ISSN 1120-1770

ITALIAN JOURNAL OF FOOD SCIENCE

Rivista italiana di scienza degli alimenti

IJFS

Volume XVII
Number 4
2005

Spedizione in ab. post. comma 26 - art. 2 - legge 549/95 n. 4/2005 - Torino

CHIRIOTTI EDITORI

ITALIAN JOURNAL OF FOOD SCIENCE

(RIVISTA ITALIANA DI SCIENZA DEGLI ALIMENTI)

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

Editor-in-Chief:
Paolo Fantozzi
Dipartimento di Scienze Economico-Estimative e degli Alimenti, Università di Perugia,
S. Costanzo, I-06126 Perugia, Italy
Tel. +39 075 5857910 - Telex 662078 UNIPG - Telefax +39 075 5857939-5852067
E-mail: paolofan@unipg.it

Scientific Editor:
S. Mary F. Traynor, F.S.E.
Dipartimento di Scienze Economico-Estimative e degli Alimenti, Università di Perugia,
S. Costanzo, I-06126 Perugia, Italy
Tel. +39 075 5857912 - Telex 662078 UNIPG - Telefax +39 075 5857939-5852067
E-mail: ijfs@unipg.it

Publisher:
Alberto Chiriotti
Chiriotti Editori s.a.s., 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 in food science (chemistry, analysis, microbiology), food technology (engineering, processing) and related areas (nutrition, safety, toxicity, physiology, dietetics, economics, etc.). 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 Advisory Board 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 from the "IJFS Official Referee List" composed of 200 qualified Italian or foreign scientists. Acceptance of a paper rests with the referees.

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

Impact Factor: 0.387 published in the 2004 Journal of Citation Reports, Institute for Scientific Information

Subscription Rate: 2006: Volume XVIII

Ordinary	€	120.00
Supporting	€	500.00

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

IJFS has a page charge of € 20.00 up to 5 pages; extra pages are € 30.00.
Reprints (100) will be sent free of charge.

ITALIAN JOURNAL OF FOOD SCIENCE

ADVISORY BOARD

PAPER

DETERMINATION OF TRANSGENIC MATERIAL ON THE ITALIAN FOOD MARKET USING A NEW MULTIPLEX PCR METHOD

DETERMINAZIONE DI MATERIALE TRANSGENICO NEL MERCATO ALIMENTARE ITALIANO MEDIANTE L'UTILIZZO DI UN NUOVO METODO DI MULTIPLEX PCR

A. GERMINI*, C. SALATI[1], G. QUARTAROLI and R. MARCHELLI
Dipartimento di Chimica Organica e Industriale, Università di Parma,
Parco Area delle Scienze 17/A, 43100 Parma, Italy
[1] Laboratorio Biotecnologie, Progeo spa, Via Asseverati 1, 42029 Masone, RE, Italy
* Corresponding author: Tel. +39 0521 905406, Fax +39 0521 905472
e-mail: andrea.germini@nemo.unipr.it.

ABSTRACT

A recently developed multiplex PCR method, allowing for the simultaneous detection of five different transgenes (Roundup Ready soybean and MON810, Bt176, Bt11 and GA21 maize), was applied to 40 food products available on the Italian market claiming to contain maize and/or soybean as an ingredient. Samples were tested for the presence of GM material using screening methods able to detect DNA traits common to the main transenes and the samples which were

RIASSUNTO

Un metodo di PCR multiplex, recentemente sviluppato per l'analisi simultanea di cinque transgeni (soia Roundup Ready e mais MON810, Bt176, Bt11 e GA21), è stato applicato all'analisi di 40 campioni alimentari ammessi al commercio in Italia e contenenti mais e/o soia tra gli ingredienti. I campioni sono stati sottoposti ad uno screening utilizzando protocolli usati per determinare tratti di DNA comuni ai principali transgeni, ed i campioni trovati positivi sono stati sottoposti all'analisi median-

- Key words: Foodstuff, GMO, maize, multiplex PCR, PCR, soybean -

found to be positive were further tested by to the multiplex PCR analyses. Seven products, some of which claimed to be GMO-free or from organic cultivation, were found to contain traces of GM material, although the levels were always under the legal limit set for compulsory labelling (0.9%).

te multiplex PCR. In sette prodotti, compresi alcuni venduti come OGM free o da coltivazione biologica, sono state trovate tracce di materiale geneticamente modificato, anche se il tenore di OGM non superava la soglia stabilita per l'etichettatura obbligatoria (0,9%).

INTRODUCTION

The presence on the market of food products obtained by biotechnology has generated considerable debate among scientists and consumers over the last few years. While scientific evidence suggests that genetically modified organisms (GMOs) and their derived products should be considered as safe for human consumption as their traditional counterparts, a big gap still exists between this evidence and the general attitude of consumers towards the acceptance of engineered products as components of their diet. To respect the need for consumer choice, the European Union (EU) has enacted legislation requiring strict control of the raw materials used for food and feed production throughout all the food chain and compulsory labelling of foods "containing material which contains, consists of or is produced from GMOs in a proportion higher than 0.9% of the food ingredients considered individually..." (EU, 2003). To date the EU has only allowed a few GMOs and their derivatives to be used for animal or human consumption. However, around 80 GMOs have been approved for cultivation worldwide (source: http://www.agbios.com/dbase.php) and most of these will probably enter the European food chain in some form as a consequence of adventitious or fraudulent contamination.

In order to comply with the strict European regulations and respond to increasing consumer demand for clear label information, it is of great importance for both the producers of raw materials and the food industry, to be able to detect the presence of approved and unapproved transgenic products in food and feed. Moreover, in the case of organic food producers, accurate traceability is crucial because of the total ban of any GM crops from their products.

Although different approaches have been used to develop methods for GMO detection (ANKLAM *et al.*, 2002), the systems used most frequently for routine analyses are those based on specific DNA sequence detection by means of PCR techniques (MEYER, 1999) due to their speed and sensitivity. Amplification of DNA by PCR can detect the presence of trace amounts of GMO products in raw materials and processed foods (AHMED, 2002; VOLLENHOFER *et al.*, 1999). Routine protocols for detecting GMOs involve the use of screening PCR methods to detect DNA traits common to the main transgenes (such as specific promoters and terminators) followed by event-specific PCR to detect the single transgenes.

The availability of the sequences of many transgenic elements used to generate various GMOs (MATSUOKA *et al.*, 2002) together with the development of modified DNA polymerases, such as hot start enzymes, have allowed new, more specific PCR protocols to be developed that can simultaneously investigate several targets with a multiplex PCR approach (LÖFFER *et al.*, 1999). Although

less sensitive, this approach combines several primer pairs in one reaction tube and allows several targets to be detected simultaneously with less consumption of reagents. Methods for the simultaneous detection of four (MATSUOKA *et al.*, 2000) and five (MATSUOKA *et al.*, 2001) transgenic types of maize have been described. Due to the worldwide distribution of transgenic soybean and its importance as an ingredient or source for derivatives used in the food industry, we recently developed and validated a multiplex PCR method for the simultaneous detection of Roundup Ready soybean and MON810, Bt11, Bt176 and GA21 maize in raw material and processed foods (GERMINI *et al.*, 2004).

This new multiplex PCR, in combination with a published PCR method for the screening of GMOs (LIPP *et al.*, 2001), was used to evaluate the occurrence of transgenic material in foodstuffs currently available on the Italian market. The performance of the multiplex PCR method is compared to that of a single PCR for each transgene.

MATERIALS AND METHODS

Food samples

Samples, purchased in different food stores, were chosen on the basis of different parameters: declared composition containing soybean, maize or their derivatives as main ingredients; widespread availability to consumers in different food stores; and being representative of different food processes. Biscuits were the main products available on the market that specifically claimed to contain maize and/or soybean as the main ingredient.

Extraction of genomic DNA

All the products were solid and were ground in a Moulinex blender before extraction in order to enhance homogeneity. Two samples were taken for every product. DNA extraction was performed using the Wizard Plus Minipreps System (Promega Italia, Milan, Italy) following the manufacturer's specifications with two main modifications: first the quantity of starting material was increased from 0.35 to 5 g; and second, lipids were removed by extraction with chloroform in an additional purification step. The extraction procedure is summarized below: 5 g of ground sample were transferred to a 25 mL falcon tube and mixed with 12 mL of extraction buffer (10 mM TRIS, 150 mM NaCl, 2 mM EDTA, 1% SDS, pH 8) and 3 mL of 5M guanidine-HCl and incubated at 60°C for 3 hours. Lipids were removed from each sample by extraction with 5 mL chloroform followed by centrifugation at 7,000 rpm for 10 min. The supernatant was then collected in 2 mL plastic tubes and 8 µL of Proteinase K (20 mg/mL) were added to each tube; the samples were incubated at 60°C for 10 min and then centrifuged at 14,000 rpm for 1 min. The supernatant was transferred to a new 2 mL plastic tube and 5 µL of RNase (100 mg/mL) were added; digestion was carried out at 40°C for 10 min. The solution transferred to a 14 mL falcon tube and 2 mL DNA-binding resin (Wizard Plus Minipreps DNA purification system, Promega Italia, Milan, Italy) was added and thoroughly mixed by inverting several times. The resin was then transferred to a minicolumn using a 2 mL syringe and subsequently washed with 2 mL of 80% isopropanol according to the manufacturer's instructions. The purified DNA was eluted from the column with 100 µL of pre-warmed water (70°C) and stored at -20°C before use.

Evaluation of purity and concentration of extracts

The DNA concentration was measured by UV absorption at 260 nm, while the DNA purity was evaluated on

the basis of the UV absorption ratio at 260/280 nm. All the samples showed a 260/280 nm ratio ranging from 1.6 to 2.0. The extracted materials were then diluted to a final concentration of 50 ng/µL and used as stock solutions for PCR analysis.

Oligonucleotide primers

All the oligonucleotide primers (Table 1) were purchased as purified and desalted from Sigma Genosys (Cambridge, U.K.), then diluted to a final concentration of 20 µM with double distilled water and stored at -20°C until use.

PCR conditions

All the qualitative PCR analyses were performed with a PCR Sprint Thermal Cycler (Thermo Hybaid, Basingstoke, U.K.). Thirteen primers were used in the multiplex PCR at final concentrations of: 0.2 µM for MZfor, MZrev, SLfor, SLrev, Ev176for and Ev176rev; 0.4 µM for MON810rev, RRrev, Bt11for, Bt11rev, GA21for, GA21rev; 0.6 µM for P-E35Sfor. Reactions were performed in a final volume of 25 µL with the following reagent concentrations: genomic DNA 150 ng, PCR reaction buffer 1× (provided with the enzyme) (Qiagen s.p.a, Milan, Italy), 3.5 mM $MgCl_2$, 0.4 mM dNTPs, HotStartTaq DNA Polymerase 0.15 U/µL (Qiagen s.p.a, Milan, Italy). Thermal cycler conditions were as follows: pre-incubation at 95°C for 10 min; 40 cycles consisting of 95°C for 50 s, 60°C for 50 s, 72°C for 50 s; and final elongation at 72°C for 5 min. All the event-specific single PCRs were performed using the same thermal cycler conditions and the above-mentioned concentrations apart from the concentration of forward and reverse primers which were 0.4 µM.

All the single PCRs specific for chloroplast, 35S promoter and NOS terminator (LIPP *et al.*, 2001) were carried out in a final volume of 25 µL at the following reagent concentrations: 120 ng of genomic

Table 1 - Primer names, sequence and target specificity of the set of primers used.

Primer	Sequence (5'-3')	Primer specificity
P-E35s for	CAT TTC ATT TGG AGA GGA CAC G	MON810 maize / Roundup Ready soybean
MON810 rev	GCA TTC AGA GAA ACG TGG CAG TA	MON810 maize
RR rev	TGG GGT TTA TGG AAA TTG GAA	Roundup Ready soybean
MZ for	CGC CAG AAA TCG TTT TTC AT	Maize zein
MZ rev	GGT GGT GTC CTT GCT TCC TA	Maize zein
SL for	ATG GGC TTG CCT TCT TTC T	Soybean lectin
SL rev	CCG ATG TGT GGA TTT GGT G	Soybean lectin
Bt11 for	CTG GGA GGC CAA GGT ATC TAA T	Bt11
Bt11 rev	GCT GCT GTA GCT GGC CTA ATC T	Bt11
Ev176 for	CCC TTC AAC TTC AGC AAC GGC A	Bt176
Ev176 rev	TAG TCG GTC ACG TCG GTC TTC AGG	Bt176
GA21 for	TCT CCT TGA TGG GCT GCA	GA21
GA21 rev	ACG GTG GAA GAG TTC AAT GTA TG	GA21
CLO1	TTC CAG GGT TTC TCT GAA TTT G	Chloroplast
CLO2	TAT GGC GAA ATC GGT AGA CG	Chloroplast
35S-cf3	CCA CGT CTT CAA AGC AAG TGG	35S promoter
35S-cr4	TCC TCT CCA AAT GAA ATG AAC TTC C	35S promoter
HA-nos-118f	GCA TGA CGT TAT TTA TGA GAT GGG	NOS terminator
HA-nos-118r	GAC ACC GCG CGC GAT AAT TTA TCC	NOS terminator

DNA, PCR reaction buffer 1× (provided with the enzyme) (Euroclone, Milan, Italy), 3.0 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 µM forward primer, 0,4 µM reverse primer, 0.1 U/µL Hot Start Blue Taq (Euroclone, Milan, Italy). Thermal cycler conditions were as follows: 95°C for 5 min; 40 cycles of 95°C for 50 s, 60°C for 50 s, 72°C for 50 s; and final elongation at 72°C for 5 min.

Quantitative assays were performed using a real time PCR protocol for GMO quantification according to a published method (VAITILINGOM *et al.*, 1999). Amplification reactions were performed in a final volume of 30mL and carried out using the TaqMan Universal Master-Mix (Applied Biosystems, Monza, Italy) consisting of reaction buffer, dUTP, AmpliTaqGold DNA polymerase, AmpErase uracil N-glycosilase (UNG) and $MgCl_2$. The concentration of the primers was 900 nM both for the transgenic target and the endogenous control. The concentration of the TaqMan probes was 200nM both for the transgenic target and the endogenous control. 2% Soybean Powder certified reference material ERM-BF410e (Fluka, Sigma Aldrich, Milan, Italy) was used as standard, using dilutions in water of the extracted DNA ranging from 150 to 9.37 ng. All the samples were analyzed in triplicate.

Agarose gel electrophoresis

PCR products were analyzed by agarose gel electrophoresis. The gel was prepared with 2.0 and 3.0% agarose (MetaPhor, BioWhittaker 5018, Vallensbaek Strand, DK), for single and multiplex PCR, respectively, in Tris Borate EDTA (TBE) 0.5× with 0.5 µg/mL of Ethidium Bromide (EtBr). The molecular size ladder used was a 50 bp marker (Amersham Biosciences Europe GMBH, Cologno Monzese, Italy). The running conditions were constant voltage at 120 V for 1 h in TBE 0.5× buffer.

RESULTS AND DISCUSSION

DNA was extracted from 40 chemical products and first screened by PCR to detect a chloroplast sequence in order to verify the presence of plant material and amplifiable DNA in the extracts; a GMO screening method was then used that is based on two single PCRs for the detection of the 35S promoter and the NOS terminator sequences (LIPP *et al.*, 2001). All the samples which gave negative results for chloroplast amplification (data not shown) were excluded from further analyses because of the lack of amplifiable plant DNA which was probably the result of degradation during processing. The remaining samples underwent a preliminary test for the presence of the 35S promoter and the NOS terminator. Eighteen samples were chosen (Table 2) to undergo the full analysis process because of: (a) a clear positive response in at least one of the two screening PCRs used (8 samples); (b) very weak amplification signals or; (c) the samples were difficult to ascribe (10 samples). The 18 samples selected were then tested by the full analysis process.

The 35S promoter and NOS terminator screenings were repeated and the results showed that seven samples (2, 4, 6, 11, 12, 14, 15) were positive for the presence of the 35S promoter [Fig. 1 (expected amplicon length 123 bp)] which is present in GM-soybean and GM-maize, and three samples (9, 11, 14) were positive for the presence of the NOS terminator [Fig. 2 (expected amplicon length 118 bp)] which is only present in GM-soybean and Bt11 and GA21 maize. Eight samples (2, 4, 6, 9, 11, 12, 14 and 15) had at least one positive result during the screening.

In order to evaluate which GMOs were responsible for the positive results observed during the screening tests, the multiplex PCR method was applied to the eight samples which had at least

Table 2 - Samples selected after preliminary screening to be tested with the multiplex PCR.

Sample	Product	Note*
1	Maize biscuits	Total maize content 16%
2	Maize fettuccine	Maize flour (100%)
3	Ready to cook polenta	Maize flour (100%)
4	Soybean drink	Soybean proteins. Infant formula
5	Soybean sauce	Soybean seeds
6	Soybean burger	Soybean protein (40%). "OGM-free" product
7	Maize biscuit	Maize flour (20%). From organic production
8	Maize flakes	Maize seeds
9	Maize chips	Maize flour
10	Soybean lecithin	Pure
11	Soybean biscuits	Soybean flour (6.1%). From organic production
12	Soybean biscuits	Soybean flour (15.5%)
13	Maize biscuit	Total maize content 6%
14	Soybean biscuits	Soybean flour (8%)
15	Maize biscuits	Maize flour
16	Soybean biscuits	Soybean flour and proteins
17	Maize and soybean biscuits	Maize starch and flour, soybean flour
18	Maize biscuits	Maize flour (6.8%)

* Where not stated, the quantity was not declared on the label.

one positive result. While the presence of maize or soybean was correctly identified in all samples by means of their target sequences [zein gene (expected amplicon length 139 bp) and lectin gene (expected amplicon length 157 bp) respectively], only three of the eight samples that were positive in the preliminary screening were found to contain GM material, namely Roundup Ready soybean (expected amplicon length 125 bp). Specifically, samples 2, 6, 9, 11, 12 and 15 were found to contain maize; samples 4, 6, 11, 12 and 14 were found to contain soybean, and among these, samples 4, 6 and 14 contained Roundup Ready soybean (Fig. 3).

Because of the low number of positive results obtained by the multiplex PCR compared with what observed during the screening with the specific sequences for the 35S promoter and the

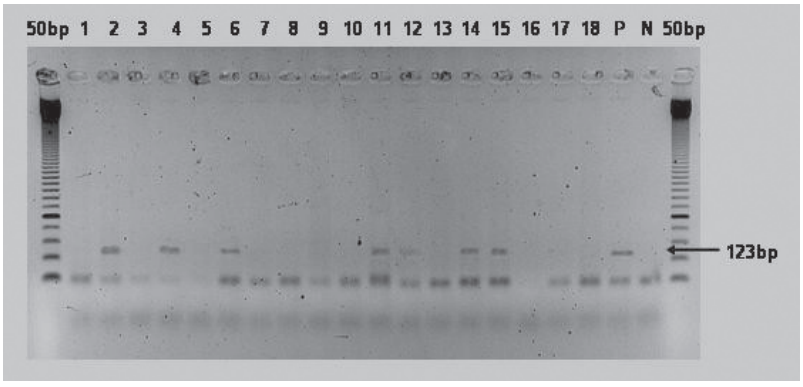

Fig. 1 - Agarose gel electrophoresis of the PCR amplification products for the detection of a DNA sequence specific for the 35S promoter (expected amplicon length 123 bp). Samples from 1 to 18 are as reported in Table 2; P: positive control; N: negative control; 50 bp marker.

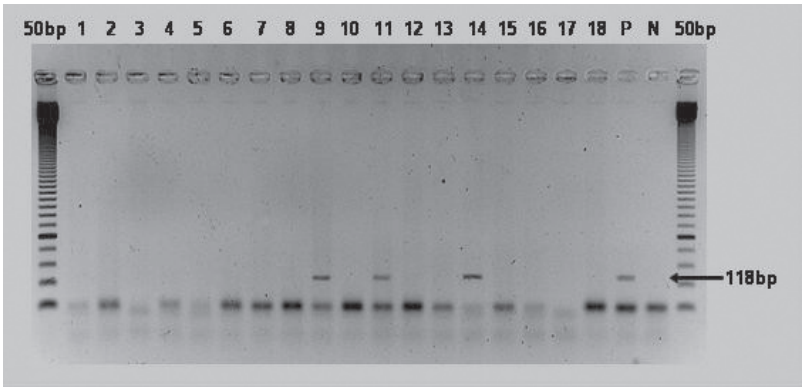

Fig. 2 - Agarose gel electrophoresis of the PCR amplification products for the detection of a DNA sequence specific for the NOS terminator (expected amplicon length 118 bp). Samples from 1 to 18 are as reported in Table 2; P: positive control; N: negative control; 50 bp marker.

NOS terminator, it was decided to test the whole pool of 18 samples by single PCR with the same primer pairs specific for each transgene used during the multiplex test. This was done because multiplex PCRs, while allowing for faster analyses due to multiple simultaneous DNA amplifications, are reported to be less sensitive than single end point PCRs. The multiplex PCR used here was previously found to have a detection limit of 0.25% for each transgene which is in agreement with other similar methods (GERMINI *et al.*, 2004).

Thus the 18 samples reported in Table 2 underwent specific single PCR amplification for the detection of MON810, Bt11, Bt176 and GA21 maize and Roundup Ready soybean. No traces of MON810, Bt11 or GA21 were found among the samples tested (data not shown), while positive results were observed for the presence of Roundup Ready soybean and Bt176 maize. In particular, Roundup Ready soybean was found in three other samples [Fig. 4 (expected amplicon length 125 bp)], not detected by the multiplex PCR (samples 12, 16 and 17). In order to better evaluate these results, the content of GM soybean in all six positive samples was determined, using a standard real time PCR protocol (VAITILINGOM *et al.*, 1999) (complete data not shown). Using this method, samples 4 and 6 were found to contain levels of Roundup Ready greater then the 0.25% detection limit for the multiplex assay (0.5% and 0.6%, respectively), whereas samples 12, 14, 16 and 17 were found to have levels of Roundup Ready low-

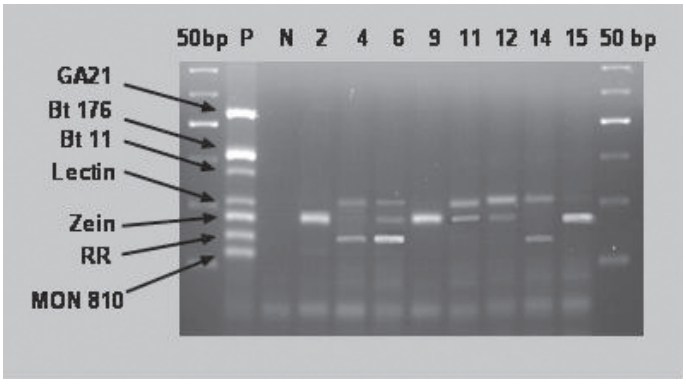

Fig. 3 - Results of the application of the multiplex PCR method for the detection of maize, soybean and MON810, Bt11, Bt176, GA21 maize and RR soybean to eight commercial samples found positive after 35S and/or NOS screening (2: maize *fettuccine*; 4: soybean drink; 6: soybean burger; 9: maize chips; 11, 12, 14: soybean biscuits; maize biscuits). P: positive control; N: negative control; 50 bp marker.

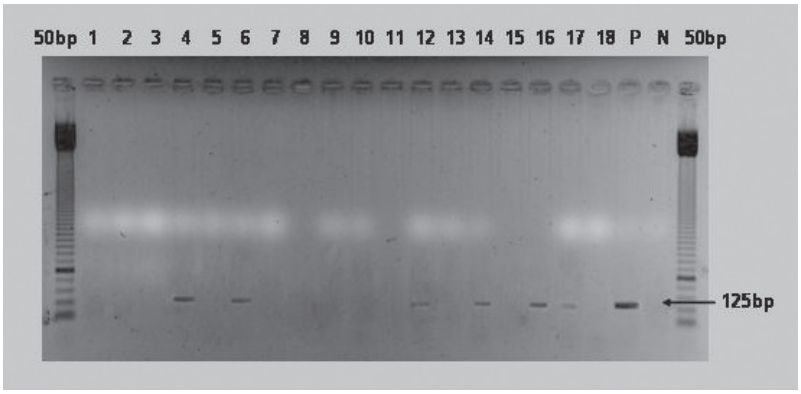

Fig. 4 - Results of the application of the single PCR for the detection of Roundup Ready soybean (expected amplicon length 125 bp). Samples from 1 to 18 are as reported in Table 2; P: positive control; N: negative control; 50 bp marker.

er then 0.25% (0.2% or less). These results closely match and would support the results obtained with the multiplex PCR method which correctly identified samples 4 and 6, and also sample 14, but failed to identify the presence of the transgene in samples 12, 16 and 17. It must be noted that, despite the greater sensitivity of the single PCRs, the previous screening test failed to detect the presence of Roundup Ready soybean in samples 16 and 17. This was the reason why these samples were excluded from the multiplex analysis; however, the single PCR detected it.

A similar interpretation could be given for the results of the single PCR specific for Bt176 maize [Fig. 5 (expected amplicon length 209 bp)]. Sample 7 was the only one found to be positive during the single PCR analysis, although it had been excluded from the multiplex PCR test on account of its negative response during the preliminary screening test.

The results obtained using screening (35S and NOS) and transgene-specific PCRs (multiplex, RoundupReady and Bt176) are summarized in Table 3.

CONCLUSIONS

In conclusion, the multiplex PCR correctly identified the presence of every

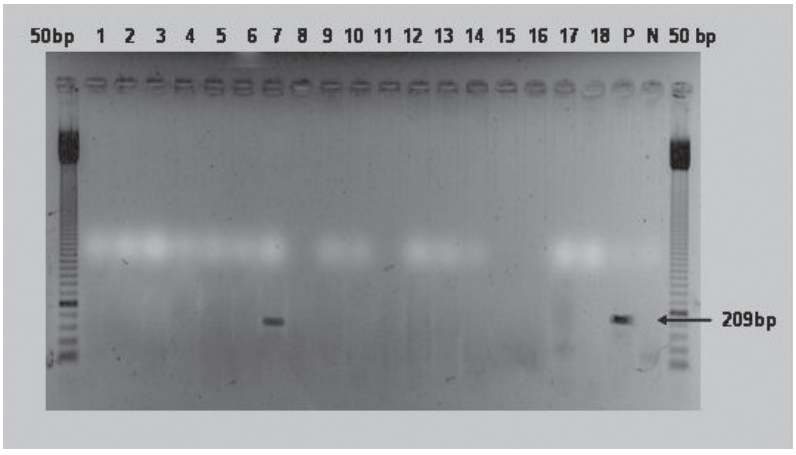

Fig. 5 - Results of the application of the single PCR for the detection of Bt176 maize (expected amplicon length 209 bp). Samples from 1 to 18 are as reported in Table 2; P: positive control; N: negative control; 50 bp marker.

Table 3 - Results of GMO analysis by screening, multiplex and event-specific PCRs.

	Screening PCRs		Transgene specific PCRs		
Sample	35S	NOS	Multiplex	RR	Bt176
1					
2	+				
3					
4	+		+	+	
5					
6	+		+	+	
7					+
8					
9		+			
10					
11	+	+			
12	+			+	
13					
14	+	+	+	+	
15	+				
16				+	
17				+	
18					

target sequence in the samples when the amount of the product was above or around its detection limit (0.25%). The method is therefore suitable and reliable for a fast analysis of foodstuffs when the presence of GMOs must be labelled above the 0.9% level. However, as already reported in the literature, the multiplex method is less sensitive than the single PCR (LOD 0.25% against 0.10%).

Regarding the single PCR method used to detect the 35S promoter and the NOS terminator in the preliminary screening, it must be noted that, although the analyses were repeated twice, once during the pre-screening phase and again during the full analysis process, conflicting results, were obtained when compared with the data obtained from the transgene specific PCRs. In particular four samples which were positive for 35S and/or NOS could not be confirmed by the transgene specific PCRs. This is probably not ascribable to viral or bacterial contamination of the tested products due to the unknown origin of the different raw materials used during manufacturing of the foodstuffs. It is probably due to either the presence of trace amounts of GMOs authorized in the EU under Reg. 258/97/CE but other than those targeted by the multiplex PCR (e.g. NK 603 maize contains both 35S and NOS, and T25 maize contains NOS) or to trace amounts of target DNA close to the LOD of the PCR methods employed. Furthermore, the screening procedure failed to correctly identify its target in three out of the 18 cases but these were later identified by their transgene-specific single PCR. This can be probably ascribed to the different sensitivity of these screening assays was compared to the transgene specific PCRs. The abovementioned considerations raise doubts about the reliability of screening protocols. Although they are fast, relatively easy routine analyses for testing a large number of samples, they can lead to false negative results.

In contrast, using the combined PCR methods reported above it was possible to verify that seven food samples out of 40 (17.5%) randomly chosen from the store shelves contained traces of transgenic material. All the results obtained, however, were consistent with the information reported on the label. Although none of the labels on the products examined claimed to contain GMOs, the content of transgene found during the analysis was always under the 0.9% legal limit for compulsory labelling. On the other hand, two products with labels that claimed to be "GMO-free" and "Organic Production" were found to contain Roundup Ready and Bt176, respectively.

Finally, among the set of samples analysed, the only transgenes found were Roundup Ready soybean and Bt176 maize, both allowed by the European regulations.

ACKNOWLEDGEMENTS

This work was supported by the EU V Framework as part of the European project "DNA-TRACK: traceability of DNA fragments throughout the food chain by DNA/PNA technologies" (OLK1-2000-01658). We would like to thank Dr. Lucia Liviero, Dipartimento di Scienze Ambientali, Università di Parma, who kindly provided the sequence of the primers for the chloroplast analysis.

REFERENCES

Ahmed F.E. 2002. Detection of genetically modified organisms in foods. Trends Biotechnol. 20: 215.

Anklam E., Gadani F., Heinze P., Pijnenburg H. and Van Den Eede G. 2002. Analytical methods for detection and determination of genetically modified organisms in agricultural crops and plant-derived food products. Eur. Food Res. Technol. 214: 3.

EU, Reg. CE 1829/2003.

Germini A., Zanetti A., Salati C., Rossi S., Forré C., Schmid S. and Marchelli R. 2004. Development of a seven-target multiplex PCR for the simultaneous detection of transgenic soybean and maize in feeds and foods. J. Agr. Food Chem. 52: 3275.

Lipp M., Bluth A., Eyquem F., Kruser L., Schimmel H., Van de Eede G. and Anklam E. 2001. Validation of a method based on polymerase chain reaction for the detection of genetically modified organism in various processed foodstuffs. Eur. Food Res. Technol. 212: 497.

Löffer D., Karger S., Twieling G., Ulber V. and Kang J. 1999. Optimization of multiplex PCR. Qiagen News 2.

Matsuoka T., Kawashima Y., Akiyama H., Miura H., Goda Y., Kusakabe Y., Isshiki K., Toyoda M. and Hino A. 2000. A method of detecting recombinant DNAs from four lines of genetically modified maize. J. Food Hyg. Soc. Jpn. 41: 137.

Matsuoka T., Kuribara H., Akiyama H., Miura H., Goda Y., Kusakabe Y., Isshiki K., Toyoda M. and Hino A. 2001. A multiplex PCR method of detecting recombinant DNAs from five lines of genetically modified maize. J. Food Hyg. Soc. Jpn. 42: 24.

Matsuoka T., Kuribara H., Takubo K., Akiyama H., Miura H., Goda Y., Kusakabe Y., Isshiki K., Toyoda M. and Hino A. 2002. Detection of recombinant DNA segments introduced to genetically modified maize (*Zea mays*). J. Agr. Food Chem. 50: 2100.

Meyer R. 1999. Development and application of DNA analytical methods for the detection of GMOs in food. Food Control 10: 391.

Vaitilingom M., Pijnenburg H., Gendre F. and Brignon P. 1999. Real-time quantitative PCR detection of genetically modified maximizer maize and Roundup Ready soybean in some representative foods. J. Agr. Food Chem. 47: 5261.

Vollenhofer S., Burg K., Schmidt J. and Kroath H. 1999. Genetically modified organisms in food screening and specific detection by polymerase chain reaction. J. Agr. Food Chem. 47: 5038.

Revised paper received April 12, 2005 Accepted July 22, 2005

PAPER

EVALUATION OF THE ANTIOXIDANT ACTIVITY OF RED WINES IN RELATIONSHIP TO THEIR PHENOLIC CONTENT

STUDIO DELL'ATTIVITÀ ANTIOSSIDANTE DEI VINI ROSSI IN RELAZIONE ALLA COMPOSIZIONE POLIFENOLICA

G. GIOVANELLI
DISTAM, Dipartimento di Scienze e Tecnologie Alimentari e Microbiologiche, Università degli Studi di Milano, Via Celoria 2, 20133 Milano, Italy
e-mail: gabriella.giovanelli@unimi.it

ABSTRACT

The antioxidant activity of 20 red wines (12 young wines and 7 one-year-old wines) was measured by two in vitro assays: the inhibition of coupled oxidation of β-carotene/linoleic acid mixture and the free radical scavenger activity using 2,2-diphenyl-1-picrylhydrazyl (DPPH*). Total phenolics, total flavonoids and total anthocyanins of wines were determined spectrophotometrically; individual phenolic compounds were also determined by HPLC. The antioxidant activity of the wines was correlat-

RIASSUNTO

L'attività antiossidante di 20 vini rossi (12 vini giovani e 7 vini invecchiati per 12 mesi) è stata determinata mediante due sistemi in vitro: l'inibizione dell'ossidazione accoppiata di una miscela β-carotene/acido linoleico in emulsione e l'attività di radical scavenger su 2,2-diphenyl-1-picrylhydrazyl (DPPH*). Il profilo polifenolico dei vini è stato determinato mediante valutazione dei polifenoli totali, dei flavonoidi totali e degli antociani totali, mentre i singoli composti sono stati determinati mediante anali-

- Key words: antioxidant activity, β-carotene/linoleic acid coupled oxidation, DPPH*, polypehenols, red wines -

ed to their phenolic composition: correlation models showed that the antioxidant activity (evaluated by both methods and expressed as I_{50}) was related to the total phenolics and total flavonoids by a non-linear correlation, while linear correlations were observed if the reciprocal of the I_{50} values were considered. Non-significant correlations were found between the antioxidant activity and total anthocyanins or individual phenolic compounds. The correlation model was the same when young or one-year-old wines were considered, showing that a relatively short storage time under reducing conditions does not substantially modify the antioxidant activity of the phenolic pool.

si HPLC. I dati di correlazione mostrano che l'attività antiossidante, determinata con entrambi i sistemi ed espressa come I_{50}, è correlata ai polifenoli totali e ai flavonoidi totali mediante una relazione esponenziale; la correlazione è di tipo lineare se si considerano i valori di $1/I_{50}$. Non sono state evidenziate correlazioni significative tra l'attività antiossidante e il contenuto di antociani o di singoli composti polifenolici. Dodici mesi di invecchiamento dei vini in condizioni riducenti non sembrano modificare sostanzialmente la relazione tra il potere antiossidante e il pool delle sostanze polifenoliche.

INTRODUCTION

It has been widely demonstrated that the consumption of fruit and vegetables can reduce the risk of cardiovascular disease and certain forms of cancer. The beneficial effect of food is mainly associated with its antioxidant components (HERTOG *et al.*, 1993; GOLDBERG, 1998; FRANKEL, 1999). Red wine is a rich source of polyphenols (mainly flavonoids and anthocyanidins), which have been shown to exert antioxidant activity by various mechanisms (free radical terminators, singlet oxygen quenchers, metal chelators) (BORS *et al.,* 1990; JOVANOVIC *et al.*, 1998; RICE-EVANS, 1999; KINSELLA *et al.,* 1993). Several methods have been proposed to evaluate the antioxidant activity of vegetable products, based on both *in vivo* and *in vitro* reactions. *In vitro* assays are generally based on the inhibition of various oxidation reactions, such as: low density lipoprotein oxidation (FRANKEL *et al.*, 1992; FURHMAN *et al.,* 1995; LANDBO and MEYER, 2001; YILDIRIM *et al.,* 2004), linoleic acid peroxidation in micelles of detergent (PRYOR *et al.*, 1993), methyl-linoleate oxidation (HEINONEN *et al.,* 1998) and coupled oxidation of linoleic acid/β-carotene emulsified mixtures (MARCO, 1968; BADER-SCHNEIDER *et al.,* 1999). Another approach is to evaluate the inactivation of stable synthetic radicals, such as: 2,2-diphenyl-1-picrylhydrazyl (DPPH*) (SÁNCHEZ-MORENO *et al.,* 1999), 2,2'-azinobis (3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (ABTS) (MILLER *et al.*, 1993; PELLEGRINI *et al.*, 2000; MINUSSI *et al.*, 2003) and N,N-dimethyl-p-phenylenediamine (DMPD) (FOGLIANO *et al.,* 1999). In all cases, the synthetic radical has a characteristic colour and the radical inactivation can be followed by spectrophotometry. The direct measurement of oxygen radical absorbance capacity (ORAC) has also been proposed to evaluate antioxidant activity (CAO *et al.*, 1995). Various studies have been conducted on the antioxidant activity of wines in relation to their phenolic content. BADER-

SCHNEIDER *et al.* (1999), BRENNA and PAGLIARINI (2001), MINUSSI *et al.* (2003) and SÁNCHEZ-MORENO *et al.* (2002) found a positive correlation between the antioxidant activity as measured by different methods and the total phenolic content of wines; correspondingly, red wines exert higher antioxidant activity than rosé or white wines. ZAFRILLA *et al.* (2003) and HEINONEN *et al.* (1998) did not find good correlation between total phenolics and antioxidant activity of wines and various fruit wines and liquors. MASTROCOLA *et al.* (2001) observed that white wine and red wine phenolics exert similar antioxidant activity as measured by the DPPH* assay, whereas white wine phenolics are more effective than red wine phenolics towards oxygen scavenging. GHISELLI *et al.* (1998) observed that anthocyanins are the most effective red wine phenolic fraction for scavenging reactive oxygen species and inhibiting lipoprotein oxidation. Other studies have shown some correlation between antioxidant activity and individual phenolic components (YILDIRIM *et al.*, 2004; MINUSSI *et al*, 2003). With regard to ageing, PELLEGRINI *et al.* (2000) showed that the antioxidant activity of young red wines is higher than that of aged red wines when related to total phenolic content. On the contrary, other authors (LARRAURI *et al.*, 1999; ECHEVERRY *et al.*, 2005) have observed that the antioxidant capacity of red wines increases during ageing. In most studies, the wines were tested without previous fractionation or purification, so that sulphur dioxide and other antioxidant components could have contributed to their antioxidant activity.

From literature data it is clear that the antioxidant activity of food material depends on the oxidation substrate, the oxidation mechanism and the reaction medium (BADERSCHNEIDER *et al.*, 1999; RICE-EVANS, 1999). Moreover, phenolic compounds can be evaluated by using a number of different methods, which can give different results. The aim of our study was to evaluate the antioxidant activity of red wines by using different methods and then relate the results to their phenolic profile. Therefore, 20 red wines, produced by the same cellar from different grapes in two subsequent years, were analysed for their antioxidant activity by two *in vitro* assays: the inhibition of coupled oxidation of β-carotene/linoleic acid mixture, and the free radical scavenger activity using 2,2-diphenyl-1-picrylhydrazyl (DPPH*). The phenolic profile of the samples was evaluated by spectrophotometric measurement and by HPLC and correlations between the data were studied. To avoid interference by other wine components, before the antioxidant activity and total phenolics were evalutated, samples were purified by cartridge fractionation to exclude other antioxidant components (principally sulphur dioxide, ascorbic acid and sugars).

MATERIALS AND METHODS

The 20 red wine samples were supplied by the Istituto Sperimentale per l'Enologia (Barletta, Italy). Grapes were grown in the Puglia region following organic farming or integrated pest management practices. The grape varieties were Primitivo (samples 1, 2, 11, 12 and 14-19), Negroamaro (samples 3-5, 9, 10 and 20), Montepulciano (sample 13), Sangiovese (sample 8), Bombino (sample 7) and Uva di Troia (sample 6). All wines were obtained by microvinification in similar and standard conditions (6 to 10 days maceration, use of active dry yeast inoculum, sulphur dioxide addition and no addition of stabilizers or fining agents). The final products were bottled in 750 mL dark glass bottles sealed by crown caps and stored at 15°C. The wines were from two sub-

sequent years (samples from 1 to 7 were one-year-old samples and samples from 8 to 20 were the young wines); samples identified by * were produced from organically grown grapes.

All chemicals and reagents were analytical grade or HPLC grade. Standard compounds used were: gallic acid, (+)catechin, quercetin, *trans*-resveratrol, trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and ascorbic acid (Sigma Aldrich, Italia); procyanidin B2, epicatechin, quercetin-3-glucoside, malvidin-3-glucoside (Extrasynthèse, France); *trans*-caffeic acid, *p*-coumaric acid, myricetin (Fluka, Italia).

Analytical methods

Free and total sulphur dioxide were determined following the simplified iodometric official method (GAZZETTA UFFICIALE CE, 1990).

Total anthocyanin index (TAI) and flavonoid index (FI) were determined spectrophotometrically as described by DI STEFANO *et al.* (1989). Total anthocyanin index is obtained by absorbance at 540 nm and results are expressed as mg/L malvidin-3-glucoside; the flavonoid index is obtained by the relative height of the peak at 280 nm on the absorbance curve and results are expressed as mg/L catechin.

Most data in the literature about antioxidant activity of wines were obtained without any sample treatment, so that relationships between the antioxidant activity and the phenolic content of wines cannot be fully interpreted.

In this study total phenolics (TP) and antioxidant activity analyses were performed on samples previously purified by SepPak C18 cartridge (Waters, Italia) as described by DI STEFANO *et al.* (1989). Wine was diluted 1:1 with 0.01 N H_2SO_4. An aliquot of 2 mL diluted sample was loaded on a C18 SepPak cartridge, previously activated with 2 mL methanol and 3 mL 0.01 N H_2SO_4. After washing the cartridge with 3 mL 0.01 N H_2SO_4, polyphenols were eluted with 2 mL methanol. The methanolic eluate represents the purified sample. Total phenolics were determined by the Folin-Ciocalteau method, and results are expressed as mg/L catechin.

Determination of antioxidant activity

Linoleic acid/β-carotene coupled oxidation.

The method proposed by BADERSCHNEIDER *et al.* (1999) was followed with minor modifications. The reaction mixture was prepared as reported in the literature but using Tween 20 (Sigma, Italia) instead of Tween 40. An aliquot of 5 mL of the mixture was mixed with 250 µL of properly diluted purified sample (methanol was used as dilution solvent). Absorbance at 500 nm was read (1 cm optical path) at the initial time and after incubation at 50°C for 30 min. Antioxidant activity is expressed as percentage inhibition with respect to a control reaction (no sample added) and is calculated as:

% inhibition = (Δ Abs control - Δ Abs sample) / Δ Abs control * 100, where:

Δ Abs control = Abs control at initial time – Abs control after 30 min

Δ Abs sample = Abs sample at initial time – Abs sample after 30 min.

At least four different dilutions were assayed for each sample, in order to obtain a percentage inhibition between 20 and 80% with respect to the control reaction. Results were plotted to obtain a dose-response curve and the sample volume corresponding to 50% inhibition was calculated by interpolation. Two dose-response curves were built for each sample. The antioxidant activity of samples is then expressed as I_{50}, i.e. the original wine volume (µL) corresponding to 50% inhibition of the control reaction.

DPPH* assay

The radical scavenger activity of wines was determined using the radical 2,2-diphenyl-1-picrydazyl (DPPH*), following the method proposed in the literature (SÁNCHEZ-MORENO *et al.*, 1999). A 25 mg/L DPPH* methanolic solution was prepared daily and stored protected from the light. A 30 µL sample (properly diluted with methanol) was added to 3 mL DPPH* solution and incubated at 40°C for 50 min. The DPPH* discoloration reaction was followed by measuring absorbance at 515 nm (1 cm optical path). Absorbance was read on the blank (Abs_{blank}) (30 µL sample + 3 mL methanol), on the control ($Abs_{control}$)(30 µL methanol + 3 mL DPPH* solution) and on the sample solution (30 µL sample + 3 mL DPPH* solution) after the incubation time ($Abs_{sample\ t50}$). The antioxidant activity is expressed as percentage residual DPPH*, calculated as follows:

$$\% \text{ residual DPPH*} = (Abs_{sample\ t=50} - Abs_{blank}) / Abs_{control} * 100$$

Five sample dilutions were tested, to obtain the % residual DPPH* in the range of 20 to 70%. In this case too, dose-response curves (sample amount vs % residual DPPH*) were built to calculate the original sample amount (µL) corresponding to 50% residual DPPH*, which corresponds to I_{50}. Determinations were carried out in duplicate (two dose-response curves for each sample).

HPLC determination of individual phenolic substances was performed using a Waters HPLC apparatus equipped with gradient pumps (mod. 600E) and photodiode array detector (mod. 996). A Symmetry C18 column (4.6x250 mm, 5 µm, Waters, Italia) was used. The chromatographic system was controlled by Millennium 2010 software. Wines were diluted 1:1 with 0.2 M *o*-phosphoric acid and injected into the system (injection volume 20 µL). Binary gradient elution was carried out at 35°C, at 1 mL/min flow rate (eluent A: 0.2 M *o*-phosphoric acid; eluent B: 20% A + 80% acetonitrile), according to the following pattern:

Time (min)	%A	%B
0	90	10
10	80	20
25	60	40
35	40	60
45	30	70
46	0	100
60	90	10

Individual compounds were identified by retention time and spectral analysis.

Statistical analysis of data

Each analytical determination was performed at least in duplicate and values are given as the mean value ± standard deviation. Analytical data were processed by one-way ANOVA and by the least significant difference test (LSD), using Statgraphics Plus v. 5 (Manugest KS Inc, Rockville, USA) to determine significant differences among samples.

Correlations between parameters were evaluated using Table Curve 2D v. 4 (Jandel Scientific, CA, USA).

RESULTS AND DISCUSSION

Several trials were carried out to define the best conditions for determining the antioxidant activity of wines by the DPPH* and linoleic acid/β-carotene assays. For the DPPH* assay, reaction kinetics with various sample concentrations was followed to determine the final reaction time (or equilibrium time). For all concentrations, 50 min at 40°C allowed completion of the reaction. Repeatability of the assay was tested by performing 10 determinations on the same sample, obtaining an I_{50} value of 6.38±0.49 µL (standard deviation).

Similar trials were carried out to optimise the linoleic acid/β-carotene assay. Since the substrate mixture is quite unstable (coupled oxidation occurs even at room temperature), the mixture was prepared daily just before the determination. The control reaction (no antioxidant added, incubation at 50°C) was completed within 30 min. The reaction kinetics in the presence of various sample concentrations was followed: linear reaction kinetics was observed at high sample levels, while asymptotic kinetics was observed at low sample levels. A final reaction time of 30 min was chosen, which corresponds to linear kinetics (i.e. constant reaction rate) in all cases. This fact was critical in that various sample concentrations were to be tested in order to interpolate the I_{50} concentration. Repeatability of the method was determined by performing the assay eight times on the same sample: an I_{50} value of 8.49±1.66 µL (standard deviation) was obtained.

To compare the performance of the two methods with respect to different antioxidant substances, the antioxidant activities of four representative antioxidant compounds were determined: trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, the water-soluble analogous of vitamin E), ascorbic acid, (+)catechin and malvidin-3-glucoside. Dose-response curves obtained with catechin in the two assays are shown in Fig. 1. The antioxidant activity of the standard compounds is reported in Table 1.

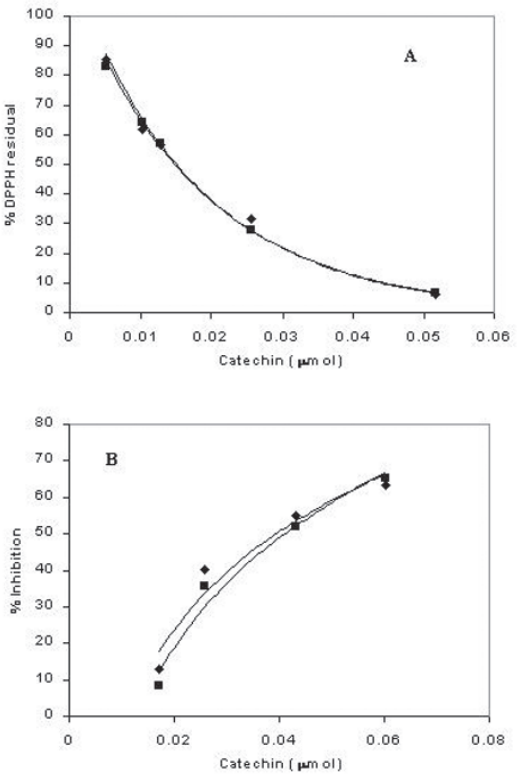

Fig. 1 - Dose-response curves obtained with (+)catechin in the DPPH* (A) and in the linoleic acid/β-carotene (B) assays. Different symbols represent duplicate analyses.

The data show that in the DPPH* assay similar I_{50} values were obtained for the four compounds tested, with catechin being the most effective and trolox the least effective. These data are comparable to those found in the literature (SÁNCHEZ-MORENO *et al.*, 1999).

Table 1 - Antioxidant activity (I_{50}) of standard compounds as measured by the DPPH* and linoleic acid/β-carotene assays.

	DPPH* assay I_{50} (µmol/L)	linoleic acid/β-carotene assay I_{50} (µmol/L)
trolox	0.041±0.001	0.014±0.001
ascorbic acid	0.033±0.001	1.86±0.04
(+)catechin	0.015±0.001	0.040±0.001
malvidin-3-glucoside	0.029±0.001	-

On the contrary, linoleic acid/β-carotene coupled oxidation was more inhibited by trolox and catechin than by ascorbic acid (high antioxidant activity corresponds to low I_{50} values and *vice-versa*). It was not possible to evaluate the antioxidant effect of malvidin-3-glucoside in this assay, because there was an antioxidant effect at low concentrations (<0.1 µmol/L), which became a pro-oxidant effect at higher concentrations. In this model system, water-soluble compounds (such as ascorbic acid) are less effective than lipophilic ones (BADERSCHNEIDER *et al.*, 1999). The rank of antioxidant activity (trolox>catechin>ascorbic acid) can be ascribed to the different solubilities of these compounds in the emulsion and at the surface of lipid particles, where the oxidation reaction takes place.

The analytical data of the 20 wine samples are shown in Table 2. Samples differed significantly one from another as demonstrated by ANOVA analysis. All wines contained sulphur dioxide within the legal limit (150 mg/L). The

Table 2 - Analytical data and antioxidant activity of red wines. Different letters in the same column correspond to significantly different values ($p<0.05$).

Sample	Free SO_2 (mg/L)	Total SO_2 (mg/L)	Total phenolics (mg/L catechin)	Anthocyanin index (mg/L malvidin-3-glucoside)	Flavonoid index (mg/L catechin)	I_{50} linoleic acid/β-carotene (µL sample)	I_{50} DPPH* (µL sample)	Ethanol (1) (mL/100mL)
1	8.00±0.01^{c}	9.60±0.01^{a}	2,568±20^{q}	117.2±5.7^{e}	2,091±15^{o}	4.82±0.07^{a}	3.71±0.01^{b}	14.75
2	10.40±1.13^{d}	16.80±1.13^{b}	3,834±2^{r}	106.3±1.7^{d}	3,920±27^{p}	4.43±0.69^{a}	2.06±0.02^{a}	14.68
3	6.40±0.01^{b}	23.20±1.13^{c}	1,998±25^{p}	95.0±0.6^{c}	1,532±1^{l}	6.26±1.05^{ab}	4.42±0.04^{c}	11.41
4	6.40±0.01^{b}	31.20±1.13^{ef}	970±8^{s}	49.7±2.9^{a}	849±1^{c}	10.18±0.86^{cde}	10.92±0.09^{n}	10.50
5	4.80±0.01^{a}	40.00±0.01^{hi}	885±20^{ab}	42.8±2.3^{a}	721±1^{a}	12.07±0.11^{efg}	14.72±0.09^{p}	12.18
6	8.00±0.01^{c}	16.80±1.13^{b}	1830±9^{n}	61.0±1.7^{b}	1,351±18^{h}	9.09±1.65^{cd}	3.60±0.05^{b}	10.17
7	8.00±0.01^{c}	29.60±1.13^{e}	888±12^{b}	50.1±1.1^{a}	689±9^{a}	19.29±0.10^{l}	7.99±0.05^{h}	10.55
8	16.00±0.01^{f}	31.20±1.13^{ef}	1,399±2^{l}	208.6±0.1^{h}	1,101±9^{e}	10.70±0.03^{cde}	5.86±0.15^{d}	12.59
9	20.00±1.13^{g}	40.80±1.13^{il}	1,128±5^{e}	302.8±0.6^{m}	1,081±1^{e}	13.42±0.39^{g}	6.63±0.07^{e}	12.80
10	10.40±1.13^{d}	33.60±0.01^{g}	1,082±5^{d}	294.7±5.1^{m}	1,069±18^{e}	12.10±1.58^{efg}	9.21±0.12^{l}	12.95
11	16.00±0.01^{f}	32.80±1.13^{fg}	1,249±10^{g}	289.4±4.6^{l}	1,152±9^{f}	11.47±0.59^{def}	7.63±0.21^{g}	13.33
12	23.20±1.13^{h}	61.60±1.13^{o}	1,201±24^{f}	426.5±12.0^{q}	1313±72^{gh}	13.48±1.50^{gh}	6.63±0.09^{e}	16.11
13	55.20±1.13^{j}	96.00±0.01^{q}	1,768±42^{m}	603.1±4.6^{r}	1838±1^{n}	13.98±1.18^{gh}	3.60±0.03^{b}	11.38
14*	6.40±0.01^{b}	46.40±0.01^{n}	846±7^{a}	191.2±4.0^{g}	798±1^{b}	21.86±2.09^{m}	11.23±0.21^{o}	10.65
15*	23.20±1.13^{h}	27.20±0.01^{d}	1,931±19^{o}	367.5±2.9^{n}	1,723±1^{ml}	8.56±0.43^{bc}	4.19±0.01^{c}	14.62
16*	22.40±0.01^{h}	68.00±1.13^{p}	1,441±10^{j}	390.5±0.0^{o}	1,455±18^{l}	12.31±1.19^{efg}	7.14±0.12^{f}	15.39
17*	13.60±1.13^{e}	43.20±0.01^{m}	1,038±10^{c}	253.5±0.6^{i}	997±9^{d}	15.91±0.51^{hi}	10.10±0.33^{m}	11.33
18*	16.00±0.01^{f}	42.40±1.13^{lm}	1,321±3^{n}	407.1±7.4^{p}	1,275±18^{g}	14.12±3.05^{ghi}	7.13±0.19^{f}	13.32
19*	9.60±0.01^{d}	38.40±0.01^{h}	1,304±40^{h}	254.3±2.7^{i}	1,024±9^{d}	16.04±0.70^{j}	8.41±0.06^{i}	12.80
20*	10.40±1.13^{d}	32.80 ±1.13^{fg}	1,093±7de	174.3±2.3^{f}	824±1^{bc}	12.52±0.47^{efg}	10.26±0.15^{m}	11.23

(1) data supplied by the producer; * indicates organically grown grapes.

total phenolics (TP) (measured by the Folin-Ciocalteau method) varied widely among samples, ranging from 846 to 3,800 mg/L. Accordingly, the flavonoid index (FI) (spectrophotometric determination) varied from 689 to 3,920 mg/L. The total anthocyanin index (TAI) also varied widely, from 43 to 600 mg/L, and was not related to TP or FI. The statistical analysis shows that differences between samples cannot be ascribed to the farming system and the storage period, except for anthocyanin concentration, which was lower in aged samples.

With regard to the antioxidant activity, the I_{50} values obtained by the two methods are consistent, although the variation was greater for the linoleic acid/β-carotene assay (ranging from 4 to 22 µL) than for the DPPH* assay (from 2 to 15 µL). The data also show that the I_{50} values were lower in samples with high TP and FI; the average phenolic content was higher in the young wines than in the one-year-old wines, which corresponded to higher antioxidant activity.

HPLC analysis of the wines allowed various compounds to be identified and quantified; the most representative compounds were: gallic acid, *trans*-caftaric acid, resveratrol, myricetin, quercetin, cyanidin-3-glucoside and malvidin-3-glucoside. HPLC data were also used to determine the monomeric anthocyanins (MA), measured as the sum of the peak areas determined at 520 nm and referred to as malvidin-3-glucoside. Relevant data are reported in Table 3. *Trans*-caftaric acid (*trans*-caffeil-tartaric acid) was the most abundant compound, followed by malvidin-3-glucoside and gallic acid. Resveratrol was determined in most samples in low quantities (0.25-1.5 mg/L). For most parameters, the one-year-old wines did not differ from the young wines and no differences can be ascribed to the farming system. The only discriminant parameter was the anthocyanin content, as already shown by the spectrophotometric evaluation: the one-year-old wines (samples from 1 to 7) had a much lower anthocyanin content due to the fact that anthocyanins undergo condensation reactions with other polyphenols, which is a typical consequence of wine ageing.

The analytical data were analysed to determine significant correlations between parameters. The results obtained by the different analytical methods for determining the phenolic compounds were examined first. There were good linear correlations between TP (determined by the Folin-Ciocalteau method) and FI (determined by spectrophotometry): FI = 0.974 TP – 109.7; R^2 = 0.935. Good linear correlation was also found between TAI (spectrophotometric analysis) and MA (determined by HPLC): TAI = 0.814 MA – 55.988; R^2 = 0.929.

The two *in vitro* assays used to measure the antioxidant activity were then compared (Fig. 2). There was poor correlation between the I_{50} values obtained by the two methods (linear correlation coefficient R^2=0.321). As already observed with standard compounds, the two methods give different responses, and therefore this result was partly expected.

The I_{50} values were then related to the phenolic composition of the wines, in order to determine which of the analytical indexes correspond better to the antioxidant activity. Fig. 3 shows the correlations between the I_{50} values obtained by the two methods and TP and FI, respectively. One-year-old samples (empty symbols) showed correlations similar to those of young wines (full symbols), so the data were pooled to obtain the model. Correlation parameters, reported in Table 4, show that the relationship is similar for the two methods used to measure the antioxidant activity and for the two parame-

Table 3 - Phenolic content of red wines as determined by HPLC. Different letters in the same column correspond to significantly different values ($p<0.05$).

Sample	Gallic acid (mg/L)	Trans-caftaric acid (mg/L)	Trans-resveratrol (mg/L)	Myricetin (mg/L)	Quercetin (mg/L)	Cyanidin-3-glucoside (mg/L)	Malvidin-3-glucoside (mg/L)	Monomeric anthocyanins (mg/L malvidin-3-glu)
1	83.6±2.5^{l}	19.2±0.1^{b}	nd	1.9±0.1^{e}	5.0±0.1^{e}	nd	nd	14.4±4.5^{a}
2	208.7±1.4^{m}	nd	nd	nd	nd	nd	nd	7.9±1.2^{a}
3	45.4±1.0^{i}	218.5±0.3^{p}	nd	2.5±0.1^{g}	4.3±0.2^{cd}	nd	nd	14.6±0.1^{a}
4	17.4±0.8^{cde}	187.1±0.4^{o}	0.8±0.1^{e}	1.8±0.1^{d}	4.1±0.1^{c}	nd	nd	12.4±1.5^{a}
5	15.8±0.1^{cd}	162.3±0.5^{n}	0.5±0.1^{bcd}	nd	2.8±0.1^{b}	nd	nd	9.5±0.7^{a}
6	39.4±1.8^{h}	nd^{b}	1.1±0.1^{fg}	nd	4.4±0.4^{cd}	nd	nd	14.3±0.2^{a}
7	10.6±2.6^{b}	8.6±0.1^{a}	1.5±0.1^{h}	1.9±0.1^{de}	2.9±0.2^{b}	nd	nd	15.1±0.7^{a}
8	39.2±2.5^{h}	nd	nd	1.3±0.1^{c}	5.5±0.1^{f}	22.8±0.6^{d}	50.5±0.9^{abc}	95.8±1.5^{bc}
9	9.5±0.1^{ab}	144.7±1.6^{m}	0.3±0.1^{a}	2.2±0.1^{f}	4.4±0.1^{cd}	nd	92.9±0.5^{bcde}	185.4±1.6^{fg}
10	10.0±0.4^{ab}	131.3±1.2^{l}	0.3±0.1^{a}	2.7±0.1^{h}	7.7±0.7^{g}	10.8±1.6^{bc}	94.7±1.0^{cde}	207.7±10.6^{g}
11	2230±2.4^{u}	67.5±20.6^{d}	0.7±0.1^{e}	1.8±0.1^{de}	1.9±0.1^{a}	7.5±0.1^{ab}	69.3±1.5^{abcd}	142.3±18.9^{de}
12	20.7±3.3^{efg}	87.0±2.5^{g}	1.0±0.3^{f}	3.4±0.1^{i}	2.8±0.1^{b}	30.6±0.5^{e}	131.3±1.2^{ef}	270.9±23.2^{h}
13	23.3±0.7^{g}	91.9±2.1^{h}	1.2±0.1^{fg}	2.4±0.1^{g}	4.0±0.1^{c}	29.6±1.4^{e}	217.1±3.2^{g}	497.5±0.3^{i}
14*	14.4±1.1^{c}	70.2±0.1^{e}	0.2±0.1^{a}	1.1±0.1^{b}	nd	nd	31.2±0.5^{a}	67.5±2.1^{b}
15*	21.2±0.9^{efg}	104.6±0.4^{i}	0.6±0.1^{cde}	nd	4.8±0.2^{de}	10.4±0.2^{bc}	105.6±2.7^{de}	181.0±3.7^{fg}
16*	18.9±0.3^{def}	67.2±0.8^{d}	0.5±0.1^{bc}	nd	4.5±0.3^{cd}	28.8±1.2^{e}	161.6±2.4^{f}	307.2±13.5^{i}
17*	15.6±1.0^{cd}	93.0±0.1^{h}	0.7±0.1^{de}	1.7±0.1^{d}	1.6±0.2^{a}	3.5±0.5^{a}	40.5±56.1^{a}	167.8±3.3^{ef}
18*	17.4±0.4^{cde}	78.0±1.1^{f}	0.3±0.1^{ab}	nd	1.7±0.2^{a}	15.2±1.5^{c}	138.2±2.9^{ef}	260.6±9.1^{h}
19*	15.3±1.1^{cd}	25.9±0.7^{c}	nd	0.9±0.1^{a}	nd	nd	45.2±1.2^{ab}	98.2±55.8^{bc}
20*	6.8±0.1^{a}	228.1±1.6^{q}	1.2±0.1^{g}	nd	10.0±0.2^{h}	3.6±0.1^{a}	47.9±0.6^{abc}	123.6±4.9^{cd}

n.d.: not detectable; * indicates organically grown grapes.

ters, TP and FI. The I_{50} values are related to TP and FI by a power equation, and the DPPH* assay provides results which fit the mathematical model better. The linear correlation is also highly significant ($p<0.01$). Our data are in agreement with those of BADERSCHNEIDER *et al.* (1999) and BRENNA and PAGLIARINI (2001), who found a significant correlation between total phenolics and antioxidant activity measured by the linoleic acid/β-carotene assay in white wines and by an electrochemical method in red wines, respectively. The power equations indicate a reciprocal correlation (exponent value close to -1), so the data were rearranged to obtain the linear equations shown in Fig. 4. In this case, the phenolic concentration is related to the $1/I_{50}$ values and the data fit the linear model (correlation parameters are given in Table 4).

No significant correlations were found between antioxidant activity and TAI: for the linear model, R^2= 0.235 (DPPH*) and R^2= 0.221 (linoleic

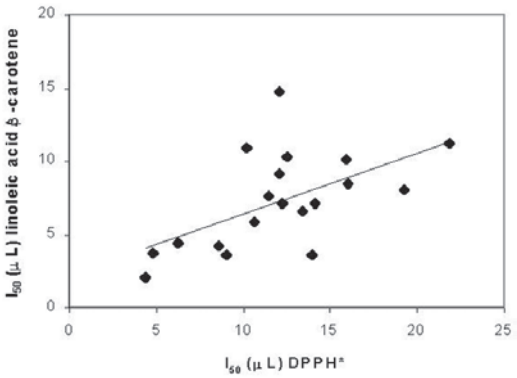

Fig. 2 - Correlation between I_{50} values obtained by the DPPH* and the linoleic acid/β-carotene assays.

acid/β-carotene assay), corresponding to p>0.1 in both cases. It can thus be concluded that the antioxidant activity, measured by both the DPPH* and by the linoleic acid/β-carotene assays, is strongly correlated to TP and FI. If the $1/I_{50}$ values are used, the relationship is described by a linear model. Even in this case, the data obtained by the DPPH* assay fit the mathematical model better and the correlation coefficients are higher when the TP are considered.

Correlations with the I_{50} values were also examined with regard to individual phenolic compounds (as determined by HPLC). The data are shown in Table 5: significant relationships (p<0.01) were only observed for gallic acid and malvidin-3-glucoside, while the cyanidin-3-glucoside concentration was correlated to the antioxidant activity as measured by the DPPH* method only (p>0.01). This result was partly expected, in that single phenolics make up a very small part of the antioxidant pool and behave differently towards different evaluation systems. Monomeric anthocyanins (MA) were not correlated to the I_{50} values (p>0.1).

The general conclusion of the study is that the DPPH* and the linoleic acid/β-carotene assays are suitable for evaluating the antioxidant activity of red wines: both methods are simple, can be executed rapidly and provide sufficiently accurate results. As expected, the two assays behaved differently towards standard compounds, depending on their chemical characteristics. Nevertheless, when applied to red wines, the two assays performed in a similar way. When the antioxidant activity was tested on purified samples, so that interference or side-reactions from wine components other than polyphenols

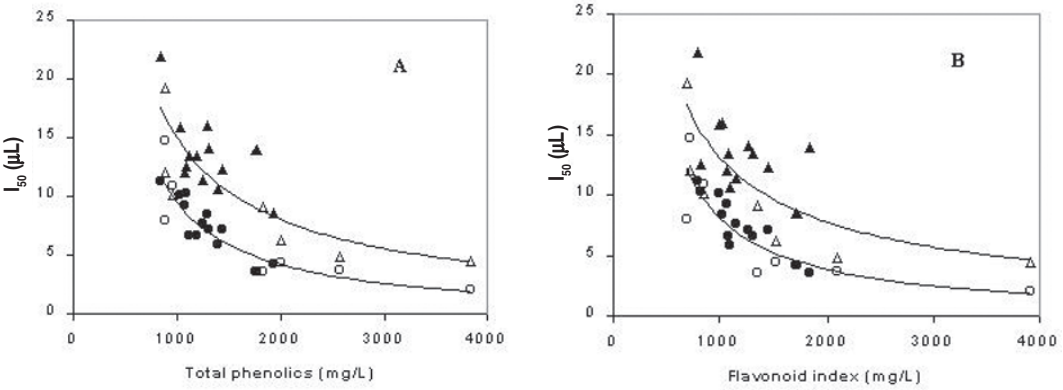

Fig. 3 - Correlation between total phenolic concentration (A) and flavonoid index (B) and the antioxidant activity determined by the DPPH* (●) and the linoleic acid/β-carotene (▲) assays. Open symbols refer to one-year-old samples.

Table 4 - Correlation models of antioxidant activity (I_{50} values) measured by the DPPH* and the linoleic acid/β-carotene assays and phenolic content of red wines.

	Correlation model	R^2, (p)	Parameter value
I_{50} vs total phenolics			
DPPH* assay	power $y = a+b*x^c$	0.808 ($p<0.01$)	a = 0.109; b = 59803; c = -1.263
	linear $y = a+b*x$	0.602 ($p<0.01$)	a = 12.472; b = -3.493 10^{-3}
linoleic acid/β-carotene	power $y = a*x^b$	0.616 ($p<0.01$)	a = 4395; b = -0.821
assay	linear $y = a+b*x$	0.558 ($p<0.01$)	a = 18.965; b = -4.591 10^{-3}
I_{50} vs flavonoid index			
DPPH* assay	power $y = a*x^b$	0.747 ($p<0.01$)	a = 12466; b = -1.058
	linear $y = a+b*x$	0.531 ($p<0.01$)	a = 11.637; b = -3.257 10^{-3}
linoleic acid/β-carotene	power $y = a+b*x^c$	0.478 ($p<0.01$)	a = 1.907; b = 3278; c = -0.819
assay	linear $y = a+b*x$	0.424 ($p<0.01$)	a = 17.455; b = -3.973 10^{-3}
$1/I_{50}$ vs total phenolics			
DPPH* assay	linear $y = a+b*x$	0.914 ($p<0.01$)	a = -2.552 10^{-2}; b = 1.332 10^{-4}
linoleic acid/β-carotene assay	linear $y = a+b*x$	0.813 ($p<0.01$)	a = 6.281 10^{-3}; b = 6.091 10^{-5}
$1/I_{50}$ vs flavonoid index			
DPPH* assay	linear $y = a+b*x$	0.878 ($p<0.01$)	a = -9.047 10^{-4}; b = 1.296 10^{-4}
linoleic acid/β-carotene assay	linear $y = a+b*x$	0.665 ($p<0.01$)	a = 2.266 10^{-2}; b = 5.468 10^{-5}

R^2: determination coefficient; p: probability level.

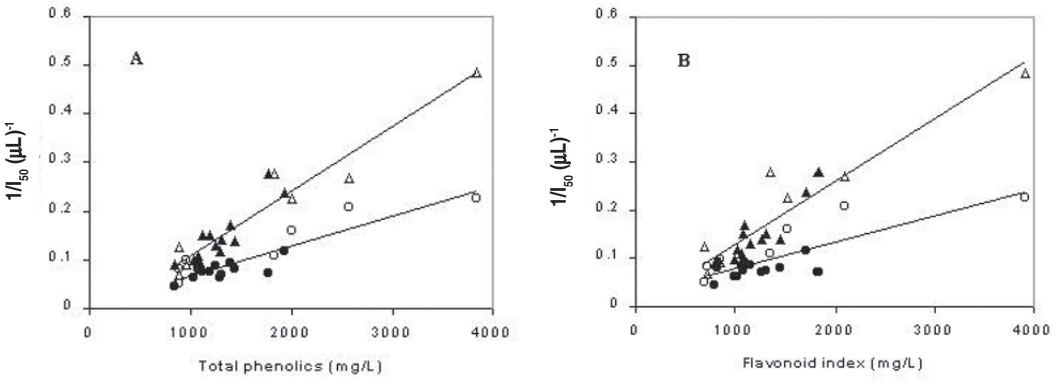

Fig. 4 - Correlation between total phenolic concentration (A) and flavonoid index (B) and $1/I_{50}$ values determined by the DPPH* (●) and the linoleic acid/β-carotene (▲) assays. Open symbols refer to one-year-old samples.

Table 5 - Significant correlation models ($p<0.01$) of antioxidant activity (I_{50} values) measured by the DPPH* and the linoleic acid/β-carotene assays and individual phenolic components.

	Correlation model	R^2, (p)
I_{50} vs gallic acid		
DPPH* assay	power $y = a+b*x^c$	0.368 ($p<0.01$)
linoleic acid/β-carotene assay	power $y = a+b*x^c$	0.427 ($p<0.01$)
I_{50} vs malvidin-3-glu		
DPPH* assay	power $y = a+b*x^c$	0.380 ($p<0.01$)
linoleic acid/β-carotene assay	power $y = a+b*x^c$	0.445 ($p<0.01$)
I_{50} vs cyanidin-3-glu		
DPPH* assay	power $y = a+b*x^c$	0.320 ($p<0.01$)
$1/I_{50}$ vs gallic acid		
DPPH* assay	linear $y = a+b*x$	0.715 ($p<0.01$)
linoleic acid/β-carotene assay	linear $y = a+b*x$	0.698 ($p<0.01$)
$1/I_{50}$ vs malvidin-3-glu		
DPPH* assay	linear $y = a+b*x$	0.375 ($p<0.01$)

R^2: determination coefficient; p: probability level.

were minimised, it was strictly correlated to the total phenolics and flavonoid index. The results of this study also show that one-year storage under non-oxidative conditions does not seem to influence the antioxidant activity of red wines. This is principally due to the fact that anthocyanins, the phenolic components which undergo major changes during this kind of storage, are minimally involved in antioxidant activity.

ACKNOWLEDGMENTS

The author wishes to thank Prof. R. Di Stefano, who provided assistance and helpful advice for the application of the analytical methods.

REFERENCES

Baderschneider B., Luthria D., Waterhouse A.L. and Winterhalter P. 1999. Antioxidants in white wine (cv. Riesling): I. Comparison of different testing methods for antioxidant activity. Vitis 38: 127.

Bors W., Heller W. and Michel C. 1990. Flavonoids as antioxidants. Determination of radical scavenging efficiencies. Methods Enzymol. 186: 343.

Brenna O.V. and Pagliarini E. 2001. Multivariate analysis of antioxidant power and polyphenolic composition in red wines. J. Agric. Food Chem. 49: 4841.

Cao G., Verdon C.P., Wu A.H.B., Wang H.E. and Pryor R.L. 1995. Automated oxygen radical absorbance capacity assay using the COBAS FARA II. Clin. Chem. 41: 1738.

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

Echeverry C., Ferreira M., Reyes-Parada M., Abin-Carriquiry J.A., Blasina F., Gonzalez-Neves G. and Dajas F. 2005. Changes in antioxidant capacity of Tannat red wines during early maturation. J. Food Engin. 69: 147.

Fogliano V., Verde V., Randazzo G. and Ritieni A. 1999. Method for measuring antioxidant activity and its application to monitoring the antioxidant capacity of wines. J. Agric. Food Chem. 47: 1035.

Frankel E.N. 1999. Natural phenolic antioxidants and their impact on health. In "Antioxidant Food

Supplements in Human Health". L. Packer (Ed.), p. 385. Academic Press, London.

Frankel E.N., German J.B. and Davis P.A. 1992. Headspace gas chromatography to determine human low-density lipoprotein oxidation. Lipids 27: 1047.

Furhman B., Lavy A. and Aviram M. 1995. Consumption of red wine with meals reduces the susceptibility of human plasma and low-density lipoprotein to lipid peroxidation. Am. J. Clin. Nutr. 61: 549.

Gazzetta Ufficiale delle Comunità Europee N. 272, 3/10/90.

Ghiselli A., Nardini M., Baldi A. and Scaccini C. 1998. Antioxidant activity of different phenolic fractions separated from Italian red wine. J. Agric. Food Chem. 46: 361.

Goldberg D.M. 1998. The role of wine phenolics in disease prevention. Riv. Vitic. Enol. 3: 15.

Heinonen I.M., Lethonen P.J. and Hopia A.I. 1998. Antioxidant activity of berry and fruit wines and liquors. J. Agric. Food Chem. 46: 25.

Hertog M.G., Feskens E.J.M., Hollman P.C., Katan M.B. and Kromhout D. 1993. Dietary antioxidant flavonoids and risk of coronary heart disease: the Zutphen elderly study. Lancet 342: 1007.

Jovanovic S., Steenken S., Simic M. and Hara Y. 1998. Antioxidant properties of flavonoids: reduction potentials and electron transfer reactions of flavonoid radicals. In "Flavonoids in Health and Disease". C. Rice-Evans and L. Packer (Ed.). p. 137. Marcel Dekker, New York.

Kinsella J.E., Frankel E.N., German J.B. and Kanner J. 1993. Possible mechanism for the protective role of antioxidants in wine and plant foods. Food Technol. 4: 85.

Landbo A.K. and Meyer A.S. 2001. Ascorbic acid improves the antioxidant activity of European grape juices by improving the juices ability to inhibit lipid peroxidation of human LDL in vitro. Int. J. Food Technol. 36: 156.

Larrauri J.A., Sánchez-Moreno C., Ruperez P. and Saura-Calixto F. 1999. Free radical scavenging capacity in the aging of selected red Spanish wines. J. Agric. Food Chem. 47: 1603.

Marco G.J. 1968. A rapid method for evaluation of antioxidants. J. Am. Oil Chem. Soc. 45: 594.

Mastrocola D., Manzocco L. and Nicoli M.C. 2001. Antioxidant activity of wine: effect of winemaking technology and ageing. Industrie delle Bevande 30: 375.

Miller N.J., Rice-Evans C., Davies M.J., Gopinathan V. and Milner A. 1993. A novel method for measuring antioxidant capacity and its application to monitoring the antioxidant status in premature neonates. Clin. Sci. 84: 407.

Minussi R.C., Rossi M., Bologna L., Cordi L., Rotilio D., Pastore G.M. and Duran N. 2003. Phenolic compounds and total antioxidant potential of commercial wines. Food Chem. 82: 409.

Pellegrini N., Simonetti P., Gardana C., Brenna O., Brighenti F. and Pietta P. 2000. Polyphenol content and total antioxidant activity of Vini Novelli (young red wines). J. Agric. Food Chem. 48: 732.

Pryor W.A., Cornoicelli J.A., Deva L., Tait B., Trivedi B., Witiak D.T. and Wu M. 1993. Rapid screening test to determine the antioxidant potencies of natural and synthetic antioxidants. J. Org. Chem. 58: 3521.

Rice-Evans C. 1999. Screening of phenolics and flavonoids for antioxidant activity. In "Antioxidant Food Supplements in Human Health". L. Packer (Ed.). p. 239. Academic Press, London.

Sánchez-Moreno C., Larrauri J.A. and Saura-Calixto F. 1999. Free radical scavenging capacity of selected red, rosè and white wines. J. Sci. Food Agric. 79: 1301.

Sánchez-Moreno C., Jiménez-Escrig A. and Saura-Calixto F. 2002. LDL oxidizability indexes in measurement of antioxidant activity in selected Spanish wines. Nutr. Res. 22: 507.

Yildirim H.K., Akcay Y.D., Guvenc U. and Sozmen E.Y. 2004. Protection capacity against low-density lipoprotein oxidation and antioxidant potential of some organic and non-organic wines. Int. J. Food Sci. Nutr. 55: 351.

Zafrilla P., Morillas J., Mulero J., Cayuela J.M., Martinez-Cacha A., Pardo F. and Lopez Nicolas J.M. 2003. Changes during storage in conventional and ecologial wine: phenolic content and antioxidant activity. J. Agric. Food Chem. 51: 4694.

Paper received March 21, 2005 Accepted June 14, 2005

PAPER

EVALUATION OF ANTIOXIDANT ACTIVITY OF SPIRITS USING TWO DIFFERENT FREE RADICAL SCAVENGING TESTING METHODS

ATTIVITÀ ANTIOSSIDANTE DI AMARI DETERMINATA CON DUE DIFFERENTI METODI BASATI SULL'EFFETTO SCAVENGER DI RADICALI LIBERI

K. HÖNER* and R. CERVELLATI[1]
Institut für Fachdidaktik der Naturwissenschaften,
Abt. Chemie und Chemiedidaktik, Technische Universität Braunschweig,
Pockelsstraße 11, 38106 Braunschweig, Germany
[1]Dipartimento di Chimica "G. Ciamician", Università di Bologna,
Via Selmi, 2, 40126 Bologna, Italy
*Corresponding author: Tel. +49 531 3912876, Fax +49 531 3912845,
e-mail: k.hoener@tu-bs.de

ABSTRACT

The relative antioxidant activities of 17 commercial herbal spirits were investigated by using two different analytical assays: the Briggs-Rauscher oscillating reaction method (carried out at pH ≈ 2) and the Trolox Equivalent Antioxidant Capacity method (carried out at pH = 7.4). The total phenolic content of these spirits was also determined using the Folin-Ciocalteu reagent. The results show that the antioxidant activity

RIASSUNTO

L'attività antiossidante relativa di 17 amari è stata valutata usando due diversi saggi in vitro: il metodo della reazione oscillante di Briggs-Rauscher (che lavora pH ≈ 2) e il metodo TEAC (Trolox Equivalent Antioxidant Capacity, che lavora a pH = 7,4). È stato anche determinato il loro contenuto totale di fenoli con il reagente di Folin-Ciocalteu. I risultati mostrano che l'attività antiossidante delle bevande alcoliche esami-

- Key Words: antioxidants, Briggs-Rauscher, herbal spirits, total phenolic content, Trolox Equivalent Antioxidant Capacity -

of the herbal spirits was similar to that of white wines, while their total phenolic content was somewhat higher than that of white wines. A comparison of the ranking order of the antioxidant capacity obtained with the different methods is reported and discussed.

nate è simile a quella dei vini bianchi, mentre il loro contenuto totale di fenoli è più alto rispetto a quello dei vini bianchi. Viene infine riportato e discusso un confronto degli ordini di attività ottenuti con i diversi metodi utilizzati.

INTRODUCTION

Cardiovascular diseases and cancer are ranked as the first and second leading causes of death in most industrialized countries. Regular consumption of fruit and vegetables is associated with reduced risk of cancer, cardiovascular diseases, stroke, Alzheimer disease, cataracts and some of the functional declines associated with ageing. Prevention is a more effective strategy than pharmacological treatment in chronic diseases. Food that contains significant amounts of bioactive components provides desirable health benefits as well as basic nutrition, and plays an important role in the prevention of chronic diseases (LIU, 2003; FREI, 1994; MADHAVI *et al.*, 1996; CADENAS and PACKER, 1996; HANNUM, 2004; HEBER, 2004; BOYER and LUI, 2004; PARK and SURH, 2004). Antioxidants found in many foods strengthen the endogenous antioxidant system by reducing oxidative stress and thereby help protect against pathological events (PACKER, 1996; SIMONETTI *et al.*, 1997).

Several studies have shown that a moderate consumption of wine, especially red wine, may have protective effects (RENAUD and DE LORGERIL, 1992; CRIQUI and RINGEL, 1994; KROENKE *et al.*, 2003; SATO *et al.*, 2002; CORDER *et al.*, 2001; STOCLET, 2001; SUN *et al.*, 2002). The results indicate that ethanol itself has potential positive effects but that wine with its phenolic compounds may give additional benefits due to its greater antioxidant activity. The antioxidant defence depends specifically on the radical-scavenging properties of these phenolic substances (FRANKEL *et al.*, 1993; TEISSEDRE *et al.*, 1996; PELLEGRINI *et al.*, 2000; LANDRAULT *et al.*, 2001; SIMONETTI *et al.*, 1997).

Besides the possible positive effects of some alcoholic beverages, the metabolism of alcohol might lead to the production of hydroxyl radicals (NORDMANN, 1994; ALBANO *et al.*, 1996; IMARK *et al.*, 2001; CEDERBAUM, 2001). Hydroxyl radicals are very reactive among the radicals and other reactive oxygen species that play a role in human metabolism. Their presence can readily lead to uncontrolled reactions, resulting in oxidative damage to important biological macromolecules such as nucleic acids, proteins, and lipids (ROBERFROID and BUC CALDERON, 1995; ALBANO *et al.*, 1996).

Commercially-sold herbal spirits or liquors are known for the positive effects they have on the gastroenteric system, such as aiding digestion and strengthening the stomach wall. Some studies have been carried out using various methods to determine the antioxidant activity of herbal and other spirits in order to evaluate the total phenolic content and the antioxidant activities (IMARK *et al.*, 2001; GOLDBERG *et al.*, 1999).

To produce herbal spirits, selected herbs are extracted with a mixture of alcohol and water. The exact recipe is a se-

cret of the producer. In some spirits the number of herbs and/or plants used is reported on the bottle label. Besides their positive digestive effects, phenolic compounds can also react with radicals in the upper part of the gastroenteric tract, i.e. the stomach (KANNER *et al.*, 2003; TALAVERA *et al.*, 2003; PASSAMONTI *et al.*, 2003). In fact phenolic compounds have an inhibiting effect on chemical-induced tumors in the stomach and in other organs (COHEN, 1988).

Several chemical testing methods are available for measuring *in vitro* the antioxidant capacity of beverages and/or plant extracts (FRANKEL *et al.*, 1992; MARCO, 1968; PRYOR *et al.*, 1993; MILLER *et al.*, 1993, BRAND-WILLIAMS *et al.*, 1995; FOGLIANO *et al.*, 1999). Many of these methods are carried out at the same pH as that of blood (pH = 7.4). A commonly used test is the Trolox Equivalent Antioxidant Capacity (TEAC) assay, of which there are different modifications (RE *et al.*, 1999).

Some of these testing methods were recently compared on the basis of the ranking order of the antioxidant capacity of some pure substances and beverages (BADERSCHNEIDER *et al.*, 1999; SCHLESIER *et al.*, 2002). The results showed that the methods were not fully comparable because the ranking order differed from assay to assay (SCHLESIER *et al.*, 2002). The authors concluded that the differences were probably caused by the different pH values at which the methods were performed. They recommended using at least two methods when studying the protective *in vitro* efficacy of the samples.

A method that is still largely used to determine the total phenolic content is based on the Folin-Ciocalteu (FC) reagent (DI STEFANO *et al.*, 1989; CHU *et al.*, 2002), even if more selective, precise methods are now available such as ^{1}HNMR (DEL CAMPO MARTINEZ *et al.*, 2002) or micro methods (ROMANI *et al.*, 1996). The results of the Folin Ciocalteu method are sometimes used to get an indication of the antioxidant capacity of the samples (MADHAVI *et al.*, 1996; IMARK *et al.*, 2001; BADERSCHNEIDER *et al.*, 1999; SCHLESIER *et al.*, 2002; HEINONEN *et al.*, 1998).

The aim of the present study was to evaluate the antioxidant activity of seventeen herbal spirits. The Briggs-Rauscher oscillating reaction method (BR) was used under acidic conditions, and the results were compared with those obtained with the TEAC assay under physiological conditions (pH = 7.4). The total phenolic content was also determined using the Folin-Ciocalteu reagent method, which is based on the redox reaction between phenolate anions and a mixture of phosphotungstate and phosphomolybdate.

The BR method like other methods is based on the generation of free radicals in the reaction mixture. Antioxidant scavengers of free radicals added to an active oscillating BR mixture immediately quench the oscillations, but, after some time (inhibition time that linearly depends on the amount of antioxidant added) the oscillations resume (CERVELLATI *et al.*, 2000; 2001). The BR reaction method was tested successfully on white and red wines (HÖNER *et al.*, 2002; PRENESTI *et al.*, 2005), several fruit and vegetable extracts (HÖNER and CERVELLATI, 2002; 2003) and some natural polyphenolic compounds (CERVELLATI *et al.*, 2002). In the present work the Briggs-Rauscher oscillating reaction method was used to determine the antioxidant activities. The BR method works at an acidic pH and could therefore give some further information about antioxidant activity at low pH values, similar to the pH of human gastric fluids.

The TEAC assay analyses the ability of antioxidants to reduce the radical cation $ABTS^{\bullet+}$. One version of this assay uses a pre-formed $ABTS^{\bullet+}$ radical and determines the decrease in absorbance (RE *et al.*, 1999).

MATERIALS AND METHODS

Malonic acid (Merck, reagent grade, >99%), manganese (II) sulfate monohydrate (Merck, reagent grade, >99%), $NaIO_3$ (Merck, reagent grade, >99.5%), Na_2CO_3 anhydrous (Merck; reagent grade, >99,9%), gallic acid (= 3,4,5-trihydroxy benzoic acid, Fluka; reagent grade, ≥98%), 2,6-DHBA (= 2,6-dihydroxy benzoic acid, Aldrich; reagent grade ≥98%), $K_2S_2O_8$ (Fluka; reagent grade ≥99%), ABTS (2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid), Fluka No. 11557), Trolox (6-hydroxy-2,5,8-tetrametylchroman-2-carboxylic acid, Aldrich No. 39,192-3) were used without further purification. Folin-Ciocalteu reagent (Fluka No. 47641), $HClO_4$, H_2O_2, and other chemicals were of analytical grade. All stock solutions were prepared with double distilled, deionized water. Perchloric acid was analyzed by titration vs. a standard 0.1 M NaOH solution (from Merck). H_2O_2 was standardized daily by manganometric analysis.

Seventeen herbal spirits were tested. They were kept in subdued lighting and were diluted in a suitable way with double distilled, deionized water prior to the measurements.

BR measurements

Oscillations in the BR mixtures were followed potentiometrically by recording the potential of a combined redox electrode (Mettler Toledo InLab 501, Giessen, Germany). The electrode was connected to a multimeter (WTW, model pH 540 GLP, Weilheim i. OB, Germany) controlled by an IBM-compatible PC. The accuracy of the multimeter was ±1 mV. The data-acquisition program Multi Achat II (WTW, Weilheim i. OB, Germany) was used. The multimeter was equipped with a temperature sensor with an accuracy of ±0.1°C. All solutions and reaction mixtures were maintained at constant temperature (25.0°C) using a thermostating system (accuracy ±0.1°C).

One millilitre of diluted spirit was added to 30 mL of an active, well-stirred BR mixture (initial composition: $[H_2O_2]$ = 1.5 M, $[HClO_4]$ = 0.0266 M, $[IO_3^-]$ = 0.0667 M, [malonic acid] = 0.05 M, $[Mn^{2+}]$ = 0.0063 M) after the third oscillation. The order of addition was malonic acid, $MnSO_4$, $HClO_4$, $NaIO_3$, and H_2O_2. The oscillations started after H_2O_2 was added and then the inhibition times were measured.

All sample dilutions were analyzed at least in triplicate and the results are expressed as the mean values of 2,6-DHBA equivalents (mg/L) ± standard deviation. 2,6-DHBA was chosen as standard because the concentration intervals explored for all the samples fell within the concentration interval for 2,6-DHBA. The spirits were suitably diluted according to their activity. Dilutions from 3:10 to 5:1000 were used.

TEAC measurements

For the TEAC measurements, the $ABTS^{\bullet+}$ radical cation was prepared by mixing ABTS stock solution (7 mM in water) with 2.45 mM potassium persulfate. The mixture must be kept in the dark for 12 to 24 h until the reaction is complete and absorbance is stable. For the measurements, the $ABTS^{\bullet+}$ solution was diluted with ethanol to an absorbance of 0.800±0.020 at 734 nm. The herbal spirits were diluted from 1:1 to 2:10. For the photometric assay 3.0 mL of diluted $ABTS^{\bullet+}$ solution and 30 µL of the sample solution were mixed in a photometric cuvette (1.00 cm optical path length) for 45 s and the absorbance was measured after exactly 1 min at 734 nm. A blank with distilled water was measured in the same way. The difference between the results of the blank and the sample gave ΔE60 ($E60_{blank}$-$E60_{sample}$ = ΔE60), the value used for further calculations of the Trolox equivalents (TEAC) in mg/L. A stock solution of Trolox 0.25 mg/mL was prepared and diluted to an amount ranging from 0.05 to 0.1875 mg/mL. Absorbance was measured by using

a CECIL 3000 spectrophotometer (W.S.B. Electronic, Worpswede, Germany).

Ethanol does not interfere in either the TEAC (GOLDBERG *et al.*, 1999) or the BR (CERVELLATI *et al.*, 2002 b) assays.

Determination of total phenolics using the Folin-Ciocalteu reagent

This test is based on the oxidation of phenolic groups by phosphomolybdic and phosphotungstic acids (Folin-Ciocalteu reagent). After oxidation the absorbance of a green-blue complex can be measured at 765 nm. The procedure was used for the total volume (20 mL) of the reacting mixture (SINGLETON and ROSSI, 1965).

Two millilitres of a suitably diluted sample solution were mixed with 10 mL of Folin-Ciocalteu reagent (diluted 1:10 with distilled water) in a 20 mL volumetric flask and allowed to stand at 24°C for exactly 5 min. Then eight millilitres of a sodium carbonate solution containing 0.6 g anhydrous Na_2CO_3 were added to the mixture. After exactly 2 hours at 24°C, absorbance was measured at 765 nm. The blank (2 mL of distilled water) was treated in the same way. Gallic acid was used as standard and the total phenolic content is expressed as gallic acid equivalents (GAE) in mg/L.

A stock solution of gallic acid (1 g/L) was prepared and diluted to an amount ranging from 10 to 70 mg/L just before the measurements were taken. The spirits were diluted from 3:10 to 1:100. All measurements were made in triplicate. Because the spirits were diluted, the resulting sugar content in the reacting mixture did not interfere with the measurements; this was controlled with stock sugar solutions up to 300 g/L. The ethanol content did not interfere with the measurements (SINGLETON and ROSSI, 1965). Absorbance was measured by using a CECIL 3000 spectrophotometer.

RESULTS

Antioxidant activity in acidic conditions

Table 1 reports the origin, alcoholic strength, sugar content and the number

Table 1 - Origin, alcoholic strength, sugar content and the number of herbs used (when reported) of the herbal spirits tested.

N.	Origin	Alcohol vol.%	Sugar content g/L	N. of herbs used
1	Germany	35	≥100	n.r.
2	Italy	16	≥100	n.r.
3	Germany	32	231	60
4	Germany	44	<20	n.r.
5	Germany	32	≥100	n.r.
6	Germany	16.5	261	n.r.
7	Italy	40	16	40
8	Germany	30	≥100	n.r.
9	Germany	38	<30	n.r.
10	Germany	35	≥100	n.r.
11	Germany	35	≥100	50
12	Germany	35	167	n.r.
13	Germany	58	60	n.r.
14	Italy	30	196	n.r.
15	Germany	35	<100	n.r.
16	Germany	32	≥100	n.r.
17	Germany	44	<30	43

n.r. = not reported.

of herbs in the herbal spirits. For each spirit studied, the inhibition time depended on the dilution. The t_{inhib} vs. dilution values for some spirits are reported in Fig. 1a.

The experimental data are well-fitted by straight lines. The parameters of the straight lines together with their confidence limits and the R-squared values are reported in Table 2.

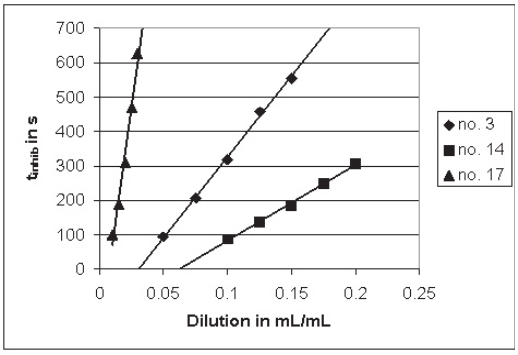

Fig. 1a - Effect of dilution on inhibition time of three herbal spirits (BR measurements).

The most suitable standard was 2,6-dihydroxybenzoic acid (2,6-DHBA). The equation for the straight line of 2,6-DHBA is:

$$t_{inhib}\ (s) = 62.31 \times conc\ (mg/L) - 220\ (s) \qquad R^2 = 0.994$$

The relative antioxidant activity was then calculated as equivalents of 2,6-DHBA in mg/L (DHBAE).

Antioxidant activity at pH = 7.4

For each spirit, the ΔE60 at different dilutions was measured. The results of some ΔE60 vs dilution are reported in Fig. 1b. The experimental data are well-fitted by straight lines. The parameters of the straight lines together with their confidence limits and the R-squared values are reported in Table 3.

Trolox was used as standard; the equation of its straight line is:

$$\Delta E60 = 0.250 \times conc\ (mmol/L) + 0.005 \qquad R^2 = 0.990$$

Table 2 - Parameters of the straight lines of the BR measurements, confidence limits and R-squared values for the spirit samples.

N. spirit	Slope, m [s mL mL^{-1}]	95% Confidence limit on m [s mL mL^{-1}]	intercept, q [s]	95% Confidence limit on q [s]	R^2
1	18,875.0	18,641.05 <μ< 19,108.95	-194.72	-200.79 <β< -188.64	0.999
2	10,840.90	9,654.02 <μ< 12,027.78	-454.29	-550.71 <β< -357.87	0.956
3	4,699.76	4,582.81 <μ< 4,816.71	-143.59	-155.99 <β< -131.19	0.998
4	3,474.40	3,295.64 <μ< 3,653.16	-137.04	-156.00 <β< -118.08	0.990
5	3,514.52	3,301.98 <μ< 3,727.06	-264.38	-297.14 <β< -231.63	0.986
6	3,338.00	3,228.69 <μ< 3,447.31	-151.90	-166.10 <β< -137.70	0.996
7	3,190.68	3,121.63 <μ< 3,259.73	-83.83	-94.48 <β< -73.19	0.998
8	19,008.80	18,554.46 <μ< 19,463.14	-185.37	-197.18 <β< -173.57	0.998
9	2,012.36	1,910.61 <μ< 2,114.11	-144.87	-160.55 <β< -129.19	0.990
10	3,096.92	2,972.15 <μ< 3,221.69	-191.38	-210.61 <β< -172.16	0.994
11	4,785.36	4,455.66 <μ< 5,115.06	-209.30	-244.27 <β< -174.33	0.982
12	1,884.00	1,801.73 <μ< 1,966.27	-171.96	-188.67 <β< -155.26	0.993
13	1,637.36	1,585.38 <μ< 1,689.34	-71.94	-79.95 <β< -63.93	0.996
14	2,204.80	2,150.27 <μ< 2,259.33	-137.82	-146.22 <β< -129.42	0.998
15	2,552.00	2,356.08 <μ< 2,747.92	-118.40	-148.59 <β< -88.21	0.978
16	44,766.40	42,446.39 <μ< 47,086.41	-121.18	-159.65 <β< -82.71	0.990
17	26,794.60	25,229.18 <μ< 28,360.02	-196.89	-230.11 <β< -163.69	0.987

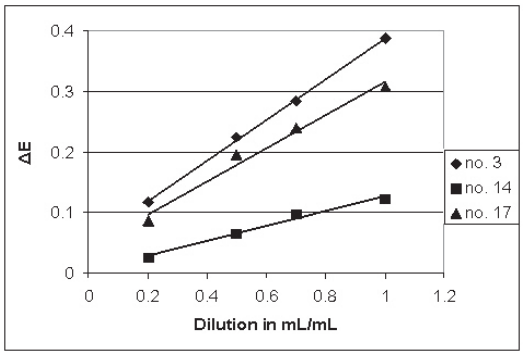

Fig. 1b - Effect of dilution on ΔE60 of three herbal spirits (TEAC measurements).

The relative antioxidant activity was calculated as Trolox equivalents in mmol/L.

Total phenolic content

For each spirit the absorbance of different FC reagent dilutions was measured. Some results of Abs vs dilution are reported in Fig 1c. The experimental data are well-fitted by straight lines. The parameters of the straight lines together with their confidence limits and the R-squared values are reported in Table 4.

It can be seen that the slopes of the straight lines are different (Figs. 1a-c), so the calculation of the relative antioxidant activites or total phenolic content depends on the substance chosen as standard and the sample concentration. The slopes of the straight lines are given in Table 4.

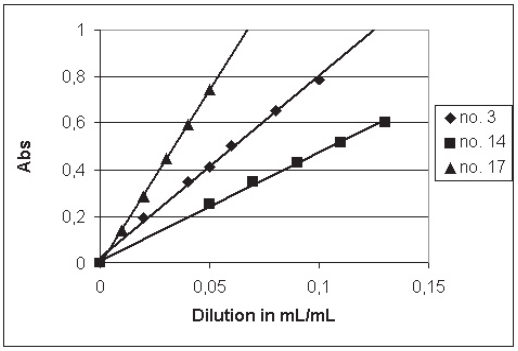

Fig. 1c - Effect of dilution on absorbance of three herbal spirits (total phenolic content measurements).

Table 3 - Parameters of the straight lines of the TEAC measurements, confidence limits and R-squared values for the spirit samples.

No. spirit	Slope, m [ΔE60 mL mL^{-1}]	95% Confidence limits on m [ΔE60 mL mL^{-1}]	Intercept, q [ΔE60]	95% Confidence limits on q [ΔE60]	R^2
1	0.3885	0.3786 <μ< 0.3985	0.0146	0.008 <β< 0.0213	0.999
2	0.1029	0.0968 <μ< 0.1091	0.0038	-0.0079 <β< 0.0004	0.993
3	0.3362	0.3287 <μ< 0.3437	0.0518	0.0468 <β< 0.0568	0.999
4	0.2476	0.2331 <μ< 0.2621	0.0126	-0.0222 <β< -0.0029	0,993
5	0.1288	0.1240 <μ< 0.1337	0.0032	0.0 <β< 0.0064	0.997
6	0.1065	0.0999 <μ< 0.1130	0.0019	-0.0025 <β< 0.0063	0.992
7	0.2097	0.1970 <μ< 0.2224	0.0109	0.0024 <β< 0.0194	0.992
8	0.2256	0.2131 <μ< 0.2381	0.0784	0.0701 <β< 0.0867	0.994
9	0.1112	0.1038 <μ< 0.1185	0.0035	-0.0014 <β< 0.0085	0.991
10	0.1041	0.0969 <μ< 0.1113	0.0011	-0.0041 <β< 0.0062	0.996
11	0.2009	0.1940 <μ< 0.2078	0.0025	-0.0021 <β< 0.0071	0.998
12	0.1629	0.1567 <μ< 0.1692	-0.006	-0.0102 <β< -0.0018	0.997
13	0.1782	0.1689 <μ< 0.1875	-0.0079	-0.0141 <β< -0.0018	0.994
14	0.1235	0.1136 <μ< 0.1335	0.0036	-0.0030 <β< 0.0103	0.987
15	0.1171	0.1109 <μ< 0.1233	0.0228	0.0187 <β< 0.0269	0.994
16	0.5515	0.5150 <μ< 0.5879	0.0646	0.0403 <β< 0.0889	0.991
17	1.9033	1.8059 <μ< 2.001	-0.0036	-0.0151 <β< 0.0079	0.995

Table 4 - Parameters of the straight lines of the total phenolic measurement, confidence limits and R-squared values for the spirit samples.

No. spirit	slope, m [ABS mL mL^{-1}]	95% Confidence limit on m [ABS mL mL^{-1}]	Intercept, q [ABS]	95% Confidence limit on q [ABS]	R^2
1	7.2429	7.1474 <μ< 7.3383	0.0018	-0.0011 <β< 0.0046	0.999
2	2.3324	2.2843 <μ< 2.3806	0.0016	-0.0007 <β< 0.004	0.998
3	7.8071	7.6304 <μ< 7.9839	0.0238	0.0131 <β< 0.0345	0.997
4	3.5529	3.5092 <μ< 3.5965	-0.0025	-0.0051 <β< 0.0002	0.999
5	2.9800	2.8597 <μ< 3.1003	-0.0167	-0.0271 <β< -0.0063	0.990
6	3.5958	3.5112 <μ< 3.6804	0.0105	0.0032 <β< 0.0178	0.997
7	6.6886	6.6352 <μ< 6.7419	0.0049	0.0017 <β< 0.0081	0.999
8	7.0171	6.8974 <μ< 7.1369	-0.0061	-0.0097 <β< -0.0025	0.998
9	2.3414	2.3168 <μ< 2.366	0.0021	0.0 <β< 0.0041	0.999
10	3.0451	2.9988 <μ< 3.0915	0.0195	0.0130 <β< 0.0260	0.999
11	4.1986	4.1176 <μ< 4.2795	0.0029	-0.0020 <β< 0.0078	0.998
12	2.3309	2.2836 <μ< 2.3781	0.0074	0.0029 <β< 0.0120	0.997
13	1.5451	1.5244 <μ< 1.5658	0.0018	0.0 <β< 0.0035	0.999
14	4.6493	4.5680 <μ< 4.7306	0.0113	0.0043 <β< 0.0183	0.998
15	2.8321	2.7979 <μ< 2.8663	0.0021	-0.0009 <β< 0.0051	0.999
16	6.5163	6.2595 <μ< 6.7730	-0.0357	-0.0578 <β< -0.0136	0.990
17	14.9857	14.8584 <μ< 15.1130	-0.0061	-0.010 <β< -0.0023	0.999

Gallic acid was used as standard; the equation of its straight line is

$$Abs = 0.0147 \times conc\ (mg/L) + 0.0055 \qquad R^2 = 0.998$$

The total phenolic content was then calculated as gallic acid equivalents (GAE) in mg/L.

The calculated mean values of the relative antioxidant activities and total phenolic content are reported in Table 5. All the results are the average of at least three measurements for each dilution. Mean values are given along with the standard deviation; the variation coefficient is reported in %.

Correlations between radical-scavenging properties and total phenolic content

Correlations were obtained by Pearson correlation coefficient in bivariate correlation. A high correlation coefficient (0.859, $p<0.01$) was found between the TEAC and DHBAE values; the ranking order of activity was almost the same for the two free-radical scavenging testing methods. A significant correlation was found between the DHBAE and GAE values (0.552, $p<0.05$) and also between the TEAC and GAE values (0.676, $p<0.01$). In this case the total phenolic content can be used as an indication of antioxidant capacity, but this it is not true in other cases (HÖNER *et al.*, 2004; HEINONEN *et al.*, 1998).

DISCUSSION AND CONCLUSIONS

Even though the results of the chemical *in vitro* assays cannot be directly transferred to human body processes, they at least give an idea about the free radical scavenging activity of pure antioxidants or of those in matrices.

To better compare the ranking orders of antioxidant activity obtained by the BR and the TEAC assays, herbal spirit 4 was chosen as a standard because it had a mid-range activity in relation to all other samples. All the activities were

Table 5 - Antioxidant activity given as DHBAE, TEAC and GAE values together with the coefficients of variation.

No. spirit	$DHBAE_{mean}$±std (mg/L)	cv%	$TEAC_{mean}$±std (mmol/L)	cv%	GAE_{mean}± (mg/L)	cv%
1	321±7	2.2	1.61±0.08	4.93	484±11	2.3
2	126±11	8.6	0.32±0.08	25.97	154±5	3.4
3	90±7	7.6	1.7±0.3	17.98	573±41	7.1
4	72±9	13	0.8±0.1	15.49	226±19	8.5
5	51±2	4.1	0.49±0.01	2.5	178±19	11
6	63±4	6.0	0.39±0.02	5.6	249±16	6.3
7	67±4	6.2	0.88±0.08	9.2	456±5	1.0
8	330±12	3.6	1.5±0.5	30	431±48	11
9	41±3	7.2	0.42±0.04	9.1	157±2	1.6
10	53±1	1.9	0.37±0.02	6.4	222±8	3.8
11	79±6	7.3	0.77±0.03	3.5	280±18	6.6
12	34±1	2.8	0.54±0.06	11	162±5	2.9
13	43±5	11	0.58±0.09	16	102±2	1.8
14	45±3	6.4	0.46±0.04	9.1	324±9	2.9
15	52±3	5.9	0.6±0.1	20	190±4	2.2
16	870±133	15	2.7±0.3	11	395±43	11
17	454±34	7.5	1.4±0.2	13	973±44	4.5

calculated in percentages with respect to this standard (Fig. 2).

The activities of the herbal spirits 1, 2, 8, 16 and 17 were higher with the BR method. Only spirit 3 showed significantly higher activity with the TEAC method. The differences could depend on the pH of the testing methods. It seems that the antioxidants in herbal spirits are more active in an acidic medium, which could depend on the dissociation constants of the phenolic acid compounds. Similar results have also been found for several German white wines (HÖNER *et al.*, 2002).

The $TEAC_{mean}$ value for the examined herbal spirits (0.91 mmol/L) is in line with that found for German white wines (0.87 mmol/L); it is more than one order of magnitude less than the $TEAC_{mean}$ for red wines (16.7 mmol/L) (PAGANDA *et al.*, 1999; HENN and STEHLE, 1998). The GAE_{mean} value for the herbal spirits (327 mg/L) was greater than that for white wines (197 mg/L). Herbal spirits contain very complex mixtures of compounds with different antioxidant behaviours and this complexity may cause varied responses in the tests used which make it difficult to establish relationships between the scavenging activity

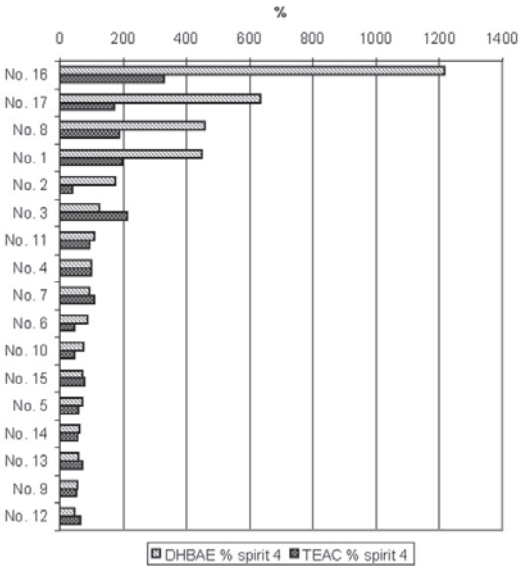

Fig. 2 - Comparison of the different herbal spirits with respect to spirit number 4, used as standard.

and the polyphenolic content profile of the samples.

KANNER and LAPIDOT (2001) investigated reactions that occur in the acidic pH of the stomach that accelerate the generation of lipid hydroperoxides and co-oxidation of dietary constituents. Given that other dietary constituents, such as antioxidants, probably reach the stomach during the same meal, there is evidence that the stomach acts as a bioreactor in which many chemicals can interact (KANNER and LAPIDOT, 2001). KANNER *et al.* (2003) also studied the inhibition of lipid peroxidation by polyphenols in simulated and human gastric fluid. They reported the potentially harmful effects of oxidized fat intake in the presence of endogenous catalysts found in foods, and the major benefit of including plant-derived antioxidants in the meal (KANNER *et al.*, 2003). In addition, recent *in vivo* studies demonstrated that an important amount of dietary anthocyanins are promptly absorbed in the stomach (RICE-EVANS *et al.*, 1996; TALAVERA *et al.*, 2003; PASSAMONTI *et al.*, 2003). Since there are anthocyanins in the examined herbal spirits (HÖNER, 2005) that are quite stable at low pH levels, their absorption would be favoured by the acidic gastric environment. An organic anion carrier, bilitranslocase, expressed in the gastric epithelium, may be involved in the absorption of anthocyanins at the gastric level (PASSAMONTI *et al.*, 2003). These results indicate the importance of investigating the chemical *in vitro* antioxidant activity of phenolic compounds at acidic pH levels as a first step.

The TEAC assay and the determination of the total phenolic content are usually performed in triplicate at only one concentration or dilution of the sample. As can be seen from Table 5 there is a noticeable variation in the vc% of the measurements at different dilutions. More realistic TEAC and total phenolic content values can be obtained using different dilutions. Four different dilutions (each in triplicate) were measured with the TEAC assay, and five different dilutions (each in triplicate) were used to determine the total phenolic content. When the BR reaction method is used several different sample concentrations must be measured (CERVELLATI *et al.*, 2001). Consequently, in this study five different sample dilutions (each in triplicate) were used to determine the inhibition times.

Since different testing methods give different ranking orders of antioxidant capacity due to different experimental conditions (radicals produced in the reacting mixture, pH of the mixture, solvent of the system, etc.) (SCHLESIER *et al.*, 2002), at least two different testing methods should be used in order to obtain a realistic estimate of the antioxidant activity of pure polyphenolic substances or mixtures.

For the study of antioxidative activity some factors of the methods have to be considered. i.e. the practicability, instrumental requirements and the time, expertise and cost necessary for the analysis. Concerning these aspects, the BR method has many advantages: the analysis is inexpensive and rapid, and reagents and apparatus are in common use in all chemical laboratories. Another advantage is the type of radical ($HOO^{\bullet}$) produced in the reaction mixture because it is a radical that is also present in the human body. We are obviously aware that on no account can *in vitro* chemical antioxidant assays mimic physiological conditions. On the other hand, recent *in vivo* experiments on rats and mice have shown that some plant extracts with *in vitro* antioxidant activity (BR method) show gastrointestinal protective effects (SPERONI *et al.*, 2005). Thus we can conclude that a moderate consumption of herbal spirits after a meal could reduce some stomach diseases.

REFERENCES

Albano E., Clot P., Morimoto M., Tomasi A., Ingelman-Sundberg M. and French S.W. 1996. Role of cytochrome P4502E1-dependent formation of hydroxyethyl free radical in the development of liver damage in rats intragastrically fed with ethanol. Hepatology 23: 155.

Baderschneider B., Luthria D., Waterhouse A.L. and Winterhalter P. 1999. Antioxidants in white wine (cv. Riesling): I. Comparison of different testing methods for antioxidant activity. Vitis 38: 127.

Boyer J. and Lui R.H. 2004. Apple phytochemicals and their health benefits. Nutr. J. 3: 5.

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

Cadenas E. and Packer L. 1996. "Handbook of Antioxidants". Marcel Dekker, Inc. New York.

Cederbaum A.I. 2001. Introduction-serial review: Alcohol, oxidative stress, and cell injury. Free Rad. Biol. Med. 31: 1524.

Cervellati R., Crespi-Perellino N., Furrow S.D. and Minghetti A. 2000. Inhibitory effects by soy antioxidants on the oscillations of the Briggs-Rauscher reaction. Helv. Chim. Acta 83: 3179.

Cervellati R., Höner K., Furrow S.D., Neddens C. and Costa S. 2001. The Briggs-Rauscher reaction as a test to measure the activity of antioxidants. Helv. Chim. Acta 84: 3533.

Cervellati R., Renzulli C., Guerra M.C. and Speroni E. 2002a. Evaluation of antioxidant activity of some natural polyphenolic compounds using the Briggs-Rauscher reaction method. J. Agric. Food Chem. 50: 7504-7509. also cited in Gastroenerology week, February 16/2003.

Cervellati R., Höner K., Furrow S.D. and Mazzanti F. 2002b. Inhibitory effects by antioxidants on the oscillations of the Briggs-Rauscher-Reaction in mixed EtOH/H_2O medium. Helv. Chim. Acta 85: 2523.

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

Cohen L.A. 1988. Ernährung und Krebs. Spektrum der Wissenschaft 1: 108.

Corder R., Douthwaite J.A., Lees D.M., Khan N.Q., Dos Santos A.C.V., Wood, E.G. and Carrier M.J. 2001. Health: Endothelin-1 synthesis reduced by red wine. Nature 414: 863.

Criqui M.H. and Ringel B.L. 1994. Does diet or alcohol explain the French paradox? Lancet 334: 1719.

Del Campo Martínez G., Santos Gonzáles J.I. and Berregi Abalde I. 2002. Determination of total phenolic compounds by ^{1}HNMR in apple juices. Abstract C29, I Reunión Bienal del Grupo Especializado de RMN de la RSEQ, Calella (Spain), 20-23 October.

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

Fogliano V., Verde V., Randazzo G. and Ritieni A. 1999. Method for measuring antioxidant activity and its application to monitoring the antioxidant capacity of wines. J. Agric. Food Chem. 47: 1035.

Frankel E.N., German J.B. and Davis P.A. 1992. Headspace gas chromatography to determine human low-density-lipoprotein oxidation. Lipids 27: 1047.

Frankel E.N., Kanner J., German J.B., Parks E. and Kinsella J.E. 1993. Inhibition of oxidation of human low-density lipoprotein by phenolic substances in red wine. Lancet 341: 454.

Frei B. 1994. "Natural Antioxidants in Human Health and Disease". Academic Press. San Diego, California.

Goldberg D.M., Hoffmann B., Yang J. and Soleas G.J. 1999. Phenolic constituents, furans, and total antioxidant status of distilled spirits. J. Agric. Food Chem. 47: 3978.

Hannum S.M. 2004. Potential impact of strawberries on human health: a review of the science. Crit. Rev. Food Sci. Nutr. 44: 1.

Heber D. 2004. Vegetables, fruits and phytoestrogens in the prevention of disease. J. Postgrad Med. 50: 145.

Henn T. and Stehle P. 1998. Gesamtphenolgehalte und antioxidative Kapazität handelsüblicher Getränke. Ernährungs-Umschau 45: 308.

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

Höner K. and Cervellati R. 2002. Measurements of the antioxidant capacity of fruits and vegetables using the BR reaction method. Eur. Food Res. Technol. 215: 437.

Höner K., Cervellati R. and Neddens C. 2002. Measurements of the antioxidant activity of German white wines using a novel method. Eur. Food Res. Technol. 214: 356.

Höner K. and Cervellati R. 2003. Antioxidative Kapazität von Äpfeln mit der Briggs-Rauscher Methode. Ernährungs-Umschau 50: 13.

Höner K., Cervellati R., Costa S. and Sartini E. 2005. Unpublished data. Effectiveness of the determination of the total phenolic content with the Folin-Ciocalteu reagent. Chemie und Chemiedidaktik, Technische Univ. Braunschweig, Braunschweig, Germany.

Höner K. 2005. Unpublished results. The total anthocyanin content of the examined herbal spirits ranges from 1.8 mg/L (Nr. 6) to 94.5 mg/L (Nr. 1). Chemie und Chemiedidaktik, Technische Univ. Braunschweig, Braunschweig, Germany.

Imark C., Kneubuhl M. and Bodmer S. 2001. Occurrence and activity of natural antioxidants in herbal spirits. Innov. Food Sci. Emerg. Technol. 1: 239.

Kanner J. and Lapidot T. 2001. The stomach as a bioreactor: Dietary lipid peroxidation in the gastric fluid and the effects of plant-derived antioxidants. Free Radic. Biol. Med. 21: 1388.

Kanner J., Lapidot T. and Gorelik S. 2003. Lipid peroxidation inhibited by polyphenols in simulated and human gastric fluid: implication on health effects. Communication presented at the 1st International Conference on Polyphenols and Health, 18-21/11/2003, Book of Abstracts, p. 87, Vichy, France.

Kroenke C.H., Chu N.F., Rifai, N., Spiegelman D., Hankinson, S.E., Manson J.E. and Rimm E.B. 2003. Moderate alcohol consumption has beneficial glycemic effects. Diabetes Care 26: 1971.

Landrault N., Poucheret P., Ravel P., Gasc F., Cros G. and Teissedre P.-L. 2001. Antioxidant capacities and phenolic levels of French wines from different varietis and vintages. J. Agric. Food. Chem. 49: 3341.

Liu R.H. 2003. Health benefits of fruit and vegetables are from additive and synergistic combinations of phytochemicals. Am. J. Clin. Nutr. 78 (suppl.): 517S.

Madhavi D.L., Deshpande S.S. and Salunkhe D.K. 1996. "Food Antioxidants: Technological, Toxicological, and Health Perspectives". p. 1-5, Marcel Dekker Inc. New York.

Marco G.J. 1968. A rapid method for evaluation of antioxidants. J. Am. Oil Chem. Soc. 45: 594.

Miller N.J., Rice-Evans C.A., Davies M.J., Gopinathan V. and Milner A. 1993. A novel method for measuring antioxidant capacity and its application to monitoring the antioxidant status in premature neonates. Clin. Sci. 84: 407.

Nordmann H. 1994. Alcohol and antioxidant systems. Alcohol. 29: 513.

Packer L. 1996. Antioxidant defences in biological systems: an overview. In "Proceedings of the International Symposium on Natural Antioxidants". Packer L., Traber M.G., Xin W., p. 9-23, AOCS Press. Arlington, Virginia.

Paganda G., Miller N. and Rice-Evans C.A. 1999. The polyphenolic content of fruits and vegetables and their antioxidant activities. What does a serving constitute? Free Rad. Res. 30: 153.

Park O.J. and Surh Y.J. 2004. Chemopreventive potential of epigallocatechin gallate and genistein: evidence from epidemiological and laboratory studies. Toxicol. Lett. 150: 43.

Passamonti S., Vrhosek U. and Mattivi F. 2003. The stomach as a site for anthocyanins absorption from foods. FEBS Lett. 544: 210.

Pellegrini N., Simonetti P., Gardana C., Brenna O., Brighenti F. and Pietta P. 2000. Polyphenol content and total antioxidant activity of Vini Novelli (Young Red Wines). J. Agric. Food Chem. 48: 732.

Prenesti E., Toso S. and Berto S. 2005. Redox chemistry of red wine. Quantification by an oscillating reaction of the antioxidant power as a function of the temperature. J. Agric. Food Chem. 53:4220.

Pryor W.A., Cornoicelli J.A., Deva L.J., Tait B., Trivedi B.K., Witiak D.T. and Wu M. 1993. A rapid screening test to determine the antioxidant potencies of natural and synthetic antioxidants. J. Org. Chem. 58: 3521.

Re R., Pellegrini N., Proteggente A., Pannala A., Yang M. and Rice-Evans C.A. 1999. Antioxidant activity applying an improved ABTS$^{\bullet+}$ radical cation decolorization assay. Free Radic. Biol. Med. 26: 1231.

Renaud S. and de Lorgeril M. 1992. Wine alcohol, platelets, and the French paradox for coronary heart disease. Lancet 339: 1523.

Rice-Evans C.A., Miller N.J. and Paganga G. 1996. Anthocyanins are efficiently absorbed from the stomach in anesthetized rats. Free Radic. Biol. Med. 20: 933.

Roberfroid M. and Buc Calderon P. 1995. "Free Radicals and Oxidation Phenomena in Biological Systems". Marcel Dekker Inc., New York.

Romani A., Mancini P., Tatti S. and Vincieri F.F. 1996. Polyphenols and polysaccharides in Tuscan grapes and wines. Ital. J. Food Sci. 1: 13.

Sato M., Maulik N. and Das D.K. 2002. Cardioprotection with alcohol. Role of both alcohol and polyphenolic antioxidants. Annals N. Y. Acad. Sci. 957: 122.

Schlesier K., Harwat M., Böhm V. and Bitsch R. 2002. Assessment of antioxidant activity by using different *in vitro* methods. Free Rad. Res. 36: 177.

Simonetti P., Pietta P. and Testolin G. 1997. Polyphenol content and total antioxidant potential of selected Italian wines. J. Agric. Food Chem. 45: 1152.

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

Speroni E., Schweiger S., Egger P., Berger A-T, Cervellati R., Govoni P., Guerra M.C. and Stuppner H. 2005. Efficacy of different extract of *Leontopodium alpinum* cass. (*Edelweiss*) in the rat. J. Ethnopharmacology, in press.

Stoclet J.C. 2001. Bonum vinum laetificat hominum. Med. Sci. Monit. 7: 842.

Sun A.Y., Simony A. and Sun G.Y. 2002. The "French paradox" and beyond: neuroprotective effects of polyphenols(1,2). Free Radic. Biol. Med. 32: 314.

Talavera S., Felgines C., Texier O., Besson C., Lamaison J.L. and Remsey C. 2003, Anthocyanins are efficiently absorbed from the stomach in anestetized rats. J. Nutr. 133: 4178.

Teissedre P.L., Frankel E.N., Waterhouse A.L., Peleg H. and German J.B. 1996. Inhibition of in vitro human LDL oxidation by phenolic antioxidants from grapes and wines. J. Sci. Food Agric. 70: 55.

Revised paper received April 27, 2005 Accepted September 16, 2005

PAPER

EFFECT OF MICROWAVE HEATING ON SOLUBILITY AND DIGESTIBILITY OF PROTEINS AND REDUCTION OF ANTINUTRIENTS OF SELECTED COMMON BEAN (*PHASEOLUS VULGARIS* L.) VARIETIES GROWN IN ETHIOPIA

EFFETTO DEL RISCALDAMENTO A MICROONDE SULLA SOLUBILITÀ E SULLA DIGERIBILITÀ DELLE PROTEINE E SULLA DIMINUZIONE DI COMPOSTI ANTINUTRIZIONALI DI VARIETÀ SELEZIONATE DI FAGIOLO COMUNE (*PHASEOLUS VULGARIS* L.) COLTIVATO IN ETIOPIA

E.A. SHIMELIS and S. RAKSHIT*
Food Engineering and Bioprocess Technology Program, Asian Institute of Technology, P.O. Box 4 Klong Luang, Pathumthani 12120, Bangkok, Thailand
* Corresponding author: Tel. +66 2 5246215, Fax +66 2 5246200, e-mail: rakshit@ait.ac.th

ABSTRACT

The effects of microwave heating on antinutrients, raffinose family oligosaccharides, sucrose, *in vitro* protein digestibility and solubility of three selected varieties of whole common bean (Roba, Awash and Beshbesh) were evaluated. The increase in *in vitro* protein digestibility as a consequence of microwave heating depends on the time

RIASSUNTO

Gli effetti del riscaldamento a microonde sui composti antinutrizionali, oligosaccaridi della famiglia del raffinosio e del saccarosio, sulla digeribilità e sulla solubilità delle proteine di tre varietà selezionate di fagiolo comune (Roba, Awash and Beshbesh) sono state saggiate *in vitro*. L'incremento della digeribilità proteica *in vitro* come con-

- Key words: Antinutrients, common bean, *in vitro* protein digestibility, microwave heating, *Phaseolus vulgaris*, Raffinose family oligosaccharides, solubility -

of heating. The best *in vitro* protein digestibility due to reduced antinutrients such as tannins, phytic acid and trypsin inhibitor activity was obtained with a 3-minute exposure. Protein solubility, tannins and trypsin inhibitor activity of treated whole bean samples decreased as microwave time increased. The results indicate that microwave heating can be used to effectively reduce some antinutrients and improve protein quality.

seguenza del riscaldamento a microonde dipende dal tempo di riscaldamento. La migliore digeribilità proteica *in vitro*, dovuta alla riduzione di composti antinutrizionali quali tannini, acido fitico e dell'attività dell'inibitore di tripsina, è stata riscontrata dopo una esposizione al riscaldamento di 3 min. La solubilità proteica, i tannini e l'attività dell'inibitore di tripsina dei campioni di fagiolo trattati diminuisce all'aumentare del tempo di trattamento con microonde. I risultati indicano che il riscaldamento con microonde può efficacemente essere utilizzato per ridurre la concentrazione di alcuni composti antinutrizionali e per migliorare la qualità proteica.

INTRODUCTION

Lowland pulses provide variety to the human diet and, more importantly, are an economical source of protein for many people in developing countries, especially in Africa, lacking animal protein in their diet. Among the commonly consumed lowland pulses, common bean (*Phaseolus vulgaris* L.) is an important element in the human diet in the eastern and Great Lakes regions of Africa. Common beans are a major staple food being the least expensive protein source for resource-poor people who cannot afford to buy expensive meat. The beans however, also contain various antinutrients which elicit adverse nutritional, biological, and physiological responses in humans and animals (RACKIS, 1972). Common beans are not free from flatulence-causing factors and some other antinutritional factors such as, tannins, phytic acid, saponins, phytohaemagglutinins and enzyme inhibitors, which in turn affect the digestibility of protein and starch. Thus, beans should be consumed after being treated to reduce the antinutritional substances and increase protein quality.

In order to increase the biological benefit and digestibility and decrease antinutrient compounds in beans, traditional procedures such as heating or blanching, soaking, germination, cooking, steaming, roasting and dehulling are generally used. A heating process to remove antinutritional factors is essential for the use of beans as a food or feed (WRIGHT, 1968; RACKIS, 1974). This is particularly important in the preparation of common bean for consumption, from the point of view of acceptability as well as improved protein digestibility. Acceptability of thermally processed common bean is influenced not only by its taste, color, aroma and texture but also by its protein quality and the presence of antinutritional factors such us polyphenolic compounds, trypsin inhibitors and phytates. The presence of enzyme inhibitors in common bean varieties can reduce protein digestibility.

Various methods of heating, including microwaving, have been used to improve the nutritional value of beans (WHITE *et*

al., 1967; SIMOVIC *et al.*, 1972; POUR-EL and PECK, 1973; HILL and HARSHBURGER, 1979; COLLINS and BEATY, 1980; HAFEZ *et al.*, 1983; HERNÁNDEZ-INFANTE *et al.*, 1998). The literature indicates that heating may not facilitate the complete hydrolysis of proteins, leaving about 5.6% protein unhydrolyzable (VAINTRAUB *et al.*, 1979). Various authors have suggested using microwave heating to more rapidly eliminate deleterious components (POUR-EL *et al.*, 1981; CHUNG *et al.*, 1981; RUNNELS *et al.*, 1978; HAFEZ *et al.*, 1983, 1984,1985; RAJKO *et al.*, 1997). Microwave energy is non-ionizing radiation that leads to instantaneous heat generation within the product due to molecular motion (the migration of ions and the rotation of dipoles) but does not cause changes in the molecular structure (MUDGETT, 1985). The characteristic features of microwave heating are that it ensures homogeneous operation in the whole volume of the substance, has great penetrating depth and selective absorption (RAJKO *et al.*, 1997).

The aim of this study was to assess the microwave conditions required to improve protein digestibility, reduce antinutrients and specifically inactivate trypsin inhibitor activity in three common bean varieties grown in Ethiopia.

MATERIALS AND METHODS

Materials, transportation and preparation

The common bean (*Phaseolus vulgaris* L.) varieties Roba, Awash and Beshbesh were grown at the Nazareth Agricultural Research Center, Ethiopia. The beans are improved and export and food-type varieties, and nationally released from lowland pulse breeding and crop protection research programs. They were grown by the lowland pulse breeding and food science and post-harvest technology research program of the Ethiopian Agricultural Research Organization (EARO) under similar field conditions and normal agronomic practices required for bean crops. These varieties were selected from among many other varieties due to their low, medium and high levels of antinitritional factors and *in vitro* protein digestibility. The physico-chemical properties and proximate composition of these improved dry bean varieties were determined by SHIMELIS and RAKSHIT (2005). Bean varieties were transported from Ethiopia by air to the Food Engineering and Bioprocess Technology Laboratory of the Asian Institute of Technology (AIT), Bangkok, Thailand. The bean samples were dried in a ventilated oven at 55°C for 24 h, and ground in an analytical mill (Cole-Parmer, Cole-Parmer Instrument Company, Model 4301-02, Illinois, USA) to a fine powder that was able to pass through an 80-mesh screen for the analyses of the unprocessed bean samples. The bean powder obtained after grinding was placed in plastic containers and stored at room temperature (20°C) until experiments for untreated bean samples were conducted. Sub-samples were then taken for subsequent analysis of each variety of common beans. Similarly, after microwave heat treatment of the three improved bean varieties, standard procedures and methods were used for the analyses of antinutrients, protein and its solubility, *in vitro* protein digestibility and some other sugars. Most chemicals and reagents were purchased from Sigma Chemical Co. Ltd. (St. Louis, MO, U.S.A.). All chemicals used were analytical and reagent grade.

Microwave heating procedure

The effect of microwave heating on protein solubility and digestibility of common bean samples was determined by the modified procedure of HAFEZ *et al.* (1985). One hundred-fifty grams of whole common beans of the three vari-

eties (Roba, Awash and Beshbesh) were microwaved for 0.0, 1.0, 2.0, 3.0, 4.0, 6.0 and 10.0 min. A domestic size microwave oven (Hitachi, Convection, Model MR-8290, 1.18 kW power at 2450 MHZ, Stuttgart, Germany) was used. The oven was rated to 800 watts at 2450 MHz and operated at full power. In order to obtain uniform heating, the micro-oven turntable was used to slowly rotate the sample in the direction opposite of the built-in microwave stirrer. The three bean samples were microwaved in their containers (2.85 mm thick microwavable circular plastic), in tripilicate. The effect of microwave heating on reducing the antinutritional factors, protein solubility and *in vitro* digestibility of proteins were then analyzed using standard procedures.

Protein composition and solubility

Crude protein content (N x 6.25) of raw and microwaved bean samples was performed according to standard AOAC (2003) methods by micro-Kjeldhal digestion.

Microwave treated samples (about 100 g) were ground in an analytical mill (Model 4301-02 Cole-Parmer Instrument Co., Illinois, USA) and passed through an 80-mesh screen. The solubility of common bean protein was determined by extraction with distilled water for 2 h at room temperature (23°C) using the method of BETSCHART (1974). Protein solubility profiles of the microwave-treated common bean flours were measured according to the adopted method of ARABA and DALE (1990). About 1.5 g of the flour were dispersed in 75 mL of distilled water (1:50 w/v) in a stoppered conical flask. The pH of the dispersion was adjusted to the desired value pH = 2-10 by adding a few drops of 0.1N or 1.0 M sodium hydroxide or hydrochloric acid solution, respectively. The dispersion was then shaken in a Gallenkamp shaker at 100 oscillations min^{-1} for 45 min at room temperature (18°-20°C). The pH of the mixture was checked at intervals and adjusted, if necessary, to the original value. After shaking, the dispersion was then centrifuged at 3,000 x g for 30 min using a high-speed centrifuge (Model, Centrikon T-324, Kontron Instruments, Milan, Italy). The supernatant was decanted, avoiding centrifuge, and filtered through a Whatman No. 1 filter paper to obtain a clear solution. Finally, about 40 mL of solution was recovered in a 50 mL beaker. Subsequently, 15 mL in triplicate from each filtrate was transferred to Kjeldahl tubes giving a 0.3 g aliquot of the original sample (1.5 g x 15 mL per 75 mL). Concentrated H_2SO_4 (12.5 mL), two Kjeltab and 2 mL of 30% H_2O_2 were added to each tube. Then, soluble protein (N) was then determined with the micro-Kjeldahl method. Protein solubility is expressed as the percentage of the amount of protein (N) in the supernatant compared to the amount of protein (N) in the sample.

In vitro protein digestibility

The procedure used to isolate proteins from common beans for *in vitro* protein digestibility analysis was described by SATTERLEE *et al.* (1975). Dialysis tubing, with an average flat width of 32 mm, benzoylated with product no. D-7884 from Sigma Chemical, Co. (St. Louis, Mo, USA, via U & V Holding Co. Ltd., Thailand), was used to isolate proteins from the common bean varieties. The fractions obtained (albumin and globulin) were recombined to get a bean protein concentrate (BPC) in the proportions that exist in the common bean (1.00 part albumin to 1.85 parts of globulin). The *in vitro* protein was digestibility of raw and processed common bean varieties determined using the method of HSU *et al.* (1977). In this method the decrease in pH is measured, which occurs following the digestion of the bean protein concentrate with a mixture of 3 enzymes: trypsin (porcine pancreatic tryp-

sin type IX-S, with 14,900 units per mg solid of enzymatic activity, product no. T-0303), α-chymotrypsin (bovine pancreatic chymotrypsin, type II, 66 units per mg solid, product no. C-4129) and peptidase (porcine intestinal mucosa, grade III, 102 units per gm solid, product no. P-7500). All these enzymes were procured from Sigma Chemical Co. (St. Louis, MO, USA via U&V Holding Co. Ltd., Thailand). Percent digestibility (Y) was calculated according to the regression equation (Y = 210.464 - 18.103X, where X is the pH of the suspension) of HSU *et al.* (1977). The decrease in pH of the suspension was recorded automatically over a 10-minute period of digestion with a freshly prepared multi-enzyme solution. Its activity was determined using casein (from bovine milk, purchased from Sigma Chemical Co., St. Louis, MO, USA) of known *in vivo* apparent digestibility.

Determination of trypsin inhibitor activity (TIA)

Trypsin inhibitor activity (TIA) of raw and microwaved common beans was determined by the methods of KAKADE *et al.* (1974) and SMITH *et al.* (1980). The method involves extraction of the trypsin inhibitors from the samples at pH 9.5 and mixing unfiltered suspension with bovine trypsin. Bovine trypsin (trypsin from bovine pancreas with 9,600 units/mg solid, purchased from Sigma Chemical Co., St. Louis, Mo, USA) and N-benzoyl-DL-arginine-P-nitroanilide (BAPNA) hydrochloride (Sigma Chemical Co., St. Louis, Mo, USA) was used as a synthetic trypsin substrate. The activity of the remaining trypsin was then measured by offering it BAPNA under standard conditions; the *p*-nitroaniline released (product of reaction) is measured spectrophotometrically. Greater color intensity of the clear solution indicates less trypsin inhibition. One trypsin inhibitor unit (TIU) was expressed as an increase of 0.01 absorbance units per 10 mL of reaction mixture at 410 nm. Trypsin inhibitor activity was expressed in terms of trypsin units inhibited per mg sample (TIU mg^{-1}) on a dry weight basis.

Determination of tannins

Tannin contents were determined according to the vanillin-HCl method of PRICE *et al.* (1978). Catechin [(+) - Catechin hydrate, purity greater than 98%, product no. C1251, Sigma, Chemical, St. Louis, MO, USA] was used as the reference standard; the minimum tannin values were expressed in mg of D-catechin equivalents per g of sample (mg g^{-1} catechin equivalents, dry weight basis).

Phytic acid analysis

Phytic acid was determined using the method of HAUG and LANTZSCH (1983). After the samples were extracted with 0.2N HCl, ferric solution was added and centrifuged; the supernatant was then transferred to another test tube. Finally, 2,2'-bipyridine (Merck Art.3098) solution was added. The absorbance was then read at 519 nm on a spectrophotometer (UV/Vis spectrophotometer, model 6405, Jenway Ltd. UK, 1999) against distilled water. Sodium salt of phytic acid, (type V, 97% purity and containing about 15% water) obtained from Sigma (P-5756) was used as phytate reference solution for the standard calibration curve. The phytic acid was estimated as phytate in mg g^{-1} dry weight basis (DWB).

Extraction and chromatographic analyses of sugars by HPLC

The raffinose family oligosaccharides (verbascose, stachyose, and raffinose) and other sugars (glucose, sucrose, fructose and maltose) were extracted from raw and processed common bean seeds using AOAC official method 982.14 (AOAC, 2003). About 5

g of fine powdered sample in a 250 mL Pyrex flask were treated with 60 mL of 60% ethanol to water (v/v; aqueous ethanol) for the first extraction. The sample was thoroughly mixed using a vortex mixer and then shaken in a water-bath at 60°C for 1h with occasional stirring. The mixture was then cooled to room temperature and centrifuged for 5 min at 653xg using a high speed centrifuge (Model Centrikon T-324, Kontron Instruments, Milan, Italy) and the supernatant was transferred to another 80 mL polyethylene tube collector. The sample residue in the first centrifuge tube was treated with 10 mL of 60% ethanol to water (v/v) and the extractions were repeated as previously described. Finally, 10 mL of 60% ethanol: water (v/v) were added to the same sample residue and the extraction was repeated as in the two previous steps. The three supernatants were combined in a second polyethylene centrifuge tube, and 10 mL of 10% zinc sulphate: water (w/v) were added in order to deproteinize the solution. The mixture was shaken using a vortex mixer until a homogeneous mixture was obtained and then centrifuged for 10 min at 653xg. The extract obtained was transferred to a 100 mL volumetric flask, and brought up to 100 mL with ethanol to water (60:40,v/v). The extract obtained was filtered through 0.45 µm Pall nylon filter and then analyzed by liquid chromatography.

The analyses of raffinose family oligosaccharides and other sugars were carried out by high-performance liquid chromatography (HPLC) according to the method of DOYON *et al.* (1991). A Hewlett-Packard 1100 series liquid chromatograph (Hewlett Packard, GmbH, Germany) equipped with a LiChrospher 100-NH2 analytical column (5 µm, 250x4 mm, LxID) and a refractive index detector (RID, Hewlett Packard, Waldbronn, Germany) were used. Solute elution was monitored using a differential refractive index (RI) detector. The prepared extracts and solvents were purified by passing them through a Sep-pak C_{18} cartridge (Waters Associates, Milford, MA, USA) and a Millipore (Milford, MA, USA) system, cellulose acetate membrane filters with a pore size of 0.45 µm, before injection into the column. Individual sugar standards were allowed to dry for 12 h at 60°C under vacuum. Subsequently, standard solutions were prepared of raffinose family oligosaccharides; HPLC-grade raffinose with product no. R-0250 and purity minimum 98%, stachyose with product no. S-4001 and product purity minimum 98% from Sigma Chemical (St. Louis, MO, USA) and verbascose with product no. 56217, purity of greater than 98% was from Fluka Chemika, Buchs AG, Switzerland; and other sugars (HPLC-grade glucose, sucrose, fructose and maltose; purchased from Sigma Chemical, St. Louis, MO, USA) were prepared at a concentration of 0.0625 to 1.000 mg mL^{-1} for each of the raffinose series oligosaccharides and 0.250 to 2.500 mg mL^{-1} for the other sugars used for calibrate a selected plot.

HPLC grade acetonitrile and water (from Fisher Scientific, Leicestershire, UK) purified with a Milli-Q system (Millipore, Bedford, Waters Co., MA, USA) were used as mobile phase with 50:50 (v/v), flow rate of 1mL min-1 for raffinose series oligosaccharide detection. Other sugars (sucrose, fructose, maltose and glucose) were eluted with acetonitrile/water 73:27 (v/v) as a mobile phase at a pump rate of 1 mL min^{-1} and injection volume of 10 µL to meet the column criteria. The prepared extracts (unknown samples) were then injected into the HPLC column, was quantified by comparison with the standard sugars and expressed as mg g^{-1}.

Statistical analysis

Data were analyzed using a two-way analysis of variance (ANOVA) followed by least significant difference (LSD) for

multiple comparison among treatment means at a 5% level of significance. Statistical analyses were performed using SPSS/12 software for Windows.

RESULTS AND DISCUSSION

Two-way ANOVA showed that total microwave heating had significant effect ($P<0.05$) on tannins, *in vitro* protein digestibility, protein solubility, trypsin inhibitor activity, stachyose, raffinose, total α-galactosides and oligosaccharides, phytic acid, sucrose and soluble protein in the bean varieties, but, had no significant effect ($P>0.05$) on total protein. Multiple comparisons showed that Awash had less soluble protein than the Beshbesh and Roba varieties after 10 min of microwave heating. Roba and Beshbesh did not differ. Beshbesh had significantly lower protein solubility after 4 min of microwave heating compared to the Awash and Roba varieties. Roba and Awash did not differ in protein solubility.

The total protein of common beans was not affected by microwave heating (Table 1). Microwave heating for up to 3 min, however, drastically reduced the protein solubility (soluble protein) in beans in deionized H_2O (Table 1 and Fig. 1). Earlier reports (CLARK *et al.*, 1977); BERRIG *et al.*, 1974; KING and PUWASTIEN, 1984) indicated that heat treatment, either dry or moist, considerably decreased solubility of bean protein. As the common bean was heated, the relative proportion of the albumin and globulin fractions decreased with a corresponding reduction in nitrogen solubility (CLARK *et al.*, 1977). These studies also demonstrated that heating caused an irreversible disruption of the quaternary structure of globulin, the major component of bean proteins (YAMAGISHI, *et al.*, 1980; MORI *et al.*, 1981).

The results of the present study indicate that microwave heating has the same effect as other heat treatments. *In vitro* protein digestibility for microwave-heated common beans is illustrated in Fig 2. The protein digestibility increased for microwave-heating periods of 1 to 3 min; 81.84 to 85.75%, 75.78 to 83.78% and 71.63 to 80.75%, for Roba, Awash and Beshbesh varieties, respectively, and then declined after 3 min exposure. The decrease in protein digestibility may be attributed to an increase in browning substances. A dark brownish color, indicating caramelization of the

Table 1 - Effect of microwave heating on the protein solubility of common bean varieties[m].

Microwave time (Min)	Total protein [n](g/100 g)			Protein solubility (%)		
	Roba	Awash	Beshbesh	Roba	Awash	Beshbesh
0	20.55[a]	21.95[b]	20.44[c]	80.49[a]	81.15[a]	79.45[a]
1	20.56[a]	21.95[b]	20.43[c]	76.79[b]	76.86[b]	76.22[b]
2	20.48[a]	21.95[b]	20.44[c]	60.05[c]	60.86[c]	58.81[c]
3	20.55[a]	21.94[b]	20.45[c]	20.78[d]	20.91[d]	21.38[d]
4	20.55[a]	21.95[b]	20.44[c]	19.90[ex]	19.95[ex]	19.42[e]
6	20.55[a]	21.95[b]	20.45[c]	18.93[f]	18.17[f]	18.25[f]
10	20.55[a]	21.95[b]	20.45[c]	16.89[g]	15.58[g]	17.17[g]

[a-g] Means with the same superscript letters within a column are not significantly different at $P \leq 0.05$.
[m] Whole common bean seeds were microwaved in 150 g batches.
[n] Total protein (soluble and insoluble) content of common bean expressed per 100 g dry seeds (wet basis).
All values are means of three replicates.

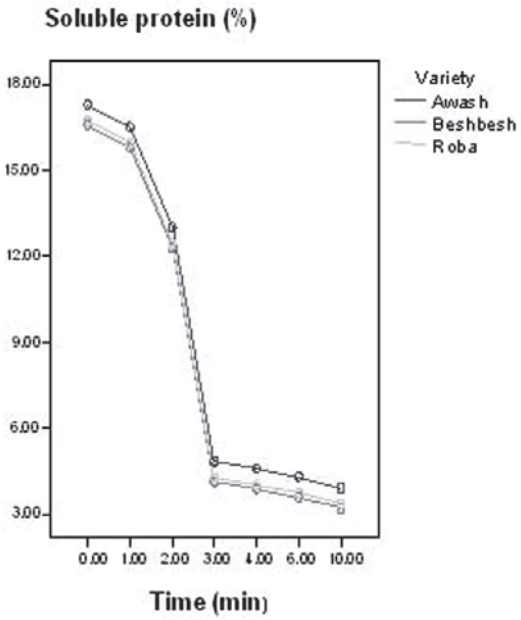

Fig. 1 - Effect of microwave heating on soluble protein.

sugar released during microwave heating, and burnt odor became apparent after 10 min in common beans that were not water soaked. A decrease in the sol-

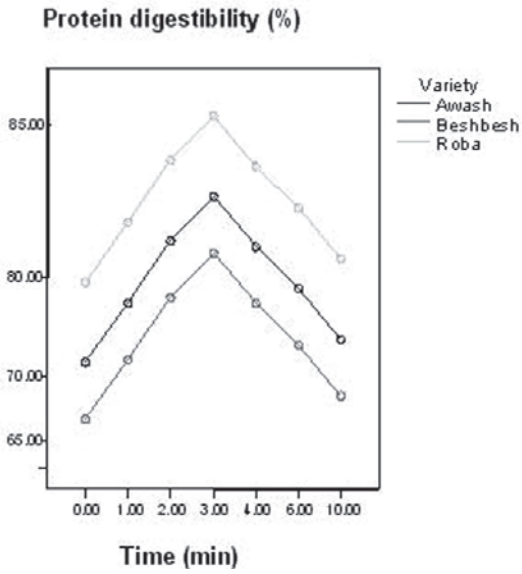

Fig. 2 - Influence of microwave heating on *in vitro* protein digestibility.

ubility of common bean protein with increasing microwaving time was accompanied by increased digestibility. These results agree with the fact that proper heat treatment increases the digestibility of legume proteins reported by HSU *et al.* (1977) and RHEE and RHEE (1981).

The reduction in digestibility after 3 min of microwave treatment was probably due to the Maillard reaction and the formation of browning substances which were found after microwave heating. The Maillard or browning reaction is a type of non-enzymatic browning which involves the reaction of simple sugars (carbonyl groups) and amino acids (free amino groups). The early stage of the Maillard reaction consists of the process where by an Amadori rearrangement product is formed via 1,2-eminal after Schiff base formation between the amino group of protein and the aldehyde group of the reducing sugar (KAWAKISHI *et al.*, 1991). The late stage involves oxidation, dehydrogenation and condensation followed by formation of fluorescent proteins containing advanced glycation end products (AGEs- are a generic name for advanced glycation end products by the Maillard reaction) and intra- and intermolecular cross linkage of the proteins (HAYASE *et al.*, 1989). Therefore, Maillard browning destroy essential amino acids during microwave heating.

The tannin content of the microwaved common bean sample followed a negative trend to the protein digestibility of beans. The results show that bean tannins adversely affect the nutritive value of beans by decreasing the digestibility of the bean proteins. As mentioned above, the decrease in protein digestibility did not continue after 3 min of microwave heating, even though tannin content continues to decrease (Table 2). This could have been due to the Maillard reaction of the proteins. RHEE and RHEE (1981) reported a decrease in the *in vitro* digestibility of protein when browning substances increased. HSU *et al.* (1977)

Table 2 - The impact of microwave heating on tannins and phytic acid of common bean seeds.

Microwave time (Min)	Tannins (mg g^{-1})			Phytic acid (mg g^{-1})		
	Roba	Awash	Beshbesh	Roba	Awash	Beshbesh
0	5.38[a]	17.55[a]	28.78[a]	23.52[a]	24.07[a]	17.33[a]
1	5.21[b]	17.04[b]	27.44[b]	23.24[b]	23.67[b]	17.12[b]
2	4.84[c]	16.45[c]	25.03[c]	22.83[c]	23.28[c]	16.66[c]
3	2.50[d]	12.35[d]	20.45[d]	22.34[d]	22.94[d]	16.37[d]
4	2.37[e]	11.89[e]	19.17[e]	22.02[e]	22.60[e]	15.73[e]
6	2.32[e]	11.57[f]	18.35[f]	21.75[f]	22.22[f]	15.68[f]
10	2.25[f]	11.39[g]	18.02[g]	21.41[g]	21.87[g]	15.27[g]

[a-g] Means with different superscript letters within a column indicate statistically significant differences ($P \leq 0.05$). All values are means of triplicates.

reported that soy grits or soy flour with lower solubility index and lower protein dispersion index values contain less active trypsin inhibitor and more denatured protein which increases the protein digestibility *in vitro*. The phytic acid values changed minimally after microwave heating (Table 2), in agreement with MÁNEZ *et al.* (2002).

Mean values respectively of stachyose, raffinose and sucrose for microwave heated and raw beans ranged from 15.79 to 15.82 mg g^{-1}, 2.73 to 2.87 mg g^{-1} and 27.39 to 23.12 mg g^{-1}. The results of the analyses for raffinose family oligosaccharides after processing showed minimum changes (Table 3). However, the sucrose content increased in the microwave-heated samples. Sugars decompose easily on heating because of their tendency to lose water (HEIMAN, 1980). Stachoyse was not affected by microwave heating, but raffinose was reduced considerably after 3 min of treatment. Therefore, results of the quantification analyses for raffinose and stachyose show that, heating is an inadequate method for destroying oligosaccharides. However, HAFEZ *et al.* (1983) reported that microwave heating of whole beans in 150 g batches with 10.9% moisture content in dry matter basis for two minutes destroyed trypsin inhibitor by 70.3%. The results of our study are in agreement, indicating that microwave heating of 3 different common bean varieties as a whole in 150 g batches with moisture contents of 10.0, 10.5 and 11.0 g/100 g dry matter basis at about 3 minutes of microwave heating, destroyed 57.52, 55.29 and 50.68% of the trypsin inhibitor activity for Roba, Awash and Beshbesh varieties, respectively (Fig. 3). It should be pointed out that the microwaving time would vary according to the weight of the sample to be microwaved, moisture content and the power of the oven.

Evidently, heat treatment induces some denaturation changes in trypsin inhibitors whereby the amino acid residues at reactive sites become inaccessible for the formation of the trypsin-trypsin inhibitor complex (LIENER and KAKADE, 1969). This observation is also in agreement with the findings of ESAKA *et al.* (1987) and YOSHIDA and KAJIMOTO (1988) who reported that the percentages of destroyed trypsin inhibitor activity and protein digestibility reached maximum levels only within the optimum microwaving time. HERNANDEZ-INFANTE *et al.* (1998), however, reported that microwave heating of common beans failed to destroy hemagglutinins and trypsin inhibitors, and consequently their digestibility and protein efficiency ratio (PER)

Table 3 - Influence of microwave heating on stachyose, raffinose and sucrose contents (mg g^{-1}) of common bean varieties.

Microwave time (Min)	Stachyose			Raffinose			Total α-galactosides			Sucrose			Total oligosaccharides		
	Roba	Awash	Beshbesh	Roba	Awash	Beshbesh	Roba	Awash	Beshbesh	Roba	Awash	Beshbesh	Roba	Awash	Beshbesh
0	12.38^{a}	16.67^{a}	18.41^{a}	3.36^{a}	2.35^{a}	2.89^{a}	15.74^{a}	19.02^{a}	21.30^{a}	26.84^{e}	25.24^{e}	17.27^{g}	42.58^{f}	44.26^{g}	38.57^{g}
1	12.36^{a}	16.65^{a}	18.41^{a}	3.29^{a}	2.30^{a}	2.75^{b}	15.65^{b}	18.95^{b}	21.16^{b}	26.95^{d}	25.89^{d}	20.78^{d}	42.60^{f}	44.84^{f}	41.94^{f}
2	12.37^{a}	16.62^{a}	18.41^{a}	3.28^{b}	2.29^{a}	2.70^{b}	15.65^{b}	18.91^{b}	21.11^{b}	27.65^{c}	26.78^{c}	23.21^{c}	43.30^{e}	45.69^{c}	44.32^{e}
3	12.37^{a}	16.62^{a}	18.39^{a}	3.24^{b}	2.27^{a}	2.69^{b}	15.61^{b}	18.89^{b}	21.08^{b}	28.71^{c}	27.26^{b}	26.21^{b}	44.32^{a}	46.15^{a}	47.29^{a}
4	12.28^{b}	16.49^{b}	18.31^{b}	3.10^{c}	2.12^{b}	2.42^{c}	15.38^{c}	18.61^{c}	20.73^{c}	28.81^{b}	27.29^{ab}	26.29^{a}	44.19^{b}	45.90^{b}	47.02^{b}
6	12.19^{c}	16.32^{c}	18.29^{b}	2.95^{c}	1.98^{c}	2.27^{d}	15.14^{d}	18.30^{d}	20.56^{d}	28.90^{a}	27.31^{a}	26.30^{a}	44.04^{c}	45.61^{d}	46.86^{c}
10	12.12^{c}	16.28^{c}	18.25^{b}	2.74^{d}	1.71^{d}	2.11^{e}	14.86^{e}	17.99^{e}	20.36^{e}	28.97^{a}	27.37^{a}	26.32^{a}	43.83^{d}	45.36^{e}	46.68^{d}

$^{a-g}$ Means with different superscript letters within a column indicate statistically significant differences (P≤0.05).
Verbascose, Fructose, Glucose and Maltose were not detected in all raw and processed samples.
All values are means of triplicates.

Fig. 3 - The impact of microwave heating on TIA in whole common bean varieties by using zero time as a control.

values were poor. The literature indicted that the browning substances have proteolytic inhibitor activity which inhibit proteolytic enzymes *in vitro* (HSU *et al.*, 1977; RHEE and RHEE, 1981), but their *in vivo* mechanism needs to be elucidated. In general, the extent to which trypsin inhibitor activity is destroyed by heating is a function of temperature, duration of heating, particle size and moisture conditions.

CONCLUSIONS

The present study showed that microwave treatment is an effective way to inactivate trypsin inhibitor in whole common bean seeds. The increase in protein digestibility as a consequence of microwave heating depends on the time of heating. Initially, protein digestibility of beans increased with microwave heating time and the tannins and trypsin inhibitor activity decreased. However with prolonged heating, the Maillard reaction has been presumed to affect the quality of the protein due to the blockage of

amino acids in the Amadoric compounds and the browning compound formed has proteolytic inhibitor activity that reduces its *in vitro* protein digestibility. Microwave heating of common beans failed to destroy raffinose family oligosaccharides due to their heat-stable nature, and as a result, the composition of stachyose remained the same and that of raffinose changed slightly.

The use of only a single method cannot eliminate all of the antinutrients, flatulence-causing and toxic factors such as, tannins, phytic acid, saponins, phytohaemagglutinins, protease inhibitor activities and raffinose family oligosaccharides in beans. A combination of two or more methods seems to be a practical approach for changing the seed composition and improving palatability. Proper processing of beans under well-defined and controlled conditions renders them palatable and removes or lowers most of the antinutritional factors to a level that eliminates any deleterious physiological, biological and nutritional effects.

Simple, inexpensive processing methods which can reduce undesirable sugar content and antinutritional compounds will help overcome the problem of protein-energy malnutrition (PEM) which is very common in Africa and Asia. These methods are under study in many laboratories including ours and will be used to make appropriate recommendations to increase the protein quality of common beans. Microwave heating along with some other processing techniques can be expected to play an important role, especially in industrial applications.

ACKNOWLEDGMENTS

The authors express their profound gratitude and sincere appreciation to the Ethiopian government, Addis Ababa and Asian Institute of Technology, Bangkok for providing financial support for this study. Gratitude is also expressed for support from the National Food Institute (NFI) of Thailand, Laboratory Services Department, for the HPLC sugar analyses. Thanks are also due to Miss Bernice Buot Polohan (AIT, AARM), Mr. Vivek Sharma (AIT, FEBT) and Miss Nguyen Thi Hong Hoa (AIT, FEBT) for their valuable support and encouragement during the experiments.

REFERENCES

Araba M. and Dale N.M. 1990. Evaluation of protein solubility as an indicator of over processing soybean meal. Poultry Sci. 69:76.

AOAC. 2003. "Official Methods of Analysis" Association of Official Analytical Chemists. Official method 982.14. Food composition; additives; natural contaminants. William, H.(ed). Volume II, 17th ed., 2nd revision. Gaithersburg, MD, USA.

Agbo N.G., Uebersax M.A. and Hosfield G.L. 1985. Efficient extraction techniques of sugars from dry edible beans (*Phaseolus vulgaris* L.) and estimation of HPLC.Ann.Univ. Nation de Cote d'Ivoire, Se'rie C (Sciences), tome XXI-B: 167.

Berrig R.G., Clark H.H. and Davis C.L. 1974. Effect of abomasal infusion of sodium caseinate on milk yield, nitrogen utilization and amino acid nutrition of the dairy cow. J. Nutr. 104:151.

Betschart A. 1974. Nitrogen solubility of alfalfa protein concentrate as influenced by various factors. J. Food Sci. 39:1110.

Chung S.Y., Morr C.V. and Jen J.J. 1981. Effect of microwave and conventional cooking on the nutritive value of colossus peas (*Vigna unguiculata).* J. Food Sci. 46:272.

Clark J.H., Spires H.R., Berrig R.G. and Bennik M.R. 1977. Milk production, nitrogen utilization and glucose synthesis in lactating cows infused postruminally with sodium caseinate and glucose. J. Nutr. 107:632.

Collins J.L. and Beaty B.F. 1980. Heat inactivation of trypsin inhibitor in fresh green soybeans and physiological responses of rats fed the beans. J. Food Sci. 45:542.

Doyon G., Gaudreau G., St-Gelais D., Beaulieu Y. and Randall J.C. 1991. Simultaneous HPLC determination of organic acids, sugars and alcohols. Can. Inst. Sci. Technol. J. 24:87.

Esaka M., Suzuki K. and Kubota K. 1987. A research note. Impact of microwave heating on lipoxygenate and trypsin inhibitor activities, and water absorption of winged bean seeds. J. Food Sci. 52:1738.

Hafez Y.S., Singh G., Mclellan M.E. and Monroe-Lord L. 1983. Impact of microwave heating on nutritional quality of soybeans. Nutr. Rept. Intern. 28:413.

Hafez Y.S. and Mohamed A.I. 1983. Presence of non protein trypsin inhibitor in soy and winged beans. J. Food Sci. 48:75.

Hafez Y.S., Mohammed A.I. and Hearth W. 1984.

Nutritive composition of different varieties of winged bean seeds and tuber. Nutr. Rept. Intern. 29:253.

Hafez Y.S., Mohamed A.I., Hewedy F.M. and Singh G. 1985. Impact of microwave heating on solubility, digestibility and metabolism of soy protein. J. Food Sci. 50:415-423.

Haug W. and Lantzsch H.J. 1983. A sensitive method for the rapid determination of phytate in cereals and cereal products. J. Sci. Food Agric. 34:1423.

Hayase F., Nagaraj R.H., Miyata S., Njoroge F.G. and Monnier V.M. 1989. Aging of proteins: Immunological detection of a glucose-derived pyrrole formed during Maillard reaction *in vivo*. J. Biol. Chem. 264:3758.

Heimann W. 1980. "Fundamentals of Food Chemistry". Ellis Horwood Series in Food Science and Technology. Ellis Horwood Limited Publishers. AVI Publishing Company, West Port, USA.

Hernández-Infante M., Sousa V., Montalvo I. and Tena E. 1998. Impact of microwave heating on hemagglutinins, trypsin inhibitors and protein quality of selected legume seeds. Qual. Plant. Pl. Foods Hum. Nutr. 52:199.

Hill P.R. and Harshbarger K.E. 1979. Impact of moisture content and amino acid supplementation of protein nutritive value of microwave processed whole soybeans. Fed. Proc. 38:283.

Hsu H.W., Vavak D.L., Satterlee L.D. and Miller G.A. 1977. A multienzyme technique for estimating protein digestibility. J. Food Sci. 42:1269.

Kakade M.L., Rackis J.J., McGhee J.E. and Pusk G. 1974. Determination of trypsin inhibitor activity of soy products: A collaborative analysis of an improved procedure. Cereal Chem. 51:376.

Kawakishi S., Tsunehiro J. and Uchida K. 1991. Autoxidative degradation of Amadori compounds in the presence of copper ion. Carbohydr. Res. 211:167.

King R.D. and Puwastien P. 1984. Effect of blanching and soaking on winged beans (*Psophocarpus tetragonolobus*). J. Sci. Food Agric. 35:441.

Liener I.E. and Kakade M.L. 1969. Protease inhibitors. In "Toxic Constituents of Plant Foodstuffs". p. 7.

Mánez G., Alegría A., Farré R. and Frígola A. 2002. Effect of traditional, microwave and industrial cooking on inositol phosphate content in beans, chickpeas and lentils. Int. J. Food Sci. Nutr. 53:503.

Mori T., Nakamura T. and Utsumi S. 1981. Gelation mechanism of soybean 11S globulin: Formation of soluble aggregates as transient intermediates. J. Food Sci. 47:26.

Mudgett R.E. 1985. Dielectric properties of foods. In "Microwaves in the Food Processing Industry". Decareau. R.V. (Ed.) p. 15. Academic Press. Orlando, Fl.

Pour-El A. and Peck E.E. 1973. Functionality of soybean protein as affected by heat processing presented at the symposium on quality improvement of the plant protein. 165th National Meeting of the American Chemical Society, Dallas, TX, April 9.

Pour-El A., Nelson S.O., Peck E.E., Tjhio B. and Stetson L.E. 1981. Biological properties of VHF and microwave-heated soybeans. J. Food Sci. 46:880-895.

Price M.L., Van Scoyoc S. and Buttler L.G. 1978. A critical evaluation of vanillin reaction as an assay for tannin in sorghum grain. J. Agric. Food Chem. 26:1214.

Rajko R., Szabo G., Vidal-Valverde C. and Kova'cs E. 1997. Designed experiments for reducing antinutritive agents in soybean by microwave energy. J. Agric. Food Chem. 45:3565.

Rackis J.J. 1972. Biologically active components. In "Soybeans: Chemistry and Technology", A.K. Smith and S.J. Circle (Ed.), Vol. 1. AVI, Publishing Co., West Port, CT.

Rackis J.J. 1974. Biological and physiological factors in soybeans. J. Amer. Oil Chem. Soc. 51:161A.

Rhee K. and Rhee K.C. 1981. Nutritional evaluation of the protein in oilseed products heated with sugars. J. Food Sci. 46:164.

Runnels T.B., Malone G.W. and Wutoh J.G. 1978. Microwave heated soybeans as an ingredient in broiler diets. Poultry Sci. 57:1185.

Satterlee L.D., Bembers M. and Kendrick J.G. 1975. Functional properties of the great northern bean (*Phaseouls vulgaris*) protein isolate. J. Food Sci. 40:81.

Shimelis A.E. and Rakshit S.K. 2005. Proximate composition and physico-chemical properties of improved dry bean (*Phaseolus vulgaris* L.) varieties grown in Ethiopia. LWT-Food Sci. Technol. 38:331.

Simovic R., Summers J.D. and Bilanski W.K. 1972. Heat treatment of full-fat soybeans. Can. J. Anim. Sci. 52:183.

Smith C., Megen W.V., Twaalfhoven L. and Hitchcock C. 1980. The determination of trypsin inhibitor levels in foodstuffs. J. Sci. Food Agric. 31:341.

Vaintraub I.A., Seliger P. and Shutor A.D. 1979. The action of pepsin on the reserve proteins of some leguminous seeds. Die Nahrung. Food. 23:15.

White C.L., Green D.E., Waldroup P.W. and Stephenson E.L. 1967. The use of untreated soybeans for chicks. Poultry Sci. 46:1180.

Wright K.N. 1968. Determination and quality of soybean meal. Feed Stuffs. 4:21.

Yamagishi T., Tamarchi F. and Shibasaki K. 1980. Isolation and partial characterization of heat-denatured products of soybean 11S globulin and their analysis by electrophoresis. Agric. Biol. Chem. 44:1575.

Yoshida H. and Kajimoto G. 1988. Impact of microwave treatment on the trypsin inhibitor and molecular species of triglycerides in soybeans. J. Food Sci. 53:1756.

Revised paper received April 25, 2005 Accepted July 20, 2005

PAPER

EFFECT OF DIETARY ADMINISTRATION OF ROSEMARY EXTRACT ON THE OXIDATIVE STABILITY OF PIGEON MEAT

EFFETTO DELL'INTEGRAZIONE ALIMENTARE CON ESTRATTO DI ROSMARINO SULLA STABILITÀ OSSIDATIVA DELLA CARNE DI PICCIONE

A. DAL BOSCO*, C. CASTELLINI and R. CARDINALI
Dipartimento di Biologia Vegetale e Biotecnologie Agroambientali e Zootecniche, Università degli Studi di Perugia, Borgo XX Giugno 74, 06121 Perugia, Italy
* Corresponding author: dalbosco@unipg.it

ABSTRACT

The oxidative status and meat quality of pigeons fed a standard diet or a diet with the addition of rosemary extract were studied. The trial was carried out on 45 pairs of breeders per treatment. Pigeons were fed the two diets *ad libitum* for 120 days, after which 30 fryer pigeons per group were slaughtered. Half of the refrigerated carcasses were used for analyses of the fresh meat and the remainder was stored under vacuum conditions for 90 days at -18°C. The *pectoralis major* muscles were re-

RIASSUNTO

È stato studiato l'effetto dell'integrazione alimentare di estratto di rosmarino sulla stabilità ossidativa e sulla qualità della carne di piccione. La prova è stata effettuata su 45 coppie di riproduttori per trattamento (rosmarino e controllo). I piccioni sono stati alimentati *ad libitum* per 120 giorni; alla fine del periodo sperimentale sono stati macellati 30 piccioncini. Metà carcassa refrigerata è stata subito utilizzata per le analisi mentre l'altra metà è stata conservata sotto vuoto per 90 giorni

- Key words: Oxidative stability, pigeon, rosemary extract -

moved and analysed for pH, colour, water-holding capacity, tenderness, fatty acids and oxidative stability. The oxidative status of the young birds was affected by the dietary treatment. Generally, the physical traits were better in the supplemented group. The positive effect was more relevant in frozen meat as also denoted by the significant diet x storage interactions. The positive effect of rosemary extract during storage was also confirmed by the lipid oxidation level which, after freezing-thawing, was lower in pigeons in the supplemented group.

a -18°C. Sul muscolo *pectoralis major* sono state determinate le caratteristiche fisiche (pH, colore, capacità di ritenzione idrica, perdite di liquidi, tenerezza, umidità) e chimiche (stabilità ossidativa, composizione acidica). Lo stato ossidativo dei piccioncini è stato influenzato dal trattamento alimentare. Per ciò che concerne le caratteristiche fisiche l'effetto positivo è stato osservato in maniera più rilevante dopo la conservazione della carne. L'effetto dell'estratto di rosmarino è stato confermato dai livelli di ossidazione lipidica che dopo lo scongelamento sono stati significativamente più bassi nel gruppo supplementato.

INTRODUCTION

The consumption of pigeon meat is widespread in some areas of central and northern Italy, with a production of about 50,000 tons/year (ISTAT, 2004). To satisfy this demand, there are specialized pigeon farms, with their own slaughterhouses. In order to guarantee the availability of the meat during the critical period when the productive performance decreases, farmers freeze pigeon carcasses for two to three months. This can promote autoxidation of lipids that, in turn, can alter the sensorial and nutritional quality of the meat. It is known that some of the major changes that occur during food processing and storage are due to lipid oxidation that initiates reactions which affect the nutritional quality, wholesomeness, safety, colour, flavour and texture of the food (MORRISSEY *et al.*, 1998; SHAHIDI, 2001).

Consumers greatly appreciate the organoleptic characteristics of pigeon meat which is lean and rich in protein and with intramuscular fat that contains high proportions of monounsaturated and polyunsaturated fatty acids (CASTELLINI *et al.*, 1996). While polyunsaturated fatty acids are very important for human health (WARRIS, 2000; HARRIS, 2004), they are very susceptible to oxidation and therefore particular attention must be given to storage (STANLEY, 1991; O'KEEFE *et al.*, 1995).

Antioxidants can be use to reduce oxidation processes. The food industry has used synthetic antioxidants for over 50 years (CUVELIER *et al.*, 1994; BRAND-WILLIAMS *et al.*, 1995), but these compounds have been shown to be related to health risks, so on their use in food is now strictly regulated (HETTIARACHCHY *et al.*, 1996). BARLOW (1990), cautioning about the long-term effect of some commonly used synthetic antioxidants (BHT, propyl gallate), reported that consumer preference for "natural" ingredients has motivated extensive research on effective plant-derived antioxidants. It has been shown that the addition of spices in sensorially acceptable amounts improves the oxidative stability of cooked meat products (HUISMAN *et al.*, 1994). The compounds responsible for the anti-

oxidative effect have been identified for many spices, including rosemary (INATANI *et al.*, 1983). The antioxidative effect is due to the phenolic compounds (HERMANN, 1973), mainly carnosic and rosemarinic acids with carnosol being the major antioxidant.

Few studies have been conducted on the use of antioxidants in pigeon meat. The present study was carried out in response to an urgent need to find antioxidants capable of maintaining the characteristics of fresh meat, as much as possible, during storage. The aim of this study was to use rosemary extract as an additive in diets of breeder pigeons on commercial farms and to evaluate the oxidative stability of fresh and frozen meat.

MATERIALS AND METHODS

Animals and diets

The trial was carried out on a commercial farm (10,000 breeder couples) in central Italy, using 90 couples of White King breeder pigeons randomly assigned (45 couples/group) to two different dietary treatments: a) Control – standard commercial pellets + a grain mixture (cereals and legumes); b) Rosemary – standard commercial pellets with the addition of 1,000 mg/kg of rosemary (*Rosmarinus officinalis* L.) extract + a grain mixture. The characteristics of the commercial pellets are reported in Table 1. Rosemary powder extract (6% - Sochim International, Milano, Italy) contains 6.23% carnosic acid, 1.20% rosmarinic acid and 2.87% ursolic acid.

Breeder pigeons were fed diets ad libitum for 120 days and the fryer pigeons were slaughtered at the end of the experimental period. Feed consumption was evaluated daily to assess the intake of both pellets and grain (28.7±5 and 78.2±8 g/d/couple, respectively).

Sample collection and analytical determinations

At 30 days-of-age (standard slaughtering age in Italy for fryer pigeons), 15 young pigeons were randomly taken from each group. The birds were electrically stunned, killed by manual exanguination, plucked and eviscerated (elimination of non-edible viscera: intestines, proventriculus, gall bladder, spleen, oesophagus and full crop).

Immediately before slaughter, blood samples were collected in heparinized vacutainers and centrifuged at 1,500 x g for 10 min at +4°C, to measure the oxidative status *in vivo* using the BAP Test (Biological Antioxidant Potential) and d-ROMs tests produced by Diacron s.r.l. (Italy) (CESARONE *et al.*, 1999).

Briefly, the BAP test evaluates the efficacy of a solution of ferric ions (Fe^{3+}) tied to a chromogen to decolor when they are reduced to ferrous ions (Fe^{2+}) when an antioxidant system, like plasma, is added.

The d-ROMs test is a spectrophotometric test that allows the concentration of hydroperoxides (ROOH) in a biological sample to be assessed. Such compounds are generated in the cells by the oxidative attack of reactive oxygen substances on a number of organic substrates (e.g. carbohydrates, lipids, amino acids, proteins, nucleotides, etc.). The initials "ROMs" indicate that the analites measured by this test, i. e. hydroperoxides, belong to the reactive oxygen metabolites.

The edible viscera (heart, liver, and gizzard) were removed from the refrigerated carcasses (24 hours at +4°C) in order to obtain ready-to-cook carcasses. The *pectoralis major* muscles were removed from the carcasses and half of them were used for the fresh meat analyses and the remainder were stored (-18°C) under vacuum conditions for 90 days.

Proximate chemical analyses of diet and meat were done according to AOAC methods (1995).

Table 1 - Formulation and chemical composition of the diet.

	%
Vicia faba	40.00
Wheat wholemeal	30.00
Wheat bran	12.40
Wheat germ	5.00
Calcium carbonate	3.50
Dicalcium phosphate	3.00
Molasses	3.00
Vitamin-mineral premix**	1.80
Sodium bicarbonate	0.50
Salt	0.50
Methionine	0.20
Lysine	0.10
	% d.m.
Crude protein	17.90
Ether extract	2.56
Crude fibre	5.67
Ash	9.80
NDF - Neutral Detergent Fibre	9.74
ADF - Acid Detergent Fibre	2.81
ADL - Acid Detergent Lignin	0.95
Cellulose	3.56

* Added per kg: Vit. A 11.000 IU; Vit. D_3 2.000 IU; Vit. B_1 2.5 mg; Vit. B_2 4 mg; Vit. B_6 1.25 mg; Vit. B_{12} 0.01 mg; α-tocopheryl acetate 50 mg; Biotin 0.06 mg; Vit. K 2.5 mg; Niacine 15 mg; Folic acid 0.30 mg; Pantothenic acid 10 mg; Choline 600 mg; Mn 60 mg; Fe 50 mg; Zn 15 mg; I 0.5 mg; Co 0.5 mg.

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

To evaluate cooking loss, samples of muscle (about 20 g) were placed in open aluminium pans and roasted in an electric oven (pre-heated to 200°C) for 15 min to an internal temperature of 80°C (CYRIL *et al.*, 1996). Cooking loss was estimated as the percentage of weight of the roasted samples (cooled for 30 min to about 15°C and dried on the surface with a paper towel) with respect to the weight of the raw samples.

Ultimate pH (pH*u*) was measured at 24 h with a Knick digital pHmeter (Broadly Corp., Santa Anna, CA, USA) after homogenization of 1 g of raw muscles for 30 sec in 10 mL of 5M iodoacetate (KORKEALA *et al.,* 1986).

The water holding capacity (WHC) was estimated (NAKAMURA and KATOH, 1985) by centrifuging 1 g of muscle placed on tissue paper inside a tube for 4 min at 1,500xg. The water remaining after centrifugation was quantified by drying the samples at 70°C overnight. WHC was calculated as follows: (weight after centrifugation - weight after drying)/initial weight x 100.

Shear force was evaluated on cores (1.25x2 cm) obtained from the mid-portions of the roasted samples (as above) by cutting them perpendicularly to the fibre direction, using an Instron, model 1011, equipped with a Warner-Blatzler Meat Shear Apparatus (Instron International Ltd., Great Britain).

The colour parameters (L*, a*, b*) were measured on the raw muscles using a tristimulus analyser (Minolta Chroma Meter CR-200, Azuchi-Machi Higashi-Ku, Osaka 541, Japan), with the CIE-LAB Colour System (1976).

The extent of lipid oxidation was evaluated in fresh and stored meat as TBA-RS (Thio Barbituric Acid Reactive Substances) according to the modified method of KE *et al.* (1977). Ten grams of minced muscles were homogenised for 2 min with 95.7 mL of distilled water and 2.5 mL of 4N HCl. The mixture was distilled until 50 mL were obtained. Five milliliters of the distillate and 5 mL of TBA reagent (15% trichloroacetic acid, 0.375% thiobarbituric acid) were then heated in a boiling water bath for 35 min. After cooling under running tap water for 10 min, the absorbance was measured at 538 nm against a blank. Oxidation products were quantified as malondialdehyde equivalents (MDA mg/kg muscle).

The fatty acid composition of the *pec-*

toralis major muscle was determined on lipids extracted from samples of about 5 g in a homogeniser with 20 mL of 2:1 chloroform/methanol (FOLCH *et al.*, 1957), followed by filtration through Whatman No. 1 filter paper. Fatty acids were determined as methyl esters with a Mega 2 Carlo Erba Gas Chromatograph, model HRGC (Milano, Italy), using a D-B wax capillary column (0.25 mm ∅, 30 m long). The fatty acid percentages were calculated with the Chrom-Card software. The mean value of each fatty acid was used to calculate the sum of saturated (SFA), monounsaturated (MUFA), polyunsaturated (PUFA) and peroxidability index (PI) with the equation of ARAKAWA and SAGAI (1986): PI = (% monoenoic x 0.025) + (% dienoic x 1) + (% trienoic x 2) + (% tetraenoic x 4) + (% pentaenoic x 6) + (% hexaenoic x 8).

Quantitative fatty acid composition was used to calculate atherogenicity and thrombogenicity indexes as proposed by ULBRICHT and SOUTHGATE (1991):

- atherogenicity index = (C12:0 +4xC14:0 + C16:0) / [∑MUFA + ∑ (n-6) + ∑ (n-3)];
- thrombogenicity index = (C14:0 + C16:0 + C18:0) / [0.5 x ∑MUFA + 0.5 x (n-6) + 3 x (n-3) + (n-3)/(n-6)].

Statistical analyses

Data of qualitative traits were analysed with a linear model (COPYRIGHT, 2002, The R development Core Team) including the effect of diet x storage. Significance of differences was evaluated by the t-test.

RESULTS AND DISCUSSION

Regarding the oxidative status of the young birds (Table 2), after just 30 days of dietary supplementation, the rosemary extract significantly improved the plasma antioxidant capacity (BAP test; P<0.01), with a significant increase in the ROMs level (P<0.05).

Table 2 - *In vivo* oxidative status of fryer pigeons.

		Control	Rosemary	SED
ROMs	mg H_2O_2 100 mL^{-1}	6.41a	7.94b	1.33
BAP	µEQ/L	1,290A	1,749B	625

n=15 for each treatment. Level of significance: a.b: P<0.05; A: P<0.01.

The reason for the increase in ROMs is not completely clear but it can be hypothesised that carnosic acid has an aspecific stimulating effect on body metabolism (SINGLETARY, 1996; KOSAKA and YOKOI, 2003). Although there are many factors that affect oxidative stress, these findings confirm the positive effect of rosemary extract on the *in vivo* antioxidant capacity and demonstrate the ability of the parent birds to transfer the antioxidant compounds ingested with their diet to the offspring.

Many researchers have demonstrated the antioxidant activity of the phenolic compounds of *R. officinalis* both *in vivo* or *in vitro*. One of the mechanisms of the proposed *in vivo* antioxidant effect is related to the chelation of iron by rosemary extracts and thus to the lower adsorption of non-haeme iron (SAMMAN *et al.*, 2001). In this case, the variation in antioxidant capacity could also be associated with a different iron level in the body.

AUROMA *et al.* (1992) measured the inhibition power of rosemary on lipid peroxidation in microsomial and liposomial systems, emphasising the relevant scavenger activity versus peroxylic radicals and H_2O_2. In comparing various antioxidant substances ZHAO *et al.* (1989) found that rosemary has less scavenger activity than vitamin C but more than vitamin E. In humans, ZENG *et al.* (2001) confirmed the inhibitory ability of carnosic and rosmarinic acids on LDL oxidation and

their scavenger effect against free radicals and superoxide anions. Comparing the antioxidant activities of some spices and spice extracts with two different laboratory assays, MADSEN *et al.* (1996) observed good antioxidant properties of rosemary.

Different hypotheses can be proposed regarding the transfer of antioxidants from parent diets to the offspring: a) compounds are transferred through the digestive apparatus-blood-reproductive system to the egg; b) modification of the "crop milk" produced by the parents and fed to offspring; c) feed regurgitated by parents to offspring contains the antioxidants.

In hens, GALOBART *et al.* (2001) observed a good transfer of antioxidant compounds from eggs to chicks which explains the better reproductive performance recorded, whereas LEMONICA *et al.* (1996) did not find any effect of rosemary extract on embryonic and post-natal development.

The chemical composition and the amount of cholesterol in breast meat was not affected by dietary treatments (Table 3).

Concerning the physical traits of the meat (Table 4), the positive effect of rosemary was more relevant in frozen meat of the supplemented group as shown by the significant diet x storage interactions. The dietary effect was significant for pH and colour parameters, particularly of the skin; the results were very significant after freezing as shown by the significant diet x storage interaction.

The supplemented group, in both fresh and stored meat, showed higher pH values, so the water holding capacity improved. On the other hand, after freezing, the pH increased less in the rosemary group than in the control (+0.04 *vs.* 0.07), presumably, because the increased protection of the cell membranes reduced the dispersion of free fatty acids caused by lipolysis.

Another important effect of rosemary extract was that the red parameter (a*) of the meat was lower in the supplemented group than in the control. It is known that meat colour depends on the state of the myoglobin (reddish) and on the degree of iron oxidation in the haeme (HULOT and OUHAYOUN, 1999). The antioxidant compounds of rosemary reduced such processes and consequently reduced the production of meta-myoglobin (brownish colour). This trend was more pronounced in fresh meat probably due to the above-mentioned positive effect of the antioxidant, while after storage the differences were less. A decrease in redness was also found by MCCARTHY *et al.* (2001), who added rosemary powder to frozen pork patties kept under chilled display conditions for 9 days. Another possible hypothesis could be related to the above-mentioned iron chelation by

Table 3 - Chemical characteristics of breast meat.

		Control		Rosemary		Significance			SED
		Fresh	Frozen	Fresh	Frozen	Diet	Storage	Diet x Storage	
Moisture	%	76.23	76.17	75.86	75.72	n.s.	n.s.	n.s.	2.14
Protein	%	21.18	20.91	21.502	21.45	n.s.	n.s.	n.s.	1.98
Ether extract	%	1.54	1.49	1.41	1.43	n.s.	n.s.	n.s.	0.43
Ash	%	1.05	1.43	1.23	1.40	n.s.	n.s.	n.s.	0.36
Cholesterol	mg/100 g	38.71	37.45	39.27	38.42	n.s.	n.s.	n.s.	1.78

n=30 for each treatment. Level of significance: **: P<0.01; *: P<0.05; n.s. = not significant.

Table 4 - Physical characteristics of breast meat.

		Control		Rosemary		Significance			SED
		Fresh	Frozen	Fresh	Frozen	Diet	Storage	Diet x Storage	
pH		5.75	5.85	5.82	5.89	*	*	*	0.14
Moisture	%	80.03	79.81	79.82	79.08	n.s.	*	*	2.98
WHC	%	58.02	56.97	58.79	57.45	n.s.	*	*	2.03
Cooking loss	%	37.58	38.77	37.01	37.87	n.s.	n.s.	n.s.	1.45
Shear force	kg/cm^2	1.52	1.48	1.37	1.23	n.s.	n.s.	n.s.	0.25
Skin Colour									
L*		66.53	52.61	69.06	61.35	*	**	**	2.41
a*		10.42	7.43	8.98	7.79	**	**	**	1.02
b*		10.16	8.61	12.59	10.74	**	**	**	1.24
Meat Colour									
L*		49.46	45.13	48.35	43.69	n.s.	**	**	2.04
a*		18.87	16.53	17.98	17.02	**	**	**	1.23
b*		-4.55	-0.62	-3.89	-0.17	n.s.	**	**	0.15

n=30 for each treatment. Level of significance: **: P<0.01; *: P<0.05; n.s. = not significant.

rosemary extracts (SAMMAN *et al.*, 2001) and to the subsequent reduction of myoglobin.

Brightness (L^*) was improved by the herb only in the skin and the increase was more relevant in frozen meat than in fresh meat (+8.74 *vs* 2.53).

The fatty acid composition of the muscle was improved by rosemary supplementation (Table 5). In fact, the amount of SFA and MUFA decreased in both fresh and frozen meat, while that of PUFA increased (P<0.01). Evidently, the rosemary exerted a protective action on the long chain PUFA (≥20 carbon chain), that are more susceptible to oxidative processes. Although present in small amounts, the differences in these fatty acids (≥20 and with more than 3 double bonds) were significant and more pronounced.

The analyses of the quantitative fatty acid composition of breast meat clearly showed the positive effect of rosemary extract after freezing (Table 6); the decrease of unsaturated fatty acids in the control group was particularly evident. This trend was also confirmed by the TBA-RS level, which was lower (P<0.01) in the pigeon meat of the supplemented group after thawing.

After feeding broilers a diet containing 500 mg/kg of rosemary extract LOPEZ-BOTE *et al.* (1998) observed a high oxidative stability of lipids and a significant reduction of microsomial and cholesterol oxidation in frozen, as well as fresh and refrigerated meat. These results were better than ours and were obtained with less rosemary extract (500 *vs* 1000 mg/kg). This could be explained by the fact that the antioxidant present in fryer pigeons came from an indirect pathway being modulated by the ingestion and/or metabolism of their parents.

The peroxidability index showed a higher level of unsaturation in meat of the supplemented group but it was not correlated with an unbalanced oxidative status as confirmed by the TBA-RS analysis. Moreover, the rosemary group had a lower thrombogenicity (P<0.01; 0.84 *vs.* 0.94 fresh meat; 0.85 *vs.* 0.98

Table 5 - Fatty acid composition (% of fatty acids) of breast meat.

	Control		Rosemary		Significance			SED
	Fresh	Stored	Fresh	Stored	Diet	Storage	Diet x Storage	
C14:0	0.70	0.63	0.78	0.67	n.s.	*	n.s.	0.05
C16:0	22.58	23.06	22.01	22.45	*	*	n.s.	2.51
C18:0	10.62	10.15	10.14	9.67	n.s.	n.s.	*	1.54
Others	2.05	3.13	1.68	2.54	*	**	n.s.	0.62
SFA	35.95	36.97	34.61	35.33	*	*	*	2.89
C14:1n-6	0.23	0.20	0.32	0.30	n.s.	n.s.	n.s.	0.14
C16:1n-7	7.56	7.73	8.74	8.55	*	n.s.	*	0.89
C18:1n-9	20.95	20.70	18.12	17.43	**	n.s.	**	1.05
Others	3.35	3.24	1.97	2.27	**	*	*	0.59
MUFA	32.09	31.90	29.15	28.55	**	n.s.	**	2.47
C18:2n-6	22.05	21.92	24.81	24.67	**	n.s.	**	1.84
C20:3n-6	0.27	0.25	0.24	0.28	n.s.	n.s.	n.s.	0.08
C20:4n-6	6.37	6.15	6.81	6.96	*	n.s.	n.s.	0.95
C18:3n-3	0.56	0.45	0.69	0.70	n.s.	n.s.	n.s.	0.12
C20:3n-3	0.27	0.22	0.33	0.28	n.s.	n.s.	*	0.10
C20:5n-3	0.15	0.17	0.25	0.23	*	n.s.	*	0.08
C21:5n-3	0.69	0.46	1.24	1.20	**	n.s.	**	0.36
C22:5n-3	0.52	0.49	0.71	0.66	*	n.s.	*	0.47
C22:6n-3	0.22	0.19	0.41	0.36	**	n.s.	*	0.16
Others	0.75	0.74	0.67	0.80	n.s.	n.s.	n.s.	0.29
PUFA	31.96	31.17	36.32	36.12	**	n.s.	*	2.94

n=30 for each treatment. Level of significance: **: P<0.01; *: P<0.05; n.s. = not significant.

Table 6 - Quantitative fatty acid composition (mg/100 g meat), oxidative stability and nutritional indexes of breast meat.

	Control		Rosemary		Significance			SED
	Fresh	Stored	Fresh	Stored	Diet	Storage	Diet x Storage	
SFA	481.66	450.29	424.56	433.39	**	*	*	65.21
MUFA	429.54	388.54	357.58	350.27	**	*	**	41.25
∑ n-6	395.39	354.44	399.63	398.11	**	*	**	45.89
∑ n-3	32.29	24.12	44.53	42.08	**	**	**	8.47
PUFA	428.20	379.65	445.54	431.08	**	**	**	56.81
TBA-RS mg MDA/kg	0.53	1.65	0.66	0.79	*	**	**	0.58
Peroxidability index	61.84	60.21	69.95	69.49	**	n.s.	n.s.	3.41
Atherogenicity index	0.41	0.42	0.40	0.40	n.s.	n.s.	n.s.	0.09
Thrombogenicity index	0.94	0.98	0.84	0.85	**	*	*	0.15

n=30 for each treatment. Level of significance: **: P<0.01; *: P<0.05.

stored meat) index, compared with the control due to a higher PUFA concentration.

CONCLUSIONS

In conclusion, the study showed that the use of a natural extract of rosemary as an additive in the diets of pigeons improved the meat quality, particularly in frozen samples. The protective action of rosemary on the oxidative status *in vivo* of young birds was not immediately evident; after slaughtering, the supplementation increased the lipid stability and improved the colour of the carcasses and the fatty acid composition of the meat, especially after freezing.

Further research, is needed to study the main pathways of antioxidant transfer from parent to offspring in order to determine if the major antioxidant mechanism of rosemary is linked to a scavenger action or decreased dietary adsorption of iron.

ACKNOWLEDGEMENTS

The authors wish to thank Colombo Italia (Beroide, PG) for technical assistance.

REFERENCES

AOAC 1995. "Official Methods of Analysis" 15th ed. Association of Official Analytical Chemists. Washington, DC. USA.

Arakawa K. and Sagai M. 1986. Species differences in lipid peroxide levels in lung tissue and investigation of their determining factors. Lipids 21: 769.

Auroma O.I., Halliwell B., Aeschbach R. and Loligers J. 1992. Antioxidant and pro-oxidant properties of active rosemary constituents carnosol and carnosic acid. Xenobiotica 22: 257.

Barlow S.M. 1990. Toxicologic aspects of antioxidants used as food additives. In "Food Antioxidants". Hudson B.J.F. (Ed.) Elsevier Applied Science. London and New York.

Boehringer Mannheim Manual 1995. "Methods of Enzymatic BioAnalysis and Food Analysis". Mannheim, Germany.

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

Castellini C., Battaglini M., Lattaioli P. and Dal Bosco A. 1996. Effetto della razza e della stagione sulla produttività del colombo e sulle caratteristiche qualitative della carne. Zoot. Nutr. Anim. 2: 89.

Cesarone M.R., Belcaro G., Carratelli U., Corneli U., De Sanctis M.T., Incandela L., Barsoiti A., Terranova R. and Nicolaide S.A. 1999. A simple test to monitor oxidative stress. Int. Angiol. 18: 127.

CIELAB 1976. "Colour System. Commission International de l'Eclairage". CIE, p. 231 Publication 36, Paris.

Cyril H.W., Castellini C. and Dal Bosco A. 1996. Comparison of three cooking methods of rabbit meat. Ital. J. Food Sci. 8: 337.

Copyright 2002. The R development Core Team Version 1.6.1 2002-11-01. www.R-project.org. The R Foundation for Statistical Computing. Vienna University of Technology, Vienna, Austria.

Cuvelier M.E., Berset C. and Richard H. 1994. Antioxidant constituent in sage (*Salvia officinalis*). J. Agric. Food Chem. 42: 665.

Folch J., Lees M. and Sloane-Stanley H. 1957. A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. 226: 497.

Galobart J., Barroeta A.C., Baucells M.D., Codony R. and Ternes W. 2001. Effect of dietary supplementation with rosemary extract and α-tocopheryl acetate on lipid oxidation in eggs enriched with omega-3 fatty acids. Poultry Sci. 80: 460.

Harris W.S. 2004. Fish oil supplementation: evidence for health benefits. Cleve Clin. J. Med. 71: 212.

Hermann K. 1973. Phenolische Pftanzeninhaltssoffe als naturliche abtioxidantien. Fette, Seifen, Anstrichmittel 75: 499.

Hettiarachchy N.S., Glenn K.C., Gnanaesbandam R. and Johnson M.G. 1996. Natural antioxidant extracts from fenugreek (*Trigonella fenunmgraecum*) for ground beef patties. J. Food Sci. 61: 516.

Huisman M., Madsen H.L., Skibsted L.H. and Bertelsen G. 1994. The combined effect of rosemary (*Rosmarinus officinalis* L.) and modified atmosphere packaging as protection against warmed over flavour in cooked minced pork meat. Z. Lebensm. Unters Forsch. 198: 57.

Hulot F. and Ouhayoun J. 1999. Muscular pH and related traits in rabbits: a review. World Rabbit Sci. 7: 15.

Inatani R., Nakatami N. and Fuwa H. 1983. Anti-

oxidative effect of the constituents of rosemary (*Rosmarinus officinalis* L.) and their derivates. Agric. Biol. Chem. 47: 521.

Istat 2004. Istituto Nazionale di Statistica - Via Cesare Balbo 16 - 00184 Roma, Internet site: www.istat.it.

Ke P.J., Ackman R.G., Linke B.H. and Nash D.M. 1977. Differential lipid oxidation products in various parts of frozen mackerel. J. Food Techn. 12: 37.

Korkeala H., Mäki-Petais O., Alanko T. and Sorvettula O. 1986. Determination of pH in meat. Meat Sci. 18: 121.

Kosaka K. and Yokoi T. 2003. Carnosic acid, a component of rosemary (*Rosmarinus officinalis* L.), promotes synthesis of nerve growth factor in T98G human glioblastoma cells. Biol. Pharm. Bull. 26: 1620.

Lemonica I.P., Damasceno D.C. and di Stasi L.C. 1996. Study of the embryotoxic effects of an extract of rosemary *(Rosmarinus officinalis* L.). Braz. J. Med. Biol. Res. 29: 223.

Lopez-Bote C.J., Gray J.I., Gomaa E.A. and Flegal C.J. 1998. Effect of dietary administration of oil extracts from rosemary and sage on lipid oxidation in boiler meat. Brit. Poultry Sci. 39: 235.

Madsen H.L., Nielsen B.R., Bertlesen G. and Skibsted L.H. 1996. Screening of antioxidative activity of spices. A comparison between assays based on ESR spin trapping and electrochemical measurement of oxygen consumption. Food Chem. 57: 331.

McCarthy Y.L., Kerry J.P., Kerry J.F., Lynch P.B.and Buckley D.J. 2001. Evaluation of the antioxidant potential of natural food/plant extracts as compared with synthetic antioxidant and vitamin E in raw and cooked pork patties. Meat Sci. 57: 45.

Morrissey P.A., Sheehy P.J.A., Galvin K., Kerry J.P. and Buckley D.J. 1998. Lipid stability in meat and meat products. Meat Sci. 49: S73.

Nakamura M. and Katoh K. 1985. Influence of thawing method on several properties of rabbit meat. Bulletin of Ishikawa Prefecture College of Agriculture 11: 45.

O'Keefe S.F., Proudfoot F.G. and Ackan R.G. 1995. Lipid oxidation in meats of omega-3 fatty acid-enriched broiler chickens. Food Sci. Techn. 28: 417.

Samman S., Sandtrom B., Toft M., Bukhave K., Jensen M., Soresen S. and Hansen M. 2001. Green tea or rosemary extract added to foods reduces nonheme-iron adsorption. Am. J. Clin. Nutr. 73: 607.

Shahidi F. 2001. Headspace volatile aldehydes as indicators of lipid oxidation in foods. Adv. Exp. Med. Biol. 488: 113.

Singletary K.W. 1996. Rosemary extract and carnosol stimulate rat live glutathione-S-transferase and quinone reductase activities. Cancer Lett. 27: 139.

Stanley D.W. 1991. Biological membrane deterioration and associated quality losses in food tissues. Crit. Rev. Food Nutr. 30: 487.

Warris P.D. 2000. "Meat Science. An Introductory Text". CABI Publishing, Oxon.

Ulbricht T.L. and Southgate D.A.T. 1991. Coronary heart disease: seven dietary factors. The Lancet 338: 985.

Zhao B.L., Li XJ, He R.G., Cheng S.J. and Xin W.J. 1989. Scavenging effect of extracts of green tea and natural antioxidants on oxygen radicals. Cell. Biophys. 14: 175.

Zeng H., Tu Peng F., Zhou K., Wang Bao-Hi. and Lu Jing. 2001. Antioxidant properties of phenolic diterpenes from *Rosmarinus officinalis*. Acta Pharmacol. 2: 1092.

Paper received January 13, 2005 Accepted May 30, 2005

PAPER

SIGNIFICANCE OF FUROSINE INDEX IN TALEGGIO CHEESE-MAKING

SIGNIFICATO DELL'INDICE DI FUROSINA NELLA CASEIFICAZIONE A TALEGGIO

F. MASOTTI* and I. DE NONI
Dipartimento di Scienze e Tecnologie Alimentari e Microbiologiche,
Università degli Studi di Milano, Via Celoria 2, 20133 Milano, Italy
* Corresponding author: Tel. +39 02 50316665, Fax +39 02 50316672,
e-mail: fabio.masotti@unimi.it

ABSTRACT

The furosine (FUR) level of Taleggio cheese was investigated with respect to different cheese-making parameters used in 11 factories. Taleggio made from raw milk according to traditional cheese-making and submitted to continuous-flow pasteurization (72°-74°C/15 s) had a mean FUR level of 8.1 mg/100 g protein (n=6). This level was mainly affected by the amount of starter culture, composition of growth medium and ripening time. When additional batch heat treatment was ap-

RIASSUNTO

È stato studiato l'effetto dei principali parametri tecnologici di caseificazione sul valore di furosina (FUR) del formaggio Taleggio prodotto presso 11 aziende. Il valore medio di FUR del Taleggio ottenuto secondo la tradizionale tecnologia di caseificazione e a partire da latte crudo pastorizzato in flusso continuo (72°-74°C/15 s) è risultato pari a 8.1 mg/100 g proteine (n=6). Quantità e tipo di innesto ed il periodo di maturazione sono i fattori che maggiormente influenzano il livello di FUR

- Key words: Cheese-making, furosine, heat treatment, Taleggio cheese -

plied to pasteurized milk, the corresponding cheese showed a FUR value about 2 mg/100 g protein higher. A further significant increase was observed in cheese samples manufactured from milk heated by injecting steam through a pipe (mean value 20.3 mg/100 g protein; n=5). In these cases, the major effect on the FUR level was exerted by processing conditions used in batch or steam heating of the milk. Analyses of commercial Taleggio cheese demonstrated that 21 out of 22 samples had a FUR value consistent with the cheese-making technology described in the product specifications.

nel Taleggio. Un aggiuntivo trattamento termico in batch del latte pastorizzato ha comportato un aumento (circa 2 mg/100 g proteine) del livello di FUR nel formaggio. Caseificazioni a partire da latte riscaldato mediante iniezione manuale di vapore nel latte in caldaia hanno determinato valori di FUR nel formaggio significativamente superiori (media = 20.3 mg/100 g proteine; n=5). In queste ultime due tecnologie, il livello di FUR del formaggio è stato principalmente determinato dalle condizioni di riscaldamento del latte in batch o con vapore. L'analisi di 22 campioni commerciali di Taleggio ha evidenziato che per 21 di essi i valori di FUR sono compatibili con il processo di caseificazione descritto nel Disciplinare di produzione.

INTRODUCTION

Taleggio is a soft, short-ripened Italian cheese (BATTISTOTTI and CORRADINI, 1993; SALVADORI DEL PRATO, 1998) that received the Protected Designation of Origin (PDO) in 1996 (C. R., 1996). This square-shaped cheese is produced in a restricted area of northern Italy from whole milk inoculated with a culture of lactic acid bacteria and rennet coagulated at 34°-36°C. After a two-stage cut, the curd is drained, placed into moulds and kept at 22°-25°C/90% RH for 8-16 h ("warming up"). The cheese is then salted (either brine or dry) and ripened at 2°-6°C for at least 35 d (85-90% RH).

According to the product specifications for Taleggio cheese (E. U., 1993) either raw or pasteurized milk can be used in cheese-making. Currently, all industrial-scale Taleggio manufacturers sanitize the milk through continuous-flow pasteurization at 72°-74°C for 15 s. Heat treatment by injecting steam into the milk through a pipe is sometimes used in small factories. This procedure, however, cannot be considered as "a traditional and fair practice".

Heating milk promotes the Maillard reaction which leads to the formation of the Amadori compound lactulosyl-lysine (FINOT *et al.*, 1981). Upon warm acid hydrolysis, this molecule is converted into furosine (FUR) which can be detected by using IP-RP HPLC as proposed by RESMINI *et al.* (1990). This method has been adopted as the official method by Italian law (M. D., 1996) and has now become an ISO-IDF International Standard (ISO, 2004). The FUR level has proven to be an objective parameter for evaluating quality and genuineness of both raw and pasteurized milk, as well as of other dairy products (RESMINI *et al.*, 1994). In this regard, the Italian law states the maximum levels of FUR for peroxidase-positive continuous-flow pasteurized milk and for Mozzarella cheese (8.6 and 12 mg/100 g protein, respectively).

The same criterion has been adopted to guarantee the genuineness of "traditional speciality guaranteed Mozzarella" by fixing an upper level of 10 mg/100 g protein (C. R., 1998). At present, the FUR level of Taleggio cheese must not exceed 14 mg/100 g protein, as stated in the product specifications (E. U., 1993).

The aim of this work was to investigate the effects that different technological parameters adopted in cheese-making have on the FUR level of Taleggio. In particular, the effect of different milk heating practices was evaluated with respect to the maximum FUR value provided in the product specifications.

MATERIALS AND METHODS

Taleggio cheese was made at the industrial level using continuous-flow (conventional) pasteurized milk in factories 1 to 6. The technological parameters are reported in Table 1 and correspond to those included in the product specifications (E. U., 1993). Raw milk subjected to conventional pasteurization followed by batch heat treatment was used to make Taleggio cheese in factories 7 and 8 (Table 1). Other Taleggio cheese was made using milk heated by steam injection (factories 9 to 11). In this case, the steam (pressure and flow rate were not given) was manually injected into the milk in a vat using a pipe and under continuous stirring. In factories 10 and 11, cheese was made at 1-month intervals (trials B in Table 2). In all the factories, the milk was then cooled to coagulation temperature (34°-36°C) by regenerative heat exchange. Starter cultures, grown in reconstituted skimmed milk powder (SMP), were used in factories 1, 2, 4 and 9.

A total of 98 samples from 11 factories were analyzed: raw milk (n=13); heat-treated milk (n=13); starter culture preparation (n=12); inoculated milk (n=12); curd under whey (n=11); curd after "warming up" (n=11); salted curd (n=13) and cheese 35-40 d ripened (n=13). Curd and cheese were sampled as specified in ISO 707 (1997). Cheeses were also analyzed by taking test portions (n=26, 20 mm-side cube) from both the core and the straight side of the outer part after rind removal.

Further analyses were carried out on 22 commercial samples of Taleggio (35-40 d ripened) supplied by the "Consorzio per la tutela del formaggio Taleggio".

The FUR content was determined according to ISO 18329 - IDF 193 International Standard (ISO, 2004). All experimental and commercial samples were analyzed in duplicate and mean values are reported. ANOVA was carried out using the Stat-View software, version 5.1 (SAS Institute Inc., Cary, NC, USA).

RESULTS AND DISCUSSION

The different technological parameters adopted for cheese-making are reported in Table 1. The FUR level of raw milk samples ranged from 4.4 to 5.7 mg/100 g protein (Table 2). This level increased (about 0.5 mg/100 g protein) with conventional milk pasteurization used in factories 1 to 6. This finding agrees with results reported by RESMINI *et al.* (1992) for conventional pasteurization of raw milk. Pasteurized milk was direct-inoculated with 0.5-1.5% (v/v) of liquid starter culture. As expected, starter cultures which were grown in reconstituted SMP (factories 1, 2 and 4) had FUR values comparable to those reported for high-heat milk powders (RESMINI *et al.*, 1994). The addition of starter (l.0-1.5%, v/v) significantly increased the FUR value of inoculated milk in factories 1 and 2 (9.0 mg and 9.1 mg/100 g protein, respectively). In contrast, the lower (0.5%, v/v) amount of liquid starter added to the milk in factory 4 resulted in a FUR level of the inoculated milk that was below 7 mg/100 g protein. Although the

Table 1 - Technological parameters of Taleggio cheese-making in eleven factories.

Factory		1	2	3	4	5	6	7	8	9	10	11
Milk heating:	Type	Continuous-flow pasteurization						Continuous flow pasteurization (P) + batch treatment (B)		Steam injection through a pipe		
	Conditions (°C/s)	74/15	73/15	74/15	72/15	72/15	72/15	P: 73/19 + B: 65/1500	P: 73/19 + B: 65/840	60-65/1800 + 70-80/300	70-80/300	65-70/900 + 70-80/300
Starter inoculation (% v/v)		1.5	1.0	0,5	0,5	n.k.	n.k.	2.0	2.0	2.0	1.0	2.0
Salting		Dry	Dry	Brine	n.k.	n.k.	n.k.	Brine	Brine	Brine	Brine	Brine

n.k.: not known.

Table 2 - Furosine level (mg/100 g protein) determined at different steps of Taleggio cheese-making from Table 1 (Mean value of duplicate analyses).

Factory Trial	1	2	3	4	5	6	7	8	9	10 A	10 B	11 A	11 B
Raw milk	4.4	5.3	5.6	4.6	5.2	5.1	4.8	4.9	5.1	4.6	5.0	5.7	5.4
Heated milk	4.9	5.7	6.0	5.3	5.5	5.7	8.7	8.0	16.7	14.5	13.7	23.0	22.9
Starter culture	302	348	n.k.	257	16.7	15.6	33.7	40.0	362	33.6	27.7	41.7	48.3
Inoculated milk	9.0	9.1	6.8	6.5	6.1	6.0	9.4	9.0	24.4	14.2	n.k.	22.2	23.8
Curd under whey	5.5	6.9	5.2	5.1	4.6	4.6	n.k.	n.k.	20.5	11.2	12.4	20.0	22.0
“Warmed up” curd	6.8	7.5	6.4	5.2	5.5	5.0	n.k.	n.k.	19.1	14.7	14.3	22.4	24.4
Salted curd	6.8	7.5	6.8	5.3	6.0	5.4	9.1	8.6	21.6	15.2	14.3	24.3	24.4
Cheese	8.9	9.2	8.2	8.4	7.3	6.6	10.4	10.0	22.5	14.5	14.4	25.4	24.5
Outside[1]	7.7	8.8	7.7	8.2	6.6	6.4	9.7	9.5	22.3	14.5	14.2	25.5	24.2
Core[2]	9.7	9.4	8.6	8.4	7.6	6.7	10.5	10.0	22.5	14.3	14.2	25.6	24.4

n.k.: not known. [1]: test portion taken from the straight side below the rind. [2]: test portion taken from the core of the cheese.

inoculation rate used in factories 5 and 6 was not given, use of selected starter preparations with low FUR values led to a slight (0.3-0.6 mg/100 g protein) increase of the FUR index of inoculated milk. The increase (0.8 mg/100 g protein) of the FUR level observed in cheese from factory 3 was probably due to the addition of starter culture not propagated in reconstituted SMP. The FUR values in samples of curd under whey were lower than those of the corresponding inoculated milk samples. This behaviour could be explained by the lower extent of Maillard reaction in the casein fraction with respect to the whey fraction when milk is submitted to pasteurization (PELLEGRINO *et al.*, 1993). "Warming up" of the curd increased the FUR value by 0.1-1.3 mg/100 g protein, whereas the effect of salting, either dry or brine, was negligible. During ripening, the FUR level in Taleggio increased by 1.2-3.1 mg/100 g protein. CORZO *et al.* (2000) reported a FUR value increase from 8 to 22 mg/100 g protein during 45-d ripening of Manchego cheese. This increase was consistent with the presence of residual galactose in cheese. PELLEGRINO *et al.* (1997a) did not observe any increase in the FUR value during ripening of Grana Padano since reducing sugars disappeared within a few hours after curd extraction. Samples of Taleggio from factories 1 to 6 showed FUR levels from 6.6 to 9.2 mg/100 g protein. VILLAMIEL *et al.* (2000) reported FUR levels ranging from 4.2 to 12.8 mg/100 g protein for ripened cheeses with moulds made from pasteurized milk. The mean FUR level of ripened Taleggio was 8.1 mg/100 g protein (SD ± 0.98 mg/100 g protein; n=6). Taking these results into account, a maximum FUR level of 11.0 mg/100 g protein at 99.7% confidence level (mean + 3 SD) could be considered as characteristic of Taleggio cheese manufactured from conventional pasteurized milk. A lower level could be established if more detailed rules concerning the inoculation step would be provided in the product specifications. Indeed, this limit would probably be exceeded if more than 1.5% (v/v) starter cultures grown in SMP were added to milk.

The values in the outer part of Taleggio from factories 1 to 6 were always lower (0.2-2.0 mg/100 g protein) than those in the core. PELLEGRINO *et al.* (1997b) reported that Grana Padano cheese is characterized by a steep centripetal gradient of FUR values which increases by about 60-70 mg/100 g protein from the surface to the core. Such a gradient is the result of the Maillard reaction that takes place in parallel with the temperature gradient occurring within the curd once it is extracted from the vat (53°-54°C) and kept in the mould at room temperature. The slow cooling of this big-size cheese allows the Maillard reaction to occur extensively in the core (PELLEGRINO *et al.*, 1997a). Due to the small size of Taleggio and the low curd temperature (34°-36°C), small differences (0.2-2.0 mg/100 g protein) in the FUR values were observed between the outer and inner part of the cheese.

Experimental cheese-making in factories 7 and 8 was carried out using milk that had been submitted to conventional pasteurization followed by batch heat treatment (Table 1). Such conditions would be representative of the actual situation in factories where, because of particular plant design, pasteurized milk is temporarily stored in tanks. Heat-treated milk from factories 7 and 8 was characterized by FUR levels that coincided with the threshold value fixed by Italian law for peroxidase-positive pasteurized milk (i.e. 8.6 mg/100 g protein). The use of starter cultures characterized by low heat-damage caused a minimal increase in the FUR level in inoculated milks. Subsequent FUR formation (about 1.3 mg/100 g protein) during ripening was comparable to that found for experimental cheese samples made from

conventional pasteurized milk. The FUR level of cheeses from factories 7 and 8 were 10.4 and 10.0 mg/100 g protein, respectively; the core value was higher in both samples.

Injection of steam into milk in the vat, carried out at factories 9 to 11, caused the FUR values of milk to range from 13.7 to 23.0 mg/100 g protein (Table 2). According to ERBERSDOBLER and DEHN-MULLER (1989) and RESMINI *et al.* (1990), similar values were found in raw milk submitted to laboratory-scale treatment at 90°-100°C for 360-600 s. The FUR level of inoculated milk from factory 9 was consistent with the heat-treatment and the use of starter grown in SMP (Table 2). In the other cases, the FUR level was associated with the severity of the applied heat process. "Warming up" of the curd increased the FUR value with the exception of cheese from factory 9 (Table 2). The subsequent salting caused a further increase in the FUR value, whereas there were no changes during ripening, at the end of which all cheeses had FUR values > 14.4 mg/100 g protein.

The significant differences among FUR levels in successive steps of Taleggio cheese making are reported in Table 3. Inoculation ($P<0.05$), draining in vat ($P<0.05$) and ripening ($P<0.01$) affected the FUR level in cheeses made from conventional pasteurized milk (factories 1 to 6). The significant increase ($P<0.05$) of the FUR level due to inoculation was related to the use of starter cultures grown in SMP. In contrast, only the heat treatment of raw milk significantly changed the FUR value during cheese-making in factories 7-8 and 9-11 (Table 3).

When the same steps of technology were taken into consideration, the FUR level of the corresponding product (from heated milk to cheese) differed significantly in the cheese-making that used the steam injection practice compared with either conventional pasteurization or conventional pasteurization plus batch treatment ($P<0.001$ and $P<0.01$, respectively). Accordingly, the ripened cheeses could be univocally distinguished. No significant differences were observed in the FUR value of either heated milk or cheese obtained from conventional pasteurization or conventional pasteurization plus batch treatment.

Table 3 - Significant differences in the FUR level among different steps of Taleggio cheese-making from Table 1.

Factory	1-6	7-8	9-11
Raw milk vs Heated milk	ns	**	***
Heated milk vs Inoculated milk	*	ns	ns
Inoculated milk vs Curd under whey	*		ns
Curd under whey vs "Warmed up" curd	ns		ns
"Warmed up" curd vs Salted curd	ns	ns	ns
Salted curd vs Cheese	**	ns	ns

* $P<0.05$; ** $P<0.01$; *** $P<0.001$; ns: not significant.

The FUR values were then determined in 22 commercial samples of Taleggio cheese (Table 4). The values ranged from 4.9 to 10.1 mg/100 g protein in 16 samples suggesting that the cheeses were probably manufactured from milk that had not been submitted to steam injection. Six samples were between 11.7 and 13.5 mg/100 g protein and hence below the maximum level (i.e. 14 mg/100 g protein) allowed by the product specifications (E. U., 1993). The addition of high amounts of starter cultures grown in reconstituted SMP could account for these sample values. Over-heating of the milk could explain the FUR level in sample V (22.2 mg/100 g protein). However, the partial use of reconstituted milk powder for cheese-making cannot be excluded. Three of the 22 samples were characterized by a reverse FUR gradient from the outside to the core of the cheese (Table 4). This phenomenon can be explained by a forced "warming up" of the curd in order to accelerate acidification.

Table 4 - Furosine value (mg/100 g protein) in 22 samples of commercial Taleggio cheese. (Mean value of duplicate analyses).

Sample	Cheese	Outside[1]	Core[2]
A	4.9	4.7	5.1
B	6.1	5.7	6.4
C	6.3	6.1	6.8
D	6.9	6.5	7.2
E	6.9	6.8	6.8
F	7.3	6.9	7.5
G	7.6	7.2	7.9
H	7.7	7.4	8.0
I	8.0	7.5	8.2
J	8.8	8.2	9.3
K	9.1	8.1	9.7
L	9.1	8.6	9.4
M	9.3	10.6	8.9
N	9.5	9.5	9.7
O	10.0	9.7	10.2
P	10.1	10.2	9.9
Q	11.7	11.4	11.8
R	12.5	12.3	12.9
S	12.9	13.8	12.6
T	13.2	12.8	13.4
U	13.5	12.8	13.6
V	22.2	21.7	22.3

1: test portion taken from the straight side below the rind.
2: test portion taken from the core of the cheese.

CONCLUSIONS

The results obtained in this study show that the FUR index is a useful indicator of the extent of the Maillard reaction during Taleggio manufacturing. A FUR value lower than 11.0 mg/100 g protein is characteristic of Taleggio made according to traditional cheese-making and from pasteurized milk. Higher values are associated with the use of overheated milk or high amounts of starter cultures grown in SMP.

ACKNOWLEDGEMENTS

The authors thank professors P. Resmini and L. Pellegrino for helpful discussion and the "Consorzio per la Tutela del formaggio Taleggio" for the samples.

REFERENCES

Battistotti B. and Corradini C. 1993. Italian cheese. Ch. 7. In "Cheese: Chemistry, Physics and Microbiology". P.F. FOX (Ed.), Vol. II, p. 221, 2nd ed. Chapman & Hall, London, UK.

Corzo N., Villamiel M., Arias M., Jiménez-Pérez S. and Morales F.J. 2000. The Maillard reaction during the ripening of Manchego cheese. Food Chem. 71: 255.

C. R. (Commission Regulation EC) N. 1107/96 June 12, 1996, on the registration of geographical indications and designations of origin under the procedure laid down in Article 17 of Council Regulation (EEC) N. 2081/92. Off. J. Eur. Communities L 148, June 21, 1996.

C. R. (Commission Regulation EC) N. 2527/98 November 25, 1998, supplementing the Annex to Regulation (EC) N. 2301/97 on the entry of certain names in the "Register of certificates of specific character" provided for in Council Regulation (EEC) N. 2082/92 on certificates of specific character for agricultural products and foodstuffs. Off. J. Eur. Communities. L 317 November 26, 1998.

Erbersdobler H.F. and Dehn-Muller B. 1989. Formation of early Maillard products during UHT treatment of milk. Int. Dairy Fed., Special Issue 238, p. 153, Brussels, Belgium.

E. U. (European Union) 1993. Disciplinare di produzione del formaggio Taleggio, attached to application of registration to use the protected designation of origin according to Council Regulation (CEE) N. 2081/92. July 22, 1993. Prot. N. 367/93.

Finot P.A., Deutsch R. and Bujard E. 1981. The extent of the Maillard reaction during the processing of milk. Ch. 5. In "Progress in Food and Nutrition Science". C. Eriksson (Ed.), p. 345. Pergamon Press, New York, NY.

ISO 707. 1997. Milk and milk products. Guidance on sampling.

ISO 18329 - IDF 193. 2004. Milk and milk products – Determination of furosine content – Ion-pair reverse-phase high performance liquid chromatography method.

M. D. (Ministerial Decree), May 16, 1996. Approvazione del metodo ufficiale di analisi per la determinazione diretta della furosina nel latte e nel formaggio. Italian Off. J. (G.U.) N. 162 (August, 12, 1996).

Pellegrino L., Oreglio M. and De Noni I. 1993. Riconoscimento del latte in polvere nel formaggio Mozzarella mediante valutazione dell'intensità della reazione di Maillard. Chiriotti Ed., Proc. 1st CISETA, Parma, Italy.

Pellegrino L., Battelli G. and Resmini P. 1997a. Andamento centripeto della maturazione nei formaggi Grana Padano e Parmigiano Reggiano. Sci. Tecn. Latt.-Cas. 48: 73.

Pellegrino L., Battelli G., Resmini P., Ferranti P.,

Barone F. and Addeo F. 1997b. Effects of heat load gradient occurring in moulding on characterization and ripening of Grana Padano. Lait 77: 217.

Resmini P., Pellegrino L. and Battelli G. 1990. Accurate quantification of furosine in milk and dairy products by a direct HPLC method. Ital. J. Food Sci. 3: 173.

Resmini P., Pellegrino L., Masotti F., Tirelli A. and Prati F. 1992. Determinazione del latte in polvere ricostituito nel latte crudo ed in quello pastorizzato, mediante HPLC della furosina. Sci. Tecn. Latt.-Cas. 43: 169.

Resmini P., Pellegrino L. and Masotti F. 1994. Evaluation of the Maillard reaction extent for quality control of low-heat treated dairy products. In "Protein and Fat Globule Modifications by Heat Treatment, Homogenization and Other Technological Means for High Quality Dairy Products", p. 153, Proc. IDF Seminar held in Munich., D. Int. Dairy Fed., Special Issue 9303, Brussels, Belgium.

Salvadori Del Prato O. 1998. Ch. 18. Taleggio. In "Trattato di Tecnologia Casearia". Edagricole Ed., p. 676. Bologna, Italy.

Villamiel M., Arias M., Corzo M. and Olano A. 2000. Survey of the furosine content in cheeses marketed in Spain. J. Food Protect. 63: 974.

Paper received February 1, 2005 Accepted June 30, 2005

PAPER

ELECTROANALYTICAL QUANTIFICATION OF RHODIUM IN LENTILS EXPOSED TO INCREASING Rh(III) CONCENTRATIONS

QUANTIFICAZIONE ELETTROANALITICA DEL RODIO
IN LENTICCHIE ESPOSTE A CONCENTRAZIONI CRESCENTI DI Rh(III)

B. BRUNETTI, R. CATTANEO and E. DESIMONI*
Dipartimento di Produzione Vegetale, Università degli Studi di Milano,
Via Celoria, 2, 20133 Milano, Italy
* Corresponding author: e-mail: elio.desimoni@unimi.it

ABSTRACT

The quantification of trace/ultra-trace levels of rhodium in the environment and in food requires extremely sensitive analytical techniques. Catalytic Adsorptive Stripping Voltammetry (CAdSV) allows the concentration of rhodium to be determined at sub-µg/L levels in an easy and fast way. The best operational procedure was determined for the CAdSV quantification of rhodium in real food samples. In particular, adjusting the deposition times as a function of the rhodium concen-

RIASSUNTO

La quantificazione del rodio a livello di tracce/ultra-tracce nell'ambiente e negli alimenti richiede tecniche analitiche estremamente sensibili. La voltammetria catalitica di adsorbimento e dissoluzione (CAdSV) permette di determinare la sua concentrazione a livello di sub-µg/L in maniera facile e veloce. Questo lavoro presenta la messa a punto di una procedura CAdSV per la quantificazione del rodio in campioni reali di interesse alimentare. In particolare, si è potuto dimostrare che variando i tempi

- Key words: CAdSV, electroanalysis, food plants, NMKL, platinum group metals, ultra-trace analysis, voltammetry -

tration and using a weighted, linear, least-square regression substantially improved the quality of the results. The method was applied to the analysis of in-house contaminated lentils. The results confirmed that rhodium could enter the food chain at rather high concentrations.

di deposizione in funzione della concentrazione del rodio ed usando la regressione lineare pesata ai minimi quadrati è possibile migliorare sostanzialmente la qualità dei risultati sperimentali. Il metodo è stato applicato all'analisi di lenticchie opportunamente contaminate in laboratorio. I risultati hanno confermato che il rodio potrebbe entrare nella catena alimentare a livelli di concentrazione abbastanza alti.

INTRODUCTION

Platinum Group Elements (PGE) are widely used in three-way catalytic converters (TWC) to limit the emission of pollutants from combustion engines. The overall concentration of platinum, palladium and rhodium in conventional TWCs usually ranges from 0.10 to 0.15%. Unfortunately, TWC emissions, due to surface abrasion of the catalyst while the car is running, have been high enough since the early 1980s to cause slow but constantly increasing environmental contamination (LUSTIG *et al.*, 1998; PALACIOS *et al.*, 2000; RAVINDRA *et al.*, 2004; HOPPSTOCK and SURES, 2004; ZIMMERMANN and SURES, 2004). As already reported (RAVINDRA *et al.*, 2004; RAUCH and MORRISON, 2000), a significant uptake of TWC-emitted noble metals by different plants growing in the surrounding contaminated highway soils indicates a higher than expected mobility of PGE. This is probably due to the fact that, when PGE metal/oxide particles come in contact with soil, they can be transformed to soluble PGE salts (LUSTIG *et al.*, 1998; ECKARDT *et al.*, 2000; EK *et al.*, 2004). In particular, while the fraction of soluble PGE salts was less than 10% of the total amount emitted from fresh catalysts, significantly higher Pd and Rh fractions were emitted from aged catalysts (MOLDOVAN *et al.*, 2002). According to an evaluation of the ratio between the concentration in the plant and the concentration in the soil (SCHÄFER *et al.*, 1998), Pd was as mobile as Zn, while Pt and Rh were as mobile as Cu.

The PGE metal/oxide particulate emitted during TWC operation can be regarded as virtually inert from a biological point of view. However, soluble PGE compounds, probably emitted as carbonyl complexes (MOLDOVAN *et al.*, 2002) can contaminate vegetables and food because of their bioavailability.

Few data exist about the toxicological activity of PGE (GEBEL, 2000; KATSAROS and ANAGNOSTOPOULOU, 2002; ARTELT *et al.*, 1999). Regarding the contamination of plants, it seems that the order of toxicity is Pt(II) > Pd(II) > Pt(IV) > Rh(III) (FARAGO and MULLEN, 1979; FARAGO and PARSONS, 1994).

At present, PGE concentrations in most environmental samples are at trace or ultra-trace levels (LUSTIG *et al.*, 1997; VERSTRAETE *et al.*, 1998; ARTELT *et al.*, 1999; LUSTIG and SCHRAMEL, 2000; HOPPSTOCK and ALT, 2000; HUTCHINSON *et al.*, 2000; RAUCH and MORRISON, 2000; SCHIERL, 2000) and, consequently, the quantification of PGE requires extremely sensitive analytical techniques. This justifies the attention given to electroanalytical techniques, since they offer

an appealing compromise between sensitivity and cost per analysis. Even if some papers have dealt with the electroanalysis of PGE using differential pulse polarography (ZHAO and FREISER; 1986, DUBEY *et al.*, 1998; KOLPAKOVA *et al.*, 2003) and linear sweep voltammetry (ENSAFI and ZAREI, 1998), the method based on Catalytic Adsorptive Stripping Voltammetry (CAdSV) at a Hanging Mercury Drop Electrode (HMDE) (HOPPSTOCK *et al.* 1989; LEON *et al.*, 1997) seems the best suited to determine the overall concentration of PGE at sub-µg/L levels. Of all the electroanalytical techniques it allows the most appealing combination of very high sensitivity and ease of use. Reported limits of detection range from 5.6 pg/L (LEON *et al.*, 1997) to 2.0 ng/L (HELMERS and MERGEL, 1998). Its only serious drawback is high sensitivity to traces of organic compounds that requires drastic mineralisation of the real samples.

Several researchers have successfully applied the CAdSV method to PGE quantification in real samples (see, for example, NYGREN *et al.*, 1990; VAUGHAN and FLORENCE, 1992; HONG *et al.*, 1994, KÜMMERER and HELMERS, 1997; HELMERS and MERGEL, 1998; ZIMMERMANN *et al.*, 2003; SURES *et al.*, 2003 a). While performing a systematic validation study of the CAdSV method for determining platinum in several real matrices of environmental and food concern (DESIMONI *et al.*, 2002; 2003; 2004a), attention was also given to rhodium. The quantification of Rh causes additional experimental difficulties. Considering that in current TWC the usual ratios range from Pt/Rh = 5/1 (in USA) to Pt/Rh = 10/1 (in Europe), and that the two metals exhibit similar mobilities (SCHÄFER *et al.*, 1998), the expected concentration level of rhodium in real matrices could be even lower than that of platinum (PALACIOS *et al.*, 2000; LUSTIG and SCHRAMEL, 2000).

The literature about the CAdSV analysis of PGE (HOPPSTOCK *et al.*, 1989; HONG *et al.*, 1994; LEON *et al.*, 1997; HELMERS and MERGEL, 1998) reports partially discordant information about the convenience of quantifying rhodium alone or rhodium and platinum in the same run. In this work, some experiments were performed in an attempt to set up the best operational procedure for quantifying rhodium by CAdSV. The method was then applied to quantify the rhodium concentration in lentil samples grown on rhodium-contaminated substrates.

MATERIALS AND METHODS

Reagents

Ultrapure (UP) water (resistivity level: 18.2 MΩ·cm at 25°C; TOC level: lower than 15 µg/kg) was produced by Simplicity 185 water purification system (Millipore S.A., Molsheim, France). Hydrochloric acid (30%) and hydrogen peroxide (30%) were of Suprapur grade; formaldehyde (37%) was of pro-analysis grade. All reagents were purchased from E. Merck (Darmstadt, Germany). Rhodium(III) and platinum(II) concentrated standard solutions (991±10 mg/L and 1,004±5 mg/L, respectively) were also purchased from E. Merck (Darmstadt, Germany). In-house standard solutions containing 20, 40, 60, 80 and 100 ng/L of rhodium were prepared daily by diluting the concentrated standard solution with 0.1 M HCl.

Lentil samples

Lentil samples (*Lens esculenta*), obtained from a local store, were contaminated in-house. To this aim about ten grams of lentils, corresponding to about 190-200 lentils, were soaked in Petri dishes containing cotton-wool imbibed with 0.0 µg/L (control), 10.0, 50.0, 100.0, 500.0 µg/L, 1.0 mg/L and 5.0 mg/L solutions of rhodium(III) (obtained

by diluting the concentrated standard with UP water). The water volume in each Petri dish was held constant by daily refilling with UP water. After ten days, the lentils were sufficiently germinated to allow the lentil radicle, seed coat and shoot sub-samples to be collected. These sub-samples were carefully washed with UP water, dried at 80°C until a constant weight and then mineralised (see below). When necessary, care was taken to dilute the mineralised lentil sub-samples in such a way that the rhodium concentration in the voltammetric cell was less than 60 ng/L. This was necessary in order to avoid a possible loss of precision (as can happen when the deposition times are too short) and to work in a linear range sufficiently below the upper limit (the Application Bulletin Metrohm AB 220/3e reports 500 ng/L by using an enrichment time of 60 s). Rhodium concentrations and the uncertainties (see the Results and Conclusion section) were corrected by multiplying them by the proper dilution factors.

Mineralisation procedures

Lentil sub-samples (radicles, seed coats and shoots) were mineralised by UV photolysis performed with a 705 Digester (Metrohm Ltd, Herisau, CH). Each sub-sample was prepared by adding 6 mL of 30% hydrogen peroxide plus 2 mL of ultrapure water to 10-30 mg (dry weight) of the mass of radicles, seed coats or shoot sub-samples and irradiating the resulting suspension for four hours. The rhodium concentration in the mineralised samples did not change during this time of treatment. This result does not prove that mineralisation was complete, since the percentage of mineralisation could remain constant after such a time. However, it was accepted as evidence of reasonably complete mineralisation since, as already observed throughout this work, even minor traces of organic materials regularly led to unreal analytical results. All sub-samples used for replicating the analysis were prepared independently.

Voltammetric analysis

A 693VA-Processor was used in conjunction with a 694VA Stand (Metrohm Ltd, Herisau, CH) equipped with a mercury drop multimode working electrode (Metrohm Ltd, Herisau, CH), a glassy carbon auxiliary electrode and a double-junction $Ag/AgCl/Cl^-$ (3.0 M) reference electrode. The use of mercury as a working electrode was not of any environmental concern, since the minimum amount of the metal necessary for any determination was completely recovered from the reaction vessel by using the Metrohm silver wire mercury drop catcher (capturing mercury drops by amalgamation). To avoid contamination from memory effects, voltammetric measurements were performed by using a series of measuring vessels previously stored in 1:1 hydrochloric acid for at least 24 hours.

Software

Voltammetric results were analysed by using a spreadsheet prepared as template in Mathcad 7.02a Professional (MathSoft Inc., Cambridge, Massachusetts, USA) (DESIMONI, 1999). Whatever the confidence level, the number of analysed standard solutions and their available replicates, the spreadsheet allowed i) unbalanced response arrays to be managed, ii) the scedasticity (by Bartlett test), linearity (by lack-of-fit test), outliers (by an F-test) and normality (by Shapiro-Wilk test) to be tested, iii) the slope and intercept of the calibration function to be evaluated along with their confidence intervals by weighted, linear, least square regression and iv) unknown concentrations to be evaluated along with their confidence intervals.

Each solution, whether standard or

obtained by mineralising real samples, was analysed by the standard addition method. The analysis of standard solutions involved up to four independent repetitions of any standard solution as received, as well as after eight standard additions. The analysis of lentil sub-samples involved two repetitions of the mineralised solutions as received, as well as after four standard additions.

RESULTS AND CONCLUSIONS

Preliminary experiments performed in the course of this study by analysing in-house standard solutions confirmed that insufficiently reproducible results were obtained by quantifying platinum and rhodium in the same run, as previously reported (HOPPSTOCK *et al.*, 1989; LEON *et al.*, 1997; HELMERS and MERGEL, 1998). Therefore, in all subsequent experiments, measurement conditions were set up to quantify rhodium only. In particular, the composition of the support electrolyte was according to HELMERS and MERGEL (1998) that is, 0.42 M HCl plus 0.02 M formaldehyde. Moreover, experiments were performed to evaluate the dependence of deposition time on rhodium concentration (LEON *et al.*, 1997) using a mercury drop having a constant dimension (4 a.u. according to HELMERS and MERGEL, 1998 and to METROHM AB 220/3e). To this aim aliquots of UP water and of standard solutions containing 20, 40, 60, 80 and 100 ng/L of rhodium were repeatedly analysed using different deposition times. The optimum deposition times were chosen as those that allowed the best agreement between the average experimental concentrations and the standard solution concentrations. Fig. 1 shows the best fit of the experimental results. Table 1 reports the results obtained using the optimum deposition time. The uncertainties were estimated as both Instrumental Confidence Interval (ICI; $\alpha = 0.05$) and as NMKL expanded uncertainties (NMKL, 1997) by using a coverage factor k = 2.0 for associating the 95% confidence level to expanded uncertainties.

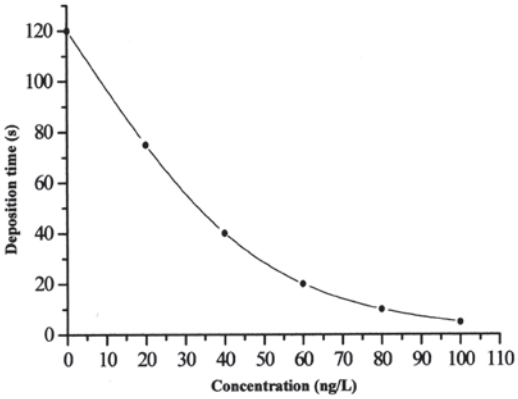

Fig. 1 - Experimental dependence of optimal deposition time on rhodium concentration. Deposition times at each rhodium concentration were evaluated over five independent repetitions of standard solutions of the analyte.

As expected, NMKL uncertainties of the results relevant to solutions contain-

Table 1 - Results of the analysis of the rhodium (ng/L) in five stock solutions and ultra pure (UP) water.

Expected		0 (UP)	20	40	60	80	100
Found	a	0.013±0.019	20.25±0.31	40.82±0.86	60.60±0.62	81.1±2.2	101.2±2.0
	b	0.013±0.074	20.2±2.0	40.8±2.5	60.6±2.7	81.1±2.2	101.2±2.1
r^2		0.9962	0.9985	0.9990	0.9997	0.9980	0.9989

a: instrumental confidence interval ($\alpha = 5\%$); b: NMKL expanded uncertainty (k = 2.0). As suggested by ISO (GUM., 1995) and EURACHEM (QUAM., 2000) experimental results are rounded to the second significant figure of their uncertainty.

ing 0-60 ng/L of rhodium were 3-7 times higher than those estimated as ICIs. At higher rhodium concentrations, the ICIs increased noticeably because the high analyte concentration required the use of deposition times that were too low or dilutions that were to high. According to this result, when analysing unknown samples, a preliminary analysis was always performed to obtain a rough estimate of the rhodium content.

The Bartlett test of the Mathcad spreadsheet (DESIMONI, 1999) showed a clear heteroscedasticity of the analytical system. This finding, never reported before, indicates that the use of weighted, linear, least-square regression, as done throughout this work, is mandatory when analysing the data obtained by the CAdSV method for rhodium. Using the ordinary, linear, least-square regression could lead to more or less significant systematic errors. The same was also observed with platinum only (DESIMONI *et al.*, 2003; 2004a). Typical differences between the parameters obtained by the two regression methods are shown in Table 2.

The average concentration of rhodium in UP water samples was not significantly different from zero. To estimate the limit of detection (LOD), UP water was spiked with 1.0 ng/L of rhodium(III) and analysed by weighted, linear, least-square regression (five independent repetitions of the spiked water as such, as well as after seven standard additions, up to a final rhodium concentration in the cell equal to 7.94 ng/L). The result was LOD= $(3.3 \cdot s_{y/x})/b$ = 0.4 ng/L, where $s_{y/x}$ is the residual standard deviation and b the slope of the regression line. This approach, detailed in a recent review (DESIMONI *et al.*, 2004b) limits the probability of both false positive and false negative errors at 5.0%. The result seems to be in good agreement with that reported by Metrohm (0.5 ng/L in the Application note Metrohm AB 220/3e, where no information is given about the adopted approach).

Analysis of lentil sub-samples

Rh-contaminated lentils were grown in-house after preliminary tests failed to detect rhodium in some real samples (tap-water, white wine, skimmed milk). Mineralised solutions of all lentil sub-samples were independently analysed in duplicate by the standard addition method (mineralised sub-sample as such, as well as after four standard additions). The lentils soaked in a solution of 5.0 mg/L rhodium were not analysed because they did not germinate at all. Fig. 2 shows the voltammograms relevant to the analysis of the sample grown on 100 µg/L rhodium. The over-

Table 2 - Comparison of the results obtained by analysing UP water by ordinary, linear, least-square regression and by weighted, linear, least-square regression.

Regression method	Linear least-square	Weighted least square
degrees of freedom	38	38
$s_{y/x}$	18.4703	10.6455
r^2	0.9919	0.9952
slope/std. dev.	87.5363/1.2862	87.3550/0.9862
Intercept/std. dev.	-23.2776/6.4543	-22.6035/2.7289
residual sum of square	$1.2964 \cdot 10^3$	$4.3064 \cdot 10^3$
residual mean square	341.1502	113.3256
F statistic	4,669.8780	7,883.9973
$LOD = \frac{3.3 \cdot s_{y/x}}{slope}$	0.6963	0.4022

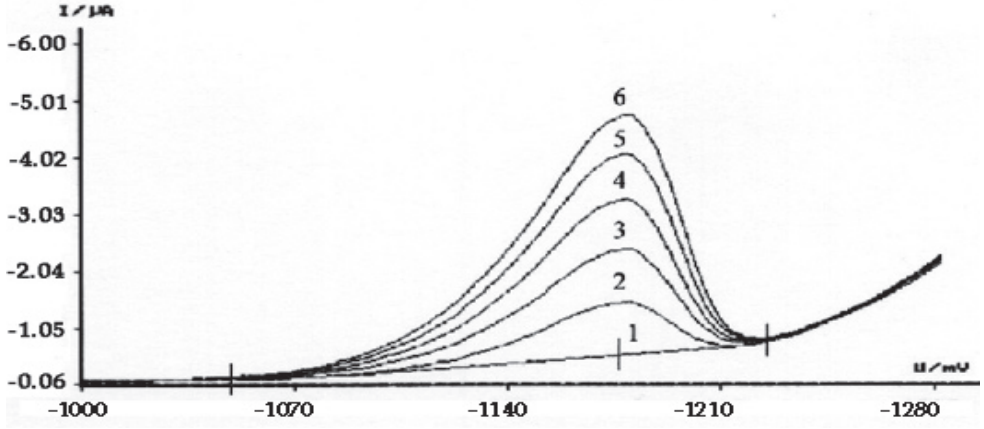

Fig. 2 - CAdSV profiles relevant to the analysis of a diluted mineralised solution of a shoot sub-sample of lentils grown over 100 µg/L rhodium before and after four additions of a standard solution containing 10 µg/L of the metal. 1: background; 2: sample; 3: sample plus 1 addition; 4: sample + 2 additions; 5: sample + 3 additions; 6: sample + 4 additions. Each curve is the average of two repetitions.

all results are summarised in Table 3. Soaking with 1,000 µg/L rhodium solution led to the maximum rhodium uptake by all sub-samples under the experimental conditions used.

As can be seen in Table 3, the rhodium concentration in the control samples was not significantly different from zero, while those in all the other samples increased with the concentration of rhodium in the soaking solution. Moreover, radicles showed the highest rhodium uptake (almost four times higher than in seed coats and shoots). The highest rhodium concentration observed in radicles soaked in aqueous solutions containing 1,000 µg/L rhodium was 19.9±1.7 µg/g dry weight. The observed ratios of the chemical concentration of rhodium in the sub-samples to the concentration in the exposure source are very high (but consider that they refer to dry weight) and suggest that lentils grown on rhodium(III) solutions significantly accumulate rhodium species. A few papers have recently reported results of rhodium bioaccumulation (MOLDOVAN *et al.*, 2001; SURES *et al.*, 2003a, b; ZIMMERMANN *et al.*, 2003,

Table 3 - Results of the analysis of rhodium (ng/g dry weight) in lentil sub-samples. All sub-samples were independently analysed in duplicate by the standard addition method (mineralised sub-sample as such, as well as after four standard additions).

Soaking Rh concentration (µg/L)	Shoots	Radicles	Seed coats
0.0 (control)	0.010±0.012	0.011±0.014	0.0069±0.0078
10.0	0.073±0.054	0.157±0.027	0.1303±0.0090
50.0	0.135±0.043	0.622±0.033	0.228±0.023
100.0	0.694±0.049	2.119±0.090	0.683±0.014
500.0	4.21±0.36	18.1±1.7	5.47±0.37
1,000.0	5.35±0.52	19.9±1.7	6.72±0.30

As suggested by ISO (GUM., 1995) and EURACHEM (QUAM., 2000) experimental results are rounded to the second significant figure of their uncertainty.

2004; EK *et al.*, 2004) and only one paper (SURES *et al.*, 2003b) refers to bioaccumulation factors as high as 1600, but this was in an aquatic biosphere.

In conclusion, the above findings show that the CAdSV method permits a reliable quantification of traces of rhodium in real samples, provided that the experimental results, obtained by the standard addition method, are elaborated by using a weighted, linear, least-square regression. Moreover, real samples containing very high rhodium concentrations can also be analysed by CAdSV with reasonable precision but, of course, after proper dilutions. The above findings confirm that rhodium can enter the food chain at concentration levels that are quite high. Whether this poses some health problems is still under debate (RAVINDRA *et al.*, 2004; ZIMMERMANN and SURES, 2004).

ACKNOWLEDGEMENTS

The work was supported by the Ministero dell'Istruzione, dell'Università e della Ricerca (MIUR, Rome, I).

REFERENCES

Artelt S., Creutzemberg O., Kock H., Levsen K., Nactigall D., Heinrich U., Ruhle T. and Schlogl R. 1999. Bioavailability of fine dispersed platinum as emitted from automotive catalytic converters: a model study. Sci. Total. Environ. 228: 219.

Desimoni E. 1999. A program for the weighted linear least square regression of unbalanced responses arrays. Analyst. 124: 1191

Desimoni E., Brunetti B. and Bacchella R. 2002. Cathodic stripping voltammetric determination of platinum in some foods and beverages at ng/g level under statistical control. Electroanal. 14: 459.

Desimoni E., Brunetti B. and Cattaneo R. 2003. Validation of a procedure for quantifying platinum at sub-mg/L level in matrices of alimentary and environmental concern by catalytic adsorptive stripping voltammetry. J. Phys. IV. 107: 373.

Desimoni E., Brunetti B. and Cattaneo R. 2004a. Validation of some procedures for quantifying platinum at sub-g/L level in some real matrices by catalytic adsorptive stripping voltammetry. Electroanal. 16: 304.

Desimoni E., Brunetti B. and Cattaneo R. 2004b. Comparing some operational approaches to the limit of detection. Annali Chim. (Rome) 94: 555.

Dubey R.K., Bhalotra A., Gupta M.K. and Puri B.K. 1998. Differential pulse polarographic determination of rhodium(III) and ruthenium(III) in synthetic samples after preconcentration of their quinolin-8-olate complexes onto microcrystalline naphthalene. Microchem. J. 361: 103.

Eckardt J.D., Schäfer J., Puchelt H. and Stüben D. 2000. Environmental PGE contamination in Southwest Germany. In "Anthropogenic Platinum Group Element Emissions. Their Impact on Man and Environment". F. Zereini and F. Alt (Ed.) p. 47. Springer, Berlin.

Ek K.H., Morrison G.M. and Rauch S. 2004. Environmental routes for platinum group elements to biological materials - a review. Sci. Total. Environ. 21: 334.

Ensafi A.A. and Zarei K. 1998. Highly selective catalytic determination of ultra trace amounts of rhodium by linear sweep voltammetry. Fresenius J. Anal. Chem. 361: 103.

Farago M.E. and Mullen W.A. 1979. The uptake of platinum group metals by tomato, bean and corn. Inorg. Chim. Acta. 34: 151.

Farago M.E. and Parsons P.J. 1994. The effects of various platinum metal species on the water plant *Eichornia crassipes* (MART.). Chem. Speciation Bioavail. 6/1: 1.

Gebel T. 2000. Toxicology of platinum, palladium, rhodium and their compounds. In "Anthropogenic Platinum Group Element Emissions. Their Impact on Man and Environment". F. Zereini and F. Alt (Ed.) p. 245. Springer, Berlin.

GUM. 1995. "Guide to the Expression of Uncertainty in Measurement". ISO, Geneva, Switzerland.

Helmers E. and Mergel N. 1998. Platinum and rhodium in a polluted environment: studying the emissions of automobile catalysts with emphasis on the application of CSV rhodium analysis. Fresenius J. Anal. Chem. 362: 522.

Hong T., Czae M., Lee C., Kwon Y. and Hong M. 1994. Determination of ultratraces of rhodium by adsorptive stripping voltammetry of formaldehyde complex. Bull. Korean Chem. Soc. 15: 1035.

Hoppstock K., Alt F., Cammann K. and Weber G. 1989. Determination of platinum in biotic and environmental materials. Part II: a sensitive voltammetric method. Fresenius J. Anal. Chem. 335: 813.

Hoppstock K. and Alt F. 2000. Voltammetric determination of ultratrace platinum and rhodium in biological and environmental samples.

In "Anthropogenic Platinum Group Element Emissions. Their Impact on Man and Environment". F. Zereini and F. Alt (Ed.) p. 145. Springer, Berlin.

Hoppstock K. and Sures B. 2004. Platinum-Group Metals. In "Elements and Their Compounds in the Environment". Merian E., Anke M., Ihnat M., Stoeppler M. (Eds.) Wiley-VCH, Weinheim, Germany.

Hutchinson E.J., Farago M.E. and Simpson T.R. 2000. Changes in platinum concentrations in soils and dusts from UK cities. In "Anthropogenic Platinum Group Element Emissions. Their Impact on Man and Environment". F. Zereini and F. Alt (Eds.) p. 57. Springer, Berlin.

Katsaros N. and Anagnostopoulou A. 2002. Rhodium and its compounds as potential agents in cancer treatment. CRC Cr. Rev. Oncol-hem. 42: 297.

Kolpakova N.A., Smyshlyaeva E.A., Tuzikov S.A. and Doroshenko A.V. 2003. Determination of platinum in biological materials by stripping voltammetry. J. Anal. Chem. 58: 303.

Kümmerer K. and Helmers E. 1997. Hospital effluents as a source for platinum in the environment. Sci. Total. Environ. 193: 179.

Leon C., Emons H., Ostapczuk P. and Hoppstock K. 1997. Simultaneous ultratrace determination of platinum and rhodium by cathodic stripping voltammetry. Anal. Chim. Acta. 356: 99.

Lustig S., Zang S., Michalke B., Schramel P. and Beck W. 1997. Platinum determination in nutrient plants by inductively coupled plasma mass spectrometry with special respect to the hafnium oxide interference. Fresenius J. Anal. Chem. 357: 1157.

Lustig S., Michalke B., Beck W. and Schramel P. 1998. Platinum speciation with hyphenated techniques: high performance liquid chromatography and capillary electrophoresis on-line coupled to an inductively coupled plasma-mass spectrometer - application to aqueous extracts from a platinum treated soil. Fresenius J. Anal. Chem. 360: 18.

Lustig S. and Schramel P. 2000. Platinum bioaccumulation in plants and overview of the situation for palladium and rhodium. In "Anthropogenic Platinum Group Element Emissions. Their Impact on Man and Environment". F. Zereini and F. Alt (Eds.) p. 95. Springer, Berlin.

Metrohm Application Bulletin 220/3e. Voltammetric detection of platinum and rhodium in the ultratrace range. Metrohm AG, Herisau, Switzerland.

Moldovan M., Rauch S., Gomez M., Palacios A. and Morrison G.M. 2001. Bioaccumulation of palladium, platinum and rhodium from urban particulates and sediments by the freshwater isopod *Asellus aquaticus*. Water Res. 35: 4175.

Moldovan M., Palacios M.A., Gómez M.M., Morrison G., Rauch S., McLeod C., Ma R., Caroli S., Alimenti A., Petrucci F., Bocca B., Schramel P., Zischka M., Pettersson C., Wass U., Luna M., Saenz J.C. and Santamaria J. 2002. Environmental risk of particulate and soluble platinum group elements released from gasoline and diesel engine catalytic converters. Sci. Total Environ. 296: 199.

NMKL Procedure N. 5. 1997. "Estimation and Expression of Measurement Uncertainty in Chemical Analysis". NMKL Secretariat. Oslo, Norway.

Nygren O., Vaughan G.T., Florence T.M., Morrison G.M.P., Warner L.M. and Dale L.S. 1990. Determination of platinum in blood by adsorptive voltammetry. Anal. Chem. 62: 1637.

Palacios M.A., Moldovan M. and Gomez M.M. 2000. The automobile catalyst as an important source of PGE in the environment. In "Anthropogenic Platinum Group Element Emissions. Their Impact on Man and Environment". F. Zereini and F. Alt (Eds.) p. 3. Springer, Berlin.

QUAM. 2000. "Quantifying Uncertainty in Analytical Measurement". 2nd Ed. EURACHEM Secretariat. Teddington, UK.

Rauch R. and Morrison G.M. 1999. Platinum uptake by the freshwater isopod *Asellus aquaticus* in urban rivers. Sci. Total Environ. 235: 261.

Ravindra K, Bencs L and Van Grieken R. 2004. Platinum group elements in the environment and their health risk. Sci. Total Environ. 318: 1.

Schäfer, J., Hannker, D., Eckhardt J.D. and Stüben D. 1998. Uptake of traffic-related heavy metals and platinum group elements (PGE) by plants. Sci. Total Environ. 215: 59.

Schierl R. 2000. Environmental monitoring of platinum in air and urine. Microchem. J. 67: 245.

Sures B., Zimmermann S., Messerschmidt J. and von Bohlen A. 2003a. Relevance and analysis of traffic-related platinum group metals (Pt, Pd, Rh) in the aquatic biosphere, with emphasis on palladium. Ecotoxicology 11: 385.

Sures B., Zimmermannn S., Sonntag C., Stüben D. and Taraschewki H. 2003b. The acanthocephalan *Paratenuisentis ambiguus* as a sensitive indicator of the precious metals Pt and Rh from automobile catalytic converters. Environ. Pollut. 122: 401.

Vaughan G.T. and Florence T.M. 1992. Platinum in the human diet, blood, hair and excreta. Sci. Total Environ. 111: 47.

Verstraete D., Riondato J., Vercauteren J., Vanhaecke F., Moens L., Dams R. and Verloo M. 1998. Determination of the uptake of $[Pt(NH_3)_4](NO_3)_2$ by grass cultivated on a sandy loam soil and by cucumber plants, grown hydroponically. Sci. Total Environ. 218: 153.

Zhao Z. and Freiser H. 1986. Differential pulse polarographic determination of trace levels of platinum. Anal. Chem. 58: 1498.

Zimmermann S., Messerschmidt J., von Bohlen

A. and Sures B. 2003. Determination of Pt, Pd and Rh in biological samples by electrothermal atomic absorption spectrometry as compared with adsorptive cathodic stripping voltammetry and total-reflection X-ray fluorescence analysis. Anal. Chim. Acta. 498: 93.

Zimmermann S. and Sures B. 2004. Significance of platinum group metals from automobile ehaust gas converters for the biosphere. Environ Sci, Pollut. Res. 11: 194.

Zimmermann S., Baumann U., Taraschewski H. and Sures B. 2004. Accumulation and distribution of platinum and rhodium in the European eel *Anguilla anguilla* following aqueous exposure to metal salts. Environ. Pollut. 127: 195.

Paper received April 15, 2005 Accepted July 15, 2005

SHORT COMMUNICATION

TRIACYLGLYCEROL PROFILE BY HPLC/ELSD AS A DISCRIMINANT PARAMETER OF VARIETAL OLIVE OILS FROM PORTUGAL

PROFILO TRIACILGLICEROLICO MEDIANTE HPLC/ELSD QUALE PARAMETRO DISCRIMINANTE DI OLI DI OLIVA MONOVARIETALI PORTOGHESI

S.C. CUNHA[1], S. CASAL and M.B.P.P. OLIVEIRA*
REQUIMTE, Serviço de Bromatologia, Faculdade de Farmácia da Universidade do Porto, R. Aníbal Cunha 164, 4099-030 Porto, Portugal
[1]I.S.E.I.T-M. Av. 25 de Abril, 5370 Mirandela, Portugal
* Corresponding author: Tel. +351 222 078927, Fax +351 222 003977, e-mail: beatoliv@ff.up.pt

ABSTRACT

An HPLC/ELSD method was used to quantify the triacylglycerols in varietal virgin olive oils from *Cvs*. Verdeal Transmontana, Madural, and Cobrançosa. The triacylglycerol profiles showed that *Cv*. Verdeal Transmontana had the highest values for OOO (66.8%) and POO (23.4%) and the lowest for LOO (3.6%), the major triacylglycerols in the samples studied. Using PCA statistical analysis the oils could be discriminated, showing the potential of this method for characterizing varietal olive oils.

RIASSUNTO

È stata impiegata una metodica HPLC/ELSD per quantificare i triacilgliceroli in oli di oliva vergini monovarietali da *Cv*. Verdeal Transmontana, Madural e Cobrançosa. I profili triacilgliceroli rivelano che la *Cv*. Verdeal Transmontana presenta i valori più elevati in OOO (66.8%) e POO (23.4%) e quelli più bassi in LOO (3.6%) fra le principali specie di triacilgliceroli nei campioni considerati. Gli oli possono essere discriminati utilizzando l'analisi statistica PCA, evidenziando il potenziale di questa metodica per la caratterizzazione monovarietale degli oli di oliva.

- Key words: Authenticity, HPLC/ELSD, olive oil, triacylglycerols -

INTRODUCTION

Olive oil is the principal lipid source of the Mediterranean diet (SACCO *et al.*, 2000) and consumption is increasing mainly due to its potential health benefits (STARK and MADAR, 2002; GIMENO *et al.*, 2002; MANNA *et al.*, 2002).

Because it has a high market price compared to other edible oils, it is a potential target for adulteration (SYNOURI-URETTAKOU *et al.*, 1984; DOWNEY *et al.*, 2002). Characterisation of vegetable oils obtained from different origins and assessment of their identity is a challenge to the food industry and quality control authorities, in order to protect the consumer, verify the authenticity of the products and prevent unfair trade (FIRESTONE and REINA, 1996).

Various attempts have been made to determine the genuineness of olive oil and to detect its adulteration (SYNOURI-URETTAKOU *et al.*, 1984; DOWNEY *et al.*, 2002; SPANGENBERG and OGRINC, 2001; MANNINA *et al.*, 2001). Triacylglycerols (TAGs) are the major components of olive oil and its profile has proved to be important in the detection of olive oil adulteration. Given the importance of the composition of TAG, an accurate and reproducible method that allows them to be simultaneously identified and quantified, and which involves minimal sample preparation and is suitable for routine analysis is therefore desirable. Even though the position of fatty acids in the glycerol backbone is important for biological and nutritional reasons and for complete evaluation of the quali-quantitative composition of the TAG fraction, laborious methodologies are not desirable in quality control (BUCHGRABER *et al.*, 2004). For separating TAGs, several approaches, mainly involving reversed-phase high-performance liquid chromatography (RP-HPLC) and RP-HPLC combined with silver chromatography (Ag-RP-HPLC), have been described (CHOBANOV *et al.*, 1991; DOBSON *et al.*, 1995; CÁRDENAS *et al.*, 1999; APARICIO and APARICIO-RUIZ, 2000; ANDRIKOPOULOS, 2002; BUCHGRABER *et al.*, 2004). Although Ag-RP-HPLC permits the separation of TAGs based solely on the degree of unsaturation, it cannot separate TAGs that differ only in the chain length of their fatty acids. However, in RP-HPLC, the TAGs are separated according to increasing carbon number (CN) and decreasing unsaturation (DB), i.e. according to partition number (PN; PN= CN-2DB), providing better separation of individual TAG molecules (RUIZ-GUTIÉRREZ and BARRON, 1995).

The quantitative analysis of TAGs by RP-HPLC can be performed with several detectors e.g. ultraviolet (UV) (FLOR *et al.*, 1993), refraction index (RI) (PARCERISA *et al.*, 1995) and evaporative light-scattering (ELSD) (AMARAL *et al.*, 2004; CARELLI and CERT, 1993; STOLYHWO *et al.*, 1984). However, the UV detector presents problems with the isomerization and conjugation of double bonds (APARICIO and APARICIO-RUIZ, 2000). The RI seems to be the most appropriate for quantitative analysis, but is not compatible with gradient elution and is sensitive to temperature and pressure changes. An alternative is ELSD, which has the following advantages: a) possible use of gradient elution with improvement in separation; b) it is not affected by small temperature changes; and c) it provides a better signal to-noise ratio (CÁRDENAS *et al.,* 1999). Furthermore, the detector response is proportional to the compound mass, not being limited by their spectral feature (PERONA *et al.*, 2001).

This paper describes an HPLC/ELSD method for the quantification of TAGs applied to varietal olive oils (*Cvs.* Verdeal Transmontana, Madural and Cobrançosa). These cultivars are the most important in the Trás-os-Montes region of Portugal, and are the most significant in quantitative terms of "Trás-os-Montes olive oil", a product with protected designation of origin (PDO) (EU, 1992;

M. D., 1994). Furthermore the commercialisation of varietal olive oils is becoming more common, which also implies knowledge of its chemical characteristics to control unfair trade. This information can also be important in the cultivar composition of new orchards, rejecting the ones with possibly more quality problems. The proposed methodology could be a useful tool to help in these objectives.

MATERIALS AND METHODS

Reagents and standards

Triacylglycerols (TAG): 1,2,3-trilinoleoylglycerol (LLL), 1,2,3-trimyristoylglycerol (MMM), 1,2,3-trioleoylglycerol (OOO), 1,2,3-tripalmitoylglycerol (PPP), 1,2,3-tristearoylglycerol (SSS), 1,2,3-trilinolenoylglycerol (LnLnLn), 1,2,3-triarachidoylglycerol (AAA), 1,2,3-tripalmitoleoylglycerol (PoPoPo), 1,2-dilinoleoyl-3-palmitoyl-rac-glycerol (LLP), 1,2-dilinoleoyl-3-oleoyl-rac-glycerol (LLO), 1,2-dipalmitoyl-3-oleoyl-rac-glycerol (PPO), 1,2-dioleoyl-3-stearoyl-rac-glycerol (OOS), 1-palmitoyl-2-oleoyl-3-linoleoyl-rac-glycerol (POL) and 1,2-dioleoyl-3-palmitoyl-rac-glycerol (OOP) of 98% purity were purchased from Sigma (St. Louis, MO, USA). Acetonitrile and acetone were HPLC grade from Merck (Darmstad, Germany). All the other reagents were analytical grade.

Olive samples preparation

The *Olea europaea* L. cultivars were *Cvs*. Verdeal Transmontana, Madural and Cobrançosa. The trees were identified and carefully marked in an orchard in northeastern Portugal. The olives were handpicked in the four orientations of the trees, at operator height in 2002. After harvest, the fruit was divided into 18 lots (6 for *Cv*. Verdeal Transmontana, 5 for *Cv*. Madural and 7 for *Cv*. Cobrançosa), immediately transported to the pilot extraction plant and processed separately for oil extraction.

An Abencor analyzer (Comercial Abengoa S.A., Sevilla, Spain) was used to process the olives. After being processed, the oils were put into dark glass bottles and stored in the dark at 4°C. Before the analytical determinations the samples were dehydrated with anhydrous sodium sulphate and subsequently filtered through filter paper.

A 0.2 g oil sample was dissolved with 4.0 mL of acetone and homogenised by stirring. The solution was filtered through a 0.22 µm disposable LC filter disk and injected into the chromatographic system.

HPLC and chromatographic conditions

The chromatographic analyses were carried out using a Jasco (Japan) high performance liquid chromatograph, equipped with a quaternary pump (model PU-1580) and an auto sampler (model AS-950) with a 10 µL loop. The detector was an evaporative light scattering detector, ELSD (model 75-Sedere, France). Data were analysed using Borwin-PDA Controller Software (JMBS, France).

The chromatographic separation of TAGs was achieved with a reverse phase Kromasil 100 C18 (5 µm; 250 x 4.6 mm) column (Teknokroma, Spain) operating at ambient temperature (~20°C).

The eluent was a gradient of acetone (A) and acetonitrile (B), at a flow rate of 1 mL/min, with a linear gradient as follows: 0 min 30% B, 20 min 25% B, 35 min 20% B, maintaining these conditions for 20 min and returning to the initial ones within 3 min. Detection was accomplished with an ELS detector programmed with the following settings: evaporator temperature 40°C; air pressure 3.5 bar; and photo multiplier sensitivity 6.

Taking into account the selectivity (α,

relative retention time to the 1,2,3-trioleoylglycerol) peaks were identified according to the logarithms of α *versus* the number of double bonds in the TAG (PARCERISA *et al.*, 1995; HERNÁNDEZ *et al.*, 1991). Quantification was based on the internal normalization method, assuming that the detector response was the same for all compounds.

Statistical analysis

Data are reported as mean ± standard deviation (n=4) and were analysed by ANOVA. Differences were considered significant for $p<0.05$. Data were also processed by principal component analysis (PCA). Bartlett's shericity and Kraiser-Meyer-Olkin tests were used to check that PCA might be applied to data set. PCA was applied under the following conditions: Kaiser`s normalisation and varimax rotation. Statistical analyses were carried out with SPSS for Windows version 11.5 (SPSS Inc. Chicago, IL, U.S.A.).

RESULTS AND DISCUSSION

A mixture of acetone/acetonitrile was chosen, since these solvents are the most widely used as organic modifier and organic base solvent, respectively (RUIZ-GUTIÉRREZ and BARRON, 1995), permitting the best separation of TAGs with the gradient program described. This mobile phase allowed a reduction in the retention times of the compounds with higher CN, achieving simultaneously good peak resolution.

The sensitivity of this method is affected by three instrumental parameters the best conditions being obtained with an evaporator temperature of 40°C, nebulization with an air pressure of 3.5 bar and photo multiplier sensitivity of 6.

In order to obtain reliable PNs for peak identification, TAGs were injected and the positional distribution relative to trioleine was estimated. Fig. 1 shows the chromatogram of the TAG profile of *Cv.* Madural olive oil obtained with the described methodology. Table 1 summarizes the mean values of TAG composition obtained for *Cvs* Verdeal Transmontana, Madural and Cobrançosa and ANOVA results. The main TAGs were OOO, POO, LOO, and SOO. Other minor TAGs, POP, OLP, LLO, SOP, LLP, LnOP, LLL and OOA, were also detected and quantified.

Concerning the main triacylglycerols (OOO, POO, LOO) *Cv.* Verdeal Transmontana had the highest percentages for OOO (66.8%) and POO (23.4%) and the lowest for LOO (3.6%). The levels of these TAGs in *Cv.* Verdeal Transmontana were higher than what has been reported for other Spanish varieties. *Cv.* Verdial has been reported to have 41.0% for OOO (GRACIANI, 1988) and *Cv.* Verdial from Badajoz, mean values below 43.0% for OOO and 30.3% for POO (OSORIO *et al.*, 2003). These results can be explained by the high levels of oleic acid ($C_{18:1}$) in this cultivar (PEREIRA *et al.*, 2002).

Cv Madural oil had the lowest content for OOO (50.5%), POO (20.2%) and SOO (2.2%) but the highest content of LOO (18.4%) compared with the other cultivars studied. The values for OOO and LOO for *Cv.* Cobrançosa oil (57.6% and 9.5%, respectively) are different from the values reported by GOUVEIA (1997) for the same variety grown in southern Portugal (44.1% for OOO and 10.7% for LOO). These differences can be due to the different methodology used but the influence of specific factors related to environmental conditions (soil, climate and cultural practices) and physiological conditions of the olive fruit must not be disregarded (APARICIO and APARICIO-RUIZ, 2000).

The one-way analysis of variance (ANOVA) was used to determine if there were significant differences between the mean scores of each TAG profile of the three cultivars. In general, TAG compo-

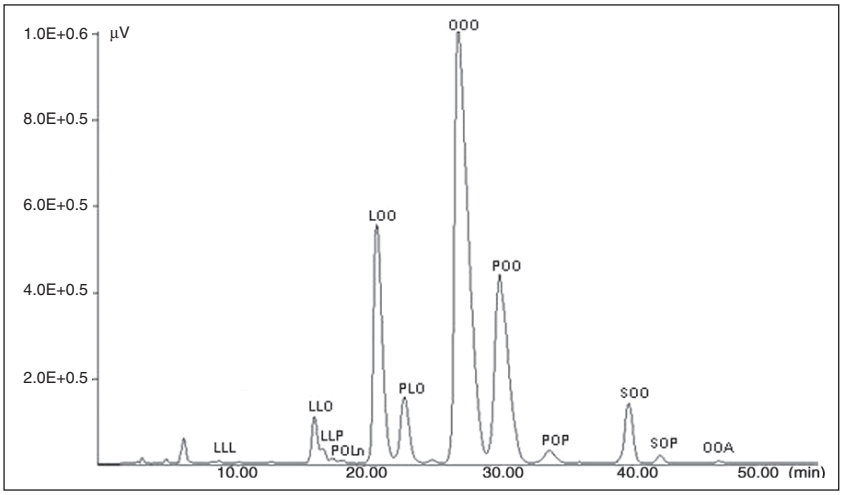

Fig. 1 - Triacylglycerol profile of *Cv* Madural olive oil obtained by HPLC/ELSD.

sitions were normally distributed and homoscedasticity was observed. Significant differences were found for the varietal TAG profiles. Tuke´s post-hoc test was used to find out where such differences were located (Table 1).

PCA was used to compare the TAG compositions of these 3 cultivars. As shown in Table 2, communality among the variables was high, ranging from 0.427 to 0.935. Therefore, PCA could be applied to the results without deterioration or loss of information.

The results of PCA are depicted on a three dimensional plot, which is able to explain 72.36% of total variance. Thus,

Table 1 - Triacylglycerol profile of three varietal olive oils (VD= Verdeal Transmontana; MD= Madural and CB= Cobrançosa) and ANOVA results.

TAG	VD X±SD (n=24)	MD X±SD (n= 20)	CB X±SD (n= 28)	ANOVA results F	p
LLL	0.06±0.16^{a}	0.03±0.01^{a}	0.03±0.02^{a}	0.71	0.496
LLO+PoLO	0.05±0.03^{a}	2.09±0.31^{c}	0.38±0.10^{b}	137.19	0.000
LLP+LnOO	0.42±0.03^{a,b}	0.40±0.15^{a}	0.56±0.20^{b}	4.76	0.013
LnOP	0.07±0.02^{a}	0.76±0.80^{b}	0.05±0.02^{a}	11.02	0.000
LOO+PoOO	3.56±0.15^{a}	18.44±1.23^{c}	9.45±1.43^{b}	126.15	0.000
OLP+PPoO	0.60±0.10^{a}	4.31±0.23^{b}	3.08±2.59^{b}	13.78	0.000
OOO	66.81±0.79^{a}	50.52±0.92^{c}	57.57±2.26^{b}	101.19	0.000
POO+SOL	23.44±0.70^{b}	20.20±1.07^{a}	21.01±1.04^{a}	33.43	0.000
POP	1.08±0.42^{b}	0.83±0.45^{a}	0.68±0.16^{a}	10.23	0.000
SOO+AOL	3.65±0.29^{b}	2.15±0.37^{a}	6.54±0.87^{c}	199.13	0.000
SOP	0.25±0.03^{b}	0.17±0.03^{a}	0.53±0.09^{c}	150.10	0.000
OOA	0.05±0.01^{a}	0.00±0.00^{a}	0.13±0.15^{b}	13.33	0.000

^{a,b,c} Values in the same line with different letters are significantly different at $p<0.05$.

Table 2 - Principal components (PC) rotated loadings for triacylglycerols.

Triacylglycerols	Component			Communalities
	1	2	3	
LLL			0.864	0.837
LLO+PoLO	0.960			0.935
LLP+LnOO		0.374		0.197
LnOP	0.609			0.427
LOO+PoOO	0.952			0.928
OLP+PPoO	0.662			0.552
OOO	-0.903			0.929
POO+SOL	-0.713	-0.532		0.803
POP		-0.708		0.680
SOO+AOL		0.772		0.918
SOP		0.836		0.916
OOA		0.722		0.563

the results were reduced from 12 variables to 3 principal components, with 10.92% loss of variance. The first component (PC1) by itself condensed 35.66% and the second component (PC2) represented almost 25.78% of the total information.

Loading coefficients obtained from the application of PCA to the data are shown in Table 2. Component PC1 was high in LLO, LOO, OLP (positive values) OOO and POO (negative values). Component 2 was high in SOO, SOP, OOA (positive values) and POP (negative values).

Olive oil samples as a function of the first 2 principal components are plotted in Fig. 2. The most important triacylglycerols for the definition of these components are shown on the edges of the axes, following the direction in which TAG values increase, as is conventionally done in PCA.

The differences observed allowed the formation of three groups corresponding to the cultivars in evaluation. PC1 separated *Cvs*. Madural (MD) and Verdeal Transmontana (VD). All MD

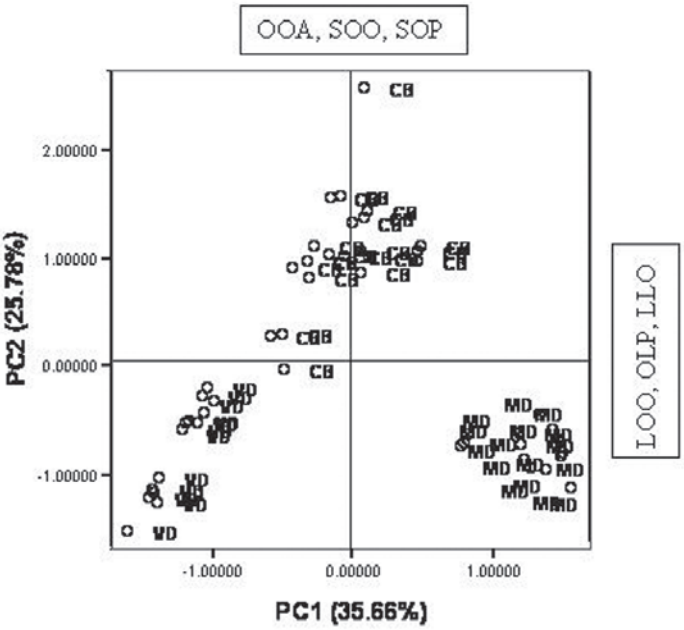

Fig. 2 - Score plot of the 1st and 2nd dimensions from PCA of the data set (Table 1) constituted by olive oil samples from three cultivars (VD = Verdeal Transmontana; MD = Madural and CB = Cobrançosa).

oils were located on the right side of the map showing higher contents of LOO, OLP, and LLO. Samples from VD were located on the left side of the map. They had the highest contents of OOO and POO. The *Cv*. Cobrançosa oils (CB) differed from all the other counterparts on PC2. All CB oils were located at the top of the map as a result of the high contents of SOP, SOO and OOA. The TAG profiles of these cultivars confirm and are in accordance with the fatty acid composition reported by PEREIRA *et al.* (2002).

In conclusion, reversed-phase HPLC using a solvent gradient and ELSD seems to be an appropriate technique for discriminating the olive oil cultivars in relation to the triacylglycerol profile. The comparative study of these profiles revealed: i) significant differences among the cultivars analysed; ii) a higher content of triolein in *Cv*. Verdeal Transmontana than the other two cultivars and some cultivars reported in the literature. The characteristic profiles of varietal olive oils probably contributes to the genuineness of Trás-Os-Montes olive oil. This study must be continued for several crop years to characterize the varietal olive oils from these cultivars.

ACKNOWLEDGMENT

Sara Cunha is grateful to Fundação para a Ciência e Tecnologia (SFRH/BD/8822/2002). The authors are grateful for financial support of Acção 8.1 from PO AGRO n. 482.

REFERENCES

Amaral J.S., Cunha S.C., Alves M.R., Pereira J.A., Seabra R.M. and Oliveira B.P.P. 2004. Triacylglycerol composition of walnut (*Juglans regia* L.) cultivars: characterization by HPLC-ELSD and chemometrics. J. Agric. Food Chem. 52: 7964.

Andrikopoulos N.K. 2002. Chromatographic and spectroscopic methods in the analysis of triacylglycerol species and regiospecific isomers of oils and fats. Crit. Rev. Food Sci. Nut. 42: 743.

Aparicio R. and Aparicio-Ruíz R. 2000. Authentication of vegetable oils by chromatographic techniques. J. Chrom. A. 881: 93.

Buchgraber M., Ulberth F., Emons H. and Anklam E. 2004. Triacylglycerol profiling by using chromatographic techniques. Eur. J. Sci. Technol. 106: 621.

Cárdenas S., Gallego M. and Valcárcel M. 1999. Evaporative light scattering detector: a new tool for screening purposes. Anal. Chem. Acta. 402: 1.

Carelli A.A. and Cert A. 1993. Comparative study of the determination of triacylglycerol in vegetable oils using chromatographic techniques. J. Chromatogr. A. 630: 213.

Chobanov D., Amidzhin B. and Nikolova-Damyanova B. 1991 Densitometric determination of triacylglycerols separated by reversed phase thin-layer chromatography. Application to natural oils. Riv. Ital. Sostanze Grasse 68: 357.

Dobson G., Christie W.W. and Nikolova-Damyanova B. 1995. Silver ion chromatography of lipids and fatty acids. J. Chromatogr. B 671: 197.

Downey G., McIntyre P. and Davies A.N. 2002. Detecting and quantifying sunflower oil adulteration in extra virgin olive oils from the Eastern Mediterranean by visible and Near-Infrared Spectroscopy. J. Agric. Food Chem. 50: 5520.

E.U. 1992. Commission Regulation EC2081/92, July 1992. Official Journal of the European Communities. L 208: 1.

Firestone D. and Reina R.J. 1996. Authenticity of vegetable oils. In "Food Authentication". Ashurt, P.R. and Dennis, M.J. (Eds), p. 198 Chapman & Hall, London.

Flor R.V., Hecking L.T. and Martin B.D. 1993. Elaboración de los criterios sobre cromatografía líquida de alta definición para la clasificación de los aceites de oliva. Ciencia y Tecnica 48: 37.

Gimeno E., Fitó M., Lamuela-Raventós R.M., Castellote A., Covas M., Farré M., Torre-Boronat M.C. and López-Sabater M.C. 2002. Effect of ingestion of virgin olive oil on human low-density lipoprotein composition. Europ. J. Clinical Nutrition 56: 114.

Gouveia J.M.B. 1997. Comparación de los aceites de oliva de los cultivares Cobrançosa, Blanqueta, Azeiteira y Picual con los del Cv. Galega Vulgar, producidos en el Norte del Alentejo. Olivae 66: 34.

Graciani E. 1988. Caracterización del aceite de oliva virgen espanõl. II Posibilidad de caracterización por variedades de aceituna o por zonas de produción de acuerdo con su contenido en triacilgliceroles. Grasas y Aceites 39: 105.

Hernández B., Castellote A. and Permonyer J.J. 1991. Triglyceride analysis of cocoa beans from different geographic origins. J. Food. Chem. 41: 269.

Manna C., D'Angelo S., Migliardi V., Loffredi E., Mazzoni O., Morrica P., Galletti P. and Zappia V. 2002. Protective effect of the phenolic fraction

from virgin olive oils against oxidative stress in human cells. J. Agric. Food Chem. 50: 6521.

Mannina L., Patumi M., Proietti N., Bassi D. and Segre A.L. 2001. Geographical characterization of Italian extra virgin olive oils using High-field ^{1}H NMR Spectroscopy. J. Agric. Food Chem. 49 (6): 2687.

M.D. 1994. Ministerial dispatch n. 34/94 of the Portuguese Government. D.R. II Serie, 3rd February, 28:1036

Osorio E., Sanches J.J., Martinez Cano M. and Montanõ A.M. 2003. Estudio del contenido en triglicéridos de aceites monovarietales elaborados a partir de aceitunas producidas en la región extremenã. Grasas y Aceites 54: 1.

Parcerisa J., Boatella J., Codony R., Rafecas M., Castellote A.I., Gracía J., López A. and Romero A. 1995. Comparison of fatty acid and triacylglycerol composition of different hazelnut varieties (*Corylus avellana* L.) cultivated in Catalonia (Spain). J. Agric. Food Chem. 43: 13.

Pereira J.A., Casal S., Bento A. and Oliveira M.B.P.P. 2002. Influence of Olive storage period on quality of three Portuguese cultivars of *Olea europaea*, Cobrançosa, Madural, and Verdeal Transmontana. J. Agric. Food Chem. 50: 6335.

Perona J.S., Barrón L.J.R. and Ruiz-Gutiérrez V. 2001. Determination of rat liver triglycerides by gas-liquid chromatography and reversed-phase high-performance liquid chromatography. J. Chromatogr. A 706: 173.

Ruiz-Gutiérrez V. and Barron L.J.R. 1995. Methods for the analysis of triacylglycerols. J. Chrom. B 671: 133.

Sacco A., Brescia M.A., Liuzzi V., Reniero F., Guillou C., Ghelli S. and Meer P. 2000. Characterization of Italian olive oils based on analytical and nuclear magnetic resonance determinations. J. AOCS 77: 619.

Spangenberg J.E. and Ogrinc N. 2001. Authentication of vegetable oils by bulk and molecular carbon isotope analyses with emphasis on olive oil and pumpkin seed oil. J. Agric. Food Chem. 49: 1534.

Stark A.H. and Madar Z. 2002. Olive oil as functional food: epidemiology and nutritional approaches. Nutrition Rev. 60: 170.

Stolyhwo A., Colin H., Martin M. and Guiochom G. 1984. Study of the qualitative and quantitative properties of the light-scattering detector. J. Chromatogr. A 288: 253.

Synouri-Urettakou S., Komaitis M.E. and Voudouris E.C. 1984. Triglyceride composition of olive oil, cottonseed oil and their mixtures by low temperature crystallization and gas Liquid Chromatography. J. AOCS 61: 1051.

Revised paper received January 26, 2005 Accepted July 18, 2005

SHORT COMMUNICATION

CHEMICAL AND NUTRITIONAL CONSTITUENTS OF SEA BUCKTHORN (*HIPPOPHAE RHAMNOIDES* SSP. *TURKESTANICA*) BERRIES FROM PAKISTAN

COSTITUENTI CHIMICI E NUTRIZIONALI DELLE BACCHE DI OLIVELLO SPINOSO (*HIPPOPHAE RHAMNOIDES* SSP. *TURKESTANICA*) PROVENIENTE DAL PAKISTAN

S.M. SABIR*, H. MAQSOOD, S.D. AHMED, A.H. SHAH and M.Q. KHAN
AJK University, Faculty of Agriculture Rawalakot, 12350 Azad Kashmir, Pakistan
*Corresponding author: Fax +92 05871042826, e-mail: mubashersabir@yahoo.com

ABSTRACT

Sea buckthorn (*Hippophae rhamnoides* L. ssp. *turkestanica*) is a very important multipurpose plant in areas of northern Pakistan. The berry of sea buckthorn is rich in nutrients and medicinal compounds such as vitamins, carotenoids, flavonoids, oil, carbohydrates, organic acids, amino acids and minerals. The chemical composition and nutritive value of sea buckthorn berries from eight populations from different areas of northern Pakistan were compared. The concentra-

RIASSUNTO

L'olivello spinoso (*Hippophae rhamnoides* L. ssp. *turkestanica*) è una pianta polifunzionale molto importante nelle zone del Pakistan del nord. La bacca di olivello spinoso è ricca in nutrienti e composti medicinali quali vitamine, carotenoidi, bioflavonoidi, olii, carboidrati, acidi organici, aminoacidi e sali minerali. Sono state confrontati la composizione chimica e il valore nutrizionale delle bacche di olivello spinoso proveniente da otto differenti aree del nord del Pakistan. La concentrazione di vita-

- Key words: Anthocyanin, *Hippophae rhamnoides* L, minerals, oil content, phytosterol vitamin C -

tion of vitamin C in the berries was 250-333 mg/100 g; the seed oil was 7.69-13.7%, oil in dried pulp was 19.2-29.1% and the pytosterol content of the seed oil was 3.3-5.5%; there were 0.5-25 mg/L anthocyanins in the fruit juice. The potassium (2.8-7.2 g/kg), sodium (0.065-0.8 g/kg), calcium (0.7-1.25 g/kg), magnesium (139-240 mg/kg), iron (40-225 mg/kg) and phosphorus (110-133 mg/kg) contents were high in dried berries. Sea buckthorn berries from Pakistan are a good source of phytonutrients and minerals.

mina C nelle bacche è risultata di 250-333 mg/100 g; l'olio del seme è risultato il 7,69-13,7%, l'olio della polpa essiccata era il 19,2-29,1% e il contenuto in fitosterolo dell'olio del seme era del 3,3-5,5%; gli antociani nel succo erano 0,5-25 mg/L. I contenuti di potassio (2,8-7,2 g/kg), sodio (0,065-0,8 g/kg), calcio (0,7-1,25 g/kg), magnesio (139-240 mg/kg), ferro (40-225 mg/kg) e fosforo (110-133 mg/kg) sono risultati alti nelle bacche essiccate. Le bacche di olivello spinoso del Pakistan sono una valida fonte di nutrienti e sali minerali.

INTRODUCTION

Sea buckthorn is a shrub or small tree of the genus *Hippophae*. The genus belongs to the family Elaeagnaceae which consists of six species and ten sub-species, among which *Hippophae rhamnoides* L., commonly known as sea buckthorn, is the most economically important (RONGSEN, 1992). The only sub-species found in the northern areas of Pakistan is *H. rhamnoides* ssp. *turkestanica*, widely found in Central and Western Asia, including Afghanistan, Tajikistan, Turkmenistan, Uzbekistan, Kirghisistan, the Xinjiang province of China and northern India. It is the only sub-species which can withstand the harsh biophysical conditions characterized by hot arid summers and cold winters (RONGSEN, 1992).

H. rhamnoides fruit contains 60 to 80% juice that is rich in sugar, organic acids, amino acids and vitamins. The vitamin C content is 200-1,500 mg/100 g in the fruit which is 5 to 100 times higher than any other fruit or vegetable (RONGSEN, 1992). The oil content ranges from 1.5-3.5% in fresh fruit and about 9.9-19.5% in seeds (RONGSEN, 1992). Oil from the juice and pulp is rich in palmitic (16:0) and palmitoleic acids (16:1), while the oil from the seed contains the essential fatty acids linoleic (18:2) and linolenic (18:3) acid. MARK and PETER (2004) reported that the pomace of sea buckthorn which is generally considered waste, contains about 15% palmitoleic acid in its oil and may be utilized as a raw material for the production of a palmitoleic acid (*cis*-Δ9-C16) methyl ester concentrate. The oil from the seed and juice also contains vitamin E and carotenoids (BERNATH and FOLDESI, 1992; MA and CUI, 1989).

There are 24 chemical elements in sea buckthorn juice including calcium, magnesium, phosphorous, iron, manganese, sodium, potassium, aluminum and others (ZHANG *et al.*, 1989; TONG *et al.*, 1989). In addition sea buckthorn berries, leaves and bark contain β-sitosterol, tocopherol and many other bioactive compounds (MIRONOV, 1989). NEGI *et al.* (2005) screened the crude extracts of sea buckthorn seeds for antioxidant and antibacterial activity and reported the highest activity for methanol extract.

Many health claims associated with sea buckthorn such as preventive effects against flu, cardiovascular problems, mucosa injuries and skin prob-

lems (XIAO, 1980; LIU *et al.*, 1985) are related to the high contents of nutritive and protective agents. In order to optimize the composition and sensory quality of various sea buckthorn products and to identify the mechanisms behind the physiological effects, the composition of sea buckthorn berries was investigated in detail. The aim of this study was to determine the contents of vitamin C, oil, plant sterols, flavonoids and minerals in sea buckthorn berries growing in different areas of northern Pakistan.

MATERIALS AND METHODS

Berry samples were harvested after full maturation from eight populations of sea buckthorn plants selected from different areas of the Gilgit District (Danyore, Jalalabad, Nagar, Shigar, Murtazabad, Hussain abad, Gilgit City and at the Chinese border). The berries were harvested in mid August, 2004 on three successive days. Five plants from each population were randomly selected for harvesting the fruits. Berries from eight populations of sea buckthorn comprised of 40 replicate samples were subjected to biochemical analysis. The fruits from each selection were collected in separate plastic bags and immediately frozen at -20°C and stored.

The vitamin C content was determined using the phenol indophenol dye method described by AOAC (1984). Ten grams of fresh berries were blended with metaphosphoric-acetic acid-extracting solution. Five millilitres of the filtrate extract were then titrated against the standard indophenol dye to the pink end point. The method was also validated by the indophenol-xylene extraction method reported by ROBINSON and STOTZ (1945). Oil from the berries of different plants was extracted according to standard methods described by AACC (1983). Fruits were dried in an oven at 105°C for 6-12 hours to constant weight. Ten grams of dried sample were extracted for oil in a Soxhlet apparatus (30°-40°C) for 6 h using diethyl ether as solvent. The solvent was removed under vacuum and the residual oil was dried over anhydrous Na_2SO_4. Analytical grade chemicals (Merck, Darmstadt, Germany) were used for extracting the oil.

Sterol estimation was carried out by the Lieberman-Burchard method (SAID *et al.*, 1995). Samples of 1 g of oil were obtained after Soxhlet extraction and diluted with chloroform to 10 mL. Three mL of diluted sample solutions were taken and their absorbance was determined after adding 2 mL of chloroform and Lieberman-Burchard reagent, containing 0.5 mL of sulfuric acid dissolved in 10 mL of acetic anhydride. The Lieberman-Burchard reagent reacted with the sterol to produce a characteristic green color whose absorbance was determined on a UV-VIS spectrophotometer (Shimadzu, Japan) at 640 nm. Ten mg of standard cholesterol were dissolved in 10 mL chloroform. Choles-5-en-3-β-ol was used as a standard whose minimum purity was 95%. The results were obtained using a standard curve which was linear over the concentration range of 0 to 3.0 (mg/mL) for standard cholesterol against absorbance values. The total anthocyanin content in berry juice was determined by the pH differential method described by WROLSTAD (1976). Two dilutions of the sample were prepared, one with potassium chloride buffer, pH 1.0, and the other with sodium acetate buffer, pH 4.5 and equilibrated for 15 minutes. Absorbance of each dilution was measured on a UV-VIS spectrophotometer (Shimadzu, Japan) at 510 and 700 nm (to correct for haze), against a blank cell filled with distilled water. Anthocyanin pigment was calculated as cyanidin-3-glucoside mg/L using the extinction coefficient of 29,600 and molecular weight of 449.2.

The mineral content was determined on 1 g sample of the dried fruit. Ten mL

of the conc. HNO_3 were added to each sample in a digestion flask and allowed to stand overnight. The samples were heated carefully until the production of a brown nitrogen (IV) oxide fume had ceased. The flasks were cooled and 2-4 mL of 70% perchloric acid was added. Heating was continued till the solution became colorless. The solutions were then diluted to 50 mL with distilled water. The extract was then used to estimate the mineral content. Calcium and magnesium contents were determined by titrating the sample against EDTA solution using Mureoxide and Erichrome Black T indicators, respectively, as described by RICHARDS (1969). The phosphorus content was determined following the method of OSER (1976). KH_2PO_4 solution was used as a standard phosphorus solution. Five millilitres of mineral extract were reacted with H_2SO_4, 0.25% ammonium vandate and 5% ammonium molybdate. The inorganic phosphorus reacted with ammonium molybdate to produce a yellow color complex of molybdenum which was estimated at 470 nm on a UV-VIS spectrophotometer (Shimadzu, Japan). The results were obtained using a standard curve which was linear over the concentration range of 0 to 2.5 (mg/mL) for standard phosphorus against absorbance values. The iron contents of the berries was determined by reacting the sample with potassium thiocyanate. Five mL of potassium thiocyanate (1.5 M) and 2 M HCl were added to the 3 mL mineral extract. A red iron complex (FeSCN) was formed which was estimated at 447 nm on a UV-VIS spectrophotometer (Shimadzu, Japan) according to the method described by RANGANNA (1978). The results were obtained using a standard curve which was linear over the concentration range of 0 to 0.3 (mg/mL) for standard iron against absorbance values. The sodium and potassium contents were determined on a flame photometer (Model 410, Corning, New York). Standard solutions of Na and K were prepared and the emission intensity for each standard, blank (deionized water) and sample were determined at 589 nm for Na and 766.5 nm for K. The results were obtained using a standard curve which was linear over the concentration range of 0 to 30 (ppm) for standard potassium against emission intensity.

Statistical analyses

The results of the chemical analyses are expressed as the mean of five determinations (n=5) ±SD.

RESULTS AND DISCUSSION

When the populations of sea buckthorn were compared based on the contents of vitamin C, fatty oil, phytosterol, minerals and anthocyanins, there was a wide range of variation.

The vitamin C content ranged from 250 to 333 mg/100 g (Table 1). The highest vitamin C content was found in SBT-1, while, the lowest was observed in SBT-3. In our previous investigation we reported the vitamin C content of sea buckthorn berries collected from District Skardu and Khaplu (northern areas) to be in the range of 150 to 250 mg/100 g (SABIR *et al.*, 2003). The vitamin C concentration ranged from 28 to 310 mg/100 g of berries in berries of the European subspecies *Rhamnoides* (YAO *et al.*, 1992; ROUSI and AULIN, 1977), from 460 to 1,330 mg/100 g in berries of *Fluviatilis* ssp. (DARMER, 1952) and from 200 to 2,500 mg/100 g in berries of Chinese *Sinensis* ssp. (ZHENG and SONG 1992; YAO *et al.*, 1992). Harvesting time affects the concentration of vitamin C considerably (ANTONIO *et al.*, 2004). The concentration of vitamin C was highly variable among the populations as reported earlier (KARHU *et al.*, 1999) and was lower than that reported in some other studies. YAO *et al.* (1992) reported the vitamin C content

Table 1 - Concentration of phytochemicals in eight populations of Sea buckthorn from Gilgit Pakistan.

Populations	Locality	Color of berries	Vitamin C content of fruit (mg/100 g)	Moisture in berries (%)	Oil content of seed (g/100 g)	*Pulp oil (g/100 g)	Sterol content of seed oil (g/100 g)	Anthocyanin content of fruit juice (mg/L)
SBT-1	Danyore	yellow	333±0.4	24.0±0.2	13.5±0.2	29.1±0.2	5.5±0.01	5.0±0.12
SBT-2	Jalalabad	yellow	330±0.1	30.0±0.12	11.4±0.1	25.5±0.3	5.3±0.17	10.0±0.56
SBT-3	Nagar	orange-red	250±0.54	22.0±0.02	7.7±0.5	21.3±0.4	3.3±0.13	1.4±0.03
SBT-4	Shigar	light-yellow	275±0.3	20.0±0.24	13.7±0.2	26.4±1.1	4.1±0.05	0.5±0.01
SBT-5	Murtazaabad	red	290±0.4	28.0±0.13	8.3±0.3	17.8±0.9	3.9±0.12	25.0±0.12
SBT-6	Hussainabad	red	315±0.28	25.0±0.25	9.3±0.8	19.2±0.1	4.5±0.20	12.5±0.1
SBT-7	Gilgit city	orange	302±0.35	32.0±0.21	11.2±0.6	22.3±0.4	5±0.15	8.31±0.14
SBT-8	China border	yellow	260±0.14	27.0±0.5	12.1±0.1	27.0±1.5	4.2±0.13	15.1±0.3

SBT = sea buckthorn, ± standard deviation (SD);
*pulp oil derived from berries dried to constant weight.

of Chinese *H. rhamnoides* in the range of 460-1,330 mg/100 g. The lower vitamin C concentration in the present investigation could be due to the specific geographical nature of the area where a short growing season prevails (YAO and TIGERSTEDT, 1995).

The oil content in sea buckthorn seeds also varied among the different populations compared (Table 1). Earlier studies reported an oil content of 5.3-15.7% in the seed of *turkestanica* (YANG, 2001). The present investigation reports seed oil in the range of 7.7-13.7% in *turkestanica,* with the maximum amount in the yellow berries of SBT-4, while the minimum amount was found in the orange-red berries of SBT-3. Yellow and orange-yellow fruits have been reported to have higher levels of oil than orange and orange-red fruits (DAIGATIV *et al.*, 1985), which corresponds to our observations. Dried pulp of *turkestanica* was reported to be the richest source of oil, containing 17.8-34% oil (YANG, 2001). In this study the oil content of dried pulp was in the range of 17.8-29.1% (Table 1). The maximum amount of oil was found in SBT-1, while the minimum was in SBT-5. The higher oil concentration of ssp. *turkestanica* may be very important for its use in medicines.

Phytosterols are plant sterols with structures related to cholesterol and when consumed they can lower plasma cholesterol. Since elevated blood cholesterol is one of the well established risk factors for coronary heart disease, the lowering of blood cholesterol could reduce the risk of heart disease (THURNHAM, 1999). Phytosterols are the major constituents of the unsaponifiable fraction of *H. rhamnoides* oils. The major phytosterol in *H. rhamnoides* oil is β-sitosterol, with 5-avenasterol being the second. Other phytosterols are present in relatively minor quantities. Our earlier investigations reported the phytosterol content of pulp oil in the range of 1.3-2% (SABIR *et al.*, 2003). In this study the phytosterol content of seed oil was in the range of 3.3-5.5% (Table 1). The maximum amount of sterol was found in SBT-1, while the minimum was found in SBT-3. Thus, oil from *turkestanica* seed contains more phytosterol than the oil derived from the pulp.

Anthocyanin pigments are responsible for the attractive red, purple and blue colors of many fruits and vegetables.

They may play a role in reducing coronary heart disease (BRIDLE and TIMBERLAKE, 1996) and increasing visual acuity (TIMBERLAKE and HENRY, 1988). They also have antioxidant (WANG *et al.*, 1997) and anticancer properties (KAMEI *et al.*, 1995). Anthocyanins are also used in the food industry as safe and effective food colorants (STRACK and WRAY, 1994). Table 1 shows that the anthocyanin content of sea buckthorn berries ranged from 0.5 to 25 mg/L in berry juice. The maximum amount was estimated in SBT-5, while the minimum was in SBT-4. Red berries (25 mg/L) were found to contain more anthocyanins than yellow (5 mg/L), orange (8.31 mg/L) and light yellow (0.5 mg/L) berries. The anthocyanin content of sea buckthorn berries was less than that in apple (100 mg/L), bilberries (3,200 mg/L), blackberries (3,600 mg/L), black currants (4,000 mg/L), cherries (4,500 mg/L), cranberries (2,000 mg/L) and strawberries (350 mg/L) (TIMBERLAKE, 1980). This is because the color of sea buckthorn is mainly derived from carotenoids.

When the dried sea buckthorn berries from different populations were compared on the basis of mineral content, a wide range of variation was observed (Table 2). These differences could be due to the natural content of elements in the soil, as well to contamination in both soil and air. Potassium was the most abundant element found and this is in line with other studies (CHEN 1988; TONG *et al.*, 1989; ZHANG *et al.*, 1989). The concentrations of potassium (2.8-7.2 g/kg), sodium (0.065-0.8 g/kg), calcium (0.7-1.25 g/kg), magnesium (139-240 mg/kg), iron (40-225 mg/kg) and phosphorus (110-133 mg/kg) were high. KALLIO *et al.* (1999) reported that the Chinese sea buckthorn berries contained potassium (6.44-12.2 g/kg), calcium (0.8-1.48 g/kg), magnesium (0.47-0.73 g/kg), iron (64-282 mg/kg), zinc (8.8-27 mg/kg) and copper (3.8-12 mg/kg).

CONCLUSIONS

The results of the investigation provide data about the contents of various phytochemicals and minerals among eight populations of sea buckthorn. The large amount of seed and pulp oil in the *Turkestanica* ssp. could be of commercial importance for the local community to market their farm produce with additional benefits. Due to the high vitamin C content sea buckthorn berries can be used in making fruit juices and soft drinks. Sea buckthorn oil is a concentrated source of phytosterols that compete with the absorption of cholesterol in the body. The use of sea buckthorn

Table 2 - Concentration of minerals in different populations of sea buckthorn in Gilgit, Pakistan.

Populations	Locality	Color of berries	K (g/kg)	Na (g/kg)	Ca (g/kg)	Mg (mg/kg)	Fe (mg/kg)	P (mg/kg)
SBT-1	Danyore	yellow	7.2±0.15	0.6±0.01	0.8±0.2	240±0.15	225±0.21	131±0.11
SBT-2	Jalalabad	yellow	6.3±1.1	0.5±0.01	1.0±0.5	225±0.2	140±0.14	128±0.04
SBT-3	Nagar	orange-red	3.3±0.17	0.6±0.02	0.98±0.15	139±0.9	120±0.21	133±0.12
SBT-4	Shigar	light-yellow	3.4±0.23	0.4±0.01	0.7±0.03	229.5±0.2	115±0.13	115±0.05
SBT-5	Murtazaabad	red	2.8±0.1	0.8±0.03	1.11±0.16	150±0.31	40±0.05	110±0.12
SBT-6	Hussainabad	red	5.8±0.32	0.08±0.2	1.17±1.1	195±1.32	110±1.2	122±0.2
SBT-7	Gilgit city	orange	6.2±1.9	0.065±0.1	0.98±0.2	202±0.8	150±0.02	119±0.1
SBT-8	China border	yellow	6.44±2.1	0.075±1.2	1.25±0.2	210±1.3	170±1.5	126±0.2

SBT = sea buckthorn, ± standard deviation.

oil could help decrease the incidence of heart disease. The medicinal components of sea buckthorn berries could be a very cheap raw material for the pharmaceutical industries.

REFERENCES

Antonio R., Flavio P. and Macro A. 2004. Changes in sugar, organic acid, flavonol and carotenoid composition during ripening of berries of three sea buckthorn (*Hippophae rhamnoides* L.) cultivars. Eur. Food Res. and Tech. 219:360.

A.A.C.C. 1983. Approved Methods of American Association of Cereal Chemists. The American Association of Cereal Chemist Inc., St. Paul. Minnesota, USA.

A.O.A.C. 1984. Vitamins and Other Nutrients. "Official Methods of Analysis", 14th ed. William S (Ed.) p. 838. Association of Official Analytical Chemists, Virginia.

Bernath J and Foldesi D. 1992. Sea buckthorn (*Hippophae rhamnoides* L.): A promising new medicinal and food crop. J. Herbs Species Med. Plants 1:27.

Bridle P. and Timberlake C.F. 1996. Anthocyanins as natural food colors-selected aspects. Food Chem. 58:103.

Chen T. 1988. Studies of the biochemical composition of *Hippophae* and its quality assessment in Gansu Prpvince. In Hippophae 1:19.

Daigativ D.D., Muratchaeva P.M. and Magomedmirzaev M.M. 1985. Correlation of some fruit characteristics with lipid and tocopherol content in *Hippophae rhamnoides* L. Rastit. Resur. (Russian) 21:283.

Darmer G. 1952. "Der sanddorn als Wild-und Kulturepflaze". p. 89. Leipzig, Germany.

Kamei H., Kojima T., Hasegawa M., Koide T., Umeda T., Yukawa T. and Terabe K. 1995. Suppression of tumor cell growth by anthocyanins *in vitro.* Cancer Invest. 13:590.

Kallio K., Yang B.R., Tahvonen R. and Hakala M. 1999. Composition of sea buckthorn berries of various origins. Proceeding of International Symposium on Sea Buckthorn (*Hippophae rhamnoids* L.) Beijing, China.

Karhu S.T., Ulvinen S.K., Hagg M., Ahvenainen R. and Evers A.M. 1999. Vitamin C: A variable quality factor in sea buckthorn breeding. Agri. Food Quality II: Quality Management of Fruits and Vegetables. p. 360. Royal Society of Chemistry, Cambridge, UK.

Liu B., Wu Zh., Liu W., Kuang Y., Lang P., Wang E., Zhang T., Xu C., Chen L. and Jiang S. 1985. A preliminary research on the effects of sea buckthorn juice on hyperlipeamia and cardiovascular disease. Shanxi Med. Research 2:68.

Ma Z. and Cui Y. 1989. Studies on the fruit character and bio-chemical composition of some forms within the Chinese Sea buckthorn (*H. rhamnoides* ssp. *sinesis*) in Shanxi, China. Proc. Int. Symp. Sea Buckthorn (*H. rhamnoides* L.), p. 106. Xian, China.

Mark R.G. and Peter U.M. 2004. A palmitoleic acid ester concentrate from sea buckthorn pomace. Europ. J. Lipid Sci. Tech. 106:412.

Mironov VA. 1989. Chemical composition of *Hippophae rhamnoides* of different populations of USSR. Int. Symp. Sea Buckthorn (*H. rhamnoides* L.) p. 67. Xian, China.

Negi P.S., Chauhan A.S., Sadia G.A., Rohinishree Y.S. and Ramteke R.S. 2005. Antioxidant and antibacterial activities of various sea buckthorn (*Hippophae rhamnoides* L.) seed extracts. Food Chem. 92:119.

Oser B.L. 1976. "Hawk's Physiological Chemistry". 14th Ed. Tata McGraw-Hill Publishing Co. Ltd. New Dehli, India.

Robinson W.G. and Stotz E. 1990. The indophenol-xylene extraction method for ascorbic acid modification for interfering substances. J. Bio. Chem. 160:217.

Rousi A. and Aulin H. 1977. Ascorbic acid content in relation to ripeness in fruits of six *Hippohae rhamnoides* clones from Pyhäranta, SW Finland. Ann. Agric. Fenn. 16:80.

Richards L.A. 1969. Handbook of Agriculture No. 60, U.S. Department of Agriculture, Washington D.C.

Ranganna S. 1978. "Manual of Analysis of Fruit and Vegetable Products". Tata McGraw-Hill Publishing Co. Ltd. New Dehli, India.

Rongsen A. 1992. "Sea Buckthorn a Multi-Purpose Plant Species for Fragile Mountains". ICIMOD occasional paper No. 20, P.6,18. Kathmandu, Nepal.

Strack D. and Wray V. 1989. Anthocyanins. In "Methods in Plant Biochemistry", Vol. I, Plant Phenolics (P.M. Dey and J.B. Harborne. eds.). Academic Press, San Diego.

Said I.M, Din L.B, Samsudin M.W, Yusoff N.I, Latif A, Mat-Ali R. and Hadi A.H.A. 1995. A Phytochemical survey of Sayap-Kinabalu park, Sabah. In G. Ismail and L.B. Din (Eds.) p. 137. "A Scientific Journey through Borneo". Sayap Kinabalu Park, Sabah. Pelanduk Publications, Malaysia.

Sabir S.M., Ahmed S.D. and Lodhi N. 2003. Morphological and biochemical variation in *Hippophae rhammnoides* ssp. *turketanica*, a multipurpose plant for fragile mountains of Pakistan. S. Afr. J. Botany 69:587.

Tong J., Zhang C., Zhao Z., Yang Y. and Tian K. 1989. The determination of physical-chemical constants and sixteen mineral elements in sea buckthorn raw juice. Intern. Symp. Sea Buckthorn (*H. rhamnoides* L.), p. 132 Xian, China, Oct. 19-23.

Thurnham D.I. 1999. Functional foods: Cholesterol-lowering benefits of plant sterols. Br. J. Nutr. 82:255.

Timberlake C.F. and Henry B.S. 1988. Anthocyanins as natural food colorants. Prog. Clin. Biol. Res. 280:107.

Timberlake C.F. 1980. Anthocyanins, occurrence, extraction and chemistry. Food Chem. 5:69.

Wang H., Cao G. and Prior R.L. 1997. Oxygen radical absorbing capacity of anthocyanins. J. Agric. Food Chem. 45:304.

Wrolstad R.E. 1976. Color and pigment analyses in fruit products. Oregon St. Univ. Agric. Exp. Stn., Bulletin 624:1.

Xiao Z. 1980. Chemical study on the flavonoids in *Hippophae rhamnoides*. Acad. J. Sichuan Med. University 11:174.

Yao Y., Tigerstedt P.M.A. and Joy P. 1992. Variation of vitamin C concentration and character correlation between and within natural Sea buckthorn (*Hippophae rhamnoides* L.) populations. Acta Agric. Scand. 42:12.

Yao Y. and Tigerstedt P.M.A. 1995. Geographical variation of growth rhythm, height and hardiness, and their relations in *Hippophae rhamnoides*. J. Am. Soc. Horticult. Sci. 120:691.

Yang B.R. 2001. Lipophilic components of Sea buckthorn (*Hippophae rhamnoides*) seeds and berries and physiological effects of sea buckthorn oils. PhD dissertation, Turku University, Finland.

Zhang W., Yan J., Duo J., Ren B. and Guo J. 1989. Preliminary study of biochemical constitutes of berry of sea buckthorn growing in Shanxi Province and their changing trend. Intern. Symp. Sea Buckthorn (*H. rhamnoides* L.), p. 96 Xian, China, Oct. 19-23.

Zheng X.W. and Song X.J. 1992. Analysis of the fruit nutrient composition of nine types of sea buckthorn in Liaoning, China. Northern Fruits of China 3:22.

Revised paper received April 27, 2005 Accepted June 13, 2005

SHORT COMMUNICATION

AROMA CHARACTERISATION OF WHITE TRUFFLE BY GC-MS AND GC-O

CARATTERIZZAZIONE DELL'AROMA DEL TARTUFO BIANCO CON GC-MS E GC-O

M. PILONI, L. TAT, A. TONIZZO and F. BATTISTUTTA
Dip. Scienze degli Alimenti, Università degli Studi di Udine,
Via Marangoni 97, 33100 Udine, Italy
Tel. +39 0432 590738, Fax +39 0432 590719, e-mail: massimiliano.piloni@uniud.it;
franco.battistutta@uniud.it; lara.tat@uniud.it

ABSTRACT

Headspace analysis of white truffle (*Tuber magnatum* Pico) using solid-phase micro extraction (SPME) together with gas chromatography-olfactometry (GC-O) and gas chromatography-mass spectrometry (GC-MS) allowed some typical compounds of these fungi to be identified and characterised. Bis(methylthio)methane and dimethyl sulphide (DMS) were the most important compounds from both a quantitative and qualitative point of view. Dimethyl sulphoxide (DMSO), dime-

RIASSUNTO

L'analisi dello spazio di testa del tartufo bianco (*Tuber magnatum* Pico), con la tecnica di microestrazione in fase solida (SPME) abbinata all'analisi gascromatografica-olfattometrica (GC-O) ed alla gascromatografia-spettrometria di massa (GC-MS), ha permesso l'identificazione e la caratterizzazione di alcuni composti tipici di questi funghi. Il bis(metiltio)metano ed il dimetilsolfuro (DMS) erano i composti più importanti sia dal punto di vista quantitativo sia qualitativo. Sono stati anche identificati

- Key Words: Bis(methylthio)methane, dimethyl sulphide, olfactometry, sulphur compounds, *Tuber magnatum* Pico, white truffle -

thyl sulphone and (methylsulfinyl)(methylthio)methane were identified for the first time in white truffle. The results of headspace analysis of fresh and stored white truffles showed very important changes in the aroma profile, with the formation or increase of several compounds including alcohols and acids.

nuovi composti solforati nell'aroma del tartufo bianco, tra cui il dimetilsolfossido (DMSO) il dimetilsolfone ed il (metilsulfinil)(metiltio)metano. L'analisi dello spazio di testa del tartufo fresco e dopo un breve periodo di conservazione, ha mostrato importanti cambiamenti del profilo dell'aroma, con la formazione o l'incremento di diversi composti come acidi ed alcoli.

INTRODUCTION

White truffle (*Tuber magnatum* Pico), a prized Italian product, which is also widespread in some regions of southern France, typically matures from October to the end of December. The tuber carpophore has a smooth, thin peridium (skin) and a hazel-brown, sometimes reddish, gleba (pulp) with thin white veins. White truffle was first analysed by FIECCHI *et al.* (1967), who indicated that bis(methylthio)methane is the most important component of the aroma (POLESELLO *et al.*, 1989; BIANCO *et al.*, 1988). Later, other sulphur compounds were identified in the aroma, including dimethyl sulphide (DMS), a substance which is very common in nature, dimethyl disulphide (DMDS), dimethyl trisulphide (DMTS), tris(methylthio)methane and methyl(methylthio)methyl disulphide (PELUSIO *et al.*, 1995; POLESELLO *et al.*, 1989). PELUSIO *et al.* (1995) found 1,2,4-trithiolane in some samples of white truffle; this sulphur substance is also present in other kinds of fungi, e.g. *Marasmius alliaceus* (RAPIOR *et al.*, 1997).

Ethanol, 2-methylbutanol, hexanal and butanone were found to be important, non-sulphur compounds (BELLESIA *et al.*, 1996; PELUSIO *et al.*, 1995); their presence depended essentially on the storage time and storage conditions of the truffles (BELLESIA *et al.*, 1996). Non-sulphur compounds are responsible for a minor part of the aroma in fresh samples but their importance increases over time as a function of storage conditions, while the concentration of bis(methylthio)methane decreases (BELLESIA *et al.*, 1996; BALESTRIERI *et al.*, 1988).

The number of compounds and their abundance in the headspace clearly indicate that sulphur substances play a key role in white truffle aroma. This characteristic makes *T. magnatum* very different from other truffle species, such as *Tuber melanosporum* Vitt. (Black truffle) (TALOU *et al.*, 1987; 1989; DIAZ *et al.*, 2003) *Tuber borchii* Vitt. (BELLESIA *et al.*, 1998; 2001) or *Tuber aestivum* (DIAZ *et al.*, 2002; 2003). Dimethyl sulfoxide (DMSO) is one of the sulphur compounds identified for the first time in white truffle; it is largely used as a solvent in several applications and is industrially produced by DMS oxidation. DMSO is approved by the FDA for the palliative treatment of interstitial cystitis and for limited veterinary use. It cannot be used as a dietary supplement.

The aim of this work was to qualitatively and quantitatively characterise the headspace aroma of fresh and refrigerated white truffles, using the stat-

ic headspace - solid-phase micro extraction (SH-SPME) sampling technique. Gas chromatography-olfactometry analysis was also used to confirm the presence and flavour characteristics of each of the compounds.

MATERIALS AND METHODS

Materials

White truffle samples (*T. magnatum* Pico) were collected from Alba, Italy in November 2002. They were stored at +4°C in sealed jars during the period of analysis.

Headspace sampling

Four truffles, each weighing about 10 g, were placed in a 300 mL glass vial made up of two parts that were joined together, so as to allow the whole tuber to be inserted. Headspace sampling was performed by SPME. The fibres used were 2 cm long and 30 to 50 µm thick (Supelco) and were coated with a layer of divinylbenzene (DVB)/carboxen/polidimethylsiloxane (PDMS). The sampling temperature was +30°C with a 30 min equilibration time. Exposition time of the fibre was 15 min.

Gas Chromatography-Mass Spectrometry (GC-MS)

A VARIAN 3400 gas chromatograph coupled with an ion trap mass spectrometry (SATURN ITMS) (Varian, Palo Alto, CA, USA) was used. The chromatographic column was an EC-WAX from ALLTECH (30 m x 0.25 mm I.D. with a 0.25 µm film thickness). The initial column temperature program was set at 35°C maintained for 3 min and heated to 190°C (maintained for 2 min) at a rate of 3°C/min. Injection was at 250°C in splitless mode (120 s valve closed). Helium was used as the carrier gas, at a flow rate of 1.5 mL/min into the column. The ion trap was operated at 170°C in the electron impact mode. Quantification was performed on the basis of total ion current (TIC), while compounds were identified by comparing the spectra with those in a mass spectrometry library (Wiley and Nist). For some compounds, analytical standards were used to give further confirmation.

Four different truffles were tested at time 0 and after eight days, in order to determine differences in the headspace of truffles stored in sealed jars at +4°C. The same analysis was carried out at intermediate times for each truffle.

Gas Chromatography-Olfactometry (GC-O)

An HRGC Mega 2 Series (Carlo Erba Instruments) was used. Injection and separation conditions were the same as those for the GC-MS analysis. The flow of helium with analytes, coming from the GC column, was split 1:1 between the FID and the sniffing port. A mixture of mostly air and make up gas (nitrogen) also flowed into the olfactometric chamber together with the carrier gas. The operators recorded the perceived odour while pushing and holding a button throughout the perception; they also tried to describe the characteristic note of the odour. The analysis was divided into four parts and the operators took turns at the sniffing port. This turnover was done to avoid tiring of the operators. Three repetitions for each truffle sample were performed on fresh truffles and after 8 days of storage. Only the odours of the compounds detected in each test and identified by all the operators are reported.

RESULTS AND DISCUSSION

Twenty compounds were identified in white truffle headspace (Table 1). Fig. 1

shows a typical total ion current (TIC) chromatogram of white truffle headspace, analysed by the SPMEGC-MS technique. The chromatogram for ion 62, the parent ion of dimethyl sulphide, is also reported.

The results from the SPMEGC-MS analysis showed that the most abundant compounds in the headspace were bis(methylthio)methane, dimethyl sulphide and 2-acetyl-5-methylfuran. In contrast to most other studies, DMS appeared to be an important component of the aroma and, quantitatively, was the second most common compound in the headspace. DMSO, commonly found in coffee, tomatoes and tea leaves (THOMAS *et al.*, 1981), was detected for the first time in these fungi. Traces of dimethyl sulfone, a possible oxidation product of DMSO, were detected in one of the samples. Another newly identified sulphur compound, (methylsulfinyl)(methylthio) -methane, was clearly detected by both GC-MS and GC-O analysis.

The GC-O analysis gave information about the impact of volatile components on the aroma; five sulphur compounds were detected using this technique (Table 1). Dimethyl sulphide odour was described to be like broccoli, cauliflower, truffle, and sulphur; bis(methylthio)methane had a very strong, penetrating sulphurous gas odour, while tris(methylthio)methane odour was described as sulphur and garlic. Among the six other sulphur compounds identified, only DMTS and (methylsulfinyl)(methylthio)methane were detected and described in the GC-O analysis; DMDS was never perceived. These data indicate that bis(methylthio)methane plays a key role in the perceived aroma

Table 1. Mean value (4 samples) of the areas (TIC; % on total) on the first and last day of analysis and odour description for some compounds.

Compound name	N.	Time	Identification*	Day 0	Day 8	Odour
Dimethyl sulphide	1	1:57	1,2,3	11.60	8.44	Broccoli, cauliflower, truffle, sulphur
Ethanol	2	4:01	1,2	0.23	3.22[c]	
Dimethyl disulphide	3	8:07	1	0.08	0.29	
2-methyl-1-propanol	4	9:17	1,2	0.01	0.35[c]	
Limonene	5	13:23	1,2	0.06	0.07	
2-methyl-1-butanol	6	14:13	1,2	0.32	4.52[c]	
Bis(methylthio)methane[a]	7	17:41	1,2,3	41.83	25.11	Sulphur, very strong
Dimethyl trisulphide	8	21:41	1,3	0.05	0.12[b]	Rotten, cooked turnip
1-methoxy-3-methylbenzene	9	24:50	1	0.17	0.12	
Acetic acid	10	25:26	1,2	0.04	0.38[b]	
2-acetyl-5-methylfuran	11	29:28	1	2.38	2.79	
Linalol		29:41	1,2	n.d.	0.04[c]	
Dimethyl sulfoxide	12	29:45	1	0.07	0.03	
3-ethyl-4-methyl-3-penten-2-one	13	31:03	1	0.62	0.15	
3,4-dimethyl-3-hexen-2-one	14	33:07	1	0.10	0.19	
Methyl(methylthio)methyldisulphide		33:42	1	0.03	0.02	
Tris(methylthio)methane	15	41:02	1,3	0.46	0.49	Sulphur, garlic
Dimethyl sulfone		42:53	1	tr.	tr.	
Phenylethyl alcohol	16	43:18	1	0.02	0.26[b]	
(methylsulfinyl)(methylthio)methane	17	49:03	1,3	0.07	0.09	Sulphur

([c,b]) Significant differences between means at day 0 and after 8 days ($p \leq 0.05$ and $p \leq 0.01$ for "b" and "c", respectively).
([a]) Column overloaded and detector saturated.
(*) 1, identification by comparing mass spectra with those of public library; 2, injection of pure compound; 3, odour confirmation by GC-O analysis.

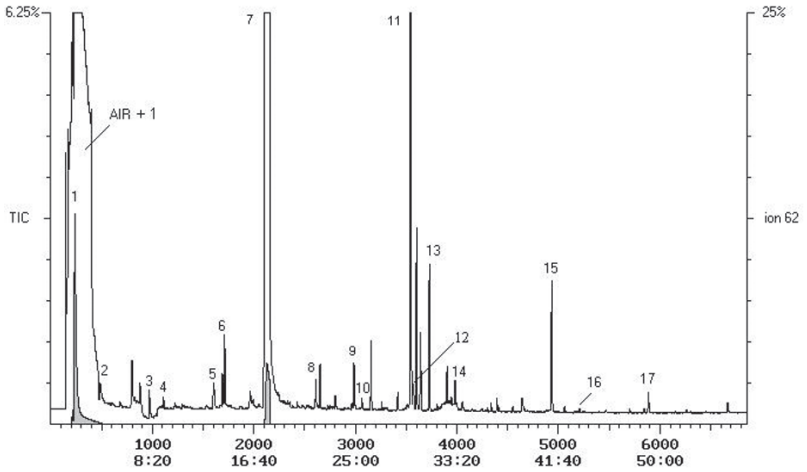

Fig. 1 - SPME GC-MS analysis. White truffle Total Ion Chromatogram (TIC) and ion 62 chromatogram (parent ion of DMS; grey area) superimposed upon one another. Peak numbers correspond to N. identification in Table 1. Sample after 3 days of storage.

and that dimethyl sulphide is also important. The results of the GC-O analysis indicated that the perceived odour of DMS in truffles was less after eight days of storage compared to the fresh sample; the bis(methylthio)methane concentration was still high so no differences could be noted at the sniffing port. This was confirmed by the trend shown in Table 1.

Table 2 - Area value (TIC; % of total) after eight days for a single truffle.

Compound name	Day 0	Day 1	Day 2	Day 3	Day 6	Day 8
Dimethyl sulphide	9.63	8.59	9.54	8.56	3.49	3.15
Ethanol	0.09	0.01	0.02	0.08	1.45	3.36
Dimethyl disulphide	0.09	0.05	0.06	0.06	0.04	0.07
2-methyl-1-propanol	0.02	0.01	0.01	0.00	0.21	0.58
Limonene	0.12	0.03	0.04	0.01	0.01	0.02
2-methyl-1-butanol	0.18	n.d.	0.01	0.01	2.36	4.74
Bis(methylthio)methane[a]	60.29	55.43	57.44	50.19	48.86	47.17
Dimethyl trisulphide	0.08	0.05	0.05	0.09	0.06	0.07
1-methoxy-3-methylbenzene	0.13	0.14	0.13	0.07	0.01	0.09
Acetic acid	0.04	0.03	0.07	0.05	0.19	0.48
2-acetyl-5-methylfuran	1.87	1.79	1.77	1.41	1.67	1.87
Linalol	n.d.	n.d.	n.d.	n.d.	0.04	0.07
Dimethyl sulfoxide	0.07	0.06	0.09	0.09	0.02	0.03
3-ethyl-4-methyl-3-penten-2-one	0.36	0.31	0.29	0.22	0.13	0.11
3,4-dimethyl-3-hexen-2-one	0.08	0.13	0.08	0.10	0.08	0.16
Methyl(methylthio)methyldisulphide	0.01	0.02	0.01	0.01	0.00	0.00
Tris(methylthio)methane	0.37	0.41	0.40	0.23	0.35	0.21
Dimethyl sulfone	tr.	tr.	tr.	tr.	tr.	tr.
Phenylethyl alcohol	0.01	0.01	0.02	0.02	0.13	0.13
(methylsulfinyl)(methylthio)methane	0.06	0.06	0.10	0.07	0.08	0.07

([a]) Column overloaded and detector saturated.

Static truffle headspace analysis was also carried out over eight days, in order to determine the behaviour of the fungi stored in sealed jars at +4°C. Bis(methylthio)methane and dimethyl disulphide decreased during storage, while the alcohols and acid compounds (e.g. ethanol, 2-methyl-1-propanol, 2-methyl-1-buthanol and acetic acid) increased. The other chemical classes did not show similar trends (Tables 1 and 2). Aldehydes, were detected with GC-MS in some samples, but only at low concentrations. The concentrations of these compounds probably increase at a later time and make the product unacceptable. In fact, the results of the GC-O analysis showed only slight changes in the aromatic profile of the truffle headspace between the first and last day of evaluation.

GC-O was useful for confirming the sulphur odour compounds identified by GC-MS. One weakness of this technique however was associated with the characterisation of odours; different concentrations of a given compound, i.e., bis(methylthio)methane, resulted in different responses. For example, when the concentration was increased by SPME, the odour very strong and unpleasant, but the same odour was not detected by sniffing the truffle.

REFERENCES

Balestrieri F., Marini D. and Vietti M.M. 1988. Sulla variazione dell'aroma del *Tuber magnatum* Pico durante il processo di conservazione. Ind. Alimentari 27: 423.

Bellesia F., Pinetti A., Bianchi A. and Tirillini B. 1996. Volatile compounds of the white truffles (*Tuber magnatum* Pico) from middle Italy. Flavour Fragrance J. 11: 239.

Bellesia F., Pinetti A., Bianchi A. and Tirillini B. 1998. The volatile organic compounds of black truffle (*Tuber melanosporum* Vitt.) from middle Italy. Flavour Fragrance J. 13: 56.

Bellesia F., Pinetti A., Tirillini B. and Bianchi A. 2001. Temperature-dependent evolution of volatile organic compounds in *Tuber borchii* from Italy. Flavour Fragrance J. 16: 1.

Bianco L., Marucchi M. and Cossa P. 1988. Aromi naturali ed artificiali nei tartufi e negli alimenti. Ind. Alimentari 27: 518.

Diaz P., Senorans F.J., Reglero G. and Ibanez E. 2002. Truffle aroma analysis by headspace solid phase microextraction. J. Agric. Food Chem. 50: 6468.

Diaz P., Ibanez E., Senorans F.J. and Reglero G. 2003. Truffle aroma characterization by headspace solid-phase microextraction. J. Chromatogr. A 1017: 207.

Fiecchi A., Galli Kienle M., Scala A. and Cabella P. 1967. Bis-methylthiomethane, and odorous substance from white truffle, *Tuber magnatum*, Pico. Tetrahedron Lett. 18: 1681.

Pelusio F., Nilsson T., Montanarella L., Tilio R., Larsen B., Facchetti S. and Madsen J. Ø. 1995. Headspace solid-phase microextraction analysis of volatile organic sulfur compounds in black and white truffle aroma. J. Agric. Food Chem. 43: 2138.

Polesello A., Di Cesare L.F. and Nani R. 1989. Recupero degli aromi dagli ortofrutticoli mediante estrazione in fase solida. Ind. Bevande 18: 93

Rapior S., Breheret S., Talou T. and Marie B.J. 1997. Volatile flavour constituents of fresh *Marasmius alliaceus* (Garlic *Marasmius*). J. Agric. Food Chem. 45: 820.

Talou T., Delmas M. and Gaset A. 1987. Principal constituent of black truffle (*Tuber melanosporum*) aroma. J. Agric. Food Chem. 35: 774.

Talou T., Delmas M. and Gaset A. 1989. The volatile components of tinned black perigord truffles *Tuber melanosporum* Vitt. Flavour Fragrance J. 4: 109.

Thomas W.P., Howard J.D. and Homer B.L. 1981. Natural occurring levels of dimethyl sulfoxide in selected fruits, vegetables, grains, and beverages. J. Agric. Food Chem. 29: 1089.

Paper received January 31, 2005 Accepted July 7, 2005

A SURVEY OF THE MICROBIOLOGICAL QUALITY OF SOME FOOD CATERING SERVICES IN GREECE

LA QUALITÀ MICROBIOLOGICA NEGLI ALIMENTI DA CATERING IN GRECIA

E.H. DROSINOS*, M. GIALITAKI, S. PARAMITHIOTIS and J. METAXOPOULOS

Laboratory of Food Quality Control and Hygiene, Department of Food Science and Technology, Agricultural University of Athens, Iera Odos 75, 11855 Athens, Greece

*Corresponding author: Tel. +30 210 5294713, Fax +30 210 5294683, e-mail: ehd@aua.gr

ABSTRACT

The microbiological quality of three catering services was assessed. Food samples (108) and samples (12) from surfaces and personnel in contact with the foodstuff were collected and divided into four categories. Results indicate that the meals supplied could be considered of satisfactory microbiological quality. The catering services seemed to comply with the instructions given, as far as the hygienic conditions of their facilities and personnel were concerned, but at times a lack of aware-

RIASSUNTO

È stata valutata la qualità microbiologica di tre servizi da catering. 108 campioni di alimenti e 12 campioni prelevati dalle superfici e dal personale a contatto con gli alimenti sono stati raccolti e suddivisi in quattro categorie. I risultati indicano che gli alimenti forniti possono essere considerati di soddisfacente qualità microbiologica. I servizi da catering sono risultati in linea con i capitolati di fornitura per quanto riguarda le condizioni igienico-sanitarie degli impianti e del personale; al tem-

- Key words: Catering services, GHP, HACCP, microbiological quality -

ness in food preparation was evident, leading to a variation in the microbiological quality. Close attention should be given to milk products, especially trimmed cheese and the cheese used as a filling in cheese-pies, salads and some cooked foodstuff like spaghetti and rice-containing goods.

po stesso è risultato evidente che una mancanza di accuratezza nella preparazione degli alimenti stessi comporta una variazione della qualità microbiologica. Maggiore attenzione dovrebbe essere riservata ai prodotti lattiero-caseari, in modo particolare al formaggio cubettato, a quello impiegato nella preparazione di torte al formaggio, di insalate e di alcuni alimenti cotti come spaghetti e preparati a base di riso.

INTRODUCTION

The increasing rhythm of modern life has reduced the amount of time for eating. This rapidly escalating trend has given rise to numerous small catering businesses that provide small ready-to-eat meals. Foodborne disease outbreaks are also increasing throughout the world and have become the most widespread health problem (WHO, 1984). It is estimated that in the UK up to 9.4 million people a year suffer from intestinal illness with 100-200 deaths (WALKER *et al.*, 2003). In 1992 in Sweden, there were more than 5000 reported cases of salmonellosis alone (EUFIC, 2005). In the United States, each year an estimated 76,000,000 people experience a foodborne infection, of which 5,000 incidents are lethal (TAUXE, 2002). In Greece a total of 2,244 cases of foodborne diseases were reported by the district health authorities and the hospital microbiological laboratories in 2000. Salmonellosis accounted for 40% of the reported cases, followed by brucellosis (24%) and campylobacteriosis (12%) (WHO, 2000). As a result, the annual costs related to these infections account for a substantial amount of financial resources not only in direct medical expences but also in lost productivity.

The prevention of foodborne infections requires effort throughout the food production chain and continuous consumer updating. It is thus imperative that food preparation or manufacturing facilities adhere to hygienic measures by following good hygienic practices (GHP), good manufacturing practices (GMP) and stringently implement hazard analysis critical control point (HACCP) throughout the whole food chain (PANISELLO *et al.*, 2000; LEGNANI *et al.*, 2004; FRIEDHOFF *et al.*, 2005), not only to ensure the hygienic quality of the food produced but also to eliminate the possibility of recontamination (REIJ and DEN AANTREKKER, 2004).

In this study, the microbiological quality of one medium-size enterprise supplying ready-to-eat meals (Service B) and two micro-size enterprises supplying milk products (Service A) and dining services (Service C) was examined. Classification of the enterprises was based on their staff headcount, annual turnover and annual balance sheet, as specified by the European Community (E.C., 2005).

MATERIALS AND METHODS

Sampling

Sampling took place randomly over a period of six months without advance no-

tification. Food samples were collected aseptically from the sales point, put into sterile plastic bags and transported to the laboratory in an insulated and refrigerated box. The microbiological examinations were performed the same day. Surfaces (dishes, glasses and benches) in contact with the food and personnel involved in its preparation were examined in place using the swab-rinse technique. A sterile swab was rubbed for 20 s over an area of 100 cm^2, in the case of surfaces and over the whole surface of the hand in the case of the personnel. The swab was then placed in a test tube containing a known volume of sterile saline and transferred to the laboratory.

Microbiological analysis

Representative samples (10 g) were aseptically homogenized with 90 mL sterile Ringer solution (Merck, Darmstadt, Germany), using a Stomacher apparatus (Seward Medical, London, UK) and serially diluted with the same diluent. Pour plating technique was performed by mixing 1 mL of the appropriately diluted sample with molten media. Surface spreading technique was performed by spreading 0.1 mL of the appropriately diluted sample on the surface of the media. In all cases, duplicate plates were prepared. Total aerobic count was estimated by spreading on Plate Count agar (Merck) and incubating at 30°C for 48 hours. Total coliforms and *E. coli* were determined and distinguished by pouring in Chromocult® agar (Merck), according to the manufacturer's instructions, and incubated at 35°C for 24 hours. *S. aureus* determination was carried out by spreading 0.1 mL on Baird Parker selective agar (Merck) and incubation at 35°C for 24-48 hours. Sulfur-reducing clostridia were determined by pouring 10 mL aliquots into 20 mL of molten SPS agar (Merck). After solidification, the agar was overlaid with 5 mL of sterile paraffin. Incubation was carried out at 35°C for 24 hours. Qualitative determination of *Listeria* sp. and *Salmonella* sp. was performed as follows: for *Listeria* sp. a pre-enrichment in Fraser broth (Merck) was performed followed by inoculation on Palcam agar (Biolife, Milano, Italy). For *Salmonella* sp., a pre-enrichment step in Salmosyst broth (Merck) was followed by inoculation on XLD agar (Merck), according to the manufacturer's instructions. In the case of the presence of *Listeria* sp. in the sample, the API *Listeria* biochemical test (Biomerieux, Marcy l'Etoile, France) was applied for further characterisation. The presence of *L. monocytogenes* is reported separately. Microbial counts less than the detection limits of the applied methods are conventionally taken as 0 log cfu g^{-1}, or 0 log cfu 100 cm^{-2} in order to assist quantification.

RESULTS

Surface-personnel

The microbiological quality of surfaces in contact with the food is shown in Table 1. The benches used for the food preparation were in excellent microbiological condition since the total aerobic count exhibited the virtual absence of microorganisms. The dishes and glasses examined were in satisfactory microbiological condition with the exception of one dish sample where the total aerobic count reached 4.98 log cfu 10x10 cm^{-2}. The same sample was the only one in which coliforms were detected at a log cfu 10x10 cm^{-2} of 3.27, but with absence of *E. coli*. The hands of the personnel were clean and the total aerobic count did not exceed 1.97 log cfu 10x10 cm^{-2}.

Raw foodstuff

Raw foodstuff can be divided into four categories. Milk products were considered to belong to the first category. The

Table 1 - Microbiological quality of various foodstuffs produced by Service C.

Type of sample	Number of samples	Microbiological determination	Geometric mean	SD	Minimum	Maximum
Swab-samples (surfaces, dishes, glasses, personnel)	12	Total count	1.08	4.39	-[1]	4.98
		Total coliforms	0.21	2.69	-	3.27
		E. coli	-	-	-	-
		S. aureus	-	-	-	-
Oven-baked foodstuff (lamb, chicken, potatoes, T-bones, steaks)	15	Total count	0.24	3.04	-	3.63
		Total coliforms	-	-	-	-
		E. coli	-	-	-	-
		S. aureus	-	-	-	-
		Clostridium sp.	-	-	-	-
		Listeria sp.	absent			
		Salmonella sp.	absent			
Cooked foodstuff (spaghetti, sauce, rice, mange-tout)	14	Total count	1.40	4.84	-	5.34
		Total coliforms	1.08	5.42	-	6.00
		E. coli	0.72	3.62	-	4.20
		S. aureus	-	-	-	-
		Clostridium sp.	-	-	-	-
		Listeria sp.	absent			
		Salmonella sp.	absent			
Raw foodstuff-milk based (trimmed cheese)	4	Total coliforms	4.80	4.57	4.39	5.00
		E. coli	3.90	4.90	3.41	5.23
		S. aureus	-	-	-	-
		Clostridium sp.	-	-	-	-
		Listeria sp.	present in one sample			
		Salmonella sp.	absent			
Raw foodstuff-green salads	7	Total count	6.36	6.95	5.23	7.34
		Total coliforms	4.84	5.58	3.36	6.00
		E. coli	0.46	2.85	-	3.27
		S. aureus	-	-	-	-
		Clostridium sp.	-	-	-	-
		Listeria sp.	present in one sample			
		Salmonella sp.	absent			
Raw foodstuff-combination salads (tzatziki)	3	Total count	4.81	4.91	4.20	5.25
		Total coliforms	2.18	3.29	-	3.54
		E. coli	1.85	2.28	-	2.57
		S. aureus	-	-	-	-
		Clostridium sp.	-	-	-	-
		Listeria sp.	absent			
		Salmonella sp.	absent			

Values expressed as log cfu g^{-1} except for swab-samples were values expressed as log cfu 100 cm^2
[1]: values below the detection limit.

milk products examined were trimmed cheese from Service C, yoghurt, ice cream, various creams and Feta cheese from Service A and cheese pie fillings from Service B. Only feta-cheese supplied by Service A was lacking in total coliforms and pathogens (Table 2). All samples of trimmed cheese from Service C contained significant amounts of coliforms and *E. coli*, reaching 5.00 and 5.23 log cfu g^{-1}, respectively (Table 1), indicating a serious lack in hygienic conditions during its manufacture and/or storage. One sample also contained *Listeria* sp. As far as the yoghurt samples supplied by Service A were concerned (Table 2), coliforms were detected in only one sample (2.6 log cfu g^{-1}). All of the ice-cream samples contained coliforms, but none of the milk-based cream samples did (Table 2). As far as the cheese pie fillings supplied by Service B were concerned, 40% contained coliforms, 20% of them had *E. coli* and one sample had *Salmonella* sp. (Table 3). The second raw foodstuff category examined was meat products supplied by Service B. In all samples the total aerobic count was significant but only one contained coliforms (Table 3). Salads were the third category of raw foodstuffs. All samples of green salads were heavily contaminated in terms of total aerobic count and coliforms, and one sample contained both *E. coli* and *Listeria* sp

Table 2 - Microbiological quality of various foodstuffs produced by Service A.

Type of sample	Number of samples	Microbiological determination	Geometric mean	SD	Minimum	Maximum
Yoghurt	10	Total coliforms	0.26	2.10	-[1]	2.60
		E. coli	-	-	-	-
		S. aureus	-	-	-	-
		Clostridium sp.	-	-	-	-
		Listeria sp.	absent			
		Salmonella sp.	absent			
Ice-cream	4	Total count	4.35	4.25	3.91	4.69
		Total coliforms	3.32	3.60	2.47	3.94
		E. coli	-	-	-	-
		S. aureus	-	-	-	-
		Clostridium sp.	-	-	-	-
		Listeria sp.	absent			
		Salmonella sp.	absent			
Milk-based creams	5	Total count	1.81	4.28	-	4.56
		Total coliforms	-	-	-	-
		E. coli	-	-	-	-
		S. aureus	-	-	-	-
		Clostridium sp.	-	-	-	-
		Listeria sp.	absent			
		Salmonella sp.	absent			
Feta cheese	5	Total coliforms	-	-	-	-
		E. coli	-	-	-	-
		S. aureus	-	-	-	-
		Clostridium sp.	-	-	-	-
		Listeria sp.	absent			
		Salmonella sp.	absent			

Values expressed as log cfu g^{-1};
[1]: values below the detection limit.

(Table 1). On the other hand, combination salads were found to be less contaminated and only one sample had coliforms and *E. coli* 3.54 and 2.57 log cfu g^{-1}, respectively.

The fourth category of raw foodstuff was ready-to-bake goods of a cheese-pie type. An increased total aerobic count was found in the cheese filling. This could explain the presence of coliforms in three samples and *E. coli* in two of them (Table 2).

Cooked foodstuff

The cooked goods that were examined were divided into oven-baked and cooked goods (boiled). The former were in excellent microbiological condition; a total aerobic count of 3.63 log cfu g^{-1} was only found in one steak sample (Table 1). Aerobic counts were found in 35.7% of the cooked goods, i.e. half of the spaghetti and rice-containing samples, one of each being heavily contaminated. Coliforms were present in the same samples, whereas *E. coli* was present in two spaghetti and one rice-containing sample.

DISCUSSION

The results of the examination of three catering services indicate that some points deserve special attention. The microbiological quality of the meals supplied by Service A was good. On the other hand, the variation in the microbiological quality of the edible material seemed to be the most significant

Table 3 - Microbiological quality of various foodstuffs produced by Service B.

Type of sample	Number of samples	Microbiological determination	Geometric mean	SD	Minimum	Maximum
Cheese-pie filling	20	Total coliforms	1.91	5.67	-[1]	6.27
		E. coli	1.06	4.92	-	5.54
		S. aureus	-	-	-	-
		Clostridium sp.	-	-	-	-
		Listeria sp.	absent			
		Salmonella sp.	present in one sample			
Unbaked foodstuff (cheese-pies)	4	Total count	6.44	7.29	4.97	7.62
		Total coliforms	4.40	5.13	2.17	5.47
		E. coli	2.36	4.67	-	5.00
		S. aureus	-	-	-	-
		Clostridium sp.	-	-	-	-
		Listeria sp.	absent			
		Salmonella sp.	absent			
Meat products (ham, frankfurters, bacon)	17	Total count	5.65	7.98	3.00	8.51
		Total coliforms	0.12	1.49	-	2.11
		E. coli	-	-	-	-
		S. aureus	-	-	-	-
		Clostridium sp.	-	-	-	-
		Listeria sp.	absent			
		Salmonella sp.	absent			

Values expressed as log cfu g^{-1};
[1]: values below the detection limit.

aspect of meals supplied by Services B and C particularly in the total microbial count of the meat products (Service B), the total coliform count of the cheese pie fillings, unbaked cheese-pies (Service B) and swab samples (Service C). The microbiological quality of these samples was satisfactory (PHLS, 2000) on the basis of the geometric mean, despite the fact that a few samples were found to be unsatisfactory. Although the personnel of all the enterprises seemed to comply with the instructions given concerning their personal hygiene and the hygienic preparation of edible material, a lack of flexibility and prompt response to problems was evident; this led thus to the above-mentioned variation in the microbiological quality; e.g. a malfunctioning dish washer, that was acknowledged by the personnel during sampling, was responsible for the decrease in the quality of the served meals due to the use of contaminated dishes. Another point was the handling of starchy foodstuffs after cooking. Starch is very susceptible to microbial contamination because it is already gelatinised and thus susceptible to enzymatic attack. Therefore, the mishandling of spaghetti and rice after cooking can lead to coliform and *E. coli* contamination. Attention should be given to the preparation of trimmed cheese and the cheese used as a filling in cheese-pies because many samples were heavily contaminated by both coliforms and *E. coli*. Regarding the preparation of trimmed cheese, if the microbiological quality of the raw material is satisfactory, a more meticulous cleaning of the trimming device would probably provide a safer product. Concerning the cheese-pie fillings, either the selected raw materials were not always of high quality, or they were not stored under proper conditions. The abovementioned weak points could have been eliminated if a proper training program had been provided. Since no personnel-training records were kept by the enterprises, it can be assumed that these problems arose due to unsatisfactory training.

Finally, salad is a special kind of foodstuff, requiring special attention. The variability of raw materials, the fact that it is eaten raw and that large quantities are often exposed to the environment, are factors that could lead to occasional food-borne diseases. Indeed, some of the green salads were found to be heavily contaminated by coliforms and *E. coli* and one sample was even contaminated with *Listeria* sp. Salads should be prepared in such a way that additional contamination is avoided.

The results of the present study indicated that the unsatisfactory training of the personnel involved in the handling of edible material can lead to an unpredictable variability in its microbiological quality. Thus there is an imperative need for an integrated educational program for the employees that should include detailed instructions for the handling of specific types of foods (e.g. starchy foods, salads) as well as procedures for handling any problem that may arise.

REFERENCES

E. C. (European Commission-Enterprise Directorate General). 2005. The New SME definition. User Guide and Model Declaration. Office for Official Publications of the European Commission, Luxemburg, Luxemburg.

EUFIC (The European Food Information Council). 2005. Foodborne Illness: its origins and how to avoid it. (www.eufic.org/pt/safe/safe03.htm).

Friedhoff R.A., Houben A.P.M., Leblanc J.M.J., Beelen J.M.W.M., Jansen J.T. and Mossel D.A.A. 2005. Elaboration of microbiological guidelines as an element of codes of hygienic practices for small and/or less developed businesses to verify compliance with Hazard Analysis Critical Control Point J. Food Prot. 68: 139.

Legnani P., Leoni E., Berveglieri M., Mirolo G. and Alvaro N. 2004. Hygienic control of mass catering establishments, microbiological monitoring of food and equipment. Food Control 15: 205.

Panisello P.J., Rooney R., Quantick P.C. and Stanwell-Smith R. 2000. Application of foodborne disease outbreak data in the development and

maintenance of HACCP systems. Int. J. Food Microbiol., 59: 221.

PHLS 2000. Guidelines for the microbiological quality of some ready-to-eat foods sampled at the point of sale. Commun. Dis. Public Health 3: 163.

Reij M.W. and Den Aantrekker E.D. 2004. Recontamination as a source of pathogens in processed foods. Int. J. Food Microbiol., 91: 1.

Tauxe R.V. 2002. Surveillance and investigation of foodborne diseases; roles for public health in meeting objectives for food safety. Food Control 13: 363.

Walker E., Pritchard C. and Forsythe S. 2003. Hazard analysis critical control point and prerequisite programme implementation in small and medium size food businesses. Food Control 14: 169.

WHO. 1984. The Role of Food Safety in Health and Development. Report of a Joint FAO/WHO Expert Committee on Food Safety WHO Technical Report Series 705. WHO, Geneva.

WHO. 2000. Country Reports: Greece. WHO Surveillance Programme for Control of Foodborne Infections and Intoxications in Europe 8th Report 1999-2000 WHO, Geneva.

Paper received January 17, 2005 Accepted June 16, 2005

AUTHOR INDEX

VOLUME XVII (2005)

SUBJECT INDEX

VOLUME XVII (2005)

CONTENTS OF VOLUME XVII - 2005

GUIDE FOR AUTHORS

ITALIAN JOURNAL OF FOOD SCIENCE - IJFS

1. Manuscript Preparation

(1) Manuscripts must be typed, double-spaced and **two** copies submitted along **with** the computer disk. There should be liberal margins on top, bottom and sides (2.5 cm). English is the official language. Authors who are not fluent in written English must seek help from a person fluent in scientific English. The Assistant Editor reserves the right to make literary corrections and to make suggestions to improve brevity, but **the paper must be revised by a native English speaker** before submission.

Pages and lines on all pages, including those pages for "References" and figure legends, **must be electronically numbered in the left margin**, beginning with number one at the top of the page.

The paper must also be submitted by e-mail or on a digital support (cd-rom or floppy disk). Indicate which word processor was used to generate the file and save the file also in format "Text only", DCA-RTF or ASCII, if you do not have programs for Macintosh; **graphics, pictures and diagrams must be saved at 300 dpi in TIF, JPEG, EPS or PICT formats** (not included in MsWord documents).

(2) Every 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 as a footnote 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. An abstract and title in Italian (corresponding to the English) must also be included.

Keywords. 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, article number or thesis fulfillment may be included.

Units. A list of units particular to the paper may be included.

References. References should be arranged alphabetically, and for the same author should be arranged consecutively by year, typed double-spaced. Each individual citation 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 name and year in

parentheses (only the initials in capital letters). If there are more than two authors, mention the first author and add *et al.*

(3) 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.

(4) Figures must be drawn and saved by separate in TIF, JPEG, EPS or PICT formats (300 dpi resolution). They should be drawn so that on 50% reduction, lines, figures and symbols will be clearly legible and not overcrowded. A photocopy of how the figure should appear must be included. Photographs must be unmounted, glossy prints or slides. All figures must be given Arabic numbers, e.g. Fig. 3, in the text and in the final copy only on the back where the title of the paper, the senior author's surname and the top of the illustration must also be marked; for reviewing procedures, do not include this information in the first submitted copies. Legends for figures must be self-explanatory and should be typed on a separate sheet under "Legends to Figures".

(5) 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 is 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 that 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.

2. Review Policy

Scientific contributions in one of the following forms may be submitted:

Opinions and Reviews - Papers may be sent directly to the Editor-in-Chief who will decide upon publication or articles will be requested directly from the authors by the Editor-in-Chief.

Short Communications and Surveys - They do not need to have the formal organization of a research paper; they will receive priority in publication;

Papers - The paper must follow manuscript preparation.

Short Communications, Surveys and Papers will be subjected to critical review by the referees. Upon receiving papers from authors, the Advisory Board with the Editor-in-Chief will select papers in relationship to innovation and originality and send copies to the referees. A letter stating that the paper has been accepted for refereeing will be sent to the authors. Papers needing revision will be returned to the author, and the author must return the revised manuscript to the Editor-in-Chief, otherwise the paper will be considered as withdrawn. Papers not suitable for publication will be returned to the author with a statement of reasons for rejection.

3. Editorial Policy

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 as

soon as possible, usually within 4 weeks. The identity and the report of the referees are made known to the Editor-in-Chief, but only the anonymous report is routinely sent to the author. If all referees recommend acceptance or rejection, the decision stands. If the opinions of the referees tie, the Editor-in-Chief has the freedom to decide upon acceptance or rejection of the paper. Manuscripts will be edited in the order received and accepted papers will be published as closely as possible in this order. A letter announcing the issue of publication will be sent to the author after the manuscript has been accepted by the Editor-in-Chief. Each paper is accepted with the understanding that it is the sole document under active consideration for publication covering the work reported (it has NOT been previously published, accepted or submitted for publication elsewhere). Upon acceptance of the paper for publication, the author agrees to pay the page charges as published on the first page of each issue. Authors take full responsibility for all opinions stated in their papers and published in this journal.

4. Mailing Instructions

Papers for publication and communications regarding editorial matters should be sent to:
Prof. Paolo Fantozzi or Dr. Mary F. Traynor, F.S.E.
Dipartimento di Scienza degli Alimenti, Università di Perugia, S. Costanzo, I - 06126 Perugia, Italy
E-mail: ijfs@unipg.it or paolofan@unipg.it
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 reguest when the paper is accepted for publication.
The revised proofs must be returned by fax or mail to: Chiriotti Editori - P.O. Box 167 - 10064 Pinerolo (To) - Italy - 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 Ruber Co. Cleveland, OH.

(Bulletin, circular)
Willets C.O. and Hill, C.H. 1976. Maple syrup producers manual Agric. Handbook No. 134, U.S. Dept. of Agriculture, Washington, DC.

(Chapter of book)
Hood L.F. 1982. Current concepts of starch structure. Ch. 13. In "Food Carbohydrates". D.R. Lineback and G.E. Inglett (Ed.), p. 217. AVI Publishing Co., Westport, CT.

(Journal)
Cardello A.V. and Maller O. 1982. Acceptability of water, selected beverages and foods as a function of serving temperature. J. Food Sci. 47: 1549.
IFT Sensory Evaluation Div. 1981a. Sensory evaluation guide for testing food and beverage products. Food Technol. 35 (11): 50.
IFT Sensory Evaluation Div. 1981b. Guidelines for the preparation and review of papers reporting sensory evaluation data. Food Technol. 35(4): 16.

(Non-English reference)
Minguez-Mosquera M.I., Franquelo Camacho A, and Fernandez Diez M.J. 1981. Pastas de pimiento. I. Normalizacion de la medida del color. Grasas y Aceites 33 (1): 1.

(Paper accepted)
Bhowmik S.R. and Hayakawa, K. 1983. Influence of selected thermal processing conditions on steam consumption and on mass average sterilizing values. J. Food Sci. In press.

(Paper presented)
Takeguchi C.A. 1982. Regulatory aspects of food irradiation. Paper No. 8, presented at 42nd Annual Meeting of Inst. of Food Technologists, Las Vegas, NV, June 22-25.

(Patent)
Nezbed R.I. 1974. Amorphous beta lactose for tableting U.S. patent 3,802,911, April 9.

(Secondary source)
Sakata R., Ohso M. and Nagata Y. 1981. Effect of porcine muscle conditions on the color of cooked cured meat. Agric. & Biol. Chem. 45 (9): 2077. (In Food Sci. Technol. Abstr. (1982) 14 (5): 5S877).
Wehrmann K.H. 1961. Apple flavor. Ph. D. thesis. Michigan State Univ., East Lansing. Quoted in Wehrmann, K.H. (1966). "Newer Knowledge of Apple Constitution", p. 141, Academic Press, New York.

(Thesis)
Gejl-Hansen F. 1977. Microstructure and stability of freeze-dried solute containing oil-in-water emulsions. Sc. D. Thesis, Massachusetts Inst. of Technology, Cambridge.

(Unpublished data/letter)
Peleg M. 1982. Unpublished data. Dept. of Food Engineering., Univ. of Massachusetts, Amherst.
Bills D.D. 1982. Private communication. USDA-ARS. Eastern Regional Research Center, Philadelphia, PA.

CONTRIBUTORS

Gratitude is expressed to the following entities for contributing to the realization of the Journal by being supporting subscribers for 2005.

Si ringraziano i seguenti Enti, Ditte ed Istituti per aver voluto contribuire fattivamente alla realizzazione della Rivista, sottoscrivendo un abbonamento sostenitore per il 2005.

ASSOCIATIONS

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

INDUSTRIES

Corial scpa - Foggia	Fax +39-0881-680077
Kraft Foods Italia spa - Milano	Fax +39-02-41337595
Soremartec Italia srl - Alba	Fax +39-0173-313966
Tecnoalimenti scpa - Milano	Fax +39-02-67077405

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 +39-0432-590719

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 € 6.00

ISSN 1120-1770 © 2005

CHIRIOTTI EDITORI sas - 10064 Pinerolo - Italy