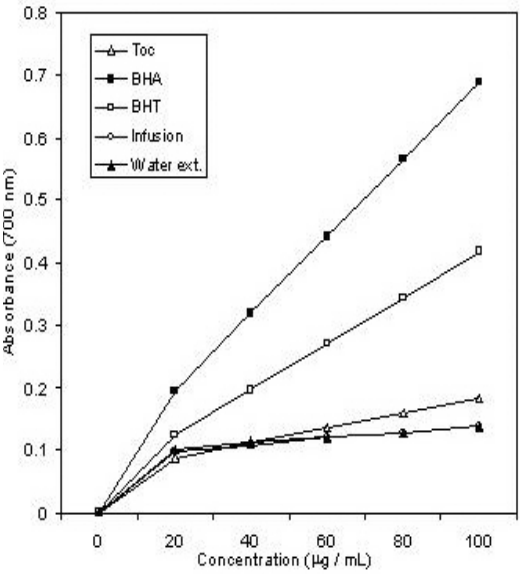

Fig. 2 - Reducing power of an infusion and aqueous extract from purslane

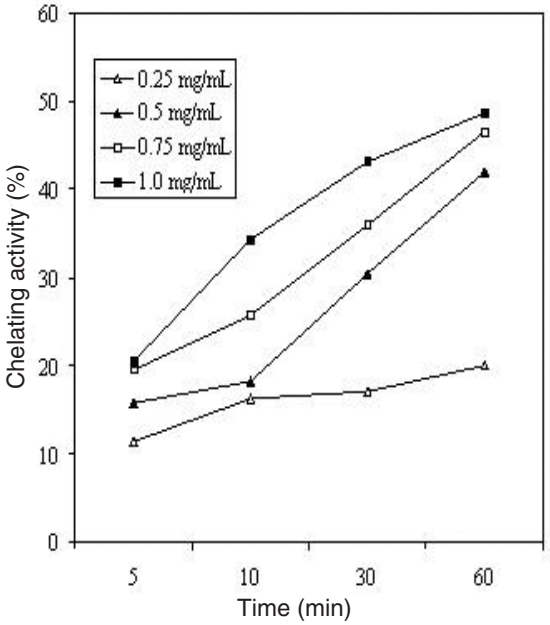

Fig. 3a - Chelating effects of different concentrations of an infusion on Fe^{2+} ions at different incubation times with $FeCl_2$.

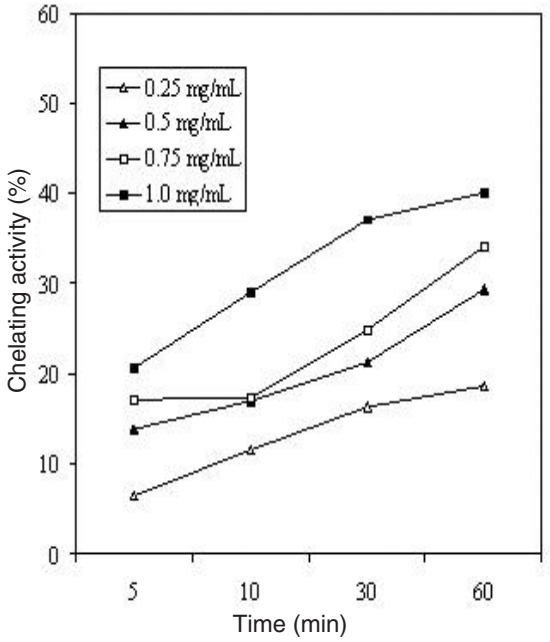

Fig. 3b - Chelating effects of different concentrations of an aqueous extract on Fe^{2+} ions at different incubation times with $FeCl_2$.

fects of purslane extracts are presented in Fig. 4. All the samples showed appreciable free radical scavenging activities. The free radical scavenging activity of the water extract was higher than infusions of purslane at 4 and 6 mg/mL. Infusions and aqueous extracts of 6 mg/mL had the lowest radical scavenging activity when compared with 0.06 mg/mL trolox. From these results, it can be stated that infusions and aqueous extracts from purslane have the ability to scavenge free radicals and could serve as a weak free radical inhibitor or scavenger according to trolox. On the other hand, such dietary antioxidants from purslane may be particularly important in protecting cellular DNA, lipids and proteins from free radical damage.

Total antioxidant activity determination

The total antioxidant activity of purslane was determined by the thiocyanate method in linoleic acid emulsion. The antioxidative activities of purslane were compared with commercial antioxidants such as α-tocopherol (Toc), BHT, BHA and trolox. The effects of 100 mg/L of infusions and aqueous extracts of purslane on peroxidation of linoleic acid emulsion are shown in Fig. 5. Infusions and aqueous extracts of purslane showed higher antioxidant activity when compared trolox, BHA, and BHT. However, total antioxidant activity of aqueous extract was also higher than infusion. Total antioxidant activity of extracts and both standards decreased in the order of α-tocopherol > aqueous extract > infusion > trolox > BHA > BHT.

Superoxide radical scavenging activity

Superoxide radical, known to be very harmful to cellular components

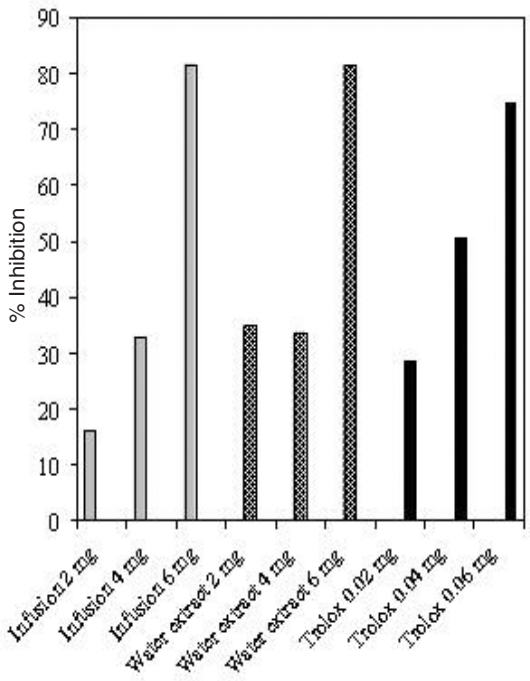

Fig. 4 - Scavenging activities of infusions and aqueous extracts against the 1,1-diphenyl-2-picryl-hydrazil (DPPH•) radical.

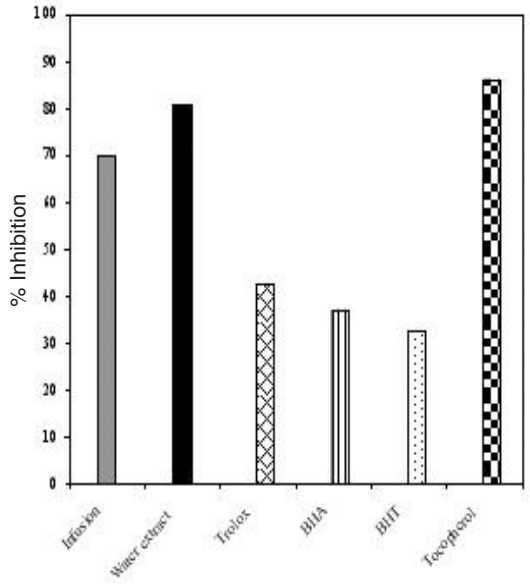

Fig. 5 - Total antioxidant activities of infusions, aqueous extracts, trolox, BHA, BHT and α-tocopherol (100 mg/L concentration) on peroxidation of linoleic acid emulsion.

as a precursor of the more reactive oxygen species, contributes to tissue damage and various diseases. In a biological system, its toxic role can be eliminated by superoxide dismutase (CHUNG *et al.*, 2005). The radicals may also play an important role during the peroxidation of unsaturated fatty acids and other potential susceptible substances (SAKANAKA *et al.*, 2005). In the PMS-NADH-NBT system, superoxide anion derived from dissolved oxygen by PMS-NADH coupling reaction reduces NBT. The decrease of absorbance at 560 nm with antioxidants indicates the consumption of the superoxide anion in the reaction mixture (JUNTACHOTE and BERGHOFER, 2005). Fig. 6 shows the superoxide radical scavenging activity by 0.1-1.0 mg/mL of infusion and aqueous extract of purslane in comparison to the same amount of BHA, epicatechin, trolox and ascorbic acid. At 0.1-0.5 mg/mL concentrations, infusions and aqueous extracts of purslane showed higher superoxide radical scavenging activity than trolox, while they showed weaker activity than BHA, epicatechin and ascorbic acid. At 1.0 mg/mL concentration, infusions and aqueous extracts of purslane showed lower superoxide radical scavenging activity than BHA, epicatechin, trolox and ascorbic acid.

Hydrogen peroxide scavenging activity

Hydrogen peroxide is an intermediate during endogenous oxidative metabolism and mediates radical oxygen formation such as OH, which may be used to predict the scavenging capability of antioxidants in biological systems (CHEN *et al.*, 2006). H_2O_2 has only a weak activity to initiate lipid peroxidation, but its activity as an active oxygen species comes from its potential to produce the highly reactive hydroxyl radical through the Fenton reaction. Figure 7 presents the scavenging activity

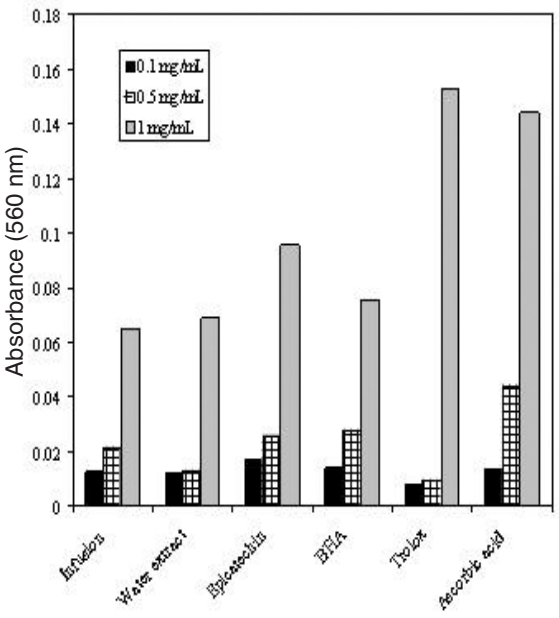

Fig. 6 - Superoxide radical scavenging activity of infusions and aqueous extracts, epicatechin, BHA, ascorbic acid and trolox at different concentrations.

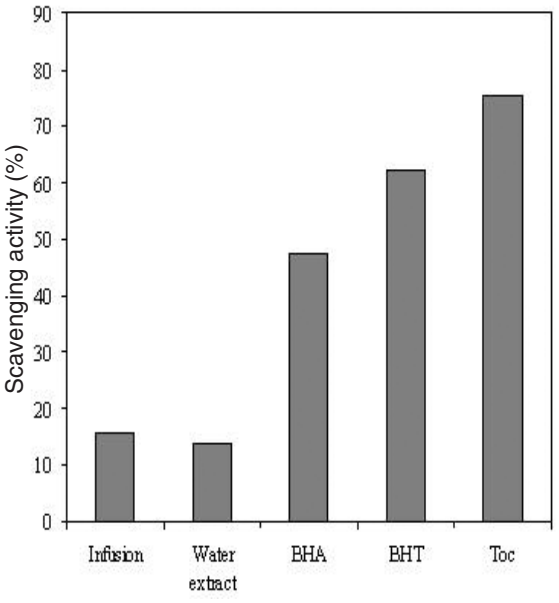

Fig. 7 - Hydrogen peroxide scavenging activity of infusions and aqueous extracts of purslane, BHA, BHT, and α-tocopherol (Toc) at 100 μg/mL concentration.

of samples on H_2O_2. The results are compared with BHA, BHT, and α-tocopherol as standards. Infusions and aqueous extracts from purslane were capable of scavenging activity in a concentration–dependent manner. At 100 μg/mL, aqueous infusions and water extracts exhibited 15.84% and 13.75% scavenging activity, respectively. On the other hand, BHA, BHT, and α-tocopherol exhibited 47.53%, 62.17%, and 75.54%, respectively, of H_2O_2 scavenging activity at the same concentration. These results showed that purslane extracts had a weak H_2O_2 scavenging activity. At 100 μg/mL concentration, H_2O_2 scavenging activity of extracts and both standards decreased in the order of α-tocopherol > BHT > BHA > infusion > aqueous extract.

Hydroxyl radical scavenging activity

The hydroxyl radical scavenging effects of infusions and aqueous extracts were determined at the concentration of 20-100 μg/mL. Fig. 8 shows the hydroxyl radical scavenging effects determined by the 2-deoxyribose oxidation method. The scavenging effect of infusions and aqueous extracts on hydroxyl radical was concentration dependent. At 20-100 μg/mL concentrations, infusions and aqueous extracts of purslane exhibited lower hydroxyl radical scavenging activity than ascorbic acid. At 20-100 μg/mL concentrations, aqueous extracts of purslane showed higher hydroxyl radical scavenging activity than infusions.

Among the oxygen radicals, hydroxyl radical is the most reactive chemical species known. The hydroxyl radical induces some oxidative damage to biomolecules such as all proteins, DNA, nucleic acid, and almost any biological molecule it touches,

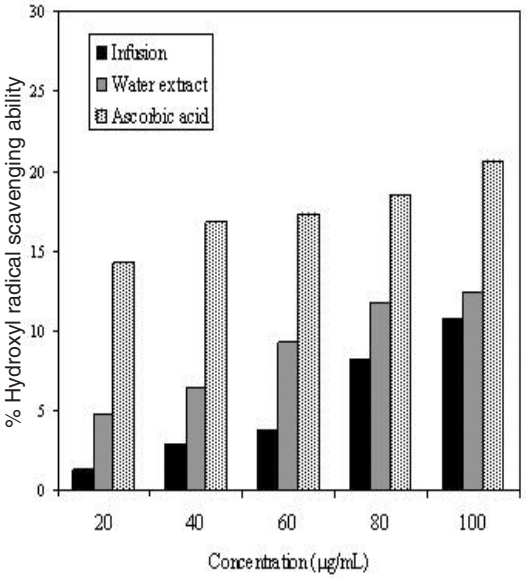

Fig. 8 - Hydroxyl radical scavenging activities of infusions and aqueous extracts. Ascorbic acid was used as the positive control.

and this damage causes ageing, cancer and several diseases (ARUOMA, 1998). According to the present findings, purslane may be used to provide a hydroxyl radical scavenger for humans.

This study showed that purslane extracts exhibited different levels of antioxidant activity in all tests. The results indicate that the purslane extracts have a significant antioxidant activity, metal chelating ability, superoxide and hydroxyl radical scavenging activity against various antioxidant systems in vitro. The antioxidant effects of the purslane extracts can be attributed to its high phenolic, flavonoid and anthocyanin contents. Purslane can be used as an easily accessible source of natural antioxidants. Consumption of purslane that contains various antioxidants has been recommended in order to prevent or slow oxidative stress caused by free radicals. Besides its natural properties, purslane is common, inexpensive and effective. Therefore, it would be interesting to do further studies using purslane extracts as food additives in order to increase the shelf life of foods by preventing lipid peroxidation. However, the components responsible for the antioxidative activities of the extracts are still unknown. Therefore, further research requires isolation and identification of the antioxidative compounds present in the extracts.

ACKNOWLEDGEMENTS

The authors would like to thank Professor Dr. Kerim Alpinar from the Faculty of Pharmacy of Istanbul University for identification of the plant.

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Revised paper received February 1, 2006 Accepted June 2, 2006

SHORT COMMUNICATION

CAROTENOID, CHLOROPHYLL AND CHLOROPHYLL-DERIVED COMPOUNDS IN PISTACHIO KERNELS (*PISTACIA VERA* L.) FROM SICILY

CAROTENOIDI, CLOROFILLE E COMPOSTI DERIVATI DALLE CLOROFILLE NEI NOCCIOLI DEL PISTACCHIO (*PISTACIA VERA* L.) SICILIANO

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ABSTRACT

The composition of carotenoid, chlorophyll, and chlorophyll-derived compounds in Sicilian pistachio kernels (*Pistacia vera* L.) was investigated. In all, 13 compounds were identified and quantified using reversed-phase liquid chromatography with photodiode array detection with a C-30 column. Internal kernel colour is an important visual quality characteristic for the food processing industry. Chlorophyll a was the major component (54.14 ppm), followed by chlorophyll b (30.2 ppm).

RIASSUNTO

La composizione in carotenoidi, clorofille e composti derivati dalle clorofille, è stata studiata nei noccioli del pistacchio siciliano. In totale sono stati identificati e quantificati 13 composti, incluso un estere. È stata inoltre dimostrata l'applicazione della cromatografia liquida ad alta prestazione a fase inversa con rivelazione a fotodiodi e l'uso di una colonna C-30, nell'analisi quali-quantitativa simultanea dei pigmenti dei noccioli. Il colore del nocciolo è un'importante caratteristica nell'industria della tra-

- Key words: carotenoids, chlorophylls, Sicilian pistachio nuts -

Lutein (29.14 ppm) and pheophytin a (25.68 ppm) were also well represented. Pheophytins a and b, neoxanthin, luteoxanthin and violaxanthin were identified in pistachio nuts for the first time. The presence of a carotenoid ester was also detected. The ratio between the two isochromic pigment fractions, namely the chlorophyll and the carotenoid fractions, was 2.7, clearly showing the prevalence of the green colour; the chlorophyll a/chlorophyll b and the lutein/β-carotene ratios were of 1.8 and 4.1, respectively. These parameters, along with other analytical parameters, could be used as indicators of typicality in Sicilian pistachio nuts. The presence of a specific pigment profile in nuts could, in fact, be used to guarantee the typicality of the product, since the quality control of food also requires a precise knowledge of the pigment composition of the original product.

sformazione degli alimenti. La clorofilla a, è il componente presente in maggiore quantità (54,14 ppm), seguita dalla clorofilla b (30,2 ppm). La luteina (29,14 ppm) e la feofitina a (25,68) sono anche presenti in buone quantità. Le feofitine a e b, la neoxantina, la luteoxantina e la violaxantina sono state identificate per la prima volta nei noccioli di pistacchio. È stata anche riscontrata la presenza di esteri di carotenoidi. Il rapporto fra le due frazioni isocromiche, e cioè le frazioni delle clorofille e dei carotenoidi, è stato di 2,7; tale valore chiaramente mostra la prevalenza del colore verde. I rapporti clorofilla a/clorofilla b, e luteina/β-carotene, sono stati rispettivamente di 1,8 e 4,1. Questi parametri, insieme ad altri parametri analitici, potrebbero essere utilizzati come indicatori di tipicità dei noccioli di pistacchio siciliani. Infatti, la presenza di uno specifico profilo di pigmenti nei pistacchi potrebbe essere utilizzato come indice delle genuinità del prodotto, dato che il controllo della qualità degli alimenti richiede anche una precisa conoscenza della composizione in pigmenti dei prodotti originali.

INTRODUCTION

The pistachio nut, *Pistacia vera* L., is a member of the Anacardiaceae family. Iran, followed by Turkey and the USA, are the leading world producers. In Italy, the pistachio nut production industry is mainly located in Sicily where they are mainly cultivated on lava-rich soils in the eastern part around Mount Etna in the area of "Bronte". They are sold as green, unripened products and are consumed as snack food, usually dried. They are also used in the food industry as an ingredient in ice-cream, pastries, fermented meats and puddings. The "Pistachio of Bronte", also locally known as "Green Gold" represents just 1% of the world pistachio production, that is, about 500,000 tons (ANONYMOUS, 2003). It has become an important economic product for the region, being exported and appreciated overseas for its qualities such as colour, nutrients and organoleptic properties.

Internal kernel colour is an important visual quality characteristic, and the food processing industry prefers intense green kernels. There have been a limited number of studies on the chemical constituents of pistachio nuts (OKAY, 2002; YALPANI and TYMAN, 1983; YILDIZ *et al.*, 1998; DANESHARED and AYNEHCHI, 1980; PHILLIPS *et al.*, 2005; AN-

DERSON and SMITH, 2005; TOKUSOGLU *et al.*, 2005.) which have shown their important nutritive value. Very few studies are available on the pistachio kernel pigment composition (MINIATI, 1981; WU and PRIOR, 2005; GEBHARDT and THOMAS, 2002; AGAR *et al.*, 1998; GIOVANNINI and CONDORELLI, 1958; GIOVANNINI and CONDORELLI 1956.). Chlorophylls and carotenoids are very common pigments that play key roles in photosynthesis and give colour to vegetables and several fruits. Neither chlorophylls nor carotenoids can be synthesized by animal tissues, though animal cells can chemically modify them for assimilation. Hence they must be obtained from plant-derived food.

Several reports have demonstrated that plant pigments play important roles in health (MAYNE *et al.*, 1996; FRANCESCHI *et al.*, 1994). Recent studies have shown the potential health benefits of a carotenoid-rich diet due to their role as antioxidants, and as agents preventing cardiovascular diseases and degenerative eye pathologies (KRITCHEVSKY, 1999; LANDRUM and BONE, 2001). In addition to the provitamin A value of the carotenoids with a β-ionone ring, numerous studies have demonstrated the anticancer activity of β-carotene and other carotenoids (VAN POPPEL and GOLDBOHM, 1995). Their presence in pistachio nuts may depend on genetic factors (variety), the degree of ripeness, environmental conditions and geographical origin. Compared to the classical C-18 stationary phase, the use of the much more hydrophobic C-30 phase gives a better resolving power for compounds with a clearly hydrophobic character, such as carotenoids and chlorophylls; the C-30 stationary phase provides sufficient phase thickness to enhance interaction with long chain molecules.

The aim of this study was to evaluate the carotenoid, chlorophyll and chlorophyll-derived compounds present in pistachio kernels from the cultivar " Napoletana", produced in Sicily in the area of Bronte and to assess their levels. To our knowledge, no such thorough investigation is available in the literature. Moreover, the presence of a specific pigment profile in pistachio nut and the ratio between the pigments could be used to guarantee product authenticity (GANDUL-ROJAS *et al.*, 2000).

MATERIALS AND METHODS

Chemicals

HPLC-grade solvents, organic solvents and reagents were purchased from Sigma-Aldrich (Milan, Italy), as were the chlorophyll a and b standards. Pheophytins a and b were obtained by acidification with hydrochloric acid from the respective solutions of chlorophylls (SIEVERS and HYNNINEM, 1977). Chlorophyll a and b C-10 epimers, were obtained according to the method of WATANABE *et al.*(1984). β-carotene and lutein were purchased from Extrasynthese (Genay, France). Violaxanthin and neoxanthin were obtained after extraction with cold acetone and purification by OCC (Open Column Chromatography) from curly lettuce, as described by KIMURA and RODRIGUEZ-AMAYA (2002). To avoid contamination among the bands during OCC, only the main portion of each carotenoid band was collected and the standard purity, evaluated by HPLC, was around 95% for neoxanthin and 96% for violaxanthin. Standards were stored under nitrogen in the dark at -18°C.

Materials and pigment extraction

Pistachio nut samples (*Pistacia vera* L., cultivar "Napoletana") were purchased from local markets in the Bronte area of Sicily and were analysed soon after arrival in the laboratory. They were shelled and stored in the dark at room temperature, around 10°C, until they

were analysed in triplicate. Therefore, minimal or no pigment degradation had occurred in the intact pistachio kernels prior to analyses. Eight different samples of green kernels of around 37 g each were ground and exhaustively extracted with acetone; after solvent removal in a rotary vacuum (Buchi rotavapor R-200, Catania, Italy) evaporator, an oil was obtained with a 38% yield. Fresh oil samples were stored at –20°C in the dark and under nitrogen, until analysis. Each oil sample was then extracted by liquid-phase distribution (LPD) between N,N-dimethylformamide (DMF) and hexane, according to a method described by MINGUEZ-MOSQUERA *et al.* (1992). The oil samples (5 g) were dissolved directly in DMF (30 mL) and treated with five successive 10 mL portions of hexane in a decanting funnel. Chlorophylls, chlorophyllic derivatives and xanthophylls were retained in the DMF phase. The hexane phase contained lipids and carotenes. The DMF phase was treated with a 2% Na_2SO_4 solution at 0°C and transferred to 100 mL of a mixture of hexane/ethyl ether (1:1; *v*/*v*). The aqueous phase was discarded, eliminating polyphenols and other water-soluble compounds. The organic phase was evaporated to dryness in a rotovapor at 30°C. The dry residue was dissolved in an appropriate volume of methanol and analysed by HPLC. Using the standard technique of enrichment, 91% lutein and 89% pheophytin a were recovered. The five hexane phases were combined, concentrated, filtered, and made up to a known volume of hexane, to spectrophotometrically measure the β-carotene concentration directly, using a previously prepared calibration curve, as this phase only contains β-carotene.

Both chlorophyll and carotenoid molecules have long chains of conjugated double bonds and react easily with acid, base, oxygen and light. Therefore care should be taken during extraction and analyses; oils containing such pigments should be stored in the dark, at reduced temperature and if possible, under nitrogen. Amber glassware or glassware covered with aluminium foil should be used and the laboratory should be dimly lit.

Pigment analyses

Chlorophylls, chlorophyllic derivatives and xanthophylls present in the oil extracts were analysed using an HPLC-PDA Shimadzu system (Shimadzu, Milan, Italy) equipped with two LC-10AD-Vp pumps, a SCL-10A-Vp system controller, a SPD-M10Avp photodiode array detector and a Rheodyne injector with a 5 µL loop. For HPLC separation, a YMC 30 (YMC Europe, Schermbeck, Germany) analytical column (250 x 4.6 mm i.d.) packed with 5 µm C-30 reversed-phase material, and a precolumn (YMC 30; S-5 µm, 10 x 4.0 mm i.d.) were used. The mobile phase consisted of a binary gradient of methanol, MeOH (A) and methyl-*ter*t-butyl ether, MTBE (B), starting with 5% B, followed by a linear gradient to 95% B in 45 min, then re-equilibrating the column to the initial B concentration, at a flow rate of 0.8 mL/min; chromatograms were recorded at five different wavelengths, λ_1 410 nm (Pheo a), λ_2 430 nm (Chl a), λ_3 444 nm (Lutein), λ_4 465 nm (Chl b), λ_5 665 nm, and UV-Vis spectra were recorded in the range of 325-750 nm. Pigments in the samples were identified by comparison with standards and from their spectral characteristics, both absorption maxima and peak ratios. For chlorophylls and derivatives, the characteristic peak ratio is that between the absorbance of the Soret band I and the absorption maximum III; for carotenoids, the height of the longest wavelength absorption band III is expressed as a percentage of the middle absorption band II, the base line in each case being the minimum between the two maxima (100III/II) (DAVIES, 1975). HPLC quantification was carried out using the external standard method; standard curves were calculated by linear regression analyses, based

on the calibration curves of the available standards, and the mean values are reported. β-carotene was quantified using a Shimadzu UV-2410PC UV-Vis spectrophotometer and an appropriate previously prepared calibration curve; the mean value is reported.

RESULTS AND DISCUSSION

The composition of carotenoid, chlorophyll, and chlorophyll-derived compounds in monovarietal ("Napoletana") Sicilian pistachio kernels from the "Bronte" area was investigated. In all, 13 different pigments were identified and quantified, including an ester. Reversed phase liquid chromatography with photodiode array detection using a C-30 column in the simultaneous chemical characterization of chlorophyll and carotenoid pigments present in pistachio nuts was applied for the first time. The qualitative study of the pigments in pistachio nut samples confirmed the presence of lutein, β-carotene, chlorophyll a and chlorophyll b, previously reported by GIOVANNINI and CONDORELLI (1958) and AGAR *et al.*, (1998). Neoxanthin and its ester, the 5,8-furanoid isomer luteoxanthin of the original xanthophyll violaxanthin bearing the 5,6-epoxide group, and violaxanthin itself, were detected for the first time in pistachio nuts. Among the chlorophylls, the pheophytins a and b and the chlorophlls a' and b' C-10 epimers, were also detected for the first time in pistachio nuts. Under mild conditions, chlorophylls are subject to various alteration processes, such as the loss of the central Mg ion, epimerisation, photochemical degradation and molecular-oxygen oxidation. In natural environments, chlorophylls and their derived products are often present as complex mixtures. A typical chromatogram of a pigment extract from Sicilian monovarietal "Pistachio of Bronte" kernels is shown in Fig. 1. Since the pigments considered have a characteristic UV-Vis spectra, the photodiode array detector (PDA) has been used extensively to study these pigments. Moreover, carotenoid esterification does not change the shape of the respective absorption spectrum. Table 1 shows the chromatographic and spectroscopic characteristics of the various pigments separated, including retention factor, absorption maxima measured by PDA and peak ratios. The chromatographic and spectroscopic characteristics of these pigments after alkaline hydrolysis confirmed the

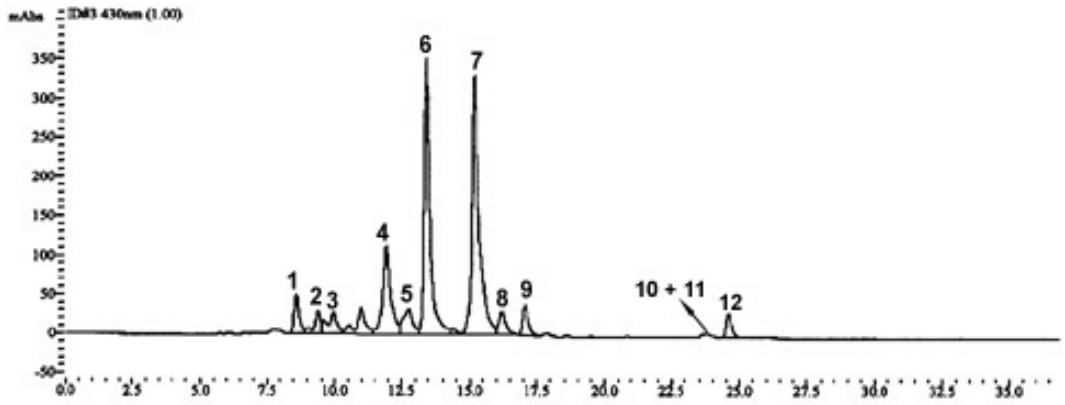

Fig. 1 - Typical HPLC profile on a C-30 column at 430 nm, of pigments separated in Sicilian "Pistachio of Bronte" kernels. Peak numbers as in Table 1.

Table 1 - Chromatographic and spectroscopic characteristics of pigments separated in Sicilian "Pistachio of Bronte" kernels by HPLC.

Peak number	Pigment	*k;(a)*	Spectral data in HPLC eluent				
			Position of peak (nm);			Peak height relationship;*(b)*	
			I	II	III	100III/II	I/III
1	Neoxanthin	2.8	415	438	467	91	-
2	Luteoxanthin	3.3	398	420	447	100	-
3	Violaxanthin	3.4	413	434	464	93	-
4	chlorophyll b	4.3	467	602	650	-	3.5
5	chlorophyll b'	4.7	467	602	650	-	3.5
6	Lutein	4.9	423	444	471	60	-
7	chlorophyll a	5.5	430	620	666	-	1.1
8	chlorophyll a'	5.9	430	620	666	-	1.1
9	Neoxanthin ester	6.4	415	438	467	91	-
10	Pheophytin b	9.3	434	601	652	-	4.1
11	Pheophytin a'	9.4	406	506	665	-	2.1
12	Pheophytin a	9.7	406	506	665	-	2.1

(a) Retention factor (*k*) = tr - tm/tm, where tr is the retention time of the pigment peak and tm is the retention time of an unretained component. *(b)* Peak ratio I/III for chlorophylls and 100III/II for carotenoids.

tentative identification of a neoxanthin ester. Table 2, shows the quantitative composition of chlorophylls and carotenoids in Sicilian "Pistachio of Bronte" kernels. This is important for the accurate determination of the dietary intake of these micronutrients and the development of comprehensive food tables. The CV% was less than 9% in all HPLC measurements and less than 1% in the spectrophotometric measurements.

In agreement with the report by AGAR *et al.* (1998), the pistachio nut studied in this work has a high pigment content, with green being the prevailing colour. This was clearly indicated by the value of 2.7 for the ratio between the two isochromic pigment fractions, namely the chlorophyll and carotenoid fractions. Chlorophylls represented 73% of the total pigments identified; chlorophyll a, was the major component (54.14 ppm; 30.8% of total pigments), followed by chlorophyll b (30.2 ppm; 17.2%). Lutein (29.14 ppm; 16.6%) and pheophytin a (25.68 ppm; 14.64%) were also well represented. The β-carotene content (7.1

Table 2 - Concentrations (ppm) and CV% (coefficient of variation %) of individual carotenoids and chlorophylls and ratios between pigment fractions from Sicilian "Pistachio of Bronte" kernels.

Pigment	ppm	CV%
Neoxanthin	3.04	4.2
Luteoxanthin	2.75	4.4
Violaxanthin	2.81	4.1
chlorophyll b	30.2	8.2
chlorophyll b'	7.8	5.1
Lutein	29.14	5.8
chlorophyll a	54.14	6.7
chlorophyll a'	5.2	5.2
Neoxanthin ester	2.48	6.2
Pheophytin b	0.71	4.8
Pheophytin a'	4.33	5.5
Pheophytin a	25.68	7.3
β-carotene	7.1	0.6
Total Chlorophylls	128.06	
Total Carotenoids	47.32	
Ratios		
Chl a/Chl b	1.8	
Lutein/β-carotene	4.1	
Chlorophylls/Carotenoids	2.7	

Mean (ppm) value of eight samples, each sample in triplicate.

ppm) was consistent with the value (7.9 ppm) reported by GIOVANNINI and CONDORELLI (1956 and 1958), whereas the lutein amount (29.14 ppm) was higher than reported by the same authors (8.4 ppm). This difference may be attributed to varietal differences with characteristic biosynthetic or catabolic pathways and/or to geographical differences. It is generally known that pigment concentration in fruit decreases during fruit ripening when the synthesis of anthocyanins intensifies, therefore maximum pigment contents are found in green pistachio kernels (ROCA and MINGUEZ-MOSQUERA, 2001). The lutein/β-carotene ratio was 4.1 and the chlorophyll a/chlorophyll b ratio was 1.8, which could be useful for distinguishing nuts from a single cultivar. The presence of a specific pigment profile in the "Pistachio of Bronte" kernels could be used to guarantee the typicality of the product, since the quality control of foods also requires a precise knowledge of the pigment composition of the original products.

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Revised paper received May 5, 2006 Accepted May 30, 2006

SHORT COMMUNICATION

EFFECTS OF POST-HARVEST TREATMENT AND STORAGE TIME ON THE ORGANIC ACID CONTENT IN ASSARIA AND MOLLAR POMEGRANATE (*PUNICA GRANATUM* L.) FRUIT

EFFETTI DEL TRATTAMENTO POST-RACCOLTA ED AL TEMPO DI IMMAGAZZINAMENTO SUL CONTENUTO IN ACIDI ORGANICI NEL FRUTTO DI MELOGRANI ASSARIA E MOLLAR (*PUNICA GRANATUM* L.)

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ABSTRACT

The effects of postharvest treatment and storage at 5ºC on changes in the organic acid content in the juice of 'Assaria' and 'Mollar' pomegranate were monitored. The fruit was chill stored after the following treatments: covering boxes with a 25-μ thick low-density polyethylene film and spraying with 1.5% $CaCl_2$; untreated fruit was used as the control. The results showed that citric acid is the main organic acid in the 'Mollar' cultivar, followed by tartaric acid, whereas three organic acids were

RIASSUNTO

Sono stati monitorati gli effetti del trattamento successivo alla raccolta e dell'immagazzinamento a 5°C, sul contenuto in acidi organici del succo di melograno 'Assaria' e 'Mollar'. I frutti sono stati immagazzinati al freddo dopo i seguenti trattamenti: imballaggio con un film, di 25 μ di spessore, di polietilene a bassa densità e trattati con una soluzione spray all'1,5% di $CaCl_2$; i frutti non trattati sono stati utilizzati come controllo. I risultati mostravano che l'acido citrico risultava essere l'acido orga-

- Key words: 'Assaria' and 'Mollar', cold storage, organic acids, pomegranate, *Punica granatum* -

present at similar levels in 'Assaria' pomegranate: citric, oxalic and tartaric acids. Differences in the main organic acid contents were detected when fruit was submitted to different treatments prior to cold storage. The different pre-treatments did not significantly interfere with the accumulation of organic acids in either cultivar. There were two exceptions. The ascorbic acid content was significantly higher in the 'Mollar' cultivar when the fruit was treated with calcium, while the pyruvic acid content was significantly higher in the 'Assaria' cultivar in the untreated fruit.

nico principale nella cultivar "Mollar", seguito dall'acido tartarico, mentre tre acidi organici erano presenti in concentrazioni simili nella melograno 'Assaria': l'acido citrico, l'ossalico ed il tartarico. I differenti pre-trattamenti non interferivano significativamente con l'accumulo di acidi organici in entrambe le cultivar. Sono state riscontrate due eccezioni. Il contenuto in acido ascorbico era significativamente più alto nella cultivar "Mollar" quando i frutti erano stati trattati con il calcio, mentre il contenuto di acido piruvico era significativamente più alto nella cultivar "Assaria" nei frutti non trattati.

INTRODUCTION

Pomegranate trees (*Punica granatum* L.) have been undervalued in Portugal. Interest in this type of crop has begun to increase due to the process of desertification, particularly in southern Portugal. Pomegranate trees are particularly adapted to the adverse soil conditions in semiarid climates so they could be a good alternative in areas that are at risk of becoming desertified. An increase consumer demand for exotic fruit, as well as for nutritional, high quality food has aroused interest in pomegranate production.

At least two pomegranate cultivars, 'Mollar' and 'Assaria' are grown in Algarve, a southern province of Portugal. Its edible seeds are sweet and tender and the fruit is mainly used for direct consumption. Several studies have shown that the chemical composition of pomegranate juice depends on several factors: cultivar type, environmental and postharvest conditions, and storage and processing (HOLCROFT *et al.*, 1998; GIL *et al.*, 2000; MELGAREJO *et al.*, 2000; HESHI *et al.*, 2001; NANDA *et al.*, 2001; OZKAN, 2002).

The pre- and post-harvest application of calcium salts has been successfully used in many types of fresh fruit to reduce the loss of firmness and to slow down the ripening process (SOUTY *et al.*, 1995). Pomegranates are traditionally consumed on Christmas and New Year's Day. Since they lose moisture easily, it is important to avoid water loss.

In this study, organic acids in two pomegranate cultivars, 'Mollar' and 'Assaria', and the effect of storage conditions on their accumulation were determined.

MATERIAL AND METHODS

Fruit and treatments

Sweet pomegranates (*Punica granatum*) were harvested in an orchard in eastern Algarve (Portugal) with 14.0% °Brix cv 'Assaria' and 14.8% °Brix cv 'Mollar'. The fruit was transported to the laboratory at the University of Algarve on the same day. After selection (diseased, bruised and injured fruit were rejected), healthy fruit of uniform size and appearance was randomly put into plastic al-

veolated boxes (20 pieces per box) and treated: a) control (no covering); b) boxes were covered with a 25 μ low-density polyethylene film and c) fruit was sprayed with a solution of 1.5% $CaCl_2$ over the whole surface and left to dry for 1 hour at ambient temperature. All the fruit was then stored at 5°C in the dark at 90-95% relative humidity.

At harvest and then at monthly intervals for 3 or 4 months, 10 pieces of fruit from each replication were removed and the concentration of organic acids was measured. There were 4 replications for each sampling time. Pomegranate cv. 'Mollar' decayed more quickly than pomegranate cv. 'Assaria'; so by the fourth month of storage there were not enough 'Mollar' pomegranates left to continue the assay.

Standards and reagents

Formic, oxalic, tartaric, pyruvic, malic, ascorbic, citric acids and sulphuric acid were purchased from Fluka (Steinheim, Germany). Ultra-Pure Water was purified with the system MilliQ, from Millipore (Billerica, MA, USA).

Organic acids

To determine the organic acid content in the juice, samples (1 mL) were centrifuged for 20 minutes at 13000 rpm and filtered through a 0.45 μm filter (Millipore). The organic acid composition was determined with an HPLC (Beckman, San Ramon, CA, USA) equipped with a Jasco Refractive Index (RI) 1530 detector. The column: Polyspher OA HY (30 cm x 0.65 cm i.d.; 9 μm particle size) from Merck (Darmstadt, Germany) was operated at 35°C. The mobile phase consisted of 0.0025N H_2SO_4 applied at a flow rate of 0.4 mL/min. The injection volume was 20 μL using an injector with a 20 μL loop (Rheodyne, Cotati, California, USA). The different organic acids were identified by comparing their retention times with those of pure standards. The concentrations of these compounds were calculated from standard curves of the respective organic acids.

Organic acid values are the means of four replicates. Analysis of variance was performed by ANOVA procedures (SPSS 10.0 Windows). The data obtained were subjected to a one-way analysis of variance and Duncan's test.

RESULTS AND DISCUSSION

Citric, oxalic, tartaric, pyruvic, malic and ascorbic acids were found in the raw pomegranate juice. The organic acid contents varied significantly between cultivars. For 'Mollar' cultivar, citric acid was the predominant organic acid in the juice, ranging from 96.9 mg/100 mL at harvest time to 343.9 mg/100 mL in the juice from fruit sprayed with a solution 1.5% $CaCl_2$ and stored for two months at 5°C in the dark (Table 1). The second most important organic acid was tartaric acid (74.9-171.5 mg/100 mL) in fruit covered with low-density polyethylene film after one and two months of cold storage, respectively. The only exception was found in the juice from the fruit treated with calcium after two months of cold storage (81.0 mg/100 mL). In this case, the malic acid content was 214.5 mg/100 mL, which exceeded the tartaric acid content.

Three organic acids predominated in 'Assaria' cultivar juice: citric acid (112.2-332.7 mg/100 mL) after one month of storage in control fruits and in those covered with low-density polyethylene film, respectively; oxalic acid (95.5-374.6 mg/100 mL) in fruit treated with calcium and in the control fruits respectively, after one month of storage in both cases; and tartaric acid (71.3-341.6 mg/100 mL) in fruit covered with low-density polyethylene film after three months of storage at 5°C and in the control fruit stored for one month, respectively (Table 2).

Table 1 - Concentration of organic acids present in 'Mollar' pomegranate fruits subjected to different treatments and stored in the dark at 5°C[a].

Organic acids (mg/100 mL)						
Control						
Time (months)	Oxalic acid	Tartaric acid	Pyruvic acid	Malic acid	Ascorbic acid	Citric acid
0	16.6±11.7a	97.7±32.5a	1.9±1.8ab	43.8±29.3a	4.2±1.7ab	96.9±54.5a
1	-	153.6±32.5a	0.7±1.8a	56.2±29.3ab	2.9±1.7a	267.3±54.5bc
2	-	140.5±32.5a	5.4±1.8b	63.9±29.3ab	13.5±1.7c	336.0±54.5c
3	-	110.8±32.5a	5.7±1.8b	117.2±29.3b	7.3±1.7b	193.2±54.5ab
Average	-	126.6±17.2	3.4±1.4	69.2±23.5	6.7±2.1	232.0±37.8
Film covering						
0	16.6±11.7a	97.7±36.9a	1.9±1.5a	43.8±11.9a	4.2±1.4a	96.9±35.8a
1	-	74.9±36.9a	1.4±1.5a	39.3±11.9a	1.7±1.4a	158.5±35.8a
2	-	171.5±36.9a	5.5±1.5a	65.1±11.9a	8.9±1.4b	271.7±35.8b
3	-	158.9±36.9a	11.3±1.5b	65.1±11.9a	1.5±1.4a	152.5±35.8a
Average	-	121.0±17.2	4.3±1.4	49.9±23.5	4.7±2.1	170.4±37.8
Calcium treatment						
0	16.6±11.7a	97.7±18.3a	1.9±1.5a	43.8±39.4a	4.2±3.8a	96.9±30.1a
1	-	101.4±18.3a	9.2±1.5b	21.5±39.4a	8.6±3.8a	240.0±30.1b
2	-	81.0±18.3a	1.6±1.5a	214.5±39.4b	18.0±3.8b	343.9±30.1c
3	-	116.2±18.3a	7.1±1.5b	90.0±39.4a	8.8±3.8a	179.0±30.1ab
Average	-	98.0±17.2	4.8±1.4	87.0±23.5	10.4±2.1	225.9±37.8

[a] Data are the mean of four replicates ± standard error. For each treatment, in each column, means with different letters are significantly different ($P<0.05$). - = below detection level of 0.05 mg/100 mL.

The organic acids in the two cultivars were practically the same as those already reported by other authors for 40 different Spanish pomegranate cultivars (MELGAREJO *et al*, 2000). These include citric, malic, oxalic, acetic and tartaric acids. No quinic or succinic acids were found in the two cultivars as had been reported by POYRAZOĞLU *et al.* (2002) for thirteen pomegranate cultivars obtained from four different regions in Turkey. In contrast with the Spanish cultivars, in which citric acid was the major organic acid followed closely by malic acid, the Turkish cultivars showed a great variation in the organic acids. Citric and malic acids were also predominant in the majority of Turkish cultivars, but in some cultivars large amounts of oxalic and tartaric acids were found. In our case, citric acid was dominant in the 'Mollar' cultivar juice, while in the 'Assaria' cultivar three organic acids were determined in practically the same amounts: citric, malic and tartaric acids.

These results are very similar to those already reported (MIGUEL *et al.* 2004) for the 'Assaria' cultivar when the effect of different methods of pomegranate juice extraction on quality was tested. However, for these fruit only the two main organic acids were considered: oxalic and tartaric acids. It should be noted that these fruit were collected in another orchard and in a different year. The chemical composition also seems to depend on the growing region and climate, among other factors (POYRAZOĞLU *et al.*, 2002) but, in our opinion, this has not been well established.

Except for oxalic acid, that was only detected at the time of harvest, the amounts of the other organic acids in the

'Mollar' cultivar generally increased over time, independent of the type of treatment. The maximum values were recorded after 2 or 3 months of storage (Table 1). The amount of citric acid, the most important organic acid in pomegranate fruit, increased significantly from the beginning of the assay up to two months of cold storage, independent of treatment, and then decreased (Table 1). The accumulation of organic acids in the fruit, independent of the treatment, is difficult to explain. ARTÉS *et al.* (2000) observed a decrease in the pH in samples of 'Mollar Elche' pomegranate after 12 weeks of storage. The samples had been packed in perforated polypropylene film and stored at 5°C.

The organic acid contents in the 'Assaria' cultivar also increased over time, reaching the maximum values after one to three months of storage (Table 2). However an increase in the amounts of the main organic acids in the juices after two months of cold storage, as had been observed in the 'Mollar' cultivar, was not evident. The citric acid levels decreased in the untreated fruits as well as in those sprayed with 1.5% $CaCl_2$, during the first month of storage. This decrease was not observed in the fruit covered with low-density polyethylene film. The levels of oxalic and tartaric acids in the fruit sprayed with 1.5% $CaCl_2$ decreased during the first month of storage; in contrast, the levels in the other treatments increased during the same period of time. These results are difficult to explain.

Table 2 - Concentration of organic acids present in 'Assaria' pomegranate fruits subjected to different treatments and stored in the dark at 5°C[a].

Organic acids (mg/100 mL)						
Control						
Time (months)	Oxalic acid	Tartaric acid	Pyruvic acid	Malic acid	Ascorbic acid	Citric acid
0	130.0±43.5a	180.0±77.5a	2.5±3.3a	18.6±39.3a	5.7±4.3ab	227.4±26.7bc
1	374.6±43.5b	341.6±77.5a	4.2±3.3ab	66.3±39.3ab	1.9±4.3a	112.2±26.7a
2	148.4±43.5a	329.1±77.5a	16.1±3.3c	158.4±39.3b	17.2±4.3b	276.4±26.7c
3	178.6±43.5a	169.0±77.5a	14.5±3.3bc	151.6±39.3b	17.6±4.3b	188.3±26.7ab
4	102.6±43.5a	186.6±77.5a	5.8±3.3abc	43.8±39.3a	10.5±4.3ab	159.2±26.7ab
Average	181.4±32.2	233.3±36.9	8.9±1.9	90.6±23.3	10.8±2.2	192.7±25.3
Film covering						
0	130.0±77.8a	180.0±82.8a	2.5±3.1a	18.6±30.4a	5.7±3.6a	227.4±51.2ab
1	256.8±77.8a	242.2±82.8a	1.9±3.1a	92.6±30.4b	4.1±3.6a	332.7±51.2b
2	133.3±77.8a	231.0±82.8a	6.9±3.1a	102.4±30.4b	12.0±3.6a	260.5±51.2ab
3	194.9±77.8a	71.3±82.8a	6.1±3.1a	114.9±30.4b	11.5±3.6a	171.8±51.2a
4	98.3±77.8a	198.2±82.8a	5.9±3.1a	71.9±30.4ab	3.5±3.6a	156.1±51.2a
Average	162.8±32.2	180.6±36.9	4.7±1.9	79.3±23.3	7.2±2.2	233.5±25.3
Calcium treatment						
0	130.0±45.1ab	180.0±65.4ab	2.5±4.0a	18.6±39.7a	5.7±3.4a	227.4±44.2ab
1	95.5±45.1a	87.2±65.4a	8.3±4.0ab	35.8±39.7a	10.8±3.4ab	160.5±44.2a
2	150.0±45.1ab	250.5±65.4b	6.5±4.0ab	158.4±39.7b	14.9±3.4b	268.4±44.2b
3	242.5±45.1b	131.4±65.4ab	15.0±4.0b	69.8±39.7ab	14.6±3.4b	159.8±44.2a
4	102.1±45.1a	152.7±65.4ab	10.3±4.0ab	55.6±39.7a	9.4±3.4ab	122.7±44.2a
Average	144.8±32.2	160.9±36.9	8.5±1.9	68.2±23.3	11.2±2.2	189.2±25.3

[a] Data are the mean of four replicates ± standard error. For each treatment, in each column, means with different letters are significantly different (P<0.05).

To our knowledge this is the first study on the effects of post-harvest treatments on the accumulation of organic acids. The results of this study show that the type of treatment did not significantly interfere with the average of organic acids in the two cultivars (Tables 1 and 2). One exception was the ascorbic acid content in the 'Mollar' cultivar which was significantly higher when the fruit was treated with calcium; the pyruvic acid content was also significantly higher in the untreated fruit of the 'Assaria' cultivar.

The results of this study show that:

- Citric acid is the main organic acid in the 'Mollar' cultivar, followed by tartaric acid, whereas the 'Assaria' cultivar has almost equal levels of citric, oxalic and tartaric acids;

- With a few exceptions, the main organic acid contents increased over time until a certain point after which they decreased (3 or 4 months), independent of the type of treatment used and the cultivar;

- In general the three treatments assayed, the control (no covering); covering the boxes with a 25 μ low-density polyethylene film; and spraying the entire surface of the fruits with a solution of 1.5% $CaCl_2$, did not greatly influence the contents of the main organic acids.

ACKNOWLEDGMENT

This work was supported by the Centro de Desenvolvimento de Ciências e Técnicas de Produção Vegetal, Portugal (CDCTPV).

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Revised paper received January 20, 2006 Accepted March 24, 2006

SHORT COMMUNICATION

VOLATILE COMPOSITION OF THE *VITIS VINIFERA* ALBARIÑO MUSTS ACCORDING TO GEOGRAPHIC AREAS FROM RÍAS BAIXAS D.O. (SPAIN)

CARATTERIZZAZIONE DI COMPOSTI VOLATILI DEI MOSTI OTTENUTI DA *VITIS VINIFERA* ALBARIÑO IN BASE ALL'AREA GEOGRAFICA DELLA RÍAS BAIXAS AOC (SPAGNA)

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ABSTRACT

The volatile composition of musts from different geographical areas within the Rías Baixas Denomination of Origin is reported. Volatile compounds were identified and quantified by gas chromatography/mass spectrometry. The results demonstrate that the Albariño must from O Rosal should be the most aromatic must since it had a significantly higher volatile compound content, and was comprised of the largest group of bound compounds. Non-terpenyl compounds were the

RIASSUNTO

È stata relazionata la composizione in composti volatili di mosti provenienti da differenti aree geografiche appartenenti alla AOC Rías Baixas. I composti volatili sono stati identificati e quantificati con la gascromatografia accoppiata alla spettrometria di massa. I risultati dimostrano che il mosto di Albariño proveniente da O Rosal dovrebbe essere quello maggiormente aromatico in quanto presenta un contenuto in composti volatili significativamente maggiore e com-

- Key words: Albariño wine, characterization, differentiation, must, volatile composition -

most abundant compounds in the Albariño musts for the three geographical areas.

prende il più ampio gruppo di composti minori. I composti non-terpenici sono quelli più abbondanti nei mosti di Albariño provenienti dalle tre aree geografiche.

INTRODUCTION

Albariño, a *Vitis vinifera* L. grape variety, is a typical white variety from Galicia, a region in northwestern Spain, located on the Atlantic Ocean. Rías Baixas Denomination of Origin is the most important production area of the *Vitis vinifera* Albariño grape in Spain. Twelve native varieties are allowed to be grown in this area, but Albariño makes up 90% of the vineyards. The Rías Baixas area was originally made up of three subzones, Val do Salnés, O Rosal and Condado do Tea.

The aromatic Albariño grape grows in very small clusters and is distinguished from other types of grapes by its high sugar content and high degree of acidity. Traditionally the vines are cultivated on granite pergolas called 'parrales', to lift them off the damp ground and give them greater exposure to the sun. They are trained to a double-curtain system. The vintage, which varies according to the subzones, takes place in mid-September in O Rosal and Condado do Tea and at the end of September in Val do Salnés.

The Atlantic climate, characterized by wet winters and sea mist, varies among the subzones. Differences of climate and geography make the wines from the different subzones unique. This variety has been the object of various studies (CORTÉS, 1997; FERNÁNDEZ *et al.*, 1999; CARBALLEIRA *et al.*, 2001; VILANOVA and MASNEUF, 2005). The Albariño variety is characterized by a high intensity of floral descriptors (CARBALLEIRA *et al.*, 2001; DIEGUEZ *et al.*, 2003).

The objective of this work was to identify and quantify the aromatic compounds of the *Vitis vinifera* cv. Albariño must according to geographic area: Val do Salnés, O Rosal and O Condado do Tea.

MATERIALS AND METHODS

Must samples

The samples of the Albariño grape variety were obtained from *Vitis vinifera* cv Albariño grapes during the 2004 vintage. The grapes were collected from different cultivars of each geographic area from Rías Baixas AOC: Val do Salnés, Condado do Tea and O Rosal. Three samples of 10 kg were analyzed from each area (in triplicate). The grapes from different cultivars from each geographic area were mixed, crushed, destemmed, racked and pressed and the musts obtained were analyzed in triplicate. The maturation index (sugar content/total acidity) in Albariño musts was very similar: O Rosal (22.22), Condado do Tea (22.19) and Val do Salnés (21.32).

Analysis of free and bound compounds

Each sample was fractionated by selective retention on SepPak Vac C-18 (Waters, Milford, MA, USA) according to the procedure described by DIEGUEZ *et al.* (2003).

The extracts were analysed using a Hewlett-Packard 5890 Series II gas chromatograph equipped with Chrompack CP-Wax57CB (50 m x 0.25 mm i.d. x

0.20 mm film thickness) (Varian, Houten, The Netherlands) a fused silica capillary column with a flame ionisation detector. The conditions used for chromatographic analysis were: injector temperature (250°C), temperature program (60°C for 5 min, increased to 200°C), temperature detector (260°C), injection type (Splitless, 30s) and injection size (1 mL).

Aromatic compounds were identified by comparing the retention times with those of pure compounds and confirmed by GC-MS using a HP5890 Series II coupled to a HP 5989 A mass spectrometer in the EI mode (ionization energy, 70 eV, source temperature 250°C). The acquisition was made in the scanning mode from 10 to 1,000 m/z at 5 scan/s. Internal standards were used to quantify concentrations of individual compounds.

The analyses were made in triplicate.

Statistical analyses

To determine if there were significant differences among the musts from the different areas the data were analyzed by ANOVA, using the Enterprise Guide 3 System Software (SAS Institute, Cary, NC, USA).

RESULTS AND DISCUSSION

Table 1 shows the free and bound volatile compounds of Albariño musts, expressed as the mean (mg/L) from the GC analysis. A total of 17 aroma compounds were identified and quantified in the three geographic areas, including terpenes, C_{13}-norisoprenoids and benzyl alcohol. Significant differences were found in 14 of the volatile compounds in the three geographic areas. In the Albariño

Table 1 - Volatile compounds in Albariño musts (mg/L) and standard deviation.

Compounds	Condado do Tea	O Rosal	Val do Salnés	Sig.
Free compounds				
Limonene	29.57±0.28	42.14±0.51	21.53±0.35	***
Linalool	6.63±0.45	9.46±0.15	3.57±1.05	***
Citronellol	1.77±0.11	2.23±0.36	1.83±0.39	ns
Nerol	42.52±1.06	47.27±0.44	50.42±0.57	***
α-Ionone	5.97±0.44	8.67±0.21	6.89±0.46	***
2-Phenylethanol	178.81±2.34	180.36±4.63	185.34±2.80	ns
α-Damascenone	26.96±0.36	27.05±1.57	26.57±0.35	ns
β-Damascenone	31.08±1.80	31.71±0.29	34.79±0.02	*
Total free compounds	323.31	348.89	330.94	
Bound compounds				
Limonene	46.49±0.92	53.80±0.26	54.38±2.53	**
Linalool	45.20±0.09	51.96±0.26	37.98±4.47	**
α-terpineol	1.24±0.14	3.02±0.26	0.00±0.00	***
Citronellol	5.78±0.23	6.26±0.26	8.06±0.55	***
Nerol	11.22±1.26	14.59±0.26	13.35±1.45	*
α-Ionone	15.49±0.38	26.30±0.26	15.58±1.92	***
2-Phenilethanol	260.12±9.08	319.38±0.26	176.23±7.39	***
α-Damascenone	7.90±0.05	9.06±0.26	9.19±0.33	**
β-Damascenone	0.28±0.00	0.51±0.26	0.35±0.79	***
Total bound compounds	393.72	484.88	315.12	

Data are means of triplicates. Sig.: significance in which means differ as shown by ANOVA: *, **, *** = significance at p<0.05, p<0.01and p<0.001 respectively; ns: not significant.

musts from O Rosal, the concentration of volatile compounds (836.69 mg/L) was higher than the Albariño must from Val do Salnés (647.63 mg/L) and Condado do Tea (716.81 mg/L).

Free aroma fraction

Non-terpenyl compounds were the most abundant compounds in the free volatile fraction of the Albariño must from the three geographic areas, with 2-phenylethanol being the highest. This compound is characterized by a floral aroma. This concurs with previous data obtained by DIEGUEZ *et al.* (2003), who observed a floral character in the Albariño variety. Of the C_{13}-norisopresnoids, β-damascenone was present in the highest concentrations for the three areas. The terpene content of a variety is considered to be a positive quality factor. The Albariño must from O Rosal showed a high free terpene content (101.10 mg/L). Nerol was the most abundant monoterpene in the three areas. The next most abundant free monoterpene was limonene.

Bound aroma fraction

The total amounts of bound compounds were higher than those of the free forms in O Rosal and Condado do Tea (Table 1), which would be expected in a quality variety. These glycosidically bound forms can be converted into the free odorous forms by hydrolysis with glycosidases or by yeast with b-glycosidase activity, modifying the aromatic profile of wines and even enhance their varietal character (MENDES FERREIRA *et al.*, 2001). This author has confirmed that yeast have the ability to increase free terpenols especially in aromatic wine varieties such as Muscat. However in two other Portuguese varieties, no free terpenols were released, probably due to their neutral character. Among the bound compounds, 2-phenylethanol was the most abundant compound, followed by terpenes. Terpenols and norisoprenoids are very important volatile compound that are synthesized during berry maturation; their concentration depends on various factors such as cultivar, the area and the climatic conditions (WILLIAMS *et al.*, 1989). The two most important terpenols identified were linalool and limonene. DIEGUEZ *et al.* (2003) demonstrated a high content of bound forms in Albariño, where linalool, geraniol benzyl alcohol and 2-phenylethanol predominate. Similar results were found by CARBALLEIRA *et al.* (2001). From norisoprenoids, α-ionone (violet aroma) was the most abundant. The highest concentration of bound terpenes was found in the Albariño musts from O Rosal (Table 1). Skin contact treatment could be used to enrich this variety in free and bound aroma compounds. SELLI *et al.* (2006) showed that the skin contact treatment increased the total concentration of free and bound compounds in Narince wines.

CONCLUSION

Non-terpenyl compounds were the most abundant compounds in the free aroma fraction of the Albariño musts from the three geographic areas, of these, 2-phenylethanol (rose aroma) was the most important. The Albariño must from O Rosal should be the most aromatic must since it had a significantly higher volatile compound content, with the bound compounds making up the largest group, quantitatively. The Albariño must from Val do Salnés had the lowest concentrations of volatile compounds and should therefore be the least aromatic; this could be related to its lower maturation index.

ACKNOWLEDGEMENTS

Excma. Diputación Provincial de Pontevedra and CSIC (I3P Program) supported by Fondo Social European.

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Revised paper received April 7, 2006 Accepted July 4, 2006

SHORT COMMUNICATION

SOLID PHASE MICROEXTRACTION FOR THE ANALYSIS OF IRRADIATED RAW CHICKEN THIGH

MICROESTRAZIONE IN FASE SOLIDA PER L'ANALISI DI COSCE DI POLLO CRUDE SOTTOPOSTE A RADIAZIONI

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ABSTRACT

Volatile compounds of irradiated raw chicken thighs were extracted by solid phase microextraction (SPME) and analyzed by GC-FID and GC-O. The olfactometry experiments correlated characteristic sensory descriptors of raw chicken thighs to volatile compounds identified by GC-MS. Hexanal, octanal, nonanal, 2,4-decadienal, and gamma-butyrolactone were the main volatile compounds produced by the ox-

RIASSUNTO

I composti volatili delle cosce di pollo crude e sottoposte a radiazioni sono stati estratti attraverso la microestrazione in fase solida (SPME) ed analizzati via GC-FID e GC-O. Gli esperimenti olfattometrici correlavano i descrittori sensoriali caratteristici della carne di cosce di pollo cruda ai composti volatili identificati via GC-MS. L'esanale, l'ottanale, il nonanale, il 2,4-decadienale ed il gamma butirrolattone erano i princi-

- Key words: gamma radiation, gas chromatography, hexanal, microextraction, olfactometry, poultry -

idation of the lipids in the raw chicken thighs. Other non-identified volatile compounds were detected as the gamma radiation dosage increased. There was a clear sigmoidal relationship between the gamma radiation dosage and volatile hexanal.

pali composti volatili prodotti dall'ossidazione dei lipidi nella carne di cosce di pollo cruda. Altri composti volatili non identificati sono stati rilevati con l'incremento del dosaggio di radiazioni gamma. Si osservava una evidente relazione sigmoidale fra il dosaggio di radiazioni gamma e l'esanale volatile.

INTRODUCTION

Lipid oxidation, which is responsible for flavor and off-flavor development, is an important factor influencing the quality and acceptance of chicken meat (DU *et al.* 2001). During this process, free radicals can be formed from simple and complex lipids by temperature, metallic ions and ultraviolet radiation. The oxidative deterioration of food lipids involves autoxidative reactions of unsaturated fatty acids, accompanied by various secondary reactions. The major initial products are hydroperoxides, which degrade into alkanes, alkenes, aldehydes and ketones (RAMARATHNAM, 1991). Unsaturated fatty acids in chicken meat cuts are susceptible to these reactions and thus, contribute significantly to its quality during storage (LADIKOS and LOUGOVOIS, 1990).

AHN *et al.* (1998) and DU *et al.* (2001) demonstrated that the precursors of undesirable odors in irradiated meat were soluble in water and the respective chemical structures had nitrogen and sulfur atoms. The GC-MS analysis carried out by PATTERSON and STEVENSON (1995) showed that dimethyl sulfite was one of the most powerful volatile compounds that caused off-flavors in irradiated raw chicken meat. Sweet and blood odors in irradiated raw chicken meat were also reported by HASHIM *et al.* (1995). Other chemical groups found in irradiated chicken meat, such as ketones, alcohols, aldehydes, hydrocarbonides, nitrogen and sulfur compounds have also been shown to be important (MOTTRAM, 1997).

After irradiation of triacylglycerides, MORSEL and SCHMIEDL (1994) detected the formation of 2-dodecilcyclobutanone and 2-tetracilcyclobutanone, which are derived from the cleavage of the carbon-oxygen bonds from palmitic and stearic acids, respectively. The 2-alkylcyclobutanones were detectable only in irradiated foods, thus making them potential chemical markers of irradiated food products.

The utilization of ionizing radiation is a promising technique for the preservation of stored foods (MOSELEY, 1990). One of the most important aspects of this technology is the establishment of the best ionizing parameters for each type of food. These parameters must be capable of significantly reducing microbial growth without promoting the loss of the sensory characteristics of the food (LAMUKA, 1992).

Solid phase microextraction (SPME) is an absorption technique that has gained widespread acceptance in the analysis of volatile compounds (STEFFEN and PAWLISZYN, 1996). SPME involves the extraction of analytes from the headspace above a sample onto a fused silica fiber; the analytes are then desorbed into a GC for analysis (ZHANG and PAWLISZYN, 1993). SPME has been used to detect volatile compounds in a number of meat products such as turkey (BRUNTON *et al.*, 2002), pork (ELMORE *et al.,* 2000) and ham (RUIZ *et al.,* 1998).

The objective of the present work was to evaluate the effect of gamma radiation on the volatile compounds of raw chicken meat using SPME/GC-MS and relate the results to sensory analysis from olfactometry analyses.

MATERIALS AND METHODS

Sample preparation

Raw chicken thighs with skin from just-slaughtered birds were placed on extended-polyestyrene (Cryovac®) commercial trays (240 mm x 180 mm x 30 mm) and covered with an oxygen-permeable stretchable and thermo-shrink film. Each tray contained 200 grams of the sample. Ten trays were put into five cardboard boxes (600 mm x 420 mm x 100 mm) for a total of fifty trays, which were stored at 4±1°C. One box was kept as the control and the others were exposed to a Cobalt 60 gamma radiation source at a strength of 1,200,000 Ci at a dose of 4.5 kGy.h^{-1}. The radiation time was calculated to reach doses of 1.5 kGy; 3.0 kGy; 5.0 kGy and 7.0 kGy gamma radiation.

Dosimetry

The gamma radiation dose received by the samples was determined by transmittance readings from polymethyl methacrylate (Harwell Amber® 3042N, Oxfordshire, U.K.) dosimeters uniformly distributed in the cardboard boxes with the samples.

Solid phase microextraction procedure

A carboxen SPME fiber (1 cm x 100 μm; Supelco, Bellefonte, PA) was exposed in a (40 mL) vial assembled with open-top screw caps and septa containing 15.0 g of raw chicken thigh meat 10 minutes after the pre-equilibrium at 23±2°C. Before use, the fiber was preconditioned in the GC injection port at 250°C for 30 min. Fiber coated with carboxen showed affinity for detecting the compounds of lipid oxidation products such as aldehydes and ketones. Time extraction was evaluated between 10 and 80 minutes. The volatile compounds desorption was carried out in an injector at 200°C, SPME insert of 0.75 mm and a splitless period of 3 min.

Gas chromatography-olfactometry analysis (GC-O)

The analyses were carried out in a Shimadzu GC-17A Gas Chromatograph equipped with an injector at 200°C (splitless 1:20), FID at 250 °C and a DB-Wax column (30 m x 0.25 mm i.d. x 0.25 μm) kept at 105 kPa. Program temperature: 50°C (2 min.); 5°C min^{-1} → 210°C (5 min.). A flow divider was placed at the outlet of the chromatographic column dividing the effluent between a silica tube and the FID. The effluent was evaluated by trained tasters, who described the olfactory sensations in their own words.

Mass spectrometry analysis

The mass spectra of the volatile compounds were obtained from the gas chromatograph equipped with a Shimadzu QP-5000 quadrupole mass detector with direct interface at 230°C, electron-beam ionization source at 70eV and 35 – 350 m/z scanning.

RESULTS AND DISCUSSION

The effect of the extraction time on the isolation of the volatile compounds through SPME is shown in Fig. 1. The assays conducted at different times showed an increase in the peak areas of the volatile compounds with increases in the extraction time. Moreover, increases in the extraction time starting at 60 min did

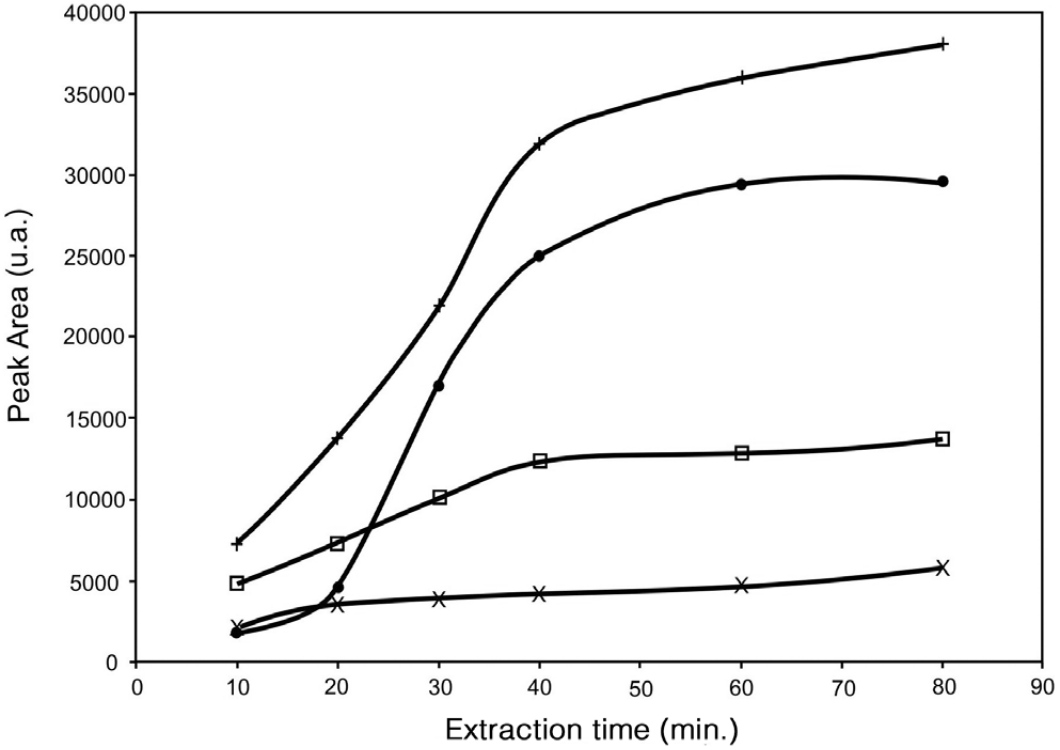

Fig. 1 - Peak area versus extraction profiles for hexanal (+), octanal (•), nonanal (*) and 2,4-decadienal (□) obtained with carboxen SPME fiber.

not significantly increase the content of volatile compounds.

The main sensory perceptions noticed by the tasters in the evaluation of the aroma of raw chicken meat submitted to gamma radiation were described as stored, sweet, fresh meat, fermented, burnt and rancid. In order to evaluate which of these sensory descriptors were related to one or more volatile compounds present in the chicken meat, olfactometry experiments were carried out to identify which peaks represented the described aromas. These volatile compounds were subjected to chemical identification by mass spectrometry and compared to standard available substances.

The mass spectra obtained in this study showed characteristic fragmentations of saturated and unsaturated aliphatic aldehydes, resulting from the breakdown of C-C bonds near the carboxyl, thus causing the formation of 44+, 56+, and 72+ ions. The presence of ion (M-1)+ was rarely observed for most of the aldehydes, including those with long chains, whereas the loss of the water molecule (M-18), ethylene (M-28), radical (M-43), and of the structure (M-44) showed fragments of aliphatic aldehydes. Table 1 summarizes the mass spectrometry data and other characteristics of the volatile compounds identified in the irradiated raw chicken thighs.

Some volatile compounds were present in trace amounts with low intensity and no directed m/z fragmentation, which would allow them to be identified. The re-

Table 1 - Characteristics of the volatile compounds isolated by SPME and identified by GC-MS in the gamma irradiated raw chicken thighs.

	Compound	Retention index [A]	M/z	Olfactometry
1	Hexanal	801	82^+, 72^+, 56^+, 44^+, 41^+	Grass, insect
2	Octanal	1006	95^+, 84^+, 69^+, 57^+, 41^+	-----
3	Nonanal	1104	114^+, 98^+, 56^+, 72^+, 44^+	Soap
4	2,4-decadienal	1284	152^+, 81^+, 67^+, 55^+, 41^+	Fried food, rancid
5	γ- butyrolactone	1299	86^+, 56^+, 42^+, 41^+	Sweet
6	Undecanal	1311	126^+, 96^+, 82^+, 57^+, 41^+	-----

A – On DB-Wax column (30 m x 0.25 mm i.d. x 0.25 µm) kept at 105kPa.

tention indices did not allow these compounds to be identified but helped organize them into an increasing order at a stationary phase. Although some of these unidentified volatile compounds were detected in the samples after irradiation, none of them gave noticeable odors in the olfactometry experiments.

The identification of hexanal, octanal, nonanal, 2,4-decadienal and gamma-butyrolactone established some correlation with the sensory descriptors observed in the olfactometry experiments. The gamma-butyrolactone composition of the furanone class was related to the "sweet" aroma perceived in the sensory analysis. Gamma-butyrolactone has been previously described in the literature as a "sweet aroma" or as a "caramel aroma" (GASSER and GROSCH, 1990). Normally sweet aromas involve multiple volatile compounds, however, in raw meat, gamma-butyrolactone appears to play a role. In fact, other lactones with sweet fruity odors, such as gamma-decalactone, have been identified in chicken meat (RAWASWAMY and RICHARDS, 1982).

Hexanal, the "grass and insect aroma", has also been described in the literature (GASSER and GROSCH, 1990) as a "cut-leaf aroma". These attributes were not mentioned in the quantitative description analysis, but the characteristic odor of hexanal could possibly be masked by other typical aromas of chicken meat, or could be combined with other volatile compounds. To access this information, a fraction collector would be required to evaluate the synergic effects of the various volatile compounds. Of the volatile compounds identified, hexanal showed a sigmoidal relationship with the gamma radiation dosage applied to the raw chicken thighs, as shown in Fig. 2.

2,4-Decadienal was associated with the "rancid, fried food aroma", which correlates with the literature (GASSER and GROSCH, 1990). The elimination of a hydrogen atom next to the double bond and the introduction of an oxygen molecule at carbons 9 or 13 of the linoleic acid molecule favor the formation of hexanal and 2,4-decadienal, respectively (SHI and HO, 1994; MOTTRAM, 1994). In chicken thigh, linoleic acid accounts for about 20% of the total fatty acids (TACO, 2004).

Besides stimulating lipid oxidation, gamma irradiation also caused the development of a light "burnt" odor in the chicken thigh meats at high intensity radiation. In this paper no direct relationship between this "burnt" odor and volatile compounds was observed. However, as suggested by MINOR *et al.* (1965), HEATH *et al.* (1990) and LESCANO *et al.* (1991), oxygen and sulphur-containing compounds have distinct odours and of-

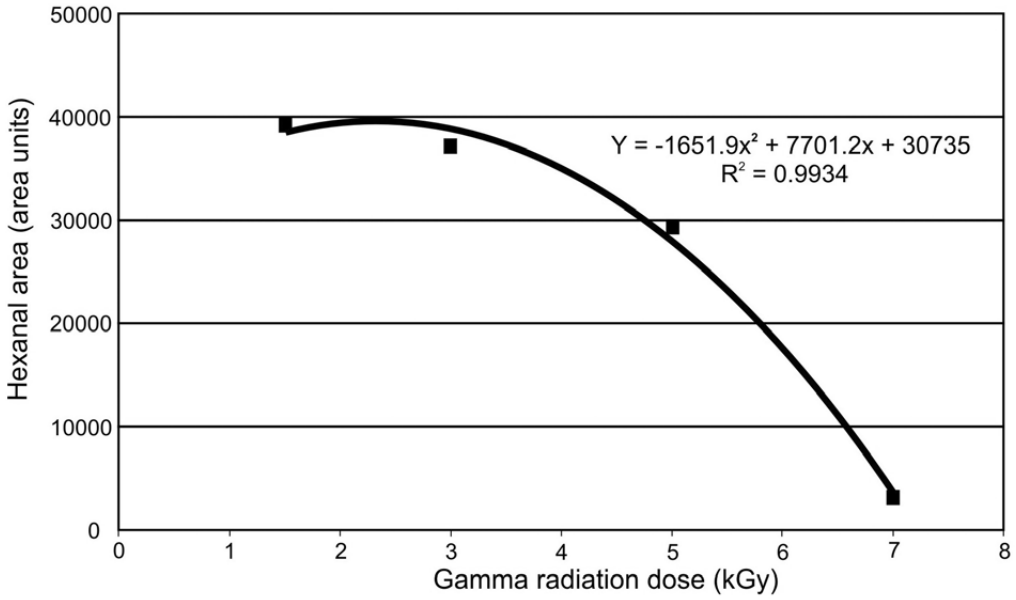

Fig. 2 - Peak area as a function of the gamma radiation dose for hexanal.

ten low odour thresholds are likely to be components of the irradiation odour.

CONCLUSIONS

The combination of SPME and GC-O showed versatility and good applicability for analyzing volatile compounds in raw chicken thigh. Six volatile compounds were identified in irradiated raw chicken thigh by gas chromatography. Hexanal, nonanal, 2,4-decadienal and gamma-butyrolactone were related, respectively, with the characteristic grass, soap, rancid and sweet odors revealed during the olfactometry tests.

ACKNOWLEDGEMENTS

The authors would like to thank the State of São Paulo Research Foundation (FAPESP – São Paulo, SP) for their financial support and the Brazilian Sterilization Company (CBE – Jarinú, SP) for the help of their professional personnel.

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Revised paper received April 29, 2006 Accepted June 1, 2006

SHORT COMMUNICATION

CHEMICAL COMPOSITION OF RAW AND COOKED TURKEY MEAT DÖNER

COMPOSIZIONE CHIMICA DI CARNE DI TACCHINO CRUDA E CUCINATA TIPO DÖNER

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ABSTRACT

The chemical composition of raw and cooked turkey meat döner was determined. Döner was prepared using turkey breast meat and chemical analyses including pH value and protein, fat, moisture and ash amounts were performed both in raw and cooked samples. Average pH value, protein, fat, moisture and ash amounts were 6.03±0.11, 19.93±0.12, 13.91±0.60, 62.39±0.84 and 2.07±0.06, respectively, for raw döner, whereas corresponding values for cooked döner were

RIASSUNTO

È stata determinata la composizione chimica della carne di tacchino, cruda e cucinata tipo Döner. Il Döner è stato preparato usando petto di tacchino e le analisi chimiche, che comprendevano determinazione del pH e delle proteine, lipidi, umidità e ceneri sono state condotte sia su campioni di carne cruda sia su campioni di carne cucinata. I valori medi di pH, proteine, lipidi, umidità e ceneri erano rispettivamente 6,03±0,11, 19,93±0,12, 13,91±0,60, 62,39±0,84 e 2,07±0,06, per il döner

- Key words: chemical composition, Döner kebab, fatty acid composition, turkey meat -

6.23±0.45, 38.00±0.29, 16.58±0.45, 39.48±1.09 and 3.03±0.08. Amounts of total saturated, monounsaturated and polyunsaturated fatty acids of raw and cooked samples were 28.52±0.23, 42.50±0.62, 28.68±0.44 and 30.17±0.61, 41.34±0.11, 25.92±0.24, respectively. Results indicated that the effect of cooking on chemical properties of döner was significant (P<0.05).

crudo, mentre i corrispondenti valori per il döner cucinato erano 6,23±0,45, 38,00±0,29, 16,58±0,45, 39,48±1,09 e 3,03±0,08. L'ammontare di acidi grassi saturi, monoinsaturi e poliinsaturi era rispettivamente 28,52±0,23, 42,50±0,62, 28,68±0,44 e 30,17±0,61, 41,34±0,11, 25,92±0,24. I risultati indicavano che l'effetto della cottura sulle proprietà chimiche del Döner risultava essere significativa (P<0,05).

INTRODUCTION

Since processed poultry products are rich in nutritive value, they are widely consumed and have been gaining popularity in fast food buffets and markets. Döner is a well-known traditional Middle Eastern product (KAYISOGLU *et al.*, 2003; KAYAHAN and WELZ, 1992). It is also known as gyros and souvlaki in Greece, shawirma in Saudia Arabia and other Arabic countries, and donna kebab in England. To produce döner, lamb, beef, pork or poultry meat is used. The meat is marinated with onion and spices for 6 hours at refrigerator conditions. It is then shaped by impalating it on a stainless steel döner stick (KAYAHAN and WELZ, 1992; SEEGER *et al.*, 1986). Döner is grilled vertically in front of a heating device. Cooked döner is sliced thinly with a special döner knife. It can be kept frozen until cooking.

Since döner is consumed widely in many countries of the world, determining its chemical composition and nutritive value is very important. For this purpose, in this study, the chemical composition of raw and cooked döner made from turkey breast meat was determined.

MATERIALS AND METHODS

Turkey meat döner was prepared in Ege Genetik, Çiftlik Döner Plant, Manisa, using the following formulation: turkey breast meat (5.5 kg), turkey tallow (1.2 kg), spice-mix (190 g), soybean flour (500 g) and onion extract (150 g).

Turkey meat and tallow were purchased from Carrefour Meat Store and minced together twice in a mincing machine. Then, the spice mixture and soybean flour, purchased from Çiftlik Döner Plant in Manisa, were added. Döner dough was prepared and marinated in a refrigerator at 4°C for 2 hours. After marination, the dough was impalated on stainless steel sticks to shape it (ANON., 1995). Stretch film was used as packaging material. Döner samples were kept frozen until cooking. Frozen döner was cooked vertically in front of a traditional döner cooking device. Cooked döner was sliced thinly and the chemical properties, including pH value and moisture, protein, fat and ash contents were determined according to AOAC (1990) methods. Fatty acid composition of the samples was determined by gas chromatography according to AOCS (2000) standards. The oil obtained from the döner was converted to methyl esters using a

trifluoride methanol complex (14% w/v). The mixture was maintained at 100°C for 1 h. The reaction was stopped with 0.5 mL of distilled water. Then, the extracted fatty acid methyl esters were dissolved in pure heptane (Merck) for GC analyses. GC analyses were performed on a Hewlett-Packard 5890 Series II gas chromatograph (H.P. Co., Amsterdam, The Netherlands) equipped with a flame hydrogen ionization detector and a capillary column. The column temperature was programmed from 180 to 240°C at 5°C/min and the injector and detector temperatures were set at 250°C. Nitrogen was the carrier gas. Identification of the peaks was achieved using retention times and by comparing them with authentic standards analysed under the same conditions. Peak areas of duplicate injections were measured with a HP computing integrator (AOCS, 2000; BESBES *et al.*, 2004). Results were evaluated by GLM procedure of SAS Statistical Analyses Programme (SAS, 2001). The design was completely randomized. Using the Duncan procedure, means were separated when significant (P<0.05) (SANTE and FERNANDEZ, 2000, JOHNSTON and KARLSTROM, 1981). All of the chemical analyses were repeated 3 times.

RESULTS AND DISCUSSION

According to the Turkish Standards for döner, the pH value of the fresh raw döner should not exceed 6.20. In this study, the initial pH value of döner was 6.03±0.11 (Table 1). Our results agree with those of KAYISOGLU *et al.* (2003) who reported the pH value of fresh chicken döner samples to range from 5.69 to 6.13. PALEARI *et al.* (1998) reported the pH value of fresh turkey breast meat to be 6.31±0.36, whereas BULGAY *et al.* (1992) reported that average pH values of fresh turkey thigh and breast meat were 5.79 and 6.74, respectively. VAZGECER *et al.* (2004) reported that pH values of

Table 1 - Chemical composition of raw and cooked turkey meat döner.

	Raw	Cooked
pH	6.03±0.11[a*]	6.23±0.45[b]
Moisture (%)	62.39±0.84[a]	39.48±1.09[b]
Protein (%)	19.93±0.12[a]	38.00±0.29[b]
Fat (%)	13.91±0.60[a]	16.58±0.45[b]
Ash (%)	2.07±0.06[a]	3.03±0.08[b]
Total saturated fatty acids**	28.52±0.23[a]	30.17±0.61[b]
Myristic acid (14:0)	1.17±0.43[a]	1.56±0.27[b]
Palmitic acid (16:0)	21.30±0.14[a]	22.40±0.43[b]
Stearic acid (18:0)	5.82±0.14[a]	5.99±0.42[a]
Arachidonic acid (20:0)	0.23±0.03[a]	0.22±0.04[a]
Behenic acid (22:0)	0.38±0.02[a]	0.58±0.04[b]
Total monounsaturated fatty acids**	42.50±0.62[a]	41.34±0.11[b]
Palmitoleic acid (16:1)	4.36±0.28[a]	4.25±0.32[a]
Oleic acid (20:0)	37.65±0.22[a]	36.76±0.11[a]
Gadoleic acid (20:1)	0.49±0.17[a]	0.33±0.20[b]
Total polyunsaturated fatty acids**	28.68±0.44[a]	25.92±0.24[b]
Linoleic acid (18:2)	25.65±0.51[a]	23.66±0.31[b]
Linolenic acid (18:3)	3.03±0.22[a]	2.26±0.19[b]

* Values on the same line are statistically different (P<0.05)
** (% of total fat).

cooked chicken döner samples were between 5.44 and 6.28. After cooking, the average pH value of the döner samples in this study was 6.23±0.45. Statistical analysis indicated that the effect of cooking on the pH values of the samples was significant (P<0.05).

The average moisture content of raw turkey meat döner was 62.39±0.84%. According to KAYISOGLU *et al.* (2003), the moisture content of raw chicken döners was between 59.61 and 61.61%, whereas SEEGER *et al.* (1986) reported that the amount of moisture found in chicken döners sold in Germany was between 37.9 and 68.1%. VAZGECER *et al.* (2004) indicated that average moisture content of cooked chicken döner samples was 47.27±3.59. Due to the amount of fat added during the manufacturing of döner, the moisture content of the samples may vary. The average moisture content of cooked turkey meat döner was 39.48±1.09%. Due to considerable amount of moisture loss, cooking significantly affected moisture content (P<0.05).

The average protein content of turkey meat döner was 19.93±0.12%. PALEARI *et al.* (1998). Reported a protein content of fresh turkey meat to be 20.4±0.77%. In another study, which supports the present findings, it was reported that the protein content of chicken döner samples was between 18.08 and 21.70% (KAYISOGLU *et. al.,* 2003). The average protein content of the cooked turkey meat döner was 38.00±0.29. Cooking significantly affected the average protein content of turkey meat döner (P<0.05).

As can be seen in Table 1, the average amount of fat was 13.91±0.11%. UZUMCUOGLU (2001) reported that the fat content of döner samples obtained from Ankara buffets and restaurants was between 9.48 and 22.77%. According to KAYISOGLU *et al.* (2003), the fat content of raw chicken döner ranged from 1.05 to 2.08%. They reported that the results were unexpectedly low due to improper sampling during their study. MURMANN *et al.* (1985) reported the fat content of raw döner to range from 2.5 to 22.5%. In this study, the average amount of fat of cooked turkey meat döner was 16.58±0.45%. Cooking also significantly affected the amount of fat (P<0.05).

Normally, the average amount of ash of turkey breast meat is 1.1% (BULGAY *et al.,* 1992); the ash content in this study, however, was higher due to the spices and soybean flour that were added. The initial ash content of döner was 2.07±0.06% (Table 1). KAYISOGLU *et al.* (2003) and UZUMCUOGLU (2001) found the average ash content of chicken döner samples to be 1.91% and 2.32%, respectively. The average ash content of the cooked samples was 3.03±0.08. So, the effect of cooking was significant (P<0.05) on the amount of ash.

According to the fatty acid composition of the samples, the amounts of total saturated, monounsaturated and polyunsaturated fatty acids were determined to be 28.52±0.23%, 42.50±0.62% and 28.68±0.44%, respectively. Based on results of the analyses performed after cooking, amounts of total saturated, total monounsaturated and total polyunsaturated fatty acid amounts of samples were found to be 30.17±0.61%, 41.34±0.11% and 25.92±0.24%, respectively. Because of oxidation during cooking, the amount of saturated fatty acids increased, whereas the amount of total monounsaturated and polyunsaturated fatty acids decreased. Cooking significantly affected the amounts of total saturated and polyunsaturated fatty acids of turkey meat döner (P<0.05), whereas it did not significantly affect the amounts of total monounsaturated fatty acids (P>0.05).

In conclusion, based on the data presented, turkey meat döner is rich in nutritive value and is an important poultry product for the consumer.

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Paper received December 28, 2005 Accepted March 17, 2006

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ANNOUNCEMENT

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Luxembourg
March 7-15, 2007

Course directors: E. Kampman PhD and R.P.J. van der Wielen PhD

The aim of the programme is to assist in the development of future leaders in the field of human nutrition in Europe. Emphasis is given to understanding the qualities and skills of leaders, team building, communication of nutrition information in a broader context, and to understanding the role of nutrition science in society.

These seminars are not focused on science, but on other skills and behavioural competences, which future leaders need to have. Therefore, professional trainers in team building, communication and leadership are invited to lecture and discuss individual skills and competences. The programme is organized by a group of leading European nutritionists in close collaboration with and supported by the European food industry.

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International Dairy Federation Exploration of milk genes to understand health benefits of dairy products

Experts in nutrition and genetics will gather at the Third International Symposium on Milk Genomics and Human Health in Brussels (19-21 September 2006) to present new scientific breakthroughs and the potential for research and development in understanding milk and its properties using genomics.

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The new field of Genomics provides new opportunities to understanding the role that each component plays in milk – by studying its genetic origins. The human genome is now sequenced and many other mammalian genomes are following. Those genes responsible for milk production can enlighten us as to the function of the different components in milk.

It is the first time the conference is located in Europe with many European scientists present. This gives a more European perspective on milk genomics. European initiatives on nutrition and genomics are producing the first generation of scientific research, and comparing the contrasting successes of plant-based initiatives versus milk-based initiatives is also very promising.

For further information, please contact Marylene Tucci, IDF Communications and Public Affairs, Tel. +32 2 7068644, Fax +32 2 7330413, e-mail: MTucci@fil-idf.org or

Visit the event website at http://milkgenomics.fil-idf-pr.com

BOOKS

PROBIOTIC DAIRY PRODUCTS

Edited by A.Y. Tamime
232 pages, hb, Illustrated
January 2006
ISBN: 101405121246 / 139781405121248
Price: £95.00 / US $ 169.99 / AU $ 313.50
Publisher: Blackwell Publishing

Following significant developments in recent understanding of milk systems and an explosion in interest in the use of probiotics and prebiotics as functional foods, *Probiotic Dairy Products* looks at advancements in the dairy industry and reviews the latest scientific developments in regard to the 'functional' aspects of dairy and fermented milk products and their ingredients. The first title in Blackwell Publishing's prestigious Society of Dairy Technology Series, this key text includes coverage of:

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- Physical properties associated with health claims
- Maintenance of the viability of probiotic products
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Edited by A.Y. Tamime
280 pages, hb, Illustrated
May 2006
ISBN: 100632064587 / 139780632064588
Price: £95.00 / US $ 169.99 / AU $ 313.50
Publisher: Blackwell Publishing

Highly profitable and an important range of products within the dairy industry worldwide, the economic importance of fermented milks continues to grow. Technological developments have led to a wider range of products and increased popularity with consumers. In the second book to feature in the SDT series *Fermented Milks* reviews the properties and manufacturing methods associated with products such as yoghurt, buttermilk, kefir, koumiss milk-based fermented beverages and many other examples from around the globe, offering the reader:

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344 pages, hb, Illustrated
July 2006
ISBN: 101405124601 / 139781405124607
Price: £95.00 / US $ 169.99 / AU $ 313.50
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SWEETENERS AND SUGAR ALTERNATIVES IN FOOD TECHNOLOGY

Edited by Helen Mitchell, Danisco Sweeteners Ltd, Redhill. Surrey, UK

432 Pages, Illustrated, Hardback

June 2006

ISBN: 1405134348 / 139781405134347

Price: £95.00 / US $ 169.99 / AU $ 314.00

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Gratitude is expressed to the following entities for contributing to the realization of the Journal by being supporting subscribers for 2006.

Si ringraziano i seguenti Enti, Ditte ed Istituti per aver voluto contribuire fattivamente alla realizzazione della Rivista, sottoscrivendo un abbonamento sostenitore per il 2006.

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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 © 2006

CHIRIOTTI EDITORI sas - 10064 Pinerolo - Italy

publishes the technical magazines:

VOLUME XVIII IJFS No. 3, 2006

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