ISSN 1120-1770

ITALIAN JOURNAL OF FOOD SCIENCE

Rivista italiana di scienza degli alimenti

IJFS

Volume XXII
Number 1
2010

CHIRIOTTI EDITORI

ITALIAN JOURNAL OF FOOD SCIENCE

(RIVISTA ITALIANA DI SCIENZA DEGLI ALIMENTI) 2nd series

Founded by Paolo Fantozzi under the sponsorship 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

Publisher:
Alberto Chiriotti
Chiriotti Editori sas, Viale Rimembranza 60, I-10064 Pinerolo, Italy - Tel. +39 0121 393127 - Telefax +39 0121 794480
e-mail: info@chiriottieditori.it - URL: www.chiriottieditori.it

Aim: The Italian Journal of Food Science is an international journal publishing original, basic and applied papers, reviews, short communications, surveys and opinions on food science and technology with specific reference to the Mediterranean Region. Its expanded scope includes food production, food engineering, food management, food quality, shelf-life, consumer acceptance of foodstuffs, food safety and nutrition and environmental aspects of food processing.

Reviews and surveys on specific topics relevant to the advancement of the Mediterranean food industry are particularly welcome.

Upon request and free of charge, announcements of congresses, presentations of research institutes, books and proceedings may also be published in a special "News" section.

Review Policy:

The Co-Editors with the Editor-in-Chief will select submitted manuscripts in relationship to their innovative and original content. Referees will be selected from the Advisory Board and/or qualified Italian or foreign scientists. Acceptance of a paper rests with the referees.

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

5 years Impact Factor: 0.651 published in the 2008 Journal of Citation Reports, Institute for Scientific Information ICV: 13.19 in the index Copernicus Journals Master List 2009.

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; Index Copernicus Journal Master List (PL).

IJFS has a page charge of € 25.00 each page.

Subscription Rate: IJFS is available on-line in PDF format only.
2010: Volume XXII: Ordinary € 50.00 - Supporting € 1,000.00

ITALIAN JOURNAL OF FOOD SCIENCE

IJFS

ADVISORY BOARD

R. Amarowicz
Editor-in-Chief
Polish J. Food and Nutrition Sci.
Olsztyn, Poland

A. Bertrand
Institut d'Oenologie
Université de Bordeaux
Talence Cedex, France

L.B. Bullerman
Dept. of Food Science and
Technology
University of Nebraska-Lincoln
Lincoln, NE, USA

C. Cannella
Ist. Scienza dell'Alimentazione
Università di Roma (La Sapienza)
Roma, Italy

F. Devlieghere
Dept. Food Technology and Nutrition
Faculty of Agricultural
and Applied Biological Sciences
Gent University
Gent, Belgium

S. Garattini
Ist. di Ricerche Farmacologiche
"Mario Negri"
Milano, Italy

J.W. King
Dept. Chemical Engineering
University of Arkansas
Fayetteville, AR, USA

T.P. Labuza
Dept. of Food
and Nutritional Sciences
University of Minnesota
St. Paul, MN, USA

A. Leclerc
Institut Pasteur
Paris, France

C. Lee
Dept. of Food Science and Technology
Cornell University,
Geneva, NY, USA

G. Mazza
Agriculture and Agri-Food Canada
Pacific Agri-Food Research Centre
Summerland, BC, Canada

J. O'Brien
School of Biological Sciences
University of Surrey
Guildford, Surrey, UK

J. Piggott
Dept. of Bioscience
and Biotechnology
University of Strathclyde
Glasgow, Scotland

S. Porretta
Associazione Italiana di
Tecnologie Alimentari (AITA)
Milano, Italy

J. Samelis
Dairy Research Institute
National Agricultural Research
Foundation
Ioannina, Greece

M. Suman
Food Research Lab
Barilla C.R. F.lli spa
Parma, Italy

M. Tsimidou
School of Chemistry,
Artisotle University
Thessaloniki, Greece

Prof. Emeritus J.R. Whitaker
Dept. of Food Science and Technology
University of California
Davis, CA, USA

LIFE CYCLE ASSESSMENT (LCA) OF SPANISH-STYLE GREEN TABLE OLIVES

G.M. CAPPELLETTI*, G.M. NICOLETTI[1] and C. RUSSO[1]
Dipartimento di Scienze Economico-Aziendali, Giuridiche, Merceologiche e Geografiche
(SEAGMeG), Università degli Studi di Foggia, Via Caggese, 1, 71100 Foggia, Italy
BIOAGROMED (Istituto per la Ricerca e le Applicazioni Biotecnologiche per la Sicurezza e la
Valorizzazione dei Prodotti Tipici e di Qualità), Università degli Studi di Foggia, Foggia, Italy
[1]Dipartimento di Scienze Economico-Aziendali, Giuridiche, Merceologiche e Geografiche
(SEAGMeG), Università degli Studi di Foggia, Via Caggese, 1, 71100 Foggia, Italy
*Corresponding author: g.cappelletti@unifg.it

ABSTRACT

The production of table olives is one of the important agro food sectors in the Mediterranean Area. Both the cultivation and the processing of table olives have increased in recent years. Consumption has also gone up, not only in Mediterranean countries but also in northern Europe and in the USA. As the rise in the production of table olives has caused a considerable increase in the amount of waste generated by processing industries, the environmental impact is considerable. In the table olive sector alone the amount of waste water that has to be treated and disposed of is in excess of 750,000 tons/year. Life Cycle Assessment methodology was applied to highlight the environmental hot spots arising during the production of green table olives, which are processed using the most commonly-used method world-wide: the Spanish-style. The analysis of the input and output materials and energy flows has enabled us to propose a hypothesis for reducing the impact on the environment.

- Key words: environmental impact, LCA, Spanish-style green table olives -

INTRODUCTION

In the Mediterranean area, the cultivation and processing of table olives has a very important part to play in the region's agro-industrial system. The production of table olives has increased world-wide in recent years (from 1,343,000 tons in 2000/01 to 1,796,000 tons in 2007/08) (IOOC, 2008).

The countries with the largest production of table olives in 2007/08 were Spain (493,200 tons), Turkey (270,000 tons) and Egypt (220,000 tons). Not only the production but also the consumption of table olives is concentrated in the Mediterranean region, with the largest consumers being Spain (235,000 tonnes), Egypt (200,000 tonnes) and Turkey (185,000 tonnes); however table olives are also consumed elsewhere, e.g. in the United States (228,000 t). In Italy about 66,000 tonnes of table olives are produced annually, but this amount is only able to satisfy 45% of the country's average consumption (146,000 tonnes).

Life cycle assessment (LCA) is commonly used for evaluating the environmental performance of products (VIGON et al., 1994; BAUMANN et al., 2004; GUINÉE et al., 2001; RUSSELL et al., 2005; UDO DE HAES et al., 2006; VEZZOLI et al., 2008), and recently many LCA studies have focused on the agro-food sector (SCHAU et al., 2008). As a rule, the greatest environmental impact is a result of the agricultural production and processing stages (MATTSON et al., 2003). Most of the production chains analysed have been those of milk, cheese, meat, seafood, etc. In recent times, other agro-food production chains have also been studied (RUSSO et al., 2009).

Our aim was to assess the environmental impact of the entire production chain of Spanish-style green table olives in Italy, starting from the production of the raw materials to the packaging of the finished product. Other studies have analysed the production chain of olive oil (NOTARNICOLA et al., 2004a), the agricultural phase of which has a lot in common with that of table olives.

MATERIALS AND METHODS

1. Goal and scope

LCA methodology was applied to the life cycle of Spanish-style green table olives packaged in tins and glass jars, as regulated by ISO 14040:2006 and ISO 14044:2006 standards (ISO 14040, 2006; ISO 14044, 2006).

Both the agricultural phase and the industrial phase were studied, the latter consisting of processing of the raw material and packaging in glass jars and tins. The type of container was also taken into consideration, to see how different methods of packaging (both primary and secondary) affect the environmental impact of the whole system under examination. The end life was excluded from the system boundaries. Fig. 1 shows the input and output flows of energy and materials, with the functional unit represented by 100 kg of drained packaged olives, including pit.

2. Inventory analysis

Agricultural phase

The production phase of the raw material includes all the agronomic practices connected to managing and irrigating an olive-grove, where the planting-distance for each tree is 5 m × 6 m.

The plantation is presumed to be in the southern Italy, and so the characteristics of the soil, the climatic conditions and the choice of fertilizers and plant protection products (from now on indicated as PPP) all refer to this area (INEA, 2009; SIAN, 2009; DE GENNARO et al., 2005; MILA I CANALS et al., 2007; NOTARNICOLA et al., 2004b; SALOMONE, 2008).

The agronomic practices examined were:

- three different soil-cultivation methods: one utilizing a rotary hoe and two a spring-tine harrow;
- two different fertilization plans: one including an application of mineral fertilizers (by means of mechanical spreading) to supply nitrogen (calcium nitrate), phosphorus (superphosphate) and potassium (potassium sulphate); the second fert-irrigation using an ammonium nitrate solution;
- one method of irrigation;
- a plant protection plan including: a fungicide treatment based on copper and lime, and two treatments using an organo-phosphorous compound (dimethoate) and a carbamate compound (carbaryl). The PPP were applied using a filed sprayer operated via tractor.

Industrial phase

As for the industrial phase, the Spanish-style method was the one chosen for examination (GARRIDO FERNÁNDEZ et al., 1997; SÁNCHEZ GÓMEZ et al., 2006). After the olives have been selected and calibrated, this method uses a process of "deamarization" (also called "lye treatment") to eliminate bitterness, where the olives are kept in lye (1.5-3% NaOH) for 8-12 hours and then rinsed several times with water. This is followed by a 2-3 month period of fermentation, during which the olives are kept in brine (6-8% NaCl).

Packaging

The study took into consideration the various types of glass jars and tins used for packaging the olives (Table 1). These containers are filled with olives and fresh brine, and they are then pasteurized at 90° C. After pasteurization, the glass jars and the 500 mL sized tins are put into appropriately-sized cardboard boxes (secondary packaging), in order to optimize the lo-

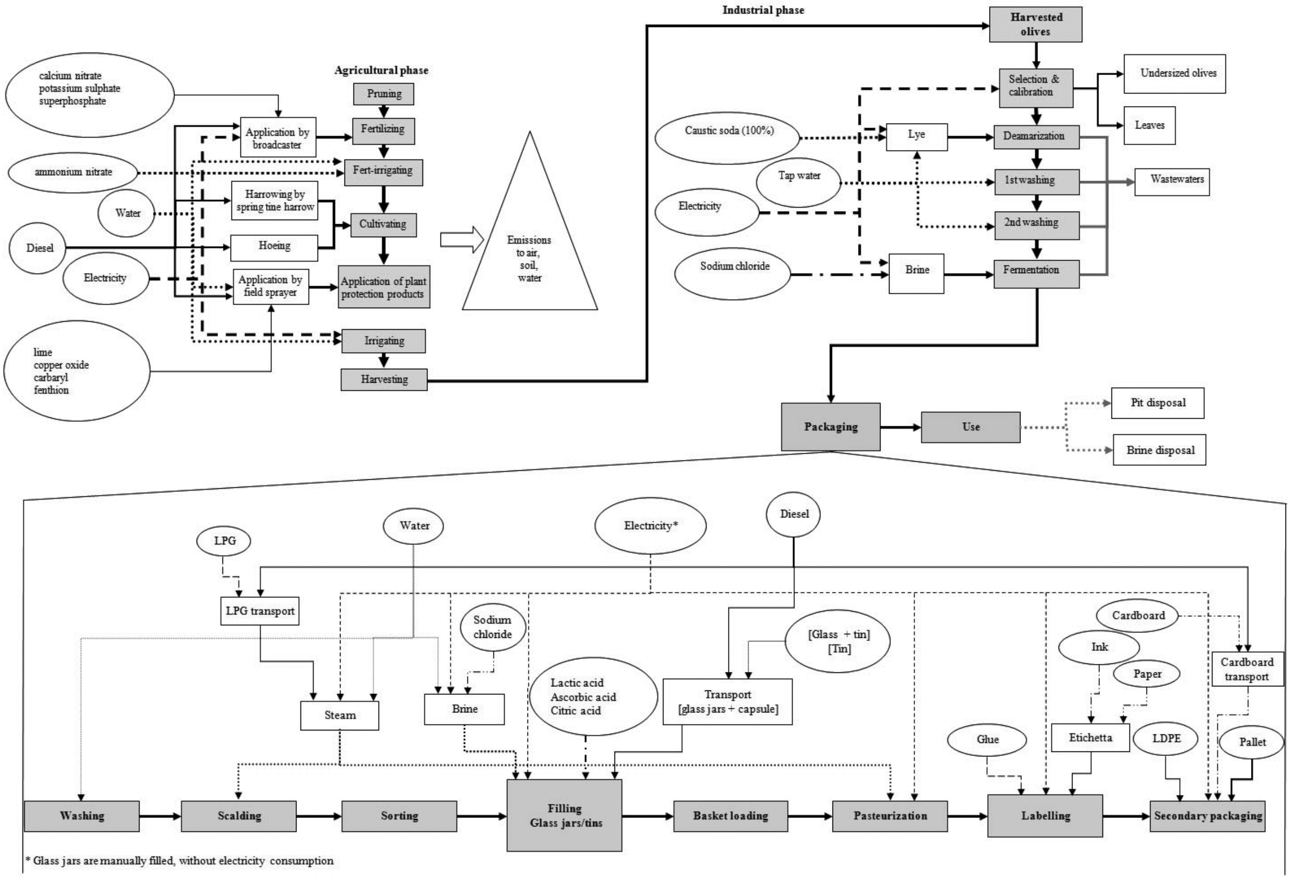


Fig. 1 - Spanish-style green table olives: layout.

gistics of transport and distribution. The 3000 mL and 4700 mL sized tins, on the other hand, are wrapped in PVC shrink film.

Data quality

The inventory data was collected from various sources. The information relating to the agricultural phase was received directly from people working in the sector. The consultation of almost forty farm-registers (made available by farmers' associations) was crucial for measuring the quantity of fertilizers, water and PPP used, and for finding out exactly which methods of soil cultivation were used. It was thus possible to estimate the consumption of fuel (for the agronomic practices) and electricity. As far as electricity production is concerned, the Italian mix was the type considered (AEEG, 2009). Emissions to water, air and soil deriving from the agricultural phase were calculated using the models available in the literature; emissions linked to the use of fertilisers were estimated using the Bentrup method (BRENTRUP et al., 2000), with the parameters being matched to the geographical context (e.g. precipitation rate); the N_2O emissions from the agricultural soil were also considered. The MacKay model (MACKAY et al., 1997) and the Hauschild dispersion model (HAUSCHILD, 2000) were used to assess the environmental distribution of plant-protection products. Ecoinvent databases were also used, as a means of assessing the impact of fertilizer and pesticide-production (FRISCHKNECHT et al., 2004) and fuel production.

The data concerning the technological processing was collected directly from two large

Table 1 - Weight of containers, capsules (glass jars), brine, types and number of packages for 100 kilograms of finished product.

100 kg of processed olives	Glass jars (mL)						Tins (mL)		
	314	580	720	1062	1700	3100	500	3000	4700
Brine (kg)	52.38	65.75	63.64	64.62	65.71	59.50	140.00	116.67	70.00
Capsule (kg)	4.29	4.11	4.09	3.38	2.67	1.40			
Container (kg)	90.48	78.08	73.86	77.69	72.29	50.50	27.00	20.92	14.28
Number of packages	476	273	227	153	95	50	500	83	40

processing companies which produce table olives, the PDO "La Bella della Daunia". Electricity consumption was measured, as well as the amount of input resources and quantities related to the primary and secondary packaging. The composition of the containers was defined, using data from the National Packaging Consortium (CONAI, 2009; COREVE, 2009; CIAL, 2009), and the percentage of the recycled material used for their manufacture was also specified. The same procedure was followed for the quantification of output; by means of laboratory analyses we assessed the pollution caused by the exhausted brines, lyes and washing waters. Emissions related to transport were calculated, while emissions related to the combustion of LPG (to produce steam) were obtained from the literature (ACUREX et al., 1993).

The data collected was processed using GaBi4 software (IKP and PE, 2002). Table 2 shows the amount of energy and materials entering the system under examination. As regards primary and secondary packaging, quantities of material and energy are given as the minimum and maximum values of the various types of glass (square brackets) and tin plate containers. Since the glass jars were packaged by hand, electricity consumption was taken as zero in this case.

Table 2 - Materials and energy input with reference to 100 kg of packaged olives.

INPUT		Agricultural phase	Industrial phase
Diesel	kg	0.3	0.2
Calcium nitrate	kg	0.4	
Potassium sulphate	kg	1.7	
Superphosphate	kg	1.2	
Ammoniun nitrate	kg	3.3	
Water	kg	50,050	333
Electricity	kWh	36.5	6
Lime	kg	0.08	
Copper oxide	kg	0.14	
Carbaryl	kg	0.07	
Dimethoate	kg	0.02	
Caustic soda (100%)	kg		1.3
Sodium chloride	kg		9.4
LPG	kg		1.3 - 1.7
Ascorbic acid	kg		0.07
Citric acid	kg		0.13
Lactic acid	kg		0.130
Tin plate	kg		14-27
[Glass/tin capsule]	kg		50-90/1.40-4.29
Paper (labels)	kg		0.07
[Cardboard]	kg		7-10
PVC	kg		0.2-0.4

RESULTS AND DISCUSSION

1. LCIA (Life Cycle Impact Assessment)

The CML 2001 impact assessment method was used to analyse the environmental impact of the input and output measured during the inventory phase. The categories of pollution considered were: abiotic depletion potential (ADP), acidification potential (AP), eutrophication potential (EP), freshwater aquatic ecotoxicity potential (FAETP inf.), global warming potential (GWP 100 years), human toxicity potential (HTP), marine aquatic ecotoxicity potential (MAETP), ozone layer depletion potential (ODP, steady state), photochemical ozone creation potential (POCP), terrestrial ecotoxicity potential (TETP) and energy use (EU).

The graph in Fig. 2.a shows the main categories of environmental damage due to the agricultural and industrial phases; the impact was normalized according to southern European factors and the different kinds of containers were considered separately.

Fig. 2.b, 2.c and 2.d show, for EP, FAETP, and GWP respectively, the contribution of the agricultural and industrial phases to the absolute values of the impact of the whole life cycle. The wastewater produced during the processing of the table olives has an effect on the EP of the industrial phase, while FATP is greatly affected by the agricultural phase due to the toxicity linked to the use of PPP. The large quantity of CO_2 produced in the industrial phase is related to LPG consumption during pasteurization, but the value varies according to the kind of primary and secondary packaging adopted. Fig. 2.d shows that the 3100 mL glass jars packaged in cardboard boxes produce the lowest amount of CO_2.

As for the analysis of total energy use, Fig. 2.d shows the MJ required for the agricultural and industrial phases. The total energy-use value varies according to the type of primary and secondary packaging. The 3000 mL tin is the type of packaging that requires the lowest amount of energy.

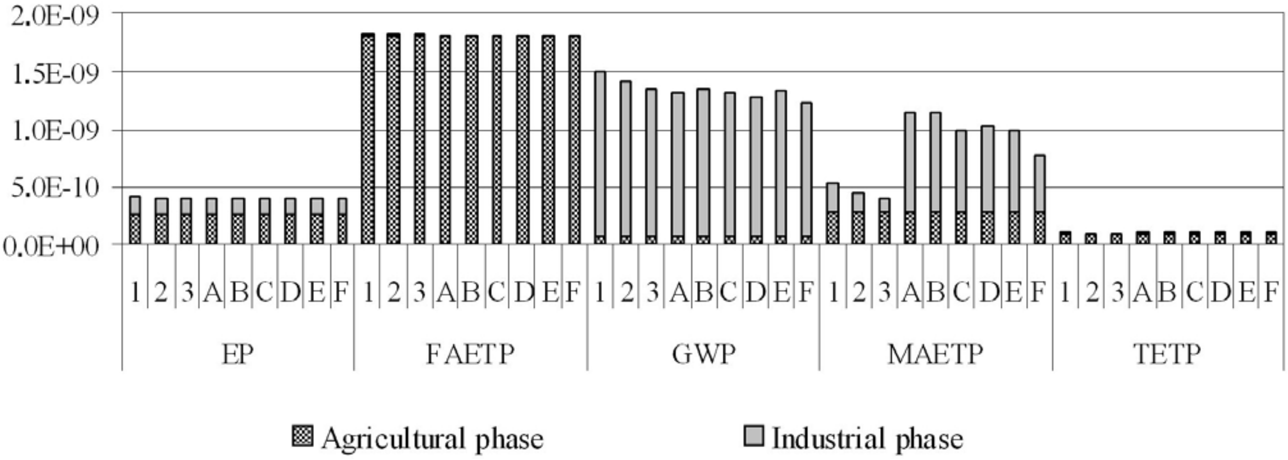
Agricultural phase

By analyzing the agricultural phase (Fig. 3), it is possible to see the contribution of the various sub-phases to the most important impact categories (Fig. 3.a). The impact of PPP greatly affects the different toxicity categories, amounting to almost 100% of the total impact when considering FAETP and TETP. Fig. 3.b indicates that such problems are attributable more to the application than to the production of chemicals.

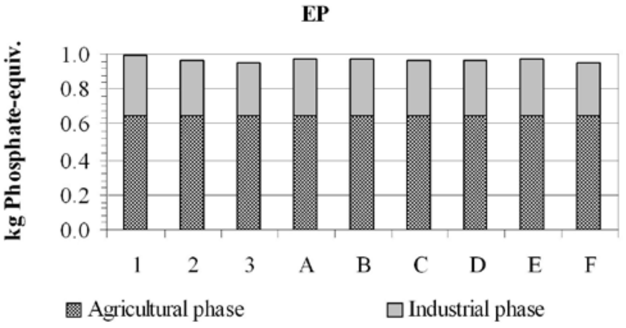
The use of fertilizers represents 99% of EP. Again, as can be seen from the graph in Fig. 3.c, this stems from the effects of their application, while other kinds of impact derive from the production of ammonium nitrate. The production and use of fertilizers (especially ammonium nitrate), combined with the electricity needed for irrigation, also contribute to GWP (Fig. 3.a). As for cultivation practices, irrigation has a higher impact than mechanical operations (soil cul-

Tin	500 mL = 1	3000 mL = 2	4700 mL = 3			
Glass jar + capsule	314 mL = A	580 mL = B	720 mL = C	1062 mL = D	1700 mL = E	3100 mL = F

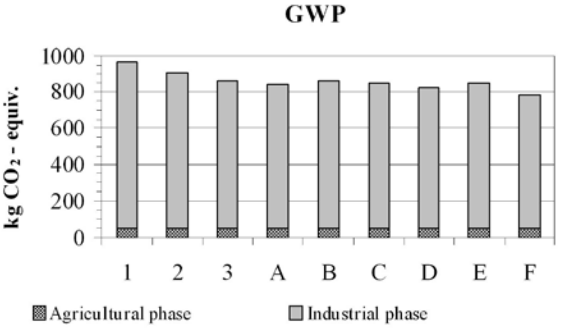
2.a: Normalized results.



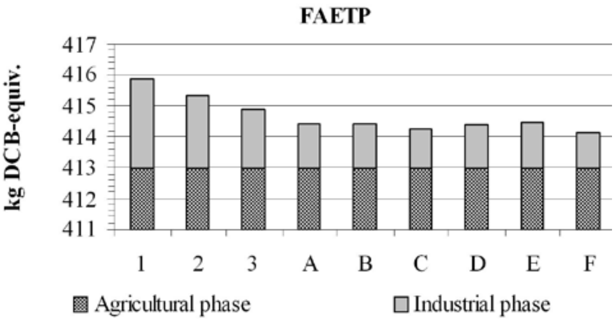
2.b: Eutrophication potential.



2.d: Global warming potential.



2.c: Freshwater acquatic ecotoxicity potetial.



2.e: Energy use.

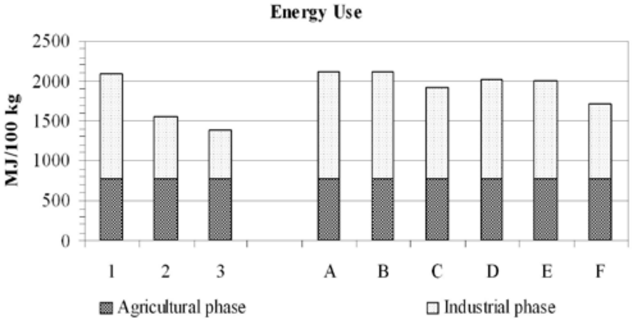
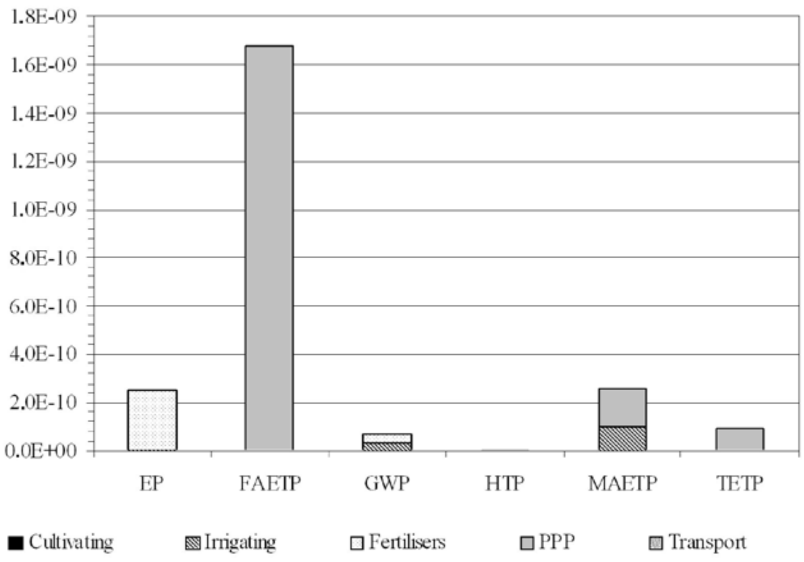


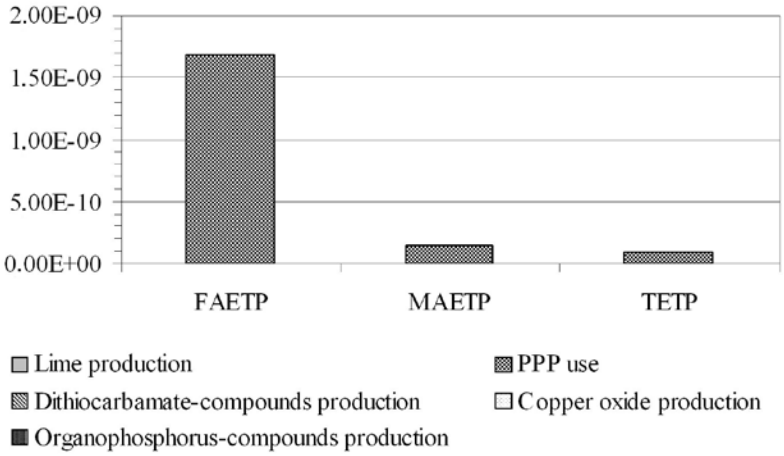
Fig. 2 - The most serious kinds of environmental impact caused by different types of packaging with reference to the functional unit.

tivation, mechanical application of plant protection products and fertilizers). This is because of the energy used to pump the enormous amounts of water needed to grow the raw material to a size that appeals to consumers.

3.a: Agricultural phase – normalized results.



3.b: Agricultural phase – plant protection products.



3.c: Agricultural phase – fertilizers.

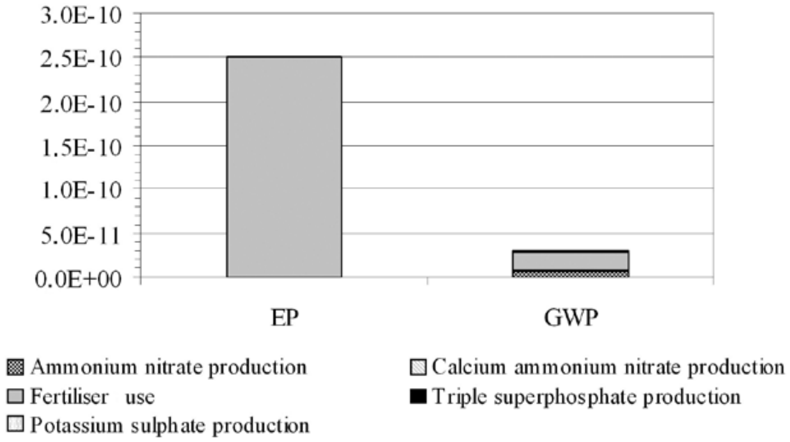


Fig. 3 - Impact of agricultural phase.

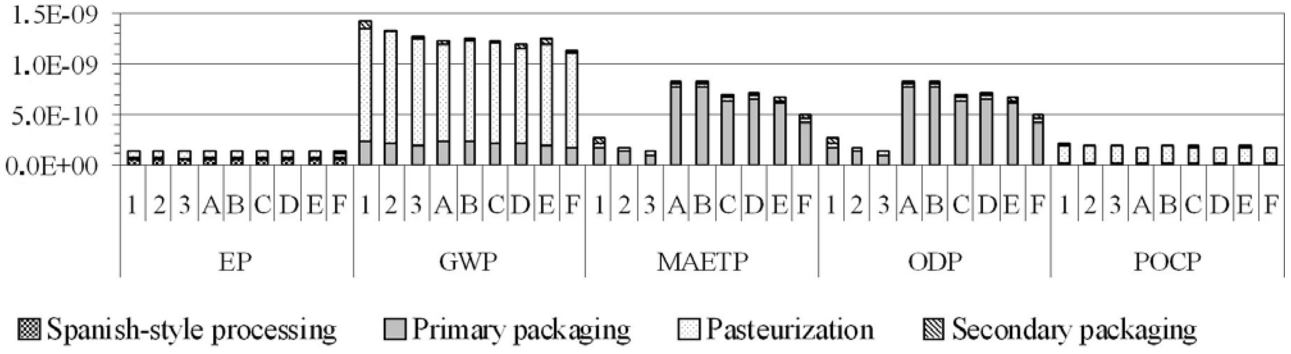
Following the study of CRUTZEN et al. (2008), the N_2O emissions linked to fertiliser use had increased by 4%. This data variation has brought about an increase of over 95% for the GWP linked to the agricultural phase; but for the overall life cycle, the total kg of CO_2 per functional unit rose by only 5%.

Industrial phase

Fig. 4 shows the environmental impact associated with the industrial phase; pasteurization accounts for a large part of the GWP. As can be seen in the graph in Fig. 5, waste water is one of the problems with the Spanish-style processing method. Lyes, washing waters and brines have high BOD5 and COD values, which affect the EP. The most important categories of environmental damage linked to the Spanish-style processing-method are also affected by the production of the caustic soda used to de-bitter the olives, and by the electricity used in the technological process (Fig. 5). This impact, however, is lower than that of pasteurization (Fig. 4.a, 4.b), a process which is necessary to guarantee the microbiological safety of the product. The choice of packaging material (the different types of tin and glass container) affects the impact of the overall system in various ways. By comparing the environmental effects of the different containers, it is immediately obvious that there are environmental benefits linked to the adoption of larger-sized containers (Fig. 6). This allows not only a lower consumption of resources, but also a reduction in waste production and emissions during packaging, transport and final disposal. As far as tins are concerned, increasing the size of the containers leads to a proportional reduction in their impact. However, this is not the case for the six types of glass jars examined. In this case, increasing the size of the container does not automatically lead to a reduction in impact. The main reason for this lies in the properties of the container itself: to

Tin	500 mL = 1	3000 mL = 2	4700 mL = 3			
Glass jar + capsule	314 mL = A	580 mL = B	720 mL = C	1062 mL = D	1700 mL = E	3100 mL = F

4.a: Normalized results.



4.b: Global warming potential.

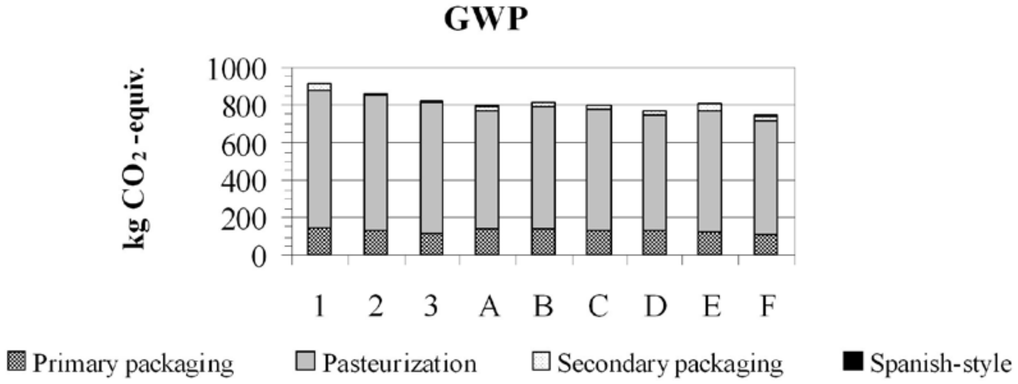


Fig. 4 - Impact of industrial phase, separated according to the different kinds of containers.

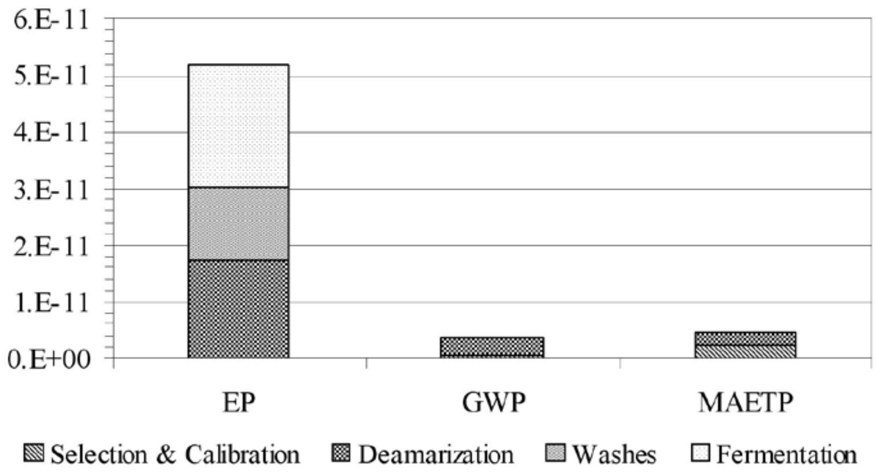


Fig. 5 - Environmental effects of Spanish-style processing.

Tin	500 mL = 1	3000 mL = 2	4700 mL = 3			
Glass jar + capsule	314 mL = A	580 mL = B	720 mL = C	1062 mL = D	1700 mL = E	3100 mL = F

Figure 6.a: ADP

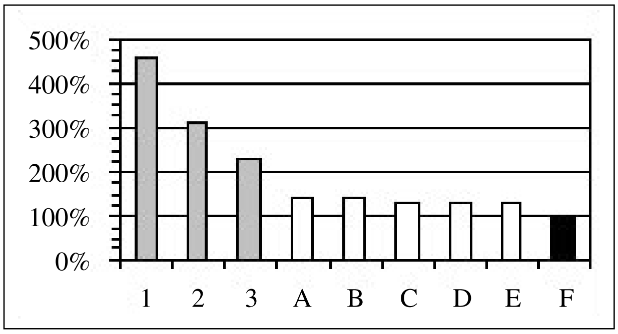


Figure 6.b: EP

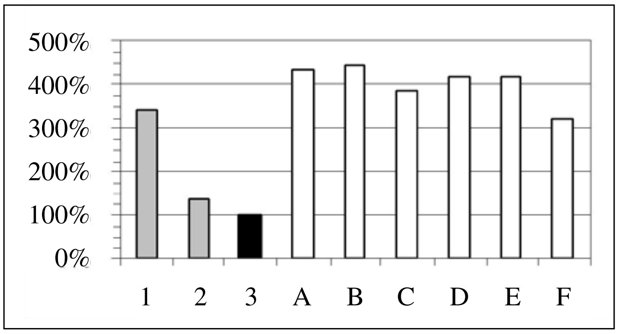


Figure 6.c: FAETP

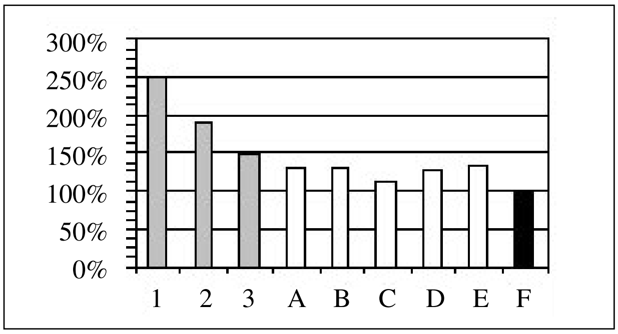


Figure 6.d: GWP

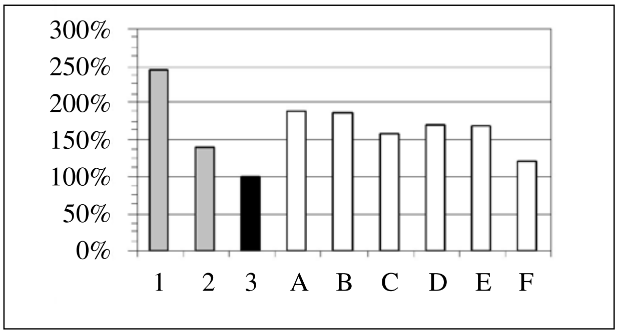


Figure 6.e: ODP

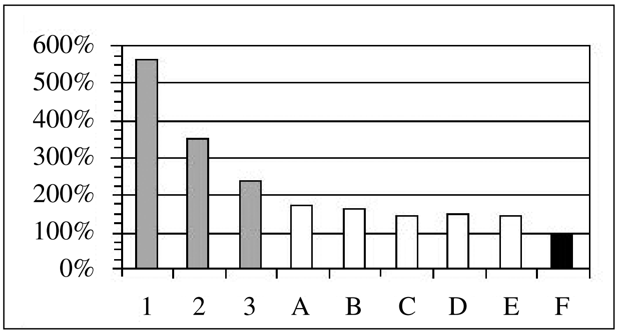


Figure 6.f: MAETP

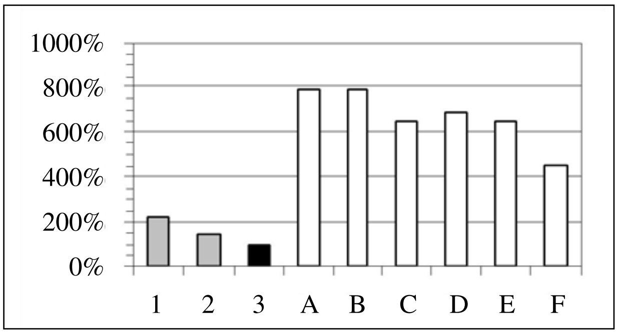


Figure 6.g: HTP

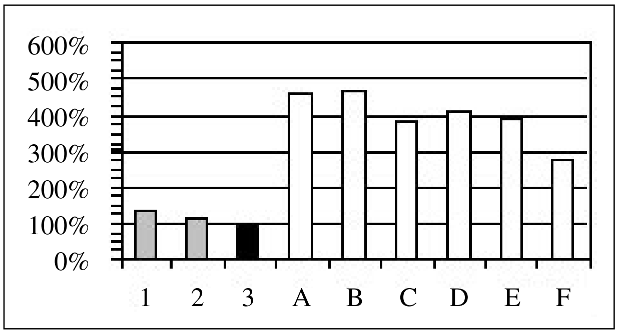


Figure 6.h: TETP

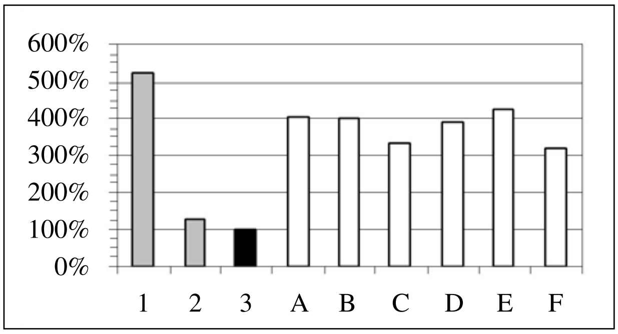


Fig. 6 - Percentage comparison of different containers relative to the container that has the least impact.

ensure the integrity of the packaging and efficiency of logistics, each container needs suitable secondary packaging. There is thus a variation in the amount of cardboard required per functional unit. The graphs in Fig. 6 show the results of comparing the different kinds of packaging regarding the most significant categories of impact they cause. In this way it was possible to quantify, with regard to each category of impact, the contributions of each type of packaging and to identify the container which has the best environmental performance.

As stated in the introduction, the various packaging systems employ different kinds and quantities of raw materials and energy: the larger-sized containers have less environmental impact.

As far as ADP, FAETP and ODP are concerned, the larger-sized glass jars are the more "sustainable" packaging choice. The reverse is true for the other categories of impact (EP, GWP, TETP, MAETP,), where the production and use of glass and cardboard have a greater negative impact, and the 4700 mL tins are more eco-compatible.

2.Improvement hypothesis

Agricultural phase

Efforts to reduce the environmental impact which results from the agricultural phase should concentrate on improving the necessary cultivation techniques, trying to reduce waste and optimising water consumption. Using manure instead of mineral fertilizers would reduce the impact arising from the production of ammonium nitrate. At the same time a reduction in the use of plant protection products would be welcome, as this would improve the defence systems of the crop and have little environmental impact. The use of traps for catching harmful insects could replace the use of organo-phosphorous insecticides, which are toxic for humans and the environment. Plantations for growing table olives need a proper, reliable water-supply, especially during the summer months when the fruits grow to a size which gives them a higher commercial value. The optimization of water resources, however, is a fundamental objective, especially in geographic areas characterized by long periods of drought. It is possible to reduce water waste by optimizing the amount of water used for irrigation. More efficient irrigation techniques could be employed, such as localized underground irrigation carried out by means of micro-droppers under the soil.

Industrial phase

In order to improve the environmental performance of the Spanish-style olive processing-system, a technological solution could be adopted with the aim of reducing the amount of caustic soda and water used (and consequently limit the negative effects connected to its production and use) (MARSILIO et al., 2008). It is also worth considering reusing the brine and the rinsing-water, as well as extracting the useful substances (phenols etc.) (BELTRÁN-HEREDIA et al., 2000a; 2000b; BELTRÁN et al., 2001; BRENES BALBUENA et al., 1989; BRENES et al., 2000; DE CASTRO et al., 1983; FERNÁNDEZ et al., 1992; GARCÍA et al., 1990; GARRIDO et al., 1992; GARRIDO FERNÁNDEZ, 1983; GARRIDO FERNÁNDEZ et al., 1997; KOPSIDAS, 1994; RIVAS et al., 2000; 2001; VEGA et al., 1982).

The emissions from thermal treatment could be significantly reduced by adopting "pasteurization programs" with the pasteurization time varying according to the size of the container. This would call for greater care on the part of the operators, who would be responsible for varying the length of the process.

In practice, however, standardized pasteurization schemes with a single programme parameterized for the larger containers are used so as to avoid insufficient pasteurization of the finished product. This results in an inefficient use of energy and increased emissions of greenhouse gases. Improved efficiency of the pasteurization process would bring about a significant reduction in the impact associated with it, with obvious advantages with regard to the following categories of impact: ADP, AP, EP, GWP, POCP. By examining the various types of glass and tin packaging, to find out how they contribute to the eight categories of impact, it is clear that using tin instead of glass means savings in terms of global warming and eutrophication, and also in terms of human, soil and marine toxicity. It must be noted, however, that the use of tins (in particular the 500 mL containers) causes a rise in abiotic depletion and ozone depletion of up to 500% and an increase in fresh water toxicity of about 250%.

As far as sustainability is concerned, the implications are that companies should use larger containers as much as possible. This could be achieved through the use of dispensers in grocery stores, where consumers could buy the bulk product in the desired quantities; special bags and reusable containers could be provided for transportation.

INTERPRETATION

At the end of the LCA, the variability of the results was analysed. By means of GaBi software, it was possible to carry out both a Sensitivity analysis and a Monte Carlo analysis; for these analyses only the damage categories GWP, FAETP, EP and Energy use were considered (because these categories proved to be the most important).

Table 3 shows the variability (as Standard deviation) of the kinds of impact with respect to the variability of the data considered in input. The variability in the amount of irrigation high-

Table 3 - Sensitivity analysis.

		Standard deviation			
	Quantity	GWP	FAETP	EP	Energy use
Number of irrigations	±100%	±2.66%	±0.01%	±0.59%	±24%
Ammonium nitrate	±180%	±4.13%	0.00%	±36.70%	±0.53%
Calcium ammonium nitrate	±100%	±0.10%	0.00%	±3.50%	±0.12%
Potassium sulphate	±70%	0.00%	0.00%	0.00%	±0.04%
Triple superphosphate	±270%	±0.23%	0.00%	±114%	±0.55%
Carbaryl	±150%	±0.04%	±118%	±0.03%	±0.40%
Copper oxide	±400%	±0.15%	±78.70%	±0.27%	±1.62%
Dimethoate	±150%	±0.06%	±1.91%	±0.02%	±0.48%
Time of pasteurization	±10%	±7.47%	0.00%	±1.28%	±0.07%

Table 4 - Table of "hot spots".

	GWP	FAETP	EP	Energy Use
Number of irrigations	X			XX
Ammonium nitrate	X		XX	
Calcium ammonium nitrate			X	
Potassium sulphate				
Triple superphosphate			XXX	
Carbaryl		XXX		
Copper oxide		XXX		X
Dimethoate		X		
Time of pasteurization	X		X	
XXX > 50%	10% ≤ XX ≤ 50%		1% ≤ X ≤ 10%	

ly influences the Energy use and GWP due to the use of electricity. The impact category EP is affected by the quantity of nitrogen and phosphate fertilisers, with ammonium nitrate also affecting GWP. The standard deviation referring to the quantity of PPP used greatly influences the variability of FAETP, with the exception of dimethoate because of its lower polluting potential. Changing the pasteurization programs results in little variability in GWP.

As regards the Monte Carlo analysis, Fig. 7 shows the Gaussian distribution of the 20 clusters deriving from the simulation. The higher probability of GWP is related to the variability of the input value (between 0% and 20%, while with that of EU the cluster is between -20% and 0%); as far as FAETP and EP are concerned, the probability is higher. Table 4 shows the hot spots - the sources of greatest impact - for each category of damage (with a variation of >50%): these are carbaryl (±118%) and copper oxide (±78.7%) for FAETP, and triple superphosphate (±114%) for EP. A significant impact is linked to the amount of irrigation (±24%) for EU and the quantity of ammonium nitrate (±36.7%) for EP (which shows a variability between 10% and 50%). The other kinds of impact have a variation between 1% and 10%. An improvement in these hot spots could bring about a consequential reduction in their environmental impact, with respect to their importance. In particular, as for the time of pasteurization, it greatly affects the GWP; Table 3 shows, in fact, that the GWP value varies almost in the same way as the time of pasteurization (a variation of the time of pasteurization between ±10% corre-

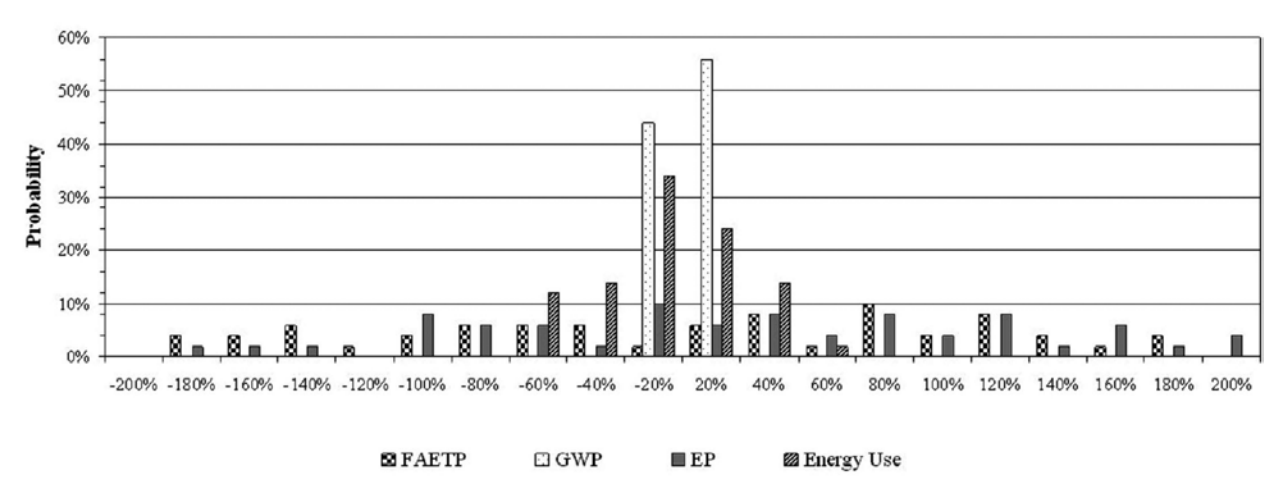


Fig. 7 - Monte Carlo analysis.

sponds to a variation of the GWP almost of the same percentage).

CONCLUSION

The analysis presented has highlighted the "hot spots" of the life cycle of Spanish-style green table olives. Although some of the data considered in the Inventory analysis (LCI) may have a degree of uncertainty, this study has painted a picture of the environmental performance that stems from the production and processing of green table olives. The most important environmental problem deriving from the olive production can be attributed to the chemicals used for pest control. This is also true for olive oil production. However with table olives the farmer has to obtain a raw material which is whole and aesthetically perfect, in order to get a better price. Damaged table olives cannot be sold to processing industries; they could be used to make olive oil, but their oil content is lower than that of the olives grown specifically to produce oil, and they also sell for less. So what is needed are sustainable solutions, such as the eco-trap. The most serious problem arising from the industrial phase is the emission of greenhouse gases. This derives mainly from the pasteurization process, but production of the containers (tins, glass jars, cardboard boxes) also results in a high impact. Something else worth mentioning is that the large quantity of steam used to pasteurize the containers is commonly produced by a boiler fed by LPG, the combustion of which results in a high value for GWP during the industrial phase.

In this study it was possible to analyse the differences between various types of packaging. The environmental impact varies significantly according to the type of package that is selected, decreasing as the size of the container increases. Generally, the choice of larger- sized containers leads to improvement in terms of reduced impact. Some solutions are also proposed for improving the agricultural and industrial phases and, in conclusion, the analysis of the system showed where improvements could be made with regard to achieving the objectives of eco-compatibility. In most cases the adoption of eco-efficient choices leads to advantages, which are not only environmental (a lower consumption of resources, a reduction in pollutants, etc.) but also economic (i.e. lower costs).

REFERENCES

Acurex Environmental Corporation 1993. Edward Aul E. & Associates Inc., Pechan E.H. & Associates Inc. Emission factor documentation for AP-42 section 1.5 Liquefied petroleum gas combustion. Supplement B., U. S. Environmental Protection Agency. Research Triangle Park, North Carolina.

Baumann H. and Tillman A.M. 2004. The hitchhiker's guide to LCA: an orientation in life cycle assessment methodology and application. Studentlitteratur, Lund, Sweden.

Beltrán-Heredia J., Torregrosa J., Dominguez J.R. and Garcia J. 2000a. Aerobic biological treatment of black table olive washing wastewaters: effect of an ozonation stage. Process Biochem. 35: 1183.

Beltrán-Heredia J., Torregrosa J., Dominguez J.R. and Garcia J. 2000b. Ozonation of black-table-olive industrial wastewaters: effect of an aerobic biological pre-treatment. J. Chem. Technol. Biotechnol. 75: 561.

Beltrán F., García-Araya J.F., Navarrete V. and Gimeno O. 2001. Treatment of wastewater from table olive industries: quantum yield of photolytic processes. Bull. Environ. Contam. Toxicol. 67: 195.

Brenes Balbuena M., De Vicente Fernández J., García García P. and Garrido Fernández A. 1989. Characteristics of waste waters from the elaboration of table olives. Grasas Aceites 40: 287.

Brenes M., García P., Romero C. and Garrido A. 2000. Treatment of green table olive waste waters by an activated-sludge process. J. Chem. Technol. Biotechnol. 75: 459.

Brentrup F., Küsters J., Lammel J. and Kuhlmann H. 2000. Methods to estimate on-field nitrogen emissions from crop production as an input to LCA studies in the agricultural sector. Int. J. Life Cycle Assess. 5: 349.

Castro Gomez-Millan A., Duran Quintana M.C., Garcia Garcia P., Garrido Fernàndez A., Gonzales Cancho F., Rejano Navarro L., Sànchez Roldàn F and Sànchez Tèbar J.C. 1983. Processing fruits of «Gordal» variety, without washing and reusing lye solutions, as Spanish style green style olives. Wastewaters study and some purification tests. Grasas Aceites 3: 162.

Crutzen P.J., Mosier A.R., Smith K.A. and Winiwarter W. 2008. N_2O release from agro-biofuel production negates global warming reduction by replacing fossil fuels. Atmos. Chem. Phys. 8: 389.

De Gennaro B., Notarnicola B. and Tassielli G. 2005. Ricadute ambientali nella filiera dell'olio di oliva. Estimo e Territorio 48: 11.

Fernández A.G., Balbuena M.B. and García P.G. 1992. Treatment for green table olive fermentation brines. Grasas Aceites 43: 291.

Frischknecht, R. and Jungbluth N., (Editors) 2007. Overview and methodology - Ecoinvent report No. 1. Dübendorf, December 2007 from http://www.ecoinvent.org/fileadmin/documents/en/01_OverviewAndMethodology.pdf

García Garcìa P., Brenes Balbuena M., Vicente Fernández J. and Garrido Fernández A. 1990. Physicochemical depuration of the waste-waters from the green table olive packing industries. Grasas Aceites 41: 263.

Garrido A., García P. and Brenes M. 1992. The recycling of table olive brine using ultrafiltration and activated carbon adsorption. J. Food Eng. 17: 291.

Garrido Fernandez A. 1983. Study of ripe olive wastewaters and their reusing possibilities. Grasas Aceites 5: 317.

Garrido Fernandez A., Fernandez Diez J. and Adams M.R. 1997. "Table Olives". Chapman & Hall. London.

Guinée J.B., Gorée M., Heijungs R., Huppes G., Kleijn R., Koning A. de, Oers L. van, Wegener Sleeswijk A., Suh S., Udo de Haes H.A., Bruijn H. de, Duin R. van and Huijbregts M.A.J. 2001. "Handbook on Life Cycle Assessment - Operational Guide to the ISO Standards". Kluwer Academic Publishers, Dordrecht.

Retrieved from http://www.leidenuniv.nl/cml/ssp/projects/lca2/lca2.html.

Hauschild M. 2000. Estimating pesticide emissions for LCA of agricultural products. In "Agricultural Data for Life Cycle Assessment" B.P. Weidema M. and Meeusen J.C. The Hague Agricultural Economics Research Institute 2: 64.

IKP and PE. 2002. GaBi 4 - Software-system and databases for life cycle engineering. Stuttgart. Echterdingen.

ISO 14040. 2006. Environmental management - Life cycle assessment - Principles and framework (ISO 14040:2006). International Standard. Geneva.

ISO 14044. 2006. Environmental management - Life cycle assessment - Requirements and guidelines (ISO 14044:2006). International Standard. Geneva.

Kopsidas G.C. 1994. Waste-water from the table-olive industry. Water Res. 28: 201.

Mackay D., Wan Ying Shi and Kuo Ching Mai 1997. Illustrated handbook of physical-chemical properties and environmental fate for organic chemicals: pesticides. CRC Press. Boca Raton, Florida, USA.

Marsilio V., Russi F., Iannucci E. and Sabatini N. 2008. Effects of alkali neutralization with CO_2 on fermentation, chemical parameters and sensory characteristics in Spanish-style green olives (*Olea europaea L.*). LWT – Food Sci. Technol. 5: 796.

Mattson B. and Sonesson U. 2003. "Environmentally – Friendly Food Processing". SIK. Göteborg, Sweden.

Mila i Canals L., Romanya J. and Cowell J.S. 2007. Method for assessing impacts on life support functions (LSF) related to the use of fertile land in Life Cycle Assessment (LCA). J. Clean. Prod. 15: 1426.

Retrieved from http://www.sciencedirect.com/science/article/B6VFX-4KB111FI/1/2/d934e973bd29b9d1479e0cd005b4ba38

Notarnicola B., Tassielli G. and Nicoletti G.M. 2004a. LCC and LCA of extra-virgin olive oil: organic vs. conventional. Life Cycle Assessment in the Agri-food sector - Proceeding from the 4th International Conference, October 6-8 2003, Bygholm, Denmark. N. Halberg. Horsens. Denmark. Danish Institute of Agricultural Sciences (DIAS): 289.

Retrieved from http://www.lcafood.dk/lca_conf/DJFrapport_paper_2_poster.pdf

Notarnicola B., Tassielli G. and Nicoletti G.M. 2004b. Environmental and economical analysis of the organic and conventional extra-virgin olive oil. New Medit 3: 28.

Rivas F.J., Beltrán F.J., and Gimeno. O. 2000. Joint treatment of wastewater from table olive processing and urban wastewater. Integrated ozonation-aerobic oxidation. Chem. Eng. Technol. 23: 177.

Rivas F.J., Beltrán F.J., Gimeno O. and Alvarez P. 2001. Chemical-biological treatment of table olive manufacturing wastewater. J. Environ. Eng.-Asce 127: 611.

Russell A., Ekvall T. and Baumann H. 2005. Life cycle assessment - introduction and overview. J. Clean. Prod. 13: 1207.

Retrieved from http://www.sciencedirect.com/science/article/B6VFX-4GDBT7K-3/2/f65de94ab5f9d393a731395c5a26be18.

Russo C., Cappelletti G.M. and Nicoletti G.M. 2009. LCA of Californian black-ripe table olives. Proceedings of the 6th International Conference on LCA in the Agri-Food Sector – Towards a Sustainable Management of the Food Chain. November 12-14, 2008, Zurich, Switzerland, Nemecek, T. & Gaillard, G. (eds.), Agroscope Reckenholz-Tänikon Research Station ART.

Russo C., Cappelletti G.M. and Nicoletti G.M. 2010. Life Cycle Assessment (LCA) used to compare two different methods of processing ripe table olives. Grasas Aceites, 61: 136.

Salomone R. 2008. "Applicazione della Life Cycle Assessment in Aziende di Produzione di Olio di Oliva: Analisi Comparativa degli Studi Italiani. In "Ecomondo 2008", vol. 2. Morselli L. (ED.), p. 454. Maggioli Editore, Santarcangelo di Romagna (RN), Italy.

Sánchez Gómez A.H., García García P. and Navarro L.R. 2006. Elaboration of table olives. Grasas Aceites, 57: 86.

Schau E.M. and Fet A.M. 2008. LCA studies of food products as background for environmental product declarations. Int. J. Life Cycle Assess. 13: 255. Retrieved from http://dx.doi.org/l0.1065/lca2007.12.372.

Udo de Haes H.A. 2006. Sustainable management of natural resources in all Life-cycle perspective. Int. J. Life Cycle Assess. 11: 2.

Retrieved from http://dx.doi.org/10.1065/lca2006.01.001.

Vega M.N., Ladrón R.V. and Ramos R.D. 1982. Utilization possibilities of by-products from table olive industry. Grasas Aceites 33: 135.

Vezzoli C. and Manzini E. 2008. "Design for Environmental Sustainability". Springer. London. U.K. Retrieved from http://dx.doi.org/10.1007/978-1-84800-163-3_12.

Vigon B.W, Tolle D.A., Cornaby B.W., Latham H.C., Harrison C.L., Boguski T.L., Hunt R.G., Sellers J.D. and U.S.E.P.A. 1994. Risk reduction engineering laboratory. Life-cycle assessment inventory guidelines and principles. Lewis publishers, Boca Raton, Florida, USA.

Websites:

AEEG, www.autorita.energia.it (Web site consulted 1st Sept 2009).

CONAI, www.conai.org, (Web sites consulted 1st Sep 2009).

COREVE, www.coreve.it, (Web sites consulted 1st Sep 2009).

CIAL, www.cial.it. (Web sites consulted Sept 1st 2009).

INEA, www.inea.it/otris (Web site consulted 1st Sept 2009).

IOOC, www.internationaloliveoil.org (Web site consulted 1st Sept 2009).

SIAN, www.sian.it (Web site consulted 1st Sept 2009).

CONTRIBUTION OF AUTHORS

This paper has been thought, discussed and written by the three authors and it is the result of their common commitment; in particular C. Russo contributed to data collection and classification, and bibliographical research, G.M. Cappelletti contributed to elaboration and comment on data, G.M. Nicoletti contributed to planning and final review of the research.

Revised paper received May 21, 2009 Accepted September 8, 2009

APPLICATION OF AN ANALYTICAL METHOD FOR THE SIMULTANEOUS DETERMINATION OF THE OFF-FLAVOUR VOLATILES GEOSMIN, 4-ETHYLPHENOL AND 4-ETHYLGUAIACOL AND OF TARGET WINE AROMA VOLATILES

M.T. LISANTI*, A. GENOVESE, P. PIOMBINO, A. GAMBUTI and L. MOIO
Dipartimento di Scienza degli Alimenti, Università degli Studi di Napoli "Federico II",
Via Università 100, 80055 Portici, NA, Italy
*Corresponding author: Tel./Fax +39 825 784678,
e-mail: mt.lisanti@hotmail.it

ABSTRACT

An analytical method, specifically developed and used to determine the presence of geosmin in wine, was evaluated for the simultaneous determination of geosmin, responsible for an earthy off-flavour, 4-ethylphenol and 4-ethylguaiacol, responsible for phenolic off-flavour and of selected volatile wine aroma compounds. The method consists of a liquid-liquid extraction with n-pentane followed by GC-MS/SIM analysis. The linearity of the response was very good ($r^2>0.9956$) in the usual concentration ranges of the volatiles in wines. The precision was also very good (%RSD less than or near 10%) and the accuracy of the method, was satisfactory. LOD and LOQ were below 1 or a few mg/L for most compounds. The method was then applied to evaluate the efficacy of a treatment with deodorant activated charcoal to decrease the amount of geosmin, 4-ethylphenol, 4-ethylguaiacol and its effect on the concentration of the volatile aroma compounds, both in red and white wines.

- Key words: aroma, 4-ethylguaiacol, 4-ethylphenol, geosmin, off-flavours, wine -

INTRODUCTION

Organoleptic quality is a fundamental aspect of the overall quality of wines. In some cases aroma is an expression of varietal or territorial identity and plays, an outstanding role in organoleptic quality. It is one of the principal evaluation criteria used by the purchasers of wine. Wine aroma is determined by the complex equilibrium of several hundreds of volatile compounds in a wide range of concentrations and belonging to different chemical classes (alcohols, esters, aldehydes, ketones, fatty acids, terpenes, phenols, etc.). This complex equilibrium can be altered due to chemical or microbiological contamination of grapes and wines. In these cases, several volatile compounds characterized by unpleasant odorous notes can result in the appearance of off-flavours if the concentrations are higher than the olfactory perception threshold. Among the off-flavours that can appear in wine, the "musty-earthy" and "phenolic" ones are particularly detrimental to the organoleptic quality of wine. Odours described as mushrooms, mouldy, camphoric or earthy are often observed in must made with rotten grapes, particularly when high rainfall and hail occurred during the vintage. In previous studies, geosmin (*trans*-1,10-dimethyl-*trans*-9-decalol) was identified as being responsible for a strong earthy odour in red and white wines even at low concentration, due to its extremely low olfactory perception threshold (10 ng/L in water, 60-65 ng/L in white wine, 80-90 ng/L in red wine (DARRIET *et al.*, 2000; DARRIET *et al.*, 2001). Geosmin originates from the metabolism of *Penicillium expansum* fungus in grapes pre-contaminated by *Botrytis cinerea* (LA GUERCHE *et al.*, 2005) and is very stable during alcoholic fermentation (DARRIET *et al.*, 2002; LA GUERCHE *et al.*, 2006).

4-Ethylphenol (4-EF) and 4-ethylguaiacol (4-EG), responsible for the phenolic off-flavour, are principally produced by the winery contaminant yeast *Brettanomyces*/*Dekkera* by means of the decarboxylation and subsequent reduction of grape-derived ferulic and *p*-cumaric acids (CHATONNET *et al.*, 1992). The aroma associated with these phenols in wine has been generally described as stable, animal and spicy (CHATONNET *et al.*, 1990; CHATONNET *et al.*, 1992). Wood casks used in wine ageing are an optimal substrate for the proliferation of *Brettanomyces*; it can also proliferate in tanks and bottles (CHATONNET *et al.*, 1992; SUÁREZ *et al.*, 2007; PÉREZ-PRIETO *et al.*, 2002). Phenolic off-flavour almost exclusively affects red wines due to their abundance in precursory phenolic acids (CHATONNET *et al.*, 1992; CHATONNET *et al.*, 1993).

Climate changes in recent years have caused increased fungal contamination of grapes and the production of musts characterized by analytical parameters (i.e. high pH and sugar content) that along with high temperature favour the proliferation of winery contaminant microorganisms. Due to the origin of geosmin and ethylphenols, wines with a high commercial value can be affected by earthy and phenolic off-flavours even if preventive actions are implemented. In these cases remedial treatments may be necessary to remove the off-flavours. A possible approach is to decrease the concentration of the responsible compounds below their olfactory perception threshold by adsorbing them on highly adsorptive materials, i.e. activated charcoal. To evaluate the applicability of treatments to reduce earthy and phenolic off-flavours, it is necessary to consider their effect not only on the off-flavour compounds but also on the volatile compounds that are important for wine aroma. A suitable method for the determination of these volatile compounds of wine is therefore necessary. Several methods for determining 4-ethylphenol and 4-ethylguaicol in wine have been previously reported. Volatile phenols are usually determined by gas chromatographic analysis, preceded by an extraction treatment of the sample. Different extraction techniques have been employed, including liquid-liquid extraction (CHATONNET and BOIDRON, 1988; CHATONNET *et al.*, 1995; POLLNITZ *et al.*, 2000), dispersive liquid-liquid microextraction (DLLME) (FARIÑA *et al.*, 2007), solid-phase extraction (SPE) (DOMÍNGUEZ *et al.*, 2002), solid-phase microextraction (SPME) (MARTORELL *et al.*, 2002; MONJE *et al.*, 2007) and stir bar sorptive extraction (SBSE) (DÍEZ *et al.*, 2004). Liquid chromatography has also been employed to determine the presence of ethylphenols in wine (CABONI *et al.*, 2007; LARCHER *et al.*, 2007; NICOLINI *et al.*, 2007). In contrast, only two methods for the determination of geosmin in wine have been previously reported. One consists in a liquid-liquid extraction with n-pentane, followed by GC-MS analysis in selected ion monitoring (SIM) mode (DARRIET *et al.*, 2000, 2001; LA GUERCHE *et al.*, 2005), while the other consists in SPME isolation followed by GC-MS analysis (DUMOULIN and RIBOULET, 2004). No methods for the simultaneous determination of geosmin, 4-ethylphenols, 4-ethylguaiacol, and the main wine aroma compounds have been previously reported.

The aim of this study was to verify the applicability of an analytical method specifically employed for the determination of geosmin in wine, to determine the selected target wine aroma volatiles as well as the contaminants geosmin and ethylphenols. Besides providing data on the level of geosmin and ethylphenol contamination of wines, this method can also be applied in studies aimed at determining remedial treatments for these off-flavours.

MATERIALS AND METHODS

Reagents, standards and samples

Dichloromethane (HPLC grade), n-pentane (analytical grade), ethanol (99%, HPLC grade), solid anhydrous ammonium sulphate and L-(+)-tartaric acid were supplied by J.T. Baker (Deventer, Holland). Water was purified through a Milli-Q system (Millipore, Bedford, MA, USA). The suppliers and purity of the chemical standards employed are reported in Table 1. 2-Octanol was the internal standard. Red wines (*cv* Aglianico) and white wines (*cv* Falanghina), both from the 2005 harvest, were obtained from a cellar in Benevento (Italy).

Extraction method

Wine (500 mL), spiked with 50 μL of a 2-octanol solution (100 mg/L in ethanol) as internal standard, was extracted in 1 L flasks by three successive extractions with n-pentane (20, 10 and 10 mL, respectively) with magnetic stirring for 10 min each time. The combined organic phases were then dried with anhydrous sodium sulphate and concentrated to 200 mL under nitrogen flow (0.5 mL/min).

Instrumentation and chromatographic conditions

Each organic extract (1.2 μL) was injected in splitless mode (injector port temperature 250°C) and analysed by Gas Chromatography-Mass Spectrometry in the Selected Ion Monitoring mode (GC/MS-SIM). An Agilent 5973N quadrupole mass spectrometer (Agilent Technologies, Palo Alto, CA, USA) coupled to an Agilent 6890 gas chromatograph (Agilent Technologies, Palo Alto, CA, USA) was employed. The ion source and quadrupole temperatures were 230° and 150°C, respectively. The GC/MS system was provided with a DB-Wax silica capillary column (60 m, 0.32 mm i.d., 0.5 mm film thickness; J&W Scientific, Folsom, CA, USA). Electron impact mass spectra were recorded with an ion-source energy of 70 eV. The oven temperature was maintained at 45°C for 1 min, and then increased 3°C/min up to 230°C and held for 10 min. Helium was employed as carrier gas with a flow of 3.5 mL/min.

Quantification

Quantitative data of the identified compounds were obtained by the interpolation of the relative areas versus the internal standard area, in the calibration graphs prepared for pure reference compounds. A model wine solution (13% v/v ethanol, 5 g/L L-(+)-tartaric acid, adjusted to pH 3.2 with 1N NaOH) containing known amounts of the analytes was properly diluted in order to obtain 6-10 calibration points. The number of calibration points and the concentration range for each compound are reported in Table 6. For each calibration point, the model solution was extracted in duplicate and analysed by GC-MS/SIM, as previously described. Peak area data were processed by the software Chemstation (Agilent Technologies, Palo Alto, CA, USA).

Table 1 - Chemical standards used in this study.

Compound	Supplier	Purity
2-octanol	Sigma-Aldrich	97%
isoamyl acetate	Aldrich	99%
3-methyl-1-butanol	Aldrich	99%
ethyl hexanoate	Aldrich	99%
1-hexanol	Aldrich	98%
(Z)-3-hexen-1-ol	Aldrich	98%
ethyl octanoate	Lancaster	99%
linalool	Lancaster	99%
ethyl decanoate	Aldrich	99%
α-terpineol	Aldrich	90%
β-damascenone	Aldrich	1% in ethanol
geosmin	Sigma	98% 2 mg/mL methanol
geraniol	Aldrich	98%
2-phenylethanol	Aldrich	99%
4-ethylguaiacol	Aldrich	98%
4-ethylphenol	Aldrich	99%
4-vinylguaiacol	Aldrich	98%
4-vinylphenol	Aldrich	10% in propylene glycol

Method validation

The extraction method, applied for the simultaneous determination of geosmin, 4-ethylphenol, 4-ethylguaiacol, and the selected volatile aroma compounds in red and white wines (reported in Table 2 along with their odours and olfactory perception thresholds), was that proposed by DARRIET *et al.* (2000) for the specific quantification of geosmin in grape juice and wines.

Solvent selection: the solvent employed in the original method, n-pentane, was compared with dichloromethane. A model wine solution (13% v/v ethanol, 5 g/L L-(+)-tartaric acid, adjusted to pH 3.2 with 1N NaOH), containing the analytes at concentrations reported in Table 3, was submitted to the liquid-liquid solvent extraction as previously described, with each of the two solvents. Each extraction was performed in triplicate. The organic extracts were analyzed by GC-MS/SIM analysis as previously described. Extraction efficiency (mean relative peak areas) for the volatile compounds and precision (%RSD), together with chromatographic performances, were considered.

The validation parameters studied were linearity, precision, accuracy, limits of detection and quantification.

Linearity was estimated by the regression coefficient (r^2) of the calibration graphs obtained as previously described.

Table 2 - Volatile aroma compounds selected for this study.

Compound	Odour*	Olfactory perception threshold (µg/L)
esters		
isoamyl acetate	sweet[1], fruity[1,2], banana[1,3,4,5,6]	30[a]
ethyl hexanoate	fruity[1,2,6,7], apple[1,8,9], banana[1], brandy[1], green apple[3,5], strawberry[6]	14[b]
ethyl octanoate	fruity[1,4,6], floral[1,5], banana[1], pineapple[1,5,7,9], brandy[1], pear[1], sweet[3,6], soap[3], anise[4], strawberry[7]	5[b]
ethyl decanoate	brandy[1], oily[1], fruity[1], grape[1], pleasant[3], soap[3], floral[5], citrus fruit[5]	200[b]
alcohols		
3-methyl-1-butanol	fusel oil[1], whiskey[1], alcohol[3], harsh[3,6], cheese[4,10], herbaceous[5]	30000[a]
1-hexanol	green[3,4,6], grass[3,5], toasty[4,6]	8000[a]
(Z)-3-hexen-1-ol	fresh[1,6], green[1], grass[1,3,4,5,6]	400[a]
2-phenylethanol	rose[1,3,4,5,6,9], honey[1], floral[1]	10000[a]
terpenes		
linalool	floral[1,3,4,5,9], orange flowers[5], citrus[1], lemon[1], orange[1,9], sweet[1]	15[a]
α-terpineol	fragrant[1], floral[1,5], lilac[1], sweet[3], anise[4]	250[b]
geraniol	sweet[1], floral[1,5], rose[1], fruity[1], citric[3], orange flowers[5]	30[a]
norisoprenoids		
β-damascenone	bark[3], baked apple[2,4], canned peach[6], tea[5,9], floral[5], dried fruit[5]	0.05[a]
phenols		
4-vinylguaiacol	phenolic[3,4], pleasant[3], smoky[4,5,9], curry[8]	130[c]
4-vinylphenol	medicinal[1,5], phenolic[1,5], almond shell[4]	180[c]

*Bibliographic odour descriptors, references are reported in brackets (1= Flavours & Fragrances catalogue 2003-2004. 2=Chisholm *et al*., 1994. 3=Ferreira *et al*., 2002. 4=Culleré *et al*., 2004. 5=Genovese *et al*., 2007. 6=Aznar *et al*., 2001. 7=Piombino *et al*., 2004. 8=Moio *et al*., 1999. 9= Moio *et al*., 2002. 10=Escudero *et al*., 2000). a=Guth, 1997, odour threshold determined in water/ethanol solution 10% v/v; b=Ferreira *et al*., 2000, odour threshold determined in water/ethanol solution 10% v/v + 7 g/L glycerine, pH 3.2; c=Chatonnet *et al*., 1993, odour threshold determined in water/ethanol solution 12% v/v + 8 g/L glycerine and several salts.

The precision of the method was estimated by the relative standard deviation of triplicate analyses both of red and of white wines spiked with 0.360 mg/L of geosmin and of the wine model solution reported in Table 3.

The accuracy of the method was evaluated by the determination of % recovery of the volatile compounds, spiked in red and white wine at three different concentrations. Each spiked wine was extracted in duplicate and analysed by GC-MS/SIM. Quantitative data were obtained by interpolation on the calibration graphs obtained following the quantification procedure previously reported. For the calculation of % recovery the following equation was used:

Table 3 - Composition of the model wine solution used for the solvent selection.

Compound	Concentration (µg/L)
isoamyl acetate	408
3-methyl-1-butanol	119,000
ethyl hexanoate	264
1-hexanol	616
(Z)-3-hexen-1-ol	100
ethyl octanoate	288
linalool	100
ethyl decanoate	300
α-terpineol	100
β-damascenone	102
geosmin	0.2
geraniol	59.2
2-phenylethanol	38,000
4-ethylguaiacol	414
4-ethylphenol	810
4-vinylguaiacol	307
4-vinylphenol	295

Recovery (%)= [concentration determined in spiked wine/ (concentration determined in wine + added concentration)] ·100%

Limit of detection (LOD) and limit of quantification (LOQ) of the volatile compounds were calculated at signal-to-noise ratios of 3 and 10, respectively.

Fining experiment

A red wine (*cv* Aglianico) and a white wine (*cv* Falanghina) were analysed. The wines were previously analysed for their concentration of geosmin and ethylphenols. Prior to spiking, no geosmin was detected in the red or white wines above the detection limit of 0.02 µg/L. Both wines were then spiked with geosmin in at a concentration of 0.360 mg/L. This concentration was chosen based on a preliminary sensory test result that determined a clearly perceptible earthy off-flavour both in red and in white wine. 4-Ethylguaiacol and 4-ethylphenols were not detected in the white wine, while the red wine had levels of 46.1±3.6 mg/L of 4-ethylguaiacol and 538.9±48.2 mg/L of 4-ethylphenol. As ethylphenol contamination almost exclusively affects red wines (CHATONNET *et al.*, 1992; 1993), white wines were not artificially contaminated with ethylphenols, in order to apply the method to a matrix as similar as possible to real cases. The treatment with deodorant activated charcoal (20 g/hL) was performed in triplicate on three aliquots each of 2 L of wine, for 14 h at 20°C. After racking and filtration, each experimental replicate was extracted in duplicate and analysed by GC-MS/SIM for the quantitative determination of the volatile compounds. The untreated wines were extracted in triplicate and considered as a control. Quantitative data of the wines treated with charcoal and those of the corresponding untreated ones were compared by Student t-test (α=0.05) after having verified the homogeneity of variances by the F-test (α=0.05).

RESULTS AND DISCUSSION

Besides the off-flavour compounds (geosmin, 4-ethylguaiacol, 4-ethylphenol), fourteen volatile compounds, reported as odour active in wines were selected (Table 2). The method was applied to quantify the analytes in the ranges of concentration expected in wine (MAARSE *et al.*, 1992; FERREIRA *et al.*, 1992). Moreover, each compound was checked to determine if the minimum concentration quantifiable by the calibration curves was lower than the olfactory threshold, reported in Table 2. For these reasons, the method was optimised by focusing principally on geosmin, a compound typically detected in wines at low concentration (4-400 ng/L; DARRIET *et al.*, 2000; LA GUERCHE *et al.*, 2003) and characterized by an extremely low olfactory threshold (0.010 mg/L in water, 0.060-0.065 mg/L in white wine, 0.080-0.090 mg/L in red wine; DARRIET *et*

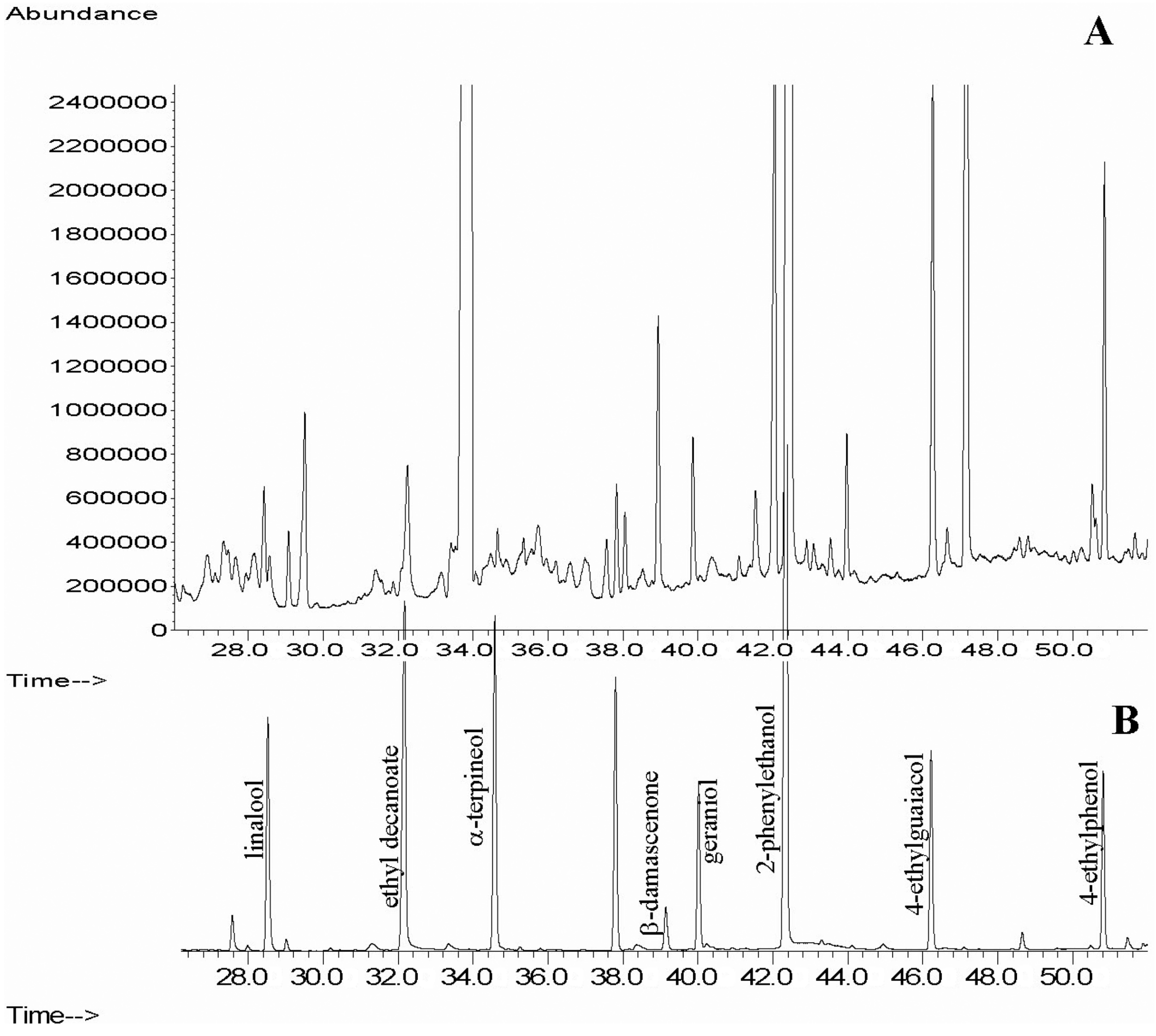


Fig. 1 - Total ion current (TIC) profile obtained from the analysis of an organic extract of red wine (A) and a model wine solution (B).

al., 2000). Fig. 1(A) reports a portion of the Total Ion Current (TIC) obtained from the analysis in full scan mode of an organic extract of red wine, spiked with geosmin (0.360 mg/L), 4-ethylguaiacol (125 mg/L), and 4-ethylphenol (1,235 mg/L) and extracted following the proposed method. It is compared with the corresponding portion of the TIC obtained by injecting an organic extract of a model wine solution (Fig. 1(B)) containing the aroma volatile compounds selected for the study, geosmin, 4-ethylguaiacol and 4-ethylphenol at concentrations comparable with those detected in wine. At the retention time of geosmin (39.55 min), situated between β-damascenone and geraniol, the peak of geosmin was not detected in the wine extract, nor in the model solution extract. This means that in these conditions the detection limit for geosmin was higher than 0.360 mg/L, a concentration that corresponds to a high level of contamination, considering the range detected in wine (DARRIET *et al.*, 2000; FERREIRA *et al.*, 2002). Moreover, several volatile target compounds, such as linalool, β-damascenone, geraniol, and 4-ethylguaiacol, could not be determined in full scan mode because of the coelution with other volatile compounds of wine or the base line noise was too high. Therefore to determine the volatile target compounds it was necessary to adopt a more selective and sensitive GC-MS method, such as Selected Ion Monitoring (SIM). The retention times and the selected ions for the analytes are reported in Table 4. For each compound, from two to four ions were selected, one of which was employed as quantifier and the others as qualifier. Concerning geosmin, only the ion *m/z* 112 was selected and it was used to identify and quantify this compound.

Two solvents were compared for the liquid-liquid extraction: n-pentane and dichloromethane. The former was chosen because it was employed in previous studies on the determination of geosmin in wine (DARRIET *et al.*, 2000, 2001; LA GUERCHE *et al.*, 2005), while the latter is a solvent widely used for liquid-liquid extraction of wine volatiles (MOIO *et al.*, 1995; ORTEGA-HERAS *et al.*, 2002; PERESTRELO *et al.*, 2006) and specifically used for volatile phenol determination (CHATONNET and BOIDRON, 1988). As criteria for the choice, the extraction efficiency (relative peak areas), the precision (%RSD), together with chromatographic performances, were considered.

Table 4 - GC-MS/SIM parameters of the volatile compounds.

Peak no.	Compound	Retention time (min)	Ions[a] *m/z*
IS[b]	2-octanol	23.23	**45**-55-97
1	isoamyl acetate	10.77	**70**-55-61
2	3-methyl-1-butanol	14.27	**69**-71
3	ethyl hexanoate	15.3	**88**-60-99
4	1-hexanol	20.45	**56**-55-69
5	(Z)-3-hexen-1-ol	21.76	**67**-55-82
6	ethyl octanoate	23.94	**88**-101-127
7	linalool	28.51	**80**-121
8	ethyl decanoate	32.23	**61**-155
9	α-terpineol	34.56	**59**-93-121-136
10	β-damascenone	39.15	**121**-190
11	geosmin	39.55	**112**
12	geraniol	40.01	**93**-123
13	2-phenylethanol	42.35	**122**-63-77-103
14	4-ethylguaiacol	46.19	**137**-122-152
15	4-ethylphenol	50.78	**107**-77-122
16	4-vinylguaiacol	51.43	**150**-135
17	4-vinylphenol	57.21	**120**-65-91

[a] bold numbers indicate quantifier ions;
[b] IS=Internal Standard.

The two solvents are characterized by a different polarity; dichloromethane is the most polar (Log P (Log octanol/water partition coefficient) n-pentane=2.80, Log P dichloromethane=1.34, estimated by EPI Suite v. 3.20). Log P of the analytes ranged from 1.26 (3-methyl-1-butanol) to 4.79 (ethyl decanoate) (Table 5). The mean internal standard area did not differ significantly in the extractions with the two solvents (n-pentane=15994435±3254494, dichloromethane=14897720±742863), therefore the differences of relative peak areas were imputable to differences in the peak areas of the analytes. The extraction efficiency was higher in the extraction with n-pentane for the four esters (isoamyl acetate, ethyl hexanoate, ethyl octanoate, ethyl decanoate), β-damascenone, the three terpenols (linalool, α-terpineol, geraniol) and geosmin, while alcohols (3-methyl-1-butanol, 1-hexanol, (Z)-3-hexenol, 2-phenylethanol) and phenols (4-ethylguaiacol, 4-ethylphenol, 4-vinylguaiacol, 4-vinylphenol) were extracted better by dichloromethane (Table 5). As expected, the extraction efficiency varied for the two solvents to the polarity of the analytes (Log P). It can be noted that the compounds having Log P greater than 3, a polarity similar or slightly lower than that of n-pentane, were extracted better by it. The analytes with a Log P less than 2 were extracted better by dichloromethane. The compounds having a Log P between 2 and 3 showed different behaviours according to their chemical class. Aliphatic esters (isoamyl acetate and ethyl hexanoate) were extracted better by n-pentane, while volatile phenols (ethyl and vinyl phenols) were extracted better by dichloromethane. Fig. 2 shows the portion of chromatograms related to geosmin, that, as previously explained, can be considered as the "limiting analyte". In the case of the extraction with n-pentane (A) the peak of geosmin had a better resolution and was higher than in the case of the extraction with dichlorometane (B). The precision was better with n-pentane, with %RSD varying between 1.1 and 8.8% (mean %RSD=4.3), while %RSD was between 1.3 and 19.8% (mean %RSD=6.7) for the

Table 5 - Extraction efficiency of n-pentane and dichloromethane.

Compound		Relative peak area[a]			
		n-pentane		dichloromethane	
	log *P*[b]	mean[c]	%RSD[d]	mean[c]	%RSD[d]
isoamyl acetate	2.26	16.9	5.8	13.6	3.6
3-methyl-1-butanol	1.26	3.86	3.2	25.4	19.8
ethyl hexanoate	2.83	16.2	5.9	10.4	7.8
1-hexanol	1.82	7.08	2.8	20.2	5.9
(Z)-3-hexen-1-ol	1.61	0.412	1.1	2.91	1.4
ethyl octanoate	3.81	15.7	7.3	10.2	5.3
linalool	3.38	1.44	5.1	1.35	3.2
ethyl decanoate	4.79	2.22	5.4	1.54	4.0
α-terpineol	3.33	1.33	1.3	1.17	2.0
β-damascenone	4.21	0.737	4.9	0.131	8.7
geosmin	3.57	0.014	8.8	0.009	10.6
geraniol	3.47	0.578	5.0	0.118	6.6
2-phenylethanol	1.57	25.6	2.2	207	8.6
4-ethylguaiacol	2.38	26.6	3.0	34.9	6.8
4-ethylphenol	2.55	14.4	1.2	60.0	5.9
4-vinylguaiacol	1.92[e]	1.95	4.2	3.44	7.5
4-vinylphenol	2.41	1.11	3.3	15.45	1.3

[a] Analyte peak area/IS peak area (2-octanol);
[b] Octanol/water partition coefficient estimated using EPI Suite v.3.20 software, U.S. Environmental Protection Agency and Syracuse Research Corp.;
[c] *n*=3;
[d] Relative standard deviation %;
[e] Log P estimated using ACD-Lab software.

dichloromethane extraction (Table 5). In conclusion, the two solvents had different extraction efficiencies, depending on the polarity and chemical structure of the compounds. n-Pentane had a better extraction efficiency for the least abundant compounds (linalool, geraniol, α-terpineol, and geosmin), a better precision, and better chromatographic performance especially for geosmin. On the basis of these results, n-pentane was chosen as the extraction sIt is also interesting to note that, in sensory tests conducted by laboratory staff, n-pentane extracts were very reminiscent of the original samples; this is an important prerequisite for any technique used to extract volatile compounds.

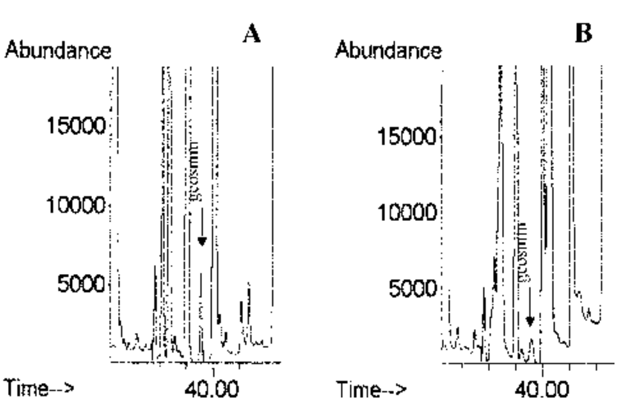


Fig. 2 - Chromatographic peak of geosmin obtained by GC/MS-SIM analysis of n-pentane (A) and dichloromethane extracts (B).

Method validation

A typical chromatogram obtained following the whole procedure can be seen in Fig. 3. The seventeen volatiles were quantified by calibration graphs based on pure reference compounds in a model wine solution. From 6 to 10 calibration points were obtained in the range of concentration reported in Table 6. These ranges of concentration are very wide, covering at least two orders of magnitude for all the compounds, and three orders of magnitude for most of the compounds. The range of concentration for geosmin covers one order of magnitude; however it includes the concentrations determined in different typologies of wine (DARRIET *et al.*, 2000). Linearity of the method, estimated by the linear correlation coefficients (r^2), was very good, in the ranges of concentration considered: r^2 values were between 0.9956 (ethyl decanoate and 2-phenylethanol) and 0.9997 (isoamyl acetate). In particular, the r^2 was greater than 0.999 for isoamyl acetate, 3-methyl-1-butanol, β-damascenone and 4-ethylguaiacol. This indicates that for all the analytes, the linear range included at least the entire concentration range studied. As the ranges of linearity cover the range of concentration typically detected in wine (MAARSE *et al.*, 1992; FERREIRA *et al.*, 2002), the method is suitable for the analysis of many typologies of wine with different levels of contamination by ethylphenols and geosmin. The slope of the straight calibration lines is a measure of method sensitivity. According to its definition (INTERNATIONAL VOCABULARY OF BASIC AND GENERAL TERMS IN METROLOGY, 1993) the

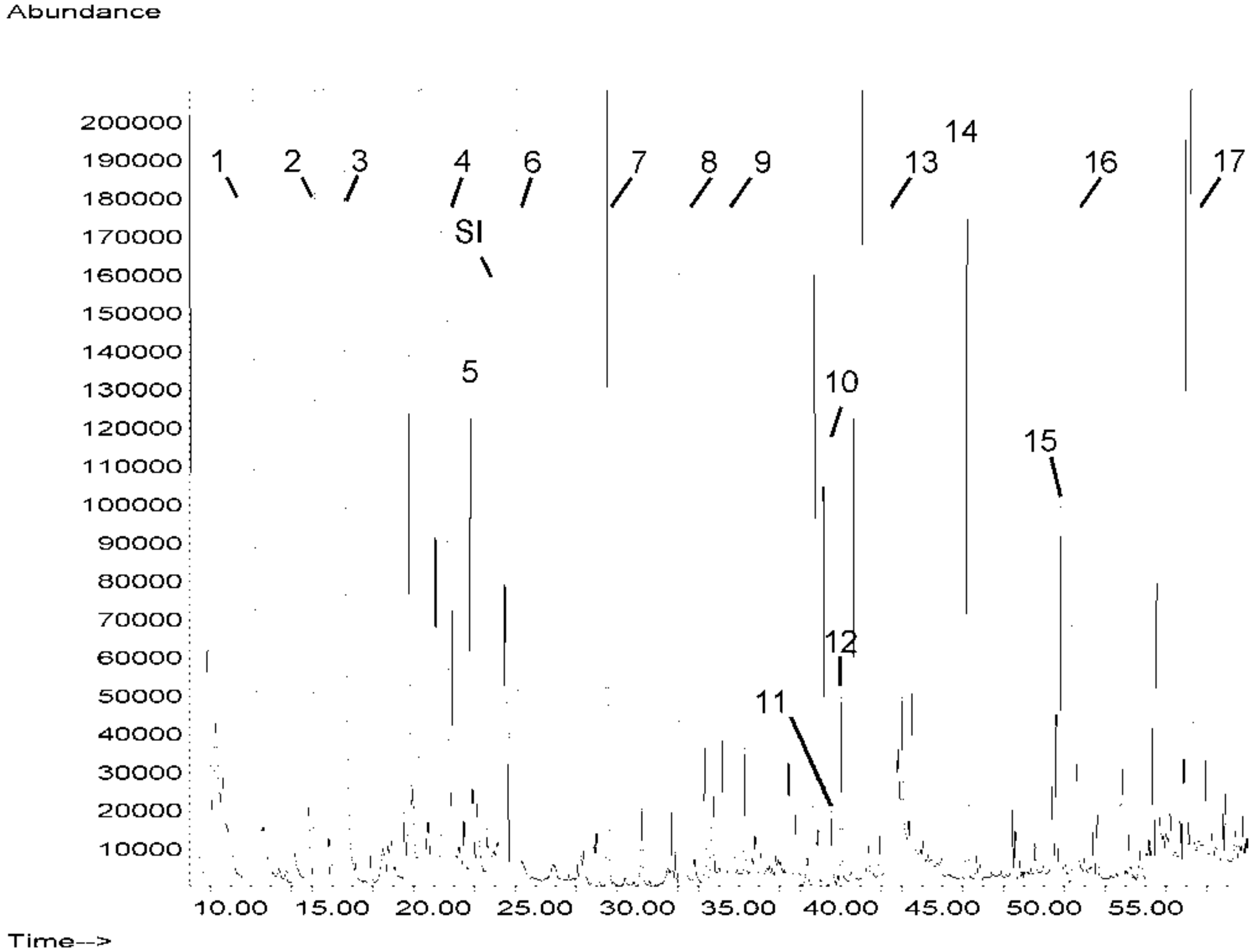


Fig. 3 - Chromatogram of a wine extract (peak numbers refer to Table 4).

Table 6 - Calibration parameters.

Compound	Intercept	Slope	r^2	Linear range (µg/L)	*n*	LOD (µg/L)	LOQ (µg/L)
isoamyl acetate	0.3883	0.1938	0.9997	2.97-2970	10	0.40	1.33
3-methyl-1-butanol	0.2360	0.0003	0.9993	3489-348975	8	435.73	1452.44
ethyl hexanoate	3.0304	0.2316	0.9977	3.6135-3613.5	9	0.76	2.53
1-hexanol	1.1999	0.0819	0.9 979	9.653-9653	10	1.11	3.72
(Z)-3-hexen-1-ol	-0.0366	0.0314	0.9981	1.5190-1519	10	2.86	9.52
ethyl octanoate	1.7249	0.3605	0.9970	3.1185-3118.5	9	0.27	0.89
linalool	0.0530	0.0894	0.9988	0.97-679	9	0.16	0.54
ethyl decanoate	0.1909	0.1182	0.9956	0.99-990	10	0.31	1.04
α-terpineol	0.2374	0.3426	0.9986	0.90-450	8	0.11	0.35
β-damascenone	-0.0158	0.7822	0.9995	0.054-54	9	0.59	1.96
geosmin	-0.0005	1.0103	0.9963	0.020-0.400	6	0.02	0.05
geraniol	-0.0050	0.0572	0.9986	0.539-539	10	0.80	2.67
2-phenylethanol	7.1645	0.0051	0.9956	4518-150268	7	39.99	133.31
4-ethylguaiacol	0.5358	1.5261	0.9994	0.49-490	10	0.08	0.27
4-ethylphenol	1.3114	0.1781	0.9983	6.435-6435	10	0.71	2.35
4-vinylguaiacol	0.0294	0.0504	0.9984	0.833-833	9	1.17	3.91
4-vinylphenol	-0.0706	0.0516	0.9988	1.50-1500	9	4.80	16.00

higher the sensitivity, the smaller the quantifiable differences in concentration. The best sensitivities were obtained for 4-ethylguaiacol and geosmin, for which the slope was greater than one, and β-damascenone. The worst sensitivities were obtained for 3-methyl-1-butanol and 2-phenylethanol. Limits of detection (LOD) and limits of quantification (LOQ) of the analytes are reported in Table 6. For most of the compounds the values were very low, being below 1 or a few mg/L. 3-Methyl-1-butanol and 2-phenylethanol presented higher detection and quantification limits. These are acceptable for the application of the method, considering that they are lower than the lowest concentrations typically detected in wine.

The precision of the method was evaluated by %RSD. In the wine model solution the precision of the method was very good; all of the values were below 8.8% (Table 5). The %RSD was be-

Table 7 - Recovery (%) of the volatile compounds in red wine.

Compound	Concentration in wine (μg/L)	Added (μg/L)	Recovery % (mean±SD)
isoamyl acetate	664.9	641.5	113.0±0.1
		481.7	105.7±2.1
		160.6	104.0±4.3
3-methyl-1-butanol	200403.8	198532.1	126.4±0.2
		148552.0	107.3±5.5
		49424.8	117.9±12.1
ethyl hexanoate	160.6	158.8	132.6±0.2
		118.8	119.8±3.9
		39.6	111.8±4.0
1-hexanol	1909.9	1938.6	134.6±1.1
		1457.1	116.6±2.1
		484.0	114.7±9.0
(Z)-3-hexen-1-ol	149.2	132.4	77.1±3.5
		99.4	70.4±0.1
		33.1	86.0±13.6
ethyl octanoate	249.6	220.9	122.1±0.8
		165.7	112.3±5.4
		55.2	108.5±2.9
linalool	16.3	15.2	59.4±1.9
		11.4	64.0±0.6
		3.8	80.0±0.2
ethyl decanoate	38.9	34.4	60.2±1.3
		29.1	58.0±6.2
		9.7	79.1±8.3
α-terpineol	12.5	12.1	78.5±1.0
		9.1	81.1±1.4
		3.0	95.7±2.0
β-damascenone	1.1	1.0	50.5±2.7
		0.8	55.0±0.7
		0.3	71.8±1.5
geosmin	0.0	0.360	73.5±2.4
		0.270	83.9±11.0
		0.090	52.6±3.0
geraniol	9.8	11.0	78.5±0.8
		8.3	79.7±0.8
		2.8	85.3±2.6
2-phenylethanol	66330.0	64929.7	126.7±1.3
		48768.2	106.2±7.1
		16275.0	112.7±13.7
4-ethylguaiacol	43.7	122.6	75.6±6.7
		91.7	67.3±3.5
		30.6	63.1±7.2
4-ethylphenol	475.0	1915.1	156.1±2.6
		1435.3	136.5±2.2
		479.8	122.1±12.2
4-vinylguaiacol	15.5	15.8	66.6±0.4
		11.8	61.5±2.7
		3.9	48.8±9.2
4-vinylphenol	27.4	28.4	132.5±3.7
		21.3	114.1±0.3
		7.1	98.1±11.4

Table 8 - Recovery (%) of the volatile compounds in white wine.

Compound	Concentration in wine (μg/L)	Added (μg/L)	Recovery % (mean±SD)
isoamyl acetate	789.3	768.2	115.4±1.1
		576.2	111.2±0.4
		192.1	109.4±4.8
3-methyl-1-butanol	140683.3	138833.6	146.9±4.4
		104125.2	144.9±3.4
		34708.4	138.0±5.3
ethyl hexanoate	978.0	974.2	124.3±6.6
		730.6	124.9±6.5
		243.5	138.3±3.8
1-hexanol	1218.7	1234.8	145.5±3.4
		926.1	138.9±4.4
		308.7	118.3±3.7
(Z)-3-hexen-1-ol	53.1	47.1	76.3±3.8
		35.3	79.9±1.5
		11.8	88.5±5.6
ethyl octanoate	1111.3	986.0	112.2±6.8
		739.5	113.3±6.4
		246.5	125.1±1.9
linalool	24.5	25.6	47.4±1.1
		19.2	51.2±0.1
		6.4	66.6±0.4
ethyl decanoate	191.1	196.9	64.1±4.0
		147.7	69.0±3.8
		49.2	95.9±2.9
α-terpineol	20.3	19.5	79.5±1.0
		14.6	81.8±0.7
		4.9	92.2±1.3
β-damascenone	2.7	3.0	42.9±2.9
		2.3	48.5±1.2
		0.8	70.4±0.3
geosmin	0.0	0.360	97.6±7.5
		0.270	95.7±2.5
		0.090	59.8±3.4
geraniol	8.7	9.9	75.6±2.0
		7.4	75.4±0.5
		2.5	78.4±0.8
2-phenylethanol	28898.9	28353.6	159.8±9.1
		21265.2	160.6±2.3
		7088.4	142.9±8.2
4-ethylguaiacol	<0.49	167.4	101.0±2.4
		125.5	93.4±4.6
		41.8	91.2±1.9
4-ethylphenol	<6.435	2059.2	136.9±5.4
		1544.4	92.6±4.4
		514.8	122.1±6.0
4-vinylguaiacol	403.4	427.3	104.4±2.6
		320.5	99.8±3.2
		106.8	86.6±3.2
4-vinylphenol	117.8	122.4	70.6±3.8
		91.8	79.0±5.9
		30.6	81.2±7.5

low 5.9% for all the compounds, except for ethyl octanoate (7.3%) and geosmin (8.8%). Triplicate extraction and analysis performed in the same batch on the untreated wines also provided precision data, reported in Table 9, therefore this figure was estimated in two different chemical environments. The precision of the overall method was also very good in the wine samples with %RSD below or near 10% for most of the compounds. Therefore, there was no effect of matrix on the precision of the method.

The accuracy of the method was estimated by the perceptual recoveries of the volatile compounds at three different concentrations, determined in both red and white wines spiked with known amounts of the analytes (Table 7-8). This measure of the accuracy of a method is often used when a standard of exactly known concentrations of the analytes and matrix identical to the sample is not available for comparison. The results for red and white wine are reported in Tables 7 and 8, respectively. For each volatile compound, the three added concentrations corresponded to 100, 75 and 25% of the concentration detected in the wine samples. The highest addition of geosmin was 0.360 μg/L and the other two corresponded to 75% and 25% of this value. In white wine the highest added concentrations of 4-ethylphenol and 4-ethylguaiacol were 2059.2 and 167.4 μg/L, respectively and the other two additions corresponded to 75 and 25% of these concentrations. In red wine, the highest concentration added was chosen to obtain a total concentration (concentration determined in wine + concentration added) comparable to that of white wine, while the other two additions were made so that a total concentration corresponding to 75 and 25% of the total higher concentration was obtained. Wines were analysed soon after spiking, in order to prevent transformations of the analytes, such as the hydrolysis of the esters, or the molecular interconversion of terpenols (SEJER PEDERSEN *et al.*, 2003).

The recoveries for all volatile compounds in red wine were between 52.6 and 156.1%, with a mean recovery of 93.2%. The lowest recoveries were obtained for linalool (59.4-80%), ethyl decanoate (58.0-79.1%), β-damascenone (50.5-71.8%), and 4-vinylguaiacol (48.8-66.6%). Concerning the volatile compounds responsible for wine taints, the percentage recovery of geosmin was between 52.6 and 83.9%, those of 4-ethylguaiacol and 4-ethylphenol were between 63.1 and 75.6%, 122.1 and 156.1%, respectively, varying with the different concentrations.

Many factors could have determined these differences in the recovery of analytes, including the partition between the aqueous and the organic phase as well as the losses due to the manipulation of the samples and during the concentration of the organic extract. The losses of compounds could partly explain the lower recoveries of the most volatile compounds.

The percentage recoveries in white wine were between 42.9 and 160.6%, with a mean recovery of 99.3%. Similar to red wine, the lowest re-

Table 9 - Application of the method to wine.

Compound	Red wine		Charcoal			White wine		Charcoal		
	mean[a]	%RSD[b]	mean[c]	%RSD[b]	%Var.[d]	mean[a]	%RSD[b]	mean[c]	%RSD[b]	Var.[d]
isoamyl acetate	651.8	7.7	408.9	6.8	37	716.9	8.2	530.5	8.2	26
3-methyl-1-butanol	240846.7	2.0	207650.0	12.8	ns	130576.7	6.6	133812.0	8.3	ns
ethyl hexarioate	158.0	14.5	37.9	21.7	76	817.0	7.6	544.5	7.4	33
1-hexanol	2146.2	4.5	1914.2	12.0	ns	1218.5	8.6	1113.6	19.2	ns
(Z)-3-hexen-1-ol	160.4	6.2	147.0	9.7	ns	55.0	6.0	51.9	12.2	ns
ethyl octanoate	248.7	10.0	74.8	11.1	70	957.6	9.9	372.7	6.2	61
linalool	16.6	10.1	15.0	7.4	ns	25.1	8.9	20.4	9.5	19
ethyl decanoate	35.7	16.4	5.1	20.0	86	168.2	14.1	8.1	32.9	95
α-terpineol	13.1	10.8	12.3	7.1	ns	21.0	3.3	18.1	10.5	14
β-damascenone	1.0	10.0	1.0	6.3	us	2.7	14.7	2.2	12.8	18
geosmin	0.4	8.7	0.4	23.5	ns	0.4	9.2	0.3	8.1	23
geraniol	9.8	8.2	9.2	5.4	ns	9.1	4.2	6.9	12.2	24
2-phenylethanol	80960.0	2.2	67440.0	12.7	17	26283.3	6.6	30446.0	8.3	16
4-ethylguaiacol	46.1	7.9	41.0	5.0	11	nd	-	nd	-	-
4-ethylpheruol	538.9	8.9	441.0	9.6	18	nd	-	nd	-	-
4-vinylguaiacol	13.2	5.8	5.1	23.1	61	393.7	9.2	236.9	11.5	40
4-vinylphenol	25.2	4.4	22.2	6.4	12	118.0	4.7	66.1	14.9	44

[a] n=3;
[b] Relative Standard Deviation %;
[c] 6 (three replicates of treatment each extracted twice);
[d] significant perceptual decreasings (t-test, α=0.05);
nd= not detected;
ns not significant.

coveries were obtained for linalool (47.4-66.6%) and β-damascenone (42.9-70.4%). The recoveries of geosmin at the three concentrations studied (59.8-97.6%) were higher in white wine than in red wine. The recoveries of geosmin in both red and white wine were comparable to those previously reported in wine (DARRIET *et al.*, 2000). The percentage recoveries of 4-ethylguaiacol and 4-ethylphenol were 91.2-101% and 92.6-136.9%, respectively. Analogous to geosmin, for most volatile compounds at the three studied concentrations, a higher recovery was obtained in white wine. There were interactions between volatile compounds, belonging to several chemical classes, and wine polyphenols, particularly tannins, anthocyanins and gallic acid, (DUFOUR and BAYONOVE, 1999; DUFOUR and SAUVAITRE, 2000; JUNG *et al.*, 2000), the lowest recoveries in red wine could be due to the retention of volatile compounds by a polyphenol-rich matrix, such as red wine.

Application of the method to wine: fining experiment

Finally, the proposed method was applied to evaluate the efficacy of a treatment with deodorant activated charcoal to decrease the contamination of geosmin and ethylphenols in red wine and of geosmin in white wine. In order to evaluate the effect of the treatment on the overall wine aroma, the concentration of the selected volatile compounds before and after the treatment was determined. The results are reported in Table 9.

In the untreated red and white wines, the concentrations of all the volatile compounds were within the quantification range of the method, thus, the method was confirmed as being suitable for the analysis of these two wine typologies. As expected, red and white wines showed several differences in the volatile composition. The alcohols determined were more abundant in red wine than in white wine and made up the largest group of volatile compounds in red wine. The origin of these alcohols is different: 3-methylbutanol and 2-phenylethanol come from yeast glucidic and amino acidic metabolism during alcoholic fermentation, while C6 alcohols 1-hexanol and (Z)-3-hexenol derive from the lipoxygenase activity on unsaturated fatty acids of grapes. Among them, only 2-phenylethanol positively contributes to wine aroma with its rose odour (ETIÉVANT, 1991). Esters, responsible for fruity notes in wine, principally arising from alcoholic fermentation, were more abundant in white wine, as were linalool and α-terpineol, originated from grape and characterized by flowery odorous notes; β-damascenone, originated from carotenoid degradation and responsible for spicy-tea notes, and vinylphenols, synthesized by *Saccharomyces cerevisiae* and whose role in white wine aroma could be positive or negative depending on the concentration (CHATONNET *et al.*, 1993; VERSINI, 1985; VERSINI *et al.*, 1992). Regarding 4-ethylguaiacol and 4-ethylphenols, the red wine was contaminated and can be defined as medium-low, considering the range of concentration typically determined in red wine (CHATONNET *et al.*, 1992; POLLNITZ *et al.*, 2000; FERREIRA *et al.*, 2002). Moreover the ratio 4-ethylphenol/4-ethylguaiacol (of 11.7), rather constant in red wine, was comparable to the ratios reported in a previous study (CHATONNET *et al.*, 1992; POLLNITZ *et al.*, 2000). The treatment with deodorant charcoal did not result in any significant decrease of geosmin concentration in red wine, while in white wine it decreased the geosmin content by 23%, with respect to the initial content. Considering the concentration range detected in wine in previous studies (4-400 ng/L; DARRIET *et al.*, 2000; LA GUERCHE *et al.*, 2003), the level of contamination of wines can be considered high. Geosmin is efficiently adsorbed by activated charcoal and this treatment is used to remove geosmin from water (COOK *et al.*, 2001). However, since compounds having a molecular weight between 200 and 600 can compete for charcoal adsorption sites (COOK *et al.*, 2001), the results of this study suggest that the complexity of the wine matrix could decrease the geosmin-adsorption capacity of charcoal. In particular, a competition between polyphenols and geosmin could have made the treatment ineffective in red wine. Regarding the ethylphenols, the treatment decreased 4-ethylguaiacol and 4-ethylphenol content in red wine by 11 and 18%, respectively. Charcoals have a great chemical affinity for apolar and benzenic compounds (FERNÁNDEZ *et al.*, 2005), particularly phenols (DĄBROWSKI *et al.*, 2005). Considering this particular affinity, the decreases obtained could seem too low. However, red wine is an extremely complex matrix, with abundant phenolic compounds that are able to compete with ethylphenols for the adsorption sites of charcoal. Unfortunately, the treatment did not show a specificity for the off-flavour compounds. The charcoal decreased the esters, 2-phenylethanol and vinylphenols in both red and white wine. In addition it decreased terpenols (linalool, α-terpineol and geraniol), and β-damascenone in the white wine. Esters, particularly ethyl decanoate, and vinylphenols decreased the most. These results suggest that the decreased concentrations of geosmin and ethylphenols could not have determined a real decrease of the off-flavour, as the loss of a masking effect by positive olfactory notes seems to have occurred.

CONCLUSIONS

The proposed method is appropriate for the simultaneous determination of geosmin, responsible for earthy off-flavour, 4-ethylphenol and 4-ethylguaiacol, responsible for phenolic off-flavour and the main aroma volatile compounds of wine. There was a very good linearity in a wide range of concentrations and the precision and accuracy were satisfactory.

The method is suitable for quantifying the contamination of wine by geosmin and ethylphenol, even at low concentrations. Moreover, it can also be used to evaluate the effect of remedial treatments not only to lower the concentration of off-flavour compounds, but also to preserve those volatile compounds that are the most important for wine aroma.

REFERENCES

Aznar M., Lopez R., Cacho J.F. and Ferreira V. 2001. Identification of impact odorants of aged red wines from Rioja. GC-Olfactometry, Quantitative GC-MS, and odor evaluation of HPLC fractions. J. Agric. Food Chem. 49: 2924.

Caboni P., Sarais G., Cabras M. and Angioni A. 2007. Determination of 4-ethylphenol and 4-ethylguaiacol in wines by LC-MS-MS and HPLC-DAD-fluorescence. J. Agric. Food Chem. 55: 7288.

Chatonnet P. and Boidron J-N. 1988. Dosages de phenols volatils dan les vins par chromatographie en phase gazeuse. Sci. Aliment. 8: 479.

Chatonnet P., Boidron J-N. and Pons M. 1990. Elevage des vins rouges en futs de chene: evolution de certains composes volatils et de leur impact aromatique. Sci. Aliment. 10: 565.

Chatonnet P., Dubourdieu D., Boidron J.N. and Pons M. 1992. The origin of ethylphenols in wines. J. Agric. Food Chem. 60: 165.

Chatonnet P., Dubourdieu D., Boidron J.N. and Lavigne V. 1993. Synthesis of volatile phenols by *Saccharomyces cerevisiae* in wines. J. Sci. Food Agric. 62: 191.

Chatonnet P., Dubourdieu D. and Boidron J.-N. 1995. The influence of *Brettanomyces/Dekkera* sp. yeast and lactic acid bacteria on the ethylphenol content of red wines. Am. J. Enol. Vitic. 46: 463.

Chisholm M.G., Guiher L.A., Vonah T.M. and Beaumont J.L. 1994. Comparison of some French-American hybrid wines with white Riesling using GC/O. Am. J. Enol. Vitic. 45: 201.

Cook D., Newcombe G. and Sztajnbok P. 2001. The application of powdered activated carbon for MIB and geosmin removal: predicting PAC doses in four raw waters. Water Res. 35: 1325.

Culleré L., Escudero A., Cacho J. and Ferreira V. 2004. Gas Chromatography-olfactometry and chemical quantitative study of the aroma of six "premium quality" Spanish aged red wines. J. Agric. Food Chem. 52: 1653.

Dąbrowski A., Podkościelny P., Hubicki Z. and Barczak M. 2005. Adsorption of phenolics compounds by activated carbon-a critical review. 2005. Chemosphere 58: 1049.

Darriet P., Pons M., Lamy S. and Dubourdieu D. 2000. Identification and quantification of geosmin, an earthy odorant contaminating wines. J. Agric. Food Chem. 48: 4835.

Darriet P., Lamy S., La Guerche S., Pons M., Dubourdieu D., Blancard D., Steliopoulos P. and Mosandl A. 2001. Stereodifferentiation of geosmin in wine. Eur. Food Res. Technol. 213: 122.

Darriet P., Pons M., Henry R., Dumont O., Findeling V., Cartolaro P., Calonnec A. and Dubourdieu D. 2002. Impact odorants contributing to the fungus type aroma from grape berries contaminated by powdery mildew (*Uncinula necator*); incedence of enzymatic activities of the yeast *Saccharomyces cerevisiae*. J. Agric. Food Chem. 50: 3277.

Díez J., Domínguez C., Guillén D.A., Veas R. and Barroso C.G. 2004. Optimisation of stir bar sorptive extraction for the analysis of volatile phenols in wines. J. Chromatogr. A 1025: 263.

Domínguez C., Guillén D.A. and Barroso C.G. 2002. Determination of volatile phenols in fino sherry wines. Anal. Chim. Acta 458: 95.

Dufour C. and Bayonove C.L. 1999. Interaction between polyphenols and aroma substances. An insight at the molecular level. J. Agric. Food Chem. 47: 678.

Dufour C. and Sauvaitre I. 2000. Interactions between anthocyanins and aroma substances in a model system. Effect on the flavour of grape-derived beverages. J. Agric. Food Chem. 48: 1784.

Dumoulin M. and Riboulet J.M. 2004. Analyse de géosmine dans les vins: apport de la microextraction en phase solide (SPME) couplée à l'analyse par GC-MS. Rev. Fr. Oenol. 208: 28.

Escudero A., Cacho J.F. and Ferreira V. 2000. Isolation and identification of odorants generated in wine during its oxidation: a gas chromatography-olfactometric study. Eur. Food Res. Technol. 211: 105.

Etiévant P.X. 1991. Wine. In: "Volatile Compounds of Food and Beverages". H. Maarse (Ed.), p. 483. Marcel-Dekker, New York, Usa.

Fariña L., Boido E., Carrau F. and Dellacassa E. 2007. Determination of volatile phenols in red wines by dispersive liquid-liquid microextraction and gas chromatography-mass spectrometry detection. J. Chromatogr. A 1157: 46.

Fernández M.J., Oliva J., Barba A. and Cámara M.A. 2005. Effects pf clarification and filtration processes on the removal of fungicide residues in red wines (var. Monastrell). J. Agric. Food Chem. 53: 6156.

Ferreira V., Lopez R., Aznar M. 2002. Olfactometry and aroma extract dilution analysis of wines. In: "Molecular Methods of Plant Analysis, Analysis of Taste and Aroma". Vol. 21. Jackson J.F. and Linskens H.F. (Ed.), p. 88. Springer Berlin.

Ferreira V., Lopez R. and Cacho J. F. 2000. Quantitative determination of the odorants of young red wines from different grape varieties. J. Sci. Food Agric. 80: 1659.

Flavours & Fragrances catalogue 2003-2004. Sigma-Aldrich, St. Louis, MO, Usa.

Genovese A., Lisanti M.T., Gambuti A., Piombino P. and Moio L. 2007. Relationship between sensory perception and aroma compounds of red wines. Acta Hortic. (ISHS) 754: 549.

Guth H. 1997. Quantitation and sensory studies of character impact odorants of different white wine varieties. J. Agric. Food Chem. 45: 3027.

International vocabulary of basic and general terms in metrology. 1993. (VIM) (BIPM, IEC, IFCC, ISO, IUPAC, IUPAP, OILM), 2nd Ed.

Jung D. M., de Ropp J. S. and Ebeler S. E. 2000. Study of Interactions between Food Phenolics and Aromatic Flavors Using One- and Two-Dimensional ^{1}H NMR Spectroscopy J. Agric. Food Chem. 48: 407.

La Guerche S., Blancard D., Chamont S., Dubourdieu D. and Darriet P. 2003. VII^{e} Symposium International d'œnologie, A. Lonvaud (Ed.)., Tec et Doc, Lavoisier, Paris.

La Guerche S., Chamont S., Blancard D., Dubourdieu D. and Darriet P. 2005. Origin of (-)-geosmin on grapes: on the complementary action of two fungi, *Botrytis cinerea* and *Penicillium expansum*. Antonie Leeuwenhoek 88: 131.

La Guerche S., Dauphin B., Pons M., Blancard D. and Darriet P. 2006. Characterization of some mushroom and earthy off-odors microbially induced by the development of rot on grapes. J. Agric. Food Chem. 54: 9193.

Larcher R., Nicolini G., Puecher C., Bertoldi D., Moser S. and Favaro G. 2007. Determination of volatile phenols in wine using high-performance liquid chromatography with a coulometric array detector. Anal. Chim. Acta 582: 55.

Maarse H., Visscher C.A., Willemsens L.C., Boelens M.H. 1992. Volatile Compounds in Food. Qualitative and Quantitative Data. Vol. 1. TNO-CIVO Biotechnology and Chemistry Institute: Zeist, The Netherland.

Martorell N., Martí M.P., Mestres M., Busto O. and Guasch J. 2002. Determination of 4-ethylguaiacol and 4-ethylphenol in red wines using headspace-solid-phase microextraction-gas chromatography J. Chromatogr. A 975: 349.

Moio L., Chambellant E., Lesschaeve I., Issanchou S., Schlich P. and Etievant P.X. 1995. Production of representative wine extracts for chemical and olfactory analysis. Ital. J. Food Sci. 3: 265.

Moio L., Del Prete G., Diana M. and Valentino A. 1999. Influenza dell'origine geografica del legno di rovere impiegato per la fabbricazione delle barrique e dell'intensità della tostatura del legno sull'aroma del vino rosso. Industria delle Bevande 158: 615.

Moio, L. Di Marzio L., Genovese A., Piombino P., Squillante E., Castellano L. and Mercurio V. 2002. I descrittori sensoriali ed i componenti volatili ad elevato impatto olfattivo dell'aroma del vino Fiano. Vignevini 4: 115.

Monje M.-C., Privat C., Gastine V. and Nepveu F. 2002. Determination of ethylphenol compounds in wine by headspace solid-phase microextraction in conjunction with gas chromatography and flame ionization detection. Anal. Chim. Acta 458: 111.

Nicolini G., Larcher R., Bertoldi D., Puecher C. and Magno F. 2007. Rapid quantification of 4-ethylphenol in wine using high-performance liquid chromatography with a fluorimetric detector. Vitis 46: 202.

Ortega-Heras M., Gonzáles-SanJosé M.L. and Beltrán S. 2002. Aroma composition of wine studied by different extraction methods. Anal. Chim. Acta 458: 85.

Perestrelo R., Fernandez A., Albuquerque F.F., Marques J.C. and Câmara J.S. 2006. Analytical characterization of the aroma of Tinta Negra Mole red wine: identification of the main odorants compounds. Anal. Chim. Acta 563: 154.

Pérez-Prieto L.J., López-Roca J.M., Martínez-Cutillas A., Pardo Mínguez F. and Gómez-Plaza E. 2002. Maturing wines in oak barrels. Effect of origin, volume, and age of the barrel in the wine volatile composition. J. Agric. Food Chem. 50: 3272Piombino P., Nicklaus S., Le Fur Y., Moio L. and Le Quere J.L. 2004. Selection of products presenting given flavor characteristics: An application to wine. Am. J. Enol. Vitic. 55: 27.

Pollnitz A.P., Pardon K.H. and Sefton M.A. 2000. Quantitative analysis of 4-ethylphenol and 4-ethylguaiacol in red wine. J. Chromatogr. A 874: 101.

Sejer Pedersen D., Capone D.L., Skouroumounis G.K., Pollnitz A.P. and Sefton M. A. 2003. Quantitative analysis of geraniol, nerol linalool, and α-terpineol in wine. Anal. Bioanal. Chem. 375: 517.

Suárez R., Suárez-Lepe J.A., Morata A. and Calderón F. 2007. The production of ethylphenols in wine by yeast of the genera *Brettanomyces* and *Dekkera*: a review. Food Chem. 102:10.

Versini G. 1985. Sull'aroma del vini Traminer Aromatico o Gewürztraminer. Vignevini 12: 57.

Versini G., Dalla Serra A., Falcetti M. and Sferlazzo G. 1992. Role du clone, du millesime et de l'epoque de la recolte sur le potentiel aromatique du raisin de Chardonnay. Rev. Oenol. 18:19.

Revised paper received July 9, 2009 Accepted July 23, 2009

DETERMINATION OF BIOGENIC AMINE AND HEAVY METAL CONTENTS IN SICILIAN WINE SAMPLES

G.L. LA TORRE*, R. RANDO, M. SAITTA, M. ALFA, R. MAISANO[1] and G. DUGO
Dipartimento di Scienze degli Alimenti e dell'Ambiente "Prof. G. Stagno d'Alcontres",
Università di Messina, Salita Sperone 31, 98166 Messina, Italy
[1] Dipartimento di Matematica, Università di Messina, Salita Sperone 31, 98166 Messina, Italy
*Corresponding author: Tel. +39 090 6765435, Fax +39 0906765436,
e-mail: llatorre@unime.it

ABSTRACT

The purpose of this study was to determine the biogenic amine and heavy metal contents in Controlled Denomination of Origin (DOC) and Protected Geographical Indication (IGP) *Passito*, white and red wines from Sicily. The samples analyzed included wines derived from biological and traditional agriculture in the province of Messina. Potentiometric stripping analysis (PSA) was used to determine simultaneously the content of Cd (II), Cu (II), Pb (II) and Zn (II), whereas biogenic amines were determined as dansyl derivatives by reverse phase HPLC. The results provide new insight into the composition of commercial Sicilian wines from the province of Messina. The wines examined contained moderate quantities of biogenic amines, while the heavy metal content was so low that they do not represent a toxicological risk for the health of the consumer.

- Key words: biogenic amines, dansyl chloride, heavy metals, HPLC -

INTRODUCTION

Current oenological research is oriented at defining wine quality in relationship to its chemical composition, pharmacological properties and safety. In fact, while some wine micro-constituents, such as polyphenols, are recognized for their health value, other chemical compounds can influence the health aspect of the product.

For a number of years, interest has been focused on the presence of biogenic amines in food, and particularly in wines, due to their pharmacological properties and physiological disorders they could provoke at high concentrations in the human body (WANTKE *et al.*, 1993; MARINE-FONT *et al.*, 1995). These compounds are derived essentially from the decarboxylation of the respective precursor amino acids through substrate-specific enzymes resulting from the microorganisms present in the food (VIDAL-CAROU *et al.*, 1991; COTON *et al.*, 1999; MORENO-ARRIBAS and POLO, 2008). Edible foods that are frequently associated with the presence of large amounts of biogenic amines are protein-rich foods in which a microbial proteolytic activity exists and that increases the amount of free amino acids. Wines are not rich in protein, although they contain free amino acids (VIDAL-CAROU *et al.*, 1991). There are three possible origins of biogenic amines in wines: a) they can be present in the must, b) they can be formed by yeasts during alcoholic fermentation and c) they can result from the action of bacteria involved in malolactic fermentation (ARCE *et al.*, 1998). Biogenic amines have been incriminated as being responsible for intolerance reaction to wines (JARISH, *et al.*, 1992; BAUZA *et al.*,1995), even though a study has shown that the amount of histamine in wine has no clinical or biological effect in healthy subjects (KANNY *et al.*, 1999).

In recent years, great importance has been given to monitoring food products for heavy metal contamination and assessing the source of food contamination. The presence of trace elements in wine depends on many factors such as the type of soil, fungicide treatments, wine processing equipment and vinification methods (HSIA *et al.*, 1975; GOLIMOWSKY *et al.*, 1979; KARADJOVA *et al.*, 2002; LA PERA *et al.*, 2008). Traces of metals in wine (including Cu and Zn, essential for humans, animals and plants) and particularly Zn at low concentrations, play an important role in wine fermentation (RIBÉREAU-GAYON *et al.*, 1976). On the other hand, high concentrations of Cu participate significantly in the destabilization of wine and in their oxidative evolution (BENÌTEZ *et al.*, 2002). At the same time, the concentrations of Pb and Cd in wine are important because they are toxic in the case of excessive intake (RIBÉREAU-GAYON *et al.*, 1976; GENNARO *et al.*, 1986; FERNANDEZ PEREIRA, 1988; MCKINNON and SCOLLARY 1997).

A considerable amount of data on biogenic amines and metals in wine has been published but, to the best of our knowledge, no such data have been reported for Sicilian Controlled Denomination of Origin (DOC) wines and for Sicilian Protected Geographical Indication (IGP) wines.

Sicily is considered one of the oldest European viticultural regions, and with its warm temperatures, hilly terrain, sea breezes and rich soil it has more area under vine production than any other major winemaking region in Italy. Sicily produces at least 17% of all the Italian wines, most of which are now protected by D.O.C. regulation. In recent years, wine has been one of the few Sicilian agricultural products that has seen a rise in export levels, both in quality and average unit value compared to previous years (CRESCIMANNO *et al.*, 2002). Among the Sicilian white wines, the most distinctive ones are Insolia, Damaschino or Grillo, sometimes blended with Chardonnay. Among the red wines, the most diffused native varieties are Nero d'Avola and Nerello Mascalese that are vinified in their purest varietal form or blended with indigenous varieties such as Cabernet Sauvignon, Merlot and Syrah.

The concept of wine quality must be extended beyond sensory analysis guaranteeing, as well as the organoleptic quality, the safety of the product and protection of the consumer health. The purpose of this study was to determine the content of biogenic amines and heavy metals in DOC and IGP wines produced in the province of Messina.

Red, white and sweet (*Passito*) wines, produced from grapes that are grown exclusively in this area, under biological and traditional agriculture, were analyzed.

MATERIALS AND METHODS

Samples

The wine samples were Sicilian DOC wines and Sicilian IGP wines, produced in the province of Messina during the 2000-2003 crop years according to the production regulations (ITALIAN REPUBLIC, DPR 20/09/1973; ITALIAN REPUBLIC, DPR 04/03/1976; ITALIAN REPUBLIC, DPR 11/09/2004). The classification of the samples and the related codes are presented in Table 1; Table 2 provides information about the cultivation techniques used in the vineyards. Seven of the samples (Table 2) were produced under biological agriculture conditions, which allows the use of specific products for plant protection and organic fertilizers of biological origin for maturing of the vineyard.

The wines were very heterogeneous and had

Table 1 - Wine samples analyzed.

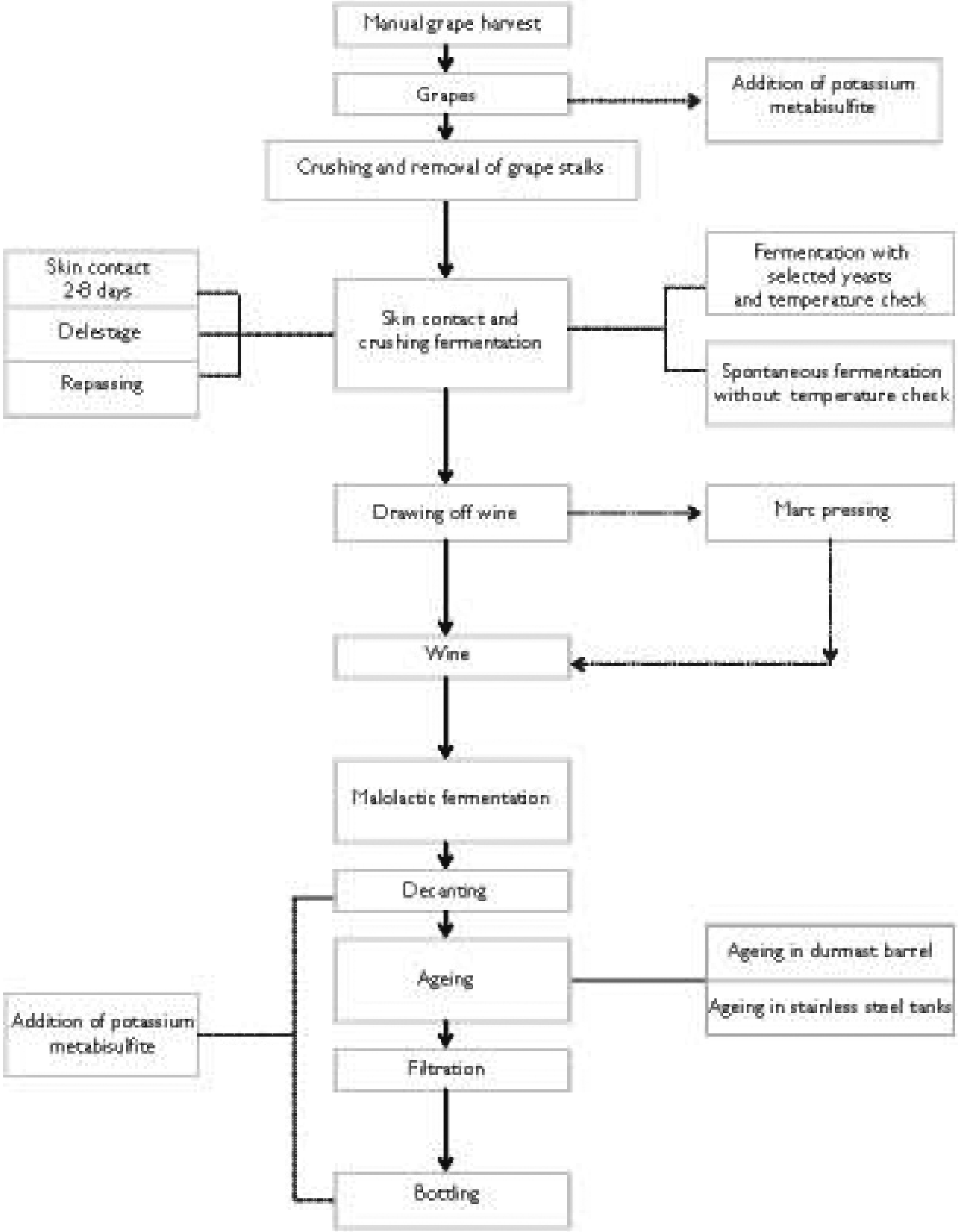
Sample	Vintage denomination and year	Grape variety
***Passito* Wine**		
F01	Malvasia delle Lipari DOC, 2003	95% Malvasia, 5% Corinto Nero
F02	Malvasia delle Lipari DOC, 2002	95% Malvasia, 5% Corinto Nero
F03	Malvasia delle Lipari DOC, 2003	95% Malvasia, 5% Corinto Nero
M03	Malvasia delle Lipari DOC, 2003	95% Malvasia, 5% Corinto Nero
C02	Malvasia delle Lipari DOC, 2002	95% Malvasia, 5% Corinto Nero
Red Wine		
FG0	Faro DOC, 2000	60% N. Mascalese, 30% N. Cappuccio, 10% Corinto & Nero D'Avola
FC1	Faro DOC, 2001	60% N. Mascalese, 30% N. Cappuccio, 10% Corinto & Nero D'Avola
MV1	Mamertino IGT, 2001	100% Nero D'Avola
MV3	Mamertino IGT, 2003	100% Nero D'Avola
MG1	Mamertino IGT, 2001	100% Nero D'Avola
NC4	Nero D'Avola IGT, 2004	100% Nero D'Avola
White Wine		
MV3	Mamertino IGT, 2003	45% Catarratto, 20% Inzolia,15% Grillo, 20% Altre
MS3	Mamertino IGT, 2003	45% Catarratto, 20% Inzolia, 15% Grillo, 20% Altre
TC3	Trebbiano IGT, 2004	100% Trebbiano
IC4	Inzolia IGT, 2004	100% Inzolia

been produced according to an, as yet, unstandardized method. They differed from one case to another. Some samples were made using traditional wine-making techniques, while others were made with a more modern process that, unlike the first ones, utilized thermal-conditioned wine-making and selected yeasts to control the alcoholic fermentation. Fig. 1-3 show the flowcharts of the wine-making process and the elaboration technique of

Table 2 - Cultivation techniques used in vineyards.

Samples	Agriculture type	Cultivation and pruning techniques	Manuring
***Passito* wines**			
F01	Biological Agriculture	"Controspalliera" with long pruning	Biological mulch
F02			
V03	Traditional Agriculture		Ternary fertilizers and green manure with clovers
M03			
C02			
Red wines			
FG0	Traditional Agriculture	"Controspalliera"	Ternary fertilizers
FC1	Biological Agriculture		Biological mulch and ternary fertilizers
MV1			
MV3			
MG1	Traditional Agriculture	"Cortina" with long pruning	Ternary fertilizers
NC4			
White wines			
MV3	Biological Agriculture	"Controspalliera" with short pruning	Biological mulch and ternary fertilizers
MS3			
TC3	Traditional Agriculture	"Cortina" with long pruning	Ternary fertilizers
IC4			

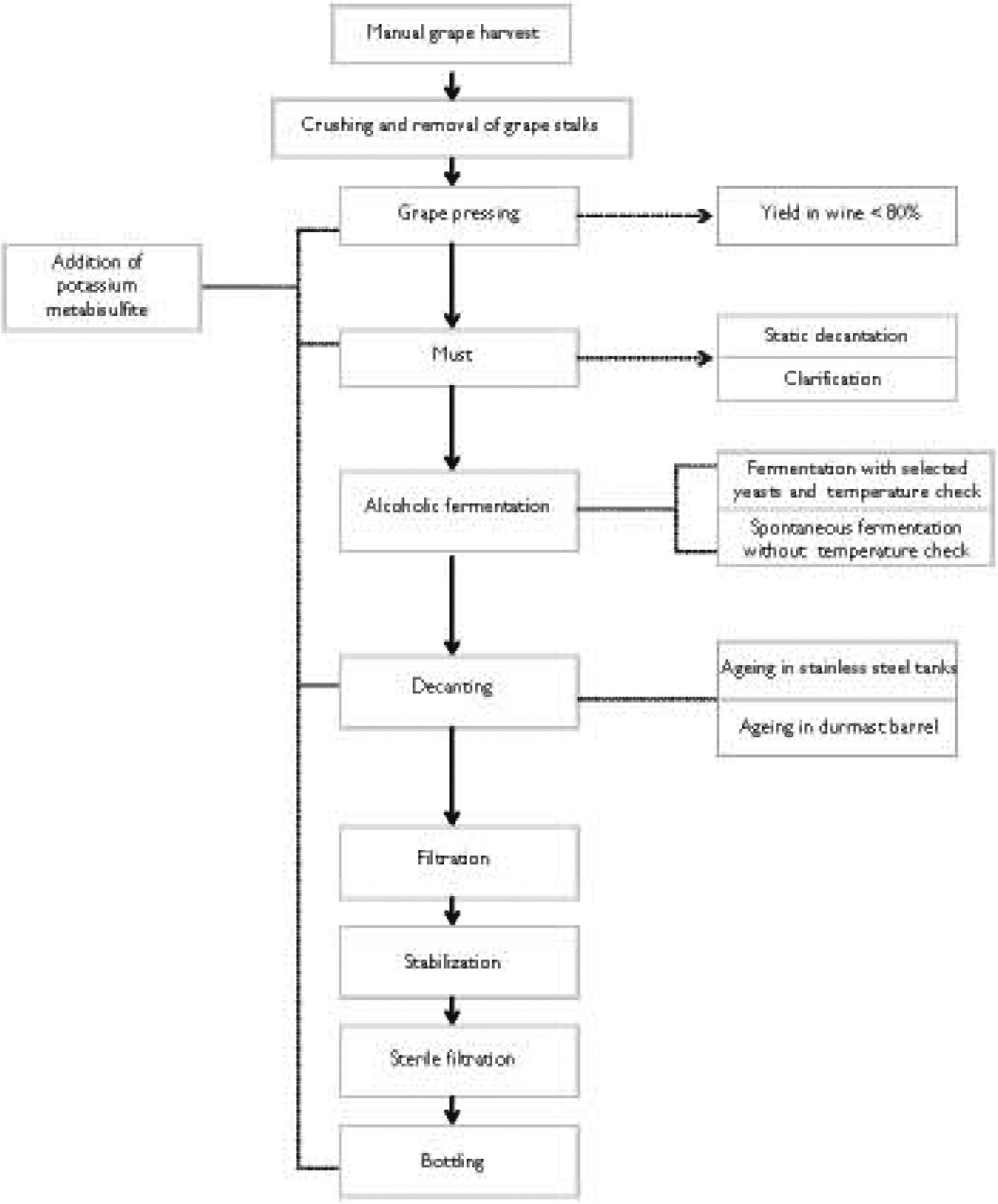
"Controspalliera" = vertical-trellised training system
"Cortina" = hanging down training system



RED WINE	SHORT SKIN CONTACT	FERMENTATION WITH SELECTED YEASTS	THERMO-CONDITIONED WINE-MAKING	MLF*	BARREL AGEING
FG0		X	X	X	X
FC1				X	X
MV1				X	X
MV3				X	X
MG1		X	X	X	X
NC4	X	X	X	X	X

*MLF = malolactic fermentation

Fig. 1 - Flowchart related to the elaboration of each red wine sample and description of processing technique (the symbol X means a positive response).



WHITE WINE	MUST CLARIFICATION	FERMENTATION WITH SELECTED YEASTS	THERMO-CONDITIONED WINE-MAKING	AGEING IN STAINLESS STEEL TANKS
MV3				X
MS3				X
TC3	X	X	X	X
IC4	X	X	X	X

Fig. 2 - Flowchart related to the elaboration of each white wine sample and description of processing technique (the symbol X means a positive response).

the red, white and *Passito* wines, respectively. After sampling, all of the wines were stored in the dark at 4°C, and each one was opened just prior to analysis.

Reagents

For the simultaneous chronopotentiometric determination of cadmium, copper, lead and zinc, all glassware was rinsed with 10% (v/v) nitric acid. Ultra-pure water (18.2 Mohm/cm) was obtained from a Pure Lab RO and a Pure Lab UV system USF (Ransbach-Baumbach, Germany). Ultra pure hydrochloric acid (34-37%), Hg (II) (1000 µg/mL, 1M in hydrochloric acid) and Cd(II), Cu(II), Pb(II) and Zn(II) (1000 µg/mL, 0.5 N in nitric acid) standard solutions were purchased from Panreac Quimica (Barcelona, Spain). Solutions of 1.0 µg/mL Cd (II), 2.5 µg/mL Cu (II), 1.0 µg/mL Pb (II) and 2.5 µg/mL Zn (II) were prepared by diluting with ultra pure water. Supel-ENVI carbon columns were obtained from Supelco (Bellefonte, PA, USA).

1,5-Diaminepentane (DAP), 1,4-diaminebutane (DAB), histamine (HIS), tyramine (TYR), tryptamine (TRY), 2-phenylethylamine (PHE), 1,7-diamineheptane (DAE), dansyl chloride and Na_2CO_3 were obtained from Sigma Aldrich (Milan, Italy); spermidine (SPD), spermine (SPM) were supplied by Fluka (Milan, Italy). Acetonitrile and water of HPLC grade were obtained from Carlo Erba (Milan, Italy).

HPLC analysis of biogenic amines

The HPLC determination of the biogenic amines was performed with a Shimadzu liquid chromatographic system coupled with a system controller SCL-10A-Vp; two LC-10A-Vp pumps; DAD detector SPD-M 10A Vp.; a GT-154 degasser, Rheodyne injector (model 7725i) with a 20 µL loop, and a C-18 Supelco Discovery column (150 x 2.1 mm, 5 µm), with a precolumn of the same material. The determination of the biogenic amines was performed according to a method reported in a previous study (DUGO *et al.*, 2006). The mobile phase was a gradient prepared from water (W) and acetonitrile (A): 0.01-6.00 min 65-35% W; 6.01-10.00 min, 35-20% W; 10.01-15.00 min, 20-10% W. The gradient was reduced to the initial condition in another 5 min; ten min of equilibration were required before the subsequent injection. The flow rate was 0.4 mL/min and the analyses were performed at 20°C.

Wine samples were analyzed after derivatization with dansyl chloride solution, as previously described (DUGO *et al.*, 2006). The reaction was carried out by adding 1.6 mL of dansyl chloride solution (10 mg/mL in acetone) to 1.5 mL of wine adjusted to pH 8.2 with Na_2CO_3 solution (40 g/L). The mixture was heated in a water-bath for an hour at 40°C. After the reaction, the acetone was removed in a slight stream of N_2. Then, the volume was made up to 1 mL with acetonitrile, and before HPLC analysis, the sample was filtered through a 0.45 µm membrane millipore filter. The resulting sample was analyzed three times. A wavelength of 254 nm was used.

The observed analytes were identified according to their retention time compared with those of the amines tested, as well as their spectral characteristics. Using a wine sample spiked with the biogenic amine standard solutions, the retention time of each amine was confirmed.

For each biogenic amine a stock of standard solution was prepared by adding an accurately weighed amount of each amine (*c.*10 mg) to a 10 mL volumetric flask and brought to the mark with 0.1 *M* HCl. The standard solutions were stored at 4°C until used. A standard mixture of biogenic amines was prepared by adding 0.1 mL of each amine stock solution in a 10 mL volumetric flask and bringing it up to mark with 0.1 *M* HCl. These solutions were stored at 4°C until used. A calibration line for each dansylated amine was obtained by analyzing the standard solutions diluted at different concentrations.

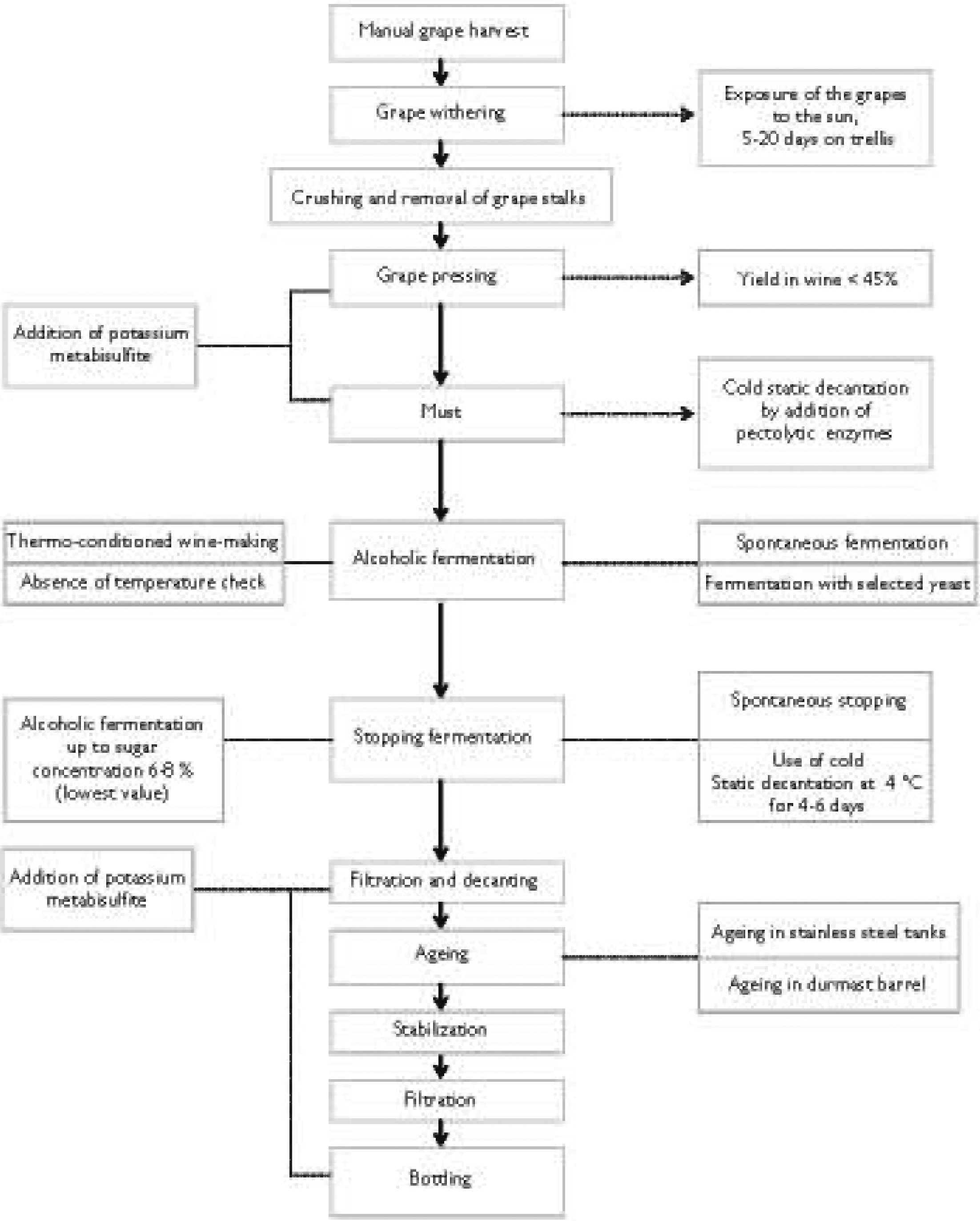
Chronopotentiometric stripping analysis of Cd, Cu, Pb and Zn

The analysis of Cd(II), Cu(II), Pb(II), Zn(II) was carried out using a PSA ION 3 stripping chronopotentiometric analyzer (Steroglass, S. Martino in Campo, Perugia, Italy), connected to a PC. The analyzer operated under the control of the NEOTES 2.0.1 software package (Steroglass); it was equipped with a conventional three-electrodes cell: the working electrode was glassy carbon one, coated with a thin film of mercury; the reference electrode was an Ag/AgCl electrode (3M KCl) and a platinum wire was used as the auxiliary electrode, as described in previous papers (LA PERA *et al.*, 2003; SALVO *et al.*, 2003; DUGO *et al.*, 2004a,b; DUGO *et al.*, 2005a,b,c; MATARESE PALMIERI *et al.*, 2005).

Wine samples were directly analyzed after acidification to pH 2 with 5M HCl and filtration through a carbon column, as described in previous studies (SALVO *et al.*, 2003; DUGO *et al.*, 2005b).

Cd(II), Cu(II), Pb(II) and Zn(II) were determined simultaneously in wine, as described in previous papers (SALVO *et al.*, 2003; DUGO *et al.*, 2005b,c; MATARESE PALMIERI *et al.*, 2005).

Before starting each analysis, the working electrode was cleaned with absolute methanol and filter paper; then the plating was carried out by, putting a 20 mL volume of a 1000 µg/mL Hg (II) standard solution in the electrochemical cell and carrying out the electrolysis, at –950 mV against the reference electrode, for 1 min. Cd



PASSITO WINE	MUST CLARIFICATION	FERMENTATION WITH SELECTED YEASTS	THERMO-CONDITIONED WINE-MAKING	BARREL AGEING
F01	X	X	X	
F02	X	X	X	
F03				X
M03				X
C 02	X	X	X	

Fig. 3 - Flowchart related to the elaboration of each *Passito* wine sample and description of processing technique (the symbol X means a positive response).

(II), Cu (II), Pb (II) and Zn (II) were determined simultaneously putting the acidified wine (2.0-5.0 mL) in the electrochemical cell together with 10.0 mL of ultrapure water and 1.0 mL of 1000 μg/mL Hg (II) as oxidizing agent.

Method performance for Cd, Cu, Pb and Zn chronopotentiometric analysis in wines and vegetables has been reported in previous papers (LA PERA *et al.*, 2003; SALVO *et al.*, 2003; DUGO *et al.*, 2004a,b; DUGO *et al.*, 2005a,b,c; MATARESE PALMIERI *et al.*, 2005).

Statistical analysis

The data were analyzed using Statistica StatSoft, Inc. 6.0. Initially, the Mann-Whitney non-parametric test was applied to determine any differences between wine samples from biological or traditional agriculture according to their amine and metal concentrations. Successively, Linear Discriminant Analysis (LDA) was performed to characterize the wine samples.

RESULTS AND DISCUSSION

Determination of biogenic amines

The biogenic amines levels in a total of five red, six white and four Passito wines from the province of Messina were investigated for the first time. The mean concentrations of biogenic amines in the wine samples are reported in Table 3; the wines are grouped according to typology. The analyses were carried out in triplicate, and the results are reported as the average value. The coefficient of variation (CV%) of the three analyses was always less than 5%.

Among the studied amines, 1,4-diaminobutane (DAB) was detected in all the samples at concentrations ranging from 0.33 mg/L (sample NC4) to 3.53 mg/L (sample MV1) with an average value of 1.94 mg/L for the red wines, 1.01 mg/L for the white wines and 0.94 mg/L for the *Passito* wines.

High levels of histamine (HIS) were detected in the red wines (average content 1.23 mg/L) but they never exceeded 1.52 mg/L (sample NC4). All the white wines had a low histamine content and often values were near the detection limit; the only exception was sample MV3 that had a histamine content of 1.16 mg/L. Among the *Passito* wines, the concentration of histamine never exceeded 1.20 mg/L (sample CO2) and, with the exception of samples VO3 and MO3 (1.11 and 0.14 mg/L, respectively), the histamine contents were always near the detection limit.

All of the red wines contained tyramine (TYR); the average content was 1.77 mg/L. Only sample MV3 contained high concentrations of tyramine (3.82 mg/L); white wines showed an average tyramine content that was much lower (0.08 mg/L) than that of the red ones, while in *Passito* wines, it was only detected in samples FO3, FO2 and CO2 and at values near the detection limit.

Spermidine (SPD) was only found in two *Passito* wines (samples FO2 and MO3) and was near the detection limit.

Among the biogenic amines studied, spermine (SPE) levels were lower than the detection limit in all the *Passito* and white wines and was only detected in two red wines at low concentrations (0.15 and 0.12 mg/L, respectively, in samples MG1 and NC4).

Most of the wines that were analyzed had a prevalence of 1,4-diaminobutane, histamine and tyramine; among the other amines studied, tryptamine, 2-phenylethylamine, 1,5-diaminopentane, 1,7-diaminoeptane, spermine and spermidine, were present in the wines in very small amounts that were often below the instrumental detection limit. While cadaverine, putrescine, histamine and tyramine were the most common amines found in the wines (ADAMS *et al.*, 1990; ERTAN ANLI *et al.*, 2009), nevertheless in these wine samples 1,5-diaminopentane (cadaverine) was often found at values near the detection limit.

The data show that the total biogenic amine content was higher in the red wines than in the white and *Passito* wines and ranged from 3.74 to 7.22 mg/L. This peculiarity could be explained by the difference in the vinification techniques; prolonged contact with the skin implicates an enrichment of the must with amino acids that are precursors of biogenic amines (RIBÉREAU-GAYON *et al.*, 2003a; ANCÍN-AZPILICUETA *et al.*, 2008).

Each sample of red wine, derived from a wine-making process that did not make use selected yeasts and where the fermentation temperatures were not controlled, had higher concentrations of biogenic amines, confirming the observations of some authors (RIBÉREAU-GAYON *et al.*, 2003b).

The total biogenic amines content of the *Passito* wines ranged from 0.89 to 2.35 mg/L and was mainly represented by putrescine and histamine. Moreover, biogenic amine content was high in the wines produced according to the traditional method, that uses spontaneous fermentation of turbid musts with native yeasts and refinement in chestnut casks. In contrast, the Passito wines made according to a modern wine-making technique, that opts for selected yeasts and the control of the fermentation temperatures, had low concentrations of biogenic amines.

The total biogenic amine content in the white wines ranged from 0.73 to 2.38 mg/L and 1,4-diaminobutane was the principal biogenic amine. The results also show that, for the white wines, the spontaneous fermentation of the musts with no control of the temperatures results in a high amine content.

Table 3 - Mean concentration of biogenic amines (mg/L ± SD), detection limit (DL in mg/L) and total content (mg/L) in the Sicilian wine samples.

Amine	TRY	PHE	DAB	DAP	HIS	DAE	TYR	SPD	SPE	Total content
DL	0.02	0.01	0.02	0.01	0.01	0.01	0.01	0.01	0.02	
***Passito* wines**										
FO1	0.03 ± 0.00	0.02 ± 0.00	1.01 ± 0.01	0.02 ± 0.00	0.03 ± 0.00	nd	0.02 ± 0.00	nd	nd	1.13
FO2	nd	nd	0.73 ± 0.01	0.04 ± 0.00	0.04 ± 0.00	nd	0.04 ± 0.00	0.04 ± 0.00	nd	0.89
VO3	nd	nd	1.21 ± 0.01	0.03 ± 0.00	1.11 ± 0.00	nd	nd	nd	nd	2.35
MO3	nd	0.03 ± 0.00	0.84 ± 0.01	0.01 ± 0.00	0.14 ± 0.00	0.03 ± 0.00	nd	0.02 ± 0.00	nd	1.07
CO2	nd	0.02 ± 0.00	0.82 ± 0.00	0.03 ± 0.00	1.20 ± 0.01	nd	0.04 ± 0.00	nd	nd	2.11
Red wines										
FG0	0.04 ± 0.00	nd	2.33 ± 0.05	0.04 ± 0.00	1.23 ± 0.01	nd	0.45 ± 0.01	nd	nd	4.09
FC1	nd	nd	1.52 ± 0.03	0.04 ± 0.00	1.25 ± 0.01	nd	1.32 ± 0.01	nd	nd	4.13
MV1	nd	nd	3.53 ± 0.08	0.02 ± 0.00	1.14 ± 0.02	nd	1.86 ± 0.01	nd	nd	6.55
MV3	nd	nd	2.15 ± 0.05	0.03 ± 0.00	1.22 ± 0.03	nd	3.82 ± 0.06	nd	nd	7.22
MG1	nd	0.02 ± 0.00	1.81 ± 0.04	0.04 ± 0.00	1.07 ± 0.01	0.10 ± 0.01	0.55 ± 0.01	nd	0.15 ± 0.01	3.74
NC 4	0.03 ± 0.00	0.03 ± 0.00	0.33 ± 0.00	0.02 ± 0.00	1.52 ± 0.02	nd	2.64 ± 0.06	nd	0.12 ± 0.00	4.69
White wines										
MV3	nd	nd	1.05 ± 0.01	nd	1.16 ± 0.01	nd	0.17 ± 0.00	nd	nd	2.38
MS3	nd	nd	1.22 ± 0.01	nd	0.07 ± 0.00	0.14 ± 0.00	0.14 ± 0.00	nd	nd	1.57
TC4	nd	nd	0.54 ± 0.01	0.04 ± 0.00	0.06 ± 0.00	0.04 ± 0.00	0.05 ± 0.00	nd	nd	0.73
IC4	nd	nd	1.26 ± 0.01	nd	0.04 ± 0.00	nd	nd	nd	nd	1.30

n.d., not detected
TRY = tryptamine, PHE = 2-phenylethylamine, DAB = 1,4-diaminobutane, DAP = 1,5-diaminopentane, HIS = histamine, DAE = 1,7-diaminoeptane, TYR = tyramine, SPD = spermidine, SPE = spermine

Determination of heavy metals Cd, Cu, Pb and Zn

Considering the wide use of wine in the world and the fact that, in recent years, Sicilian wines have finally made it onto the international stage, it was of interest to determine the Cd(II), Cu(II), Pb(II) and Zn(II) concentrations. Table 4 shows the concentration of metals found in fifteen wine samples produced in the province of Messina. Zn was the most abundant metal, followed by Cu, Pb and Cd.

In particular, Cd was present in very small amounts in almost all of the samples and the mean concentration ranged from 0.002 to 0.007 mg/L, without significant differences among the wine typologies. The Cd concentrations in two white wine samples (MV1 and MG1) were below the instrumental detection limit.

The mean concentration of Pb was 0.004-0.078 mg/L; sample FC1 (0.078 mg/L) had the highest level among the red wines, followed by the white wine sample MS3 (0.073 mg/L) and the Passito wine CO2 (0.066 mg/L).

The Cu concentration ranged from 0.020 to 0.740 mg/L with an average content of 0.288 mg/L for the Passito wines, 0.337 mg/L for the red wines and 0.168 mg/L for the white wines. With the exception of sample MS3, that had the lowest Cu content (0.020 mg/L), the wines made according biological agriculture techniques were the only ones for which the Cu concentration was greater than that of the other samples produced according to a more traditional process.

The Zn content varied from 0.341 to 2.182 mg/L with the *Passito* wine samples having the highest values. In particular, *Passito* wines had an average Zn content of 1.459 mg/L and sample FO1 had the highest concentration (2.182 mg/L). The average Zn content in the red wines was 0.816 mg/L and the concentration of this metal never exceeded 1.300 mg/L (sample FG0). The wines made according to biological agriculture techniques had the lowest Zn concentrations (0.453, 0.560 and 0.652 mg/L in samples FC1, MV1 and MV3, respectively). The Zn content in the white wine samples was below 0.560 mg/L (sample MS3)

Table 4 - Heavy metal concentrations (mg/L) and detection limit (DL in mg/L) in Sicilian wine samples. Each value is the mean of three determinations, the range of relative standard deviation is: 0.8% for Cd (II), 1.5% for Cu (II), 1.0% for Pb (II) and 0.7% for Zn (II).

Metals	Pb (II)	Cd (II)	Cu (II)	Zn (II)
DL	0.07	0.05	0.05	0.05
***Passito* wines**				
F01	10.48	6.50	611.34	2182.21
F02	15.34	1.02	509.88	1270.92
V03	12.06	1.83	92.43	1253.02
M03	14.12	4.98	80.96	1439.88
C02	65.80	4.16	150.16	1150.10
Red wines				
FG0	12.00	1.03	72.39	1300.04
FC1	77.95	1.97	420.11	453.31
MV1	20.18	nd	740.25	560.40
MV3	27.10	2.02	640.90	652.11
MG1	14.96	nd	93.10	1100.06
NC 4	4.16	7.14	59.88	860.79
White wines				
MV3	4.11	1.02	540.97	341.08
MS3	73.05	5.10	20.12	560.13
TC4	13.12	1.06	44.35	362.33
IC4	15.16	2.98	70.31	480.16

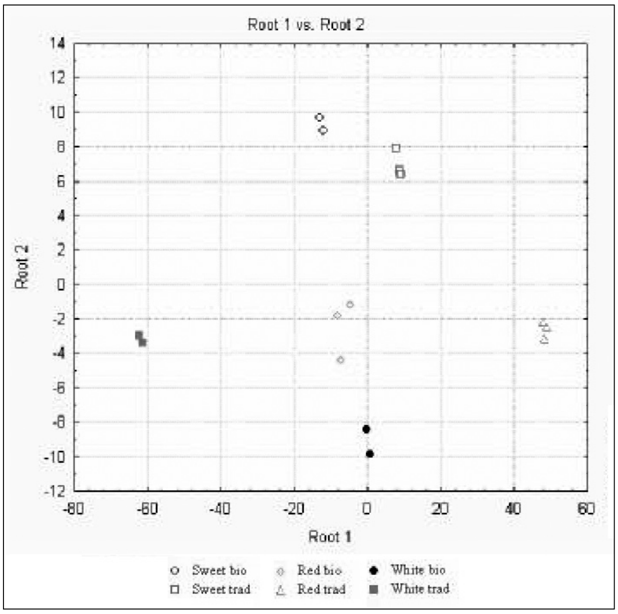


Fig. 4 - Scatterplot of canonical scores. Discriminant functions for grape type and agricultural practice according to five amines (HIS, SPD, DAE, TRY, DAP) and three metal (Zn, Cu, Cd) concentrations.

with no relevant differences in the concentrations (average value 0.435 mg/L).

Statistical analysis

The non-parametric Mann-Whitney test was applied to determine differences between wine samples from biological or traditional agriculture, based on their amine and metal concentrations. Table 5 shows the results from the Mann-Whitney test; the p-level was significant only for Cu; this suggests a difference between the two group samples that were either grown according to traditional or biological agriculture practices.

The concentrations of the nine amines (TRY, PHE, DAB, DAP, HIS, DAE, TYR, SPD and SPE), considered alone and together with the metals (Zn, Pb, Cd and Cu), were then studied using Linear Discriminant Analysis (LDA). This was done in order to create a model that, through some classification functions, could separate samples in relation to a particular type and agriculture, cultivation or manuring. Indeed, the two types of agriculture, the four types of grapes, the two types of cultivation and the two types of pruning techniques could contribute to the presence of one or more of the thirteen variable concentrations measured in the fifteen samples.

Linear Discriminant Analysis was initially performed grouping wines by grape type (*Passito*, white or red) and agriculture type (traditional or biological) using all nine amines and four metal concentrations in a stepwise forward Linear Dis-

Table 5 - Mann-Whitney test to compare two independent groups (biological and traditional agriculture samples) by their amines and heavy metal concentrations. Marked tests are significant at p<0.05 level.

variable	Rank Sum biological	Rank Sum tradizional	U	Z	p-level	Z adjusted	p-level	2°1 sided exact p
TRY	52.50000	67.50000	24.50000	-0.40505	0.685444	-0.58012	0.561835	0.694328
PHE	44.50000	75.50000	16.50000	-1.33087	0.183234	-1.58665	0.112594	0.189277
DAB	64.00000	56.00000	20.00000	0.92582	0.354540	0.92582	0.354540	0.396892
DAP	50.00000	70.00000	22.00000	-0.69473	0.487454	-0.69686	0.485892	0.535820
HIS	54.00000	66.00000	26.00000	-0.23146	0.816961	-0.23146	0.816961	0.866511
DAE	51.00000	69.00000	23.00000	-0.57864	0.562834	-0.74261	0.457718	0.612587
TYR	67.00000	53.00000	17.00000	1.27300	0.203018	1.27757	0.201401	0.231857
SPD	57.00000	63.00000	27.00000	0.11573	0.907869	0.19562	0.844911	0.955089
SPE	49.00000	71.00000	21.00000	-0.81009	0.417888	-1.36931	0.170904	0.463403
Pb	65.50000	54.50000	18.50000	1.09941	0.271590	1.10535	0.269009	0.280963
Cd	53.00000	67.00000	25.00000	-0.34718	0.728454	-0.35225	0.724650	0.778866
Cu	76.00000	44.00000	8.00000	**2.31455**	**0.020638**	**2.31869**	**0.020412**	**0.020513**
Zn	49.00000	71.00000	21.00000	-0.81009	0.417888	-0.81082	0.417471	0.463403

Table 6 - Summary of stepwise analysis: variables entered in the model according to their discriminant power.

Variable Enter/Remove	Step	F to entr/rem	df 1	df 2	p-level	No.of vars. In	Lambda	F-value	df 1	df 2	p-level
^{Zn}-(E)	1	9.243274	5	9	0.002380	1.000000	0.162995	9.24327	5	9	0.002380
^{Cu}-(E)	2	6.341483	5	8	0.011429	2.000000	0.032839	7.22924	10	16	0.000286
^{HIS}-(E)	3	7.114594	5	7	0.011398	3.000000	0.005400	7.40216	15	20	0.000033
^{SPD}-(E)	4	6.127290	5	6	0.023693	4.000000	0.000884	7.64121	20	21	0.000010
^{DAE}-(E)	5	6.120593	5	5	0.034279	5.000000	0.000124	8.23715	25	20	0.000005
^{TRY}-(E)	6	7.490555	5	4	0.036878	6.000000	0.000012	9.59776	30	18	0.000003
^{DAP}-(E)	7	3.919368	5	3	0.145093	7.000000	0.000002	9.84646	35	15	0.000013
^{Cd}-(E)	8	3.617521	5	2	0.230635	8.000000	0.000000	10.16381	40	12	0.000061

criminant Analysis. At each step all of the variables are reviewed and evaluated according to which one will contribute most to the discrimination between groups.

Zn and Cu are the first variables in the model followed by HIS and SPD based on their discriminating power (Table 6). According to the Partial Wilks' Lambda value and the F value, the Pb, DAB, PHE, SPE and TYR variables are not included in the model. Five discriminant functions provide 100% correct classification.

As can be seen (Fig. 4) samples are grouped according to their grape type and agriculture method through two discriminant functions which explain 99.2% of the total variance. In particular, samples coming from biological agriculture are plotted along the zero value for function 1 (Root 1).

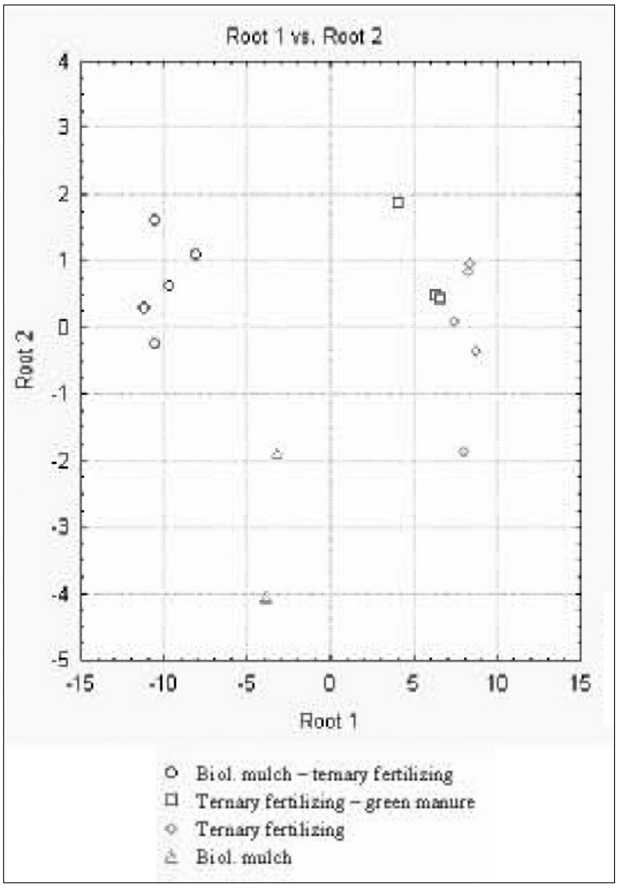


Fig. 5 - Scatterplot of canonical scores. Discriminant functions for cultivation type according to eight amines (TRY, DAB, DAE, SPE, HIS, SPD, TYR and PHE) and two metals (Zn, Cu) concentrations.

In addition, two discriminant analyses were conducted to investigate a possible classification in relation to the cultivation type and manuring type. Regarding the cultivation type, *controspalliera* long pruning, *controspalliera* short pruning and *cortina* long pruning were considered.

The variables in the model and ordered according to their ability to separate the type of cultivation are: Zn, TRY, DAB, DAE, SPE, HIS, SPD, Cu, TYR and PHE.

According to this model, 100% of the cases were correctly classified. In fact, two discriminant functions, that explained 100% of the total variance, allow a good separation between the samples in relation to their cultivation type.

Finally, a stepwise forward Linear Discriminant Analysis was performed to discriminate between samples coming from different types of manuring: ternary fertilizers and green manure with clover, ternary fertilizers and biological mulch, and ternary fertilizers.

The analysis of variance and the Partial Wilks' Lambda test showed that the variables, ordered in relation to the highest discriminating power are: Cu, Pb, DAP, SPD, Cd, PHE and DAE. The other variables were excluded from the analysis. Three discriminant functions were created that allowed 93.3% correct classification. A scatterplot in Fig. 6 shows a good separation with Root 1 of the samples coming from ternary or biological mulch. In this case, the two functions explain 99.5% of the total variance.

CONCLUSIONS

The definition of wine quality should include the threshold quantities when toxicological effects are caused directly by the ingestion of biogenic amines, but this value is still under discussion because there are differing views among different countries. To date, the OIV ("Organisa-

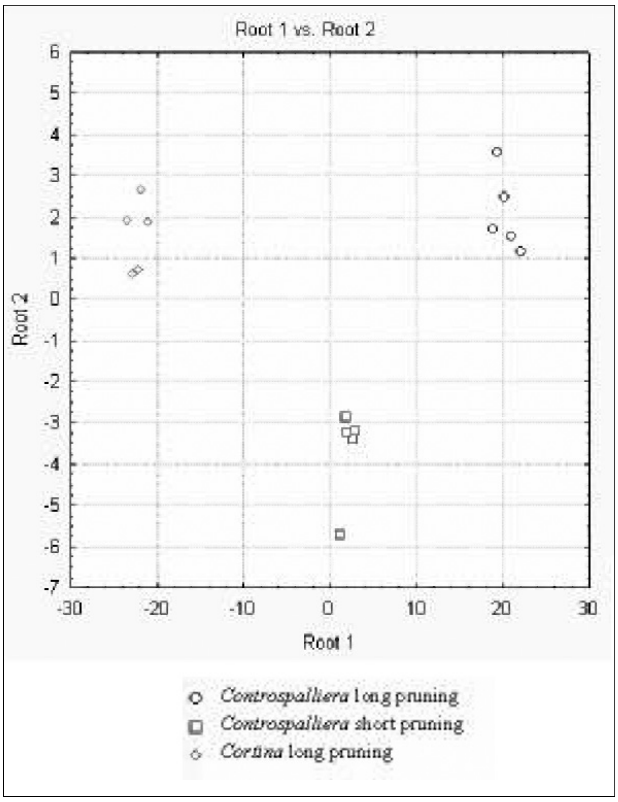


Fig. 6 - Scatterplot of canonical scores. Discriminant functions for manuring type according to four amines (DAP, SPD, PHE and DAE) and three metals (Cu, Pb and Cd) concentrations.

tion Internationale de la Vigne et du Vin") has not set any maximum limits concerning the level of biogenic amines in wine, which could be used to draft future European legislation. Nevertheless, some countries have, arbitrarily, recommended maximum histamine levels for wine (BUSTO *et al.*, 1996).

Although there are no legal limits, the wines analyzed in this study showed moderate quantities of biogenic amines. In comparison to the values reported in the literature (LONVAUD-FUNEL and JOYEUX, 1994; BUSTO *et al.*,1996), they do not constitute a health risk for the consumer. In fact, all the examined wines, produced according to a modern wine-making technique, had low concentrations of biogenic amines.

With respect to the metal contents found in the wines, the red wines showed elevated average concentrations of copper and zinc. Moreover, the samples derived from biological agriculture techniques, except white wine sample MS3, had higher copper contents with respect to those obtained for wine samples derived from traditional agriculture.

Based on the data obtained, a moderate daily consumption of the analyzed wines would provide a Cu and Zn intake that is below the recommended daily allowances (RDAs) established by the Food and Drug Administration (for a 70-kg adult, 18 mg Zn day^{-1}, and 3 mg Cu day^{-1}) and the Cd and Pb levels would be within the legal limits established by the Italian Republic (ITALIAN REPUBLIC 1986, EUROPEAN COMMUNITY 2006). Therefore, it can be affirmed that the analyzed wine samples do not represent a toxicological risk for human health.

REFERENCES

Adams D.O., Frankel K.E. and Christensen L.P. 1990. Elevated putrescine levels in grapevine leaves that display symptoms of potassium deficiency. Am. J. Enol. Vitic. 41: 121.

Ancín-Azpilicueta C., González-Marco A. and Jiménez-Moreno N. 2008. Current knowledge about the presence of amines in wine. Crit. Rev. Food Sci. Nutr. 48: 257.

Arce L., Rìos A. and Valcárcel M. 1998. Direct determination of biogenic amines in wine by integrating continuous flow clean-up and capillary electrophoresis with indirect UV detection. J. Chromatogr. A 803: 249.

Bauza T., Kanny G., Blaise A. *et al.* 1995. Les amines biogènes des vins: métabolisme et toxicitè. Bull. OIV , 767-768: 42.

Benìtez P., Castro R. and Barroso G.C. 2002. Removal of iron, copper and manganese from white wines through ion exchange techniques: Effects on their organoleptic characteristics and susceptibility to browning. Anal. Chem. Acta 458: 197.

Busto O., Guasch J. and Borrull F. 1996. Biogenic amines in wine: a review of analytical methods. J. Int. Sci. Vigne Vin 30: 85.

Coton E., Torlois S., Bertrand, A. and Lonvaud-Funel A. 1999. Biogenic amines and wine lactic acid bacteria. Bull. OIV, 815-816: 22.

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

Dugo G., La Pera L., La Torre G.L and Giuffrida D. 2004a. Determination of Cd(II), Cu(II), Pb(II), and Zn(II) content in commercial vegetable oils using derivative potentiometric stripping analysis (dPSA). Food Chem. 87: 639.

Dugo G., La Pera L., Lo Turco V., Giuffrida D. and Restuccia S. 2004b. Determination of Cu, Zn, Se, Pb and Cd using potentiometric stripping methods in potatoes (*Solanum tuberosum* L.). Food Addit. Contam. 21: 649.

Dugo G., La Pera L., Lo Turco V. and Di Bella G. 2005a. Speciation of inorganic arsenic in alimentary and environmental aqueous samples by using derivative anodic stripping chronopotentiometry (dASCP). Chemosphere 61: 1093.

Dugo G., La Pera L., Pellicanò T.M., Di Bella G. and D'Imperio M. 2005b. Determination of some inorganic anions and heavy metals in D.O.C. Golden and Amber Marsala wines: Statistical study of the influence of the ageing period, colour and sugar content. Food Chem. 91: 355.

Dugo G., La Pera L., Lo Turco V., Matarese Palmieri R. and Giuffrida D. 2005c. Effect of boiling and peeling on manganese content of some vegetables determined by derivative anodic stripping chronopotentiometry (dASCP). Food Chem. 93: 703.

Dugo G., Vilasi F., La Torre G.L. and Pellicanò T.M. 2006. Reverse phase HPLC/DAD determination of biogenic amines as dansyl derivatives in experimental red wines. Food Chem. 95: 672.

Ertan Anli R. and Bayram M. 2009. Biogenic amines in wines. Food Rev. Int. 25: 86.

European Community. 2006. Rule n° 1881/2006. European Official Gazette n. 364, December 2006.

Fernandez Pereira C. 1988. The importance of metallic elements in wine. A literature survey. Z. Lebensm. Unters Forsh. 186: 295.

Gennaro M.C., Mentasti E., Sarzanini C. and Pesticcio A. 1986. Undesirable and harmful metals in wines - determination and removal. Food Chem. 19: 93.

Golimowsky J., Valenta P. and Wolfgang-Nurnberg H. 1979. Toxic trace of metals in food. II. A comparative study of the levels of toxic trace metals in wine by differential pulse anodic stripping voltammetry and electrothermal atomic absorption spectrometry. Z. Lebensm. Unters Forsh. 168: 439.

Hsia C., Planck R.W. and Nagel C.W. 1975. Influence of must processing on iron and copper contents of experimental wines. Am. J. Enol. Vitic. 26: 57.

Italian Republic. 1973. DPR 20 September 1973. Published in the Italian Official Gazette n. 28s, 30 January 1974.

Italian Republic. 1976. DPR 03 December 1976. Published in the Italian Official Gazette n. 61s, 04 March 1977.

Italian Republic. 1986. Caratteristiche e limiti di alcune sostanze nel vino. D.M. 29 December 1986.

Italian Republic. 2004. DPR 03 September 2004. Published in the Italian Official Gazette n. 214, 11 September 2004.

Jarish R., Pirker C., Mdslinger T. and Gdtz M. 1992. The role of histamine in wine intolerance. J. Allergy Clin. Immunol. 89: 197.

Kanny G., Bauza T., Fremont S., Guillemin F., Blaise A., Daumas F., Cabanis J.C., Nicolas J.P. and Moneret-Vautrin D.A. 1999. Histamine content does not influence the intolerance of wine in normal subject. Allerg. Immunol. 31: 45.

Karadjova I., Izgi B. and Gucer S. 2002. Fractionation and speciation of Cu, Zn and Fe in wine samples by atomic absorption spectrometry. Spectrochim. Acta, Part B 57: 581.

La Pera, L. Saitta M., Di Bella G. and Dugo G. 2003. Simultaneous determination of Cd(II), Cu(II), Pb(II) and Zn(II) in citrus essential oil by derivative potentiometric analysis. J. Agric. Food Chem. 51: 1125.

La Pera L., Dugo G., Rando R., Di Bella G., Maisano R., Salvo F. 2008. Statistical study of the influence of fungicide treatments (mancozeb, zoxamide and copper oxychloride) on heavy metal concentrations in Sicilian red wine. Food Addit. Contam. 25: 302.

Lonvaud-Funel A. and Joyeux A. 1994. Histamine production by wine lactic-acid bacteria. Isolation of a histamine-producing strain of leuconostoc-oenos. J. Appl. Bacteriol. 77: 401.

Marine-Font A., Vidal-Carou C., Izquierdo-Pulido M., Veciana-Nogues T. and Hernandez-Jover T. 1995. Biogenic amines in foods: significance and analysis. Annales des Falsifications de l'Expertise Chimique et Toxicologique 88: 119.

Matarese Palmieri R., La Pera L., Di Bella G. and Dugo G. 2005. Simultaneous determination of Cd(II), Cu(II), Pb(II) and Zn(II) by stripping chronopotentiometry in *Pittosporum* Zn(II) by stripping chronopotentiometry in *Pittosporum tobira* leaves: A measurement of local atmospheric pollution in Messina (Sicily, Italy). Chemosphere 59: 1161.

McKinnon A.J. and Scollary G.R. 1997. Size fractionation of metals in wine using ultrafiltration. Talanta 44: 1649.

Moreno-Arribas M.V. and Polo M.C. 2008. Occurrence of lactic bacteria and biogenic amines in biologically aged wines. Food Microbiol. 25: 875.

Ribéreau-Gayon J., Peynaud E. and Ribéreau-Gayon P. 1976. Precipitation dans le vins. In "Sciences et techniques du vin" 2nd Edn., Vol. 3, p. 567. Ed. Bordas, Paris, France.

Ribéreau-Gayon P., Glories Y., Maujean A. and Dubourdieu D. 2003a. Chimica del vino. Stabilizzazione trattamenti. In "Trattato di enologia II" 3rd Ed., Vol. 2, p. 107. Ed. Edagricole, Bologna, Italy.

Ribéreau-Gayon P., Dubourdieu D., Donèche B. and Lonvaud A. 2003b. Microbiologia del vino e vinificazioni. In "Trattato di enologia I", Vol. 1, p. 154. Ed. Edagricole, Bologna, Italy.

Salvo F., La Pera L., Di Bella G., Nicotina M. and Dugo G. 2003. Influence of mineral and organic pesticides treatments on of Cd(II), Cu(II), Pb(II) and Zn(II) content determined by Derivative Potentiometric Stripping Analysis in Italian red and white wines. J. Agric. Food Chem. 51: 1090.

Vidal-Carou M.C., Codony-Salcedo R. and Mariné-Font A. 1991. Changes in the concentration of histamine and tyramine during wine spoilage at various temperatures. Am. J. Enol. Vitic. 42: 145.

Wantke F., Götz M. and Jarisch R. 1993. Histamine-free diet. Treatment of choice for histamine. Induced food intolerance and supporting treatment for chronical headaches. Clin. Exp. Allergy 23: 982.

Revised paper received May 13, 2009 Accepted June 4, 2009

DETERMINATION OF VARIETAL VOLATILES AS QUALITY AND SHELF-LIFE MARKERS/ORIGIN AND TYPICALNESS TRACERS IN SOUTHERN ITALIAN WINES

A. NASI, T. DE GENNARO, V. AVARA, V. NICOLELLA, A. MONACO[1], V. MERCURIO, G. ADDIMANDA, T.M. GRANATO, L. CHIANESE* and P. FERRANTI
Dipartimento di Scienze degli Alimenti, Università "Federico II" di Napoli,
Parco Gussone, 80055 Portici, Napoli, Italy
[1]Dipartimento di Arboricoltura, Università di Napoli "Federico II", 80055 Portici, Napoli, Italy
*Corresponding author: chianese@unina.it

ABSTRACT

The composition of wine varietal volatiles (terpenes, norisoprenoids, etc.), expressions of distinctive vine genetic characteristics, was determined in some southern Italian wines. The aim was to tentatively identify specific quality and shelf-life molecular tracers, among compounds related to the grapevine biodiversity, and to observe their modifications under different storage conditions. During the shelf-life of bottled Fiano wine samples, high temperatures produced an increase of terpene interconversion and degradation. p-Cymene and some terpenyl oxides (linalool oxide and limonene oxide) were also produced which appeared as shelf-life molecular markers. In Aglianico wine samples, some terpene compounds (α-pinene, β-myrcene, 3-carene, 4-carene, limonene and linalool) acted as possible process markers during the ageing step. In autochthonous Ischia varieties, terpene compounds also acted as suitable indicators of geographical origin. The data obtained showed that some volatile compounds could be potential analytical tools for checking, saving and improving the quality of typical wines.

- Key words: aging, terpenes, shelf-life, varietal volatile compounds, wine quality -

INTRODUCTION

High quality wines acquire a meaningful importance on the market through the diversification of their specific identifiable qualitative characteristics. During the last 30 years, particularly in the traditional winemaking countries (Italy, France, Spain, etc.) consumer preferences have become more oriented towards products with a strong cultural and territorial identity. In particular, consumer preferences have been oriented towards authochthonous wines with quality trademarks such as the European Appellation of Origin designations (e.g., Italian Controlled and Guaranteed Denomination of Origin, i.e. DOCG, or Controlled Denomination of Origin, i.e. DOC, etc.). The typicalness of these wines is related to the grape variety and specific characteristics of the *terroir* (i.e. soil, location, climate, and specific vineyard-environment interaction).

Several studies of viticultural zoning have been carried out by using a multidisciplinary approach to define the best qualitative potential of the oenological production. As high quality products have always been the main aim, technological innovation in the oenological sector has been coupled with tradition and typicalness (CHRISTAKI and TZIA, 2002).

Using analytical tools to identify quality, authenticity and typicalness, molecular markers provide new information and when combined with conventional analytical parameters give a better description of the typicalness and quality of food products.

For this reason several studies have been carried out to determine the trace elements in order to correlate the composition of these elements and the origin, *terroir* and authenticity of the wine (THIEL *et al.*, 2004). Several studies have identified and quantified the molecular typicalness markers in wines such as anthocyanins, norisoprenoids, pyrazines and terpenes (CÂMARA *et al.*, 2004; CARRO *et al.*, 1996; ROSILLO *et al.*, 1999; OLIVEIRA *et al.*, 2004; SÁNCHEZ-PALOMO *et al.*, 2005; MAMEDE *et al.*, 2006; MATEO *et al.*, 2000; FLAMINI, 2005; MAZZUCA *et al.*, 2005; NASI *et al.*, 2008, 2006; VILANOVA and SIEIRO, 2006).

The quali-quantitative composition of terpenes, which are odorous molecules with low perception threshold, is strictly related to the varietal origin (CÂMARA *et al.*, 2004; CARRO *et al.*, 1996; MATEO *et al.*, 2000; FLAMINI, 2005; PEŤKA *et al.*, 2006; RAPP, 1998; LÓPEZ *et al.*, 2002; ROSILLO *et al.*, 1999; OLIVEIRA *et al.*, 2004). Specific genes encode for enzymes that catalyse the monoterpene biosynthesis that produce these compounds that show a large structural diversity (LUCKER *et al.*, 2001; HARBORNE, 1991).

Conservation modalities during the ageing step and storage conditions during the shelf-life of wines could affect the quality and original potential varietal expression of a wine. Some researchers have investigated the modifications that occur in the volatile components in wines during shelf-life (PÉREZ-COELLO *et al.*, 2003; LAMBROPOULOS and ROUSSIS, 2007). Very few studies have reported the possible modification and interconversion of terpenes due to the effects of temperature and pH in aqueous solutions (MAICAS and MATEO, 2005). No study has been reported that is specifically aimed at identifying potential shelf-life and quality markers from among varietal volatiles and related degradation products.

In view of the potential contribution of varietal volatile compounds to wine quality and aroma, the varietal volatile components of some southern Italian autochthonous red and white wines (Fiano white wine and Aglianico red wine) were investigated under different storage conditions during the shelf-life in bottle and the ageing step. The aim was to identify potential quality markers and other modifications which could potentially affect their specific characteristics in order to save and reinforce the quality characteristics of these wines that are well known on the international market.

The composition of volatile varietal markers of autochthonous wines (Don Lunardo and Arilla white wines) from different areas on the Isle of Ischia (specifically chosen because of its small, defined geographical area) was also determined in order to identify potential tracers of geographical origin.

MATERIALS AND METHODS

After fermentation following a traditional winemaking protocol in a winery in Taurasi (Avellino, Italy), the Aglianico wine was separated into 3 different tanks for ageing (9 months): a) a 5000 L inox tank; b) a 3000 L oak barrel; and c) a 228 L oak barrel.

The Fiano wine was produced in a winery in Atripalda (Avellino, Italy). After bottling the sample was divided into 5 parts (each part= 11 bottles of 750 mL), each part was stored at 4 different temperatures (5, 14, 20 and 35 °C) and at two different light exposures: light and dark.

The Don Lunardo and Arilla grapes and wines were produced in different areas on the Isle of Ischia. The same winemaking conditions were used to produce the wines.

Liquid/liquid extraction of volatile molecules was carried out with 2.5 mL of dichloromethane and 50 mL of wine using a vortex for 1 h.

The SPME (Solid Phase Micro-Extraction) analysis was effected with the holder and fibres (50/30 μm divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS)) purchased from Supelco (Aldrich, Bornem, Belgium). It was carried out on 100 mL of sample (wine and must) with 30 g of NaCl added and also on 3 g of skins homogenised in 10 mL of water with 4 g of NaCl. Thermal desorption of the analytes from the fibre inside the GC injection port was carried out in the split mode (1/10) at a desorption temperature of 250°C for 1 min.

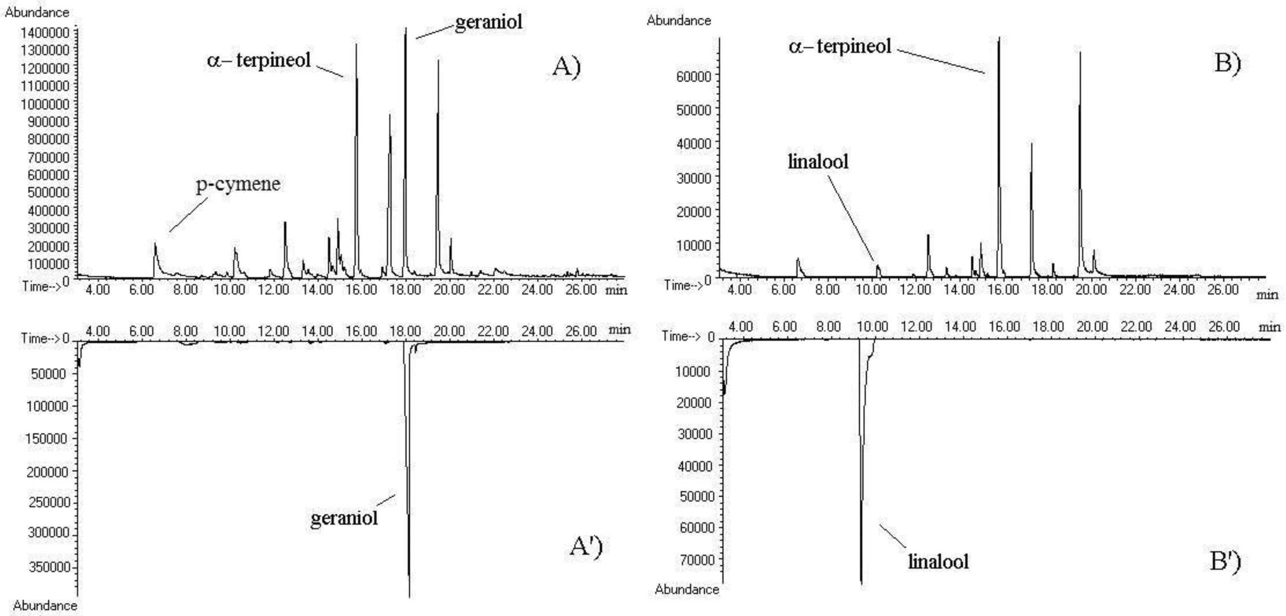


Fig. 1 - TIC chromatograms obtained through GC/MS analysis on an extract in dichloromethane from hydroalcoholic solution containing geraniol and linalool after (A, B) and before (A', B') acid hydrolysis (pH 3) at 35°C.

All samples were analysed with an HP 6890 coupled to a 5973N quadrupole HP mass spectrometer (Agilent Technologies, Palo Alto, CA, USA). The gas chromatograph was equipped with an HP-5ms capillary column (30 m x 0.25 mm ID; 0.25 μm Film Thickness) and the carrier gas used was helium.

For the analysis of the volatile molecules, the GC oven temperature was programmed from 40°C (held for 7 min) to 180°C at 5°C/min (6°C/min for the analysis in wine model hydroalcoholic solutions (pH=3)). The masses were scanned on m/z range of 45-350 amu. In other cases, a SIM method was used (for terpene compounds m/z 59, 69, 93, 121, 136). The NIST library and comparison with spectra and retention times of standards (Sigma-Aldrich; Acros Organics) were used to identify the odorous compounds.

Quantitative determinations of terpenes were obtained by means of calibration curves, in the concentration ranges typical of wines for each compound. In the range of linearity verified seven concentration levels and five replicates per level were used. Multiple replicates (n=3-6) of the samples were analysed.

RESULTS AND DISCUSSION

Varietal volatiles as shelf-life tracers in Fiano wines

The composition of the varietal volatile component was investigated during the shelf-life (seven months) of the white DOCG Fiano wine.

Some modifications of the terpene compounds were observed during the storage of Fiano wine bottles. These changes were also detected *in vitro*, i. e. in hydroalcoholic wine model solutions (pH=3), containing terpene molecules and stored at 35°C, for 5, 35 and 45 days; Fig. 1). The relative concentration of the α-terpineol increased and the relative concentration of the other ter-

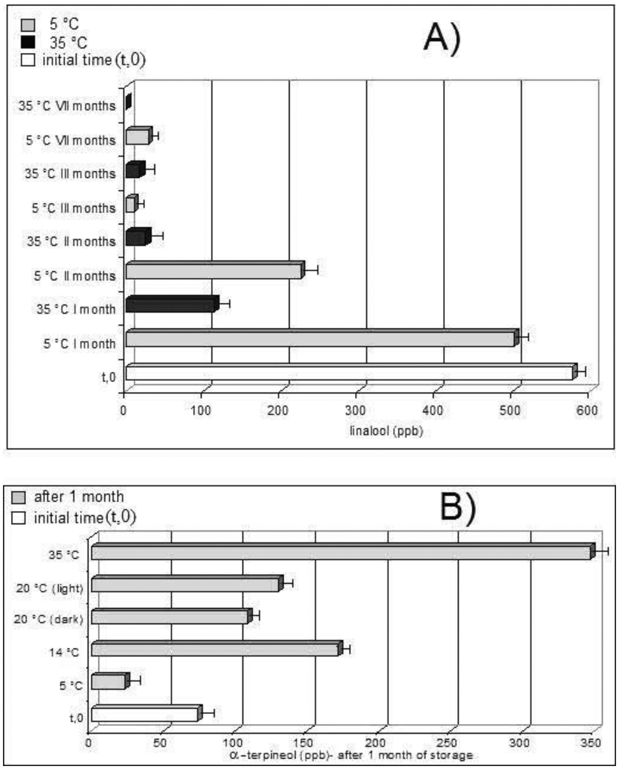


Fig. 2 - Concentrations in ppb of linalool (A) at 5°C and 35°C during different storage times, and α-terpineol (B) after the first month of storage at different storage temperatures.

Table 1 - Qualitative terpene component in Fiano wine samples stored under different conditions for different times. * Potential shelf-life markers (present only for higher storage temperatures and for longer storage times); L. Light exposure, D. Dark exposure, tr. in traces.

Storage Temperature (°C)	Storage time (months)	limonene	linalool	α-terpineol	geraniol	α-ionone	p-cymene	limonene epoxide	linalool oxide
5	1	+	+	+	+	+	-	-	-
5	2	+	+	+	+	+	-	-	-
5	3	+	+	+	+	+	-	-	-
14	1	+	+	+	+	+	-	-	-
14	2	+	+	+	+	+	-	-	-
14	3	+	+	+	+	+	-	-	-
20	1 (L)	+	+	+	+	-	-	-	-
20	2 (L)	+	+	+	+	+	+ (tr)*	-	-
20	3 (L)	+	-	+	-	+	+ (tr)*	-	-
20	1 (D)	+	+	+	+	-	-	-	-
20	2 (D)	+	+	+	+	+	+ (tr)*	-	-
20	3 (D)	+	+	+	+	+	+ (tr)*	-	-
35	1	+	+	+	+	+	+*	-	-
35	2	+	+	+	+	+	+*	-	-
35	3	+	+	+	+	+	+*	+*	+*
Initial conditions		+	+	+	+	+	-	-	-

penes decreased in the wine samples during the storage time. These modifications, which derived presumably from an interconversion and degradation of the terpenes, were particularly complex at higher temperature, longer storage times and under light conditions (Fig. 2, Table 1).

p-Cymene was only detected at higher temperatures and longer storage times (Fig. 3); p-cymene has already been identified as a degradation product in some other matrices such as tea-tree oil formulations used in therapeutic products (SHABIR, 2005).

Linalool and limonene oxides were also present only at higher temperature and after longer storage times. These compounds (p-cymene, linalool and limonene oxides) could serve as potential shelf-life molecular markers which are strictly related to terpene degradation depending on the time, temperature and light exposure (Fig. 2, 3, Table 1).

After 7 months of storage, the terpene molecules were only present above their odour threshold in the samples stored at 5°C and 14°C (25 μg/L, 10 μg/L, 250 μg/L, 130 μg/L, for linalool, limonene, α-terpineol and geraniol respectively; LÓPEZ *et al.*, 2002). In the samples stored at 20°C, in the light and in the dark, and at 35°C, these compounds were present but at concentrations below the reported odour threshold, Fig. 2.

These data appeared to correspond to the sensory evaluation carried out through a panel test. The results indicated that the varietal aroma composition of the Fiano wine samples was maintained better at 4°C during the shelf-life (data not shown).

Varietal volatiles as process markers during the ageing step in Aglianico wines

Different winemaking conditions can affect the varietal volatile component of a wine. This study investigated possible modifications in an autoch-

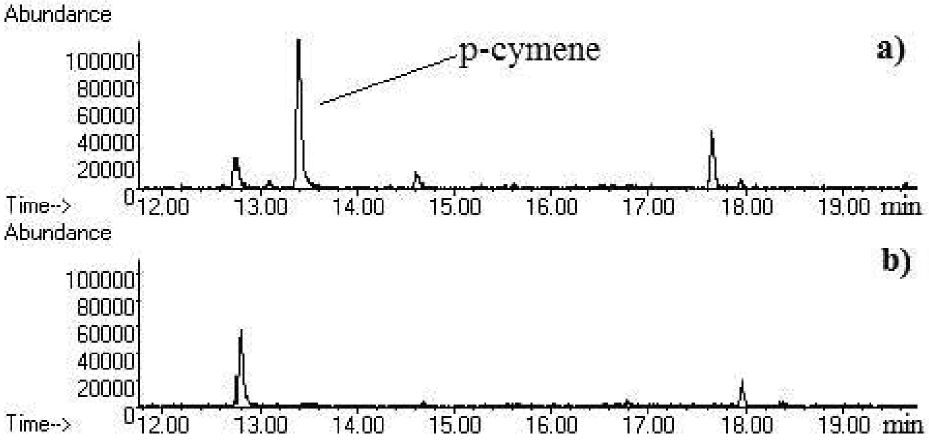


Fig. 3 - TIC chromatogram (ion trace at m/z 119) obtained through HS-SPME-GC/MS analysis on a wine sample stored at 35°C (a) and at 5°C (b) after 3 months of storage.

thonous red wine (Aglianico wine) obtained under different ageing conditions [inox tanks (5000 L) and oak barrels (3000 L and 228 L)].

The quantitative composition of varietal molecules in the Aglianico wine aged (9 months) in inox (5000 L), in a 3000 L oak barrel and in a 228 L oak barrel, is reported in Fig. 4. The wine aged in inox had the highest quali-quantitative level of varietal volatile components in comparison with the wine aged in oak barrels; some of the volatile compounds in the oak barrel wine were present but below the reported odour threshold (e. g. β-myrcene; PLOTTO *et al.*, 2004). The lower concentration of varietal compounds in the oak barrel wines is in agreement with the results obtained in previous studies carried out *in vitro* on model wine, i. e. hydroalcoholic solutions containing terpene molecules in contact with wood of oak barrel (RAMIREZ *et al.*, 2001; 2004). In these previous *in vitro* studies the wood surface/solution volume ratio appeared to be crucial.

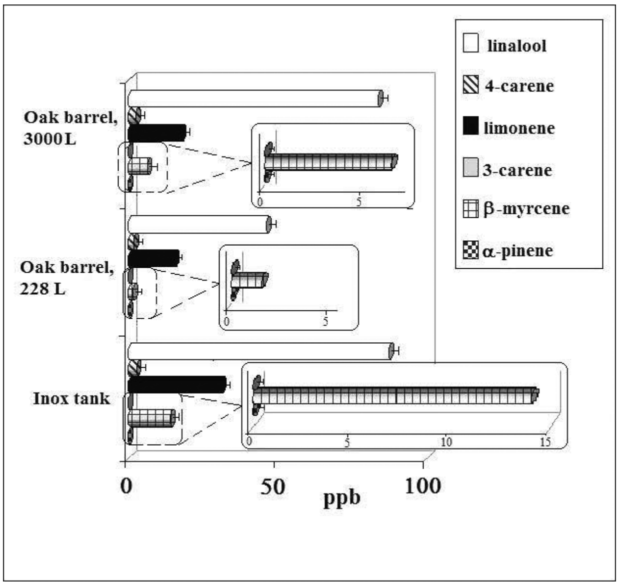


Fig. 4 - Concentrations in ppb of some terpenes detected in Aglianico wine samples aged for 9 months in different modalities: in oak barrels (3000 L and 228 L) and in inox tanks (5000 L).

The quantitative differences observed between the oak barrel wines (Fig. 4) can be attributed to the internal surface/volume ratio which is smaller in the 3000 L oak barrel than in the 228 L oak barrel. The differences could be due to possible gas exchange or the adsorption process of the volatile molecules effected by the wood surface.

The sensory evaluation of the Aglianico wine samples indicated that the varietal aroma was better preserved in the inox tanks compared to the oak barrels (data not shown). The greatest quantities of varietal components were found in the wine aged in the inox tanks.

Varietal volatiles as typicalness and origin tracers in Don Lunardo and Arilla grapes and wines

The terpene composition of Don Lunardo grapes (b) and wines (a) from Cuotto (Isle of Ischia), and Arilla grapes (b) and wines (a) from Cuotto and Spadara (two geographical areas on the Isle of Ischia) is reported (Fig. 5).

As expected, the most notable differences in the terpene composition were related to the varietal origin. In fact, Don Lunardo grapes and wines had a more complex qualitative and quantitative terpene composition in comparison to the Arilla grapes and wines.

The quantitative differences observed for the Arilla grapes and wines from Cuotto and Spadara were related to their different geographical origins, and were presumably due to different salinity stress and water deficit conditions and to different ripening rates. The terpene compounds in Arilla grapes and wines that came from Spadara tended to have higher concentrations compared to those from Cuotto. This result was related to earlier ripening in Spadara in comparison to Cuotto (data not shown). In Arilla wines the dominant terpene was linalool which was not

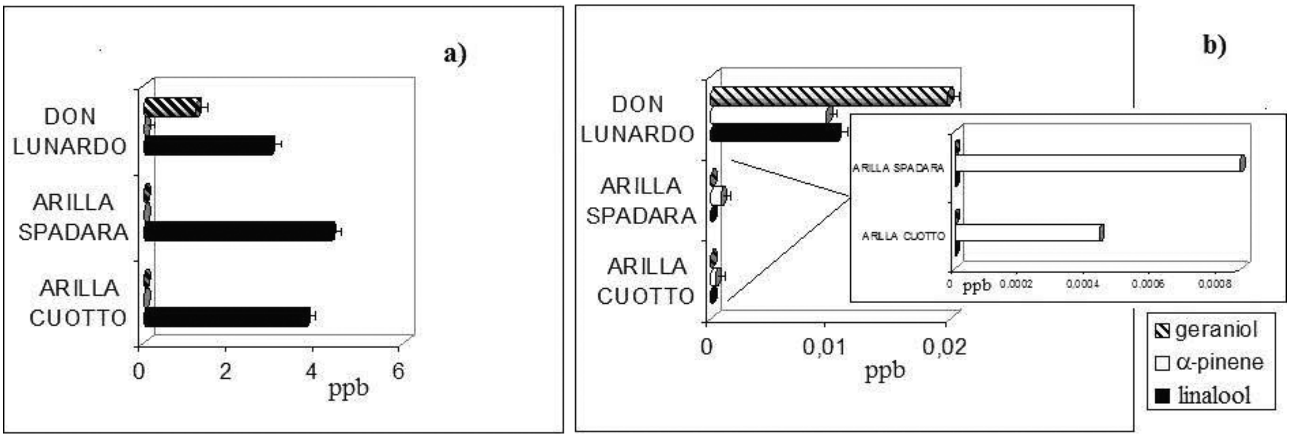


Fig. 5 - Concentrations in ppb of some terpenes detected in some autochtonous grapes and wines from Ischia (Italy): Don Lunardo grapes (b) and wines (a) from Cuotto area, Arilla grapes (b) and wines (a) from Cuotto and Spadara areas.

detected in the grapes but was produced in the wine during the winemaking process through acid and enzymatic hydrolysis from its glycoside precursors. The dominant terpene in Arilla grapes was α-pinene but it was lost during winemaking because it is not present in the glycoside form.

The sensory evaluation of the samples analysed indicated that the aroma expression was different for the Arilla wines produced from the two different geographical areas of the Isle of Ischia even though the same winemaking conditions were used to make them (data not shown).

CONCLUSIONS

Suitable quality and authenticity molecular markers of typical foods and drinks can furnish useful analytical tools which can provide quality control and commercial protection. The metabolomic approach carried out in this study on some varietal volatile compounds (terpenes and norisoprenoids) of autochthonous wines from the Italian region of Campania indicated that the varietal metabolites identified which express the distinctive vine genetic characteristics and grapevine biodiversity, could act as quality molecular tracers for typical oenological products.

Further work and statistical treatments are being conducted in our laboratories in order to verify systematically the effective suitability of these compounds to serve as tracers of quality and typicalness. If suitable, these tracers could be new tools for checking and improving the quality of autochthonous oenological products.

REFERENCES

Câmara J.S., Herbert P., Marques, J.C. and Alves M.A. 2004. Varietal flavour compounds of four grape varieties producing Madeira wines. Analytica Chimica Acta. 513: 203.

Carro N., López E., Günata, Z.Y., Baumes R.L. and Bayonove C.L. 1996. Free and glycosidally bound aroma compounds in grape must of four non-floral *Vitis vinifera* varieties. Analusis. 24: 254.

Christaki T. and Tzia C. 2002. Quality and safety assurance in winemaking. Food Control. 13: 503.

Flamini R. 2005. Some advances in the knowledge of grape, wine and distillates chemistry as achieved by mass spectrometry. J. Mass Spectrom. 40: 705.

Harborne J.B. 1991. "Ecological Chemistry and Biochemistry of Plant Terpenoids". University Press, Oxford.

Lambropoulos, I. and Roussis I. G. 2007. Inhibition of the decrease of volatile esters and terpenes during storage of a white wine and a model wine medium by caffeic acid and gallic acid. Food Res. Intern. 40: 176.

López R., Aznar M., Cacho, J. and Ferreira V. 2002. Determination of minor and trace volatile compounds in wine by solid-phase extraction and gas chromatography with mass spectrometric detection. J. Chromatogr. A. 966: 167.

Lucker J., Bouwmeester H.J., Schwab W., Blaas J., Van Der Plas L. H. W. and Verhoeven, H.A. 2001. Expression of Clarkia S-linalool synthase in transgenic petunia plants results in the accumulation of S-linalyl-β-D-glucopyranoside. Plant J. 27: 315.

Maicas S. and Mateo J.J. 2005. Hydrolysis of terpenyl glycosides in grape juice and other fruit juices: a review. Appl. Microbiol. Biotechnol. 67: 322.

Mamede M. E. O. and Pastore G.M. 2006. Study of methods for the extraction of volatile compounds from fermented grape must. Food Chemistry. 96: 586.

Mateo J.J. and Jiménez M. 2000. Monoterpenes in grape juice and wines. J. Chromatogr. A. 881: 557.

Mazzuca P., Ferranti P., Picariello G., Chianese L. and Addeo F. 2005. Mass spectrometry in the study of anthocyanins and their derivatives: differentiation of *Vitis vinifera* and hybrid grapes by liquid chromatography/electrospray ionization mass spectrometry and tandem mass spectrometry. J. Mass Spectrom. 40: 83.

Nasi A., Ferranti P., Amato S. and Chianese L. 2008^{a}. Identification of free and bound volatile compounds as typicalness and authenticity markers of non-aromatic grapes and wines through a combined use of mass spectrometric techniques. Food Chem. 110: 762.

Nasi A., Mercurio V., Ferranti P. and Chianese L. 2008^{b}. Determination of varietal odorous markers on winemaking and conservation conditions in autochthonous wines of the Campania Region. Ing. Al. 8: 19.

Nasi A., Ferranti P. and Chianese L. 2006. Study of aromatic characterization of Falanghina grape by mass spectrometric techniques. Riv. Vitic. Enol. 4: 23.

Oliveira J.M., Araújo I.M., Pereira, O.M., Maia J.S., Amaral, A.J. and Maia, M.O. 2004. Characterization and differentiation of five "Vinhos Verdes" grape varieties on the basis of monoterpenic compounds. Analytica Chimica Acta. 513: 269.

Pérez-Coello M.S., González-Viñas M.A., García-Romero E., Díaz-Maroto M.C. and Cabezudo M.D. 2003. Influence of storage temperature on the volatile compounds of young white wines. Food Control. 14: 301.

Peťka J., Ferreira V., González-Viñas M.A. and Cacho J. 2006. Sensory and chemical characterization of the aroma of a white wine made with Devín grapes. J. Agric. Food Chem. 54: 909.

Plotto A., Margaría C.A., Goodner K.L., Goodrich R. and Baldwin E.A. 2004. Odour and flavour thresholds for key aroma components in an orange juice matrix: terpenes and aldehydes. Flavour Frag. J. 19: 491.

Ramirez G.R., Lubbers S., Charpentier C., Feuillat M., Voilley A. and Chassagne D. 2001. Aroma compound sorption by oak wood in a model wine. J. Agric. Food Chem. 49: 3893.

Ramirez G.R., Chassagne D., Feuillat M., Voilley A. and Charpentier C. 2004. Effect of wine constituents on aroma compound sorption by oak wood in a model system. Am. J. Enol. Vitic. 55: 2.

Rapp A. 1998. Volatile flavour of wine: correlation between instrumental analysis and sen-sory perception. Nahrung. 42: 351.

Rosillo L., Salinas M.R., Garijo J. and Alonso G.L. 1999. Study of volatiles in grapes by dynamic headspace analysis application to the differentiation of some *Vitis vinifera* varieties. J. Chromatogr. A. 847: 155.

Sánchez-Palomo E., Díaz-Maroto M. C. and Pérez-Coello M.S. 2005. Rapid determination of volatile compounds in grapes by HS-SPME coupled with GC-MS. Talanta. 66: 1152.

Shabir G.A. 2005. Method development and validation for the GC–FID assay of p-cymene in tea tree oil formulation. J. Pharm. Biomed. Anal. 39: 681.

Thiel G., Geisler G., Blechschmidt I. and Danzer K. 2004. Determination of trace elements in wines and classification according to their provenance. Anal. Bioanal. Chem. 378: 1630.

Vilanova M. and Sieiro C. 2006. Determination of free and bound terpene compounds in Albariño wine. J. Food Compos. Anal. 19: 694.

Revised paper received March 31, 2009 Accepted September 9, 2009

EFFECT OF PREFERMENTATIVE COLD MACERATION ON THE AROMA AND PHENOLIC PROFILES OF A MERLOT RED WINE

D. DE SANTIS* and M.T. FRANGIPANE
DISTA, Dipartimento di Scienze e Tecnologie Agroalimentari, Università degli Studi della Tuscia,
Via Camillo De Lellis s.n.c., 01100 Viterbo, Italy
*Corresponding author: desdiana@unitus.it

ABSTRACT

The effect of prefermentative cold maceration (PCM) on Merlot winemaking was studied in order to evaluate its influence on the content of phenolic and volatile compounds. The results showed that PCM increased the concentration of phenolic compounds in Merlot red wine. This is particularly important for the extraction of anthocyanins, mainly malvidine-3-glucoside, which contributes to the colour stability. PCM also increased the concentration of the volatile compounds, particularly the esters, α-terpineol, geraniol and 2-phenylethanol which are fingerprints for this wine. The application of the cold maceration process proved to be successful. In fact the concentration of the esters responsible for the typical flavour of Merlot increased by more than 20% in the PCM-wine with respect to the traditional wine.

- Key words: cold maceration, phenolic compounds, red wine, volatile composition -

INTRODUCTION

The traditional wine-making process for most red wines is sufficient to extract enough phenolic compounds from the grape skins to ensure the best aging conditions. This extraction, however, is influenced by various factors such as cultivar, time, enzymes and seeds as well as the temperature of the process (GÓMEZ-MÍGUEZ *et al.*, 2007). Sometimes, the extraction of a high concentration of anthocyanins is followed by a problem of stabilization (BOULTON, 2001).

The use of low maceration temperatures (5°-15°C) before fermentation, known as "cold-maceration" or "cold-soak", delays the fermentation process, thus improving the extraction of pigments, tannins and aroma compounds from the grape skins in the absence of ethanol.

Prefermentative cold maceration (PCM) is a procedure traditionally used for white winemaking (HEATHERBELL, 1996; FLANZY, 1998). It is, currently gaining ground in red wine-making to achieve better extraction and stabilization of phenolic compounds, particularly anthocyanins.

The use of prefermentative cold maceration allows a better-structured product to be obtained, one that is richer in phenolic and aromatic compounds, thus preserving a strong connection with the area of production (ALVAREZ *et al.*, 2006). Moreover, PCM can potentially promote a selective and significant extraction of phenols; this results in a less aggressive taste and reduces the use of sulphur dioxide as an antimicrobic-antioxidant (AMATI *et al.*,1992; ARFELLI, 1990).

The temperature can be reduced in different ways:

- using a heat exchanger to reach a sufficiently uniform average temperature throughout the must;
- a direct contact of crushed grapes with a cryogen so that, in the contact zones, the temperature is lower than the average temperature of the must (liquid CO_2, solid CO_2, liquid N_2). The use of a cryogen, however, increases the cost of the process.

Prefermentative cold maceration using direct contact of the crushed grapes with a cryogen is an innovative vinification technique since the temperatures in the area of contact between the must and the cryogen are less than zero degrees centigrade. These temperatures cause freezing in part of the grape intracellular water that increases the volume resulting in the breaking of plasmic membranes and dissolution of the cellular compounds into the juice.

The extraction of phenolic compounds depends on various technological and biochemical factors. The cell walls of the peels of a ripe grape are more likely to favour the spillage of the enclosed compounds (FEUILLAT, 2000).

By using PCM, many compounds are extracted that greatly influence colour stabilization (GÒMEZ-MÌGUEZ, 2007). PCM provides immediate gentle cooling of the pressed grapes and maintains them at low temperatures for a short time. This is done by dipping a tubular heat exchanger in the must in order to delay alcoholic fermentation and extend the time of contact between peels and the watery contents (MCMAHON *et al.*, 1999; COUASNON, 1999a; b).

Recent studies (BOULTON, 2001) have shown that the solubilization of phenolic compounds, which are involved in the co-pigmentation phenomenon, is facilitated in the watery phase. Such co-pigmentation has a double effect on the wine: it stabilizes the anthocyanins which, in a free form, would be subjected to oxidation and increases colour intensity because the anthocyanin-cofactor complex has more colour than the anthocyanin in its free form.

GIL-MUÑOZ *et al.* (1999) reported that the anticipated extraction of anthocyanins during the prefermentative phase does not always result in a final enhancement of the wine colour due to a modest extraction of polyphenolic substances at low temperatures.

However, since PCM may increase the final concentration of polyphenols, fast cooling would exert a mechanical action on the skin cells and cause micro lesions of the tissues resulting in the dissolution of the phenolic contents.

Regarding aroma, terpenols are very important aroma compounds in wine. These compounds are synthesized during berry maturation and their concentration in grapes depends on various factors such as cultivar, region and climatic conditions (MATEO and JIMENZ, 2000; CAMARA *et al.* 2004). PCM can also improve the fruity and flowery attributes of wines (CABAROGLU *et al.*, 1997), depending on the grape variety and experimental conditions (temperature and time).

Similar to previous studies (GURBUZ *et al.*, 2006), the major compounds in the Merlot aroma were ethyl octanoate, ethyl decanoate, 3-methyl-1-butanol, ethyl hexanoate, ethyl acetate and 2-phenylethanol. These esters, in particular, hardly contribute to the aroma due to their low perception thresholds (PEINADO *et al.*, 2004). The concentration of these volatile compounds differs according to cultural practices, grape maturity and climatic or biological factors. The wine obtained with cold-maceration treatment had a higher concentration of volatile compounds than the one obtained without the maceration treatment. Esters are very important flavour compounds that give wine a fruity odour and a characteristic fingerprint.

In this study prefermentative cold maceration was used in Merlot winemaking in order to evaluate its influence on the content of phenolic and volatile compounds.

MATERIALS AND METHODS

Wine samples and protocols

Grapes of Merlot variety grown in a vineyard located in Umbria, a region in central Italy, were harvested in 2007 at optimum maturity (density of 1,088 g/mL, total acidity of 7.1 g/L and a pH of 3.45) and in good sanitary conditions.

The grapes were destemmed and crushed, and the must was put into four 250 hL stainless steel tanks and potassium bisulfite was added (70 mg SO_2/L).

Two different lots were prepared: one lot (A) underwent a traditional fermentation process (normal-temperature vinification), while the other lot (B) underwent prefermentative cold maceration (8±2°C).

(A) Three replicates of a control wine were made using traditional maceration (TM) with fermentation on-skin, inoculated with 2% (v/v) of a pure culture of *Saccharomyces cerevisiae* yeast strain (Lallemand Fermented Beverages, Verona, Italy). Fermentation was conducted at a controlled temperature, 25°C.

Fermentation caps were punched down twice a day during the 6-day on-skin maceration period. When alcoholic fermentation was almost finished, the mash was drawn off and the free-run musts were left to mature under the same conditions. After 15 days, alcoholic fermentation was completely finished (≤3 reducing sugars/L).

Samples (250 mL) were collected from each replicate during the alcoholic fermentation until the end of the fermentation process, more or less 5 samples every 2 days. All the samples were analysed in triplicate.

(B) Three replicates of pre-fermentative cold maceration wine (PCM) were brought out through a two-stage process: 4 days of prefermentative cold maceration at 8°C, followed by 9 days of traditional vinification.

To reach a temperature of 8°C, the initial must was first cooled by passing it through a stainless steel refrigerator unit before pumping it into the tanks. The temperature of the mixture (must and skins) was kept at around 8°C by the refrigeration system. The fermentation caps were punched down twice a day. After this period, the temperature was rapidly raised to 21°C, and then vinification followed the same process as the TM wine.

The samples (250 mL) for analysis were collected from each replicate during the alcoholic fermentation in protocol A.

Physico-chemical and spectrophotometric parameters

The physico-chemical parameters, pH, sugars, total acidity, alcohol, tartaric and malic acids, were determined according to the EU Official Methods. The method of DI STEFANO *et al.* (1989) was followed for sample extraction. The total anthocyanin and polyphenol concentrations were determined on the skin extracts (DI STEFANO and GUIDONI, 1989); polyphenols were also determined in the juice.

Liquid chromatographic determination

The anthocyanins in the skins were determined by HPLC, according to the method of RITCHEY and WATERHOUSE (1999). After filtration through Millipore filters (0.22 µm diameter) the samples were injected directly into a Dionex chromatograph (20 µm) loop with a photodiode detector (Dionex S.p.A., Milano, Italy). A Waters Nova Pak C18 column, 300 mm x 3.9 mmØ, (Waters S.p.A., Milano, Italy) kept at 22°C was used as stationary phase, with a mobile phase flow rate of 0.5 mL/min. Solvent A = 50 mM dihydrogen ammonium phosphate adjusted to pH 2.6 with orthophosphoric acid; solvent B = 20% A with 80% acetonitrile; solvent C = 0.2 M orthophosphoric acid adjusted to pH 1.5with NaOH. Two wavelengths were monitored: 280 nm for phenolic acids and 520 nm for anthocyanins. Peak identification was carried out by comparing the results with standards as well as with standards previously characterized on the HPLC system with both relative elution times and spectral matching.

Gas chromatographic conditions

Analysis of volatile compounds was performed by applying the SPME (Solid Phase Micro extraction) technique gas chromatographic method described by ROCHA *et al.* (2001). Five millilitres of wine were transferred to a 25 mL glass vial (Supelco, Sigma-Aldrich Co, St Louis, MO, USA) containing 3 g of NaCl. Each sample was spiked with 200 µL of a solution of 1-penten-3-one as internal standard (5 gL^{-1}in Milli-Q water). A small magnetic stirring bar was also added. The vial was tightly capped with a PTFE (Polytetrafluoroethylene) faced silicone septum and placed in a thermostatic block on a stirrer.

The sample was equilibrated for 15 min at the sampling temperature in a Thermo Haake DL30-V15B water bath (ENCO Spinea, Ve, Italy). The temperature was maintained at 20 ± 2°C; the SPME fibre (PDMS 100 µm) was then inserted into the headspace. During the sampling time (30 min.), the sample was stirred at a constant speed (140 rpm).

The polydimethylsiloxane fibre (PDMS 100 µm) (Sigma-Aldrich) was conditioned in the GC injection port at 250°C for 2 h prior to use. After the selected extraction time, the SPME fibre was transferred into the GC injection port and thermally desorbed at 230°C for 7 min. The splitless injector was mounted on a mod-

Table 1 - Oenological parameters during prefermentative cold maceration (PCM) and traditional maceration (TM).

Samples	Tartaric acid (g/L)		Malic acid (g/L)		pH		Sugar (g/L)		Total acidity (g/L)	
	PCM	TM	PCM	TM	PCM	TM	PCM	TM	PCM	TM
22/09/2007	4.40±0.13	4.49±0.25	1.24±0.08	1.29±0.08	3.43±0.02*	3.36±0.01	226±10*	212±15	7.7±0.10	7.1±0.20
24/09/2007	3.24±0.08	2.94±0.10	1.15±0.08	1.31±0.09	3.43±0.01*	3.39±0.01	198±12*	102±14	7.6±0.11	7.1±0.19
27/09/2007	3.45±0.10	2.67±0.9	1.07±0.10	1.15±0.08	3.30±0.01*	3.40±0.02	75±8*	9±1	7.4±0.18	6.9±0.10
29/09/2007	3.88±0.11	2.54±0.13	1.26±0.06	1.50±0.10	3.29±0.02	3.30±0.03	34±5*	2±0.6	7±0.12	6.8±0.16
01/10/2007	2.56±0.07	2.43±0.11	1.32±0.09	1.13±0.11	3.32±0.01	3.40±0.02	11±2	2±0.4	6.8±0.10	6.8±0.10
04/10/2007	2.60±0.08		1.24±0.07		3.33±0.01		4±0.8		6.5±0.11	
06/10/2007	2.45±0.06		1.03±0.08		3.25±0.02*		3±0.5		6.5±0.10	

* Indicates significant differences (P<0.05) between the means of the two macerations.

el 5300 Mega Series gas chromatograph (Carlo Erba Instruments, Milan, Italy) equipped with a fused silica capillary column impregnated with a polar phase of Carbowax 20M (Alltech Assoc, Inc, Deerfield, IL,USA), 60 m long × 0.25 mm id and 0.25 μm film thickness. Helium was used as carrier gas. The temperature was maintained at 40°C for 7 min, then programmed to reach 230°C at a rate of 3°C min^{-1}, with a final isotherm of 30 min. A high sensitivity flame ionisation detector (FID) at 260°C was used. The signal was recorded and integrated by a Mega Series integrator. Compounds were identified using a Shimadzu 17A GC/MS and a Shimadzu QP 5050A MS and matching against the NIST 107 and NIST 21 libraries, and by matching the GC retention times against standards.

Statistical analysis

All analyses were carried out in triplicate and the analytical results were submitted to statistical analysis. Variance homogeneity was analysed with the Student-Newman-Keuls test. All pair-wise multiple comparison procedures were performed at the p<0.05 significance level (Underwood, 1997). The presence of an asterisk (*) indicates significant differences at 5% (P<0.05).

RESULTS AND DISCUSSION

The results obtained from the statistical analysis of the cold macerated and the control must and wines were compared to determine the influence of PCM on the characteristics of the final products.

The evolution of the average values of the common oenological parameters during PCM and during traditional maceration (TM) is shown in Table 1. The first sample (22/09) was collected just after grape crushing; the values for all the parameters were similar in the two lots. The tartaric acid and sugar values decreased more slowly in PCM due to the delayed onset of alcoholic fermentation.

Table 2 shows the analytical parameters of the PCM and TM Merlot wines; the PCM had a positive effect on the total concentration of the polyphenolic compounds. The anthocyanin concentration was slightly higher in wines obtained with cold maceration. This significant increase could have a positive effect on the colour density as well as on the colour stability. In contrast, no significant differences were found in the pH and alcohol values among the wines produced.

A chromatogram of the anthocyanins in Merlot wine produced with prefermentative cold maceration is reported in Fig. 1. The extraction of an-

Table 2 - Analytical parameters of Merlot wines produced by prefermentative cold maceration and by traditional maceration.

Analytical parameters	Wine produced by prefermentative cold maceration	Wine produced by traditional maceration
alcohol (% vol.)	13.3 ± 0.2	13.6 ± 0.3
total acidity (g/L)	6.2 ± 0.14*	6.5 ± 0.12
pH	3.2 ± 0.02	3.3 ± 0.01
total polyphenols (mg/L gallic acid)	2416 ± 132*	2090 ± 129
total anthocyans (mg/L malvin)	682 ± 22*	632 ± 15

* Indicates significant differences (P<0.05) between the means of the two macerations.

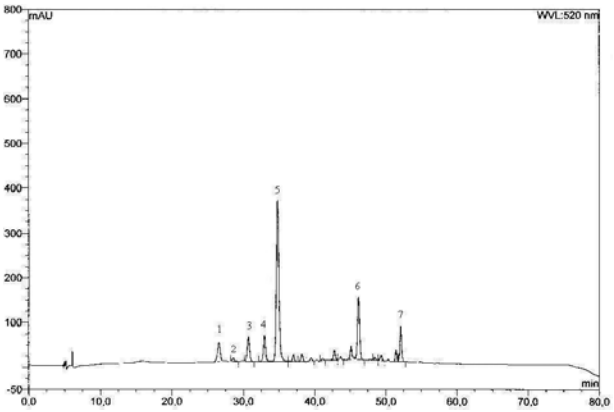


Fig. 1 - Chromatogram of anthocyanins in a Merlot wine produced by prefermentative cold maceration. See Tab. 3 for peak identification.

Table 3 - HPLC mean values of anthocyanins (mg/L) in a Merlot wine produced by prefermentative cold maceration and by traditional maceration.

N°	Compound	Prefermentative cold maceration	Traditional maceration
1	delphinidin-3-G	101 ± 13	101 ± 13
2	cyanidin-3-G	13 ± 2	13 ± 1
3	petunidin-3-G	110 ± 13	111 ± 14
4	peonidin-3-G	111 ± 12	89 ± 5
5	malvidin-3-G	819 ± 28	707 ± 30
6	malvidin acetate	264 ± 25	224 ± 27
7	malvidin coumarate	123 ± 14	102 ± 12

thocyanins was nearly constant throughout the entire cold maceration period; the total anthocyanin content of the must increased just before the onset of alcoholic fermentation.

Compared to the TM, the cold treatment speeded up the anthocyanin extraction (Table 3), particularly that of malvidine-3-glucoside (819±60 vs. 707±47 mg/L), which contributes to colour stability. The same result was previously reported for wines of other grape varieties (ALVAREZ *et al.*, 2006).

Fig. 2 and 3 show the effect of the different treatments on the anthocyanin concentration and total polyphenol content during maceration. During PCM, the anthocyanin concentration increased quickly and the peak was reached between September 22 and 24. A similar increase in anthocyanins was noted during the same period in the TM.

The high levels of anthocyanins in PCM during the first three days were due to the low temperature of the pressed mass (8°C). Although the anthocyanin concentration decreased, by the end of the experiment, it was higher in the PCM samples. The total polyphenol concentration behaved very differently in PCM with respect to TM; it increased quickly and steadily throughout the entire PCM, while it was lower in the TM.

In Merlot wine, esters are responsible for the majority (68-69%) of the total non-ethanol GC peak area, followed by alcohols (nearly 30%) and acids and aldehydes (<1%). In the traditionally macerated Merlot, the total concentration of esters and acids was lower than in the corresponding cold-macerated wine.

With the skin contact treatment, the concentrations of several esters increased significantly (Fig. 4), as also reported by SELLI *et al.* (2006a). The esters responsible for the Merlot wine flavour (ethyl hexanoate, ethyl octanoate, ethyl decanoate, diethyl succinate, and ethyl laurate) increased by more the 20% (Fig. 5) in the PCM-wine with respect to the TM-wine. The amount of hexyl acetate seems to decrease with the skin contact process. Previously reported results (SELLI *et al.*, 2006b) showed a higher alcohol content, that did not seem to be influenced by PCM (data not reported).

Octanoic acid and decanoic acid were the pre-

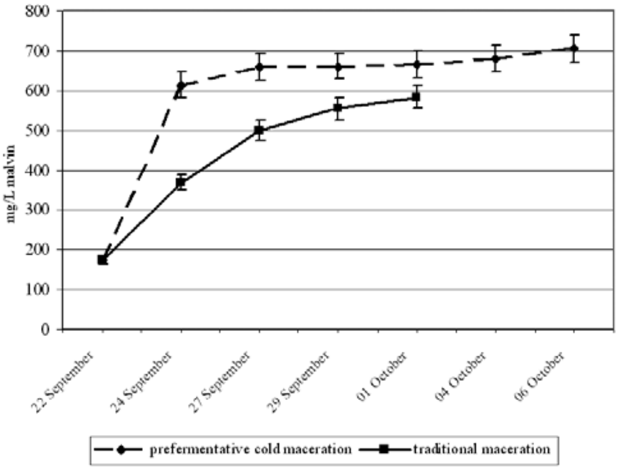


Fig. 2 - Evolution of total anthocyans during traditional and prefermentative cold maceration.

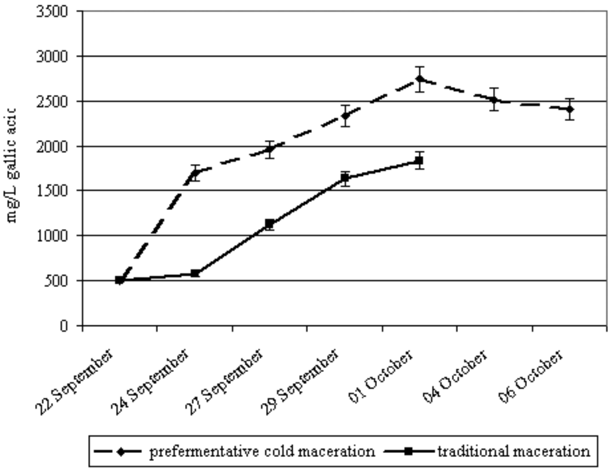


Fig. 3 - Evolution of total polyphenols during traditional and prefermentative cold maceration.

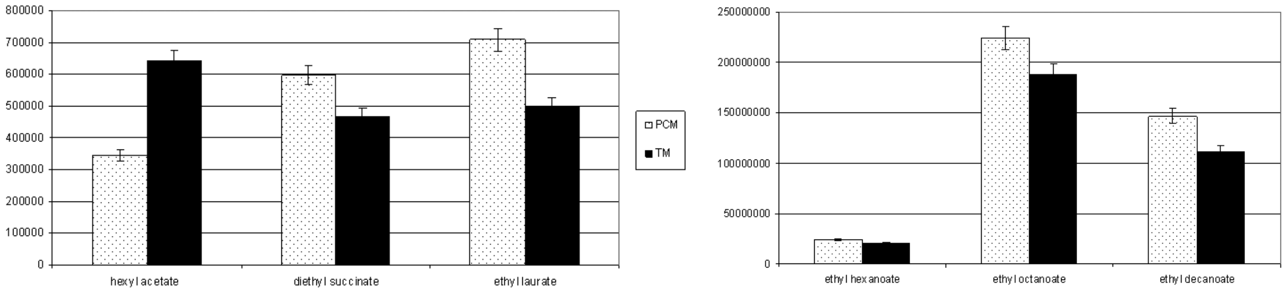


Fig. 4 - Esters in wines produced with traditional maceration (TM) and prefermentative cold maceration (PCM) (chromatographic peak area).

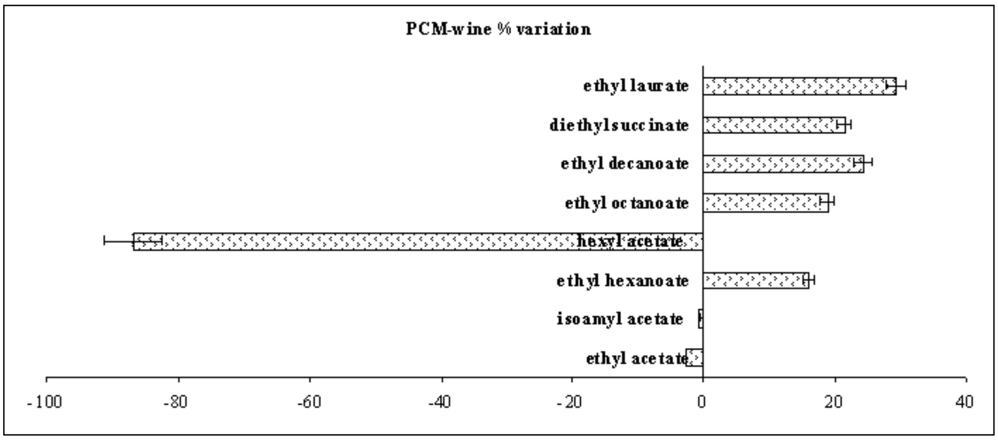


Fig. 5 - Variation in relative percentages of esters detected in a Merlot wine produced with PCM with respect to those produced with traditional maceration.

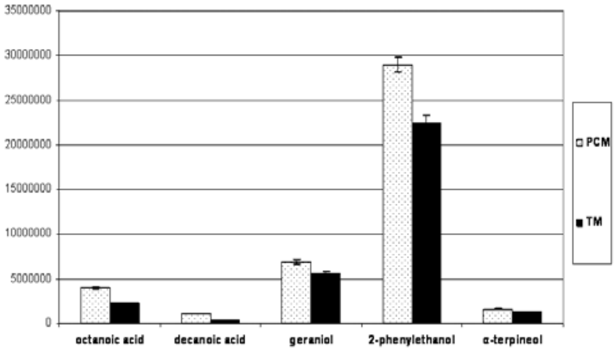


Fig. 6 - Acidic and terpenic content in head-space of PCM and TM wines (chromatographic peak area).

dominant fatty acids in the PCM Merlot wine; their levels increased 43% and 67%, respectively, in PCM-wine (Fig. 6).

Two terpenols were identified in the Merlot wines: α-terpineol and geraniol. Their contribution to the aroma of the Merlot wine cannot be considered relevant because this grape is a neutral variety (MATEO and JIMÉNEZ, 2000). Compared to traditional Merlot winemaking, the concentration of these compounds increased significantly (>20%) due to PCM (Fig. 6). ROSI *et al.* (1994) suggested that the low temperature treatment promoted the growth of yeasts able to synthesize enzymes such as the β-glucosidase, which, in turn, hydrolysed monoterpenic alcohols in the must.

CONCLUSIONS

The results obtained show that the prefermentative cold maceration technique increases phenolic compounds in Merlot red wine. This is important for the extraction of anthocyanins, particularly malvidine-3-glucoside, which contributes to the colour stability. Therefore, a Merlot wine produced with PCM has a higher concentration of phenolic compounds than wines produced with traditional maceration. The advantages of using cold maceration are a reduced level of oxidation, increased concentrations of polyphenols and anthocyanins, as well as a reduction in the oxidation of aroma compounds. On the other hand, the effect of prefermentative cold maceration on the most common oenological parameters was not significant. As regards aroma, Merlot is a neutral variety with a low monoterpene content and a typical flowery odour due to a relevant concentration of esters and phenylethanol; cold maceration could be a good application for Merlot wine because the amount of esters responsible for its flavour (ethyl hexanoate, ethyl octanoate, ethyl decanoate, diethyl succinate, and ethyl laurate) increased by more than 20% in the PCM-wine with respect to the traditional wine.

The results of this study can contribute to the currently poor literature, offer new ways to understand winemaking reactions and contribute to the expression of the varietal and territorial characteristics of the wines.

REFERENCES

Alvarez I., Aleixandre J.L., Garcìa M.J. and Lizama V. 2006. Impact of prefermentative maceration on the phenolic and volatile compounds in Monastrell red wines. Analitica Chimica Acta. 563: 109.

Amati A., Carnicini A., Monti R. and Zironi R. 1992. Vinificazione per macerazione delle vinacce a temperatura controllata: influenza del tempo e della temperatura in un impianto pilota. Vignevini. 9: 29.

Arfelli G. 1990. La tecnica di vinificazione per macerazione a freddo del pigiato. Vignevini. 9: 15.

Boulton R. 2001. The copigmentation of anthocyanins and its role in the colour of red wine: a critical review. Am. J. Enol Vitic 52: 67.

Cabaroglu T., Canbas A., Baumes R.L., Bayonove C.L., Lepoutre J.P. and Gunata Z. 1997. Aroma composition of a white wine of *Vitis vinifera* L. cv. Emir as affected by skin contact. J. Food Sci. 62: 680.

Câmara J.S., Herbert P., Marques J.C. and Alves M.A. 2004. Varietal flavour compounds of four grape varieties producing Madeira wines Anal Chim Acta. 513: 203.

Couasnon M. B. 1999. Une nouvelle technique: la macération préfermentaire à froid. Extraction à la neige carbonique (part 1: résultats oenologiques). Revue des Œnologues. 92: 26.

Couasnon M. B. 1999. Une nouvelle technique: la macération préfermentaire à froid. Extraction à la neige carbonique (part 2: la tecnologie de la neige carbonique). Revue des Oenologues. 92: 28.

Di Stefano R. and Guidoni S. 1989. La determinazione dei polifenoli totali nei mosti e nei vini. Vignevini. 1: 47.

Di Stefano R., Cravero M. C. and Gentilizi N. 1989. Metodi per lo studio dei polifenoli nei vini. L'Enotecnico, 5: 83.

Feuillat M. 2000. Vinification du pinot noir en bourgogne par macération préfermentaire à froid. Revue des Œnologues. 82: 29.

Flanzy C. 1998. "Oenologie - Fondaments Scientifiques et Technologiques". Tec&Doc Paris, London, New York.

Gil-Muñoz R., Gòmez-Plaza E., Màrtinez, A. and Gòmez Roca J.,M. 1999. Evolution of phenolic compounds during wine fermentation and post-fermentation: influence of grape temperature. J. Food Compos. Anal. 12: 259.

Gòmez-Mìguez M., Lourdes Gonzàlez-Miret M.L. and Heredia F.J. 2007. Evolution of colour and anthocyanin composition of Syrah wines elaborated with pre-fermentative cold maceration. J. Food Eng. 79: 271.

Gurbuz O., Rouseff J.M. and Rouseff R.L. 2006. Comparison of aroma volatiles in commercial Merlot and Cabernet Sauvignon Wines using Gas Chromatography-Olfactometry and Gas Chromatography-Mass Spectrometry. J. Agric. Food Chem. 54: 3990.

Heatherbell D. 1996. Effect of prefermentative cold maceration on the composition, colour and flavour of Pinot Noir wine. In 4th Int. Symp.: Cool Climate Viticulture and Enology. Rochester, New York, July 16-20.

Ministero dell'Agricoltura e Foreste (MAF) 1986. Metodi ufficiali di analisi per i mosti, i vini, gli agli di vini (aceti) e i sottoprodotti della vinificazione. Suppl. ordin. n. 58, Roma.

Mateo J.J. and Jiménez M. 2000. Monoterpenes in grapejuice and wines. J. Chromatogr. A. 881: 557.

Mcmahon H.M., Zoecklein B.W. and Jasinski W. 1999. The effect of prefermentative maceration temperature and percent alcohol (v/v) at press on the concentration of Cabernet sauvignon grape glycosides and glycoside fractions. Am. J. Enol. Vitic 50: 385.

Peinado R.A., Moreno J., Bueno J.E., Moreno J.A. and Maurcio J.C., 2004. Comparative study of aromatic compounds in two young white wines subjected to pre-fermentative cryomaceration. Food Chem. 84: 585.

Rocha S., Ramalheira V., Barros A., Delgadillo I. and Coimbra M. A. 2001. Headspace solid phase microextraction (SPME) analysis of flavor compounds in wines. Effect of the matrix volatile composition in the relative response factors in a wine model. J. Agric Food Chem. 49: 5142.

Rosi I., Vinella M. and Domizio P. 1994. Characterization of B-glucosidase activity in yeasts of oenological origin. J. Appl. Bacteriol. 77: 519.

Selli S., Canbas A., Cabaroglu T., Erten H. and Gunata Z. 2006a. Aroma components of cv. Muscat of Bornova wines and influence of skin contact treatment. Food Chem. 94: 319.

Selli S., Canbas A., Cabaroglu T., Erten H., Lepoutre J.P. and Gunata Z. 2006b. Effect of skin contact on the free and bound aroma compounds of the white wine of *Vitis vinifera* L. cv Narince. Food Control. 17: 75.

Underwood A. J. 1997. "Experimental in Ecology. Their logical Design and Interpretation Using Analyses of Variance". P. 504. Cambridge Univ. Press. UK.

Revised paper received August 4, 2009 Accepted October 15, 2009

PECORINO OF APPENNINO REGGIANO CHEESE: EVALUATION OF RIPENING TIME USING SELECTED PHYSICAL PROPERTIES

M. RINALDI, E. CHIAVARO* and R. MASSINI
Dipartimento di Ingegneria Industriale, Università degli Studi di Parma,
Viale G.P. Usberti 181/A, 43124 Parma, Italy
*Corresponding author: Tel. +39 0521 905888, Fax +39 0521 905705,
e-mail: emma.chiavaro@unipr.it

ABSTRACT

Physico-chemical properties (texture, colour and image analysis) of Pecorino of Appennino Reggiano cheese were evaluated on six cheese samples at different ripening times (0, 80, 90, 150, 180 and 240 days). Hardness values, obtained by means of texture profile analysis and fracture test, as well as Young's modulus values, significantly increased during ripening. Among other textural parameters, cohesiveness decreased significantly from 0 to 80 days and then remained unchanged for the rest of the ripening time, whereas adhesiveness and springiness did not exhibit a linear trend. L*, b* and C* values decreased significantly with ripening, whereas the percentage area occupied by holes remained unaltered. A statistical model was developed to predict ripening time by means of a step-wise multiple linear regression analysis based on five textural properties (hardness, cohesiveness, chewiness, hardness at fracture and Young' modulus) and three colour parameters (L*, b* and C) ($R^2 = 0.984$). The model was applied to a validation set of six samples from different milk lots and successfully predicted ripening time with good reproducibility and accuracy ($R^2 = 0.971$).

- Key words: colour, pecorino cheese, ripening time, statistical model, texture -

INTRODUCTION

Cheese is very diverse category of food product and its final quality differs depending on various parameters: milk origin, cheese-making process, cheese size, ripening time and conditions. The ripening of cheese is a very complex, often slow, biochemical process that involves three primary reactions: glycolysis, lipolysis and proteolysis. Thus, such physical properties as texture, colour and gas hole development may be used to differentiate among cheeses and describe their changes during ripening (CASIRAGHI *et al.*, 1989; CACCAMO *et al.*, 2004; DUFOSSÈ *et al.*, 2005), as they are related to milk type and/or cheese-making procedures. Evaluation of the parameters could be of great interest for the protected designation of origin (PDO) or geographical indication (PGI) for the European Union (EU) in order to avoid misuse, imitation or evocation of the registered name (ROSEIRO *et al.*, 2003), as consumers take these properties into primary consideration when making purchasing decisions. These important physical parameters could be used to develop a statistical model for ripening prediction; such a tool could be used to avoid misleading consumers, especially with regard to cheese that is artisanally produced using raw milk where the variability in quality can be high (POVEDA *et al.* 2003; PINHO *et al.* 2004; ALVARENGA *et al.*, 2008).

Pecorino of Appennino Reggiano is an Italian semi-hard cheese that is traditionally produced from whole sheep milk with the addition of selected starters or natural microbiological cultures (obtained from sheep milk or whey from the restricted area of production). In 2000, the production process of Pecorino of Appennino Reggiano was established and is limited to a restricted area of production (Comunità Montana dell'Appennino Reggiano) in the region of Emilia Romagna (northeastern Italy), by a Code of Rules (OFFICIAL BULLETIN OF EMILIA ROMAGNA REGION, 2005) in order to obtain the PDO. Since 2000, Pecorino of Appennino Reggiano has been included in the list of the Traditional Food Products (TFP) by the Italian Ministry of Agriculture. Moreover, the International Slow Food Association had established an ad-hoc presidium in the production area of this cheese (Slow Food, http://www.presidislowfood.it/ita) to preserve its characteristics and to protect the product against imitations.

According to product specifications, this cheese is marketed as two commercial categories. Soft cheese can be marketed from 30 days after production up to 60 days of ripening and is produced from pasteurised milk. Semi-hard cheese can be marketed from 90 days of ripening up to 12 months and is generally manufactured from raw milk, although pasteurised milk can be used. These two categories obviously exhibit different sensory properties and quality parameters depending on the degree of ripening (CASTAGNETTI *et al.*, 2004; PIGNEDOLI, 2005). Pecorino of Appennino Reggiano must also be cylindrically shaped (the height always less than the radius) and should weigh 1-2.5 kg; the colour is from white to yellow, with green reflexes that appear with ripening due to mould growth, and the possible presence of gas holes that are irregularly distributed. It can be manufactured in different sizes (diameter x height) and weight, according to degree of ripening: 150 mm x 60 mm and about 1-1.2 kg for the soft cheese; 180 mm x 100 mm and about 2-2.5 kg for the semi-hard cheese.

The aim of this preliminary work was to evaluate changes in the selected physical properties (texture, colour and gas holes) of Pecorino of Appennino Reggiano cheese during ripening of a semi-hard cheese made from raw milk that is the main commercial category. In addition, an estimation model was developed on the basis of these parameters by means of step-wise multiple linear regression analysis (MLR) in order to predict ripening time of Pecorino of Appennino Reggiano cheese for marketing purposes.

MATERIALS AND METHODS

Sampling and cheese-making

Ten Pecorino of Appennino Reggiano samples manufactured under conditions specified in the production rules were obtained from a local dairy (Succiso - Reggio Emilia); they were produced the same day using the same lot of raw milk in August 2008. Two other samples produced from different lots of milk in 2009 were purchased from the same manufacturer.

All the cheeses were manufactured according to the code rules. The cheese was manufactured by adding 11 mL L^{-1} of natural starter and 28 mg L^{-1} of commercial bovine rennet to whole raw sheep milk at 39°C. The natural starter was obtained from pasteurised sheep milk of the previous day, which was incubated at 45°C until the acidity value reached 10°SH 50mL^{-1}. The coagulation was completed within 20-25 min. Then, the curd was cut into maize-grain-size pieces with a standard wire cage equipped with a handle, subjected to several agitation steps and then was partially cooked at 44-46°C for 10-15 min. The curd was placed in moulds, pressed and dried for 5 h at 30°C. Dry salting was then carried out for 48 h at 8°C. All the samples were ripened under the same temperature and humidity conditions (12°C and RH 70%).

Six cheese samples, ripened for 0, 80, 90, 150, 180 and 240 days, were selected as standard cheeses to obtain the estimation model. The other six were used as a validation set for the prediction model: four, ripened for 80, 150, 180 and 240 days, were produced on the same day as the calibration set, and the other two of 80 and 150 days of ripening, were obtained from other milk lots in a different period.

Proximate composition at time 0 of ripening (moisture 41.3%, protein 22.4%, fat 30.3%, salt 1.7% and ash 3.2%) was confirmed using Standard Association of Official Analytical Chemists (AOAC) methods (1995).

Texture analysis

Texture evaluation was performed using a TA.XT2 Texture Analyzer equipped with a 25 kg load cell (Stable Micro Systems, Goldalming, U.K.) and Texture Expert for Windows software (version 1.22) for data analysis. The load cell calibration was performed daily according to the TA.XT2 manual (Stable Micro Systems). A TPA (Texture Profile Analysis) test was performed. The compression test was performed with a 35 mm diameter aluminium cylindrical probe and cheese samples were compressed to approximately 67% of the original height with a constant speed of 2 mms^{-1} and a 5 s delay between the first and second bite. Five slices were cut from each cheese sample and three cylindrically-shaped samples were obtained from each slice (approximately 25 mm diameter and 15 mm thick). The TPA parameters considered were: hardness (H, peak force of the first compression cycle in N), cohesiveness (CO, ratio between the positive force area during the second compression over that during the first compression, dimensionless), springiness (SP, ratio between the time duration of force input during the second compression over that during the first compression, dimensionless), adhesiveness (AD, negative force area for the first bite representing the work necessary to pull the plunger away from the food sample, N x mm) and chewiness (CH, hardness multiplied by cohesiveness multiplied by springiness in N) (BOURNE, 1978).

Fracture tests were obtained using a Guillotine blade (3 mm thick) compressing cheese slices (15 mm thickness) until the breaking point at a constant speed of 2 mms^{-1}. The maximum force of the force/distance peak was taken as a measure of hardness of the sample (H_f in N). Measurements were carried out on 5 slices from each sample.

The elastic modulus or Young's modulus (Y) was calculated according to DEL NOBILE *et al.* (2007), as the slope of the stress-strain curve, converting the instantaneous values of specimen height H(t) and compression charge applied F(t) into engineering (σ_E), true (σ_T) stress vs. engineering (ε_E) and Hencky (ε_T) strain (MANCINI *et al.*, 1999) according to the following equations:

$$\sigma_E = \frac{F(t)}{S_0}, \qquad \varepsilon_E = \frac{H_0 - H(t)}{H_0},$$

$$\sigma_T = \sigma_E \cdot (1-\varepsilon_E), \qquad \varepsilon_T = -\ln(1-\varepsilon_E). \tag{1}$$

The elastic modulus was then calculated by fitting the following mathematical model to the whole true tension–true strain curves (DEL NOBILE *et al.,* 2007) according to the following equations:

$$\sigma_T(\varepsilon_T) = E_C \cdot \varepsilon_T \cdot \exp(\varepsilon_T \cdot K) \tag{2}$$

where E_C is the elastic modulus (i.e. the tangent to the stress strain curve at the origin); K is a constant and is considered to be a fitting parameter. Measurements were carried out on 5 slices of each cheese, 3 samples (30 mm x 30 mm x 30 mm) were extracted from each slice. All samples were equilibrated at room temperature (25°C) for at least 30 min before each test.

Colour analysis

Colour determinations were carried out on three central slices (30 mm thickness) of each cheese using a Minolta Colorimeter (CM 2600d, Minolta Co., Osaka Japan) on a CIE L* a* b* system equipped with a standard illuminant D65 on 10 pre-selected locations for each slice. L* [lightness: black = 0, white = 100], a* [redness: a* > 0, green < 0], b* [yellowness: b* > 0, blue <0], C [Chroma or colour saturation = $(a^{*2} + b^{*2})^{1/2}$: 0 completely unsaturated (neutral grey, black or white), 100 maximum saturation] were quantified on each sample by using the 2nd position of the standard observer. The instrument was calibrated before each analysis with white and black standard tiles. A total of 30 determinations were performed at each ripening time.

Image analysis

For each cheese sample, digital pictures of both faces of 2 central slices (30 mm thickness) were taken using a flatbed scanner (Model Scanjet 8200, HP, Cupertino, CA, USA), with a resolution of 600 dots per inch (dpi) and converted from true colour to 256 level grey scale. The images were calibrated, standardized and optimised applying appropriate filters to measure pore number using the Image-Pro Plus 4.5 (Media Cybernetics Inc., Bethesda, MD, USA) software with a procedure previously reported (INNOCENTE and CORRADINI, 1998). Cheese hole distribution was characterized by means of percent ratio between the area occupied by holes and total slice area, as previously reported (CACCAMO *et al.*, 2004). A total of 4 determinations were performed at each ripening time.

Statistical analysis

Means and standard deviations (SD) were calculated with STATISTICA (Release 5.5, StatSoft, Tulsa, OK, USA) statistical software. STATISTICA was used to perform one-way-analysis of variance (ANOVA) and Least Significant Difference test (LSD) at a 95 % confidence level ($p \leq 0.05$) to identify differences among samples and regression analysis. Step-wise multiple linear regression analysis (MLR), which combines the backward elimination and forward selection regression methods (ALVARENGA *et al.*, 2008) was used to estimate the length of the ripening period of Pecorino of Appennino Reggiano. The level of significance of an independent variable for entry and stay in the calibration model was set at 0.05 during the execution of step-wise variable selection.

RESULTS AND DISCUSSION

TPA parameters and hardness at fracture were measured during ripening time and the results are summarized in Tab. 1 for standard cheeses. Ripening significantly increased the TPA hardness of cheese and also significantly decreased the cohesiveness from 0 to 80 days. On the contrary, adhesiveness and springiness remained substantially unaltered during ripening. Hardness at fracture significantly increased from 0 to 240 days of ripening.

Biochemical changes of cheese during ripening such as proteolysis and/or water availability to bond components were found to be related to cheese texture, although the mechanism of this relation was not completely understood (LUCEY *et al.*, 2003). In particular, the formation of ionic groups generated by peptide bond cleavage that could compete with protein for bonding available water were related to the increase of both cheese hardening and resistance to deformation during ripening (LAWRENCE *et al.*, 1986).

Changes of Young's modulus (Y) during ripening are reported in Fig. 1. Values of Y increased significantly with ripening until 150 days and continued to increase until the end of ripening time. An increase of Y with ripening of cheese was previously reported in the literature (DEL NOBILE *et al.*, 2007: GONZÀLEZ VIÑAS *et al.*, 2007); it was related to the decrease in moisture content and the strengthening of the protein matrix due to maturation (GONZÀLEZ VIÑAS *et al.*, 2007).

Table 1 - TPA and fracture test results of standard cheeses at each ripening time.

Days	H (N)	CO	SP	AD	CH (N)	H_f (N)
0	57.69^{d} (10.17)	0.37^{a} (0.06)	0.58^{a} (0.06)	1.43^{a} (0.31)	12.36^{b} (3.67)	10.67^{d} (1.25)
80	158.58^{c} (27.01)	0.19^{b} (0.02)	0.39^{b} (0.03)	1.72^{a} (0.29)	11.76^{b} (2.22)	21.92^{cd} (7.78)
90	239.86^{b} (47.10)	0.21^{b} (0.02)	0.46^{ab} (0.06)	1.86^{a} (0.85)	23.17^{a} (5.07)	33.26^{bc} (6.39)
150	242.72^{b} (48.47)	0.20^{b} (0.03)	0.46^{ab} (0.10)	0.95^{a} (1.40)	22.27^{a} (7.83)	50.83^{a} (9.38)
180	234.41^{b} (29.44)	0.19^{b} (0.02)	0.50^{ab} (0.19)	1.08^{a} (0.75)	22.29^{a} (3.81)	39.44^{ab} (16.86)
240	303.63^{a} (35.35)	0.17^{b} (0.02)	0.46^{ab} (0.06)	0.96^{a} (0.57)	23.73^{a} (3.92)	40.25^{ab} (12.67)

Values with same letters $^{a, b, c, d}$ within each column are not significantly different (p≤0.05), standard deviation given in parentheses (n= 1, samples size = 15 for TPA parameters; n= 1, sample size = 5 for fracture test). ^{a}Abbreviations: H, hardness; CO, cohesiveness; SP, springiness; AD, adhesiveness; CH, chewiness; H_f, hardness at fracture.

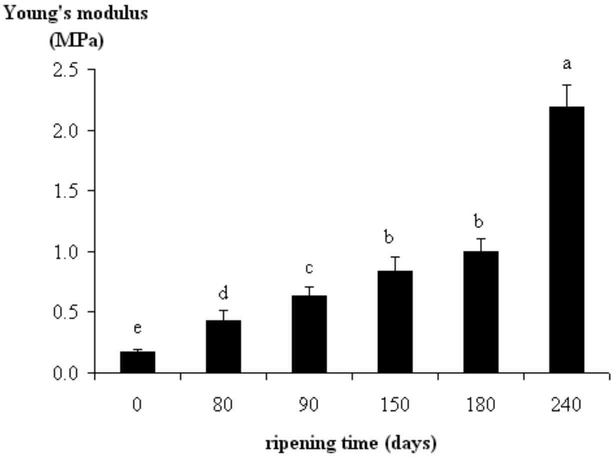


Fig. 1. Changes in Young's modulus of standard cheeses during ripening. Error bars represent +/- 1 standard deviation (n= 1, sample size = 15). Histogram bars with the same letter are not significantly different (p ≤ 0.05).

Table 2 - Colour parameter results of standard cheeses at each ripening time.

Days	L*	a*	b*	C
0	87.8^{a} (0.6)	-2.5^{d} (0.1)	16.4^{a} (0.6)	16.5^{a} (0.6)
80	80.4^{b} (1.5)	-2.4^{c} (0.1)	16.2^{a} (0.7)	16.4^{a} (0.7)
90	76.5^{c} (1.2)	-1.9^{b} (0.2)	12.3^{b} (0.7)	12.5^{b} (0.8)
150	72.5^{d} (1.4)	-2.0^{bc} (0.3)	12.5^{b} (1.0)	12.6^{b} (1.1)
180	68.0^{e} (1.6)	-2.2^{cd} (0.3)	11.8^{b} (0.9)	12.2^{b} (0.9)
240	57.7^{f} (2.0)	-1.4^{a} (0.2)	7.5^{c} (1.1)	7.6^{c} (1.1)

Values with same letters $^{a, b, c, d, e, f}$ within each column are not significantly different (p≤0.05), standard deviation given in parentheses. (n= 1, sample size = 30).

Colorimetric indices for Appenino Reggiano cheese during ripening are summarized in Tab. 2. Cheese samples became less bright (L*) and yellow (b*) as ripening time increased. A loss of colour saturation (C) was also observed during ripening.

Ripening was previously found to induce changes in the colour of cheeses produced with raw cow (ROHM and JAROS, 1996) and goat milk (BUFFA *et al.*, 2001). In particular, the L* values were found to decrease (ROHM and JAROS, 1996; BUFFA *et al.*, 2001) whereas the b* values generally increased during cheese-ripening (ROHM and JAROS, 1996) but a slight decrease after a long ripening time (more than 60 days) was also reported for ewe cheese (PINHO *et al.* 2004).

The description of cheese appearance at 0 and 240 days is reported in Fig. 2a and 2b, respectively. The Pecorino samples examined in this study exhibited an appearance typical of this cheese, i.e. a compact structure with few

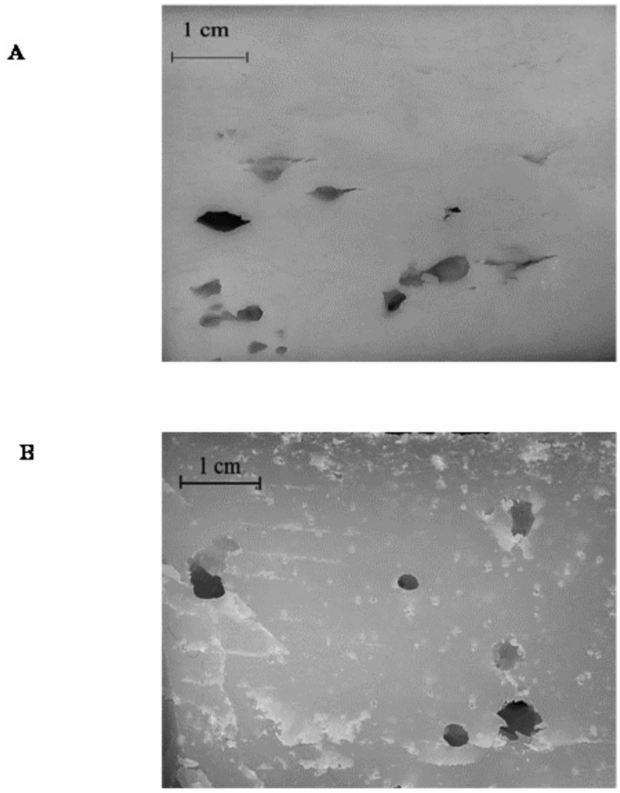


Fig. 2. Image of standard cheeses obtained at 0 (A) and 240 (B) days of ripening.

holes. A low percentage of the area was occupied by holes at 0 time of ripening (approximately 0.2%) and this value remained substantially unchanged during ripening. Thus, cheese appearance may not be representative of change during ripening.

A step-wise multiple linear regression (MLR) analysis using textural and colour data was performed to develop a statistical model that would allow the ripening time of Pecorino of Appennino Reggiano to be estimated. A summary of the step-wise analysis is reported in Tab. 3.

The MLR analysis showed that five variables from the texture determinations (hardness, cohesiveness and chewiness from the TPA test, Young's modulus and hardness at fracture) and three colour parameters (L^*, b^* and C) were useful for predicting ripening time of Pecorino cheese, as based on the high R^2 value and the estimation error of calibration. The following equation was used:

$$RT = 631.4 + 0.001\ H - 121.3\ CO + 0.75\ CH - 19.4\ Y + 0.5\ Hf - 5.6\ L^* - 0.2\ b^* - 5.9\ C \qquad (3)$$

with $R^2 = 0.984$ and an estimation error of 8.2 days.

The estimation error of ripening time was similar to that previously reported in the literature for a similar statistical model developed by means of textural and colour variables on other types of cheeses (POVEDA *et al.*, 2003; PINHO *et al.*, 2004; ALVARENGA *et al.*, 2008).

This model was applied to a validation set of six samples, four produced on the same day as the standard cheeses and ripened for 80, 150, 180 and 240 days, the other two samples were obtained from another lot of milk (80 and 150 days ripened); the effect that the variability in the composition of the milk would have on the predictive model was considered.

The texture and colour parameters of the validation set of cheeses were similar to those of standard samples (data not shown). Small differences between the predicted and the real ripening times (about 10 days) were obtained. The correlation coefficient obtained between the predicted and the real ripening times was slightly lower than that of the calibration set of samples ($R^2 = 0.971$).

These results show that the textural and colour parameters considered in this study can give practical and useful information to describe changes in Pecorino of Appennino Reggiano semi-hard cheese during ripening. This information can be used to regulate the productive process in order to obtain the PDO recognition of the cheese.

The predictive model based on these selected physical parameters can differentiate samples of Pecorino of Appennino Reggiano according to rip-

Table 3 - Summary of MLR with textural and colourimetric data as independent variables.

Step	Regression equation	R^2
1	$RT = -30.2 + 0.73\ H$	0.715
2	$RT = 69.1 + 0.52\ H - 257.3\ CO$	0.737
3	$RT = 93.1 + 0.35\ H - 362.0\ CO + 1.8\ CH$	0.748
4	$RT = 74.1 + 0.08\ H - 276.8\ CO + 1.2\ CH + 80.3\ Y$	0.892
5	$RT = 32.7 + 0.09\ H - 156.2\ CO + 0.49\ CH + 80.0\ Y + 1.1\ H_f$	0.914
6	$RT = 576.9 - 0.10\ H - 158.7\ CO + 0.83\ CH + 7.6\ Y + 0.8\ H_f - 6.0\ L^*$	0.975
7	$RT = 619.7 - 0.04\ H - 128.0\ CO + 0.75\ CH - 11.9\ Y + 0.6\ H_f - 5.8\ L^* - 4.7\ b^*$	0.981
8	$RT = 631.4 + 0.001\ H - 121.3\ CO + 0.75\ CH - 19.4\ Y + 0.5\ H_f - 5.6\ L^* - 0.2\ b^* - 5.9\ C$	0.984

[a]Abbreviations: RT, ripening time; H, hardness; CO, cohesiveness; CH, chewiness; Y, Young's modulus; H_f, hardness at fracture; L^*, lightness; b^*, yellowness; C^*, chroma; R^2, adjusted coefficient of determination.

ening time and the level of quality. The application of these predictive models is of great interest for Pecorino of Appennino Reggiano, as well as for other similar cheeses made with raw milk, where the addition of natural autochthonous microflora starters obtained from milk, the environment and artisanal cheese-making procedures and tools can lead to variable quality.

These initial findings must be confirmed by analysing a larger set of semi-hard samples that takes into consideration the effect of different producers, manufacturing conditions and time of production on reproducibility of the model. The model must also be improved by analysing soft Pecorino of Appennino Reggiano cheeses in order to differentiate between samples of the two commercial categories according to ripening time.

REFERENCES

Alvarenga N., Silva P., Garcia J.R. and Sousa I. 2008. Estimation of Serpa cheese ripening time using multiple linear (MLR) considering rheological, physical and chemical data. J. Dairy Res. 75: 233.

AOAC. 1995. "Official Methods of Analysis" 16th ed., Association of Official Analytical Chemistry, Washington, DC.

Bourne M.C. 1978. Texture profile analysis. Food Technol. 32: 62.

Buffa M.N., Trujillo A.J., Pavia M and Guamis B. 2001. Changes in textural, microstructural, and colour characteristics during ripening of cheese made from raw, pasteurized or high-pressure-treated goats'milk. Int. Dairy J. 11: 927.

Caccamo M., Melilli C., Barbano D.M., Portelli G., Marino G. and Licita G. 2004. Measurement of gas holes and mechanical openness in cheese by image analysis. J. Dairy Sci. 87: 739.

Casiraghi E., Lucidano M. and Pompei C. 1989. Correlation among instrumental texture, sensory texture and chemical composition of five Italian cheeses. Ital. J. Food Sci. 1: 53.

Castagnetti G.B., Arlotti G., Bertolini L. and Pignedoli S. 2004. Caratterizzazione e valorizzazione del formaggio Pecorino dell'Appenino Reggiano. Proceedings of XVI National Congress of Italian Society of pathology and breeding of ovine and caprine cattle (SIPAOC), Siena, Italy, p. 254. September 29th- 2nd October.

Del Nobile M.A., Chillo S., Falcone P.M., Laverse J., Pati S. and Baiano A. 2007. Textural changes of Canestrello Pugliese Cheese measured during storage. J. Food Eng. 83: 621.

Dufossé L., Galaup P., Carlet E., Flamin C. and Valla A. 2005. Spectrocolorimetry in the CIE L*a*b* color space as useful tool for monitoring the ripening process and the quality of PDO red-smear soft cheeses. Food Res. Int. 38: 919.

González Viñas M.A., Ballesteros C., Martín-Alvarez P.J. and Cabezas L. 2007. Relationship between sensory and instrumental measurements of texture for artisanal and industrial Manchego cheeses. J. Sensory Studies 22: 462.

Innocente N. and Corradini C. 1998. Impiego di una tecnica di analisi dell'immagine nel controllo di qualità del formaggio Montasio. Sci. Tecn. Lattiero-Casearia 49: 8294.

Lawrence R.C., Creamer L.K. and Gilles J. 1986. Symposium: cheese ripening technology. Texture development during cheese ripening. J. Dairy Sci. 70: 1748.

Lucey J.A., Johnson M.E. and Horne D.S. 2003. Invited review: perspectives on the basis of the rheology and texture properties of cheese. J. Dairy Sci. 86: 2725.

Mancini M., Moresi M. and Rancini R. 1999. Mechanical properties of alginate gels: empirical characterisation. J. Food Eng. 39: 369.

Official Bulletin of Emilia Romagna Region of 30 March 2005. Code of Rules of Pecorino of Appenino Reggiano. Off. Bull. Emilia Romagna Region 59: 194.

Pignedoli S. 2005. Caratterizzazione del formaggio "Pecorino dell'Appennino Reggiano". Doctoral Fellowship Thesis in Food Biotechnology, University of Bologna, Bologna, Italy.

Pinho O., Mendes E., Alves M.M. and Ferreira I.M.P.L.V.O. 2004. Chemical, physical and sensorial characteristics of "Terrincho" ewe cheese: change during ripening and intravarietal comparison. J. Dairy Sci. 87: 249.

Pollard A., Sherkat F., Seuret M.G. and Halmos A.L. 2003. Textural changes of natural cheddar cheese during the maturation process. J. Food Sci. 68: 2011.

Poveda J.M., García A., Martín-Alvarez P.J. and Cabezas L. 2004. Application of partial least squares (PLS) regression to predict the ripening time of Manchego cheese. Food Chem. 84: 29.

Rohm H. and Jaros D. 1997. Colour of hard cheese. 2. Factor of influence and relation to compositional parameters. Zeit. Leben. Unter. Forsch. 204: 259.

Roseiro L.B., Wilbey R.A. and Barbosa M. 2003. Serpa cheese: Technological, biochemical and microbiological characterisation of a PDO ewe's milk cheese coagulated with *Cynara cardunculus* L. Lait 83: 469.

Slow Food. http://www.presidislowfood.it/ita

Revised paper received June 25, 2009 Accepted September 10, 2009

PAPER

PHYSICOCHEMICAL, MICROBIOLOGICAL AND SENSORY CHARACTERISTICS OF BALADI, A TRADITIONAL JORDANIAN CHEESE MADE FROM RAW SHEEP MILK

J. HADDADIN* and R. AL-TAHIRI
Department of Nutrition and Food Technology, Faculty of Agriculture, Mutah University,
P.O. Box 7, Karak, Jordan
*Corresponding author: jean@mutah.edu.jo

ABSTRACT

Baladi cheese samples were produced from raw sheep milk made according to traditional and modified methods. The main changes in the modified method were: pasteurization (73°C for 16s) of the milk, storing the cheese at low temperature (8°C for 48 h) during cheese sprinkling with dry salt, and achieving 86°C at the centre of the cut cheese blocks through the final cheese boiling stage. Generally, chemical parameters of the cheese were not affected by the modification, while the microbiological quality of the cheese improved significantly; coliforms, fecal coliforms and staphylococci were destroyed completely. The final cheese produced by the modified method had a homogeneous structure with firmly packed curd which was not easily broken, with no pinhole gas opening. In contrast, the cheese manufactured with unpasteurized sheep milk had a sponge-like structure, in which the curds were not firmly packed and there were many pinhole-gas openings that caused a major disruption of the matrix.

- Key words: Boiling in brine solution, Jordanian Baladi cheese, microbiological and organoleptic properties, and raw sheep milk -

INTRODUCTION

The province of Karak in the south of Jordan has very long tradition of artisanal cheese production. The climate in this region is considered tropical; it is hot and dry from April to October, with maximum daily temperatures of 30-40°C in this period. Baladi artisanal cheeses have been manufactured by local farmers on a small scale for decades. Raw sheep milk is used and the traditional techniques that are employed have been passed down from generation to generation. Baladi cheese has a white milky colour, high fat, high protein and high dry material contents, a cream-like aroma, obvious sweet and salty taste, and a homogeneous structure that is semi-hard and not easily broken. Baladi cheese is usually soaked in soft water overnight before being consumed in order to remove the excess quantity of salt. The cheese obtained from the market may have a variety of physicochemical characteristics depending on many factors, including the breed, animal-to-animal variability, age, stage of lactation, the feed, season of the year, time of milking and manufacturing location.

Raw milk is used to produce Baladi cheese so it is not easy to control all the factors during the manufacturing process. This leads to a lot of irregularities. It is necessary to improve the hygienic quality of the cheese by respecting its organoleptic properties (visual aspects, texture, and taste). These properties account for the typical characteristic of this cheese.

Microbial counts in the raw milk are much higher during the warm summer months than in the cool winter months which have implications for the resulting cheese (MENDIA *et al.*, 2007). TUNICK *et al.* (2007) confirmed that microbes flourish in raw milk especially during warmer months. They reported that raw milk cheese contained more aerobic and anaerobic bacteria than the pasteurized milk cheese, with the total counts differing by two orders of magnitude.

Msherfa is a governmental station located in Karak for breeding Awassi sheep; currently there are around 500 head of sheep. Baladi cheese in Msherfa station is made from evening and morning milk collected in the spring and summer, when milk production is abundant. A heat treatment is not applied to the cheese produced by the traditional method. No starters are added. SERT *et al.* (2007) reported that when the cheese is produced by the traditional method, generally a heat treatment is not applied to the cheese milk. No starter or additives are added.

Baladi cheese, still produced traditionally in the Karak district, is highly prized. The cheese is considered to be of premium value because of its flavour characteristics, it plays an important role in the local farmer's economy. In the Mediterranean area, many of these cheeses are produced from raw ewe's or goat's milk and have very characteristic organoleptic properties (HAJIKHANI *et al.*, 2007). Baladi cheese does not yet have a standard of identity.

During the summer, Baladi cheese had a problem of pinhole-sized gas formations due to the microbial activity in the raw milk cheeses. TUNICK *et al.* (2007) confirmed that bacterial fermentation in raw milk cheeses releases gases such as CO_2 and H_2 which create openings in the cheese. When the cheese cut blocks are sprinkled with dry salt for two days at room temperature (30°C) then drained, these pinhole gas formations became larger and eventually caused major disruption of the matrix, and the cheese develops a sponge-like structure in which has a very negative effect on its quality.

The aim of this work was to characterize an artisanal cheese produced in the Msherfa sheep station/province of Karak, and modify the traditional manufacturing method to improve the microbial and structural characteristics of Baladi cheese.

MATERIAL AND METHODS

Milk and cheese making

Sheep milk was obtained from the Msherfa station in the province of Karak in southern Jordan. Raw milk was pasteurized according to the modified method at 73°±0.5°C for 16 s and then cooled to 33°C with a Mini-Pasteurizer (PLADOT, Israel) designed especially for small- and medium-sized producers. The system controls temperature and flow by using manually controlled devices; this ensures a high quality end product. The basic configuration of the system is set to flow rates of 200-300 L/hour.

The Baladi cheeses were made in the dairy plant at the Msherfa station. They were made with the same milk and manufacturing was timed such that the modified and traditional processes would finish on the same day. Three experiments using traditional and modified methods for cheese-making were carried out in three weeks time. The experiments of traditional and modified methods were carried out in June 2008. The steps of the traditional and modified manufacturing methods steps are shown in Fig. 1.

Baladi cheese in the Msherfa station is made by mixing together fresh and cold raw overnight milks (8°C), brought in from the farm, and warmed to approximately 33°C. It was kept in a small plastic rectangular vat (150 L capacity) without addition of starter or colour. Two grams of microbial rennet powder per 100 kg milk (2:100000) were added, diluted with clean water and stirred for 4 min, then the milk was left to rest. A soft homogeneous curd formed in 40 min. The curds were broken into cubes using horizontal and vertical wire knives with a distance of 2 cm between each of the two wires; the temperature of the curds and the whey was maintained for 15 min. The curd and whey contents in the vat were emptied into simple perforated, stainless steel boxes, 45 x 45 x 5 cm, which had a perforated, detachable stainless steel top and a bottom plate. Each box was lined with a double-layer of cheesecloth. The cloth was folded over the top of the curd and covered with the perforated plate. A 20 kg stone plate was placed on top of the box to increase the pressing efficiency. After one h a solid block of curd formed, the weight and cloth were removed, and the large curd block was cut into smaller blocks, of approximately 6 cm x 4 cm and 2-3 cm using a sharp knife. Immediately after cutting, each curd cut blocks was sprinkled with dry salt (5 kg dry salt for each 25 kg of curd) and left to drain for 2 days at room temperature. Salting the curd 2 h after the rennet addition slows down the bacterial activity, reduces the development of acidity in the curd, and prevents the early blowing of the curd by predating the moment of salt addition. After 2 days of salting, the salted cut blocks are solid enough to undergo the boiling process.

The salted cut blocks were washed with clean tap water and then the cheese was boiled in brine (16% salt W/V), using 30 l of brine for each 20 kg of cheese. The temperature of the brine was kept constant at 100-102°C. The temperature at the centre of the cheeses produced by the traditional method after 2-3 minutes of boiling in the brine ranged from 72-75°C.

The hot brined cheeses were then cooled by laying the cheese cut blocks on a stainless steel table for 20 min at room temperature. On hot summer days an electrical air fan can be used to accelerate the cooling process. The 5 kg salted cheeses were then carefully placed in a new tin can, covered nearly to the top with 4.5 l of a 16% cold salt brine solution (8°C), and then the tin can was covered with a tin lid and stored at 8°C. The cheese can be consumed directly on the second day or can be stored for 6-12 months at this temperature before consumption.

Physicochemical analysis

Fat content of the milk was determined by the Gerber method (DAVIS, 2002). Protein content of the milk and the cheese were determined by the Kjeldahl method using Vapodest 20 (Gerhard, Königswinter, Germany). Lactose content, specific gravity and freezing point tests for milk were carried out using the Lactoscan 90 (Milk ana-

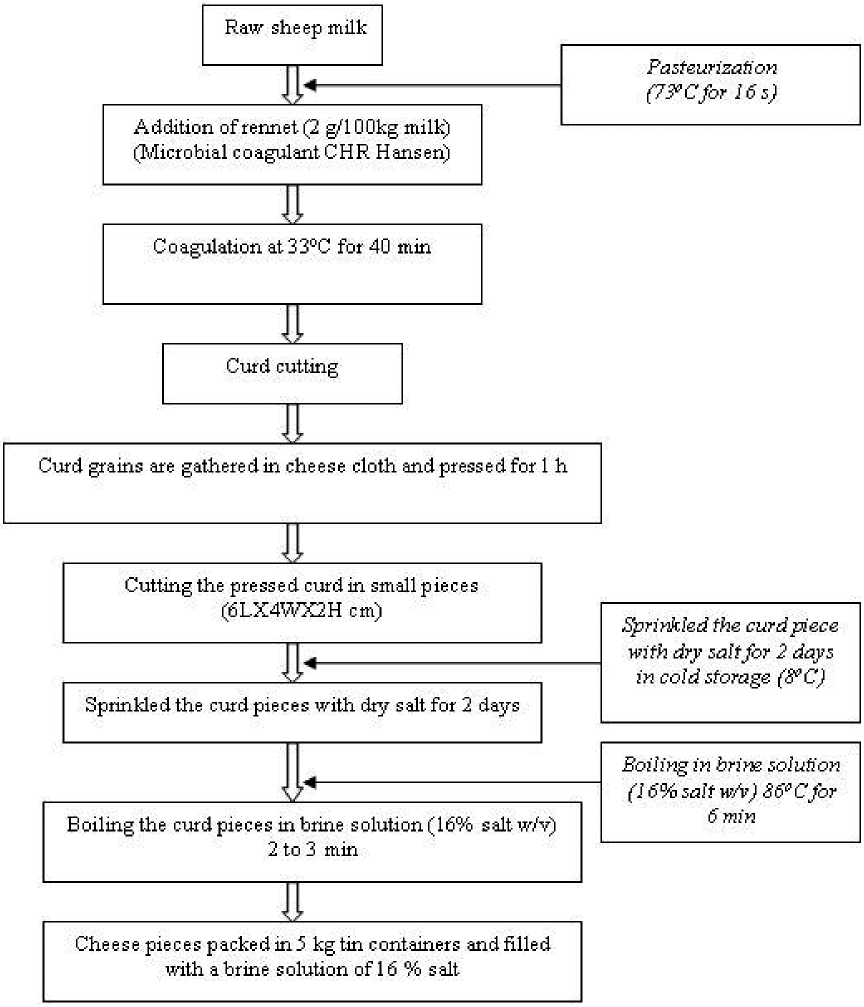


Fig. 1 - Traditional and modified processing methods of Baladi cheese. The text in italics represents the modified steps of the traditional method.

lyzer) (Serial No. 4960, Milkotronic LTD, Nova Zagora, Bulgaria). The pH was measured with a pH meter (MP 220 pH meter, Mettler-Toledo, AG CH-8603, Schwerzenbach, Switzerland) at 25±1°C. Cheese pH was determined using an InLab 413 electrode on slurry prepared by thoroughly blending 10 g of grated cheese with 10 ml of deionized water using a mortar and pestle (SERT *et al.*, 2007).

Fat content of the cheese was determined according to IDF reference method 5B (1985). Total solids were estimated using the open dish method in which samples (approximately 3 g) were accurately weighed on a dry dish and placed in an oven at 102°C. The following day the plates were re-weighed, and the total solids of samples were estimated (HALLAB *et al.*, 2007). Salt content was determined by the modified Volhard test (KOSIKOWSKI, 1977). All chemical analyses were performed in triplicate and the results are presented as mean and standard deviation.

Microbiological analysis

The cheese sample (25 g) was homogenized for 1 min in 225 mL of a sterile solution of 0.9% (w/v) NaCl using stomacher (Easy Mix, AES Laboratoire, Bruz, France.) Further decimal dilutions of milk and cheese were prepared with the same diluents (CEYLAN *et al.*, 2007). Total bacterial counts were enumerated on plate agar (Criterion, Hardy diagnostics, Santa Maria, CA, USA), using the pour plate technique to enumerate anaerobic and facultative anaerobic microorganisms. The plates were incubated at 30°C for 72 h. The total aerobic bacterial counts were enumerated on plate agar using the surface spread technique to enumerate strict aerobic microorganisms; the plates were incubated at 30°C for 72 h. Fecal and total coliform group bacteria were enumerated on violet red bile agar (Criterion, Hardy diagnostics, Santa Maria, CA, USA), after being incubated for 48 h at 44°C and 37°C, respectively (ROSA *et al.*, 2008). Staphylococci were enumerated on Baird-parker agars (Hi Media Laboratories Pvt. Ltd. Mumbai, India) with egg yolk according to the method for staphylococci count proposed by ANDREWS (1992). All microbiological analyses were performed in triplicate and the results are presented as mean and standard deviation.

Sensory analysis

A six-member panel of Msherfa employees, well experienced and familiar with Baladi cheese, assessed the cheese samples organoleptically. They evaluated the cheese samples for colour, aroma, taste, texture, gas opening, and general acceptability using a hedonic scale. Each attribute was graded on a 1-5 scale, where 1 was the lowest quality and 5 the highest quality (CEYLAN *et al.*, 2007).

Statistical analysis

The count of colony-forming units per gram or ml (CFU) was transformed to logarithms (log CFU/g or mL) prior to statistical treatment. The SPSS statistical package, Evaluation version 15 (SPSS Inc., Chicago, Illinois, USA) was used for the statistical analysis. Analysis of variance (ANOVA) and Independent Samples Test / t-test for Equality of means were applied to determine the differences ($P \leq 0.05$ and $P \leq 0.001$) between the studied parameters of the two manufacturing methods.

RESULTS AND DISCUSSION

Physico-chemical analysis

The results regarding the composition, specific gravity, freezing point and pH (Table 1) of the sheep milk are in agreement with most of the literature; the values for the raw sheep milk from the Msherfa breeding station are within the physicochemical standard specifications (WONG *et al.*, 1998; BELITZ *et al.*, 2004).

Results obtained from the physicochemical analyses of the final cheeses after one day in

Table 1 - Physicochemical parameters of raw sheep milk. The values are the mean ± standard deviation of triplicates.

Parameter	N	*Exp.1 Mean ± S.D	*Exp.2 Mean ± S.D	*Exp.3 Mean ± S.D
Total solid (g/100 g)	3	16.18 ± 0.04	15.99 ± 0.07	16.06 ± 0.07
Fat (g/100 g)	3	06.87 ± 0.06	06.77 ± 0.06	6.83 ± 0.06
Protein (g/100 g)	3	04.43 ± 0.03	04.87 ± 0.01	4.50 ± 0.01
Non Fat Solids (g/100 g)	3	09.31 ± 0.012	09.29 ± 0.01	9.30 ± 0.01
Specific gravity	3	01.03 ± 0.001	01.03 ± 0.001	1.03 ± 0.001
Freezing point °C	3	-0.54 ± 0.001	-0.54 ± 0.001	-0.54 ± 0.00
Temperature °C	3	11.83 ± 0.29	10.67 ± 0.29	11.33 ± 0.29
pH	3	06.96 ± 0.05	07.09 ± 0.04	6.87 ± 0.04

N= replicate numbers, S.D = Standard deviation, df = degrees of freedom.

the brine are presented in Table 2. The analysis shows that manufacturing methods have a significant effect on the organoleptic and microbiological properties. All of the values for the physicochemical parameters in both methods increased during the manufacturing period except for the pH which decreased from 6.96 in the raw milk to 5.90 and 6.25 in the cheeses produced by the traditional and modified methods, respectively; the results are in agreement with other studies (ŞENGÜL 1995; ÇAĜLAR 2001). The increases in the values for the physicochemical parameters were due to decrease in moisture during the manufacturing time. The moisture decreased due to syneresis of the cheeses. The results in Table 2 show that there are few differences between the moisture content of the traditional and modified methods. In addition the differences in the moisture content, even though statistically significant ($P<0.05$), are of little interest (0.012%) from any practical point of view in artisanal cheese production.

The results reported in Table 2 indicate that there were highly significant differences between the pH values in the traditional and modified methods at ($P<0.001$). The lowest pH was found in the samples produced with the traditional manufacturing method. In the traditional method, the lactic flora intervenes in the reduction of pH. There was no heat treatment on the milk.

According to the physicochemical results of the final cheeses (Table 2), Baladi cheese may be classified as a medium moisture (KOSIKOWSKI, 1977), high fat cheese (22-26%), with 24-28% protein and a high salt (5-10%) content. The final products yield was about 18-20 %, with a final pH ranging from 5.5 to 6.5.

Microbiological analysis

Results obtained from the microbiological analyses of the raw and pasteurized sheep milk are presented in Table 3. It is not surprising that microbiological counts were significantly low ($P<0.001$) due to the pasteurization process. Total pouring and aerobic counts declined from 5.65 to 3.64, and from 6.87 to 3.8 log colony forming units (CFU/mL) respectively, The coliforms and fecal coliforms declined from 5.46 to zero and from 2.62 to zero (CFU/mL), respectively. The results indicate a high staphylococci count in raw sheep milk; this was mostly due to a lack of hygienic condition and/or to mastitis.

The results obtained from the microbiological analyses of samples after the sprinkling of the cheese with dry salt for two days are presented (Table 4). The results indicate highly significant differences ($P<0.001$) in the microbial load between the traditional and the modified methods, because of milk pasteurization and the use of cold storage (8°C) during the sprinkling of the cheeses with dry salt. Pasteurization and cold storage improved the microbiological quality of the cheese and produced differences in the pouring and aerobic total counts. The differences were 1.7 log CFU/g in the pouring count technique and 2.1 log CFU/g for the aerobic count technique. The coliforms and fecal coliforms did not grow when the modified technique was used;

Table 2 - Physicochemical parameters of Baladi cheese manufactured by the traditional and modified methods after one day in the brine.

Parameter	Type of method	N	Mean ± S.D	df (2-tailed)	Sig. difference	Mean
Fat	Traditional	9	25.07 ± 0.11	16	0.06	-0.21
	Modified	9	25.28 ± 0.28			
Protein	Traditional	9	26.13 ± 0.12	16	0.26	0.17
	Modified	9	25.96 ± 0.43			
Salt	Traditional	9	08.07 ± 0.33	16	0.43	0.09
	Modified	9	07.98 ± 0.05			
Moisture	Traditional	9	38.36 ± 0.14	16	0.04*	0.12
	Modified	9	38.24 ± 0 .07			
pH	Traditional	9	05.90 ± 0.01	16	0.0001**	0.35
	Modified	9	06.25 ± 0.02			
FDM	Traditional	9	40.67 ± 0 .17	16	0.11	0.28
	Modified	9	40.94 ± 0.46			
PDM	Traditional	9	42.42 ± 0 .22	16	0.78	0.02
	Modified	9	42.40 ± 0 .10			
SM	Traditional	9	20.77 ± 0.19	16	0.23	0.10
	Modified	9	20.87 ± 0.14			
Yield	Traditional	9	18.30 ± 0.23	16	0.11	0.16
	Modified	9	18.14 ± 0 .16			

Independent Samples Test / t-test for Equality of means (*significance at $P<0.05$ and ** significance at $P< 0.001$). N= number of samples, S.D = Standard deviation, df = degrees of freedom.
FDM=Fat in Dry Matter. PDM=Protein in Dry Matter. SM=Salt in Moisture.

this was due to the pasteurization of the milk. In the traditionally produced cheese the coliforms were 4.75 log CFU/g and fecal coliforms were 2.63 log CFU/g. In a study on Queso Chihuahua cheese done in the spring, BRICKER *et al.* (2005) found coagulase-positive staphylococci (4.43-6.86 log CFU/g). Coliforms (2.84-5.47 log CFU/g), and enterococci (3.91-6.98 log CFU/g) were present in the raw milk cheese but not in pasteurized milk cheese. Although staphylococci were still present in the cheese produced by the modified method, the results show highly significant differences (P<0.001) in the staphylococci count between the cheeses produced by the traditional method and those produced by the modified method. The differences were 3.2 log CFU/g.

The microbial profiles for pouring and aerobic plate count, coliforms and fecal coliforms and staphylococci in the final cheeses produced by the traditional and modified methods are shown in Table 5. The results show a notable reduction in the bacterial counts in all the microbial tests due to the boiling of the final products. The results presented in Table 5 show that the mean values of coliforms, fecal coliforms, and staphylococcus counts were 1.55, 1.4, 1.17 log CFU/g of cheese, respectively. CELIK and TURKOGLU (2007) reported that Őrgü cheese is a semi-hard cheese variety produced mainly from raw ewe milk containing high levels of fat. Its curd is scalded and kneaded, and is ripened in brine. They also confirmed that because Őrgü cheese is produced from raw milk, it may contain many microorganisms, which may create a potential public health risk. OZDEMIR *et al.* (1998) reported mean coliform, *Staphylococcus aureus* and yeast-mould counts of 2.57, 1.34, 5.0 log CFU/g, respectively, in traditional Őrgü cheeses collected from the Karacadag region of Turkey. The results of this study also showed significant differences (P<0.05) in the pouring total count between the cheeses produced by the traditional and those produced by modified methods. The differences were 0.32 log CFU/g· (Table 5). From bacteriological viewpoint, there

Table 3 - Changes of microbiological parameters (log CFU/mL) of raw sheep milk due to the pasteurization process.

Parameter	Type of milk	N	Mean ± S.D	df	Sig. (2-tailed)	Mean difference
TCC / pouring	Raw	9	5.65 ± 0.02	16	0.0001	2.0168
	Pasteurized	9	3.64 ± 0.02			
TCC / aerobic	Raw	9	6.87 ± 0.01	16	0.0001	3.0698
	Pasteurized	9	3.80 ± 0.01			
Coliforms	Raw	9	5.46 ± 0.02	16	0.0001	5.4636
	Pasteurized	9	0.00 ± 0.00			
Fecal coliforms	Raw	9	2.62 ± 0.02	16	0.0001	2.6226
	Pasteurized	9	0.00 ± 0.00			
staphylococci	Raw	9	4.21 ± 0.04	16	0.0001	1.4691
	pasteurized	9	2.74 ± 0.02			

Independent Samples Test / t-test for Equality of means (significance at P< 0.001). N= number of samples, S.D = Standard deviation, df = degrees of freedom.

Table 4 - Changes of microbiological parameters (log CFU/g) of the cheeses produced by the traditional and modified methods, after the sprinkling of the cheeses with dry salt for two days.

Parameters	Type of milk	N	Mean ± S.D	df	Sig. (2-tailed)*	Mean difference
TCC / pouring	Traditional	9	5.41 ± 0.03	16	0.001	1.71
	Modified	9	3.70 ± 0.03			
TCC / aerobic	Traditional	9	5.89 ± 0.01	16	0.001	2.13
	Modified	9	3.75 ± 0.02			
Coliforms	Traditional	9	4.75 ± 0.02	16	0.001	4.75
	Modified	9	0.00 ± 0.00			
Fecal coliforms	Traditional	9	2.63 ± 0.01	16	0.001	2.63
	Modified	9	0.00 ± 0.00			
staphylococci	Traditional	9	4.48 ± 0.25	16	0.001	3.20
	Modified	9	1.29 ± 0.16			

Independent Samples Test / t-test for Equality of means (*significance at P<0.001). N= number of samples, S.D = Standard deviation, df = degrees of freedom.

are differences if there is at least 1 log gap between them. The differences were highly significant (P<0.001) between the traditional method and the modified method in aerobic total (1.4 log CFU/g), coliform (1.5 log CFU/g) and fecal coliform (1.4 log CFU/g) counts. The results showed that boiling the cheese (5-6 min) until the temperature reached 86°C in the center of the cheese cut blocks in the modified method completely destroyed all the staphylococcal bacteria. While, boiling cheese cut blocks for 2 min in the traditional method however was not enough to kill all the coliforms, fecal coliforms and staphylococci.

Pasteurization of milk in the modified method without adding any starter can be a risk factor for the quality of the cheese. The very short time (2 h) between rennet addition and the time of salting at low temperature (8°C) can reduce the growth of lactic acid bacteria, but there is also the risk of microfloral growth that cause post-contaminating the milk. The NaCl helps to counteract unwanted microbial growth and reduce the structural defects due to gas-producing fermentation.

Appearance and physico-chemical characteristics of cheeses

The results of the organoleptic analysis of the cheeses are presented (Table 6). The results show the superiority of the cheeses produced by the modified method over the cheeses produced by the traditional method. The results are highly significant (P<0.001) for most of the sensory parameters (aroma, taste, texture, gas opening, and acceptability) of the cheeses, except for colour, where the differences were not significant (P>0.05). The cheeses manufactured with full fat pasteurized sheep milk by using the modified method were a milky white colour, cream like aroma, obvious sweet taste, and a homoge-

Table 5 - Changes in microbiological parameters (log CFU / g) of the cheeses produced by the traditional and modified methods, after the boiling process.

Parameters	Type of milk	N	Mean ± S.D	df	Sig. (2-tailed)	Mean difference
TCC / pouring	Traditional	9	1.50 ± 0.34	16	0.014*	0.318
	Modified	9	1.18 ± 0.05			
TCC / aerobic	Traditional	9	3.60 ± 0.03	16	0.001**	1.44
	Modified	9	2.17 ± 0.04			
Coliforms	Traditional	9	1.55 ± 0.05	16	0.001**	1.55
	Modified	9	0.00 ± 0.00			
Fecal coliforms	Traditional	9	1.40 ± 0.04	16	0.001**	1.40
	Modified	9	0.00 ± 0.00			
staphylococci	Traditional	9	1.17 ± 0.06	16	0.001**	1.17
	Modified	9	0.00 ± 0.00			

Independent Samples Test / t-test for Equality of means (*significance at P<0.05 and ** significance at P< 0.001). TCC = Total colony count. N= number of samples, S.D = Standard deviation, df = degrees of freedom.

Table 6 - Organoleptic parameters of Baladi cheese manufactured by the traditional method and by the modified method.

Parameter	Method	N	Mean ± S.D	Sig. (2-tailed)
Color	Traditional	18	4.44 ± 0.51	0.19
	Traditional	18	4.67 ± 0.49	0.19
Aroma	Traditional	18	2.00 ± 0.59	0.00*
	Modified	18	4.28 ± 0.46	0.00*
Taste	Modified	18	1.83 ± 0.51	0.00*
	Modified	18	4.56 ± 0.51	*0.00
Structure	Traditional	18	1.33 ± 0.49	*0.00
	Modified	18	4.56 ± 0.51	0.00*
Gas opening	Traditional	18	1.39 ± 0.50	0.00*
	Modified	18	4.50 ± 0.51	0.00*
Acceptability	Traditional	18	2.50 ± 0.51	0.00*
	Modified	18	4.78 ± 0.43	0.00*

Independent Samples Test/t-test for Equality of means (*significance at P< 0.05). Hedonic scale: 5 Excellent; 4 Good; 3 Accepted; 2 Marginally accepted; 1 Rejected
S.D = Standard deviation.

Table 7 - Comparison between Lebanese Baladi cheese and Jordanian Baladi cheese.

Milk	Lebanese Baladi cheese[a] Cow's milk fat %	Jordanian Baladi cheese Sheep's milk fat %
	3.21±0.003-3.97±0.00	6.77±0.06-6.87±0.06
Milk standardization	Milk standardized (3% fat)	No standardization
Pasteurization	68°C for 30 min	No pasteurization[b]
Coagulation temperature	38°C	33°C
Coagulation time	60 min	40 min
Starter culture	1% starter	No starter
Pressure (g/cm^2)	1.04	9.9
Pressure time	14.5 h	1 h
Cheese total solids % (TS) (hot season)	47	61.32
Cheese total solids retention (hot season)[c]	44.5	72.7
Cheese fat content	18.5-20%	25%
Boiling in brine	No boiling	boiling
pH	5.0	5.9-6.25

[a] The data for the Lebanese Baladi cheese is from KAMLEH *et al.,* (2006) and this study.
[b] Pasteurization at 73°C for 16 s applied only in the modified method.
[c] The percentage of total solids retention (TSR %) in the cheese was calculated according to KAMLEH *et al.,* (2006).

neous structure with firmly packed curd, not easily broken with no pinhole gas openings. The cheeses manufactured with full fat unpasteurized sheep milk by the traditional method had a sponge-like structure, the curds were not firmly packed and the cheese had many pinhole-sized gas openings that caused a major disruption of the matrix. The analysis showed that manufacturing methods have a significant effect on the organoleptic and microbiological properties.

Table 7 shows a comparison between a Lebanese Baladi cheese (KAMLEH *et al.*, 2006) and the Jordanian Baladi cheese manufactured in this study. The comparison showed that there are many differences between the two cheeses: source of milk, (cow and sheep) and processing conditions (standardization of milk, heat treatment, coagulation conditions, addition of starter and pressure conditions). The two cheeses have very different physicochemical and organoleptic characteristics.

CONCLUSION

The results of this study indicate that the modified method improves the colour, aroma, taste and structure of Baladi cheese. The chemical characteristics of the cheese produced by the modified method were very similar to those of the traditional cheeses. The modified method reduced the levels of the total bacterial count, and completely destroyed coliforms, fecal coliforms and staphylococci. Consequently, using the modified method to produce Baladi cheese improves the microbiological and organoleptic quality of the cheese and improves its compliance with European hygienic standards.

REFERENCES

Andrews A. 1992. "Manual of Food Quality Control" 4. Rev. 1.Microbiological analysis. p. 131. FAO, Rome.

Belitz H.D., Grosch W. and Schieberle P. 2004. "Food Chemistry", 3rd ed. P. 505. Springer-Verlag Berlin Heidelberg, Germany.

Bricker A.L., Van Heklen D.L., Guerrero V.M. and Gardea A.A. 2005. Microflora isolated from Queso Chihuahua cheeses. Food Protection Trends 25: 29.

Çağlar A. 2001. Change in some microbiological properties of Erzincan tulum cheese made from raw milk and ripened in various casing material. Agriculture Faculty. Atatürk University 32: 285.

Celik S. and Turkoglu H. 2007. Ripening of traditional Őrgü cheese manufactured with raw or pasteurized milk: Composition and biochemical properties. Inter. J. Dairy Technol. 60: 253.

Ceylan Z.G., Çaglar A. and Çakmakçi S. 2007. Some physicochemical, microbiological, and sensory properties of tulum cheese produced from ewe's milk via a modified method. Inter. J. Dairy Technol. 60: 191.

Davis J.G. 2000 . "Milk Testing". Agrobios Jodhpur, India.

Hallab R., Kohen C., Grandison M.A., Lewis M.J. and Grandison A.S. 2007. Assessment of the quality of cottage cheese produced from standard and protein-fortified skim milk. Inter. J. Dairy Technol. 60: 69.

Hajikhani R., Beyatli Y. and Aslim B. 2007. Antimicrobial activity of enterococci strains isolated from white cheese. Inter. J. Dairy Technol. 60: 105.

International Dairy Federation. 1985. Milk and milk products. Methods of sampling, IDF standard 50B. Brussels, Belgium.

Kamleh R., Tannous R. and El Maydaa E. 2006. Cows milk quality in two regions in Lebanon and its effect on Baladi (local) cheese quality. J. Food, Agricul. Environ. 4: 75.

Kosikowski V.F. 1977. "Cheese and Fermented Milk Foods". 2nd ed. p, 570. Edwards Brothers, Inc. Ann Arbor, MI, USA.

Mendia C., Ibañez F.C., Torre P. and Barcina Y. 2000. Influence of the season on proteolysis and sensory characteristics of Idiazábal cheese. J. Dairy Science 83: 1899.

Ozdemir S., Celik S., Ozddemir C. and Sert S. 1998 . Diyarbaker in Karacadag yöresinde mahalli olarak yapilan örgü peynirinin mikrobiyolojik özellikleri. V. süt ve Süt Ürünleri Sempozyumu. 21-22. Mays, 1998, Tekirdağ, Turkey.

Rosa T.D., Wassermann G.E., Volken De Souza C.F., Caron D., Carlini C.R. and Ayub M.A.Z. 2008. Microbiological and physicochemical characteristics and aminopeptidase activities during ripening of Serrano cheese. Inter. J. Dairy Technol. 61: 70.

Şengül M. 1995. The determination of some quality criteria of Tulum cheese produced from raw and pasteurized milk and ripening in various packaging materials, Master Thesis, Erzurum, Turkey: atatürk University, Graduate School of Natural and Applied Science, Turkey.

SERT D., AYAR A. and AKIN N. 2007. The effects of starter culture on chemical composition, microbiological and sensory characteristics of Turkish Kaşar Cheese during ripening. Inter. J. Dairy Technol .60: 245.

Tunick M.H., Van Hekken D.L., Call J., Molina-Corral F.J. and Gardea A.A. 2007. Queso Chihuahua: effects of seasonality of cheesemilk on rheology. Inter. J. Dairy Technol. 60: 13.

Wong NP., Jenness R., Keeney M. and Marth E.H. 1998. "Fundamentals of Dairy Chemistry". P. 1. CBS Publishers and Distributors, New Delhi, India.

Revised paper received March 24, 2009 Accepted June 18, 2009

CHARACTERIZATION OF TRIACYLGLYCEROLS IN *PISTACIA VERA* L. OILS FROM DIFFERENT GEOGRAPHIC ORIGINS

G. BALLISTRERI, E. ARENA and B. FALLICO*
Dipartimento di OrtoFloroArboricoltura e Tecnologie Agroalimentari (DOFATA),
Università di Catania, Via S. Sofia 98, 95123 Catania, Italy
*Corresponding author: Tel. +39 0957580214, Fax +39 0957141960,
e-mail: bfallico@unict.it

ABSTRACT

Pistacia vera L. is the only species of the *Pistacia* genus that produces edible nuts. It is mainly cultivated in the USA, western Asia and some Mediterranean countries. In this study, the triacylglycerol (TGs) composition of the oil extracted from the pistachio kernels coming from seven different countries (Italy, Turkey, Iran, Syria, Tunisia, Greece and the USA), as well as the distribution of the fatty acids (FAs) attached to the glycerol molecule were determined by HPLC. The major FAs were oleic (C18:1), linoleic (C18:2) and palmitic (C16:0) acids forming more than 90% of the total FAs. The main TGs found in the *P. vera* oil were: OLO, OLL, OOO, LLL, OLP, LLP and OOP, accounting for more than 85% of the total TGs. Principal component analysis and linear discriminant analysis (PCA and LDA) were successfully used and indicate that the TG characteristics studied are suitable for authenticating geographical origin of pistachio seeds.

- Key words: fatty acids, HPLC, linear discriminant analysis, pistachio oil, principal component analysis, triacylglycerols -

INTRODUCTION

Pistachio (*Pistacia vera* L.) is a member of the *Anacardiaceae* family. The genus *Pistacia* contains only 11 species, of which *P. vera*, cultivated for its edible nuts, is the most important. Its seeds are of high economic value due to their balanced composition, characterized by a low carbohydrate content of about 10%, a protein content of more than 20% and a lipid content varying from 40 to 63%, all on a dry weight basis (CHAHED *et al.*, 2006). The pistachio nut is mainly used as a snack food, both raw and toasted, and is a confectionery ingredient in salami, ice cream, bread and sauces. It has been proposed that the shells can be used to prepare activated charcoal (GALVANO *et al.*, 1996).

The *P. vera* tree is native to arid zones of central and west Asia. It was only during the last century that its cultivation was extensively increased in some Mediterranean countries, and spread to other countries. The USA and Iran are now the most important producers of pistachio (LUA and YANG, 2005).

The top worldwide producers are: Iran (230,000 tons), followed by United States of America (11,0000 tons), Turkey (78,409 tons) and Syria (29,000 tons). Less important producers are Greece (9000 tons), Italy (2000 tons) and Tunisia (800 tons) (FAO, 2007).

In Italy, pistachio is grown mainly in Bronte, an area around the Etna volcano, where the lava and climate allow the production of an intensely green nut with a very aromatic taste that is highly prized on the international markets. Italian production is very low in comparison to that of Asia and the USA; however, it is compensated by the very high quality (DI MARCO, 1987). The annual pistachio production in Bronte is 1550 tons, which represents 80% of the total production in Sicily (BELLIA, 2007).

Many studies have been conducted to characterize pistachio nuts of different geographic origins. Differences in the profiles of inorganic anions and organic acids, in fatty acids and in colour among pistachios of different origins and varieties have been reported in the literature (ANDERSON and SMITH, 2005; ARENA *et al.*, 2007; BELLOMO and FALLICO, 2007). Differences in the fatty acid profiles would be expressed more clearly in the triglyceride profiles of the nut oils (DYSZEL and PETTIT, 1990; HOLCAPEK *et al.*, 2003). The fatty acid composition can indicate the nutritional quality of oils, while the type and the amount of the various triglyceride species constitute criteria for the physical and functional properties of the oil.

Oils are complex mixtures containing mainly triacylglycerols (TGs) formed by various fatty acids (FAs), differing in the acyl chain length and their position *sn*-1, 2 or 3 on the glycerol skeleton, and in the number and position of the double bonds on the acyl chains.

HPLC may be considered the method of choice for the separation and determination of TGs (DUGO *et al.*, 2006). Examination of the literature reveals a number of points of agreement among the various authors regarding the general strategies, major difficulties and limitations of the methods that include the appropriate stationary phase, correct mixtures in gradient elution and detectors (ADEBOWALE and NICHOLSON, 2002; HOLCAPEK *et al.*, 2003).

The aim of this work was to characterize the TG composition of pistachios from different countries by building supervised classifiers (PCA and LDA) for the discrimination of the country of origin of pistachio seed samples and the elucidation of the FA distribution on the glycerol molecule. Monoacylglycerols, diacylglycerols and free FAs, were not considered because TGs constitute the main acylglycerols in pistachio oil (more than 90% of the total glycerolipids, CHAHED *et al.*, 2008). They also have more possible combinations in the FA distribution on the glycerol molecule, and are therefore more discriminating.

MATERIALS AND METHODS

Samples

Pistachio samples (*n* = 37), from Italy (Bronte; var. Bianca), Turkey (Gaziantep; var. Siirt), Iran (var. Akbari), Syria (var. Jalab), Tunisia (var. Mateur), Greece (var. Aiginis) and the USA (California; var. Kerman), were picked at ripeness.

Each sampling was taken from different pistachio producers and importers: 14 Italian samples, 7 Turkish, 4 Iranian, 3 Syrian, 3 Tunisian, 3 Greek and 3 American. The Italian samples were representative of 1000 kg of pistachio kernels from which, 1 kg of pistachio was taken. The other samples were 1 kg each, coming from lots between 1000 and 40000 kg.

All samples were produced and collected in 2007, stored at 4°C in darkness, at least up to 48 h before the analyses.

Reagents and standards

Acetonitrile and 2-propanol were purchased from Lab Scan (Dublin, Ireland), *n*-hexane and water from JT. Baker (Deventer, Holland). All the reagents used were of the required HPLC purity grade.

All the standards used for the study were purchased from the Sigma-Aldrich Chemical Company (St. Louis, MO, USA) and Larodan Fine Chemicals (Malmö, Sweden).

Oil extraction and chemical analyses

Moisture content (%) was determined on pistachio kernels, acidity (% oleic acid) and peroxide number (meq/kg) were determined from pistachio oil according to a standard method (BALESTRIERI and MARINI, 1996). All the analyses were conducted in triplicate.

Pistachio oil was extracted using the following procedure. First, nuts were shelled and 10-15 g of kernels were carefully crushed in a mortar until fine particles were obtained these were then mixed with 30 mL of *n*-hexane and stirred for 30 min. The *n*-hexane extract was filtered and solvent was evaporated under reduced pressure, using a rotary evaporator at 40°C.

For HPLC analysis, oil samples were dissolved (1:10, w/v) in acetonitrile:2-propanol (1:1, v/v) and filtered with a PTFE 0.45 μm filter (Albet).

HPLC analysis of TGs

The chromatographic apparatus consisted of an HPLC Spectra System equipped with a P4000 pump with a quaternary gradient pump system, a SCM1000 vacuum membrane degasser, a UV6000LP diode-array UV detector, an AS3000 autosampler with a thermostated column compartment (Thermo Electron Corporation, Waltham, Massachusetts, USA). The column was an Alltech Alltima C_{18} (250 mm x 4.6 mm, 5μm particle size). Analysis conditions were the following: 10 μL injection volume, flow rate 1mL/min, column temperature 40°C, spectra were recorded from 200 to 300 nm and UV detection at 205 nm. The mobile phase and the gradient program were set up according to HOLCAPEK *et al.*, (2003). TGs were identified by the correspondence of retention times and ECN (equivalent carbon number) with standard compounds.

The HPLC was connected to Chromquest Chromatography Manager 4.2 (Rev.A.97202) software (Thermo Electron, San Jose, CA, USA) for determination of peak areas and percentages of TGs. Analyses were carried out in triplicate.

Statistical analysis

Multiple range test and multivariate analysis of experimental data, principal component analysis (PCA) and linear discriminant analysis (LDA), were performed using the Statgraphic Plus 4.1 software (Manugistic Inc. Rockville, MD, USA).

RESULTS AND DISCUSSION

Table 1 shows the moisture content of pistachio nuts and the acidity and the peroxide number of the extracted oils. As expected, the seeds had a low moisture content (mean value 2.5%), ranging from 1.0% for Iranian samples up to 3.6% for Italian samples; indeed, pistachio seeds with a water content above 6.5 % are not suitable for the market (CEE, 2004).

Low moisture content is important for maintaining the quality and shelf life of seeds because it decreases the probability of microbial growth, unwarranted fermentation, premature seed germination, and many other biochemical changes such as phospholipase activity.

The low level of acidity and peroxide number indicates the freshness of the samples, the good quality of the oil, and no chemical or enzymatic hydrolysis of glycerides

These results are in agreement with the literature data (GARCIA *et al.*, 1992; MASKAM and KARATAS, 1998; KUCUKONER *et al.*, 2003; SATIL *et al.*, 2003; ARENA *et al.*, 2007).

The considerable resolving power of HPLC in reversed-phase mode has been used to separate TG species in natural oils and fats. The elution order is based on the equivalent carbon number increase ECN = CN – 2DB (equivalent carbon number = carbon number – twice the double bond number). Under optimized separation conditions, the separation of some TGs with the same ECNs is also possible, for example, the pair LLL and OLLn, or the group of OOO, OOP, POP and PPP can be resolved.

Fig. 1 reports an example of the HPLC chromatogram of TGs of P. vera oil from Italy, monitored at 205 nm. By comparing retention times and ECNs with those of standard compounds, 18 TGs were identified.

Percentages of the different triacylglycerol types in the oil of P. vera from seven origins are reported in Table 2.

The main TGs in the P. vera oil were: OLO, OLL, OOO, LLL, OLP, LLP and OOP; these accounted for more than 85% of the total TGs. The most abundant TG was OLO, with a mean percentage of 18.9% ± 1.0 varying from 17.4 % in the American samples to 20.6 % in the Syrian ones. The second most abundant TG in the P. vera oil was OLL, with an average content of 16.5% ± 1.7 and a range from 14.3% in the Italian samples to 18.9% in the Iranian ones. A similar OLL content (16.1%) was reported by DYSZEL *et al.*, (1990) for an Iranian variety. The average value of OOO (14.0% ± 3.6) was significantly higher than that reported by HOLCAPEK *et al.*, (2003). The next three TGs were: LLL, OLP and LLP with mean contents of 11.4% ± 2.0, 10.9% ± 0.6 and 8.7% ± 1.3, respectively. The LLL percentage ranged from 8.9% in the Italian samples up to 14.2% for the Iranian ones; OLP content ranged between 10.2% for Syrian sam-

Table 1. Moisture content (%) of pistachio nut and acidity (% oleic acid) and peroxide number (meq/kg) of Pistacia vera oils.

Samples	Moisture (%)	Acidity (% oleic acid)	Peroxide number (meq/kg)
Italy	3.6	0.4	0.7
Turkey	3.0	0.6	1.3
Iran	1.0	0.6	1.2
USA	3.0	1.3	2.6
Syria	2.0	0.6	1.1
Tunisia	2.0	1.1	2.1
Greece	3.0	0.9	1.7
Mean ± SD	2.5 ± 0.9	0.8 ± 0.3	1.5 ± 0.7

SD, standard deviation.

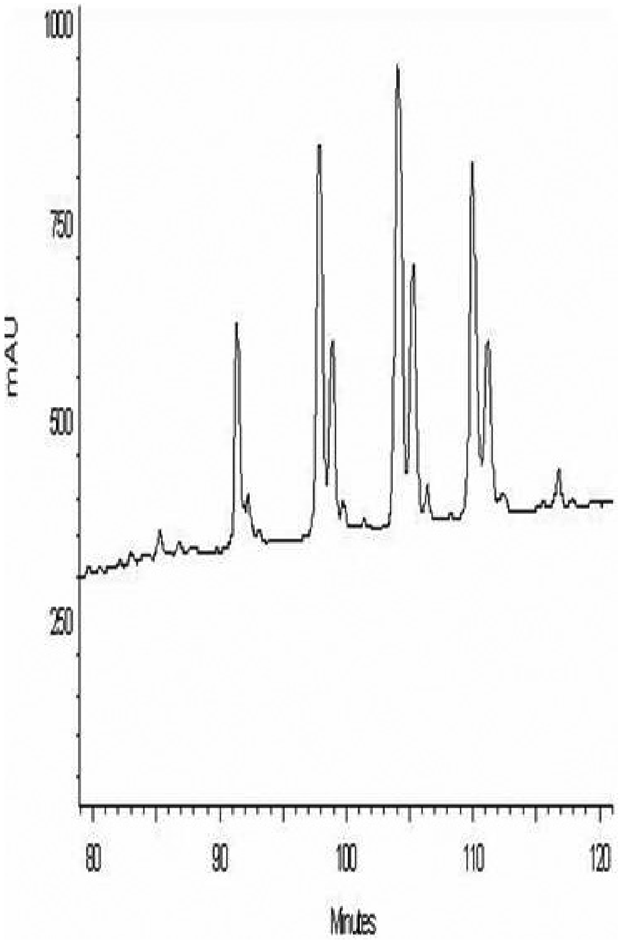


Fig. 1 - HPLC profile of TGs of *Pistacia vera* oil from Italy, monitored at 205 nm.
See Table 2 for peak numbers.

ples and 11.8% for the American ones; the LLP content varied from 7.4% to 11.1% for the Syrian and American samples, respectively. These values were similar to those reported in the literature (HOLCAPEK *et al.*, 2003). The average content of OOP was 6.9% ± 1.3 with a range from 5.1 % to 8.4 % in the Iranian and Italian samples, respectively.

A previous paper concerning the origin of pistachio seeds, verified that fatty acid and phytosterol composition are significant discriminating criteria (GARCIA *et al.*, 1992; SEFEROGLU *et al.*, 2006; ARENA *et al.*, 2007).

The FA composition was calculated from TGs distribution in pistachio oils according to HOLCAPEK *et al.*, (2005) (Table 3). The FAs of the TGs were palmitic (C16:0), margaroleic (C17:1), stearic (C18:0), oleic (C18:1), linoleic (C18:2), linolenic (C18:3) and gadoleic (C12:1) acids. Unsaturated FAs such as oleic and linoleic acids showed a preference for the internal position: with proportions of 33.3% and 61.1%, respectively. In P. vera oil, palmitic acid was esterified at the 1- and 3-positions in all of the TGs. These results are in agreement with the general distribution pattern of the unsaturated and saturated FAs reported for vegetables oils (MATTSON and VOLPENHEIN, 1961; MATTSON and VOLPENHEIN, 1963). This FA distribution is more suitable for the eukaryotic pathway in the endoplasmic reticulum (OHLROGGE and BROWSE, 1995).

The major fatty acids were oleic (37.40-47.63%), linoleic (36.45-47.38%) and palmitic (10.93-13.10%) acids; these accounted for more than 90% of the total FAs. The high content of C18:1 in pistachio oil suggests that there is an

Table 2 - Content (%) of the most relevant triacylglycerol types in *Pistacia vera* oil from different geographic origins.

TGs	ECN	*F*-ratio	Italy (n=14)	Turkey (n=7)	Iran (n=4)	USA (n=3)	Syria (n=3)	Tunisia (n=3)	Greece (n=3)	Mean ± SD
1. LLLn	40	2.37	1.4 a	1.2 a	1.9 a	1.0 a	1.6 a	1.2 a	0.7 a	1.4 ± 0.5
2. LLL	42	40.30	8.9 a	10.1 b	14.2 d	13.9 d	10.3 bc	11.2 c	9.8 ab	11.4 ± 2.0
3. OLLn	42	3.24	2.7 a	2.7 ab	3.3 bc	3.6 c	2.4 a	2.8 abc	2.2 a	2.9 ± 0.4
4. LnLP	42	1.62	1.1 a	0.8 a	1.3 a	1.3 a	0.7 a	1.2 a	0.6 a	1.0 ± 0.3
5. OLL	44	32.52	14.3 a	15.3 b	18.9 d	18.1 d	16.0 bc	16.9 c	15.1 ab	16.5 ± 1.7
6. LLP	44	36.36	7.6 a	7.9 ab	10.5 c	11.1 c	7.4 a	8.3 b	8.0 ab	8.7 ± 1.3
7. OLnP	44	1.15	1.6 a	1.3 a	1.1 a	1.3 a	1.4 a	1.4 a	1.3 a	1.3 ± 0.2
8. OLMo	45	0.55	0.6 a	0.4 a	0.4 a	0.5 a	0.2 a	0.7 a	0.4 a	0.4 ± 0.2
9. OLO	46	3.83	18.5 ab	19.2 bc	18.2 ab	17.4 a	20.6 c	19.6 bc	19.4 bc	18.9 ± 1.0
10. OLP	46	5.01	10.6 a	10.5 a	11.6 b	11.8 b	10.2 a	10.6 a	11.0 ab	10.9 ± 0.6
11. PLP	46	3.16	2.1 b	1.6 a	2.1 ab	2.8 c	1.7 ab	2.1 ab	2.1 ab	1.9 ± 0.4
12. GLO	48	0.59	0.6 a	0.3 a	0.4 a	0.3 a	0.3 a	0.6 a	0.4 a	0.4 ± 0.1
13. OOO	48	38.04	16.8 c	16.3 c	8.9 a	9.2 a	16.3 c	13.9 b	17.1 c	14.0 ± 3.6
14. OOP	48	15.85	8.4 c	7.8 c	5.1 a	5.7 ab	6.7 b	6.3 b	8.1 c	6.9 ± 1.3
15. POP	48	1.56	1.6 a	1.0 a	1.0 a	1.1 a	1.2 a	1.1 a	1.4 a	1.1 ± 0.3
16. GOO	50	2.14	0.7 a	0.9 a	0.5 a	0.2 a	0.9 a	1.1 a	0.9 a	0.8 ± 0.3
17. SOO	50	10.50	2.0 c	1.8 bc	0.7 a	0.5 a	1.4 ab	0.9 ab	1.4 bc	1.3 ± 0.6
18. SOP	50	1.59	0.5 a	0.2 a	0.3 a	0.3 a	0.6 a	0.0 a	0.2 a	0.3 ± 0.2

n, number of samples.
Mean values with different letters within the same row are statistically different (p-value < 0.05).

Table 3 - FA composition (%) of TGs in *Pistacia vera* oils from different geographic origins.

Samples	P (C16:0)	Mo (C17:1)	S (C18:0)	O (C18:1)	L (C18:2)	Ln (C18:3)	G (C20:1)
Italy	12.39	0.20	0.83	47.42	36.45	2.27	0.44
Turkey	11.37	0.13	0.68	46.88	38.46	2.05	0.43
Iran	11.97	0.14	0.32	37.48	47.38	2.54	0.28
USA	13.10	0.17	0.27	37.40	46.60	2.40	0.17
Syria	10.93	0.07	0.67	46.80	39.00	2.03	0.40
Tunisia	11.40	0.23	0.30	43.87	41.33	2.20	0.57
Greece	12.07	0.13	0.53	47.63	37.70	1.60	0.43
Mean ± SD	11.89 ± 0.73	0.15 ± 0.05	0.51 ± 0.22	43.92 ± 4.60	40.99 ± 4.36	2.16 ± 0.30	0.39 ±0.13

SD, standard deviation.

important β-ketoacyl-ACP (KAS II) activity, which acts on palmitoyl-ACP to yield stearoyl-ACP, as well as, an important activity of stearoyl-ACP Δ9-desaturase (CHAHED *et al.*, 2006).

In the current study it was found that TGs of pistachios contain an average of 44% oleic acid; in other papers oleic acid ranged from 50 to 58% (RUGGERI *et al.*, 1998; SATIL *et al.*, 2003; VENKATACHALAM and SATHE, 2006) and up to 74% (YILDIZ *et al.*, 1998; OKAY, 2002; KUCUKONER and YURT, 2003; ARENA *et al.*, 2007; ACAR *et al.*, 2008) of the total FA content.

Part of this variation may be due to cultivar. In addition, other main factors that are known to affect total fatty acid composition, and especially oleic acid content, are latitude, climatic conditions, and the ripening stage of the fruit when harvested (RANALLI *et al.*, 1997; APARICIO and LUNA, 2002; CHAHED *et al.*, 2008).

All samples, except the Iranian and American ones, had about 46% oleic acid and 39% linoleic acid, whereas the Iranian and American samples showed an inverted distribution, i.e. 37% oleic acid and 47% linoleic acid. The fatty acid composition of the oils from the Italian and Turkish samples were similar to each other and could be easily distinguished from those of the Iranian and American samples; the latter are also similar with respect to the TG distribution, as also reported by DYSZEL and PETTIT (1990).

ANOVA (by means of the Fischer F-ratio) and PCA are normally employed to reduce the number of variables to be included in the mathematical model (MARINI *et al.*, 2003).

There were statistically significant differences (p-value<0.05) among the seven different geographic origins studied for most of the triacylglycerols based on their Anova F-ratio and content, as reported in Table 2. The major statistically significant differences, among the different geographic origins, were found in: LLL (F = 40.30), OOO (F = 38.04), LLP (F = 36.36), OLL (F = 32.52), OOP (F = 15.85), SOO (F = 10.50), OLP (F = 5.01), OLO (F = 3.83), OLLn (F = 3.24) and PLP (F = 3.16).

PCA and LDA showed that the variables LLL and OLLn were the most significant combinations for classifying the geographic origins of the P. vera oil.

PCA of the ten major statistically significant TGs gave two linear combinations which explain 78.6% of the variance, in particular, 62.1% for the first component and 16.5% for the second. Fig. 2 shows the vectors of each variable and the distribution of the oil samples in the plan defined by the values of the two principal components.

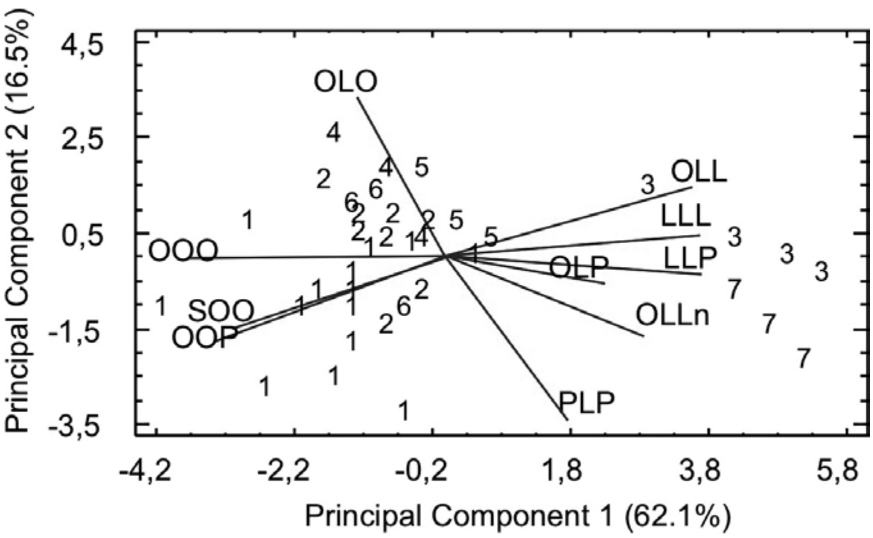


Fig. 2 - Plot of principal components. (1) Italy; (2) Turkey; (3) Iran; (4) Syria; (5) Tunisia; (6) Greece; (7) USA.

Table 4 - Linear discriminant analysis of TGs: statistics and classification of results.

FD	Eigenvalue	Variance (%)	Canonical correl.	P-Value
Triacylglycerols				
1	33.36	78.52	0.985	0.0000
2	5.88	13.86	0.924	0.0143
		Classification of results		
Groups	Cases	Cases correct	Cases not correct	Total cases correct (%)
Triacylglycerols				
Italy	14	14	-	100
Turkey	7	7	-	100
Iran	4	3	1[a]	92
Syria	3	3	-	100
Tunisia	3	3	-	100
Greece	3	3	-	100
USA	3	3	-	100
Total	37	36	1	97.29

[a] Classified as American.

Italian, Turkish, Syrian, Tunisian and Greek pistachio samples were similar and differed from the other samples with respect to the high percentage of OLO, OOO, OOP and SOO. Iranian and American samples were located in a completely different zone of the plan, characterized by high percentages of LLL, OLLn, OLL and LLP.

LDA was performed using TGs having F-ratios > 1 and p-values < 0.05. The linear combinations were constituted by LLL, OLLn, OLL, LLP, OLO, OLP, PLP, OOO, OOP and SOO as variables in the 37 samples. Each origin was considered as a class (7 different ones).

Two discriminating functions of the variables were used to distinguish samples of different origins (Table 4). Together these discriminating functions explain 92.4% of the variance and show multiple correlation coefficients and p-values with a confidence level greater than 95%. The standardized coefficient values of the functions indicate that all the variables were important for differentiating the oil. From the relative magnitude of the coefficients in the functions it is possible to determine how the independent variables are being used to discriminate among the different origins.

The classification of samples was very good since among the 37 samples, 36 were correctly classified (97.3%) according to the classification matrix (Table 4), calculated by the LDA. Only 1 case was not correctly classified: one sample from Iran was classified as American. This can be supported by the literature; in fact, American pistachios are originated from the Kerman province (CRANE and IWAKIRI, 1981; CRANE and MARANTO, 1988; CHANG, 1990; KAFKAS *et al.*, 2006).

The robustness of the statistical models was evaluated by building a training data set to validate the predictive ability of the optimized mathematical model. In this case, ten samples (two for each group of origin with n > 3 and one for each group with n < 3) were randomly left out from the original data set and the model was calibrated on the remaining data (27 samples). Among the 27 observations used to fit the model, 25 were correctly classified (92.59%). The statistical results were similar to those of the previous model (37 samples). The ten samples left out from the model were then introduced into the data set as an unknown group. Eight samples were correctly classified; only

Table 5 - Performance of linear discriminant analyses.

Samples	Group size	Predicted Groups						
		Italy	Turkey	Iran	Syria	Tunisia	Greece	USA
Triacylglycerols								
Italy	12	12	-	-	-	-	-	-
Turkey	5	-	5	-	-	-	-	-
Iran	2	-	-	2	-	-	-	-
Syria	2	-	-	-	2	-	-	-
Tunisia	2	-	-	-	-	2	-	-
Greece	2	-	-	-	-	-	2	-
USA	2	-	-	-	-	-	-	2
Unknown	10	2	2	-	1	1	1	3*

* Two samples not correctly classified.

two samples coming from Iran were classified as American (Table 5).

CONCLUSION

PCA and LDA analyses of TG types highlighted the possibility of distinguishing the geographic origin of pistachios. The Mediterranean area samples were similar to each other with respect to the FA distribution in the TGs; Iranian and American samples were completely different from the other samples, but similar to each other for this criterion, i.e. the FA distribution in the TGs.

For more accurate conclusions, this investigation should be extended to a wider number of samples. In any case, HPLC, when combined with LDA proved to be a suitable method for the discrimination of pistachio oils of different origins according to the FA distribution in the TGs.

REFERENCES

Acar I., Kafkas E., Ozogul Y., Dogan Y. and Kafkas S. 2008. Variation of fat and fatty acid composition of some pistachio genotypes. Ital. J. Food Sci. 20: 273.

Adebowale K. O. and Nicholson G. 2002. Triacylglycerols in some underutilized tropical seed oils I. Systematic studies of ten oils. Riv. Ital. Sostanze Gr. 79: 267.

Anderson K. and Smith B. W. 2005. Use of chemical profiling to differentiate geographic growing origin of raw pistachio. J. Agric. Food Chem. 53: 410.

Arena E., Campisi S., Fallico B. and Maccarone E. 2007. Distribution of fatty acids and phytosterols as a criterion to discriminate geographic origin of pistachio seeds. Food Chem. 104: 403.

Aparicio R. and Luna G. 2002. Characterization of monovarietal virgin olive oils. Eur. J. Lipid Sci. Tech. 104: 614.

Balestrieri F. and Marini D. 1996. Metodi di Analisi Chimica dei Prodotti Alimentari, p. 182, 223, 249, 270. Monolite editrice srl, Rome, Italy.

Bellia F. 2007. Indagini territoriali ed aziendali sulla frutticoltura etnea: risultati economici e prospettive. Principali risultati del Progetto di Ricerca Miglioramento e Valorizzazione delle Produzioni Frutticole Etnee, Vol 1, p. 125. Emme Erre Grafica srl, Tremestieri Etneo, Italy.

Bellomo M. G. and Fallico B. 2007. Anthocyanins, chlorophylls and xantophylls in pistachio nuts (*Pistacia vera*) of different geographic origin. J. Food Compos. Anal. 20: 352.

CEE 2004. Norme CEE-ONU DDF-09 concernant la commercialisation et le contrôle de la qualité des pistaches en coque. Available on: http://www.unece.org/trade/agr/meetings/standard/dry/dry_f/09unpist.pdf.

Chahed T., Hamrouni I., Dhifi W., Msaada K., Kchouk M. E. and Marzouk B. 2006. Lipid evaluation during the development of pistacchio seed from the region of Kairouan (middle of Tunisia). J. Food Lipids. 13: 375.

Chahed T., Bellila A., Dhifi W., Hamrouni I., M'handi B., Kchouk M. E. and Marzouk B. 2008. Pistachio (Pistacia vera) seed oil composition: geographic situation and variety effects. Grasas Aceites. 59 (1): 51.

Chang K. 1990. Market prospects for edible nuts. Nut production and industry in Europe, Near East and North Africa. REUR Technical Series. 13: 47.

Crane J. C. and Iwakiri B. T. 1981. Morphology and reproduction of pistachio. Hort. Rew. 3:376.

Crane J. C. and Maranto J. 1988. Pistachio Production, publication No: 2279, p. 15. University of California, Agriculture and Natural Resources, Oakland, USA.

Di Marco L. 1987. Il pistacchio in Sicilia: situazione e prospettive. Agricoltura Ricerca. 79: 9.

Dugo P., Kumm T., Crupi M. L., Cotroneo A. and Mondello L. 2006. Comprehensive two dimensional liquid chromatography combined with mass spectometric detection in the analyses of triacylglycerols in natural lipidic matrixes. J. Chromatogr. A. 1112: 269.

Dyszel S. M. and Pettit B. C. 1990. Determination of the country of origin of pistachio nuts by DSC and HPLC. JAOCS. 67: 947.

FAO. 2007. FAOSTAT database. Available on: http://faostat.fao.org/site/567/default.aspx#ancor.

Garcia J. M., Agar I. T. and Streif J. 1992. Analysis of fat content and fatty acid composition in individual seeds in pistachio varieties grown in Turkey. Gartenbauwissenschaft. 57: 130.

Galvano F., Pietri A., Fallico B., Bertuzzi T., Scirè S., Galvano M. and Maggiore R. 1996. Activated carbons: in vitro affinità for aflatoxin B_1 and relation of absorption ability to physicochemical parameters. J. Food Protect. 59: 545.

Holcapek M., Jandera P., Zderadicka P. and Hruba L. 2003. Characterization of triacylglycerol and diacylglycerol composition of plant oils using high-performance liquid chromatography-atmospheric pressure chemical ionization mass spectrometry. J. Chromatogr. A. 1010: 195.

Holcapek M., Lisa M., Jandera P. and Kabatova N. 2005. Quantitation of triacylglycerols in plant oils using HPLC with APCI-MS, evaporative light-scattering, and UV detection. J. Sep. Sci. 28: 1315.

Kafkas S., Ozkan H., Ak B. E., Acar I., Atli H. S. and Koyuncu S. 2006. Detecting DNA polymorphism and genetic diversity in a wide pistachio germplasm: Comparison of AFLP, ISSR, and RAPD markers. J. Am. Soc. Hortic. Sci. 131: 522.

Kucukoner E. and Yurt B. 2003. Some chemical characteristics of *Pistacia vera* varieties produces in Turkey. Eur. Food Res. Technol. 217: 308.

Lua A.C. and Yang T. 2005. Characteristics of activated carbon prepared from pistachio-nut shell by zinc chloride activation under nitrogen and vacuum conditions. J. Colloid Interf. Sci. 290: 505.

Marini F., Balestrieri F., Bucci R., Magrì A. L. and Marini D. 2003. Supervised pattern recognition to discriminate the geographical origin of rice brain oils: a first study. Microchem. J. 74: 239.

Maskam M. and Karatas S. 1998. Fatty acid oxidation of pistachio nuts stored under variation atmospheric conditions and different temperatures. J. Sci. Food Agr. 77: 334.

Mattson F. H. and Volpenhein R. A. 1961. The specific distribution of fatty acids in the glycerides of vegetable fats. J. Biol. Chem. 236: 1891.

Mattson F. H. and Volpenhein R. A. 1963. The specific distribution of unsaturated fatty acids in the triglycerides of plants. J. Lipid. Res. 4: 392.

Ohlrogge J. and Browse J. 1995. Lipid biosynthesis. Plant Cell. 7: 957.

Okay Y. 2002. The comparison of some pistachio cultivars regarding their fat, fatty acids and protein content. Gartenbauwissenschaft. 67: 107.

Ranalli A., de Mattia G., Ferrante M. L. and Giansante L. 1997. Incidence of olive cultivation area on the analytical characteristics of the oil. Note 1. Riv. Ital. Sostanze Gr. 74: 501.

Ruggeri S., Cappelloni M., Gambelli L. and Carnevale E. 1998. Chemical composition and nutritive value of nuts grown in Italy. Ital. J. Food Sci. 10: 243.

Satil F., Azcan N. and Baser K. H. C. 2003. Fatty acid composition of pistachio nuts in Turkey. Chem. Nat. Compd. 39: 322.

Seferoglu S., Seferoglu H. G., Tekintas F. E. and Balta F. 2006. Biochemical composition influenced by different locations in Uzun pistachio cv. (*Pistacia vera* L.) grown in Turkey. J. Food Compos. Anal. 19: 461.

Venkatachalam M. and Sathe S. K. 2006. Chemical composition of selected edible nut seeds. J. Agric. Food Chem. 54: 4705.

Yildiz M., Turcan G. S. and Ozdemir M. 1998. Oil composition of pistachio nuts from Turkey. Eur. J. Lipid Sci. Tech. 100: S. 84.

Revised paper received June 30, 2009 Accepted September 15, 2009

DISCRIMINANT ANALYSIS OF ALMOND CULTIVARS USED IN *TURRÓN*

A. VERDÚ, L. VÁZQUEZ-ARAUJO[1], A. MIQUEL[1], F. MARTÍNEZ-SÁNCHEZ[1]
and A.A. CARBONELL-BARRACHINA[1,*]
Consejo Regulador de las Denominaciones Específicas Jijona y Turrón de Alicante,
C/ Font del Moratell, s/n. 03100 Jijona, Alicante (España/Spain)
[1]Universidad Miguel Hernández, Departamento Tecnología Agroalimentaria,
Carretera de Beniel, km 3.2. 03312-Orihuela, Alicante (España/Spain)
*Corresponding author: Tel. +34 966 749754, Fax +34 966 749677,
e-mail: angel.carbonell@umh.es

ABSTRACT

Turrón is a typical Spanish confectionery product made from almonds, honey and sugar. Discriminant analysis was applied to 45 Alicante *turrón* and 9 Jijona *turrón* samples to establish which quality parameters could be applied to obtain a mathematical model for classifying *turrón* according to the almond cultivar used to manufacture it. Three different cultivars were studied: "Comuna" and "Marcona" (Spain) and "California" (USA). The parameters used for establishing the mathematical model were: palmitoleic acid, linoleic acid, arachidic acid, stigmasterol, β-sitosterol and δ-stigmasterol. The model developed was able to classify correctly 100% of the samples. This model will be a very useful control tool (Control Organizations) and *turrón* traders will be able to prove that they have used a specific almond cultivar in their products.

- Key words: almond, discriminant analysis, fatty acids, Protected Geographical Indication, sterols, torrone -

INTRODUCTION

Alicante *turrón* is a typical Spanish confectionery product made in a traditional way using high quality toasted almonds, honey, sugar and egg whites (CHIRALT *et al.*, 1991). The high quality raw materials used lead to final products with very typical sensory characteristics (flavor and texture) that are very appealing to consumers. This confection provides consumers with a considerable intake of nutrients, including magnesium, potassium, oleic and linoleic acids and phytosterols (e.g. β-sitosterol and campesterol).

While Alicante and Jijona *turrón* have been mainly marketed in Spain for many years, nowadays these Spanish confections are sold all over the world. The countries with the highest *turrón* consumption are the USA and Venezuela, with total exportations from Spain in 2007 of 247 and 83 t, respectively (VERDÚ *et al.*, 2009).

In Spain, *turrón* is commercially classified according to its almond percentage. In fact, Spanish legislation, through the Technical-Sanitary Regulation for the Production and Selling of *Turrón* and Marzipans (RTS, 1982) only establishes the limits for the almond content. In this way, Supreme products (the only ones protected by Geographical Indications "*Jijona*" and "*Turrón de Alicante*") require a minimum almond percentage of 60 % in Alicante *turrón*. However, the Regulating Council of the Protected Geographical Indications of Jijona and *Turrón* de Alicante (RCPGIJTA) also establishes the minimum requirements for honey content, 10%. Products made under the control and supervision of the RCPGIJTA are named "protected *turrón*" (BOE, 1996).

The almond percentage and cultivars used in protected *turrón* are two of the main quality criteria used in commercial transactions. These parameters are quite easy to quantify and identify in Alicante *turrón*, in which almonds are found intact or in large pieces. After removing the caramel matrix with hot water, the almond cultivar can be identified by visual inspection and after filtering and drying of the remaining almonds, it is possible to quantify the almond content. However, the identification of the almond cultivar and quantification of the almond content are not possible in Jijona *turrón* samples because thealmonds have been crushed in a stone mill and ground to a very small particle size.

Almond (*Prunus amygdalus* L.) production occupies an important place in Spanish agriculture; mild weather conditions are favorable for its growth. Nevertheless, in recent years, Spanish almond production has decreased considerably, while production in the USA has increased rapidly due to the investment of large amounts of money to improve agricultural techniques and the selection of new almond cultivars. This situation has led to competition between American and Spanish almond producers, with some Spanish varieties, such as "Marcona", being more expensive because of their prized sensory properties and low production (PRATS-MOYA *et al.*, 1997). During the 2006-2007 season, about 6895 t and 5129 t of peeled almonds of Spanish and North-American cultivars, respectively, were marketed in Spain (CRIGPJTA, 2009).

Three almond cultivars are used to manufacture *turrón*. Two cultivars, "Marcona" and "Comuna", are widely grown in eastern Spain, while the third one, "California", is grown in the USA. The first two cultivars are mainly used to make protected *turrón*, while the American variety is used in non-protected *turrón*. Even though the Spanish varieties are grown in the vicinity of the *turrón* industries, the cost is significantly higher than that of the American variety due to lower production. The cost of the almonds used in this experiment were: "Marcona" 6.71 € kg^{-1}, "Comuna" 5.71 € kg^{-1}, and "California" 5.00 € kg^{-1} (COLEFRUSE, 2009)]. According to *turrón* manufacturers, the cultivar "Marcona" has superior sensory qualities compared to the other cultivars, which justifies the higher price.

Given that the "Marcona" almonds as well as the *turrón* prepared using this almond cultivar are more expensive, traders need a reliable method to differentiate this almond cultivar from the other ones in Jijona *turrón*. The RCPGIJTA needs a method to determine whether the correct almond cultivars are being used to manufacture protected products. To date, there is no analytical method for identifying the specific almond cultivar that is being used. The main objective of this work was to establish a mathematical model that would discriminate which parameters of the Alicante *turrón* composition are the most suitable for classifying Alicante *turrón* samples according to the almond cultivar used. Only quality parameters carried out in routine analyses (almond, moisture, fiber, protein, fat, and total sugars content and fatty acid composition) were included in the first mathematical modeling. More sophisticated and expensive parameters (sterol composition) were included later after the first attempt failed. The model developed for Alicante *turrón* was tested on samples of Jijona *turrón*.

MATERIALS AND METHODS

Materials

The samples of Alicante *turrón* (AT) analyzed in this study were manufactured by three companies for the RCPGIJTA; each company manufactured five samples (5 bars per sample) for each of the almond cultivars ("Marcona", "Comuna", and "California"). This was the only way to be sure that only one almond cultivar was used to manufacture the samples. The "Marcona" and "Comuna" samples were grown in the area of Valencia (Spain), while the "California" samples were from California (U.S.A). All 45 *turrón* samples (3 almond cultivars × 5 samples × 3 companies) were manufactured during the 2008 season; the factor of company was not used in the statistical study because all of the companies were instructed to follow the same manufacturing procedure.

Physico-chemical analyses

Samples of AT were treated as recommended by the Spanish Ministry of Health and Consumption (MSC, 1985) to obtain a homogeneous product for analyses. The initial samples were cut into small cubes and then ground using a domestic grinder.

Moisture was calculated by drying approximately 3 g of fresh sample at 95°-100°C until constant weight (AOAC Official Method 925.40) (HORWITZ, 2000c).

Nitrogen was determined by the Kjeldahl method and the protein (crude) content was obtained by multiplying the N result by 5.18 for almonds and almond-based products (AOAC Official Method 950.40) (HORWITZ, 2000d).

Crude fiber is the dried residue that remains after digesting the sample with 1.25 % (mass/volume, m/v) H_2SO_4 and 1.25% (m/v) NaOH solutions under specific conditions (AOAC Official Method 935.53) (HORWITZ, 2000b). The method can be used with materials from which the fat has been previously extracted. The digestion was carried out in a Selecta digester, model Dosi-Fiber 4000623 (J.P. Selecta, S.A., Barcelona, Spain).

Fat was extracted from 2 g of test sample for 16 h in a Soxhlet-type extractor with petroleum ether (AOAC Official Method 948.22) (HORWITZ, 2000a).

Sugars were analyzed according to AOAC Official Method 977.20 (HORWITZ, 2000e) for separating sugars in honey and honey-based products. Five grams of *turrón* samples were mixed with 25 mL H_2O, diluted to 50 mL with CH_3CN and filtered through a 0.45 μm filter. Samples were injected into a HPLC (Hewlett Packard model 1100 Series) connected to a refractive index detector, RID (HP Model 1100 Series) with an auto-sampler (HP Model 1100 Series), operated by HP ChemStations software (Hewlett Packard, Waldbronn, Germany). Sugars were separated using a stainless steel Supelcogel C-610H column (30.0 cm × 7.8 mm id) with a Supelguard C-610H guard column (5.0 cm × 4.6 mm id) using LC grade acetonitrile diluted with ultrapure water (83+17) at a flow rate of 1.0 mL min^{-1}. Authenticated standards of sucrose, glucose, fructose, and maltose were used to identify and quantify the sugars.

The toasted almond content in Alicante *turrón* was determined after dissolving the sugar matrix in water, filtering it and then weighing the dried residue (MSC, 1985).

Even though the AT samples were supposed to have been prepared using only one almond cultivar, this was checked in the final products (it was imperative that all of the samples were independent and no sample belonged to two groups). The almonds that remained after dissolving the sugar matrix were used for the almond cultivar identification by the trained panel of RCPGIJTA (VÁZQUEZ-ARAÚJO *et al.*, 2008). Details about panel selection and training can be found in VÁZQUEZ-ARAÚJO *et al.* (2005). Experimental results showed that the proper almond cultivar was used in all of AT samples manufactured.

Fatty acids were analyzed by GC-MS after derivatization to fatty acid methyl esters (FAMEs) with 2N KOH in methanol, according to the IUPAC standard method (IUPAC, 1992). A Shimadzu GC-17A coupled with a Shimadzu mass spectrometer detector GC-MS QP-5050A and a Supelcowax™-10 column (60 m × 0.25 mm × 0.25 μm film thickness) were used. Details about GC and MS conditions can be found in (FULLANA *et al.*, 2004). Authenticated standards of methyl esters of the following acids: palmitic, palmitoleic, stearic, oleic, linoleic, and arachidic, were used to identify and quantify the fatty acids.

Sterols were analyzed by GC-MS according to the MSC (MSC, 1985; IOOC, 2001), equivalent to the UNE norm 55-019-73. The fatty substance, with β-cholestanol added as an internal standard, was saponified with potassium hydroxide in the ethanolic solution and the unsaponifiable compounds were extracted with ethyl ether. The sterol fraction was separated from the unsaponifiable extract by chromatography on a basic silica gel plate. The sterols recovered from the silica gel were transformed into trimethyl-silyl ethers and were analyzed by GC-MS using the previously described, Shimadzu GC-MS QP-5050A system. Authenticated standards of cholesterol, campesterol, stigmasterol, δ-sitosterol, and β-stigmasterol were used to identify and quantify the sterols.

All of the physico-chemical analyses were run at least in triplicate and are expressed as the "mean ± standard error".

Mathematical and statistical analyses

Discriminant analysis is a multivariate technique that is used to classify items into pre-existing categories (defined by a discrete dependent variable). A mathematical function is developed using the set of continuous independent variables that best discriminate the categories from which the items arise. For instance, descriptive attribute data might be used to classify a finished product as being "acceptable" or "unacceptable" from a quality control perspective or descriptive and/or instrumental measures can be used to determine the source (e.g., country or manufacturer) of a raw ingredient (MEILGAARD *et al.*, 1999).

The SPSS statistical package version 11.5 (SPSS Inc., Chicago, IL, USA) was used to develop mathematical models, using discriminate analysis, to classify samples of Alicante *turrón* according to the almond cultivar used in their manufacture ("Comuna", "Marcona", and "California"); quality data were used (almond, moisture, fiber, protein, fat and sucrose contents and fatty acid and sterol composition). The criterion used to decide which variables should be included in the model was to minimize the Wilks' lambda. Figures were prepared using Sigma Plot 9.0 (SPSS Science, Chicago, USA).

RESULTS AND DISCUSSION

Forty-five samples of Alicante *turrón* were classified according to the three almond cultivars ("Comuna", "Marcona" and "California") used in this confection using quality control data: almond, moisture, fiber, protein, fat and total sugars and fatty acid composition (Table 1). The individual sugar contents were assayed but the results did not improve the quality of the models developed. Therefore, only the total sugar content was used. The main sugars, fructose, glucose, sucrose and maltose, found in Alicante *turrón* were 7.61±0.16%, 9.04±0.23%, 19.8±0.3% and 0.90±0.15%, respectively.

The discriminant analysis showed that all 45 samples were apt for the mathematical modeling and that the first mathematical model could be built using only 4 (fiber, C16:1, C18:2, and C20:0) of the 12 parameters provided (routine analyses: almond, moisture, fiber, protein, fat, sugars; fatty acids: C16:0, C16:1, C18:0, C18:1, C18:2, C20:0). The fact that the Wilks' lambda values were not very low (not close to zero, $0.468 > \lambda > 0.151$), indicated that the categories ("Comuna", "Marcona" and "California") of the dependent variable (almond cultivar) were probably not completely discriminated. The *p*-values (equal to 0) as well as the statistics of the "exact *F*" of the Wilks' lambda (greater than 15) certified the significance of the discriminant functions. The categories of the almond cultivars could be discriminated. The discriminant functions (DF) developed were as follows:

$DF_1 = 0.108 \times \text{fiber} - 8.093 \times \text{palmitoleic acid} + 0.455 \times \text{linoleic acid} - 0.513 \times \text{arachidic acid} - 5.892$

$DF_2 = 0.243 \times \text{Fiber} + 18.361 \times \text{palmitoleic acid} + 0.078 \times \text{linoleic acid} - 0.418 \times \text{arachidic acid} - 13.109$

The first discriminant function (DF_1) explained 68.4 % of the variability of the model, while DF_2 explained the remaining 31.6%. According to the *p*-values of the Wilks' lambda both functions were significant for the canonical discriminant functions.

Centroids for the three almond cultivars were: a) "Comuna" DF_1 -1.288 and DF_2 -1.084,

Table 1 - Main characteristics of Alicante *turrón* samples (2008 season).

Parameter	Almond Cultivar		
	"Comuna"	"Marcona"	"California"
ROUTINE ANALYSES (%)			
Almond	63.6±1.1	65.1±0.9	61.2±0.9
Moisture	3.73±0.18	3.61±0.26	3.34±0.15
Fiber	2.86±0.50	4.49±0.82	6.79±0.59
Protein	14.40±0.52	14.80±0.34	13.82±0.30
Fat	37.7±0.9	38.6±0.7	35.3±0.6
Sugars	40.7±0.9	39.2±1.1	40.8±1.1
FATTY ACIDS (%)			
Palmitic acid, 16:0	6.38±0.12	6.71±0.10	7.29±0.70
Palmitoleic acid, 16:1	0.55±0.01	0.64±0.01	0.51±0.01
Stearic acid, 18:0	2.25±0.06	3.61±1.33	2.14±0.06
Oleic acid, 18:1	70.5±1.3	69.1±0.5	66.0±0.6
Linoleic acid, 18:2	20.4±0.6	21.7±0.5	25.0±0.3
Arachidic acid, 20:0	0.95±0.06	0.07±0.01	0.13±0.04
STEROLS [mg (100 g^{-1})]			
Cholesterol	0.32±0.02	0.24±0.04	0.31±0.06
Campesterol	6.41±0.19	5.74±0.21	5.40±0.15
Stigmasterol	1.14±0.06	0.62±0.21	0.66±0.04
β-Sitosterol	215±6	217±5	211±6
δ-Stigmasterol	1.73±0.26	1.73±0.13	1.83±0.08

b) "Marcona" DF_1 -0.737 and DF_2 1.300, and c) "California" DF_1 2.025 and DF_2 -0.216 (Fig. 1A). The centroid values implied that the "California" samples could be distinguished from the Spanish cultivars, but the discrimination between "Comuna" and "Marcona" samples was not as good as required.

Table 2 summarizes the results of the discriminant classification of the 45 Alicante *turrón* samples; the model successfully classified 82.2% of the original samples: 80.0, 66.7, and 100% of "Comuna", "Marcona" and "California" samples, respectively. The best results were for "California" samples, while the results obtained for the other two almond cultivars were not as good. It is important to distinguish between non-Spanish (only for non-protected products) and Spanish cultivars (mainly used in protected products); in this way, only 2 of the 45 samples (4.4 %) were not classified correctly by the model.

To improve this initial model, all Alicante *turrón* samples were analyzed for sterols. The results are summarized in Table 1. This analysis is not usually carried out in the routine quality control analyses of the RCPGIJTA.

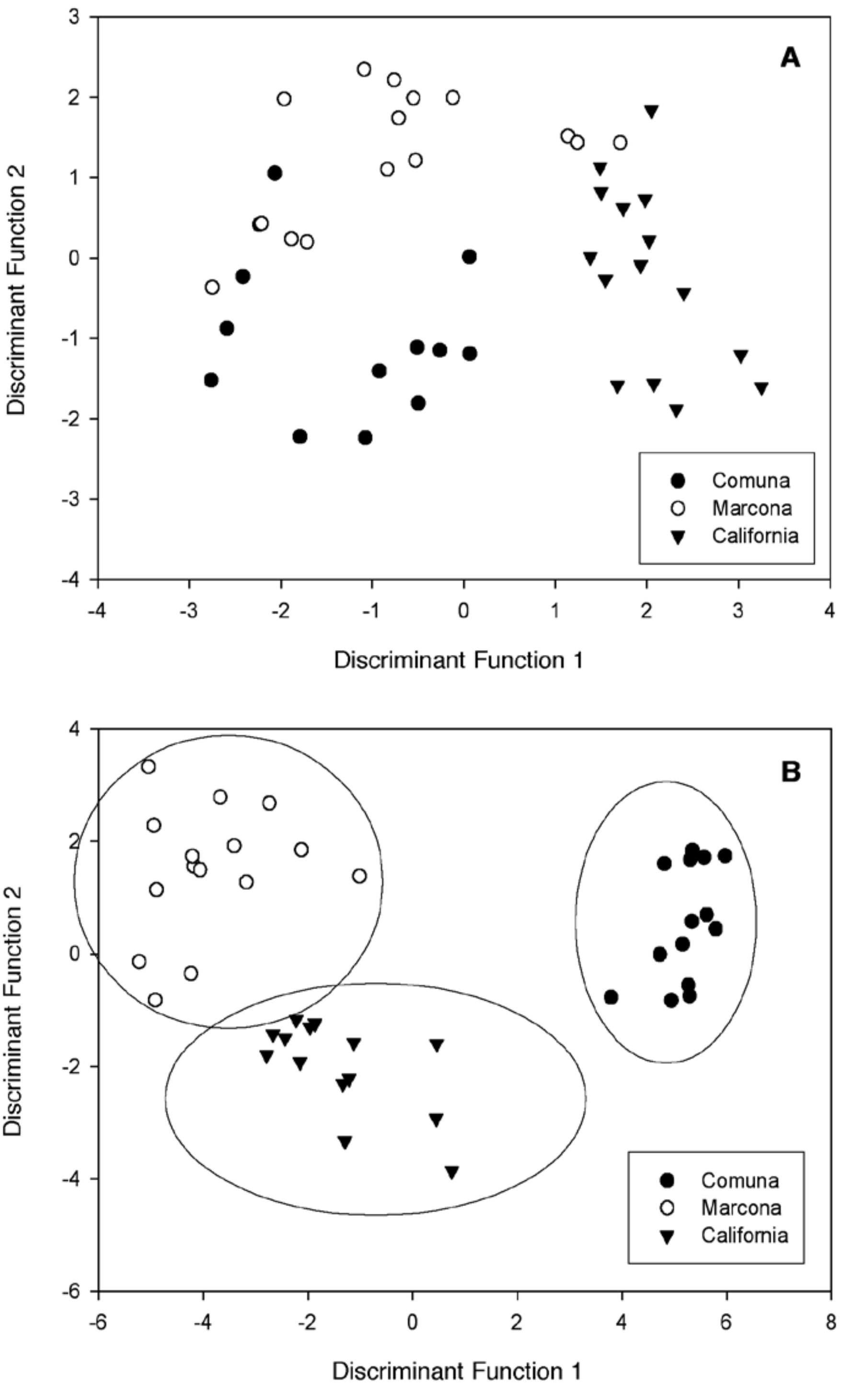


Fig. 1 - Scatter-plot for Alicante *turrón* prepared using three almond cultivars (upper figure (A): first model; lower figure (B): second model).

All 45 samples were again used to build the second model; 6 parameters (C16:1, C18:2, C20:0, stigmasterol, β-sitosterol, and δ-stigmasterol) of the 17 provided (previous 6 routine parameters; previous 6 fatty acids; cholesterol, campesterol, stigmasterol, β-sitosterol, and δ-stigmasterol). The Wilks' lambda values ($0.344 > \lambda > 0.018$) were lower than in the first model, indicating that the discrimination among the almond cultivars was better than before. The *p*-values (equal to 0) and statistics of the "exact *F*" of the Wilks' lambda (higher than 37) certified the significance of the discriminant functions of this second model. The discriminant functions (DF) developed were as follows:

DF_1' = – 13.658 × palmitoleic acid – 0.160 × linoleic acid + 1.171 × arachidic acid + 12.591 × stigmasterol – 0.061 × β-sitosterol + 0.993 δ-stigmasterol + 12.062

DF_2' = 16.081 β palmitoleic acid – 0.449 δ linoleic acid + 0.305 × arachidic acid – 0.902 × stigmasterol – 0.007 × β-sitosterol + 0.474 δ-stigmasterol + 2.308

The first discriminant function (DF_1') explained 87 % of the data variability, while the second one (DF_2') explained the remaining 13 %; both func-

Table 2 - Classification matrix of almond samples from different cultivars based on the main physico-chemical parameters (almond, moisture, fiber, protein, fat and sucrose contents) and fatty acid composition.

Group	Percent correct	Number of cases classified into group		
		"Comuna"	"Marcona"	"California"
"Comuna"	80.0	**12**	3	0
"Marcona"	66.7	3	**10**	2
"California"	100	0	0	**15**

Table 3 - Classification matrix of almond samples from different cultivars based on the main physico-chemical parameters (almond, moisture, fiber, protein, fat and sucrose contents) and fatty acid and sterol compositions.

Group	Percent correct	Number of cases classified into group		
		"Comuna"	"Marcona"	"California"
"Comuna"	100	**15**	0	0
"Marcona"	100	0	**15**	0
"California"	100	0	0	**15**

tions were significant according to the Wilks' lambda *p*-values for the canonical discriminant functions.

The centroids for three almond cultivars were: a) "Comuna" DF_1 5.235 and DF_2 0.553, b) "Marcona" DF_1 -3.854 and DF_2 1.478, and c) "California" DF_1 -1.380 and DF_2 -2.031 (Fig. 1B).

Table 3 summarizes the results of the discriminant classification of the 45 Alicante *turrón* samples with complete analyses (routine analyses + fatty acids + sterols). The model successfully classified 100% of the original samples.

As expected, the model did not use any of the routine composition analyses (almond content, moisture, etc.) because the differences among the *turrón* samples from the different almond cultivars were too small and not statistically significant. A previous study (MIQUEL SIRVENT, 2008) in which more than 200 AT samples from three different seasons (2004-2006) were used concluded that it is impossible to discriminate among almond cultivars using only routine analyses. The final model developed to classify the almond cultivar in the AT samples included three variables from the fatty acid analyses and another three from the sterol analyses. Alicante *turrón* made with "Comuna" almonds had the highest values for arachidic acid and stigmasterol and the lowest linoleic acid value. Alicante *turrón* made with "Marcona" almonds had the highest palmitoleic acid and β-sitosterol values, and the lowest arachidic acid and stigmasterol values. Finally, AT made with "California" almonds had the highest linoleic acid and δ-stigmasterol values and the lowest palmitoleic acid and β-sitosterol values.

To validate the goodness of the model based on the composition data from Alicante *turrón* samples, it was applied to 9 Jijona *turrón* samples (3 *turrón* samples made with each of the 3 almonds cultivars), randomly selected from those collected by the RCPGIJTA during the 2008 season. The model was able to discriminate all 9 samples.

CONCLUSIONS

The first mathematical model which was developed using only routine quality parameters (almond content, moisture, fiber, protein, fat, sugars and fatty acid) was not able to correctly classify all the Alicante *turrón* samples according to the almond cultivar used. However, when sterol composition was used, the model was able to successfully classify 100% of the samples according to the almond cultivar used to manufacture this confection. The mathematical model included the following variables: palmitoleic acid, linoleic acid, arachidic acid, stigmasterol, β-sitosterol and δ-stigmasterol. These results could be quite valuable for the RCPGIJTA to establish a proper control for Alicante *turrón*, and especially for Jijona *turrón* since the almond cultivar used cannot be visually identified. Traders around the world will now have a tool that can be used to prove that legally established almond cultivars have been used to make Alicante and Jijona *turrón*.

REFERENCES

BOE. 1996. Orden de 22 de Marzo de 1996 por la que se ratifica el Reglamento de las Denominaciones Específicas "Jijona" y "Turrón de Alicante" y su Consejo Regulador Común (BOE núm. 98 de 23 de abril). Boletín Oficial del Estado (BOE). Madrid, Spain.

Chiralt A., Galotto M.J. and Fito P. 1991. Changes in rheological properties and particle-size distribution during the manufacture of "Xixona turrón". J. Food Eng. 14: 117.

Colefruse. 2009. Colefruse Internacional. http://www.colefruse.es Accessed 15th July, 2009.

CRIGPJTA (Consejo Regulador Indicaciones Geográficas Protegidas Jijona y Turrón de Alicante). 2009. http://www.jijona.com Accessed 15th July, 2009.

Fullana A., Carbonell-Barrachina A.A. and Sidhu S. 2004. Volatile aldehyde emissions from heated cooking oils. J. Sci. Food Agric. 84: 2015.

Horwitz W. 2000a. Fat (crude) in nuts and nut products. Gravimetric method (AOAC Official Method 948.22). In "Officials Methods of Analysis of AOAC International", 17th ed. AOAC International. Gaithersburg, MD.

Horwitz W. 2000b. Fiber (crude) in nuts and nut products (AOAC Official Method 9235.53). In "Officials Methods of Analysis of AOAC International", 17th ed. AOAC International. Gaithersburg, MD.

Horwitz W. 2000c. Moisture in nuts and nut products (AOAC Official Method 925.40). In "Officials Methods of Analysis of AOAC International", 17th ed. AOAC International. Gaithersburg, MD.

Horwitz W. 2000d. Protein (crude) in nuts and nut products. Improved Kjeldahl method (AOAC Official Method 950.48). In "Officials Methods of Analysis of AOAC International", 17th ed. AOAC International. Gaithersburg, MD.

Horwitz W. 2000e. Separation of sugars in honey. Liquid chromatographic method (AOAC Official Method 977.20).

In “Officials Methods of Analysis of AOAC International”, 17th ed. AOAC International. Gaithersburg, MD.

IOOC (International Olive Oil Council). 2001. Determination of the composition and content of sterols by capillary-column gas chromatography. COI/T.20/Doc. No. 10/ Rev. 1. http://www.internationaloliveoil.org. Accessed 30th April, 2009.

IUPAC (International Union of Pure and Applied Chemistry). 1992. Standard methods for the analysis of oils, fats and derivatives, 7th ed. Pergamon Press. Oxford, UK.

Meilgaard M., Civille G.V. and Carr B.T. 1999. “Sensory Evaluation Techniques”, 3rd ed. CRC Press. Boca Raton, FL.

Miquel Sirvent MMA. 2008. Aplicación del análisis discriminante para la caracterización y clasificación del turrón de Alicante y de Jijona y la almendra [Master Thesis]. Universidad Miguel Hernández. Orihuela, Alicante, Spain.

MSC (Ministerio de Sanidad y Consumo). 1985. Turrón y mazapán. In: Análisis de alimentos. Métodos oficiales y recomendados por el Centro de Investigación y Control de la Calidad. MSC. Madrid, Spain.

Prats-Moya S., Grané-Teruel N., Berenguer-Navarro V. and Martín-Carratalá M.L. 1997. Inductively coupled plasma application for the classification of 19 almond cultivars using inorganic element composition. J. Agric. Food Chem. 45: 2093.

RTS. 1982. Reglamentación Técnico Sanitaria para la elaboración, circulación y venta de turrones y mazapanes. Real Decreto 1787/82. (BOE 2/08/82) modified by Real Decreto 1167/90 (BOE 26/09/90). Boletín Oficial del Estado (BOE). Madrid, Spain.

Vázquez-Araújo L., Enguix L., Verdú A., García-García E. and Carbonell-Barrachina A.A. 2008. Investigation of aromatic compounds in toasted almonds used for the manufacture of *turrón*. Eur. Food Res. Technol. 227: 243.

Vázquez-Araújo L., Pérez-Castejón V., Verdú A. and Carbonell-Barrachina A.A. 2005. Reclutamiento, selección, entrenamiento y validación de un panel de catadores especializado en turrón y sus materias primas. Alim. Equip. Tecnol. 10: 92.

Verdú A., Vázquez Araújo L., Miquel A., Martínez Sánchez F., Carbonell Barrachina A.A. 2009. Calidad de los turrones de Jijona y Alicante. Comparativa entre los destinados al mercado nacional e internacional. Alim. Equip. Tecnol. 240: 24.

Paper received May 4, 2009 Accepted August 3, 2009

EVALUATION OF RHEOLOGICAL BEHAVIOUR OF WHOLE RYE AND BUCKWHEAT BLENDS WITH WHOLE WHEAT FLOUR USING MIXOLAB

I. BANU*, I. VASILEAN and I. APRODU
Dunarea de Jos Galati University, Faculty of Food Science and Engineering,
111, Domneasca St., 800201, Romania
*Corresponding author: iuliana.banu@ugal.ro

ABSTRACT

Knowledge of the rheological properties of flour is essential for establishing baking parameters. The effect of adding different amounts of whole rye and buckwheat flours (10, 20 and 30%) on the rheological behaviour of wheat dough subjected to mixing and thermal constraints was studied using the Mixolab system. The results showed that the addition of buckwheat and rye induces significant changes in the physical properties of whole wheat flour dough. A synergetic effect was observed in both wheat/rye and wheat/buckwheat blends, in terms of peak torque (C3), minimum torque reached during cooling (C4) and torque after cooling (C5), indicating the suitability of the tested blends for bread making.

- Key words: buckwheat, Mixolab, rheological behaviour, rye, wheat -

INTRODUCTION

Whole grain cereal products are important components of the daily diet worldwide. Health effects of diets rich in whole grain and cereal fibre are well known. A high consumption of these products is associated with a decreased risk of diabetes; many epidemiological studies clearly point out the protective effect of whole grain cereals on myocardial infarctions, and certain cancers (JACOBS and GALLAHER, 2004; VENN and MANN, 2004; LARSSON *et al.,* 2005). Rye and buckwheat can be used to enhance the nutritional quality of bread.

Rye (*Secale cereale*) is an important source of dietary fibre and bioactive compounds such as alkyresorcinols, lignans, phenolic acids, phytosterols, tocopherols, tocotrienols and folates (KUJALA, 2002, LIUKKONEN *et al.*, 2003, KATINA *et al.*, 2007). Pentosans and enzymes are constituents of rye that have special significance for baking quality. A high pentosan content and particularly high amounts of water-extractable pentosans are beneficial for rye bread processing, but are detrimental for feeding purposes (WEIPERT, 1995). Resistance to pre-harvest sprouting is considered the most important condition for rye baking quality, as well as pentosans and their corresponding hydrolytic enzymes (GUNNARSSON, 1995, VINKX and DELCOUR 1996).

Buckwheat (*Fagopyrum esculentum*) is an important source of proteins with high biological value (BV) (92.3% of the BV of nonfat dried milk and 81.5% of the BV of dried whole egg, DUARTE *et al.*, 1998). The amino acid profile of these proteins is well balanced and nutritionally superior to other cereals; in particular, the lysine content of buckwheat proteins is significantly higher than other cereals (6.1% compared to 2.4-4.0%, RENZETTI *et al.*, 2008). In the United States, Japan and the Eastern European countries, buckwheat flour is currently mixed in different proportions (10-50%) with other cereal flours and used in different cereal-based products (DUARTE *et al.*, 1998, PRUSKA-KEDZIOR *et al.*, 2008).

Knowledge of the rheological properties of flour is essential for establishing baking parameters. Different empirical methods based on classical extensograph, alveograph, farinograph and mixograph instruments are currently used to obtain accurate data on baking properties of flour, by simulating the bread-making steps (UTHAYAKUMARAN *et al.*, 2002, DOBRASZCZYK *et al.*, 2003, TRONSMO *et al.*, 2003, COLLAR *et al.*, 2003, CHIOTELLI *et al.*, 2004).

Different rheological methods have been developed to evaluate the baking quality of rye meal flours (REPECKIENE *et al.*, 2001). Concerning buckwheat, the effect of the protein concentrates on wheat dough properties was studied by BEJOSANO and CORKE (1998), while the rheological and thermal properties of the starch were analysed by LI *et al.* (1997) and QIAN *et al.* (1998).

Recently, the rheological behaviour of buckwheat flour and rye was studied using Mixolab, a new system which measures the torque associated with dough during mixing and heating (Mixolab Applications Handbook, 2006). COLLAR *et al.* (2007) analyzed the potential of the Mixolab device to determine the rheological behaviour of formulated wheat bread dough. ROSELL *et al.* (2007) investigated the effects of several hydrocolloids with different molecular structure on wheat dough behaviour using the Mixolab device. Important studies on the use of Mixolab to predict the bread and cookie baking quality of different wheat flours were carried out by HAROS *et al.* (2006), KAHRAMAN *et al.* (2008), OZTURK et al. (2008) and CATO and MILLS (2008). MARCO and ROSELL (2008) investigated the mixing and thermal characteristics of soybean and rice dough to obtain gluten-free breads using hydroxypropylmethylcellulose as structuring agent and transglutaminase as processing aid. Similar experiments were performed by BONET *et al.* (2006) and ROSELL *et al.* (2009) on wheat dough enriched with different protein sources (soy flour, lupin flour, egg albumin, gelatin powder, protein-rich beer yeast flour) and highly nutritious Andean crops quinoa, kaniwa, kiwicha and tarwi.

The present study was aimed at providing detailed information about the rheological behaviour of whole rye and buckwheat flours mixed with whole wheat flour by using the Mixolab system.

MATERIALS AND METHODS

Five different commercial rye flours from different milling companies and five buckwheat grains sold on the local markets were used in this study. The rye and buckwheat flours were mixed in different percentages (10, 20, and 30%) with the whole wheat flour.

The physical-chemical characteristics of the whole wheat flour were evaluated as follows:

- the moisture content with the AACC 44-51 method;
- the wet gluten content with the SR ISO 21415-2:2007 method;
- the gluten index with the SR ISO 21415-2:2007 method;
- the falling number value with the AACC 56-81B method.

The whole rye flours were analysed in terms of falling number value.

The rheological properties were evaluated using Mixolab Chopin (Tripette & Renaud Chopin, Villeneuve La Garenne, France) in terms of protein quality by determining the water absorption, stability, elasticity and weakening properties, and starch behaviour during gelatinization and retrogradation. The running parameters of the device during the tests are reported in Table 1. The typical curve recorded by the Mixolab is shown in Fig. 1. The recorded parameters were water absorption required for a constant torque of 1.1 Nm; C1 (Nm), maximum torque during mixing; C2 (Nm) measures the protein weakening based on the mechanical work and temperature; C3 (Nm) expresses the starch gelatinization; C4 (Nm) indicates the stability of the starch gel formed; C5 (Nm) measures the starch retrogradation during the cooling stage; α represents the slope of the curve between the end of the period of 30°C and C2 indicates the rate of protein thermal weakening; β represents the slope of the curve between C2 and C3, which gives indications about the gelatinization rate; γ represents the slope of the curve between C3 and C4, that gives indications about the rate of enzymatic hydrolysis.

All tests were carried out in triplicate; the average values are reported together with standard deviation (SD) or coefficient of variation (CV).

RESULTS AND DISCUSSION

The characteristics of the commercial whole wheat flour were: wet gluten content 26.9% (CV = 6.7%), gluten index 93% (CV = 7.5%), falling number value 439 s (CV = 7.5%). The whole rye flours had a falling number value of 277 s (CV = 7.3%).

Table 1 - Settings of the Mixolab.

Parameter	Value
Mixing rate (rpm)	80
Dough weight (g)	75
Tank temperature (ºC)	30
Temperature of the first plateau (ºC)	30
Duration of the first plateau (min)	8
Temperature of the second plateau (ºC)	90
First temperature gradient (ºC/min)	4
Duration of the second plateau (min)	7
Second temperature gradient (ºC/min)	4
Temperature of the third plateau (ºC)	50
Duration of the third plateau (min)	5
Total analysis time (min)	45

The whole rye flours were characterized by low water absorption capacity (62%, CV = 3.3%), comparable to soft whole wheat flour. The water absorption in wheat dough depends on the quantity and quality of the protein, whereas in rye dough the water-holding capacity of the pentosans and their influence on the water distribution in the dough is decisive.

The water absorption capacity of the whole buckwheat flours (80%, CV = 4%) was higher with respect to the wheat. The results of BEJOSANO and CORKE (1998) indicated that the high water absorption capacity of the buckwheat is due to a large insoluble fraction of the proteins. HROMADKOVA et al. (2007) attributed the high

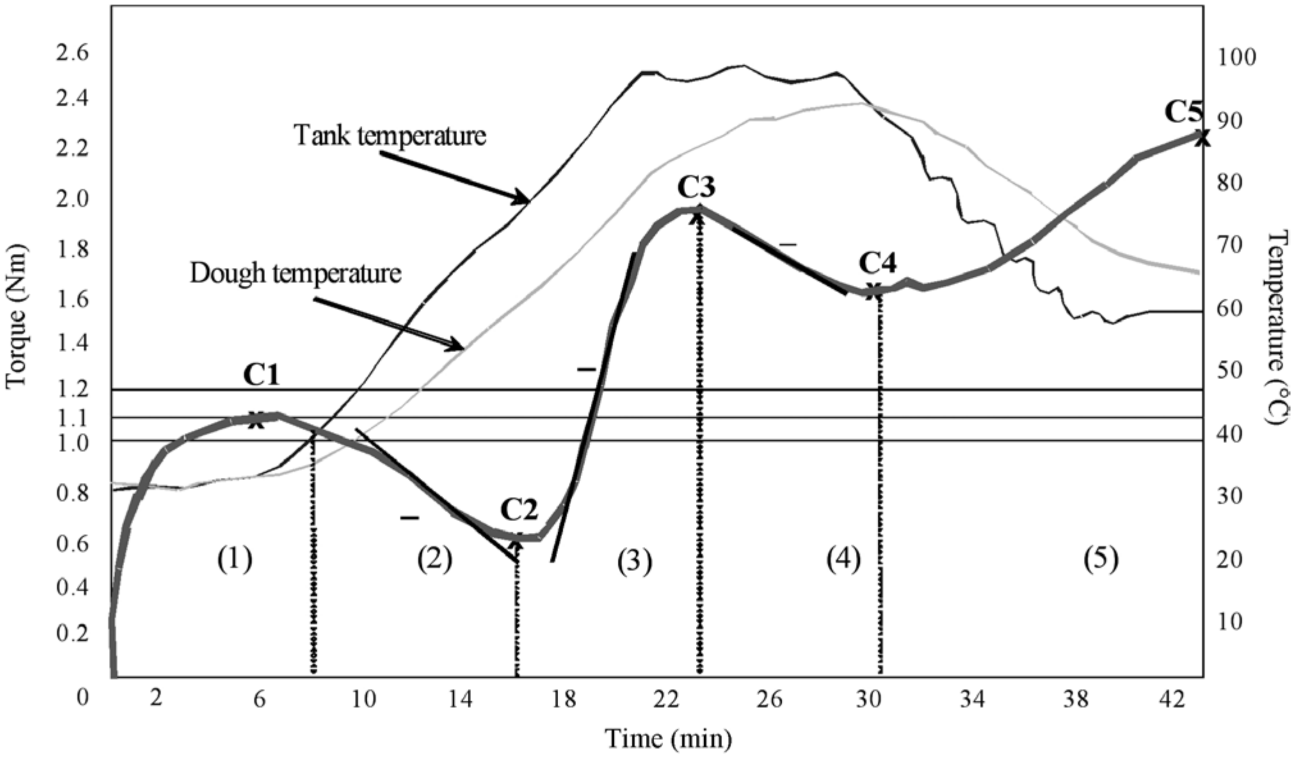


Fig. 1 - Typical curve recorded by the Mixolab.
Stage (1) - dough development (constant temperature. 30°C); stage (2) - thermal weakening of proteins; stage (3) - starch gelatinization; stage (4) - enzymatic activity, constant heating rate; stage (5) - starch retrogradation.

water absorption capacity to the rich glucuronoxylan content.

In order to determine the influence of buckwheat and rye on the rheological properties of the wheat dough, the real-time dough behaviours of wheat, buckwheat, rye and their blends was measured using the Mixolab system at a constant consistency of 1.1 Nm. The whole rye flour showed the lowest development time (1:15 min:s); the maximum torque during mixing was reached after 2:55 min for buckwheat and 4:22 min for wheat.

The Mixolab curves of the wheat/rye and wheat/buckwheat blends are presented in Fig. 2 and Fig. 3, respectively. The first stage of the Mixolab curves indicates the characteristics of dough blends during mixing at a constant temperature of 30°C. After achieving the maximum torque at a constant temperature of 30°C, the dough is stable. The time when the torque and temperature are constant corresponds to a stable dough. Mixolab analysis showed that the addition of buckwheat and rye causes significant changes in the physical properties of wheat whole flour dough. Increasing the rye content from 10% to 30% increased the development time from 2:55 min to 5:29 min (Fig. 2). The same behaviour was observes in the buckwheat samples (Fig. 3) when the development time increased from 4:33 min (10% buckwheat) to 6:10 min (30% buckwheat). These results are in agreement with the observations of HROMADKOVA *et al.* (2007) who showed that, by incorporating buckwheat hull hemicelluloses into wheat flour, the dough development time increased from 2.5 min to 4 min.

The rise of the dough temperature in the Mixolab implies protein denaturation (HAROS *et al.*, 2006; ROSELL *et al.*, 2007), which releases large quantities of water. The α-slope of the curve between the end of the 30°C period and C2 (Fig. 1) gives indication about the rate of protein thermal weakening. In the case of rye and buckwheat, the C2 torque was higher than in the wheat (Table 2). The α-slope was lower for whole buckwheat flour (-0.122 Nm/min) compared to the whole wheat and whole rye flours (-0.06 Nm/min and -0.04 Nm/min,

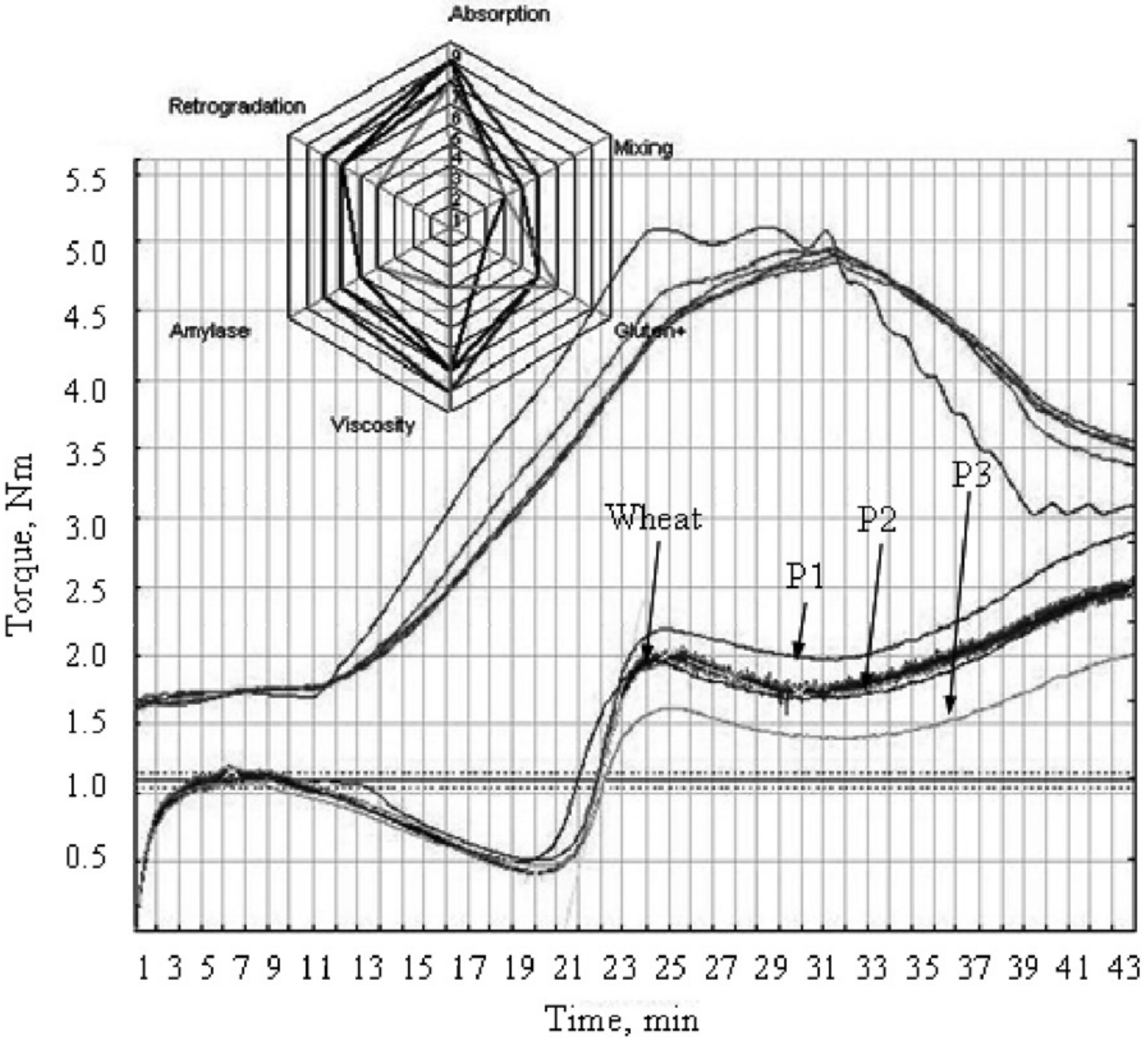


Fig. 2 - Mixolab curves of whole wheat flour with different percentages of rye: P1 = wheat/rye blend (90/10), P2 = wheat/rye blend (80/20), P3 = wheat/rye blend (70/30).

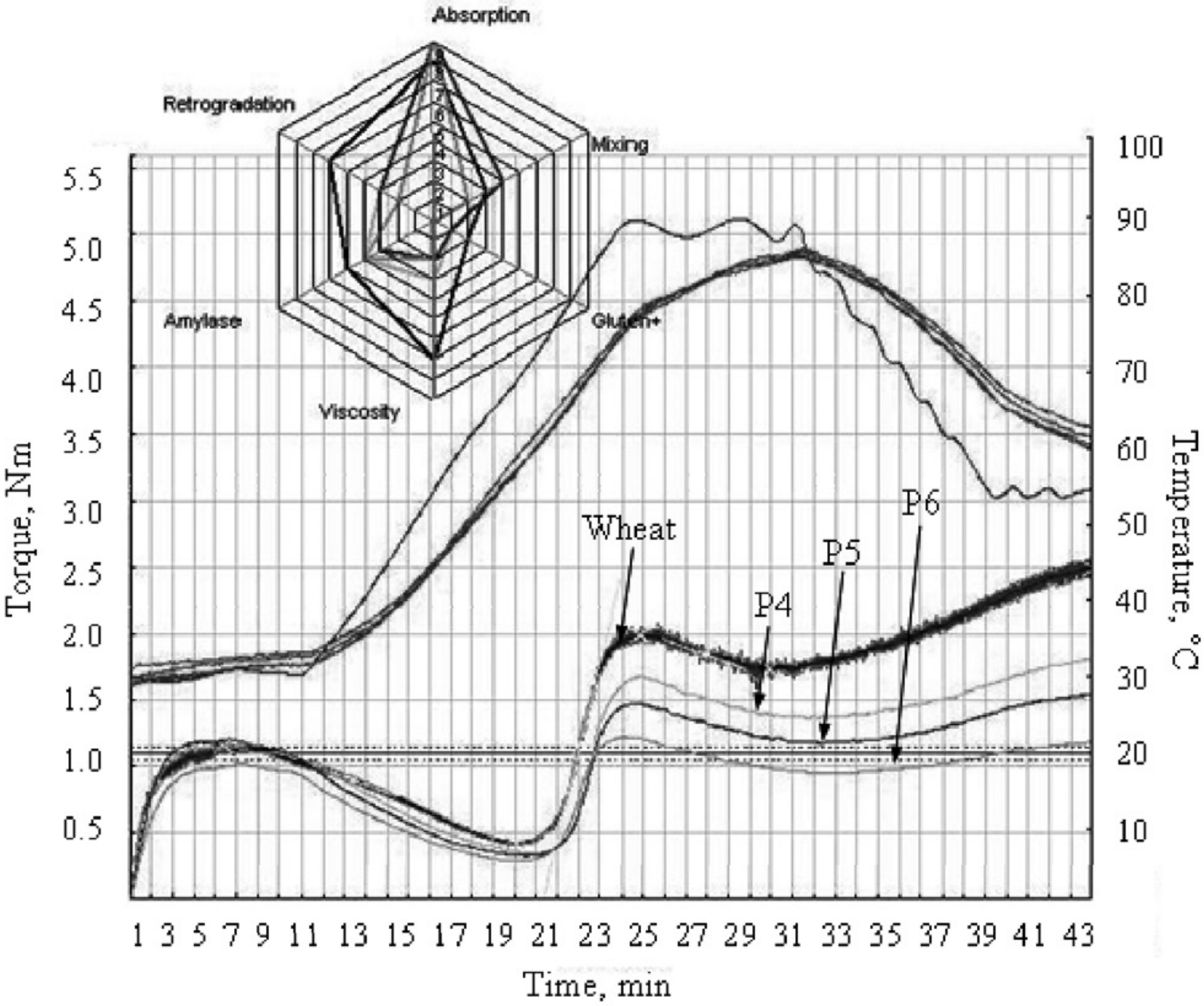


Fig. 3 - Mixolab curves of whole wheat flour with different percentages of buckwheat: P4 = wheat/buckwheat blend (90/10), P5 = wheat/buckwheat blend (80/20), P6 = wheat/buckwheat blend (70/30).

respectively). When mixing the wheat flour with high levels of rye and buckwheat, C2 decreased and α-slope increased (Fig. 2 and Fig. 3). In the case of wheat, the C2 torque was 0.42 Nm (Table 2) whereas in the rye and buckwheat blends the C2 ranged from 0.51 to 0.47 Nm (Fig. 2) and from 0.34 to 0.45 Nm (Fig. 3), respectively.

The dough heating coupled with the water released by the thermally denatured proteins caused starch gelatinization. At the minimum torque (C2), the dough reached the specific temperature for the beginning of starch gelatinization. The minimum torque (C2) was reached in the wheat and rye after 18.00 min at a temperature of 57.6°C, while the buckwheat starch ge-

Table 2 - Mixolab characteristics of whole wheat, rye and buckwheat flours.

Mixolab parameters	Wheat	Rye	Buckwheat
Water absorption (%)	61.9±1.10	59.9±1.50	80.2±1.90
Development time (min:s)	4.22±0.96	1.15±0.98	2.55±1.50
C2 (Nm)	0.42±0.10	0.53±0.15	0.91±0.34
C3 (Nm)	1.99±0.14	2.36±0.23	1.08±0.28
C4 (Nm)	1.74±0.15	1.4±0.27	nd
C5 (Nm)	2.49±0.21	1.86±0.25	1.42±0.37
α (Nm/min)	-0.060±0.05	-0.040±0.04	-0.122±0.05
β (Nm/min)	0.670±0.12	0.764±0.12	0.060±0.14
γ (Nm/min)	-0.060±0.02	-0.124±0.02	nd

latinization (61.6°C) occurred after 31.5 min. These results agree with the observations of QIAN *et al.* (1998) who measured the gelatinization temperature of the buckwheat and wheat starch (61.1-80.1°C and 57.1-73.5°C, respectively) using differential scanning calorimetry. Moreover KIM *et al.* (1977) used the Brabender ViscoAmylograph to study buckwheat starch behaviour and reported a gelatinization temperature of 61-65°C.

Swelling and hydration of starch granules induce an increase in dough consistency until the mechanical shear forces and temperature lead to the physical division of the granules (ROSELL *et al.*, 2007). The lowest peak torque (C3) and minimum torque reached during cooling (C4) was obtained for the whole buckwheat (1.08 Nm and 0.92 Nm, respectively), while the whole wheat flour was characterised by C3 of 1.99 Nm and C4 of 1.73 Nm. The highest C3 torque was recorded for the whole rye flour (Table 2). The rate of dough consistency decrease, given by the γ slope was much lower for buckwheat (0.06 Nm/min) than for the wheat (0.67 Nm/min). Concerning the torque values of the buckwheat flour, the Mixolab Applications Handbook (2006) reports a wide range of values of C3, between 2.15 Nm/min and 1.94 Nm/min and C4 between 2.19 Nm/min and 1.86 Nm/min. This behaviour is due to the buckwheat starch which is very particular with respect to rye, corn and wheat starches. Buckwheat starch has a higher amylose content, water binding capacity and peak viscosity. Consequently it has a lower intrinsic viscosity when compared with corn and wheat starches (QIAN *et al.*, 1998).

The cooking stability was estimated as the difference between C3 and C4 (COLLAR et al. 2007) and the results indicate that the whole rye flour had the lowest stability at heating (0.96 Nm), while the whole wheat flour was more stable (0.26 Nm). In the wheat/rye (Fig. 2) and wheat/buckwheat (Fig. 3) blends, the C3 and C4 torques decreased as the percentage of rye and buckwheat decreased.

The torque after cooling (C5) that indicates the end of the starch retrogradation period, varied with the type of flour analysed. The value obtained for whole buckwheat (1.43 Nm) was lower than that of whole wheat (2.49 Nm) which is in agreement with the Mixolab Applications Handbook (2006). A reduction of starch retrogradation was observed in the wheat-rye and wheat-buckwheat blends when the amounts of rye or buckwheat increased (Table 3). The setback (C5-C4) of whole rye flour was 0.46 Nm and that of whole wheat flour was 0.76 Nm.

CONCLUSIONS

The rheological properties of the whole rye and buckwheat flours and blends with whole wheat flour were studied using the Mixolab system. The addition of different amounts of rye and buckwheat flour (10, 20 and 30%) to whole wheat flour is reflected in the values of the parameters that define dough mixing behaviour, starch gelatinization stage and setback. Decreases in the peak torque (C3), the minimum torque reached during cooling (C4) and torque after cooling (C5) were observed when the amounts of rye and buckwheat, were increased showing that wheat/rye and wheat/buckwheat blends can be successfully used in bread making.

ACKNOWLEDGMENTS

This research was supported by Contract PN-II-ID-PCE-2008, Exploratory Research Projects, no. 1046/2009 (ID 500/2008), financed by the National University Research Council.

Table 3 - Mixolab characteristics of whole wheat flour with different percentages of rye and buckwheat.

Mixolab parameters	Sample					
	P1	P2	P3	P4	P5	P6
Water absorption (%)	61.9±1.34	60.2±1.40	60.8±1.41	66.8±1.64	75.5±1.69	82.8±1.71
Development time (min:s)	2:55±0.90	3:59±0.95	5:29±0.96	4:33±1.20	5:20±1.22	6:10±1.39
C1 (Nm)	1.14±0.10	1.10±0.12	1.08±0.15	1.16±0.13	1.21±0.13	1.02±0.15
C2 (Nm)	0.51±0.10	0.50±0.11	0.47±0.13	0.34±0.21	0.32±0.21	0.29±0.22
C3 (Nm)	2.19±0.19	1.96±0.20	1.62±0.21	1.68±0.23	1.47±0.24	1.23±0.25
C4 (Nm)	1.96±0.21	1.67±0.22	1.40±0.24	1.36±0.28	1.18±0.28	0.95±0.30
C5 (Nm)	2.90±0.20	2.52±0.22	2.01±0.23	1.81±0.28	1.55±0.29	1.19±0.31
α (Nm/min)	-0.040 ±0.04	-0.068 ±0.04	-0.062 ±0.05	-0.078 ±0.04	-0.110 ±0.05	-0.066 ±0.05
β (Nm/min)	0.678±0.12	0.486±0.12	0.514±0.12	0.708±0.13	0.470±0.14	0.346±0.14
γ (Nm/min)	-0.052 ±0.02	-0.056 ±0.02	-0.030 ±0.02	-0.066 ±0.02	-0.050 ±0.02	-0.060 ±0.02

P1 = wheat/rye blend (90/10), P2 = wheat/rye blend (80/20), P3 = wheat/rye blend (70/30), P4 = wheat/buckwheat blend (90/10), P5 = wheat/buckwheat blend (80/20), P6 = wheat/buckwheat blend (70/30).

REFERENCES

AACC International. 2000. Approved Methods of the American Association of Cereal Chemists, 10th Ed. Method 44-51. The Association. St. Paul, MN, USA.

AACC International. 2000. Approved Methods of the American Association of Cereal Chemists, 10th Ed. Method 56-81B. The Association. St. Paul, MN, USA.

ASRO. 2008. Metode de analiza a cerealelor si produselor de macinis. SR ISO 21415-2:2007. Bucuresti, Romania.

Bejosano F.P. and Corke H. 1998. Effect of amaranthus and buckwheat proteins on wheat dough properties and noodle quality. Cereal Chem. 75: 171.

Bonet A., Blaszczak W. and Rosell C.M. 2006. Formation of homopolymers and heteropolymers between wheat flour and several protein sources by transglutaminase-catalyzed cross-linking. Cereal Chem. 83:655.

Cato L. and Mills C. 2008. Evaluation of the Mixolab for assessment of flour quality. Food Australia. 12:577.

Chiotelli E., Rolee, A. and Le Meste M. 2004. Rheological properties of soft wheat flour doughs: effect of salt and triglicerides. Cereal Chem. 81: 459.

Chopin Mixolab User's Manual. 2005. Tripette & Renaud Chopin., Villeneuve La Garenne, France.

Collar C. 2003. Significance of viscosity profile of pasted and gelled formulated wheat doughs on bread staling. Eur. Food Res. Technol. 216: 505.

Collar C., Bollain C. and Rosell C.M. 2007. Rheological behaviour of formulated bread doughs during mixing and heating. Food Sci. Technol. Int. 13: 99.

Dobraszczyk B.J. and Morgenstern M.P. 2003. Rheology and the breadmaking process. J. Cereal Sci. 38: 229.

Duarte P.R., Majewska K. and Doetkott C. 1998. Effect of extrusion process parameters on the quality of buckwheat flour mixes. Cereal Chem. 75: 338.

Gunnarsson E. 1995. Cultivar variation and breeding goals for rye in the nordic countries, International Rye Symposium: Technology and Products. VTT Symposium 161: 26. Espoo, Finland.

Haros M., Ferrer A. and Rosell C.M. 2006. Rheological behaviour of whole wheat flour. p. 1139 in Proc. IUFoST World Congress, 13th World Congress of Food Science & Technology, Nantes, France, 17-21 September.

Hromádková Z., Stavová A., Ebringerová A. and Hirsch J. 2007. Effect of buckwheat hull hemicelluloses addition on the bread-making quality of wheat flour. J. Food Nutr. Res. 46:158.

Jacobs Jr. D.R. and Gallaher D. D. 2004. Whole grain intake and cardiovascular disease: A review. Current Atherosclerosis Reports. 6:415.

Kahraman K., Sakyyan O., Ozturk S., Koksel H., Sumnu G. and Dubat A. 2008. Utilization of Mixolab to predict the suitability of flours in terms of cake quality. Eur. Food Res. Technol. 227:565.

Katina K., Liukkonena K.H., Kaukovirta-Norja A., Adlercreutz H., Heinonen S.M., Lampic A.M., Pihlavad J.M. and Poutanen K. 2007. Fermentation-induced changes in the nutritional value of native or germinated rye. J. Cereal Sci. 46:348.

Kim S.K., Hahn T.R., Kwon T.W. and D'Appolonia B.L. 1977. Physicochemical properties of buckwheat starch. Kor. J. Food Sci. Technol. 9: 138.

Kujala T. 2002. Rye & Health, rye.vtt.fi/rye&health.pdf.

Larsson S. C., Wolk A., Giovannucci E. and Bergkvist L. 2005. Whole grain consumption and risk of colorectal cancer: A population-based cohort of 60000 women. Brit. J. Cancer. 92: 1803.

Li W., Lin R. and Corke H. 1997. Physicochemical properties of common and tartary buckwheat starch. Cereal Chem. 74: 79.

Liukkonen K.H., Katina K., Wilhelmsson A., Myllymaki O., Lampi A.M., Kuriluoto S., Piironen V., Heinonen S.M., Nurmi T., Adlercreutz H., Peltoketo A., Pihlava J.M., Hietaniemi V. and Poutanen K. 2003. Process-induced changes on bioactive compounds in whole grain rye. Proceedings of the Nutrition Society. 62: 117.

Marco C. and Rosell CM. 2008. Breadmaking performance of protein enriched, gluten-free breads. Eur. Food Res. Technol. 227: 1205.

Mixolab Applications Handbook: Rheological and Enzymatic Analysis. 2006. Chopin Applications Laboratory. Villeneuve La Garenne. France.

Ozturk S., Kahraman K., Tiftik B. and Koksel H. 2008. Predicting the cookie quality of flours by using Mixolab. Eur Food Res. Technol. 227:1549.

Pruska-Kedzior A., Goracy M., Pietrowska, K. and Spychalska K. 2008. Comparison of rheological, fermentative and baking properties of gluten-free dough formulations. Eur. Food Res. Technol. 227:1523.

Qian J., Duarte R.P. and Grant L. 1998. Partial characterization of buckwheat (*Fagopyrum esculentum*) starch. Cereal Chem. 75: 365.

Renzetti S., Dal Bello F. and Arendt E.K. 2008. Microstructure, fundamental rheology and baking characteristics of batters and breads from different gluten-free flours treated with a microbial transglutaminase. J. Cereal Sci. 48:33.

Repeckiene A., Eliasson A.C., Juodeikiene G. and Gunnarsson E. 2001. Predicting baking performance from rheological and adhesive properties of rye meal suspensions during heating. Cereal Chem. 78: 193.

Rosell C.M., Collar C. and Haros M. 2007. Assessment of hydrocolloid effects on the thermo-mechanical properties of wheat using the Mixolab. Food Hydrocolloids, 21: 452.

Rosell C.M., Cortez G. and Repo-Carrasco R. 2009. Breadmaking use of Andean crops quinoa, kaniwa, kiwicha, and tarwi. Cereal chem. 86:386.

Tronsmo K.M., Magnus E.M., Baardseth P., Schofield J.D., Aamondt A. and Faergestad E. M. 2003. Comparison of small and large deformation rheological properties of wheat dough and gluten. Cereal Chem. 80: 587.

Uthayakumaran S., Newberry M., Phan-Tien N. and Tanner R. 2002. Small and large strain rheology of wheat gluten. Rheol. Acta 41: 162.

Venn B. J. and Mann J. I. 2004. Cereal grains, legumes and diabetes. Eur. J. Clinical Nutr. 58:1443.

Vinkx C.J. and Delcour J.A. 1996. Rye (*Secale cereale* L.) Arabinoxylans: A Critical Review. J. Cereal Sci. 24: 1Weipert D. 1995. Processing performance of rye as influenced by sprouting resistance and pentosan contents, International Rye Symposium: Technology and Products. VTT Symposium 161: 39. Espoo, Finland.

Revised paper received July 31, 2009 Accepted September 8, 2009

EFFECTS OF REHMANNIA (JIWHANG) POWDER ON THE PROPERTIES OF KOREAN WHEAT-TYPE NOODLES

J-W. RHIM, J-H. KIM, H. KIM[1] and P.K.W. NG[2]*
Department of Food Engineering, Mokpo National University,
860 Muanro, Chungkyemyon, Muangun, 534-729, Jeonnam, Republic of Korea
[1]Korea Flour Mills Co., Ltd. 1568 Sanjungdong, Mokpo, Jeonnam, Republic of Korea
[2]Department of Food Science and Human Nutrition, Michigan State University,
East Lansing, MI 48824-1224, USA
*Corresponding author: ngp@msu.edu

ABSTRACT

Korean-type dry noodles were prepared with composite flours mixed with various amounts of freeze-dried rehmannia (jiwhang) powder. Composition of the composite flours affected flour and final product qualities. Gelatinization temperature of the composite flour increased linearly, while peak viscosity decreased logarithmically, with increases in the rehmannia powder content. Water absorption determined by both a Farinograph test and cooking test decreased linearly with increases in the rehmannia powder content. Swelling index (SI) also decreased, while cooking loss and turbidity of cooking water increased progressively, with increasing rehmannia powder content. In terms of turbidity of cooking water, rehmannia noodles with rehmannia powder content of up to 4% (w/w) were comparable to control wheat flour noodles. Sensory evaluation tests also indicated a similar result in that rehmannia noodles with rehmannia powder content up to 4% (w/w) were more acceptable than or comparable to the control wheat flour noodles.

- Key words: cooking quality, dry noodles, flour blend, sensory evaluation, rehmannia -

INTRODUCTION

Noodles, a traditional food product made from wheat, originated in northern China as early as 5000 B.C. (CORKE and BHATTACHARYA, 1999). They are favored by consumers for their convenience, ease in handling and cooking, and storage properties as well as nutritional qualities. In recent years, noodles have been recognized as a functional food product with a low glycemic index (JENKINS *et al.*, 1988; BJÖRK *et al.*, 2000; WOLEVER, 1990; HAN *et al.*, 2007), a means of quantifying the effect of a food product on human blood glucose levels as compared to that of glucose.

Oriental noodles differ widely from pasta in many aspects, including the raw materials used, preparation method, and quality attributes. Pasta usually refers to Italian-style extruded products, such as spaghetti and macaroni, which are made from coarse semolina milled from tetraploid (or durum) wheat, while noodles are made from hexaploid (or common) wheat. The basic process in the manufacture of noodles includes mixing the ingredients to obtain a stiff dough of relatively low water content, passing this dough repeatedly between sheeting rolls till the desired thickness is attained, and finally cutting the dough sheet into strips. Noodles can be broadly classified into three main groups: white salted noodles, popular in Korea, Japan and China; yellow alkaline noodles, popular in Malaysia, Singapore, Indonesia, Thailand and southern China; and instant noodles, popular in East and Southeast Asia (CORKE and BHATTACHARYA, 1999). Korean noodles are one type of white salted noodle. The Japanese *Udon*, also a white salted noodle, is perhaps the most extensively studied among the Oriental noodles. Korean noodles are white or creamy white in appearance, have a soft and elastic texture, and are usually made from a formula of flour (100 parts), water (32-35 parts) and salt (2-3 parts). The flour used is predominantly milled from relatively soft wheat of low to medium protein level (8-10% w/w), low flour ash content (0.36-0.40% w/w), low damaged starch, and a good color grade, giving the bright creamy tone and desirable texture to the noodles (NAGAO, 1995; CROSBIE, 1991). During preparation of the noodles, wheat gluten and starch play a critical role in creating a gel network and setting the noodle structure.

Recently, there has been renewed interest in developing composite flour noodles or pasta, where wheat flour is mixed with other starch or dietary fiber-based materials, and natural ingredients with functional properties (COLLADO and CORKE, 1996; Lee *et al.*, 2000; TUDORICA *et al.*, 2002; BRENNAN *et al.*, 2004; PARK and CHO, 2006; KIM, 1998; PARK, and CHO, 2004). They are mainly intended to exploit the low glycemic effect of dietary fiber, through slow digestion of the carbohydrates, or the beneficial functional properties (e.g., antioxidation, prevention of obesity or a specific disease) of the natural ingredients obtained from traditionally used medicinal herbs or plants. One of such traditionally used medicinal plants, rehmannia (*Rehmannia glutinosa*), has a high potential for use as a new food ingredient. Rehmannia, which belongs to the Scrophulariaceae family, also known as Chinese foxglove or Jiwhang in Korea, is one of the most widely used medicinal herbs in Oriental medicine especially as a hematopoietic or tonic. Functional ingredients of rehmannia include iridoid glucosides such as catalpol, leonuride, aucubin, and melittoside as well as rehmannoside A, rehmannoside B, rehmannoside C, and rehmannoside D (OSHIO and INOUYE, 1981). Though rehmannia has a great potential for being used as a food ingredient, it has not been widely used in the food processing industry except for some minor uses, such as the utilization of rehmannia extract in making alcoholic beverages or teas.

Therefore, the objectives of this research were to prepare rehmannia noodles and to test their processing and quality properties in order to increase the potential applicability of rehmannia as a new food-processing ingredient.

MATERIALS AND METHODS

Materials

Fresh rehmannia, which is a perennial herb with slender, tuberous, orange roots (ZHANG *et al.*, 2008; Brown, 2002), was obtained directly from a local farm in South Korea. Rehmannia root samples were freeze-dried using a freeze-drier (EYELA vacuum freeze dryer, FDU-1100, Tokyo Rikakikai Co., Ltd., Tokyo, Japan) at -40°C, 760 mm Hg vacuum for 2 days, then powdered using a high-speed hammer mill (Myungsung Machine Co., Ltd., Seoul, Korea), passed through a 160-mesh sieve, and finally packed in airtight polyethylene bags and stored in a refrigerator until studies were conducted. High quality patent flour, produced from an Australian soft wheat, was obtained from Korea Flour Mills Co., Ltd. (Mokpo, Korea) and commercially available refined salt (Hanjoo Salt Co. Ltd., Woolsan, Korea) with high purity (99% NaCl) were used for preparation of the noodles. Composite flour samples with varying amounts of freeze-dried rehmannia powder were prepared by mixing 2, 4, 6, 8, 10, and 20 g of rehmannia powder with 98, 96, 94, 92, 90, and 80 g of wheat flour, respectively, just before manufacturing the noodles.

Proximate analysis

Proximate composition, namely moisture, crude protein (N×5.70), crude lipid, and crude ash contents, of the freeze-dried rehmannia and wheat flour were determined following AOAC methods (AOAC 930.04, 987.04, 930.09, 930.05, 1995). Carbohydrate content was determined by the difference.

Preparation of noodles

Proper water absorption values for the control flour or composite flours (flour blends) were determined by the appearance and handling properties of each dough sample necessary to make a uniform dough sheet. They were 32, 32, 33, 33, 33, 34 and 34 parts of water (wt of water/wt of total flour blend) for 0 (the control wheat flour), 2, 4, 6, 8, 10 and 20% rehmannia powder, respectively. Experimental noodles were prepared according to the method of TOYOKAWA *et al.* (1989). Three hundred grams of wheat flour or premixed composite flour, 96-102 mL of water depending on the type of flour blend, and 6 g of salt were mixed with a flat paddle in a Hobart mixer for 6 min on slow speed, helping to distribute water evenly throughout the flour particles. The resulting crumbly dough was sheeted between steel rolls (180 mm diameter, 5 mm gap) of a Yamato (Tokyo, Japan) laboratory-type noodle machine; it was then folded end-to-end and put through the sheeting rolls, then folded and sheeted again. The sheet was rested for 1 h in a plastic bag to allow gluten development and then put through the sheeting rolls nine times at progressively smaller gap settings from 5.0 mm to 1.0 mm to reduce the sheet to a final thickness of 1.0 mm. The sheet was then cut with a cutting roll (Okuba 1.0 m/m A30) into strips with 1.0×1.0 mm cross section raw noodles. The strips of noodles were dried by hanging in a convection oven controlled at 25-30°C for 48 h, and cut to approximately 25 cm lengths after drying (referred to as dry noodles).

Surface color

Surface color values of rehmannia powder, control and composite flours, and dry noodles were measured using a Hunter *Lab* color scale with a colorimeter (CR-300 Minolta Chroma Meter, Minolta Camera Co., Ltd., Osaka, Japan). Total color difference (ΔE) was calculated as

$$\Delta E = (\Delta L^2 + \Delta a^2 + \Delta b^2)^{1/2}$$

where $\Delta L = L_{\text{standard}} - L_{\text{sample}}$, $\Delta a = a_{\text{standard}} - a_{\text{sample}}$, and $\Delta b = b_{\text{standard}} - b_{\text{sample}}$. A standard white plate with values of $L = 98.86$, $a = -0.02$, and $b = 1.99$ was used for the calibration of the colorimeter.

Gelatinization properties

Gelatinization characteristics of the control flour and rehmannia composite flours were evaluated using a Brabender Visco-Amylograph (Model 800245010, Brabender OHG, Duisburg, Germany) following the method of AACC (AACC 22-10, 1995). Each flour suspension was prepared by mixing 64.85 g of individual flour sample into 450 mL of distilled water using a homogenizer, and then transferred to the Amylograph bowl. Temperature of the bowl was raised from 25°C to 95°C at a heating rate of 1.5°C/min and then maintained for 15 min at 95°C. Gelatinization temperature (°C), peak viscosity (B.U.: Brabender Units), viscosity at 95°C (B.U.), and viscosity at 95°C after 15 min (B.U.) were determined.

Dough properties

Dough forming ability and physical properties of the formed dough of the flour and rehmannia composite flours were tested using a Farinograph (Model 8101, Brabender OHG, Duisburg, Germany) according to the method of AACC (AACC 54-21, 1995).

Cooking qualities

The noodles were cooked in boiling water until the zone of gelatinization had extended through the noodles; they were then evaluated for cooking characteristics following the methods of TUDORICA *et al.* (2002). Each of the dry noodle samples (20 g) was cooked for 4 min in 240 mL of boiling distilled water. After cooking and draining, samples were analyzed for swelling index (SI), dry matter (DM), water absorption (WA), cooking loss (CL), and turbidity of cooking water (T).

The SI (g of water/g of dry noodle) of cooked noodles was evaluated by drying the cooked noodle samples to a constant weight at 105°C, and expressed as

$$SI = (W_1 - W_2)/W_2$$

where W_1 and W_2 are the weight of freshly cooked and drained product and weight after drying, respectively. The DM of noodles was determined by drying the cooked noodle samples to a constant weight at 105°C; results are expressed as percentage of dry noodle weight. The WA (g of water absorbed/100 g of raw noodle) of drained noodle samples was determined as follows:

$$WA = [(W_1 - W_3)/W_3] \times 100$$

where W_1 and W_3 are the weights of cooked and raw noodles, respectively.

Cooking loss into the cooking water collected from each sample was determined by evaporation to constant weight in an air oven at 105°C. The residue was weighed and results are reported as percentage of the weight of the original noodle sample (DEBBOUZ and DOETKOTT, 1996). Turbidity of the cooking water collected from each sample by draining after cooking was determined by measuring absorbance at 675 nm using a spectrophotometer following the method of PARK and CHO (2006).

Statistical analysis

The measurements of the proximate analysis, cooking qualities, and color characteristics were obtained for individually prepared noodles in triplicate, as the replicated experimental units. Statistics on a completely randomized design were determined using the General Linear Models (GLM) procedure in the SAS program (SAS, 1995). Duncan's multiple range test was conducted to determine the significant difference ($p<0.05$) between each type of noodle.

Sensory evaluation

Sensory quality of the control and rehmannia composite flour noodles was evaluated through a sensory test (LEE *et al.*, 2000; PARK and CHO, 2006). Test samples were prepared by cooking 100 g of each type of dry noodle in 500 mL boiling water for 4 min and rinsing under running cold tap water for 1 min. Small portions of cooked noodle samples placed in ceramic-ware for sensory evaluation testing were provided to each of 8 members of a trained panel composed of 4 female and 4 male graduate students at the Department of Food Engineering of Mokpo National University. Sensory qualities of cooked noodles such as color, appearance, taste, texture and overall acceptability were tested using a 5-point hedonic scale (1: least preferred; 2: less preferred; 3: moderately preferred; 4: more preferred; 5: most preferred). Test results were evaluated by ANOVA and the significant differences between treatments groups were determined by Duncan's multiple range tests.

RESULTS AND DISCUSSION

Proximate composition

Proximate compositions of the freeze-dried rehmannia and the control flour were determined (Table 1), however, since the starch content of rehmannia is low, total carbohydrate content was determined and is reported as a difference. The freeze-dried rehmannia powder was lower in protein but higher in ash and carbohydrate contents than the control wheat flour. The proximate composition of freeze-dried rehmannia is in good agreement with composition results reported for *R. radix preparata* (LEE *et al.*, 2005) and *R. glutinosa* Liboschitz (AHN *et al.*, 2000). LEE *et al.* (2005) reported 6.89, 2.89, 83.52, 1.55 and 5.12% for moisture, crude ash, carbohydrate, crude lipid and crude protein, respectively, for *R. radix preparata.* AHN *et al.* (2000) reported 7.3, 4.3, 78.9, 5.4, 0.6 and 3.5% for moisture, ash, carbohydrate, total protein, total lipid and total dietary fiber, respectively, of *R. glutinosa* Liboschitz. It is known that rehmannia contains various sugars (ZHANG *et al.*, 2008; AHN *et al.*, 2000; TOMODA *et al.*, 1971). AHN *et al.* (2000) reported dried *R. glutinosa* Liboschitz contains 14% mono- and di-saccharides, such as glucose, fructose, maltose, sucrose and galactose. TOMODA *et al.* (1971) reported total sugar content of *R. radix* to be 22%, mainly tetra-saccharide and stachyose. The contents of starch and protein in the flours or blends are of great importance since each plays a major role in governing textural properties, which are critical characteristics for consumer-acceptability of oriental noodles (TOYOKAWA *et al.*, 1989). In addition, other components present in the rehmannia powder, and hence present in the composite flours, were expected to affect greatly not only the physicochemical properties of the main ingredient, wheat flour, but also the appearance, color, taste and texture of the final products (LEE *et al.*, 2000; PARK and CHO, 2006; KIM, 1998).

Surface color of flours

The surface color of the wheat flour was creamy white and the rehmannia powder was yellowish brown (Table 2). Generally, the surface color of the flour blends was greatly affected by the rehmannia powder, i.e., with increasing amounts of rehmannia powder, flour blend lightness decreased, with increases in greenness and yellowness, as indicated in Hunter *L*, *a*, and *b*-values, respectively. Consequently, total color difference values (ΔE) of the flour blend samples increased with increasing content of rehmannia powder.

Gelatinization properties

Gelatinization properties were determined using an Amylograph; the Amylogram of the control wheat flour had a typical graph of a patent flour (data not shown) with a peak viscosity of 1040 B.U. (Table 3). Gelatinization temperature of the control flour was 63.25°C, and it increased linearly as the content of rehmannia

Table 1 - Proximate composition[1] of freeze-dried rehmannia powder and a commercial wheat flour.

Components	Rehmannia powder (g/100 g)	Wheat flour (g/100 g)
Moisture	5.7±0.1	12.5±0.1
Crude Protein	6.3±0.1	10.4±0.1
Crude Lipid	0.9±0.2	0.1±0.2
Crude Ash	3.1±0.02	0.4±0.02
Carbohydrates	84.0±0.2	76.6±0.4

[1] Values represent means of three replicates±SD. Carbohydrate content was determined by difference.

Table 2 - Surface color values[1] of freeze-dried rehmannia powder, control wheat flour, and composite flours containing various amounts of rehmannia powder.

Flour[2]	*Hunter L*	*Hunter a*	*Hunter b*	Δ*E*
rehmannia	70.45±5.54^{a}	1.12±1.70^{f}	40.73±0.96^{e}	47.14±2.68^{e}
F-0 (control)	97.57±0.28^{e}	-1.36±0.10^{b}	8.26±0.22^{a}	6.46±0.20^{a}
CF-2	95.48±1.06^{d}	-1.11±0.10^{cd}	8.54±0.20^{a}	6.85±0.33^{a}
CF-4	93.65±2.10^{d}	-1.04±0.10^{de}	8.60±0.19^{a}	7.61±1.04^{a}
CF-6	91.12±2.90^{c}	-0.90±0.23^{e}	11.94±1.13^{b}	11.80±2.60^{b}
CF-8	91.16±0.40^{c}	-1.17±0.04^{cd}	14.02±0.43^{c}	13.37±0.46^{c}
CF-10	90.05±1.43^{c}	-1.26±0.16^{bc}	14.73±0.58^{c}	14.54±1.02^{c}
CF-20	86.85±0.39^{b}	-1.74±0.10^{a}	23.03±0.30^{d}	23.37±0.34^{d}

[1]Values represent means of three replicates±SD. Within the same column, values with the same letter are not significantly different ($p>0.05$).
[2]F-0: control wheat flour; CF-2 ~ CF-20: composite flours containing from 2 to 20 g of rehmannia powder per 100 g flour blend.

powder was increased (T = 0.339C + 63.185; $R^2 = 0.98$, where T is the gelatinization temperature and C is the rehmannia powder content in g/100 g flour). In general, gelatinization properties of flour, as determined by Amylography are mainly influenced by the amount and quality of starch, as well as amylase enzyme activity (KIM *et al.*, 1996). The increase in gelatinization temperature of the composite flours may be attributed to the relatively lower amount of starch and more fiber, which is competing with starch for water. The peak viscosity of composite flour decreased from 1040 B.U. (control) down to 800 B.U. when 2% (w/w) rehmannia powder was present, and then decreased logarithmically (P.V. = -174.6 *ln* C + 889.8; $R^2 = 0.96$, where P.V. is the peak viscosity and C is the rehmannia powder content in g/100 g flour) as the content of rehmannia powder increased. Such a decrease in peak viscosity of the composite flour is due to the lower amount of wheat flour in the composite flour samples compared with the control wheat flour and could be due to hindering of gelatinization of starch by the added rehmannia powder. Similar phenomena of increases in gelatinization temperature with decreases in peak viscosity of composite flours have been reported for other composite flours blended with other materials such as *Dioscorea japonica* powder (PARK and CHO, 2006), mushroom powder (KIM, 1998), rice bran dietary fiber (KIM *et al.*, 1997) and buckwheat powder (KIM *et al.*, 2000). In particular, gelatinization properties of flour blends are closely related to the physical properties and cooking quality of noodles (LEE *et al.*, 2000; TUDORICA *et al.*, 2002; BRENNAN *et al.*, 2004; PARK and CHO, 2006; KIM, 1998; KIM *et al.*, 1996; OH *et al.*, 1985).

Table 3 - Amylograph test results on control wheat flour and composite flours containing various amounts of rehmannia powder.

Flour[1]	Gelatinization Point (°C)	Peak Viscosity (B.U.)
F-0	63.25	1040
CF-2	64	800
CF-4	64.75	639
CF-6	64.75	550
CF-8	65.5	520
CF-10	67	465
CF-20	70	403

[1]See Table 2 for details.

Generally, wheat flours with peak viscosities ranging from 500 to 800 B.U. are known to be suitable for making Korean-type dry noodles (KIM and KIM, 1997). Flour with a higher peak viscosity than this level will make a soft noodle, without any other adverse effects. However, flour with too low a peak viscosity is not suitable for making noodles. The amylase enzyme activity of such flour is generally high, a weak sheet of dough with lack of elasticity is formed, and the resulting noodle strands are easily solubilized during cooking. In the present study, the peak viscosity of the control wheat flour was higher than those of ordinary wheat flours. It is a characteristic feature of Australian wheat flour, known to be good noodle-making flour, which has a peak viscosity known to be greater than 1,000 B.U. (SHIN and KIM, 1993; PARK and CHO, 2006).

Dough properties

Dough properties of the control and rehmannia flour blends were tested using a Farinograph and the results are listed in Table 4. Water required for developing optimum dough (water absorption, WA) with the control flour was 63.1 g/100 g; it decreased linearly (WA = -0.69 *C* + 62.62; $R^2 = 0.99$, where C is the rehmannia powder content in g/100 g flour blend) with increases in the rehmannia content. Dough development time (time for the consistency of the dough to reach 500 B.U.) for the control flour was 2.4 min and it decreased with the presence of rehmannia powder. In general, dough development time is used as an index for the quality evaluation of wheat flour (BOYCIOGLU and D'APPOLONIA, 1994). Based on the Farinograph data, the flour blend samples containing 2 or 4% (w/w) rehmannia powder appeared to have similar dough development times to that of the control flour. Above 4% (w/w), however, rehmannia powder had a much greater effect on the dough development time.

Surface color of noodles

Compared to the surface colors of the flour samples (Table 2), those of the respective counterpart noodles changed significantly and the degree of this change increased further with increasing rehmannia powder content (Table 5). Specifically, the redness (Hunter *a*-value) and the yellowness (Hunter *b*-value) of dry noodles increased significantly, while the lightness (Hunter *L*-value) decreased. Generally, noodle color is known to come from two main sources: fragments of bran and enzymatic browning products that accumulate during processing of the noodle dough (OH *et al.*, 1985). Any discoloration, visible specks, or dull gray hue is considered to be unacceptable quality in ordinary flour noodles. In the case of flour blend noodles, high concentrations of oxidative enzymes, phenolics, and pigments in the rehmannia powder probably caused further color changes in the noodle. However, as consumer demand for nutraceutical foods with assorted functional properties has increased, variously colored noodles, prepared with natural functional ingredients, are increasingly being accepted by consumers.

Cooking qualities

As expected from the results of proximate composition (Table 1), the DM of noodles increased with increases in the rehmannia powder content since the DM of rehmannia was greater than that of wheat flour (Table 6). The WA and SI decreased progressively with increases in the rehmannia powder content (Table 6). The lower WA and SI are mainly due to the lower protein and starch contents in the flour blend samples. On the other hand, CL and T increased with increasing rehmannia powder content (Table 6), which indicates that increasing amounts of soluble solids or non-admixed particles leaked out from the noodles and was transferred into the cooking water during cooking. Note that the T values of the cooking water of composite flour noodles did not change significantly from 0 to 4% (w/w) of rehmannia powder in the blends, but then increased profoundly at and above 6% (w/w). Based on the results of the cooking tests, a content of 4% (w/w) or less of rehmannia powder in the flour blend seems to be suitable for the preparation of rehmannia noodles.

Table 4 - Farinograph test results on control wheat flour and composite flours containing various amounts of rehmannia powder.

Flour[1]	Water Absorption (g/100 g)	Dough Arrival Time (min)	Dough Development Time (min)
F-0	63.1	1.2	2.4
CF-2	60.8	1.2	2.0
CF-4	60.2	1.0	2.0
CF-6	58.3	1.0	1.5
CF-8	56.7	1.0	1.5
CF-10	55.7	1.0	1.5
CF-20	49.0	0.7	0.9

[1] See Table 2 for details.

Table 5 - Surface color values[1] of dry noodles made from control wheat flour and rehmannia composite flour blends.

Noodle[2]	*Hunter L*	*Hunter a*	*Hunter b*	ΔE
N-0	84.6±0.83^{e}	-2.2±0.11^{a}	19.8±0.26^{a}	21.8±0.59^{a}
N-2	69.7±1.26^{d}	1.1±0.22^{b}	34.1±1.46^{b}	42.1±1.6^{b}
N-4	61.7±0.10^{c}	1.8±0.17^{c}	35.9±1.02^{c}	50.5±1.4^{c}
N-6	59.6±2.75^{b}	2.75±0.35^{d}	38.6±0.84^{d}	52.9±0.89^{d}
N-8	58.7±1.19^{b}	2.5±0.33^{d}	40.3±0.47^{e}	52.1±0.35^{d}
N-10	58.3±0.96^{b}	2.53±0.15^{d}	41.2±0.67^{e}	55.1±0.32^{e}
N-20	48.7±1.83^{a}	3.73±0.51^{e}	39.9±1.57^{e}	61.5±0.76^{f}

[1] Values represent means of three replicates±SD. Within each column, values with the same letter are not significantly different (p>0.05) from each other.

[2] N-0: noodles made with the control wheat flour; N-2 ~ N-20: noodles made with composite flour containing from 2 to 20 g of rehmannia powder per 100 g flour blend.

Sensory evaluation

The criteria for judging noodle quality is cooked noodle texture (eating quality); followed by color, taste, surface appearance, cooking loss, and noodle yield (TOYOKAWA *et al.*, 1989). Traditionally, the noodle should have a white opaque appearance and be free of checking. However, recent consumer preferences for functional foods dictate that various natural food ingredients sometimes be added to produce colored noodles rather than white noodles. The noodles should be cooked as quickly as possible, remain firm and not lose solids in the cooking water, and should not become sticky and soggy when standing after cooking. Upon sensory evaluation, for noodle qualities such as color and appearance, rehmannia noodles with 2% (w/w) rehmannia powder for the color and up to 4% (w/w) for the appearance were preferred to the control wheat flour noodle (Table 7).

The texture of a cooked noodle is perceived by the resistance of the noodle to chewing and by the mouth-feel of its surface. Textural properties of noodles are usually determined by objective testing using a texture measuring device such as the General Foods Texturometer, Autograph S-100, Texturecorder, a viscoelasticity meter, Instron and Rheometer, or by subjective testing with sensory evaluation by trained panelists (OH *et al.*, 1985). Though the objective test methods are widely used in the quantitative measurements of textural properties, actual sensory testing of noodle texture is a direct and ultimate method for evaluating the final product. In the sensory evaluation of eating quality of rehmannia noodles in the present study, 2% (w/w) rehmannia for the texture and up to 4% (w/w) for the taste were not significantly different from those of the control noodle (Table 7). Consequently, in the overall acceptability, the rehmannia noodles with up to 4% (w/w) rehmannia powder in flour blends were preferred by the panelists. This result corresponded with that of the cooking quality tests in which noodles of good quality were obtained with up to 4% (w/w) rehmannia powder in the flour blends.

CONCLUSION

Physicochemical properties, such as surface color, gelatinization properties determined by Amylograph, and dough properties determined by Farinograph, of the flour blends composed of wheat flour and freeze-dried rehmannia powder

Table 6 - Cooking characteristics[1] of dry noodles made from control wheat flour and rehmannia composite flour blends.

Noodle[2]	DM (g/100 g noodle)	WA (g/100 g noodle)	SI (g water/g noodle)	CL (g/100 g noodle)	T (A_{675nm})
N-0	89.37±0.24^{a}	286.5±10.3^{d}	5.49±1.47^{b}	8.84±1.42^{a}	1.78±1.23^{a}
N-2	89.58±0.02^{a}	264.4±16.2^{cd}	4.52±0.25^{a}	9.91±1.37^{a}	1.11±1.02^{a}
N-4	89.58±0.02^{a}	253.5±32.4^{bc}	4.35±0.39^{a}	9.26±1.55^{a}	1.96±0.92^{a}
N-6	89.78±0.05^{a}	231.1±7.5^{b}	4.09±0.11^{a}	9.92±1.56^{a}	9.80±1.47^{b}
N-8	89.91±0.02^{a}	241.5±22.4^{bc}	4.25±0.21^{a}	10.71±1.46^{ab}	11.0±1.52^{bc}
N-10	90.13±0.15^{a}	236.5±12.8^{bc}	4.26±0.17^{a}	12.25±1.19^{b}	13.0±1.21^{c}
N-20	91.26±0.10^{b}	193.9±6.7^{a}	3.78±0.07^{a}	14.75±0.65^{c}	42.35±1.22^{d}

[1] DM: dry matter; WA: water absorption; SI: swelling index; CL: cooking loss; T: turbidity of cooking water. Values represent means of three replicates±SD. Within the same column, values with the same letter are not significantly different (*p*>0.05) from each other.

[2] See Table 5 for details.

Table 7 - Sensory evaluation scores[1] of cooked noodles made from control wheat flour and rehmannia composite flour blends.

Noodle[2]	Color	Appearance	Texture	Taste	Overall Acceptability
N-0	4.25±0.70^{d}	4.11±1.02^{c}	4.57±1.12^{d}	4.50±1.07^{d}	4.69±0.10^{d}
N-2	4.45±1.08^{de}	4.72±1.42^{d}	4.41±1.06^{d}	4.20±1.42^{d}	4.50±1.01^{d}
N-4	3.86±1.00^{c}	4.54±1.07^{d}	4.12±1.42^{c}	4.03±1.52^{d}	4.12±1.22^{d}
N-6	4.61±0.98^{de}	4.12±1.15^{c}	3.98±1.21^{bc}	3.61±1.19^{c}	3.53±1.31^{c}
N-8	4.10±1.21^{d}	3.32±1.12^{b}	3.20±1.01^{b}	2.62±1.33^{b}	2.85±1.08^{b}
N-10	2.21±1.02^{ab}	3.12±1.03^{b}	2.41±0.87^{a}	1.28±0.21^{a}	2.21±1.05^{b}
N-20	1.98±0.93^{a}	2.09±0.12^{a}	2.01±0.93^{a}	1.01±0.10^{a}	1.45±1.14^{a}

[1] Values represent means of three replicates±SD. Within the same column, values with the same letter are not significantly different (*p*>0.05) from each other.

[2] See Table 5 for details.

were strongly dependent on the content of the rehmannia powder, but the results indicated that a content of up to 4% (w/w) in flour blends did not substantially affect flour blend quality. Cooking quality, determined as water absorption, swelling index, cooking loss, and turbidity of cooking water, of Korean-type dry noodles prepared with the same composite flours, also indicated that a content of up to 4% (w/w) rehmannia powder was suitable for the preparation of acceptable rehmannia noodles. Additionally, sensory evaluation results revealed that the rehmannia noodles with a content of up to 4% (w/w) in flour blends were better than or comparable to the control wheat flour noodles. In conclusion, the maximum amount of rehmannia powder for the preparation of the rehmannia noodles of acceptable cooking and sensory quality was found to be 4% (w/w) in flour blends.

REFERENCES

AACC. 1995. "Approved Methods of the AACC", 9th ed., American Association of Cereal Chemists: St. Paul, MN, USA.

AOAC. 1995. "Official Methods of Analysis of AOAC Intl." 16th ed., Association of Official Analytical Communities, Arlington, VA, USA.

Ahn S.W., Kim M.H., Chung W.T., Hwang B., Seong N.S. and Lee H.Y. 2000. Enhancement of alcohol fermentation yield by adding the extract of dried *Rehmannia glutinosa* Liboschitz. Kor. J. Med. Crop Sci. 8: 351.

Björk L., Liljeberg H. and Ostman E. 2000. Low glycaemic index foods. British J. Nutr. 83: S149.

Boycioglu M.H. and D'Appolonia B.L. 1994. Characterization and utilization of durum wheat for bread making. 1. Comparison of chemical, rheological and baking properties between bread wheat flours and durum wheat flours. Cereal Chem. 71: 21.

Brennan C.S., Kuri V. and Tudorica C.M. 2004. Inulin-enriched pasta: effects on textural properties and starch degradation. Food Chem. 86: 189.

Brown D. 2002. Rehmannia. In "Encyclopedia of Herbs and Their Uses". p. 341. The Royal Horticulture Society, London, UK.

Collado L. and Corke H. 1996. Use of wheat-sweet potato composite flours in yellow-alkaline and white-salted noodles. Cereal Chem. 73: 439.

Corke H. and Bhattacharya M. 1999. Wheat products: 1. Noodles. In "Asian Foods", C.Y.W. Ang, K. Liu and Y.W. Huang (ED.), p. 43. Technomic Publishing Co., Ltd. Lancaster, PA, USA.

Crosbie G. 1991. The relationship between starch swelling properties, paste viscosity and boiled noodle quality in wheat flours. J.Cereal Sci. 13: 145.

Debbouz A. and Doetkott C. 1996. Effect of process variables on spaghetti quality. Cereal Chem., 73: 672.

Han J.A., Seo T.R., Lee S.J. and Lim S.T. 2007. *In vitro* digestibility of cooked noodle products. Food Sci. Biotechnol. 16: 1078.

Jenkins D.J., Wolever T.M. and Jenkins A.L. 1988. Starchy foods and glycemic index. Diabetes Care. 11: 149.

Kim B.R., Choi Y.S. and Lee S.Y. 2000. Rheological properties of buckwheat-wheat flour mixture. J. Kor. Soc. Food Sci. Nutr. 29: 369.

Kim H.K. and Kim I.S. 1997. Flour test. In "Wheat and Flour", p. 103. Korea Flour Industry Association, Seoul, Korea.

Kim Y.H., Choi K.S., Son D.H. and Kim J.H. 1996. Rheological properties of dough with whole wheat flour. J. Kor. Soc. Food Sci. Nutr. 25: 817.

Kim Y.S. 1998. Effects of *Poria cocos* powder on wet noodle qualities. Agric. Chem. Biotechnol. 41: 539.

Kim Y.S. 1998. Quality of wet noodle prepared with wheat flour and mushroom powder. Kor. J. Food Sci. Technol. 30: 1373.

Kim Y.S., Ha T.Y., Lee S.H. and Lee H.Y. 1997. Effect of rice bran dietary fiber on flour rheology and quality of wet noodles. Kor. J. Food Sci. Technol. 29: 90.

Lee B.K., Jung J.E. and Choi Y.H. 2005. Optimization of microwave-assisted extraction process of *Rehmannia Radix preparata* by response surface methodology. Food Eng. Prog. 9: 283.

Lee J.W., Kee H.J., Park Y.K., Rhim J.W., Jung S.T., Ham K.S., Kim I.C. and Kang S.G. 2000. Preparation of noodle with laver powder and its characteristics. Kor. J. Food Sci. Technol. 32: 298.

Nagao S. 1995. Wheat usage in East Asia. In " Wheat End Uses Around the World", H. Faridi and J.M Faubion (ED.), p. 167. Am. Assoc. of Cereal Chem., St. Paul, MN, USA.

Oh N.H., Seib P.A., Ward A.B. and Deyoe C.W. 1985. Noodles. IV. Influence of flour protein, extraction rate, particle size, and starch damage on the quality characteristics of dry noodles. Cereal Chem. 62: 441.

Oshio H. and Inouye H. 1981. Iridoid glycosides of *Rehmannia glutinosa*. Phytochemistry 21: 133.

Park B.H. and Cho H.S. 2006. Quality characteristics of dried noodle made with *Dioscorea japonica* flour. Kor. J. Food Cook. Sci. 22: 173.

Park S.I. and Cho E.J. 2004. Quality characteristics of noodle added with chlorella extract. Kor. J Food Nutr. 17: 120.

SAS Institute, Inc. 1995. "SAS User's Guide". Statistical Analysis Systems Institute, Cary, IN, USA.

Shin S.Y. and Kim S.K. 1993. Cooking properties of dry noodles prepared from HRW-WW and HRW-ASW wheat flour blends. Kor. J. Food Sci. Technol. 25: 232.

Tomoda M, Tantka N. and Kondo N. 1971. Water-soluble constituents of *Rehmanniae* Radix. II. On the constituents of roots of *Rehmannia glutinosa* var. purpurea. Chem. Pharm. Bull. 19: 2411.

Toyokawa H., Rubenthaler G.L., Powers J.R. and Schanus E.G. 1989. Japanese noodle qualities. I. Flour components. Cereal Chem. 66: 382.

Tudorica C.M., Kuri V. and Brennan C.S. 2002. Nutritional and physicochemical characteristics of dietary fiber enriched pasta. J. Agric. Food Chem. 50: 347.

Wolever T.M.S. 1990. Relationship between dietary fiber content and composition in foods and the glycemic index. Am. J. Cli. Nutr. 51: 72.

Zhang R.X., Li M.X. and Jia Z.P. 2008. *Rehmannia glutinosa*: Review of botany, chemistry and pharmacology. J. Ethnopharmacol. 117: 199.

Revised paper received July 7, 2009 Accepted October 8, 2009

SHORT COMMUNICATION

EFFECTS OF DRY MATTER STANDARDIZATION ORDER ON BIOCHEMICAL AND MICROBIOLOGICAL CHARACTERISTICS OF FRESHLY MADE PROBIOTIC DOOGH (IRANIAN FERMENTED MILK DRINK)

A.M. MORTAZAVIAN*, R. KHOSROKHAVAR[1], H. RASTEGAR[1] and G.R. MORTAZAEI[2]
Department of Food Science and Technology, Faculty of Nutrition Sciences,
Food Science and Technology/National Nutrition Sciences
and Food Technology Research Institute, Shaheed Beheshti University of Medical Sciences,
P.O. Box 19395-4741, Tehran, Iran
[1]Food and Drug Control Laboratories, and Food and Drug Laboratory Research Center,
Ministry of Health and of Medical Education, P.O. Box 11136, Tehran, Iran
[2]Department of Food Technology Research, National Nutrition Sciences and Food Technology
Research Institute, Shaheed Beheshti University of Medical Sciences,
P.O. Box 19395-4741, Tehran, Iran
*Corresponding author: Tel. +98-912-7114977, Fax +98-21-22360657
e-mail: mortazvn@sbmu.ac.ir / mortazvn@yahoo.com

ABSTRACT

The effects of the order of dry matter standardization (i.e., dilution of milk with potable water before fermentation or first, production of yogurt and then, addition of potable water) on the microbiological and biochemical characteristics of a typical Iranian probiotic fermented milk drink (doogh) containing *Lactobacillus acidophilus* LA-5, *Bifidobacterium lactis* BB-12, *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus* were studied. Experimental and calculated parameters included the rates of pH decline, acidity increase, and redox potential increase during the fermentation period, incubation time, viability of probiotic microorganisms immediately after fermentation, growth/death indices of probiotics during the fermentation period and acetic acid concentration (percentage) in fresh samples. Standardization after fermentation (dilution of yogurt) led to a longer incubation time (1.42-fold) compared with standardization before fermentation. The treatment with standardization after fermentation showed a lower pH decline and redox potential increase rates as well as a lower pH-acidity modulus (rate of acidity increase versus pH drop), but a faster rate of acidity increase and a higher final acidity content. Viability of both probiotic microorganisms was significantly higher when standardization was done after fermentation. The acetic acid percentage was 0.04% with standardization before fermentation, while it was 0.11%, with standardization after fermentation.

- Key words: bifidobacteria, doogh, fermented milk, *Lactobacillus acidophilus,* probiotic, viability -

INTRODUCTION

The manufacture of dairy products containing probiotic bacteria is an important issue with industrial and commercial consequences; many products of this kind are available for consumption by humans, farm animals and pets (HOIER, 1992; HOLZASPFEL and SCHILLINGER, 2001; O'GRADY and GIBSON, 2005; SANDERS, 1999; SHAH, 2001). Presently, species of *Lactobacillus* and *Bifidobacterium* are the most important bacteria used in probiotic products. Among bifidobacteria, *Bifidobacterium lactis* (*B. animalis* ssp. *lactis*) is currently offered by starter culture-producing companies and is used by manufacturers to produce many different kinds of fermented probiotic dairy products because it has good tolerance of detrimental environmental factors such as acid, low pH and molecular oxygen (IWANA *et al.*, 1993; MEILE *et al.*, 1997; MORTAZAVIAN and SOHRABVANDI, 2006a; YAESHIAMA *et al.*, 1996).

Probiotic microorganisms must be viable in the final product until they are consumed. This is the most important qualitative parameter of probiotic products since it determines their pharmaceutical effectiveness. Although there is no overall agreement regarding the minimum number of viable probiotic cells per gram or milliliter of probiotic products, 10^6 cfu/mL or cfu/g is generally the accepted minimum level and 10^7 and 10^8 cfu/mL or cfu/g are considered satisfactory levels. Probiotic products should also be consumed regularly (approximate 100 g/d) in order to deliver about 10^9 viable cells into the intestine (HICKEY, 2005; KAILASAPATHY and RYBKA, 1997; KURMANN and RASIC, 1991; LOURENS-HATTINGH and VILJOEN, 2001; MORTAZAVIAN and SOHRABVANDI, 2006a; SHAH, 2000). It is not easy to maintain these standards up to the expiration date of the product due to the poor viability of probiotic microorganisms during the fermentation time and storage period, especially in fermented products (MORTAZAVIAN and SOHRABVANDI, 2006a; SHAH, 2000). Various compositional and process factors have a notable effect on the viability of probiotic microorganisms in products: pH, titrable acidity, molecular oxygen, redox potential, hydrogen peroxide, bacteriocins, short-chain fatty acids, some flavouring agents, microbial competition, packaging materials and packaging conditions, rate and proportion of inoculation, step-wise/stage-wise fermentation, microencapsulation, supplementation of milk with nutrients, heat treatment of the product, incubation temperature, storage temperature, carbonation, addition of salt, sugar and sweeteners, cooling rate of product and scale of production (MORTAZAVIAN et al., 2008; 2007b; 2006; MORTAZAVIAN and SOHRABVANDI, 2006; SHAH, 2000; TAMIME et al., 2005).

Doogh is a typical traditional Iranian fermented milk drink. It is exported (about 150,000 tones/year) and is consumed in other countries such as Afghanistan, Azerbaijan, Armenia, Iraqi, Syria, Turkey, and the Balkans, and to a lesser extent in other countries of the Middle East and central Asia (ANON, 2008). Doogh is composed of non-fat milk solids (more than 3.2% and normally 4 to 6% W/W in range), milk fat (not more than 50% of dry non-fat milk), salt (at most 1%) and flavouring compounds (such as mint, ziziphora or cucumber essences) with a pH less than 4.5. Carbon dioxide can be naturally (by starter cultures) or artificially added to the product (ANON, 2008).

In the production of drinks based on fermented milk, there are two possible ways to standardize the dry matter. First, milk dry matter is standardized to the desired content by adding sterilized-demineralized potable water before fermentation. Fermentation is then carried out which leads directly to the formation of the final product. Second, milk or dry matter-fortified milk is fermented to produce the fermented milk base (such as yogurt) and then, this product is diluted by adding potable water (with/without dissolved additives). The impact of this sequence (dry matter standardization before or after fermentation) on the viability of probiotic microorganisms has not been reported elsewhere. The aim of this study was to investigate the effect that the order of dry matter standardization has on the microbiological and biochemical characteristics of ABY-type doogh (containing *Lactobacillus acidophilus* LA-5, *Bifidobacterium lactis* BB-12, *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus*).

MATERIALS AND METHODS

Starter culture

Fifty-unit pouches of commercial lyophilized ABY culture (containing *Lactobacillus acidophilus* LA-5, *Bifidobacterium lactis* BB-12, *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus*) that are known as 'FD-DVS ABY-1', were supplied by Chr-Hansen (Horsholm, Denmark). This culture is currently used by the dairy industry to produce probiotic fermented dairy products. According to the manufacturer's instructions, the cultures were maintained at -18°C.

Production of doogh

For those samples with dry matter standardization before fermentation (indicated by the abbreviation of "MD", "milk to doogh"), the dry matter content was standardized at 6% by reconstitution of skim milk powder. In the case of the samples with dry matter standardization after fermentation (specified by the abbreviation of "YD", "yogurt to doogh"), first, a yogurt milk (reconstituted skim milk) was prepared with a non-fat dry matter content of 12%. After fermentation and cooling to 5°C, the amount of sterilized-demineralized-distilled water at the same temperature required to obtain a final non-fat dry matter concentration of 6% was added under sterile conditions. Reconstituted skim milk samples of both treatments were heated at 90°C for 15 min. After cooling down to fermentation temperature and inoculating the starter cultures (commercial lyophilized ABY culture containing *L. acidophilus*, *B. lactis* Bb-12, *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus*, according to Chr. Hansen's construction), the milk was poured into several 250 mL PET containers under sterile conditions and incubated at 40°C±0.2 until pH 4.5±0.02 (for some treatments) or pH 4.2±0.02 (for other treatments) was reached. Biochemical parameters including the rates of the pH decrease as well as acidity and redox potential increase, and incubation time were measured during the fermentation period of both treatments. The pH, titrable acidity and redox potential during fermentation were recorded at 30 min intervals. The samples were kept at 5°C until the probiotic organisms were counted and the acetic acid content was determined.

Microbiological analysis

MRS-bile agar medium (MRS agar from Merck, Darmstadt, Germany and bile from Sigma-Aldrich (St. Louis, MO, USA) was used for the selective count of *L. acidophilus* and bifidobacteria in the ABY culture composition according to MORTAZAVIAN *et al.* (2007a), using the "subtractive enumeration method (SEM)". The plates were incubated aerobically and anaerobically at 37°C for at least 72 h. Anaerobic conditions were produced using a GasPac system (Merck, Darmstadt, Germany). Growth indexes of probiotic microorganisms during the fermentation period were calculated as follows (MORTAZAVIAN, 2008):

- Growth proportion index (GPI) = Final cell population/initial cell population
- Growth rate index (GRI) = final cell population – initial cell population/incubation time [cells/min]

Chemical analysis

The pH values and redox potentials of the samples were measured at room temperature using a pH meter (MA235, Mettler, Toledo, Switzerland).

The titrable acidity was determined after mixing 10 mL of sample with 10 mL of distilled water and titrating with 0.1 N NaOH using 0.5% phenolphthalein according to DAVE and SHAH (1996).

The pH drop rate, acidity increase and redox potential increase as well as the pH-acidity modulus were calculated as follows (MORTAZAVIAN, 2008):

- pH drop rate = (final pH value – initial pH value)/incubation time [pH value/min]
- Acidity increase rate = (final acidity value – initial acidity value)/incubation time [Dornic degree/min]
- Redox potential increase rate = (final value – initial value) / incubation time [mV/min]
- pH-acidity modulus = pH drop rate/acidity increase rate [pH value/ Dornic degree]

The above parameters were calculated according to how the break/turn points appeared on the respective kinetic curves (pH drop, acidity increase and redox potential increase versus time as well as acidity increase versus pH drop). Break points observed on the curves determined distinct zones which could be attributed to different phases of bacterial growth.

Quantification of the acetic acid content was carried out by High Performance Liquid Chromatography (Varian Analytical Instruments, CA, USA) according to a modified method of AKALIN *et al.* (2004). To extract acids, 4.0 g of doogh were diluted to 25 mL of 0.1 N H_2SO_4, homogenized and centrifuged at 5000 *g* for 10 min. The supernatant was filtered through Whatman #1 filter paper and through a 0.20 μm membrane filter, and 2 mL aliquots were stored in HPLC vials at -20°C until HPLC analysis. A Jasco UV-980 detector and a Nucleosil 120-5C_{18} column (Macherey Nagel, Duren, Germany) were used. The mobile phase was 0.009 N H_2SO_4 at a flow rate of 0.5 mL min^{-1}. The detection wavelength was optimized at 210 nm. The standard solution of acetic acid (Sigma-Aldrich, St. Louis, MO, USA) was prepared in distilled water. The retention time for acetic acid was 7.25 min and the regression coefficient of the standard curve was 0.997.

Statistical analysis

Experiments were performed four times and the significant differences among means were analyzed using the ANOVA test using Minitab software.

RESULTS AND DISCUSSION

Changes in pH, acidity and redox potential during fermentation

Table 1 shows the pH drop, acidity increase and redox potential increase rates of MD and YD treatments at various time intervals during fermentation. Table 2 indicates the acidity increase rates of the YD and MD treatments/pH drop intervals during fermentation. The means of the pH drop rate, acidity increase rate, redox potential rate and pH-acidity modulus of MD and YD treatments throughout the fermentation period are presented in Table 3. As shown in Table 1, for the MD treatment, the greatest pH drop and the greatest acidity increase and redox potential increase rates were observed within 1.5-4.0 hours of fermentation. This area indicates the logarithmic (exponential) growth phase of the starter bacteria. At the start of the fermentation process, the rates of the pH decrease and acidity increase were generally slow; because the bacteria were in the lag or pre-log phase of growth and the buffering capacity of the doogh milk inhibited a sharp pH decline. The buffering capacity of doogh milk significantly influenced the incubation time and the final titrable acidity of the product (Table 3). Increasing dry matter content from 6% (MD treatment) to 12% (YD treatment) increased the fermentation time from 360 to 510 min and increased the final titrable acidity from 64.9 to 74.4 (Table 3). According to Table 1 and considering the rate of pH decline per time interval, the logarithmic growth of the starter bacteria in the YD treatment was within the 1.5-5.5 hours of fermentation. In the MD treatment, the logarithmic growth phase started at h 1.5, but finished 1.5 hours sooner (h 4 after the start of fermentation). The starting points of logarithmic growth were also similar for the acidity increase and redox potential increase rates (Table 1). For both YD and MD treatments, the lowest rate in the decline of pH and the minimum increase rates of acidity and redox potential were observed in the first time interval of fermentation. This time interval represents the lag phase of bacterial growth. Considering the fact that the logarithmic growth of starter bacteria in both treatments started at the same time points (Table 1), it can be concluded that the changes in dry matter of doogh milk did not affect the starting point of logarithmic phase in bacterial growth stages. As shown in Table 2, the greatest acidification rates for MD and YD treatments were observed within the pH drop ranges of 5.89-4.58 and 5.96-4.55, respectively. The similarity between these two ranges indicates that the logarithmic phase of bacterial growth in both treatments ended at pH values of about 4.50-4.60. As can be seen in Table 2, the minimum acidification rates in both treatments were observed within the first pH drop interval, that is, the lag phase of bacterial growth. The lower mean of rate of pH decline in YD compared to that of MD indicates a higher buffering capacity of the fermenting media in YD. The greater buffering capacity stimulated the acidification rate of the by starter bacteria because they are inhibited more slowly during fermentation due to a slower rate of pH

Table 1- pH drop, acidity increase and redox potential increase rates of MD and YD treatments per time interval during fermentation period until final pH of 4.2*.

Treatment	pH drop rate per time interval (h)					Acidity increase rate per time interval (h)					Redox potential increase rate per time interval (h)		
MD	0.0-0.5	0.5-1.5	1.5-4.0	4.0-5.5	5.5-6.0	0.0-1.0	1.0-2.0	2.0-4.0	4.0-5.0	5.0-6.0	0.0-1.5	1.5-4.0	4.0-6.0
	-0.001^{e} (min)	-0.005^{b}	-0.009^{a} (max)	-0.004^{bc}	-0.002^{d}	0.01^{e} (min)	0.10^{c}	0.29^{a} (max)	0.16^{b}	0.06^{d}	0.18^{c} (min)	0.65^{a} (max)	0.22^{b}
YD	0.0-0.5	0.5-1.5	1.5-5.5	5.5-7.5	7.5-8.5	0.0-1.0	1.0-2.0	2.0-6.0	6.0-8.5	0.0-1.0	1.0-1.5	1.5-4.5	4.5-5.5
	0.000^{d}	-0.001^{c}	-0.007^{a}	-0.003^{b}	-0.002^{c}	0.02^{d}	0.09^{c}	0.41^{a}	0.17^{b}	0.04^{e}	0.20^{c}	0.45^{a}	0.26^{b}
The means followed by different letters are significantly different ($p<0.05$).													

Table 2 - Acidity increase rate of MD and YD treatments per pH drop interval during fermentation period until final pH of 4.2*.

Treatment	pH drop interval								
MD	6.51 - 6.31 0.01^{e}		6.31 - 5.89 0.10^{c}		5.89 - 4.58 0.29^{a}		4.58 - 4.36 0.16^{b}		4.36 - 4.22 0.07^{d}
YD		6.31 - 6.27 0.02^{d}		6.27 - 5.96 0.09^{c}		5.96 - 4.55 0.41^{a}		4.55 - 4.21 0.17^{b}	
* The means followed by different letters are significantly different ($p<0.05$).									

Table 3 - Means of pH drop rate, acidity increase rate, redox potential rate and pH-acidity modulus of MD and YD treatments throughout the fermentation periods as well as incubation time, final acidity and concentration of acetic acid at the end of fermentation (until final pH of 4.2)*.

Treatment	Parameters						
	pH-DR**	A-IR	RP-IR	pH-A-M	Incubation time	Final acidity	Acetic acid percentage
MD	0.006^a	0.15^b	0.39^a	0.04^a	360^b	64.9^b	0.04^b
YD	0.004^b	0.26^a	0.25^b	0.01^b	510^a	74.4^a	0.11^a

* Means in the same column with different letters are significantly different ($p<0.01$).

** pH-DR = pH drop rate, A-IR = acidity increase rate, RP-IR = redox potential increase rate; pH-A-M = pH-acidity modulus.

decline. The mean acidification rate for the YD treatment was significantly higher than that in the MD treatment (Table 3). According to Table 3, the pH-acidity modulus of the YD treatment was smaller than that of the other treatment, representing a slow decrease in pH with a parallel rapid increase in acidity. The mean value of redox potential increase rate in the MD treatment was greater than that in the YD treatment. Production of organic acids during the fermentation period causes an increased redox potential (MORTAZAVIAN and SOHRABVANDI, 2006). The YD treatment had a significantly greater acidity increase rate during fermentation compared with the MD treatment (Table 3). Therefore, the redox potential increase rate in the former treatment should have been significantly greater than the other treatment; however, the opposite was observed. This finding can be attributed to the non-fat milk solid enriched media in the YD treatment (12%) compared to that of MD (6%). Milk proteins (especially sulfur-containing amino acids) efficiently lower the redox potential of the media and keep it low after sufficient heat treatment (MORTAZAVIAN and SOHRABVANDI, 2006). There could also be less penetration of molecular oxygen in the YD treatment during fermentation because the matrix is much more condensed.

Viability of probiotic bacteria

The viability of the probiotic microorganisms and their growth/death indices (growth proportion index = GPI and growth rate index = GRI) in MD and YD treatments immediately after fermentation are reported in Table 4. The viable counts of each probiotic bacteria (*L. acidophilus* and bifidobacteria) and the total probiotic count were significantly higher in the YD compared to the MD treatment. In each treatment, the viable cell population of bifidobacteria was significantly higher than *L. acidophilus*. The viable cell population of *L. acidophilus* immediately after fermentation was 1.90 (190%) of its initial population in the YD treatment. The viable cell population for bifidobacteria was 1.58 (158%) at the end of fermentation. Considering the growth rate index (GRI) in Table 4, on average, the cell populations of *L. acidophilus* and bifidobacteria in the YD treatment (YD-pH4.2) increased by 2879.4 and 3158.7 viable cells/min during fermentation, respectively. In the MD treatment, the GRI values were negative for both probiotic bacteria. The final populations of both probiotic bacteria had fewer viable cells than were initially inoculated into non-fermented milk. On average, the viable cell populations of *L. acidophilus* and bifidobacteria in the MD treatment decreased by 3585.2 and 4153.6 viable cells/min during termination, respectively. The population of bifidobacteria in the YD-pH4.2 treatment was 3.47 times higher than in MD (Table 4). Comparing the amounts of acetic acid in both treatments at the end of fermentation (0.15 in YD compared with 0.04 in MD), the viability of bifidobacteria was greater in the YD treatment. Bifidobacteria form acetic acid in a molar proportion of 3:2. Therefore, the increase in acetic acid is proportional to the growth of these bacteria (GOMES and MALCATA, 1999; MORTAZAVIAN, 2008).

A significantly lower viability of probiotics in the MD treatment compared to the YD treatment can be attributed to several factors. A higher buffering capacity in the YD treatment led to a longer fermentation time and the growth inhibition of probiotic starter bacteria was delayed until the final pH value of fermentation (pH4.20±0.02) (Table 3). Consequently, the time of bacterial multiplication was longer. The lower buffering capacity of the media in the MD treatment led to a sharp decrease in pH during the fermentation period (Table 3). This might gave caused a pH drop shock to probiotics, particularly because they are susceptible to pH decline (GOMES and MALCATA, 1999; MORTAZAVIAN and SOHRABVANDI, 2006). In the YD treatment, the amount of final acidity as well as the mean acidity increase rate (Table 3) was higher than that of the MD treatment (higher buffering capacity of the former media). This did not have a notable influence on the viability of probiotic bacte-

Table 4 - Viability of probiotic microorganisms and their growth/death indexes in MD and YD treatments immediately after fermentation*.

	Initial population (log cfu/mL)***			Final population (log cfu/mL)			GRI (cfu/min)			GPI		
Treatment	A**	B	A+B	A	B	A+B	A	B	A+B	A	B	A+B
MD (pH4.2)	6.21	6.44	6.64	5.52^{cB}	6.10^{cA}	6.20^{c}	-3585.2^{cA}	-4153.6^{cB}	-7722.9^{c}	0.20^{cB}	0.46^{cA}	0.36^{c}
YD-pH4.2***	6.21	6.44	6.64	6.49^{bB}	6.64^{bA}	6.88^{b}	+2879.4^{bB}	+3158.7^{bA}	+6332.6^{b}	1.90^{bA}	1.58^{bB}	1.74^{b}
YD-pH4.5	6.21	6.44	6.64	6.79^{aB}	6.93^{aA}	7.16^{a}	+13939.1^{aB}	+17659.9^{aA}	+30948.6^{a}	3.80^{aA}	3.09^{aB}	3.31^{a}

* Means shown with small and capital letters represent significant differences (p<0.05) in the same columns (between MD and YD treatments) and rows (between the probiotic bacteria in each treatment), respectively;
** A = *L. acidophilus*, B = bifidobacteria, A + B = total probiotics; *** YD-pH4.2 = YD treatment with pH4.2.

ria (Table 4). In other words, the role of pH decline (both pH value and pH drop rate) on the loss of viability in probiotics might be stronger than the impact of acidity increase, although organic acids are also bacteriocidic agents (JAY, 1992; MORTAZAVIAN and SOHRABVANDI, 2006). This hypothesis is consistent with some previous reports in which the final pH at the end of yogurt fermentation has been reported to be the most important factor affecting the growth and viability of *L. acidophilus* and especially *Bifidobacterium* spp. (LAROIA and MARTIN, 1991; SHAH and RULVA, 2000). To test this hypothesis, the microbiological characteristics of the YD treatment-pH4.2 (at the end of fermentation) and YD treatment-pH4.5 containing equal amounts of solid non-fat contents were compared (Table 4). The viability of the probiotics and their relevant growth indices in the YD treatment-pH4.5 were much greater than in the YD treatment-pH4.2. The logarithmic bacterial growth phase in both treatments ended in the pH range of 4.55-4 (Table 2). Therefore, the greatest viable count of probiotics was observed at a pH limit of about 4.50. When the pH falls below 4.20, the starter bacteria must be at the end of the stationary phase or in the death phase (remarkably reduced rates of acidity increase and pH decrease, Table 1), resulting in a dramatic loss in probiotic viability. However, pH values higher than 4.2 are not suitable for doogh production because the taste of final product would not be satisfactory.

In the YD treatment, the yogurt gel matrix formed during the fermentation exhibits a physical and probably chemical property against molecular oxygen, hydrogen ions and organic acid molecules (MORTAZAVIAN and SOHRABVANDI, 2006), the viability of the probiotics within such a matrix probably increases. The lower redox potential increase rate of YD compared with the MD treatment also influenced the viability of probiotics in the YD treatment (Table 3). Higher values of redox potential inhibit or restrict the growth and/or activity of bifidobacteria (GOMES and MALCATA, 1999; MORTAZAVIAN and SOHRABVANDI, 2006).

In the YD treatment, a higher concentration of non-fat milk solids had a positive effect on the growth and/or activity of the probiotics compared to MD due to the higher nutritional value of the YD treatment. Lower non-fat solids content in the fermenting media enhances bacterial competition that results in the loss of probiotic viability. This is particularly important in the case of antagonistic effects of *L. delbrueckii* ssp. *bulgaricus* and probiotic bacteria (MORTAZAVIAN *et al.*, 2006; 2007b; MORTAZAVIAN and SOHRABVANDI, 2006). It has been reported that in the ABY culture composition in fermented milks, there is a dramatic loss of viability in *L. acidophilus* due to hydrogen peroxide produced by *L. delbrueckii* ssp. *bulgaricus* (DAVE and SHAH, 1997; MORTAZAVIAN *et al.*, 2006; 2007b; SHAH *et al.*, 1995).

Although the final population and growth rate index (GRI) of bifidobacteria was significantly greater than that of *L. acidophilus* in the YD treatment, their growth proportion indices were significantly smaller (Table 4). *L. acidophilus* grew and multiplied more efficiently in the samples related to this treatment and the significantly higher viable count and GRI and GPI of bifidobacteria at the end of fermentation was due to the considerably longer initial inoculated population (Table 4). In the MD treatment, the rate of viability loss of bifidobacteria was greater than that of *L. acidophilus* (Table 4, high negative GRI). Therefore, the production conditions of the MD treatment compared with YD must have been more detrimental for the bifidobacteria rather than the *L. acidophilus* (greater death rate). Greater sensitivity and drastic viability loss of bifidobacteria in the MD treatment compared with the YD treatment can be attributed to the susceptibility of these bacteria to sharper pH drop and redox potential increase rates in the MD treatment as well as the lack of proteolytic and β-galactosidase activities (GOMES and MALCATA, 1999; MORTAZAVIAN and SOHRABVANDI, 2006). Moreover, low pH values (e.g., pH4.2) have a strong adverse impact on the viability of bifidobacteria (GOMES and MALCATA, 1999; MORTAZAVIAN and SOHRABVANDI, 2006). It has been reported that the growth of bifidobacteria is restricted at pH<5.0 (SHAH, 1997; GOMES and MALCATA, 1999).

CONCLUSIONS

In the present research, the effects of the order of dry matter standardization on the production of ABY-type probiotic doogh and on its microbiological and biochemical characteristics were studied. The results showed that standardization after fermentation (YD treatment) resulted in significantly higher viable counts of *L. acidophilus* and bifidobacteria compared with standardization before fermentation (MD treatment). However, standardization after fermentation led to longer incubation times that could be unfavorable from a technological point of view. In both treatments (YD and MD), the growth rates of bifidobacteria were lower than *L. acidophilus*. The values of growth rate index (GRI) were negative for both probiotics in the MD treatment compared with YD. This study showed that Chr-Hansen's ABY-type probiotic starter culture which is widely applied for yogurt production was not suitable for manufacturing of fermented milk drinks with lower pH values including doogh. In order to select between the two treatments (MD or YD), the colloidal stability (comprising phase separation) as well as the rheological and sensorial characteristics of doogh made by both procedures must be compared in a separate study.

REFERENCES

Akalin A.S., Fenderya S. and Akbulut N. 2004. Viability and activity of bifidobacteria in yogurt containing fructooligosaccharide during refrigerated storage. Int. J. Food Sci. Tech. 39: 613.

Anon 2008. Doogh: Characteristics and methods of analysis. Iran National Standard (ISIRI: #(2453)). Available on www.isiri.org.

Dave R.I. and Shah N.P. 1997. Viability of yoghurt and probiotic bacteria in yoghurts made from commercial starter cultures. Int. Dairy J. 7: 31.

Dave R.I. and Shah N.P. 1996. Viability of yogurt and probiotic bacteria in yogurts made from commercial starter cultures. Int. Dairy J. 7: 31.

Gomes A.M.P. and Malcata F.X. 1999. *Bifidobacterium* spp. and *Lactbacillus acidophilus*: biological, biochemical, technological and therapeutical properties relevant for use as probiotics. Trends Food Sci. Tech. 10: 139.

Hickey M. 2005. Current legislation of probiotic products. In "Probiotic Dairy Products". Tamime A.Y. (Ed). P. 73. MP. 234. UK.

Hoier E. 1992. Use of probiotic starter cultures in dairy products. Food Aust. 44: 418.

Holzaspfel W.H. and Schillinger U. 2001. Introduction to pre- and probiotics. Food Res. Int. 35:109.

Iwana H., Masuda H., Fujisama T., Suzuki H. and Mitsuoka T. 1993. Isolation of *Bifidobacterium* spp.- In commercial yogurts sold in Europe. Bifidobacteria Microflora 12: 39.

Jay J.M. 1992. "Modern Food Microbiology". Chapman & Hall, London.

Kailasapathy K. and Rybka S. 1997. *Lactobacillus acidophilus* and *Bifidobacterium* spp.- Their therapeutic potential and survival in yogurt. Aust. J. Dairy Tech. 52: 28.

Kurmann J.A. and Rasic J.L. 1991. The health potential of products containing bifidobacteria. In "Therapeutic Properties of Fermented Milks" Robinson P.K. (Ed). P. 117. MP. 4320. UK.

Laroia S. Martin J.H. 1991. Effects of pH on survival of *Bifidobacteriumj bifidum* and *Lactobacillus acidophilus* in frozen fermented dairy desserts. Cult. Dairy Prod. J. 26: 13.

Lourens-Hattingh A. and Viljoen B. 2001. Yoghurt as a probiotic carrier food. Int. Dairy J. 11: 1.

Meile L., Ludwig W. and Rueger U. 1997. *Bifidoacterium lactis* sp. Nov., a moderately oxygen tolerant species isolated from fermented milk. System. Appl. Microbiol. 6: 263.

Mortazavian A.M. 2008. Effects of principle compositional factors and microencapsulation of probiotics on qualitative parameters of probiotic Doogh. PhD Thesis, University of Tehran, Tehran.

Mortazavian A.M., Ehsani M.R., Azizi A., Razavi S.H., Mousavi S.M. and Sohrabvandi S. 2008. Viability of calcium alginate-microencapsulated probiotic bacteria in Iranian yogurt drink (Doogh) during the refrigerated storage period and under the simulated gastrointestinal conditions. Aust. J. Dairy Technol. 63: 24.

Mortazavian A.M., Sohrabvandi S. and Reinheimer J.A. 2007a. MRS-bile agar: Its suitability for the enumeration of mixed Probiotic cultures in cultured dairy products. Milchwissenschaft 62: 270.

Mortazavian A.M., Ehsani M.R., Mousavi S.M., Rezaei K., Sohrabvandi S. and Reinheimer, J.A. 2007b. Effect of storage temperature on the viability of probiotic microorganisms in yogurt. Int. J. Dairy Tech. 60:123.

Mortazavian A.M. and Sohrabvandi 2006. "Probiotics and Food Probiotic Products; Based on Dairy Probiotic Products". Eta Publication, Tehran [In Persian].

Mortazavian A.M., Ehsani M.R., Mousavi S.M., Reinheimer J.A., Sohrabvandi S. and Rezaei K. 2006. Preliminary investigation of the combined effect of heat treatment and incubation temperature on the viability of the probiotic microorganisms in freshly made yogurt. Int. J. Dairy Tech. 59: 8.

O'Grady B.G and Gibson R. 2005. Microbiota of the human gut. In "Probiotic Dairy Products" Tamime A.Y. (Ed). P. 1. MP. 234. UK.

Sanders M.E. 1999. Probiotics. Food Tech. 53: 67.

Shah N.P. 2001. Functional foods from probiotics and prebiotics. Food Tech. 55(11): 46.

Shah N.P. 2000. Probiotic bacteria: selective enumeration and survival in dairy foods. J. Dairy Sci. 83: 894.

Shah N.P. and Rulva R.R. 2000. Influence of water activity on fermentation, organic acids production and viability of yogurt and probiotic bacteria. Aust. J. Dairy Tech. 55: 127.

Shah N.P. 1997. Bifidobacteria: characteristics and potential for application in fermented milk products. Milchwissenschaft 52: 16.

Shah N.P., Lankaputhra W.E.V., Britz M. and Kyle W.S.A. 1995. Survival of *L. acidophilus* and *Bifidobacterium bifidum* in commercial yogurt during refrigerated storage. Int. Dairy J. 5: 515.

Tamime A.Y., Saarela M., Korslund Sondergaard A., Mistry V.V., and Shah N.P. 2005. Production and maintenance of viability probiotics micro-organism in dairy products. In "Probiotic Dairy Products" Tamime A.Y. (Ed). P. 39. MP. 234. UK.

Yaeshiama T., Takahashi S., Ishibashi N. and Shihamura S. 1996. Identification of bifidobacteria from dairy products and evaluation of a microplate hybridization method. International J. Food Microbiol. 30: 303.

Revised paper received May 20, 2009 Accepted July 14, 2009

SHORT COMMUNICATION

BODY PROPORTIONS AND CHEMICAL COMPOSITION OF WILD AND REARED EDIBLE SNAILS OF IVORY COAST

A. OTCHOUMOU*, M. DUPONT-NIVET[1], L. OCHO ANIN ATCHIBRI[2] and H. DOSSO[3]
Laboratoire de Biologie et Cytologie Animales, UFR des Sciences de la Nature,
Université d'Abobo-Adjamé, 26 BP 623 Abidjan 26, Côte d'Ivoire
[1]Institut National de la Recherche Agronomique, Laboratoire de Génétique des Poissons,
78352 Jouy en Josas Cedex, France
[2]Laboratoire de Nutrition et Sécurité Alimentaire, UFR des Sciences et Technologies Alimentaires,
Université d'Abobo-Adjamé, 52 BP 801 Abidjan 52, Côte d'Ivoire
[3]Centre de Recherches en Ecologie (CRE),
Université d'Abobo-Adjamé, 52 BP 801 Abidjan 52, Côte d'Ivoire
*Corresponding author: Tél. (225) 21-24-43-33, Fax (225) 21-24-43-33,
e-mail: albertameri@yahoo.fr or atcho@aviso.ci

ABSTRACT

The chemical composition and body proportions of *Achatina fulica (Bowdich)*, *Achatina achatina (Linné)* and *Archachatina ventricosa (Gould)* fed a concentrated diet were investigated and compared with wild *A. fulica*, *A. achatina* and *Arch. ventricosa.* Wild and artificially reared snail shell proportion were 42.09 and 46.91%; 47.20 and 52.96%; 48.02 and 51.96% of the whole body, respectively, for *A. fulica*, *A. achatina* and *Arch. ventricosa.* The pedal mass proportion was 41.36 and 36.66%; 27.80 and 26.62% than 36.59 and 32.71%, respectively, for wild and reared *A. fulica*, *A. achatina* and *Arch. ventricosa.* The pedal mass mean energy, dry matter, proteins, lipid and mineral matter content were 53.21cal/100g; 12.86% by weight of deshelled snails; 63.98/DM (% of dry matter); 15.80/DM and 8.96/DM in *A. achatina.* For *A. fulica* pedal mass aver-

- Key words: chemical composition, edible snails, Ivory Coast, pedal mass, visceral mass -

age energy, dry matter, proteins and mineral matter content were 47.82 cal/100 g; 13.63% by weight of deshelled snails; 58.94/DM and 14.92/DM. *Arch. ventricosa* pedal mass mean energy, dry matter, proteins, lipid and mineral matter content were 50.32 cal/100g; 14.19% in weight of deshelled snails; 66.36/DM; 13.43/DM and 10.25/DM. The protein contents differed slightly between artificially reared and wild snails.

INTRODUCTION

The rural populations of under-developed countries and in particular the Ivory Coast suffer from a chronic deficit in animal protein. The Ivory Coast initially imported meat (sheep and oxen) from the Sahelian African countries (Mali, Burkina Faso and Niger), the European Union (sheep, poultry and dairy products) and certain Latin American countries which was a significant expenditure. Therefore, it was necessary to seek new sources of animal protein. Edible terrestrial gastropods occur naturally in the dense forests of sub-Saharan Africa and particularly in the Ivory Coast. These snails belong to the *Achatina* genus, particularly the species *A. achatina* Linné, 1758 and *A. fulica* Bowdich, 1820 and the *Archachatina* genus with the species *Arch. ventricosa* Gould, 1850 and *A. marginata* Swainson, 1821. In the past, snails belonging to the genus *Achatina* were often consumed, while those of the genus *Archachatina* were rarely used as food because of various taboos. Today both juveniles and adults of these genera are consumed (ABOUA and BOKA, 1996). The soft bodies of these snails are rich in amino acids, proteins, minerals and iron and constitute a potentially valuable source of animal protein and iron for rural populations (ABOUA, 1990, ADEYEYE *et al.*, 1996).The snail meat is relished by the forest populations of the Ivory Coast for its taste and especially its aroma. It is consumed in various forms: cooked in sauces, steamed or roasted and served with rice, semolina of cassava, yams and plantain bananas (ABOUA and BOKA, 1996). The visceral mass, which is usually not consumed, could constitute a source of protein and minerals in animal feed. The artificial raising of snails is necessary to make up for seasonal deficits in snail availability and to preserve the species from the pressures that result from the destruction of the forests, the abusive use of pesticides and over-collecting (ZONGO *et al.*, 1990; OTCHOUMOU *et al.*, 1989-1990; 2003a;b; 2004a;b; 2005). However artificially raised snails are not greatly appreciated by consumers compared to wild specimens (KOUASSI *et al.*, 2007).

The aims of the present study were to compare the proportions of some of the body parts (shell, pedal mass and visceral mass) of reared and wild snails and to determine and compare the chemical composition of the meat of reared and wild snails in order to determine if snail culture should be encouraged for human consumption.

MATERIALS AND METHODS

Snail material

One hundred and fifty (50/species) wild snails of the species *A. achatina* (Linné), *A. fulica* (Bowdich) and *Arch. ventricosa* (Gould) were used. The average live weights were 32.05±10.5 g; 32.50±3.44 g and 32.10±9.98 g, respectively, for *A. achatina*, *A. fulica* and *Arch. ventricosa*. They were collected in the forests in the south-west of the Ivory Coast from an area of approximately 3 hectares. For the reared species, 150 snails (50/species) were used. The average live weights were 32.06±7.48 g; 32.61±4.88 g and 32.11±11.58 g, respectively, for *A. achatina*, *A. fulica* and *Arch. ventricosa*. The reared snails were raised at the experimental farm of the University of Abobo-Adjamé. They were fed a concentrated flour diet in accordance with the requirements for the species, as reported by OTCHOUMOU *et al.* (2004a, b) for six months (from June 2006 to December 2007). The formulation of the diet is given in Table 1. The principal characteristics of the diet were checked by chemical analysis (AOAC, 1984) and the results are reported in Table 2.

Sample preparation

Fifty snails were randomly chosen according to species; twenty-five wild snails and twenty-five reared snails. The remainder of the wild snails were released in the forest. They were weighed after a 24-hour fast and sacrificed and the flesh

Table 1 - Meal diet components (g) and characteristics.

Corn	Cotton cattle-cake	Soya grains	Bran soft wheat	Calcium phosphate	Vitamins	Calcium carbonate	Salt	Trace elements	Total
10.00	16.00	16.00	15.00	4.00	0.50	39.20	0.40	0.10	100
Characteristics/DM									
Crude energy	Total Nitrogen	Total calcium	Fat	Starch	Free sugars	crude cellulose	Ash		
2727	17.14	16.01	4.61	12.24	3.04	4.67	43.35		
/DM: Results expressed as % dry matter									

Table 2 - Diet components determined by chemical analysis.

		Components (% of dry matter)				
Sample weight	Dry matter	Proteins	Total Lipids	Calcium	Mineral	Energy (cal/g)
44.66	83.13	18.35	3.34	15.80	45	2.380

was extracted from the shell using a silver fork. The pedal mass (the head and edge of the mantle) was then separated from the visceral mass (digestive glands, gonads, gland of albumin, liver, pancreas, genitals, heart and kidney). The shell, pedal mass and visceral mass were weighed. After drying in an oven at 60°C for 24 h, the snail diet, pedal mass and visceral mass were ground and the chemical composition was determined using 50 g of dry ground snail meat for each sample. The chemical analysis of each sample was repeated three times.

Proximate composition

Dry matter

The diet sample was dried and the loss of mass was determined by weighing. At least 50 g of the sample were mashed without any variation in moisture. A mashed sample (5 g) was then put into a tarred container and heated at 103°C for four h in a drying oven. The mass of dry matter obtained is expressed as a percentage of the sample.

Mineral matter

A 5 g sample was weighed and put in an incineration crucible which was gradually heated until the sample was carbonized. The crucible was then placed in a muffle oven at 550°±5°C until white ash was obtained. The crucible was immediately weighed after cooling. The weight of the residue was calculated by subtracting the tare and is expressed as a percentage of the sample.

Crude proteins

The crude proteins were determined according to the Kjeldahl method. The sample was mineralized by the wet process. The acid solution was alkalized with a solution of sodium hydroxide. The ammonia released was extracted by distillation and collected in a volume of sulphuric acid whose excess was titrated with sodium hydroxide. One mL of sulphuric acid (0.1 N) corresponds to 1.4 mg of nitrogen; the quantity of nitrogen obtained was multiplied by 6.25 and the result are expressed as a percentage of the sample.

Total lipids

The total lipids were determined according to the method of FOLCH *et al.* (1957).

The tissues were homogenized with a 2:1 mixture of chloroform and methanol and the extract was washed by adding pure water (0.2% in volume). The mixture was separated into two phases by centrifuging. The lower phase was the extract of pure total lipids.

Crude energy

The crude energy was determined by the coefficient of ATWATER and ROSA (1899). According to them 1g of carbohydrates and 1 g of protein provides 17 joules and 1 g of lipids 38 joules. One calorie corresponds to 4.18 joules. Crude energy was calculated as follows:

$$CE = \frac{38[\text{Lipids }\%]+17[\text{Proteins }\%]+17[\text{Carbohydrates }\%]}{4.18}\,Cal/100g$$

Starch

The sample was extracted with ethanol (40%). After acidification of the substrate with hydrochloric acid, precipitation and filtration, the optical activity of the solution was measured by polarimetry.

Free sugars

Free sugars were determined by the sulphuric phenol test (DUBOIS *et al.*, 1956).

Crude cellulose

A mixture of 3 g of sample, 2 g of an asbestos, 125 mL of sulphuric acid (1.02N) and 75 mL of distilled water was boiled for 10 min and then filtered. One hundred-twenty mL of potassium hydroxide (0.89N) and 75 mL of distilled water were added to the residue. The solution was filtered after boiling for 10 min on an asbestos. The residue was rinsed with warm water and acetone for drying. The asbestos residue containing cellulose was then placed in a porcelain crucible and dried in an oven at 130°C for 2 h. The mixture was then weighed with the crucible and incinerated at 900°C for 30 min. The crucible and the dry residue were then weighed. The crude cellulose content is expressed as a percentage of the sample.

Statistical analyses

The statistical analyses were carried out with the SAS program (1987). Averages of shell proportions, pedal mass, visceral mass, total mass of flesh proportions, energy, dry matter, proteins, lipids and mineral matter were compared by analysis of variance with ANOVA (5% threshold of confidence). The following model was used: $Y_{ijk} = \mu + R_I + E_{ijk}$, where Y_{ijk} is the measured variable, μ the general average, R_I the fixed effect of snail origins and E_{ijk} the residual.

RESULTS AND DISCUSSION

Various parts of the body proportions

The results concerning the proportions of the various parts of the body of the three snail species are given in Table 3. The statistical analyses showed that there were no significant differences (P>0.05) between the wild and reared snails with respect to the average proportions of the various parts of the snail body. The consumable pedal mass was equivalent to 33% of the live weight of the three species; the visceral mass of *A. achatina* and *Arch. ventricosa* accounted for 16% of the live weight. The visceral mass of *A. fulica* made up about 20% of its live weight.

Table 3 - Body portions are percentage of body weight (%) of edible snails of the Ivory Coast.

	Achatina achatina	
Body weight (g)	Wild	32.05 ± 10.5
	Reared	32.06 ± 7.48
Shell	Wild	$42.09^{a} \pm 4.5$
	Reared	$46.91^{a} \pm 14.00$
Pedal mass	Wild	$41.36^{a} \pm 6.23$
	Reared	$36.66^{a} \pm 7.20$
Visceral mass	Wild	$16.53^{b} \pm 2.89$
	Reared	$16.44^{b} \pm 7.94$
Total flesh mass	Wild	$57.91^{a} \pm 3.6$
	Reared	$53.09^{a} \pm 14.00$
	Achatina fulica	
Body weight (g)	Wild	32.50 ± 3.44
	Reared	32.61 ± 4.88
Shell	Wild	$47.20^{a} \pm 3.56$
	Reared	$52.96^{a} \pm 5.53$
Pedal mass	Wild	$27.80^{b} \pm 2.44$
	Reared	$26.61^{b} \pm 1.49$
Visceral mass	Wild	$25.00^{b} \pm 1.43$
	Reared	$20.43^{b} \pm 6.63$
Total flesh mass	Wild	$52.80^{a} \pm 7.5$
	Reared	$47.04^{a} \pm 5.55$
	Archachatina ventricosa	
Body weight (g)	Wild	32.10 ± 9.98
	Reared	32.11 ± 11.58
Shell	Wild	$48.02^{a} \pm 13.99$
	Reared	$51.95^{a} \pm 4.47$
Pedal mass	Wild	$36.59^{b} \pm 4.3$
	Reared	$31.71^{b} \pm 3.64$
Visceral mass	Wild	$15.37^{c} \pm 1.2$
	Reared	$16.34^{c} \pm 1.82$
Total flesh mass	Wild	$51.98^{a} \pm 5.88$
	Reared	$48.05^{a} \pm 5.19$

NB: Mean values in the same column followed by the same letter have no significant difference (P>0.05).

Chemical composition

The results of the chemical analysis of some body parts of the three snail species are given in Table 4. Statistical analyses showed that regardless of species, there were no significant differences (P>0.05) between the chemical composition of the wild specimens and those of reared specimens. Regardless of the species and origin, the visceral mass contained more energy, lipids and mineral matter than the pedal mass. The pedal mass was richer in proteins than the visceral mass and reared species had higher protein contents than those of the wild ones.

In Africa and particularly in the Ivory Coast, snails are important for the quantity of meat that they provide and not for their gastronomical finesse (ZONGO *et al.*, 1990). The pedal mass is the part of the body that is consumed (ABOUA and BOKA, 1996). The results of this study should encourage the consumption of reared snail meat and use of the shell and visceral mass. The results indicate that the shell accounts for half of the live snail weight, while the pedal and visceral masses account for 33% and 17% of the body weight, respectively. These results are similar to those of PA-

CHECO *et al.* (1998). GOMOT (1998) studied the proportions of the various parts of the body of Helix snails and reported 10.3 to 11.5% of the live snail weight was constituted by the shell of reared specimens, while the shell constituted 17.5 to 22% of the live weight in the wild individuals. The high proportion of shells of wild specimens of Achatinidae and Helicidae are due to the nature of the soil, the vegetation and the influence of the season. The shell and the visceral mass could be used as a source of calcium and protein in animal feed (RISTIC *et al.*, 2000). ABOUA (1990; 1995) studied the chemical composition of *A. fulica* and showed that the flesh was very rich in protein. The pedal mass had from 53.36 to 74.6 g of protein for the three studied species which was slightly higher than the value (72 g) reported by ABOUA (1990). SALDANHA *et al.* (2001) reported 14.87 g on wet matter. The pedal mass contained more proteins than the visceral mass which was probably due to the components. Pedal mass is primarily made up of contractile proteins (actins and myosin). However, the pedal mass of *Arch. ventricosa*, an object of various taboos, was the richest in proteins compared to the other two species. This explains why it is highly consumed in Benin and Nigeria (EBENSO, 2002). Traces of lipids were found in pedal and visceral masses of *A. fulica* in contrast to what ABOUA (1990) found, 2.10% of the body weight. In the two other species, the lipid contents were low in the pedal mass. This explains why snail meat is recommended for low lipid diets (SALDANHA *et al.*, 2001). The results of this study were slightly higher due to the concentrated diet. In addition the visceral mass was rich in lipids and was more energetic compared to the pedal mass because the lipids are highly energetic. The mineral matter of the pedal and visceral masses exceeded 19.6 g per 100 g of sample regardless of the species. These values were double those obtained by ABOUA (1990) in *A. fulica* and even greater than those of WATT and MERRILL (1975). These results could be explained by the type of diet used to rear the snails. The results of this study have shown that the visceral mass is richer in mineral matter than the pedal mass. It is thought that the mineral content absorbed by the snails is transferred to the internal organs where a storage threshold exists. This threshold differs according to the snail species.

The edible snails of the Ivory Coast are very rich in protein, minerals and energy. For this reason they are gathered by the forest populations of the Ivory Coast. This study has shown that the chemical quality of wild snails differs a little from that of the reared ones. An improvement of the various parameters for raising snails, and particularly diet components could improve the quality of reared snail meat. Thus it is recommended that reared snail meat be consumed in order to preserve wild snail biodiversity and to protect snail consumers from possible intoxication due to frequent use of pesticides and heavy metals.

Table 4 - Chemical composition of the pedal and visceral mass of edible snails of the Ivory Coast

	Achatina achatina Pedal mass		Visceral mass	
	Wild snails	Reared snails	Wild snails	Reared snails
Energy (cal/100g)	$5135^{ab}\pm77.78$	$5027.5^{a}\pm175.79$	$5370.5^{ab}\pm27.58$	$5614.5^{b}\pm472.56$
Dry matter (%)	$10.99^{a}\pm0.30$	$13.51^{b}\pm2.75$	$11.90^{a}\pm0.56$	$14.74^{b}\pm5.2$
Proteins (%DM)	$74.6^{b}\pm0.14$	$62.6^{ab}\pm7.61$	$65.76^{ab}\pm0.62$	$53.36^{a}\pm6.06$
Lipids (%DM)	$7.17^{a}\pm0.04$	$7.54^{ab}\pm0.73$	$21.04^{ab}\pm0.66$	$24.44^{b}\pm8.02$
Mineral matter (%DM)	$7.39^{a}\pm0.13$	$7.38^{a}\pm2.25$	$10.53^{a}\pm0.25$	$9.54^{a}\pm3.01$
	Achatina fulica			
Energy (cal/100 g)	$4958^{a}\pm172.53$	$4950^{a}\pm323$	$4561.50^{a}\pm2.12$	$5002.83^{a}\pm355.48$
Dry matter (%)	$12.76^{a}\pm0.65$	$14.51^{a}\pm1.3$	$13.47^{a}\pm2.08$	$14.08^{a}\pm8.96$
Proteins (%DM)	$57.18^{a}\pm0.82$	$66.22^{a}\pm10.25$	$51.67^{a}\pm1.52$	$59.56^{a}\pm10.65$
Lipids (%DM)	Traces	Traces	Traces	Traces
Mineral matter (%DM)	$10.25^{a}\pm6.39$	$10.25^{a}\pm6.39$	$19.6^{a}\pm0.79$	$13.48^{a}\pm4.10$
	Archachatina ventricosa			
Energy (cal/100g)	$4777^{a}\pm80.61$	$4816.5^{a}\pm82.03$	$5288^{a}\pm16.97$	$5267.66^{a}\pm556.28$
Dry matter (%)	$15.74^{b}\pm1.38$	$14.32^{b}\pm2.42$	$11.82^{a}\pm1.11$	$16.57^{b}\pm5.02$
Proteins (%DM)	$64.36^{a}\pm1.19$	$68.37^{a}\pm5.85$	$64.39^{a}\pm0.58$	$58.24^{a}\pm12.3$
Lipids (%DM)	$6.95^{a}\pm1.39$	$5.69^{a}\pm0.61$	$17.75^{ab}\pm0.78$	$21.18^{b}\pm7.31$
Mineral matter (%DM)	$8.55^{a}\pm0.72$	$10.99^{a}\pm2.53$	$10.54^{a}\pm0.64$	$11.95^{a}\pm3.09$

NB: Mean values in the same line followed by the same letter are not significantly different (P>0.05)
DM: Dry Matter.

REFERENCES

Aboua F. 1990. Chemical composition of *Achatina fulica*. Tropicultura. 8: 121.

Aboua F. 1995. Proximate analysis and mineral content of two giant African snails consumed in the Ivory Coast. Trop. Sci. 35: 220.

Aboua F. and Boka K. 1996. Les escargots géants comestibles d'Afrique: quelques aspects physiques et préparation en Côte d'Ivoire. Nature et Faune. 12: 2.

Adeyeye I. E. 1996. Waste yield, proximate and mineral composition of three different types of land snails found in Nigeria. Inter. J. Food Sci. Nutr. 47: 111.

Ashaye O. A., Omole J. A., Adetoro F. O. and Kehinde O. F. 2001. Effect of processing on chemical and sensory properties of two West-African snails (*Achatina fulica* and *Limicolaria spp.* Moor J. Agric. Res. 2: 51.

AOAC. 1984. "Official Methods of Analysis". 14th Ed. Association of Official Analytical Chemists. Washington D.C.

Atwater W.O. and Rosa E. B. 1899. A new respiration calorimeter and experiments on the conservation of energy in human body. Phys. Rev. 9: 129.

Dubois M., Gilles K., Hamilton J. K., Rebers P. and Smith F. 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. 28: 350.

Ebenso I. E. 2002. Consumption and sales of domesticated snails *Archachatina marginata* in rural southern Nigeria. Trop. Sci. 4: 485.

Folch J., Lee M. and Sloane-Stanley G. H. 1957. A simple method for the isolation and purification of total lipid from animal tissues. J. Biol. Chem. 226: 297.

Gomot A. 1998. Biochemical composition of Helix snails: influence of genetic and physiological factors. J. Molluscan Studies. 64: 173.

Kouassi K. D. Otchoumou A. and Gnakri D. 2007. Le commerce des escargots (*Achatina achatina*), une activité lucrative en Cote d'Ivoire. Livestock Research for Rural Development http://www.cipav.org.co./lrrd120/4/koua20058.htm.

Otchoumou A., Zongo D. and Dosso H. 1989-1990. Contribution à l'étude de l'escargot géant africain *Achatina achatina (Linné*). Annales d'Ecologie, Université Nationale de C.I. 21: 31.

Otchoumou A., Dosso H. and Fantodji A. 2003a. The edible African giant snails: fertility of *Achatina Achatina* (Linné, 1758), *Achatina fulica* (Bowdich, 1820) and *Archachatina ventricosa* (Gould, 1850) in humid forest; influence of animal density and photoperiod on fertility in breeding. Bollettino Malacologico 39: 179.

Otchoumou A., Dosso H. and Fantodji A. 2003b. Elevage comparatif d'escargots juvéniles *Achatina achatina* (Linné, 1758), *Achatina fulica* (Bowdich, 1820) et *Archachatina ventricosa* (Gould, 1850): Influence de la densité animale sur la croissance, l'ingestion alimentaire et le taux de mortalité cumulée. Revue Africaine de Santé et Productions Animales 1: 146.

Otchoumou A., N'da K., Dosso H. and Kouassi K. D. 2004a. Inventaire de végétaux sauvages consommés par l'escargot géant africain *Archachatina ventricosa* (Gould, 1850): préférences alimentaires. Haliotis 33: 13.

Otchoumou A., Dupont-Nivet M. and Dosso H. 2004b. Les escargots comestibles de Côte d'Ivoire: Effets de quelques plantes, d'aliments concentrés et de la teneur en calcium alimentaire sur la croissance d'*Archachatina ventricosa* (Gould, 1850) en élevage hors sol en bâtiment. Tropicultura 22: 127.

Otchoumou A., N'da K. and Kouassi K. D. 2005. L'élevage des escargots géants comestibles d'Afrique: Inventaire de végétaux sauvages consommés par *Achatina achatina* (Linné, 1758) et préférences alimentaires. *Livestock Research for Rural Devlopment* http://www.cipav.org.co/lrrd17/3/otch17028.htm.

Pacheco P., Fatima-Martins M., De Battemarque V., Rodriguez P. H. M., Ghion E. and Spers A. 1998. Diferentes fontes de calcio na dieta do escargot gigante africano (*Achatina fulica*) e su efeito no crescimento e rendimento de carcaca. Higiene Alimentar. 12: 43.

Ristic M., Sakac M., Kormanjos S. and Filipovic S. 2000. The investigation of the quality of snail inedible by-products. Tehnologija Mesa, 41: 163.

Saldanha T., Gaspar A., Santana D. M. and Da N. 2001. Composicao centesimal da carne de escargot (*Achatina fulica*) criado em Iguape, SP. Higiene Alimentar. 15: 69.

SAS. 1987. "SAS User's Guide: Statistics". Version 6, Volumes 1 and 2. SAS Institute, Inc, Cary, NC.

Watt B. K. and Merril A. L. 1975. "Composition of Foods". Agriculture Handbook, 8: U.S. Department of Agriculture, Washington D. C.

Zongo D., Coulibaly M., Diambra O. H. and Adjiri E. 1990. Note sur l'élevage de l'escargot géant africain *Achatina achatina* (Linné). Nature et Faune. 6: 32.

Revised paper received January 30, 2009 Accepted May 19, 2009

GUIDE FOR AUTHORS

ITALIAN JOURNAL OF FOOD SCIENCE - IJFS

1. Manuscript Preparation

Manuscripts must submitted as an electronic version by e-mail to ijfs@unipg.it or paolofan@unipg.it (www.chiriottieditori.com/ijf/papersub.htm). The word processor used to generate the file should be indicated and the files should be saved in format “Text only”; **graphs, pictures and diagrams must be saved at 300 dpi in TIF, JPG or EPS formats** (not included in MsWord documents).

Manuscripts must be typed, double-spaced and pages should be in A4 format using Times New Roman 12 pt as the advised font. Top, bottom and side margins should be 25 mm. Pages and lines on all pages, including those for References and figure legends, **must be electronically numbered in the left margin.**

English is the official language. The Editor-in-Chief and/or Co-Editors reserve the right to make literary corrections and to make suggestions to improve brevity, but **the paper must be previously revised for English by the authors**. Authors who are not fluent in written English **must seek** help from one of the following agencies:

www.journalexperts.com
www.sciencedocs.com
www.internationalscienceediting.com
www.writescienceright.com
www.genedits.com

2. Manuscript Preparation

(1) The paper should be divided under the following headings in this order:

Title. Informative of the content of the article (<50 characters + spaces).

Author(s). Initials and Surname, omit professional and official titles. The institute and address where the research was carried out and the current address of each author should be given on the title page.

Abstract. Clearly state the objective of the study, give a concise description of experiment(s), observations, results and conclusions. No references should be cited. **Do not exceed 100 words**.

Key words. Up to six words, in alphabetical order, which describe the document must be given to aid data retrieval and indexing.

Introduction. Review pertinent previous work and cite appropriate references. State the purpose of the investigation.

Materials and Methods. Indicate apparatus, instruments, reagents, etc., giving sufficient detail to allow the work to be repeated.

Results and Conclusions. Results and Conclusions may be presented together or separately. Concisely present results using tables and figures to help justify conclusions (do not present the same information in both forms). Use statistical analysis when appropriate. Unsupported hypotheses should be avoided. Conclusions should point out the significance of the findings and, if possible, relate the new findings to some problem in Food Science and Technology.

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

Units. A list of units particular to the paper may be included.

References. References in the Reference list should be arranged alphabetically (initials of first name, only), and, for the same author, should be arranged consecutively by year, typed double-spaced. Each individual reference should begin flush left (no indentation). Refer to attached examples taken from “Style Guide for Research Papers” by the Institute of Food Technologists (Chicago - Illinois - USA). Literature citations in the text should be referred to by Surname and year in parentheses. If there are more than two authors, give the surname of the first author and add et al. and the year in parentheses. Examples: (SMITH, 2007), (SMITH and JONES, 2008) (SMITH et al., 2008).

(2) Tables should be as few and as simple as possible and include only essential data. Each table must be saved and printed on a separate sheet and have an Arabic number, e.g. Table 4 NOT Tab.

4. Legends must be self-explanatory and on a separate sheet. Use lower-case letters for footnotes in tables and explain below the table in the order in which they appear in the table.

(3) Figures must be prepared and saved separately in **TIF, JPEG, EPS (300 dpi resolution).** They should be prepared so that on 50% reduction, lines, figures and symbols will be clearly legible and not overcrowded. All figures must be given Arabic numbers, e.g. Fig. 3. Legends for figures must be self-explanatory and should be typed on a separate sheet under "Legends to Figures".

(4) Standard Usage, Abbreviations and Units. The Concise Oxford and Webster's English Dictionaries are the references for spelling and hyphenation. Statistics and measurements should always be given in figures, e.g. 10 min, except when the number begins a sentence. When the number does not refer to a unit of measurement it should be spelled out unless it is 100 or greater. Abbreviations should be used sparingly, only when long or unwieldy names occur frequently, and never in the title; they should be given at the first mention of the name. International Standard abbreviations should generally be used except where they conflict with current practice or are confusing. For example, 3 mm rather than $3x10^{-3}$ m. Abbreviations should be defined the first time they are used in the text and they should be used consistently thereafter. Temperatures should be expressed in the Celsius (centigrade) scale. Chemical formulae and solutions must specify the form used, e.g. anhydrous or hydrated, and the concentration must be in clearly defined units.

Common species names should be followed by the Latin binomial (italics) at the first mention. For subsequent use, the generic name should be contracted to a single letter if it is unambiguous.

3. Editorial and Review Policy

Scientific contributions in one of the following forms may be submitted:

Reviews – They can be submitted directly to the Editor-in-Chief or articles can be requested directly by the Editor-in-Chief.

Short Communications, Surveys and Opinions – They do not need to have the formal organization of a research paper; they will receive priority in publication; maximum of five pages allowed.

Papers – The paper must follow the guidelines as specified under the section Manuscript Preparation.

Reviews, Papers, Short Communications and Surveys will be **subjected to critical review by referees.**

(1) Manuscripts will be processed in the order received. The Editor-in-Chief will select papers to enter into the reviewing system based on originality and innovation. A letter will be sent to the authors acknowledging receipt of the manuscript along with a Declaration form stating that it has NOT been previously published, accepted or submitted for publication elsewhere and agreeing to the page charges upon acceptance of the paper. On receipt of the signed Declaration form, the Editor-in-Chief will send the manuscript to a Co-Editor and/or referees for evaluation.

(2) Referees may not be from the same institution as the author. Referees should make their comments and questions in detail and return the paper to the Editor-in-Chief and/or Co-Editor as soon as possible, usually within two weeks. The identity and report of the referees are made know to the Editor-in-Chief, but only the anonymous referee report is sent to the author(s). If all referees recommend acceptance or rejection, the decision stands. If

the opinions of the referees tie, the Editor-in-Chief and/or Co-Editors have the freedom to decide upon acceptance or rejection of the paper.

(3) The results of the refereeing process, accompanied by a letter from the Editor-in-Chief or the Co-Editor, will be sent to the author(s). Papers needing revision must be returned to the Co-Editor within the timeframe suggested, otherwise the paper will be considered as withdrawn. A letter announcing acceptance of the manuscript will be sent to the author(s) upon acceptance by the referees.

(4) The authors **will receive galley proofs** of the manuscript along **with the invoice** for the page charges (stated on the first page of each issue) which **must be paid in order to allow for publication**. The proofs will be sent to the corresponding author as a PDF file by e-mail, only. A hard copy will be sent by mail only if the author makes this request when the paper is accepted for publication.

The revised galley proofs must be returned by fax or mail to Chiriotti Editori – 10064 Pinerolo (TO) – Italy – Fax: +39 0121 794480; e-mail: info@chiriottieditori.it

REFERENCE EXAMPLES

EXAMPLES of use in a Reference list are given below. The bold-faced parenthetical type of citation above the example is indicated ONLY for information and is NOT to be included in the reference list.

(Anonymous)

Anonymous. 1982. Tomato product invention merits CTRI Award. Food Technol. 36(9): 23.

(Book)

AOAC. 1980. "Official Methods of Analysis" 13th ed. Association of Official Analytical Chemists, Washington, DC.

Weast, R.C. (Ed.). 1981 "Handbook of Chemistry and Physics" 62nd ed. The Chemical Rubber Co. Cleveland, OH.

(Bulletin, circular)

Willets C.O. and Hill, C.H. 1976. Maple syrup producers manual Agric. Handbook No. 134, U.S. Dept. of Agriculture, Washington, DC.

(Chapter of book)

Hood L.F. 1982. Current concepts of starch structure. Ch. 13. In "Food Carbohydrates". D.R. Lineback and G.E. Inglett (Ed.), p. 217. AVI Publishing Co., Westport, CT.

(Journal)

Cardello A.V. and Maller O. 1982. Acceptability of water, selected beverages and foods as a function of serving temperature. J. Food Sci. 47: 1549.

IFT Sensory Evaluation Div. 1981a. Sensory evaluation guide for testing food and beverage products. Food Technol. 35 (11): 50.

IFT Sensory Evaluation Div. 1981b. Guidelines for the prepaation and review of papers reporting sensory evaluation data. Food Technol. 35(4): 16.

(Non-English reference)

Minguez-Mosquera M.I., Franquelo Camacho A, and Fernandez Diez M.J. 1981. Pastas de pimiento. Normalizacion de la medida del color. Grasas y Aceites 33 (1): 1.

(Paper accepted)

Bhowmik S.R. and Hayakawa, K. 1983. Influence of selected thermal processing conditions on steam consumption and on mass average sterilizing values. J. Food Sci. In press.

(Paper presented)

Takeguchi C.A. 1982. Regulatory aspects of food irradiation. Paper No. 8, presented at 42nd Annual Meeting of Inst. of Food Technologists, Las Vegas, NV, June 22-25.

(Patent)

Nezbed R.I. 1974. Amorphous beta lactose for tableting U.S. patent 3,802,911, April 9.

(Secondary source)

Sakata R., Ohso M. and Nagata Y. 1981. Effect of porcine muscle conditions on the color of cooked cured meat. Agric. & Biol. Chem. 45 (9): 2077. (In Food Sci. Technol. Abstr. (1982) 14 (5): 5S877).

Wehrmann K.H. 1961. Apple flavor. Ph. D. thesis. Michigan State Univ., East Lansing. Quoted in Wehrmann, K.H. (1966). "Newer Knowledge of Apple Constitution", p. 141, Academic Press, New York.

(Thesis)

Gejl-Hansen F. 1977. Microstructure and stability of Freeze dried solute containing oil-in-water emulsions Sc. D. Thesis, Massachusetts Inst. of Technology, Cambridge.

(Unpublished data/letter)

Peleg M. 1982. Unpublished data. Dept. of Food Engineering., Univ. of Massachusetts, Amherst. Bills D.D. 1982. Private communication. USDA-ARS. Eastern Regional Research Center, Philadelphia, PA.

CONTRIBUTORS

Gratitude is expressed to the following entities for contributing to the realization of the Journal by being supporting subscribers for 2010.

ASSOCIATIONS and COMPANIES

Associazione Italiana di Tecnologia Alimentare (A.I.T.A.) - Milano	Fax +39-02-2365015 www.aita-nazionale.it
Società Italiana di Scienze e Tecnologie Alimentari (S.I.S.T.Al) - Perugia	Fax +39-075-5857939 www.sistal.org
Soremartec Italia srl - Alba	Fax +39-0173-313966

RESEARCH INSTITUTES

Dipartimento di Scienze Tecnologie Agroalimentari (D.I.S.T.A.), Facoltà di Agraria, Università degli Studi della Tuscia, Viterbo	Fax +39-0761-357498
Dipartimento di Ingegneria e Tecnologie Agro-Forestali, Università di Palermo, Palermo	Fax +39-091-484035
Dipartimento di Scienze degli Alimenti, Università di Udine, Udine	Fax +39-0432-558130
Dipartimento di Scienze e Tecnologie Agroalimentari e Microbiologiche (DI.S.T.A.A.M.), Università del Molise, Campobasso	Fax +39-0874-404652
Dipartimento di Scienze e Tecnologie Alimentari e Microbiologiche (DI.S.T.A.M.), Università di Milano, Milano	Fax +39-02-50316601
Dipartimento di Valorizzazione e Protezione delle Risorse Agroforestali (DI.VA.P.R.A.), Sezione Microbiologia ed Industrie Agrarie, Università di Torino, Grugliasco	Fax +39-011-6708549

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 € 20.00

ISSN 1120-1770

CHIRIOTTI EDITORI sas - 10064 Pinerolo - Italy

publishes the technical magazines:

INDUSTRIE ALIMENTARI
INDUSTRIE DELLE BEVANDE
TECNICA MOLITORIA MANGIMIFICI - PASTIFICI - MOLINI - SILI
AROMI ADDITIVI SEMILAVORATI INGREDIENTI ALIMENTARI
ITALIAN FOOD TECHNOLOGY
ITALIAN BEVERAGE TECHNOLOGY
PASTICCERIA INTERNAZIONALE
ITALIAN JOURNAL OF FOOD SCIENCE

VOLUME XXII IJFS No. 1, 2010

CONTENTS

PAPERS

SHORT COMMUNICATIONS