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Editor-in-Chief:
Paolo Fantozzi
Dipartimento di Scienze 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

Assistant Editor:
S. Mary F. Traynor, F.S.E.
Dipartimento di Scienze 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:
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PAPER

DISTINGUISHING RAW, PASTEURIZED, UHT-TREATED AND BATH- STERILIZED COMMERCIAL MILKS BY THEIR INTERACTION WITH IMMUNOPROBES AGAINST CASEINS AND WHEY PROTEINS

RICONOSCIMENTO DI LATTE CRUDO, LATTE PASTORIZZATO, LATTE UHT E LATTE STERILIZZATO IN BASE ALL'INTERAZIONE CON IMMUNOSONDE SPECIFICHE PER CASEINA E PROTEINE DEL SIERO

L. FUKAL*, L. KARAMONOVÁ, M. VÍTKOVÁ, P. RAUCH and G.M. WYATT[1]
Department of Biochemistry and Microbiology, Faculty of Food and Biochemical Technology of Prague ICT, Technická 5, 166 28 Prague 6, Czech Republic
[1]Institute of Food Research, Colney Lane, NR4 7UA Norwich, United Kingdom
*Corresponding author: Fax +420 2 2435 5167, e-mail: Ladislav.Fukal@vscht.cz

ABSTRACT

Polyclonal antibodies were raised against ten immunogens (five native and the same five pasteurized milk proteins: α+β-casein, κ-casein, whole casein, α-lactalbumin, and β-lactoglobulin). Indirect competitive enzyme immunoassays with these antibodies were used to assess their ability to distinguish the type of heat treatment that the milk had undergone. Measuring the immunoreactivity of raw milk and commercial heat-treated milks resulted in the finding that the use of a combina-

RIASSUNTO

Dieci diversi immunogeni, cinque proteine native del latte e le stesse cinque proteine ottenute da latte pastorizzato (α+β-caseina, κ-caseina, caseina, α-lattoalbumina e β-lattoglobulina) sono state impiegate per sintetizzare anticorpi policlonali. Gli anticorpi sono stati poi impiegati in saggi immunoenzimatici per verificarne la capacità di distinguere tra diversi tipi di trattamenti termici subiti dal latte. La misura della immunoreattività del latte crudo e di quello termicamente trattato, median-

- Key words: caseins, enzyme immunoassay, heat treatment, milk, whey proteins -

tion of the two most suitable antibodies (against native α-lactalbumin and β-lactoglobulin) allowed significant ($p < 0.001$) categorization of milks as raw, pasteurized, UHT-treated or bath-sterilized.

te la combinazione dei due anticorpi rivelatisi più adatti, ha permesso la significativa distinzione ($p < 0,001$) tra latte crudo, latte pastorizzato, latte UHT e latte sterilizzato.

INTRODUCTION

During processing, cow milk is submitted to various heat-treatments, which are used primarily for milk preservation and shelf-life extension. However, changes in its composition during heating may affect its nutritional, organoleptic and rheological properties. Therefore, for process control or safety regulation purposes, it is important to know the extent of the heat treatment that the milk has undergone.

The extent of thermal processing can be assessed by measuring either the inactivation of indicator indigenous enzymes, or the denaturation of whey proteins, or the increase in products formed during heat treatment, such as furosine and lactulose. Unfortunately, most of these indicators do not allow all kinds of heat treatments to be evaluated. For example, the activity of alkaline phosphatase and lactoperoxidase can be used for temperatures from 65° to 80°C (GRIFFITHS, 1986), while lactulose and furosine are formed in high-heated milks (UHT-milk, sterilized milk), only (CATALDI *et al.*, 1999; RESMINI *et al.*, 1990).

Milk proteins consist of two phases, colloidal aggregates known as casein micelles (consisting α-, β- and κ-casein), which are surrounded by the more soluble, essentially globular whey proteins. The caseins have comparatively little secondary and tertiary structures, but on heating they undergo changes in the ways they associate within the micelles and with the denatured whey proteins. On heating milk above 60°C, the whey proteins increasingly lose their globular conformation, unfold, and become associated with the κ-casein through disulfide bridges or hydrophobic interactions. Thus, in contrast to the native fraction, these denatured whey proteins precipitate together with caseins when the milk pH is lowered to 4.6 (DALGLEISH, 1990; CORREDIG and DALGLEISH, 1999).

For pasteurized milk the most reliable index of thermal damage is the ratio of soluble whey proteins to total whey proteins (PAGLIARINI *et al.*, 1990; RESMINI *et al.*, 1989). Individual whey proteins (α-lactalbumin, β-lactoglobulin, serum albumin, and immunoglobulins) and caseins can be quantitatively determined by several chromatographic and electrophoretic techniques (LAW *et al.*, 1994; MORALES *et al.*, 2000). However, these techniques are unable to give much useful information on changes in protein conformation caused by thermal processing. Alternative techniques, gaining in popularity for quality assurance in food analysis, are immunoassays. They have many advantages in terms of speed, ease of use, and the fact that they do not require very much equipment. They can be programmed either for high sample throughput or single analyses and are increasingly receiving industrial and regulatory approval. Antibodies, also, are a much simpler and cheaper alternative to spectroscopic methods for analysing conformational changes in proteins. From the immunochemical point of view it can be

assumed that depending upon the time/temperature ratios used for heat processing of milk, the protein structure can be modified and thus with the immunochemical approach it should be possible to design antibodies specific for epitopes which appear or disappear during the heating process. Monoclonal antibodies produced against β-lactoglobulin have been used for its epitopic characterization (KAMINOGAWA *et al.*, 1989; VENIEN *et al.*, 1997) and to investigate the denaturation of the β-lactoglobulin molecule during heating (KAMINOGAWA *et al.*, 1989; NEGRONI *et al.*, 1998). Enzyme-linked immunosorbent assay (ELISA) has been developed to quantify the native and heat-denatured form of α-lactalbumin in heat-treated milks (DURANTI *et al.* 1991, JEANSON *et al.* 1999). Severely heated milk samples such as UHT and sterilized milks can be identified with this method.

Recently, changes in immunoreactivity of caseins and whey proteins during model thermal treatment of buffer solutions of protein standards and raw milk have been recognized using ELISAs with polyclonal antibodies (KARASOVÁ *et al.*, 1999; VÍTKOVÁ *et al.*, 2002). The immunoreactivity of milk proteins was shown to be a sensitive indicator of heat-related changes, prompting the investigation in commercial milks, also. This paper reports the use of immunochemical probes to discriminate various categories of heat-treated milk, with a view to establishing analytical references for commercial milk in the Czech Republic.

MATERIALS AND METHODS

Materials

Standards of α-, β-, and κ-caseins, α-lactalbumin (LA), β-lactoglobulin B (LGB), o-phenylendiamine dihydrochloride (OPD) and Tween 20 were purchased from Sigma (St. Louis, USA). Gelatin was obtained from Fluka AG (Buchs, Switzerland). The secondary antibody labelled with enzyme (the conjugate of swine anti-rabbit IgG and peroxidase (SwAR/Px)) was from Sevapharma (Prague, Czech Republic). Microtitration plates Costar 9018 were purchased from Corning Costar Europe (Badhoevedorp, Netherlands). Prosep A columns were from Bioprocessing Technologies (Darhem, UK).

Milk samples

Samples of fresh raw milk were obtained from dairy plants in the Czech Republic. They were stored frozen or freeze-dried at -20°C before ELISA. Heat-treated milk samples were obtained from shops at two different collection times with a one-month difference in the date of production. Milk samples were from seven different producers. Fourteen samples, each, of raw, pasteurized and UHT-treated milk and 5 bath-sterilized milk samples were analyzed. Each sample was analysed 3 times. Heat-treatment conditions used in Czech dairy plants are 72°-84°C / 15-20 sec for pasteurized milk; 137°-138°C / 4-5 sec (above 100°C for 15 s; indirect type of treatment) for UHT-milk; 115°C / 15 min for sterilized milk (in bottles). Commercial milk samples were diluted from 100-times to 100,000-times and analysed immediately by ELISA.

Immunogens

Native α-lactalbumin (LA) and β-lactoglobulin B (LGB) were obtained from Sigma (St. Louis, USA). Native and thermally treated casein fractions (α+β-casein, κ-casein, whole casein) and thermally treated LA and LGB were isolated from unheated fresh raw milk and from pasteurized milk (72°C, 20 sec) using the procedure described by WAL *et al.* (1995). Homogeneity of isolated proteins was checked by SDS-PAGE in 15% gels and ion-exchange chromatography using Fast Flow Q Sepharose

and MQ columns. Pure fractions were freeze dried and stored at -20°C.

Antibodies

Five fractions of native milk proteins (α+β-casein, κ-casein, whole casein, LA and LGB) and the same proteins, but pasteurized, were used as immunogens.

Polyclonal antisera were raised against each of these proteins in New Zealand White rabbits using two different procedures:

a) Against all forms of caseins and pasteurized LA and LGB

A solution (200 µL) of 250 µg of protein in PBS (pH 7.4) was emulsified in 200 µL of complete Freund's adjuvant. This emulsion was administered to the animals subcutaneously in the rear legs. After 21 days a booster injection was given in the same way, but 250 µg of protein were emulsified in an adjuvant containing aluminium hydroxide (160 µL of PBS, 160 µL of Al-span Oil I and 480 µL of Al-Span Oil II). This injection was repeated twice at intervals of 21 days. On day 90, a final injection was given with 500 µg of protein emulsified as described for the booster injections.

b) Against native LA and LGB

The protein emulsified in complete Freund's adjuvant (0.5 mL of PBS pH 7.4 and 0.5 mL of adjuvant) was administered at doses of 100 µg per rabbit by subcutaneous injection. This injection was repeated after 35 days with incomplete Freund's adjuvant. After 23 days a booster injection was given intramuscularly with 200 µg of protein emulsified in 800 µL of Al-Span Oil adjuvant. Finally, after 14 days an intravenous booster was given with 100 µg of protein in PBS.

Animals were bled seven days after the final injection. The IgG fraction from each antiserum was isolated by affinity chromatography on protein A (Prosep A column). Lactose was added as a cryoprotective agent at a ratio 1:1 (w:w) and fractions were lyophilized.

Enzyme immunoassay (ELISA)

For indirect competitive ELISA, the wells of polystyrene microtitration plates were coated overnight at 4°C with 200 µL of the milk protein solution (concentration in the range of 0.2-2.0 µg per mL) in 0.05M carbonate/bicarbonate buffer (pH 9.6). (In all ELISAs, microtitration plates were coated with native milk protein corresponding to the antibody used.) After incubation for 16 h at 5°C the unbound protein was removed and the wells were washed four times with 0.01M PBS (pH 7.4) containing 0.05% Tween 20 (PBS-T). Aliquots (50 µL) of antigen solution in PBS-T containing 1% gelatin (standard solution in the range of 0.5 ng - 500 µg per mL, or diluted milk sample) and 50 µL of antibody solution in the same buffer (the dilution was adjusted for each antibody in the range of 1:2,000 to 1:10,000) were pipetted into coated wells. After incubation for 1 h at 37°C the plates were washed four times with PBS-T, and a conjugate of secondary antibody (swine anti-rabbit IgG) with horseradish peroxidase diluted 1:4,000 in PBS-T containing 1% gelatin was added (100 µL). Plates were then incubated for 1 h at 37°C and washed four times with PBS-T. Then 100 µL of a solution of peroxidase substrate and chromogenic compound (50 mg of o-phenylendiamine in 100 mL of 0.1M citrate/phosphate buffer pH 5.0 containing 0.03% hydrogen peroxide) were added to each well. Following incubation for 15 min at 37°C the enzyme reaction was stopped by pipetting 50 µL of 2M sulfuric acid. The absorbance at 492 nm was measured using a microplate reader (Multiscan MCC/340, Labsystems, Finland). The absorbance was inversely proportional to the amount of antigen in solution.

Standard curve and calculation of results

Sigmoid calibration curves were fitted using the Microsoft Excel computer pro-

gramme with a four-parameter regression function:

$$A = C + \frac{D-C}{1+\exp(-2\cdot(\alpha+\beta\cdot x))} \quad (1)$$

where C is the level of the bottom asymptote, D is the level of the top asymptote, α is the curve characteristic of the position of the linear nonasymptote part from the view of antigen concentration coordinates, β is the curve characteristic of the slope of the linear nonasymptote part, x is the decadal logarithm of antigen concentration and A is the absorbance at 492 nm.

The sigmoid inhibition curves were constructed not only for protein standards but also for all samples tested for immunoreactivity. For these purposes various dilutions of samples in the wide range were used in ELISA. All standards and samples were analysed in triplicate.

From the absorbance measured the corresponding antigen concentration ($c_{50\%}$) was calculated using the equations:

$$\log(c_{50\%}) = \frac{\ln\dfrac{(D-A_{50\%})}{(A_{50\%}-C)} - \alpha}{\beta}$$

where

$$A_{50\%} = \frac{(C+D)}{2} \quad (2)$$

Cross-reactions were calculated as a percentage using the equation:

$$CR\% = \frac{(c_{50\%})_1}{(c_{50\%})_2} \times 100 \quad (3)$$

where $(c_{50\%})_1$ was the concentration at $A_{50\%}$ of the corresponding immunogen and $(c_{50\%})_2$ the concentration at $A_{50\%}$ of the cross-reacting antigen.

Immunoreactivity was calculated as a percentage in the same way:

$$IR(\%) = \frac{(c_{50\%})_S}{(c_{50\%})_T} \times 100 \quad (4)$$

where $(c_{50\%})_S$ was the concentration at $A_{50\%}$ of the standard (reference) sample and $(c_{50\%})_T$ the concentration at $A_{50\%}$ of the treated sample or the sample compared.

The relative standard deviation was less than 12% in intra-assay and less than 9% in inter-assay for the analyte concentration in raw milk close to $c_{50\%}$ (12 replicates; 4 microtitration plates).

RESULTS AND DISCUSSION

Heat-treated milks from different producers were screened. The immunoreactivity of milk proteins in milk samples was determined by ELISA using rabbit polyclonal antibodies raised against ten immunogens – five native proteins and the same five proteins thermally treated (α+β-casein, κ-casein, whole casein, LA and LGB). Fig. 1 illustrates the standard curves of ELISA for κ-casein and LGB. The standard curves for the other monitored milk proteins had a similar shape. Values of $c_{50\%}$ (see equation 2) for individual native and pasteurized protein in ELISA using appropriate antibodies were at α-casein 2.48 and 6.35 μg/mL, β-casein 0.42 and 9.64 μg/mL, κ-casein 0.56 and 0.80 μg/mL, whole casein 4.30 and 4.55 μg/mL, LA 0.11 (0.10) and 0.19 μg/mL, LGB 1.51 (1.81) and 0.72 μg/mL. Cross-reactions of all antibodies against LA and LGB were <0.01% and <0.1% with the other proteins, respectively. ELISAs of caseins were specific without interference from whey proteins (cross-reactions <0.01%), however, with proportional interference between caseins. The most

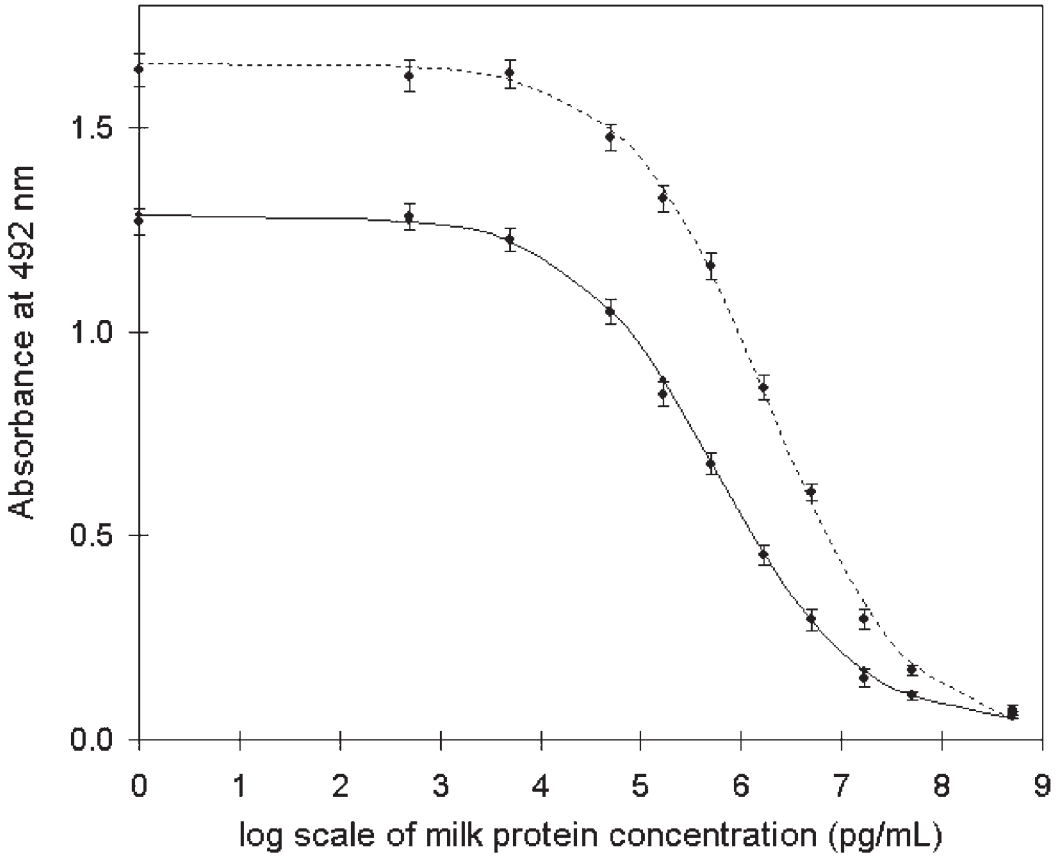


Fig. 1 - Standard curves for determination of native κ-casein (—) and native β-lactoglobulin B (- - - -) by ELISA.

specific was ELISA for κ-casein (cross-reaction of α-casein 15%, and β-casein 2%). Immunoreactivity of fresh raw milk did not significantly change during storage at -20°C for 3 months with any anti-whey-protein antibodies. The same was found for the storage of lyophilized fresh raw milk and anti-casein antibodies. Using all the antibodies the immunoreactivity of different raw milks varied only at a level ≤8%. Thus raw milk with mean values of immunoreactivities was chosen as the reference.

Tables 1 and 2 show differences in the immunoreactivity of commercial milks with respect to the type of heat treatment and the antibody specificity. The immunoreactivities with most of the anti-casein antibodies did not differ significantly. It is well known that caseins have little secondary and tertiary structures compared with whey proteins (ANDREWS *et al.*, 1979; LOUCHEUX-LEFEBVRE *et al.*, 1978). But, on heating they undergo changes including association with denatured whey proteins, removal of phosphate from phosphoserine (BELEC and JENNES, 1962), formation of lysinoalanine (MANSON and CAROLAN, 1980), and reaction of ε-amino groups of lysine with the carbonyl group of lactose (PIZZANO *et al.*, 1998). All of these modifications were not great enough to modify the immunodominant epitopes in

Table 1 - Immunoreactivities (%) of commercial milk samples with respect to raw milk measured by ELISA with anti-casein antibodies.

Analysed milk samples	Antibodies used against					
	α+β-casein	α+β-casein pasteurized	κ-casein	κ-casein pasteurized	whole casein	whole casein pasteurized
raw milk	100±6	100±6	100±7	100±6	100±8	100±8
pasteurized milk	91±9	131±21	164±24	168±25	96±16	136±41
UHT-milk	72±6	115±24	113±27	96±16	105±22	109±29
sterilized milk	41±17	66±24	37±12	41±16	56±15	47±12

Table 2 - Immunoreactivities (%) of commercial milk samples with respect to raw milk measured by ELISA with anti-whey-protein antibodies.

Analysed milk samples	Antibodies used against					
	LA native (1)[a]	LA native (2)[a]	LA pasteurized	LGB native (1)[a]	LGB native (2)[a]	LGB pasteurized
raw milk	100±5	100±6	100±6	100±7	100±5	100±5
pasteurized milk	130±32	100±19	123±22	1,401±312	2,258±841	516±110
UHT-milk	44±22	18±2	44±8	563±138	1,240±611	211±78
sterilized milk	1.4±0.2	1.0±0.1	1.4±0.5	140±21	163±20	67±23

[a] = two rabbits immunized with the same immunogen.

the casein molecules and significantly change their immunoreactivity. Moreover, the range of measured values was too wide for one type of milk and antibody.

On the other hand, the immunoreactivity of most of the milk samples with all anti-whey-protein antibodies differed significantly with respect to the type of heat treatment (Tables 2 and 3). Compared with raw milk, the immunoreactivity of pasteurized milk was slightly higher with all anti-LA antibodies. But this immunoreactivity progressively decreased (5- and

Table 3 - Level of significance in distinguishing commercial milks with different types of heat-treatment using their immunoreactivities (results were calculated using values from Table 2).

Pairs of Milk samples	Antibodies used against					
	LA native (1)[a]	LA native (2)[a]	LA pasteurized	LGB native (1)[a]	LGB native (2)[a]	LGB pasteurized
raw / pasteurized	ns	ns	ns	+++	+++	++
raw / UHT	++	+++	++	++	+++	+
raw / sterilized	+++	+++	+++	ns	+	ns
pasteurized / UHT	+	+++	++	+	ns	+
pasteurized / sterilized	+++	+++	+++	+++	+++	++
UHT / sterilized	+++	+++	+++	++	++	+

ns, +, ++, +++ = non significant or significant at $p \leq 0.05$, 0.01 or 0.001, respectively;
[a] = two rabbits immunized with the same immunogen.

100-times) with UHT processing and in-bottle sterilization. Denaturation of LA during pasteurization probably uncovered epitopes from the inner part of the molecule, while more severe heat processing mainly caused damage to immunodominant epitopes. During sterilization these epitopes were almost completely destroyed. Milk immunoreactivity with anti-LGB antibodies dramatically increased in pasteurized and UHT-milk (up to 22-times) and only minor changes were found in sterilized milk. Probably, slight denaturation caused unfolding of the LGB molecule. This conformational modification induced the appearance of new epitopes at the surface (CASAL *et al.*, 1988; MANDERSON *et al.*, 1999). However, most of these epitopes were damaged by severe heating during sterilization. Therefore, the immunoreactivity decreased to the level of raw milk. KAMINOGAWA *et al.* (1989), VENIEN *et al.* (1997), and NEGRONI *et al.* (1998) described a similar phenomenon. They also obtained antibodies recognizing denatured LGB, while immunization was done with native LGB. The question is, what causes antibodies to be raised against denatured immunogen by immunization with the native one. It may be due to denaturation of part of the native protein during its isolation, during emulsification in Freund's adjuvant, or *in vivo* during the immune response of the immunized animal.

In spite of known differences in the protein content of raw milks (GRAY and MACKENZIE, 1987) and differences in conditions of heat processing in dairy plants, immunoassays can distinguish the type of heat treatment that the milk has undergone. There are at least six immunoprobes that can distinguish all the heat-treated milk samples. Raw, pasteurized, UHT-treated or bath-sterilized milk can be significantly ($p < 0.001$) categorized using combinations of two suitable antibodies (antibody (2) against native LA and antibody (2) against native LGB).

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REFERENCES

Andrews A.L., Atkinson D., Evans M.T.A., Finer E.G., Green J.P., Phillips M.C. and Robertson R.N. 1979. The conformation and aggregation of bovine β-casein A. 1. Molecular aspects of thermal aggregation. Biopolymers 18: 1105.

Belec J. and Jenness R. 1962. Dephosphorization of casein by heat treatment. II. In skim milks. J. Dairy Sci. 45: 20.

Casal H.L., Köler U. and Mantsch H.H. 1988. Structural and conformational changes of β-lactoglobulin B: an infrared spectroscopic study of the effects of pH and temperature. Biochim. Biophys. Acta. 957: 11.

Cataldi T.R.I., Angelotti M. and Bufo S.A. 1999. Method development for the quantitative determination of lactulose in heat-treated milks by HPAEC with pulsed amperometric detection. Anal. Chem. 71: 4919.

Corredig M. and Dalgleish G. 1999. The mechanisms of the heat-induced interaction of whey proteins with casein micelles in milk. Int. Dairy J. 9: 233.

Dalgleish G. 1990. Denaturation and aggregation of serum proteins and caseins in heated milk. J. Agric. Food Chem. 38: 1995.

Duranti M., Carpen A., Iametti S. and Pagani S. 1991. α-Lactalbumin detection in heat-treated milks by competitive ELISA. Milchwissenschaft 46: 230.

Gray R.M. and Mackenzie D.D. 1987. Effect of plane nutrition on the concentration and yield of whey proteins in bovine milk. N. Z. J. Dairy Sci. Technol. 22: 157.

Griffiths M.W. 1986. Use of milk enzymes as indexes of heat-treatment. J. Food Protect. 49: 696.

Jeanson S., Dupont D., Grattard N. and Rolet-Répécaud O. 1999. Characterization of the heat treatment undergone by milk using two inhibition ELISAs for quantification of native and heat denatured α-lactalbumin. J. Agric. Food Chem. 47: 2249.

Kaminogawa S., Shimizu M., Ametani A., Hattori M., Ando O., Hachimura S., Nakamura Y., Totsuka M. and Yamauchi K. 1989. Monoclonal antibodies as probes for monitoring the denaturation process of bovine β-lactoglobulin. Biochim. Biophys. Acta 998: 50.

Karasová L., Rauch P. and Fukal L. 1999. Construction of competitive enzyme immunoassay for determination of α-lactalbumin and β-lactoglobulins of cow's milk. Czech J. Food Sci. 17: 5.

Law A.J.R., Horne D.S., Banks J.M. and Leaver J. 1994. Heat-induced changes in the whey proteins and caseins. Milchwissenschaft 49: 125.

Loucheux-Lefebvre M.H., Aubert J.P. and Jolles P. 1978. Prediction of the conformation of the cow and sheep κ-caseins. Biophys. J. 23: 323.

Manderson G.A., Creamer L.K. and Hardman M.J. 1999. Effect of heat treatment on the circular dichroism spectra of bovine β-lactoglobulin A, B, and C. J. Agric. Food Chem. 47: 4557.

Manson W. and Carolan T. 1980. Formation of lysinoalanine from individual bovine caseins. J. Dairy Res. 47: 193.

Morales F.J., Romero C. and Jiménez-Pérez S. 2000. Characterization of industrial processed milk by analysis of heat-induced changes. Inter. J. Food Sci. Technol. 35: 193.

Negroni L., Bernard H., Clement G., Chatel J.M., Brune P., Frobert Y., Wal J.M. and Grassi J. 1998. Two-site enzyme immunometric assays for determination of native and denatured beta-lactoglobulin. J. Immunol. Meth. 220: 25.

Pagliarini E., Iametti S., Peri C. and Bonomi F. 1990. An analytical approach to the evaluation of heat damage in commercial milks. J. Dairy Sci. 73: 41.

Pizzano R., Nicolai M.A., Siciliano R. and Addeo F. 1998. Specific detection of the Amadori compounds in milk by using polyclonal antibodies raised against a lactosylated peptide. J. Agric. Food Chem. 46: 5373.

Resmini P., Pellegrino L., Hogenboom J.A. and Andreini R. 1989. Thermal denaturation of whey protein in pasteurized milk. Fast evaluation by HPLC. Ital. J. Food Sci. 1: 51.

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. 2: 173.

Venien A., Levieux D., Astier C., Briand L., Chobert J.M. and Haertle T. 1997. Production and epitopic characterization of monoclonal antibodies against bovine β-lactoglobulin. J. Dairy Sci. 80: 1977.

Vítková M., Rauch P. and Fukal L. 2002. Optimization of indirect competitive ELISAs of α-, β-, and κ-caseins for the recognition of thermal and proteolytic treatment of milk and milk products. Czech J. Food Sci. 20: 53.

Wal J.M., Bernard H., Yvon M., Peltre G., David B., Creminon C., Frobert Y. and Grassi J. 1995. Enzyme immunoassay of specific human IgE to purified cows' milk allergens. Food Agric. Immunol. 7: 175.

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PAPER

FATTY ACID PROFILE OF CHEESE PRODUCED WITH MILK FROM COWS GRAZING ON MOUNTAIN PASTURES

SIGNIFICATO DEL PROFILO DEGLI ACIDI GRASSI NELLA CARATTERIZZAZIONE DI FORMAGGI OTTENUTI CON LATTE DI VACCHE ALIMENTATE AL PASCOLO IN MONTAGNA

N. INNOCENTE*, D. PRATURLON and C. CORRADINI
Dipartimento di Scienze degli Alimenti, Università di Udine, Via Marangoni 97, 33100 Udine, Italy
* Corresponding author: Tel. +39 0432 590771, Fax +39 0432 590719, e-mail: Nadia.Innocente@DSA.Uniud.it

ABSTRACT

The fatty acid profile of mountain cheese produced in the summer, when the dairy cows are put out to summer alpine pasture, was compared with that of cheese from cows kept inside cowsheds and fed harvested forage and grain. The mountain cheese was characterised by a higher unsaturated fatty acid content and lower levels of saturated fatty acids with respect to the corresponding cheeses produced in the plains. The unsaturated fatty acid content in the mountain cheese was 44.10% of the total fatty acids and 33.21% of the total fatty acids for the

RIASSUNTO

Il profilo degli acidi grassi del formaggio di montagna prodotto nel periodo estivo, quando il bestiame lattifero viene monticato presso i pascoli d'alta quota, è stato confrontato con quello di un formaggio prodotto da latte di animali mantenuti in stabulazione fissa ed alimentati con foraggi conservati. I formaggi di montagna sono risultati essere caratterizzati da un maggior contenuto in acidi grassi insaturi e da più bassi livelli di acidi grassi saturi rispetto ai corrispettivi formaggi di pianura. Il contenuto in acidi grassi insaturi è risultato del 44,10 e del 33,21% sul totale degli acidi

- Key words: authenticity, fatty acids, low- and highland fodder, milk fat -

cheeses from the plains. Conversely, the saturated fatty acid contents were 53.74% and 63.44% of the total fatty acids for the mountain cheeses and those from the plains, respectively. The C18:1/C16 ratio was always higher in mountain cheese (1.37) than in cheese from the plains (0.85). The c9,t-11 isomer of the C:18 fatty acid was found in appreciably higher amounts in the mountain cheese (1.63% of the total fatty acids measured) compared to that from the plains (0.75% of the total). Indexes useful for identifying the dairy products from the mountain have been specified. Results show that cheese manufactured from milk produced by cows grazing in the mountain pastures was rich in unsaturated fatty acids and the c9,t-11 isomer of conjugated linoleic acid that have been shown to have potential health benefits for humans compared with cheese from milk produced by cows fed stored forage and grain.

grassi rispettivamente per i formaggi di montagna e per i formaggi di pianura; viceversa, per quanto riguarda gli acidi grassi saturi, sono stati determinati un contenuto del 53,74 e del 63,44% rispettivamente per i formaggi di montagna e per i formaggi di pianura. Anche il rapporto C18:1/C16 è risultato sempre più elevato nei formaggi di montagna (1.37) che in quelli di pianura (0,85). Infine, l'isomero c9,t11 dell'acido grasso C:18 è stato rilevato in quantità decisamente più elevate nel formaggio di montagna (1,63% sul totale degli acidi grassi misurati) rispetto a quello di pianura (0,75% sul totale degli acidi grassi misurati). Sono stati definiti degli indici utili alla valorizzazione dei prodotti lattiero-caseari di montagna. I risultati della ricerca mostrano che i formaggi di montagna ottenuti esclusivamente da latte di vacche alimentate al pascolo sono più ricchi in acidi grassi insaturi e nell'isomero C9, t-11 dell'acido linoleico coniugato, che risulta potenzialmente benefico per la salute umana, rispetto ai formaggi di pianura ottenuti da latte di vacche alimentate con foraggi conservati.

INTRODUCTION

It is a commonly-held opinion of consumers that the genuineness and the nutritional value of cheese produced in mountain farmhouses in the summer is associated with the environment where the cows graze, feeding on grass only, without diets based on stored forage and additives common in the intensive dairy farming methods used in the plains. In this light, it is should be of interest to the scientific community to define an index for analytically specifying these products according to the characteristics of the raw material and to their origin. It is known that the fatty acid composition of milk is influenced by the environment and feed. A diet based exclusively on pasture-grazing on the mountains could be distinguished by the fatty acid profile of the lipid fraction of the cheese compared with the same profile of similar cheeses from the plains and from farms where stored forages are included in the diet (BEAULIEU and PALMQUIST, 1995; DHIMAN *et al.*, 1995; CHOUINARD *et al.*, 1997; COLLOMB *et al.*, 1999). Determining the composition of the fat in cheese is also interesting from a nutritional point of view if the importance attributed to increasing the intake of mono and polyunsaturated fatty acids and the consequent reduction in saturated fatty acids is considered (GRUM-

MER, 1991). There have been several reports on the existence of a relationship between the botanical composition of the forage and the composition of the fatty acids in the triglycerides, especially regarding the saturated/unsaturated ratio (HERMANSEN, 1995; MANSBRIDGE and BLAKE, 1997; COLLOMB *et al.*, 1999; BOUGAUD *et al.*, 2001). All these authors studied the fatty acid profile of milk obtained from cows grazing on alpine pastures and that of milk obtained from cows kept permanently in cowsheds and fed with forage from the hills or plains, and, if necessary, with mixtures of cereals and maize silage. It was noted that the saturated fatty acids with six to sixteen carbon atoms were present in significantly higher concentrations in the milk of cows kept permanently in cowsheds, while the level of unsaturated fatty acids (oleic acid and polyunsaturated) was higher in the milk of cows grazing on alpine pastures (COLLOMB *et al.*, 1999). The aim of this study was to ascertain the possibility of using the composition of the total fatty acids of the lipid fraction as a tracer to identify the origin of traditional mountain dairy products and as a possible index for promoting mountain products.

MATERIALS AND METHODS

Cheese samples

Twenty-six semi-hard cheeses produced in the summer from 11 different alpine farmhouses in north-eastern Italy were analyzed. The cheeses were made in the months of June (eleven samples), July (eleven samples) and August (five samples) and then ripened for thirty days before analysis. The cheese-making involved processing the raw milk, exclusively from cows grazing on pastures; it was coagulated with bovine rennin, with subsequent breaking of the curd and heating at 46°C (INNOCENTE *et al.*, 2000).

Twenty-six cheeses from 10 different cheese-makers located on the plains of north-eastern Italy were also analyzed. They were made using technology similar to that described for the mountain cheeses, the only variable being the raw material which, in this case, came from cows kept permanently in cowsheds and fed alfalfa and field forage, and, if necessary, the addition of oil flours (maximum 20%), oats, rye and wheat (maximum 25%), maize (maximum 15%) or barley (maximum 40%). The cheeses were produced at different times during 2001 (about two or three cheese samples per month) in order to monitor any seasonal differences.

Analysis of total fatty acids

The cheese sample (0.5 g) was mixed with 3 mL of distilled water, acidified with 2.5 mL of 2.5 M H_2SO_4 and shaken for 2 min in an electric homogeniser. The acidified homogenate was mixed with 15 mL of a 1:1 mixture of diethyl ether and heptane and then centrifuged at 1,330 g for five min at 0°C. The upper organic phase was put in a bottle containing 1 g anhydrous Na_2SO_4 prior to transmethylation of the total fatty acids (TFA) in order to absorb the water in the cheese slurries (MARTINEZ-CASTRO *et al.*, 1986). After 5 min., 3 mL of the organic layer were put in a 10 mL screw-capped vial, 0.5 mL of 2M KOH in methanol was added and the mixture was centrifuged. The upper layer containing the methyl esters from glycerides was then directly injected into the gas chromatograph.

High-resolution gas chromatography was done using a Carlo Erba 5160 Mega series instrument (Carlo Erba, Milano, Italy) equipped with an FID detector, an on-column injector and a 30 m x 0.32 mm id SP 2320 capillary column (Supelco, Bellefonte, PA, USA). The carrier gas was helium with a column flow rate of 1.5 mL/min. Both injector and detector temperatures were 210°C. The column temperature ranged from 60° to 190°C, with a 10°C/min. rate (CONTE *et al.*, 1989).

The value of each individual fatty acid was expressed as a relative percentage, calculating the percentage ratio between the area corresponding to its peak and the sum of the areas of the measured fatty acids.

Statistical analysis

The data of fatty acid profiles for cheeses produced from milk from cows grazing on pasture or from cows fed with stored forages plus grain were statistically processed using the Statistical Discovery JMP 3.0 software. To ascertain normality of the distribution of the data relating to the percentage of the individual fatty acids in the cheeses analysed, the Shapiro-Wilk test was used (DAGNELIE, 1986). The distributions examined were considered normal for values of $P > 0.05$, where P is the probability that the distribution observed is equal to a normal distribution. To assess whether the differences between the means were statistically significant, the non-parametric Wilcoxon test (valid for comparison between two means) was used in the case of non-normal distributions, and one-way analysis of variance (F-test) for normal distributions. In all cases the differences between the means were considered statistically significant for values of $P < 0.05$.

RESULTS AND DISCUSSION

The following fatty acids were determined: C6, C8, C10, C12, C14, C14:1, C15, C16, C16:1, C17, C18, C18:1, C18:2, C18:3 and C18:2,c9,t11-isomer. Tables 1 and 2 report the median values, 25 and 75% quantiles, means, standard deviations and coefficients of variation calculated from the data obtained from 26 cheese samples produced from the milk of cows grazing on summer pastures and cows fed on stored forage and grain and kept inside cowsheds, respectively. To check whether there was a statistically significant difference between the mean values of single fatty acids with respect to the diet of the animals, the Wilcoxon test was used in the case of non-normal distributions, while for the saturated fatty acids with 10 and 17 carbon atoms, one-way analysis of variance was used as the data were distributed according to a Gaussian curve. The relative percentage of the individual fatty acids determined in the cheese produced with milk from cows grazing on pastures differed statistically from that of the cheese produced from the milk of animals fed stored forage plus grain (Table 3). The C15, C16:1 and C18 fatty acids were an exception as the differences between the two types of cheeses were not statistically significant.

The unsaturated fatty acids (C18:1; C18:2; C18:3 and C18:2,c9,t11) were always present in higher quantities in the cheese produced on the mountains. Hence, the sum of the relative percentages of saturated and unsaturated fatty acids was calculated in addition to their ratio. Table 4 shows that there was a good balance between saturated and unsaturated fatty acids in cheese produced from cows grazing on pastures, while in cheese obtained from the milk of cows kept permanently in cowsheds and fed stored forage and grain, the saturated fatty acids were almost twice as much as the unsaturated ones. This indicates that the ratio between unsaturated fatty acids and saturated fatty acids could be an initial index for defining the influence of dietary factors on the compositional characteristics of the raw material and the derived products. This is important from the point of view of promoting mountain dairy products. From a nutritional and health point of view, it is important to reduce the level of saturated fatty acids with respect to unsaturated ones in the diet. Among the unsaturated fatty acids determined in the cheeses analysed, the c9,t-11 C18:2

Table 1 - Statistical results (mean, 25 and 75% quantiles) of the individual fatty acids determined in cheeses produced with milk from cows grazing on pastures during summer. The data are expressed as the relative percentage of the total measured fatty acids.

		Quantiles				
Fatty acids	Median	25%	75%	Mean	SD	CV
C6	1.59	1.42	1.71	1.59	0.24	15.09
C8	1.03	0.85	1.14	1.03	0.20	19.42
C10	2.29	1.86	2.72	2.35	0.66	28.08
C12	2.39	2.15	2.73	2.47	0.46	18.62
C14	8.79	8.30	9.28	8.73	0.86	9.85
C14:1	1.51	1.41	1.59	1.51	0.21	13.37
C15	1.24	1.08	1.37	1.18	0.23	19.49
C16	23.46	21.80	24.65	23.46	2.02	8.61
C16:1	2.58	2.07	2.80	2.57	0.70	27.24
C17	1.25	0.98	1.46	1.32	0.23	17.42
C18	10.46	8.79	12.10	10.67	2.48	23.25
C18:1	32.14	30.70	36.55	33.80	4.32	12.78
C18:2	2.70	2.49	3.07	2.84	0.57	20.07
C18:3	1.10	0.90	1.32	1.10	0.26	23.64
C18:2,c9,t11	1.67	1.28	1.96	1.63	0.48	29.45

SD = standard deviation;
CV = coefficient of variation.

Table 2 - Statistical results (mean, 25 and 75% quantiles) of the individual fatty acids determined in cheeses produced with milk from cows fed stored forages plus grain and kept in cowsheds. The data are expressed as the relative percentage of the total measured fatty acids.

		Quantiles				
Fatty acids	Median	25%	75%	Mean	SD	CV
C6	1.67	1.62	1.76	1.71	0.17	9.94
C8	1.13	1.05	1.22	1.16	0.15	12.93
C10	2.70	2.49	3.03	2.82	0.43	15.25
C12	3.36	3.11	3.72	3.46	0.42	12.14
C14	11.74	11.63	12.34	11.68	0.87	7.45
C14:1	1.66	1.54	1.72	1.65	0.16	9.70
C15	1.26	1.21	1.34	1.27	0.10	7.87
C16	30.38	29.86	31.27	30.53	1.03	3.37
C16:1	2.661	2.25	2.84	2.54	0.49	19.29
C17	1.03	0.91	1.12	1.08	0.31	28.70
C18	9.22	9.78	10.68	9.83	0.97	9.87
C18:1	25.27	24.66	26.07	25.32	0.96	3.79
C18:2	2.50	2.28	2.71	2.50	0.29	11.60
C18:3	0.48	0.41	0.54	0.50	0.13	26.00
C18:2,c9,t11	0.71	0.61	0.84	0.75	0.19	25.33

SD = standard deviation;
CV = coefficient of variation.

Table 3 - Comparison between the means of the individual fatty acids of cheese produced with milk from cows grazing on pastures and from cows fed and kept permanently in cowsheds. For the non-parametric Wilcoxon test and for the F-test (one-way analysis of variance), the differences are statistically significant for $P < 0.05$.

Fatty acid	P level Wilcoxon test	P level F-test
C6	0.0167	
C8	0.0106	
C10		0.0034
C12	<0.0001	
C14	<0.0001	
C14:1	0.0032	
C15	0.4231	
C16	<0.0001	
C16:1	0.6826	
C17		0.0444
C18	0.2511	
C18:1	<0.0001	
C18:2	0.0049	
C18:3	<0.0001	
C18,c9,t11	<0.0001	

isomer of the conjugated linoleic acid is of particular importance. As the data in Tables 1 and 2 show, there are higher amounts of this isomer (more than double) in the mountain cheeses. This fatty acid has become very significant due to its possible anticarcinogenic as well as its antiatherogenic, immunomodulatory and growth-promoting properties (MACDONALD, 2000; COLLOMB *et al.*, 2001). This is an additional factor for promoting mountain cheese.

Since in all the cheese samples analysed, the fatty acids C16 and C18:1 were always the most representative from the quantitative point of view, the C18:1/C16 ratio was also calculated (Table 5). In cheeses produced on the mountains, the unsaturated fatty acid C18:1 was always more abundant than C16 and their ratio is therefore higher than one. The result is inverted in the case of similar cheeses obtained from milk produced in cowsheds. Therefore the determination of these two fatty acids and their ratio could be sufficient for recognising cheese produced using milk from different feeding regimes. It is important to note that there can also be high levels of unsaturated fatty acids when cows are fed forages to which unsaturated oils (soybean, corn or rapeseed oil), or seeds containing unsaturated oils, have been added. For this reason, with the objectives of recognizing and differentiating the mountain cheeses obtained from the milk of pasture-grazed cows, the evalu-

Table 4 - Unsaturated and saturated fatty acids and their ratio in cheese produced with milk from cows grazing on pastures and in cheese produced with milk from cows fed and kept permanently in cowsheds.

		Quantiles				
	Median	25%	75%	Mean	SD	CV
Mountain						
Unsaturated fatty acids, %	46.26	40.87	48.38	47.20	4.02	8.51
Saturated fatty acids, %	53.74	50.56	55.33	52.80	3.69	6.70
Unsaturated/saturated ratio	0.86	0.74	0.99	0.90	0.04	4.65
Plains						
Unsaturated fatty acids, %	36.56	31.98	34.34	36.47	1.46	4.00
Saturated fatty acids, %	63.44	62.59	64.49	63.53	1.32	2.07
Unsaturated/saturated ratio	0.58	0.50	0.55	0.57	0.03	5.76

SD = standard deviation;
CV = coefficient of variation.

Table 5 - C18:1 and C16 fatty acids and their ratio in cheese produced from the milk of cows grazing on pastures and from the milk of cows fed stored forages plus grain and kept inside cowsheds.

	Milk of cows grazing on pastures during summer			Milk of cows fed stored forages plus grain in cowsheds		
	C16	C18:1	C18:1/C16	C16	C18:1	C18:1/C16
Median	23.46	32.14	1.37	30.38	25.27	0.85
25% quantile	21.80	36.55	1.20	29.86	24.66	0.78
75% quantile	24.65	30.70	1.65	31.27	26.07	0.87
Mean	23.46	33.80	1.46	30.53	25.32	0.83
SD	2.02	4.32	0.30	1.03	0.96	0.05
CV	8.61	12.78	20.55	3.37	3.79	6.02

SD = standard deviation;
CV = coefficient of variation.

ation of the unsaturated/saturated ratio, the C18:1/C16 ratio and C18:2,c9,t11 content must be accompanied and completed by other analytical indexes or by appropriate sensory profiles. For example it could be interesting to conduct a sensory evaluation using a panel of expert tasters to determine if mountain pasture grazing confers typical aromatic characteristics to the cheese.

CONCLUSIONS

Determination of the fatty acid profile proved to be a useful analysis for contributing to the recognition of the cheeses produced in mountain farmhouses during the summer, using as raw material milk from cows grazing on the pastures. With the exception of C15, C16:1 and C18 fatty acids there was a statistically significant difference between the content of fatty acids of mountain cheese and cheese produced on the plains.

Cheese from the milk of cows grazing on pastures was characterised by a higher unsaturated fatty acid content and lower saturated fatty acid level. The ratio between unsaturated and saturated fatty acids was 0.86 for the mountain cheeses and 0.58 for the cheeses produced on the plains. The determination of the C18:1/C16 ratio was also important; it was always higher in mountain cheeses and could therefore represent a further useful index for promoting these traditional products. From the nutritional point of view, the higher content of the C18:2,c9,t-11 isomer in the mountain cheeses can be considered particularly interesting.

REFERENCES

1. Beaulieu A.D. and Palmquist D.L. 1995. Differential effects of high fat diets on fatty acid composition in milk of Jersey and Holstein cows. J. Dairy Sci. 78: 1336.
2. Bougaud C., Buchin S., Colon J.B., Hauwuy A. and Dupont D. 2001. Influence of the nature of alpine pastures on plasmin activity, fatty acid and volatile compound composition of milk. Le Lait 81:401.
3. Chouinard P.Y., Girard C. and Brisson G.L. 1997. Performance and profiles of milk fatty acids of cows fed full fat, heat-treated soybeans using various processing methods. J. Dairy Sci. 80: 334.
4. Collomb M., Buetikofer U., Sieber R., Spahni M., Jeangros B. and Bosset J.O. 1999. Composition en acides gras et en glycerides de la matiere grasse du lait de vache en zones de montagne et de plaine. Sciences des Alimentes 19: 97.
5. Collomb M., Buetikofer U., Sieber R., Bosset J.O. and Jeangros B. 2001. Conjugated linoleic acid

and trans fatty acid composition of cows' milk fat produced in lowlands and highlands. J. Dairy Res. 68: 519

6. Conte L.S., Zironi R. and Lercker G. 1989. Application of CGC to the analysis of butter fat: a study of several gas chromatographic parameters related to the fatty acid composition determination. Proc. Tenth International Symposium on Capillary Chromatography. Riva del Garda, Italy, May 22-25.
7. Dagnelie P. 1986. Théorie et Méthodes Statistiques: Applications Agronomiques. Vol. II. Ed. Les Presses Agronomiques de Gembloux, Gelmbloux, Belgium.
8. Dhiman T.R., Zanten K.V. and Satter L.D. 1995. Effect of dietary fat source on fatty acid composition of cow's milk. J. Sci. Food Agric. 69: 101.
9. Grummer R.R. 1991. Effect of feed on the composition of milk fat. J. Dairy Sci. 74: 3244.
10. Hermansen J.E. 1995. Prediction of milk fatty acid profile in dairy cows fed dietary fat differing in fatty acid composition. J. Dairy Sci. 78: 872.
11. Innocente N., Stefanuto O. and Corradini C. 2000. Chemical characterization of cheese produced in the mountains of Friuli Venezia Giulia. Sci. Tecn. Latt.-Cas. 51: 213.
12. Mansbridge R.J. and Blake J.S. 1997. Nutritional factors affecting the fatty acid composition of bovine milk. British J. Nutr. 78: 537.
13. Martinez-Castro I., Alonso L. and Juarez M. 1986. Gas-chromatographic analysis of free fatty acids and glycerides of milk fat using tetramethylammonium hydroxide as catalyst. Chromatographia 21: 37.
14. MacDonald H.B. 2000. Conjugated linoleic acid and disease prevention: a review of current knowledge. J. Am. Coll. Nutr. 19:111S.

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PAPER

EFFECT OF HIGH PRESSURE ON OLIVE OIL OXIDATION AND THE MAILLARD REACTION IN MODEL AND FOOD SYSTEMS

EFFETTI DELL'ALTA PRESSIONE SULL'OSSIDAZIONE DI OLIO DI OLIVA E SULLA REAZIONE DI MAILLARD IN SISTEMI MODELLO E IN FORMULATI ALIMENTARI

C. SEVERINI, A. BAIANO*, P. ROVERE[1], G. DALL'AGLIO[1] and R. MASSINI[2]
Istituto di Produzioni e Preparazioni Alimentari, Facoltà di Agraria, Università di Foggia, Via Napoli 25, 71100 Foggia, Italy
[1] Stazione Sperimentale per l'Industria delle Conserve Alimentari, Viale F. Tanara 31/A, 43100 Parma, Italy
[2] Dipartimento di Ingegneria Industriale, Università di Parma, Parco Area delle Scienze 181/A, 43100 Parma, Italy
* Corresponding author

ABSTRACT

The effect of high pressure on olive oil oxidation in emulsified model systems and in oil tomato food systems were compared with an equivalent heat treatment. The high pressure treatment reduced both early and final Maillard reaction products as well as lipid oxidation in the emulsified model systems. Similar results were obtained in the oil tomato food system although to a different degree, probably because of the influence of lycopene and ascorbic acid in the tomato-sauce and of the complexity of the food matrix.

RIASSUNTO

Gli effetti delle alte pressioni sull'ossidazione dell'olio di oliva in sistemi modello emulsionati e in formulati alimentari a base di pomodoro sono stati confrontati con gli effetti di un trattamento termico equivalente. Il trattamento iperbarico ha rallentato sia la formazione dei composti precoci e finali della reazione di Maillard sia l'ossidazione lipidica rispetto al trattamento termico. I risultati sono stati confermati nel formulato alimentare anche se in misura diversa, probabilmente per l'effetto antiossidante del licopene e dell'acido ascorbico contenuti nella passata di pomodoro e a causa della complessità della matrice alimentare.

- Key words: heat treatment, high pressure, Maillard reaction, olive oil, oxidation, tomato -

INTRODUCTION

The use of high pressure, as a food technology, not only for research purposes, but also in food processing, is quite recent. High pressure is an alternative treatment to traditional pasteurization and sterilization because of its capability of extending the shelf life and modifying the functional properties of foods. Moreover, high pressure treated foods are accepted by consumers because of their organoleptic features, which are like those of fresh foods (HEREMANS, 1995; ROVERE and MAGGI, 1995; SEVERINI and LERICI, 1995; CHEFTEL and CULIOLI, 1997; VELEZ-RUIZ *et al.*, 1998).

The effects of high pressure on lipid oxidation have been studied by some researchers. According to TANAKA *et al.* (1991), lipid oxidation was slower in high pressure treated sardines than in untreated ones. CHEAH and LEDWARD (1996) reported that pork meat treated at 800MPa for 20 min at 20°C and stored at 4°C for 8 days oxidized faster than untreated and heat treated pork meat; no significant increase in the rate of oxidation was observed in pork mince treated at a pressure lower than 300Mpa, but above this value, the oxidation rate increased. Furthermore, minced meat samples pressure treated in air had higher initial TBA values than samples pressure treated under nitrogen; with storage, both samples showed a higher oxidation than the control. In studying the effects of a_w on pressure induced oxidation, CHEAH and LEDWARD (1995) observed the inhibition of oxidation at a_w values outside the range of 0.40-0.55, while, within this range, other factors, such as the freeing of transition metals, may override peroxide destruction. The effect of pressures higher than 500MPa on lipid oxidation of turkey thigh muscle has been reported to be as strong as the effect of heat treatment (DISSING *et al.*, 1997). High pressure induced a decrease in the oxidative stability of seed and olive oils, in terms of induction time, but it did not produce remarkable changes in the peroxide value and volatile hydrocarbons (SEVERINI *et al.*, 1997). The effect of high pressure treatment on the Maillard reaction was studied by TAMAOKA *et al.* (1991) and HILL *et al.* (1996; 1997). High pressure treatments slowed or inhibited the Maillard reaction at low pH (5.1 and 6.5), while the effect of pressure was negligible at pH 7.0-7.5. Furthermore, the Maillard reaction was significantly enhanced in solutions with an initial pH of 8.0 and 10.1. HILL *et al.* (1996, 1997) asserted that high pressure inhibits the Maillard reaction which, beginning with the first steps, needs an increase in volume. The effectiveness of high pressure compared to heat treatment has also been studied (ROVERE *et al.*, 1996; 1998). These authors reported that an increase in pressure corresponded to a decrease in D_T values.

The aim of this investigation was to study the effect of a high pressure treatment on both lipid oxidation and the Maillard reaction in an emulsified model system containing olive oil and a glucose-glycine solution and in an oil tomato food system. In fact these two reactions occur at different rates when they take place simultaneously (SEVERINI and LERICI, 1995; SEVERINI *et al.*, 2000).

MATERIALS AND METHODS

Materials

Glucose, glycine and citric acid were supplied by Carlo Erba (Milan, Italy), whereas xanthan gum was supplied by Sigma-Aldrich (Steinheim, Germany). Tomatoes and fresh refined olive oil were purchased at a local market.

Methods

Tests were carried out on the following systems:

Model systems

- An emulsified model system (o/w), consisting of a 7 g/100g glucose-glycine (1:1) water solution, commercial refined olive oil (10 g/100g emulsion) and xanthan gum (2 g/100g solution) was prepared; the pH was adjusted to 4.1-4.2 with citric acid in order to simulate the pH of pasteurized vegetable foods. A Stephan D8 homogenizer (Hameln, Germany) at 2,500 r.p.m. under light vacuum conditions (50 mmHg) was used to make the emulsion.

- A 7 g/100g glucose-glycine water solution (1:1) at pH 4.1-4.2 was prepared.

Food systems

- An oil-tomato food system, made of tomato-sauce from fresh tomatoes and commercial refined olive oil (10 g/100g oil tomato food system) was prepared; the pH was adjusted to 4.1-4.2 with citric acid. Ingredients were mixed in a Braun Multiquick 300 hand blender (Kronberg, Germany).

- tomato-sauce (the same as used in the oil-tomato food system) at pH 4.1-4.2.

Commercial refined olive oil

- The same as used in the emulsion.

Samples (100 g) of each model system were packed under vacuum conditions in welded nylon/PE bags and submitted to a high pressure treatment (HP) with a pasteurizing effect at 600MPa for 5 min with a QFP6 hydrostatic press (Asea Brown Boveri, Milan, Italy) or to traditional pasteurisation (75°C, 15 min in a water bath) which is used for canned foods having a pH≤4.2. Twenty-four bags (control) received no treatment.

Three samples per treatment were submitted to rapid aging: (a) in a water bath at 95°C for 0, 2, 4, 6, 9, 12h or (b) at temperatures varying from 65° to 80°C for 0, 1, 2h in a device called a "Meteosol" (Bertuzzi, Brugherio, Milan, Italy) having the following technical features: source of light with lamps covering the complete light spectrum; maximum radiation higher than natural summer radiation; shaking with a rotation of 30 r.p.m. and 30 mm run.

Glucose-glycine water solutions, tomato sauces and aqueous phases, separated from both emulsions and food systems by centrifugation at 78,246 xg for 15 min, (A.L.C. 4239R centrifuge, 7 cm radius, Milan, Italy), were submitted to the following analyses:

- optical density (O.D.) at 294 and 420 nm (LERICI *et al.*, 1990) with a UV/VIS Beckman DU 640 (Fullerton, CA, U.S.A.) spectrophotometer. The solutions had been previously diluted with distilled water and microfiltered through a UFP2THK24 filter (Millipore, Milan, Italy); the tomato sauces were diluted with distilled water in the ratio of 6 to 50 g and filtered through filter paper (Whatman N. 2, Maidenstone, England, UK); O.D. values were estimated considering dilution coefficients;

- colour was analyzed, using a tristimulus colorimeter (Chromameter-2 Reflectance, Minolta, Osaka, Japan) equipped with a CR-300 measuring head; the instrument was standardised against a white tile before each measurement; colours are expressed in L* (brightness), a* (red point), b* (yellow point) Hunter scale parameters (MASTROCOLA and LERICI, 1991); at least five measurements were carried out on each sample and the coefficients of variation were less than 10%;

- reducing power, using a platinum electrode and a Ag/AgCl saturated Cl electrode connected to a voltameter (Hanna Instruments 8417, Singapore). The instrument was standardised with a standard solution having a potential of 220 mV at 25°C (Titolchimica, Pontecchio Pol., Ro, Italy). Before analysis, each sample was diluted with distilled water in the ratio of 6 to 50 g and filtered through filter paper (Whatman N. 2, Maidenstone, England, UK). The measurements were carried out in 50 mL size cells filled with 20 mL of sample. Before analysis, each sample was stripped with a flow of N_2 for 10 min. The analyses were carried out at 25°C and values were recorded for at least 30 min and considered constant if variations were less than 1 mV within the successive 5 min.

After filtration through anhydrous sodium sulphate, the commercial refined olive oil and the oils separated from both emulsions and food systems were submitted to the following analyses:

- peroxide values (P.V.) expressed as milliEquivalents (mEq) of active oxygen per kg of oil, according to AOAC (1984);
- p-anisidine values (p-A.V.) according to IUPAC (1997).

With the exception of colour measurements, analyses were carried out at least in duplicate and the difference between the two analyses on the same sample did not exceed 6%.

RESULTS AND DISCUSSION

The oxidative activity of early uncoloured Maillard reaction products is well known (NAMIKI and HAYASHI, 1983), as is the antioxidant activity of intermediate and final coloured Maillard reaction products (EICHNER, 1981; YAMAGUSHI *et al.*, 1981; TANAKA *et al.*, 1988; GORDON, 1990). The early ones (including 5-hydroxymethyl-2-furancarboxyaldehyde and other products) absorb at 285-300 nm and have the maximum absorption peak at 294 nm (LERICI *et al.*, 1990). Fig. 1 shows the comparison between O.D. values at 294 nm of glucose-glycine solutions and glucose-glycine solutions from emulsified model systems with respect to the different treatments. Early, uncoloured Maillard reaction products were induced only by heat treatment. Solutions from emulsified model systems had the highest O.D. values (ten times those of the solutions only), because of the strong homogenization even if carried out under light vacuum conditions. In fact, the increase in specific surface area and the consequent increase in contact with O_2 caused a rapid increase in oxidation. Moreover, it is well known that the Maillard reaction proceeds rapidly in the presence of lipids (ARNOLDI *et al.*, 1990), because lipids and their oxidation compounds can take part in the reaction. Furthermore, it is possible to exclude interference due to the presence of oil because of the oil-solution filtration system used.

The high pressure treated emulsions showed an absorption lower than the heat treated emulsions. Final Maillard reaction products did not appear in spite of the increase in temperature (3°C/100MPa)

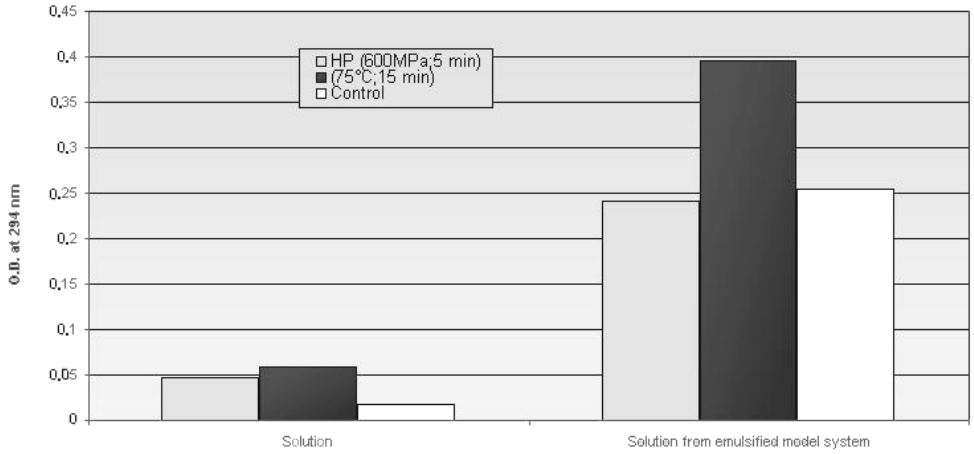


Fig. 1 - Optical density at 294 nm of glucose-glycine solutions and glucose-glycine solutions from emulsified model systems as a function of the different treatments.

caused by the treatment because of the inhibition of the Maillard reaction.

O.D. values at 294 nm of tomato-sauce and tomato-sauce from oil tomato food systems are shown in Fig. 2. Early, uncoloured Maillard reaction products were present both in HP and in heat treated tomato-sauce. The O.D. values were higher in the tomato-sauce than in tomato-sauces from HP and heat treated food systems. These results can be explained by supposing an interaction between early Maillard reaction products and oil, even if this is in contrast with HILL *et al.* (1996, 1997).

According to LERICI *et al.* (1990), both solutions and solutions from emulsified model systems have very low O.D. values at 420 nm (< 0.005), which is the maximum absorption peak of final coloured Maillard reaction products. O.D. values at 420 nm of heat treated tomato-sauce were higher than the values of HP tomato-sauce (Fig. 2). This means that browning was induced in the heat treated samples and not in the HP treated ones.

There were no differences in the L*, a* and b* values of the HP, heat treated and untreated samples for both solutions and solutions from emulsified systems (data not shown). Likewise, there were no substantial differences between the colour data for tomato-sauce and tomato-sauce from the food system and among colour data of HP, heat treated and untreated samples (data not shown).

Data concerning the reducing power of the solutions are reported in Fig. 3. Solutions from emulsions had the highest redox pontential values, probably because of homogenization. For both solutions and solutions from emulsions, the redox potential of HP and untreated samples was lower than the redox potential of the heat treated ones.

For both tomato-sauce and tomato-sauce from food systems (Fig. 3), the redox potential of HP samples was slightly lower than the redox potential of the untreated and heat treated ones. The presence of lycopene and ascorbic acid in the tomato-sauce probably decreased differ-

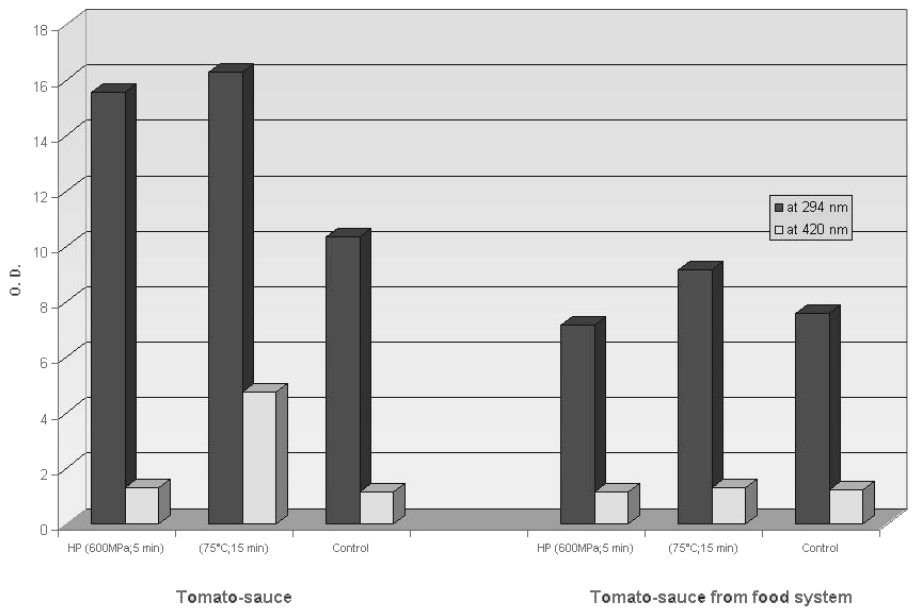


Fig. 2 - Optical density at 294 and 420 nm of tomato-sauce and tomato-sauce from an oil tomato food system as a function of the different treatments.

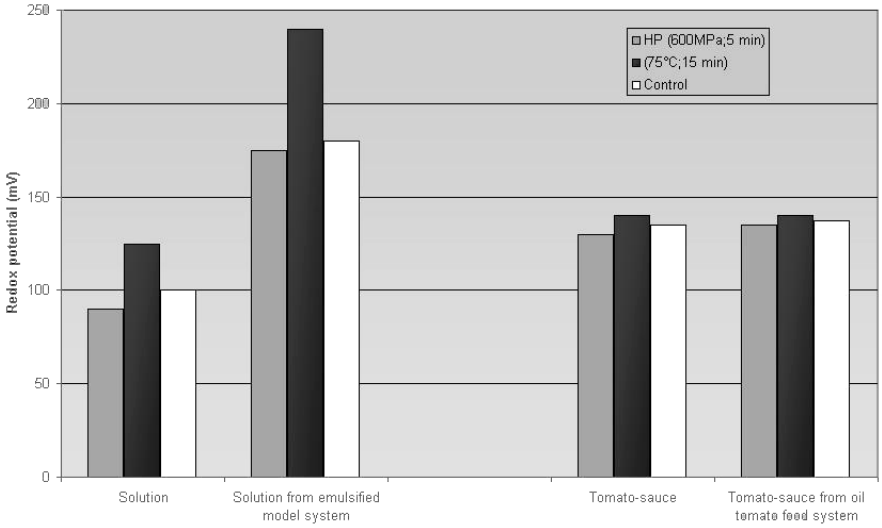


Fig. 3 - Redox potentials of the glucose-glycine solution, glucose-glycine solution from the emulsified model system, tomato-sauce and tomato-sauce from the oil tomato food system as a function of the different treatments.

ences between HP, untreated and heat treated samples.

Peroxide and p-anisidine values were determined to estimate the effect of high pressure on olive oil oxidation. The trends of P.V. values of oils and oils from emulsions during aging in a water bath at 95°C are shown in Fig. 4. Peroxide values of oils from emulsions just after homogenization were higher than the

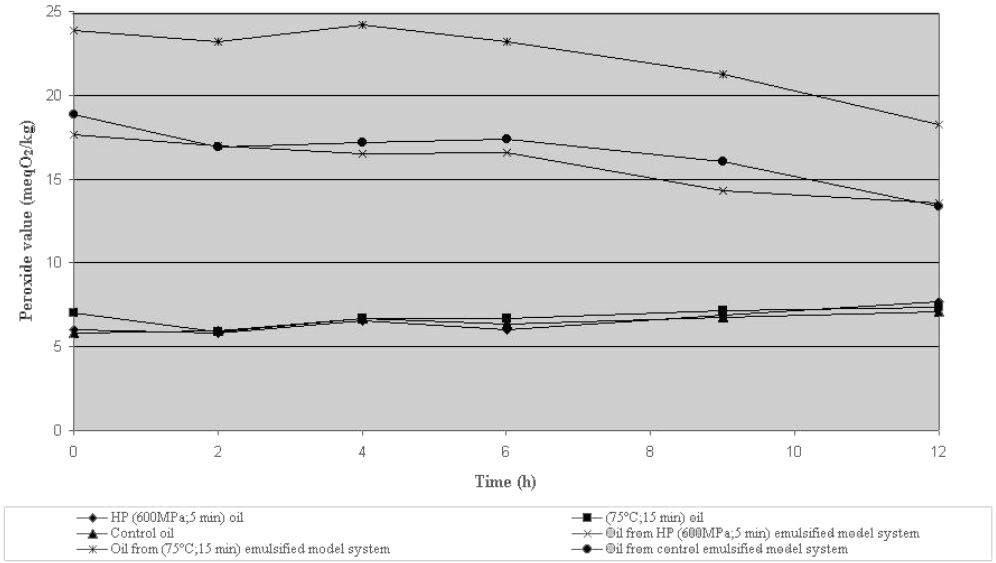


Fig. 4 - Peroxide values of oil and oil from the emulsified model systems with respect to time at 95°C.

others. There were no differences among the peroxide values of untreated, HP and heat treated oils. Furthermore, there was no significant increase in P.V. during aging as shown by the K values reported in Table 1. The peroxide value of oil from heat treated emulsified model systems was always higher than those of oils from untreated and HP emulsions. All the oil samples from emulsified model systems showed a decrease in P.V. during aging, as demonstrated by the K values in Table 1. All these results are in agreement with data reported in the literature. In particular, it is well known that heating is responsible for inducing lipid oxidation. Moreover, TAUSCHER (1995) and SEVERINI *et al.* (1997) demonstrated that high pressure inhibits oxidation which needs an increase in volume.

Peroxide values (Fig. 5) of oils only were higher than peroxide values of the oils from the food systems. This is probably because of the antioxidant effect of lycopene and ascorbic acid from the tomato (NICOLI *et al.*, 1999). HP and untreated oil samples from food systems had the lowest peroxide values. Peroxide values of all samples showed no substantial increase (Table 1). This is probably due to the presence of lycopene and ascorbic acid, too.

P-anisidine values of oils from HP samples were lower than those of the oils from untreated and heat treated samples. Moreover, p-anisidine values of oils from emulsions were higher than p-anisidine values of the oils only and the values increased with time of aging (Fig. 6 and Table 1). P-anisidine values of oils only and oils from food systems had similar trends. The trends of P.V. and p-A.V. were confirmed by submitting the samples to rapid aging conditions using the "Meteosol" device (Fig. 7 and 8).

CONCLUSIONS

The results concerning model systems show that high pressure treatment was able to inhibit the Maillard reaction starting with the first steps. In spite of the fact that the heat treatment was carried out at a temperature and pH typical of canned foods, it induced the generation of early, uncoloured Maillard reaction

Table 1 - Kinetic constant (K), correlation coefficient (R) and significance (α) calculated from linear regression of curves in Figs. 4, 5 and 6.

			K	R	α
P.V.	Oil only	Control	0.105	0.921	0.01
		(75°C; 15 min)	0.069	0.605	n.s.
		HP (600MPa; 5 min)	0.137	0.881	0.05
	Oil from emulsified mod. system	Control	-0.375	0.904	0.01
		(75°C; 15 min)	-0.445	0.883	0.05
		HP (600MPa; 5 min)	-0.349	0.963	0.001
	Oil from food system	Control	-0.048	0.841	0.05
		(75°C; 15 min)	-0.035	0.402	n.s.
		HP (600MPa; 5 min)	-0.043	0.484	n.s.
p-A.V.	Oil only	Control	0.046	0.993	0.001
		(75°C; 15 min)	0.037	0.931	0.01
		HP (600MPa; 5 min)	0.047	0.993	0.001
	Oil from emulsified mod. system	Control	0.396	0.972	0.001
		(75°C; 15 min)	0.415	0.978	0.001
		HP (600MPa; 5 min)	0.411	0.973	0.001

n.s.: not significant.

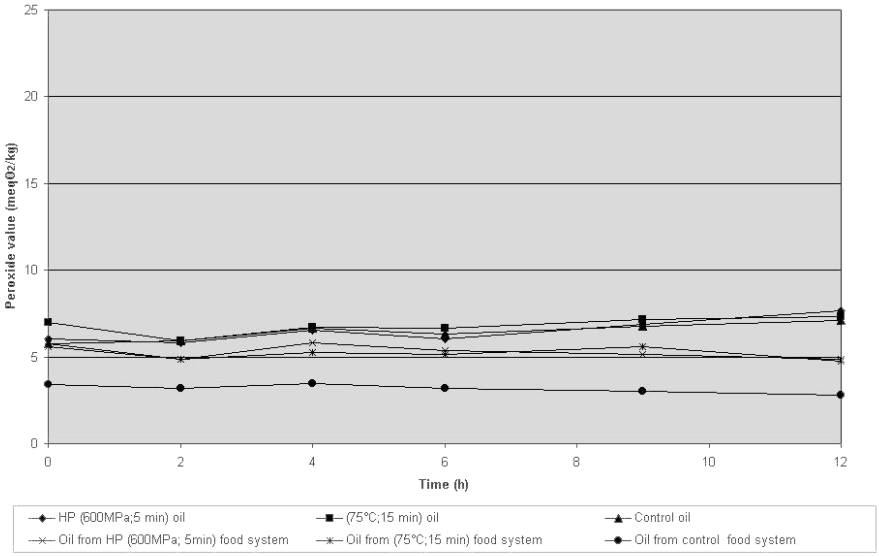


Fig. 5 - Peroxide values of oil and oil from the food systems with respect to time at 95°C.

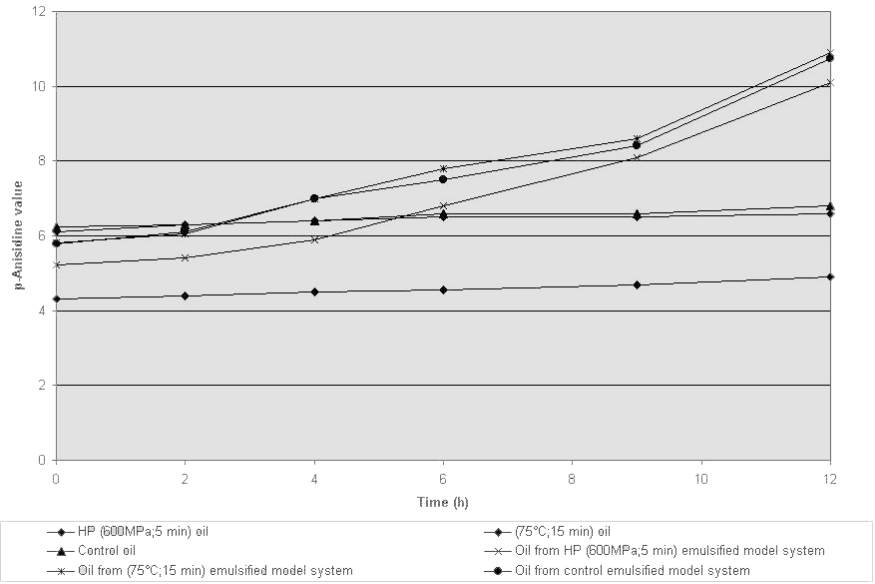


Fig. 6 - p-Anisidine values of oil and oil from the emulsified model systems with respect to time at 95°C.

products, especially in the emulsified model systems, because of homogenization and the presence of oil. Likewise, high pressure treatment reduced oil oxidation, while heat treatment induced it.

In lipid-containing foods, the Maillard reaction and lipid oxidation are affected by interactions among the food components, but not necessarily in a negative way. Consequently, it is more difficult to interpret the results.

The results obtained for the model sys-

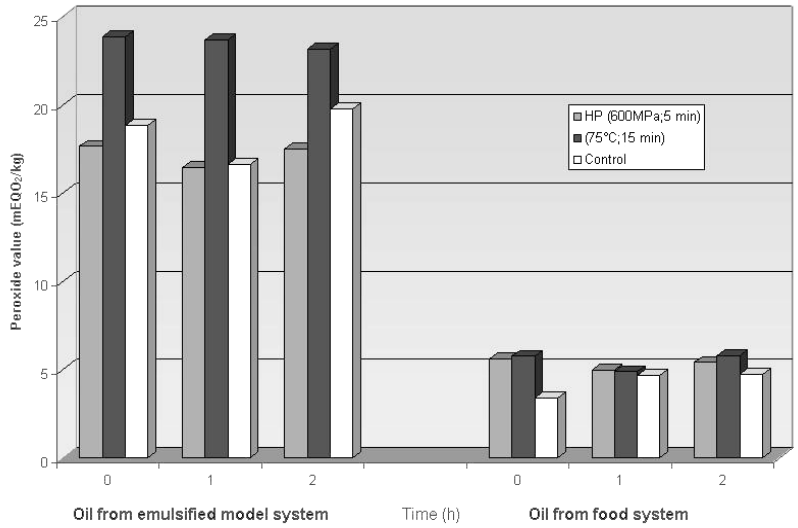


Fig. 7 - Peroxide values of oil from the emulsified model systems and food systems with respect to time (aging in the "Meteosol" device).

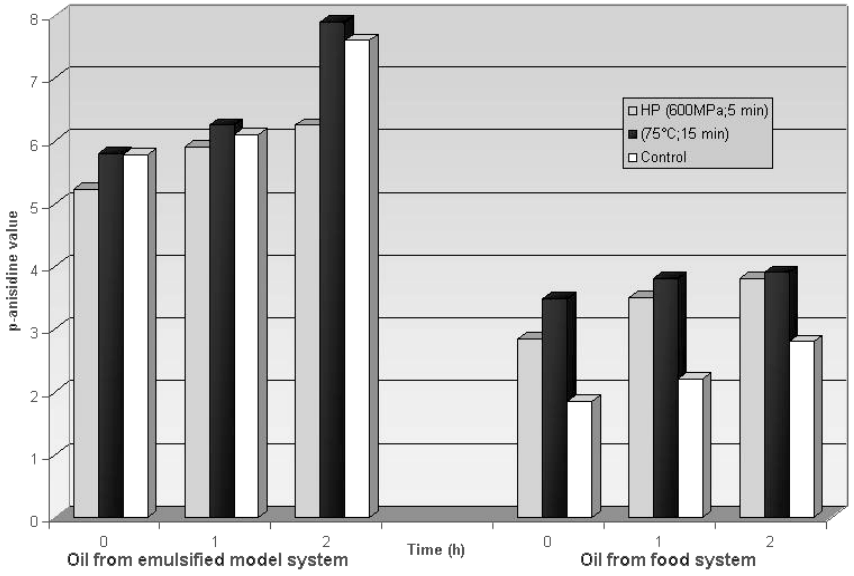


Fig. 8 - p-Anisidine values of oil from the emulsified model systems and food systems with respect to time (aging in the "Meteosol" device).

tems were substantially confirmed by the results obtained from the food systems, even if the effect of both high pressure and heat treatment were less evident, because of the presence of antioxidant compounds, such as lycopene, in the tomato-sauce. In spite of the fact that high pressure treatment was able to inhibit the Maillard reaction, the presence of early Maillard reaction products in HP samples may be due to the interactions among the components of the food matrix.

REFERENCES

AOAC 1984. "Official Methods of Analysis". Association of Official Analytical Chemists, Washington, D.C.

Arnoldi A., Arnoldi C., Baldi O. and Ghizzoni C. 1990. Effects of lipids in the Maillard reaction. In "The Maillard Reaction, Advances in Life Sciences", Birkhauser Verlag, Basel.

Cheah P.B. and Ledward D.A. 1995. High pressure effects on lipid oxidation. JAOCS. 72:1059.

Cheah P.B. and Ledward D.A. 1996. High pressure effects on lipid oxidation in minced pork. Meat Sci. 43:123.

Cheftel J.C. and Culioli J. 1997. Effects of high pressure on meat: a review. Meat Sci. 46:211.

Dissing J., Bruun-Jensen L. and Skibsted L.H. 1997. Effect of high pressure treatment on lipid oxidation in turkey thigh muscle during chill storage. Z. Lebensm. Unters. Forsch. 205:11.

Eichner K. 1981. Antioxidative effect of Maillard reaction intermediates. Progr. Food Nutr. Sci. 5:441.

Gordon M.H. 1990. The mechanism of antioxidant action in vitro. In "Food Antioxidants", B.J.F. Hudston (Ed.), p. 8, Elsevier Applied Science, London.

Heremans K. 1995. High pressure effects on food components: concepts and applications. In "L'Impiego delle Alte Pressioni nell'Industria Alimentare". G. Dall'Aglio, C.R. Lerici and A. Zamorani p. 27, Stazione Sperimentale Industria delle Conserve Alimentari e Università di Udine, Parma.

Hill V.M., Ames J.M. and Ledward D.A. 1997. Investigation of the effect of high hydrostatic pressure on the Maillard reaction. Proceedings of the Sixth International Symposium "The Maillard Reaction" 27-30th July 1997, Royal College of Phisicians, London, UK.

Hill V.M., Ledward D.A. and Ames J.M. 1996. Influence of high hydrostatic pressure and pH on the rate of Maillard browning in a glucose-lysine system. J. Agric. Food Chem. 44:594.

IUPAC. 1987. Standard Methods for the Analysis of Oils, Fats and Derivatives, 7th Ed., Method number 2504, Blackwell Scientific Publications, Boston, MA and Oxford, UK.

Lerici C.R., Barbanti D., Manzano M. and Cherubin S. 1990. Early indicator of chemical changes in foods due to enzymic or non enzymic browning reactions. 1: Study on heat treated model systems. Lebensm. – Wiss. U. Tech. 23:289.

Mastrocola D. and Lerici C.R. 1991. Colorimetric measurements of enzymatic and non enzymatic browning in apple purees. Ital. J. Food Sci. 3:219.

Namiki M. and Hayashi T. 1983. A new mechanism of the Maillard reaction involving sugar fragmentation and free radical formation. In "The Maillard Reaction in Food and Nutrition", G.R. Waller and M.S. Feather (Ed.), P. 21. American Chemical Society, Washington, D.C..

Nicoli M.C., Anese M. and Manzocco L. 1999. Oil stability and antioxidant properties of an oil tomato food system as affected by processing. Adv. Food Sci. 21:10.

Rovere P. and Maggi A. 1995. Approccio alle alte pressioni: una nuova tecnologia a disposizione dell'industria alimentare. Ind. Conserve 70:45.

Rovere P., Maggi A., Scaramuzza N., Gola S., Miglioli L., Carpi G. and Dall'Aglio G. 1996. Trattamenti termici ad alte pressioni: valutazione dell'effetto sterilizzante e del danno termico. Ind. Conserve 71:473.

Rovere P., Miglioli L., Lonnerborg N.G., Scaramuzza N. and Gola S. 1998. Modellazione e calcolo dell'effetto sterilizzante nei trattamenti termici ad alta pressione. Ind. Conserve 73:303.

Severini C. and Lerici C.R. 1995. Interactions between the Maillard reaction and lipid oxidation in model systems during high temperature treatment. Ital. J. Food Sci. 7:189.

Severini C., Baiano A., Romani S., Massini R. and Lerici C.R. 2000. Maillard reaction and lipid oxidation as affected by temperature. Res. Adv. In Food Sci. 1:33.

Severini C., Romani S., Dall'Aglio G., Rovere P., Conte L. and Lerici C.R. 1997. High pressure effects on lipid oxidation of extra virgin olive oils and seed oils. Ital. J. Food Sci. 9:183.

Tamaoka T., Itoh N. and Hayashi R. 1991. High pressure effect on Maillard reaction. Agric. Biol. Chem. 55:2071.

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

Tanaka M., Xueyi Z., Nagashima Y. and Taguchi T. 1991. Effect of high pressure on the lipid oxidation in sardine meat. Nippon Suisan Gakkaishi 57:957.

Tauscher B. 1995. Pasteurization of food by hydrostatic high pressure: chemical aspects. Z. Lebensm. Unters. Forsch. 200:3.

Velez-Ruiz J.F., Swanson B.G. and Barbosa-Canovas G.V. 1998. Flow and viscoelastic properties of concentrated milk treated by high hydrostatic pressure. Lebensm. – Wiss. U. Tech. 31:182.

Yamagushi N., Koyama Y. And Fujimaki M. 1981. Fractionation and antioxidative activity of browning reaction products between D-xylose and glycine. Prog. Food Nutr. Sci. 5:429.

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PAPER

STUDY OF BREAD STALING BY X-RAY DIFFRACTION ANALYSIS

STUDIO DEL RAFFERMAMENTO DEL PANE
MEDIANTE ANALISI DI DIFFRAZIONE DEI RAGGI X

L. MANZOCCO*, M.C. NICOLI and T.P. LABUZA[1]
Dipartimento di Scienze degli Alimenti, Università degli studi di Udine,
Via Marangoni 97, 33100 Udine, Italy
[1]Department of Food Science and Nutrition, University of Minnesota,
1354 Eckles Avenue, St. Paul, MN 55108, USA
* Corresponding author

ABSTRACT

The crystallinity of bread stored at -18°, 4° and 25°C for up to 20 days was studied using X-Ray diffraction analysis. Relative crystallinity of bread was calculated from the total diffraction at specific angles. Firmness and crystallinity both increased during storage. When moisture migration from the crumb to the crust occurred during staling, the development of firmness was well described by a simple equation accounting for the changes in crystallinity, moisture and storage temperature.

RIASSUNTO

L'analisi di diffrazione dei raggi X è stata utilizzata per studiare la cristallinità di campioni di pane conservati a -18°, 4° e 25°C fino a 20 giorni. La cristallinità del pane è stata espressa sulla base della diffrazione totale a specifici angoli. L'aumento di consistenza osservato durante la conservazione era associato ad un progressivo incremento di cristallinità. In presenza di fenomeni di migrazioni di umidità dall'interno del pane verso la superficie esterna, l'incremento di consistenza è risultato essere ben descritto dai cambiamenti di cristallinità, umidità e temperatura di conservazione.

- Key-words: bread, crystallisation, moisture migration, staling, XRD -

INTRODUCTION

The study of the reactions leading to the quality depletion (staling) of bakery products is a topic with considerable technological and commercial implications. For example, the amount of bread that stales in a shorter time than required by the market is estimated to be approximately 8% (ZOBEL and KULP, 1996).

Changes in firmness and flavour, as well as in soluble starch content, are among the events associated with staling. Concomitant modifications in water holding capacity and mobility are also detectable. Abundant literature data indicates that the major cause of this phenomenon is the reversible aggregation of the starch fraction. In fact, starch segments, mainly represented by amylopectin, progressively re-associate to form a molecular structure with increased order, which can then be assimilated to a crystalline one (KATZ, 1928; KATZ and VAN ITALLIE, 1930; SCHOCH and FRENCH, 1947; KULP and PONTE, 1981, 1996; MILES *et al.*, 1985; LIN and LINEBACK, 1990; ZOBEL, 1992). Despite the broad consensus developed regarding the relation between staling and crystallisation, the contribution of non-starch components to the mechanism of this process is not yet clearly understood. In fact, circumstantial evidence suggests that the physico-chemical properties of proteins can exert a critical role in modifying staling kinetics (LEUNG *et al.*, 1979; ZELEZNAK and HOSENEY, 1986; CZUCHAJOWSKA and POMERANZ, 1989). In particular, rheological changes in bakery products during storage have been attributed to the formation of hydrogen bonds between the continuous protein matrix and the discontinuous lattice formed by the gelatinised starch granules (MARTIN *et al.*, 1991; HOSENEY and ROGERS, 1993; SEOW and TEO, 1996).

The molecular associations in starch undergoing retrogradation have been studied by rapid-scanning Raman spectroscopy and Fourier transform infrared analysis (WINTER and KWAK, 1987; VAN SOEST et al., 1994). In addition, nuclear magnetic resonance analyses have provided information about staling mechanisms by studying the state and the mobility of water (COLWELL et al., 1969; LEUNG *et al.*, 1979; MORGAN et al., 1992; WU and EADS, 1993; CHEN *et al.*, 1997; SLADE and LEVINE, 1991; KIM-SHIN *et al.*, 1991). The determination of structure formation has been performed by calorimetric analyses of staling endotherm (CHAMPENOIS *et al.*, 1995; SCHIRALDI *et al.*, 1996; COOKE and GIDLEY, 1992).

X-ray diffraction analysis (XRD), which is widely used in pharmacological chemistry and material science to investigate the degree of molecular order, has also been adopted to evaluate the crystallinity of food compounds (SURYANARAYANAN, 1995; GROTENHUIS *et al.*, 1999; WRIGHT *et al.*, 2000). Although small or imperfect crystalline regions are limiting factors to its use, it has been used to obtain primary evidence for the development of crystallinity in starch gels and baked products (ZOBEL and KULP, l996; PISESOOKBUNTERNG et al., 1983). Different authors have observed that storage of starch gels is associated with an increase in diffraction intensity and that progressive crystallisation occurs following a B-type XRD pattern (DRAGSDORF and VARRIANO-MARSTON, 1980; MILES *et al.*, 1985; ROULET *et al.*, 1988; I'ANSON *et al.*, 1990; HOOVER and SENANAYAKE, 1996; JEONG *et al.*, 1997).

The aim of this study was to provide further information about bread staling kinetics by XRD analysis. Bread samples from a local bakery were tested to evaluate the possibility of obtaining significant information about their staling kinetics. In the first part of the work, a methodology to quantitatively determine the degree of crystallinity of bread from its XRD pattern was established. Secondly, the changes in bread firmness,

were correlated to crystallinity, water content and storage temperature.

MATERIALS AND METHODS

Sample preparation

Fresh "French" style bread was purchased from a local bakery and cut into 10 cm thick pieces discarding the two end slices of the loaves. One aliquot of bread pieces was manually cut to remove the crust and obtain bread crumb samples. Bread with crust and bread crumb samples were then put into closed glass jars, which were stored at room temperature (25±2°C), in a refrigerator (4±1°C) and in a freezer (-18±1°C). At times up to 20 days, samples were removed from the storage rooms and equilibrated at room temperature for 2 h before analysis.

Firmness was measured on sliced bread and bread crumb. Moisture content, water activity and XRD analyses were carried out on the ground crumb of the samples. XRD analysis was also performed on six-year-old wheat starch stored in our flour laboratory.

Moisture content

Moisture content was determined using the AOAC method (AOAC, 1995), in which samples were weighed and oven-dried at 101°C for about 20 h.

Bread firmness

Firmness was measured by a puncture test using a TA.XT2 Texture Analyser (Texture Technologies Corp., New York, U.S.A.) (AACC, 1983). The instrumental settings and operation of the instrument were accomplished using a PC with package version 3.7 XTRAD software (Texture Technologies Corp., NY/ Stable Micro System, Godalming, Surrey, U.K.). On the test day, 20 mm thick slices were compressed with a 20 mm diameter cylindrical probe, which can move downward and upward. Crosshead speed was set at 10 mm/min. Force-distance curves were obtained from the puncture tests and firmness was taken as the force (N) required to compress the slices 6 mm (25% compression).

X-ray diffraction analysis

A Siemens D 5005 X-ray diffractometer (Siemens, Munich, Germany) was used. Copper radiation (Kα radiation, λ=1.540) was selected to analyse the bread samples. Operation was at 45 kV and 40 mA. Aliquots of 0.5 g of ground sample were exactly weighed in the XRD holder and pressed to a thickness of 1 mm so that a smooth surface was achieved. Samples were scanned from 5 to 60 2-θ (step of 0.1, dwell 2 sec). XRD patterns were processed by Jade® 5.0 software (Materials Data Inc., Livermore, CA, USA). Distances between the planes of the crystal d (Å) where calculated from the angle of diffraction θ (°) obtained by the XRD pattern, according to Bragg's law:

$$n\lambda = 2d\sin\theta \qquad (1)$$

where λ is the wavelength of the X-ray beam and n is the order of reflection.

Statistical analysis

Data are the average of at least three replicates for each determination. In the case of firmness, at least six measurements were carried out at each sampling time. The coefficients of variation, expressed as the percentage ratio between the standard deviation and the mean value, were less than 4% for moisture, 10% for firmness and 8% for relative crystallinity. Linear regression analysis was performed using Statistica for Windows software (Version 5.1, Statsoft, Inc, 1997, Tulsa, USA).

RESULTS AND DISCUSSION

Fig. 1 shows the X-ray diffraction patterns of wheat flour (a) and starch (b). It can be observed that the wheat flour and starch had similar patterns. For these samples, the most intense peaks were identified and distances between the planes of the crystallites d (Å) were calculated from the X-ray diffraction angle according to Bragg's law. Values of d-spacings and intensities of the main peaks in the wheat derivatives were compared to those of potato and corn starch taken from the literature (Jade® 5.0 database, Materials Data Inc., Livermore, CA, USA) (Table 1). On the basis of d-spacings and diffraction intensities, wheat starch and flour showed a predominant A crystallographic pattern (KATZ and VAN ITALLIE, 1930; KERR, 1950; HELLMAN *et al.*, 1954; ZOBEL, 1964; HOSENEY, 1994). In fact, the strong 3.8, 4.9, 5.1 and 5.8 Å spacings are consistent with the presence of crystallites characterised by left-handed parallel-stranded double helices packed in monoclinic space group B2 (WU and SARKO, 1978a, b; IMBERTY et al., 1988; PARIS et al., 1999). Since A-diffraction patterns are typical of cereal starch crystallites, similar d-spacings with different diffraction intensities can also be recognised in corn starch. On the other hand, tuber and amylose-rich starches, whose structural element is the double helix packed in a hexagonal unit cell with the P6 space group, exhibit a B crystallographic pattern (WU and SARKO, 1978b; IMBERTY and PEREZ, 1988). The presence of an intense 4.4 Å spacing (2θ=19.76) in the X-ray diffractograms of wheat starch and flour (Fig. 1) also suggests the presence of crystallites with a V-type pattern, which are unusual in native starch granules but may be formed during starch extraction (BILIADERIS and GALLOWAY, 1989).

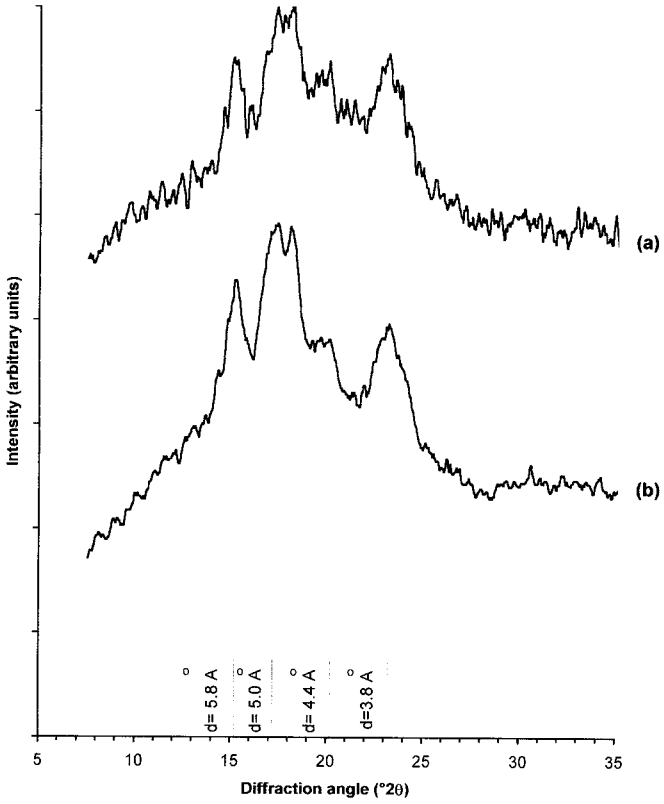


Fig. 1 - X-ray diffractograms of wheat flour (a) and wheat starch (b). The d-spacings calculated from the angle of diffraction of the most intense peaks are also reported.

Fig. 2 shows the changes in the XRD pattern of bread crumb during storage in sealed jars for up to 20 days. Wheat flour, used as the control, is also shown. No significant peaks were observed in the XRD pattern of fresh bread (day 0), while a progressive increase in crystallinity occurred with storage. According to literature data, staling is associated with the development of the B-type crystallographic pattern, which is clearly indicated by the development of a

Table 1- Values of d-spacings and diffraction intensities associated with the main XRD peaks of wheat flour and starch. Corn and potato starch patterns were obtained from Jade® 5.0 database (Materials Data Inc., Livermore, CA, USA).

Sample	d-spacing (Å)	Intensity (arbitrary units)
Wheat starch	3.85	76
	4.41	71
	4.86	100
	5.10	100
	5.81	88
Wheat flour	3.84	87
	4.43	84
	4.87	100
	5.10	100
	5.84	88
Corn starch	3.79	100
	4.37	50
	4.87	75
	5.18	100
	5.79	100
Potato starch	3.71	40
	4.00	50
	4.55	30
	5.15	100
	6.15	50

sharp peak at 17.2°2θ and by the presence of two peaks at ≈22.5 and ≈24.4°2θ (HELLMAN *et al.*, 1954. MILES *et al.*, 1985; HOSENEY, 1994; ZOBEL and KULP, 1996).

A rigorous methodology for determining the degree of crystallinity of polymers exhibiting both crystalline and non-crystalline XRD behaviours was developed by HERMANS and WEIDINGER (1948). Although widely applied in the pharmaceutical sector, this procedure cannot always be implemented, especially in a complex food matrix, since it requires the demarcation and the quantitative evaluation of the crystalline and amorphous intensities from the XRD pattern (SURYANARAYANAN, 1995). In relatively pure compounds, crys-

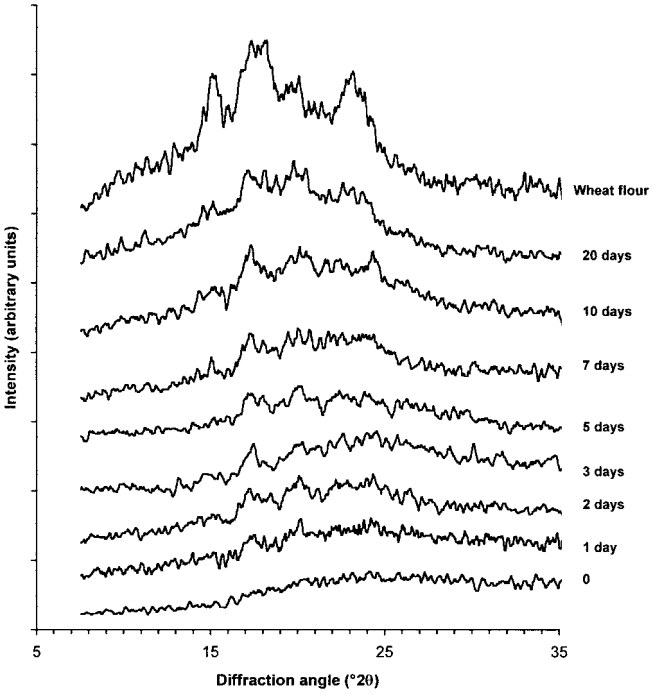


Fig. 2 - X-ray diffractograms of bread crumb stored at 25°C for up to 20 days. Wheat flour was used as the control.

talline and amorphous areas under the XRD pattern can be separately integrated. In the case of bread samples, due to the low crystalline content, the separation of the intensity area attributable to the amorphous phase from that due to the crystalline one hardly leads to statistically acceptable results.

For this reason, the degree of crystallinity is expressed on the basis of the total diffraction at specific angles rather than of the integrated intensities. In particular, three representative XRD angles of diffraction were selected according to the occurrence of the main crystalline peaks ($2\theta_i$ = 17.14, 22.39, 23.59) and each peak height (ΔH_i) was calculated by subtraction of the eventual background noise:

$$\Delta H_i = (H_{2\theta i} - H_{2\theta o}) \qquad (2)$$

where $H_{2\theta i}$ is the total diffraction at the selected XRD angle i and $H_{2\theta o}$ is the signal intensity at the beginning of the pattern ($2\theta_o$=10.00). This methodology gave highly reproducible ΔH_i since XRD patterns performed on the same sample did not show significant variations.

Relative crystallinity (RC) is expressed as the mean value of the ratio between the height of peak i in the sample and the height of the same peak in the standard:

$$RC = \frac{1}{n}\sum_{i=1}^{n}\frac{\Delta Hi_{sample}}{\Delta Hi_{standard}} \qquad (3)$$

In general terms, the ideal standard should be the maximally crystallised sample. In the case of bread, the ideal standard would undoubtedly be the maximally staled bread, accounting for the specific crystalline structures formed upon staling. However, it is evident that the identification of the completely crystallised bread sample is rather difficult, if not impossible. In order to express the relative degree of crystallinity of pure starch during gelatinisation, some authors have suggested the use of unheated starch or flour, which have identical degrees of crystallisation (NARA *et al.*, 1978; VARRIANO-MARSTON *et al.*, 1980). The relative crystallinity of bread is thus expressed using flour as the reference standard.

The increase in crystallinity of bread crumb stored for up to 20 days at different temperatures is shown in Fig. 3. As

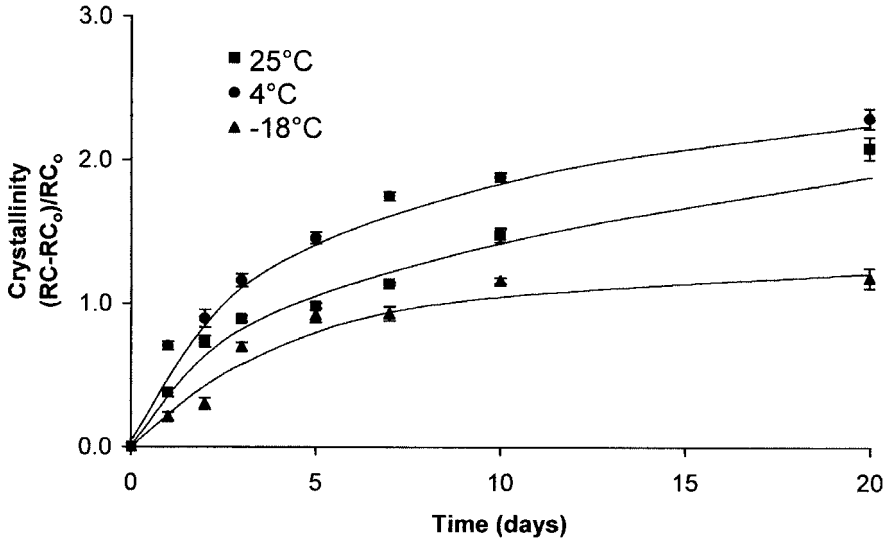


Fig. 3 - Crystallinity of bread crumb as a function of storage time at -18°, 4° and 25°C.

expected, crumb showed increasing crystallinity in the temperature order -18°<25°<4°C and a similar trend was also observed when considering firmness (Fig. 4). The changes in firmness were then plotted as a function of the increase in crystallinity (Fig. 5). Although there was a positive relationship between firmness and RC, the determination of bread crystallinity, only, did not allow firmness to be estimated by a simple model. Despite a slight increase in crystallinity, samples stored at -18°C showed similar firmness over time. Even if crystallinity can be considered one of the major causes of staling, it is not the sole predictive parameter of firmness since changes in other components and their interactions with starch, which are probably temperature dependent, may play an important role (ROGERS *et al.*, 1988; KROG *et al.*, 1989).

Further experiments were carried out by storing bread for up to 20 days at dif-

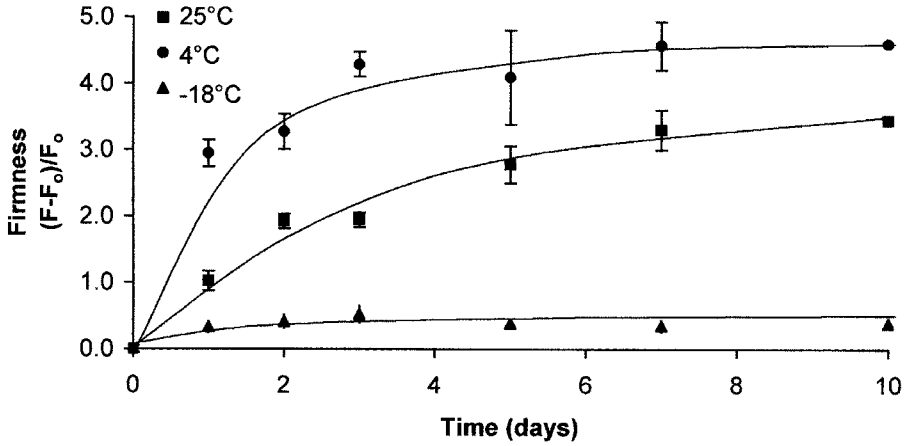


Fig. 4 - Firmness of bread crumb as a function of storage time at -18°, 4° and 25°C.

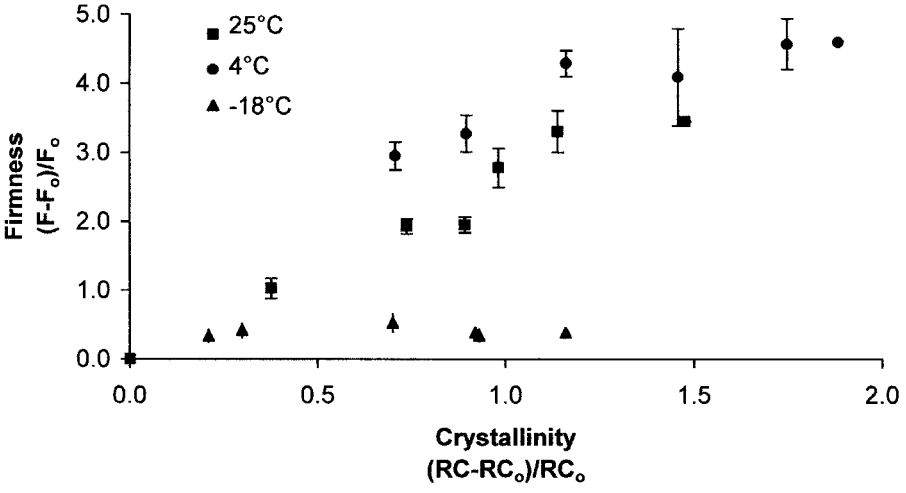


Fig. 5 - Firmness of bread crumb as a function of crystallinity.

ferent temperatures in sealed jars without removing the crust. Also in this case, crystallinity and firmness were determined (Figs. 6 and 7). While the changes in crystallinity appeared to be of the same order of magnitude as those observed for the crumb (Fig. 3), the increase in firmness was significantly higher in the case of bread stored with the crust. This difference was particularly evident for the samples stored at 4° and 25°C. As expected, crystallinity of bread stored at -18°C was lower than that at higher temperatures. In addition, bread samples stored at 4° and 25°C showed similar changes in crystallinity. Although no moisture changes were observed for the -18°C stored sample, water content of the crumb separated from the bread stored for 20 days at 4° and 25°C changed from 41.6% (w/w) to 28.9% and 17.3%, respectively. By comparing staling development in bread stored with crust (Fig. 7) with that observed during storage of bread crumb only (Fig. 4), it is evident that water mobility can greatly affect the development of firmness. These results are in agreement with previous

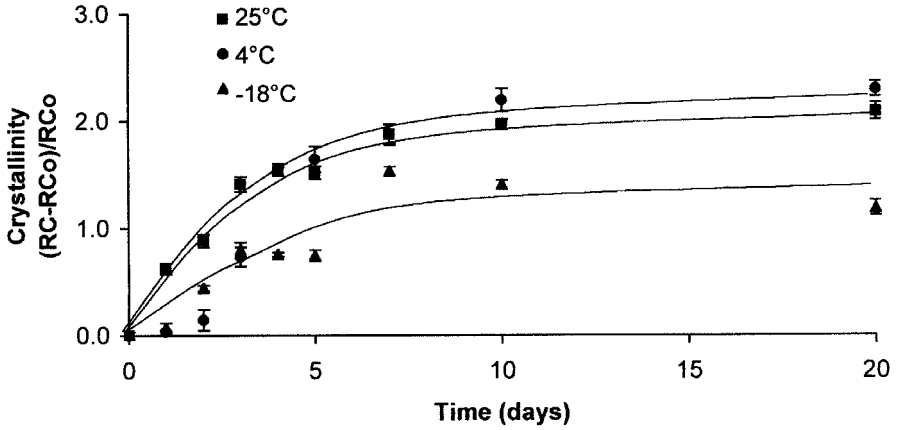


Fig. 6 - Crystallinity of bread as a function of storage time at -18°, 4° and 25°C.

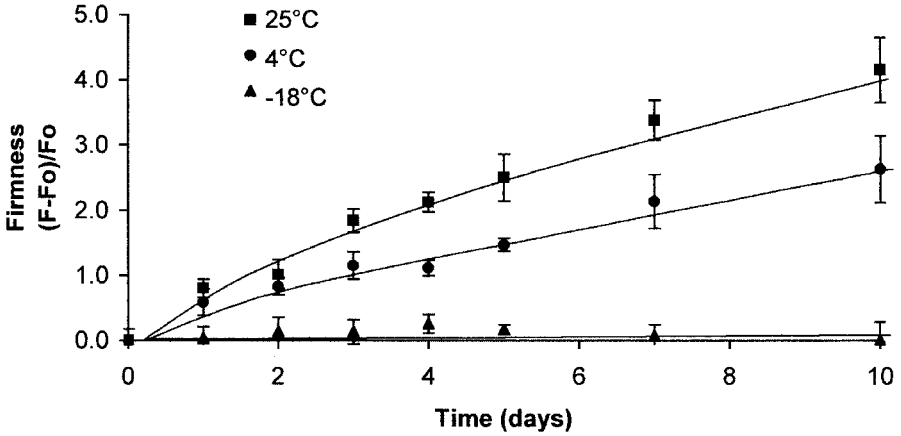


Fig. 7 - Firmness of bread as a function of storage time at -18°, 4° and 25°C.

data showing that firmness during bread staling increases with the degree of moisture loss from the crumb to the crust (BAIK and CHINACHOTI, 2000). Water migration from the crumb to the crust is expected to modify water activity and hence staling kinetics, shifting the maximum of crystallisation to higher temperatures (ROOS, 1995; ZELEZNAZ and HOSENEY, 1986; SLADE and LEVINE, 1989, 1991). The greater the decrease in moisture, the greater the increase in the temperature of maximum crystallisation. In the case of bread, dehydration which occurs during storage, can shift the maximum of crystallisation from refrigeration temperature to higher temperatures so that a maximum of firmness may not be detectable below room temperature. For this reason, it can be inferred that the high water content observed in bread crumb at 25°C counterbalanced the expected decrease in the rate of crystallisation, leading to changes in firmness which were higher than those detected at 4°C (Fig. 7).

When the increase in crystallinity during staling at different temperatures occurs in concomitance with moisture changes, firmness is likely to be the result of the combined changes in these parameters. For this purpose, a linear regression analysis of bread firmness as a function of crystallinity, moisture (M) and temperature (T) was performed. It was carried out to identify individual as well as interactive effects of the independent variables on firmness. However, the latter were found to be not significant, leading to the following equation:

$$\text{Firmness} = 3.12 + 0.74 \cdot \text{Crystallinity} - 0.08 \cdot M + 0.02 \cdot T \quad (r = 0.92,\ p < 10^{-6}) \qquad (4)$$

In our experimental conditions, firmness of bread slices stored with crust at different temperature was negatively affected by crumb moisture and positively affected by crystallinity and temperature, suggesting that these parameters exerted significant and independent effects on firmness. However, it must be noted that equation (4) does not account for the contribution of the crumb alveolar structure to firmness. In fact, while firmness was evaluated on bread slices, moisture and crystallinity were assessed on the ground crumb. Even with this limitation, such a relationship described well the changes in firmness observed as a consequence of the combined changes of moisture content and storage temperature in bread slices.

CONCLUSIONS

The results clearly demonstrate that important qualitative and quantitative information about the physico-chemical phenomena occurring during staling of bakery products can be obtained using XRD analysis. Qualitative data can be obtained by comparison of the total diffraction at specific 2-θ angles relevant to the main diffracted peaks. In contrast, quantitative information about complex matrices, such as bread, can be achieved by evaluating the height of the main diffracted peaks in relation to that of a reference sample. It must be noted that the selection of the proper reference is critical. In fact, in bread, as in most food polymers, the maximally crystallised sample, which is likely to be the ideal reference, is hardly identifiable and a careful choice is necessary.

The index of crystallinity can be used to follow the structural changes occurring during bread staling and, potentially, during a number of food processes in which changes from the amorphous to the crystalline state, or *visa-versa*, are involved. In the case of bread staling, a parallel increase in firmness and crystallinity was observed, but a simple mod-

el was not achieved. When moisture changes occurred during bread storage at different temperatures, the development of firmness was described well by the changes in crystallinity, moisture and storage temperature.

REFERENCES

AACC. 1983. "Approved Methods of the AACC". Method 79-09, Bread Firmness by Universal Testing Machine. American Association of Cereal Chemists, St. Paul, Minnesota, USA.

AOAC. 1995. "Official Methods of Analysis". Cunniff P. (Ed.). Association of Official Analytical Chemists International, Arlington, Virginia, USA.

Baik M.-Y. and Chinachoti P. 2000. Moisture redistribution and phase transition during bread staling. Cereal Chem.77: 484.

Biliaderis C.G. and Galloway G. 1989. Crystallization behavior or amylose-V complexes: structure-property relationships. Carbohydr. Res. 189: 31.

Champenois Y., Colonna P. Buleon A. Della Valle G. and Renault A. 1995. Gélatinisation et rétrogradation de l'amidon dans le pain de mie. Sci. Aliments 15: 593.

Chen P.L., Long Z., Ruan R. and Labuza T.P. 1997. Nuclear magnetic resonance studies of water mobility in bread during storage. Lebensm.-Wiss-u-Technol. 30: 178.

Colwell K.H., Axford D.W.E., Chamberlain N. and Elton G.A.H. 1969. Effect of storage temperature on the ageing of concentrated wheat starch gels. J. Sci. Food Agric. 20: 550.

Cooke D. and Gidley M.J. 1992. Loss of crystalline and molecular order during starch gelatinisation: origin of the enthalpic transition. Carbohydr. Res. 227: 103.

Czuchajowska Z. and Pomeranz Y. 1989. Differential scanning calorimetry, water activity, and moisture content in crumb center and near-crust zones of bread during storage. Cereal Chem. 66: 305.

Dragsdorf R.D. and Varriano-Marston E. 1980. Bread staling: X-ray diffraction studies on bread supplemented with α-amylases from different sources. Cereal Chem. 57: 310.

Grotenhuis E., Van Aken G.A., Van Malssen K.F. and Schenk H. 1999. Polymorphism of milk fat studied by differential scanning calorimetry and real-time X-ray powder diffraction. J. Am. Oil Chem. Soc. 76: 1031.

Hellman N.N., Fairchild B. and Senti F.R. 1954. The bread staling problem. Molecular organization of starch upon aging of concentrated starch gels at various moisture levels. Cereal Chem. 31: 495.

Hermans P.H. and Weidinger A. 1948. Quantitative X-ray investigations on the crystallinity of cellulose fibers. A background analysis. J. Appl. Phys. 19: 491.

Hoover R. and Senanayake N. 1996. Effects of sugars on the thermal and retrogradation properties of oat starches. J. Food Biochem. 20: 65.

Hoseney R.C. and Rogers D.E. 1993. Mechanism of sugar functionality in cookies. In "The Science of Cookie and Cracker Production". E. Faridi (Ed.), p.203. Chapman & Hall, New York, U.S.A.

Hoseney R.C. 1994. "Principles of Cereal Science and Technology", p. 29. American Association of Cereal Chemists, Inc., St. Paul, Minnesota, USA.

I'Anson K.J., Miles M.J., Morris V.J., Besford L.S., Jarvis D.A. and Marsh R.A. 1990. The effects of added sugars on the retrogradation of wheat starch gels. J. Cereal Sci. 11: 243.

Imberty A. and Perez S. 1988. A revisit to the three-dimensional structure of B-type starch. Biopolymers 27: 1205.

Imberty A., Chanzy H., Perez S., Buleon A. and Tran V. 1988. The double helical nature of A-starch. J. Mol.Biol. 201: 365.

Jeong O.K., Wan S.K. and Mal S.S. 1997. A comparative study on retrogradation of rice starch gels by DSC, X-ray and alpha-amylase methods. Starch/Staerke 49: 71.

Katz J.R. 1928. "A comprehensive Survey of Starch Chemistry". R.P. Walton (Ed.). Chemical Catalog Co. Inc., New York, U.S.A.

Katz J.R and van Itallie T.B. 1930. Abhandlungen zur physikalischen chemie der staerke und der brotbereitung. Zeit. Physik. A 150: 90.

Kerr R.W. 1950. "Chemistry and Industry of Starch" 2nd Ed. p. 159. Academic Press, Inc., New York.

Kim-Shin M.S., Mari F., Rao P.A., Stengle T.R. and Chinachoti P. 1991. ^{17}O Nuclear magnetic resonance studies of water mobility during bread staling. J. Agric. Food Chem. 39: 1915.

Krog N., Olesen S.K., Toernaes H. and Joensson T. 1989. Retrogradation of the starch fraction in wheat bread. Cereal Foods World 34: 281.

Kulp K. and Ponte J.G. 1981. Staling of white pan bread: Fundamental causes. Crit. Rev. Food Nutr. 15: 1.

Leung H.K., Magnuson J.A. and Bruinsma B.L. 1979. Nuclear magnetic resonance studies of water mobility in flour doughs. J. Food Sci. 44: 1408.

Lin W. and Lineback D.R. 1990. Changes in carbohydrate fractions in enzyme-supplemented bread and the potential relationship to staling. Starch/Staerke 42: 385.

Martin M.L., Zeleznak K.J. and Hoseney R.C. 1991. A mechanism of bread firming. I. Role of starch swelling. Cereal Chem. 68: 498.

Miles M.J., Morris V.J., Orford P.D. and Ring S.G. 1985. The role of amylose and amylopectin in the gelation and retrogradation of starch. Carbohydrate Res. 135: 271.

Morgan K.R., Ferneaux R.H. and Stanley R.A. 1992. Observation by solid-state -3C CP MAS NMR spectroscopy of the transformations of wheat starch associated with the making and staling of bread. Carbohydr. Res. 235: 15.

Nara S., Mori A. and Komiya T. 1978. Study on relative crystallinity of moist potato starch. Starch/Staerke, 30: 111.

Paris M., Bizot H., Emery J., Buzaré J.Y. and Buléon A. 1999. Crystallinity and structuring role of water in native and recrystallized starches by 13C CP-MAS NMR spectroscopy. 1: Spectral decomposition. Carbohydr. Polym. 39: 327.

Pisesookbunterng W., D'Apollonia B.L. and Kulp K. 1983. Bread staling studies. II. The role of refreshening. Cereal Chem. 60: 301.

Rogers D.E., Zeleanak K.J., Lai C.S. and Hoseney R.C. 1988. Effect of native lipids, shortenings, and bread moisture on bread firmness. Cereal Chem. 65: 398.

Roos Y.H. 1995. "Phase Transition in Foods". p. 235. Academic Press, Inc., San Diego, CA.

Roulet P.A., MacInnes W.M., Wuersch P., Sanchez R.M. and Raemy A. 1988. A comparative study of the retrogradation kinetics of gelatinised wheat starch in gel and powder form using X-rays, differential scanning calorimetry and dynamic mechanical analysis. Food Hydrocolloids 2: 381.

Schiraldi A., Piazza L. and Riva M. 1996. Bread staling: a calorimetric approach. Cereal Chem. 73: 32.

Schoch T.J. and French D. 1947. Studies on bread staling. I. The role of starch. Cereal Chem. 24: 231.

Seow C.C. and Teo C.H. 1996. Staling of starch-based products: a comparative study by firmness and pulsed NMR measurements. Starch/Staerke 3: 90.

Slade L. and Levine H. 1989. A food polymer science approach to selected aspects of starch gelatinization and retrogradation. In "Frontiers in Carbohydrate Research – 1: Food Applications". R.P. Millane, J.N. BeMiller and R. Chandrasekaran (Eds.). p. 215. Elsevier, London.

Slade L. and Levine H. 1991. Beyond water activity: Recent advances based on an alternative approach to the assessment of quality and safety. CRC Crit. Rev. Food Sci. Nutr. 30: 115.

Suryanarayanan R. 1995. X-ray powder diffractometry. In "Physical Characterisation of Pharmaceuticals Solids". H.G. Brittain (Ed.), p. 188. Marcel Dekker, Inc., NY.

Van Soest J.J.G., de Wit D., Wageningen H.T. and Vliegenthart J.F.G. 1994. Retrogradation of potato starch as studied by Fourier transform infrared spectroscopy. Starch 46: 453.

Varriano-Marston E., Ke V., Huang G. and Ponte J.Jr. 1980. Comparison of methods to determine strach gelatinization in bakery foods. Cereal Chem. 57: 242.

Winter W.T. and Kwak Y.T. 1987. Rapid-scanning Raman spectroscopy: a novel approach to starch retrogradation. Food Hydroc. 1: 461.

Wright A.J., McGauley S.E., Narine S.S., Willis W.M., Lencki R.W. and Marangoni A.G. 2000. Solvent effects on the crystallisation behaviour of milk fat fractions. J. Agric. Food Chem. 48: 1033.

Wu H.C. and Sarko A. 1978a. The double helical molecular structure of crystalline A-amylose. Carbohydr. Res. 61: 27.

Wu H.C. and Sarko A. 1978b. The double helical molecular structure of crystalline B-amylose. Carbohydr. Res. 61: 7.

Wu J.Y. and Eads T.M. 1993. Evolution of polymer mobility during ageing of gelatinized waxy maize starch: a magnetization transfer -1H NMR study. Carbohydr. Polym. 20: 51.

Zeleznak K.J. and Hoseney R.C. 1986. The role of water in the retrogradation of wheat starch gels and bread crumb. Cereal Chem. 63: 407.

Zobel H.F. 1964. X-ray analysis of starch granules. Methods Carbohydr. Chem. 4: 109.

Zobel H.F. 1992. "Developments in Carbohydrate Chemistry". R.J. Alexander and H.F. Zobel (Eds.) p. 1. American Association of Cereal Chemists, St. Paul, Minn.

Zobel H.F. and Kulp K. 1996. The staling mechanism. In "Baked Goods Freshness". Hebeda R.H. and Zobel H.F. (Eds.). p. 1. Marcel Dekker, Inc., New York, U.S.A.

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PAPER

CHARACTERISATION OF VOLATILE COMPOUNDS IN THREE ACETIFICATION BATTERIES USED FOR THE PRODUCTION OF "ACETO BALSAMICO TRADIZIONALE DI REGGIO EMILIA"

STUDIO DEI COMPOSTI VOLATILI IN TRE BATTERIE DI ACETIFICAZIONE PER LA PRODUZIONE DI ACETO BALSAMICO TRADIZIONALE DI REGGIO EMILIA

G. ZEPPA*, M. GIORDANO, V. GERBI and G. MEGLIOLI[1]
Dipartimento di Valorizzazione e Protezione delle Risorse Agroforestali, Sezione Industrie Agrarie, Università degli Studi di Torino, Via L. da Vinci 44, 10095 Grugliasco (To), Italy
[1] Istituto Tecnico Industriale di Stato "L. Nobili", Via Makallè 10, 42100 Reggio Emilia, Italy
* Corresponding author: Tel. +39 011 6708550, Fax +39 011 6708549, e-mail: giuseppe.zeppa@unito.it

ABSTRACT

In contrast to common vinegar Traditional Balsamic Vinegar is produced from acetic acid fermentation of cooked grape must in a "battery", a series of 5 or 7 wooden casks called "*vaselli*". The aim of this work was to study the effect of the acetification battery on the volatile components of the product obtained with the same acetification process. Then the volatile fraction of the vinegar from three acetification batteries operating under the same conditions but with different wooden casks and used

RIASSUNTO

A differenza dell'aceto comune l'Aceto Balsamico Tradizionale viene prodotto mediante la biossidazione acetica di mosto cotto in una "batteria", una serie di 5 o 7 botti di legno chiamate "vaselli". Lo scopo di questo lavoro è stato quello di studiare qual è l'effetto della batteria di acetificazione sui componenti volatili del prodotto finito mantenendo costante il processo di acetificazione. É stata quindi esaminata la frazione volatile di tre batterie di acetificazione operanti nelle stesse condizioni,

- Key words: Aceto Balsamico Tradizionale di Reggio Emilia, Maillard reaction, traditional balsamic vinegar, vinegar, volatile compounds -

for the production of Traditional Balsamic Vinegar (Aceto Balsamico Tradizionale di Reggio Emilia, Italy) was analysed. From among over one hundred detected compounds ketones, aldehydes, ethyl esters and acetates from alcoholic and acetic fermentation and the furan derivates from Maillard reactions, such as furfural, 5-methyl-2-furaldehyde, 5-hydroxymethylfurfural or HMF, 5-acetoxymethylfurfural and 2-furoic acid were the most abundant compounds identified. In every battery the compound concentrations produced during alcoholic fermentation decreased from the first barrel to the last barrel and, on the contrary, the concentration of derivatives of the acetic acid fermentation, the oxidative ageing and the Maillard reactions products increased from the first barrel to the fifth barrel. Also the wood used for manufacturing the casks and their age seems to have a significant effect on the aroma components.

ma costituite da differenti "vaselli" ed utilizzate nella produzione di Aceto Balsamico Tradizionale di Reggio Emilia. Fra gli oltre cento composti isolati i più abbondanti sono risultati essere i chetoni, le aldeidi, gli esteri etilici e gli acetati provenienti dalla fermentazione alcolica e dalla biossidazione acetica ed i derivati furanici provenienti dalle reazioni di Maillard quali il furfurale, il 5-metil-2-furfurale, il 5-idrossimetilfurfurale, il 5-acetossimetilfurfurale e l'acido 2-furoico. In tutte le batterie la concentrazione dei composti formatisi durante la fermentazione alcolica diminuisce passando dal primo all'ultimo "vasello", mentre al contrario quella dei derivati della biossidazione acetica, dell'invecchiamento ossidativo e delle reazioni di Maillard aumentano dal primo all'ultimo "vasello". Anche il tipo di legno utilizzato per la costruzione del "vasello" e la sua età sembrano avere grande effetto sui componenti della frazione volatile.

INTRODUCTION

Traditional balsamic vinegar is a special vinegar which dates back to the X-XI century and is produced exclusively in the regions of Modena and Reggio Emilia (Italy). According to Italian laws, traditional balsamic vinegar is made from the acetic acid fermentation of cooked grape must of Trebbiano, Spergola, Lambrusco and other local grapevine varieties. It must be aged at least 12 years in barrels of different sizes and wood, but it is possible to find vinegar aged 40-50 years and even up to 200 years. Unlike common vinegar it is not made from wine but grape must. The production process is very complex and often varies among producers. Generally after grape crushing, the seeds and the skins are separated and the must is concentrated by boiling at atmospheric pressure directly exposed to heat and reduced 20-30% of the starting volume. This cooked must is then transferred into a cask taking care to not exceed 80% of its capacity. In these casks the must undergoes partial alcoholic fermentation with an ethanol production of 40-70 g L^{-1}. After 5 to 6 months, the must is blended with wine vinegar containing a large number of acetic acid bacteria and then divided into 5 to 7 smaller casks called *vaselli*, again taking care not to exceed 80% of its capacity. The *vaselli* are made of different types of wood (oak, chestnut, mulberry or juniper) and come in different sizes.

Due to evaporation during ageing, the volume of the liquid decreases, and the *vaselli* are refilled using the "Solera" method. For example in a battery of five

vaselli, the fifth *vasello* (the last and smallest) is refilled from *vasello* number 4, then number 4 is refilled from number 3 and so on until *vasello* number 1 is refilled with fresh cooked must. These refills must be made once a year, in the spring, for the entire life of the battery. Only from the twelfth year onward, is it possible to take the finished product from the last *vasello* for consumption.

The battery needs a long time to consistently produce a balsamic vinegar with sufficiently stable composition and sensorial characteristics. Alcoholic fermentation occurs in the cooked must before blending it into the battery, while acetic acid fermentation usually takes place in the first two *vaselli*. In the others there is no longer any microbiological activity, but only chemical and enzymatic activities favoured by temperature changes that happen during the year in the storage places, usually lofts. The aroma of the product is also influenced by the cooking of the must that causes the development of Maillard reactions or non-enzymatic browning between the amino acids and the reducing sugars (GOGUS *et al.*, 1998). These reactions include the formation of many products most of which have an impact on the flavour and appearance of the vinegar.

The first step in the amino acid-reducing sugar reaction is a sugar-amine condensation in equimolar ratios leading to N-glycosylamines or N-fructosylamines. The isomerisation of these compounds leads to 1-amino-1-deoxy-2-ketoses or 1-amino-2-deoxy-2-aldoses also known as Amadori and Heyns intermediates which are essential precursors of heterocyclic compounds (VERNIN and PÁRKÁNYI, 1982).

Two traditional balsamic vinegars, *Aceto Balsamico Tradizionale di Modena* (ABTM) and *Aceto Balsamico Tradizionale di Reggio Emilia* (ABTRE) are available on the market. They differ due to the area of production and the grape used for the must production.

Many studies have been carried out on traditional balsamic vinegars (COPPINI *et al.*, 1978; TURTURA and ROSETTI, 1984; PLESSI *et al.*, 1988, 1989; STACCHINI *et al.*, 1990; TURTURA and BORTOLOTTI, 1991; GIUDICI, 1993; CORRADINI *et al.*, 1994; GIUDICI *et al.*, 1994; MASINI and GIUDICI, 1995; DEL SIGNORE, 1997; 1998; 2000; 2001a,b; MEGLIOLI *et al.*, 1997; ANKLAM *et al.*, 1998; CHIAVARO *et al.*, 1998; DEL SIGNORE *et al.*, 1998, 2000; MASCOLO and GAVIOLI, 1998; STANCHER *et al.*, 2001). These studies have shown that the chemical composition of traditional balsamic vinegar is highly variable and depends on the acetification process (type of grape must, cooking modality, temperature and humidity during aging) and the acetification battery it originates from (battery age, wood used for the *vaselli*, *vaselli* age). In fact, its acidity, expressed as acetic acid, is generally between 30 to 120 g kg^{-1}; the reducing sugars are between 100 to 500 g kg^{-1} and the ethanol is between 0.4 to 14 mL kg^{-1} (MEGLIOLI *et al.*, 1997; MASCOLO and GAVIOLI, 1998).

Among the volatile compounds, particular attention has been given to substances found exclusively in the finished product with the aim of determining possible commercial frauds: 5-acetoxy-methyl-2-furaldehyde, a characteristic substance present only in aged vinegars (GIACCIO *et al.*, 1996; GIACCIO and DEL SIGNORE, 1997), 5-hydroxymethylfurfural (THEOBALD *et al.*, 1998), ethyl acetate (STACCHINI *et al.*, 1990), and some principal volatile compounds (DEL SIGNORE, 2001b).

Since the chemical composition of balsamic vinegar is determined by the acetification process and the acetification battery the aim of this work was to study the effect of the acetification battery on the volatile compounds of the product obtained with the same acetification process. Then the volatile fraction of the vinegar from three acetification batteries with different wooden casks used for the production of ABTRE (*Aceto*

Balsamico Tradizionale di Reggio Emilia, Italy) was analysed.

MATERIALS AND METHODS

Samples

The studies in this field are very difficult because an acetification battery can produce balsamic vinegar only after 12 years as established by regulation (G.U., 1986; G.U., 1987) but only after at least 20 years is the vinegar production stable. Then the lengthy production times prohibit planning repetitive experimentation and the research on the trend of acetification can only be carried out on the battery already in production. In this study three acetification batteries (A, B and C) at the Reggio Emilia firm, each with 5 *vaselli* and initiated contemporarily 50 years ago were studied (Table 1). In batteries A and C almost all the *vaselli* were made of oak, while in battery B there were also chestnut and mulberry *vaselli.* Mulberry was also present in battery C and in both the batteries it was used for the 4th *vasello* required by tradition. The ages of the *vaselli* varied a lot. Batteries B and C were formed by *vaselli* that replaced the original ones in the 1980s. Battery A, instead, was formed largely by the original *vaselli* that were over 200 years old. Five 50 mL samples were obtained from each battery.

Chemical analysis

Acidity was determined according to the official Italian methods (MAF, 1986). The dry extract content was evaluated with a refractometer and is reported as °Brix.

Extraction of volatile compounds

The volatile compounds were extracted by reverse-phase Solid Phase Extraction with a C_{18} cartridge (DI STEFANO, 1991; KARAGIANNIS *et al.*, 2000). Internal standard (1-heptanol; 50 µL of a 1.7 g L^{-1} solution) was added to vinegar samples (10 g) and diluted up to 50 mL with deionised water. Each sample was poured into a 2 g C_{18} cartridge (Bakerbond SPE,

Table 1 - Type of wood used and age of casks and vinegar characteristics for each cask.

Battery	Barrel (*vasello*) code	Barrel wood	Barrel age	°Brix	Titrable acidity (acetic acid g kg^{-1})
A	1	Oak	1980	24.5	20.2
	2	Chestnut	1700	40.1	55.1
	3	Oak	1800	47.5	63.9
	4	Oak	1960	61.1	72.8
	5	Oak	1800	64.5	70.6
B	1	Oak	1980	32.0	15.1
	2	Chestnut	1980	33.0	29.9
	3	Chestnut	1980	37.5	51.8
	4	Mulberry	1980	45.5	70.2
	5	Oak	1980	58.5	70.8
C	1	Oak	1980	27.5	21.1
	2	Oak	1990	29.1	24.2
	3	Oak	1980	35.5	39.6
	4	Mulberry	1986	45.0	68.6
	5	Oak	1985	57.1	63.6

J.T. Baker, Deventer, Holland) previously activated with methanol (20 mL) and washed with water (20 mL). After washing with 30 mL of water, volatile compounds were eluted by gravity with 40 mL of dichloromethane (Merck, Darmstadt, Germany). The solution obtained was dried with anhydrous sodium sulphate then concentrated under nitrogen at room temperature to a final volume of 0.5 to 1 mL and injected. Three extractions were performed for each sample.

Gas Chromatography-Mass Spectrometry (GC-MS)

Compound identification was achieved with a Shimadzu GC-17A gas chromatograph (GC) coupled with a Shimadzu QP-5000 quadrupole mass spectrometer (Shimadzu, Tokyo, Japan). The GC was equipped with a J&W fused silica column (DB-WAX, 30 m x 0.25 mm i.d., 0.25 µm film thickness, J&W Scientific Inc., Folsom, CA, USA) and a split-splitless injector. The injected volume was 2 µL. The temperature program and the operating conditions were as follow: injector temperature 250°C; injection in splitless mode for 0.30 min and then split 1:60; carrier gas helium at 1 mL min^{-1}; the column was maintained at 35°C for 5 min then ramped at 2°C min^{-1} to 210°C and finally 10 min at 210°C.

Mass spectra were recorded in the electron impact mode at an ionisation voltage of 70 eV in the 29-250 amu mass range. The ion source and the interface were maintained at 220°C. Compound identification was carried out with the mass spectra and retention times of standard compounds, when available, or the NIST 12 and NIST62 mass spectral data base.

Quantitative measurements of each compound were carried out with a Varian GC-3400 gas chromatograph (Varian, Walnut Creek, CA, USA) equipped with a split-splitless injector and a flame ionisation detector held at 250°C. Data integration was performed with EZCHROM™ data system (Scientific Software Inc., San Ramon, CA, USA) with no consideration of extraction recoveries, i.e. calibration factors F=1.00 for all compounds. The column and the operating GC-FID chromatographic conditions were the same as GC-MS. Injections of standard compounds in the GC-FID system gave identical peak sequences as GC-MS.

RESULTS AND DISCUSSION

The increase in acidity in the three batteries was similar, but battery C had values slightly lower than batteries A and B (Table 1).

The dry extract content was very different in the three batteries reaching 64.5°Brix in battery A, but only 58°Brix in the other two. There was a noticeable increase of the °Brix values in batteries B and C from the *vaselli* made of mulberry to the *vaselli* made of oak. The mulberry, being more porous, facilitates evaporation and thus concentration of the product.

In the fifteen products studied, over one hundred volatile substances were detected (Table 2). They originated from alcoholic and acetic acid fermentations, Maillard reactions, pyrolysis reaction during cooking of the must, oxidative ageing, extraction from wooden barrels and enzymatic reactions that occur in balsamic vinegar production. In battery A, there were many more compounds than in batteries B and C and in greater quantities. The coefficient of variation of these compounds for each *vasello* varied from 2 to 19%.

In every battery, the concentrations of the compounds produced during alcoholic fermentation decreased from the first barrel (where the fresh cooked must was added) to the last barrel (where the balsamic vinegar was taken from). On the contrary, the concentration of the derivatives of the acetic acid fermenta-

Table 2 - Mean concentration (μg kg^{-1}; three injections) and retention time (min) of aroma compounds detected in the three batteries of Aceto Balsamico Tradizionale di Reggio Emilia (I) and calculated by GC-FID signals using 1-heptanol as internal standard. For the unknown and unidentified compounds the m/z value of the base peak is reported.

	Battery A	Rt (min)	Vasello				
			1	2	3	4	5
ketones							
	diacetyl*	5.6		2830	2289	1408	878
	2-pentanone	5.8	442				
	cyclopentanone	15.2	25				
	3-hydroxy-3methyl-2-butanone	19.4	344	238	200	144	167
	acetoin*	22.2	35799	37172	42940	24695	3089
	1-hydroxy-2-propanone	22.9	45	56	50		
	3-hydroxy-2-pentanone	25.8	524	392	350	249	204
	3,4-dimethyl-2,5-furandione	49.5		48		169	112
	1-ethoxy-2-heptanone	56.4	336	216			
	dihydro-2-methyl-3(2H)-furanone	20.7		54		89	195
	dihydro-2,5-furandione	71.2	481				
esters							
	ethyl isobutyrate	5.5		122			
	ethyl isovalerate*	9.5	188	175			
	ethyl pyruvate*	21.6	1487	638	67		
	ethyl lactate*	26.5	24842	13120	350	441	348
	ethyl glycolate	31.4	1351	5798	1042		
	ethyl 2-hydroxyisovalerate	31.8		74			
	ethyl 3-hydroxybutyrate	37.8	434	262			
	diethyl succinate*	47.4	8371	2847			
	ethyl benzeneacetate	53.2	285	1000			
	monoethyl succinate	83.1	38830	35582	9400	2005	3107
	diethyl malate	67.2	15020	8408	484		521
	diethyl tartrate	80.6	1817	1988	530		
acetates							
	isobutyl acetate*	7.1		271			
	1,3-propanediol, monoacetate	51	450	761	1625	2602	5370
	1,3-propanediol, diacetate	46.2				249	592
	isopentyl acetate*	12.3	517	758			15
	2-ethoxyethyl acetate	23.4				232	418
	meso 2,3-butanediol, diacetate	29	283	1874	2360	2200	2121
	1,2-ethanediol, diacetate	34.4					394
	dl 2,3-butanediol, monoacetate + isobutyric acid*	41	8366	16893	30052	33364	32242
	meso 2,3-butanediol, monoacetate	41.8		6828	15490	22074	21872
	2-phenylethyl acetate	54.9	tr	1182	1162	1323	2040
	1,2,3-propanetriol, triacetate	68.9				327	690
	1,2-ethanediol, monoacetate	45.1	22	288	733	1270	689
	1,2,3-propanetriol, diacetate (isomer 1)	76.5	191	4074	17956	25809	48907
	1,2,3-propanetriol, diacetate (isomer 2)	77.9		1210	5520	9345	15523
	1,2,3-propanetriol, monoacetate	78.6		2008	6600	7352	10304
aldehydes							
	2-methyl-pentanal	6.2	688	745	747		471
	benzaldehyde*	37.2		48			10
alcohols							
	1-propanol*	8.3	1471	422			

	Battery A	Rt (min)	Vasello				
			1	2	3	4	5
	2-methyl-1-propanol	10.9	17326	3644	121		
	1-butanol*	13.9	445	131	tr		
	trans 3-penten-2-ol*	15.5			65		
	3-methyl-1-butanol*	17.9	60603	12372	1257	284	240
	2-ethoxy-ethanol	18.4		102	270	2153	2175
	2-hexanol	24	1295	1137	1018	1171	834
	2-heptanol	25.5	100				
	1-hexanol*	27.5	732				
	3-ethoxy-1-propanol*	28.8	1839	1464	277	122	164
	cis-3-hexen-1-ol*	29.4	39				
	2-butoxyethanol + unknown (*m/z* 54)	30.3				219	263
	dl 2,3-butanediol*	39.6	10219	13128	20554	20063	9260
	1-acetoxy-2-propanol	41.6	147	1703	15490	7513	8179
	meso 2,3-butanediol*	42	2501	3688	4772	5922	4185
	2-acetoxy-1-propanol	44.2		785	1624	3047	4236
	butoxyethoxyethanol	54.1				732	
	benzyl alcohol*	58.4	347				
	2-phenyl-ethanol*	60.3	19113	14721	13749	9339	10550
furan derivates							
	furfural* + unknown (*m/z* 45)	33.9		4262	5411	8253	11763
	5-methyl-2-furfural	40.7	47	903	724	743	1223
	methyl 3-furoate	64.6	266	2158	4720	5120	9078
	5-acetoxy-methyl-2-furaldehyde	74.3		3920	7068	10294	28755
	2-furoic acid	85.2	122	985	216	979	1559
	5-(hydroxymethyl)-2-furaldehyde	88.8	11349	164529	357213	305083	287233
	2-furyl methyl ketone + unknown (*m/z* 44)	36.4	15385	8332	2453	2534	
	2-furyl methyl ketone	36.4					2025
enolic derivates							
	cyclotene*	62.3	405	1770	3264	4748	2953
	maltol*	62.8		194	358		1021
	5-hydroxy-maltol	78.7	138	6314	14335	9383	11482
lactones							
	α-angelica lactone	31.6		49	59	61	114
	γ-methyl-γ-butyrolactone	42.4	721	560	92		435
	γ-butyrolactone*	43.5	13484	12933	18679	17728	9498
	γ-caprolactone	47.9				136	tr
	γ-crotonolactone	50.8		182	392	711	302
	γ-ethoxybutyrolactone	49.4	18				
	α-methyl-γ-crotonolactone	58		56	100	104	163
	cis-β-methyl-γ-octalactone	62	77			222	
	dehydromevalonic lactone	64.8	tr	2070	8415	9509	21763
	pantolactone	66.1	764	1384	1670	1674	1699
	solerone	67.4	tr	54	442	353	816
miscellaneous							
	ethyl 4-hydroxynonanoate	68.4	167				
	ethyl 4-hydroxydecanoate	73	361	169			
	2-methyl-1,3-dioxane	9.1	191				
	2,2,4,5-tetramethyl-1,3-dioxolane	37.4				79	129
	1,3,5-trioxane	14.6	10				
	trans furan linalool oxide*	32.8	149	207	223	153	223

	Battery A	Rt (min)	Vasello				
			1	2	3	4	5
	cis furan linalool oxide*	34.7		108	106	78	122
	cis pyran linalool oxide + unknown (*m/z* 95)	52.5				185	128
nitrogen compounds							
	N-ethylacetamide	45.6	186	308	286	288	
	N-amylacetamide	58.4			844		1097
	N-amylacetamide + benzyl alcohol*	58.4		734		700	
	2-pyrrolidinone	66.5		149	207	204	367
	1-methyl-1H-pyrrole-2-carboxaldehyde	69.8		168			66
	1H-pyrrole-2-carboxyaldehyde + allomaltol	65.6		tr		tr	848
hydrocarbons							
	undecane	11			74		669
	dodecane	17		46	148		
	tridecane	23.6	240	195	456		
fatty acids							
	acetic acid*	33.4	773	2328	9400	2282	1906
	propanoic acid*	39	584	1067	1436	1564	250
	butanoic acid*	44.6	1197	1016	1103	1418	1571
	isovaleric acid*	47	9741	18926	24517	17787	19972
	2-butenoic acid	52.8	35	101	153	580	213
	hexanoic acid*	57	1589	1677	1820	1201	1599
	2-ethylhexanoic acid	62.6				701	
	octanoic acid*	68.1	130	700	430	189	523
	benzoic acid*	64.8	62	490	223		935
sulfur compounds							
	methionol	49.7	33				
phenols							
	2-methoxy-phenol	57.4				142	122
	phenol	65.1	96				
	4-ethyl-phenol*	73.5	83				
	syringol	77.5		260			7974
	tyrosol	83.9	963	4219	4081	13431	2187
unidentified compounds							
	m/z 75	7.8	8869	10498	24844	26315	17398
	m/z 43	16.4	388	476			
	m/z 45	27		439	290	238	
	m/z 73	31.3				433	295
	m/z 43	33	1072	1305	808	783	816
	m/z 43	33.2	1081	1827	1840	1346	1283
	m/z 45	33.7	1489				
	m/z 43	36				232	444
	m/z 43	38.4			131	118	598
	m/z 43	38.8	29	424			82
	m/z 45	42.4	12581	6882			
	m/z 45	44.8				93	99
	m/z 43	45.6					823
	m/z 43	47.3				573	399
	m/z 45	46.6					2038
	m/z 43	53.2			740	580	845
	two compounds with *m/z* 45 and *m/z* 41	55.7	10551	4389	1550	2176	3299

	Battery A	Rt (min)	Vasello				
			1	2	3	4	5
	m/z 76	55.1	392	1387	1216	936	1191
	m/z 43	57.3	658	1076	354		
	m/z 43	58.8				556	1037
	m/z 183	59.1		85	253	207	399
	m/z 97	61		351	278	175	482
	m/z 124	63.3		97	571	233	270
	m/z 45	67.9					778
	m/z 45	68.3					675
	m/z 43	69.3	2129	2864	928		
	m/z 45	71	8607	4382	237		
	m/z 111	72.8	3571	708			
	m/z 43	74.1	1260				
	m/z 55	71.9				168	349
	m/z 55	72.2				115	313
	m/z 126	73.8				162	673
	m/z 85	76	139				
	m/z 43	75.3				1123	7595
	m/z 45	75.1	19273	15502			
	m/z 97	82.6	321	68			
	m/z 140	89	42			145	8844
	m/z 45 (compound A)	80	1550	5661	12012	13856	17434
	m/z 45 (compound B)	82.4	1522	2360	2740	2465	3283
	Battery A						
ketones							
	2-pentanone	5.8	526				
	cyclopentanone	15.2	44				
	3-hydroxy-3-methyl-2-butanone	19.4	207	174	164	70	86
	acetoin*	22.2	13611	39262	29409	130	143
	3-hydroxy-2-pentanone	25.8	156	320	383		
	1-hydroxy-2-butanone	27.9		27	24		
	4-cyclopentene-1,3-dione	41.2	102	18			
	1-ethoxy-2-heptanone	56.5	539	537	292		
esters							
	ethyl isovalerate*	9.5	50	88	55		
	ethyl caproate*	19.2	27				
	ethyl pyruvate*	21.6	1112	789	272		
	ethyl lactate*	26.5	10366	7509	4987	344	79
	ethyl glycolate	31.4	2083	4471	3047		
	ethyl 2-hydroxyisovalerate	31.8		34			
	ethyl 3-hydroxybutyrate	37.8	370	208			
	ethyl laevulinate	43.3	362	547			
	diethyl succinate*	47.4	5150	3180	821		
	diethyl malate	67.2	14414		1727	200	
	diethyl malate + solerone	67.2		13184			
	diethyl tartrate	80.6	1457	2158	496	384	149
	monoethyl succinate	83.1	42325	35052	16629	5486	2059
	monoethyl malate	87.2	992	958	403		
	ethyl benzeneacetate + unknown (*m/z* 45)	53.2		482			

	Battery B	Rt (min)	Vasello				
			1	2	3	4	5
acetates							
	isobutyl acetate*	7.1	118	157			
	butyl acetate*	9.7		26		56	
	isopentyl acetate*	12.3	438	399	200		
	2-ethoxyethyl acetate	23.4					420
	2,3-butanediol, diacetate	29	158	947	1919	2426	2549
	1,2-ethanediol, diacetate	34.4				81	121
	dl 2,3-butanediol, monoacetate	42	1119	tr		15327	24205
	dl 2,3-butanediol, monoacetate + isobutyric acid*	41			16538		
	meso 2,3-butanediol, monoacetate	41.8	4759	2599	6290	10293	18351
	1,2-ethanediol, monoacetate	45.1		161	424		
	1,3-propanediol, monoacetate	51	278	285	713		
	1,3-propanediol, diacetate	46.2				99	
	2-phenylethyl acetate*	54.9		119	795	1289	1544
	1,2,3-propanetriol, triacetate	68.9				371	
	1,2,3-propanetriol,diacetate (isomer 1)	76.5	938	1013	5123	9455	15577
	1,2,3-propanetriol,diacetate (isomer 2)	77.9	242	321	1571	3789	6914
	1,2,3-propanetriol, monoacetate	78.6	215	1098	3100		
alcohols							
	tert-pentyl alcohol	7.3		1000	1039		
	1-propanol*	8.3	1552	869	106		
	2-methyl-1-propanol	10.9	8640	4392	658		
	1-butanol*	13.9	471	159			
	trans-3-penten-2-ol*	15.5	138	142	142	949	1469
	3-methyl-1-butanol*	17.9	38855	15977	2917	420	173
	2-ethoxy-ethanol	18.6	106	41	197		
	2-hexanol	24	1451	1210	1188	1060	1371
	3-methyl-2-buten-1-ol	25.2				164	243
	1-hexanol*	27.5	293	151			
	3-ethoxy-1-propanol*	28.8	868	1339	744	162	83
	2-butoxyethanol	30.3	62	133	164		301
	dl 2,3-butanediol*	39.6	8098	12237	13323	538	637
	1-acetoxy-2-propanol	41.6	447	698	1851		
	meso 2,3-butanediol*	42	1511	3202	3606		
	2-acetoxy-1-propanol	44.2		271	732		
	α-terpineol*	48.7	tr				
	butoxyethoxy ethanol	54.1	727	918	1020		
	benzyl alcohol*	58.4	186				
	2-phenyl-ethanol*	60.3	12406	11700	10616	11089	10092
	1-dodecanol	63.7				83	
	octadecanol	71.5				1056	1035
aldehydes							
	hexanal*	10				22	
	2-methyl-crotonaldehyde	10.4		35			
	heptanal*	15.8				34	
	octanal*	22.6				91	
	nonanal*	29.5				69	
	benzaldehyde*	37.2		24	32		
	4-hydroxy-benzaldehyde	86.8			197		

	Battery B	Rt (min)	Vasello				
			1	2	3	4	5
furan derivates							
	furfural*	33.9	100	929	3608	1020	1744
	2-furaldehyde ethylmethyl acetal	54.3	317				
	methyl 3-furoate	64.6	794	1675	2237		
	2-furyl methyl ketone	36.4				395	448
	2-furyl methyl ketone + unknown (*m/z* 44)	36.4	6848	8690	2573		
	5-methyl-2-furfural	40.7	368	1071	1126	633	
	5-acetoxy-methyl-2-furaldehyde	74.3			4270	5948	10857
	2-furoic acid	85.2	114	925	933		
	5-(hydroxymethyl)-2-furaldehyde (HMF)	88.8	51286	150464	197559	4476	7164
enolic derivates							
	cyclotene*	62.3		1537	2668	21	36
	maltol*	62.8				497	645
	allomaltol	65.6				208	113
	5-hydroxy-maltol	78.7	621	3439	11952	7930	6509
lactones							
	5-methyl-2(3H)-furanone (α-angelica lactone)	31.6	32	40	41		
	γ-methyl-g-butyrolactone	42.4	605	582	307		
	γ-butyrolactone*	43.5				87	90
	γ-butyrolactone* + unknown (*m/z* 43)	43.5	7084	9730	12047		
	γ-crotonolactone	50.8		85	240		
	α-methyl-γ-crotonolactone	58			75		
	cis-β-methyl-γ-octalactone	62					106
	solerone	67.4			355		
	pantolactone	66.1	559	1343	1521		
miscellaneous							
	1,3,5-trioxane	14.6			28		
	trans furan linalool oxide*	32.8	127	142	187	249	251
	cis furan linalool oxide*	34.7	39	32	66		
	ethyl 4-hydroxydecanoate (*m/z* 85)	73	313	221			
nitrogen compounds							
	N-ethylacetamide	45.6	349	138	301		
	N-amylacetamide + benzyl alcohol*	58.4		363	580	1785	1862
	phenyl carbamate	65.1			682	151	13
hydrocarbons							
	undecane	11				50	
	dodecane	17	88	36	136		
	tridecane	23.6	241	142	406	130	136
fatty acid							
	acetic acid*	33.4	820	1925	3663	903	1504
	propanoic acid*	39	274	958	1278		
	isobutyric acid*	41	4360	9748			
	butanoic acid*	44.6	649	729	1044	97	154
	isovaleric acid*	47	2239	12179	21122	22755	24346
	valeric acid*	51				245	
	2-butenoic acid	52.8		48	128		
	hexanoic acid*	57	1161	1203	1795	2059	1668
	2-ethylhexanoic acid	62.6	606	814	974		
	octanoic acid*	68.1	456	551	484	505	332
	homovanillic acid	81	150				
	benzoic acid*	84.8	86	288	703	697	644

	Battery B	Rt (min)	Vasello				
			1	2	3	4	5
phenols							
	syringol	77.5		148		1151	2005
	tyrosol	83.9	1439	59	1511		
	p-guaiacol	57.3				71	22
unidentified compounds							
	m/z 75	7.8	6258	9962	10255	10532	36828
	m/z 43	16.4	1262	1147	490		
	m/z 43	33.1	177	771	572	2304	1045
	m/z 43	33.2	352	1024	1047	903	2009
	m/z 45	33.8	435	379			
	m/z 47	36.7	54	76			
	m/z 97	51.4	2073	257			
	m/z 87	55	906				
	m/z 76	55.2	222	535	753	720	599
	m/z 45	55.8	4293	4061	1309		
	m/z 43	57.4	798		947		
	m/z 97	61.1	111	371	408	343	246
	m/z 124	63.3	44	104	463		
	m/z 43	69.6	2105		3435		
	m/z 45	71	1202	5518	840		
	m/z 111	72.9	3962	659			
	m/z 45	75.2	33826	21692	4610		
	m/z 85	75.7	626				
	m/z 45 (compound A)	80	806	2248	6481		
	m/z 45 (compound B)	82.4	1177	2601	2020		
	m/z 43	87.4	1582	2712			
	m/z 41	88.7	114			144	395
	Battery C						
ketones							
	3-hydroxy-3-methyl-2-butanone	19.4	88	105	19		84
	acetoin*	22.2	316	300	173	527	105
	1-ethoxy-2-heptanone	56.4	599	492	451		
	4-hydroxy-2-methyl-acetophenone	74.2	921	tr			
esters							
	ethyl isobutyrate*	5.5		256	164		
	ethyl isovalerate*	9.5		161	148		
	ethyl lactate*	26.5	3114	3237	3752	3231	230
	ethyl 2-hydroxyisovalerate	31.8		42	23		
	ethyl 3-hydroxybutyrate	37.8		438	126		
	ethyl laevulinate	43.3	108	492	456		
	diethyl succinate*	47.4	1976	3833	1660	450	
	ethyl benzeneacetate + unknown (*m/z* 45)	53.2			394	324	
	diethyl malate	67.2	8040	19841	9841	1432	133
	diethyl tartrate	80.6	762	2446	1476	439	
	monoethyl succinate	83.1	19763	39746	31820	9477	3598
acetates							
	isobutyl acetate*	7.1		267	236		
	isopentyl acetate*	12.3	147	632	586	212	

	Battery C	Rt (min)	Vasello				
			1	2	3	4	5
	2-ethoxyethyl acetate	23.6					78
	2,3-butanediol, diacetate	29	99	353	1036	1878	2421
	1,2-etanediol,diacetate	34.4					58
	dl 2,3-butanediol, monoacetate	41	1625	3113	6425	17685	22745
	meso 2,3-butanediol, monoacetate	41.8	667	1249	2969	9041	16048
	1,3-propanediol,diacetate	46.2					163
	2-phenylethyl acetate*	54.9			740	790	1142
	1,2,3-propanetriol, triacetate	68.9					193
	1,2,3-propanetriol,diacetate (isomer 1)	76.5	891	tr	1189	6746	14720
	1,2,3-propanetriol,diacetate (isomer 2)	77.9	190		238	1671	5838
alcohols							
	tert-pentyl alcohol	7.3			97		
	2-methyl-1-propanol	10.9	3218	2675	1197		
	1-butanol*	13.9	151	160	43		
	trans-3-penten-2-ol*	15.5	1527	1077	1384	1582	1196
	3-methyl-1-butanol*	17.9	51602	33280	15598	3838	263
	2-hexanol	84	1489		1426	1334	1195
	2-heptanol	25.5	235				
	3-methyl-2-buten-1-ol	25.2	228				
	1-hexanol*	27.5	285	175			
	3-ethoxy-1-propanol*	28.8	635	798	767	480	137
	dl 2,3-butanediol*	42	465	641	428	736	514
	1-acetoxy-2-propanol	41.6					160
	2,5-hexanediol	57.5	60	145			
	2-phenyl-ethanol*	60.3	11870	13212	11580	9936	9607
aldehydes							
	4-hydroxy-benzaldehyde	86.8	tr				
	2,4-dihydroxy-benzaldehyde	85.6				11359	
furan derivates							
	furfural*	33.9	57	21	563	1533	1817
	2-furyl methyl ketone	36.4	109		278	281	268
	5-methyl-2-furfural	40.7	351	128	1112	763	567
	2-acetyl-5-methyl-furan	41.4		31			
	5-acetoxy-methyl-2-furaldehyde	74.3			1676	3237	7284
	2-furaldehyde ethylmethyl acetal	54.3	487				
	5-(hydroxymethyl)-2-furaldehyde	88.8	4131	4305	7720	16845	12067
enolic derivates							
	maltol*	62.8					475
	allomaltol	65.6					153
	5-hydroxy-maltol	78.7	95	1202	2527	5635	7047
lactones							
	γ-methyl-γ-butyrolactone	42.4		20			
	δ-valerolactone	42.6		82			74
	cis-β-methyl-γ-octalactone	62		85	114		124
	dehydromevalonic lactone	64.8				210	659
miscellaneous							
	ethyl 4-hydroxydecanoate (*m/z* 85)	73	186	372	118		
	trans furan linalool oxide*	32.8		158	175	185	197
	cis furan linalool oxide*	34.7	35	75	59	98	146

Battery C	Rt (min)	Vasello				
		1	2	3	4	5
nitrogen compounds						
N-amylacetamide	58.4		401	715	1028	1426
phenyl carbamate + (amine) *m/z* 44	65.1			94		
phenyl carbamate	65.1					117
hydrocarbons						
tridecane	23.6	166	108	97		79
fatty acids						
2-hydroxy-2-methyl propanoic acid	22.7					125
acetic acid*	33.4	178	165	475	3328	766
butanoic acid*	44.6	91	146	152	246	147
isovaleric acid*	47	3462	7537			
isovaleric acid* + 2-methyl butanoic acid	47			15722	24103	20776
hexanoic acid*	57	902	1043	1200	1311	1465
octanoic acid*	68.1	147	318	388	213	281
benzoic acid*	84.8	441	50	225		
phenols						
syringol	77.5		98	72		1689
tyrosol	83.9		11514	3265	11468	9843
unidentified compounds						
m/z 75	7.8	41763	15953	26228	57312	20511
m/z 43	33	406	796	618	604	967
m/z 43	33.2	583	1107	992	1254	2015
m/z 45	42.6	881	1464	559	260	
m/z 97	51.4	1379	1420	149		
m/z 87	54.9	608	1110			
m/z 76	55.1	15	311	355	488	633
m/z 69	55.8					954
m/z 76	59.8					95
m/z 97	61.1		183	467	263	160
m/z 44	65.1		139			
m/z 109	66		469			
m/z 43	66.6		283			
m/z 45	67.9					444
m/z 45	68.4					337
m/z 85	68.3	222	268			
m/z 45	71	3309	6451	3163	494	
m/z 111	72.8	11447	4198	325		
m/z 45	75.1	5209	8294	3912		
m/z 85	75.6	318	563	384		
m/z 125	89				6233	1121
m/z 120	89.2	4321	451	889		

(tr - trace level ≤10 μg kg^{-1}; * identified also by comparing the retention time with those of the standard compound).

tion, the oxidative ageing and the Maillard reaction products increased from the first to the fifth barrel. This was particularly evident in battery A.

The volatile compounds were subdivided into 15 groups: ketones, ethyl esters, acetates, aldehydes, alcohols, furan derivatives, enolic derivatives, lactones, nitrogen compounds, hydrocarbons, fatty acids, sulphur compounds, phenols, miscellaneous and unidentified compounds.

There were few ketones and they generally decreased from the first to the fifth barrel. Only acetoin was very

abundant and increased until *vasello* 3. This compound is initially produced by alcoholic fermentation (DUBOIS, 1994), it then increases due to the transformation of acetic acid bacteria of lactate, α-acetolactate and 2,3-butanediol (ASAI, 1968).

3-hydroxy-2-pentanone has already been found in vinegars and may be derived from the reduction of 2,3-pentanedione by yeasts (CHARLES *et al.*, 2000).

Diacetyl was present only in battery A. This compound may be produced either by the fragmentation of rearranged Amadori and Heyns intermediate compounds derived from the Maillard reactions (VERNIN and PÁRKÁNYI, 1982) or by the acetic acid bacteria from acetoin (ASAI, 1968). The fragmentation of rearranged Amadori and Heyns intermediate compounds leads to other α-dicarbonyl compounds such as 1-hydroxy-2-propanone (acetol) (VERNIN and PÁRKÁNYI, 1982) which are also present in the volatile fraction of ABTRE.

The ethyl esters were abundant only in the first three *vaselli* and were generally absent in the fourth *vasello* due to the shift in esterification equilibrium caused by the small amount of ethanol and probably to volatilisation. Monoethyl succinate was an exception since it was also present in *vasello* n. 5.

The acetates increased from the first to the fifth *vasello* due to the increase in esterification during ageing, by reaction of acetic acid with mono-, di- and polyhydroxy alcohols, which then generally decreased from the first to the fifth *vasello*, and which had already disappeared in barrel n. 3 (Fig. 1a).

Some acetates which have already been found in wine vinegars (CHARLES *et al.*, 2000) are derived from the reaction between acetic acid and some diols (1,3-propanediol, 2,3-propanediol and 2,3-butanediol). Due to the long ageing of traditional balsamic vinegar, esterification is however more forced than in wine vinegar and forms large amounts of mono-, di- and tri-acetates.

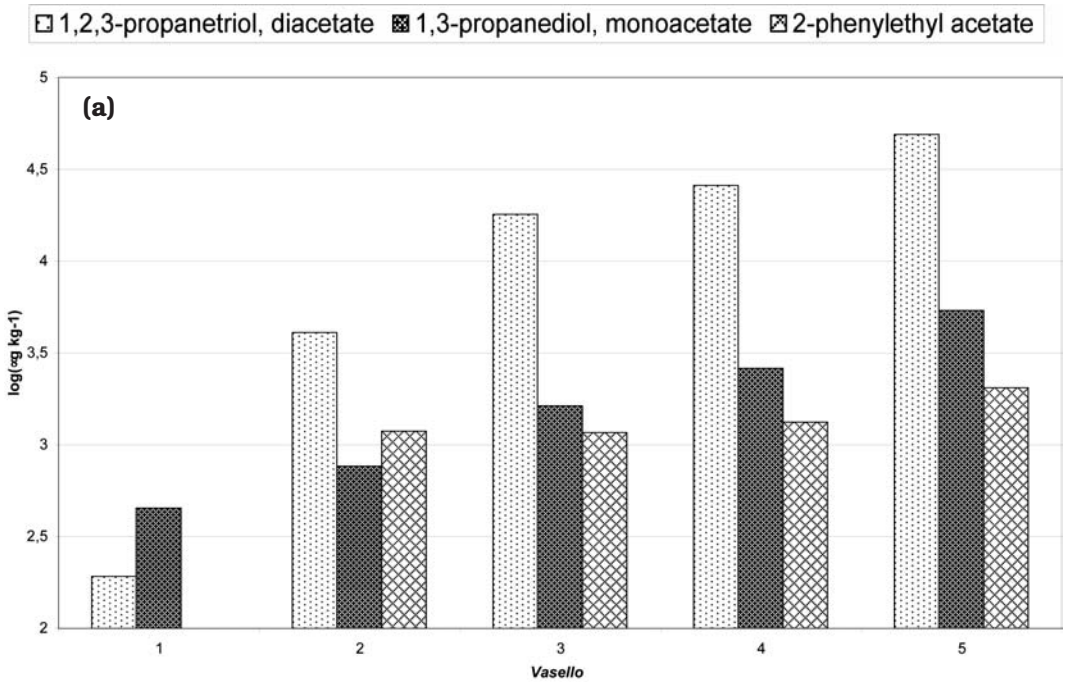
Two isomers of 1,2,3-propanetriol diacetate were also found. As indicated by CHARLES *et al.* (2000), they are formed by reaction between glycerol and acetic acid. It may hypothesized that the compound labelled "isomer 1" in Table 2 is the 1,3-diacetate isomer, while "isomer 2" is the 1,2-diacetate isomer. In fact, isomer one is more abundant in the three batteries, with respect to the second because it has less steric hindrance. Some aldehydes present in battery A could be derived from the corresponding alcohols by oxidation.

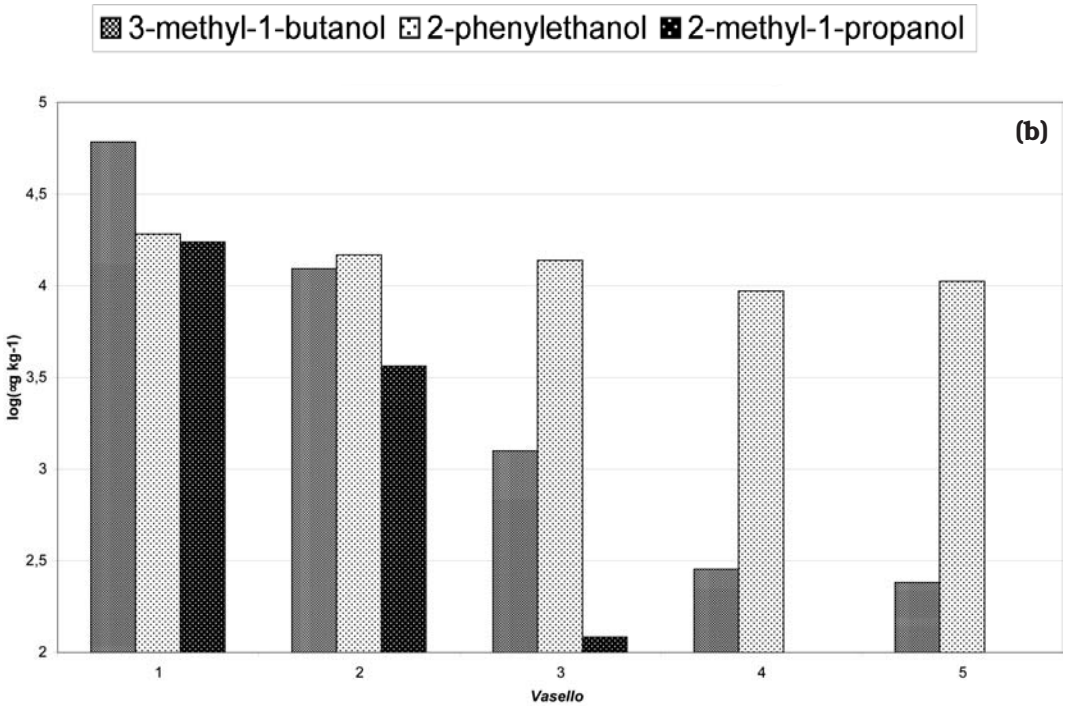
The alcohols generally decreased from the first to the fifth *vasello* in all batteries (Fig. 1b). 2-methyl-1-propanol and 3-methyl-1-butanol have already been identified in wine vinegars (CHARLES *et al.*, 2000) and their concentration decreases as acetic acid fermentation progresses. It is interesting to note the presence of phenylethyl alcohol also found in wine vinegars (CHARLES *et al.*, 2000) and in many products obtained from fermentation (wine, beer, cider, port, sherry, etc.) as by-products of alcoholic fermentation. Its production is due to amino acid transformation and mainly to phenyl-alanine deamination (Ehrlich reaction).

2,3-butanediol was very abundant and increased until *vasello* n. 3 due to the alcoholic fermentation of sugar by yeasts.

1-acetoxy-2-propanol and 2-acetoxy-1-propanol have not yet been reported. The former is more common in esterification due to steric action.

Furan derivatives partially produced during the Maillard reactions are characteristic of these vinegars, produced with cooked must (Fig. 1c). The most important ones are furfural from pentose sugars, 5-methyl-2-furaldehyde and 5-hydroxymethylfurfural (HMF) from hexose sugars, 5-acetoxymethylfurfural from the esterification between acetic acid and HMF and 2-furoic acid from ascorbic acid (VERNIN and PÁRKÁNYI, 1982). The most important is HMF (THEOBALD *et al.*, 1998), also found in sweet


1,2,3-propanetriol, diacetate
1,3-propanediol, monoacetate
2-phenylethyl acetate
(a)
log(=g kg-1)
5
4,5
4
3,5
3
2,5
2
1
2
3
4
5
Vasello


3-methyl-1-butanol
2-phenylethanol
2-methyl-1-propanol
(b)
log(=g kg-1)
5
4,5
4
3,5
3
2,5
2
1
2
3
4
5
Vasello

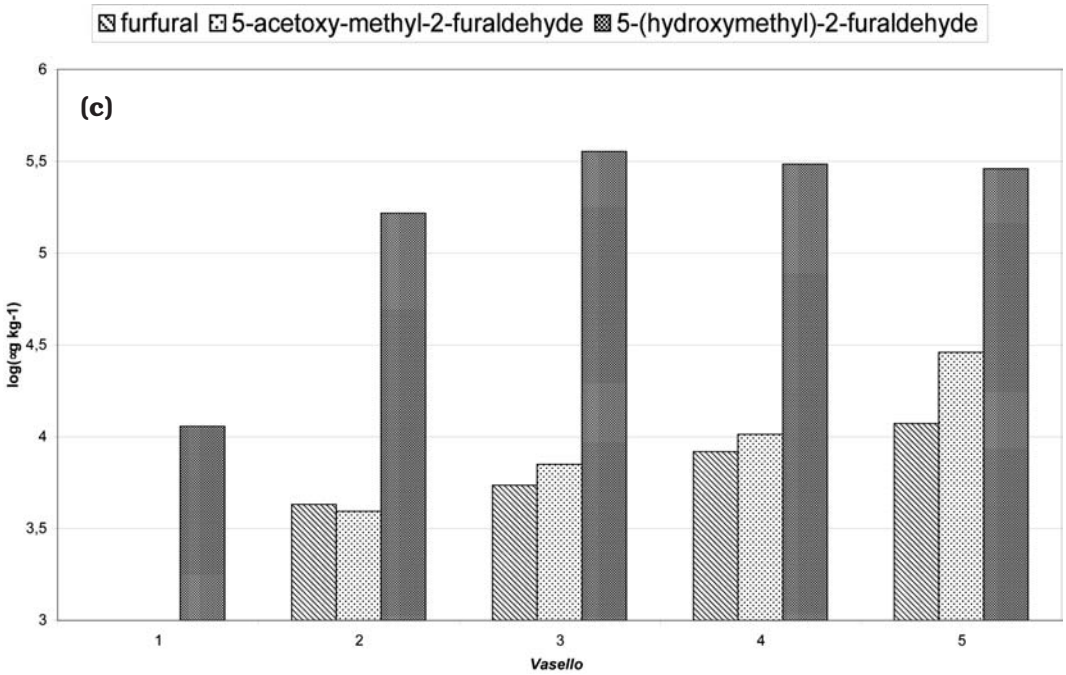


Fig. 1 - Evolution of some acetates (a), alcohols (b) and products of Maillard reactions (c) in battery A. The concentrations are reported in logarithmic form.

fortified wines (CUTZACH *et al.*, 1999). This molecule seems to be stable in the absence of oxygen, but in the presence of oxygen it may produce 5-methyl-2-furfural (CUTZACH *et al.*, 1999). It was also found in the volatile compounds of traditional balsamic vinegar. 5-ethoxymethylfurfural was not found since it is formed from HMF only in the absence of oxygen.

The same furfural found in white wine also comes from the toasting of the wooden barrel (FEUILLAT, 1999). With furfural there is a co-elution of a not yet identified compound (relative abundance in brackets) [m/z: 29(16), 43(57), 44(87), 45(100), 57(8), 58(10), 71(7), 88(12), 117(3), 131(2)]. This unknown compound was present only in *vasello* n. 1, in the absence of furfural and its concentration decreased in the other *vaselli*, while the furfural concentration increased. The situation is analogous for 2-furyl-methyl-ketone. In fact, together with this compound, there is a co-elution of a compound that has not yet been identified but may be attributed to a cyclic acetal (relative abundance in brackets) [m/z: 29(28), 31(13), 43(97), 44(100), 45(96), 57(17), 88(6), 103(17), 117(5)] whose quantity decreased from the first to the fourth *vasello*. In the fifth *vasello* only 2-furyl methyl ketone was present.

Among the enolic derivatives it is interesting to note the presence of cyclotene, maltol and 5-hydroxy-maltol also found in sweet wines (CUTZACH *et al.*, 1998a,b). Cyclotene can be formed by the Maillard reaction and also from lactic acid or lactic aldehyde formed from fructose (CUTZACH *et al.*, 1999). Instead maltol and 5-hydroxy-maltol are related to the use of red grape and the presence of oxygen. These molecules have been reported to be responsible for a slight "toasty" aroma of wines aged in the cask (CUTZACH *et al.*, 1997). Maltol and 5-hy-

droxy-maltol were also found, due to the thermal degradation of 2,3-dihydro-3,5-dihydroxy-6-methyl-4(H)-pyran-4-one, one of the most important intermediates of Maillard reactions in model systems (KIM and BALTES, 1996).

Considerable quantities of lactones were detected. γ-Lactones and solerone come from cyclization of hydroxylated fatty acids during alcoholic fermentation (DUFOSSÉ *et al.*, 1994). Pantolactone has also been identified in aged sweet wines (CUTZACH *et al.*, 1998b) and sherry (WEBB *et al.*, 1967). Dehydromevalonic lactone which was quantitatively very important in battery A has never been reported for wine and vinegar aroma.

α-Angelica lactone from sugar degradation (VERNIN and PÁRKÁNYI, 1982) and whiskey lactone (β-methyl-γ-octalactone) from wooden oak barrels (FEUILLAT, 1999) were also present.

γ-Nonalactone and γ-decalactone were not found in the balsamic vinegars although they have been found in wines (DUFOSSÉ *et al.*, 1994; CUTZACH *et al.*, 1998b). On account of ring cleavage these compounds may have given origin to γ-hydroxyacid. The corresponding esters come from the esterification between γ-hydroxyacid and ethanol. In fact the presence of two compounds identified as ethyl 4-hydroxynonanoate (relative abundance in brackets) [m/z: 29(60), 57(18), 73(7), 85(100), 102(3), 131(20)] and ethyl 4-hydroxydecanoate (relative abundance in brackets) [m/z: 29(52), 57(11), 73(3), 85(100), 102(4), 131(25), 159(5)] were found in the first barrels.

The other identified compounds were the nitrogen-containing heterocyclic products (e.g. pyrroles and 2-pyrrolydinone) produced from Strecker oxidative degradation of amino acids in the presence of α-dicarbonyl compounds (VERNIN and PÁRKÁNYI, 1982).

The small quantities of hydrocarbons found may come from the wooden barrel (SEFTON *et al.*, 1990), and they were more abundant in batteries B and C.

Considerable quantities of acids were also found. Their concentrations generally increased until the third *vasello*, after which they decreased due to esterification. Syringol and tyrosol were among the phenols found in all the batteries. Tyrosol may come from tyrosine transformation due to the action of yeast during alcoholic fermentation or from the wooden barrel (FABIOS *et al.*, 2000). Volatile phenols are more easily leached from the younger *vaselli* (CUTZACH-BILLARD, 2000) and so greater amounts were found in vinegars from battery C and in particular in *vaselli* n. 2 and n. 4.

4-ethyl-phenol was practically absent as there was no lactic bacteria action nor *Brettanomyces* to explain the production of this phenol (FLANZY, 1998).

Linalool oxides produced by the oxidation of diols or linalool (VERNIN, 1997), normally present in grape varieties used for vinegar production, were present in all batteries.

Oxygenated compounds such as glycol acetates and cyclic acetals were among the numerous unidentified compounds.

An unknown compound (relative abundance in brackets) [m/z: 29(95), 31(15), 39(16), 43(67), 45(100), 57(33), 58(30), 85(76), 86(80), 87(4), 88(4)], present in two isomer structures (labelled as "Compound A" and "Compound B" in Table 2), increased from *vasell*o n. 1 to *vasello* n. 5 and was found mostly in battery A. This compound probably contains a sulphur atom as shown by the characteristic isotopic ratio between ions m/z 86 and m/z 88.

CONCLUSIONS

Balsamic vinegar flavour is very complex and is still largely unknown. However the products of alcoholic and acetic fermentation such as ketones, aldehydes, ethyl esters and ethyl acetates are very important. Also the products of the

Maillard reactions are very interesting and probably formed during cooking of the must and the long aging in warm places and with oxygen. Of particular importance are furfural, 5-methyl-2-furaldehyde, 5-hydroxymethylfurfural or HMF, 5-acetoxymethylfurfural and 2-furoic acid. The acetification techniques and acetification battery (*vaselli* number, wood, *vaselli* age, their position within the battery) are important for their formation. In fact although the three batteries examined were started simultaneously and handled in the same manner, the volatile composition was very different. There was a small number of volatile compounds in the products of the younger *vaselli* with respect to those present in the older battery and also their concentration was less. The volatile compounds from the wooden barrel were absent in the battery with older *vaselli*. Although it was not possible to study the effect of the wood on the composition of the balsamic vinegars, the effect of mulberry is evident because its porosity leads to concentration of the product and oxidation.

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REFERENCES

Anklam E., Lipp M., Radovic B., Chiavaro E. and Palla G. 1998. Characterisation of Italian vinegar by pyrolysis-mass spectrometry and a sensor device ('electronic nose'). Food Chem. 61: 243.

Asai T. (Ed.). 1968. "Acetic Acid Bacteria" University Tokyo Press, Tokyo.

Charles M., Martin B., Ginies C., Etievant P., Coste G. and Guichard E. 2000. Potent aroma compounds of two red wine vinegars. J. Agric. Food Chem. 48: 70.

Chiavaro E., Caligiani A. and Palla G. 1998. Chiral indicators of ageing in balsamic vinegars of Modena. Ital. J. Food Sci. 10: 329.

Coppini D., Plessi M. and Monzani A. 1978. Ulteriore contributo allo studio dell'aceto balsamico. Riv. Vitic. Enol. 31: 351.

Corradini F., Marcheselli L., Marchetti A., Preti C. and Biancardi C. 1994. Analysis of heavy metals in Aceto Balsamico Tradizionale di Modena by flame atomic absorption spectroscopy. J. AOAC Int. 77: 714.

Cutzach-Billard I. 2000. Évolution de l'arôme des vins doux naturels non muscatés au cours de leur élevage et de leur vieillissement. Revue Fr. Oenol. 27: 13.

Cutzach I., Chatonnet P., Henry R. and Dubourdieu D. 1997. Identification of volatile compounds with a "toasty" aroma in heated oak used in barrelmaking. J. Agric. Food Chem. 45: 2217.

Cutzach I., Chatonnet P. and Dubourdieu D. 1998a. Study on sweet natural non muscat wine aroma. 1rst part: qualitative analysis of sweet natural wines aroma found during ageing. J. Int. Sci. Vigne Vin 32: 99.

Cutzach I., Chatonnet P., Henry R., Pons M. and Dubourdieu D. 1998b. Study in aroma of sweet natural non muscat wines. 2nd part: quantitative analysis of volatile compounds taking part in aroma of sweet natural wines during ageing. J. Int. Sci. Vigne Vin 32: 211.

Cutzach I., Chatonnet P. and Dubourdieu D. 1999. Study of the formation mechanisms of some volatile compounds during the aging of sweet fortified wines. J. Agric. Food Chem. 47: 2837.

Del Signore A. 1997. Il colore degli aceti Balsamici. Riv. Merceol. 36: 137.

Del Signore A. 1998. Differentiation of balsamic vinegars using physical indexes. J. Commodity Sci. 37: 51.

Del Signore A. 2000. Infrared spectra (Mid-Ir) classification of balsamic vinegars. J. Commodity Sci. 40:159.

Del Signore A. 2001a. Chemometric analysis and chemical and physical parameters of Traditional Balsamic Vinegars from Modena. J. Commodity Sci. 41: 33.

Del Signore A. 2001b. Chemometrics analysis and volatile compounds of traditional balsamic vinegars from Modena. J. Food Eng. 50: 77.

Del Signore A., Campisi B. and Di Giacomo F. 1998. Characterization of balsamic vinegar by multivariate statistical analysis of trace element content. J. AOAC Int. 81: 1087.

Del Signore A., Stancher B. and Calabrese M. 2000. Characterisation of balsamic vinegars by amino acid content using a multivariate statistical approach. Ital. J. Food Sci. 12: 317.

Di Stefano R. 1991. Proposition d'une méthode de préparation de l'échantillon pour la détermination des terpènes libres et glycosides des raisins et des vins. Bull. OIV 64: 219.

Dubois P. 1994. Les arômes des vins et leurs défauts. Revue Fr. Oenol. 145: 27.

Dufossé L., Latrasse A. and Spinnler H.E. 1994. Importance of lactones in food flavours: structure, distribution, sensory properties and biosynthesis. Sci. Aliments 14: 17.

Fabios M., Lopez-Toledano A., Mayen M., Merida J. and Medina M. 2000. Phenolic compounds and browning in sherry wines subjected to oxidative and biological aging. J. Agric. Food Chem. 48: 2155.

Feuillat M. 1999. Élevage des vins blancs: incidence aromatique des fermentations en fût et de l'autolyse des levures. Revue Fr. Oenol. 174: 18.

Flanzy C. (Ed.) 1998. "Oenologie. Fondements scientifiques et technologiques" Lavoisier Ed. Tec DOC, Paris.

Giaccio M. and Del Signore A. 1997. Il contenuto di 5-acetossimetil-2-furaldeide nell'Aceto Balsamico Tradizionale di Modena e di Reggio Emilia. Riv. Merceol. 36: 91.

Giaccio M., Del Signore A., Cichelli A. and Morgante A. 1996. Differenziazione dell'Aceto Balsamico Tradizionale dall'aceto balsamico comune mediante una sostanza caratteristica. Riv. Merceol. 35: 17.

Giudici P. 1993. Acido gluconico: criterio di genuinità dell'aceto balsamico tradizionale. Industrie Bevande 22: 123.

Giudici P., Barbagallo R.N., Altieri C. and Masini G. 1994. Origine ed evoluzione degli acidi organici durante l'invecchiamento dell'Aceto Balsamico Tradizionale. Industrie Bevande 23: 569.

Gogus F., Bozkurt H. and Eren S. 1998. Kinetics of Maillard reactions between the major sugars and amino acids of boiled grape juice. Leben. Wissen. Technol. 31: 196.

G.U. 1986. Law n. 93, April 3, 1986. Riconoscimento della produzione dell'Aceto Balsamico Tradizionale di Modena o di Reggio Emilia. Italian Official Journal April 9, 1986.

G.U. 1987. Law n. 191, March 3, 1987. Disciplina della produzione dell'Aceto Balsamico Tradizionale di Reggio Emilia e delle modalità di controllo. Italian Official Journal May 18, 1987.

Karagiannis S., Economou A. and Lanaridis P. 2000. Phenolic and volatile composition of wines made from *Vitis vinifera* cv. Muscat Lefko grapes from the island of Samos. J. Agric. Food Chem. 48: 5369.

Kim M.O. and Baltes W. 1996. On the role of 2,3-dihydro-3,5-dihydroxy-6-methyl-4(H)-pyran-4-one in the Maillard reaction. J. Agric. Food Chem. 44: 282.

MAF (Ministero dell'Agricoltura e delle Foreste). 1986. Metodi ufficiali di analisi per i mosti, i vini e gli aceti. Gazzetta Ufficiale della Repubblica Italiana n. 161 del 14.07.1986. Istituto Poligrafico dello Stato, Roma.

Masini G. and Giudici P. 1995. Ricerca e innovazione nella produzione di Aceto Balsamico tradizionale. L'Enotecnico 31: 73.

Mascolo A. and Gavioli G. 1998. Valutazione soggettiva ed obiettiva dell'Aceto Balsamico Tradizionale di Modena. L'Enotecnico 34: 77.

Meglioli G., Parisini G., Tedeschi M. and Pesenti G. 1997. L'aceto balsamico tradizionale di Reggio Emilia. Studio delle caratteristiche chimiche e chimico-fisiche. Industrie Bevande 26: 41.

Plessi M., Monzani A. and Coppini D. 1988. Determination of the monosaccharide and alcohol content of balsamic and other vinegars by enzymatic methods. Agric. Biol. Chem. 52: 25.

Plessi M., Monzani A. and Coppini D. 1989. Quantitative determination of acids and derivatives in balsamic and other vinegars. Sci. Aliments 9: 179.

Sefton M.A., Francis I.L. and Williams P.J. 1990. Volatile norisoprenoid compounds as constituents of oak woods used in wine and spirit maturation. J. Agric. Food Chem. 38: 2045.

Stacchini A., Draisci R. and Lorusso S. 1990. Tecnologia e caratterizzazione degli aceti balsamici. Industrie Bevande 19: 212.

Stancher B., Calabrese M. and Saccù D. 2001. Caratteristiche di qualità dell'aceto balsamico e dell'Asperum. Industrie Bevande 30: 233.

Theobald A., Müller A. and Anklam E. 1998. Determination of 5-Hydroxymethylfurfural in vinegar samples by HPLC. J. Agric. Food Chem. 46: 1850.

Turtura G.C. and Rosetti A.M. 1984. Caratterizzazione dell'aceto balsamico naturale. Industrie Bevande 13: 197.

Turtura G.C. and Bortolotti P.P. 1991. Caratteristiche microbiologiche e chimiche dell'aceto balsamico naturale. II. Relazioni con le tecniche di preparazione. Ann. Microbiol. 41: 169.

Vernin G. and Párkányi C. 1982. Mechanisms of formation of heterocyclic compounds in Maillard and pyrolysis reactions. In "Chemistry of Heterocyclic Aroma Compounds in Flavours and Aromas" Vernin G. (Ed.) p. 151. Ellis Horwood, Chichester, U.K.

Vernin G. 1997. Terpénoides et norisoprenoides dans les arômes des baies de raisins et dans les vins. 1. Origines et formation. Riv. Ital. EPPOS 22: 19.

Webb A.D., Kepner R.E. and Maggiora L. 1967. Sherry aroma. VI. Some volatile components of flor sherry of Spanish origin. Neutral substances. Am. J. Enol. Vitic. 18: 190.

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PAPER

AROMA COMPOUNDS IN MORRÃO DE CANDEIA (*CROTON* SP.) AND ASSA-PEIXE (*VERNONIA* SP.) HONEYS

COMPONENTI AROMATICI DEI MIELI BRASILIANI DI *CROTON* SP. E *VERNONIA* SP.

L.M.C. MATOS, R.F.A. MOREIRA, L.C. TRUGO[1] and C.A.B. DE MARIA*
Laboratório de Química de Alimentos, Instituto Biomédico,
Universidade do Rio de Janeiro (UNIRIO),
Rua General Sampaio 71, apt. 911 bl. 2 Caju, 20931-350 Rio de Janeiro, Brazil
[1] Instituto de Química, Universidade Federal do Rio de Janeiro, Brazil
* Corresponding author: Fax +55 21 25319678, e-mail: carreb@uol.com.br

ABSTRACT

Aroma compounds in two different types of Brazilian honey, morrão de candeia and assa-peixe, were studied by gas chromatography/flame ionization detection (GC/FID), GC/mass spectrometry (GC/MS), GC/SNIFFING and aroma extract dilution analysis (AEDA). Volatiles in morrão de candeia (51) and assa-peixe (31) honeys were tentatively identified by GC/MS analysis. Volatiles identified in both floral types were alcohols, hydrocarbons, acids, esters and aromatics. The morrão de candeia honeys were very rich in higher hydrocarbons while aromatics and esters predominated in

RIASSUNTO

Sono stati studiati i componenti aromatici di due differenti mieli brasiliani, appartenenti alle specie *Croton* e *Vernonia* mediante diverse vie gascromatografiche: GC/FID, GC/MS, GC/SNIFFING ed AEDA (aroma extract dilution analysis). I 51 componenti volatili dei mieli di Croton ed i 31 dei mieli di Vernonia sono stati identificati tentativamente mediante GC/MS. I componenti identificati in entrambi i mieli erano riferibili ad alcoli, idrocarburi, acidi, esteri e aromi diversi. I mieli di Croton si sono rivelati ricchi in idrocarburi a lunga catena, mentre gli aro-

- Key words: AEDA, aroma compounds, GC/MS, honey, impact volatiles -

the assa-peixe ones. Odour notes were perceived by means of GC/SNIFFING. Morrão de candeia honeys had some 31% more odour notes than the assa-peixe honeys. Important odourants were tentatively identified by comparison of the AEDA results with those from GC/MS and standards. 2-phenylethanol was a potent odourant of both honeys, while 2-butyl-1-octanol and benzonitrile seemed to have a powerful impact on morrão de candeia aroma, only. 2,3-butanedione may be considered as an odour-active compound for assa-peixe aroma. A methylbutanoic acid isomer seemed to provide a remarkable off-odour to assa-peixe honeys.

mi e gli esteri predominavano nei mieli di Vernonia. Le note aromatiche sono state rilevate mediante GC/SNIFFING e dall'analisi è emerso che i mieli di Croton presentano circa il 31% di note aromatiche in più di quelli di Vernonia. Tra i composti aromatici più importanti identificati mediante comparazione tra AEDA e GC/MS il 2-fenil etanolo appare il più potente aroma nei due mieli, mentre il 2-butilottanolo ed il benzonitrile sembra avere un forte impatto aromatico solo nel miele di Croton. Il 2-3 butanedione ed l'isomero dell'acido metilbutanoico si dimostrano componenti aromatici attivi nel miele di Vernonia.

INTRODUCTION

Brazil produces about 30 million kg of apiary honey annually, making it the fifth world producer (SOMMER, 1998). Due to the vast number of floral species visited by the honeybees, there is a substantial number of Brazilian unifloral honeys with peculiar sensorial properties. These honeys with exotic flavours are greatly appreciated by foreign consumers. However, the aromas of typical Brazilian honeys have not yet been characterized.

More than 300 different compounds have been identified in the volatile fraction of honeys of diverse botanical origins from different countries. These volatile compounds are probably derived from the original nectar sources and post-collection processing (BOUSETA *et al.*, 1992; 1996). The volatile compounds of honey aroma have been identified by gas chromatography (GC) coupled with mass spectrometry (MS). BICHI *et al.* (1983) reported the identification of volatile compounds in some Piedmontese floral honeys. The volatiles were isolated by a two-step extraction with a modified Likens-Nickerson apparatus. They concluded that some flavour compounds were present in all the samples (e.g., phenylacetaldehyde, benzyl alcohol), while others varied from one sample to another (e.g., phenylethyl alcohol, 4-terpinenyl acetate), thus contributing considerably to the distinctive bouquets of the different honeys.

BLANK *et al.* (1989) used simultaneous distillation and extraction and aroma extract dilution analysis (AEDA) to investigate the impact volatile compounds in *Tilia cordata* (linden) honey from Rumania. They found 21 odour compounds such as 1-hexen-3-one and phenylacetaldehyde having high factors of dilution (FD). TAN *et al.* (1988; 1989; 1990) isolated volatiles from New Zealand unifloral honeys by means of continuous liquid/liquid extraction with diethyl ether. Aliphatic and aromatic acids, phenols and/or degraded carotenoids were suggested as chemical markers for *Thymus vulgaris* (thyme), *Salix* sp. (willow), *Calluna vulgaris* (heather), *Trifolium repens* (white clover), *Leptospermum scoparium* (manuka) and *L. ericoides* (kanuka) honeys. Aroma com-

pounds from *Rhus succedanea* (haze) honey from Japan were separated by adsorptive column chromatography, packed with Porapak, and analysed by GC/MS and SNIFFING (SHIMODA and OSAJIMA, 1996). From a total of 130 compounds identified, phenylacetaldehyde, linalool, phenylethyl alcohol, lilac aldehydes among others, appeared to contribute to haze honey aroma. Volatile profiles of 43 authentic unifloral honey samples from 8 different European countries were adsorbed on Tenax trap and subsequently thermally desorbed and transferred to the GC (RADOVIC *et al.*, 2001). These authors suggested the existence of certain marker compounds for lavender (presence of heptanal), *Rosmarinus officinalis* (rosemary) (absence of 2-acetylfuran), rape (presence of dimethyl disulphide) and *Helianthus annuus* (sunflower) (presence of α-pinene or 3-methyl-2-butanol). Furthermore, possible marker compounds were set for the honeys from England (presence of 1-penten-3-ol) and Denmark (absence of 3-methyl-butanal).

The identification of the specific volatile compounds of Brazilian honey aroma would be useful to help monitor the basic level of quality of the finished product. In this paper, the results of analyses of the aroma compounds of some morrão de candeia and assa-peixe honeys produced in Brazil are given.

MATERIALS AND METHODS

Chemicals

Methanol and acetonitrile were of HPLC grade from Fluka (Buchs, Switzerland). Standards of fructose, glucose, oligosaccharides and 3-methylbutanoic acid were from Sigma (Missouri, USA). Benzoic acid was from Carlo Erba (Milan, Italy). Hydroxymethylfurfural (HMF), hydrocarbons, 1-hydroxy-2-propanone, 2,3-pentanedione, 2,3-butanedione, 2-cyclohexen-1-one, 2-methylpropanoic acid, 2-methyl-2,4-pentanediol, 3,7-dimethyl-1-octanol, (R)-(-)-2,2,-dimethyl-1,3-dioxolane-4-methanol, octanoic acid, decanoic acid, 2-(2-butoxyethoxy)-ethanol, 2-butyl-1-octanol, phenylmethanol, 2-phenylethanol, benzonitrile, 2,2'-oxy-bis-ethanol, ethyl 9-octadecenoate, dodecanoic acid and 1-octadecanol were purchased from Aldrich (Wisconsin, USA). 2-octanol was from Baker (New Jersey, USA). hexadecanoic, octadecanoic and 9-octadecenoic acids and other reagents (p.a. grade) were supplied by Merck (Darmstadt, Germany). Hydrocarbon standards were dissolved in benzene at a concentration of 1 mg/mL. Other standards were dissolved in acetone at the same concentration.

Samples

Authentic *Apis mellifera* honey samples of morrão de candeia (2 samples) (*Euphorbiaceae)* and assa-peixe (2 samples) (*Compositae)* were obtained from an apiarist from the State of Espírito Santo, Brazil. All 4 unifloral honeys were harvested between 1997 and 1998 and stored in small plastic bottles at -18°C under N_2 atmosphere. Before analysis, crystallized samples were defrosted at 30°C and homogenized by gently stirring for 3 min.

Non-volatile fraction

Screening for floral purity was based on pollen analyses described by LOUVEAUX *et al.* (1978). Analyses of free acidity, pH, moisture content, free proline and diastase activity were carried out according to AOAC (1984). Monosaccharides and HMF analyses were carried out in a HPLC system as described by COSTA *et al.* (1999). The oligosaccharide composition was also determined by HPLC in accordance with work reported elsewhere (DA COSTA LEITE *et al.* 2000). The

HPLC system consisted of a pump and a UV monitor (Knauer, Berlim, Germany), an injection valve with a 20 µL loop (Rheodyne, California, USA) and a refractive index detector (Waters, Milford, USA).

Isolation of the volatile fraction

The method for isolating the volatiles was based on the work of SHIMODA and OSAJIMA (1996). This technique was modified, as follows: 100 mL of an aqueous honey solution (20 g%, w/v) was passed through a glass column (0.5 cm i.d. x 18 cm) packed with Porapak Q adsorbent (50/80 mesh) (Supelco, Milford, USA) previously conditioned at 225°C for 3 h under N_2 at a flow rate of 0.9-1.0 L/min. Deionized water (20 mL) was then passed through the column to remove sugar compounds. The desorption of the volatiles was performed with 100 mL of acetone, and the eluate was dried in a rotary evaporator at 20°C and then diluted with 200 µL of acetone. Two different extracts were prepared for each honey sample.

GC analysis

A 30 m x 0.25 mm (id) Supelcowax™ 10 fused-silica capillary column with a film thickness of 0.25 µm (Supelco, Milford, USA) was installed on a Carlo Erba model 4300 gas chromatograph (Carlo Erba, Milan, Italy). Injection was in the split mode with a 1:20 ratio. The flame ionization detector (FID) and injector temperatures were 240° and 230°C, respectively. The oven temperature was 50°C for the first minute, programmed to 240° at 3°C/min and then held at 240°C for 20 min. The linear flow rate of helium gas was 28 cm/s. Modified Kóvatz Indices (KI) were calculated for the Supelcowax capillary column on the GC system in accordance with VAN DEN DOOL and KRATZ (1963).

GC/MS

GC/MS was carried out using a Shimadzu GC-17A/QP-5050 instrument (Shimadzu, Kyoto, Japan) with National Institute of Standards and Technology (NIST) 12.lib and 62.lib data systems, a cyclic scan of 1 s and mass range of *m/z* 20-300. Both ionization voltage of 70 eV and an ion source temperature of 240°C were used. The column and the chromatographic conditions were the same as described for GC analysis. The identification of the isolated volatile compounds was achieved by comparing mass spectra of unknown peaks with those stored in the NIST. Where possible the results were confirmed by comparison with authentic substances.

SNIFFING and AEDA

For GC/SNIFFING and AEDA, the same capillary column mentioned earlier was used, splitting the effluent 1:10 with a glass-cap-cross for FID, as well as for simultaneous odour perception by means of a SNIFFING PORT. Olfactory perception of the separated volatiles from honey samples was performed independently by three assessors. In order to identify key odours within the overall aroma, the stepwise dilutions (2^1-2^6) (AEDA) of honey extracts in acetone were injected until no odour could be perceived by the assessors. External standards were sniffed from the containers directly. AEDA analysis was based on the report described by ACREE and TERANISHI (1993).

RESULTS AND DISCUSSION

A descriptive analysis of the non-volatile fraction of the honey samples is given in Table 1. Variable levels of individual oligosaccharides were found which agreed with results reported elsewhere (DA COSTA LEITE *et al.*, 2000). The hon-

Table 1 - Free acidity, pH, moisture content, proline, HMF, diastase activity and sugar contents of honey samples (n=2).

Parameters	Morrão de candeia	Assa-peixe
free acidity	29.1/31.0	25.0/29.1
pH	3.6/3.3	3.2/3.2
moisture content (%)	18.9/18.1	17.5/18.3
proline	657/771	737/633
HMF	6.1/4.5	2.6/3.8
diastase activity	15.0/13.2	12.1/18.2
fructose	41.1/38.9	38.6/41.0
glucose	31.6/33.0	31.4/32.8
sucrose	0.6/0.2	0.9/1.3
turanose	4.9/5.3	nd
maltose	4.7/3.7	3.4/4.0
nigerose	nd	nd
palatinose	nd	nd
melibiose	0.2/0.8	nd
erlose	0.3/0.1	0.3/0.1
maltotriose	0.2/0.2	0.5/0.1
raffinose	0.2/0.4	0.2/0.4
panose	0.5/1.5	0.5/0.9
isomaltotriose	0.7/0.3	nd

Each value is the average of two determinations performed on wet matter for each honey sample.
Free acidity is expressed as meq/kg of honey.
Proline and HMF are expressed as mg/kg of honey.
Diastase activity is expressed in the Goethe scale (^{0}G).
The amount of each sugar is expressed as g in 100 g of honey.
nd = Not detected.

ey samples were within the limits of the European Codex Honey Standards and Brazilian legal regulations that set current maximum levels of 20-21% for moisture content, 40 millieq./kg total acidity, 40 mg/kg HMF, a minimum of 8°G for diastase activity, maximum of 5 g/100 g sucrose and the sum of fructose plus glucose above 60 g/100 g of honey.

The identified volatile compounds and their experimental KI and relative area percentages are listed in Table 2. The more abundant fragments from the compounds found in honey for the first time or unidentified are also shown. Due to the masking effect of the peak from acetone, compounds eluting between 0-10 min could not be identified by GC/MS. The absence of furan derivatives in both floral types reflected no heat damage during processing and storage of the samples. A group of compounds (48%) was identified by comparison of retention and mass spectral data with those of authentic reference compounds. Using GC/MS 51 and 31 volatiles were identified in morrão de candeia and assa-peixe samples, respectively. Among them, 24 compounds were identified in both unifloral honeys. A total of 23 compounds were reported as honey constit-

Table 2 - Volatile compounds in morrão de candeia and assa-peixe honeys.

Volatiles			% Area	
Name	KI[a]	RT[b]	Morrão de candeia	Assa-peixe
1-hydroxy-2-propanone[c]	1,285	12.90	nd[f]	tr[g]
tetradecane[c]	1,410	18.00	tr	nd
2-cyclohexen-1-one[c]	1,446	19.53	0.01/0.03	tr
4-methyl-3-hexanol[d,e] 43(100), 27(62), 31(45), 29(40), 41(37), 72(24), 73(17)	1,452	19.73	0.22/0.52	1.25/0.55
unidentified 43(100), 45(48), 42(30), 58(24), 84(22), 29(20), 26(18)	1,457	20.00	40.8/29.7	29.2/65.1
1,2-ethanediol diacetate[d,e] 43(100), 86(15), 41(8), 73(6), 44(3), 29(2)	1,470	20.54	1.4/3.6	1.1/0.10
2-ethyl-1-hexanol[d]	1,499	21.75	0.10/0.62	1.4/0.42
pentadecane[c]	1,505	22.00	tr	0.32/01
4-methyl-3-heptanol[d,e] 59(100), 43(25), 31(24), 27(20), 9(18), 41(15), 83(6)	1,529	23.00	0.51/1.1	0.31/0.17

(Continue)

Tab. 2

Volatiles			% Area	
Name	KI[a]	RT[b]	Morrão de candeia	Assa-peixe
unidentified 43(100), 29(48), 26(35), 31(30), 58(24), 57(23), 87(22)	1,541	23.50	23.7/36.9	20.2/12.1
propanoic acid[d]	1,553	24.00	0.30/0.70	0.04/0.08
2-methylpropanoic acid[c]	1,560	24.27	0.30/0.14	nd
unidentified 43(100), 87(22), 27(14), 41(12), 55(9), 57(8), 73(5)	1,584	25.25	2.0/4.2	5.8/4.7
hexadecane[c]	1,608	26.25	0.08/0.02	nd
2,4-dimethyl-1,3-dioxolane-2-methanol[d,e] 43(100), 101(25), 58(20), 41(18), 32(15)	1,619	26.35	nd	0.47/0.13
2-methyl-2,4-pentanediol[c,e] 59(100), 43(90), 56(28), 45(25), 41(29), 29(17), 85(11)	1,652	28.00	0.16/0.50	tr
2-methyl-hexadecane[d,e] 57(100), 71(49), 43(32), 85(25), 55(21), 99(18), 41(16)	1,663	28.46	nd	0.17/0.07
3,7-dimethyl-1-octanol[c,e] 73(100), 69(57), 55(40), 43(27), 129(10), 111(7), 83(3)	1,669	28.72	0.09/0.05	nd
4-hydroxy-4-methyl-2-pentanone[d]	1,695	29.76	nd	1.8/1.2
heptadecane[c]	1,727	31.00	0.04/0.06	nd
2-(2-butoxyethoxy)-ethanol[c,e]	1,804	34.00	0.05/0.15	nd
octadecane[c]	1,811	34.26	0.01/0.05	nd
hexanoic acid[d]	1,838	35.25	0.17/0.13	nd
2-butyl-1-octanol[c,e]	1,863	36.20	0.61/0.33	nd
unidentified 43(100), 87(32), 42(10), 44(8), 27(6), 28(5), 29(4), 55(2)	1,885	37.00	1.6/3.4	4.8/2.7
phenylmethanol[c] (benzyl alcohol)	1,891	37.30	0.59/0.37	0.04/0.08
nonadecane[c]	1,904	37.72	0.05/0.07	nd
2-phenylethanol[c] (phenylethyl alcohol)	1,911	37.95	0.09/0.15	0.59/0.83
benzonitrile[c] 117(100), 90(58), 116(41), 89(30), 51(28), 39(26), 63(22)	1,940	39.00	0.01/0.05	nd
(Z)-3-hexenyl hexanoate[d]	1,962	39.76	0.17/0.09	1.2/1.3
2-tridecanol[d,e] 45(100), 41(48), 43(47), 55(45), 69(39), 57(38), 83(32)	1,968	40.00	2.2/4.8	nd
2,2'-oxy-bis-ethanol[c,e] (diethylene glycol) 45(100), 75(27), 32(25), 44(22), 76(17), 43(13), 42(10)	1,997	41.00	0.36/0.68	nd
2,5-dimethyl-1,5-hexadiene-3,4-diol[d,e] 43(100), 27(46), 55(45), 84(18), 41(11), 72(8), 73(5)	2,008	41.39	nd	0.05/0.13
eicosane[c]	2,018	41.72	0.04/0.02	nd
hexadecanal[d,e] 43(100), 57(98), 82(94), 41(70), 55(68), 96(67), 68(59)	2,167	46.75	0.24/2.0	nd
1-heptadecanol[d,e] 43(100), 55(93), 57(85), 42(71), 83(62), 69(61), 41(55)	2,175	47.00	0.24/1.2	nd
nonanoic acid[d]	2,197	47.71	0.06/0.12	nd
ethyl hexadecanoate[d]	2,280	50.52	1.9/2.2	2.3/1.5

(Continue)

Tab. 2

Volatiles			% Area	
Name	KI[a]	RT[b]	Morrão de candeia	Assa-peixe
tricosane[c]	2,322	52.00	0.26/0.78	0.04/0.08
2,6-dimethyl-?,?-octadiene-?,?-diol[d] 43(100), 71(61), 67(45), 55(40), 67(36), 68(26), 41(25)	2,325	52.10	0.03/0.01	nd
tetracosane[c]	2,391	54.50	0.01/0.07	nd
benzoic acid[c]	2,438	56.23	0.26/0.68	0.09/0.03
ethyl octadecanoate[d]	2,459	57.00	0.8/1.2	tr
ethyl 9-octadecenoate[c]	2,468	57.33	1.7/2.1	0.15/0.09
dodecanoic acid[c,e] 60(100), 73(98), 43(75), 41(71), 55(50), 57(49), 129(18)	2,476	57.64	nd	0.06/0.12
pentacosane[c]	2,493	58.26	1.1/1.4	3.1/0.38
phenylacetic acid[d]	2,542	59.73	0.10/0.22	7.3/2.2
1-octadecanol[c,e] 42(100), 41(90), 57(85), 55(82), 28(50), 83(49), 29(48)	———	60.65	0.34/0.74	3.0/0.98
hexacosane[c]	———	61.00	0.40/0.90	nd
3-hydroxy-4-methoxy-benzaldehyde[d,e] (isovanillin)	———	61.50	nd	3.2/0.32
heptacosane[d]	———	63.50	1.0/1.5	nd
2-octadecanol[d]	———	65.15	nd	tr
octacosane[d]	———	65.28	0.02/1.0	nd
pentadecanoic acid[d,e] 43(100), 60(94), 73(93), 42(91), 55(61), 57(59), 29(52)	———	68.50	nd	0.05/0.07
nonacosane[d]	———	70.30	0.51/1.9	tr
hexadecanoic acid[c] (palmitic acid)	———	70.60	0.34/2.0	nd
1-heneicosanol[d]	———	70.76	1.1/1.0	0.12/0.04
1-docosanol[d,e] 43(100), 57(85), 55(81), 41(74), 97 (71), 83(57), 69(52)	———	73.17	0.61/0.13	0.08/0.10
octadecanoic acid[c] (stearic acid)	———	78.87	0.02/0.50	nd
1-tetracosanol[d,e] 43(100), 41(75), 55(70), 57(67), 69(47), 83(45), 29(37)	———	79.83	0.66/0.36	nd
9-octadecenoic acid[c] (oleic acid)	———	83.67	tr	nd
1-hexacosanol[d,e] 43(100), 57(77), 55(72), 41(61), 83(45), 69(43), 97(30)	———	85.92	tr	0.12/0.06
17-pentatriacontene[d,e] 43(100), 57(91), 55(58), 83(49), 69(48), 97(46)	———	88.83	0.48/0.22	nd
1-heptacosanol[d,e] 43(100), 57(93), 55(61), 71(52), 41(50), 69(48), 97(45)	———	89.84	tr	nd

[a] KI = Modified Kóvatz Index according to VAN DEN DOOL and KRATZ (1963);
[b] RT = Retention time (min) recorded by GC/FID;
[c] Identification via mass spectra + reference compound;
[d] Tentative identification: based on mass spectra data only;
[e] Not yet reported as a constituent of honey aroma;
[f] nd = Not detected;
[g] tr =Traces.

uents for the first time. All the 4 unidentified compounds showed a base peak at m/z 43 (Table 2).

The volatile compounds in morrão de candeia and assa-peixe honeys were predominantly alcohols (33.3, 38.7%), hydrocarbons (29.4, 16.1%), acids (11.8, 9.7%), esters (7.8, 12.9%) and aromatics (9.8, 16.1%), respectively (Table 2). Both phenylmethanol and 2-phenylethanol have been identified in different unifloral honeys from different countries (BICCHI *et al.,* 1983; BLANK *et al.,* 1989; TAN *et al.,* 1989, 1990; BOUSETA *et al.,* 1996; SHIMODA and OSAJIMA, 1996). (Z)-3-hexenyl hexanoate has been detected in haze from Japan (SHIMODA and OSAJIMA, 1996), while phenylacetic and benzoic acids have been identified in lavender honeys from France and Spain (BOUSETA *et al.* (1996). 4-hydroxy-4-methyl-2-pentanone, 2,4-dimethyl-1,3-dioxolane-2-methanol and 3-hydroxy-4-methoxy-benzaldehyde (isovanillin) were particularly characteristic of the assa-peixe honeys. The presence of one ether (2-(2-butoxyethoxy)-ethanol) and one aldehyde (hexadecanal) on the other hand was a typical feature of the morrão de candeia samples. These results indicated that the morrão de candeia honeys were very rich in higher hydrocarbons while aromatics and esters predominated in the assa-peixe honeys.

An adsorption of volatiles on Porapak Q followed by desorption with acetone, led to extracts with a strong overall honey-like aroma, very similar to that of the original samples. The perceived odour qualities obtained by means of the GC-SNIFFING technique and their respective perception times for each unifloral honey, along with the odour characteristics of standards, are showed in Table 3. A large number of odours were sniffed in all the samples, however morrão de candeia honeys had some 31% more odour notes than assa-peixe honeys. This difference was due to the larger amount of odour notes perceived in the morrão de candeia samples in the range from 1,733 to 2,073 in modified KI on the Supelcowax column. All the honey samples had fewer odour characteristics in the range 70-90 min (Table 3).

Column effluent SNIFFING was performed on a series of dilutions (AEDA) of the concentrated extract in order to identify important odours. It was considered that odours perceived at 2^3 or more dilutions by assessors are of greater importance for overall honey aroma. On the basis of the results obtained from AEDA (Table 3), it appeared that important odours in the morrão de candeia honeys were in the range from 1,850 to 2,330 (e.g. bitter, green, unpleasant odours/KI = 1,850-1,861/FD = 2^4; bitter, sweet, clover-like odours/KI = 2,184-2,199/Fd = 2^5; pungent, floral odours/KI = 2,311-2,322/Fd = 2^4). Assa-peixe samples presumably provided a smaller total number of key odours that were sniffed in the range from 1,000 to 1,004 and from 1,660 to 1,930 (e.g. sweet odour/KI = 1,906-1,923/Fd = 2^5). The perception of pleasant key odours in morrão de candeia samples was higher than in assa-peixe honeys. Indeed, morrão de candeia honeys are widely appreciated by consumers due to their distinctive and pleasant bouquet.

A comparison of the AEDA analysis (Table 3) with the data from reference substances and GC/MS (Table 2) allowed the tentative identification of 2-butyl-1-octanol, 2-phenylethanol and benzonitrile as odour-active compounds for the overall aroma of the honeys studied. The more abundant fragments of the MS-spectral analysis of these compounds are listed in Table 2. In the fragmentation process of 1-alkanols, α-clevage is the most useful characteristic reaction (McLAFFERTY and TURECEK, 1993). Benzonitrile fragmentation provides a m/z 117 peak which is its molecular ion. Unlike aliphatic nitriles, aromatic nitriles have many abundant molecular ions (McLAFFERTY and TURECEK, 1993). The

Table 3 - Odour characteristics of the GC/SNIFFING effluents.

Perception time (min)		Odour perception							
		Morrão de candeia		Assa-peixe		Reference compound			
KI	RT	Odour note	Fd[a]	Odour note	Fd	Name	Odour note	KI[b]	RT[c]
982	3.5	bitter, herb-like	X[d]	———	—-	———	———	—-	—-
1000-1004	4.0-4.1	green, buttery	2^1	rancid, buttery	2^3	2,3-butanedione	rancid, buttery	1,003	3.90
1007	4.6	———	—-	banana-like	x	———	———	—-	—-
1025	5.0	bitter	x	———		———	———	—-	—-
1043	5.4	———	—-	herb-like	x	———	———	—-	—-
1070-1079	6.0-6.2	slightly rancid, buttery	2^1	———	—-	2,3-pentanedione	buttery	1,076	5.90
1112	7.0	———	—-	pleasant, green	x	———	———	—-	—-
1128	7.5	———	—-	floral	x	———	———	—-	—-
1178	9.0	———	—-	unpleasant	2^1	———	———	—-	—-
1259	11.9	putrid	x	———	—-	———	———	—-	—-
1283-1285	12.8-12.9	bitter, herb-like	x	burnt	x	1-hydroxy-2-propanone	pungent, irritant, herb-like	1,285	12.90
1357-1362	15.8-16.0	burnt	x	burnt, bitter	x	———	———	—-	—-
1369-1371	16.3-16.4	———	—-	pungent, bedbug-like	x	———	———	—-	—-
1383	16.9	sweet, caramel	x	———	—-	———	———	—-	—-
1440-1455	19.3-19.9	burnt, metallic	x	baked bread	x	2-cyclohexen-1-one	unpleasant, solvent	1,446	19.53
1622	26.8	———	—-	rancid	x	2,4-dimethyl-dioxolane-2-methanol	unpleasant, solvent	1,619	26.35
1637-1647	27.4-27.8	———	—-	honey-like	x	———	———	—-	—-
1666-1689	28.6-29.5	———	—-	isovaleric acid-like, putrid-fruit, sour	2^5	3-methylbutanoic acid	unpleasant, putrid fruit	1,666	28.60
1714	30.5	———	—-	anise-like	x	———	———	—-	—-
1733	31.2	pyrazine-like, green	x	———	—-	———	———	—-	—-
1742	31.6	unpleasant	x	sour	x	———	———	—-	—-
1761	32.3	———	—-	green	x	———	———	—-	—-
1779-1794	33.0-33.6	bitter, burnt	x	pungent	x	———	———	—-	—-
1804-821	34.0-34.6	honey-like, alcohol, sweet, ice tea	x	———	—-	2-(2-butoxyethoxy)-ethanol	herb-like, green, irritant	1,804	34.00
1831	35.0	sweet	x	———	—-	———	———	—-	—-
1837-1845	35.2-35.5	bitter, herb-like	x	———	—-	———	———	—-	—-
1850-1861	35.7-36.1	bitter, green, unpleasant	2^3	bitter, unpleasant	2^3	———	———	—-	—-
1866-1877	36.3-36.7	floral	2^3	———	—-	2-butyl-1-octanol herb-like	pleasant, floral	1,863	36.20
1885	37.0	black-currant-like	x	———	—-	———	———	—-	—-
1893	37.3	propolis-like	x	———	—-	phenylmethanol	irritant, pungent	1,893	37.30

(Continue)

Tab. 3

Perception time (min)		Odour perception							
		Morrão de candeia		Assa-peixe		Reference compound			
KI	RT	Odour note	Fd[a]	Odour note	Fd	Name	Odour note	KI[b]	RT[c]
1906-1923	37.8-38.4	green, sweet, pungent	2^5	slightly bitter, sweet	2^3	2-phenylethanol	floral, herb-like, pungent	1,911	37.95
1940-1943	39.0-39.1	metallic	2^5	———	—-	benzonitrile	metallic, unpleasant	1,940	39.00
1997-1999	41.0-41.1	herb-like	x	———	—-	2,2'-oxy-bis-ethanol	solvent, sweet, alcohol	1,997	41.02
2011	41.5	green	x	———	—-	———	———	—-	—-
2029-2038	42.1-42.4	floral, sweet	2^3	———	—-	———	———	—-	—-
2043-2052	42.6-42.9	burnt, wet earthy	2^1	roasted bread	x	———	———	—-	—-
2061-2073	43.2-43.6	burnt sugar	2^1	sweet, herb-like	2^1	———	———	—-	—-
2126	45.4	black tea	x	———	—-	———	———	—-	—-
2157	46.4	green, bitter	x	———	—-	———	———	—-	—-
2184-2199	47.3-47.8	slightly bitter, clover-like, sweet	2^3	slightly bitter, sweet	x	———	———	—-	—-
2208-2226	48.1-48.7	methyl-pyrrol-like, bitter	2^3	———	—-	———	———	—-	—-
2235-2273	49.0-50.3	honey-like, sweet	x	Floral, bitter	x	———	———	—-	—-
2285-2294	50.7-51.0	burnt	x	green, unpleasant	x	———	———	—-	—-
2311-2322	51.6-52.0	floral, pungent	2^4	———	—-	———	-———-	—-	—-
2336	52.5	———	—-	floral	x	———	———	—-	—-
2375-2383	53.9-54.2	———	—-	bitter, burnt	x	———	———	—-	—-
2435	56.2	fruit-like, pungent	x	———	—-	benzoic acid	pungent, sour	2,438	56.23
———	60.0-60.1	green	x	bitter	x	———	———	—-	—-
———	60.5	bread- like	x	———	—-	———	———	—-	—-
———	61.1	bitter, phenolic, herb-like	x	———	—-	———	———	—-	—-
———	63.5	bitter, burnt, weak honey-like	2^1	———	—-	———	———	—-	—-
———	64.6	———	—-	pleasant, herb-like	x	———	———	—-	—-
———	64.7-65.1	———	—-	honey-like	x	———	———	—-	—-
———	65.6-65.8	roasted	x	———	—-	———	———	—-	—-
———	66.0-66.4	sweet	x	———	—-	———	———	—-	—-
———	68.7	———	—-	sour	x	———	———	—-	—-
———	68.9	———	—-	adstringent	x	———	———	—-	—-
———	69.3	floral, alcohol	x	green	x	———	———	—-	—-
———	69.9	plum-like	x	———	—-	———	———	—-	—-
———	70.6	———	—-	slightly rancid	x	hexadecanoic acid	rancid	—-	70.59
———	74.3	———	—-	slightly green	x	———	———	—-	—-
———	75.1-76.0	slightly honey-like, sweet	x	sweet, slightly honey-like	x	———	———	—-	—-
———	84.0	rancid, sour	x	———	—-	9-octadecenoic acid	rancid	—-	83.79
———	85.5	slightly bitter	x	———	—-	———	———	—-	—-

[a] Fd = Maximal factor of dilution where odour was perceived; [b] KI = Modified Kóvatz Index according to VAN DEN DOOL and KRATZ (1963); [c] RT = Retention time recorded by GC/FID; [d] Odour only was perceived in the undiluted sample; AEDA fell within the range of 2^1-2^6; Standards were sniffed directly from the containers.

peak at m/z 90 appeared to be formed by loss of the HCN group. This loss can occur if a labile hydrogen is available in the molecule (McLAFFERTY and TURECEK, 1993). 2-phenylethanol, with sweet, floral and pungent odours (KI = 1911) appeared to be a potent odourant to the aroma of both unifloral sources (Table 3). This volatile has already been reported as an aroma-active compound in linden (BLANK *et al.*, 1989) and haze honeys (SHIMODA and OSAJIMA, 1996). 2-butyl-1-octanol (KI = 1,863), with a floral note and benzonitrile (KI = 1940), with a metallic odour, would have a powerful impact on morrão de candeia aroma, only. Five key odours sniffed in the range of 1850-2226 remained unidentified in morrão de candeia honeys (Table 3), probably due to their very low concentration or overlapping peaks as can be seen by GC/MS analysis. Further investigations on their sensory characteristics as well as their identification should be done.

Besides, 2-phenylethanol, 2,3-butanedione (KI = 1,003), with rancid, buttery notes, may be considered a potent odour contributor to assa-peixe aroma (Table 3). This diketone, with a similar odour note, was reported as an important odourant of roasted coffee (HOLSCHER *et al.*, 1990). One remarkable off-odour (putrid, sour, 3-methylbutanoic acid-like odours/1,666-1,689/2^5) was perceived in assa-peixe honeys. It was suggested to be a methylbutanoic acid isomer because the 3-methylbutanoic acid standard had similar odour notes and retention time as those from the samples. However, this volatile was not detected by GC/MS, indicating that it could be present in a very low concentration. Although certain honey-like odours had been sniffed they were perceived only in the concentrated extracts. Some odourants as e.g., 2-(2-butoxyethoxy)-ethanol, with a herb-like, irritant note; 2-cyclohexen-1-one, with an unpleasant odour; phenylmethanol, with a pungent note and benzoic acid, with a sour and pungent note were also identified in the concentrated extracts, only. As a consequence of AEDA, these compounds played a minor role in morrão de candeia and assa-peixe aromas. AEDA gave no information about the contribution of synergism or additional effects of compounds with low Fd-factors. These compounds may have more importance for overall honey aroma than AEDA indicates.

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REFERENCES

Acree T.E. and Teranishi R. (Eds.). 1993. "Flavor Science, Sensible Principles and Techniques" 1 st ed. American Chemical Society, Washington, DC.

AOAC 1984. "Official Methods of Analysis" 14th ed. Association of Official Analytical Chemists, Washington, DC.

Bicchi C., Belliardo F. and Frattini C. 1983. Identification of the volatile components of some Piedmontese honeys. J. Apic. Res. 22:130.

Blank I., Fischer, K.H. and Grosch W. 1989. Intensive neutral odourants of linden honey. Z. Lebensm.-Unters.-Forsch. 189:426.

Bouseta A., Collins S. and Dufour J.P. 1992. Characteristic aroma profiles of unifloral honeys obtained with a dynamic headspace GC-MS system. J. Apic. Res. 31:96.

Bouseta A., Scheirman V. and Collins S. 1996. Flavor and free amino acid composition of lavender and eucalyptus honey. J. Food Sci. 61:683.

Costa L.S.M., Albuquerque M.L.S., Trugo L.C., Quinteiro L.M.C., Barth O.M., Ribeiro M. and De Maria C.A.B. 1999. Determination of non-volatile compounds of different botanical origin Brazilian honeys. Food Chem. 64:1.

Da Costa Leite J.M., Trugo L.C., Costa L.S.M.,

Quinteiro L.M.C., Barth O.M., Dutra V.M.L. and De Maria C.A.B. 2000. Determination of oligosaccharides in Brazilian honeys of different botanical origin. Food Chem. 70:93.

Holscher W., Vitzthum O.G. and Steinhart H. 1990. Identification and sensorial evaluation of aroma-impact-compounds in roasted Colombian coffee. Café Cacao Thé 34:205.

Louveaux J., Maurizio A. and Worwohl G. 1978. Methods of melissopalynology. Bee World 59:139.

McLafferty F.W. and Turecek F. (Eds.) 1993. "Interpretation of Mass Spectra" 4 th ed. University Science Books, California, USA.

Radovic B.S., Careri M., Mangia A., Musci M., Gerboles M. and Anklam E. 2001. Contribution of dynamic headspace GC-MS analysis of aroma compounds to authentic testing of honey. Food Chem. 72:511.

Shimoda M. and Osajima Y. 1996. Aroma compounds from aqueous solution of haze (*Rhus succedanea*) honey determined by adsorptive column chromatography. J. Agric. Food Chem. 40:3913.

Sommer P.G. 1998. O Desenvolvimento da Apicultura Brasileira. 12 th Bienal Brasileira de Apicultura. Salvador, Brazil, November: 173.

Tan S.To, Holland P.T., Wilkins A.L. and Molan P.C. 1988. Extractives from New Zealand honeys. 1. White clover, manuka, and kanuka unifloral honeys. J. Agric. Food Chem. 36:453.

Tan S.To, Wilkins A.L., Holland P.T. and McGhie T.K. 1989. Extractives from New Zealand honeys. 2. Degraded carotenoids and other substances from heather honey. J. Agric. Food Chem. 37:1217.

Tan S.To, Wilkins A.L., Holland P.T. and McGhie T.K. 1989. Extractives from New Zealand honeys. 3. Unifloral thyme and willow honey constituents. J. Agric. Food Chem. 38:1833.

Van den Dool H. and Kratz P.D. 1963. A generalization of the retention index system including linear temperature programmed gas-liquid partition chromatography. J. Chromatogr. 11:463.

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PAPER

BIOLOGICAL ACTIVITY OF SALIVA AGAINST *IN VITRO* LDL OXIDATION AFTER CHEWING COMMERCIAL CHEWING GUMS

ATTIVITÀ BIOLOGICA DELLA SALIVA CONTRO L'OSSIDAZIONE DELLA LDL *IN VITRO* DOPO MASTICAZIONE DI GOMME DA MASTICARE COMMERCIALI

N.K. ANDRIKOPOULOS, A.C. KALIORA, A.N. ASSIMOPOULOU[1] and V.P. PAPAGEORGIOU[1]

Laboratory of Food Chemistry-Biochemistry-Physical Chemistry, Department of Science of Dietetics-Nutrition, Harokopio University, 70 El. Venizelou Av., 176 71 Kallithea, Athens, Greece

[1] Laboratory of Organic Chemistry, Department of Chemical Engineering, Faculty of Engineering, Aristotle University of Thessaloniki, 540 06 Thessaloniki, Greece

ABSTRACT

The biological activity of the saliva from five different chewing gums, collected from six healthy volunteers, on the inhibition of low density lipoprotein (LDL) oxidation, produced *in vitro* by copper ions, was demonstrated and quantitatively expressed as % protection (% Pr). Vitamin E (20 μM, 71.7% Pr), extract from the spearmint plant (50 mg, 63.0% Pr) and different concentrations of BHT synthetic antioxidant (0.014 μg/mL, 9.7% Pr) were used for comparisons. Chewing quantities (0.6, 1.5 and 3 g) and length of chewing time (first 0.25, 1, 3 h, between

RIASSUNTO

È stata dimostrata l'attività biologica della saliva da cinque diversi tipi di cingomme da masticare, prelevata da sei volontari sani, sull'inibizione dell'ossidazione della lipoproteina a bassa densità LDL, prodotta *in vitro* da ioni di rame che è stata espressa quantitativamente come la protezione % (% Pr). Sono state usate per confronto vitamina E (20 μM, 71,7% Pr), estratta dalle piante di menta verde (50 mg, 63,0% Pr) e le varie concentrazioni dell'antiossidante di sintesi BHT (0,014 μg/mL, 9,7% Pr). Sono state valutate delle quantità di gomme (0,6, 1,5 e 3 g) e lunghezza del tempo di ma-

- Key words: antioxidants, chewing gums, Low density lipoproteins, (LDL), *Pistacia lentiscus*, polyphenols, saliva, spearmint -

0.25th and 3rd h or between 6th and 7th h) were evaluated. Under the usual chewing conditions (1.5 g, 1 h) crude Chios mastic gum was found to be the most effective (74.6% Pr) followed by commercial Chios mastic gum (64.3% Pr) and spearmint-flavored gum (61.2% Pr), while the strawberry-flavored gum had a low effect (11.5% Pr). Biologically active substances present in Chios mastic gum (3 g) extracts and in the respective saliva (1 h chewing) were characterized as (poly)phenolic compounds in quantities of 0.3 and 0.2 mg, respectively.

sticazione (all'inizio 0,25, 1 e 3 ore, fra 0,25 fino a 3 ore e fra 6,0-7,0 ore). Sotto condizioni normali (1,5 g, 1 ora), è stata trovata che la pura mastica Chios è la più effettiva (74,6% Pr) seguita poi dalla mastica commerciale Chios (64,3% Pr) e poi dalle cingomma aromatizzate al mentolo (61,2% Pr), mentre si è dato alla cingomma aromatizzata alla fragola un effetto basso (11,5% Pr). Le sostanze biologicamente attive presenti negli estratti nella gomma (mastica) Chios (3 g) e nella rispettiva saliva (1 ora di masticazione) sono state caratterizzate come composti (poli)fenolici in quantità di 0,3 e 0,2 mg, rispettivamente.

INTRODUCTION

There is increasing evidence that the association of blood total cholesterol with coronary heart disease appears to derive chiefly, if not entirely, from the low density lipoprotein (LDL) fraction with which it is highly correlated (GOLDSTEIN and BROWN, 1997). Furthermore, the oxidatively modified LDL stimulates the formation of foam cells, which lead to the formation of atherosclerotic plaques (STEINBRECHER *et al.*, 1990). Adequate dietary intake of antioxidants can help inhibit LDL oxidation and thus the development of atherosclerotic lesions.

The Mediterranean diet, rich in olive oil and in natural antioxidants (polyphenols, vitamins C and E, carotenoids) (BOSKOU, 1996a), has been shown to play a protective role against cardiovascular disease. For example, several polyphenols of the phenolic fraction of olive oil (VISIOLI and GALLI, 1994; VISIOLI *et al.*, 1998) and red wine (FUHRMAN *et al.*, 1995; KERRY and ABBEY, 1997) have been shown to inhibit the *in vitro*-induced LDL oxidation by metallic ions, while aqueous extracts of edible herbs from Crete, rich in polyphenols, have been shown to protect cultured lung cells from *in vitro* oxidation (LIONIS *et al.*, 1998).

Crude Chios mastic gum is a natural resinous exudate obtained from the mastic tree (*Pistacia lentiscus* var. Chia cr. *Anacardiaceae*), which grows almost exclusively on the Island of Chios, Greece. It is obtained by making longitudinal incisions at close intervals from the base of the trunk up to the thicker branches. The resin has been used in traditional medicine for the treatment of various diseases such as gastralgia, dyspepsia and gastric ulcer (AL-SAID *et al.*, 1986), while its definite antibacterial activity against *Helicobacter pylori* has been well documented (HUWEZ *et al.*, 1998). In food processing it has been proven useful as a food preservative (WILLBLOCK, 1999) and antioxidant (ABDEL-RAHMAN and SOAD, 1975). Crude Chios mastic gum is supplied commercially either in the crude form as white or light yellow jelly crystals (1-10 mm diameter) or as sugar-coated or sugar-free chewing packs (1.5 g each). Other commercial chewing gums, also in 1.5 g packs, as sugar-free or sugar-coated forms, usually do not have a natural base; the majority of them

consist of synthetic polymeric bases, flavorings and other additives.

Millions of people chew commercial chewing gum every day, swallowing a considerable amount of saliva enriched with the extracts of the substances contained in the chewing materials. The possible positive or negative *in vitro* biological effect of this action against LDL oxidation was evaluated in the present study.

MATERIALS AND METHODS

Materials

All reagents were of analytical grade and solvents were of high performance liquid chromatography (HPLC) grade. Sterilized LDL lyophilised from 1 mL of LDL solution in 0.15 M NaCl and 0.01% EDTA at pH 7.4, with 6.3 mg protein per 400 mg LDL and phosphate buffer saline (PBS) tablets were purchased from Sigma (St. Louis, MO). Vitamin E (95%, prepared from synthetic phytol) and butylated hydroxytoluene (BHT) were also from Sigma. Malonaldehyde was from Aldrich (Steinheim, Germany). Thiobarbituric acid (TBA) was from Serva (Heidelberg, Germany). Folin Ciocalteau reagent was from Merck (Darmstadt, Germany). Plant spearmint was purchased from a local flea market in Kallithea (Athens, Greece). Chewing gums in commercial packs of approx. 1.5 g (sugar-free or sugar-coated) were bought from local stores in Kallithea, while the crude Chios mastic gum (jelly crystals) was kindly donated by the Mastic Gum Growers' Association (Chios, Greece). The spearmint-flavored chewing gum consisted of edulcorantes (manitol, sorbitol, xylitol, aspartame and akesulfame K), gum base, E322 (lecithin), E422 (glycerine), E321 (BHT) and spearmint flavor. The strawberry-flavored chewing gum consisted of the same edulcorantes and gum base, plus E321, E322, E422, E330 (citric acid), E296 (malic acid), E334 (tartaric acid), E472a (acetic acid esters of mono- and di- glycerides) and strawberry flavor.

Saliva collection

The Research Committee of the Harokopio University (Athens, Greece) approved the protocol for saliva collection. Saliva was collected at a dental clinic from six 25 to 65-year-old non-smoking, healthy volunteers with good mouth hygiene. Before being checked by the dentist, the participants had breakfast and brushed their teeth. Afterwards, they performed the chewing test, during which time eating, drinking or any other activities, except reading magazines, were not allowed. The tests took place on different days. All six volunteers participated in Tests No. 7 and 13 (Fig. 1), while five of them participated in Test No. 6 and only four of them in Tests No. 8-12, 14 and 15. All the tests were performed once for each volunteer. Several combinations of lengths of chewing time and chewing quantities were investigated. The saliva was not swallowed, but was collected in sterilized 20 mL glass tubes with glass cups, in volumes of approximately 15±2 mL during the first 15 min (0.25 h) of chewing, 40±5 mL during the first hour (1.0 h) of chewing or between the 6^{th} and 7^{th} hours of chewing, (6.0-7.0 h) and 60±5 mL during the lengths of chewing time between 15 min and 3 hours (0.25-3.0 h) and 75±5 mL during the entire period of chewing between zero-time and 3 hours (3 h) (the times given in parentheses are the denotations used hereafter). In accordance with a previous study (BOROS *et al.*, 1999) the gum chewing stimulation, which was common to all subjects, tended to minimize the differences in the rate of flow of saliva between the subjects. Tests No. 8 and 10 were performed on the same day and the saliva secreted during 0.25 h was collected separately from saliva se-

creted during 3.0 h. The same procedure described above was followed for Tests No. 11 and 12. For Test No. 2, which was defined as "blank saliva" test, secreted saliva was collected only during the last hour of a total seven-hour period of chewing. The saliva samples collected were immediately evaporated under a stream of nitrogen and evaporation was accomplished in a 37° C water bath, at a rate of approximately 5-6 mL per hour. The walls of the tubes were washed down with small volumes of ethanol and evaporated again. The residue was taken up in 0.01 mL ethanol and afterwards subjected to the LDL oxidation test.

LDL biological tests

LDL was kept at 2°C and its working suspension (200 μg protein/mL PBS) was prepared just before use. These precautions were taken since commercial LDL might be less stable than fresh LDL isolated from freshly drawn blood. The LDL oxidation test (LDL-ox) was performed as described by BALLA *et al.* (1991) with slight modifications. In brief, 1.0 mL of LDL working solution was mixed with 1.0 mL $CuSO_4$ (10 μM) as oxidizing agent, incubated at 37°C for 1, 3 and 6 h, and the resulting thiobarbituric acid reactant substances (TBA-RS), mainly malonaldehyde, were measured spectrophotometrically at 532 nm, after reaction with 2 mL of 1% alkaline TBA solution. Thiobarbituric Acid Reactive Substances (TBA-RS) were evaluated using a standard curve of standard malonaldehyde at different concentrations versus absorption at 532 nm. An LDL-blank test was also performed without the presence of copper ions. The LDL-ox and LDL-blank tests (Tests No. 1 and 16, respectively, Table 1) were carried out four times.

In Tests No. 2-15, the samples tested (saliva residues or standard samples of spearmint plant extract, vitamin E and BHT) were added as 0.01 mL ethanol solutions with simultaneous addition of 0.99 mL $CuSO_4$, (instead of the addition of 1.0 mL only in the LDL-ox test described above). Saliva residues collected during Tests No. 2 (Table 1) and 6-15 (Fig. 1) were examined against LDL oxidation once, while Tests No. 3, 4 and 5 of reference samples were tested four times. The aforementioned 0.01 mL ethanol in the test mixture was found to exhibit no effect on the test results, which is in agreement with GORDON and WENG (1992).

Polyphenolic content

Total polyphenol content of saliva residue ethyl-acetate extract (3x5 mL) and of methanol/water (6:4, v/v) extracts (3 x 3 mL) from crude Chios mastic gum (0.5 g) and plant spearmint (0.5 g) were measured spectrophotometrically, as previously described (GUTFINGER, 1981). In brief, the above extracts were evaporated under a stream of nitrogen and the residues were diluted in 1 mL of ethyl-acetate or methanol, respectively. A portion of 0.1 mL of the 1 mL solutions reacted with the Folin Ciocalteau reagent and the products were measured spectrophotometrically at 725 nm. The results, expressed as ppm of caffeic acid, were calculated using a standard curve of caffeic acid in different concentrations (0-200 ppm) versus absorption at 725 nm. HPLC of the polyphenols from the saliva and crude Chios mastic gum extracts was performed according to a quaternary solvent gradient method, as previously described (ANDRIKOPOULOS *et al.*, 1989).

Statistical analysis

Statistical treatment of the results was performed by standard methods of statistical analysis (WALPOLE and MYERS, 1978), using a computer program for commuta-

Table 1 - Inhibitory effect against *in vitro* LDL oxidation, expressed as TBA-RS (μM), and % Protection (% Pr) of Chios mastic gum (CMG) saliva residues (SR).

Test No. (i)	Samples	Incubation period	1 h	3 h	6 h
1 (n=4)¶	LDL-ox	TBA-RS [a]	1.412±0.292	2.294±0.352	2.694±0.345
		TBA-RS net [a, b]	1.412±0.292	2.193±0.437	2.471±0.387
2 (n=4)*	SR, blank-saliva§	TBA-RS [a]	1.396±0.206	2.279±0.479	2.672±0.256
		TBA-RS net [a, b]	1.396±0.206	2.179±0.544	2.449±0.309
		% Pr [a, c]	1.1±25.1	0.6±31.8	0.9±19.8
9 (n=4)*	SR after chewing (1.0 h) CMG (1.5 g), sugar coated	TBA-RS [a]	0.835±0.250	1.050±0.307	1.050±0.355
		TBA-RS net [a, b]	0.835±0.250	0.950±0.402	0.882±0.396
		% Pr [a, c]	40.8±27.2	56.7±27.0	64.3±22.4
15 (n=4)*	SR after chewing (1.0 h) crude CMG (1.5g)	TBA-RS [a]	0.700±0.288	0.740±0.027	0.850±0.358
		TBA-RS net [a, b]	0.700±0.288	0.640±0.260	0.627±0.399
		% Pr [a, c]	50.4±29.0	70.8±23.1	74.6±22.5
16 (n=4)¶	LDL-blank	TBA-RS [a]	0.000±0.001	0.100±0.259	0.223±0.175

Significance tests (*t*-Tests)

Test 1 versus Test 9	$p < 0.00007$	$p < 0.000003$	$p < 0.0000008$
Test 1 versus Test 15	$p < 0.00003$	$p < 0.00006$	$p < 0.0000003$

For % Pr, LDL-ox Test, LDL-blank Test and [a, b, c] superscripts see "Materials and Methods" section.
TBA-RS = thiobarbituric acid reactant substances.
¶ n=number of replications.
* n=number of subjects.
§ Saliva collected during 6.0-7.0th h of a total seven-hour continuous chewing of 3.0 g sugar coated CMG.

tions (ANOVA Toolkit application of MS Excel). The results in Table 1 are presented as 99% confidence intervals, calculated using the following formulae (in the order of [a, b, c] superscripts in Table 1):

$$^{a}\ \text{TBA-RS}_i \pm (99\%\ \text{Confidence Limits})_i$$
$$(99\%\ \text{Confidence Limits})_i = t_{v,a} \times SD_i / n^{1/2}$$

$$^{b}\ \text{TBA-RS}_{\text{net } i} = \text{TBA-RS}_i - \text{TBA-RS}_{16}$$
$$SD_{\text{net } i} = (SD_i^{\,2} + SD_{16}^{\,2})^{1/2}$$
$$\text{and } (99\%\ \text{Confidence Limits})_{\text{net } i} =$$
$$= t_{v,a} \times SD_{\text{net } i} / n^{1/2}$$

$$^{c}\ \%Pr_i = (\%\ \text{Protection of test i}) =$$
$$= (\text{TBA-RS}_{\text{net } 1} - \text{TBA-RS}_{\text{net i}}) \times 100 / \text{TBA-RS}_{\text{net } 1}$$
$$\%SD_i = (SD_{\text{net } 1}^{\,2} + SD_{\text{net } i}^{\,2})^{1/2} \times 100 /$$
$$/ \text{TBA-RS}_{\text{net } 1}$$
$$\text{and } (99\%\ \text{Confidence Limits})_{\%Pr\ i} =$$
$$= t_{v,a} \times \%SD_i / n^{1/2}$$

where: the subscript *i* is the serial number of the test (see first column of Table 1); TBA-RS_i = mean value of TBA-RS obtained in Test No. *i*; SD_i = standard deviation of all the experimental TBA-RS values of Test No. *i* (so $SD_i / n^{1/2}$ equals the

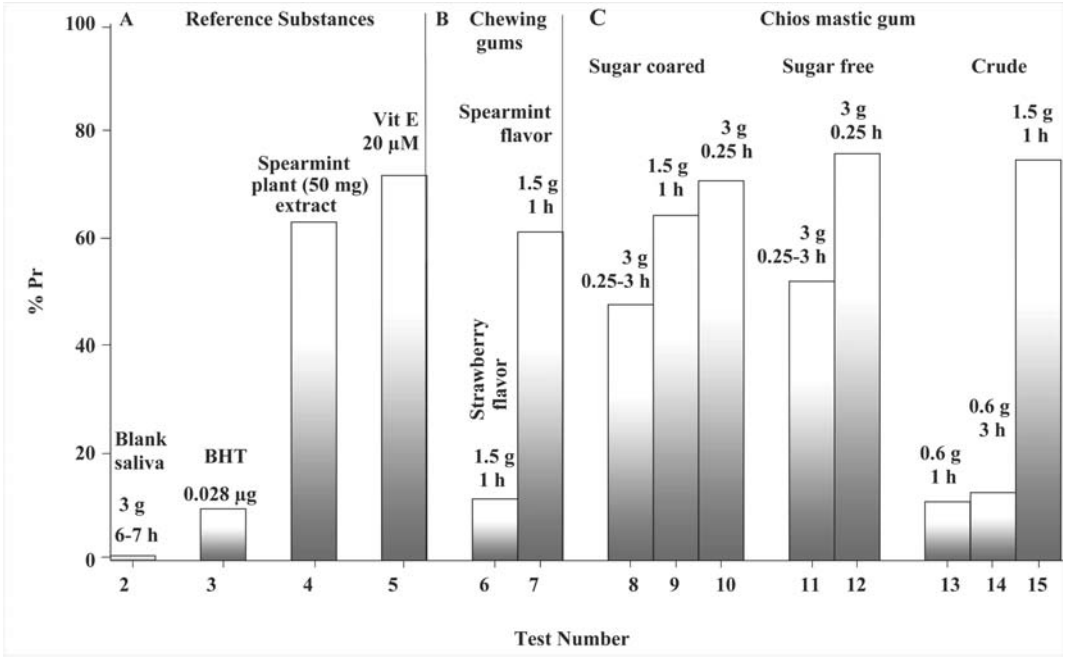


Fig. 1. - Protective (%) effect of (A) reference substances, (B) chewing gum saliva residues and (C) Chios mastic gum saliva residues, against the *in vitro* LDL oxidation.
% Pr: % Protection based on results of 6 h-incubation (see Table 1 and "Statistical analysis" subsection). The quantities (g) of gums used for chewing and the respective lengths of chewing time (h) are given above the bars. BHT: Butyl-hydroxy-toluene, 0.028 µg in the test cuvette (equivalent to 200 ppm in chewing gum transferred in blood circulation). Vit E: Vitamin E, 20 µM in the test cuvette. Tests No. 1 (LDL-ox) and No. 16 (LDL-blank) exhibited the lowest and highest % Pr, respectively, and were used as the lower and upper limits for the evaluation of the other Tests (No. 2-15).
The 99% confidence limits (computed as described in the subsection "Statistical Analysis") are as follows: Test No. 2 = ±19.8; Test No. 3 = ±20.1; Test No. 4 = ±19.6; Test No. 5 = ±19.6; Test No. 6 = ±20.1; Test No. 7 = ±20.9; Test No. 8 = ±20.7; Test No. 9 = ±22.4; Test No. 10 = ±20.1; Test No. 11 = ±19.5; Test No. 12 = ±20.1; Test No. 13 = ±26.3; Test No. 14 = ±35.0; Test No. 15 = ±22.5.

standard deviation of the mean value TBA-RS$_i$; $t_{v,a}$ = critical t-distribution factor for $v = (n - 1)$ degrees of freedom and 100 $(1 - 2\alpha)$ % confidence (e.g. for 99% confidence limits applied throughout the present report, $v = (n - 1) = 3$ and $t_{v,a} = 5.841$); TBA-RS$_{net\ i}$ and SD $_{net\ i}$ = respective values of the net of Test No. *i* oxidation derived by subtracting the mean value of TBA-RS of the LDL-blank from the respective mean value of Test No. *i*; and, %Pr$_i$ and %SD$_i$ = respective "relative" values of the percent protection of Test No. *i*. % Pr$_i$ is an indicative value expressing the relative inhibitory action of saliva, collected after chewing gum *i*, on the LDL oxidation produced *in vitro* by copper ions (Test No. 1).

Thus, e.g. for Test No. 9 (6 h incubation):

Average TBA-RS$_i$ = 1.050, SD$_i$ = 0.1215 and 99% Confidence Limits for TBA-RS$_i$ = (5.841 x 0.1215) / 2 = 0.355. Net oxidation TBA-RS$_{net\ 9}$ = (1.050 - 0.223) = 0.882, SD $_{net\ 9}$ = $(0.1215^2 + 0.0599^2)^{1/2}$ = 0.1350, and 99% Confidence Limits for TBA-RS$_{net\ 9}$ = (0.1350 x 5.841) / 2 = 0.396. % Pr$_9$ = (2.471 - 0.882) x 100 / 2.471 ≈ 64.3, %SD$_9$ = $(0.1350^2 + 0.1326^2)^{1/2}$ x 100 / 2.471 = 7.658, and 99% Confidence Limits for % Pr$_9$ = (7.658 x 5.841) / 2 = 22.4).

RESULTS AND DISCUSSION

The biological tests for two representative saliva residue samples after chewing different Chios mastic gums under the usual chewing conditions (1.5 g, 1.0 h), are presented in Fig. 2, together with the LDL-ox test, the "blank-saliva" test and LDL-blank test. The protecting effect of the saliva residue from Chios mastic gum is evident. The "blank-saliva" results are reported, because, after six hours of continuous chewing, all the biologically active compounds from the mastic gum are expected to be swallowed. This was confirmed by the absence of any protective effect on LDL protection from the respective saliva secreted between the 6th and the 7th hour of continuous chewing. This is also indicative of the upper limit of length of chewing time, beyond which the protective effect is negligible. The choice for the collection of the saliva secreted during chewing as "blank saliva", instead of the resting saliva, was made, because the masticatory movements provide stimuli to the human parotid gland, which enhances saliva secretion (KERR, 1960). More importantly, mastication produces almost the same amount of secretion among the subjects under a certain stimulation (BOROS *et al.*, 1999). In Table 1 the percentage of protection (% Pr) calculated for some indicative samples of saliva residues from Chios mastic gum are also presented. After six-hours of incubation, TBA-RS values tended to stabilize (data not shown), thus no further incubation times were used. Therefore, the % Pr refers to 6 h incubation time.

The protective effect against the *in vitro* LDL oxidation of the saliva residues from six volunteers after chewing five different chewing gums, in different quantities and for different lengths of

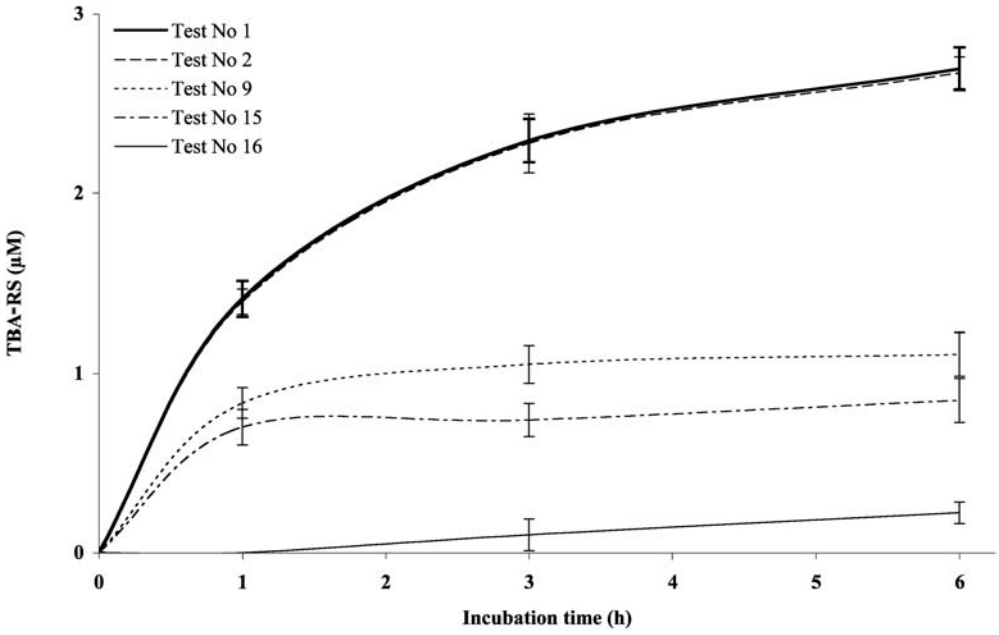


Fig. 2 - Biological activity (TBA-RS) against copper sulfate induced LDL oxidation of representative samples of Table 1: Test No. 1 = LDL-ox; Test No. 2 = SR "blank-saliva"; Test No. 9 = SR from CMG, sugar coated; Test No. 15 = SR from crude CMG; Test No. 16 = LDL-blank. Abbreviations and other conditions as in Table 1.

time, is reported in Fig. 1. Equal chewing amounts and lengths of chewing time of the chewing gums examined (1.5 g, 1.0 h) showed that the crude Chios mastic gum (Test No. 15) gave the highest protective activity on LDL oxidation, followed by the sugar-coated Chios mastic gum (Test No. 9), the commercial spearmint-flavored gum (Test No. 7) and the commercial strawberry-flavored gum (Test No. 6). The inhibitory effect of the spearmint-flavored gum is probably due to the spearmint, as shown by the percentage protection of Tests No. 7 and 4. Since the BHT content was not reported on the spearmint-flavored gum label, the additive was tested against LDL oxidation in the different final concentrations 0.014, 0.035, 0.07, 0.14, 0.28 and 0.56 μg/mL. These concentrations correspond to a possible migration of BHT in human blood (4.5 L) circulation (200 mg LDL / dL blood) from 200, 500, 1,000, 2,000, 4,000 and 8,000 ppm BHT in gum, respectively. These BHT concentrations exhibited dose dependent % Pr values as follows: 9.7±20.1, 14.3±21.6, 22.7±20.1, 28.7±19.5, 47.9±19.5 and 55.6±19.8, respectively. The 200 ppm concentration was the most appropriate for comparison, since it represents the usual upper limit in foods. It seems that the spearmint flavor contributed the most to the inhibitory effect, unless the BHT in the gum was close to the unusual 8000 ppm concentration. Whether the E422 (glycerin) additive contributed to the antioxidant activity requires further investigation. The E322 (lecithin) additive, which is also contained in the strawberry-flavored gum, appeared to have a negligible effect, along with the strawberry-flavored gum (Test No. 6) which had only 11.5% Pr, the lowest among the tested gum saliva residues.

Comparing the different chewing quantities (Tests No. 13 and 15) on the % Pr effect under similar conditions indicated that the protective effect depends on the chewing quantity. Thus, an increase in the chewing quantity of crude Chios mastic gum (jelly crystals) from 0.6 g to 1.5 g resulted in an approx. 54% higher protective effect. For comparable quantities and types of Chios mastic gum (2 packs = 3 g, sugar-coated), by comparing the effects during the first 0.25 h of chewing (Test No. 10) with those in the period between 0.25 and 3.0 h of continuous chewing (Test No. 8), 59.7% of the protective compounds were eluted from the chewing material during the first 15 min of chewing. These results are almost identical to the results obtained in Tests No. 11 and 12 (59.3%), using sugar-free gum under respective chewing conditions. By also taking into account the results of Tests No. 13 and 14, it appears that about 85% of the protective substances are eluted from the chewing material during the first hour of chewing.

Comparing Tests No. 8 and 11 (0.25-3.0 h chewing) and Tests No. 10 and 12 (0.25 h chewing), it can be concluded that after chewing 2 packs of commercial Chios mastic gum, the sugar-free gum gave approx. 5% higher protection against LDL oxidation than the sugar-coated gum. Since the sugar-free Chios mastic gum also has a coating of natural sweeteners, it still has to be investigated if the slight variation is due to the sugar or the sweeteners. Additionally, in comparing Tests No. 9 and 15 the commercial and crude Chios mastic gum gave similar % Pr. The slight difference (10.3%) is probably due to the slight differences in weight (approx. 1.5 g) or the additives in the sugar-coated pack.

The inhibitory effect of Chios mastic gum (Test No. 15) under the usual conditions of chewing was found to be almost equal to that of the optimum dose of vitamin E (Test No. 5) (GORDON and WENG 1992). After six hours of chewing (and probably much earlier), all the bio-

logically active substances in Chios mastic gum had disappeared (Test No. 2) also indicating that pure saliva has no protective effect on LDL oxidation.

The active substances in Chios mastic gum and in saliva after chewing Chios mastic gum were isolated by solvent extraction. In these extracts the presence of phenolic compounds was determined colorimetrically, while the similarity of the individual phenols in both extracts was proven by the same peak sequence and retention times after HPLC analysis (data not shown). HPLC peak identification is now in progress, combined with the respective bioavailabilities, and the results will be published in the future. Both extracts also exhibited biological activity in LDL oxidation tests, comparable to Tests No. 9 and 15 in Fig. 2.

Total polyphenols (0.3 mg) from Chios mastic gum (3 g, 2 packs) were comparable to the amount (0.2 mg) found in the saliva from chewing the same amount of Chios mastic gum (3 g, 1 h chewing). The latter corresponds to 0.1 mg polyphenols from 1.5 g, 1 h chewing (64.3% Pr, Test 9).This is almost equal to the amount of polyphenols estimated (0.066 mg) in the 50 mg of spearmint plant extract (63.0% Pr, Test 4). This indicates a possible relationship between the percentage protection and the polyphenol content in both Chios mastic gum and the spearmint plant extract.

Recently, a distinct effect of several polyphenol-rich extracts of Chios mastic gum against *in vitro* LDL oxidation has been documented (ANDRIKOPOULOS *et al.*, 2002). Compared to olive oil, which is the main source of fat in Mediterranean countries (20-40 g olive oil/day/person) and which is known to be rich in polyphenols (50-200 mg/kg) (BOSKOU, 1996a), the phenolic contribution of Chios mastic gum to the daily intake of phenols is indeed valuable. It amounts to approximately 10% of that from olive oil, considering that the amount from olive oil is 1 (VISIOLI *et al.*, 1998) to 2 mg/day/person (BOSKOU, 1996b). In addition, chewing two packs of Chios mastic gum amounts to 1-5% of the overall daily intake of polyphenols, when considering an intake of 23 mg flavonols/day/person (HERTOG *et al.*, 1993), but quite a bit lower when considering 1 g/day/person (SCALBERT and WILLIAMSON, 2000). In Mediterranean countries, the dietary antioxidants, when consumed over long time periods, have been suggested as being able to reduce mortality due to cardiovascular heart disease (HERTOG *et al.*, 1993).

These findings support the relationship of Chios mastic gum to the beneficial effects of the polyphenol-containing foods and beverages e.g. olive oil, wine, herbs and species and other vegetables most of them present in the Mediterranean diet. For example, the protection of LDL by olive oil polyphenols has been well documented by *in vitro* experiments (VISIOLI and GALLI, 1994) and by *ex-vivo* experiments on rabbits (WISEMAN *et al.*, 1996) and humans (AVIRAM and EIAS, 1993). Other polyphenolic compounds isolated from black or green tea extracts (YANG *et al.*, 2000) and from human saliva after consumption of tea (YANG *et al.*, 1999a) have also been reported with biological activities, *in vitro* e.g. in cancer prevention. Bioavailability of olive oil polyphenols has been partially estimated for tyrosol (MIRO CASAS *et al.*, 2001; VISIOLI *et al.*, 2000) and hydroxytyrosol (VISIOLI *et al.*, 2000) and for tea polyphenols for catechins (YANG *et al.*, 1999b). The aforementioned biological properties, combined with respective bioavailabilities, whenever demonstrated unequivocally *in vivo*, may partially explain the beneficial effect of phenolic and polyphenolic compounds, as already pointed out by SCALBERT and WILLIAMSON (2000). Until now, estimation of some certain biological activities *in vitro* remains the only evidence for a possible link between the phenolic content of Chios mastic gum and health benefits, while *in vivo* and *ex*

vivo investigations are an area with promising prospects.

Chios mastic gum is a local Mediterranean product and, apart from its consumption as chewing gum, also has several other uses, especially in Greek, Turkish and Arabic cooking, e.g., in powder form as a food additive, in the form of a sugar containing gel as a table-sweetener and in the form of mastic oil as a beverage and sweet additive. It would be expected that these uses of Chios mastic gum would give greater protective activity than chewing gum, as in these cases the gum is directly swallowed. Nevertheless, saliva from chewing could also be considered to make a marked contribution to the average intake of Chios mastic gum products, because for many people chewing is a daily habit in contrast to the other products, which are consumed only occasionally. The polyphenolic intake from chewing Chios mastic gum also has the advantage of the "net intake" e.g. without the triglyceride content of olive oil, which contributes to the daily intake of lipids.

CONCLUSIONS

In conclusion, saliva secreted during chewing of crude Chios mastic gum was proven to be the most effective in protecting LDL from *in vitro* oxidation, compared to other commercial chewing gums with synthetic polymeric base and flavor/antioxidant additives. To obtain the greatest possible health benefits, the length of chewing time should be over 15 min, though not more than 1 h.

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REFERENCES

Abdel-Rahman A.H.Y. and Soad A.M.Y. 1975. Mastich as antioxidant. J. Am. Oil Chem. Soc. 52:423.

Al-Said M.S., Aggel A.M., Parmar N.S. and Tariq M. 1986. Evaluation of mastic, a crude drug obtained from *Pistacia lentiscus* for gastric and duodenal anti-ulcer activity. J. Ethnopharmacol. 15:271.

Andrikopoulos N.K., Brushweiler H. Felber, H. and Taeschler C.h. 1989. HPLC analysis of phenolic antioxidants, tocopherols and triglycerides. J. Am. Oil Chem. Soc. 68:359.

Andrikopoulos N.K., Kaliora A.C., Assimopoulou A.N. and Papageorgiou V.P. 2002. Biological activity of some naturally occuring resins, gums and pigments against the *in vitro* LDL oxidation. Phytother. Res. In press.

Aviram, M. and Eias K. 1993. Dietary olive oil reduces low-density lipoprotein uptake by macrophages and decreases the susceptibility of the lipoprotein to undergo lipid peroxidation. Ann. Nutr. Metab. 37:75.

Balla G., Jacob H.S., Eaton J.W. and Vercellotti G.M. 1991. Hemin. A possible physiological mediator of low density lipoprotein oxidation and endothelial injury. Arterioscler. Thromb. 11:1700.

Boros I., Keszler P. and Zelles T. 1999. Study of saliva secretion and salivary fluoride concentration of the human minor labial glands by a new method. Arch. Oral Biol. (44 Suppl.) 1: S59.

Boskou D. 1996a. Olive oil composition. In "Olive Oil. Chemistry and Technology". D. Boskou (Ed.): p. 52. AOCS Press. Champaign (IL).

Boskou D. 1996b. History and characteristics of the olive tree. In "Olive Oil. Chemistry and Technology". D. Boskou (Ed.). p. 1: AOCS Press Champaign (IL).

Fuhrman B., Lavy A. and Aviram M. 1995. Dietary supplementation of red wine reduces the susceptibility of human plasma and LDL to undergo lipid peroxidation. Am. J. Clin. Nutr. 61:539.

Goldstein J.L. and Brown M.S. 1997. The low-density lipoprotein pathway and its relation to atherosclerosis. Ann. Rev. Biochem. 46:897.

Gordon M.H. and Weng X.C. 1992. Antioxidant properties of extracts from tanshen (*Salvia miltiorrhiza* Bunge). Food Chem. 44:119.

Gutfinger T. 1981. Polyphenols in olive oil. J. Am. Oil Chem. Soc. 58:966.

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

Huwez F.U., Thirlwell D. and Al'Aldeen D.A.A. 1998. Mastic gum kills *Helicobacter pylori*. New Engl. J. Med. 339: 1946.

Kerr AC. 1960. "The Physiological Regulation of Salivary Secretion in Man". International Series of Monographs on Oral Biology. Pergamon Press, Oxford.

Kerry L.N.S. and Abbey M. 1997. Red wine and fractionated phenolic compounds prepared from red wine inhibit low-density lipoprotein oxidation in vitro. Atherosclerosis 135:93.

Lionis C., Faresjö A., Skoula M., Kapsokefalou and Faresjö T. 1998. Antioxidant effects of herbs in Crete. Lancet 352:1987.

Miro Casas E., Farre Albadalejo M., Cavas Planells M.I., Fito Colomer M., Lamuela-Raventos R.M. and De La Torre Fornell R. 2001. Tyrosol biovailability in human after ingestion of virgin olive oil. Clin. Chem. 47:341.

Parthasarathy S., Steinberg D. and Witztum J.L. 1992. The role of oxidized low-density lipoproteins in the pathogenesis of atherosclerosis. Annu. Rev. Med. 43:219.

Scalbert A. and Williamson G. 2000. Dietary intake and bioavailability of polyphenols. J. Nutr. 130(8S Suppl.):2073S.

Steinbrecher U.P., Zhang H. and Lougheed M. 1990. Role of oxidatively modified LDL in atherosclerosis. Free Rad. Biol. Med. 9:155.

Visioli F. and Galli C. 1994. Oleuropein protects low density lipoprotein from oxidation. Life Sci. 55: 1965.

Visioli F., Bellomo G. and Galli C. 1998. Free radical-scavenging properties of olive oil polyphenols. Bioch. Biophys. Res. Commun. 247:60.

Visioli F., Galli C., Bornet F., Mattei A., Galli G. and Caruso D. 2000. Olive oil phenolics are dose-dependently absorbed in humans. FEBS Lett. 468:159.

Walpole R.E. and Myers R.E. 1978 Probability and statistics for engineers and scientists. In "Tests Concerning Means and Variances". 2nd edition: p. 248. Collier Macmillan Publishers, London.

Willblock I. 1999. Discovering antibacterial mastic. LIFE Enhancem. April: 5.

Wiseman S.A., Mathot J.N.N.J., de Fouw J.N. and Tijburg L.B.M. 1996. Dietary non-tocopherol antioxidants present in extra virgin oil increase the resistance of low density lipoproteins to oxidation in rabbits. Atherosclerosis 120:15.

Yang C.S., Lee M.J. and Chem L.S. 1999a. Human salivary tea catechin levels and catechin esterase activities: implication in human cancer prevention studies. Cancer Epidimiol. Biomark. Prev. 8:83.

Yang C.S., Kim S., Yang G.Y., Lee M.J., Liao J., Chung J.Y. and Ho C.T. 1999b. Inhibition of carcinogenesis by tea: bioavailability of tea polyphenols and mechanisms of action. Proc. Soc. Exp. Biol. Med. 220:213.

Yang C.S., Chung J.Y., Yang G., Chabra S.K. and Lee M.J. 2000. Tea and tea polyphenols in cancer prevention. J. Nutr. 130(2S Suppl.):472S.

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SHORT COMMUNICATION

PHENOLIC COMPOSITION OF APPLE JUICE TREATED BY LACCASE IMMOBILIZED ON COPPER-CHELATE REGENERABLE CARRIER

COMPOSIZIONE FENOLICA DEL SUCCO DI MELA TRATTATO CON LACCASI IMMOBILIZZATA SU CARRIER RIGENERABILE

P. PIACQUADIO, G. DE STEFANO and V. SCIANCALEPORE
Dipartimento di Scienze e Tecnologie Agro-Alimentari Ambientali e Microbiologiche, Università del Molise, Via De Sanctis, 86100 Campobasso, Italy

ABSTRACT

Phenolic composition of apple juice treated with laccase immobilized on copper-chelate carrier was determined by HPLC analysis. Significant reductions were obtained for all the phenolic compounds identified with respect to the untreated juice. The greatest extent of reduction was for chlorogenic acid and (-)-epicatechin (47.1 and 36.4%, respectively).

RIASSUNTO

La composizione fenolica del succo di mela trattato con laccasi immobilizzata su carrier rigenerabile è stata determinata mediante HPLC. I risultati ottenuti hanno mostrato riduzioni significative di tutti i componenti fenolici identificati rispetto al succo non trattato. L'acido clorogenico e l'epicatechina sono risultati i composti fenolici maggiormente ridotti a seguito del trattamento enzimatico, con una percentuale di riduzione pari a 47,1 e 36,4%, rispettivamente.

- Key words: apple juice, immobilization, laccase, phenolic composition, regenerable carrier -

INTRODUCTION

The technique of immobilizing laccase (EC.1.10.3.2.) on metal-chelate regenerable carriers has been used to remove phenolic compounds from model solutions (DE STEFANO *et al.*, 1996). The same technique was then successfully applied to apple juice (PIACQUADIO *et al.*, 1997) and to grape must (SERVILI *et al.*, 2000) to prevent the undesirable browning phenomenon that causes unpleasant colours and flavours and loss of nutrients (GOUPY *et al.*, 1990; TRONC *et al.*, 1997). This technique has been shown to be advantageous for decreasing the cost of stabilization treatment and for reducing technical and available materials because of the carrier reuse (PIACQUADIO *et al.*, 1997).

In this paper results are reported on this new stabilization treatment in relation to the phenolic composition of apple juice.

MATERIALS AND METHODS

Apple (*Malus domestica*, cv. *Golden delicious*) juice preparation, enzyme-carrier preparation using laccase (EC. 1.10.3.2.) and collection of the enzyme-treated samples were carried out according to the procedures reported by PIACQUADIO *et al.* (1997).

Phenolic composition of untreated (control) and enzyme-treated apple juice samples was determined. The extraction of phenolic compounds from the juice was performed using an Extract-Clean highload C_{18} cartridge (5 g; Alltech Italia S.r.l., Sedriano, Milano) which was preconditioned using a sequence of 100 mL of methanol and 40 mL of water adjusted to pH 7.0. The optimal recovery conditions of the phenols were the following: 10 mL of apple juice sample was adjusted to pH 7.0 with NaOH, loaded onto the C_{18} preconditioned column and washed with 10 mL of pH 7.0 water. The adsorbed fractions were eluted with 40 mL of methanol and evaporated under vacuum at a nitrogen flow rate of 30 mL min^{-1} at 35°C. The phenolic extract was dissolved in 0.5 mL of methanol, filtered through a 0.45 μm filter (Whatman International Ltd., Springfield Mill, Maidstone, Kent, England) and then 20 μL were injected into the HPLC, using a Prostar Solvent Delivery Modules (Varian Analytical Instruments, Walnut Creek, CA) chromatograph with a model 230 pump system controller and a model 330 photodiode array detector. The column was an Inertsil ODS-3 (150x4.6 mm I.D.) (Alltech Italia S.r.l., Sedriano, Milano). The mobile phase used was the same programmed blend of two solvents used by SERVILI *et al.* (2000); A was 0.2% (v/v) acetic acid in water and B was methanol. The initial mobile phase composition was 5% B/A for 19 min, then 50% B/A for 10 min, then 100% B for 10 min. The final composition was held for 5 min. Each compound was identified by comparing its retention time and UV spectrum with standards *i.e.* chlorogenic acid, *(-)*-epicatechin, ferulic acid, vanillic acid and phloridzin. These standards were all purchased from Sigma Chemical Co. (St. Louis, MO).

The mean values ± standard deviations of triplicate trials are reported in Table 1. To evaluate the significance of differences between mean values of the control and the treated sample, one way analysis of variance (ANOVA) using the paired *t*-test was used. Statgraphics Version 6.1 (Statistical Graphics Corp., 1992, Manugistics, Inc., Rockville, MD) was used to perform all statistical analyses.

RESULTS AND CONCLUSIONS

The enzymatic treatment was performed using optimized conditions (pH, flow rate, temperature) as previously reported (SERVILI *et al.*, 2000). The total phenol content of the enzyme-treated

apple juice was significantly lower than that of untreated juice (data not shown), confirming our previous results (PIACQUADIO *et al.*, 1997).

The predominant phenolic compounds in the apple juice (Fig. 1) were chlorogenic acid and *(-)*-epicatechin, which is in agreement with the results of SCHIEBER *et al.* (2001). All the phenolic compounds identified were significantly reduced after the enzymatic treatment (Table 1). Chlorogenic acid and *(-)*-epicatechin were reduced to the greatest extent (47.1 and 36.4%, respectively), followed by vanillic acid (22.7%). Ferulic acid and phloridzin were reduced to the lowest extent (13.9 and 14.2%, respectively). These differences in reduction are due to different laccase activity probably as a consequence of different links between the enzyme and the substrate (phenol) at active enzyme sites.

These results indicate that the ortho-

Table 1 - Phenolic composition of apple juice before (control) and after treatment with laccase immobilized on copper-chelate carrier.

Phenols*	Control	Enzyme-treated
Vanillic acid	10.07±1.13[a]	7.78±1.03[b]
Chlorogenic acid	101.91±2.11[a]	53.86±1.88[b]
(-)-epicatechin	71.18±1.94[a]	45.25±1.75[b]
Ferulic acid	14.46±1.31[a]	12.44±1.22[b]
Phloridzin	7.63±1.06[a]	6.54±0.95[b]

Values are means ± SD from three independent determinations. Values in each row bearing different superscripts are significantly different ($P < 0.05$).
* Evaluated by HPLC and expressed as mg L^{-1}.

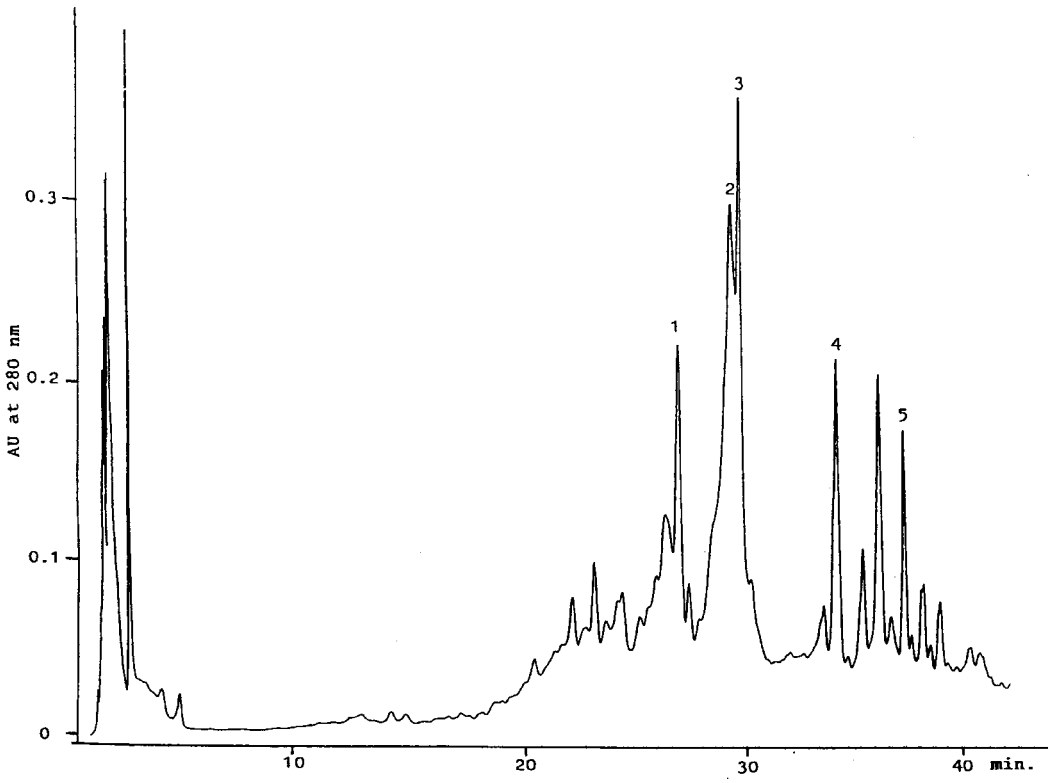


Fig. 1 - HPLC chromatogram of apple juice. Peak numbers: (1) vanillic acid; (2) chlorogenic acid; (3) *(-)*-epicatechin; (4) ferulic acid; (5) phloridzin.

diphenolic structures, chlorogenic acid and *(-)*-epicatechin, were more oxidizable than the monophenolic structures.

Work is in progress to determine if the treatment is efficient in terms of browning, considering that the amount of ortho-diphenolic compounds is still high in the enzyme-treated apple juice.

REFERENCES

De Stefano G., Piacquadio P. and Sciancalepore V. 1996..Metal-chelate regenerable carriers in food processing. Biotechnol. Techn. 10:857.

Giovanelli G. and Ravasini G. 1993. Apple juice stabilization by combined enzyme-membrane filtration process. Lebensm.-Wiss.-Technol. 26:1.

Goupy P., Amiot M.J., Richard-Forget F., Duprat F., Aubert S. and Nicolas J. 1995. Enzymatic browning of model solutions and apple phenolic extracts by apple polyphenoloxidase. J. Food Sci. 60:497.

Piacquadio P., De Stefano G., Sammartino M. and Sciancalepore V. 1997. Phenols removal from apple juice by laccase immobilized on Cu^{2+}-chelate regenerable carrier. Biotechnol. Techn. 11:515.

Schieber A., Keller P. and Carle R. 2001. Determination of phenolic acids and flavonoids of apple and pear by high-performance liquid chromatography. J. Chrom. A, 910:265.

Servili M., De Stefano G., Piacquadio P. and Sciancalepore V. 2000. A novel method for removing phenols from grape must. Am. J. Enol. Vitic. 51:357.

Tronc J.S., Lamarche F. and Makhlouf J. 1997. Enzymatic browning inhibition in cloudy apple juice by electrodialysis. J. Food Sci. 62:75.

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SHORT COMMUNICATION

IDENTIFICATION OF SULPHATHIAZOLE BY HPLC/APCI-MS IN EDIBLE TROUT TISSUE

IDENTIFICAZIONE DI SULFATIAZOLO PER HPLC/APCI-MS IN CARNE DI TROTA

M. BONONI, E. DE DOMINICIS[1], A. FOSSATI and F. TATEO*
Laboratorio Analisi Alimenti HRGC/MS/HPLC, Di.Pro.Ve, Dipartimento di Produzione Vegetale, Facoltà di Agraria, Università degli Studi di Milano, Via Celoria 2, 20133 Milano, Italy
[1] Laboratori Chelab s.r.l., 31023 Resana, Tv, Italy
*Corresponding author: e-mail: fernando.tateo@unimi.it

ABSTRACT

HPLC coupled with APCI (Atmospheric Pressure Chemical Ionisation)-MS seems to be the preferable technique for detection of sulphonamides. It was used to identify the sulphathiazole in edible trout tissue. A detection limit of 10 µg/kg and a quantitation limit of 30 µg/kg were obtained. MS data useful for the identification, confirmation and quantitation of sulphathiazole by APCI-MS have not been reported previously.

RIASSUNTO

L'analisi HPLC accoppiata alla spettrometria di massa a trappola ionica (APCI-MS) sembra essere la tecnica da preferirsi allo scopo di rilevare la presenza di sulfamidici residui in prodotti destinati all'alimentazione. Questa tecnica è stata utilizzato dagli Autori per la identificazione del sulfatiazolo in carne di trota. È stato possibile ottenere un limite di rilevabilità pari a 10 µg/kg ed un limite di quantificazione di 30 µg/kg. In questo lavoro sono riportati dati MS utili per la identificazione, conferma e quantificazione del sulfatiazolo.

- Key words: APCI-MS, HPLC, sulphathiazole, trout -

INTRODUCTION

Sulphathiazole is a molecule belonging to the sulphonamides, synthetic agents used for the prevention and treatment of bacterial diseases in livestock.

The widespread use of sulphonamides can lead to accumulation in meat, eggs, milk and fish and can enhance bacterial resistance in humans. According to Council Regulation n. 2377/90 (EEC, 1990), and the following modifications, the maximum residue limit (MRL) of sulphonamides, including sulphathiazole, in all food producing species is 100 μg/kg. Subsequently, a Council Directive 96/23/EC (EEC, 1996) included aquaculture products in the foods to be checked for sulphonamide residues.

Various HPLC methods have been proposed for the determination of sulphonamides in various food matrices (PLEASANCE *et al.*, 1991; HORIE *et al.*, 1992; ABIÁN *et al.*, 1993; NIESSEN, 1998; MEINERTZ *et al.*, 1999; HARTIG *et al.*, 1999; SCHWAIGER and SCHUCH, 2000). Confirmatory methods by APCI-MS have also been reported (GEHRING *et al.*, 1996; TARBIN *et al.*, 1999; COMBS *et al.* 1999; DOST *et al.*, 2000). Because of the high sensitivity required and no need of derivatisation reactions, HPLC coupled with APCI-MS seems to be the preferred technique to confirm the identity of sulphonamides.

Sulphathiazole has already been considered in the quantitative determination of the sulphonamides in swine muscle tissues (BALIZS *et al.*, 1994) using thermospray LC/MS, taking a single ion monitoring with 100 μg/kg as the limit of quantitation.

In this paper we suggest operating conditions for the application of an HPLC/APCI-MS method for sulphathiazole determination in edible trout tissue which allows a quantitation limit of 30 μg/kg to be reached.

MATERIALS AND METHODS

Hydrochloric acid (Merck, Darmstadt, Germany), acetonitrile (JT Baker, Milton Keynes, UK) and glacial acetic acid (Carlo Erba, Milano, Italy) were used for the extraction procedure. Glacial acetic acid and acetonitrile were used as the mobile phase. Sulphathiazole (>99%) was purchased from Sigma Aldrich (Milano, Italy).

Apparatus and operating conditions

An HPLC Beckman system (System Gold–Beckman, Fullerton, CA) equipped with a programmable solvent pump (Module 126, Beckman) and a C18 column (4.6x250 mm, 5 μm, Beckman) was used. The mobile phase was acetic acid/water (2:98 w/w) (A) and acetonitrile (B). The operating conditions were as follow: 6 min isocratic B 3%, followed by a gradient of 80% B in 10 min. Then 6 min isocratic B 80% was performed followed by a re-equilibration at 3% B in 0.5 min and left at the same concentration for 10 min further. The flow rate was 1 mL/min. Sample volumes of 20 μL were injected.

The HPLC system was coupled with a double quadrupole mass spectrometer (FinniganMat, Thermo Finnigan, San Josè, CA) equipped with an APCI ionisation source. The APCI probe was heated to 450°C to ensure complete vaporisation of the column effluent. Ionisation was achieved by a 3 μA discharge current at the corona pin generating proton donor ions. The relative collision energy (%) was varied depending on the ions to be acquired. Positive ions were acquired in Selected Ion Monitoring (SIM) for ion at m/z 256 and in short range scan mode for the derived ions at m/z 156, 108 and 92.

Furthermore, to confirm the fragmentation data useful for the sulphathiazole identification and quantitation, an LC/MSD Trap SL 1100 Series (Agilent Technologies, Palo Alto, CA) was used with the operating conditions as follows: ion

source type APCI (positive mode), 350°C dry temperature, 400°C APCI temperature, 1.10 V cone voltage and 5 L/min gas flow.

Extraction procedure

Trout muscle tissue (5 g) was homogenised and mixed with 20 mL of 0.1 M hydrochloric acid in acetonitrile. After shaking vigorously for 10 min, the mixture was centrifuged at 3,000 rpm for 5 min for phase separation, then the organic phase was recovered by filtration on a Whatman 4 filter (Whatman International Ltd., Maidstone, UK) in a 100 mL flask. The remaining solid residue was extracted again with 20 mL of 0.1 M hydrochloric acid in acetonitrile and operating as aforementioned.

The recovered extract was dried under vacuum with a rotory evaporator (Büchi R-200, Switzerland) at 35°C and reconstituted with 2 mL of acidified water (2% acetic acid)/acetonitrile (80:20 v/v).

After filtration through a 0.45 µm pore size filter (Millipore Corp., Bedford, MA), the sample was injected into the HPLC/APCI-MS system.

The recovery evaluation was carried out by spiking trout muscle tissue (5 g) with sulphathiazole standard solution (0.5 mL) at concentrations varying from 0.5 to 2 µg/mL.

RESULTS AND DISCUSSION

The standard solution of sulphathiazole was injected directly in the APCI using the operating conditions reported in the "Materials and Methods" section without collision energy. It produced the spectrum shown in Fig. 1.

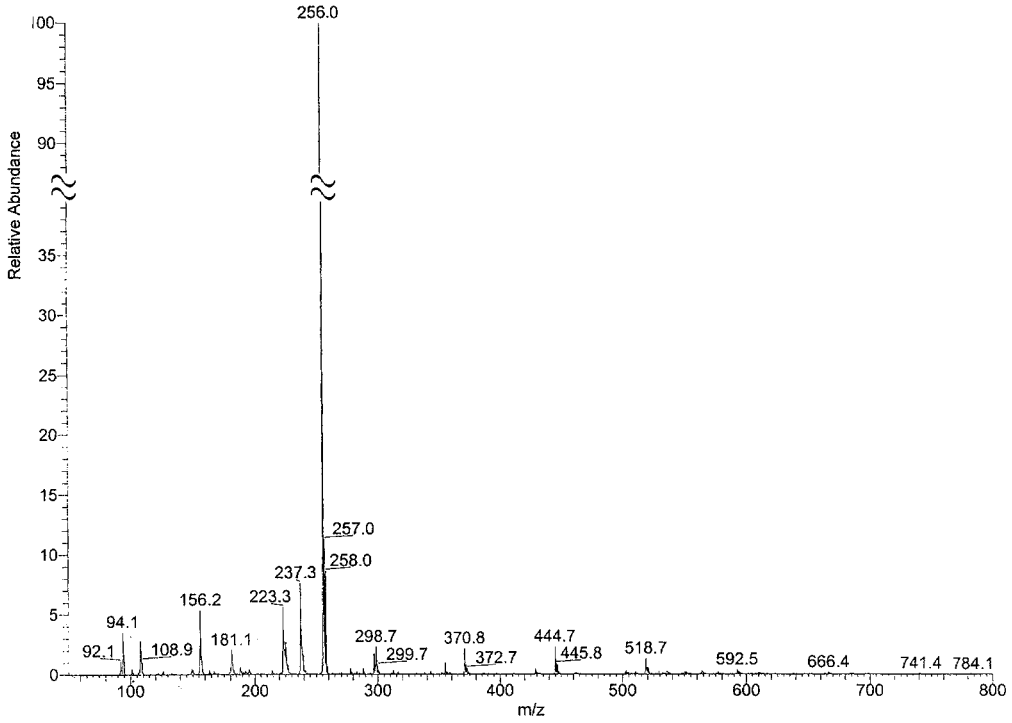


Fig. 1 - Mass spectrum of standard sulphathiazole (100 µg/L) directly injected in the APCI (Finnigan-Mat). The protonated sulphathiazole molecule is represented by the ion at *m/z* 256.

The protonated molecule of sulphathiazole was detected by monitoring the ion at *m/z* 256 in SIM mode (Fig. 2A). Then the ion at *m/z* 156 was acquired, as shown in Fig. 2B, derived from the fragmentation of ion of *m/z* 256 at a relative collision energy of 17.5%. This ion is a characteristic ion also for other sulphonamide compounds. This acquisition was performed through a limited scan range (*m/z* 155 to 257).

With a relative collision energy of 12.8%, the ion at *m/z* 156 produced the ions at *m/z* 92 and 108, as reported in Fig. 2C, working with a scan range from *m/z* 91 to 157.

The instrument used allowed the "relative collision energy" (%) and not the absolute cone voltage value (V) to be obtained.

Fig. 3 shows the result of the blank (Fig. 3A) along with the extract from the trout muscle (Fig. 3B) and the tissue spiked with sulphathiazole at a concentration of 200 µg/kg (Fig. 3C). The acquisition was performed detecting the ion at *m/z* 256 (Fig. 3B and B') then the derived ions at *m/z* 156 (Fig. 3C and C') and the ones at *m/z* 108 and 92 (Fig. 3D and D').

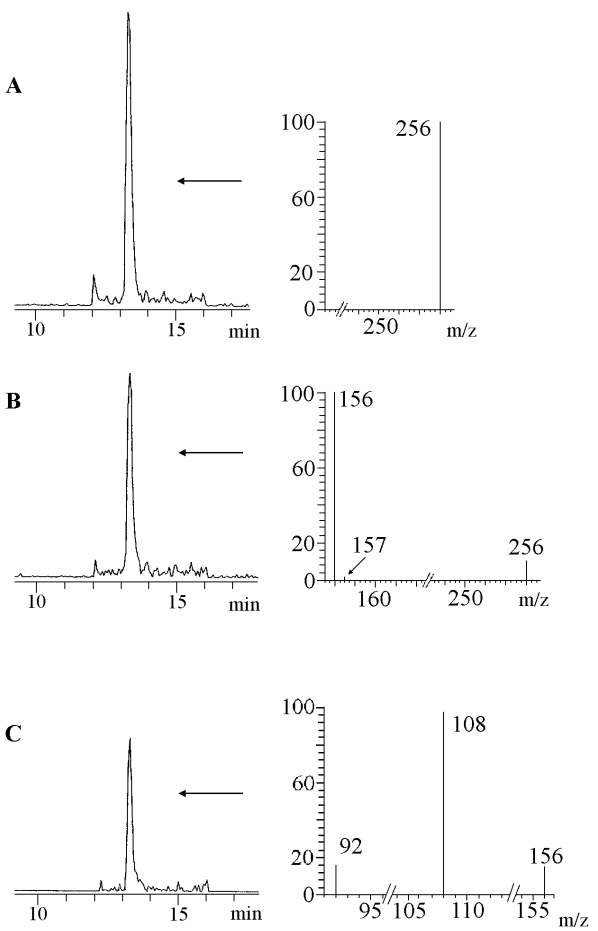


Fig. 2 - (A) Chromatogram and mass spectrum obtained by APCI (FinniganMat) from the sulphathiazole standard solution monitoring the [M+1] ion at *m/z* 256; (B) Chromatogram and mass spectrum obtained monitoring ions at *m/z* 155-257; (C) Chromatogram and mass spectrum obtained monitoring ions at *m/z* 91-157. Relative collision energies are 17.5% (B) and 12.8% (C).

Operating with the LC/MSD Ion Trap instrument it was possible to obtain the ion at *m/z* 256 and with a cone voltage value of 1.10 V to produce the same ions obtained in the previously reported conditions. The spectrum is shown in Fig. 4.

The calibration curve obtained with the sulphathiazole standard between 25 and 300 µg/L and detecting the ion at *m/z* 156 was acceptable (R = 0.9961).

Under these conditions the LOD (limit of detection) was 10 µg/kg of matrix with average recoveries between 76-85% (number of replications = 10; sulphathiazole concentration added = 50 to 200 µg/kg of trout tissue and CV between 2.5 and 7.9%). The LOQ (limit of quantitation) was assumed as 30 µg/kg of matrix, i.e. 3 times the LOD value). The concentration of sulphathiazole determined in the trout sample was 44 µg/kg (Fig. 3).

To our knowledge, there has not been spectral data reported for sulphathia-

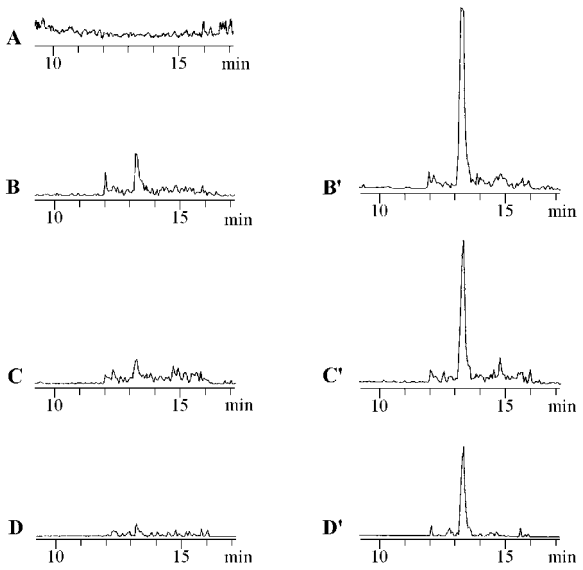


Fig. 3 - (A) Chromatogram of a blank sample; (B) Chromatogram obtained by APCI (FinniganMat) from the analysis of an extract from trout muscle monitoring the [M+1] ion at *m/z* 256; (B') Chromatogram obtained by APCI (FinniganMat) from the analysis of an extract from trout muscle spiked with 200 µg/kg monitoring the [M+1] ion at *m/z* 256; (C) Chromatogram obtained monitoring ions at *m/z* 155-257 (trout muscle); (C') Chromatogram obtained monitoring ions at *m/z* 155-257 (spiked trout muscle); (D) Chromatogram obtained monitoring ions at *m/z* 91-157 (trout muscle); (D') Chromatogram obtained monitoring ions at *m/z* 91-157 (spiked trout muscle). Relative collision energies are 17.5% (C) and 12.8% (D).

zole using the HPLC/APCI-MS method. Our results indicate that the use of this method is suitable for detecting and confirming the peak identity of sulphathiazole with a detection limit of 10 µg/kg and assuming a quantitation limit of 30 µg/kg. Furthermore the matrix considered for is sufficiently complex to affirm that the method may be of general application.

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REFERENCES

Abián J., Churchwell M.I. and Korfmacher W.A. 1993. High performance liquid chromatography-thermospray of ten sulphonamide antibiotics. Analysis in milk at ppb level. J. Chromatogr. 629:267.

Balizs G., Benesch-Girke L., Borner S. and Hewitt S.A. 1994. Comparison of the determination of four sulphonamides and their N4-acetyl metabolites in swine muscle tissue using liquid

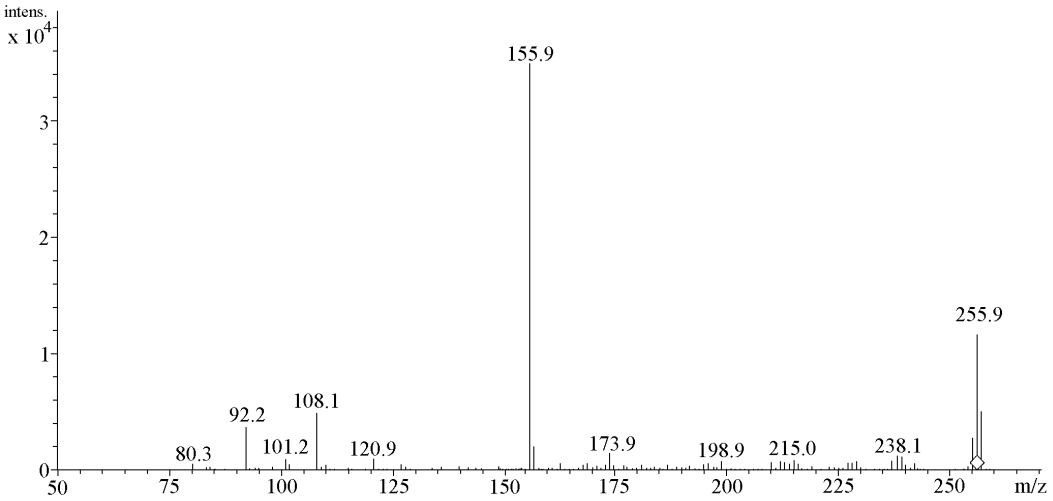


Fig. 4 - Mass Spectrum of sulphathiazole obtained from a standard solution at cone voltage of 1.10 V (LC/MSD Trap SL 1100 Series).

chromatography with ultraviolet and mass spectral detection. J. Chromatogr. B 661:75.

Combs M.T., Ashraf-Khorassani M. and Taylor L.T. 1999. HPLC/atmospheric pressure chemical ionization-mass spectroscopy of eight regulated sulphonamides. J. Pharm. Biom. An. 19:301.

EEC. 1990 Council Regulation No. 2377/90 of 26 June 1990 laying down a Community procedure for the establishment of maximum residue limits of veterinary medicinal products in foodstuffs of animal origin. (1990) Official Journal L244, 1:6.

EEC. 1996 Council Directive 96/23/EC of 29 April 1996 on measures to monitor certain substances and residues thereof in live animals and animal products and repealing Directives 85/358/EEC and 86/469/EEC and Decisions 89/187/EEC and 91/664/EEC. (1996) Official Journal L125, 10:32.

Dost K., Jones D.C. and Davidson G. 2000. Determination of sulphonamides by packed column supercritical fluid chromatography with atmospheric pressure chemical ionisation mass spectrometric detection. Analyst 125:1243.

Gehring T.A., Rushing L.G., Churchwell M.I., Doerge D.R., McErlane K.M. and Thompson H.C. 1996. HPLC determination of sulfadiazine residues in Coho salmon (*Oncorhynchus kisutch*) with confirmation by liquid chromatography with atmospheric pressure chemical ionisation mass spectrometry. J. Agric. Food Chem. 44:3164.

Hartig C., Storm T. and Jekel M. 1999. Detection and identification of sulphonamide drugs in municipal waste water by liquid chromatography coupled with electrospray ionisation tandem mass spectrometry. J. Chromatogr. A 854:163.

Horie M., Saito K., Nose N. and Nakazawa H. 1992. Simultaneous determination of sulfonamides in honey by liquid chromatography. J. AOAC Int. 75:786.

Meinertz J.R., Schmidt L.J., Stehly G.R. and Gingerich W.H. 1999. Liquid chromatographic determination of para-toluenesulfonamide in edible fish tissues from three species of fish. J. AOAC Int. 82:1064.

Niessen W.M.A. 1998. Analysis of antibiotics by liquid chromatography-mass spectrometry. J. Chromatogr. A 812:53.

Pleasance S., Blay P. and Quilliam A. 1991. Determination of sulfonamides by liquid chromatography, ultraviolet diode array detection and ionspray tandem mass spectrometry with application to cultured salmon flesh. J. Chromatogr. 558:155.

Schwaiger I. and Schuch R. 2000. Bound sulfathiazole residues in honey – need of a hydrolysis step for the analytical determination of total sulfathiazole content in honey. Deuts. Lebensm. Rund. 96:93.

Tarbin J.A., Clarke P. and Shearer G. 1999. Screening of sulphonamides in egg using gas chromatography – mass-selective detection and liquid chromatography – mass spectrometry. J. Chromatogr. B 729:127.

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DISTRIBUTION OF HYDROXYCINNAMIC ACIDS AS A CRITERION TO EVALUATE VARIETY AND GEOGRAPHICAL ORIGIN OF ITALIAN ORANGE JUICES

LA DISTRIBUZIONE DEGLI ACIDI IDROSSICINNAMICI COME CRITERIO PER VALUTARE LA VARIETÀ E L'ORIGINE GEOGRAFICA DEI SUCCHI D'ARANCIA ITALIANI

A. DI MAURO, A. PASSERINI, P. RAPISARDA[1] and E. MACCARONE*
Dipartimento di OrtoFloroArboricoltura e Tecnologie Agroalimentari (DOFATA), Università degli Studi di Catania, Via S. Sofia 98, 95123 Catania, Italy
[1] Istituto Sperimentale per l'Agrumicoltura, Corso Savoia 190, 95124 Acireale, Italy
* Corresponding author: Fax +39 095 7141960, e-mail: emacca@unict.it

ABSTRACT

Hydroxycinnamic acids (ferulic, p-coumaric, sinapic and caffeic) were determined in 113 orange juices, extracted from the most widespread pigmented and non-pigmented varieties cultivated in Italy and harvested in 1998, 1999 and 2000. The concentration of hydroxycinnamic acids (HCA) was evaluated to develop a data bank of Italian juices and use it to check variety and geographical origin of commercial products. A higher amount of HCA was found in pigmented juices, compared to non-pigmented ones, and in Sicilian juices, compared with those from oth-

RIASSUNTO

Gli acidi idrossicinnamici (ferulico, p-cumarico, sinapico e caffeico) sono stati determinati mediante analisi HPLC in 113 succhi di arance pigmentate e bionde, coltivate nelle regioni dell'Italia meridionale ed insulare, e raccolte nel triennio 1998-2000. La distribuzione del campione riflette l'entità della produzione in termini di cultivar e regione d'origine. La concentrazione di AIC è stata valutata al fine di realizzare una banca-dati dei succhi italiani, da utilizzare per scopi di controllo dei prodotti commerciali. Gli AIC sono presenti in quantità superiore nei succhi di

- Key words: geographical origin; hydroxycinnamic acids; orange cultivars; orange juices; varietal marker -

er geographical areas. The ratio between the concentrations of methoxy-substituted acids (ferulic and sinapic) and the hydroxy-substituted ones (p-coumaric and caffeic) was less than two in pigmented cultivars and greater than two in non-pigmented ones. The content of p-coumaric acid was the determining factor in differentiating the juices. The distribution of HCA was also suitable for discriminating the different clones of the Tarocco cultivar. This methodology is useful for characterizing pigmented orange juices, and could be included in the regulations of the Protected Geographical Indication (PGI) of Sicilian pigmented orange fruits and for the Protected Designation of Origin (PDO) of the corresponding juices.

arance pigmentate rispetto a quelli di arance bionde, e nei succhi siciliani rispetto a quelli di altre regioni. Il rapporto tra le concentrazioni degli acidi metossilati (ferulico e sinapico) e quelli ossidrilati (p-cumarico e caffeico) è minore di due nelle cultivar pigmentate e maggiore di due in quelle bionde. Il contenuto di acido p-cumarico è il fattore determinante per la differenziazione dei succhi. La distribuzione degli AIC discrimina anche i differenti cloni della cultivar Tarocco. La metodologia è utile per tipizzare i succhi di arance pigmentate, e potrebbe essere inserita nei disciplinari previsti per l'IGP delle arance rosse di Sicilia e per la DOP dei corrispondenti succhi.

INTRODUCTION

Sicily provides the best environmental conditions for the pigmented cultivars of *Citrus sinensis* L. (Osbeck) to express their genotypic characteristics. These oranges have a balanced sugar-to-acid ratio, a high content of vitamin C, anthocyanins and other antioxidant substances (MACCARONE, 1997).

However, the expansion of citrus cultivation in the Mediterranean countries has affected the commercial development of the Italian product because of higher production costs. Fortunately, this disadvantage has been balanced out by increased interest on the part of European consumers in pigmented orange juices (QUARLERI, 1994; LA VIA and PECORINO, 1996), considered of great health value (STURIALE, 1995a,b). Therefore, citrus processing needs to be developed and the value, quality and typicality of orange juices need to be highlighted. Hence, there is a need to safeguard the product by looking for markers which can completely characterize it, beyond its apparent colour.

Growth of the pigmented juice market carries the risk of adulteration. Intensely coloured juices could be blended with non pigmented juices of lesser value, for example, with ones reconstituted with concentrates from South America, while non pigmented juices could be coloured with generic red pigments or with anthocyanins extracted from other plant sources. These possible forms of adulteration could compromise the image of the product and damage agribusiness. So, offices in charge of checking food products must enforce effective methods in order to verify the authenticity of commercial pigmented juice or its conformity with all that is declared on the label. In this regard, the HPLC profile of anthocyanins constitutes an authentic marker for pigmented orange juices (MACCARONE *et al.*, 1983; 1985; 1998a; RAPISARDA *et al.*, 2000), and can be used for inspection purposes, but it is not always definitive, so alternative methods must be available.

Some of these other methods are the

HPLC analysis of flavanone glycosides (ROUSEFF, 1988; MOULY *et al.*, 1994, 1998; MARINI and BALESTRIERI, 1995), carotenoids (MOULY *et al.*, 1999) and the evaluation of isotopic ratios of bio-elements (H/D, $^{12}C/^{13}C$, $^{16}O/^{18}O$) in the constituents of the juice (VERSINI *et al.*, 1995). Recent studies have shown that pigmented oranges are different from non-pigmented ones, both in the qualitative and quantitative distribution of their aromatic components (MACCARONE *et al.*, 1998b), fatty acids (ARENA *et al.*, 1998a), and, in particular, *trans*-4-hydroxycinnamic acids (HCA) (RAPISARDA *et al.*, 1998).

HCA occur in orange juices mainly as esters of ferulic, p-coumaric, sinapic and caffeic acids (RISCH and HERMANN, 1988; PELEG *et al.*, 1991; FALLICO *et al.*, 1996). They take part in the process of biosynthesis of flavonoids (HELLER and FORKMANN, 1994), enhance the sensory properties of oranges and are effective antioxidants (ARENA *et al.*, 2001). The HCA content has been determined in a series of Sicilian mono-varietal juices (RAPISARDA *et al.*, 1998) and a series of Italian and foreign commercial juices (ARENA *et al.*, 1998b). The ratio between the concentrations of the methoxy and hydroxy-substituted HCA [R = (ferulic+sinapic)/(caffeic+p-coumaric)] appeared to be suitable for differentiating pigmented juices (R<2) from non-pigmented ones (R>2).

The present work is aimed at verifying, in a large sample representing a wide variety of juices, if HCA are effective markers, suitable not only for typifying different cultivars, but also for ascertaining geographical origin. Orange juices, extracted from fruits of different pigmented and non-pigmented cultivars, were analysed to evaluate the interrelationships between orange-varieties and geographical origin on HCA distribution, and also to create an Italian orange juice data bank, suitable for checking commercial products.

MATERIALS AND METHODS

Twenty-nine of the 113 orange juices were provided by Istituto Sperimentale per l'Agrumicoltura (Acireale, Italy), and came from five different clones of Tarocco fruits grown on the Palazzelli experimental Farm (Lentini, Italy) and harvested in 1998. The other 84 juices came from fruits harvested in 1999 (44) and 2000 (40), under the control of the Ispettorati Centrali Repressione Frodi, located in the regions of origin and co-ordinated by the corresponding office in Catania. Each sample had an information card with details of sample characteristics and a description of the citrus plantation. The total sample consisted of 73 juices from pigmented cultivars (50 Tarocco, 18 Moro, 5 Sanguinello) and 40 from non-pigmented cultivars (12 Valencia, 10 Washington Navel, 8 Biondo Comune, 6 Ovale, 4 Naveline). The samples came from Sicily (50), Calabria (28), Campania (14), Sardinia (10), Apulia (7) and Basilicata (4). The sample distribution roughly reflects the entity of Italian orange production, according to cultivars and region of origin (ISTAT, 1997). This is why Sicilian (44%) and Calabrian (25%) juices prevailed over the others, while pigmented ones (65%) prevailed over the other cultivars, particularly Tarocco cultivar (44%).

Juices were extracted with a laboratory skittle squeezer, and stored in 500 mL plastic food containers at -18°C until analysis. HCA standards, used for HPLC calibration, were high purity commercial products (Sigma-Aldrich, Milan). The solvents (methanol, tetrahydrofuran, water) were of HPLC grade (Merck, Milan).

Sample preparation and HPLC analysis

The juice was filtered through a 20 mesh sieve to remove the largest particles. After filtration, the juice contained

about 5% fine pulp. Subsequently, 10 mL of 2N NaOH were added to 10 mL of juice to hydrolyse the esters of HCA and obtain free acids (FALLICO *et al.*, 1996; RAPISARDA *et al.*, 1998). The hydrolysis was carried out at room temperature for 4 h in the absence of light in order to avoid *trans→cis* isomerization of HCA. The reaction mixture was acidified to pH 1.5-2.0 with 2N HCl and centrifuged at 7,000 rpm for 30 min. Then 10 mL of supernatant were removed and adsorbed on a 6 mL Supelclean LC-18 support (Supelco, Milan), previously conditioned with water. In order to elute the adsorbed HCA, acidified methanol (1% HCl) was used, which was evaporated at 35°C under vacuum. The residue was made up to known volume with a mixture of H_2O (65%), acetic acid (15%) and methanol (20%).

HPLC analyses were carried out with a Varian 9012Q chromatograph, provided with a Photo Diode Array detector (ProStar 330), equipped with a 20 µL loop and a LiChrosphere 100 RP-18 (5 µm) column (L = 25 cm; i.d. = 4 mm) (Merck, Italy) thermostatted at 30°C. Data were acquired from 220 to 400 nm, with integration to 300 nm, in 20 min, using "Star Workstation 5.31" and "Polyview 2000" processing software. An isocratic mobile phase (tetrahydrofuran/water/acetic acid, 18/80/2) was used at a flow rate of 1 mL/min (ROUSEFF *et al.*, 1992). The concentrations of the four HCA were calculated by means of calibration lines of standard compounds, and expressed as mg/L in orange juice. Fig. 1 shows a typical chromatogram of HCA in a Sicilian Tarocco orange juice, after alkaline treat-

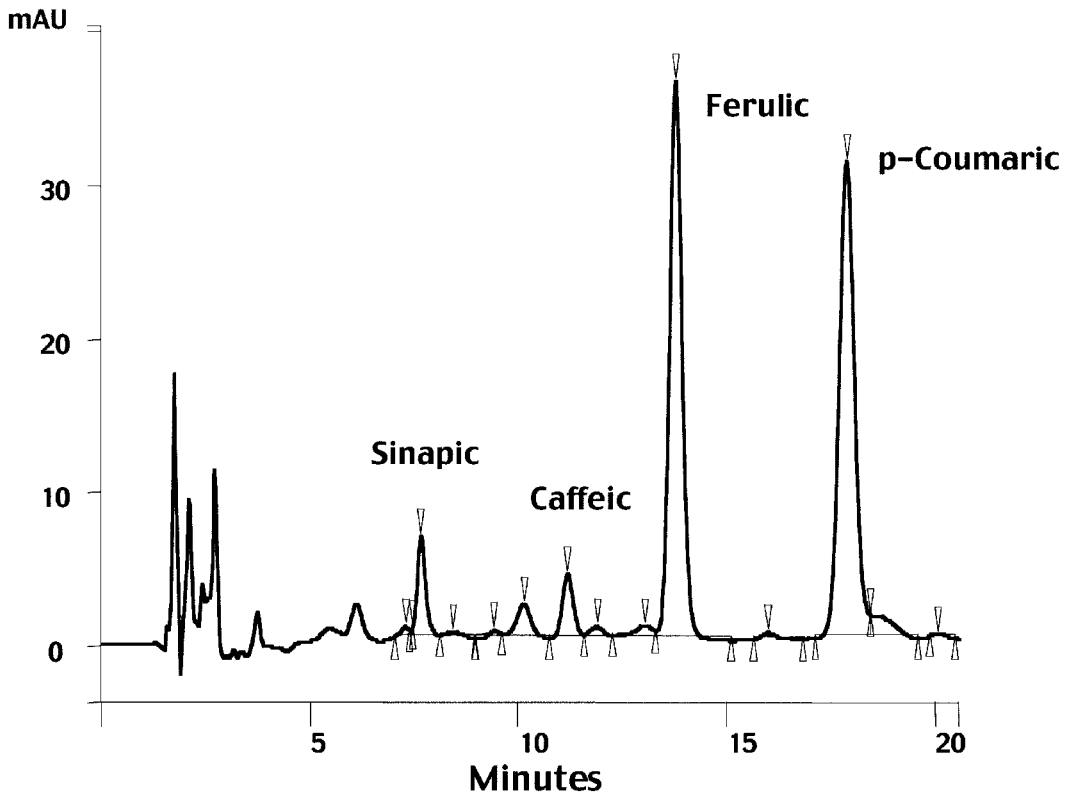


Fig. 1 - HPLC chromatogram of HCA in a Sicilian Tarocco orange juice.

ment and work up. The mean concentration from triplicate HPLC runs for each sample was used for statistical analyses. Standard errors of the replicated HPLC measurements did not exceed ±6%.

Statistical analysis

Data were analyzed by ANOVA and multivariate analyses, using "Statgraphics Plus" software for Windows 98 (Manugistic Inc. Rockville, MD, USA). Analogies or differences in the mean concentrations were determined by the Multiple Range Test. Principal Component Analysis (PCA) furnished two components which explained most the observed variance and showed the sample distribution, indicating the contribution of each variable without introducing variety or geographical origin. Linear Discriminant Analysis (LDA) gave four discriminant functions in the form of $C_1X_1 + C_2X_2 + C_3X_3 + C_4X_4$, where C_1, C_2, C_3 and C_4 are standardized coefficients of the variables X_1, X_2, X_3 and X_4, corresponding to the concentrations in mg/L of the four HCA in each juice. The discriminant functions were characterized by statistical parameters (Eigen-value, canonical correlation, P-value, etc.), which confirm the significance of multivariate regression. The software also gave the tabulation of correctly classified juices in the corresponding groups to which they belong.

RESULTS AND DISCUSSION

Influence of the cultivar

Table 1 shows the mean, minimum and maximum concentration of individual and total HCA, together with the mean ratio of 113 orange juices separated according to their cultivars. Ferulic acid prevailed in all cultivars, ranging from 22.7 mg/L in Biondo Comune to 33.4 mg/L in Valencia. The p-coumaric acid content varied significantly between pigmented cultivars (18.4 mg/L in Tarocco and Moro) and non-pigmented ones (from 7.3 mg/L in Valencia to 11.5 mg/L in Ovale), while Sanguinello was intermediate (13.9 mg/L). Sinapic and caffeic acids were the highest in Naveline, while Ovale and Biondo Comune were the lowest. Total HCA was higher in pigmented oranges than in non-pigmented ones, except for Naveline, because of the high amount of sinapic and caffeic acids. There were also significant differences in the ratio value, which was less than two in the pigmented cultivars and more than two in the non-pigmented ones, confirming the results of ARENA *et al.* (1998a). The Moro cultivar had the lowest ratio mean value (1.5), while Valencia had the highest (5.0), because of its high content of ferulic acid and low content of p-coumaric and caffeic acids. In terms of relative percentages, ferulic acid was the main contributor (56.9%), followed by p-coumaric (23.8%), sinapic (12.1%) and caffeic (7.2%) acids. There were significant differences in HCA percentages among the cultivars, particularly for p-coumaric acid, which varied from 14.7% in Valencia to 33.0% in Tarocco.

Table 2 reports the mean concentrations of HCA in 113 juices separated into two groups: the juices from pigmented cultivars (73 samples) and the juices from the non-pigmented ones (40 samples). The mean values of the concentrations of ferulic and sinapic acids were similar in both groups, while caffeic and p-coumaric acids were significantly different. Consequently, total HCA and ratio were significantly different. These results confirm that pigmented varieties are richer in HCA, and are characterised by an $R<2$ value, due to the higher content of p-coumaric acid.

Influence of geographical origin

Table 3 shows the mean concentrations of HCA in 113 juices separated into two

Table 1 - Mean, minimum and maximum concentration of HCA (mg/L) in 113 orange juices separated according to cultivars.

Acids		Tarocco (50)	Moro (18)	Sanguinello (5)	Valencia (12)	W. Navel (10)	Biondo C. (8)	Ovale (6)	Naveline (4)	F- value	P-value
Ferulic	mean	28.3^{ab}	28.8^{ab}	28.6^{ab}	33.4^{b}	30.6^{ab}	22.7^{a}	28.9^{ab}	31.8^{ab}	1.22	0.299
	SEM	1.2	2.0	3.9	2.5	2.7	3.0	3.5	4.3		
	min-max	11.1-56.4	17.3-59.2	21.4-37.5	22.8-52.9	15.3-48.6	15.0-28.2	12.9-44.1	22.6-41.1		
p-Coumaric	mean	18.4^{a}	18.4^{a}	13.9^{ab}	7.3^{b}	9.7^{b}	9.3^{b}	11.5^{b}	10.5^{b}	7.01	0.000
	SEM	1.0	1.6	3.0	1.9	2.1	2.4	2.7	3.4		
	min-max	3.0-31.2	7.6-39.7	7.1-21.6	3.20-19.1	2.80-18.6	7.30-13.7	6.0-20.8	6.50-14.5		
Sinapic	mean	5.6^{ab}	5.2^{ab}	7.5^{bc}	6.5^{ab}	6.9^{abc}	4.5^{a}	4.5^{ab}	9.7^{c}	2.63	0.015
	SEM	0.4	0.6	1.2	0.8	0.8	0.9	1.1	1.3		
	min-max	1.80-13.2	2.70-11.4	3.50-10.7	2.80-13.5	2.40-14.2	3.20-5.50	2.30-9.30	7.0-12.6		
Caffeic	mean	3.5^{a}	4.6^{bc}	5.0^{bc}	2.6^{a}	3.4^{ab}	2.6^{a}	2.6^{a}	6.0^{c}	4.32	0.000
	SEM	0.2	0.4	0.7	0.5	0.5	0.6	0.6	0.8		
	min-max	1.40-9.70	2.50-10.0	2.80-7.80	1.0-6.20	1.90-4.70	1.60-4.50	1.30-7.60	5.30-6.50		
Total HCA	mean	55.8^{a}	57.0^{a}	55.0^{ab}	49.8^{ab}	50.6^{ab}	39.1^{b}	47.5^{ab}	58.0^{a}	1.59	0.147
	SEM	2.2	3.7	6.9	4.5	4.9	5.5	6.3	7.8		
	min-max	18.5-100.4	33.3-120.3	36.0-77.6	30.8-69.4	23.5-71.8	28.0-44.0	24.2-79.1	42.6-64.2		
Ratio	mean	1.8^{a}	1.5^{a}	2.0^{ab}	5.0^{d}	3.2^{c}	2.3^{abc}	3.0^{bc}	2.6^{abc}	18.00	0.000
	SEM	0.1	0.2	0.4	0.3	0.3	0.3	0.4	0.5		
	min-max	0.9-5.0	0.9-2.5	1.60-2.40	1.57-8.42	2.16-6.50	1.70-3.48	1.72-4.15	2.10-3.21		

Number of samples in parentheses. SEM: Standard error of mean value. Means in the same line followed by a common letter are not significantly different (P<0.05).

Table 2 - Mean, minimum and maximum concentration of HCA (mg/L) in 113 orange juices separated according to pigmented and non-pigmented cultivars.

Acids		Pigmented (73)	Non-pigmented (40)	F-value	P-value
Ferulic	mean	28.4[a]	29.7[a]	0.58	0.449
	SEM	1.0	1.4		
	min-max	11.1-59.2	12.9-52.9		
p-Coumaric	mean	18.1[a]	9.2[b]	45.86	0.000
	SEM	0.8	1.1		
	min-max	3.0-39.7	2.8-20.8		
Sinapic	mean	5.6[a]	6.2[a]	1.26	0.265
	SEM	0.3	0.4		
	min-max	1.8-13.2	2.3-14.2		
Caffeic	mean	3.8[a]	3.1[b]	4.67	0.033
	SEM	0.2	0.3		
	min-max	1.4-10.0	1.0-7.6		
Total HCA	mean	55.9[a]	48.2[b]	6.33	0.013
	SEM	1.8	2.4		
	min-max	18.5-100.4	23.5-79.1		
Ratio	mean	1.7[a]	3.4[b]	59.68	0.000
	SEM	0.1	0.2		
	min-max	0.9-5.0	1.6-8.4		

SEM: Standard error of mean value. Means in the same line followed by a common letter are not significantly different ($P<0.05$).

groups according to geographical origin, independent of cultivars: Sicilian juices (50 samples) and those from other regions (63 samples). p-Coumaric acid played a major role in discriminating between Sicilian (20.2 mg/L) and juices from other regions (10.8 mg/L), while the concentrations of ferulic, sinapic and caffeic acids were similar in both groups. In particular, samples from Calabria, Campania, Basilicata and Apulia, considered separately, did not show any difference in HCA distribution, whereas the Sardinian sample (represented by 5 pigmented and 5 non pigmented juices) had the highest ratio (4.0), due to the high content of ferulic acid (32.3 mg/L) and the low amount of p-coumaric acid (8.4 mg/L).

The influence of geographical origin was tested separately for both 73 pigmented and 40 non-pigmented juices (Table 4). The analysis of 42 Sicilian juices and 31 juices from other regions showed remarkable differences in p-coumaric, sinapic and caffeic acids, total HCA and ratio values, while ferulic acid did not differ. No differences were observed in non-pigmented juices. A separate analysis for individual cultivars showed that the highest amount of total HCA was in the Sicilian Moro samples (87.7 mg/L), due to the high content of p-coumaric acid (31.8 mg/L). Results of Sicilian pigmented juices are shown in Fig. 2.

The HCA distribution in the Tarocco cultivar is typical of pigmented varieties, both for its high content of p-coumaric

Table 3 - Mean, minimum and maximum concentration of HCA (mg/L) in 113 orange juices separated according to Sicilian juices and juices from other regions.

Acids		Sicily (50)	Other regions (63)	F-value	P-value
Ferulic	mean	29.8[a]	28.1[a]	1.07	0.303
	SEM	1.2	1.1		
	min-max	19.0-59.2	11.1-56.4		
p-Coumaric	mean	20.2[a]	10.8[b]	60.38	0.000
	SEM	0.9	0.8		
	min-max	3.6-39.7	2.8-23.9		
Sinapic	mean	6.4[a]	5.4[b]	3.99	0.048
	SEM	0.4	0.3		
	min-max	1.8-13.5	1.9-14.2		
Caffeic	mean	3.4[a]	3.8[a]	1.47	0.228
	SEM	0.2	0.2		
	min-max	1.2-10.0	1.0-9.7		
Total HCA	mean	59.8[a]	48.1[b]	17.62	0.000
	SEM	2.1	1.9		
	min-max	36.5-120.3	18.5-100.4		
Ratio	mean	1.8[a]	2.7[b]	13.76	0.000
	SEM	0.2	0.2		
	min-max	0.9-8.4	1.2-7.7		

SEM: Standard error of mean value. Means in the same line followed by a common letter are not significantly different (P<0.05).

Table 4 - Mean, minimum and maximum concentration of HCA (mg/L) in pigmented and non-pigmented orange juices separated according to origin.

Acids		Pigmented juices (73)				Non-pigmented juices (40)			
		Sicily (42)	Other regions (31)	F-value	P-value	Sicily (8)	Other regions (32)	F-value	P-value
Ferulic	mean	29.2[a]	27.4[a]	0.97	0.327	33.2[a]	28.8[a]	1.15	0.289
	SEM	1.2	1.4			3.6	1.8		
	min-max	19.0-59.2	11.1-56.4			29.4-43.2	12.9-52.9		
p-Coumaric	mean	21.8[a]	13.1[b]	34.12	0.000	11.9[a]	8.6[a]	3.62	0.646
	SEM	1.0	1.1			1.6	0.8		
	min-max	8.9-39.7	3.0-23.9			3.6-20.8	2.8-19.1		
Sinapic	mean	6.2[a]	4.9[b]	5.15	0.026	7.6[a]	5.9[a]	1.96	0.170
	SEM	0.4	0.4			1.1	0.5		
	min-max	1.8-13.2	1.9-10.7			2.3-13.5	2.4-14.2		
Caffeic	mean	3.5[a]	4.4[b]	5.60	0.021	3.0[a]	3.2[a]	0.09	0.764
	SEM	0.3	0.3			0.6	0.3		
	min-max	1.4-10.0	2.3-9.7			1.2-6.5	1.0-7.6		
Total HCA	mean	60.6[a]	49.7[b]	9.32	0.003	55.7[a]	46.5[a]	2.65	0.112
	SEM	2.3	2.7			5.0	2.5		
	min-max	36.5-120.3	18.5-104.4			40.5-71.8	23.5-79.1		
Ratio	mean	1.5[a]	2.0[b]	14.99	0.000	3.5[a]	3.4[a]	0.00	0.984
	SEM	0.1	0.1			0.6	0.3		
	min-max	0.9-2.5	1.2-5.0			1.7-8.4	1.6-7.7		

Number of samples in parentheses. SEM: Standard error of mean value. Means in the same line followed by a common letter are not significantly different (P<0.05).

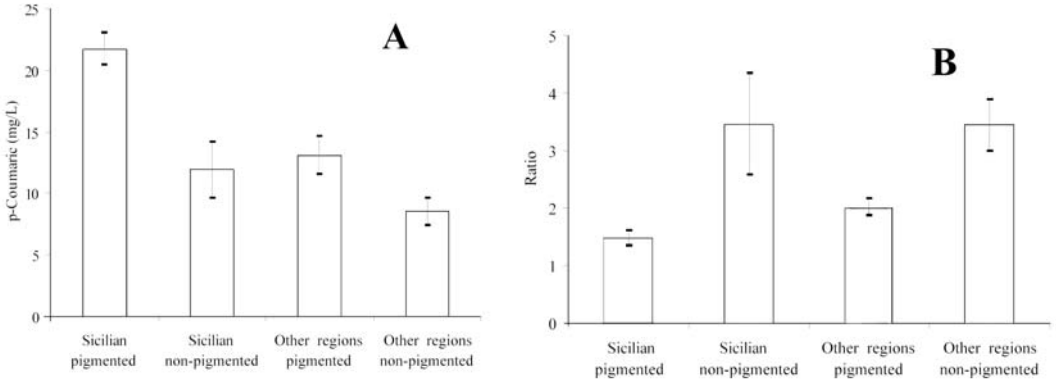


Fig. 2 - Mean concentration (mg/L) of p-coumaric acid (A) and mean value of HCA Ratio in orange juices (B). Lower and upper limits of each mean value are indicated.

Table 5 - Mean, minimum and maximum concentration of HCA (mg/L) in 50 Tarocco orange juices separated according to origin.

Acids		Sicily (38)	Other regions (12)	F-value	P-value
Ferulic	mean	28.3[a]	28.1[a]	0.01	0.911
	SEM	1.2	2.1		
	min-max	19.0-42.5	11.1-56.4		
p-Coumaric	Mean	21.2[a]	9.4[b]	39.7	0.000
	SEM	0.9	1.6		
	Min-Max	8.9-31.2	3.0-23.9		
Sinapic	Mean	5.9[a]	4.6[b]	2.62	0.112
	SEM	0.4	0.7		
	Min-Max	1.8-13.2	1.9-10.4		
Caffeic	Mean	3.3[a]	4.1[a]	2.95	0.092
	SEM	0.2	0.4		
	Min-Max	1.4-6.2	2.3-9.7		
Total HCA	Mean	58.7[a]	46.2[b]	7.81	0.007
	SEM	2.2	3.9		
	Min-Max	36.5-79.5	18.6-100.4		
Ratio	Mean	1.5[a]	2.6[b]	43.8	0.000
	SEM	0.1	0.2		
	Min-Max	0.9-2.5	1.8-5.0		

SEM: Standard error of mean value. Means in the same line followed by a common letter are not significantly different (P<0.05).

acid and for its ratio value. However, these juices are not always pigmented. Only 31 out of 50 samples had red coloured juices. The statistical analysis was carried out separately on these samples: 38 Sicilian and 12 from other regions

(Table 5). Sicilian Tarocco juice had a mean p-coumaric acid content significantly higher (21.2 mg/L) than those from other regions (9.4 mg/L), resulting in ratio values of 1.5 and 2.6, respectively. Therefore HCA analysis is useful for establishing Tarocco juice authenticity and its geographical origin even in the absence of red colouration, which is not always expressed due to environmental factors.

To verify possible sub-varietal differences, pedoclimatic conditions being equal, 29 Tarocco juices belonging to 5 different clones (Nucellare, Sciara, Gallo, Vitale and Tringale) were analysed (Table 6). Sciara, the most pigmented clone, had the highest HCA (62.6 mg/L), whereas Nucellare, the least red coloured, had the lowest amount of HCA (45.9 mg/L). In all cases the mean value of ratio was less than two.

Influence of production year

To evaluate effect of production year, 44 orange juices from 1999, and 40 orange juices from 2000 were analysed (Table 7). The juices from these years represent cultivars and geographic origin. There were no significant differences in the mean concentrations of p-coumaric and sinapic acids between the years, whereas ferulic and caffeic acids differed significantly. The juices from 1999 were generally higher in all acids

Table 6 - Mean, minimum and maximum concentration of HCA (mg/L) in 5 clones of Tarocco orange juices.

Acids		Nucellare (5)	Sciara (5)	Gallo (9)	Vitale (5)	Tringale (5)	F-value	P-value
Ferulic	mean	25.0^{a}	28.2^{bc}	30.1^{c}	28.1^{abc}	26.0^{ab}	4.47	0.008
	SEM	1.1	1.1	0.8	1.1	1.1		
	min-max	22.7-28.5	26.3-30.1	27.1-33.0	26.8-29.9	19.0-29.2		
p-Coumaric	mean	13.9^{a}	28.6^{d}	21.4^{bc}	24.0^{cd}	18.4^{ab}	10.63	0.000
	SEM	1.7	1.7	1.3	1.7	1.7		
	min-max	8.9-18.5	24.4-31.2	16.7-28.8	21.2-28.7	11.1-20.6		
Sinapic	mean	4.9^{ab}	3.6^{a}	6.1^{bc}	3.2^{a}	7.0^{c}	7.77	0.000
	SEM	0.6	0.6	0.5	0.6	0.6		
	min-max	3.4-6.12	1.8-6.7	5.5-7.3	1.9-4.4	4.7-9.0		
Caffeic	mean	2.1^{a}	2.2^{a}	3.1^{ab}	3.7^{bc}	4.8^{c}	6.87	0.001
	SEM	0.4	0.4	0.3	0.4	0.4		
	min-max	1.5-2.9	1.4-2.5	1.8-4.9	3.4-3.9	2.7-6.2		
Total HCA	mean	45.9^{a}	62.6^{b}	60.7^{b}	59.0^{b}	56.2^{b}	4.94	0.005
	SEM	3.0	3.0	2.3	3.0	3.0		
	min-max	36.5-55.8	53.9-68.3	55.1-69.3	54.3-61.5	38.0-65.0		
Ratio	mean	1.9^{d}	1.0^{a}	1.5^{c}	1.1^{ab}	1.5^{bc}	9.62	0.000
	SEM	0.1	0.1	0.1	0.1	0.1		
	min-max	1.6-2.5	0.9-1.2	1.2-1.9	0.9-1.3	1.3-1.8		

Number of samples in parentheses. SEM: Standard error of mean value. Means in the same line followed by a common letter are not significantly different ($P<0.05$).

Table 7 - Mean, minimum and maximum concentration of HCA (mg/L) in 84 orange juices separated according to year of production. Number of juices in parentheses.

Acids		1999 (44)	2000 (40)	F-value	P-value
Ferulic	mean	33.4[a]	24.5[b]	20.83	0.000
	SEM	1.3	1.4		
	min-max	14.0-59.2	11.1-41.9		
p-Coumaric	mean	12.9[a]	12.7[a]	0.03	0.867
	SEM	1.1	1.2		
	min-max	3.2-39.7	2.8-33.0		
Sinapic	mean	5.8[a]	6.4[a]	0.88	0.350
	SEM	0.4	0.5		
	min-max	1.9-11.7	2.2-14.2		
Caffeic	mean	4.2[a]	3.2[b]	5.46	0.022
	SEM	0.3	0.3		
	min-max	1.2-10.0	1.0-6.5		
Total HCA	mean	56.3[a]	46.8[b]	6.68	0.012
	SEM	2.5	2.7		
	min-max	24.0-120.3	18.5-77.2		
Ratio	mean	2.9[a]	2.3[a]	3.70	0.058
	SEM	0.2	0.2		
	min-max	1.2-8.4	0.9-7.7		

Number of samples in parentheses. SEM: Standard error of mean value. Means in the same line followed by a common letter are not significantly different ($P<0.05$).

than those from 2000, but the ratio values showed no significant differences, confirming the advantage of using the ratio value to discriminate orange juices, rather than the absolute concentration.

Multivariate analyses

Principal Component Analysis of the HCA concentration in 113 orange juices gave two components that explain cumulatively 73.5% of the total variance. Fig. 3 shows the juices distributed in the plot of these components and the positive contribution of p-coumaric acid. The horizontal component included the four acids with similar weights, while the vertical component included p-coumaric acid as the most important variable.

Table 8 reports the results of the Linear Discriminant Analyses of the data. In analysis N.1 113 juices separated into pigmented and non-pigmented varieties were analyzed. The statistical parameters were significant at the 95% confidence level, and p-coumaric acid was confirmed as the discriminating variable between the groups since it had the highest standardized coefficient. Ninety-two out of 113 juices were classified correctly (81.4%), in particular 67/73 pigmented (91.8%) and 25/40 non-pigmented (62.5%) juices. In analysis N.2 113 juices were discriminated on the basis of their geographical origin: 96 out of 113 (85.0%) cases were classified correctly, in particular 38/50 Sicilian juices

Table 8 - Linear Discriminant Analyses of HCA concentration in orange juices.

Analysis N.	Cases N.	Groups N.		Eigen value	Explained variation (%)	Canonical correlation	P-value[a]	Standardized Coefficients of Variables				Correctly classified cases (%)
								Ferulic acid	p-Coumaric acid	Sinapic acid	Caffeic acid	
1	113	2 (P, NP)		0.619	100	0.639	0.000	-0.517	1.021	-0.459	0.434	81.4
2	113	2 (S, OR)		1.029	100	0.712	0.000	-0.008	-1.122	-0.434	0.903	85.0
3	73 P	2 (S, OR)		1.213	100	0.740	0.000	0.015	-0.969	-0.542	1.016	90.4
4	40 NP	2 (S, OR)		0.274	100	0.464	0.069	-0.300	-0.881	-0.656	1.019	85.0
5	50 T	2 (S, OR)		1.974	100	0.815	0.000	0.572	-1.165	-0.730	0.744	100
6	29 T	5 (Clones)	I DF	5.875	74.4	0.924	0.000	-0.242	-1.100	0.713	0.712	89,7
			II DF	1.060	13.4	0.717	0.000	-0.394	0.201	-0.521	0.964	

[a] P-value less than 0.05 is statistically significant at the 95% confidence level. P: Pigmented cultivar juices; NP: Non-Pigmented cultivar juices; S: Sicilian juices; OR: Other Region juices; T: Tarocco; DF: Discriminant Function.

(76.0%) and 58/63 other region juices (92.1%). In analyses N.3 and N.4, 73 pigmented and 40 non-pigmented juices were discriminated on the basis of geographical origin: 66 out of 73 (90.4%) cases were correctly classified, in particular 39/42 Sicilian juices (92.0%) and 27/31 other region ones (87.1%). In analysis N.5 100% Tarocco juices were discriminated on the basis of geographical origin. In this case, both p-coumaric and caffeic acids were the discriminating variables. Fig. 4 shows the content of these acids in Tarocco juices, which clearly differentiates the Sicilian Tarocco juices from those from other regions. Finally, the discriminant analysis of 5 clones of 29 Tarocco juices (analysis N. 6) gave a highly significant canonical correlation (0.924 for the first discriminant function) and 26 juices out of 29 (89.7%) were correctly classified. Fig. 5 shows the position of juices on the plane which is defined by the values of the first two discriminant functions. Juices of different clones are located in clearly separated areas around their respective centroids, with the exception of some Gallo Tarocco juices.

CONCLUSION

HCA are important constituents of orange juices, both for their health promoting properties due to their high antioxidant activity, and for the product typicality due to their function as varietal

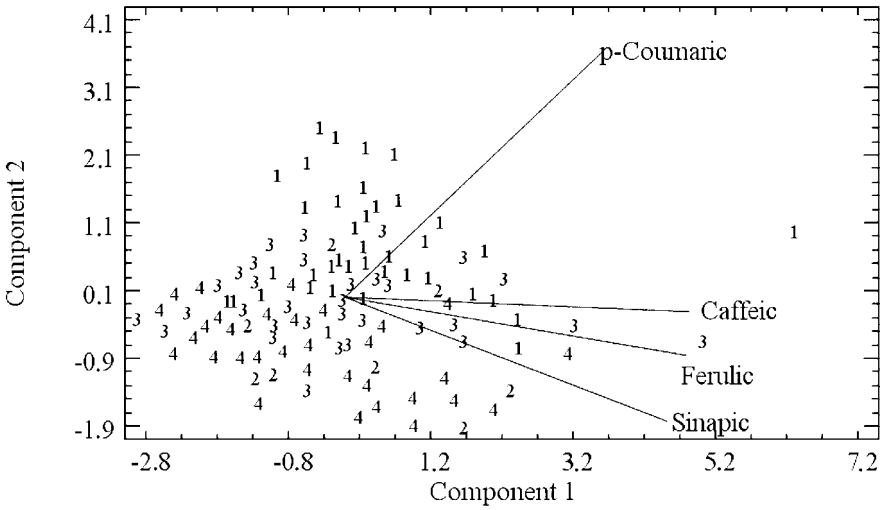


Fig. 3 - Plot of Principal Component Analysis of HCA concentrations in 113 orange juices. Key for numbers: 1, Sicilian pigmented; 2, Sicilian non pigmented; 3, Pigmented from other regions; 4, Non pigmented from other regions.

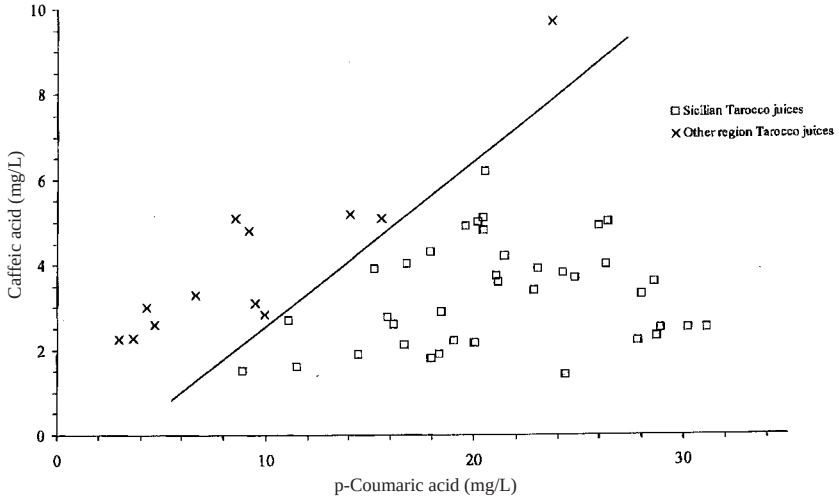


Fig. 4 - Plot of caffeic and p-Coumaric acid concentrations in 50 Tarocco orange juices.

markers. Pigmented orange juices are richer in HCA than non-pigmented ones, particularly those of Sicilian origin. The Ratio between concentrations of the methoxy and hydroxy-substituted HCA [R = (ferulic + sinapic)/(p-coumaric + caffeic)] is an important parameter to ascertain variety and geographical origin of juices. Its value is less than two in pigmented juices. The content of p-coumaric acid is the decisive component in differentiating pigmented juices from non-pigmented ones. Moreover, the juices of Sicilian origin, both pigmented and non-pigmented, were richer in p-coumaric acid than the corresponding juic-

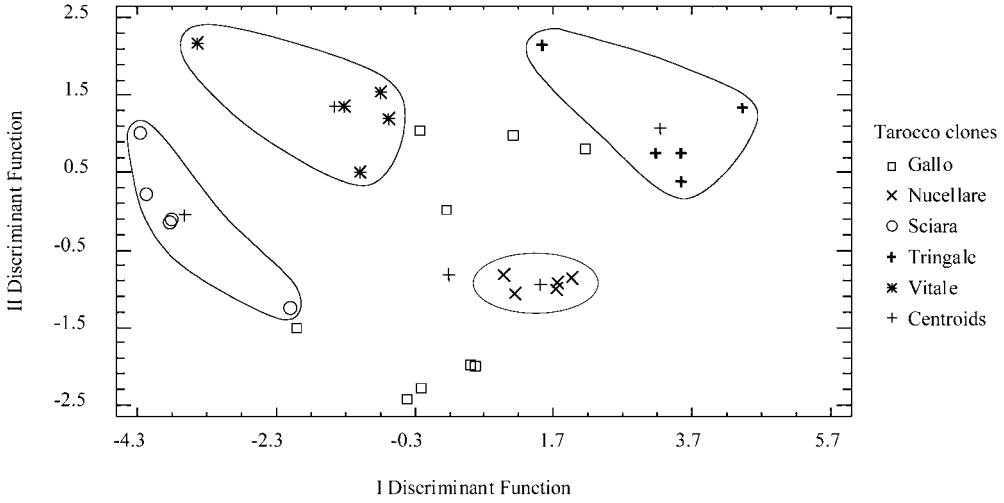


Fig. 5 - Distribution of juices from Tarocco clones in the plane defined by the values of the first two discriminant functions.

es coming from other regions. The HCA distribution can also be used to discriminate different clones of Tarocco, grown under the same pedoclimatic conditions.

In conclusion, HCA analysis allows Sicilian pigmented juices to be identified, hence it can be used in the regulations of the Protected Geographical Indication (PGI) of Sicilian pigmented orange fruits and for the Protected Designation Origin of (PDO) of corresponding juices.

A reduced form of this paper was presented at the 5th Italian Congress of Food Science and Technology, September 13-14, 2001, Cernobbio, Italy.

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REFERENCES

Arena E., Campisi S., Fallico B. and Maccarone E. 1998a. Fatty acids of Italian blood orange juices. J. Agric. Food Chem. 46: 4138.

Arena E., Fallico B., Fanella F., Maccarone E. and Rapisarda P. 1998b. Gli acidi trans-4-idrossicinnamici marker dei succhi di arancia pigmentata. Industrie Bevande 27: 609.

Arena E., Fallico B. and Maccarone E. 2001. Evaluation of antioxidant capacity of blood orange juice as influenced by constituents, concentration process and storage. Food Chem. 74: 423.

Fallico B., Lanza C.M., Maccarone E., Nicolosi Asmundo C. and Rapisarda P. 1996. Role of hydroxycinnamic acids and vinylphenols in the flavor alteration of blood orange juices. J. Agric. Food. Chem.44: 2654.

Heller W. and Forkmann G. 1994. Biosynthesis of flavonoids. In "The Flavonoids. Advances in Research Since 1986". J.B. Harborne (Ed.), p. 499, Chapman & Hall/CRC, New York.

ISTAT. 1997. Compendio Statistico Italiano. Istituto Nazionale di Statistica, Rome, Italy.

La Via G. and Pecorino B. 1996. L'industria di trasformazione degli agrumi in Italia. RAISA-CNR, Rome. Publ. N. 3049.

Maccarone E., Maccarrone A., Perrini G. and Rapisarda P. 1983. Anthocyanins of the Moro orange juice. Ann. Chim. (Rome) 73: 533.

Maccarone E., Maccarrone A. and Rapisarda P. 1985. Acylated anthocyanins from oranges. Ann. Chim. (Rome) 75: 79.

Maccarone E. 1997. Dalla Cina a Bruxelles. La lunga marcia delle arance rosse. Tecnica Agricola (Catania) 49 (4): 45.

Maccarone E., Rapisarda P., Fanella F., Arena E. and Mondello L. 1998a. Cyanidin-3-(6"-malonyl)-β-glucoside. One of the major anthocyanins in blood orange juice. Ital. J. Food Sci. 10: 367.

Maccarone E., Campisi S., Fallico B., Rapisarda P. and Sgarlata R. 1998b. Flavor components of Italian orange juices. J. Agric. Food Chem. 46: 2293.

Marini D. and Balestrieri F. 1995. Multivariate analysis of flavanone glycosides in citrus juices. Ital. J. Food Sci. 7: 255.

Mouly P.P., Arzouyan C.R., Gaydou E.M. and Estienne J.M. 1994. Differentiation of citrus juices by factorial discriminant analysis using liquid chromatography of flavanone glycosides. J. Agric. Food. Chem. 42: 70.

Mouly P., Gaydou E.M. and Auffray A. 1998. Simultaneous separation of flavanone glycosides and polymethoxylated flavones in citrus juices using liquid chromatography. J. Chromatography A 800: 171.

Mouly P.P., Gaydou E.M., Lapierre L. And Corsetti J. 1999. Differentiation of several geographical origin in single strength Valencia orange juices using quantitative comparison of carotenoid profiles. J. Agric. Food Chem. 47: 4038.

Peleg H., Naim M., Rouseff R.L. and Zehavi U. 1991. Distribution of bound and free phenolic acids in oranges (*Citrus sinensis*) and grapefruits (*Citrus paradisi*). J. Sci. Food Agric. 57: 417.

Quarleri G. 1994. Agrumi, il futuro si chiama trasformazione. Largo Consumo 10: 128.

Rapisarda P., Carollo G., Fallico B., Tomaselli F. and Maccarone E. 1998. Hydroxycinnamic acids as markers of Italian blood orange juices. J. Agric. Food Chem. 46: 464.

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

Risch B. and Herrmann K. 1988. Hydroxycinnamic acid derivatives in citrus fruits. Z. Lebensm. Unters. Forsch. 187: 530.

Rouseff R.L. 1988. Differentiating citrus juices using flavanone glycosides concentration profiles. In "Adulteration of Fruit Juices Beverages". Nagy S., Attaway J.A., Rhodes M.E. (Eds.), Dekker, New York.

Rouseff, LR., Seetharaman K., Naim M., Nagy S. and Zehavi Y. 1992. Improved HPLC determination of hydroxycinnamic acids in orange juices using solvents containing THF. J. Agric. Food Chem. 40: 1139.

Sturiale L. 1995a. Analisi dei risultati di ricerche di mercato sui succhi di arance "rosse" e prospettive di sviluppo delle relative domande: Italia e Regno Unito. RAISA-CNR, Rome. Publ. N. 2027.

Sturiale L. 1995b. Caratteristiche della domanda dei succhi di arancia "rossa" in Italia e in altri Paesi europei e relative prospettive di sviluppo. Atti del Seminario di studi "Struttura e competività dei principali comparti produttivi dell'agro-industria nel Mezzogiorno: Agrumi e derivati. CNR-Università degli Studi di Catania.

Versini G., Reniero F., Simoni M. and Ziller L. 1995. Contenuto in isotopi stabili di alcune componenti dei succhi d'arancia del commercio a base di succo concentrato non zuccherato. Atti del 2° Congresso Nazionale di Chimica degli Alimenti. p. 421. La Grafica Ed., Messina, Italy.

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ERRATA

In the Paper entitled "The Effect of Smoking, Packaging and Storage Temperature on the Bacterial Greening of Frankfurters caused by *Leuconostoc mesenteroides* subsp. *mesenteroides*" by K. Anifantaki, J. Metaxopoulos, M. Kammenou, E.H. Drosinos and M. Vlassi, published in Vol. 14, n.2, 2002, the axis on Fig. 4 should read "LAB on MRS ($\log_{10}$ cfu.g^{-1})". IJFS apologizes for the error.

FLAIR-FLOW EUROPE



HYPOALLERGENIC FOOD PRODUCTS

FFE 504/02/SME42

Using heat treatments or chemical peeling, many vegetable products may be less allergenic to humans and industry is increasingly adapting the new methods. A group of European researchers have been working on this to develop and hypoallergenic food products.

Allergens in foods are typically small proteins recognized by the IgE (immunoglobulin E) immune system and causing strong biological reactions in some humans. Among the strongest and most common natural allergens are celery, hazelnut, carrots and Prunoideae fruits such as peaches, plums and apricots.

The research team found several allergens previously unknown and determined their chemical structures, which were often quite similar across vegetable products. They then used genetic methods to produce a number of recombinant allergens to develop improved diagnostic methods.

The scientists also heat-treated hazelnut and celery and used chemical peeling in peach and apricot. Heat-treated foods did not show complete allergenicity reduction: some minor compounds were found to be heat-resistant, triggering of food allergy in some subjects. However, chemically-treated peaches and apricots provoked no adverse reactions in allergic subjects.

Food allergy has been on the headlines for several years due to its increasing incidence in the Western world (estimated to 10% of the population, or more than 40 million Europeans), and to the fear to introduction new allergens by genetic engineering of food crops. Since we know now the protein structure of the allergens, we may hope for new genetically modified vegetables where the genes expressing the allergenic proteins have been eliminated.

Project N.: FAIR-CT97-3224

Project Co-ordinator: Prof. Claudio Ortolani, Divisione Medica Bizzozero, Centro di Riferimento Regionale di Allergologia e Immunologia Clinica, Piazza Ospedale Maggiore 3, I-20162 Milan, Italy, Tel.: +39-02-64442414, Fax: +39-02-64442027, E-mail: ortolani@stnet.net.

F-FE PROJECT LEADER

Dr. T.R. GORMLEY
The National Food Centre
Dunsinea, Dublin 15, IRELAND
Tel. +353 1 383222 - Fax +353 1 383684

ITALIAN NETWORK LEADER

Dipartimento di Scienze degli Alimenti
Università di Udine, Via Marangoni 97, Udine, ITALY
Tel. +39 0432 590711 - Fax +39 0432 590719
e-mail: ffe@dsa.uniud.it
web site: www.uniud.it/ffe/welcomeflair.htm

PHYTOESTROGENS: FENDING OFF BREAST AND PROSTATE CANCER

FFE 505/02/HP40

Cancer is one of the major causes of chronic ill health and of premature death in Europe. It is estimated that diet could potentially prevent 35% of deaths from cancers. Informed dietary changes would be a very effective way to reduce the incidence of cancer. Epidemiological evidence suggests that plant-based diets may play a role in the reduction of cancer risk and research to find the substance(s) involved is being carried out

One group of substances being investigated by researchers is phytoestrogens (isoflavones, found mainly in soybeans, and lignans, found in many different foods including cereals, vegetables and berries). These compounds have weak oestrogenic and anti-oestrogenic activity and may have protective effects on cancers of the breast and prostate because they are structurally similar to the mammalian hormone oestrogen: they could have an effect on pre-cancerous lesions, preventing promotion, angiogenesis (blood supply to a tumour) and metastasis (development of secondary tumours), and thus preventing and disease and increasing patients' survival rates.

An EU-funded project called PHYTOPREVENT is now in progress to study in depth the effects of phytoestrogens (from foods, not supplements) on the development of breast and prostate cancer and the influence of individual variation in metabolism on cancer risk. Special emphasis will be placed on investigating metabolites generated by the gut microflora, such as enterolactone and equol.

The project started in April 2001 and will be completed by March 2004. The successful completion of this project will provide evidence for making widespread recommendations to European people about dietary changes leading to improved health. Further information can be seen on the website: www.phytoprevent.org where regular progress newsletters will be posted.

Project N.: QLK1-2000-00266 (PHYTOPREVENT)

Project Co-ordinator: Prof. Ian Rowland, Room G237; School of Biomedical Sciences, University of Ulster; Coleraine Campus, Cromore Road; Coleraine; Co. Londonderry; BT52 1SA, UK, Tel.: +44 (0)28 70324866, Fax: +44 (0) 28 70323023, E-mail: i.rowland@ulster.ac.uk.

HEALTH BENEFITS AND B VITAMINS FROM NUTRACEUTICALS

FFE 507/02/HP42

The term "nutraceuticals" has been used to describe a wide range of foods and food components with an "extra" medicinal or health benefit. It is an area of much interest to health professionals, the food industry and consumers alike, with potential gains to be had for all.

A new project called NUTRA CELLS is being funded under the EU 5th Framework Programme. The main objective is to improve the potential health benefits of fermented food products (such as yoghurts). It is anticipated that the food products developed will have enhanced characteristics, resulting from either production or addition of components, such as low energy (or non- metabolisable) sugars, oligosaccharides (which have stool bulking effects), or B-type vitamins (such as folic acid and riboflavin), or from the removal of "undesirable" food components, such as lactose (which can adversely affect people with lactose intolerance), or raffinose (a high content of which, e.g. in soy products, has a tendency to induce flatulence).

In order to produce these nutraceutical characteristics, micro-organisms, which are safe for use in food, will be used. In particular, the lactic acid bacteria, proprionic acid bacteria and bifidobacteria strains will be developed for use.

There are 5 specific work packages in this project:

- Production of non-metabolisable sugars;
- Galactose and / or lactose removal from dairy products;
- Removal of raffinose from soy products;
- Oligosaccharide production;
- Vitamin production.

All of these have potential implications for health. The vitamin production work package, for example, aims to increase the folic acid levels in yoghurts and other dairy products. Folic acid is specifically important in the diets of pregnant women, as it is known to reduce the incidence of babies born with neural tube defects (e.g. spina bifida). Additionally, low folic acid in the diet is associated with high homocysteine levels in the blood and subsequently with coronary diseases.

Developments on this project can be followed on the project website: www.nutracells.com.

Project N.: QLK1-2000-01376 (NUTRA CELLS)

Project Co-ordinator: Dr. Jeroen Hugenholtz, NIZO Food Research, P.O. Box 20, 6710 BA Ede, The Netherlands, Tel.: +31 318 659511, Fax: +31 318 650400, E-mail: hugenhol@nizo.nl, URL: www.nizo.com.

ALLERGY STUDIED FROM BIRTH

FFE 510/02/CG42

The incidence of allergy in Europe is increasing. One of the suggested reasons for this is the high level of hygiene in the occidental society. According to the scientists involved in the "Impact of intestinal microflora on allergy development" - project, the high level of hygiene may cause changes in the "good" bacteria that populates the intestine after birth. This can lead to allergy, which means that the body cannot tolerate some otherwise harmless food items or environmental factors such as pets or pollen. Typical symptoms well known to allergic people include eczema, irritated eyes, rhinitis and asthma.

This project examines the possible causes for the high and rising frequency of allergies in many European countries. 300 children from Sweden (Göteborg), Italy

(Rome) and Great Britain (London) are being followed from birth: samples of their intestinal flora are taken during their first year of life and environmental conditions (housing standards, family size, etc.), feeding pattern and medical illness are being assessed. Major groups of particular bacteria in the normal intestinal microflora will be established. A study nurse keeps close contact with the families and a team of paediatric allergologists examine all children at 1.5 and 3 year of age for signs of allergic sensitisation. The flora of those who become allergic by 18 months is compared to the flora of those who stay healthy.

Researchers hope that this work will enable them to identify bacteria that enhance or reduce the risk of developing an allergy.

Project N.: QLK4-2000-00538 (ALLERGYFLORA)

Project Co-ordinator: Agnes Wold, Göteborg University, Department of Clinical Immunology, Guldhedsgatan 10, SE-413 46 Göteborg, Sweden, Tel. +46 31 3424617, Fax: +46 31 826791, E-mail: agnes.wold@immuno.gu.se, URL: http://www.gu.se.

FOOD COMPONENTS AND COLORECTAL CANCER

FFE 512/01/SME44

A new in-vitro screening assay is being developed to detect preventive or inhibitory effects of food components on the initiation or development of colorectal cancer. The inventors say that the screening assay is based on detection of the genomic changes (multi-gene expression and changes in protein concentrations) involved in colorectal carcinogenesis.

The scientists in this recent European project are identifying the genes relevant for the process of colorectal cancer development and prevention in both a human and a rat model, using a number of carcinogens agents and anti-carcinogenic agents, among these last ones the flavonoid resveratrol. They also map the proteins expressed (the proteome) in healthy and tumour tissue as well as in cancer cell lines.

The model compound used, resveratrol, is a promising food component with a very potent inhibitor effect on human colon cancer cell growth in vitro. After prolonged exposure to many cancer cell lines. an increased necrosis of these cells is seen.

More than half of the incidence of gastrointestinal cancers is estimated to be related to the diet. It is, therefore, of utmost importance to identify the components in the diet that may reduce (or induce) colorectal cancer risk by various mechanisms, for instance. by preventing carcinogen formation, scavenging or modifying carcinogens, protecting the genes (DNA), antioxidation, or induction of cancer cell necrosis.

Project N.: QLK1-1999-00706 (FFACC)

Contact Person: Dr. Ruud Woutersen, TNO Voeding, Utrechtseweg 48, Postbus 360, 3700 AJ Zeist, The Netherlands, Tel.: +31 30 6944485, Fax: +31 30 6960264.

HOW CAN POLYPHENOLS PROTECT PEOPLE?

FFE 514/02/HP43

Each year in Europe 75,000 people die from colon cancer. This disease causes a lot of distress, and is a huge burden to health professionals and on resources. Epidemiological studies have shown that populations who eat diets rich in plant-based foods have a reduced risk of certain cancers, and scientists want to know why.

There is a lot of interest in the role that compounds found in plants – phytochemicals – may play in protecting cells and in preventing them from becoming cancerous. *In vitro* studies and animal models, have shown that isolated, purified phytochemicals kill cancer cells or stop normal cells from becoming cancerous, some may prevent damage to DNA or modify gut microflora and so may affect how we metabolise and digest food.

POLYBIND is a new multi-centre EU-funded project which has been set up to further investigate the most promising phytochemicals that might protect against colon cancer. Researchers will be focusing on the largest group of phytochemicals – polyphenols, and in particular the polyphenolic compounds flavanols and flavonols. These include catechins (found in foods such as cherries, tea and apricots) and quercetin (found in foods such as onions, apples and tea).

The main objectives of POLYBIND are to determine how these compounds are metabolised, and how they alter the rates of carcinogen metabolism and cell proliferation. The questions include in which chemical form polyphenols work best; if other things present in the food affect how they work; if they are actually digested and absorbed; and if so, where in the body they go, how they work and how much is needed to cause an effect? This work should lead to the development of new methods to study links between diet and health; improved dietary advice; information to allow the development of novel foods; and ultimately a reduction in the levels of chronic disease, especially colon cancer. We are expecting the final results of this important research in two years time and will keep you updated on the progress of this project in future one-pagers.

Project N.: QLK1-1999-0050S (POLYBIND)

Project Contact: Ms. Christine Hill, POLYBIND Dissemination Officer, Diet, Health and Consumer Science Division, Institute of Food Research, Norwich Research Park, Colney, Norwich, Norfolk NR4 7UA, UK, Tel.: +44 (0)1603-255000, Fax: +44 (0)1603-507723.

REDUCING THE RISK OF COLORECTAL CANCER

FFE 517/02/CG43

Colorectal cancer is a major cause of premature death among adults across Europe. As the average age of the European population increases, it will inevitably

lead to an increased incidence of this age-related cancer. Naturally, any improvement in this situation would be welcomed.

The SYNCAN project aims at determining whether the new concepts of nutrition i.e. prebiotics, probiotics and synbiotics, can effectively reduce the risk of colorectal cancer.

Probiotics are live "friendly" bacteria (such as the *Lactobacillus* bacteria in fermented dairy products), whilst prebiotics stimulate the growth of friendly bacteria in the colon (e.g. particular carbohydrates in food). The combination of probiotics and prebiotics is known as a synbiotic combination.

It has been consistently shown that prebiotics and probiotics added to the diet of animals can reduce a great deal the development of cancer. It has also been observed that synbiotic combinations are more effective than their prebiotic or probiotic components alone. The main scientific prospect of the SYNCAN project is to carry out a study with volunteers, designed to evaluate whether synbiotics can reduce the risk of colorectal cancer in humans.

These days, many types of yoghurt and other fermented milk products include probiotic bacteria. Prebiotic carbohydrates are present in many different food products (e.g. bakery products, prepared meat products, spreads, drinks, dairy products and more), both for their interesting nutritional properties and because they improve the texture of the food. Because of the growing interest, food industry and research institutes are developing new products containing prebiotics, probiotics or both.

The outcome of the SYNCAN project may contribute to the development of dietary guidelines for the prevention of colorectal cancer. Flair-Flow 4 will disseminate the results as they become available. More information will also provided on the project website

Project N.: QLKI-1999-00346 (SYNCAN)

Project Co-ordinator: Dr. Jan Van Loo, Tiense Suikerraffinaderij NV, ORAFTI, Aandorenstraat 1, B-3300 Tienen, Belgium, Tel.: +32 16 801213, Fax: +32 16 801308.

VITAMIN D FROM SUN AND FOOD

FFE 519/02/CG45

Rickets, caused by vitamin D deficiency, is reappearing in Europe. Vitamin D deficiency is also an important risk factor for hip fracture in elderly and for low bone mass in rapidly growing young. Three population groups are particularly at risk of vitamin D deficiency: the elderly, immigrants and children. There is a need to develop a new low-fat, vitamin D enriched food, like bread, says Dr. Christine Brot, co-ordinator of a new European research project looking at optimal vitamin D fortification.

There are two sources of vitamin D: sunlight and food. For most people the main source of vitamin D is exposure to sunlight. The precursor of the vitamin in the skin is converted to vitamin D when exposed to UV radiation. In the dark winter months we rely on the stores built up during the previous summer and on dietary intake.

Diet is a secondary source of vitamin D, since only few foods contain substantial amounts of vitamin D. Richest foods are fatty fish, milk products and fortified margarine. Intake is generally well below the recommended level. Vitamin D defi-

ciency is a serious health problem among large population groups in Europe. For instance, people not getting enough sunlight for social or religious reasons like enforced dress codes, or because of disability are at risk for vitamin D deficiency.

The project Dr. Brot is leading aims at a long-term improvement of vitamin D status in European population. Researchers will investigate if fortification of food with this vitamin is a feasible strategy to remedy its lack in large population groups in Europe. They will also determine at what level fortification should be pitched. Bread enriched with vitamin D is being developed in the project.

Project N.: QLK1-2000-00623 (OPTIFORD)

Project co-ordinator: Dr. Christine Brot, Department of Nutrition, Institute of Food Safety and Nutrition, Danish Veterinary and Food Administration, Moerkhoej Bygade 19, DK-2860 Søborg, Denmark, Tel.: +45 3395 6332, Fax: + 45 3395 1119.

OLIVE ANTIOXIDANTS

FFE 522/02/SME48

A team of Southern-European scientists has developed an economically feasible process to recover polyphenolic antioxidants from olive oil production waste.

In the olive mill, the olive paste is traditionally washed with water, which removes a large amount of the soluble compounds. Among others, the amphiphilic phenolic compounds, monohydroxyphenols and o-dihydroxyphenols (catechols), are extracted, while the esterfied phenols, ligstroside and oleuropein, stay in the oil phase. Catechols, also present in tea and other vegetable products, are well-known antioxidants, protecting foods from oxidation. They also are challenging biological antioxidants protecting against oxidative degradation in living cells, and so are of interest not only to food scientists but also health professionals.

According to this group of scientists, the catechols may be industrially isolated from the wash water, by acidification, enzymatic pectin degradation, and absorption on sorbent resins. Finally, the catechols are desorped: 10-20% of the total phenols are recovered in the process and the main catechol present is hydroxyltyrosol.

The researchers analysed the biological antioxidant effects of the olive phenols through *in vitro*, rat, and human studies. *In vitro,* the phenols inhibited LDL oxidation, leukocyte activation, superoxide anion production, and the enzyme glutathione-S-transferase. *In vivo* studies showed a dose-dependent absorption both in rats and in humans, an enhanced total antioxidant activity in the plasma, and significant interactions with cellular enzymes.

The studies show that the water-soluble olive oil polyphenols might be a good candidate as a functional food ingredient in the future, potentially influencing the development of cardiovascular disease, cancer and inflammatory reactions, but more human studies are need.

Project N.: FAIR<T97-3039

Project Co-ordinator: Prof. Claudio Galli, Università degli Studi di Milano, Istituto di Scienze Farmacologiche, Via Balzaretti 9, 20133 Milano, Italy, Tel.: +39 02204881.

VINES FOR HEALTHY VEINS

FFE 523/02/HP46

"Red wine and polyphenol extracts from it (RWPE) may prove to be beneficial against the major killer of European citizens", according to researchers teamed up to examine the "French Paradox" in an EU funded project, coronary heart disease is the most common single cause of death in many countries and so researchers are keen to establish how the disease can be prevented.

This project has contributed to the evidence base for the epidemiological association of moderate red wine consumption with prevention of cardiovascular disease. It focussed on the biological effects of RWPE in various *in vitro* models and *in vivo*, to identify the mechanisms by which these compounds influence atherosclerosis. RWPE showed several beneficial effects:

- it improved vascular function mainly though nitric oxide (NO) mediated mechanisms,
- it increased arterial relaxation after norepinephrine-(noradrenaline) induced contraction,
- it interfered with and reduced monocytes' adhesion to endothelial walls, and so atheroschlerotic plaques were prevented.

The "French Paradox" refers to the very low incidence and mortality rates from ischaemic heart disease that exist in France despite the fact that serum cholesterol and saturated fat intake and prevalence of smoking are not lower in France than in other countries. Could red wine consumption be the answer?

Furthermore, a human study showed that when 40 healthy volunteers drank red wine (equivalent to 30 g of alcohol per day) for 4 weeks there was a significant increase in HDL cholesterol (the so-called "good" cholesterol) and a decrease in oxidation of LDL cholesterol, but this effect was not observed when volunteers drank the same amount of alcohol as a spirit. In addition, when red wine was consumed, there was a significant fall in the expression of several markers of cardiovascular disease measured on circulating cell surfaces or in plasma.

Finally, researchers also carried out a meta-analysis of all available epidemiological studies and demonstrated that there was a negative association of moderate wine consumption (150-300 mL daily) with the risk of major cardiovascular events.

Project N.: FAIR-97-3261 (WCVD)

Project co-ordinator: Dr. Giovanni de Gaetano, Centro di Ricerche e Formazione ad Alta Tecnologia nelle Scienze Biomediche, Università Cattolica, Campobasso, Via Milano 56, 66034 Lanciano, Italy, Tel./Fax: +39 872 714008.

A VISION FOR FUTURE FOOD PRODUCTION?

FFE 528/02/CG48

Experts from science and consumer organisations have made a visionary picture about food production in our future Europe. Results indicated for example broader use of enzymes in food processing and beer production with genetically

engineered yeast. The German experts tended to be the most critical among the countries involved, specially the farmers, consumers and biotechnology critics.

The objective of this project finalised in 1998 was to analyse and forecast the impact of biotechnology on the agricultural and food sector in Germany, The Netherlands, Italy, Spain and Greece. Several thousands experts from these countries were selected from science, industry, farmers' and consumer organisations, politics and administration. They were asked to respond to statements about possible future "visions" on a range of issues in agriculture and the food sector such as technical development, regulation, economy, environment and health.

In Germany, the future estimations of expert groups differed clearly from each other. The extremes represented the experts from industry and research institutions on the one hand, and consumers and critics on the other. The responses of the farmers were between these two extremes, but with clear tendencies towards the consumer/critics cluster. The biggest differences between the five expert groups in Germany were found in statements dealing with the application of enzymes in the food industry. In general, industry and research experts tended to assess such a development as positive whereas most of the farmers, consumers and critics were more sceptical or rejected such a development.

Because of ethical and risk problems, most European consumers dislike the development of modern biotechnology related to food production, while they generally accept the technologies in the medical and the environmental areas.

Project N.: FAIR-CT95-0269

Project Co-ordinator: Dr. Klaus Menrad, Fraunhofer Institute for Systems and Innovation Research, Breslauer Str. 48, D-76139, Karlsruhe, Germany, Tel.: +49 721 6809330, Fax: +49 721 6809176.

IFSC, INTERNATIONAL FOOD SCIENCE CENTRE A/S

The International Food Science Control A/S in Lystrup, Denmark announces that its director, Prof. Dr. Vijai KS Shukla has been awarded the Stephen S. Chang Award 2002.

The Stephen S. Chang Award is one of the highest global recognitions for a scientist/technologist for outstanding original work in the world of food science, and was established by former AOCS President Stephen S. Chang and his wife, Lucy, to recognise a scientist, technologist, or engineer who has achieved decisive accomplishments in basic research, either by one major breakthrough or by an accumulation of publications. The knowledge that the person has produced must have been utilised by industries for the improvement or development of food products related to lipids. Eligibility for the award must be demonstrated through high-quality scientific papers, patents, or the marketing of commercial products developed through the awardee's scientific or technological efforts.

In his 30 years of research, Prof. Dr Shukla has made contributions to the basic understanding of lipids, their chemistry, physiology, and functionality. The understanding of fats in chocolate and confectionery has long been a focus of Prof. Dr Shukla's research. In addition to method development, he has examined the triacylglycerol structures of current and possible components, using that knowledge to explain how changes in composition affect the temper, viscosity, and hardness of chocolate. Prof. Dr Shukla has demonstrated that "total oxidation in bulk oils can be arrested and specialty fats delivered to customers in [an] extremely fresh state". In addition, his research has centered on the quality of encapsulated fish and vegetable oils. He has produced an ultrarefined fish oil with extremely low peroxide and anisidine values that is in commercial production. As part of that work, he designed several natural antioxidant systems for general nutrition as well as cosmetic oils that are in the process of being patented.

He has more than 80 publications in international journals and numerous book chapters. Prof. Dr Shukla is Adjunct Professor in the Department of Food Science and Human Nutrition at the University of Illinois in Urbana, Illinois. He is a member of the advisory board of INFORM, associate editor of the Journal of American Oil Chemists' Society, Lipid Technology, the Journal of Food Lipids, and is the chairman of the American Oil Chemist's Society's NMR Group. Prof. Dr Shukla's name appears in the Marquis Who's Who in the World. On behalf of the World Bank, Prof. Dr Shukla helped to develop strategies for the Malaysian Prime Minister's Vision 2020 program within food science research.

Second Announcement

NATIONAL SYMPOSIUM ON SHELF LIFE OF PACKAGED FOODS
June 11-13 2003
Milan, Italy

The Italian Scientific Group of Food Packaging (GSICA) announces the symposium on "Shelf Life of Packaged Foods" which will be organised in Milan in co-

operation with the Italian Packaging Institute. The aim of the Symposium is to present a complete picture of all the different topics related to the main theme of food quality preservation within the commercial distribution system. The theme of the Shelf Life of Packaged Foods is so wide and multi-disciplinary that it will include analytical chemistry, microbiology, food processing, food packaging, material science as well as physical chemistry. The Symposium will consist of three sessions, each opened by a foreign invited speaker, who will address:

- ***Shelf Life modelling***: models to predict the shelf life of packaged food as well as quality decay modelling; predicting of barrier performance of packaging materials; kinetics of food quality degradation, risk assessment, … The section will be opened with a lecture by Prof. Keith L. Yam - Rutgers University, NJ, USA.

- ***New technologies for Shelf Life extension***: new materials, new packaging devices, new food processing technologies, new techniques for risk reduction, … The section will be opened with a lecture by Prof. Josep H. Hotchkiss - Cornell University, NY, USA.

- ***Shelf Life testing***: non-invasive analytical techniques, freshness indicators, evaluation of packaging materials performance; risk management, … The section will be opened with a lecture by Prof. Harmen Hofstra - TNO, The Netherlands.

The three-day Symposium will include oral presentations and posters; the designation of each contribution will be decided by the Scientific Committee. All contributions should comply with the "Guide for Authors" of the Italian Journal of Food Science and will be reviewed according to the Editorial Policy of the same Journal.

Deadlines: November 30, 2002 for submission of an extended abstract
February 15, 2003 for final programme
May 31, 2003 for submission of final papers
For additional information see: http://www.packlab-jessica.it/
For additional information contact: segreteria@packlab-jessica.it

APPLICATION OF TEXTURE & RHEOLOGY FOR BAKERY PRODUCTS

School of Food Biosciences 19th-20th December 2002 Cosponsered by AACC and the University of Reading UK

A 2-day post-graduate training course at the University of Reading, this course provides an in-depth understanding of the principles and measurements of food texture and rheological measurements and their applications to bread. The course will provide leading-edge state of the art knowledge and practice on food texture and rheology measurements, especially in bakery products, presented by leading academic and industrial experts and includes applied case studies and practical sessions.

For further details contact: Dr Bogdan Dobraszczyk
Tel: +44 (0)118 9318714
Fax: +44 (0)118 9310080
e-mail: b.dobraszczyk@reading.ac.uk

2002 IDF AWARD GOES TO PATRICK F. FOX

During the IDF World Dairy Summit and the XXVI International Dairy Congress "Congrilait" in Paris (France), from 21 to 27 September 2002, the IDF President, Philippe Jachnik presented the 2002 "IDF Award" to Professor Patrick F. Fox, Ireland.

The IDF Award recognizes remarkable contributions to progress in international dairying. In granting the Award to Professor Fox, the Award committee wishes to pay tribute to the multitude of his well-known scientific achievements dedicated to dairy science.

Patrick Fox was Professor and Head of the Department of Food Chemistry at

University College, Cork (UCC), Ireland, from 1969 to 1997; he retired in December 1997 and is now Emeritus Professor of Food Chemistry at UCC.

Prof. Fox's research has focussed on the biochemistry of cheese, the heat stability of milk, physico-chemical properties of milk proteins and food enzymology. He has authored or co-authored about 400 research and review papers, authored or edited 20 text-books on Dairy Chemistry and was one of the founding editors of the International Dairy Journal.

In recognition of his work, Prof. Fox has already received the Research & Innovation Award of the (Irish) National Board for Science and Technology (1983), the Miles-Marshall Award of the American Dairy Science Association (1987), Medal of Honour, University of Helsinki (1991), the DSc degree of the National University of Ireland awarded on the basis of his published work (1993), the Senior Medal for Agricultural & Food Chemistry of the Royal Society of Chemistry (2000) and the ISI Highly Cited Award in Agricultural Science (2002).

Prof. Fox has been invited to lecture in various countries around the world. He has served in various capacities with the International Dairy Federation, including president of Commission F (Science, Nutrition and Education) from 1980 to 1983.

Prof. Fox is currently on the Advisory Board of the Italian Journal of Food Science.

For further information: contact Monique Lebeau
+32 2 7339888 – e-mail: mlebeau@fil-idf.org

BOOKS

USE OF ENZYME PREPARATIONS IN CHEESEMAKING (OTHER THAN RENNET)

20 pp - English only

This paper discusses the wide use of enzymes, other than rennet, in the cheese ripening process. It examines, in particular, the effect of certain enzymes on the flavour, texture and consistency of natural cheese. Furthermore, it covers the area of enzyme-modified cheese and its application as a food ingredient. Finally, it outlines the legislative framework for the incorporation of these enzymes into food products.

DETECTION OF VEGETABLE PROTEINS IN MILK PRODUCTS

M.A. Manso, T. M. Cattaneo, S. Barzaghi, MD. Pérez, L. Sánchez, M. Calvo, C. Olieman, G. Brett and R. López-Fandiño

26 pp - English only

International food law and consumer attitudes require that the protein content of milk products be derived exclusively from milk. Historically, the methods for detecting non-milk protein in dairy products have been unreliable. For this reason, the European Commission appointed experts to conduct a comparative study of the various testing methods currently available.

50 pp in total
Bulletin N. 371/2002 - 54 Euro

A FRESH PERSPECTIVE FOR MANAGING MILK-BORNE DISEASES

Proceedings
of the Animal Health Conference

IDF World Dairy Summit 2001 Auckland, New Zealand

59 pp - English only
Bulletin N. 372/2002 - 62 Euro

The Animal Health Conference in Auckland focused on the threat of milk-borne diseases. Particular attention was given to Foot and Mouth Disease Virus (FMDV), *Escherichia coli* (*E. coli*), paratuberculosis and mastitis. The aspects examined were the causes of diseases in milk, IT solutions for their surveillance, ways of reducing their prevalence, the EU legislative framework for somatic cell counts and import risk analysis.

FARMING FOR PROFIT FROM FRESH PASTURE

Proceedings
of the Farming Conference

IDF World Dairy Summit 2001 Auckland, New Zealand

37 pp - English only
Bulletin N. 373/2002 - 40 Euro

The Farming Conference in Auckland examined farming in the context of prof-

it, efficiency and lifestyle. It compared and contrasted seasonal, pasture-based and high-input dairy farming and their impact on profit, milk output and quality of life.

GENOMICS OF GC-RICH GRAM-POSITIVE BACTERIA

Editor: Antoine Danchin HKU-Pasteur Research Centre, Hong Kong

Series: Volume 2 in the Functional Genomics Series

Publication date: September 2002

ISBN: 0-9542464-3-8

Price: GB £75 or US $150 (hardback).

Pages: viii + 178

Eminent scientists have contributed comprehensive reviews on all aspects of the genomics of *Rhodococcus*, *Mycobacteria*, *Streptomyces* and other organisms. The authors provide an insight into the many facets of large-scale functional investigation in a variety of bacteria with the explicit goal to find common rules in the dynamic and structural organisation of these organisms. This volume is the definitive guide to current research in this increasingly important area. Aimed at microbiologists, biotechnologists and genome scientists.

GUIDE FOR AUTHORS

ITALIAN JOURNAL OF FOOD SCIENCE - IJFS

1. Manuscript Preparation

(1) Manuscripts must be typed, double-spaced and **four** 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 should seek help from a fluent person before the final version is typed. The Assistant Editor reserves the right to make literary corrections and to make suggestions to improve brevity.

The paper must also be submitted on a Macintosh or Windows 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 not saved in "OMS Word" format; graphics, pictures and diagrams must be saved in TIF, JPEG, EPS, CGM 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 must also be included. DO NOT EXCEED 200 WORDS.

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) Lines on all pages, including those pages for "References" and figure legends, must be numbered (by pen) in the left margin, beginning with number one at the top of the page.

(4) Tables should be as few and as simple as possible and include only essential data. Each table must be on a separate sheet and saved on floppy disk, 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.

(5) Figures must be drawn on separate sheets of paper and saved on floppy disk in TIF, JPEG, EPS, CGM or PICT formats. 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".

(6) 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 3x10-3m. 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 within 1 month, 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.

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Papers for publication and communications regarding editorial matters should be sent to:
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E-mail: ijfs@unipg.it or paolofan@unipg.it

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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

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ASSOCIATIONS

Associazione Italiana di Tecnologia Alimentare (A.I.T.A.) - Milano	Fax +39-02-2365015

INDUSTRIES

Besana spa - San Gennaro Vesuviano (NA)	Fax +39-081-8657651
Birra Peroni Industriale spa - Roma	Fax +39-06-22544313
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Corial scpa - Foggia	Fax +39-0881-680077
Kraft Foods Italia spa - Milano	Fax +39-02-41337595

RESEARCH INSTITUTES

Istituto di Tecnologie Alimentari, Facoltà di Agraria
Dipartimento di Agrobiologia e Agrochimica (D.A.B.A.C.),
Università 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 Ambientali Agrarie
e di Biotecnologie Agro-Alimentari (Di.S.A.A.B.A.),
Università di Sassari, Sassari — Fax +39-079-229276

Dipartimento di Scienze degli Alimenti, Università di Udine, Udine — Fax +39-0432-501637

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 +39-02-70638625

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

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