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PAPER

VALIDATION OF ANALYTICAL METHODS FOR THE DETERMINATION OF IMIDOCARB IN TISSUES AND MILK OF CATTLE, SHEEP AND GOATS

VALIDAZIONE DI METODI ANALITICI PER LA DETERMINAZIONE DELL'IMIDOCARB IN TESSUTI E LATTE DI BOVINO, PECORA E CAPRA

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ABSTRACT

In accordance with the Guidelines on Validation of Analytical Procedures suggested by the European Agency for the Evaluation of Medicinal Products (EMEA), three methods were used to assess and validate the most suitable procedure to analyse residual imidocarb concentrations in edible tissues and milk of cows, sheep and goats. Based on the criteria selected to identify the most suitable analytical method, the best performances were obtained using a hexane/isoamyl alcohol liquid-liquid extraction procedure for sheep milk; after exposing the matrices to en-

RIASSUNTO

Per individuare la metodica analitica più idonea per lo studio delle concentrazioni residuali di imidocarb nei tessuti edibili e nel latte di bovino, pecora e capra, sono stati valutati e validati, in ottemperanza con le "Guidelines on Validation of Analytical Procedures" stabilite dalla European Agency for the Evaluation of Medicinal Products (EMEA), tre metodi analitici descritti in letteratura cui sono state apportate alcune modifiche. Sulla base di criteri stabiliti per selezionare il metodo analitico più idoneo, le migliori prestazioni sono state ottenute con un metodo d'estrazione

- Key words: HPLC, imidocarb, validated method -

zymatic digestion before extraction, the same method was used for the muscle, liver, kidney and fat of sheep and goats and for goat milk; and an acetone/chloroform liquid-liquid extraction method was used for all the matrices of cows. The selected methods fulfil the validation criteria recommended by the EU authorities. The total recovery ranged from 70.1% (sheep liver) to 90.8% (cattle muscle). The accuracy of the recoveries was always <30% and the precision of the different methods was always <15% for intra-assay precision and <16% for inter-assay precision. The results obtained show that different matrices and animal species have different extraction requirements, which stresses the importance of validating routine analytical methods for appropriate combinations of species and tissues.

liquido-liquido esano/alcool isoamilico per il latte di pecora, con la stessa estrazione previa esposizione delle matrici a digestione enzimatica per il muscolo, il fegato, il rene ed il grasso di pecora e di capra e per il latte di capra e con un metodo estrattivo liquido-liquido acetone/cloroformio per tutte le matrici di bovino. I metodi selezionati soddisfano pienamente i criteri di validazione raccomandati dalle autorità della Comunità Europea. I recuperi medi ottenuti sono compresi in un intervallo tra il 70.1% (fegato di pecora) ed il 90.8% (muscolo di bovino). L'accuratezza dei recuperi è risultata sempre <30% e la precisione dei differenti metodi per le diverse matrici sempre <15% per le valutazioni intra-assay e <16% per le inter-assay. I risultati ottenuti mostrano differenze sostanziali nelle esigenze estrattive per le diverse matrici studiate e sottolineano ulteriormente l'importanza di un'accurata validazione dei metodi analitici per ciascuna matrice e ciascuna specie animale.

INTRODUCTION

Imidocarb is a chemotherapeutic agent belonging to the family of carbanilide derivatives (3.3'-bis(2-imidazolin-2-yl)-carbanilide) having antiprotozoal activity. It is usually administered as the dipropionate salt and has been used for over 20 years in the treatment and prophylaxis of some protozoal diseases such as babesiosis and anaplasmosis in domestic animals, including food-producing species (cattle, horse, sheep and goat) (KUTTLER, 1975; 1980; WHO, 1999).

Although imidocarb has proved to be an effective babesiocide and anaplasmocide (BEVERIDGE, 1969; RODRIGUEZ and TREES 1996; BRASSEUR *et al.*, 1998), there are some concerns about administering imidocarb to food-producing species. The literature concerning the chemotherapeutic properties of the drug is not supported by adequate investigations on its pharmacokinetic and residual behaviour. The available data are incomplete, limited to some aspects or some animal species, and have been obtained without sufficiently reliable and validated methods (ALIU *et al.*, 1977; TARBIN and SHEARER, 1992; COLDHAM *et al.*, 1994, 1995; GUMMOW *et al.*, 1995).

It has been reported (ALIU *et al.*, 1977), however, that large amounts of imidocarb persist in the animal's body; this has engendered concern about its impact on food for human consumption. Its prolonged persistence may be ascribed to:

1) the resistance of the drug to biotransformation processes, as reported in *in vitro* studies on cattle (COLDHAM *et al.*, 1994, 1995) and *in vivo* studies on sheep (ALIU *et al.*, 1977);

2) the binding of the drug to nuclear components, causing the formation of large deposits (particularly in the liver and kidney), that release the molecule very slowly (COLDHAM *et al.*, 1994, 1995).

In 1998 the European Agency for the Evaluation of Medicinal Products (EMEA) published the conclusions and recommendations of the Committee for Veterinary Medicinal Products with the provisional Maximum Residue Limits (MRLs) (expiration 1.1.2002) (CVMP, 1998b) for imidocarb in the edible tissues of cattle and sheep (300 μg/kg for muscle, 50 μg/kg for fat, 2,000 μg/kg for liver, 1,500 μg/kg for kidney and 50 μg/kg for milk of both species). Various documents (CVMP, 1998b; WHO, 1999) have indicated that the analytical methods suggested in the literature appear to be suitable for regulatory use, but that additional investigations would be necessary to fully validate the routine analytical methods for appropriate combinations of species and tissue matrices.

For the purpose of such validation, a series of tests were performed on different matrices of sheep and cattle using the available analytical methods with some modifications. These investigations were also extended to the milk and edible tissues of goat. Although it is considered a "minor" food-producing species, these animals are commonly raised for human food in Italy and have a considerable economic importance especially for milk and dairy products.

The overall characteristics of the extraction and analytical procedures tested (i.e. recoveries, simplicity, rapidity and convenience of the procedures, as well as the cost of the assay and the possibility of avoiding the use of dangerous solvents) were considered in order to select the best method to analyse imidocarb concentrations in the various matrices of the different animal species. The suitability of the method selected for detecting the presence of the residual concentration of imidocarb in the matrices was then confirmed by evaluating the validation parameters, namely specificity, linearity, range, accuracy, precision, detection and quantification limit according to the Guidelines on Validation of Analytical Procedures suggested by the EMEA (CVMP, 1998a; 2001). The aims of this investigation were to provide data to characterise and validate the performance and suitability of the analytical methods used to measure imidocarb in edible tissues of cattle, sheep and goats and to provide a tool for further experimental studies on residue depletion in these important food-producing animal species.

MATERIALS AND METHODS

Chemicals

Acetonitrile and water, both of HPLC grade, acetone, chloroform, hexane and methanol were obtained from LAB-SCAN Analytical Sciences (Labscan limited, Dublin, Ireland); 1-pentansulfonic acid, sodium salt 98% HPLC grade and hydrochloric acid were purchased from ROMIL Ltd. (Cambridge, CB5 9QT, UK); triethylamine, glacial acetic acid, sodium hydroxide, sodium chloride and sodium hydrogen carbonate were obtained from BDH Laboratory Supplies (Poole, BH15 1TD, UK); subtilisin Carlsberg, protease Type VIII bacterial, phosphate buffered saline (PBS), pH 7.4, isoamyl alcohol and trifluoroacetic acid were obtained from Sigma Chemical Co. (St. Louis, Mo., USA); imidocarb dipropionate analytical standard was kindly supplied by Shering Plough-Animal Health (Verona, Italy).

All solvents and reagents were of analytical-reagent grade.

Analytical procedure

Standards

The imidocarb stock standard solution (1 mg/mL) was prepared from the imidocarb dipropionate reference standard

(batch n. 400196 – 96.1% as is/via base). Five milligrams were accurately weighed, dissolved in 5 mL of distilled water and stored at 4°C. An intermediate standard solution (100 µg/mL) was prepared by diluting 200 µL of stock standard solution to 2 mL with water. To assess the linearity between the reference standard concentrations and detector response, working standard solutions (10.0 – 5.0 – 2.5 – 1.0 – 0.5 – 0.25 – 0.1 – 0.05 – 0.025 µg/mL) were obtained from progressive dilutions. Further dilutions were prepared to determine the limit of detection of the method. Intermediate and working solutions were prepared daily.

HPLC system

The HPLC system (Beckman Instruments, Inc.) was equipped with Programmable Solvent Module mod. 126, Scanning Detector Module mod. 167 and MS-DOS Epson 386sx with System Gold 2000 software; Injection valve Rheodyne mod. 7725*i* with a 50 µL loop. Separation was achieved on a C_{18} RP column, ABZ+Plus 150x4.6 mm 5 µm with pre-column (Supelco Inc., Supelco Park, Bellefonte, PA).

High-performance liquid cromatography

The high performance liquid chromatographic conditions used were based on those described by GUMMOW *et al.* (1995) for the determination of diminazene in cattle plasma with imidocarb used as an internal standard, with slight modifications. The mobile phases were acetonitrile (phase A) and 0.005M 1-pentansulfonic acid sodium salt in water containing 0.1% triethylamine, adjusted to pH 3.2 with glacial acetic acid (phase B). The mobile phases were prepared daily, filtered through a 0.45 µm filter (phase filtration apparatus with membrane filters – nylon filter 66 – 0.45 µm x 47 mm – Supelco Inc., Supelco Park, Bellefonte, PA) and degassed under vacuum.

The analysis was performed at a flow rate of 1 mL/min in linear gradient elution: 0-2 min 10% A; 2-8 min 20% A; 8-12 min 20% A; 12-15 min 10% A. The UV detector was set at 250 nm.

Extraction procedures

Protocol

The stock standard solution (1 mg/mL) was prepared and stored as described previously. Intermediate (100 µg/mL) and working solutions were prepared daily. Drug-free homogenised samples (2 g) of each tissue, or 2 mL of milk, were spiked in triplicate at 3 different final concentrations of imidocarb dipropionate (1.0, 0.5, 0.25 µg/g or mL), stored for 12 h at 4°C and then processed with the appropriate extraction method. Such trials were repeated on three different days, in order to establish the intermediate precision of the method.

Procedures

Three previously described extraction methods were used with some modifications to assess and validate the most suitable procedure for the matrices investigated.

- Method described by ALIU *et al.* (1977).

The method is based on a liquid-liquid extraction procedure. Briefly, 2 g of homogenised tissue in 2 mL of distilled water (or 2 mL of milk) were made alkaline with 2 mL of 1M NaOH, added to 8 mL hexane/isoamyl alcohol (v/v 3:2), vortex-mixed for 1 min, ultrasonicated for 20 min at 40°C and centrifuged (4,500 g for 30 min). The organic phase was collected in a fresh tube, back-extracted with 1 mL of 1N HCl by vortex-mixing for 1 min and ultrasonicated for 20 min at 40°C and then centrifuged (4,500 g for 30 min). The aqueous phase was collected, filtered through a 0.45 µm syringe filter (Chromafil Einmalfilter type 0-45/15, 0.45 µm x 15 mm – Mackery-Nagel, Duren, Germany) and analysed by HPLC.

- Method described by COLDHAM *et al.* (1994; 1995).

Before the extraction, the sample (2 g of homogenised tissue in 2 mL of distilled water or 2 mL of milk) was enzymatically digested by adding 2 mg of subtilisin in 500 µL of PBS. The sample was vortex-mixed for 1 min and placed in a thermostatic bath at 56°C for 1 h, with mixing every 10 min. After digestion, the sample was made alkaline with 2 mL of 1M NaOH. Then, the extraction procedure previously described for ALIU *et al.* (1977) was performed.

- Method described by TARBIN and SHEARER (1992).

Some modifications were made to the last phases of the above procedures.

The homogenised tissue (2 g in 2 mL of distilled water), or 2 mL of milk, 1 mL of 1M aqueous sodium hydrogen carbonate and 5 mL acetone were vortex-mixed for 2 min, placed in an ultrasonic bath for 10 min at 40°C and centrifuged for 10 min at 3,000 g. The supernatant was collected in a fresh tube and extraction of the matrix was repeated. Chloroform (10 mL), a saturated aqueous solution of NaCl (4 mL) and 10M NaOH (0.4 mL) were added to the combined supernatants and vortex-mixed for 1 min, placed in an ultrasonic bath for 10 min at 40°C and centrifuged (3,000 g for 10 min). The subnatant was collected and 1M HCl (0.8 mL) was added; it was vortex-mixed for 1 min, placed in an ultrasonic bath for 10 min at 40°C and centrifuged.

The aqueous phase (about 1 mL) was collected, filtered through a 0.45 µm syringe filter (Chromafil Einmalfilter type 0-45/15, 0.45 µm x 15 mm) and analysed by HPLC.

Validation procedures

Validation of the analytical methods was performed in accordance with the Guidelines on Validation of Analytical Procedures suggested by the EMEA (CVMP, 1998a; 2001).

Linearity of the detector response was evaluated over the range of concentrations from 0.025 to 10 µg/mL (n=3 for each concentration) by calculating a regression line using the least square method. The runs test was applied to analyse the deviation of the actual data points from the regression line.

Specificity of the analytical method for each matrix was checked by comparing the chromatograms of blank and spiked samples after processing with the extraction procedure selected for the investigated matrix (n=3 for each matrix).

The recovery was assessed by comparing the measured concentration in the spiked samples after extraction with the known amount of drug added before the extraction. For each matrix, this is expressed as a percentage and reported as the mean ± SD of 9 replicates for each concentration tested (1.0, 0.5, 0.25 µg/g or mL). The mean recovery is expressed as the mean ± SD of 27 determinations (3 concentrations/9 replicates).

Moreover, to express the closeness of agreement between the known concentrations and the measured concentrations, the accuracy of the recovery was determined by taking the difference between the mean concentration found and the known added amount of imidocarb and then expressing it as a percentage. In the range of concentrations tested, the limit for the accuracy of recovery of the methods assayed was set at 30%.

The precision of the chromatographic method was expressed as the repeatability under the same operating conditions over a 1-day time interval (intra-assay precision) and as intermediate precision under intra-laboratory variations over a 3-day period (inter-assay precision). This was calculated as the relative standard deviation (coefficient of variation) of the mean of the values recorded after extraction (triplicate) for each matrix investigated and spiked at 3 different imidocarb concentrations over a 1-day (intra-assay precision) or 3-day

(inter-assay precision) period. In the range of concentrations tested, the limit for the precision of the methods assayed was set at 15% for intra-assay precision and 16% for inter-assay precision.

Data from the inter-assay evaluation were also used to test the linearity after extraction from spiked samples by calculating the regression line with the least square method. The runs test was performed to determine whether the data differed significantly from a straight line.

The Limit of Detection (LOD) was determined by establishing the minimum level at which the drug was detected reliably, by analysis of the standard solutions with known concentrations of imidocarb (based on visual evaluation).

The Limit of Quantification (LOQ) was validated at the level of the lowest MRL established for imidocarb (50 µg/kg in fat and milk) and at one-half and twice the MRL (CVMP, 1996). The LOQ was based on the lowest concentration used to construct the standard calibration curve (0.1, 0.05 and 0.025 µg/mL; 3 replicates/5 days) and is expressed as the mean of the measured amount of standard (n= 15) plus 3 times the standard deviation of the mean.

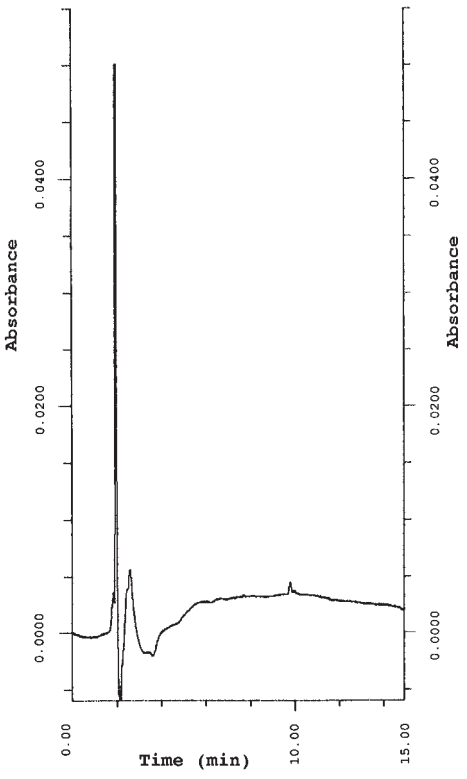


Fig. 1 - Chromatogram of the standard solution of imidocarb 0.005 µg/mL (LOD).

RESULTS

Imidocarb eluted with a retention time of 9.2±0.10 min. (n=27). The calibration curve of the detector response was linear (runs test; P>0.05) over the concentration range selected (0.025-10 µg/mL) with a regression line equation y = 4.34x + 0.042 and a correlation coefficient equal to 0.9997.

Under the experimental conditions, the LOD of the detector system was 0.005 µg/mL (Fig. 1) and the LOQ was 0.025 µg/mL. Considering the concentration factor obtained using the extraction procedures performed on 2 g (or 2 mL) matrices, the actual LOQ for imidocarb in all tissues and milk was 0.0125 µg/mL.

After a preliminary screening, based on the criteria we set for defining the best method to determine imidocarb in the different matrices analysed for the different animal species (i.e. recoveries, simplicity, rapidity and convenience of the procedures as well as cost of the assay and the possibility of avoiding the use of dangerous solvents), the method described by ALIU *et al.* (1977) proved to be suitable for obtaining a good performance for sheep milk only since the recoveries obtained for all the other matrices tested were inconsistent. The enzymatic digestion of the sample, as suggested by COLDHAM *et al.* (1994, 1995), improved the extraction potential of this hexane/isoamyl alcohol liquid-liquid

procedure, thus obtaining average recoveries that were always higher than 60% for the muscle, liver, kidney and fat of sheep and goats and for goat milk. This procedure, however, failed to give valuable extraction results for the muscle, liver, kidney, fat and milk of cows (recoveries were always inconsistent and less than 20%). For these matrices, the acetone/chloroform liquid-liquid method described by TARBIN and SHEARER (1992) appeared to be the most reliable procedure. Based on these preliminary observations, the method described by ALIU *et al.* (1977) was submitted to further validation for sheep milk, the method suggested by COLDHAM *et al.* (1994, 1995) was validated for muscle, liver, kidney and fat of sheep and for all the goat matrices investigated and the method described by TARBIN and SHEARER (1992) was validated for the cow matrices.

Imidocarb extracted from tissue eluted free of interference; typical blank and spiked sample chromatograms obtained from liver and milk, using the selected extraction conditions for each matrix, are shown in Figs. 2 and 3. The same figures also depict the specificity of the analytical procedures (lack of interference at the imidocarb retention time in the chromatograms of the blank samples) for the milk and liver of sheep, goats and cattle. The specificity was confirmed for all the matrices analysed.

The accuracy of the recoveries and the mean recovery obtained for each matrix, as well as the precision of the different methods expressed by the relative standard deviation (coefficient of variation) calculated over a 1-day (intra-assay precision) or a 3-day period (inter-assay precision) are reported for sheep matrices (Table 1), goat matrices (Table 2) and cow matrices (Table 3).

The linearity after extraction from spiked samples (runs test $P>0.05$) is reported in Table 4, where the equations for the regression analysis and correlation coefficients are reported for the tissues and milk of the three animal species.

DISCUSSION

The validation parameters obtained for the HPLC analytical procedure selected for the detection of imidocarb in food products of animal origin ensure that the method is suitable for regulatory use according to the MRLs for imidocarb in the edible tissues of cattle and sheep (CVMP, 1998b) and according to the guidelines on validation of analytical procedures suggested by the European authorities (CVMP, 1998a; 2001).

By introducing the mobile phase flow rate in linear gradient elution into the original method (GUMMOW *et al.*, 1995), a significant improvement in peak resolution was obtained. Moreover, the shift to 9.18 min of retention time *versus* 5.4 min described by GUMMOW *et al.* (1995) produced a much "cleaner-looking" chromatogram, ensured separation of imidocarb from possible co-extractives and allowed a relatively short run time which is suitable for practical purposes (routine analysis) since the run is done in 15 min.

Given that the aim of the present investigation was to validate analytical methods suitable for the regulatory evaluation of imidocarb in milk and edible animal tissue, the sensitivity of the method was not fully evaluated and the LOQ was calculated in compliance with the requirements for the LOQ/MRL ratio as established by the EU authorities (CVMP, 1996), at the lowest concentration on a standard curve including the MRL values and one-half and twice the MRL. This made it possible to obtain actual LOQs for imidocarb in all tissues and in milk (0.0125 µg/mL), ensuring good precision and detection of the drug at the MRLs set by the EU authorities (300 µg/kg for muscle, 50 µg/kg for fat, 2,000 µg/kg

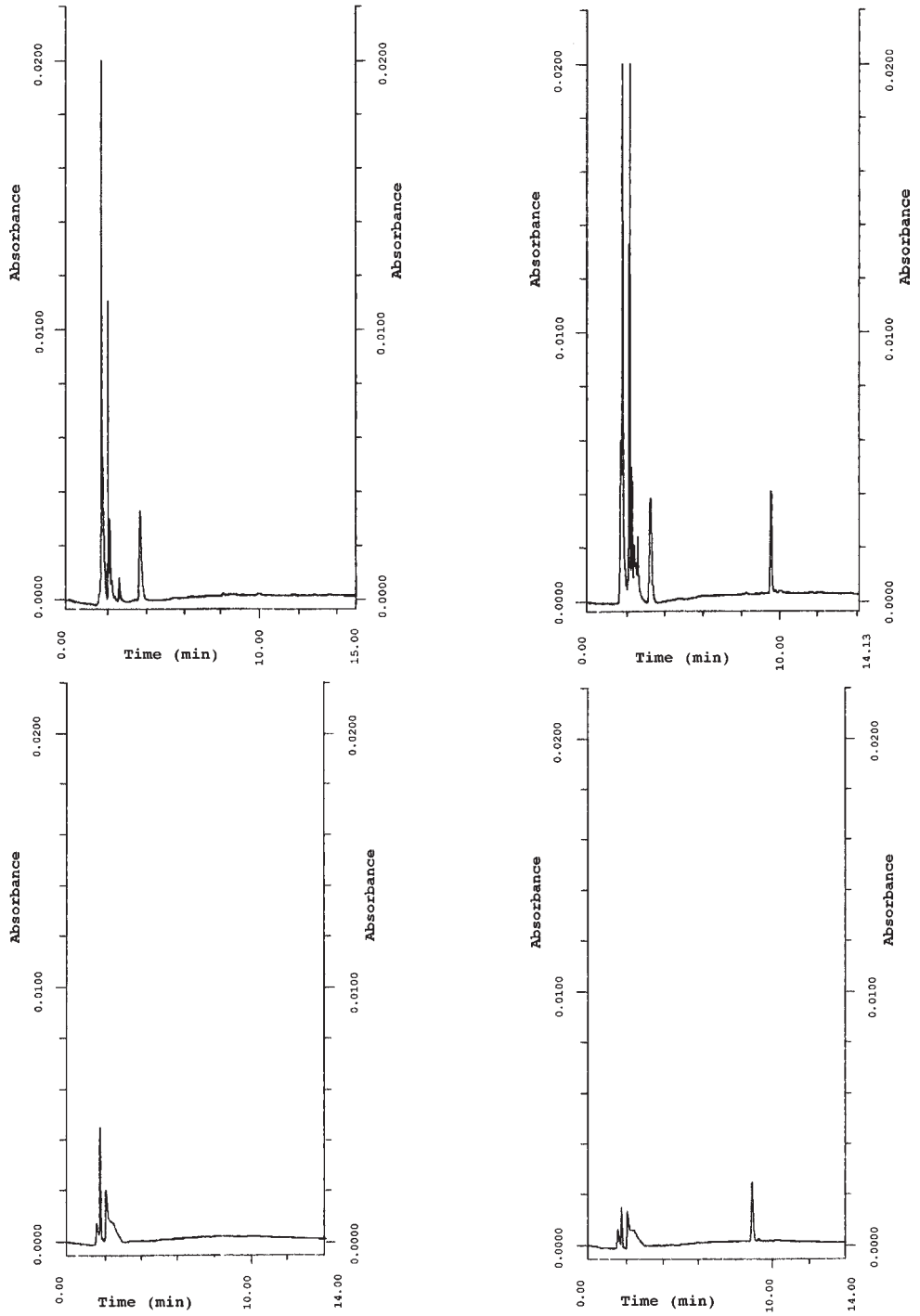


Fig. 2 - Chromatograms obtained from blanks and spiked (1 μg/g) samples of sheep liver (upper chromatograms) and milk (lower chromatograms).

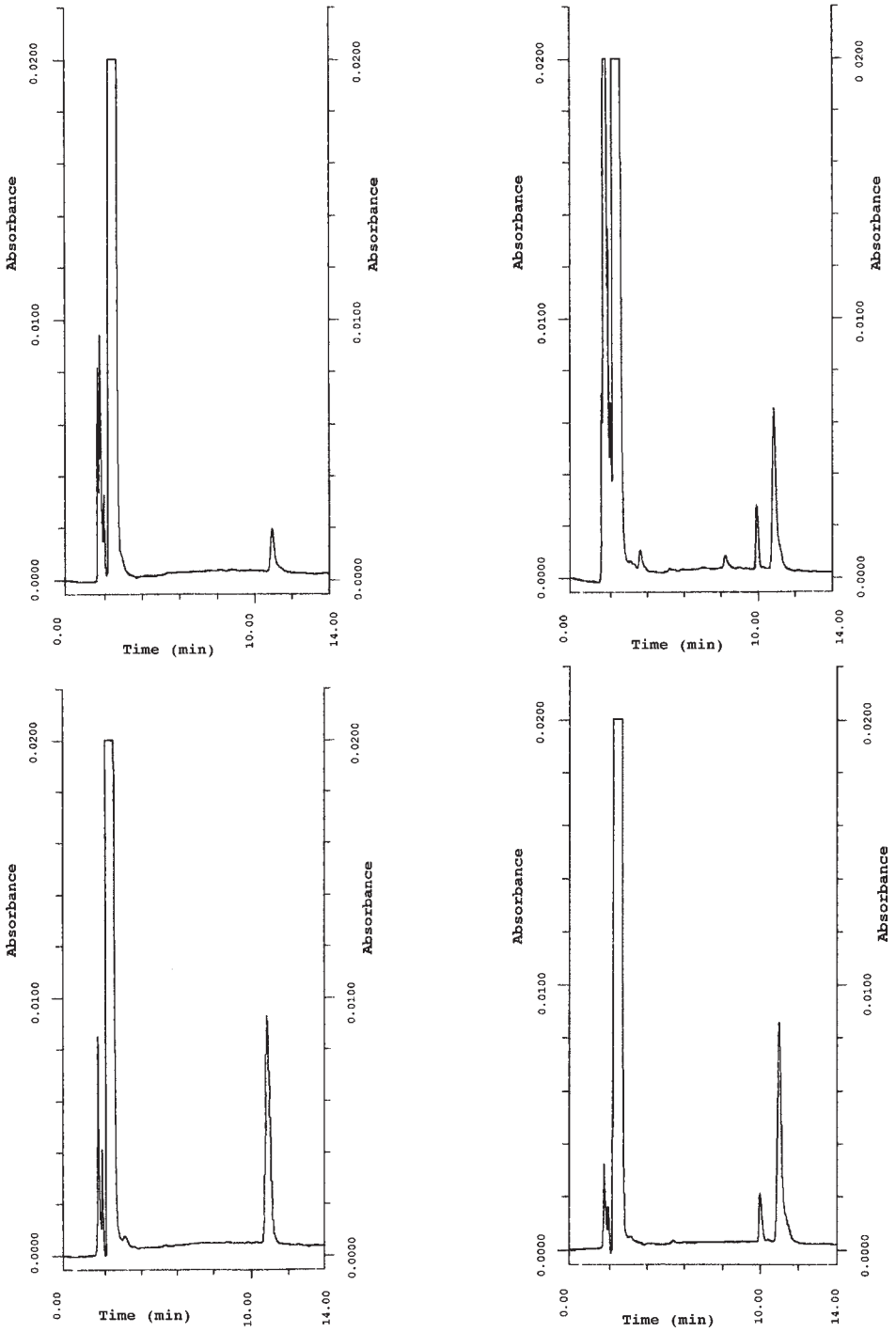


Fig. 3 - Chromatograms obtained from blanks and spiked (1 µg/g) samples of cow liver (upper chromatograms) and milk (lower chromatograms).

for liver, 1,500 µg/kg for kidney and 50 µg/kg for milk of cows and sheep).

To date, no MRL values have been established for meat and milk of a "minor species", like goat. However the EMEA has published guidelines whereby the MRL established for cattle (considered a major species) could be extrapolated to the other minor ruminant species such as goats (CVMP, 1997). Hence, the residual levels set for cattle and sheep can also apply to goats and the proposed

Table 1 - Recovery, accuracy and precision of imidocarb determination in sheep matrices spiked with different concentrations of the drug and extracted with the suitable method: liver, muscle, fat and kidney (COLDHAM et al., 1994; 1995), milk (ALIU et al., 1977).

Matrix	Fortification µg/g	Recovery ± s.d. % (n=9)	Mean recovery ± s.d. (n=27)	Accuracy %	Intra-day mean reading (n=3)	R.S.D. %	Inter-day reading (n=9)	R.S.D. %
liver	1	70.2±0.72	70.1±2.21	29.8	0.71	0.64	0.70	1.03
	0.5	69.7±3.17		30.3	0.35	9.56	0.35	4.55
	0.25	70.4±2.25		29.6	0.18	3.45	0.18	3.19
muscle	1	77.8±1.97	74.3±2.65	22.2	0.77	2.42	0.78	2.54
	0.5	76.8±2.51		23.2	0.39	2.30	0.38	3.27
	0.25	76.6±3.42		23.4	0.20	2.14	0.19	4.46
fat	1	90.7±1.84	84.91±3.76	9.3	0.92	2.73	0.91	2.03
	0.5	86.5±3.66		13.5	0.44	3.08	0.43	4.23
	0.25	87.0±4.17		13.0	0.22	4.21	0.22	4.79
kidney	1	88.5±1.20	83.8±2.33	11.5	0.89	0.86	0.88	1.36
	0.5	86.9±2.21		13.1	0.44	2.60	0.43	2.55
	0.25	85.4±2.48		14.6	0.22	4.23	0.21	2.91
milk	1	80.2±1.53	75.5±3.31	19.8	0.81	3.25	0.80	1.90
	0.5	77.4±3.69		22.6	0.38	2.27	0.39	4.77
	0.25	77.3±3.70		22.7	0.19	2.96	0.19	4.79

Table 2 - Recovery, accuracy and precision of imidocarb determination in goat matrices (liver, muscle, fat, kidney and milk) spiked with different concentrations of the drug and extracted with the suitable method (COLDHAM et al., 1994; 1995).

Matrix	Fortification µg/g	Recovery ± s.d. % (n=9)	Mean recovery ± s.d. (n=27)	Accuracy %	Intra-day mean reading (n=3)	R.S.D. %	Inter-day reading (n=9)	R.S.D. %
liver	1	71.3±0.76	71.2±2.68	28.7	0.72	0.64	0.71	1.07
	0.5	71.9±3.18		28.1	0.35	4.68	0.36	4.42
	0.25	70.5±3.42		29.5	0.18	4.22	0.18	4.85
muscle	1	76.7±1.80	72.5±2.82	23.3	0.77	2.68	0.77	2.34
	0.5	74.8±3.15		25.2	0.39	1.46	0.37	4.21
	0.25	74.0±2.92		26.0	0.19	4.01	0.19	3.94
fat	1	89.9±2.12	86.34±3.64	10.1	0.92	1.68	0.90	2.35
	0.5	88.6±3.63		11.4	0.45	3.54	0.44	4.09
	0.25	90.1±4.89		9.9	0.22	5.62	0.23	5.43
kidney	1	88.4±2.39	83.6±2.85	11.6	0.89	1.81	0.88	2.71
	0.5	85.7±2.93		14.3	0.44	2.05	0.43	3.42
	0.25	85.9±2.67		14.1	0.22	1.37	0.21	3.10
milk	1	64.7±2.59	59.6±3.83	35.3	0.62	1.45	0.65	4.01
	0.5	61.3±2.34		38.7	0.30	4.10	0.31	3.82
	0.25	59.6±4.53		40.4	0.15	7.85	0.15	7.60

method appears to be suitable for investigating residues in this species.

The findings of this investigation show remarkable differences in the extraction requirements for imidocarb among the matrices and animal species analysed. The strong retention of the drug in the liver (MOORE *et al.* 1996) was confirmed by the recoveries obtained from this matrix; the values were always lower than those of the other tissues but higher than those reported in the literature (COLDHAM *et al.*, 1994, 1995). Considering the good precision (less than 15%) of the methods used for the tissue analysed and the complexity of the matrix, accuracy of the recoveries is considered to be satisfactory.

As stated previously, the criteria for defining the most suitable method to be validated were based on the recoveries obtained in preliminary trials, as well as simplicity, rapidity, convenience, cost and safety of the procedures.

Based on these criteria, the best performances for sheep milk were obtained

Table 3 - Recovery, accuracy and precision of imidocarb determination in cow matrices (liver, muscle, fat, kidney and milk) spiked with different concentrations of the drug and extracted with the suitable method (TARBIN and SHEARER, 1992).

Matrix	Fortification µg/g	Recovery ± s.d. % (n=9)	Mean recovery ± s.d. (n=27)	Accuracy %	Intra-day mean reading (n=3)	R.S.D. %	Inter-day reading (n=9)	R.S.D. %
liver	1	75.0±8.56	76.2±8.86	25.0	0.75	4.55	0.75	11.41
	0.5	71.4±9.18		28.6	0.38	5.40	0.36	12.86
	0.25	82.1±5.49		17.9	0.21	4.82	0.21	6.69
muscle	1	89.6±10.6	90.8±7.79	10.4	0.96	9.61	0.90	11.86
	0.5	88.9±6.10		11.1	0.43	2.23	0.44	6.85
	0.25	93.7±5.7		6.3	0.23	5.75	0.23	6.08
fat	1	93.1±4.56	90.1±7.18	6.9	0.93	5.71	0.93	4.90
	0.5	91.2±6.77		8.8	0.43	13.26	0.46	7.42
	0.25	86.0±8.41		14.0	0.19	10.58	0.22	9.77
kidney	1	86.4±7.30	87.9±7.82	13.4	0.88	8.21	0.86	8.44
	0.5	84.6±8.43		15.4	0.42	12.92	0.42	9.97
	0.25	92.7±5.76		7.3	0.23	4.23	0.23	6.21
milk	1	85.7±7.50	84.8±6.78	14.3	0.83	14.62	0.86	8.75
	0.5	82.4±4.38		17.6	0.42	4.20	0.41	5.32
	0.25	86.3±8.02		13.7	0.22	12.95	0.22	9.29

Table 4 - Linearity parameters of the selected methods after extraction of imidocarb in sheep, goat and cow tissues and milk (samples spiked with 1.0, 0.5, 0.25 mg/mL imidocarb dipropionate). Runs test >0.05.

Tissue	Sheep Regression line equation	Correlation coefficient	Goat Regression line equation	Correlation coefficient	Cow Regression line equation	Correlation coefficient
Liver	y = 0.702x - 0.0002	0.9999	y = 0.711x + 0.0004	0.9999	y = 0.741x + 0.006	0.9978
Muscle	y = 0.778x - 0.002	0.9999	y = 0.768x - 0.0046	0.9998	y = 0.893x - 0.0008	0.9998
Fat	y = 0.908x - 0.0078	0.9993	y = 0.898x - 0.0012	0.9999	y = 0.935x - 0.0062	0.9998
Kidney	y = 0.887x - 0.0044	0.9999	y = 0.884x - 0.0059	0.9997	y = 0.857x - 0.0046	0.9993
Milk	y = 0.803x - 0.0058	0.9989	y = 0.649x - 0.0086	0.9989	y = 0.853x - 0.0014	0.9989

with the method described by ALIU *et al.* (1977), for the muscle, liver, kidney and fat of sheep and goats and for goat milk with the method described by COLDHAM *et al.* (1994, 1995) and for the muscle, liver, kidney, fat and milk of cows with the method described by TARBIN *et al.* (1992).

The method described by ALIU *et al.* (1977) features all the required characteristics and thus is suitable for the determination of imidocarb in sheep milk, as demonstrated by the precision and accuracy of the recovery values obtained in this study. The method was, however, totally inadequate for extracting imidocarb from tissues; this is probably due to the strong bonding to the cellular components, as described by MOORE *et al.* (1996). A similar explanation can be suggested for the unsatisfactory extraction performances when applied to cow and goat milk, which are characterised by quantitatively different cellular components (RUCKEBUSCH *et al.*, 1991; SCHODER *et al.*, 1993).

The accuracy of the extraction of imidocarb from the tissue of sheep and goats and from goat milk was improved by first enzymatically digesting the samples, as suggested by COLDHAM *et al.* (1994,1995); satisfactory parameters were obtained with a single extraction instead of the two suggested by COLDHAM *et al.* (1994, 1995), thus making the procedure faster and more cost-effective. Even if the accuracy of the recovery values calculated for goat milk were higher than the set limit (30%), the good precision and linearity of the method may ensure good analytical results. This procedure may therefore be preferred over other more complicated and more expensive methods.

The enzymatic breakdown of imidocarb bonds from the cellular components was not enough to extract the drug from the cow tissues and milk. For these matrices, some of the conditions suggested by TARBIN and SHEARER (1992) were used (clean-up with acetone in the presence of sodium carbonate and chloroform), but the modified purification procedure that was followed, resulted in a faster, easier and less expensive method than the original one. Moreover, good recovery, precision and linearity of the methods were obtained starting with less tissue (about 1/5), requiring the use of less solvent, mainly of acetone and chlorinated compounds, further curbing costs and enhancing safety.

Although data from inter-laboratory trials are also necessary for a conclusive validation, the results obtained are in good agreement with the EU validation criteria (CVMP, 1998a; 2001) and, regardless of the tissue and animal species analysed, a substantial number of samples could be processed per day with all the extraction procedures.

While no hypothesis may be put forth to explain the differences in the extraction steps required for the different tissues or, for the same matrix, among the different species, the results clearly indicate the importance of fully validating routine analytical methods for appropriate combinations of species and tissue matrices.

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PAPER

CHEMILUMINESCENT DETERMINATION OF ANTIOXIDANT CAPACITY OF BEVERAGES

DETERMINAZIONE CHEMILUMINESCENTE DELLA CAPACITÀ ANTIOSSIDANTE IN BEVANDE

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ABSTRACT

The antioxidant capacity of various beverages was determined via chemiluminescence in a rapid and sensitive manner. The method is based on the inhibition of light generated by a radical intermediate of the oxidation of luminol and catalyzed by horseradish peroxidase. The beers and wines analyzed showed that the antioxidant capacity may be related to the total polyphenol content. A low quantity of sample, short measuring times and the rapid preparation of samples are the numerous advantages of this method. Repeatability and reproducibility, ex-

RIASSUNTO

La capacità antiossidante di diverse bevande può essere determinata in un modo rapido e sensibile tramite la chemiluminescenza. Il metodo è basato sull'inibizione della luce generata da un intermedio radicalico della reazione di ossidazione del luminolo e catalizzata dalla perossidasi da rafano. Le birre e i vini analizzati hanno mostrato che la capacità antiossidante può essere messa in relazione al contenuto totale di polifenoli. Una piccola quantità di campione, tempi di misura brevi e la rapida preparazione dei campioni sono tra i vantaggi di questo metodo. L'impreci-

- Key words: antioxidant capacity, beer, chemiluminescence, luminol, tea, wine -

pressed as coefficient of variation, were normally less than 10%.

sione (ripetibilità e riproducibilità) espressa come coefficiente di variazione è normalmente inferiore al 10%.

INTRODUCTION

Substances which perform an antioxidant action are of increasing clinical and nutritional interest (SIMONETTI *et al.*, 1997), since they are capable of neutralizing the harmful effects of free radicals, that generally have a very high chemical reactivity (DUTHIE, 1999; ROBERFROID and CALDERON, 1995).

At the biological level, the mechanisms that are quantitatively most important in triggering and maintaining radicalized reactions are the monoelectronic reduction of oxygen and lipid peroxidation, mostly involving O_2, H_2O_2 and polysaturated fatty acid molecules (DUTHIE, 1999).

The life of free radicals is extremely short, and if they are not immediately neutralized by a physiological accepter, they can damage biological systems to such an extent that a chain reaction is set off, which may even cause a variety of pathological conditions (GHISELLI *et al.*, 2000A; GERMAN and WALZEM, 2000).

All aerobic organisms have developed more or less complex systems to neutralize free radicals before their potentially harmful effect is activated, most of which derive directly or indirectly from O_2. Nutritional elements are also extremely important (ROBERFROID and CALDERON, 1995).

Foods that have potential or definite antioxidant capacities are mainly vegetables and fruits, as well as beverages like red wine, tea and beer. Wine is well appreciated because of its organoleptic qualities and is also a source of antioxidant substances such as flavonoids (anthocyanins, flavonols, catechins and leucoanthocynanins) and resveratrol (NATELLA *et al.*, 2001; GERMAN and WALZEM, 2000). The beneficial effects of tea derive primarily from ingredients such as antioxidant substances (polyphenols or flavonoids), nutritional compounds and xanthine (mainly caffeine) (DUFFY *et al.*, 2001).

Beer contains tannins, which are antioxidants, as well as other antioxidants such as carotenoids and tocopherols (GOUPY *et al.*, 1999) capable of reducing the oxidization of low density lipoproteins (LDL) and neutralizing the "pro-oxidizing" effects of ethanol (GERMAN and WALZEM, 2000). The polyphenols found in beer include phenolic acids, whose antioxidant activities are greater when these substances are present in a bound form (MAILLARD and BERSET, 1995). The polyphenol content of beer varies according to the barley cultivar used and tends to decrease (ca. 30%) during the malting process (YADAV *et al.*, 2000). Hops also contribute to the antioxidant activity in beer, particularly due to the polyphenols, which are about 2-5%.

It has been reported that the consumption of food and beverages rich in flavonoids, and in particular polyphenols may increase the antioxidant capacity of human serum (WHITEHEAD *et al.*, 1992; GERMAN and WALZEM, 2000; GHISELLI *et al.*, 2000B). Black tea has considerable protective effects against oxidation of human red blood cells and the peroxidation of membrane lipids *in vitro* (HALDER and BHADURI, 1998). Moderate consumption of beer by patients with coronary illnesses tends to increase plasma levels of high-density lipoproteins (HDL) and tocopherol (GORINSTEIN *et al.*, 1997). This is controversial, however, because other studies have not shown any effect

of these beverages on plasma antioxidant capacity and LDL oxidizability (DE VRIES *et al.*, 2001; VISSERS *et al.*, 2001) and actually the real effect of these substances in vivo is yet to be established. There is no statistical evidence that coronary heart disease is reduced in areas of high red wine consumption, but wine has been shown to reduce endothelin-1 synthesis and this may help prevent coronary heart disease (CORDER *et al.*, 2001).

Meanwhile, there is growing interest in substances with antioxidant capacities, and numerous methods for determining antioxidant capacity have been developed and compared (PRIOR and CAO, 1999; DUTHIE, 1999; BENZIE and SZETO, 1999). The aim of this study was to optimize and apply a chemiluminescent (CL) method (WHITEHEAD *et al.*, 1992) to determine the antioxidant activity in various beverages.

MATERIALS AND METHODS

Reagents

Luminol (5-amino-2,3-dihydrophthalazine-1,4-dione) and horseradish peroxidase (HRP, E.C. 1.11.1.7, Grade II) were obtained from Boehringer Mannheim (Germany), and hydrogen peroxide (30%) was from Merck (Milan, Italy). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, 98% pure), a water soluble vitamin E analogue, from Fluka (Milan, Italy) was used as the reference antioxidant. All other reagents and compounds were of analytical-reagent grade. All of the solutions were prepared with pyrogen-free reagent-grade water using a Mill-Q system (Millipore, Milan, Italy).

Samples

Red wine samples were diluted 1:1,000 in 0.1 M potassium phosphate buffer (pH 7.4) and stored in the dark for about 10 min before analysis.

Tea samples were prepared directly using teabags or by weighing 1.5 g of tea (loose, the equivalent of one teabag) and pouring in 100 mL of distilled water, which had been brought to boiling. Part of the solution was filtered after 3, 5, 7 and 10 min to allow complexation of the caffeine with the tannins. It was then cooled and diluted 1:1,000 with 0.1 M potassium phosphate buffer (pH 7.4), before analysis.

The samples of sweetened tea were prepared in the same way as the tea for the calibration curves, with the addition of 1 teaspoonful of sugar (4 g), 2 teaspoonfuls (8 g) and 3 teaspoonfuls of sugar (12 g) to 150 mL of tea.

Beer was diluted 1:100 with 0.1 M potassium phosphate buffer (pH 7.4) without de-aeration.

Instrumentation

An LKB-Wallac 1250 luminometer (Turku, Finland) was used to measure light emission. It used a photomultiplier tube as detector, which converted the light produced by the sample into an electric signal. The signal was amplified and then displayed on paper by means of an LKB 2210 potentiometric recorder, which converted it into mV.

Chemiluminescent assay

The CL mixture was prepared at the time of analysis by mixing 3 mL of 10 mM hydrogen peroxide solution with 50 µL of 2 mM luminol solution, and then 200 µL of the mixture was supplemented with 20 µL of the peroxidase solution, obtained by diluting the stock solution (1 mg/mL) in a suitable manner (normally 1:7) in 0.1 M potassium phosphate buffer (pH 7.4) (GIROTTI *et al.*, 2002).

The peroxidase catalyzed the oxidation of the luminol. This mixture constituted the reference system, representing 100% of the light emission in the absence of

inhibition by the sample or standard antioxidant solutions.

To evaluate the total antioxidant capacity of the samples examined, 10 µL of the sample or the standard solutions were injected into the cuvette once the emission had reached a maximum. By recording the data on paper, the kinetics of the emission could be followed to detect the time when the reaction was at a maximum. As an analytical parameter, the time required to reach 30% of the initial light emission was measured, and the antioxidant capacity was expressed by comparing the inhibition time of the samples with those of the various points on the calibration curve, expressed as mM of Trolox in the beverage.

Calibration curves

A stock solution of 10 µM Trolox (2.5 mg/L) was diluted in 0.1 M potassium phosphate buffer (pH 7.4), and the calibration curve was drawn in the interval of 1-10 µM. In the case of wine, oenocyanine (E-163, natural coloring agent), an extract of *Vitis vinifera* (Enocianina Fornaciari, Italy), made of a mixture of grape anthocyanins, was also used as a reference standard, by diluting an aqueous solution of 60 mg/L oenocyanine to concentrations between 3 and 30 mg/L.

Standard solutions for the calibration curve of sweetened tea were made by adding 4, 8 and 12 g of sugar to three containers each containing 150 mL of black tea infusion.

Polyphenol analysis

The method was based on the reaction of the Folin Ciocalteau reagent with the phenol OH groups in an alkaline medium by adding sodium carbonate (SINGLETON and ROSSI, 1965). Measurements were made after two hours by reading the absorbance at 750 nm on a SHIMADZU PC 1204 spectrophotometer (Shimadzu, Kyoto, Japan) and calculating the concentration (g/L) according to a calibration curve using gallic acid as standard in the range 12.5-500 mg/L.

RESULTS AND DISCUSSION

The CL method suggested by WHITEHEAD *et al.* (1992) was modified and optimized for the various matrices analysed, with the aim of obtaining a method which was simple to perform, widely applicable and reliable, and which also used stable, low-cost and easily handled reagents.

Light emission occurs when luminol, the CL substrate, is oxidized by hydrogen peroxide in a catalytic reaction with HRP:

$$\text{LUMINOL} \xrightarrow[\text{Catalyst (HRP)}]{H_2O_2/OH^-} \text{PHTALATE ION} + N_2 + H_2O + \text{light (425 nm)}$$

Continuous light emission depends on the constant production of free radical intermediates derived from luminol and oxygen. For this reason light emission is sensitive to interference by radical scavenging (chain-breaking) antioxidants but will be restored when all the added antioxidants have been consumed in the reaction. If the generation of radical intermediates is constant, then the length of time of light suppression will be directly related to the amount of antioxidant present (WHITEHEAD *et al.*, 1992).

Reagents for CL determination were highly stable, could be kept for months and used for numerous experiments, apart from the hydrogen peroxide solu-

tion, which had to be prepared before the analysis, and was stable for 5-6 hours.

Evaluation of antioxidant capacity of tea samples

Various varieties of teas were prepared for infusion, and their antioxidant capacity was determined after infusion for 3, 5, 7 and 10 min. The results (not shown) showed that the antioxidant capacity generally remained constant with regard to infusion time, although maximum antioxidant activity took place after 5 min, which is in agreement with the literature (LIEBERT *et al.*, 1999).

The antioxidant capacity of tea in teabags was lower (ca. 10-30%) than that of loose leaf tea, probably because the bag material inhibited the extraction of those substances into the solution (LANGLEY-EVANS, 2000).

The antioxidant capacity of loose leaf teas corresponded to a concentration of Trolox of around 4.2±0.2 mM (standard deviation, n = 3) for green (non-fermented) tea, and 3.4±0.2 mM for black (fermented) tea, with the exception of Twinings black tea, which had a higher antioxidant capacity than other teas (around 5.1±0.3 mM) probably due to the presence of lemon as a flavouring (BENZIE and SZETO, 1999).

As well as the tea preparations made in the laboratory, various commercial bottles of flavoured tea samples were also analysed by the CL method (Fig. 1). The commercial samples had much lower values than the teas prepared by infusion. This could be due to the fact that the industrial extraction process only extracted some of the water soluble polyphenols contained in the tea (YOKOZAWA *et al.*, 2000).

In addition, various soluble teas in sachets prepared both with natural mineral water and distilled water (with dif-

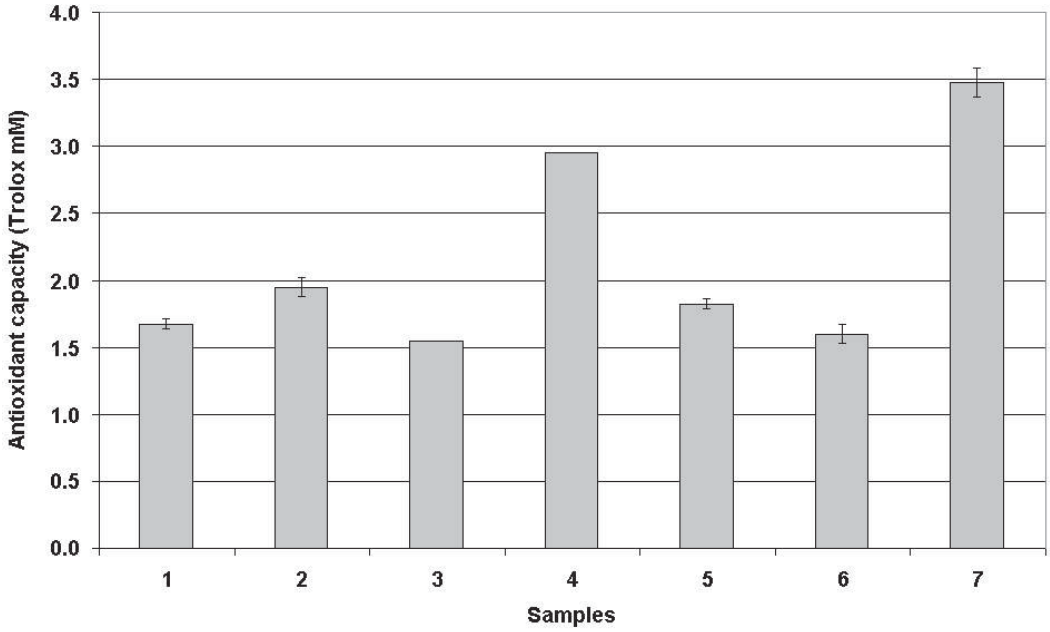


Fig. 1 - Antioxidant capacity of different commercial flavoured teas determined using the CL method. 1 - Lipton lemon; 2 - Lipton peach; 3 - San Benedetto theineless; 4 - Estathè lemon; 5 - Beltè lemon; 6 - Beltè peach; 7 - Estathè peach. Bars represent standard deviation (n=3).

ferent ion content) were analyzed. No difference in the antioxidant capacity was detected with regard to the water used, and it was therefore concluded that the ions dissolved in the water do not interfere with the analysis. As with bottled tea, the antioxidant capacity of soluble teas in sachets was far lower (between 1.4 and 1.6 mM Trolox), compared with tea infusions, which reached values higher than 5 mM of Trolox, as in the case of Twinings.

The antioxidant activity of black tea infusion was evaluated after 5 min, after adding increasing amounts of sugar (saccarose), as this substance could hinder the inhibition of CL, and therefore reduce the apparent antioxidant capacity. The addition of various amounts of sugar, equivalent to 1, 2 or 3 teaspoonfuls, reduced the antioxidant capacity, and the greater the amount of sugar added, the more was the antioxidant capacity reduced (Fig. 2). The length of time between the injection of the sample and the return of the light signal to 30% of the initial value decreased, going from 0 to 4, 8 and 12 g of sugar added. For the 12 g addition it was impossible to detect any antioxidant capacity, because it was not possible to measure the light inhibition.

The addition of an aspartame-based sweetener to the same amount of tea also reduced the antioxidant activity to a similar degree as adding one teaspoonful of sugar. The inhibition was similar to the 4 g addition (Fig. 2, thin continuous line).

Evaluation of antioxidant capacity in beer samples

Despite the high specificity and sensitivity of the chromatographic techniques normally used for the determination of total polyphenols in a variety

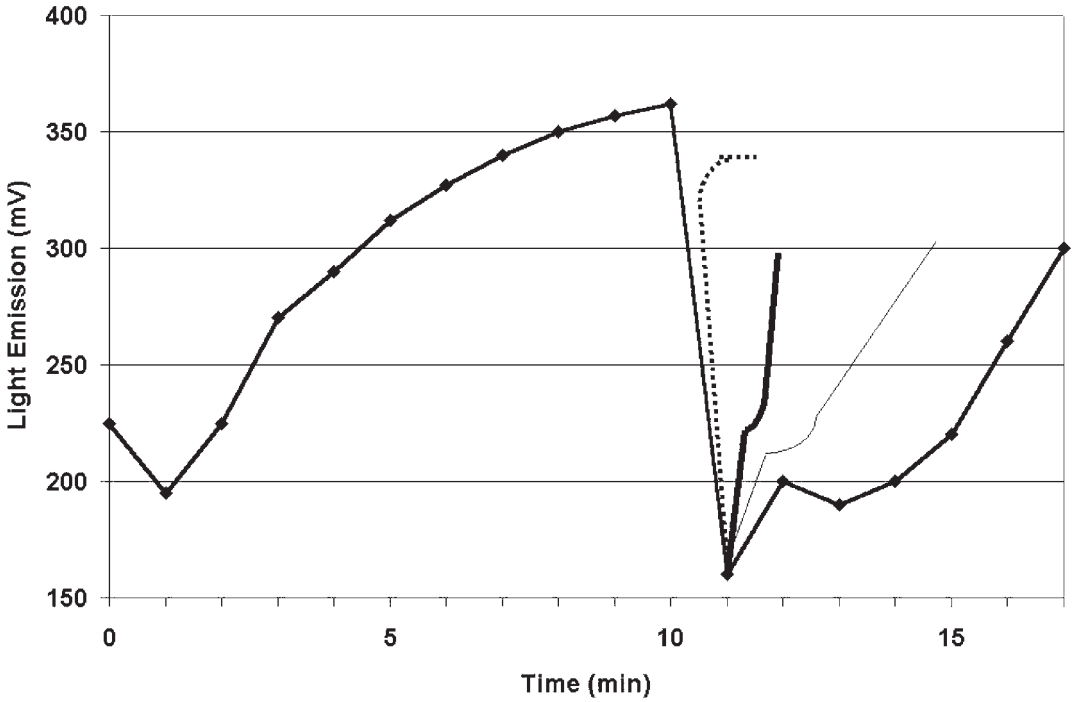


Fig. 2 - Comparative inhibition curves of tea solutions containing 4 g (——), 8 g (▬▬), 12 g (·····) of sugar and the unsugared tea solution (♦♦♦) using the CL method.

of matrices [GOUPY *et al.*, 1999], the CL method has the advantage of being simple, rapid and easy to apply to evaluate the antioxidant capacity of beer. The sample could be analysed directly, without removing gas (it was simply diluted) and without requiring specific temperature, pH, or other conditions, such as HPLC.

Fig. 3 shows the antioxidant capacity of various types of beers. These values were in accordance with the polyphenol content ($y = 0.0001x - 0.017$, $r = 0.815$, y = CL method and x = polyphenol content, $p<0.01$) derived from the raw materials (malt, maize, hops) and in particular from the different degrees of barley toasting.

As shown in Fig. 3, the antioxidant capacity was far higher in Weiss and Guinness, an almost black dark full-bodied beer having an aromatic very bitter taste, which was produced by the addition of caramel with a low alcohol content (4°-5°). In comparison to light beers, the higher antioxidant capacity of dark beer could be due to the greater number of Maillard reaction products and the greater amount of hops added (CARNOVALE, 1996). In the case of Weiss, a different base raw material (grain as opposed to barley) is used and the chemical and physical stabilization treatments are milder, therefore allowing substances with antioxidant activity to remain.

Evaluation of antioxidant capacity in wine samples

Red wine is an important food in terms of containing certain types of radical "scavenger" molecules. The correlation between the polyphenol content in wine and the CL antioxidant capacity has been previously demonstrated, $r = 0.8525$, $p<0.01$ (GIROTTI *et al.*, 2002).

To confirm the reliability of the CL method, various types of red wines were

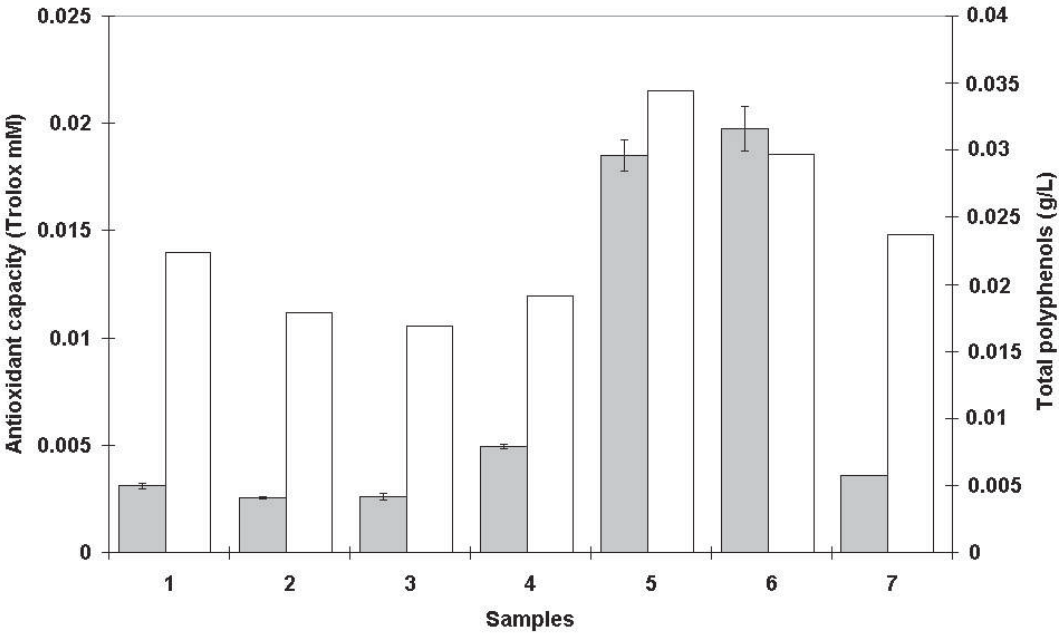


Fig. 3 - Antioxidant capacity of different beer samples determined using the CL method (gray bar) with varying polyphenol contents (white bar). 1 - Peroni; 2 - Moretti; 3 - Dreher; 4 - Beck's; 5 - Guinness extra; 6 - Weiss; 7 - Adelscott. Bars represent standard deviation (n=3).

analyzed. The antioxidant capacity of the various wines depended directly on the polyphenol content. In this respect, it was interesting to look at the differences between the various cultivars, the area of production and the winemaking technique used with regard to the same cultivar. In particular, the antioxidant capacity was lower in the "novello" wine (100% carbonic maceration) compared to the Sangiovese wines produced in the traditional manner from the same red grapes (Fig. 4). In this case too, the preparation of the wine samples was very simple, and only required dilution.

To detect the possible antioxidant effect caused by the ethanol contained in the various wine samples, ethanol solutions were prepared in buffers with the same degree of alcohol (12%) as the wines analyzed. The inhibition of the signal from such solutions was negligible (Fig. 5). White wines as references were also analyzed under the same conditions (Fig. 5). The inhibition effect was noticeably different: the signal did not fall to values close to zero after injection as with the red wine samples, and in addition it rose again very rapidly, though it did not regain the maximum emission level. The calculation of the antioxidant capacity for white wine must be slightly modified, as the signal was not inhibited up to values below 30% of the initial value. Therefore, it was necessary to use another parameter (70% of inhibition) to calculate the possible antioxidant capacity of white wine, which was, however, less significant than that of red wine.

The change in antioxidant capacity during aging of Sangiovese wine in oak barrels is shown in Fig. 6. The antioxidant capacity determined by the CL method was compared with the total

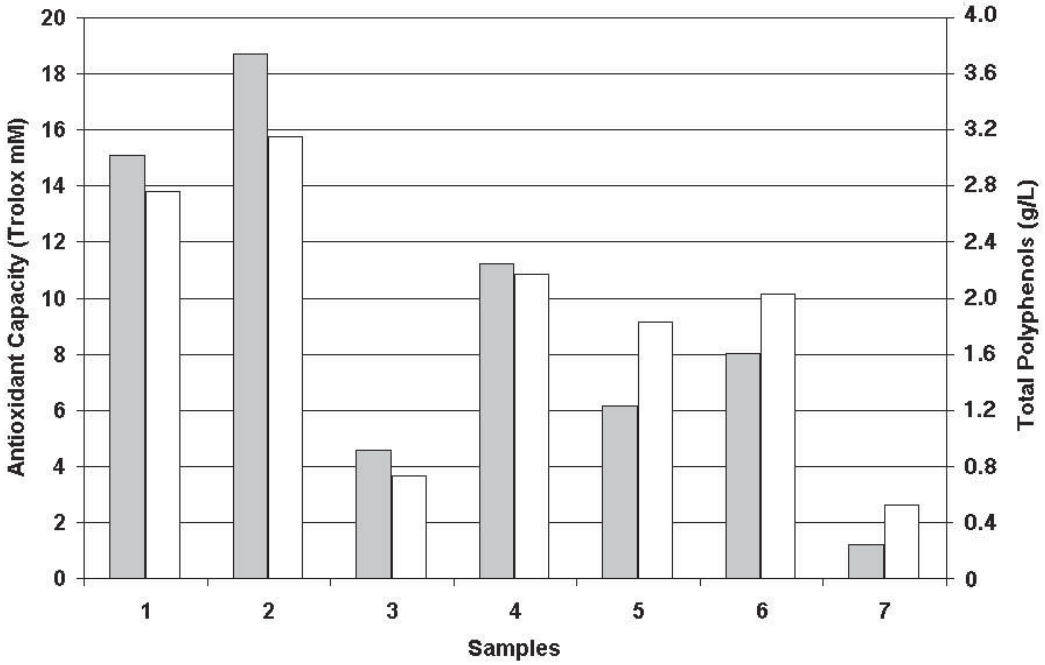


Fig. 4 - Antioxidant capacity of different wine samples determined using the CL method (gray bar) with varying polyphenol contents (white bar). 1 = Cabernet sauvignon; 2 = Montepulciano; 3 = Lambrusco di Sorbara; 4 = Sangiovese A; 5 = Sangiovese B: 6 = Sangiovese C; 7 = Sangiovese "novello". Bars represent standard deviation (n=3).

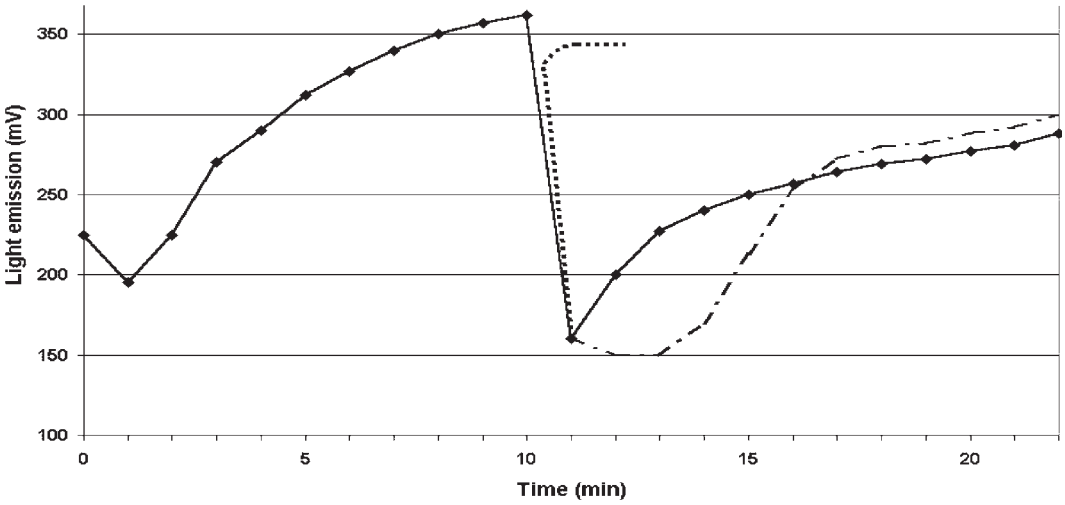


Fig. 5 - Comparative inhibition curves of a 12% ethanol solution (·····), white wine (♦-♦-♦) and red wine (—•—•—) using the CL method.

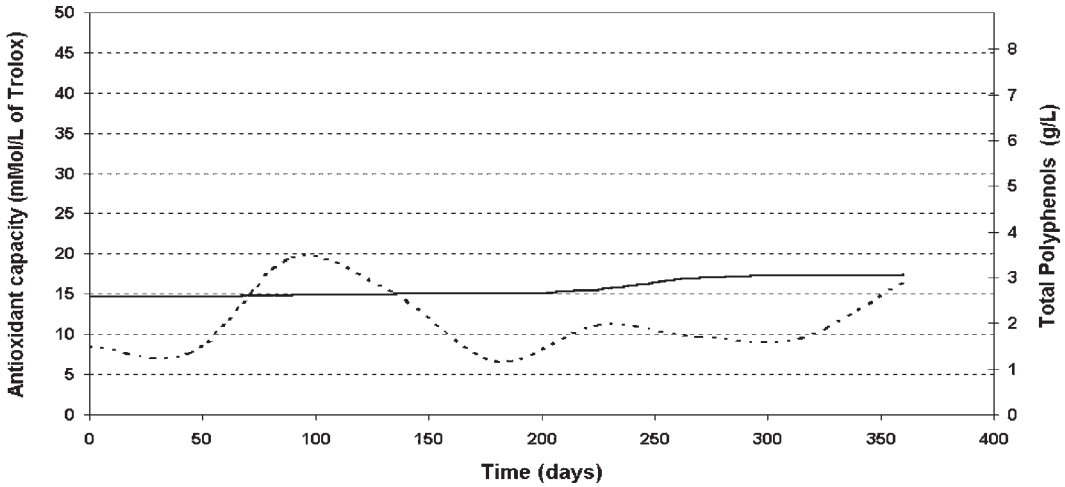


Fig. 6 - Antioxidant capacity of Sangiovese wine determined using the CL method (- - - - - - -) and its total polyphenol content (——) during affination in oak casks.

polyphenol content. The antioxidant capacity was not linear compared with that of total polyphenols, particularly between 50 to 150 days of storage, while the general increase of the antioxidant capacity with time is in accordance with the limited but constant increase in polyphenol content.

CONCLUSIONS

The CL method is suitable for determining the antioxidant capacity of beverages, and offers numerous advantages over chromatographic methods. It is simple, only requiring dilution of the sample, fast with a good detection limit (1 μM of Trolox), rel-

atively inexpensive (about 0.31 Euro per analysis) and reagents are stable over time, without the need for excessively costly instruments, or instruments which require a high degree of handling. Repeatability and reproducibility, expressed as a coefficient of variation, were normally less than 10%. Therefore, the CL system could be readily applied to study tea, beer and wine, also evaluating different production conditions, like the effect of storage and type of barrel on the antioxidant capacity of red wine.

ACKNOWLEDGEMENTS

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PAPER

BREAD-MAKING QUALITY OF ITALIAN DURUM WHEAT (*TRITICUM DURUM* DESF.) CULTIVARS

QUALITÀ PANIFICATORIA DI VARIETÀ ITALIANE DI FRUMENTO DURO

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ABSTRACT

The bread-making characteristics of 23 Italian durum wheat cultivars grown in Sicily in 1998-1999 are reported. Protein content, farinograph curves, alveographic parameters, glutenin composition and semolina colour were determined and an experimental bread-making test was carried out. Environmental conditions strongly affected the qualitative characteristics of the varieties. All the technological evaluations showed a wide range of variation also among genotypes in all environments. Loaf volume was correlated with alveographic P/L. The new cultivars Colora-

RIASSUNTO

Vengono riportati i risultati delle analisi relative alla qualità panificatoria, condotte su 23 varietà di grano duro coltivate in Sicilia nel biennio 1997/98-1998/99. Sui campioni di semola sono stati determinati il contenuto proteico, i parametri farinografici ed alveografici nonché il colore della semola; è stato inoltre effettuato il test di panificazione sperimentale. Le condizioni ambientali hanno influenzato sensibilmente le caratteristiche qualitative delle varietà in prova. I risultati delle valutazioni chimiche e tecnologiche hanno mostrato in tutti gli ambienti un'ampia varia-

- Key words: Bread-making, durum wheat, new cultivars, quality -

do, Mongibello, Varano and Svevo are well suited for bread-making and are characterized by the "7+8" or "6+8" HMW glutenins.

bilità anche fra i genotipi. Il volume del pane è risultato correlato con il P/L alveografico. Le nuove cultivar Colorado, Mongibello, Varano e Svevo sono emerse per elevata qualità panificatoria. Queste varietà sono caratterizzate dalla presenza delle glutenine APM "7+8" o "6+8".

INTRODUCTION

Early baking studies showed that durum wheat flours were unsuitable for bread-making (JAGO and JAGO, 1921; GERHARD, 1925; WIHLFAHRT, 1928). However, SHEPARD (1903) showed that many people preferred bread made from durum wheat to ordinary bread, due to its palatable and nutritional qualities. GEDDES (1932) reported that bread made from durum wheat remained fresh for a longer time than bread made from common wheat flours. He also indicated that durum wheat was used extensively for bread-making in Russia.

KALTSIKES *et al.* (1968) produced a durum wheat genotype with high bread-making quality by crossing a durum wheat variety with a common bread wheat variety. AMENDOLA and LUNDBERG (1970) reported that durum flour was used for French and Italian types of bread which require a high protein content.

The development of strong gluten durum wheat cultivars in Italy, Canada and the U.S.A. has increased their potential use as a bread flour. DEXTER *et al.* (1981) reported that some new cultivars of durum wheat have approached acceptable levels for baking. They also found no fundamental differences in the functional properties of durum wheat gluten compared with that of common wheat.

Genotype is an important factor that affects the bread-making characteristics of durum flour (JOSEPHIDES, 1982). DEXTER *et al.* (1994) showed the importance of protein content on durum bread volume and the impact that fermentation time can have on bread quality. AMMAR *et al.* (2000) have postulated that gluten extensibility is an important factor for determining durum bread-making quality. RAO *et al.* (2001) pointed out the importance of the balance between gluten elasticity and extensibility.

In a number of regions in southern Italy, bread is mainly made from durum wheat. Therefore, BOGGINI (1985) evaluated the bread-making qualities of 25 durum wheat cultivars that were grown in Italy in 1984. Further studies (BOGGINI *et al.*, 1988, 1994) showed that the best cultivars for bread-making quality were characterized by the γ "45" gliadin band. BOGGINI and POGNA (1989) investigated the bread-making quality of other Italian durum wheat cultivars and the influence of the high molecular weight (HMW) glutenin subunits on this characteristic and found that the HMW glutenin composition appeared to affect bread-making quality. The cultivars having the subunit pair "7+8" produced higher bread volumes than cultivars with the subunit "20" or the subunit pair "6+8". AMMAR *et al.* (2000) and PENA *et al.* (1994) also showed the relationship between HMW glutenin composition and bread-making quality, while LIU *et al.* (1996) reported a relationship between gluten strength and LMW glutenin alleles.

CUBADDA *et al.* (1987) and PASQUI *et al.* (1991) have demonstrated that bread volume, softness and shelf-life depend

on the alveographic characteristics of the dough and doughs with low P/L and moderate W values give high bread volume and soft crumbs.

QUICK and CRAWFORD (1983) and DICK (1988) in the U.S.A., OZEN (1986) in Turkey, BAKHSHI and BAINS (1987) in India and WILLIAMS *et al.* (1988) in Syria and Jordan evaluated the bread-making characteristics of the durum wheat varieties grown in these countries and the possibility of using durum wheat flour to improve the quality of common wheat.

BOGGINI and POGNA (1990) blended common wheat flour of poor bread-making quality with flours from 29 Italian durum wheat cultivars. The addition of 25% durum wheat flour significantly increased the loaf volume. The same results were obtained by BOYACIOGLU and D'APPOLONIA (1994 a, b, c), who also demonstrated how additives and baking procedures influenced durum bread characteristics.

This paper reports the bread-making characteristics of Italian durum wheat genotypes as well as the evolutionary trends of these characteristics in the Italian germplasm.

MATERIALS AND METHODS

Nineteen new Italian cultivars and four widespread varieties of durum wheat (Creso, Duilio, Ofanto, Simeto) were grown in four Sicilian environments: Libertinia and Mineo, in 1997-98 and Libertinia and Aragona in 1998-99. Following procedures described by BOGGINI *et al.* (1994), grain samples were milled using an experimental laboratory mill (Bona, Monza, Italy) with sieves of 54 and 42 GG and the qualitative parameters of the semolina were analyzed.

Protein content (% dry matter) was determined with an Infratec 1229 Grain Analyzer (Foss Tecator, Höganäs, Sweden) by Near Infrared Transmittance (NIT), using a calibration based on Kjeldahl nitrogen determinations. Farinograph curves were obtained using a model 810105001 Brabender instrument (Brabender OHG, Duisburg, Germany). Water absorption, mixing time, degree of stability and softening index (12 min after peak time) were measured (AACC, 1979b). Alveograph parameters, strength of the dough (W) and ratio of tenacity (P) to extensibility (L), were determined using a model MA87 Chopin alveograph (Group Tripette & Renaud, Villeneuve-La-Garenne, France) according to UNI (1995). The bread-making test was carried out according to the AACC procedure (1979a) which was modified for durum wheat by BOGGINI and POGNA (1989) to obtain two loaves from 100 g of semolina, each. The characteristics of loaf volume, weight, crust roughness and colour and crumb porosity were recorded. Crumb porosity was estimated by means of the Dallmann scale. In Italy crumb colour is considered an aesthetic advantage for durum wheat bread. Therefore in the second year of trials, the yellow semolina colour index (b*) was determined using a model CR 300 Minolta colorimeter (Minolta, Osaka, Japan), by means of a CIE colour system and using the colour space L* a* b*.

Statistical analysis of variance of the qualitative parameters was carried out and the results were then represented in "box plot" (Systat software) where the centre horizontal line indicates the median of the samples, the length of each box shows the range within which the central 50% of the values fall and the whiskers show the range of non anomalous values observed (that fall within 1.5 Hspreads of the hinges). Values between the inner and outer fences are plotted with asterisks. Values beyond the outer fences, called far outside values, are plotted with empty circles.

SDS-PAGE electrophoretic patterns of low molecular weight (LMW) and high molecular weight (HMW) glutenin subunits were determined for all genotypes,

according to the method described by DAL BELIN PERUFFO *et al.* (1981).

RESULTS AND DISCUSSION

All the varieties were characterized by LMW glutenin type 2 and HMW at the locus Glu A1 type "Nulli". The subunits at Glu B1 were quite different (Table 1). The Colosseo and Nefer cultivars had the "13+16" gluteninic component and Parsifal "6+17", which are usually only present in small quantities in Italian germplasm. The majority of cases were characterized by the "7+8", "6+8" and "20" composition. It is also important to note that the Iride and Italo varieties were heterogeneous, i.e. having equal percentages of two gluten types.

Concerning the semolina colour (Table 1), the Colorado cultivar had the highest yellow colour values, while the Gianni, Duilio and Creso varieties had the lowest values.

Regarding the semolina protein content (Fig. 1), a satisfactory average level (14.3% d. m.) was recorded at Libertinia in 1998, where the environmental conditions also allowed the genotypes to obtain high yields (6.01 t/ha). All the cultivars had a protein content greater than 13.0%. In the same year, the semolina from the Mineo site, had the lowest average percentage of protein (13.6%), which was probably due to an attack of brown rust which lowered the average yield (3.75 t/ha). The Platani and Ciccio varieties, grown at Mineo in 1998 had the lowest protein content values (12.2 and 12.4%, respectively). These two values are lower than the lowest non-anomalous values and are marked in Fig. 1 with an asterisk. In

Table 1 - Glutenin composition and yellow index of the 23 durum wheat cultivars.

Cultivars	HMW glutenins subunits at Glu – B1	Yellow index of semolina [1]
BRONTE	7+8	19.94
CICCIO	7+8	21.93
COLORADO	6+8	26.93
COLOSSEO	13+16	20.16
CRESO	6+8	18.50
DUILIO	7+8	19.43
DURFORT	6+8	23.36
FORTORE	7+8	23.02
GARGANO	7+8	20.35
GIANNI	7+8	17.57
IRIDE	*7+8/6+8	21.66
ITALO	*6+8/20	23.25
MONGIBELLO	7+8	23.77
NEFER	13+16	22.63
OFANTO	20	22.11
PARSIFAL	6+17	20.98
PLATANI	7+8	23.29
SAN CARLO	7+8	21.46
SIMETO	7+8	22.19
SVEVO	7+8	25.05
TRESOR	20	22.42
VALBELICE	20	21.60
VARANO	7+8	20.19

* Heterogeneous composition;
[1] Means of two environments, in 1998/99.

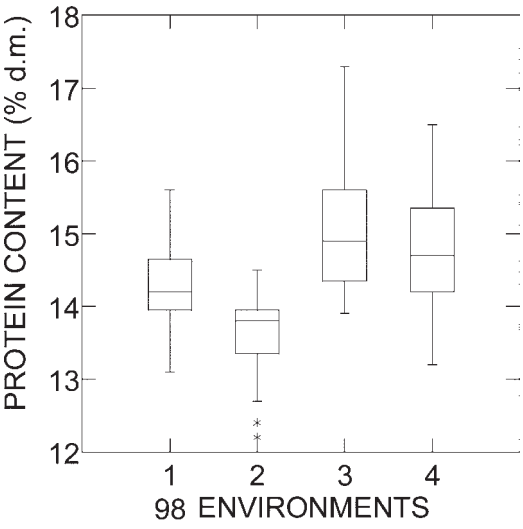


Fig. 1 - Protein content of the 23 cultivars in the four environments: 1 = Libertinia 98; 2 = Mineo 98; 3 = Libertinia 99; 4 = Aragona 99.
Values between the inner and outer fences are plotted with asterisks.

1999, rather high protein contents were recorded at Libertinia and Aragona, 15.1 and 14.8%, respectively, together with satisfactory average yields (4.98 and 5.48 t/ha, respectively). In both localities most of the varieties had protein contents over 14%; in particular, the cultivars Nefer, Colorado, Svevo and Mongibello from Libertinia and San Carlo, Svevo and Nefer from Aragona exceeded 16% protein content.

A marked difference between the two years is noted in the alveographic index P/L (Fig. 2). In the first year both localities had low average values with little variation. Only the Tresor cultivar from Mineo (marked with an asterisk in Fig. 2) had an alveographic index value (2.72) higher than the highest non-anomalous value. The same cultivar from Libertinia also had the highest non-anomalous value (2.34) of all the cultivars examined. In the second year, higher values were recorded with wider ranges of variation in both localities. This result was due to a sudden rise in the atmospheric temperature, particularly at Libertinia, during physiological maturation, which can cause an increase in gluten tenacity, as already observed in bread wheat by BORGHI *et al.* (1997).

Regarding the alveographic index W (Fig. 3), the satisfactory protein levels reached in 1998 at Libertinia and in 1999 at Aragona and Libertinia contributed to the higher values. In both test localities in 1998, the cultivar Varano had strong gluten, as shown by the two "outside values", that were higher than the highest non-anomalous value (asterisk in Fig. 3). The cultivar Valbelice from Libertinia in 1999 and even more so from Aragona had a very low dough strength, with W levels below the lowest non-anomalous value (87 and 84 10^{-4} x Joules respectively). At Aragona the value was statistically "far outside value" (empty circle, Fig. 3). At Aragona in 1999, the W mean value was around 200. San Carlo and Durfort had values that were higher than the highest non-anomalous value (300 and 268, respectively), while the values achieved by Parsifal were slightly

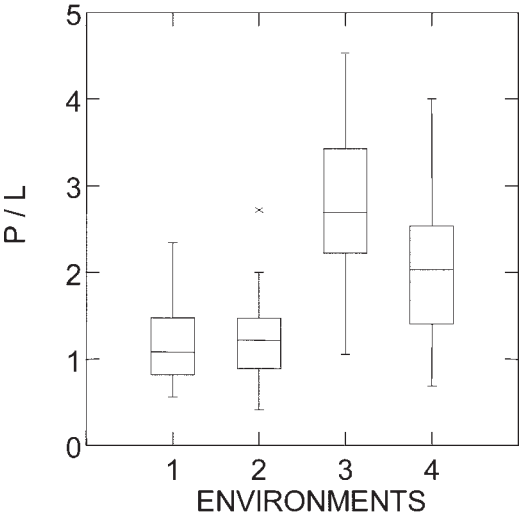


Fig. 2 - Alveographic P/L index of the 23 cultivars in the four environments: 1 = Libertinia 98; 2 = Mineo 98; 3 = Libertinia 99; 4 = Aragona 99. Values between the inner and outer fences are plotted with asterisks.

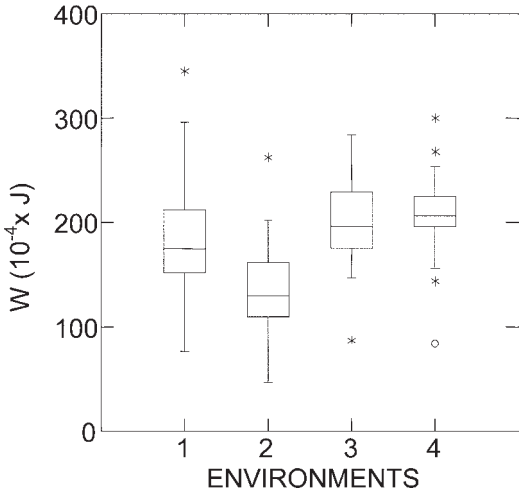


Fig. 3 - Alveographic index W of the 23 cultivars in the four environments: 1 = Libertinia 98; 2 = Mineo 98; 3 = Libertinia 99; 4 = Aragona 99. Values between the inner and outer fences are plotted with asterisks. Values beyond the outer fences, called far-outside-values, are plotted with empty circles.

lower than the lowest non-anomalous value (144).

The results from the experimental bread-making tests showed the lowest average bread volume (372 cm^3) in samples made with semolina from Libertinia in 1999, while the highest bread volume (432 cm^3) was obtained in 1998 with samples made with semolina from the same locality (Fig. 4). While there was a wide range of variation at all the environments, a value higher than the high non-anomalous value was only recorded at Libertinia in 1998 for the cultivar Colorado (550 cm^3, asterisk, Fig. 4). Comparing the data in Fig. 4 with that in the previous figures, a negative association emerges between the alveographic P/L and the bread volume. In fact, the highest bread volumes were achieved in samples from environments which showed lower tenacity/elasticity ratios. The correlations among the semolina qualitative parameters and bread characteristics show a highly significant negative relationship between loaf volume and alveographic P/L (r = -0.69 ***), which was previously demonstrated by BOGGINI *et al.* (1994). Loaf weight was positively correlated with farinographic water absorption (r = 0.57 **) and negatively correlated with loaf volume (r = -0.46 *).

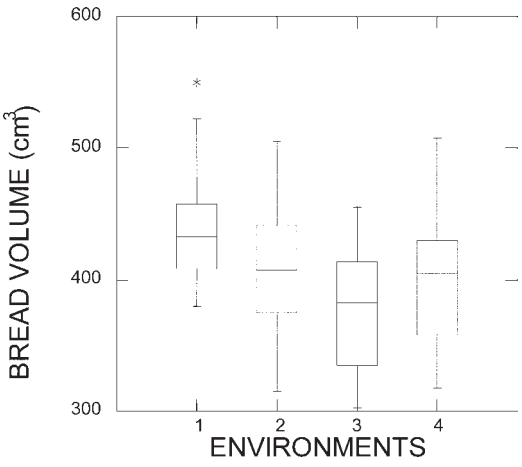


Fig. 4 - Loaf volume of the 23 cultivars in the four environments: 1 = Libertinia 98; 2 = Mineo 98; 3 = Libertinia 99; 4 = Aragona 99.
Values between the inner and outer fences are plotted with asterisks.

In Table 2, the protein content, alveographic indexes (P/L and W) and farinograph parameters (average of 4 environments) of all the varieties analyzed are reported. Regarding the protein content, the Colorado and Svevo varieties had the highest average values (15.6%) in all the environments, followed by the cultivars Nefer, Mongibello and San Carlo, with values between 15.5% and 15.0%. Platani and Ciccio, which had particularly low protein contents at Mineo in 1998 (Fig. 1), also had low protein contents at the other environments. Colorado, Valbelice and Parsifal had mean alveographic P/L values below 1, while the values for Ofanto were slightly over 1. The same performance was observed in all the environments, which indicates a greater stability of the cultivars in this respect. A high gluten tenacity was confirmed for Tresor (Fig. 2), along with Simeto, Duilio, San Carlo, Gianni and Italo. The cultivars Varano, San Carlo and Durfort (already indicated in Fig. 3) and Mongibello had the highest mean alveographic W values. These cultivars had high W values in all environments, whereas there was a large variability between years for the remaining cultivars. High mean farinographic water absorbtion values were recorded particularly for the Tresor, Italo, Varano, Svevo and Gargano cultivars, but all the varieties showed satisfactory values for this parameter. The San Carlo, Varano, Mongibello and Durfort cultivars had the best values for farinograph mixing times (>200 s). This parameter provides information about the time required to obtain optimal dough consistency. High farinographic dough stability was recorded for Mongibello, San Carlo, Varano, Svevo, Creso and Nefer. In contrast Parsifal, Gargano, Ofanto and Valbelice showed low sta-

Table 2 - Qualitative characteristics of the 23 cultivars (average of 4 environments).

Cultivars	Protein content (% d.m.)	Alveograph		Farinograph		
		P/L	W (10^{-4} J)	water absorption (%)	mixing time (s)	dough stability (s)
BRONTE	14.4 cg	1.85 ci	165.07 eh	60.93 be	156 fh	302 bd
CICCIO	13.1 j	2.10 ei	172.99 dg	61.23 ae	166 dh	254 cf
COLORADO	15.6 a	0.68 a	170.32 dg	58.87 fh	190 bf	258 cf
COLOSSEO	14.6 cf	1.75 ch	184.56 cf	61.70 ae	174 bg	288 bd
CRESO	14.8 bd	2.19 fi	191.84 ce	61.80 ae	176 bg	356 ac
DUILIO	14.1 dh	2.54 i	171.22 dg	60.85 be	168 ch	282 bd
DURFORT	14.5 cf	1.52 bg	220.71 bd	61.53 ae	202 bc	288 bd
FORTORE	14.5 cf	1.39 ae	179.50 dg	60.05 eg	174 bg	252 cf
GARGANO	14.8 bc	1.48 bf	137.52 fi	62.21 ad	150 gh	162 ef
GIANNI	14.0 eh	2.43 hi	155.81 eh	61.18 ae	156 fh	196 df
IRIDE	13.8 gh	1.97 di	183.70 cf	58.54 gh	158 eh	276 ce
ITALO	14.5 cf	2.24 gi	171.19 dg	62.79 ab	150 gh	188 df
MONGIBELLO	15.1 ac	1.34 ad	232.99 ac	61.31 ae	204 b	448 a
NEFER	15.5 ab	1.57 bg	177.31 dg	58.16 h	190 bf	346 ac
OFANTO	13.6 hj	1.12 ac	119.50 hi	58.98 fh	148 gh	158 f
PARSIFAL	14.7 ce	0.94 ab	130.82 gi	60.57 df	172 bg	164 ef
PLATANI	13.3 ij	1.77 ch	193.40 ce	59.88 eh	180 bg	322 bc
SAN CARLO	15.0 ac	2.49 hi	267.29 ab	61.23 ae	258 a	450 a
SIMETO	14.0 eh	2.56 i	186.22 cf	61.32 ae	192 be	314 bc
SVEVO	15.6 a	1.39 ae	205.28 ce	62.46 ad	182 bg	368 ac
TRESOR	14.4 cg	2.56 i	208.54 ce	63.09 a	200 bd	250 cf
VALBELICE	13.9 fi	0.74 a	93.17 i	60.69 cf	134 h	146 f
VARANO	14.8 bc	1.94 di	270.67 a	62.64 ac	236 a	397 ab
MEANS	14.46	1.76	182.16	60.96	178.96	281.09
L.S.D.	0.65	0.64	44.38	1.64	29.34	98.48

Values followed by the same letter are not significantly different at P = 0.05 according to Duncan's multiple range test.

bility values (around 160 s), confirming the low gluten strength already indicated by the low alveographic W values.

The main parameters of the bread-making test are reported in Table 3. The results demonstrate that Colorado had the highest bread volume, followed by Svevo, Mongibello and Varano. The good bread-making aptitude of these cultivars was observed in samples from each environment. Similar results, but with lower volume values, were recorded for Fortore, Valbelice and Parsifal. Loaf weight is important from an economic point of view because it indicates the bread yield. The variability of this parameter was low. San Carlo loaves were the heaviest, while Fortore, Colorado, Iride and Parsifal loaves were the lightest. High bread crumb porosity was recorded for Iride, Italo, San Carlo and Varano, whereas Tresor had the lowest value. The Italo, San Carlo and Valbelice varieties showed the highest roughness of crust values (≥2). The cultivars Ciccio, Creso, Gianni, Iride, Nefer, Ofanto and Simeto had slightly rough crusts (>1.3). The remaining varieties showed smooth, regular crusts (= 1). As for crust colour, durum wheat bread usually has a golden crust and a lighter colour than bread made with common wheat flour. Ciccio, Bron-

Table 3 - Results of bread-making test (average of 4 environments).

Cultivars	Loaf volume (cm^3)	Loaf weight (g)	Porosity [1]	Crust roughness [2]	Crust colour [3]	
					yellow index (b*)	brown index (a*)
COLORADO	492.50 a	147.8 b	6.3 ad	1.0 e	22.28 bd	14.30 ac
SVEVO	479.38 ab	151.5 ab	6.3 ad	1.0 e	24.08 bd	14.50 ac
MONGIBELLO	470.63 ac	150.0 ab	6.3 ad	1.0 e	24.72 ad	14.77 ac
VARANO	451.25 ad	148.8 ab	5.3 cd	1.0 e	21.90 bd	13.83 ac
FORTORE	438.13 be	148.0 b	6.5 ac	1.0 e	23.19 bd	14.13 ac
VALBELICE	438.13 be	148.5 ab	6.8 ab	2.0 ac	23.63 bd	13.90 ac
PARSIFAL	429.38 ce	147.3 b	6.5 ac	1.0 e	21.76 cd	13.56 bc
CICCIO	417.50 df	150.3 ab	6.5 ac	1.5 ce	30.88 a	15.28 ab
COLOSSEO	416.88 df	150.0 ab	6.0 ad	1.0 e	27.93 ac	14.41 ac
OFANTO	411.88 df	149.3 ab	6.0 ad	1.3 de	24.31 bd	14.15 ac
BRONTE	401.88 eg	151.0 ab	6.8 ab	1.0 e	28.31 ab	14.53 ac
PLATANI	399.38 eh	148.3 ab	6.5 ac	1.0 e	22.98 bd	13.30 c
DURFORT	398.13 eh	149.0 ab	5.8 ad	1.0 e	27.32 ac	15.19 ac
GARGANO	392.50 eh	150.8 ab	5.8 ad	1.0 e	23.28 bd	14.82 ac
SAN CARLO	379.38 fh	152.8 a	5.3 cd	2.3 ab	25.30 ad	14.78 ac
NEFER	377.63 fh	149.5 ab	5.5 bd	1.5 ce	20.71 d	14.54 ac
SIMETO	376.75 fh	149.8 ab	5.8 ad	1.3 de	24.40 bd	13.54 bc
IRIDE	375.00 fh	147.5 b	5.0 d	1.5 ce	28.23 ab	14.70 ac
TRESOR	365.00 gh	151.3 ab	7.0 a	1.8 bd	24.95 ad	14.32 ac
ITALO	362.63 gh	151.8 ab	5.3 cd	2.5 a	27.45 ac	15.57 a
DUILIO	359.38 gh	151.5 ab	6.5 ac	1.8 bd	23.86 bd	14.60 ac
CRESO	358.75 gh	151.5 ab	5.5 bd	1.5 ce	24.60 bd	15.57 a
GIANNI	353.13 h	150.5 ab	5.5 bd	1.8 bd	24.57 bd	13.89 ac
MEANS	406.31	149.79	6.0	1.38	24.81	14.44
L.S.D.	39.19	3.84	1.16	0.60	5.36	1.65

[1] 1: most porous; 8: least porous;
[2] 1: smoothest; 4: roughest;
[3] 1 year (1999): 2 environments.
Values followed by the same letter are not significantly different at P = 0.05 according to Duncan's multiple range test.

te and Iride, had high yellow index values, while Creso and Italo had high brown index values.

Evolutionary trends of durum wheat varieties for bread-making

From 1984 until 1999, the Catania section of the Experimental Institute for Cereal Research evaluated 90 durum wheat varieties. Fig. 5 shows the variation of the loaf volume indexes (using = 100 as the annual average loaf volume of Creso, a variety that is present in all the years) obtained in the trials in different years. There are notable changes in the Creso values in the nine years, particularly the low volumes recorded in 1984, 1998 and 1999. Such variability is associated with the late life cycle of this cultivar which consequently has a greater sensitivity to high temperature stress which occurs during the grain-filling period and which negatively affects yield. In fact, these three years were characterized by sudden temperature increases during the maturation phase, which, as previously noted, also influenced the protein quality.

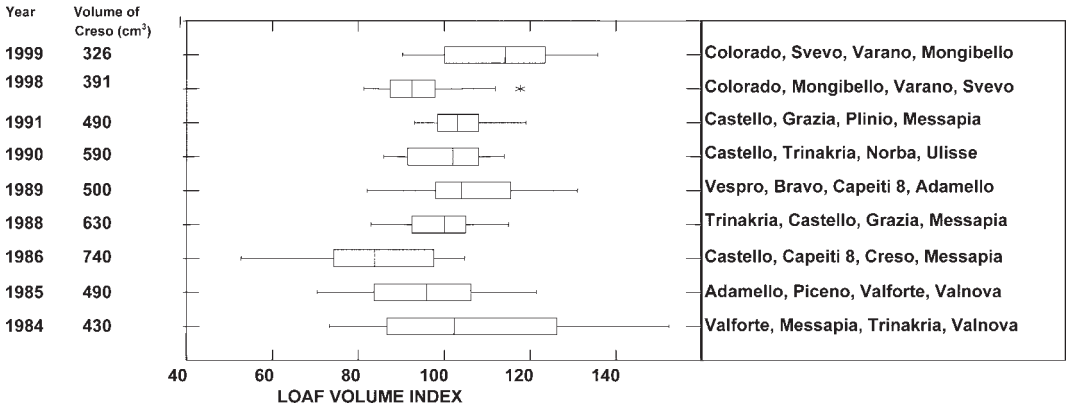


Fig. 5 - Trend of loaf volume and best cultivars during the years 1984 to 1999. (Values between the inner and outer fences are plotted with asterisks).

The environmental effect was evident in 1998 and 1999, in which the same cultivars showed the best bread-making aptitude (Colorado, Mongibello, Svevo and Varano, Fig. 5), even though the bread volumes were very different in the two years. While in 1984 there was a great variability in the bread-making aptitude of the genotypes tested, due to the wider genetic background of the most diffuse varieties, from 1988 onwards the variability decreased due to the restriction of the genetic background of the varieties cultivated in Italy (BOGGINI *et al.*, 1992).

Most of the Italian durum wheat varieties, which have been selected on the basis of their pasta-making quality, have an excessively tenacious and scarcely extensible gluten, which is unsuitable for the bread-making process. Only recently have varieties been identified which are suitable for both bread and pasta production.

CONCLUSIONS

The results obtained reveal that the best cultivars for bread-making are:

- Colorado, which has a high protein content (15.6%), along with a balanced P/L (0.68) and a reasonable W (170.32);
- Svevo and Mongibello which have high protein levels and good alveographic W values, but a relatively high P/L ratio (1.39 -1.43);
- Varano which has the highest mean W value (270.67) and a good protein content (14.8%), but a high P/L ratio (1.94);
- Fortore, even though it has a high P/L value (1.39), has good protein content and a fairly good alveographic W value (179.50).

Valbelice also has good bread-making quality, which is probably due to a well-balanced alveographic ratio, while Parsifal, also with a low P/L, shows a medium high protein content that can compensate for the lower gluten strength.

Among the cultivars with good bread-making quality, Fortore, Mongibello, Svevo and Varano have HMW glutenins "7+8", Colorado has "6+8", Valbelice has "20" and Parsifal has "6+17". The "6+17" composition is very rare in the Italian germplasm and could be important for genetically improving the bread-making quality of durum wheat (BOGGINI and PARISI, 1994).

Overall the results show that, by determining the qualitative characteristics of the different genotypes, it is possible to choose a variety that is appropriate

for the final product. In different years, in fact, some genotypes emerged that had higher bread-making quality and most of them were recurrent over several years. In particular the varieties Adamello, Capeiti 8, Messapia, Trinakria, Valforte and Valnova were noted during the 1980s; Castello and Grazia between the 1980s and 1990s; Colorado, Mongibello, Svevo and Varano in 1998-1999. These varieties can be recommended to the processing industry to help meet the increasing demand for durum wheat that is suitable for bread-making.

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PAPER

THE EFFECT OF SMOKING, PACKAGING AND STORAGE TEMPERATURE ON THE BACTERIAL GREENING OF FRANKFURTERS CAUSED BY *LEUCONOSTOC MESENTEROIDES* SUBSP. *MESENTEROIDES*

EFFETTO DELL'AFFUMICAMENTO, DEL CONFEZIONAMENTO E DELLA TEMPERATURA DI CONSERVAZIONE SULLA DECOLORAZIONE VERDE BATTERICA DI FRANKFURTERS CAUSATA DA *LEUCONOSTOC MESENTEROIDES* SUBSP. *MESENTEROIDES*

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ABSTRACT

The spoilage of frankfurters caused by lactic acid bacteria was examined. Isolates responsible for the green discolouration were identified as *Leuconostoc mesenteroides* subsp. *mesenteroides*. Naturally smoked or liquid smoke-treated frankfurters were inoculated with strains of *L. mesenteroides* and stored aerobically and under vacuum at 4° and 12°C. Greening was ob-

RIASSUNTO

Viene studiato il deterioramento dei frankfurters causato dai batteri lattici. In particolare *Leuconostoc mesenteroides* subsp. *mesenteroides* è stato identificato come responsabile della decolorazione verde. Campioni di frankfurters affumicati sia naturalmente che con affumicamento per via liquida sono stati inoculati con ceppi di *L. mesenteroides* e conservati aerobicamente e

- Key words: greening, *Leuconostoc mesenteroides*, meat products, smoking -

served under aerobic storage conditions from the third day onwards and the frankfurters were more discoloured when stored at 12°C compared to 4°C. Uninoculated samples did not discolour under aerobic conditions. Natural wood smoking, vacuum packaging and storage at 4°C were effective against frankfurter sausage discolouration.

sotto vuoto a 4° e 12°C. L'inverdimento è stato osservato sotto conservazione aerobica a partire dal terzo giorno. L'inverdimento era più pronunciato a 12°C piuttosto che a 4°C. I campioni non inoculati sotto vuoto non hanno mostrato inverdimento. L'affumicamento naturale a legna, accompagnato dal confezionamento sotto vuoto e conservazione a 4°C si è dimostrato il più effettivo nel prevenire l'alterazione verde dei frankfurters.

INTRODUCTION

The discolouration of cured meat products is a common defect that often occurs in cooked, cured meat products. A half century ago, NIVEN *et al.* (1949) described the bacterial surface discolouration of sausages, while KORKEALA and BJÖRKROTH (1997), reviewing the spoilage phenomena of vacuum-packaged, cooked sausages, did not mention discolouration.

In Greece, several studies on the microbial ecology of cooked, cured meats have demonstrated that the dominant spoilage association of these products is made up of *Lactobacillus sakei*, and atypical leuconostoc-like bacteria (SAMELIS and GEORGIADOU 2000, SAMELIS *et al.* 1998, 2000a, b), with intrinsic, extrinsic and processing factors playing an important role. Aerobic storage of "taverna" frankfurters resulted in the dominance of *Leuconostoc mesenteroides* subsp. *mesenteroides* and greening of the product. Protective cultures made up of *Lactobacillus alimentarius* and *Staphylococcus xylosus* had little effect on the colour of the product (KOTZEKIDOU and BLOUKAS 1996, 1998). The adventitious lactic acid bacteria (LAB) appear to be dominant in spoilage associations (ZHANG and HOLLEY 1999).

The green discolouration in frankfurters and other cured, vacuum-packaged, thermally processed meat products, is caused by LAB that produce H_2O_2 (HOLZAPFEL, 1998). In particular, they can be lactobacilli, leuconostocs or heterofermentative strains of streptococci (GUERRERO and CHABELA 2000, GRANT *et al.*, 1988). *Weissella viridescens* (formerly: *Lactobacillus viridescens*) that produces H_2O_2 was the first LAB to which the greening defect was attributed (NIVEN and EVANS, 1957). Subsequent studies have confirmed this (LORINCZ and INCZE, 1961; SHARPE, 1962; GARDNER, 1967). Other strains of lactobacilli also cause greening, e.g. *L. jensenii* and *L. fructovorans* (JAY, 1992). Some *Leuconostoc* spp. or *Enterococcus* spp. such as *Leuconostoc mesenteroides*, *Enterococcus faecalis* (*Streptococcus faecalis*) and *Enterococcus faecium* (*Streptococcus faecium*), as well as some *Pediococcus* strains may cause surface greening (BORCH and MOLIN, 1989).

Discolouration may also have a non-bacterial origin. Gray or green areas may occur in the center of the meat product as soon as it is cut. In most of these cases, discolouration is caused by lack of sufficient curing (GARDNER, 1967).

The aim of this study was to examine the effect of storage temperature, packaging and smoking methods on the onset of greening in frankfurters inoculated with the microorganism that is re-

sponsible for the greening defect. To do this, LAB strains were isolated from frankfurters that had already developed the greening defect. The strain that caused greening in frankfurters was identified as *Leuconostoc mesenteroides* subsp. *mesenteroides*.

MATERIALS AND METHODS

Sausage production

The meat product used in this study was an emulsion-type sausage in the form of 25 g frankfurters that were commercially manufactured by a major Greek meat producing company. The composition of the sausage was (%): pork (55), lard (25), water (10), starch (5.5), milk protein (1.3), NaCl (1.5), spices (1.0) and additives (0.7), consisting of sodium ascorbate 0.1, polyphosphates, 0.3, potassium nitrate 0.015, sodium nitrite 0.015. The raw frozen meat was thawed in a water bath and warmed to a core temperature of -4°C. After grinding, the raw meat was mixed with salt, starch, skimmed-milk powder, lard, spices and additives, and the mixture was stuffed into inedible casings. The sausages were then cooked to a core temperature of 74°C. Liquid smoke was applied just before cooking. Natural smoke was applied during cooking. After cooking, the sausages were rinsed with cold water (10°C) for 10 min until a core temperature 30°-35°C was reached. They were then drained and transferred to an air blast cooling room (-5°C) to cool them to 2°C. The sausages were then skinned and, after storage at 2°C for 18 h, were packed. The finished frankfurters were then transported, under refrigeration to the laboratory.

Isolation and identification of microorganisms

Twenty LAB strains that had been isolated from different, overtly discoloured green frankfurters were examined. The isolation and identification methods used, including biochemical tests, were previously described by SAMELIS *et al.* (1994, 2000b). The strains were grown in MRS broth (Merck, Darmstadt, Germany) at 30°C and stored in the same media supplemented with 20% (v/v) glycerol (Merck) at -18°C.

Inoculation of sausages

The organisms were subcultured twice (24 h, 30°C) in 10 mL MRS broth (Merck), 1% inoculum. The cells were harvested by centrifugation (10,844 x g for 30 min) (Biofuge 22R, Heraeus Instruments GmbH, Osterode, Germany), washed twice and resuspended in 10 mL of 50 mM sodium phosphate buffer, pH 7. The bacterial suspension was diluted with sterile 1/4-strength Ringer's solution (Oxoid), which yielded an inoculum of about 10^4-10^5 cfu mL^{-1}. The frankfurters were immersed in 2 L of bacterial suspension for 1 min at 20°C, placed on a sterile grate and the surplus solution was allowed to drip off. The inoculation was carried out in a laminar flow cabinet (Nuaire NU-425-400E, Plymouth, MN, USA).

Effect of storage temperature, packaging and smoking method

The inoculated and control samples were packaged under vacuum using a packaging machine (HENCO VAC 1900, Howden Food Equipment B.V., BA's-Hertogenbosch, The Netherlands) and a water vapour-impermeable film (type V 40-2, 40 µm thick). The oxygen transmission rate of the film was <35 $cm^3/m^2/24$ h/atm at 20°C and 65% R.H. Each packaged unit contained two sausages. Aerobic conditions were established in half of the vacuum-packed samples by making six holes with a sterile cork borer (5 mm diameter) on the package film (20x15 cm). The packages were then stored at either 4° or 12°C.

The frankfurters treated with liquid smoke were sprayed with an aqueous diluted preparation (20% v/v) for 1 min, just before cooking. Natural wood smoking was applied during cooking for 6-7 min at 65°C and 65% R.H. Smoke was produced in a smoke generator, with beech wood as fuel, at 300°-350°C and then through pipes introduced to the cooking chamber.

Microbiological analysis and pH

Frankfurters (25 g) were placed in 225 mL of 0.1% peptone water in a sterile stomacher bag, homogenized with a lab blender (Stomacher® 400, Seward Medical Ltd, London, UK) for 1 min at normal speed and 0.5 min at high speed. Decimal serial dilutions were prepared with the homogenate in sterile Ringer solution for microbiological determinations.

For the total viable counts, samples (0.1 mL) of serial homogenate dilutions were spread on Plate Count Agar (PCA, Merck) and incubated at 30°C for 72 h. The LAB were determined by the pour-plate method: 1 mL of an appropriate dilution was inoculated into 10 mL of molten MRS agar (45°C) (Merck). After setting, the plates were incubated at 30°C for 72 h under anaerobic conditions (Gas-Pack System, BBL, Becton Dickinson, Cockeysville, MD, USA). The number of colonies formed was counted and reported (log_{10} cfu g^{-1}) for each sample and each medium (SAMELIS and GEORGIADOU, 2000).

The pH value was determined by dipping the glass electrode of a pH-meter (WTW pH 526, Weilheim, Germany) into a homogenized sample: a frankfurter (10 g) was mixed with 90 mL of distilled water and homogenized with a blender (model PB500, Kenwood Ltd, Havant, U.K.). Microbiological analysis and pH measurements were conducted on duplicate samples at intervals of 0, 6 and 12 days or 0, 3 and 6 days of storage, respectively, while macroscopic evaluation was performed every three days. The coefficient of variation was <5%.

RESULTS AND CONCLUSIONS

Identification of microorganisms

Twenty LAB strains were isolated from overtly discoloured samples and numbered consecutively. The isolates were then divided into hetero- (10) and homofermentative (10) strains. Based on the identification profile of the heterofermentative strains, 3 groups were

Table 1 - Grouping of heterofermentative isolates.

Group[a]	1	2	3[b]
N. of isolates	1	5	4
Cell morphology	bC	bC	bC
Gram reaction	+	+	+
Catalase	-	-	-
CO_2 from glucose	+	+	+
NH_3 from arginine	-	-	-
Growth at 10°C	+	+	+
Production of dextran from sucrose	+	+	+
Growth in			
8% NaCl	-	-	-
10% NaCl	-	-	-
Sugar fermentation			
Maltose	+	+	-
Mannitol	+	+	(+)[c]
Lactose	+	+	+
Arabinose	(+)	(+)	+
Raffinose	+	+	+
Melibiose	+	+	+
Sucrose	+	+	+
Cellobiose	+	(+)	+
Trehalose	+	+	+
Xylose	+	+	+
Galactose	+	+	+

[a] All strains were identified as *Leuconostoc mesenteroides* subsp. *mesenteroides*.
[b] strains of this group, coded as A_5, B_8 and B_9, were used for inoculation and were correlated with extensive greening.
[c] (+), weak reaction.
bC, coccobacilli.

formed (Table 1). Although all the strains were identified as *Leuconostoc mesenteroides* subsp. *mesenteroides* / *dextranicum* by the miniaturized API 50 CH tests (Bio-Mérieux, Marcy l'Etoile, France), the different characteristics of the heterofermentative, arginine-negative, cocoid LAB reported by SAMELIS *et al.* (2000b) distinguish it from group 3 that was maltose negative. However, all the groups had a strong positive fermentation profile. An inoculation test was performed initially to determine which of the three groups of microbial strains was responsible for the greening defect. Once the third group was determined, three randomly selected strains (A_5, B_8, B_9) were then used separately for further inoculations in order to examine the effect of storage temperature and packaging method on the greening defect.

Effect of storage

Macroscopic observations

On the third day of storage, the inoculated samples that had been stored aerobically at 4°C began to develop the greening defect on their surfaces, and on the fourth day, the green discolouration had spread over the entire surface. The inoculated samples that were packaged aerobically and stored at 12°C developed extensive greening on the third day. Generally, from the fifth day on, the greening had spread from the surface inward towards the center of the frankfurters regardless of the storage temperature.

Throughout the experiment (12 days of storage), discolouration was not detected in the aerobically packaged, uninoculated samples, regardless of storage temperature. Likewise, the green discolouration was not detected in any of the vacuum-packaged samples, regardless of storage temperature or whether inoculated or not. In all the macroscopic observations, strain B_9 caused the most severe greening and therefore, this strain was used to determine the effect of the smoking method on greening. All of the above samples were naturally smoked.

Microbial analysis and pH changes

The initial total viable counts (TVC) were 10^3 cfu g^{-1}, while the inoculated samples had TVC values of 10^6-10^7 cfu g^{-1}. At the end of the storage period (12 days) the TVC were 10^8-10^9 cfu g^{-1} for the vacuum packaged samples, and an average of 10^9-10^{10} cfu g^{-1} (Fig. 1) for the aerobically stored samples. The samples stored at 4°C were very similar to those of the samples stored at 12°C.

Initially, the average LAB count for the frankfurters was 10^3-10^4 cfu g^{-1}. After inoculation, the LAB count increased to 10^5-10^6 cfu g^{-1} (Fig. 2). After 12 days of storage the LAB count reached 10^8-10^9 cfu g^{-1} for inoculated and uninoculated samples. Comparing the TVC and LAB counts it is obvious that LAB were the dominant spoilage flora. The pH values of the samples are shown in Fig. 3. The initial pH values of the frankfurters were 6.25-6.30, but they decreased during storage, in some cases reaching a final pH of 5.45 (Fig. 3b).

Comparison of smoking methods on frankfurters inoculated with strain B_9

Macroscopic observation

On the fifth day of storage, the inoculated, liquid smoked-treated samples that were packaged aerobically and stored at 4°C developed the green discolouration, particularly on the surfaces, while the samples that were stored at 12°C developed a more intense greening defect. The uninoculated, liquid-smoke treated samples that were stored aerobically did not develop any greening defect throughout the experiment, regardless of storage temperature.

In contrast, the inoculated, naturally smoked samples that were stored aerobically at 4°C developed the greening defect

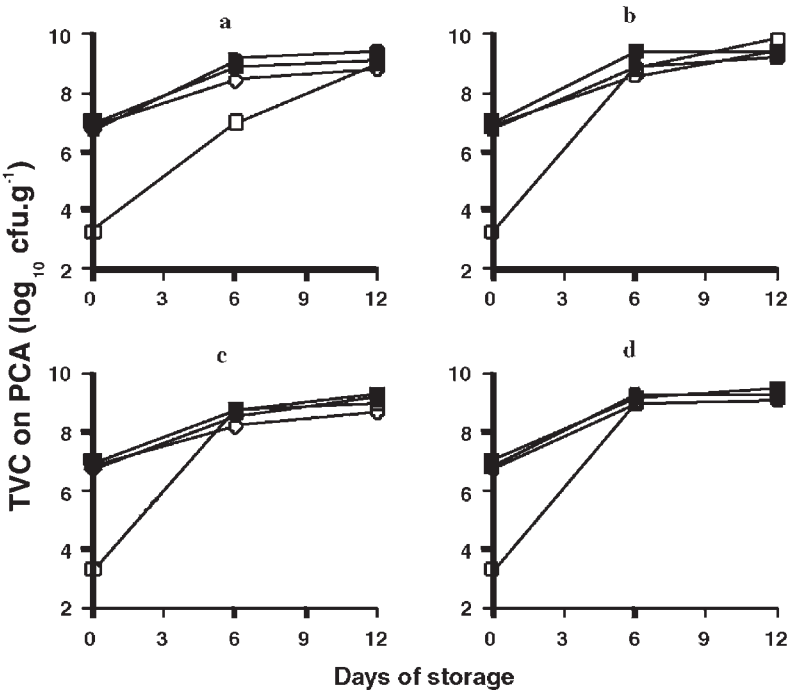


Fig. 1 - Total viable counts (TVC) of frankfurters stored under vacuum (a, c) or aerobically (b, d) at 4°C (a, b) and 12°C (c, d). Uninoculated (❑) and inoculated with three heterofermentative strains of lactic acid bacteria (A_5, ■; B_8, ❍ and B_9, ●).

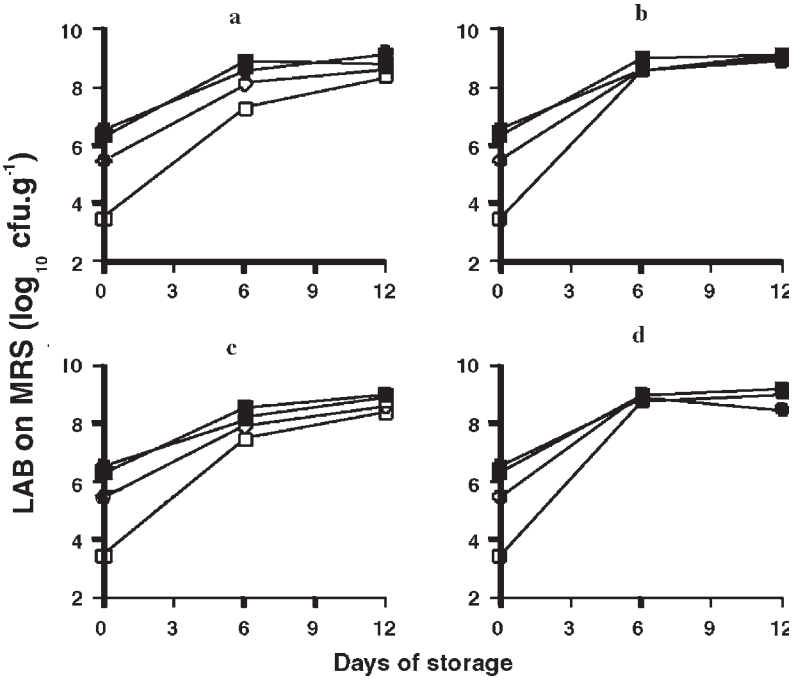


Fig. 2 - Lactic acid bacteria counts of frankfurters stored under vacuum (a, c) or aerobically (b, d) at 4°C (a, b) and 12°C (c, d). Uninoculated (❑) and inoculated with three heterofermentative strains of lactic acid bacteria (A_5, B_8, ❍ and B_9, ●).

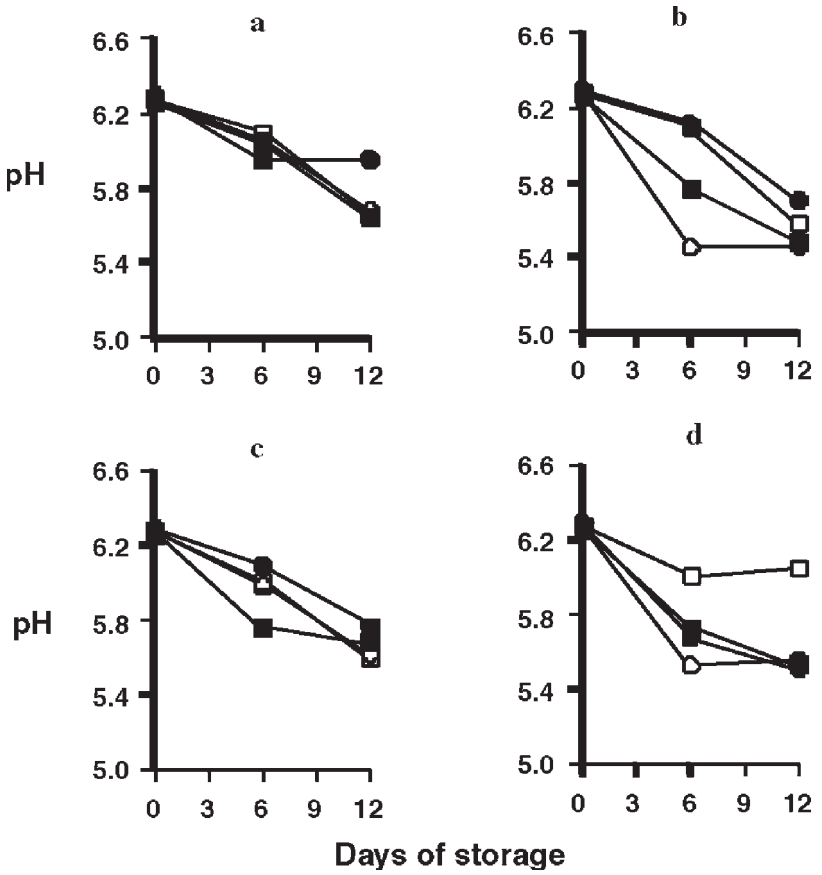


Fig. 3 - pH changes during the storage of frankfurters stored under vacuum (a, c) or aerobically (b, d) at 4°C (a, b) and 12°C (c, d). Uninoculated (❑) and inoculated with three heterofermentative strains of lactic acid bacteria (A_5, ■; B_8, ❍ and B_9, ●).

on the 14th day of storage (2 days after the end of the anticipated duration of the experiment). On the same day, the inoculated, naturally-smoked samples that were stored aerobically at 12°C developed an even more intense greening defect than the corresponding samples stored at 4°C. None of the vacuum-packaged samples developed the greening defect during the storage period, regardless of storage temperature or smoking method.

Microbial analysis, pH

The LAB population in liquid-smoked frankfurters was, on the average, less than 10^2 cfu g^{-1}. After inoculation, the population increased to 10^5 cfu g^{-1}. The LAB counts increased during sample storage and after 6 days the count reached 10^8-10^9 cfu g^{-1}. The samples stored at 12°C had slightly larger populations than those stored at 4°C (Figs. 4a and 4b).

The initial LAB population in naturally smoked frankfurters was, on the average, less than 10 cfu g^{-1}, while after inoculation, it increased to more than 10^6 cfu g^{-1}. At the end of the storage period the LAB counts were 10^8-10^9 cfu g^{-1}. In both cases, the vacuum-packed samples had smaller LAB populations than the aerobically-packaged ones (Figs. 4c and 4d). The pH values of the frankfurters ranged from 6.25 to 6.55. During storage pH values decreased in all the inoculated samples but increased in the uninoculated frankfurters. In some cases, the pH value was 5.7 (Fig. 5).

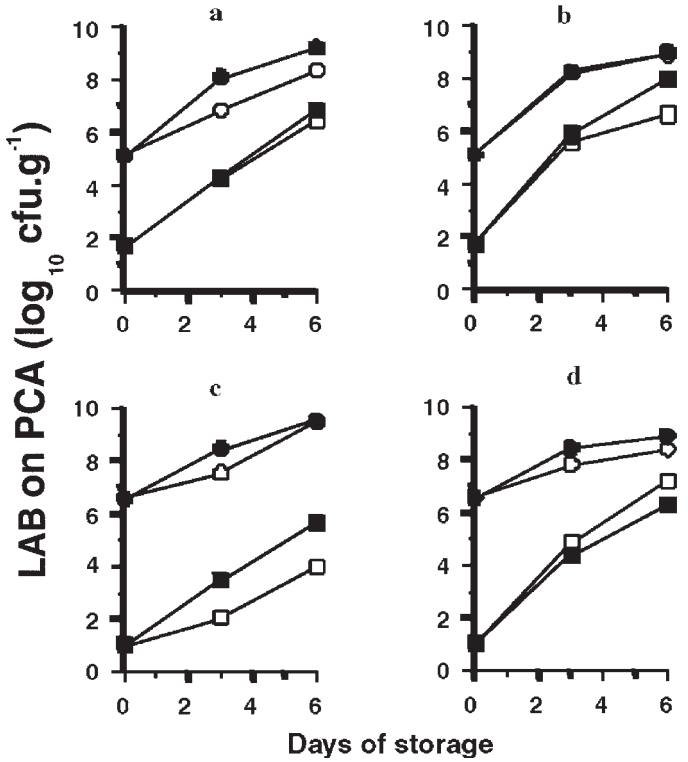


Fig. 4 - Lactic acid bacteria counts of frankfurters treated with liquid-smoke (a, b) or naturally smoked (c, d) stored under vacuum (❑, ○) or aerobically (■, ●) at 4°C (a, c) and 12°C (b, d). Uninoculated (❑, ■) and inoculated with a *Leuconostoc mesenteroides* subsp. mesenteroides strain (B_9) (○, ●).

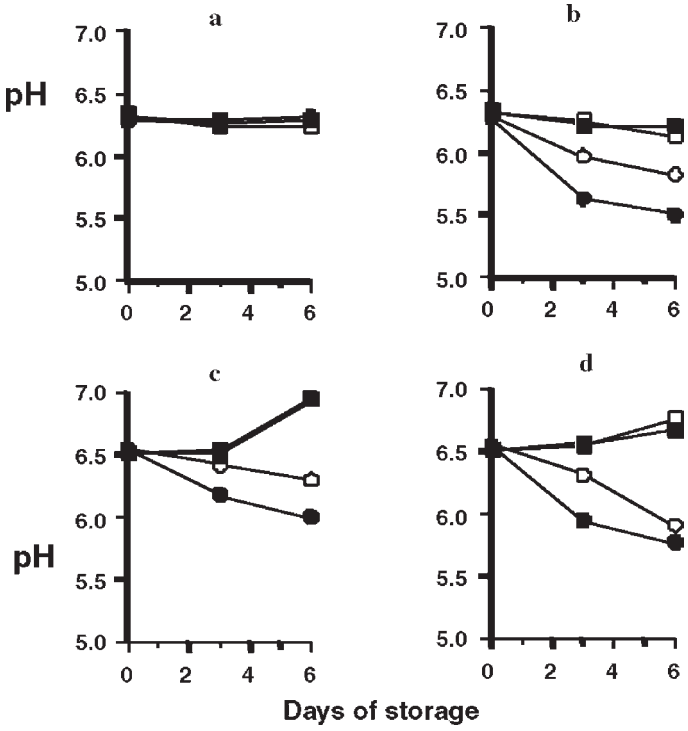


Fig. 5 - Changes in pH of frankfurters treated with liquid-smoke (a, b) or naturally smoked (c, d) stored under vacuum (❑, ○) or aerobically (■, ●) at 4°C (a, c) and 12°C (b, d). Uninoculated (❑, ■) and inoculated with a *Leuconostoc mesenteroides* subsp. *mesenteroides* strain (B_9) (○, ●).

The samples had lower ($<10^9$) total viable and LAB counts when stored at 4°C than those stored at 12°C. This finding agrees with the results from other studies which report that the maximum bacterial population that can grow on refrigerated cured meat products is 10^7-10^9 cfu g^{-1} (BORCH *et al.*, 1996).

The results of the present study confirm the association of greening with spoilage caused by heterofermentative LAB (*Weissella viridenscens*, leuconostocs). The greening in the frankfurter-type sausages was caused by *Leuconostoc mesenteroides* subsp. *mesenteroides*. These results are in agreement with studies (SAMELIS and GEORGIADOU, 2000 and SAMELIS *et al.*, 2000a, b) on the effect of different extrinsic, intrinsic and processing factors on the physiology of spoilage associated with cooked, cured meat products.

Decreased pH values during storage are in agreement with other studies in which the final pH of cooked meat products reached pH values of 5.0-5.3, due to the production of lactic acid (GARDNER, 1983). The increased pH values noted in some instances may have been caused by metabiotic phenomena (DROSINOS and BOARD, 1995). The pH value and lactic acid concentrations have been proposed as indexes of microbial quality in products (KAKOURI and NYCHAS, 1994).

This study confirms that microorganisms responsible for bacterial greening can grow and spoil in meat products even at low temperatures (4°C). Vacuum packaging is an efficient way to delay the onset of greening in meat products for a significant period of time. The smoking method used in the production of meat products is also of major importance. Natural smoking can delay the onset of the greening defect by several days. In summary, surface greening was caused by bacteria. To avoid contamination, sanitary procedures, such as during slicing (HOLLEY, 1997), are essential during the handling of cooked, cured meat products.

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PAPER

RELEASE OF VOLATILE COMPOUNDS FROM EMULSIONS: INFLUENCE OF β-LACTOGLOBULIN AND pH

RILASCIO DI COMPOSTI VOLATILI DALLE EMULSIONI: INFLUENZA DELLA β-LATTOGLOBULINA E DEL pH

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ABSTRACT

The influence of β-lactoglobulin (0, 1, 2%) and pH (3, 6, 9) on the release of 20 aroma compounds from oil-in-water emulsions was studied. The release under equilibrium and mouth conditions was measured by equilibrium headspace analysis and in a model mouth system combined with gas chromatography, respectively. Six alcohols, five ketones, four esters, three aldehydes, one terpene and one sulfur compound were investigated. Differences between compounds depended more on chain length than on functional group. The presence of β-lactoglobulin affect-

RIASSUNTO

È studiata l'influenza della β-lattoglobulina (0,1,2%) e del pH (3,6,9) sul rilascio di 20 componenti aromatici da emulsioni olio-acqua. Il rilascio all'equilibrio ed in condizioni simili a quelle della bocca è stato misurato mediante analisi dello spazio di testa all'equilibrio e con un modello di bocca artificiale combinato con la gas cromatografia. Sono stati valutati sei alcool, cinque chetoni, quattro esteri, tre aldeidi, un terpene ed un composto solforato. Le differenze tra i diversi composti dipendevano più della lunghezza della catena che dai gruppi funzionali. La presenza di β-lattoglobu-

- Key words: aroma, β-lactoglobulin, emulsion, flavor, model mouth -

ed the aroma release under both equilibrium and mouth conditions. Its concentration had a significant effect under equilibrium conditions but not under mouth conditions, which was attributed to the extinguishing effect of the artificial saliva added. The pH did not affect the aroma release at all.

lina ha, in ogni prova, influenzato il rilascio dell'aroma. La sua concentrazione ha invece effetto sotto condizioni d'equilibrio ma non in quelle della bocca artificiale, per l'effetto annullante legato alla saliva artificiale aggiunta. Il pH, infine, non ha mostrato alcun effetto sul rilascio degli aromi.

INTRODUCTION

Volatile compounds contribute to flavor perception. The odor of a food can be defined as the perception of volatile compounds prior to eating and is caused by the orthonasal passage of the compounds by inhalation through the external naris. When food is eaten, it is masticated and mixed with saliva, the volatiles are released into the oral cavity and follow the retronasal route to the receptor cells in the nose. It has been shown that the orthonasal (odor) and retronasal (aroma) volatile composition differ in quantity, which is probably because of the different release conditions (ROBERTS and ACREE, 1996).

Interactions between volatile compounds and food components play an important role in the perception of flavor. Volatile compounds may be dissolved, adsorbed, bound, entrapped, encapsulated or diffusion-limited by food components. The relative importance of each of the mechanisms varies with the properties of the volatile compounds and the physical and chemical properties of the components of the food (KINSELLA, 1988).

β-Lactoglobulin (βLG), the major bovine whey protein, is one of the most commonly used whey proteins in the food industry. Whey proteins are a potentially significant source of functional protein ingredients, for which the market is expanding. βLG has been extensively characterized and is the best described of all food proteins. βLG is known for its gelating, emulsifying, foaming and binding properties. It has been studied for its interactions with a large variety of small hydrophobic compounds, among which are aroma compounds (GUICHARD and LANGOURIEUX, 2000). It has been suggested that these compounds may bind inside the central calyx, which has been proven for fatty acids (BROWNLOW *et al.*, 1997; QIN *et al.*, 1998; WU *et al.*, 1999). Other compounds, such as aliphatic aldehydes have been shown to react with protein amino groups, causing formation of covalently bound products (STAPELFELDT and SKIBSTED, 1994).

The influence of βLG in aqueous solutions has been studied extensively (CHARLES *et al.*, 1996; JOUENNE and CROUZET, 1996; PELLETIER *et al.*, 1998; GUICHARD and LANGOURIEUX, 2000), including a collaborative experimental study as part of Cost Action 96 (LANGOURIEUX and ESCHER, 1999). A large variety of parameters have been reported to affect the binding of aroma molecules. These parameters included the commercial source of the βLG, concentration of βLG, the aroma molecule with respect to the functional group and hydrophobicity, temperature, pH and NaCl concentration.

The effect of βLG in more complicated food systems has received less attention. Many natural and processed foods exist either partly or wholly as emulsions (MCCLEMENTS, 1998). The impact of βLG in emulsified food systems has been evaluated in a few studies. The oil present in this type of system has a twofold effect. Firstly, most aroma compounds are rela-

tively hydrophobic and thus more soluble in oil than in water. Secondly, lipids interact with proteins and are likely to be in competition with aroma compounds for the same binding sites. Research in this area includes studies on binary oil/water systems and on emulsions with mostly a rather limited number of aroma compounds. A study on binary systems indicated that βLG at the interface increased the resistance to transfer of volatile compounds as a function of the hydrophobicity of the compound (ROGACHEVA *et al.*, 1999). Emulsified systems with βLG showed higher retention for specific volatile compounds than emulsions with other whey proteins (GUICHARD and LANGOURIEUX, 2000). It can be assumed, therefore, that βLG in emulsions affects both the thermodynamic (partitioning) and the kinetic (mass transfer) component of the release of volatile compounds. However, no systematic studies have been carried out so far.

In the present study the effect of βLG, its concentration and the pH on the release of 20 odor active volatile compounds from oil-in-water (O/W) emulsions were evaluated. The influence of the parameters was examined under equilibrium and mouth conditions to distinguish the thermodynamic and kinetic component of volatile release, and to determine the potential effects on odor and aroma perception separately.

MATERIALS AND METHODS

Sample materials

The lipid phase of the biphasic and emulsion samples was composed of commercial sunflower oil (Suma Wholefoods, Dean Clough, Halifax, UK). Distilled water was used for the water phase and βLG (Sigma-Aldrich, Steinheim, Germany; purity 90%) as emulsifying agent. The same oil and water was used for the samples composed of one phase only. The 20 aroma compounds included: diacetyl, 2-butanone, ethyl acetate, 2-pentanol, hexanal, 1-hexanol, 2-heptanone, heptanal, and α-pinene, which were supplied by Sigma-Aldrich. Dimethyl sulfide, ethyl butyrate, 2-octanone, and octanal were purchased from Merck (Hohenbrunn, Munich, Germany). 1-Propanol, propyl acetate, 1-butanol, butyl acetate, and 3-methyl-1-butanol were supplied by Lancaster (Walkerburn, UK), 2-nonanol and 2-decanone were obtained from Fluka Chemie. Purity of the compounds was greater than 95%.

The artificial saliva consisted of $NaHCO_3$ (5.208 g), $K_2HPO_4 \cdot 3H_2O$ (1.369 g), NaCl (0.877 g), KCl (0.477 g), and $CaCl_2 \cdot 2H_2O$ (0.441 g), NaN_3 (0.5 g), mucin (2.160 g, porcine stomach mucin; Sigma-Aldrich), and 200,000 units of α-amylase (hog pancreas α-amylase; Fluka Chemie) in 1 L of distilled water, and was adjusted to pH 7 (VAN RUTH and ROOZEN, 2000).

Sample preparation

Sunflower oil-in-water (O/W) emulsions varying in emulsifier concentration were prepared as indicated in Table 1, using a prototype single valve homogenizer. By modifying the energy during homogenization, O/W emulsions with a constant mean droplet size (D(3,2)) of 0.85 μm were made. The pH was adjusted with HCl or NaOH after emulsion preparation and the 20 aroma compounds were added in triplicate, which resulted in a final concentration of 0.001% v/v per compound. The samples, 3 replicates per sample, were stored for 12 h at 4°C in the absence of light to allow equilibration. The samples with 0% βLG were biphasic samples.

Droplet size measurements

A Malvern Mastersizer laser diffractometer (model S Ver. 2.15, Malvern Instruments, Malvern, UK) was used to determine the structural characteristics

Table 1 - Experimental samples: oil-in-water emulsions and their compositional and structural characteristics.

Sample code	Oil [% w/w]	Water [% w/w]	β-lactoglobulin concentration [% w/w]	pH	Droplet size D(3,2) [μm]	Dispersion [μm]
0%-pH3	40	60	0	3	-	-
0%-pH6	40	60	0	6	-	-
0%-pH9	40	60	0	9	-	-
1%-pH3	40	59	1	3	0.85	5.66
1%-pH6	40	59	1	6	0.85	5.66
1%-pH9	40	59	1	9	0.85	5.66
2%-pH3	40	58	2	3	0.85	5.87
2%-pH6	40	58	2	6	0.85	5.87
2%-pH9	40	58	2	9	0.85	5.87

of the emulsions: the Droplet size (D(3,2)) and the dispersion (D(v,0.9) - D(v,0.1)).

Aroma analysis under equilibrium conditions

For equilibrium headspace gas chromatography, 2 mL of sample or alternatively 1.2 mL of sample and 0.8 mL of artificial saliva were transferred to a 10 mL headspace vial for each replicate sample. The samples were incubated at 37°C and agitated at 750 rpm for 6 min in the automated headspace unit (Combipal-CTC Analytics system; JVA Analytical Ltd.) of the gas chromatograph (GC; Varian CP-3800; JVA Analytical Ltd.). After equilibration, 2 mL of headspace was automatically injected. The GC was equipped with an injector at 225°C, a BPX5 capillary column (60 m length, 0.32 mm i.d., and 1.0 μm film thickness; SGE (Kiln Farm Milton Keynes, UK); helium carrier gas 1.9 mL min^{-1}) and a flame ionization detector at 300°C. An initial oven temperature of -30°C was used for 1 min, followed by a rate of 100°C min^{-1} to 40°C. The oven temperature was maintained at 40°C for 4 min, and was subsequently programmed at 2°C min^{-1} to 90°C, further at 4°C min^{-1} to 130°C, and finally at 8°C min^{-1} to 270°C.

Five concentrations of each of the compounds were analyzed in triplicate for calibration, allowing quantification of the compounds in the air phase.

For determination of air/liquid partition coefficients of each of the compounds, air phase concentrations (w/v) under equilibrium conditions were divided by the concentrations in the liquid phase (w/v).

Aroma analysis under mouth conditions

Aroma compounds were isolated in a model mouth system, the latest version of which was reported by VAN RUTH and ROOZEN (2000). Six millilitres of sample and 4 mL of artificial saliva were transferred to the sample flask (70 mL, 37°C) of the model mouth system. The headspace was flushed with purified nitrogen gas (100 mL min^{-1}). The aroma compounds released were trapped in Tenax (Tenax TA 60/80; Supelco, Bellefonte, PA) for 1 min. Isolation of the volatile compounds was carried out with a plunger making up-and-down screwing movements (52 cycles min^{-1}) to simulate mastication.

The aroma compounds trapped on Tenax TA were quantified by combined gas chromatography (Varian Star 3400

CX, JVA Analytical Ltd., Dublin, Ireland) and mass spectrometry (MS; Varian Saturn 3, JVA Analytical Ltd.). Desorption of the volatile compounds from Tenax was performed by a thermal desorption (220°C, 4 min) /cold trap (-120°C) device (Tekmar Purge and Trap 3000 concentrator, JVA Analytical Ltd.). Through a heated transfer line, the compounds were directed to the GC column (BPX5 capillary column; 60 m length, 0.32 mm i.d., and 1.0 µm film thickness; SGE). The initial oven temperature was 40°C for 4 min, and the temperature was subsequently programmed to 90° at 2°C min^{-1}, further to 130° at 4°C min^{-1}, and finally at 8°C min^{-1} to 270°C. Mass spectra were obtained with 70 eV electron impact ionization, while the mass spectrometer was continuously scanning from m/z 25 to 250 at a scan speed of 1 scan/s.

The identity of the compounds was confirmed by comparison with spectra and retention times of single authentic compounds and bibliographic data. Six concentrations of volatile compounds in pentane were analyzed in triplicate for calibration, allowing quantification of the compounds released in the model mouth.

For quantification of aroma release, the amounts of aroma compounds released in the model mouth (w) were divided by the amount present in the sample flask of the model mouth before aroma isolation (w) and multiplied by 100 (proportion released in percent).

Statistical analysis

Data of triplicate aroma measurements of the various emulsions were subjected to non-parametric statistical tests to determine significant differences between the samples: Friedman two-way analysis of variance by ranks and sign tests.

Correlations were determined by calculation of Pearson's product moment correlation coefficients (O'MAHONEY, 1986).

RESULTS AND DISCUSSION

The influence of βLG concentration and pH of 40% oil-in-water emulsions on the release of 20 odor active volatile compounds was studied under equilibrium and mouth conditions. Although the emulsions varied in βLG concentration (1% and 2%) and pH (3, 6, 9), their droplet size characteristics were kept constant (Table 1). A control sample of a mixture of oil and water in the same ratio as the emulsions was analysed as well (0% βLG). Twenty volatile compounds were selected according to their physico-chemical and odor properties (ARCTANDER, 1969; LIDE, 1997) and used throughout the experiments. They included six alcohols: 1-propanol (alcoholic), 1-butanol (fusel-like), 3-methylbutanol (fruity-winey), 2-pentanol (winey-ethereal), 1-hexanol (chemical-winey), and 2-nonanol (oily). Five ketones were added as well as four esters: 2-butanone (ethereal), diacetyl (buttery), 2-heptanone (fruity-spicy), 2-octanone (floral), 2-decanone (citrus-like), ethyl acetate (ethereal-fruity), propyl acetate (fruity), butyl acetate (ethereal-fruity), and ethyl butyrate (fruity). Furthermore, three aldehydes, one terpene and one sulfur compound were evaluated: hexanal (grassy), heptanal (fatty-rancid), octanal (fruity), α-pinene (pine-like), and dimethyl sulfide (cabbage-like).

Equilibrium conditions

The 20 compounds in the various samples were examined under equilibrium conditions without addition of saliva and their air/liquid partition coefficients were calculated. Firstly, the partition coefficients of the aroma compounds in 100% oil, 100% water, and in oil with 1% βLG, and water with 1% βLG were determined (Table 2). The partition coefficients of the compounds differed considerably. Highest values were obtained for dimethyl sulfide and lowest for octanal through-

Table 2 - Experimental air/liquid partition coefficients (K x 1,000) of 20 aroma compounds in oil, oil with 1% β-lactoglobulin, water, and in water with 1% β-lactoglobulin (n=3) as well as predicted air/liquid partition coefficients for oil/water mixtures with and without 1% β-lactoglobulin according to the method reported by BUTTERY *et al.* (1973).

Compound	Experimental partition coefficients				Predicted partition coefficients	
	Oil	Oil + 1% βLG	Water	Water + 1% βLG	Oil/water mixture	Oil/water +1% βLG mixture
Dimethyl sulfide	8.28	8.19	48.90	42.89	16.50	15.91
1-Propanol	4.19	3.84	1.15	1.16	1.62	1.61
Diacetyl	2.76	2.55	1.86	1.76	2.14	2.01
2-Butanone	3.33	3.27	3.74	3.62	3.57	3.47
Ethyl acetate	3.47	3.44	9.86	9.49	5.67	5.57
1-Butanol	1.16	1.12	0.86	0.84	0.96	0.93
2-Pentanol	0.87	0.87	1.49	1.38	1.16	1.12
Propyl acetate	1.22	1.21	13.07	11.90	2.67	2.63
3-Methyl-1-butanol	0.69	0.69	1.18	1.11	0.92	0.89
Ethyl butyrate	0.55	0.56	20.16	17.24	1.33	1.33
Hexanal	0.38	0.37	16.81	12.46	0.92	0.90
Butyl acetate	0.50	0.50	16.30	13.07	1.20	1.19
1-Hexanol	0.48	0.49	0.48	0.45	0.48	0.47
2-Heptanone	0.22	0.23	9.08	7.21	0.54	0.54
Heptanal	0.21	0.21	19.82	12.11	0.51	0.50
α-Pinene	0.23	0.23	1.49	0.18	0.47	0.20
2-Octanone	0.21	0.21	12.51	8.56	0.51	0.50
Octanal	0.02	0.02	19.36	5.40	0.04	0.06
2-Nonanol	0.59	0.58	0.61	0.59	0.60	0.59
2-Decanone	0.28	0.28	26.13	5.95	0.69	0.66
CV[a] [%]	9.4	8.1	5.4	2.6		

[a] Average coefficient of variance.

out the samples. The combination of vapor pressure of the pure compound and the affinity of the compound for the matrix determined the concentration in the air phase and consequently the air/liquid partition coefficient (VAN RUTH *et al.*, 2000). Most compounds showed higher partition coefficients when dissolved in water, except for 1-propanol, diacetyl and 1-butanol. These compounds are relatively hydrophilic. Addition of βLG resulted generally in increased retention of the hydrophilic compounds in the oil, and increased retention of nearly all compounds in water.

Secondly, the partition coefficients of the compounds in oil/water mixtures without βLG (0% βLG) and emulsions with 1% and 2% βLG were measured (Table 3). The βLG concentration affected the partition coefficients of the volatile compounds significantly (Friedman two-way analysis of variance by ranks, $P < 0.01$). Friedman two-way analysis of variance by ranks showed that generally highest values were observed for the oil/water mixture with 0% βLG, followed by the emulsions with 1% and 2%, respectively. When comparing predicted partition coefficients (Table 2) and experimental values for the oil/water mixture without βLG (Table 3, pH6), it appears that Buttery's law (BUTTERY *et al.*, 1973) predicted quite well the partition coefficients of the compounds in the mixture. However, when comparing the predicted val-

ues based on oil with 1% βLG and water with 1% βLG (Table 2) with experimental values of the emulsion with 1% βLG (Table 3, pH6), generally the experimental values were slightly lower. This result indicates that the formation of the interface in the emulsion increased the retention of the compounds. The pH of the samples had no overall effect on the partition coefficients (Friedman two-way analysis of variance by ranks, $P < 0.05$). These results are in contrast with the effect of the pH on βLG in water as reported by JOUENNE and CROUZET (2000). The present authors also have found different results for βLG in water: the retention of volatiles was higher at pH6 and pH9, and lower at pH3 (VAN RUTH and VILLENEUVE, 2002).

The effect of the βLG depended on the chain length of the compound, as well as on their functional groups. The partition coefficients of ketones as a function of chain length is demonstrated in Fig. 1. In water, partition coefficients increased with chain length, whereas water with 1% βLG showed a less steep slope and a further decrease for 2-decanone. Table 4 shows the Pearson's correlation coefficients of the chain length and the air/liquid partition coefficients of the homologous series of alcohols, aldehydes, ketones and esters in the various matrices. The correlation coefficients for water showed that partition coefficients of ketones, esters and, to lower extent aldehydes, increased linearly with higher chain length. Alcohols did not demonstrate a significant correlation; obviously the hydrophilic group

Table 3 - Air/liquid partition coefficients (K x 1,000) of 20 aroma compounds in oil-in-water emulsions varying in β-lactoglobulin concentration and pH (n = 3).

	Compound	0% βLG			1% βLG			2% βLG		
		pH3	pH6	pH9	pH3	pH6	pH9	pH3	pH6	pH9
1	Dimethyl sulfide	15.70	15.76	15.59	11.50	12.32	13.99	14.04	15.93	14.85
2	1-Propanol	1.96	1.97	1.91	1.26	1.27	1.27	1.36	1.39	1.27
3	Diacetyl	2.10	2.11	2.10	1.98	1.98	1.72	1.58	1.59	1.51
4	2-Butanone	3.47	3.47	3.39	3.37	3.46	3.61	3.39	3.19	3.69
5	Ethyl acetate	5.67	5.67	5.61	4.45	4.56	4.80	3.85	4.26	4.77
6	1-Butanol	1.20	1.13	1.20	0.82	0.81	0.81	0.77	0.82	0.88
7	2-Pentanol	1.33	1.30	1.32	1.03	1.00	0.95	0.81	0.87	1.01
8	Propyl acetate	2.61	2.65	2.66	2.46	2.51	2.56	2.32	2.39	2.53
9	3-Methyl-1-butanol	1.25	1.25	1.27	0.86	0.87	0.87	0.99	1.03	1.20
10	Ethyl butyrate	1.35	1.35	1.34	1.21	1.22	1.22	0.99	1.00	0.99
11	Hexanal	0.85	0.85	0.80	0.83	0.77	0.69	0.50	0.32	0.14
12	Butyl acetate	1.25	1.23	1.24	1.04	1.05	1.06	1.01	1.01	1.01
13	1-Hexanol	1.22	1.22	1.22	0.60	0.61	0.62	0.65	0.68	0.67
14	2-Heptanone	0.58	0.58	0.57	0.46	0.47	0.47	0.42	0.41	0.40
15	Heptanal	0.51	0.50	0.50	0.34	0.33	0.33	0.31	0.28	0.19
16	α-Pinene	0.59	0.58	0.59	0.38	0.39	0.39	0.43	0.35	0.36
17	2-Octanone	0.50	0.52	0.53	0.30	0.29	0.30	0.27	0.28	0.27
18	Octanal	0.10	0.10	0.10	0.07	0.09	0.07	0.05	0.06	0.04
19	2-Nonanol	1.46	1.46	1.46	0.60	0.60	0.60	0.60	0.60	0.60
20	2-Decanone	0.69	0.69	0.69	0.30	0.30	0.30	0.29	0.29	0.29
	CV[a] [%]	10.0	5.8	8.3	2.5	3.9	3.8	8.6	5.7	13.6

[a] Average coefficient of variance.

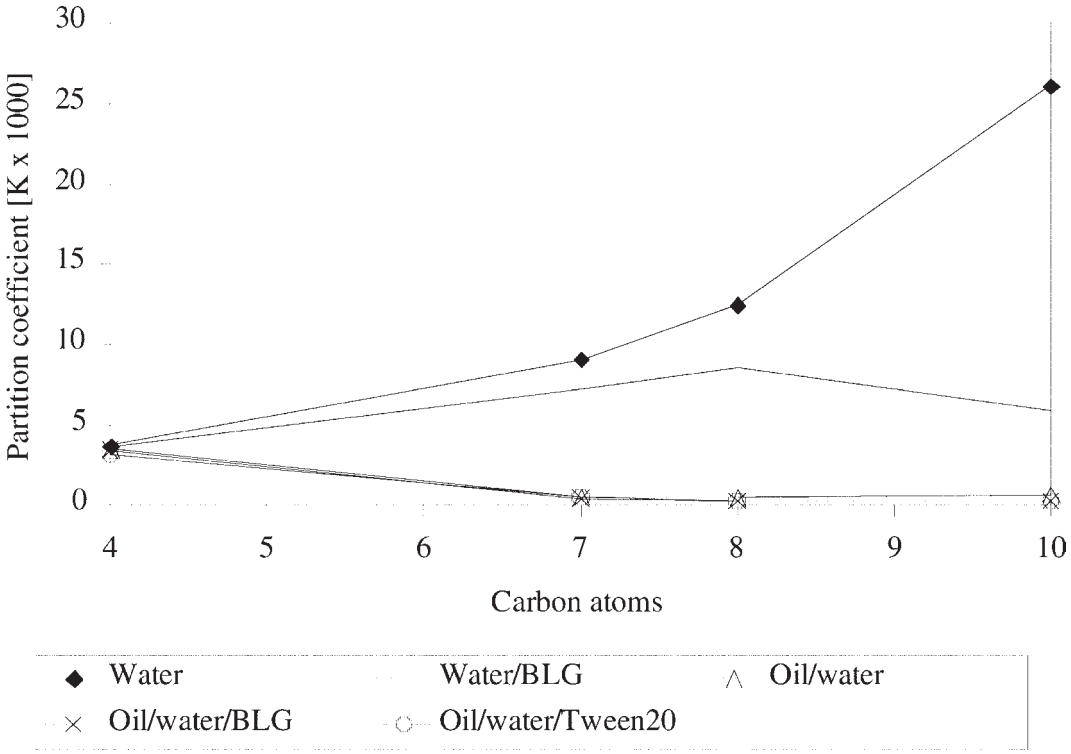


Fig. 1 - Partition coefficients of ketones (listed in Table 2) in various matrices as a function of chain length.

Table 4 - Linear relationships between the chain length of homologous series of alcohols, aldehydes, ketones, and esters and their air/liquid partition coefficients in various matrices: Pearson's product moment correlation coefficients.

Compound group	Water[a]	Water/βLG[a] (99:1)	Oil/water[b] (40:60)	Oil/water/βLG emulsion[b] (40:59:1)	Oil/water/Tween 20 Emulsion[c] (40:59:1)
Alcohols	-0.47	-0.50	-0.28	-0.79	-0.81
Aldehydes	+0.77	-0.90	-0.99	-0.99	-0.99
Ketones	+0.94	+0.87	-0.85	-0.89	-0.89
Esters	+0.97	+0.95	-0.98	-0.99	-0.91

[a] Based on data from VAN RUTH and VILLENEUVE (2002).
[b] Based on present data.
[c] Based on data from VAN RUTH *et al.* (2002).

mainly determines the properties of the molecule in water. When comparing the correlation coefficients for water with those for water with βLG, relatively slight differences for alcohols, ketones and esters were observed. However, the positive correlation coefficient for aldehydes in water changed to a negative correlation coefficient for water with βLG. These results show that aldehydes demonstrat-

ed a clear increased affinity for βLG with increasing chain length. When changing the matrix from water to an oil/water mixture, the hydrophobic character of especially the aldehydes, ketones and esters is shown, by strong negative correlations between chain length and partition coefficients. Especially the larger compounds of these groups preferred the oil to the water phase with decreased headspace concentration as a result. The functional group of the alcohols still played a major role, as alcohols yielded a low correlation coefficient between chain length and partition coefficients. This, however, changed as soon as the system was emulsified, which involved a tremendous increase in surface area. A considerable negative correlation was found in emulsified oil/water systems with both βLG and Tween 20. In these systems obviously the larger alcohols were retained to a greater extent than their lower molecular weight counterparts. The fact that an interface existed with a polar and a non-polar phase at both sides obviously played a role independent of the type of emulsifier. The other compound groups already showed a strong negative correlation for the oil/water mixture itself and remained the same for the emulsified system.

When comparing the air/liquid partition coefficients of Tween 20 and βLG emulsions, which only differ in emulsifier, it was shown that the air/liquid partition coefficients of the compounds were consistently higher for the Tween emulsions (Fig. 2). βLG reduced headspace concentrations of the volatile compounds

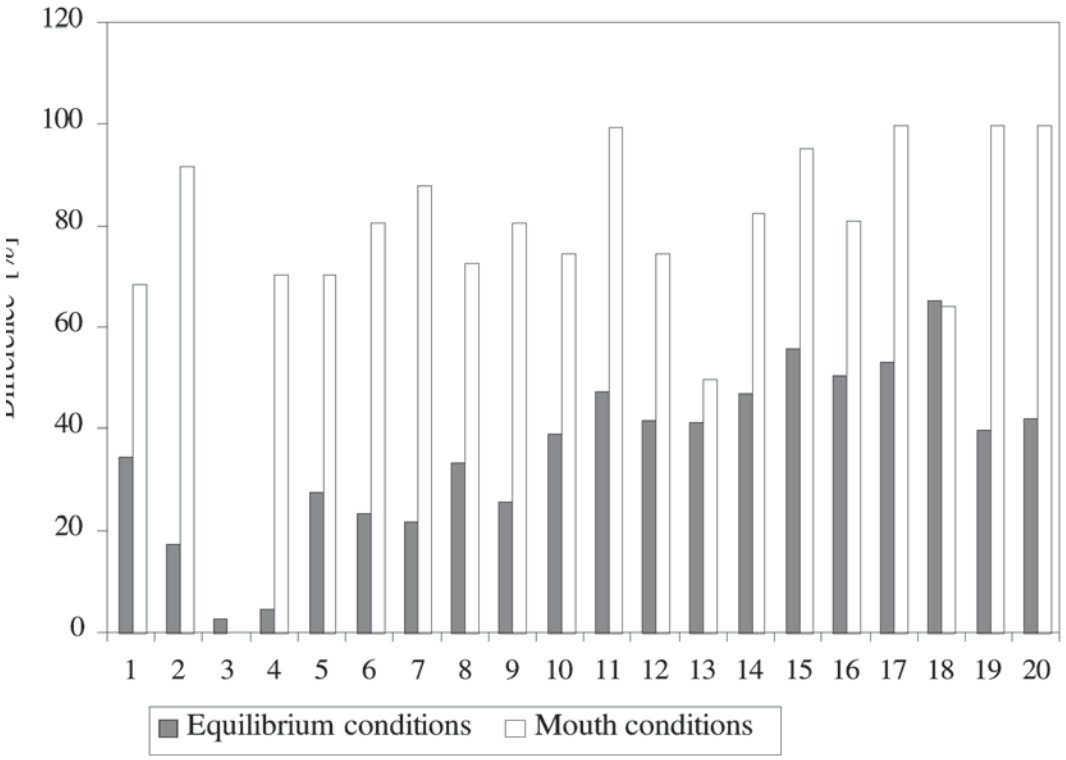


Fig. 2 - Difference in release of 20 aroma compounds from Tween 20 and β-lactoglobulin emulsions under equilibrium and mouth conditions (Tween - β-lactoglobulin). Numbers refer to compounds in Table 3.

to a much greater extent than the Tween 20. The emulsions had the same droplet size characteristics. The difference between the emulsions with the two emulsifiers increased with increasing chain length, which demonstrated the high affinity of the larger compounds for βLG. The present results confirm data of CHARLES *et al.* (2000), which showed increased selective retention of larger sized volatile compounds in βLG emulsions in comparison with α-albumine emulsions.

When looking at the single compounds, hexanal, 2-nonanol and 2-decanone showed the largest decrease in partition coefficients when changing from 0% to 2% βLG, i.e. a decrease of 62, 59 and 58%, respectively. In addition, 2-pentanol (33%), 1-hexanol (43%), heptanal (44%), α-pinene (40%), 2-octanone (46%) and octanal (40%) showed a considerable decrease. Affinity for βLG appeared to be more affected by the chain length of the compound than by their functional groups. The retention of the compounds stated above in the emulsion with 2% βLG (average of 47%) is very similar to their retention in water with 2% βLG (average of 46%; VAN RUTH and VILLENEUVE, 2002). However, absolute partition coefficients of the compounds were considerably lower for the emulsion, because of their affinity for the oil phase. From these results it can be concluded that βLG exerts a considerable effect on equilibrium headspace concentrations of volatile compounds in emulsions. These concentrations relate to the orthonasal route which links to the odor of a food.

The experiments described above were all conducted on the pure emulsions. To allow direct comparison with the mouth conditions studied, additional experiments with the 1% βLG and 2% βLG emulsion (pH6) were performed to determine the air/liquid partition coefficients of the compounds with addition of saliva. The partition coefficients of the compounds with addition of artificial saliva are presented in Table 5. As addition of saliva also changed the pH from pH6 to pH7, a 1% βLG emulsion at pH7 was analysed, to distinguish the effect of the saliva components and the effect of the change in pH (Fig. 3). It is obvious that the pH change had a minor effect compared to the overall effect of saliva. Lower partition coefficients were found for relatively hydrophilic compounds, such as 2-propanol and diacetyl, whereas compounds with a more hydrophobic nature, e.g. 2-nonanol and 2-decanone demonstrated an increase of ca. 60%. These changes are likely to be related to the higher proportion of water in the total matrix (emulsion+saliva) and are in agreement with previous studies (ODAKE *et al.*, 1998; VAN RUTH *et al.*, 2001). However, it is remarkable that each of the aldehydes showed a decrease in the par-

Table 5 - Air/liquid partition coefficients (K x 1,000) of 20 aroma compounds in oil-in-water emulsions varying in β-lactoglobulin concentration with addition of artificial saliva (ratio sample:saliva is 60:40, pH=7, n=3).

	1% βLG + saliva	2% βLG + saliva
Dimethyl sulfide	13.01	12.87
1-Propanol	1.05	0.69
Diacetyl	0.85	0.82
2-Butanone	3.28	2.77
Ethyl acetate	4.68	5.42
1-Butanol	0.86	0.93
2-Pentanol	0.78	1.02
Propyl acetate	2.93	3.04
3-Methyl-1-butanol	0.99	0.99
Ethyl butyrate	1.43	1.63
Hexanal	0.26	0.29
Butyl acetate	1.31	1.37
1-Hexanol	0.89	1.11
2-Heptanone	0.64	0.35
Heptanal	0.31	0.34
α-Pinene	0.64	0.67
2-Octanone	0.44	0.44
Octanal	0.08	0.09
2-Nonanol	0.98	0.98
2-Decanone	0.50	0.49
CV[a] [%]	10.2	12.5

[a] Average coefficient of variance.

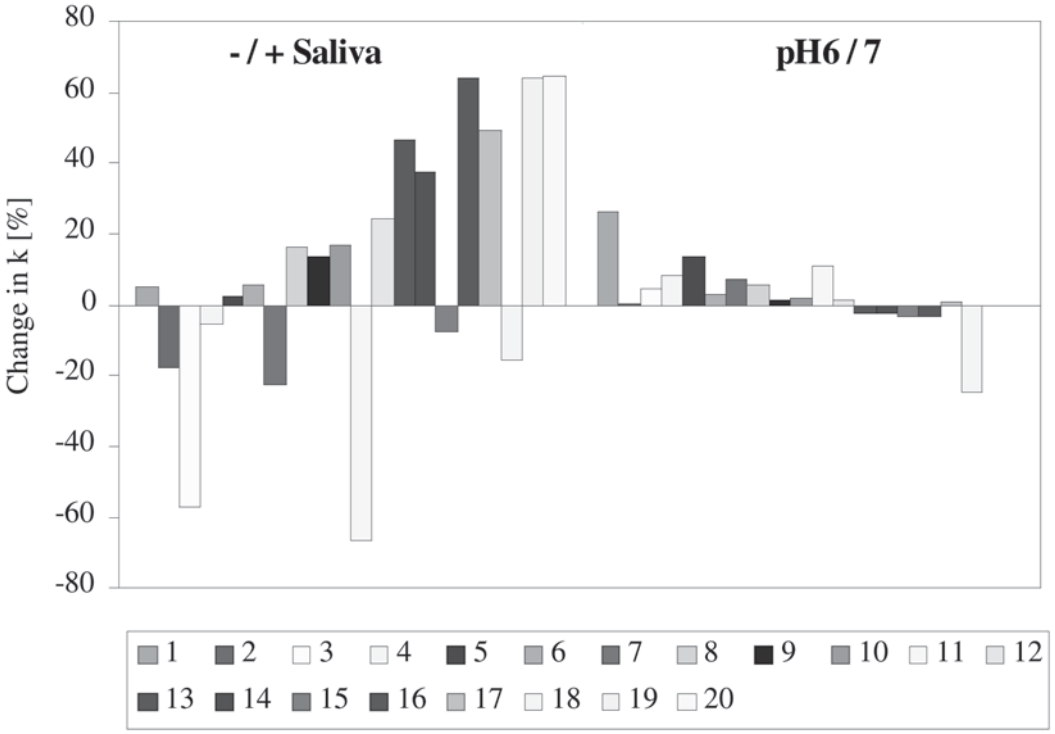


Fig. 3 - Change in air/liquid partition coefficients of 20 compounds in a 40% oil-in-water emulsion emulsified with 1% β-lactoglobulin by addition of artificial saliva and by the change in pH only associated with addition of artificial saliva.

tition coefficients with addition of saliva. From their hydrophobicity an increase in a more polar matrix would be expected. The decrease observed is likely to be due to other components of the artificial saliva than water. The apparent concentration of the aldehydes may be reduced by binding to the proteins in the saliva, such as mucin and α-amylase (VAN RUTH *et al.*, 2001).

Mouth conditions

The release of the 20 volatile compounds from the various emulsions was studied in the model mouth system. Emulsions with 1% βLG pH3, 6 and 9, as well as an emulsion with 2% βLG pH6 were evaluated. The proportions of the compounds released are presented in Fig. 4. Large differences were observed between compounds; proportions released in 1 min varied between 0.1 and 3% of the amount initially present in the emulsion. The proportions of the various compounds released from the emulsions related reasonably well to the partition coefficients of the compounds in the pure emulsions, but related slightly better to the partition coefficients of the compounds in emulsion/saliva mixtures. For instance for the 1% βLG emulsion (pH6), the correlation coefficients were r = 0.860 and r = 0.875, respectively. The thermodynamic component dictated the differences in release between compounds to a great extent.

The βLG concentration had no significant effect on the release from the emulsions at pH6 (Sign tests, $P < 0.05$) and the pH did not affect the overall release in the emulsion consisting of 1% βLG (Friedman two-way analysis of variance by ranks, $P < 0.05$).

The minor effect of βLG on volatile release under mouth conditions agrees with the equilibrium data of the emulsion when saliva was added. Although the concentration βLG had a considerable effect on retention of volatiles in pure emulsions under equilibrium conditions, this effect was extinguished by the addition of saliva. From the data it can be concluded that, the βLG concentration affected neither the thermodynamic nor the kinetic component of the release to a great extent. Although the concentration of βLG had no considerable effect, the presence of βLG had quite an effect, which can be shown by comparison with another emulsifier. The release from βLG and Tween 20 emulsions under mouth conditions is compared in Fig. 2. Release from the Tween emulsions was consistently higher than release from the βLG emulsions. The difference in release can only partially be attributed to the thermodynamic component, which is reflected by the difference under equilibrium conditions (Fig. 2). As a result, βLG must have affected the kinetic component of aroma release and thus the resistance to mass transfer.

The effect of the pH on the aroma released is in line with the thermodynamic

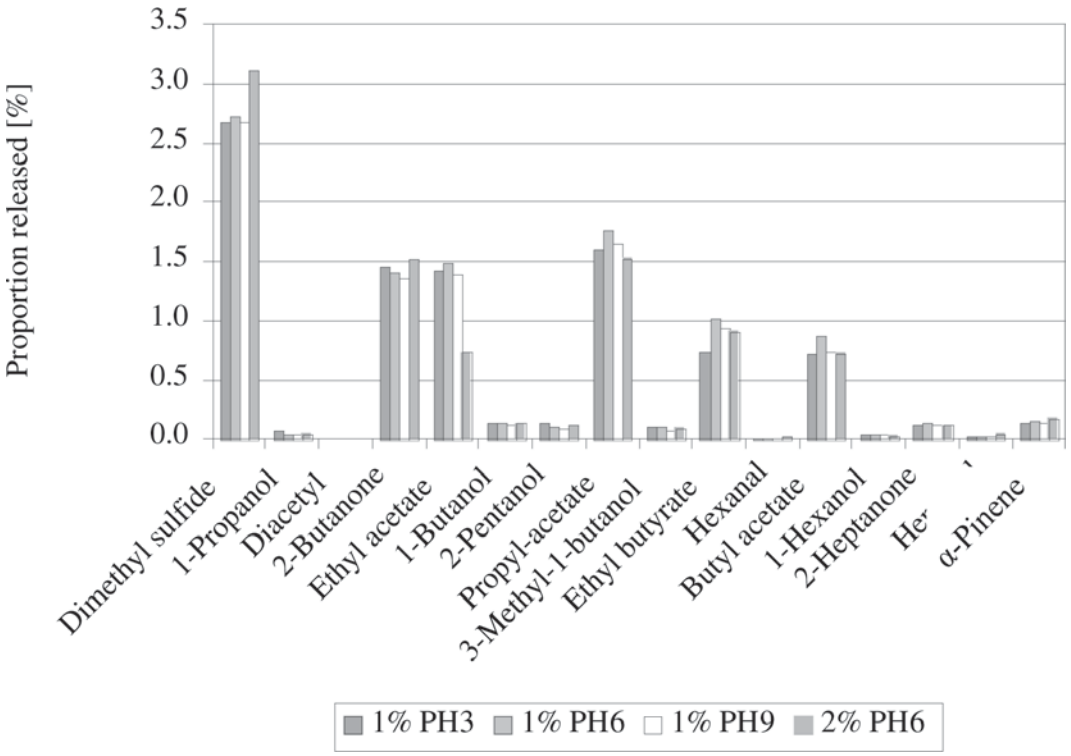


Fig. 4 - Proportions of aroma compounds released from a 40% oil-in-water emulsion under mouth conditions as a function of β-lactoglobulin concentration and pH. Numbers refer to compounds in Table 3, compounds with release <0.1% not shown.

data presented in the previous section: no effect of pH was shown under equilibrium conditions. Obviously, the pH did not alter the resistance to mass transfer, either. This is partially due to the buffering capacity of the artificial saliva affecting the final pH of the emulsions.

βLG as such affected the release of volatile compounds from oil-in-water emulsions. However, the concentration of βLG and the pH hardly affected the release, which can be attributed to a large extent to the influence of saliva. The release under mouth conditions is expected to relate to the retronasal perception of the compounds, i.e. the aroma perceived during eating.

CONCLUSIONS

The presence of βLG affected release of volatile flavour compounds from oil-in-water emulsions both under equilibrium and mouth conditions. The βLG concentration reduced the equilibrium headspace concentrations mainly of larger sized aroma compounds. The effect of βLG decreased when artificial saliva was added to the emulsion. Under mouth conditions, neither the concentration of βLG nor the pH exerted a significant effect on the release of volatiles. As a consequence, βLG concentrations could have an effect on the odor perception of the compounds in oil-in-water emulsions. However, it is likely that this parameter has a rather limited effect on the aroma perceived during eating.

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PAPER

EFFECT OF DIETARY VEGETABLE LIPIDS ON FUNCTIONAL AND SENSORY PROPERTIES OF CHICKEN EGGS

EFFETTO DI LIPIDI VEGETALI DELLA DIETA SULLE PROPRIETÀ FUNZIONALI E SENSORIALI DELLE UOVA DI GALLINA

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ABSTRACT

Trials were carried out with brown laying hens to study the effects of dietary addition of 2% palm butter (Palm), grape seed oil (Grape), flax seed oil (Flax), dried *Schizochytrium* sp. marine algae (Algae) and lard (Control) on egg quality, and on the sensory and functional properties of eggs. The weight of egg and of its components and the Haugh index were not affected by the type of lipid supplementation. Algae yolks showed a more intense colour attributable to carotenoids contained in *Schizochytrium* sp. Consistency index and flow behaviour index of yolk ho-

RIASSUNTO

Scopo della ricerca è stato studiare l'effetto dell'aggiunta ad una dieta per galline ovaiole produttrici di uova a guscio colorato del 2% di olio di palma (Palm), o olio di vinacciolo (Grape) o olio di semi di lino (Flax) o alga marina *Schizochytrium* sp. (Algae) o lardo (Control) sulla qualità e sulle proprietà funzionali e sensoriali delle uova. Il peso dell'uovo e delle sue parti, come pure l'indice di Haugh non sono stati influenzati dal tipo di integrazione lipidica della dieta. I tuorli delle uova del gruppo Algae erano colorati più intensamente probabilmente per il maggior contenuto di carotenoidi pre-

- Key words: chicken egg, dietary lipid supplements, flaxseed oil, functional and sensory properties, grape oil, palm butter -

mogenates were significantly affected by the dietary supplementation, whereas emulsion properties and baking properties of Madeira cakes were not affected by dietary treatments. The taste of hard-boiled eggs was not influenced by flax, palm, grape and lard supplements, whereas the odour of Grape eggs had the lowest score (no anomalous taste). As for taste, Grape and Flax Madeira cakes had the lowest and the highest score, respectively. The different lipid vegetable sources, with the exception of flax seed oil, did not depreciate the sensory characteristics of eggs and the baked products.

senti in *Schizochytrium* sp. Il trattamento dietetico della gallina modifica gli indici di consistenza e del comportamento del flusso degli omogenati di tuorlo, mentre non ha effetto sulle proprietà delle emulsioni e sulle caratteristiche dei prodotti da forno. Il sapore delle uova sode non è stato influenzato dagli integratori lipidici mentre per l'odore le uova del gruppo Grape hanno ottenuto la miglior valutazione degli assaggiatori (nessun odore anomalo). Le torte Madeira dei gruppi Grape e Flax hanno ottenuto rispettivamente il valore più basso (minor sapore anomalo) e più alto (più forte sapore anomalo) della scala di valutazione del sapore. Le differenti fonti lipidiche vegetali, con l'eccezione dell'olio di semi di lino, non deprezzano le caratteristiche sensoriali delle uova o dei prodotti da forno a base di uova.

INTRODUCTION

The egg is an important ingredient for the preparation of several food products such as noodles, mayonnaise, cakes and candy (FORSYTHE, 1963). It may be considered a multifunctional ingredient since it can perform several simultaneous functions by its coagulating, foaming, emulsifying, colouring, flavouring and anticrystallizing properties as well as delivering important nutrients (YANG and BALDWIN, 1995).

Many trials have been carried out to improve the nutritional properties of eggs. Eggs have been enriched with n-3 PUFA such as α-linolenic acid (CASTON and LEESON, 1990; CHERIAN and SIM, 1991) or eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids (FARRELL, 1994; HERBER and VAN ELSWYK, 1996; MELUZZI *et al.*, 1997a) and with vitamins (JIANG *et al.*, 1994; SURAI *et al.*, 1997; MELUZZI *et al.*, 2000).

Events such as bovine spongiform encephalopathy, dioxine poisoning and recurring Salmonella infections have led to changes in the feed formulation of laying hen diets. Animal meals have been banned and other animal ingredients such as lipid supplements are not wanted by large scale-distributors and consumers. Therefore, at the present, in Italy laying hen diet is formulated by using only vegetable sources. Even if the effects of different lipid supplements on egg composition are known, there is a lack of information about the effects of these sources on the sensory and functional properties of eggs and egg products which are more and more considered by the food industry for a better utilisation of eggs. Furthermore, the fortification of eggs through hen dietary manipulation, such as enrichment with n-3 PUFAs, may give a fishy flavour to the eggs (JIANG *et al.*, 1992, VAN ELSWYK *et al.*, 1995, MELUZZI *et al.*, 1997b) depending on the dose or on the type of the dietary lipid supplements. A reduc-

tion of overall acceptability and off-flavours was also detected by LEESON *et al.* (1998) when flax seed or vitamin E were added to the hen diets.

The aim of our study was to investigate the effects of vegetable lipid sources with quite different fatty acid (FA) compositions, either saturated FA, or n-6 PUFA, or n-3 PUFA or long chain n-3 PUFA, on the sensory and functional properties of eggs.

MATERIALS AND METHODS

Experimental design

One hundred and twenty 24-week-old Hyline brown laying hens, divided into 5 groups, were fed isoenergetic diets with the addition of 20 g/kg vegetable lipid supplements, of quite different fatty acid profiles: palm butter (Palm) which has a high level of saturated and monounsaturated fatty acids (45% palmitic acid and 38% oleic acid); grape seed oil (Grape) which contains more than 68% linoleic acid (n-6 PUFA) and a good amount of oleic acid (18%); flax seed oil (Flax) containing approx. 41% α-linolenic acid (n-3 PUFA) and 29% oleic acid and dried *Schizochytrium* sp. marine algae (Algae) whose lipid content (53%) was particularly rich in docosahexaenoic acid or DHA (41%). The supplement of the control group (Control) was lard containing oleic (41%), palmitic (27%), stearic (13%) and linoleic (12%) acids. The diets and the characteristics of the lipid supplements were reported by MELUZZI *et al.* (2001).

Egg quality evaluation

Four and eight weeks after the beginning of the dietary treatment, eggs collected for three consecutive days were used to evaluate yolk, albumen and shell weights and all the eggs collected in one day were assessed for the yolk index (height:width ratio; FUNK, 1948) and Haugh index to evaluate the firmness of the albumen. The Haugh index was determined according to the following equation:

$$\text{Haugh Units} = 100 * \log (h - 1.7 * P^{0.37} + 7.57)$$

where
H = height of spread egg white in mm;
P = egg weight in g.

The yolk colour was evaluated according to the CIE L* a* b* system using a Minolta colorimeter CR 300 (Minolta Camera Co., Ltd, Osaka, Japan).

Egg functional properties

Six weeks from the beginning of the dietary treatment the eggs were collected for three consecutive days to evaluate the functional properties of the yolk since the different fatty acid composition of the diets presumably only affected lipid components of the egg, i.e. yolk. Three pools of 5 yolks each were used to prepare homogenates and 10 mL per pool were used to measure the yolk viscosity by using the Stress Tech Rheometer (Reologica Instruments, AB, Lund, Sweden) equipped with a bob and cup system (CC25) at 20°C. Apparent viscosity was determined from the flow curves evaluated in the shear rate range between 0.1220 to 87.67 s^{-1}. Data from the shear stress (s) with increasing shear rate (γ) were elaborated following the power law equation ($\tau = K^* (\gamma)^n$). The values of K (Consistency Index, Pa * s^n) and n (Flow Behaviour Index, dimensionless) reported, are the average of three measurements. Three pools of 4 yolks each were used to prepare homogenates and 15 g yolk homogenate +36 g corn oil +39 g water were emulsified by an ultraturrax for 70 sec at increasing speed from 9,000 to 11,000 RPM. At 60 and 120 min the drainage was measured to evaluate the amount of non-bound emulsion water and the water released by the emulsion, respectively. The stability of emulsion was then calculated (SCHOLTYSSEK,

1995). Baking quality of the eggs was evaluated in Madeira cakes prepared according to the recipes of SCHOLTYSSEK *et al.* (1994). The dough density was calculated as the ratio of the weight of 100 mL of dough with respect to volume. Sixty grams of dough were put in steel rings (i.d.: 100 mm) and baked in a ventilated oven (SMEG, Alpha 30HX) at 200°C for 15 min. Afterwards the cooking loss of cake volume was measured. The volume was calculated by filling a container with rape seeds, then the container was emptied and filled again with the cake and the rape seeds. The volume of the remaining seeds was considered as the volume of the cake. In the middle of the cakes two rectangular samples (20 x 50 mm x height of the baked cake) were carefully cut and the maximum shear load was measured using a dynamometer Instron Universal Testing Machine, Mod. 4301, (U.T.M., Instron International Limited, High Wycombe, England) equipped with a load cell of 1 kN at a crosshead speed of 3.33 mm/s.

Sensory evaluation

The organoleptic properties of the eggs and Madeira cakes made with eggs obtained from hens receiving dietary lipid supplement such as grape seed oil, palm butter, flax seed oil and lard were evaluated. The eggs from the Algae group were not considered since both algae and flax seed oil contain n-3 PUFA even if the chains of the fatty acids have different lengths.

Hard-boiled eggs were prepared as follows: eggs were stored at 4°C for 15 days after laying, then cooked by placing in boiling water for 8 min. Once cooled to an external temperature of about 40°C, the eggs were peeled and the yolks separated; they were then divided into 4 pieces and stored in hermetically closed containers.

Madeira cakes were prepared according to SCHOLTYSSEK *et al.* (1994). To determine the dietary effect on the taste and odour of the eggs and on the taste of the Madeira cake, thirty untrained panelists were recruited from students and staff of the Course of Food Science and Technology (University of Bologna, Italy). They were asked to rate any taste or odour other than "typical egg taste or odour" on a 7-point hedonic scale (1= no anomalous taste or odour; 7= strong anomalous taste or odour) according to LAWLESS and HEYMANN (1998). Mineral water and unsalted crackers were allowed for mouth cleansing between samples. The tasting session was conducted in standard grey individual booths equipped with constant illumination in a sensory evaluation facility according to ISO 8589 (1988).

Statistical analysis

Data concerning the functional properties tested, carried out on fresh, cooked eggs, and Madeira cakes, were submitted to one way ANOVA using the General Linear Model procedure of SAS (1985), considering the dietary treatment as fixed effect. For functional data mean differences were separated by Student-Newman Keuls test, whereas sensory data were tested by means of Waller-Duncan k-ratio multiple comparison test.

RESULTS AND DISCUSSION

Egg quality

The weight of egg and its components was not affected by the diet either after 4 or 8 weeks of treatment (Table 1). HALLE (1999), feeding broiler breeder hens diets with the addition of 25 or 50 g/kg of palm butter or safflower, did not find any statistically significant effects on egg mass, yolk and albumen. Dietary levels of ground and whole flaxseed up to 10% were not found to affect egg weight (EDER *et al.*, 1998). On the contrary CASTON *et al.* (1994) detected decreases in egg

Table 1 - Effects of different dietary lipid supplements on the weight of whole egg and egg components after 4 and 8 weeks of treatment.

Group	Whole egg g	Yolk g	Albumen g	Shell g	Haugh index u	Yolk index
4 weeks						
Control	65.50	16.10	43.38	6.02	92.14	0.471
Palm	63.57	15.54	41.97	6.06	91.56	0.466
Grape	65.72	15.84	43.80	6.09	91.74	0.482
Flax	66.29	15.98	44.25	6.06	92.07	0.470
Algae	63.90	15.56	42.41	5.93	91.04	0.481
EMS	28.10	1.56	20.65	0.22	30.67	0.002
D,F,	111	111	111	111	109	109
8 weeks						
Control	64.70	15.71	42.86	6.13	90.90	0.470
Palm	63.12	15.57	41.51	6.05	90.88	0.477
Grape	64.64	15.71	42.64	6.02	91.83	0.485
Flax	65.97	15.97	43.87	6.12	90.10	0.486
Algae	64.59	16.03	42.46	6.10	90.24	0.482
EMS	23.23	1.76	17.13	0.22	37.09	0.0006
D,F,	103	102	102	102	100	99

weights after feeding 10 and 20% ground flax seed and SCHEIDELER and FRONING (1996) also reported a reduction in yolk weight after feeding 5 or 15% ground or whole flax seed. A decrease in egg weight was also observed by HERBER and VAN ELSWYK (1996) in response to 4.8% marine algae in the hen's diet. VAN ELSWYK *et al.* (1994) hypothesised that the decrease in yolk weight in response to the n-3 PUFA diet was related to the influence of n-3 PUFA on hepatic lipid metabolism and particularly to the decrease in the level of circulating lipids of birds which are the source of yolk lipids. In our study the lack of differences in egg size might be due to the low proportions of lipid supplements in the hen's diet.

Yolk index was quite stable and was not affected by the duration or the type of dietary treatment (Table 1). Haugh index, an indicator of albumen firmness, was not affected by the type of lipid supplementation of the diet (Table 1).

The results of yolk colour (Table 2) showed lower lightness values (L * axis) from the Algae group, compared to the Control, and a statistically higher redness value (a * axis) than that of all other groups. The more intense colouring of the Algae yolk is attributable to carotenoids, specifically canthaxanthin and β-carotene, occurring in *Schizochytrium* sp. (HERBER and VAN ELSWYK, 1996).

Functional properties

The values of dough density and baking properties of Madeira cakes ranged

Table 2 - Effects of different dietary lipid supplements on yolk colour.

Groups	L *	a *	b *
Control	53.21 A	0.303 b	41.67
Palm	52.32 AB	0.397 b	40.80
Grape	52.44 AB	0.206 b	41.44
Flax	52.20 AB	0.142 b	41.07
Algae	51.18 B	0.980 a	41.35
EMS[1]	3.03	0.71	4.73
D.F.	108	108	108

[1] error mean square.
Means with different letters in the same column differ significantly for $P < 0.05$, if small, and for $P < 0.01$, if capital.

little among groups and the differences were not significant. The Control eggs had a lower dough density, a lower cake volume and a higher baking loss (Table 3). The emulsifying properties of eggs, important for preparation of mayonnaise, sauces and confectionery, were not significantly affected by the different dietary treatments. Even if eggs from Flax and Algae showed a higher drainage volume at 60 min, the stability of the emulsion did not change among groups (Table 3). The different dietary lipid supplements used had a rather different fatty acid composition and the eggs had a fatty acid composition corresponding to that of the lipid supplements: grape seed oil, flaxseed oil and algae promoted the yolk deposition of linoleic, α-linolenic and docosahexaenoic acids, respectively, whereas palm oil did not increase the yolk content of palmitic acid despite its high level in the Palm diet (MELUZZI *et al.*, 2001). Probably the characteristics of the fatty acids do not affect the amount of lecithin in the yolk which is the best emulsifier of the yolk. These results are in accordance with our previous study in which only vegetable sources of n-3 PUFA were tested (TALLARICO *et al.*, 2001).

Rheological measurements showed a non-Newtonian behaviour of all the egg samples, with a shear-dependent viscosity that decreased with increasing shear rate. The apparent viscosity was higher in the Control eggs and lower in the Algae ones. Diet significantly affected both the consistency index (K) and the flow behaviour index (n) of the egg yolks (Table 4). Fatty acid composition could be responsible for the lower consistency index of the Algae and Flax samples which were significantly higher in long chain n-3 polyunsaturated fatty acids (MELUZZI *et al.*, 2001). Control and Grape homogenates had higher K values that could contribute to the stability of the emulsion.

Sensory evaluation

The effects of Palm, Grape, Flax and Lard diets on the sensory properties of

Table 4 - Consistency Index (K) and flow behavior index (n) of yolk homogenates (mean ± SD).

Groups	K (Pa.s^n)	n
Control	1.81 a±0.15	0.920 b± 0.006
Palm	1.51 b±0.09	0.930 b± 0.043
Grape	1.70 a±0.06	0.940 b± 0.017
Flax	1.31 b±0.03	0.940 ab± 0.004
Algae	1.20 b±0.07	0.960 a± 0.003

Means with different letters in the same column differ significantly $P < 0.05$.

Table 3 - Functional properties of yolk emulsion and Madeira cakes (mean ± SD).

Groups	Emulsion		Madeira cakes			
	Relative drainage[1] mL	Stability[2]	Dough density g/100 mL	Volume mL	Weight g	Baking loss %
Control	19.3±1.8	5.0±0.7	91.5±1.8	107±1	48.3±0.1	19.49±0.15
Palm	17.0±1.4	4.5±0.7	93.3±0.7	119±2	48.9±0.1	18.58±0.08
Grape	17.5±2.8	5.0±0.7	92.4±0.3	114±1	49.4±0.8	17.73±1.40
Flax	22.0±1.4	4.8±0.4	93.2±1.1	115±4	49.4±0.6	17.61±1.30
Algae	21.0±2.1	4.5±0.7	92.2±0.7	113±1	49.9±0.3	16.93±0.55

[1] (Drainage volume at 60 min x 100)/emulsion volume.
[2] Relative drainage at 120 min – Relative drainage at 60 min.

hard-boiled eggs and Madeira cakes are reported in Table 5. For clarity of presentation, we refer to Palm, Grape, Flax and Lard "samples", indicating eggs and cakes obtained from diets with the corresponding supplement. Panelists were unable to distinguish a difference ($P > 0.05$) in taste among the four different hard-boiled egg samples, the mean values ranging from 3.4 (Palm) to 4.0 (Lard). Conversely, there was a statistically significant difference ($P = 0.00002$) in egg odour, with the Grape sample having the lowest score (3.1). AHN *et al.* (1995) reported that the flavour scores of eggs from diets supplemented with 3% α-linolenic acid (the main fatty acid of flax seed) were higher, therefore less favourable, than those from the Control group. On the contrary, LEESON *et al.* (1998) found no significant difference in aroma, flavour, and overall acceptability of boiled eggs from hens whose diets contained 10 or 20% flax seed. Moreover, an off-flavour was slightly perceived in eggs from flax-fed birds during taste-panel studies involving fresh and stored eggs (CASTON *et al.*, 1994). HALLE (1999), feeding hens diets with the addition of 2.5 or 5% palm butter, gave no mention of modifications in egg flavour or odour. As regards the taste evaluation of Madeira cakes, the panelists identified a significant difference among samples ($P < 0.01$). In particular, the Grape sample obtained the lowest score (3.2) and was considered the one with the least anomalous taste, whereas the Flax sample had the highest score (5.1). These data partially confirmed the results obtained from hard-boiled eggs. In fact, Grape samples (hard-boiled eggs and Madeira cakes) had the lowest scores, thus suggesting that these samples are characterised by a less anomalous taste. Palm and Lard samples (for eggs and Madeira cakes) had intermediate scores, whereas Flax Madeira cake had the highest value, thus having the highest anomalous egg taste. Differences in evaluation between hard-boiled eggs and Madeira cakes from the Flax diet may be due to different thermal treatment. In few cases panelists reported the perception of fishy notes in Madeira cake samples. These results are the initial contribution to understanding of the sensory properties of eggs obtained from a grape seed oil based diet.

Table 5 - Sensory evaluation of boiled eggs and Madeira cakes.

Groups	Taste	Boiled eggs Odour	Madeira cakes Taste
Lard	4	4.9 A	3.9 B
Palm	3.4	4.7 A	4.2 B
Grape	3.7	3.1 B	3.2 C
Flax	3.7	4.8 A	5.1 A
P	NS	0.00002	0.000006

Means with different capital letters in a column differ significantly for $P < 0.01$.

Even if far from being conclusive, the results of the sensory analysis suggest the different vegetable sources tested, with the exception of flax seed oil, do not depreciate the sensory characteristics of eggs and the baked product. However, supplementing the laying hen diet with a low amount of flax seed oil to enrich the egg yolk with n-3 PUFA (MELUZZI *et al.*, 2001), makes eggs or egg based cakes less appreciated by ordinary consumers, particularly when they are baked at high temperatures. The functional properties of eggs were little affected by the dietary treatment of the laying hens with the exception of the consistency and flow behaviour indexes of yolk, therefore there are no negative indications for their use by the food industry.

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SHORT COMMUNICATION

EFFECT OF CHILLING ON THE VITAMIN C CONTENT OF FENNEL DURING STORAGE

EFFETTO DELLA FRIGOCONSERVAZIONE SUL CONTENUTO IN VITAMINA C DEL FINOCCHIO

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ABSTRACT

Vitamin C content was monitored in fennel during a 32-day storage period at 6°C and 90% R.H. and a rapid extraction method was developed to quickly analyse the samples. The concentration of the vitamin showed a 50% decrease during the first 4 days of storage. In the following two weeks the amount of vitamin C remained almost unchanged. Extending the storage time up to 32 days resulted in further substantial losses, with the final vitamin C concentration being 25% of the initial concentration. This phenomenon was

RIASSUNTO

È stato monitorato l'andamento del contenuto in vitamina C in finocchi conservati per 32 giorni, in condizioni controllate di temperatura e umidità (6°C e 90% UR), valutando anche l'influenza delle dimensioni del prodotto. Per minimizzare le perdite di vitamina durante l'analisi, è stata messa a punto una rapida metodologia di estrazione. I dati analitici hanno evidenziato come il contenuto in vitamina C si riduca di circa il 50% dopo i primi 4 giorni di conservazione. Successivamente la concentrazione della vitamina è rimasta pressoché costante per due settimane, ma pro-

- Key words: chilling, fennel, preservation, vegetables, vitamin C -

found to be independent of the size of the sample.

lungando la conservazione fino a 32 giorni si è osservata una ulteriore riduzione del contenuto, che ha raggiunto un valore finale pari a circa il 25% di quello del prodotto fresco. La variazione della concentrazione di vitamina C non è risultata essere influenzata dalle dimensioni del prodotto.

INTRODUCTION

The human diet should include major amounts of fruits and vegetables because of their fibre, vitamin and antioxidant contents. Vitamin C is considered one of the most important antioxidants because of its activity against free radicals, and the important role it plays against carcinogenesis and cardiovascular diseases, as well as in stimulating the immune system.

The vitamin C content can differ greatly among the numerous species and varieties of vegetable and fruits and can be affected by size, ripening stage, cultivation practices, storage conditions and packaging techniques (AGAR et al., 1997; CANO et al., 1989; GIL et al., 1998; MATTHEIS and FELLMAN, 1999; MYSHKIN et al., 1996; VELTMAN et al., 2000).

The shelf-life of vegetables can range from a few days to several weeks. The physiological changes that occur during postharvest storage include reduced respiration rate, flavour changes, losses of soluble carbohydrates, proteins and amino acids, as well as reduction of the chlorophyll and vitamin C contents. Therefore, any effort to extend the shelf-life of vegetables should also take into account maintaining the original vitamin C levels in the products (AGAR et al., 1997; NICOLI et al., 1999).

Vitamin C decreases under unfavourable storage conditions because it is very sensitive to enzymatic or chemical degradation. The vitamin C level could therefore be a quality marker for monitoring appropriate handling procedures and storage conditions (FAVELL, 1998). The quantitative analysis of vitamin C could therefore be a simple but useful tool for evaluating the quality of vegetables during storage. Of the many analytical methods that have been developed to assess the vitamin C level in edible products, high-performance liquid chromatographic methods have been shown to be selective and sensitive (GRAHAM and ANNETTE, 1992).

Fennel (*Foeniculum vulgare*) is a plant native to Italy and is usually harvested from November through June. It is widely used in the Italian diet, especially in the southern regions. Italy is the number one producer of fennel in the world and the region of Basilicata ranks seventh among the Italian fennel-producing regions. While fennel is scarcely used in the food processing industry, it is marketed in large amounts as a fresh product, being appreciated by consumers for its flavour, low caloric content and its nutritional and digestive properties. The natural shelf-life of fennel is limited to a few days, due to its very high respiratory activity, thus in order to extend the marketing time, it must be refrigerated.

There are few scientific studies dealing with the evaluation of fennel quality during storage (SOZZI and ILARDI, 1992; MENCARELLI et al., 1997). The objective of this work was to evaluate the quality loss of fresh fennel during refrigeration under controlled humidity and temperature (90% R.H. and 6°C) conditions, over an extended period of time, using

vitamin C as a marker; the influence of the size of the sample was also considered. Furthermore, in order to minimise vitamin losses during the analyses, a rapid extraction methodology for this moiety was developed.

MATERIALS AND METHODS

Sample preparation and vitamin C extraction

The study was conducted using fennel of the cultivar "Tardivo di Siracusa" harvested in the period from May to June 2000 and provided by the "Ancona" farm in Policoro (Matera). The fennels were immediately washed, trimmed and cooled in potable water before being packed in wooden boxes. Within 2 hours the samples were transported to the laboratory and immediately stored in a controlled temperature (6±1°C) and humidity (90±5% R.H.) chamber.

In order to evaluate any possible influence of the size of the sample on vitamin C degradation, the fennels were divided into two classes of 24 fennels each. Class A consisted of samples having an average weight of 285 g and class B of 486 g. Each class was then divided into 6 groups of 4 fennels each that were used to assess the degradation of vitamin C after 0, 2, 4, 8, 16 and 32 days of storage under the experimental conditions.

For the extraction of ascorbic acid from fruits and vegetables, DE SIO et al. (2000) proposed a simple methodology, in which the vegetable tissues are first chopped in a blender. An aliquot of the sample (10 g) is then brought up to 100 mL using a buffer solution (200 mM NaH_2PO_4, acidified to pH 2.14 with 1N HCl), which is then used as a mobile phase in the subsequent analytical step. Due to the high fibre content of fennel, the above procedure could not be used as such, but was partially modified. The homogenising procedure involved two steps: each fennel was sliced and about 20 g of material were chopped in a Sterilmixer homogeniser (PBI International, Milan, Italy) for 10 sec at 16,500 rpm; the chopped tissues were then homogenised using an Ultraturrax homogeniser (Model T25 Janke & Kunkel Labortechnick, Staufen, Germany) for 30 sec at 24,000 rpm. In order to prevent vitamin C degradation, and to reduce dehydroascorbic acid to ascorbic acid, potassium metabisulphite (0.2% by weight) was first added to the fennels, which were kept in ice during homogenisation. The procedure was conducted under dim light and all the glassware was wrapped in aluminium foil to shield the product from light.

About 1 g of the sample was then transferred to a 10 mL amber volumetric flask and brought up to volume using a solution of 20 mM NaH_2PO_4 acidified to pH 2.14 with 1N HCl. The flask was then stirred five times for 5 sec each using a Vortex; the solution was allowed to rest for 1 min in the dark between each stirring. The extract was then filtered through a 0.2 (µm cellulose acetate filter (Alltech, Milan, Italy) and stored in the dark at -20°C in an amber vial, under nitrogen, until analysed. Compared to the method of DE SIO et al. (2000), the buffer solution was used at a concentration of 20 mM instead of 200 mM, because at the higher concentration the ascorbic acid peak eluted at progressively longer retention times, which then required a long reconditioning of the column before each analysis. This problem was solved by using the more dilute buffer.

Reagents

All the chemicals were of suitable analytical grade and purchased from Carlo Erba (Milan, Italy). The ascorbic acid standard (>99% purity) was obtained from Fluka (Milan, Italy).

Ascorbic acid analysis

The HPLC chromatographic system consisted of a Varian 9012 pump, a Varian 9050 UV/VIS detector and a personal computer running the chromatographic software STAR 4.5 (Varian, Leini, Italy). The analytical method was a slight modification of that proposed by DE SIO et al. (2000). An aliquot of the extracted sample (20 µL) was injected onto a C18 Aqua column (5 µm, 250 x 4.6 mm, Phenomenex, Torrance, CA) with an Aqua C18 guard column (4x3 mm ID). The separation was carried out at 240 nm by isocratic elution with 20 mM NaH_2PO_4 acidified to pH 2.14 with 1N HCl (flow-rate 1 mL/min). The mobile phase acidification was required to reduce the rapid oxidation of the ascorbic acid. Each sample was prepared and analysed in duplicate. The chromatographic separation of the vitamin C extracted from a fennel sample is shown in Fig. 1. Quantitative determination of the total vitamin content in the samples was determined using the external standard method. From a stock solution of ascorbic acid (about 40 µg/mL) in 20 mM NaH_2PO_4, acidified to pH 2.14 with 1N HCl, five different working solutions were prepared (1/5, 1/10, 1/50, 1/100, 1/200) and used to build the calibration curve. The linearity of the detector response was checked over a concentration range from 0.2 to 40 µg/mL (r^2>0.999); the lowest detectable amount (42 ng/mL) was determined as the amount of standard required to give a signal-to-noise ratio of 3, while the quantification limit was considered to be three times the detection limit. The ascorbic acid analysis time was 4.9 min while the total analysis time was 20 min to allow complete elution of the co-extracted compounds.

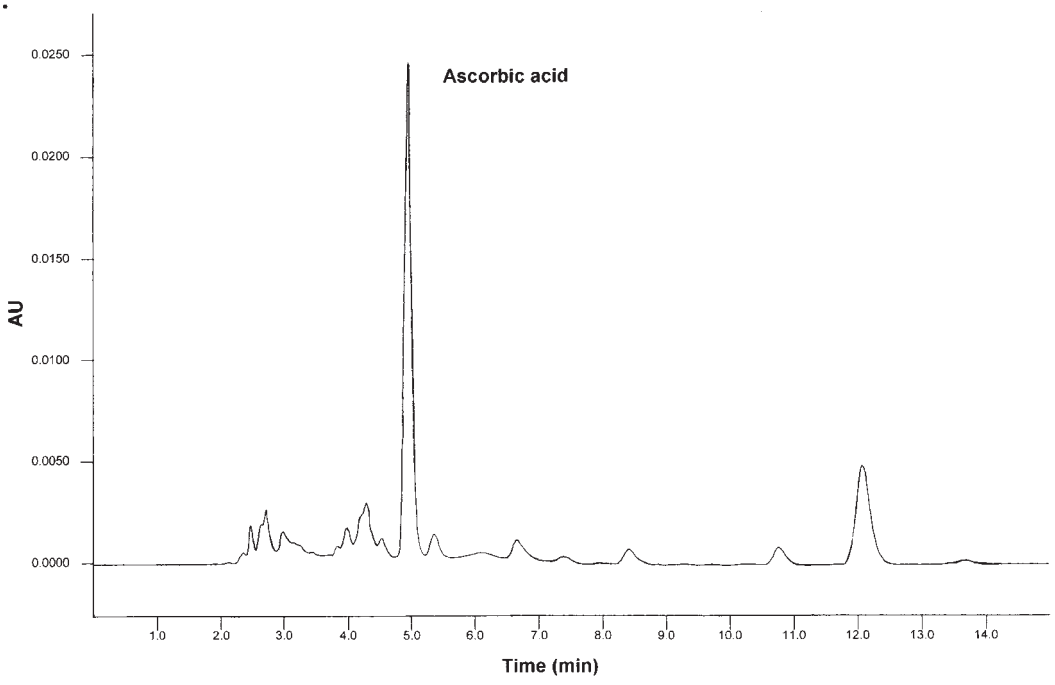


Fig. 1 - Chromatographic separation of vitamin C in a fennel sample.

Statistical analysis

The data were elaborated using the statistical package Statistica for Windows (Statsoft Inc., USA).

RESULTS AND DISCUSSION

Refrigeration is the most commonly used technique for slowing down deterioration and hence the loss of quality in fresh vegetables. However, also with refrigeration the vitamin and antioxidant contents may undergo a significant reduction (ESTEVE et al., 1995).

Different refrigeration temperatures have been suggested for postharvest storage of fennel. MENCARELLI et al. (1997), indicated that 0°C was the most suitable temperature, while SOZZI and ILARDI (1992) found that storage at 0°C did not give better results than storage at 6°C. In addition, it has been reported that slightly higher temperatures associated with high R.H. levels, have a positive effect on vitamin C retention in several fruits and vegetables (NUNES et al., 1998). For this reason the tests were conducted at 6°C.

The only available data concerning vitamin C content in fennel indicate that it is present at very low concentrations (120 μg/g of fresh weight) (INN, 1997). Therefore, in order to measure any possible loss of vitamin C during storage, a rapid extraction procedure and a sensitive analytical method were needed.

In order to optimise the vitamin C extraction, several tests were carried out. Initially the extraction procedure proposed for fruits and vegetables by DE SIO et al. (2000) was tested, but the simple use of a blender to break up the samples was not effective, due to their high fibre content. An adequate mechanical rupturing of the tissues was achieved with a two-step homogenising procedure, reported in the Materials and Methods section. As proposed by DE SIO et al. (2000), an aliquot of the homogenised sample was then brought up to volume immediately prior to the analysis, using a buffer solution, which was also used as mobile phase in the following analytical step. With this procedure, a minimal amount of vitamin C was recovered, with calculated concentrations in the sample of less than one third of that reported in the literature for fennel (120 μg/g) (INN, 1997). Therefore the influence of different times of contact between the vegetable tissues and the buffer solution was investigated.

Total contact times of 2, 5 and 10 min were tested. In order to enhance the extraction process, the samples were stirred for 5 sec every minute. The addition of a static extraction step increased the vitamin C recovery, which was maximised for a total extraction time of 5 min. An additional 10 min of contact time between the broken up tissues and the extracting solution did not substantially increase the vitamin C recovery.

Because of the longer exposure to the oxidising agents, due to the longer time of manipulation of the vegetable matrix, the addition of metabisulphite, an antioxidant, at three different concentrations was studied (0.2, 0.5 and 1.0% by weight). The results showed that a 0.2% concentration was sufficient to protect the vitamin C; however, operating at low temperatures and in dim light were also critical in order to avoid any vitamin C degradation.

The reproducibility of the extraction procedure was tested by making 10 extractions on the same sample at 0 days of storage. The results showed that the extraction method had a good reproducibility, with a mean vitamin C content of 76.86±2.60 μg/g, and a relative standard deviation of 3.38%.

In order to assess the influence of the various extraction steps on vitamin C recovery, a fresh fennel, at 0 days of storage, was sliced and divided into several portions. Some were treated as such,

according to the proposed procedure, while others were spiked with known amounts of ascorbic acid prior to the homogenising step, or prior to the extraction step. Blank extraction tests were also conducted by spiking pure water with the standard and simulating the whole extraction procedure, including the homogenising step.

The amounts of ascorbic acid added varied according to the extraction step at which time the standard was added, but the amounts were such that the final standard concentration in the extracts ranged from 0.3 to 30 ng/mL. The natural vitamin C content of the spiked sample was 76 μg/g, and the statistical analysis of the data indicated that the recoveries were above 98% in all cases, and that during the homogenising procedure there was a negligible loss of vitamin C.

The vitamin C content of the fennel samples was monitored over a 32-day period of storage at 6°C and 90% R.H.. In Table 1 the vitamin C contents are reported for class A and class B fennels as a function of storage time. In all the samples the vitamin C content decreased sharply in just a few days; after only 4 days of storage the vitamin C concentration was about 50% of the initial concentration. Similar results have been reported in the literature for other vegetables, such as green beans and spinach stored under refrigerated conditions (FAVELL, 1998). No significant changes in the vitamin C level were observed during the following 2-week period. However, when the storage time was extended to 32 days, a further loss was recorded, with the final vitamin C content of fennel being 25% of the initial value.

Variations in the vitamin C content in vegetables have been reported to be dependent on the size of the vegetables (LEE et al., 1976); however, statistical analysis (t-Student test) of the data showed that the size of the sample did not significantly affect the amount of vitamin C in the fennel, over the entire storage period.

CONCLUSIONS

This study has shown that the first 4 days are the most critical for vitamin C loss in fennel stored at 6°C and 90% R.H. The vitamin C concentration was reduced by about half the initial amount after only 4 days, while the concentration remained almost unchanged for the next 12 days of storage. Because reduction in vitamin C is strictly related to the presence of oxygen, the use of systems that can reduce or modify the gaseous exchange between the product and the atmosphere could help to limit vitamin C losses.

Table 1 - Mean values of vitamin C content in class A and class B fennels at different storage times.

Storage time (days)	Vitamin C (mg/kg fennel)		Level of significance
	Class A	Class B	
0	78.07±5.64	74.65±8.74	n.s.
2	54.98±3.39	55.26±4.73	n.s.
4	35.80±7.09	38.33±9.11	n.s.
8	41.62±6.95	37.48±4.23	n.s.
16	39.17±6.43	37.73±5.21	n.s.
32	15.51±3.15	14.20±1.71	n.s.

Each value is the mean of 4 determinations + standard deviation.
n.s. = not significant: $p<0.01$.

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SHORT COMMUNICATION

AROMA VARIATION IN TANNAT WINES: EFFECT OF MALOLACTIC FERMENTATION ON ETHYL LACTATE LEVEL AND ITS ENANTIOMERIC DISTRIBUTION

VARIAZIONE NELL'AROMA DEI VINI TANNAT: EFFETTO DELLA FERMENTAZIONE MALOLATTICA SUL TENORE IN LATTATO DI ETILE E SULLA SUA DISTRIBUZIONE ENANTIOMERICA

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ABSTRACT

The chirality of ethyl lactate was evaluated in an attempt to explain possible scents present in Tannat wines. Wines without malolactic fermentation (MLF) (ethyl lactate values from 5 to 8 mg/L) had a 69% enantiomeric excess of (R)-ethyl lactate, while with MLF (ethyl lactate values from 90 to 150 mg/L), this value decreased to 22-28%, depending on the inoculant strain. The (S)-ethyl lactate concentration increased significantly after MLF (65-130 mg/L) com-

RIASSUNTO

Si è indagata la chiralità del lattato di etile per rendere ragione della presenza di possibili particolari sentori nell'aroma dei vini tannat. I vini senza fermentazione malolattica (FML), aventi valori di lattato di etile da 5 a 8 mg/L, hanno mostrato un eccesso della forma enantiomerica (R)-lattato di etile del 69%, mentre dopo FML – con valori di lattato di etile da 90 a 150 mg/L – questo valore era diminuito al 22-28% secondo il ceppo. La concentrazione del

- Key words: enantiomeric distribution, ethyl lactate, MLF, tannat wine -

pared with the non-MLF control wine (1.5-2.5 mg/L). The aromatic impact of the (S)-enantiomer was confirmed by determining its aroma threshold in wine (110 mg/L).

(S)-lattato di etile dopo FML è aumentata in modo significativo (65-130 mg/L) in rapporto al vino di controllo, senza FML, che aveva concentrazioni di 1,5-2,5 mg/L. L'impatto sull'aroma dell'enantiomero (S) è stato confermato individuando in 110 mg/L la sua soglia di percezione.

INTRODUCTION

During growth in wine, lactic acid bacteria (LAB) metabolise L-(-)-malic acid, forming L-(+)-lactic acid [(S)-(+)-lactic acid] and CO_2 (for a review, see HENNICK-KLING, 1993). Both D- and L-lactic acid enantiomers [(R)-(-)- and (S)-(+)-enantiomers] are produced in small quantities – not more than 0.2-0.3 g/L each – during a "correct" alcoholic fermentation. the (R)-(-) isomer is produced as a metabolite of sugar fermentation by yeast, while the (S)-(+) form is produced from sugar by some bacteria. A concentration of the (S)-(+) form higher than 0.5 g/L indicates an anomalous fermentation pathway (BANDION and VALENTA, 1977). This pathway, called malolactic fermentation (MLF), is an essential part of winemaking that usually occurs after alcoholic fermentation and is used as a deacidification and acid-taste modifying technique. MLF is also necessary for developing body (mouth-feel), complex aromas and balanced flavours in red wines, some white wines and some base wines for premium sparkling wine (BERTUCCIOLI and MONTEDORO, 1981; BARTOWSKY and HENSCHKE, 1995; MCDANIEL *et al.*, 1987).

Studies carried out with Pinot noir and Chardonnay wines, with and without MLF, have shown flavor differences as determined by descriptive analysis techniques (SAUVAGEOT and VIVIER, 1997; LAURENT *et al.*, 1994; RODRIGUEZ *et al.*, 1990). These differences could be partially due to diacetyl formation and the reduction of acetates with increased levels of short-chain aliphatic aldehydes and acids (DITTRICH, 1987).

Ethyl lactate, an important aroma compound produced during MLF, is often found in concentrations exceeding its taste threshold of 60-110 mg/L (DITTRICH, 1987), which apparently gives a "broader", "fuller" taste to the wine (HENNICK-KLING, 1993). The aroma descriptor defines ethyl lactate as sweet, fruity, resembling pineapple with candy brown nuance (MOSCIANO *et al.*, 1993). Since ethyl lactate is also a by-product of yeast fermentation, its presence can indicate some yeast strain activity in the wine, e.g. *Saccharomyces cerevisiae* r.f. *uvarum*, a high ethyl lactate producer (CIOLFI and DI STEFANO, 1983).

Whereas (R)-ethyl lactate has been characterized as a genuine chiral compound of wine, whose presence is due to yeast fermentation, the corresponding (S)-enantiomer is reported to be an indicator of MLF or LAB contamination (KAUNZINGER *et al.*, 1996).

Tannat wine is a red wine made from a grape variety that originated in southern France, but is almost unknown today in Europe. Uruguay is the only country in the Americas where this grape is commonly found, and the Uruguayan wine industry has developed a strategy to produce Tannat wine using state-of-the-art viticultural technology (CARRAU, 1997). GÁMBARO *et al.* (2001) reported that MLF strongly affects the aroma profile of red wines and that Tannat wine

had a greater aroma complexity after MLF.

The aim of the present work was to study ethyl lactate chirality in order to gain some insights about the scents that are characteristic of Tannat wines and to better understand the effect that MLF has on red wine sensory characteristics in complement to the chemical aroma composition of this wine (BOIDO *et al.*, 1998, 1999; VERSINI *et al.*, 1999).

MATERIALS AND METHODS

Sample preparation

Ten batches of 20 kg each of *Vitis vinifera* Tannat grapes from different clones and vineyards were microvinified by traditional maceration during the 1999 vintage. All ten grape samples were destemmed and crushed; SO_2 was added at 5 g/hL and then each batch was inoculated with *Saccharomyces cerevisiae* strain CIVC 8130 (Gist Brocades, Santiago, Chile). The samples were allowed to ferment with the skins (vinification with maceration). Fermentation was carried out at 22°-25°C. At a density of 1,000 g/L, the wine was run off from the fermentor, pomace was pressed, and pressed wine was added to runoff wine. After the alcoholic fermentation was completed, each sample was divided into three equal portions. Two portions were taken for MLF by inoculation, in duplicate, with two different pure cultures of *Oenoccocus oeni* strains, strain DSM 7008 (Viniflora, Chr. Hansen's, Denmark), and strain D-11 (Malolactine O, Groupe Oeno-France, France). The third portion was not inoculated and served as the control. All the samples were held at 18°C and MLF was followed by determining the malic and lactic acid contents using TLC (BOIDO *et al.*, 1999). When MLF was completed, wines were sulfited with 50 mg/L SO_2, stabilized at 4°C for 20 days and sterile-filtered (0.45 µm membrane); the free SO_2 content was then adjusted to 40 mg/L. After bottling, the wines were stored at 10°C until analyzed.

Isolation of ethyl lactate

Ethyl lactate was isolated using an Isolute (IST Ltd, Mid Glamorgan, UK) ENV+ cartridge packed with 1 g of highly cross-linked SDVB (styrene divinyl benzene) polymer (40-140 µm, cod. n. 915-0100-C) as previously reported (CARLIN, 1998). Ethyl lactate was eluted together with the free aroma compounds from 50 mL of wine using dichloromethane. The extract was then anhydrified with Na_2SO_4, concentrated to 0.3 mL and used for GC and GC-MS analysis.

Sensory experiments

Pure (S)-ethyl lactate was from Aldrich (98%, Aldrich E3, 410-2, USA) and the racemate form was synthesized from lactic acid (85%, Aldrich W261106, USA) and ethanol in sulphuric media. Enantiomeric ratios were verified by multidimensional gas chromatography using chiral stationary phases as described below. The pure enantiomeric (S)-ethyl lactate and the synthesized racemate were added to a water/ethanol mixture (90 + 10, v/v) at concentrations equal to those determined in Tannat wine after MLF (90-120 mg/L). After stirring for 30 min, the model mixtures were compared by assessors who evaluated the aroma attributes of each component by smell. Sensitivity odor threshold values for the (S)-ethyl lactate were determined by using the triangle test (GUTH, 1997) in which a wine sample without MLF served as a blank. The samples were presented in the order of increasing (S)-ethyl lactate concentrations (50, 80, 110, 140, 170 mg/L) to individual panel members (six trained judges) for assessment of possible odor differences. The wines were presented in individual testing booths with "normal daylight" illumination.

Samples (60 mL) were served at 20±1°C in approximately 250 mL, clear, tulip-shaped wine glasses (ISO 3591, 1977) covered with a watch glass and identified with two digit random codes.

Identification and quantification

HRGC-FID and HRGC-MS or SIM/MS instrumental procedures, using an internal standard (1-heptanol), were applied as previously described (BOIDO *et al.*, 1999; VERSINI *et al.*, 1999). The aroma compounds were identified by comparing their Linear Retention Indices, determined in relation to a homologous series of n-alkanes, with those from pure standards or those reported in the literature. Fragmentation patterns in the mass spectra were also compared with those stored on databases (ADAMS, 1995; MCLAFFERTY and STAUFFER, 1991).

Enantioselective analysis

Enantiomeric ratios of ethyl lactate were obtained by multidimensional gas chromatography, using a developmental model (MONDELLO *et al.*, 1998) set up with two GC ovens. The first was equipped with a bonded fused-silica capillary column coated with BP 20 (SGE, Australia) (25 m x 0.25 mm i.d.) and the second with a chiral fused-silica capillary column (25 m x 0.25 mm i.d., 0.25 µm phase thickness), coated with 2,3-di-O-ethyl 6-O-t-butyldimethylsilil-b-cyclodextrin in PS 086 (13% phenylmethyl-polysiloxane) (Mega, Legnano, Italy), a hot interface, a rotary switching valve and a system for maintaining a constant flow during the transfer. With this system a heart-cut of the relevant fractions was made and these were fractions were transferred from the non-chiral column to the chiral one under previously described experimental conditions (LOAYZA *et al.*, 1999).

RESULTS AND CONCLUSIONS

The results obtained in the analysis of ethyl lactate reported in Table 1 show that the ethyl lactate values were fifteen to twenty times higher after MLF in all the Tannat wine samples analysed. The wines that did not undergo MLF (5-8 mg/L for ethyl lactate and 3-4 g/L for malic acid) had a 69% enantiomeric excess of (R)-ethyl lactate, while the wines produced with MLF (90-150 mg/L of ethyl lactate) had an enantiomeric excess of

Table 1 - Changes in the chemical composition of Tannat wines in response to malolactic fermentation (MLF).

	Samples without MLF [a]	Samples with MLF DMS 7008 strain [a]	Samples with MLF D-11 strain [a]
Malic acid (g/L)	3.73±0.29	nd[b]	nd[b]
Lactic acid (g/L)	0.77±0.35	3.31± 0.43	3.16± 0.51
Ethyl lactate (mg/L)	6.30±3.48 a	99.08±36.72 b	144.08±39.26 c
(S)- (mg/L)	1.96±1.95 a	71.79±29.29 b	111.78±18.15 c
(R)- (mg/L)	4.34±1.77 a	27.29±12.92 b	32.30±22.94 b
Ethyl esters [c] (mg/L)	0.73±0.15 b	0.51± 0.09 a	0.42± 0.09 a
Acetates [d] (mg/L)	1.96±0.37 b	1.22± 0.37 a	2.22± 0.36 b

[a] each value is the mean concentration (± standard deviation) of ten samples; letters in italics indicate differences according to Tuckey's test (95%);
[b] not detected;
[c] sum of ethyl hexanoate, ethyl octanoate and ethyl decanoate;
[d] sum of isobutyl acetate, isoamyl acetate, hexyl acetate and 2-phenyl ethyl acetate.

22-28% depending on the *O. oeni* strain used. In addition, the sensory contribution of (S)-ethyl lactate after MLF (72% enantiomeric excess for the DMS 7008 strain and 112% for the D-11 strain) was reinforced by a significant increase in its concentration (65-130 mg/L, $p \leq 0.01$) when compared with the control (1.5-2.5 mg/L). The values increased further during ageing, which is in agreement with previous results published in the literature for other varieties (VERSINI and MARGHERI, 1981; PEREZ-COELLO *et al.*, 1999).

In Tannat wine, a variety without strong spicy or floral characteristics, the contribution of MLF to the wine flavour is easily recognised. In a triangle taste test, malolactic Tannat wines were distinguished from non-malolactic Tannat wines at the $p \leq 0.001$ level (GÁMBARO *et al.*, 2001). GC-MS analysis of Tannat wine aroma showed that some fruity aroma-active substances, such as esters and acetates, were lost during MLF (BOIDO *et al.*, 1999), while a more complete aroma was developed. In particular, the sum of ethyl esters decreased from 0.73 mg/L before MLF to 0.42-0.51 mg/L after MLF, while the corresponding variation for the acetates was only significant for the DMS 7008 strain, with values changing from 1.96 to 1.22 mg/L (Table 1). The sensory tests showed that the intensity of the fresh red fruit characteristics were reduced by MLF, while dry fig and quince notes were enhanced (GAMBARO *et al.*, 2001).

The results of the olfactory comparison using alcoholic solutions of the pure (S)- enantiomer and the synthesized racemate, indicated that the (S)-enantiomer had a fruitier, more acidic and plum-like aroma. The impact of this enantiomer on the aromatic quality and intensity of the Tannat wine, as a consequence of MLF, was confirmed by the aroma threshold value (110 mg/L, $p \leq 0.05$). This value, which corresponds to that reported in the literature for the racemic form of ethyl lactate, was exceeded in many samples, particularly in those from the D-11 strain.

In conclusion, the higher ethyl lactate level, particularly the (S)-enantiomer after MLF, could explain, at least in part, the changes that occur in the fruity character of Tannat wine aroma as demonstrated through sensory analysis (GÁMBARO *et al.*, 2001). These changes could contribute to the increase of some fruit characteristics, like plum and dry fruit notes that develop during ageing of Tannat wine (BOIDO *et al.*, 1999).

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SHORT COMMUNICATION

SURVIVAL OF *ESCHERICHIA COLI* O157:H7 IN A SHORT RIPENED FERMENTED SAUSAGE

SOPRAVVIVENZA DI *ESCHERICHIA COLI* O157:H7 IN UN INSACCATO A BREVE STAGIONATURA

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ABSTRACT

The survival and toxinogenic properties of a strain of *E. coli* O157:H7 were evaluated in a typical fermented sausage during ripening. It was found that *E. coli* O157:H7 declined progressively over time even though it was still detected at the end of the ripening process. It maintained its ability to produce verocytotoxins until the end of the ripening process.

RIASSUNTO

In questo lavoro è stata valutata la sopravvivenza e la capacità di produrre verocitotossine di un ceppo di *E. coli* O157:H7 in un tipico insaccato durante la stagionatura. Gli Autori hanno rilevato una progressiva riduzione di *E. coli* O157:H7 che tuttavia era ancora presente al termine della stagionatura. La capacità del ceppo di produrre verocitotossine era presente anche al termine della stagionatura.

- Key words: *E. coli* O157:H7, fermented sausage, verocytoxicity -

INTRODUCTION

Escherichia coli O157:H7 is the cause of serious food borne illnesses. In North America and Great Britain it was identified as the etiologic agent responsible for epidemic outbreaks of hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) (RILEY *et al.*, 1983; PATON and PATON, 1998). In the U.S. about 100,000 cases of infection occur every year and 2-7% of the infected subjects develop HUS, with a mortality rate of 3 to 5%. Cases of infection due to *E. coli* O157:H7 have also been reported in Italy. From 1988 to 1999 the Enter-Net Supervision System of the Istituto Superiore di Sanità (ISS) collected data on 218 cases of infection due to verocytotoxin producing *Escherichia coli* (VTEC).

Several cases of food borne infection have been associated with the consumption of meat and meat products, and other foods of animal origin as well as vegetables (BUCHANAN and DOYLE, 1997). Yoghurt, mayonnaise, and some fermented sausages are of particular interest since they are usually considered safe from the microbiological point of view due to their low pH, although several studies have revealed that *E. coli* O157:H7 may survive in low acid foods (GLASS *et al.*, 1992; WEAGANT *et al.*, 1994; RAGHUBEER *et al.*, 1995; CLAVERO and BEUCHAT, 1996; ELLAJOSYULA *et al.*, 1998). In 1994 *E. coli* O157:H7 was responsible for a food poisoning outbreak linked to consumption of sausages, which affected 20 subjects in Washington State and 3 in California (CDC, 1995). *E. coli* O157:H7 has also been found in fresh sausages sold in Apulia (Italy) (NORMANNO *et al.*, 2002).

During ripening, a complex fermentation process takes place in the sausage due to microbial activity. For instance, lactic bacteria may be present in the product causing considerable changes in the chemical, physical and organoleptic characteristics of the raw meat, modifying the initial pH and a_w values, thus creating inhibitory conditions for the survival and growth of pathogenic and spoilage microorganisms (PALUMBO and SMITH, 1977). The aim of this study was to evaluate the survival of *E. coli* O157:H7 during the ripening of artificially contaminated sausages.

MATERIALS AND METHODS

Sample preparation

One 990 g and seven 900 g portions of pork paste were taken from a lot of pork paste produced in a sausage factory in Apulia. It had been inoculated with starter strains of *Staphylococcus carnosus* and *Lactobacillus plantarum* (Briostart SL1, Kerry Pac; 20 g/100 kg paste), according to the producer's instructions and was ready for stuffing. One of the 900 g portions was immediately stuffed into natural gut, following the normal factory procedure to produce a 70-80 g sausage called "*sfizietto*". This sausage was used as a control sample.

The 990 g portion was inoculated with 10 mL of broth culture (Nutrient Broth – OXOID) of an *E. coli* O157:H7 strain, producing verocytotoxins (VT1 and VT2), courtesy of ISS. It was incubated at 37°C overnight to a level of $8x10^8$ CFU/mL. The paste was well mixed to obtain homogenous inoculation and labelled as sample A. 100 g of sample A were added to and mixed with a 900 g portion of pork paste, thus obtaining sample B. This procedure was repeated on the other 900 g portions, to obtain samples C, D, E, F, G with decreasing logarithmic contamination (microbial counts from 10^6 to 1 CFU/g). All the samples thus inoculated were separately stuffed into natural gut, starting from the least contaminated one (sample G), thus producing sausage samples with the same weight and volume as the control sample. Each sausage sample was subdivided into 12 al-

iquots and placed individually on a steel grate to dry for 24 h (20°C and 80-90% RH) and then ripened in a storage-room at 12°C and 70-80% RH for 16 days. Starting from the time of stuffing (T0) and then at 4-day intervals (T4, T8, T12, T16), an aliquot of sample was drawn aseptically from each sausage sample and transported immediately under refrigeration (+4°C) to the laboratory.

Microbiological analysis

Ten g of each sausage aliquot were first homogenised in 90 mL of a sodium chloride solution (0.85%); decimally diluted in the same media and then submitted to the following microbiological analyses using the pour-plate method (1 mL of each dilution): Total Mesophilic Flora in Plate Count Agar (Difco); Total Enterobacteria in Violet Red Bile Glucose Agar (OXOID); Total Coliforms in Violet Red Bile Agar (OXOID). These inoculated culture media were incubated at 32°C for 48 h. For the detection of lactic acid bacteria, 10 g of each sausage aliquot were homogenised in 90 mL of Ringer's Solution; the decimal dilutions were prepared in MRS Broth (OXOID) and poured (1 mL) onto MRS Agar (OXOID) incubated at 35°C for 48 h. Quantification of *E. coli* O157:H7 was carried out in Chromogenic *E. coli O157* Agar (Biolife) incubated at 37°C for 24 h. A 25 g sample was placed in selective broth (225 mL of Modified Trypton Soya Broth -m-TSB – DID) with novobiocine (0.20 g/100 mL) and incubated at 41°C for 6 h (SCOTTER *et al.*, 2000). Afterwards, 1 mL broth aliquots underwent "immunomagnetic separation" (IMS), using Dynabeads anti *E. coli* O157 (Dynabeads M-280 anti *E. coli* O157, OXOID) and then, 50 µL bead/cell complex were recovered from each aliquot, plated on Sorbitol Mac Conkey Agar (SMAC-Difco), and incubated at 37°C for 18 h. Colonies showing morphologic characters of *E. coli* O157:H7 (sorbitol-negative, translucid, 1-3 mm in diameter, opaque centre) were replated onto Tryptone Soya Agar (TSA-OXOID) and subjected to indole testing in Peptone water (DIFCO). All the strains which tested indole positive were examined biochemically API 20E – bioMerieux) and the strains identified as *E. coli* were examined by the latex agglutination test (*E. coli* O157 Test kit – OXOID) and with anti H7 serum.

Verocytotoxicity tests

The ability to produce verocytotoxins of the strains isolated and identified as *E. coli* O157:H7 was evaluated on monolayers of VERO cells aged 24 hours (KONOWALCHUCK *et al.*, 1977).

Physical and chemical analysis

pH measurements on each sample were carried out by means of a plunge electrode pH-meter (Mod. 230A – Orion-Boston, U.S.A.), while a_w was determined by means of an ECO (Art. 17398 – PBI International, Milano Italy) water activity apparatus.

RESULTS AND CONCLUSIONS

The verocytotoxicity tests carried out on the broth culture before contamination of the sausage mixture were positive. The microbial determinations on the control sausage at time 0 showed that the microbiological condition of the paste was optimal, with a total mesophile count of 10^7 CFU/g, a lactic bacteria count of 10^6 CFU/g, a limited number of total enterobacteria, and no total coliforms and *E. coli* O157:H7. The microbiological parameters determined on the inoculated sausage samples on different days during ripening showed that the total mesophile count and the lactobacilli gradually increased during ripening with various samples having similar values; the total enterobacteria that had

been present in the paste were no longer found in the control sample from day four onwards. Amongst the various sausage samples contaminated with *E. coli* O157:H7, at time 0, the counts showed a logarithmic decrease in samples A, B, C, D, E with values ranging from 10^6 to 10^2 CFU/g. In samples F and G, *E. coli* O157:H7 could not be quantitatively determined on the plate, so only a qualitative evaluation was performed which proved to be positive. *E. coli* O157:H7 could be detected in samples A and B until day 16, samples C and D until day 8 and only at time 0 in sample E (Table 1). The samples which tested negative for the quantitative determination were later subjected to qualitative analyses where they tested positive, with the exception of sample G, that was negative from day 8 (Table 1). Thus confirming the particular resistance/survival capacity of this microorganism as already described by others (CONNER and KOTROLA, 1995; LIN *et al.*, 1996; COLOMBO *et al.*, 1997; DUFFY *et al.*, 2000). The pH and a_w of the various sausage samples had values very similar to those of the control sample; the means are shown in Fig. 1. No macroscopic alterations were found in the samples and the sensory characteristics (colour, flavour and consistency) of the various inoculated samples were identical to those of the control. A highly individual characteristic was shown when the degree of contamination of the sample was below the sensitivity of the pour-plate method since the microorganism seemed to have a greater ability to survive in these samples. Further research is required to confirm and to explain this particular behaviour. The production of verocytotoxins, constantly observed, was independent of the length of seasoning and the chemical and physical modifications of the sausage. The USDA-FSIS (1995) has required processors to either validate their processes or include a step that would ensure a 5 log reduction of the pathogen at the end of the ripening period. Since a heat conditioning phase in the process is not part of the traditional procedures used to produce fermented sausage in Italy, the competent authorities should consider the problem and recommend proper measures to be taken in

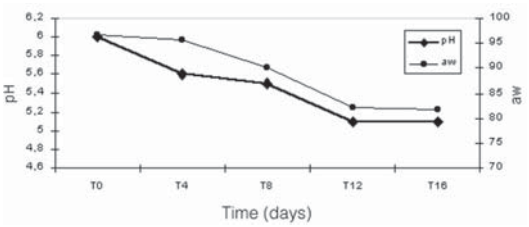


Fig. 1 - Changes in pH and water activity during ripening: means of control and inoculated samples.

Table 1 - *E. coli* O157:H7 counts (CFU/g) during ripening of an inoculated sausage sample.

Time	Control	Sample A	B	C	D	E	F	G
TO	-	$1.2x10^6$	$1.7x10^5$	$2.2x10^4$	$4x10^3$	$2.8x10^2$	+	+
T4	-	$2.8x10^5$	$4x10^4$	$1.6x10^3$	$1.5x10^2$	+	+	+
T8	-	$1.2x10^5$	$4.6x10^3$	$8x10^2$	20	+	+	-
T12	-	$2.1x10^3$	40	+	+	+	+	-
T16	-	$1.2x10^2$	10	+	+	+	+	-

+ = Qualitative presence of *E. coli* O157:H7.
- = Absence of *E. coli* O157:H7.

processing technology to produce fermented sausages that are safe for human consumption (especially for small diameter products in which pathogen reduction is difficult to obtain) (GOODFELLOW, 1999). Our suggestion is to include systematic testing for *E. coli* O157:H7 among the microbiological parameters of the control plans in order to guarantee greater safety in the production of meat.

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PLANT FOOD ALLERGENS

FFE 485/02/SME35

Food allergy is a consequence of inappropriate immune responses to some food proteins resulting in generation of antibodies (immunoglobulin E, or IgE). The main foods responsible for the majority of food allergies are peanuts and tree nuts, wheat, soya, cow's milk, egg, shellfish and fish. It has been estimated that 1-2% of adults and 5-7% of children actually having confirmed allergies to foods.

Flair-Flow previously informed about PROTALL (see http://www.flair-flow.com/docs/ffe36800.htm), a FAIR project aimed at improving our understanding of factors that may be important in predisposing certain plant food proteins to becoming allergens and reducing or removing allergenic determinants through processing. This is an update on the results from the project.

Plant proteins may be divided into 3 sub-classes: structural and metabolic proteins; protective proteins (which defend a plant against invasion by pathogens or feeding by pests, storage proteins (which act as a nutrient store to support germination in seeds). It is now clear that almost all plant food allergens are either protective or storage proteins. It is also clear that those proteins that trigger the development of an allergic response through the gastrointestinal tract belong primarily to two large protein families: The cereal Prolamin Superfamily and the Cupin Superfamily. Proteins within each family are all similar in their molecular structure.

Other important results are the development of technologies to reduce or to remove the allergens or the active sites (the epitopes) of the allergenic proteins. The work has concentrated on 3 technologies: removal of the epitopes (e.g. peeling of peaches removes a considerable part of allergenicity); their destruction (e.g. through heat treatments to unfold and deactivate them or through their hydrolysis by proteases); and their masking (e.g. by cross-linking the epitopes by transglutaminase or by other enzymatic reactions).

Further information may be obtained from the project web-site www.ifr.bbsrc.ac.uk/protall or from a synthetic report that Flair-Flow will soon be publishing.

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This one-pager was written by Mr. F. Holm, Food Group Denmark, in March 2002.

INVESTIGATING AMINES IN FOOD

FFE 489/02/HP36

It has been known for a long time that certain components of some foods called amines possess biological activity. Amines are formed during normal metabolic processes in living organisms and are, therefore, present in everyday food products. The characteristics and biological functions of amines are very diverse; they may have beneficial or harmful effects. In general, amines can be described as "biogenic amines" (such as serotonin, cadaverine and histamine) or "natural polyamines" (such as spermidine and spermine). Both polyamines and biogenic amines are present in food, but, while polyamines appear to be essential (through their involvement in growth and cell proliferation), biogenic amines are mainly detrimental (having the potential to lead to nausea, hot flushes, sweating, headaches and hyper- or hypotension). The biogenic amine content of food should therefore be kept at a very low level.

An interdisciplinary, joint European effort, which combined expertise in several scientific fields was therefore established, as a COST action, to clarify the physiological functions of biogenically active amines. The action also included investigation into medical applications, such as the formation of a low-polyamine, anticancer diet to provide a better quality of life for patients with cancer, and the development of provision of nutritional advice to people on certain types of medications (such as monoamine oxidase inhibitors, MAOI, used in some depressive illnesses) which make them sensitive to biogenic amines (specifically tyramine) in foods such as some mature cheeses, fermented foods, e.g. sauerkraut and fermented soya products, yeast extracts, pickled fish and red wine.

The COST action had 5 working groups working on the following areas:

- Physiology and metabolism of biologically active amines;
- Polyamines and tumour growth;
- Transgenic plants (with modified amine content);
- Biologically active amines in food processing;
- Production of biologically active amines by bacteria.

The fifth volume of the proceedings of this COST project, dealing with polyamines and tumour growth, and on biologically active amines in food processing and amines produced by bacteria, is now available from: http://online.eur-op.eu.int/servlet/page?_pageid=74&_dad=catdiff&_schema=PORTAL30&_mode=3.

Project No: COST 917 Action

Project contact: Dr. John Williams, European Commission; COST Scientific Officer, SDME 9/75; B-1049 Bruxelles, Belgium, Tel.: +32 (0)2 299 1599, Fax +32 (0)2 296 4289, e-mail: john-b.williams@cec.eu.int.

This one-pager was written by Dr. F. Robinson, British Nutrition Foundation, UK, in March 2002.

IN FOCUS: HEALTH IMPLICATIONS OF POLYPHENOLS

FFE 490/02/CG34

Colon cancer kills approximately 75,000 Europeans per year. Even a modest reduction would significantly benefit the EU both socially and financially.

Many studies have shown a preventing effect of naturally occurring dietary plant polyphenols on cancer formation. Polyphenols are useful compounds that protect us against harmful reactions in human cells. There are large differences in consumption of amount and type of polyphenols between North and South Europe, with lower risks associated with the Mediterranean diet rich in vegetables. Vegetables, especially onions, fruits and green tea are good sources of polyphenols. Flowers are "Non-food sources": these compounds are responsible for the colours of many of them, such as fuchsias, roses and petunias. But beware: it is eating vegetables, not flowers that is recommended!

In the year 2000, a 3-year project named "Health implications of natural non-nutrient antioxidants (polyphenols); bioavailability and colon carcinogenesis" started. For the project, a co-operative partnership was established between 13 institutions in six EU-member countries.

The expected achievements from the programme include the study of the digestion of polyphenols in human gut and their effect on gut microflora. The doses of polyphenols to optimise protection against colon cancer will also be determined. The information gained is expected to facilitate more appropriate and safe development of tasty novel foods with altered contents of polyphenols.

Project No: QLK1-1999-00505 (POLYBIND) http://www.ifrn.bbsrc.ac.uk/Polybind/default.html

Project Contact: Dr. 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, e-mail: christine.hill@bbsrc.ac.uk.

This one-pager was written by Mr. T. Kiutamo, VTT Biotechnology, Finland, in March 2002.

EATING BRASSICA VEGETABLES IS GOOD FOR OUR HEALTH

FFE 491/02/CG35

Cabbage, broccoli, cauliflower and Brussels sprouts are members of the large brassica plant family. They are widely cultivated and eaten in Europe. Besides dietary fibres and vitamins, which are common health promoting components in vegetables, they are known to contain glucosinolates, large sulphur-containing compounds specific to brassicas. Glucosinolates are responsible for the pungent flavour of these vegetables, most intense in species such as turnips, radishes and garden cress.

It has now been proven that a high consumption of common brassica vegetables is associated with a decreased risk of cancer, and that this association is most consistent for carcinomas of the lung, stomach, colon and rectum. The effect is most likely to arise from the breakdown products of the glucosinolates, among them the sulphur-containing isothiocyanates.

This conclusion is supported by the key results of a finalised 3-year FAIR project entitled "Effects of food-borne glucosinolates on human health". In the study, a combination of laboratory-based experiments and studies with human volunteers was used.

The safety of isothiocyanates was also evaluated. It was concluded that adverse effects caused by extremely high doses of some glucosinolates (i.e. in the form of concentrated supplements) could not be excluded. However, consumption of brassica vegetables does not pose any risks in humans.

The influence of food preparation (chopping, cooking in water, microwave cooking) on the level of glucosinolate breakdown products was also investigated. Findings showed that both raw and cooked brassica vegetables still contain biological activity. Consumers should be encouraged to develop a taste for the strongly flavoured brassica vegetables.

Project No: FAIR-CT97-3029 (EFGLU) http://www.ifrn.bbsrc.ac.uk/Diet/GItract_EFGLU.html

Project co-ordinator: Professor Ian T. Johnson, Institute of Food Research, Norwich Research Park, Colney, Norwich, NR4 7UA, UK, Tel.: +44 (0)1603 255330, Fax +44(0)1603 507723, e-mail: ian.johnson@bbsrc.ac.uk.

This one-pager was written by Mr. T. Kiutamo, VTT Biotechnology, Finland, in March 2002.

COLON CANCER AND NATURAL ANTIOXIDANTS

FFE 495/02/SME39

Colon cancer kills approximately 75,000 Europeans per year, and even a modest reduction would significantly benefit the EU both socially and financially.

Many studies show an effect of naturally-occurring dietary plant constituents on carcinogenesis and it has been estimated that up to 75% of the cancers of the gastrointestinal tract may be preventable by dietary modifications. There are large differences in food consumption and of amount and type of plant constituents consumed between North and South with lower risks associated with the Mediterranean diet.

Polyphenols in plants have been among the most interesting constituents. These are antioxidants which may influence important markers of carcinogenesis and may protect the DNA, cholesterol, fatty acids, nutrients, and many more against oxidation. Chemically, they constitute a very large and complex group of compounds, e.g. carotenoids, simple polyphenolics, flavonoids (inclusive isoflavonoids, lignans and catechins) and tannins.

The objectives of a Quality of Life project in progress are to answer the following key questions:

- How are dietary polyphenols metabolised?
- How do polyphenols alter the rates of carcinogen metabolism?
- What are the mechanisms whereby polyphenols affect cell signalling involved in apoptosis and proliferation?
- How do polyphenols modulate early events of colon carcinogenesis?

The scientists expect to obtain urgently-needed data to permit a risk-benefit analysis of the significance of dietary polyphenols in the modulation of colon cancer, and to know whether there might be a justification for dietary modification, the production of supplements, novel foods, etc. Dissemination of the results is carried out through information to consumers (including website) and via the 4 industrial partners (including one SME). The project website is www.ifrn.bbsrc.ac.uk/Polybind/.

Project No: QLK1-1999-00505 (POLYBIND)

Project Contact: Dr. 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, e-mail: christine.hill@bbsrc.ac.uk.

This one-pager was written by Mr. F. Holm, Food Group Denmark, in April 2002.

NO FAT OR LOW FAT FOODS

FFE 496/02/HP37

Fat in foods enhances palatability by providing texture and enhancing flavour, but excessive consumption of fat is not good for health – potentially leading to overweight and obesity, and increased risk of cardiovascular disease (CVD).

Current recommendations across Europe suggest that 25-35% of dietary energy from fat is considered consistent with good health, with an overall average less than 30% energy being suggested as optimal, although in physically inactive populations, a lower figure would be preferable.

As a result of the need to provide people with products which have reduced fat, or zero fat content which still taste as good as regular products, a project called BIOMIX was set up.

The project aimed to improve understanding and optimise the quality of reduced and zero fat foods produced using biopolymers. Biopolymers (large molecules which may consist of building blocks of protein or carbohydrate) can be used to replace liquid and solid oil in a product, thus reducing the amount of fat, and therefore potentially enabling a lower calorie (energy) product to be made. Examples of foods which use biopolymers in this way include reduced fat spreads and reduced fat salad dressings.

The projects investigated biopolymer mixture models and increased understanding of the nature of particles in biopolymer mixtures. As a result, these experiments may open up new possibilities for reduced fat foods.

Project No: FAIR-CT97-3022 (BIOMIX)

Project Co-ordinator: Prof. Wim Agterof, Unilever Research, P.O. Box 114, 3130

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This one-pager was written by Dr F. Robinson, British Nutrition Foundation, UK, in April 2002.

IDENTIFYING ALLERGENS IN FOOD

FFE 498/02/HP39

Allergic reactions to food, although relatively rare, can be life-threatening. It is therefore essential for those with a food allergy to be well informed about which foods to avoid and also to have appropriate advice on how to deal with an allergic reaction when the problematic food is eaten inadvertently (e.g. as a "hidden" component in a food).

Measures to reduce the risk of allergic reactions to food allergens (the component of the food to which a person is sensitive) include:

- Having very detailed ingredients labels on foods (pre-packaged and sold loose);
- Drawing up a list of "safe" foods;
- Using food technology to develop hypoallergenic foods.

The goal of this FAIR project was to develop methods to safeguard people who are food-allergic and who risk serious reactions if trace amounts of the allergen are ingested. Double blind placebo controlled food challenges (DBPCFC), a decisive factor in diagnosing patients with food allergy, were set up to identify patients with a true food allergic reaction. It was essential that the subsequent clinical studies were carried out in patients with real allergy so that reliable diagnostic methods could be developed for identifying the presence of an allergen. In this study, the three model foods used were: the prunoidea (peach family) food group (one of the most common causes of food allergy and anaphylactic reactions – juices and purees of these fruits are widely used in food products); hazelnuts (widely used in the production of confectionery products and ice cream); and celeriac (a very common cause of anaphylactic reactions in Central Europe where it is used in many dishes).

Using the newly developed diagnostic methods, which used sera of the patients to carry out immunological identification of major allergens in the selected food groups, allergenic molecules were isolated and characterized. As a consequence, this project has developed new methods which may be used by the food industry to produce hypoallergenic foods. By producing foods with reduced allergenicity, the risk of anaphylactic shock from inadvertently eating an allergenic food could be dramatically reduced.

A synthesis report on food allergy will be available later in 2002.

Project No: 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, 20162 Milan, Italy, Tel.: +39-02-64442414; Fax +39-02-64442027, e-mail: ortolani@stnet.net.

This one-pager was written by Dr F. Robinson, British Nutrition Foundation, UK, in April 2002.

First 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,...
- New technologies for Shelf Life extension: new materials, new packaging devices, new food processing technologies, new techniques for risk reduction,...
- Shelf Life testing: non-invasive analytical techniques, freshness indicators, evaluation of packaging materials performance; risk management,...

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: September 30, 2002 for submission of an extended paper.
December 15, 2002 for final programme.
March 31, 2003 for submission of final papers.
For additional information see: http://www.packlab-jessica.it/.
For additional information contact: segreteria@packlab-jessica.it.

First Announcement

QUALITY IN CHAINS AN INTEGRATED VIEW ON FRUIT AND VEGETABLE QUALITY
Third International and Multidisciplinary Conference
July 6-9, 2003
Wageningen, The Netherlands

Scope & Announcement

Food quality and safety is becoming an ever-increasing important feature for consumers. To stay competitive in the market of fruit and vegetables, the food providers need to create safe produce with an increased perceived quality. To meet the changing demands for quality, availability, cost, appearance and service the actors in the supply chain need to cooperate. A retailer is not capable of meeting the demand without co-operation with suppliers, wholesalers, growers, plant and seed suppliers and breeders. Servicing these consum-

er needs requires integration of quality and of quality handling throughout the entire supply chain.

To enhance and to emphasise the importance of integration of quality and quality aspects throughout the entire chain this third international conference will be structured around key questions affecting the entire supply chain and will surpass the traditional working areas. This will stimulate and motivate the exchange of ideas and techniques between different areas and disciplines involved in supply chains.

Key questions structuring the integrated view are:

- What is quality and what are quality aspects throughout the supply chain?

- How can we model quality and quality aspects relevant for consumer, provider and producer?

- How do quality and quality aspects change by the way we handle products in the chain?

- What are the opportunities for manipulating and monitoring quality and quality aspects in the supply chain?

- What are the "things" to look for to get a "measure" for quality and quality aspects?

Conference Organisation Quality in Chains: P.O. Box 17, 6700 AA Wageningen, The Netherlands
Call for papers and posters:
Contributed papers do not have to be limited to these topics and may address specific subjects or case studies, pertinent to the chain approach, to link philosophical views to technological possibilities and current practice. Authors are kindly invited to submit an extended abstract (1 page tops) by e-mail to: quic@ato.wag-ur.nl
by fax to: +31 0317 475347
More detailed information can be found on our website: www.ato.wageningen-ur.nl/quic.

AACC FORMS COMMITTEE TO EVALUATE SWEDISH STUDY ON ACRYLAMIDE IN FOODS

Researchers at Stockholm University's Department of Environmental Chemistry in cooperation with Sweden's National Food Administration, a government food safety agency, released information yesterday that acrylamide, a potential cancer-causing agent, may be formed in high concentrations when carbohydrate-rich foods such as rice, potatoes and cereals are fried or baked. However, the foods when analyzed in their raw state or when boiled showed no traces of acrylamide. The scientists who conducted the research deemed the findings so important that they released them prior to actual publication in an academic journal.

The U.S. Environmental Protection Agency classifies acrylamide as a "medium hazard probable human carcinogen". The American Association of Cereal Chemists (AACC) is taking this research very seriously and is investigating the issue further as no previous work has shown acrylamide at these levels in these foods.

AACC's Scientific Advisory Panel has convened a group of AACC experts who are looking into the claims brought forth by Stockholm University and the Swedish National Food Administration. AACC is requesting a copy of the research paper that is based on a new analytical procedure developed by the Swedish National Food Administration.

The American Association of Cereal Chemists (AACC) is an international organization of nearly 3,500 grain scientists and other professionals who study the chemistry of cereal grains and their products or work in related fields.

Contact: Susan Kohn, Director of Membership and Communication,
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E-mail: skohn@scisoc.org.
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Phone: +32 16.204035,
E-mail: hilde.keunen@scisoceurope.org.

INTERNATIONAL ASSOCIATION FOR FOOD PROTECTION ANNOUNCES FOUR WORKSHOPS

The International Association for Food Protection announces four workshops to

be held in conjunction with IAFP 2002 at the Manchester Grand Hyatt in San Diego, California. The workshops are scheduled for June 28-29, 2002.

Workshop I – Critical Steps in Laboratory Methods for the Detection of *Listeria monocytogenes* – June 28-29, 2002. This workshop is an update of the science in the isolation of *Listeria monocytogenes*. Current information on the advantages and disadvantages of technologies used in recovery of this pathogen will be presented along with laboratory demonstrations at San Diego State University.

Workshop II – Current Practices in Produce Safety: GAPs and GMPs – June 28-29, 2002. The objective of this one and a half day workshop is to discuss the impact of growing practices on the food safety of produce. Experts share current knowledge on the application of "Good Agricultural Practices" for pre- and post-harvest produce. The workshop includes a field trip to local produce operations.

Workshop III – Control of Pathogens in the Dairy Processing Environment – June 29, 2002. This workshop is intended to help dairy processing facilities design and implement an effective pathogen monitoring program. Participants will learn how data from a monitoring program provides the foundation for setting up control measures. Emphasis will be placed on determining corrective actions and follow-up.

Workshop IV – Media Training for the Scientific Community – June 29, 2002. The most common source of health and food safety information is the media, the most trusted sources include scientists. This intensive, hands-on workshop will assist in developing practical media techniques, message development and mock media interviews.

Detail descriptions and registration information regarding these workshops may be obtained by visiting our Web site at www.foodprotection.org; calling 800.369.6337; 515.276.3344, or by fax at 515.276.8655.

INTERNATIONAL ASSOCIATION FOR FOOD PROTECTION ELECTS FARBER AS EXECUTIVE BOARD SECRETARY

The International Association for Food Protection welcomes Dr. Jeffrey M. Farber to the Executive Board as Secretary. Dr. Farber will take office at the conclusion of the Awards Banquet at IAFP 2002, the Association's 89th Annual Meeting in San Diego, California. By accepting this position, he made a five-year commitment to the Association and will begin his term as President in the summer of 2005.

Dr. Farber is currently Director of the Bureau of Microbial Hazards, for the Food Directorate of Health Canada, where he is responsible for the management of research and policy development in the area of microbiological food safety. His main areas of expertise are *Listeria monocytogenes*, modified atmosphere packaging, fresh-cut produce, *Enterobacter sakazakii*, and molecular typing of foodborne pathogens. He currently holds International Life Sciences Institute (ILSI) and Biotechnology grants for work on the virulence, molecular typing and biochip detection of *L. monocytogenes* in foods.

Since joining the Association in 1986, Dr. Farber served on the Program Committee six years including Chairperson and has been involved with a number of the Professional Development Groups (PDGs). He has also been a member of the Nominating Committee, Chairperson of the Developing Scientist Award Committee, and started the Fruit and Vegetable Safety and Quality PDG.

Dr. Farber is currently the Editor of the International Journal of Food Microbiology and serves on the Editorial Board of the Journal of Food Protection and the Italian Journal of Food Science. He also serves on the Journal of Food Protection Management Committee.

BOOKS

ASEPTIC PROCESSING OF FOODS CONTAINING SOLID PARTICULATES

by Sudhir K. Sastry and Bill D. Cornelius, Ohio State University Columbus, USA

March 2002, 288 pp, Cloth
ISBN: 047136359 6
Hbk £95.00 - euro 142.50
Publisher: John Wiley & Sons Ltd, Customer Service, 1 Oldlands Way, Bognor Regis, West Sussex, PO22 9SA, UK, e-mail: customer@wiley.co.uk, Please include your postal delivery address.
How to order: UK Freefone 0800 243407, Overseas +44 1243 779777, Fax your order form to: +44 (0) 1243 843296.

This is the first in-depth book to contain state-of-the-art information on this technology, which is needed for process filing with the FDA.

- Provides comprehensive, detailed coverage of three key aspects of the technology: engineering, microbiology and statistics.
- It is the only book to cover the consensus statements from 1995-96 workshops on continuous multi-phase aseptic processing of foods.

DAIRY MICROBIOLOGY HANDBOOK: THE MICROBIOLOGY OF MILK AND MILK PRODUCTS 3rd EDITION

by Richard Robinson, University of Reading, UK

May 2002, 592 pp, Cloth
ISBN: 0471385964
Hbk £95.00 - euro 142.50
Publisher: John Wiley & Sons Ltd, Customer Service, 1 Oldlands Way, Bognor Regis, West Sussex, PO22 9SA, UK, e-mail: customer@wiley.co.uk, Please include your postal delivery address.
How to order: UK Freefone 0800 243407, Overseas +44 1243 779777, Fax your order form to: +44 (0) 1243 843296.

Now in its third edition, this book continues to be the standard reference in the field on microbiology of milk and milk products. Focusing on microbial safety issues, readers will find the latest information on public health risks, pathogens, and spoilage organisms, the factors affecting growth of these micro-organisms in plants, and good manufacturing practice.

- Provides thorough coverage of both dairy microbiology principles and practical applications.
- Emphasizes HACCP and other preventive measures that can be taken by dairy processors and manufacturers.
- Includes the latest developments in dairy starter cultures and genetic engineering techniques.

IDF Publications

NUTRITION WEEK 2000 Dairy Nutrition for a Healthy Future: Abstracts & Posters

The objective of the IDF Nutrition Week 2000 was to share current nutrition in-

formation, while increasing the scientific knowledge on the ways in which dairy products can enhance well-being at all stages of life and contribute to optimum nutrition. The three major themes covered were:

- Role of Dairy Nutrition at Different Life Stages.
- Health Effects of Milk and Other Food Components.
- Dairy Products with Nutritional Benefits.

The available papers of the IDF Nutrition Week 2000 were published in the Bulletin of IDF No. 363/2001. However, it was agreed to publish the abstracts at a later stage as more were available. Posters on related topics were presented during the meeting, the abstracts of which are presented in this Bulletin.

17 pp – English only
Bulletin N. 370/2002 – 42 euro

41, Square Vergote - B-1030 Brussels, Belgium - Tel. +32 2 7339888 - Fax +32 2 7330413 - e-mail: info@fil-idf.org - Web Site: http://www.fil-idf.org

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 you do not have programs for Macintosh; 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.

4. Mailing Instructions

Papers for publication and communications regarding editorial matters should be sent to:
Prof. Paolo Fantozzi or Dr. Mary F. Traynor, F.S.E.
Dipartimento di Scienza degli Alimenti, Università di Perugia, S. Costanzo, I - 06126 Perugia, Italy
E-mail: ijfs@unipg.it or paolofan@unipg.it

REFERENCE EXAMPLES

EXAMPLES of use in a Reference list are given below. The bold-faced parenthetical type of citation above the example is indicated ONLY for information and is NOT to be included in the reference list.

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Anonymous. 1982. Tomato product invention merits CTRI Award. Food Technol. 36(9): 23.

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AOAC. 1980. "Official Methods of Analysis" 13th ed. Association of Official Analytical Chemists, Washington, DC.
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Willets C.O. and Hill, C.H. 1976. Maple syrup producers manual Agric. Handbook No. 134, U.S. Dept. of Agriculture, Washington, DC.

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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.

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(Paper accepted)
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