ISSN 1120-1770

ITALIAN JOURNAL OF FOOD SCIENCE

Rivista italiana di scienza degli alimenti

IJFS

Volume XVII
Number 2
2005

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

CHIRIOTTI EDITORI

ITALIAN JOURNAL OF FOOD SCIENCE

(RIVISTA ITALIANA DI SCIENZA DEGLI ALIMENTI)

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

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Publisher:
Alberto Chiriotti
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Tel. +39 0121 393127 - Telefax +39 0121 794480
E-mail: info@chiriottieditori.it - URL: www.chiriottieditori.it

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

Impact Factor: 0.544 published in the 2003 Journal of Citation Reports, Institute for Scientific Information

Subscription Rate: 2005: Volume XVII

Ordinary	€	120.00
Supporting	€	500.00

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

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PAPER

PURIFICATION AND CHARACTERIZATION OF AN INTRACELLULAR FAMILY 3 β-GLUCOSIDASE FROM *LACTOBACILLUS SANFRANCISCENSIS* CB1

PURIFICAZIONE E CARATTERIZZAZIONE DI UNA β-GLUCOSIDASI DELLA FAMIGLIA 3 DA *LACTOBACILLUS SANFRANCISCENSIS* CB1

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ABSTRACT

Lactobacillus sanfranciscensis CB1 uses cellobiose and other β-glucosides (methyl-β-glucoside, arbutin, amygdalin and salicin) as carbon sources. A hexameric *ca.* 288 kDa β-glucosidase

RIASSUNTO

Lactobacillus sanfranciscensis CB1 utilizza cellobiosio ed altri β-glucosidi (metil-β-glucoside, arbutina, amigdalina e salicina) come fonte di carbonio. Una β-glucosidasi esamerica di *ca.* 288

- Key words: β-glucosidase, carbon metabolism, *Lactobacillus sanfranciscensis* -

was purified to homogeneity from *Lact. sanfranciscensis* CB1 by four chromatographic steps. The enzyme was optimally active at pH 7.5 and 40°C. It had a p*I* of ca. 4.38 and a $D_{55°C}$ value of ca. 27 sec. Almost total inhibition was found with sulfhydryl-modifying agents and divalent cations such as Cu^{2+}, Co^{2+}, Hg^{2+}, Ni^{2+} and Fe^{2+} at a concentration of 2 mM. The enzyme was active towards β-(1→4) substrates such as *p*-nitrophenyl-β-D-glucoside, -D-galactoside and -L-rhamnoside, and cellobiose. The sequencing of four internal peptides showed an elevated identity with other bacterial β-glucosides of family 3. A PCR strategy with primers designed on the basis of conserved sequences was used to partially identify the gene. The deduced amino acid sequence showed 53, 48 and 45% identity with GHF3 from *Ruminococcus albus*, *Clostridium thermocellum* and *Bifidobacterium longum*, respectively.

kDa è stata purificata da *Lact. sanfranciscensis* CB1 mediante quattro step cromatografici. L'enzima ha mostrato attività ottimale a pH 7,5 e 40°C. Il pI dell'enzima è di *ca.* 4,38 ed il valore $D_{55°C}$ di *ca.* 27 sec. Una quasi totale inibizione è stata rilevata con agenti modificanti i gruppi sulfidrilici e cationi divalenti come Cu^{2+}, Co^{2+}, Hg^{2+}, Ni^{2+} e Fe^{2+} ad una concentrazione di 2 mM. L'enzima è risultato attivo su substrati β-(1→4) come *p*-nitrofenil-β-D-glucoside, -D-galattoside e -L-ramnoside e cellobiosio. Il sequenziamento di quattro peptidi interni dell'enzima ha mostrato un'elevata identità con altre β-glucosidasi batteriche appartenenti alla famiglia 3 (GHF3). Per l'identificazione del gene è stata utilizzata una strategia di PCR con primer disegnati sulle sequenze conservate. La sequenza aminoacidica dedotta della sequenza parziale del gene ha mostrato rispettivamente il 53, 48 e 45% di identità con gli enzimi GHF3 di *Ruminococcus albus*, *Clostridium thermocellum* e *Bifidobacterium longum*.

INTRODUCTION

β-Glucosidase (β-D-glucoside glucohydrolase, EC 3.2.1.21) hydrolyses the β 1,4-glycosidic linkages of various disaccharides, oligosaccharides, alkyl- and aryl- β-D-glucosides. Based on the amino acid sequence similarities, β-glucosidases are classified into glycosyl hydrolase families 1 and 3 (GHF1 and GHF3, respectively) (COUNTINHO and HENRISSAT, 1999).

The classification of glycosyl hydrolases is available at the following site: http://afmb.cnrs-mrs.fr/CAZY. The physiological role of this enzyme varies depending on the biological system where it occurs (BHATIA *et al.*, 2002). Overall, β-glucosidase is involved in the: (i) terminal step of cellulose degradation wherein it hydrolyses cello-oligosaccharides and cellobiose; (ii) hydrolysis of toxic or bitter glucosides; (iii) release of aromatic compounds; and (iv) synthesis of various oligosaccharides, glycoconjugates, alkyl- and amino-glucosides (BHATIA *et al.*, 2002; FAURE, 2002). β-Glucosides such as cellobiose, arbutin, salicin and aesculin are usually found in foods which contain plant extracts.

Recently, interest has increased in the β-glucoside metabolism of lactic acid bacteria. In most cases, β-glucosides are hydrolyzed by extra-cellular, cell wall-associated or cytoplasmic β-glucosidases (GONZÁLES-CANDELAS *et al.*, 1989). If uncleaved, β-glucosides are transported into the cells either by spe-

cific permeases (HELASZEK and WHITE, 1991) or via the phosphoenolpyruvate-dependent phosphotransferase system (PTS) with subsequent hydrolysis of the resulting P-β-glucosides into glucose 6-P and the respective aglycons (TOBISCH *et al.*, 1997; MONEDERO *et al.*, 2001). β-glucosidases have been isolated from *Lactobacillus casei* ATCC 393 (COULON *et al.*, 1998), *Lact. plantarum* (MARASCO *et al.*, 1998; MARASCO *et al.*, 2000) and *Lact. delbrueckii* subsp. *lactis* (WEBER *et al.*, 1998).

Lact. sanfranciscensis, a key component of traditionally prepared wheat and rye sourdoughs, has a great potential for improving the nutritional quality and preservation of cereal-based products (for rev. see refs. GOBBETTI and CORSETTI, 1997; GOBBETTI, 1998). Recently, the maltose metabolism (Ehrmann and Vogel, 1998), proteolytic activity, utilization of specific amino acids and peptides, synthesis of antimicrobial compounds (GOBBETTI, 1998; DE ANGELIS, *et al.* 2002), production of exopolysaccharides (KORAKLI *et al.*, 2002) and mannitol dehydrogenase (KORAKLI and VOGEL, 2003) have been studied in *Lact. sanfranciscensis*. The responses to environmental stress such as acid and high pressure have also been reported (DE ANGELIS *et al.*, 2001; DREWS *et al.*, 2002). To our knowledge, no studies have been carried out on β-glucoside metabolism. The metabolism of β-glucosides in *Lact. sanfranciscensis* is an important mechanism to ensure survival under starvation conditions (COTE *et al.*, 2000) and, more generally, may be of interest for use in cereal-based fermented food technology. β-glucosidases have been implicated in the hydrolysis of β-glucosides (cellobiose) and β-glucan degradation producing glucose for microbial growth.

In this paper we report the purification and characterization of an intracellular β-glucosidase from *Lact. sanfranciscensis* CB1.

MATERIALS AND METHODS

Microorganism, culture conditions and fermentation of β-glucosides

Lact. sanfranciscensis CB1, from the Culture Collection of the Food Technology and Biotechnology section of the Food Science Department, University of Perugia, Italy, was routinely propagated in SDB broth (GOBBETTI, 1998) at 30°C for 24 h. Overnight cultures were harvested by centrifugation (7,000 g for 10 min at 4°C), washed twice with sterile 50 mmol L^{-1} potassium phosphate buffer, pH 7.0, and re-suspended in sterile distilled water at a 620 nm absorbance (A_{620}) of 2.5, corresponding to *ca.* log. 9.5 CFU ml^{-1}. This suspension was used to inoculate (4%) sugar fermentation broth (SFB), which is MRS broth with 0.2% (instead of 0.8%) meat extract and without glucose (WEBER *et al.*, 2000), containing 1% cellobiose, methyl-β-glucoside, arbutin, amygdalin or salicin. Lactose, limarin and esculin were also used. Incubation was at 37°C for 24 h. Under the same conditions, growth was comparatively assayed by using 1% glucose instead of β-glucosides. The kinetics of microbial growth was estimated indirectly by measuring $O.D._{620}$ and directly by plating on modified MRS agar medium (GOBBETTI, 1998). The consumption of β-glucosides in the presence of glucose was assayed by using 1% cellobiose and 1% glucose. Cellobiose and glucose were measured by high pressure-liquid chromatography using 87H Bio-Rad Column heated at 50°C, 0.5 mL of 0.13 N H_2SO_4 min^{-1}. Peaks were detected by reflective index and identified and quantified by comparison with retention times of cellobiose and glucose standard (EHRLICH *et al.*, 1981).

Sub-cellular fractionation, enzyme activity and purification

Five liters of a 24-h-old culture of *Lact. sanfranciscensis* CB1, cultivated in mod-

ified MRS broth, were harvested and subjected to sub-cellular fractionation by lysozyme treatment, as described by DE ANGELIS *et al.* (2002). Two cell fractions, cell wall and cytoplasm, were recovered and used for enzyme assays. Both fractions were dialyzed for 24 h at 4°C against 20 mmol l^{-1} potassium phosphate buffer, pH 7.0, and concentrated *ca.* 20-fold by freeze-drying. Protein concentration was determined by the Bradford method (BRADFORD, 1976).

β-glucosidase activity was measured in terms of *p*-nitrophenol released from *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG) substrate. The assay mixture contained 900 µl of 2.5 mmol L^{-1} *p*NPG (final concentration) in 0.5 mol L^{-1} potassium phosphate buffer, pH 7.5, and 100 µl of enzyme preparation. The mixture was incubated at 40°C and the reaction was stopped by heating the mixture at 95°C for 5 min. The absorbance was measured at 410 nm. One unit (U) of β-glucosidase activity was defined as the amount of enzyme required to liberate 1 nmol of *p*-nitrophenol per min under the assay conditions. The specific β-glucosidase activity was defined as U per mg of protein. The data obtained were compared to a standard curve set up by using *p*-nitrophenol.

Enzyme activity was only found in the cytoplasmic fraction, therefore this fraction was used for further enzyme purification. First, the cytoplasmic extract was applied to a DEAE-cellulose anion exchange column (Amersham Pharmacia Biotech, Uppsala, Sweden). Proteins were eluted with a linear NaCl gradient (0-0.35 mol L^{-1}) in 0.05 mol L^{-1} potassium phosphate buffer, pH 7.5, at a flow rate of 42 mL h^{-1}. Fractions with the highest β-glucosidase activity were pooled, dialyzed, concentrated *ca.* 10-times by freeze-drying and subjected to gel filtration on an FPLC Superose 12 HR 10/30 column (Pharmacia Biotech). Elution with 0.05 mol L^{-1} potassium phosphate buffer, pH 7.0, containing 0.15 mol L^{-1} NaCl, was at a flow rate of 18 mL h^{-1}. Active fractions were pooled, dialyzed, concentrated *ca.* 10-times by freeze-drying and subjected to hydrophobic interaction on an FPLC Resource 1 ml HIC column (Pharmacia Biotech). Proteins were eluted with a linear $(NH_4)_2SO_4$ gradient (1.7-0 mol L^{-1}) in 0.05 mol L^{-1} potassium phosphate buffer, pH 7.5, at a flow rate of 60 mL h^{-1}. Active fractions were pooled, dialyzed, concentrated *ca.* 10-times by freeze-drying and, finally, applied to an FPLC Mono-Q HR 5/5 column (Pharmacia Biotech) by eluting with a linear NaCl gradient from 0 to 0.5 mol L^{-1} in the same buffer at a flow rate of 24 mL h^{-1}.

Enzyme characterization

The apparent molecular mass of the purified enzyme was estimated by both FPLC gel filtration and by SDS-PAGE according to Laemmli (1970). Molecular mass marker proteins (low range 14.4–97.4 kDa, Bio-Rad Laboratories, Milan, Italy) were used as references. The SDS-PAGE was also cut into two parts, one of which was stained and the other was renaturated in 0.05 mol L^{-1} potassium phosphate buffer, pH 7.5, for 1 h at 40°C, and cut into slices corresponding to the protein band. β-glucosidase activity was detected by incubation of the slices under assay conditions (MAGBOUL and MCSWEENEY, 1999).

The isoeletric point (pI) of the enzyme was determined by two-dimensional electrophoresis (2-DE) and amyloglucosidase (pI 3.8), ovalbumin (pI 5.1), carbonic anhydrase (pI 7.0), and myoglobin (pI 7.6) were used as markers. The purified enzyme was re-suspended directly in the denaturing buffer and 2-DE electrophoresis was performed using the immobiline/polyacrylamide system as described by DE ANGELIS *et al.* (2001).

The optimum pH for the purified enzyme was determined at 40°C in the range of 3.0-9.0 by using universal buffer. The temperature dependence was de-

termined at pH 7.5 in the range of 10°-75°C. To check the thermal stability of the enzyme, aliquots of the pure enzyme preparation (100 µL) were heated in capillary glass tubes at 45° to 55°C for 1 to 30 min, and the residual activity was assayed at 40°C and expressed as a percentage of the activity of an unheated sample. Decimal reduction time (*D*) was calculated by plotting the log of the residual activity versus time. Furthermore, the enzyme preparation or cells of *Lact. sanfranciscensis* CB1 were held for 30 days at 30°, 4° and -20°C and the enzyme activity was determined during that time. To prevent bacterial growth, sodium azide was added to a final concentration of 50 mmol L^{-1}.

Substrate specificity was determined by using several *p*-nitrophenol and nonchromogenic substrates. In the latter case, β-glucosidase activity was determined by assaying the liberated glucose with an enzymatic method (Kit DIFF CHAMB, Milan, Italy).

K_m value for the activity of β-glucosidase on *p*NPG was estimated at 40°C using a range of concentration from 0.4 to 2.4 mM. A LINEWEAVER-BURK (1934) plot was used for the determination. The inhibition constant (K_i) of glucose was determined by Dixon plot using a range of concentration from 0 to 33 mM.

To assay the effect of inhibitors and divalent cations, a mixture containing purified enzyme preparation and 2-10 mmol l^{-1} (final concentration) of chemical reagents or divalent cations in 0.05 mol L^{-1} potassium phosphate buffer, pH 7.5, was incubated for 1 h at 40°C.

Glucose inhibition was studied on *p*NPG substrate in the presence of 0-33 mmol l^{-1} of glucose.

To determine the influence of β-glucosides on enzyme activities, cells were grown on SFB containing 0.5% glucose, maltose or cellobiose until OD_{620} 0.6 (WEBER *et al.*, 2000). Aliquots of 100 mL of the cultures were centrifuged and used to produce cytoplasm fraction as described above. Cytoplasm fractions were dialysed and 100 µL of each sample were mixed with 900 µl of 2.5 mmol L^{-1} of cellobiose (final concentration). The mixture was incubated at 40°C for 15 min and the reaction was stopped by heating the mixture at 95°C for 5 min. The β-glucosidase activity was determined by assaying the liberated glucose with an enzymatic method as described above. Each reaction was repeated at least three times. The enzyme expression in cells grown on maltose and cellobiose, was also analysed by 2-DE. The same amount of total protein (30 µg) was used for each electrophoresis run. 2-DE was performed using the immobiline/polyacrylamide system as described by DE ANGELIS *et al.* (2001). Three gels were analysed and spot intensities were normalised as reported by BINI *et al.*, (1997). The induction factor for β-glucosidase protein (recognized by Mr and pI values) was expressed as the ratio between the spot intensities of the same protein in cells cultivated in SFB with cellobiose and maltose.

Peptide sequencing

Purified proteins in gel SDS-PAGE were cut out and submitted, as tryptic digests, to amino acid sequencing by using an automatic Protein/Peptide Sequencer (mod 477A; Applied Biosystem Inc. Foster City, CA94404, USA) connected on-line to a phenylthiohydantoin-amino acid analyzer model 120A and a control/Data Module model 900A (Applied Biosystems Inc.). Sequence comparison was performed by using SWISS-PROT/EMBL/GenBank/DDBJ databases.

DNA extraction, PCR and cloning of β-glucosidase gene of *Lact. sanfranciscensis* CB1

Genomic DNA from *Lact. sanfranciscensis* was extracted from 2 mL of an overnight culture grown in SDB broth at 30°C (DE LOS REYES-GÀVILAN *et al.*,

1992). The concentration and purity of DNA were assayed by determining the O.D. at 260 and 280 nm. Primers, designed from the conserved sequences within bacterial β-glucosidase family 3 genes, were BglF 5'-GAATCTGAGGGATTCGAT-3' and BglR 5'-ATAATAGCGATAACCGAC-3' of the genes of *Ruminococcus albus* (SwissProt AN. O24749), *Bifidobacterium longum* (GenPept AN. NP_696910) and *Clostridium thermocellum* (SwissProt AN. P14002). PCR amplification of the β-glucosidase fragment was performed using the GeneAmp PCR System 9700 (Applied Biosystems, USA) under the following conditions: template denaturation for 4 min at 94°C followed by 30 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 53°C, and extension for 30 s at 72°C. In the last cycle, extension was for 4 min at 72°C. PCR products were separated by electrophoresis at 80 V for 1 h on 2% (w/v) agarose gel from which the only amplified *ca.* 360 bp DNA fragment was extracted and purified with NucleoSpin columns (Macherey-Nagel, Düren, Germany). The DNA was ligated directly into a dTTP tailed pGEMT vector (Promega Co., USA) and transformed into *Escherichia coli* DH5α. Computer analyses of DNA sequences were done on recombinant clone sequenced by MGW Biotech (Florence, Italy).

RESULTS AND DISCUSSION

Fermentation of β-glucosides by *Lact. sanfranciscensis* CB1

Lact. sanfranciscensis CB1, a well-known and characterized sourdough strain (GOBBETTI, 1998), mainly uses maltose to grow (EHRMANN and VOGEL, 1998; GOBBETTI, 1998). However during growth in SFB medium, *Lact. sanfranciscensis* CB1 used cellobiose, methyl-β-glucoside, arbutin, amygdalin and salicin as carbon sources (Fig. 1). Compared to the growth in the presence of glucose, the latency phase was longer with β-glucosides. Cell yield was also affected. *Lact. sanfranciscensis* CB1 reached the same final cell growth as with glucose ($O.D._{620}$ 2.5 corresponding to *ca.* $5.0x10^9$ CFU mL^{-1}) only when cellobiose was added to SFB medium. The pH at the end of fermentation was *ca.* 3.4-3.5 in the presence of all the β-glucosides used. No growth or acidification was found when lactose, limarin or esculin were used as carbon sources (data not shown). β-glucoside metabolism has been studied in several lactobacilli. CIAFARDINI *et al.* (1994) and LEI *et al.* (1999) showed that *Lact. plantarum* strains were able to hydrolyse various β-glucosidases of plant origin as salicin, arbutin, cellobiose, amygdalin and esculin. The consumption of cellobiose in the presence of glucose was also assayed by using SFB containing 1% glucose and 1% cellobiose. *Lact. sanfranciscensis* CB1 preferentially used glucose because the consumption of cellobiose decreased by *ca.* 90% when glucose was present in the medium. A glucose-repressible P-β-glucosidase gene, active on salicin, was recently identified in *Lact. plantarum* (MARASCO, *et al.* 1998). MARASCO *et al.* (2000) identified the bglGPT operon of *Lact. plantarum*, which codifies for a transcriptional antiterminator (BglG), a permease of the PTS (BglP) and a β-glucosidase (BglT). WEBER *et al.* (1998) cloned and sequenced the *arbZ* gene of *Lact. delbrueckii* subsp. *lactis*. Expression of the *arbZ* gene in *E. coli* seemed to confer the capacity to use arbutin, while *Lact. helveticus arbZ* transformants also fermented cellobiose, salicin and methyl-β-glucoside (WEBER *et al.*, 2000). *Lact. casei* ATCC393 showed β-glucosidase activity when grown with 10 g l^{-1} cellobiose as carbon source (COULON *et al.*, 1998). The assimilatory pathways of β-glucosides are well characterized for bacteria such as *E. coli* (SCHNETZ *et al.*, 1987), *Erwinia chrysanthemi* (HASSOUNI *et al.*, 1992), *Clostridium longisporium* (BROWN and THOMSON, 1998), *Lact.*

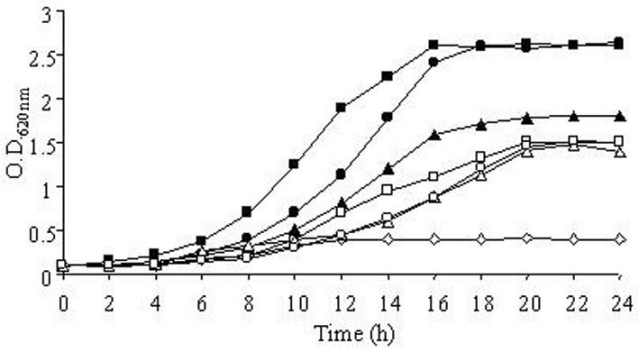


Fig. 1 - Kinetics of growth ($O.D._{620}$) of *Lactobacillus sanfranciscensis* CB1 on SFB medium with 1% glucose (■), cellobiose (●), methyl-β-glucoside (▲), arbutin (□), amygdalin (○) or salicin (▲). Growth on SFB without a carbon source was also included (◊).

plantarum (MARASCO *et al.*, 1998; 2000; MUSCARIELLO *et al.*, 2001) and *Bacillus subtilis* (LE COQ *et al.*, 1995; TOBISCH *et al.*, 1997).

Purification and characterization of β-glucosidase

The purification to homogeneity of the β-glucosidase of *Lact. sanfranciscensis* CB1 required four chromatographic steps: anion exchange on DEAE-cellulose, gel filtration on Superose 12HR 10/30, hydrophobic interaction on FPLC Resource and again anion exchange on Mono-Q HR 5/5 columns. The enzyme was purified 35-fold with a *ca.* 10% recovery yield. After the final chromatographic step on MonoQ, SDS-PAGE showed a single protein band with an apparent molecular mass of *ca.* 47.5 kDa (Fig. 2). On excision from the SDS-PAGE gel, only the protein of 47.5 kDa showed β-glucosidase activity (data not shown).

A single peak at a position corresponding to *ca.* 288 kDa was eluted by gel filtration on Superose 12HR 10/30. Non-denaturating SDS-PAGE also yielded a single protein with a molecular mass of approximately 295 kDa (data not shown). These results led to the conclusion that the enzyme consisted of six identical subunits of *ca.* 48 kDa. The pure preparation of the enzyme was used for further characterization. A hexameric structure for lactobacilli β-glucosidase was also reported for *Lact. casei* ATCC393 (COULON *et al.*, 1998). The apparent molecular masses of bacterial β-glucosidases range from 47 kDa in *B. breve* (NOMOURA *et al.*, 1996) to 480 kDa in *Lact. casei* ATCC393 (COULON *et al.*, 1998).

The activity of the β-glucosidase was optimal at pH 7.5. At pH 6.0 and 5.0, the enzyme had a relative activity of 44 and 30%, respectively, while at pH 9.0 it maintained *ca.* 72% of the maximum activity. The optimum temperature was found to be 40°C with *ca.* 64 and 18% residual activity at 30° and 45°C, respectively. As determined by 2-DE, the isoelectric point (pI) of the enzyme was found to be *ca.* 4.38. It is quite similar to those found for the β-glucosidases of *B. breve* (pI 4.3) (NOMOURA *et al.*, 1996) and *Cl. thermocellum* (pI 4.4) (ROMANIEC *et al.*, 1993). Overall, bacterial β-glucosidases have an acidic pI.

β-Glucosidase of *Lact. sanfranciscensis* CB1 retained *ca.* 83% of the maximum activity after heating at 45°C for 30 min, *ca.* 5.6% at 50°C for 30 min and no activity was detected after 30 min at 55°C. The D value calculated at 55°C was *ca.* 27 s. No loss of activity was observed after storage for 30 days at -20°C, or 4°C. When the enzyme was stored at 30°C, no loss of activity was found within 24 h and 50% of the maximum activity was maintained after 7 days.

β-Glucosidase activity was assayed on

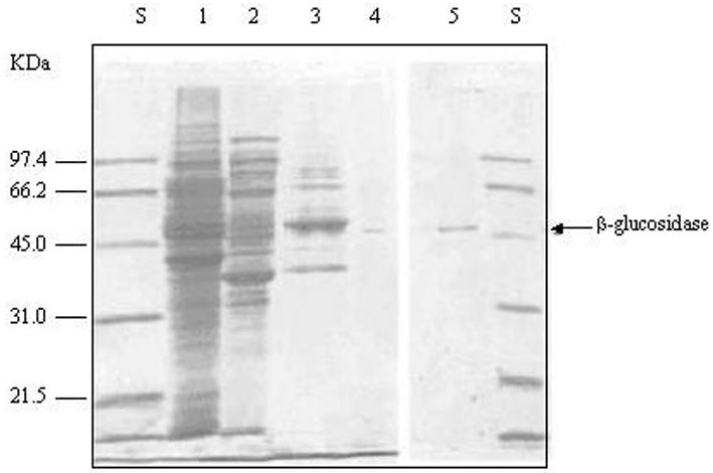


Fig. 2 - SDS-PAGE for the intracellular β-glucosidase of *Lactobacillus sanfranciscensis* CB1. Lanes: S, reference proteins; 1, cytoplasmic extract; 2, enzyme preparation after anion exchange on DEAE-cellulose; 3, after gel filtration on Superose 12HR 10/30; 4, after hydrophobic interaction; and 5, after anion exchange on Mono-Q HR 5/5.

various substrates with α or β configurations (Table 2). The enzyme was active towards *p*-nitrophenyl-β-D-glucoside, -D-galactoside and -L-rhamnoside and cellobiose. Activity was also detected on *p*-nitrophenyl- α-D-glucoside, although to a less extent, while the enzyme was not active on *p*-nitrophenyl- α-L-fucoside and -L-rhamnoside. In agreement with the results for cell growth, a very low activity was detected on lactose. This enzyme did not hydrolyse diglucosides with a (1→4)-α configuration such as maltose, the cyanogenic glycoside limarin and maltotriose, trealose, esculin and starch. This characterization agreed substantially with the substrate specificity found for the β-glucosidase of *Lact. casei* ATCC 393 (COULON *et al.*, 1998).

As determined by the linear Lineweaver-Burk plot of 1/V against 1/[S] on β-pNPG, the values of K_m and V_{max} for the β-glucosidase of *Lact. sanfranciscensis* CB1 were 7.6 mmol L^{-1} and 2.4 μmol mg^{-1} min^{-1}, respectively. These values was similar to *Lact. casei* (K_m of 16 mmol L^{-1}) (COU-

Table 1 - Activity of the β-glucosidase in the cytoplasmic extract of *Lactobacillus sanfranciscensis* CB1 cells grown on different carbohydrates.

Cell growth conditions	Amount of total protein in the assay (mg)	[a]Specific activity (%) (U/mg)
Maltose	13.5	10.05[c]
Glucose	12.7	9.74[c]
Cellobiose	13.0	54.16[d]

[a] One unit (U) of β-glucosidase activity was defined as the amount of enzyme required to liberate 1 nmol of cellobiose per 10 min under the assay conditions. The specific β-glucosidase activity was defined as U per mg of proteins The β-glucosidase activity is the average of the three replicates analysed twice.
[c-d] Values in the same column with different superscript letters differ significantly (P<0.05).

Table 2 - Specificity of the β-glucosidase of *Lactobacillus sanfranciscensis* CB1 on different substrates.

Substrate	Configuration of glycoside linkage	Relative activity (%)
p-Nitrophenyl β-D-glucoside	β (1-4)	100.00[a]
p-Nitrophenyl α-D-glucoside	α (1-4)	0.5[f]
p-Nitrophenyl β-D-galactoside	β (1-4)	18.61[c]
p-Nitrophenyl α-L-fucoside	α (1-4)	0.0[g]
p-Nitrophenyl β-D-glucuronide	β (1-4)	0.0[g]
p-Nitrophenyl β-L-rhamnoside	β (1-4)	17.0[d]
p-Nitrophenyl α-L-rhamnoside	α (1-4)	0.0[g]
Maltose	α (1-4)	0.0[g]
Maltotriose	α (1-4)	0.0[g]
Trealose	β (1-1)	0.0[g]
Cellobiose	β (1-4)	50.0[b]
Lactose	β (1-4)	4.2[e]
Limarin	β (1-4)	0.0[g]
Esculin	β (1-4)	0.0[g]
Starch	α (1-4)	0.0[g]

The β-glucosidase activity is the average of the three replicates analysed twice.
[a-g] Values in the same column with different superscript letters differ significantly ($P<0.05$).

LON *et al.*, 1998) and differed from those determined for *Flavobacterium meningosepticum* (K_m of 0.49 mmol L^{-1}) (LI *et al.*, 2002), *Leuconostoc mesenteroides* (K_m of 0.7 mmol L^{-1}) (Gueguen *et al.*, 1997) on the same substrate (β-pNPG).

The study of inhibition by glucose was performed with *p*NPG as the substrate.

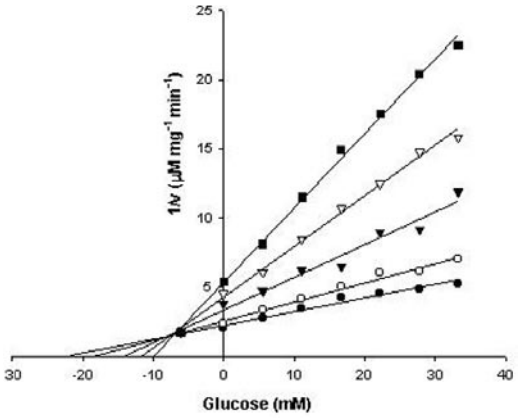


Fig. 3 - Dixon plot of inhibitory effect of glucose on *p*NPG hydrolysis by purified β-glucosidase from *Lact. sanfranciscensis* CB1. The reaction time was 1 h at pH 7.5 and 40°C. The *p*NPG concentrations used were 0.4 (■), 0.8 (△), 1.2 (▲), 1.6 (○), and 2.0 (●) mM.

Glucose acted as a competitive inhibitor of *p*NPG hydrolysis with an inhibition constant (K_i) of 6.1 mM (1.1 g L^{-1}) obtained at the intersection of the lines on the Dixon plot analysis (Fig. 3). This value was similar to other lactic acid bacteria such as *Lact. casei* (K_i of 16 mmol L^{-1}) (Coulon *et al.*, 1998) *Leuconostoc mesenteroides* (K_i of 14.3 mmol L^{-1}) (GUEGUEN *et al.*, 1997).

The chelating agent, ethylenediaminetetracetic acid (EDTA), did not inhibit the β-glucosidase of *Lact. sanfranciscensis* CB1. In contrast, the enzyme was strongly inhibited (90% inhibition) by sulphydryl-modifying agents such as N-ethylmaleimide (NEM) and iodoacetammide, especially at the highest concentration of 10 mmol L^{-1}. An increase of the activity (20-30%) was found when 2 or 10 mmol L^{-1} dithiotreitol (DTT) were added. These results suggested that –SH groups play an important role for enzyme activity. The importance of –SH groups for enzyme activity has been highlighted in other microbial β-glucosidases (OHMIYA *et al.*, 1991).

Divalent cations such as Co^{2+}, Hg^{2+}, Ni^{2+}, Cu^{2+} and Fe^{2+} were almost totally

inhibitory at 2 mmol L^{-1} and Mn^{2+} and Zn^{2+} also had a high inhibitory effect at 10 mmol L^{-1}.

The β-glucosidase activity was expressed during growth with glucose or maltose (0.5%) as carbon sources. Nevertheless, the expression was increased *ca.* five times by using cellobiose instead of maltose and glucose (Table 1). The cytoplasmic extract from cells grown with 0.5% cellobiose added, analysed by 2-DE showed an induction factor of *ca.* 3.5 compared to cytoplasmic extract from cells grown in maltose.

Overall, it has been shown that the synthesis of other bacterial β-glucosidases was subjected to a glucose-mediated catabolic repression (CR) and an induction was found in the presence of β-glucosides (CHAILLOU *et al.*, 2001).

Peptide sequencing

After tryptic digestion, the sequences of four different internal peptides of the β-glucosidase of *Lact. sanfranciscensis* CB1 were determined. The sequences were -ADKVVFFAK-, -EGLFVGYR-, -GFEK- and -AGER-. Three amino acid residues (shown in bold) of the peptide -EGLFVGYR- are strongly conserved within β-glucosidases of family 3. The whole peptide has 100% identity with the sequences of β-glucosidase characterized from *Cl. thermocellum* (SwissProt AN. P14002) and it has 85% identity with the β-glucosidases of *R. albus* (SwissProt AN. O24749), *B. longum* (GenPept AN. NP_696910), and *Thermotoga neapolitana* (SwissProt AN. Q60038). The peptides -ADKVVFFAK- and -AGER- have 77 and 75% identity, respectively, with the amino acid sequence of the β-glucosidase of *R. albus*.

Partial identification of a β-glucosidase gene

Given the above peptide similarities between the β-glucosidase of *Lact. sanfranciscensis* CB1 and the other bacte-

Table 3 - Multiple alignment of *Lactobacillus sanfranciscensis* β-glucosidase with other glycosyl hydrolase of family 3 (GHF3).

Ruminococcus (GLUA)[a]	421	**ESEGFDRKHMQLPQCQIDLIDKLSEVNPNIVVVLHNGAPVEMPFANGDEDSNS**
Clostridium (BGLB)[b]	418	**ESEGFDRTHMSIPENQNRLIEAVAEVQSNIVVVLLNGSPVEMPWID------K**
Lactobacillus sanfranciscensis[c]		**ESEGFDKTTMSLPEAQVKLLNQVLSVNPHVVVVLQNGSVVEMPWAD------Q**
Bifidobacterium (BGLB)[d]	425	**ESEGFDRDALDMPAKQITLLEQVAAANQNVVVVLSNGSVITVAPWAKN-----**
C terminal domain[e]	105	**EGEAGDRTNLTLPHNQDDLIEAVAETGKPVVVVLSVGRPVDEPWIDNP----N**
Ruminococcus (GLUA)[a]		VKAILEMYLSGQAAGEAVVRILFGEVNPSGKLAETFPLRLEDNPSYLN----FPGEADIV
Clostridium (BGLB)[b]		VKSVLEAYLGGQALGGRWR-MCYSVKSIVGKLAETFPVKLSHNPSYLN----FPGEDDRV
Lactobacillus sanfranciscensis[c]		VPAILETYLAGEAVGEATWDILSGAVNPSGKLAETFPLKLADNPTHGT----FARSYSEE
Bifidobacterium (BGLB)[d]		AKGILESWLLGQSGGPALADVIFGQVSPSGKLAQSIPLDINDDPSMLN----WPGEEGHV
C terminal domain[e]		VDAILWAWLPGQEGGNAIADVLFGKVNPSGKLPFTFPKSADDYPSPYIPKNLGPPGGFPQ
Ruminococcus (GLUA)[a]		KYSEGIFVGYRYY 542
Clostridium (BGLB)[b]		EYKEGLFVGYRYY 532
Lactobacillus sanfranciscensis[c]		NYKEGLFVGYRYY
Bifidobacterium (BGLB)[d]		DYGEGVFVGYRYY 534
C terminal domain[e]		DYEGGIGVDYRYF 229

[a] *Ruminococcus albus* (TrEMBL O66050), [b] *Clostridium thermocellum* (SwissProt accession no. P14002), [c] *Lactobacillus sanfranciscensis* CB1 (this work), [d] *Bifidobacterium longum* (GenPept accession no. NP_696910) and [e] Family 3 C terminal domain. Bold print indicates conserved amino acid residue.

rial β-glucosidases, primers (BglF and BglR) were designed from conserved sequences within GHF3.

PCR amplification of the β-glucosidase fragment was purified, ligated directly into a dTTP tailed pGEMT vector, and transformed into *Escherichia coli* DH5α. Computer analyses of DNA sequences were done on recombinant clone sequenced by MGW Biotech. The predicted translation product, a 116 amino-acid polypeptide, had 49, 46 and 39% identity with the sequences of GHF3 β-glucosidases of *R. albus*, *Cl. thermocellum* and *B. longum*, respectively (Table 3). The GHF3 exhibits a modular organization in two domains: an N-terminal catalytic A domain and a C-terminal non-catalytic, but essential, B domain. Among bacterial enzymes, the AB organization is the most frequent, but the BA and AB' (exhibiting a truncated C-terminal domain referred to as B') types have also been described. The amino acid deduced sequence of the β-glucosidase of *Lact. sanfranciscensis* had *ca.* 51% identity with the C-terminal domain of the GHF3 subclass B. Only a fragment (EGLFVGYR) determined by the N-terminal amino acid sequencing of the purified protein is identified in the gene sequence. Being that the sequence EGLFVGYR is conserved in GHF3, there is the possibility that *Lact. sanfranciscensis* CB1 has more than one GHF3 gene and further studies are necessary to confirm that the partial sequence of the identified gene codifies for the purified GHF3 protein.

To our knowledge this is the first report on the purification and characterization of a β-glucosidase family 3 within the *Lactobacillus* species. These results may facilitate the optimization of carbon breakdown during food fermentation with *Lact. sanfranciscensis* starter cultures.

ACKNOWLEDGMENTS

We are grateful to Dr. Federico Baruzzi for his help in revising the manuscript.

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Paper received June 10, 2004 Accepted March 10, 2005

PAPER

SELECTION OF FOLATE-PRODUCING LACTIC ACID BACTERIA FOR IMPROVING FERMENTED GOAT MILK

SELEZIONE DI BATTERI LATTICI PRODUTTORI DI FOLATI PER IL MIGLIORAMENTO DEI LATTI FERMENTATI CAPRINI

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ABSTRACT

Goat milk is a complete food but its low level of folic acid diminishes its nutritional efficacy. In this study, *Streptococcus thermophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lb. delbrueckii* subsp. *lactis* and *Lb. helveticus* strains were selected for folate production in goat milk to improve its quality. A suitable HPLC method was developed to directly determine both total folate and its biologically active derivatives such as 5-methyl-tetrahydrofolate (5-CH_3-H_4-PteGlu), tetrahy-

RIASSUNTO

Il latte di capra è un alimento completo, tuttavia il suo valore nutrizionale è sminuito dalla mancanza di acido folico. Al fine di migliorare dal punto di vista alimentare la qualità delle produzioni caprine sono stati selezionati ceppi di *Streptococcus thermophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lb. delbrueckii* subsp. *lactis* e *Lb. helveticus* capaci di produrre folati in latte di capra. È stata sviluppata una metodica per la determinazione dei folati in latte di capra mediante HPLC. L'anali-

- Key words: folate, goat milk, HPLC, LAB, yoghurt -

drofolate (H_4-PteGlu) and 5-formyl-tetrahydrofolate (5-CHO-H_4-PteGlu). Results showed that *S. thermophilus, Lb. delbrueckii* subsp. *lactis* and *Lb. helveticus* strains produced higher amounts of total folate than *Lb. delbrueckii* subsp. *bulgaricus*, especially 5-CH_3-H_4-PteGlu and H_4-PteGlu. Moreover the use of selected *S. thermophilus* strains in association with *Lb. delbrueckii* subsp. *bulgaricus* resulted in yoghurt with a significant quantity of folate and good organoleptic features.

si ha riguardato sia i folati totali che le forme biologicamente attive quali il 5-metil-tetraidrofolato (5-CH_3-H_4-PteGlu), il tetraidrofolato (H_4-PteGlu) e il 5-formil-tetraidrofolato (5-CHO-H_4-PteGlu). I ceppi delle specie *S. thermophilus, Lb. delbrueckii* subsp. *lactis* e *Lb. helveticus* hanno prodotto più folati totali rispetto al *Lb. delbrueckii* subsp. *bulgaricus* e in particolare 5-CH_3-H_4-PteGlu e H_4-PteGlu. L'impiego di ceppi selezionati di *S. thermophilus* in associazione con *Lb. delbrueckii* subsp. *bulgaricus* ha inoltre consentito di ottenere uno yogurt da latte di capra con un elevato tenore di folati e buone caratteristiche sensoriali.

INTRODUCTION

Folic acid and its vitamers such as 5-methyl-tetrahydrofolate (5-CH_3-H_4-PteGlu), tetrahydrofolate (H_4-PteGlu) and 5-formyl-tetrahydrofolate (5-CHO-H_4-PteGlu), commonly referred to as folate, represent a group of essential vitamins in human metabolism. These metabolites take part in DNA and RNA biosynthesis and repair (FOWLER, 2001). Moreover, during early pregnancy, a diet with insufficient folate is linked with a minor risk of neural tube defects in the foetus (LOCKSMITH and DUFF, 1998; OAKLEY, 1999; BERRY *et al.*, 1999). Folate deficiency is also the most frequent cause of megaloblastic anemia and hyperhomocysteinemia (HERCBERG and GALAN, 1992). Hence, the importance of ingesting a sufficient daily quantity of folate, as well as knowing its content and bioavailability in food has been reviewed by SARMA *et al.* (1995) and WIGERTZ *et al.* (1997).

Milk and its fermented derivatives can be an important source of folate. That is why several methods for extraction and HPLC analysis of folate from milk and dairy products have been proposed in a number of studies also aimed at defining a standard method of determination that is currently lacking (VAHTERISTO *et al.*, 1996; 1997; RUGGERI *et al.*, 1999; KONINGS, 1999; WIGERTZ *et al.*, 1997).

Folate quantity and composition in milk and dairy products is affected by several factors such as the processing technology of milk and the time and incubation temperature (WIGERTZ *et al.*, 1997; FORSSEN *et al.*, 2000; LIN and YOUNG, 2000b; CRITTENDEN *et al.*, 2003). However, differences in folate content are greatly affected by different milk sources. It is generally known that goat milk is excellent for yoghurt and fresh cheese production because of the balanced content of both essential amino acids and fatty acids, hypo-allergenicity and digestibility (PARK, 1994; HAENLEIN, 2004). Despite this, goat milk is dramatically lacking in folate (UBERTALLE, 1986). Lactic Acid Bacteria (LAB) can be another key factor in folate biosynthesis and/or consumption in dairy products (RAO *et al.*, 1984; WIGERTZ *et al.*, 1997). Several researchers have investigated the ability of different LAB spe-

cies (such as *Streptococcus thermophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactococcus lactis* subsp. *lactis* and *Lc. lactis* subsp. *cremoris*) to affect the folate content of skim milk (Oxoid) and full-cream milk (LIN and YOUNG, 2000a; CRITTENDEN *et al.*, 2003). However, there is a substantial lack of information on the folate content of different fermented products made from goat milk, e.g. yoghurt, and at the same time not much is known about the influence of different LAB species (and strains) on the folic acid content and its vitamers (SYBESMA *et al.*, 2003). The aim of this work was to assess folate production by *Streptococcus thermophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lb. delbrueckii* subsp. *lactis* and *Lb. helveticus* strains in goat milk. Moreover certain LAB strains were also characterised for suitable technological features essential for yoghurt production. Finally selected cultures of *S. thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus* were used to obtain goat yoghurt with a substantial folate content and with good sensory features.

MATERIALS AND METHODS

Bacterial strains

Different LAB strains autochthonous to goat milk (apart from the reference strains), and belonging to the collection of the Dipartimento di Scienze Ambientali Agrarie e Biotecnologie Agro-Alimentari, University of Sassari (Italy), were used. Twelve *S. thermophilus* strains (SL1, SL2, SL3, SL4, SL7, SF5, SY2, SY4, SS1, SS2, SS4 and NCDO489 as reference strain), three *Lb. delbrueckii* subsp. *bulgaricus* (LY1, LY2 and CNRZ369 as reference strain), one *Lb. delbrueckii* subsp. *lactis* strain (LF14), and one *Lb. helveticus* (LF22) were tested for folate production and characterised for some relevant technological features such as growth speed and acidifying activity. All the tests were carried out in triplicate in sterilised whole goat milk.

Folate analysis by HPLC

The folate production of *S. thermophilus*, *Lb. delbrueckii* subsp. *bulgaricus*, *Lb. delbrueckii* subsp. *lactis* and *Lb. helveticus* strains was quantified after inoculation and growth in sterile goat milk. Folate production of mixed cultures made of *S. thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus* strains was assessed in goat yoghurt. Single strains and mixed cultures (1:1 ratio) were inoculated at the final rate of 1% in sterile goat milk and folate determination was assessed after 8 hours of incubation at 42°C. The method of VAHTERISTO *et al.* (1996; 1997) was basically employed for folate analysis. However substantial modifications were introduced to adapt the methodologies to goat milk.

Folate extraction: 5 mL samples of fermented milk or yoghurt were homogenised with 25 mL of extraction buffer (0.1 M phosphate buffer containing 0.5% sodium ascorbate and 0.1% (v/v) 2-mercaptoethanol pH 6.0) using an Ultra Turrax Homogenizer (13,500 rpm for 20-30 seconds). The homogenized sample, carefully protected against light, was incubated under nitrogen atmosphere in a boiling water bath for 15 minutes. The extract was rapidly ice-cooled and centrifuged at 4°C for 30 minutes at 12,000 x *g*. The supernatant was separated and the pellet dissolved again in 10 mL of extraction buffer and centrifuged at 12,000 x *g* for another 10 minutes. Finally the supernatant was separated, added to the first supernatant and the mixture brought to volume up to 50 mL with the extraction buffer.

Deconjugation: a large quantity of folate is present in food as polyglutamates (FINGLAS *et al.*, 1999). In order to be detected and identified, they need to be converted into the respective monoglutamates using deconjugation

enzymes. For this purpose, the Human Plasma (HP) enzyme from Sigma-Aldrich Chemical was used. The enzyme activity was tested by adding 50 µL of HP to 30 µg of pteroil-γ-L-polyglutamate (PteGlu$_3$) in 1.3 mL of 0.1 M acetate buffer at pH 4.9. This mixture was incubated at 37°C for 45 minutes, filtered through a 0.45 µm filter and injected into the HPLC. The analysis showed that 90% of the pteroil-γ-L-polyglutamic acid was converted into folic acid. This 90% efficiency rate was used to adjust the total folate readings. For the deconjugation reaction, 5 mL of extract, brought to pH 4.5 with 0.1 M HCl was added to 3 mL of HP and incubated in a gently shaking water bath at 37°C for 3 hours. The extract was put into boiling water for 5 minutes in order to stop the enzyme activity, then ice-cooled and centrifuged at 10,000 x *g* for 10 minutes.

Purification: 3 mL of deconjugated extract were diluted with 6 mL of distilled water and 15 µL of 2-mercaptoehtanol to lower the salt concentration before injecting it through the strong anion exchange columns, Bakerbond SPE, Quaternary Amine (N^+).

Cartridges, previously conditioned with hexane, methanol and distilled water and treated with 0.01 M phosphate buffer containing 1% of 2-mercaptoethanol, were loaded with 3 mL of sample and washed twice with 1.5 mL of the same phosphate buffer as used for column conditioning. Finally, folate were eluted with 2.5 mL of eluant buffer (0.1 M sodium acetate) containing NaCl at 10% w/v and ascorbic acid at 1% w/v.

The eluate was filtered through 0.45 µm filters and 20 µL of this solution were injected into the HPLC. Instruments were: a pump system with three solvents and gradient control (Varian 9010), a variable wavelength uv/vis detector (Varian 9050) and a Linear Fluo LC 305 fluorometer. A Shandon Hypersil ODS (3 µm, 150x4.6 mm) column was used together with a Novapak C18 pre-column, thermostated at 30°C with a thermostat (Timberline Instruments, Inc., Boulder CO, USA).

Operating conditions were as follows: Mobile phase: A = phosphate buffer (pH 2.2); B = acetonitrile; Flow rate: 1 mL/min; Gradient: 0 min. 90% A; 9 min. 90% A; 12 min. 74% A; 20 min. 90% A; Wavelengths for fluorometric determinations: Excitement = 290 nm, emission = 356 nm for determining tetrahydrofolate (H_4-PteGlu), 5-methyl-tetrahydrofolate (5-CH_3-H_4-PteGlu) and 5-formyl-tetrahydrofolate (5-CHO-H_4-PteGlu); Wavelength for determining folic acid and pteroil-γ-L-polyglutamate (PteGlu$_3$) = 290 nm.

Quantitative folate analysis was performed using a calibration curve made up of 5 different concentrations of standard compounds. Standards were tetrahydrofolate (H_4-PteGlu), 5-methyl-tetrahydrofolate (5-CH_3-H_4-PteGlu), 5-formyl-tetrahydrofolate (5-CHO-H_4-PteGlu), and folic acid (Sigma-Aldrich) and pteroil-γ-L-polyglutamate (PteGlu$_3$) (Schircks Laboratories, Jona, CH). Standard solutions were prepared by dissolving each compound in 0.1 M phosphate buffer at pH 7; aliquots of these solutions were analysed spectrophotometrically to determine the purity level according to KONINGS (1999). Standard mixtures for the calibration curve were prepared using the rates of these solutions, brought to volume in 0.01 M acetate buffer containing 1% w/v sodium ascorbate at pH 4.9. Recovery percentages of 5-CH_3-H_4-PteGlu and folic acid were also determined in order to evaluate the substance losses during the extraction steps: they were 100% and 99% for 5-CH_3-H_4-PteGlu and folic acid, respectively.

Growth and acidification curves in goat milk

The technological suitability of different strains for yoghurt or fresh cheese production was assessed in sterilized

goat milk by inoculating each LAB strain to the final rate of about $1x10^6$ cells mL^{-1}. Growth abilities were assessed by determining the viable LAB count after 2, 4, 6, 8 and 24 h of incubation in goat milk at 42°C. Post-acidification trials were carried out by determining the viable count on plate and the acidifying activity after 8 h and 5, 15 and 30 days of storage at 4°C in goat milk. Colony Forming Units (CFUs) were determined in both the trials on solidified M17 and MRS media. Acidity determinations were carried out in 10 mL of milk titrated with 0.1 N NaOH. Phenolphthalein was used as indicator and acidity is expressed as percent of lactic acid or Dornic degrees (°D = 0.01% lactic acid). These trials were also carried out on mixed cultures (SL1 + LY1 and SL1 + LY2) inoculated in sterile goat milk at a 1:1 ratio.

Yoghurt production

Yoghurt was produced at a pilot scale using goat milk treated at 92°C for 10 min. The mixed strains SL1 + LY1 and SL1 + LY2 were used. A commercial product was also used for comparison. Mixed cultures were inoculated in goat milk at the 1:1 ratio and incubated at 42°C for 8 h. Sensory features of yoghurts were determined according to BATTISTOTTI (1983) and by a panel of untrained tasters consisting of 20 habitual yoghurt consumers.

Statistical analysis

The means and standard deviations were determined for the different folate forms detected in fermented goat milk and yoghurts. Mean values were compared by the Analysis of Variance (ANOVA) followed by the Fisher Least Significant Difference (LSD) method (significant level $p<0.05$). Data analysis were performed using the NCSS statistical analysis software.

RESULTS AND DISCUSSION

Folate production by different LAB species

Although a very low amount of folate is commonly present in goat milk (UBERTALLE, 1986), folate vitamers were not detected in the uninoculated goat milk used in this study, most likely because the goat milk was heat-sterilized before LAB inoculation. This was expected since significant folate losses during UHT treatment have been previously reported (WIGERTZ *et al.*, 1996). The folate level was then measured 8 h after LAB inoculation in goat milk. In preliminary trials this time was found sufficient for all the strains (single and mixed) to reach the maximum growth in goat milk. Moreover LIN and YOUNG (2000b) have already shown that folate production by LAB is basically growth-associated, reaching the highest (folate) content before the stationary phase and then declining during fermentation and storage. Therefore in the present study, the folate level was not assessed during the late fermentation stage and storage.

All the *S. thermophilus* strains evaluated produced different folate vitamers, especially H_4-PteGlu and 5-CH_3-H_4-PteGlu, which were the most abundant forms (Table 1). The high production of 5-CH_3-H_4-PteGlu by several strains (e.g. SL1, SL4 and SS4) is important since this vitamer plays a significant role in human metabolism converting homocysteine into methionine and thus contributing to reducing the risk of cardiovascular disease (RIDDEL *et al.*, 2000; SELHUB *et al.*, 2000; GRUNDY *et al.*, 1999). Moreover as recently reviewed by FORSSEN *et al.* (2000), there are substantial differences in the stability between various reduced folate forms (H_4-PteGlu). Generally the order of stability for the different vitamers considered in this study is as follow: 5-CHO-H_4-PteGlu > 5-CH_3-H_4-PteGlu > H_4-PteGlu (FORSSEN *et al.*,

Table 1 - Production (ng/g) of tetrahydrofolate (H_4-PteGlu), 5-methyl-tetrahydrofolate (5-CH_3-H_4-PteGlu) and 5-formyl-tetrahydrofolate (5-CHO-H_4-PteGlu) in goat milk inoculated with *Streptococcus thermophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactobacillus delbrueckii* subsp. *lactis* and *Lactobacillus helveticus* strains (average values of three determinations ± standard deviations). Values in the same column with different letters are significantly different ($p<0.05$).

	H_4PteGlu	5-CH_3-H_4PteGlu	5-CHO-H_4PteGlu	Total folate
S. thermophilus				
SL1	20.99±1.73 [b]	21.11±2.98 [b]	17.24±2.86 [a]	59.33±7.57 [b]
SL2	12.67±1.77 [d]	18.08±0.18 [c]	18.44±1.43 [a]	49.19±3.02 [c]
SL3	11.85±1.01 [d]	17.76±0.68 [c]	14.83±0.04 [b]	44.43±1.73 [d]
SL4	18.52±2.00 [b]	22.14±4.82 [b]	-	40.66±2.82 [d]
SL7	18.94 [b]	30.72 [a]	-	49.66 [c]
SS1	22.40±1.32 [b]	7.27±0.65 [d]	17.96±0.51 [a]	47.63±2.47 [c]
SS2	20.40±1.70 [b]	4.30±0.42 [e]	-	24.70±2.12 [f]
SS4	14.53±1.68 [c]	23.52±0.17 [b]	21.50±0.08 [a]	59.54±1.77 [b]
SY2	22.72±1.10 [b]	8.01±0.22 [d]	12.48±0.16 [c]	43.20±1.15 [d]
SY4	25.27±4.05 [b]	4.77±1.36 [e]	18.62±0.69 [a]	48.65±6.10 [c]
SF5	18.32±0.11 [b]	16.06±0.85 [c]	12.15±0.81 [c]	46.53±0.15 [d]
NCDO 489	6.50±0.02 [e]	6.95±0.43 [d]	3.31±0.11 [f]	16.76±0.56 [g]
Lb. bulgaricus				
LY1	21.68±0.28 [b]	7.69±0.3 [d]	3.51±0.54 [f]	32.87±0.52 [e]
LY2	16.52±0.64 [c]	5.64±0.37 [e]	5.35±0.01 [e]	27.51±0.28 [f]
CNRZ 369	-	5.45±0.15 [e]	-	5.45 [h]
Lb. lactis				
LF14	28.24±0.17 [a]	24.39±0.25 [b]	10.52±0.49 [d]	63.15±0.91 [a]
Lb. helveticus				
LF22	21.2±0.4 [b]	24.95±0.10 [b]	6.22±0.23 [e]	52.37±0.37 [c]

2000). Table 1 shows that SL1 and SS4 strains produced substantial quantities of total folate and were the most efficient *S. thermophilus* strains. The high production of H_4-PteGlu by SY4 (25.27 ng/g) has to be pointed out since H_4-PteGlu has been reported to be the prevalent form of folate in milk/dairy products (WIGERTZ *et al.*, 1997). The other strains showed less ability to produce the different vitamers especially 5-CHO-H_4-PteGlu. These findings are in contrast with SYBESMA *et al.* (2003) who reported that 5,10-methenyl and 5-formyl tetrahydrofolate were the major folate derivates of *S. thermophilus* grown on M17 medium. It must be pointed out that these authors evaluated the folate production ability of three *S. thermophilus* strains, while in this study, 12 strains were evaluated which gives a more complex picture in which 5-CHO-H_4-PteGlu and 5-CH_3-H_4-PteGlu are the major folate derivates for only some strains (e.g. SL2, SL3 and SS4). The *S. thermophilus* reference strain NCDO489 (not isolated from goat dairy products) produced a lower amount of total folate and showed some substantial difficulties to grow and survive (Table 2).

The *Lb. delbrueckii* subsp. *bulgaricus* strains generally produced a lower amount of total folate than *S. thermophilus* (Table 1). Although some authors have shown the ability of *Lb. delbrueckii* subsp. *bulgaricus* to produce folate on reconstituted Skim Milk (LIN and YOUNG, 2000b), some strains have been reported to be folate-depleting (RAO *et al.*, 1984). The ability of different *Lacto-*

Table 2 - (a): viable count (cfu mL^{-1}) and acidity curves (% of Lactic acid) of *S. thermophilus* (SL1, NCDO489), *Lb. delbrueckii* subsp. *bulgaricus* (LY1, CNRZ369), *Lb. delbrueckii* subsp. *lactis* (LF14), and *Lb. helveticus* (LF22) grown in goat milk at 42°C (average of three determinations). (b): viable count (cfu mL^{-1}) and post-acidification (% Lactic acid) of LAB strains in goat milk stored at 4°C (average of three determinations).

(a) Strain	SL1		NCDO489		LY1		CNRZ 369		LF14		LF22	
	Viable count	Acidity	Viable count	Acidity	Viable count	Acidity	Viable count	Acidity	Viable count	Acidity	Viable count	Acidity
Hours												
0	1.03E+06	0.20	4.70E+05	0.19	2.91E+06	0.16	8.00E+05	0.15	8.00E+05	0.15	6.70E+05	0.16
2	3.00E+07	0.21	1.70E+07	0.28	1.12E+07	0.32	3.00E+06	0.15	3.40E+06	0.17	3.50E+06	0.19
4	4.00E+08	0.26	3.40E+08	0.34	2.00E+07	0.65	4.00E+06	0.19	2.60E+07	0.33	2.20E+07	0.22
6	5.40E+08	0.50	5.60E+08	0.40	2.60E+08	1.10	8.10E+06	0.31	3.20E+07	0.42	2.80E+07	0.30
8	9.40E+08	0.69	8.20E+08	0.55	5.60E+08	1.13	9.00E+07	0.60	4.40E+08	0.57	3.80E+07	0.33
24	4.60E+08	0.84	8.00E+07	0.61	8.40E+06	1.71	8.00E+06	1.30	8.40E+06	0.90	4.60E+06	0.45
(b) Time												
8h	9.40E+08	0.69	8.20E+08	0.55	5.60E+08	1.13	9.00E+07	0.60	4.40E+08	0.57	3.80E+07	0.33
5 d	9.30E+08	0.61	8.10E+08	0.49	3.12E+08	1.18	7.10E+07	0.82	3.40E+08	0.70	1.70E+07	0.52
15 d	9.20E+08	0.71	2.20E+08	0.40	5.90E+07	1.19	4.20E+07	0.81	1.60E+06	0.74	3.80E+07	0.56
30 d	8.04E+08	0.71	1.30E+08	0.44	2.65E+07	1.19	1.40E+06	0.80	3.30E+06	0.81	3.50E+07	0.61

bacillus strains and species to influence the folate level in dairy products is currently under discussion. In a recent study CRITTENDEN *et al.* (2003) showed that starter cultures made of *Lactobacillus* spp. (e.g. *L. helveticus* and *L. acidophilus*) generally did not significantly influence folate levels when grown on reconstituted Skim Milk, even if *Lb. delbrueckii* subsp. *bulgaricus* CSCC2505 and CSCC5168 have been described as folate producer and depleting strains. It seems that at least within the *Lb. delbrueckii* subsp. *bulgaricus* species a certain variability for folate production exists and is strongly strain-specific.

With respect to different vitamers, the H_4-PteGlu production of *Lb. delbrueckii* subsp. *bulgaricus* was 21.68 ng/g, for the LY1 strain, and 16.52 ng/g for LY2. The reference strain CNRZ 369 (not isolated from dairy goat products) was only able to produce 5-CH_3-H_4-PteGlu. The reference cultures used in this study showed evident growth difficulties in goat milk (Table 2), thereby confirming the necessity of using autochthonous starter cultures for goat milk processing and/or improvement of probioticity or nutritional value.

Lb. helveticus produced 52.37 ng/g of total folate and the highest amount of 5-CH_3-H_4-PteGlu (24.95 ng/g). The *Lb. delbrueckii* subsp. *lactis* used in this work was by far the most productive among the tested species, for both total folate (63.15 ng/g) and H_4-PteGlu (28.24 ng/g) production. This is of particular importance since this is the first report (to our knowledge) on folate production by *Lb. delbrueckii* sub-

sp. *lactis*. However further studies are necessary to assess whether the folate-producing ability of *Lb. delbrueckii* subsp. *lactis* is strain-specific, as it seems to be for *Lb. delbrueckii subsp. bulgaricus*, or if it is common to all the members of the species, as in the case of *S. thermophilus*.

Folate production of mixed strains in yoghurt

Folate production of mixed cultures of LAB grown in goat milk is reported in Table 3. The highest production of total folate was 46.93 ng/g (SL1+LY2), significantly lower than those obtained for several *S. thermophilus* strains (Table 1). A comparison between folate contents in Tables 1 and 3 shows that folate produced by SL1 (single culture) was higher, especially for 5-CH_3-H_4-PteGlu and 5-CHO-H_4-PteGlu, than those obtained with the combined strains SL1+LY1/LY2. Total folate production was higher for the mixed cultures SL1+LY2 in which, growth and acidification of the *Lactobacillus* was reduced (Figs. 1 and 2). It is interesting that LY2 alone was found to be a lower folate producer compared to LY1 (Table 1). A lower production or accumulation of different vitamers and/or total folate in the medium could be due to the (partial) utilization of folate by *Lb. delbrueckii* subsp. *bulgaricus* as previously reported (RAO *et al.*, 1984; RAO and SHAHANI, 1987). RAO *et al.* (1984) showed that *Lb. delbrueckii* subsp. *bulgaricus* (ATCC 11842) depleted the available folate in reconstituted Skim Milk when inoculated as single and mixed culture together with *S. thermophilus* (RAO *et al.*, 1984). On the contrary in our study it was found that *Lb. delbrueckii* subsp. *bulgaricus*, inoculated as single strains, increased the total folate level of fermented goat milk while no additive effect was observed when they were inoculated together with *S. thermophilus*. CRITTENDEN *et al.* (2003), studying the synthesis and utilization of folate by yoghurt starter cultures, reported similar findings. It seems reasonable to assume that, in mixed culture, strains of *Lb. bulgaricus* influence the folate production of *S. thermophilus* through a possible inhibition mechanism or alternatively by folate consumption.

In the present study, a method, essentially adapted from VAHTERISTO *et al.* (1996, 1997), was set up for folate extraction and determination in dairy products from goat milk. Different extraction procedures have been employed or proposed by several authors to extract folates from different types of food samples (ENGELHARDT and GREGORY, 1990; VAHTERISTO *et al.* 1996; KONINGS, 1999; KONINGS *et al.*, 2001). Basically all of these authors used different γ-glutamyl hydrolases for the deconjugation such as hog kidney (HK), chicken pancreas (CP) and rat plasma (RP) conjugases. Moreover, some of these enzymes (i.e. HK) need an extraction or preparation step before use (ENGELHARDT and GREGORY, 1990). In this

Table 3 - Production (ng/g) of tetrahydrofolate (H_4-PteGlu), 5-methyl-tetrahydrofolate (5-CH_3-H_4-PteGlu) and 5-formyl-tetrahydrofolate (5-CHO-H_4-PteGlu) in goat milk inoculated with mixed cultures of *S. thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus* strains (average values of three determinations ± standard deviations). Values in the same column with different letters are significantly different (p<0.05).

S. thermophilus + *Lb. bulgaricus*	H_4PteGlu	5-CH_3-H_4PteGlu	5-CHO-H_4PteGlu	Total Folate
SL1 + LY1	20.66±0.31[a]	6.89±0.19[a]	9.21±0.01[a]	36.75±0.49[a]
SL1 + LY2	18.34±2.93[a]	10.97±1[b]	17.62±1.68[b]	46.93±5.61[b]

study, the HP (Human Plasma) enzyme was used for the deconjugation step because of its greater availability compared to the other enzymes. It is currently widely used for folate determination (HPLC methods) in milk and dairy products by several research groups (LIN and YOUNG, 2000b; SYBESMA *et al.*, 2003). KONINGS (1999) and KONINGS *et al.* (2001) determined folate concentrations in different food samples using rat plasma after treatment with protease and amylase, the latter being aimed at detecting the matrix-bound folate. In this study protease and amylase were not used since this treatment is commonly used for folate extraction from food stuffs which contain a considerable amount of protein, starch and carbohydrate matrixes. However the application of this method to different milk and dairy products did not clearly show the presence of substantial amounts of matrix-bound folate in yoghurt (KONINGS *et al.*, 2001).

Growth and acidification of *S. thermophilus* and lactobacilli in goat milk

Results concerning the growth and acidifying activities of selected *S. thermophilus* and lactobacilli strains are reported in Table 2. The growth of *S. thermophilus* in goat milk was rapid during the first hours of incubation, but later a certain slowing down occurred and the microbial population did not reach values higher than 10^9 cells mL^{-1}. The growth rates of *Lb. delbrueckii* subsp. *bulgaricus* were rather slow and reached their maxima after 8 hours of incubation, whereas acidification followed a typical trend for this species. Post-acidification trials highlighted a good tolerance of LAB strains to storage at 4°C while showing the superior ability of autochthonous lactic acid bacteria to survive in goat milk with high numbers for at least 30 days (Table 2).

Growth and acidification of mixed strains in goat milk

Both the speed of growth and the acidification ability of *S. thermophilus* SL1 mixed with *Lb. delbrueckii* subsp. *bulgaricus* LY1 (Figs. 1 and 2) showed a typical trend. Instead *S. thermophilus* SL1 associated with *Lb. delbrueckii* subsp. *bulgaricus* LY2 did not show the characteristic synergism for growth and acidification. In this case LY2 was the slowest strain for both growth and acidification. The presence in goat yoghurt of a high number of living LAB after 4 weeks (Fig. 3), or a substantial concentration of naturally-produced functional metabolites such as folate, can certainly be considered as a positive feature.

Yoghurt production and sensory analyses

Yoghurts made on a pilot scale using LAB strains selected for folate production and technological suitability showed some encouraging results when compared with a commercial product. The sensory features examined were appearance, texture, smell and taste. The experimental yoghurts had a proper thick appearance, a creamy and smooth texture; the whey was not separated and the smell was excellent and sweet. The taste of both the experimental yoghurts was generally judged as good, palatable, not too sour, and similar to the commercial goat yoghurt.

CONCLUSIONS

The ability of *S. thermophilus*, *Lb. delbrueckii* subsp. *bulgaricus*, *Lb. delbrueckii* subsp. *lactis* and *Lb. helveticus* to produce folic acid or its vitamers in goat milk and their technological suitability for improving goat milk products, particularly goat yoghurt, were examined. They all produced different folate vitam-

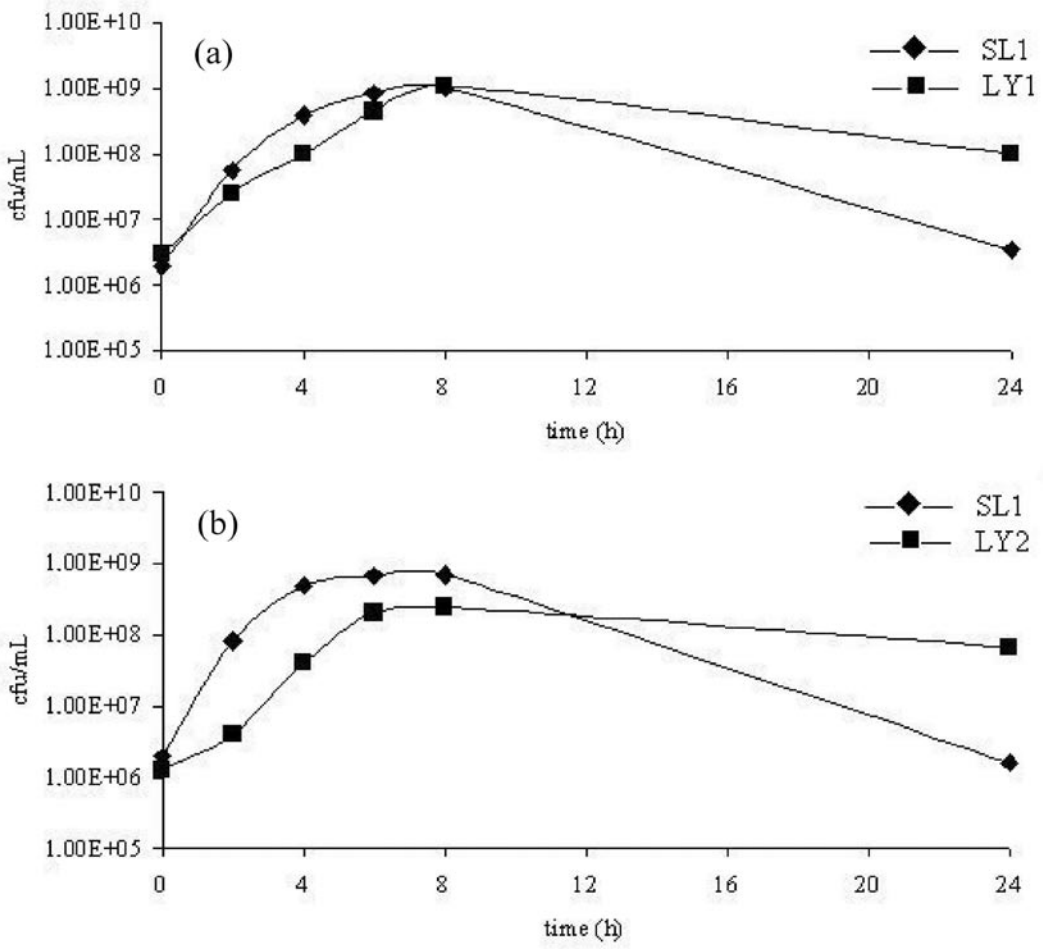


Fig. 1 - Growth curves (cfu mL^{-1}) of mixed *S. thermophilus* SL1 and *Lb. delbrueckii* subsp. *bulgaricus* LY1 (a) and LY2 (b) strains grown in goat milk at 42°C (average of three determinations).

ers (5-CH_3-H_4-PteGlu, 5-CHO-H_4-PteGlu, H_4-PteGlu). In general, 5-CH_3-H_4-PteGlu and H_4-PteGlu, the most important vitamers from a functional point of view, were produced at a higher level. Folate production did not seem to be affected by the physiological features of the LAB examined, which had a different behavior, particularly in speed of growth and acidifying activities.

Results related to folate production by yoghurt starter cultures suggest that consumption of folate by *Lb. delbrueckii* subsp. *bulgaricus* strains may occur. On the other hand, the low amount of folate produced by mixed cultures (compared to single cultures) in fermented goat milk could be related to some inhibitory effects of the lactobacillus towards the *S. thermophilus*.

These results can be considered a useful contribution to the knowledge of folate produced by lactic acid bacteria. A suitable analytical method for determining folate in goat milk and dairy products was also set up. The results from this study are encouraging, especially from an application point of view, since

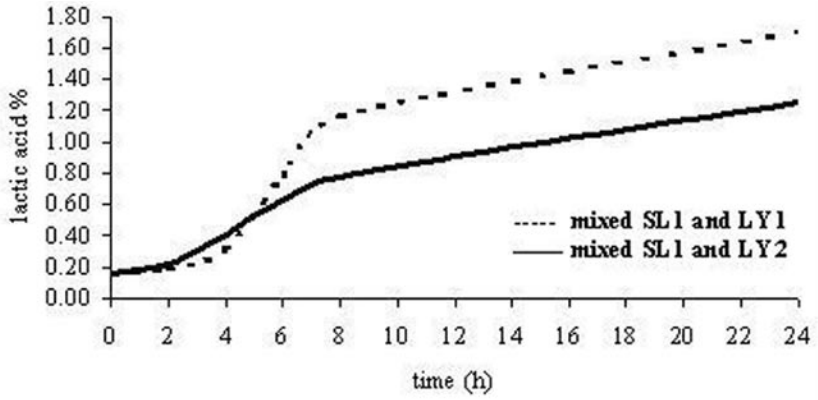
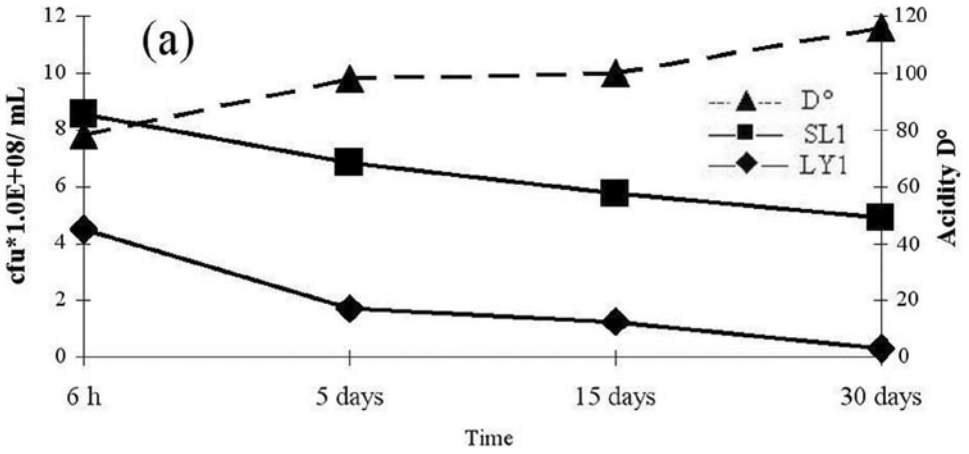


Fig. 2 - Acidity curve (% Lactic acid) of *S. thermophilus* SL1 when mixed with *Lb. delbrueckii* subsp. *bulgaricus* LY1 and LY2 strains grown in goat milk at 42°C (average of three determinations).



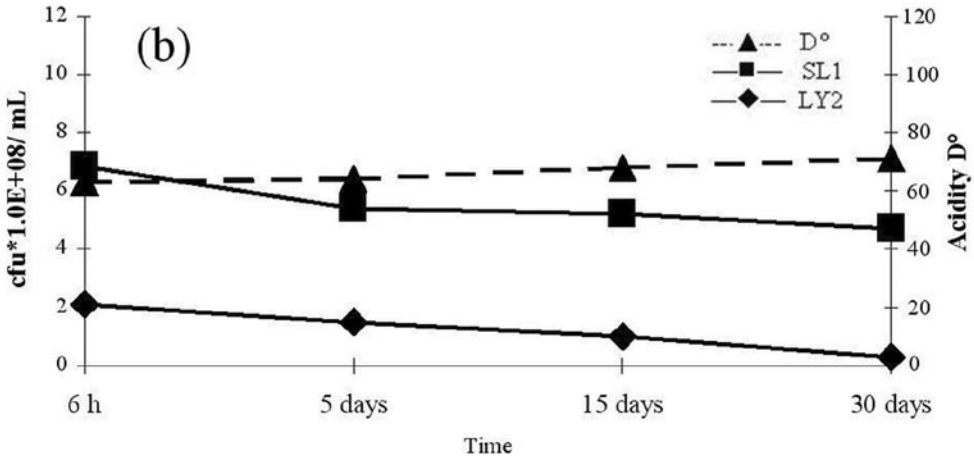


Fig. 3 - Viable count (cfu mL^{-1}) and post-acidification curve expressed as Dornic degree (D°) of mixed cultures of *S. thermophilus* SL1 and *Lb. delbrueckii* subsp. *bulgaricus* LY1 (a) and LY2 (b), during storage at 4°C in goat milk (average of three determinations).

the use of folate-producing LAB isolated from different goat dairy products could contribute to the nutritional improvement of goat milk products.

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Revised paper received September 24, 2004 Accepted December 6, 2004

PAPER

MINERALS AND TRACE ELEMENTS IN FOOD COMMONLY CONSUMED IN SLOVENIA

MINERALI ED OLIGOELEMENTI IN ALIMENTI DI LARGO CONSUMO IN SLOVENIA

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ABSTRACT

An investigation on the minerals and trace elements in foods widely consumed in Slovenia has been performed. Microwave-assisted digestion followed by flame and electrothermal atomic absorption spectrometry was used for the analysis. Validated analytical procedures were used and the accuracy was checked by using certified reference materials CRM 1570 a (spinach) and CRM 1573 a (tomato leaves). Good agreement was found between certified and determined data. Two individ-

RIASSUNTO

L'indagine ha riguardato la presenza di minerali ed elementi presenti in tracce in alcuni alimenti di uso comune in Slovenia. I campioni sono stati analizzati tramite digestione con microonde seguita da analisi allo spettrofotometro ad assorbimento atomico a fiamma ed elettrotermico. Sono stati adottati metodi analitici validati e l'accuratezza delle misure è stata verificata ricorrendo a campioni standard di riferimento (spinaci: CRM 1570, foglie di pomodoro: CRM 1573)

- Key words: Atomic absorption spectrometry, food analysis, minerals and trace elements -

ual samplings were preformed in 2002 and 2003. Data demonstrated that Zn, Cu, Cd, Pb, Cr and Ni concentrations in commonly consumed foods in Slovenia are comparable to the data reported for the European Union and other countries. The foodstuffs analysed were not contaminated with Cd, Pb or Ni. Higher Ni concentrations were found in the baby food "Čokolešnik" and were due to the cocoa content. Meat was rich in Zn (about 200 mg kg^{-1}), while cabbage, wheat, potato, egg whites, instant milk, baby food and most fish contained less than 50 mg kg^{-1}. The Cu concentrations in most of the samples were between 2 and 3 mg kg^{-1}. In general, the Cr content in the foodstuffs was below 0.05 mg kg^{-1}. Mussels, that accumulate elements from the marine environment, contained high concentrations of all the minerals and trace elements investigated.

che, sottoposti ad analisi, hanno fornito valori confrontabili con quelli certificati. I dati, ottenuti da due diverse campionature realizzate nel 2002 e nel 2003, hanno confermato che le concentrazioni di Zn, Cu, Cd, Pb, Cr e Ni, riscontrate in alimenti consumati in Slovenia, sono paragonabili a quelle disponibili in letteratura nei paesi dell'Unione Europea e a quelle di altri paesi del mondo. Gli alimenti analizzati non sono risultati contaminati con Cd, Pb e Ni. Una più alta concentrazione di Ni è stata trovata nel "Čokolešnik", alimento per la prima infanzia, come conseguenza della presenza di cacao nel prodotto. La carne é risultata ricca in Zn (circa 200 mg kg^{-1}), mentre nel cavolo, frumento, patate, tuorlo delle uova, latte in polvere, alimenti per l'infanzia e nella maggior parte del pesce analizzato ne è stato rilevato meno di 50 mg kg^{-1}. La concentrazione di Cu, nella maggior parte dei campioni analizzati, ha oscillato fra 2 e 3 mg kg^{-1}. In generale, il contenuto di Cr negli alimenti analizzati è rimasto al di sotto di 0,05 mg kg^{-1}. I molluschi, accumulando elementi dall'ambiente marino, presentano invece le concentrazioni di minerali più alte fra i campioni presi in considerazione.

INTRODUCTION

Human activities, intensified agriculture and increasing industrialization have resulted in the release of various pollutants, including metals, into the environment (PUSTIŠEK *et al.*, 2001; SVETE *et al.*, 2001; JAIN, 2003). The contaminants may enter the food chain (FICHET *et al.*, 1999) and consequently elevated concentrations of toxic metals and organic substances can be found in foodstuffs (NASREDDINE and PARENT-MASSIN, 2002). Besides the growth of plants in contaminated soils and the feeding of animals on feed containing toxic metals, storage in metal containers (SEVERUS, 1989) and food processing (ŠČANČAR *et al.*, 2004) may also contribute to contamination of foodstuffs.

Dietary factors play an important role in the amount of trace elements found in body fluids and tissues of the general population (MINOIA *et al.*, 1994). In order to verify the compliance of food products with some prerequisites for quality, numerous investigations have been carried out on the determination of toxic elements in different food products. Representative total daily diets (POKORN *et*

al., 1998; IYENGAR *et al.*, 2000; ALBERTI-FIDANZA *et al.*, 2002), traditional foods (LENDINEZ *et al.*, 2001; KUHNLEIN *et al.*, 2002) as well as dairy products (CONI *et al.*, 1994; CABRERA *et al.*, 1996) and individual foodstuffs of animal (NUURTAMO *et al.*, 1980), marine (JEWETT and NAIDU, 2000; RUPÉREZ, 2002; BUSTAMANTE *et al.*, 2003; SKINNER *et al.*, 2004) or plant origin (SCHUHMACHER *et al.*, 1993; D'ILIO *et al.*, 2002) have been investigated.

In determining minerals and trace elements by spectrometric techniques, nitric acid is commonly used to digest food samples (LENDINEZ *et al.*, 2001; KUHNLEIN *et al.*, 2002; RUPÉREZ 2002; D'ILIO *et al.*, 2002, MILAČIČ and KRALJ, 2003), while hydrochloric acid is used for the voltametric determination of metals (LOCATELLI and TORSI, 2003). To obtain reliable and representative data on minerals and trace element concentrations in foodstuffs, analytical procedures should be validated and multiple samplings performed. In our laboratory, an analytical procedure was recently optimized and validated for the determination of trace elements in food samples by flame and electrothermal atomic absorption spectrometry (FAAS, ETAAS) after microwave-assisted digestion of samples (MILAČIČ and KRALJ, 2003).

The aim of the present work was to determine the Zn, Cu, Cd, Pb, Cr and Ni contents in foods commonly consumed in Slovenia. For this purpose the analytical procedure of MILAČIČ and KRALJ (2003) was applied. To obtain representative results, two individual samplings were performed in 2002 and 2003 and various foods were selected for the analysis. The mineral and trace element concentrations were also compared with data reported for European and non-European countries. The recommended dietary allowances (RDA) are reported for the essential elements and the toxic element (Cd and Pb) concentrations were compared to those specified in the EU legislation.

MATERIALS AND METHODS

Instrumentation

Zn and Cu were determined by flame atomic absorption spectrometry (FAAS) on a Varian (Mulgrave, Victoria, Australia) SpectrAA 110 atomic absorption spectrometer. Cd, Pb, Cr and Ni were determined by electrothermal atomic absorption spectrometry (ETAAS) on a Hitachi (Tokyo, Japan) Z-8270 polarized Zeeman atomic absorption spectrometer. Microwave digestion of samples was performed on a CEM Corporation (Matthews, NC, USA) CEM MARS 5 Microwave Acceleration Reaction System. Samples were lyophilized in a Christ, Alpha 1-4 (Osterode am Harz, Germany) lyophilizer and milled in an agate mill Fritsch, Pulverisette 7 (Laval, Québec, Canada).

Reagents

Suprapur acids (Merck, Darmstadt, Germany) and water doubly distilled in quartz were used to prepare samples and standard solutions. Standard stock solutions of Zn, Cu, Cd, Pb, Cr and Ni (1,000±2 mg dm^{-3}) were obtained from Merck (Merck, Darmstadt, Germany). All other chemicals were of analytical-reagent grade.

Sampling and sample preparation

In Slovenia, each household member in the year 2001 purchased on the average 60.8 kg of bread and cakes, 24.2 kg of potatoes, 78 dm^3 of milk and 110.2 eggs (STATISTICAL YEARBOOK OF THE REPUBLIC OF SLOVENIA, 2002). Slovenians often eat cabbage (as salad or in the national dish "jota") and they commonly eat fish and meat. Therefore, it was decided to collect the most commonly consumed foods as well as infant formulas and a very popular baby food, Čokolešnik.

The food items were collected in Slov-

enia in 2001 and 2002. Potatoes and cabbage treated with appropriate agrotechnical agents were grown in Male Pece and Razori, Slovenia. Wheat, eggs, baby foods and fish were bought on the market. The wheat, eggs, instant milk, the baby food Čokolešnik, anchovy and trout were of Slovenian origin. The baby milk formulas Aptamil and Milubrei came from Germany. Mussels were imported from Spain, while mullet and gilthead beam were caught in the Adriatic Sea (Croatia). The beef was from bulls of the Brown and Simmental breeds. These two breeds make-up the majority of the breeding stock in Slovenia. Muscles were sampled 4 times over a period of 16 months in 2002 and 2003. One kg of *M. biceps* femoris and *M. longissimus* dorsi was taken from each of four animals.

All wet samples, potatoes (6 kg), cabbage (6 kg), eggs (each sample was a composite of 60 eggs), fish (6 kg), mussel (6 kg) and meat (4 kg) were frozen at -24°C, lyophilised at -50°C and 0.050 mbar, and then milled in an agate mill (speed: 6; time: 6 min). The particle size was less than 0.45 mm. Only edible parts of foodstuffs were used for the analysis, inedible parts were discarded. In order to avoid extraneous contamination, potatoes and cabbage were carefully washed with water prior to analysis.

Analytical procedure

A validated analytical procedure for the determination of minerals and trace elements in foodstuffs was applied (MILAČIČ and KRALJ, 2003). Approximately 0.35 g of lyophilized sample was weighed in a Teflon beaker and 4 mL of concentrated nitric acid (HNO_3) were added. Hydrofluoric acid (HF), 0.1 mL, was also added to the cabbage sample, CRM 1570 a (spinach leaves) and CRM 1573 a (tomato leaves) in order to dissolve silica. Samples were subjected to closed vessel microwave digestion at a maximum power of 1,200 W: ramp to temperature 20 min, 180°C, pressure 10 bars, hold 20 min, cooling 20 min. The clear solutions obtained were quantitatively transferred to 25 mL polyethylene graduated tubes and filled to mark with water doubly distilled in quartz. The same procedure (acids without food samples) was applied for the blank analysis.

Zn and Cu were determined by flame atomic absorption spectrometry (FAAS) at wavelengths of 213.8 and 324.8 nm, respectively, in an air-acetylene flame using a deuterium background correction. Cd, Pb, Cr and Ni were determined by electrothermal atomic absorption spectrometry (ETAAS) using Zeeman background correction at wavelengths of 228.8, 283.3, 359.3 and 232.0 nm, respectively. Standards used in the calibration procedure were prepared in the same acids as those used for the samples. The analyses were performed in three parallel determinations. To determine the moisture content, 1 g of lyophilized sample was kept at 80°C until constant weight and the moisture content was determined on the basis of the weight loss. The concentrations of elements in foodstuffs were calculated on a dry weight basis.

RESULTS AND CONCLUSIONS

Accuracy

The accuracy of the analytical procedure was checked by the analysis of the standard reference materials CRM 1570 a: Trace and minor elements in spinach leaves (analysis in 2002) and CRM 1573 a: Trace and minor elements in tomato leaves (analysis in 2003). The results are presented in Tables 1 and 2. The determined metal concentrations agreed well with the reported certified values, confirming the accuracy of the procedure applied.

Table 1 - Analysis of Zn, Cd, Cu and Ni in standard reference material CRM 1570 a (NIST, Trace and minor elements spinach leaves) after microwave digestion of the samples and determination of element concentrations by FAAS and ETAAS#.

	CRM 1570 a	
Element	Certified value (mg kg^{-1})	Determined value* (mg kg^{-1})
Zn	82±3	83±0.7
Cu	12.2±0.6	12.5±0.1
Cd#	2.89±0.07	2.67±0.08
Ni#	2.14±0.1	2.12±0.06

*mean of two parallel determinations ± standard deviation.

Table 2 - Analysis of Zn, Cu, Cd, Cr and Ni in standard reference material CRM 1573 a (NIST, Trace and minor elements tomato leaves) after microwave digestion of the samples and determination of element concentrations by FAAS and ETAAS#.

	CRM 1573 a	
Element	Certified value (mg kg^{-1})	Determined value* (mg kg^{-1})
Zn	30.9±0.7	31.4±0.4
Cu	4.7±0.14	4.8±0.2
Cd#	1.52±0.04	1.51±0.04
Cr#	1.99±0.06	2.04±0.04
Ni#	1.59±0.07	1.65±0.05

*mean of three parallel determinations ± standard deviation.

Analysis of Zn, Cu, Cd, Pb, Cr and Ni in foodstuffs

The foodstuffs were analyzed using the recommended analytical procedure by FAAS and ETAAS. Results for the amounts of Zn, Cu, Cd, Pb, Cr and Ni found in 22 food items sampled in 2002 and 2003 (mean of three parallel determinations ± standard deviation) are presented for in Fig. 1-6.

Standard deviations between parallel determinations, in general, did not exceed ±5%. The limit of detection (LOD) calculated on a 3 s basis (a value of three standard deviation of the blank) were 0.35 mg kg^{-1} for Zn, 1 mg kg^{-1} for Cu, 0.03 mg kg^{-1} for Cd, 0.2 mg kg^{-1} for Pb, 0.2 mg kg^{-1} for Ni and in 0.05 mg kg^{-1} for Cr.

Data in Fig. 1 show the Zn content in the foodstuffs analysed. Zn is an essential element present in more than 200 different enzymes; it provides structural integrity in many proteins (REILLY, 2002a). If Zn intake fails to meet body requirements, clinical symptoms such as growth retardation, poor and erratic food consumption, loss of appetite, alopecia and hair loss may appear. The recommended dietary allowance of Zn ranges from 5 mg for infants to 12 mg per day for adults (NATIONAL ACADEMIES, 2001). Due to its high essentiality for human beings an adequate daily intake of Zn through foodstuffs is of great importance. The data in Fig. 1 show that cabbage, wheat and potato contain about 20 mg kg^{-1} of Zn. This concentration is comparable to the Zn content in the daily dietary samples from some old people's homes in Slovenia (POKORN *et al.*, 1998); it is representative of mixed total diet composites in the USA (IYENGAR *et al.*, 2000) and the Zn concentration in various types of rice (D'ILIO *et al.*, 2002). Zn is found primarily in the egg yolk (about 70 mg kg^{-1} of Zn), while the egg white contains less than 0.35 mg kg^{-1} of Zn. The Zn concentration in baby foods and instant milk ranged from 10 to 40 mg kg^{-1}, while in fish it ranged from 20 to 90 mg kg^{-1}. The concentration of Zn (about 200 mg kg^{-1}) was the highest in mussels, while in meat (*Longissimus dorsi, Biceps femoris*) it ranged from 150 to 280 mg kg^{-1}. The Zn concentration in cabbage, wheat, potato, eggs and baby foods did not vary between the two samplings, while in mussels, fish and meat the Zn content varied up to 20% in the particular food item sampled in 2002 and 2003. The variations in

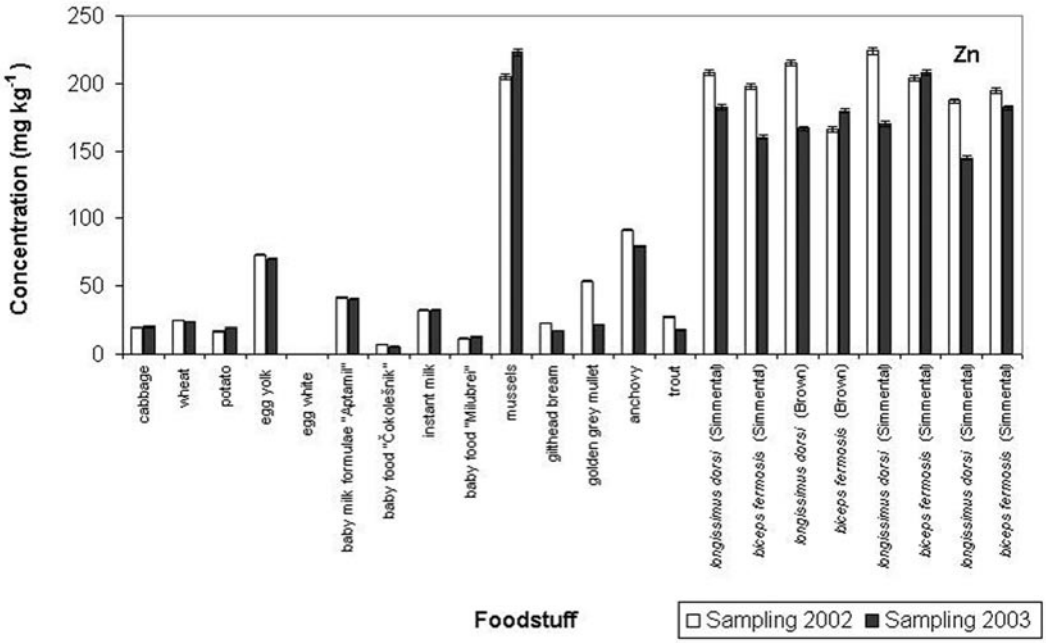


Fig. 1 - Analysis of Zn in basic foods of the Slovenian diet after microwave digestion of the samples and determination by FAAS. Results (mean of three determinations) were calculated on a dry weight basis and refer to the edible portions.

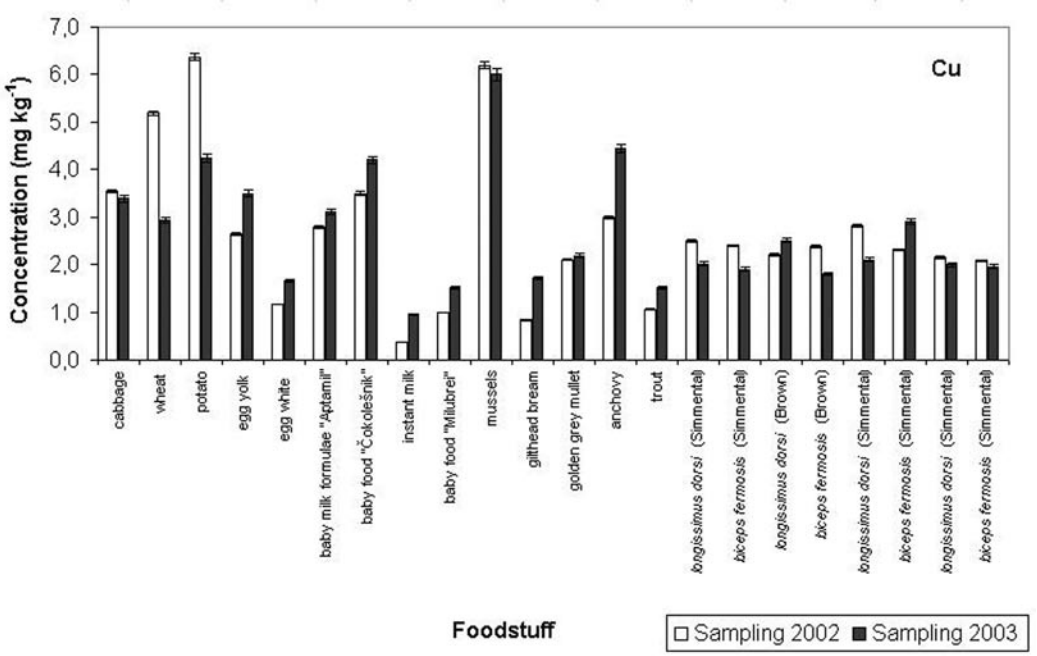


Fig. 2 - Analysis of Cu in basic foods of the Slovenian diet after microwave digestion of the samples and determination by FAAS. Results (mean of three determinations) were calculated on a dry weight basis and refer to the edible portions.

the Zn content in meat in the two samplings may have arisen from differences in the Zn content in the animal fodder and from variations in fodder intake by the animals.

Cu is a component of enzymes in iron metabolism. Cu deficiency is associated with the development of anaemia, hypopigmentation, abnormalities of glucose and cholesterol metabolism and heart function. High oral intakes of Cu may be toxic (REILLY, 2002b). The recommended dietary allowance for Cu ranges from 0.3 mg for children to 1 mg per day for adults (NATIONAL ACADEMIES, 2001). The concentrations of Cu in wheat and potato varied between the samplings in 2002 and 2003 (Fig. 2). The Cu concentrations determined in 2002 for wheat and potato were 5.1 and 6.2 mg kg^{-1}, respectively; in 2002 the values were about 40% higher than in 2003 probably due to the use of Cu-containing fungicides. The cabbage, baby milk formulae, the baby food "Čokolešnik" and egg yolk contained about 3 mg kg^{-1} Cu; the egg white contained about half the Cu concentration found in the yolk. Instant milk and the baby food "Milubrei" contained low Cu concentrations (below 1 mg kg^{-1} and about 1.5 mg kg^{-1} of Cu, respectively). The Cu content in different fish ranged from 1 to 4 mg kg^{-1} Cu, while the Cu concentration in meat (*Longissimus dorsi* and *Biceps femoris*) was about 3 mg kg^{-1}. The highest Cu concentration was found in mussels (about 6 mg kg^{-1}). The reported Cu concentrations in daily dietary samples from some old people's homes in Slovenia (POKORN *et al.*, 1998) ranged from 1 to 10 mg kg^{-1} with an average Cu concentration of 2 mg kg^{-1}, while the mean Cu concentration in the representative mixed total diet composites from the USA (IYENGAR *et al.*, 2000) was 2.8 mg kg^{-1}.

Cd is a toxic element. Its ingestion can rapidly cause vomiting, abdominal cramps and headache. Studies of industrial workers exposed to Cd have indicated a higher occurrence of prostate and lung cancer compared to the general population (REILLY, 2002c). Cd may enter the food chain through a contaminated environment. Therefore, it is very important to check the Cd content in foodstuffs. In general the Cd concentrations were very low (Fig. 3). The Cd content in egg yolk and white, baby food, fish, and meat were below the LOD (0.03 mg kg^{-1} Cd). These data are comparable to the low Cd concentrations (about 0.03 mg kg^{-1}) reported in representative mixed total diet composites from the USA (IYENGAR *et al.*, 2000), Cd concentrations in Italian students' food (0.04 to 0.05 mg kg^{-1} Cd) ALBERTI-FIDANZA *et al.*, 2002) and the Cd content found in various types of rice (about 0.02 mg kg^{-1}) (D'ILIO *et al.*, 2002). European regulations prescribe the maximum level of Cd per wet weight in foodstuffs (E.C., 1993). Beef may contain up to 0.05 mg Cd kg^{-1}, fish 0.1 mg Cd kg^{-1}, wheat and rice 0.2 mg Cd kg^{-1} and vegetables and potato 0.1 mg Cd kg^{-1}. The data reported in Fig. 3 clearly show that the Cd concentrations in most of the foodstuffs analysed were far below the maximum values permitted by law (even when expressed on a dry weight basis). The Cd concentrations in potato and mussels (Fig. 3) expressed on a wet weight basis were 0.08 and 0.09 mg Cd kg^{-1}, respectively, and were also below the legally permitted value, 0.1 mg Cd kg^{-1}.

Pb is a toxic element which primarily affects the haemopoitetic, nervous, gastrointestinal and renal systems (REILLY, 2002d). Due to its high toxicity European legislation has regulated the maximum level of Pb per wet weight in foodstuffs (E.C., 1993) Pb content in infant formulae may not exceed 0.02 mg Pb kg^{-1}, beef may contain up to 0.1 mg Pb kg^{-1}, fish 0.2 mg Pb kg^{-1} and vegetables 0.1 mg Pb kg^{-1}. The data in Fig. 4 show that Pb was not present as a contaminant in the foodstuffs analyzed. Its concentration in cabbage, wheat, potato, baby food, egg yolk and white, fish and meat was below the

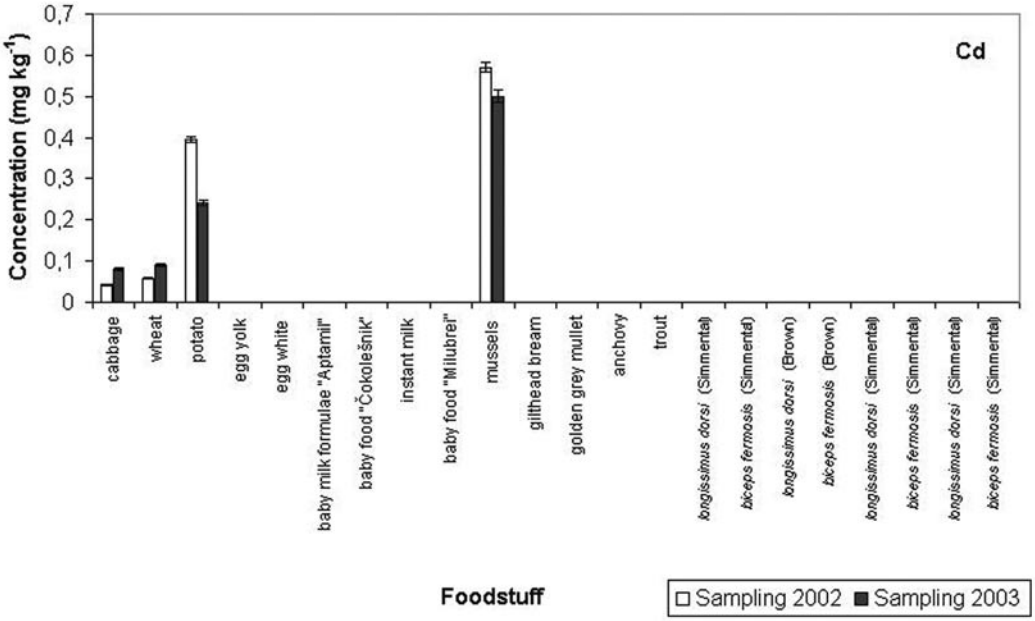


Fig. 3 - Analysis of Cd in basic foods of the Slovenian diet after microwave digestion of the samples and determination by ETAAS. Results (mean of three determinations) were calculated on a dry weight basis and refer to the edible portions.

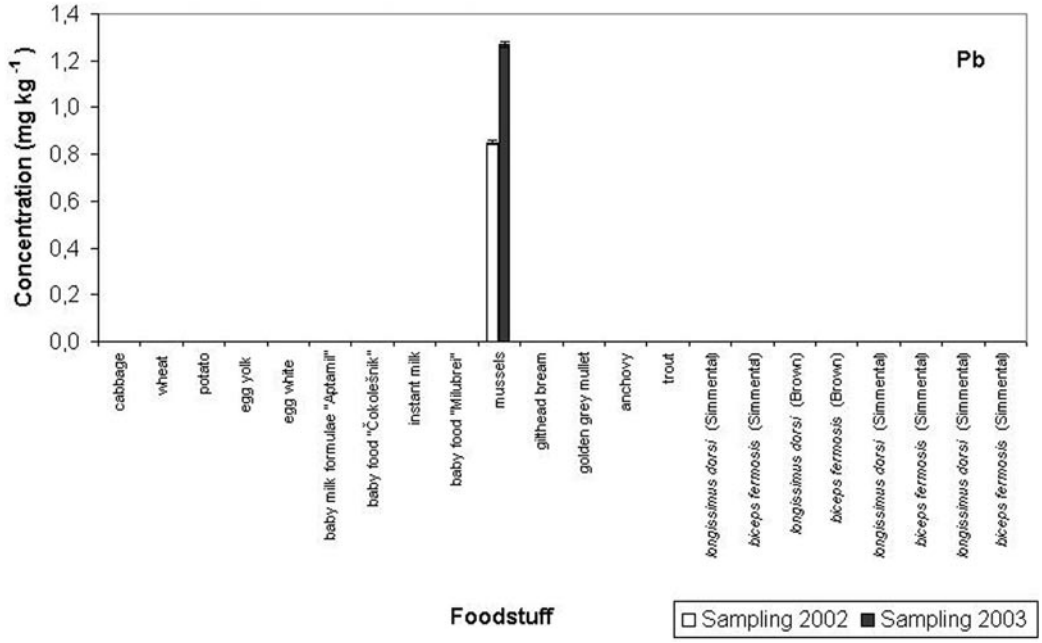


Fig. 4 - Analysis of Pb in basic foods of the Slovenian diet after microwave digestion of the samples and determination by ETAAS. Results (mean of three determinations) were calculated on a dry weight basis and refer to the edible portions.

LOD of the analytical technique applied (<0.2 mg kg^{-1} Pb). Taking into consideration the wet weight of foodstuffs, the Pb content in all of the foods analysed was below the legally permitted values. Unfortunately, the technique used was not sensitive enough to determine the Pb content in infant formulae at the low concentrations required by the EU legislation. The Pb concentrations are similar to those reported in the student cafeteria food in Italy (ALBERTI-FIDANZA *et al.*, 2002) and in representative mixed total diet composites from the USA (IYENGAR *et al.*, 2000). Mussels were the only food item analyzed that contained measurable concentrations of lead; they tend to accumulate environmental pollutants and contained 0.85 mg kg^{-1} of Pb in the 2002 sampling and 1.2 mg kg^{-1} of Pb in the 2003 sampling. The difference in Pb contents between the two samplings reflects the variations of the levels of pollution.

The toxic or essential nature of Cr is related to its chemical form. Cr(VI) compounds are highly toxic, while Cr(III) compounds are considered to be much less toxic and are even essential to humans. Cr(III) salts provide beneficial effects in carbohydrate and lipid metabolism and help maintain normal blood glucose levels (REILLY, 2002e). For normal functioning of the human body, low concentrations of Cr(III) are necessary. Recommended daily intakes of Cr range from 11 µg for children to 35 µg for adults (NATIONAL ACADEMIES, 2001). The data in Fig. 5 indicate that Cr concentrations in cabbage were about 0.07 mg kg^{-1}, in potato about 0.2 mg kg^{-1}, in wheat 0.8 mg kg^{-1} of Cr and 0.3 mg kg^{-1} in 2002 and 2003, respectively. Furthermore, it is evident that mussels accumulated appreciably high Cr concentrations, up to 1.2 mg kg^{-1}. All other foodstuffs analyzed (egg yolk and white, baby foods, fish and meat) had Cr contents that were below the LOD of the analytical procedure applied (0.05 mg kg^{-1} Cr). Other researchers have also reported generally low Cr concentrations in various foodstuffs. Daily dietary samples from some old people's homes in Slovenia contained about 0.04 mg kg^{-1} of Cr (POKORN *et al.*, 1998), while the Cr concentrations in representative mixed total diet composites from the USA (IYENGAR *et al.*, 2000) were about 0.08 mg kg^{-1}. In basic foodstuffs of Spain (LENDINEZ *et al.*, 2001) very low Cr concentrations were also observed in marine fish, milk and instant milk (below 0.05 mg kg^{-1}), while slightly higher Cr concentrations were found in corn (0.27 mg kg^{-1}) and wheat (0.33 mg kg^{-1}).

Ni is possibly an essential element for humans. It may serve as a cofactor of metalloenzymes. However, its biological function has not yet been clearly defined (REILLY, 2002f). Recommended dietary allowances for Ni have not been determined due to a lack of data regarding its adverse effects (NATIONAL ACADEMIES, 2001). The Ni content in the foodstuffs analyzed was generally below the LOD of the analytical procedure applied (below 0.2 mg kg^{-1} Ni) (Fig. 6). The exception was the baby food "Čokolešnik" which contained 0.08 to 1.4 mg kg^{-1} of Ni. The Ni in "Čokolešnik" arose from the cocoa additive, which is known to contain elevated concentrations of Ni. Variations in the Ni concentrations in "Čokolešnik" in the 2002 and 2003 samplings were due to Ni content of the cocoa. Higher Ni concentrations were also found in mussels. The Ni concentrations in mussels depended on the extent of environmental pollution and varied between 2.5 mg kg^{-1} (sampling 2002) and 1 mg kg^{-1} (sampling 2003). In general, low Ni concentrations (0.1-0.5 mg kg^{-1}) were also reported in student cafeteria food in Italy (ALBERTI-FIDANZA et el., 2002) and in representative mixed total diet composites from the USA (IYENGAR *et al.*, 2000). Higher Ni concentrations (0.8 and 3.5 mg kg^{-1}) were reported in red king crabs (JEWETT and NAIDU, 2000)

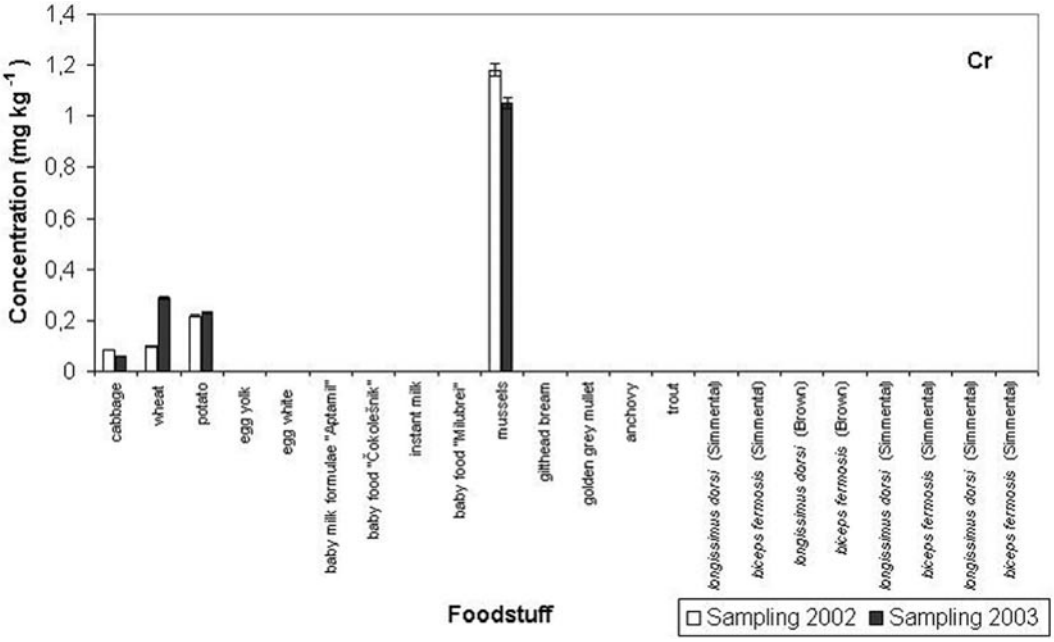


Fig. 5 - Analysis of Cr in basic foods of the Slovenian diet after microwave digestion of the samples and determination by ETAAS. Results (mean of three determinations) were calculated on a dry weight basis and refer to the edible portions.

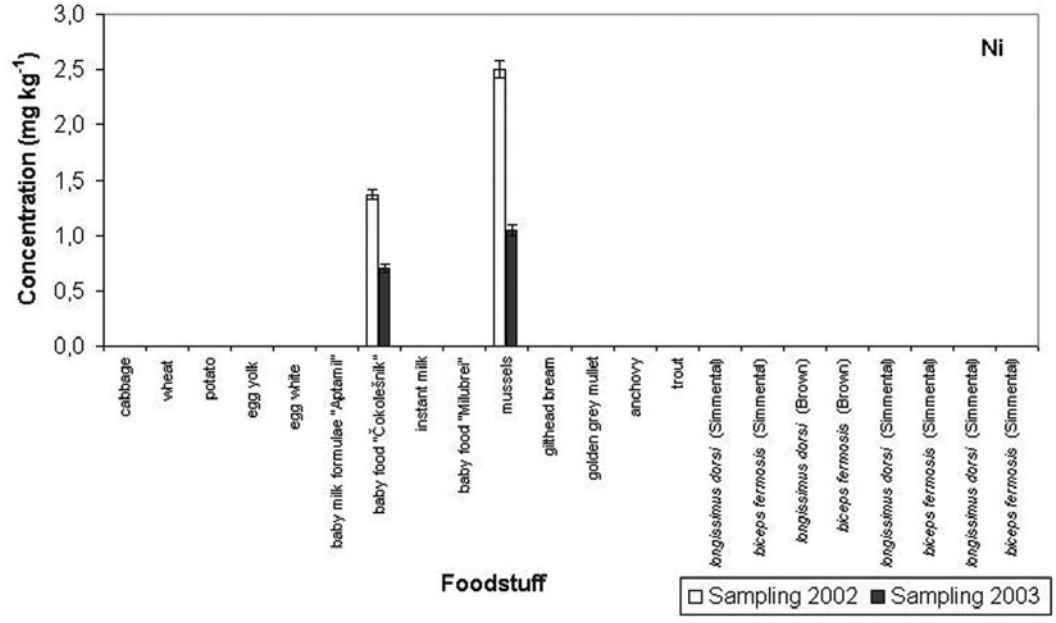


Fig. 6 - Analysis of Ni in basic foods of the Slovenian diet after microwave digestion of the samples and determination by ETAAS. Results (mean of three determinations) were calculated on a dry weight basis and refer to the edible portions.

which, similar to mussels, accumulate Ni from the marine environment.

In conclusion, data from the present investigation indicate that Cd and Pb were not present as contaminants in the foodstuffs analysed. The main sources for the daily intake of essential and trace elements are meat for Zn, wheat for Cr and cocoa for Ni, while Cu was present in similar concentrations in most of the foodstuffs studied. The data also indicate that there were no significant differences between the concentrations of the elements in the 2002 and 2003 samplings. The contents of minerals and trace elements determined were comparable to values reported for European and non-European countries.

ACKNOWLEDGEMENTS

This work was financed by the Ministry of Education, Science and Sport and the Ministry of Agriculture of the Republic of Slovenia.

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Paper received July 6, 2004 Accepted December 28, 2004

PAPER

INACTIVATION OF SPOILAGE MICROORGANISMS IN MANGO JUICE USING LOW ENERGY PULSED ELECTRIC FIELD IN COMBINATION WITH ANTIMICROBIALS

INATTIVAZIONE DI MICRORGANISMI CHE DEGRADANO IL SUCCO DI MANGO MEDIANTE APPLICAZIONE DELLA TECNICA DEL CAMPO ELETTRICO PULSATO A BASSA ENERGIA IN COMBINAZIONE CON L'USO DI SOSTANZE ANTIMICROBICHE

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ABSTRACT

The combination of various preservation hurdles such as mild heat (≤52°C), antimicrobials and pulsed electric field (PEF, batch system), to inactivate spoilage microbes in mango juice was investigated. The microbial count decreased with an increase in the process temperature above 47°C and the addition of small amounts of nisin and lysozyme. When mango juice was treated alone with mild heat and PEF, there was only 1.33 log microbial reduction. However, with the addition of small amounts of

RIASSUNTO

Il presente lavoro di ricerca ha riguardato lo studio sulla combinazione di vari metodi di conservazione, come blandi trattamenti termici (≤52°C), uso di sostanze antimicrobiche e applicazione di un campo elettrico pulsato (PEF, sistema discontinuo) sul succo di mango al fine di inattivare i microrganismi che degradano il succo. Si è evidenziata una diminuzione della carica microbica all'aumentare della temperatura di processo al di sopra di 47°C e addizionando piccole quantità di nisina e lisozima.

- Key words: fruit juice, hurdle technology, inactivation kinetics, lysozyme, nisin, nonthermal pasteurisation, pulsed electric field, spoilage microorganism -

nisin and lysozyme, a significantly large microbial reduction was observed with mild heat and PEF. There was a 4.4 log cfu/mL reduction in the microbial count with 20 instant-charge-reversal pulses at a field strength of 87 kV/cm (peak to peak) and a 52°C process temperature in the presence of nisin and lysozyme (27.5 IU of nisin and 690 IU of lysozyme per mL of juice).

Quando il succo di mango è stato sottoposto a trattamenti termici blandi associati a PEF, la riduzione microbica è risultata solo di 1,33 log. Tuttavia, blandi trattamenti termici e PEF, con l'aggiunta di piccole quantità di nisina e lisozima, hanno determinato un abbattimento significativo della carica microbica. Con 20 pulsazioni con cambio di carica istantaneo in un campo elettrico di 87 kV/cm (da picco a picco) e una temperatura di processo di 52°C, in presenza di nisina e lisozima (27,5 UI di nisina e 690 UI di lisina per mL di succo), la riduzione della carica microbica è risultata di 4.4 log UFC/mL.

INTRODUCTION

Mango (*Mangifera indica L.*) is the most popular tropical fruit in the world (SAGAR *et al.*, 2000). It is relished for its succulence, exotic flavour and delicious taste and is also a good source of vitamins A and C. Mango juice and nectar contain mango puree, sugar, water and citric acid in various proportions. Since mango juice contains a large quantity of water and added sugar, it is highly susceptible to microbial spoilage (ACHARYA and SHAH, 1999). The high sugar content and moderate acidity provide a favorable environment for yeast and molds. Heating mango juice at 70°C for 15 min eliminates most of the microorganisms, but the taste is unacceptable (ELECHI *et al.*, 1998). Interest in inactivating spoilage microorganisms in fruit juices by pulsed electric field (PEF) has increased because the flavour and nutritive value is retained.

Microbial inactivation by PEF is affected by many factors which, in decreasing order of importance, include: electric field strength, treatment time, number of pulses, pulse wave shape, processing temperature, type of microorganism, microbial growth stage, electrical conductivity of the food, viscosity, pH of the food and the presence of antimicrobials (HO and MITTAL, 2000; IU *et al.*, 2001; HODGINS *et al.*, 2002; SMITH *et al.*, 2002). Some have studies indicated that PEF could be combined with moderately high temperature (<60°C) or other processing aids such as antimicrobials to increase the microbial inactivation (HO and MITTAL, 2000). FDA regulation (FDA, 2001) states that juice processors must follow the principles of the Hazard and Critical Control Points (HACCP) for juice processing and should achieve a 5 log pathogen reduction in the product. The effect of using antimicrobials to enhance the inactivation was also studied in the above reports. Many studies have demonstrated that PEF has lethal effects on microorganisms (HODGINS *et al.*, 2002; HO and MITTAL, 2000; LIANG *et al.*, 2002). For example, a 5 log cycle reduction in counts of naturally occurring microorganisms (molds, yeast and some bacteria) was obtained by PEF treatment of orange juice (HODGINS *et al.*, 2002). A 5.35 log cfu/mL reduction in *E. coli* O157:H7 strain EC920026 population was reported by IU *et al.* (2001) in apple

cider treated with 30 pulses of 80 kV/cm (peak to peak) at 42°C using low-energy instant-charge-reversal waveform. PEF treatment, when combined with the addition of cinnamon or nisin, triggered cell death, resulting in a 6 to 8 log cfu/mL reduction in *E. coli* count. No work has been reported on the pasteurisation of mango juice using PEF.

A 4 log cycle reduction of yeast (*S. cerevisiae)* inoculated in pasteurized commercial apple juice was observed after application of 20 square-wave pulses of 260 J/pulse energy in a parallel electrode chamber with a batch volume of 25.7 mL (ZHANG *et al.*, 1994). Due to the high energy input, considerable cooling was required and the process temperature was not reported. On the other hand, QIN *et al.* (1995) reported a 6 log cycle reduction in counts of *S. cerevisiae* after PEF treatment of a commercial apple juice. The process parameters used were: exponential decay pulses, 45 kV/cm field, 2.5 :s pulse width, 1 Hz pulse frequency, and 0.6 cm electrode gap (coaxial chamber, 30 mL volume). The treatment temperature was held at 30°C by circulating chilled water. The flow rate was not reported and a suitable control treatment was not carried out so the results could not be compared with PEF effects. Due to the large energy input and removal of heat by circulating chilled water, it is difficult to distinguish between the effect of PEF and the thermal effect. Yeast was added to the juices in place of naturally occurring yeasts and moulds because the latter are difficult to inactivate. Many similar studies have been reported on fruit juices (HO and MITTAL, 2000). The combination of different hurdles such as moderately high temperatures (<55°C), antimicrobial compounds and PEF to reduce naturally-occurring microbes in freshly squeezed apple cider was explored by LIANG *et al.* (2002). Compared with counts obtained in the untreated juice at 23°C, PEF treatments of 90 kV/cm (peak to peak), with 40 pulses, at 44°C; and 90 kV/cm, with 40 pulses, at 52°C produced a 3 and >6 log cycle count reduction, respectively. When reductions due to PEF alone were calculated (by subtracting log N values at 0 and 40 pulses) at 44°, 48°, 50° and 52°C, the reductions were 2.22, 1.45, 1.73 and 1.72 log cycles, respectively.

Inactivation of *Listeria innocua*, *E. coli*, *Leuconostoc mesenteroides* and *Saccharomyces cerevisiae* in orange juice was investigated in a 100 L/h flowing PEF system at electric field strength (E_f) levels of 30 kV/cm and 50 kV/cm (McDONALD *et al.*, 2000). *L. mesenteroides*, *E. coli* and *L. innocua* were inactivated by as much as 5 log cycles at 30 kV/cm and 50°C, but a maximum reduction of only 2.5 log cycles was achieved for *S. cerevisiae* ascospores, even when an E_f of 50 kV/cm and 50°C was applied. The population of *Byssochlamys fulva* conidiospores in tomato juice decreased less than 1 log cycle when 2 pulses of 30 kV/cm were applied, and juice temperature was kept below 23°C by circulating chilled water around the treatment chamber (RASO *et al.*, 1998a). Slightly less than 4 log cycle count reductions were obtained when 15 pulses were applied. The effect of PEF treatment on grape juice inoculated with *B. fulva* conidiospores and *Neosartoria fischeri* ascospores has been reported (RASO *et al.*, 1998b). Juice temperature was controlled by passing the juice through a cooling coil immersed in iced water. The extent of inactivation of *N. fischeri* ascospores by PEF was negligible even after 20 pulses each with E_f of 44.8 kV/cm. After a PEF treatment of 8 pulses at 35 kV/cm at 20°C, the population of *B. fulva* conidiospores in grape juice decreased by 5 log cycles.

In the present study, the inactivation of naturally occurring microorganisms (mostly yeast and molds) in mango juice was investigated by studying the effects of mild process temperature with and without pulses, and the addition of nisin and lysozyme.

MATERIALS AND METHODS

Mango juice

Concentrated mango pulp was supplied by Alfresh Beverages (Toronto, Canada), and stored at -20°C in 2 L aliquots until used. Frozen mango concentrate was thawed from the stock at 6°C. About 175 mL of the concentrated pulp and 325 mL of water were mixed to make 500 mL of mango juice (suggested by Alfresh Beverages). The pH of the juice was 3.6±0.2 and electrical conductivity was 0.18±0.02 S/m. The pH and conductivity were significantly lower than apple cider and orange juice. The constituted mango juice was placed in a 1 L flask and spoiled by stirring at room temperature on a stir plate (model # 11-500-76, Fisher Scientific) for 5 days. Juice was sampled each day, and the microbial population was counted. Each time spoiled juice was re-inoculated into fresh juice. Natural spoilage microflora was used in all cases. Since fresh juice was inoculated with previously spoiled juice samples, species composition should be relatively constant between batches. A constant incubation time meant that the number and the growth stages of the microbes should be similar between different trials. A gram-stain was conducted on many samples using a microscope (Olympus BH2, Capsen Ltd., Markham, ON, Canada). The majority of cells were found to be yeasts on the media (plate count agar (PCA), potato dextrose agar (PDA), and deMan Rogosa Sharp agar (MRS)) used. Spoilage organisms in the mango juice were further identified using a Vitek Jr (Biomerieux Vitek, Inc., Hazelwood, MO) according to the manufacturer's instructions.

Heat treatment

Various instant temperatures (40°, 45°, 47°, 48°, 50° and 52°C) were applied to spoiled juice samples to study the critical temperature over which microbial count will be reduced significantly. Briefly, 3 mL of juice were transferred to a 12 mL glass tube, and then heated to the desired temperature in a water bath preheated to 70°±1°C. Temperatures were measured both before and after heating with a thermocouple probe (Temp Testr, model 35628-00, Oakton, Singapore) and the temperatures of the juice samples in the treatment chamber before and after treatment were within ±0.5°C of desired values. After reaching the desired temperature, the tube was put on ice immediately and the microbial counts in the treated juices were determined. Control samples went through the same process but without PEF application.

Pulsed electric field treatment

A batch low-energy PEF system, which consists of a high voltage pulse generator (Fig. 1) and a circular treatment chamber (Fig. 2), was used to treat the mango juice samples. The details are given by HO *et al.* (1995). The system consists of a 30 kV d.c. high voltage pulse generator, a treatment chamber, and devices for pumping and recording. The 110 V a.c. was raised in voltage through a high voltage transformer, and then rectified (Fig. 1). The d.c. high voltage supply then charges up the 0.12 μF capacitor through a series of 6 MΩ resistors (the time constant = 0.72 s). The pulse generator emits a train of 5 V pulses, and the trigger circuit serves to convert that to 500 V pulses using a silicon control rectifier (SCR). The generation of high voltage pulses relies on the discharge of the 0.12 μF capacitor through the thyratron. The batch unit can generate short-duration pulses (2 μs width, 0.5 Hz frequency) with peak-to-peak electric field strength up to 100 kV/cm. The uniqueness of this pulser is that pulses of low-energy (up to 20 J/pulse) and of instant-charge-reversal shape are generated.

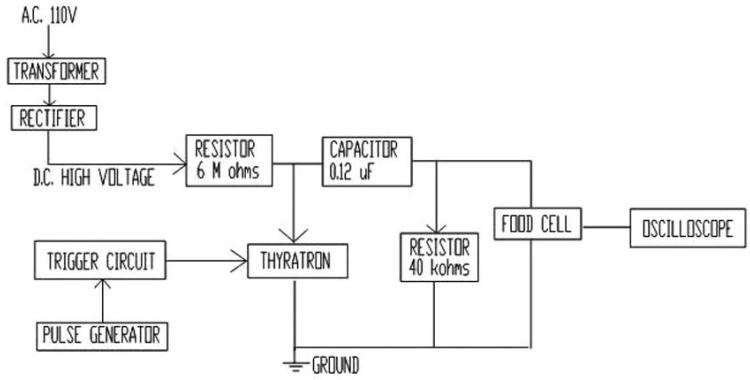


Fig. 1 - Generalised scheme of pulsed electric field equipment (HO and MITTAL, 2000).

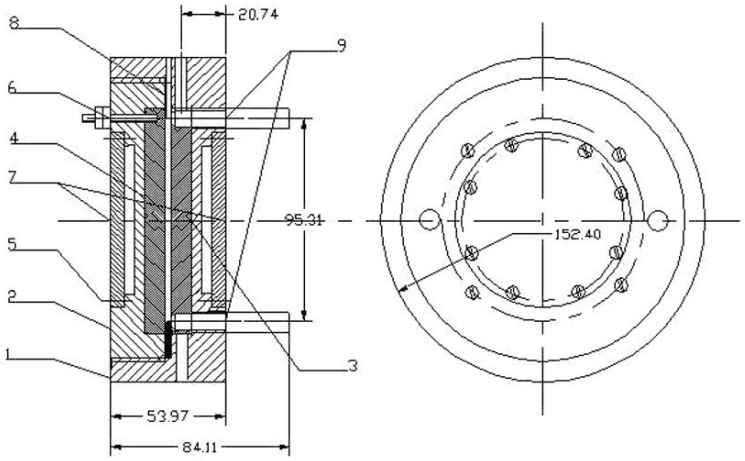


Fig. 2 - Schematic diagram of the treatment chamber showing electrodes, insulation and heating water jacket: (1) bottom insulation; (2) electrode insulation cover; (3-4) stainless steel electrodes; (5-6) bolts; (7) water jacket cover; (8) gasket; and (9) valve to seal the flow through electrodes; all dimensions are in mm.

Conventional pulsers generate pulses of much higher energy and of square or exponential decay or bipolar shape. The field strength reported here is calculated on the basis of peak-to-peak values, i.e. +ve to –ve peaks for instant-charge-reversal pulses.

The circular batch treatment chamber (total 15.0 cm diameter including insulation) has two circular and parallel stainless steel electrodes (9.5 cm) (Fig. 2). The distance between the electrodes was adjusted to 0.3 cm by a circular plate, thereby achieving a process volume of approximately 30 mL in batch mode operation. The volume is based on collected samples from the chamber after treatment. The desired temperature of the sample (>room temperature) was maintained by circulating hot water in the outer jacket around the electrodes. To eliminate air bubbles in the treatment chamber, the sample entered the chamber from the bottom through a

channel under 85 kPa vacuum. A digital oscilloscope (model TDS 340, Tektronix Inc., Beaverton, OR) and a high voltage probe (model P6015A, Tektronix Inc., Beaverton, OR) were connected to the system to monitor each pulse across the chamber.

PEF was applied to treat mango juice using 20 or 50 pulses of 87 kV/cm (peak to peak) or 26 kV (applied) at room temperature and higher. To study the effect of antimicrobials on the spoilage microorganisms in the juice, lyso-chrisin (trade mark of Canadian Inovatech, Abbotsford, Canada) containing 1:3 mixture of nisin and lysozyme (an enzyme) (23.75% w/w of total pure lysozyme with an activity of 6,900 IU/mg, and 1.69% w/w of total pure nisin with an activity of 275 IU/mg) was used. Spoiled juice was first mixed with antimicrobials, and then heated and held at the desired temperature for 30 min. The antimicrobial agent was added at 0.1 mg/mL of juice. Hence 27.5 IU of nisin and 690 IU of lysozyme per mL of juice were used. No changes in the electrical conductivity and pH of the samples were observed after adding the very small amounts of antimicrobials.

The desired temperatures were achieved by rapidly preheating the juice in a microwave oven (80% power, model NN-S759BC, Panasonic, Mississauga, Ontario, Canada) and then transferring it to a water bath where it continued to be heated until the desired temperature was reached. The temperature of the chamber was adjusted by passing hot water through the chamber. The temperature of the juice samples entering and leaving the chamber was recorded using a thermocouple probe (TempTestr, Cole-Parmer Instrument Company, Chicago, IL). Treated samples were collected in sterile containers and stored on ice until analysis. The chamber was cleaned with hot water (80°C) before and after each treatment and the microbial count of <10 cfu/mL in the wash-water after cleaning confirmed that there was no cross-contamination of the juice samples.

Microbiological analysis

For each sample, 1.0 mL of juice was serially diluted in 0.1% peptone water (Difco Laboratories, Detroit, MI, USA). Diluted juice samples were tested in triplicate for total plate counts with plate count agar (Difco, Sparks, MD, USA) and Autoplate 4000 (Spiral Biotech, Norwood, MA, USA). Plates were kept at room temperature for 5-7 days or at 37°C for 2 days, and cfu were counted.

Data analysis

All treatments were carried out at least three times and in each case, an untreated sample acted as a control. Microbial populations were transformed into log values. A Duncan analysis was used to rank the means and a t-test was conducted to determine the significance using the Statistical Analysis System (SAS, 1996).

RESULTS AND DISCUSSION

Microbial types and growth in the mango juice

The results of yeast growth in the mango juice show that from day 1 (4.69±0.26 log cfu/mL) to day 3 (9.45±0.04 log cfu/mL), there was a significant increase in yeast count followed by a slow change (10.21±0.02 log cfu/mL after 5 days), indicating that during the first 3 days of fermentation, yeast grew rapidly (Fig. 3). Thus, in this study, a 3-day fermentation was used to grow microorganisms in the juice. The mould count was 3.11±0.35 log cfu/mL after one day of fermentation, increased to 5.16±0.11 log cfu/mL after the second day, after which it was insignificant.

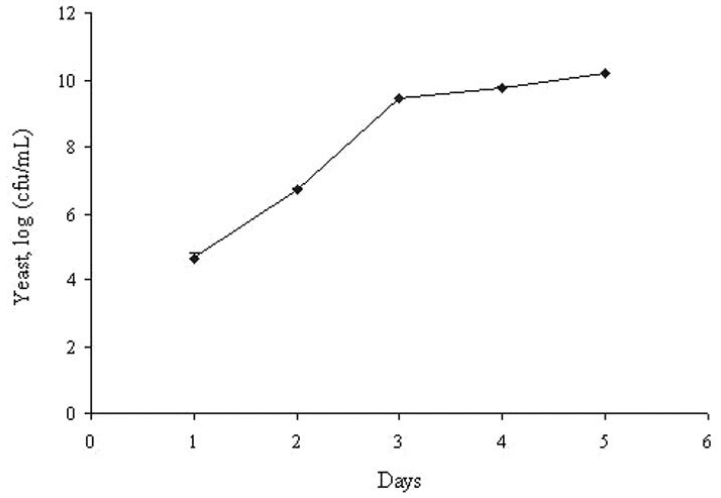


Fig. 3 - Changes in yeast number in mango juice with time.

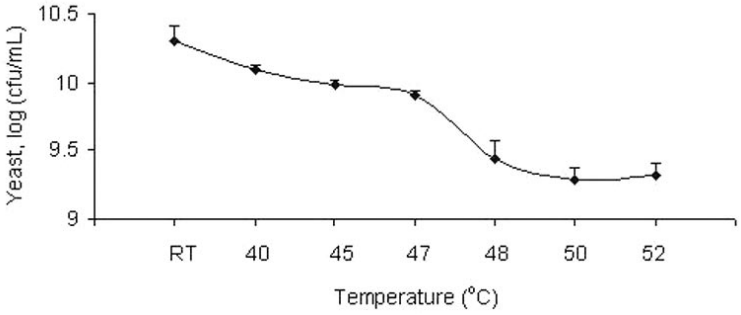


Fig. 4 - Impact of temperature on yeast count in mango juice. RT = room temperature.

Effect of temperature increase on microbial count in the juice

The results of instantaneous temperature increase indicate that the yeast count was only slightly reduced with the increase of temperature. At 47°C, the microbial count was only reduced by 0.39±0.07 log cfu/mL (Fig. 4). However, when the temperature was increased above 47°C, there was a significant decrease in the log counts (0.89±0.25 at to 48°C, and 1.03±0.15 log cfu/mL at 52°C).

Effect of PEF

Table 1 shows the effect of PEF for 20 or 50 pulses of 87 kV/cm (peak to peak) at 24° and 47°C with and without antimicrobials. PEF treatment for 20 and 50 pulses at room temperature reduced microbial counts by 1.2 and 1.33 log cfu/mL, respectively, compared with the control. PEF treatments for 20 and 50 pulses at 47°C reduced yeast counts by 1.40 and 1.49 log cfu/mL, respectively, compared to control at 24°C. The changes

Table 1 - Effects of temperature and antimicrobials with and without PEF on microbial reduction in mango juice (mean and standard deviation).

Treatments	Log (No) and log(No/N) in brackets			
	24°C	47°C	24°C with Nisin+lysozyme*	47°C with Nisin+lysozyme
Control	9.97±0.43a	9.13±0.07a (0.84)**	9.65±0.23a (0.32)	9.08±0.02a (0.89)
20 pulses of 87 kV/cm (peak to peak)	8.78±0.11b (1.19)	8.57±0.11b (1.40)	8.57±0.11b (1.40)	7.43±0.16b (2.54)
50 pulses of 87 kV/cm (peak to peak)	8.64±0.02b (1.33)	8.48±0.11b (1.49)	8.48±0.11b (1.49)	7.24±0.05b (2.73)

*1:3 ratio of nisin and lysozyme, 0.1 mg/mL;
**compared to control at 24°C;
a-b: means with the same letter in a column are not significantly different at the 95% level.

in the microbial count in the treatments were significant compared with the control ($P<0.05$), but not significant among the treatments ($P>0.05$).

Table 1 also shows the effect of PEF treatment in the presence of nisin and lysozyme and mild heat on microbial decay in the juice. The addition of nisin and lysozyme to the juice alone did not affect the microbial count ($P>0.05$). However, treatment of the juice with PEF for 20 or 50 pulses in the presence of nisin and lysozyme significantly reduced the microbial count ($P<0.05$). PEF treatments for 20 or 50 pulses at 47°C in the presence of nisin and lysozyme reduced the microbial count by 2.54 or 2.73 log cfu/mL, respectively, compared with the control at 24°C. The effects were significant ($P<0.01$). These results support the findings of microbial decay due to PEF, discussed by HO and MITTAL (1996).

Table 2 indicates that in the presence of antimicrobials and mild heat (50°C), PEF at 20 and 50 pulses of 87 kV/cm (peak to peak) reduced the microbial count by 3.07 and 3.38 log cfu/mL, respectively, compared to the control at 24°C. Increasing the temperature to 52°C, the microbial count was reduced by 4.40 log cfu/mL. Very low levels of antimicrobials (0.1 mg/mL) were used in this study, but if the levels are increased, greater microbial decay can be achieved as reported for orange juice (HODGINS *et al.*, 2002; LIANG *et al.*, 2002) and milk (SMITH *et al.*, 2002). PEF treatment had a significantly greater effect

Table 2 - Effects of antimicrobials and temperature with PEF on microbial inactivation in mango juice.

Treatments	log(No/N)*		
	50°C	50°C and nisin + lysozyme**	52°C and nisin + lysozyme
Control	1.50±0.28b	1.75±0.14b	1.90±0.20b
20 pulses of 87 kV/cm (peak to peak)	1.90±0.42b	3.07±0.22a	4.40±0.12a
50 pulses of 87 kV/cm (peak to peak)	2.30±0.42a	3.38±0.20a	-

*No = Microbial population at 24°C = 7.8±0.28 log cfu/mL;
**1:3 ratio of nisin and lysozyme, 0.1 mg/mL;

on *E. coli*, coliforms and lactic acid bacteria than on yeasts and moulds (IU *et al.*, 2001) because yeasts and moulds survive better in an acidic environment and often require higher temperatures to be destroyed (RAY, 2004). It is generally recognized that gram-negative bacteria, such as *E. coli* and coliforms, are more susceptible to the PEF process than gram-positive species, yeasts and moulds (JANDA and ABBOTT, 1998, RAY, 2004). Yeasts might have produced ascospores which are much more resistant to PEF treatment than yeasts. This requires further research.

This effect can be attributed to a reduction in the bilayer thickness due to the transition of the phospholipid molecules from a gel to a liquid-crystalline phase with a temperature increase which favors the irreversible breakdown of the cell membrane (HO and MITTAL, 1996). Nisin is a peptide that causes ion-permeable pore formation in the cytoplasmic membrane of cells (SMITH *et al.*, 2002). Nisin exhibits a broad spectrum of inhibitory activity against gram-positive organisms, including spores, but it is not generally active against gram-negative microbes. However, gram-negative microbes do show nisin sensitivity when subjected to physical treatments (heating, freezing, etc.) that affect outer membrane permeability (BOZIARIS *et al.*, 1998; STEVENS *et al.*, 1992). Thus, nisin acts on several species of gram-negative microbes if the barrier properties of the outer membrane are first disrupted (ROSS *et al.*, 2003). Hydrolysis of the cell wall by lysozyme can damage the structural integrity of the cell wall and result in the lysis of bacterial cells (PROCTOR and CUNNINGHAM, 1988). Also, a combination of nisin and lysozyme has a greater bactericidal effect than either one alone (CHUNG and HANCOCK, 2000; MASSCHALCK *et al.*, 2000). When exposed to the antimicrobial agents prior to PEF treatment, the cells suffered sublethal injury of between 0.25 and 0.4 log cfu/mL at 50° and 52°C, respectively (Table 2), and became sensitive to PEF treatment. The increased loss of cell viability after PEF treatments in the presence of antimicrobial agents is synergistic (IU *et al.*, 2001). These studies indicate that with the addition of antimicrobial agents, bacterial cell death can be increased following an increase in cell injury.

CONCLUSIONS

When mango juice was treated alone with mild heat and PEF, there was a 1.33 log cfu/mL reduction in the microbial count. However, the addition of a small amount of nisin and lysozyme plus mild heat and PEF significantly reduced the microbial count. Twenty pulses at a strength of 87 kV/cm (peak to peak), 20 pulses, at 52°C, and in the presence of nisin and lysozyme (1:3 ratio, 0.1 mg/mL), reduced the microbial count by 4.4 log cfu/mL. Further research is necessary to obtain evidence that this process is able to ensure a microbiologically safe product, i.e. a sufficient inactivation of microorganisms.

ACKNOWLEDGEMENTS

The financial support for this research was provided by the Natural Sciences and Engineering Research Council of Canada, and Alfresh Beverages, Toronto, Canada.

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Revised paper received October 5, 2004 Accepted November 29, 2004

PAPER

EVALUATION OF SAMPLING CRITERIA FOR THE DETECTION OF GM SOYBEANS IN BULK

VALUTAZIONE DI CRITERI DI CAMPIONAMENTO
PER LA DETERMINAZIONE DI SOIA GENETICAMENTE MODIFICATA
ALLO STATO SFUSO

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ABSTRACT

The aim of this study was to develop a sampling procedure to quantify genetically-modified Round-up Ready (GM RR) soybeans in a lot. Seven lots (35 kg each) fortified with three different percentages of GM RR soybeans (4 lots at 1%, 2 lots at 10%, and 1 lot at 5%) were prepared. Four sampling procedures were carried out on each lot. Different numbers and sizes of increment samples were collected from the lots tested and prepared by homogenising the sample with water. Sepa-

RIASSUNTO

La finalità di questo studio è stata quella di sviluppare uno schema di campionamento in grado di quantificare la percentuale di soia Round-up Ready (GM RR). Lo studio si è basato sulla preparazione di sette lotti (35 kg ciascuno) fortificati con tre percentuali differenti di GM RR pari all'1% (4 lotti), al 5% (1 lotto) ed al 10% (2 lotti). Da ogni singolo lotto è stato prelevato un campione globale (14,23 kg) seguendo 4 tipologie di prelievo differenti. Dopo il prelievo, ogni campione globale è sta-

- Key words: detection, GMO, labeling, sampling, soy -

rate analyses were carried out using quantitative Real Time PCR of split aliquots. The sampling procedures that used ten increment samples provided the best results, in terms of precision and accuracy.

to omogeneizzato con acqua utilizzando la tecnica dello "slurry", e suddiviso in due parti uguali analizzate sia singolarmente, sia come insieme formato dalla unione delle singole metà. In base ai risultati ottenuti la metodologia basata sul prelievo di 10 campioni incrementali ha fornito i risultati più accurati in ciascuno dei livelli di concentrazione esaminati.

INTRODUCTION

Effective sampling procedures are crucial for quantifying analytes that are not homogeneously distributed in a lot such as mycotoxins and, more recently, genetically modified organisms (GMOs). The variance associated with the sampling step represents the major contribution to the overall variance of the "analytical chain" (sampling, subsampling and analysis) (WHITAKER *et al.*, 1995), even if reliable analytical methods are employed.

Sampling errors are closely correlated with risk to the consumer, defined as the acceptance of lots that contain levels that exceed pre-established limits, and risk to the producer, defined as the rejection of lots that contain levels below the legal limits. Therefore, the use of reliable sampling procedures is very important for everyone involved (PARK *et al.*, 1998).

As far as GMO labelling is concerned (EC, 1997; 1998), the sampling procedure is a key factor for the correct implementation of the legal requirements and for preventing non voluntary contamination. Regulation 1830/2003 (EC, 2003) recognises the above - mentioned difficulties and is one of the reasons why the threshold limit of 0.9% was set as the adventitious presence of GMOs which would not require mandatory labelling. To date, several papers have been published on the theoretical basis for developing a suitable sampling plan for GMOs (MIPAF, 1993; CEN, 1998), but none of them provide a pragmatic approach that can be coupled with analytical methods currently adopted in Europe (ISTA, 1986; GILBERT, 1999; LISCHER, 2001a, b; PAOLETTI *et al.*, 2002).

This study was carried out to provide a basis for the implementation of a pragmatic sampling procedure for quantifying non homogeneously distributed GM soybeans in a bulk soybean lot. In order to evaluate the "trueness" of the experimental procedure, laboratory samples with known percentages of genetically modified (GM) material were prepared, successively sampled in different ways and then analysed in a slurried form, to decrease the variance in the analytical chain.

MATERIALS AND METHODS

Samples

Lot A, Round-up Ready (RR)-free soybean (nominal value): due to the selected low level (from 1 to 10%) of RR soybean concentrations projected for the test lots, the availability of a RR-free soybean lot was considered crucial. The 1% level was selected as the starting point considering that at the time of the study this threshold level for labelling requirements was stated by Regulation 49/2000 in the case

of the adventitious presence of GMOs in food products. Therefore, a considerable number of bags (24 bags of 27 kg each) of potentially RR-free soybeans, obtained directly from a producer, were tested with EU-validated methods (LIPP *et al.*, 2000) for their content of GM kernels. The following procedure was used: five samples of 500 grams each were randomly sampled from each sack. Each sample was thoroughly mixed with water 1:1 (slurry) and 4 test portions per analysis (300 mg each) were analysed in triplicate. Screening tests for 35 S promoter amplicon size 123 bp (LIPP *et al.*, 2000) were performed on the extracted DNA, for a total of 20 analyses per sack. The limit of detection of the method was evaluated to be 0.01% by analysis of the serial dilution of certified reference material (CRM) of RR Soybeans purchased from the Institute of Reference Materials and Measurements (IRMM, Geel, Belgium), series 410 certification level: 5%. The entire procedure was repeated twice. Whenever a single analysis was positive, the corresponding bag was discarded. There were 12 bags determined to be RR-free; they were then used for the formation of the lots at known concentrations.

Lot B, 100% RR soybean (nominal value): one lot (10 bags of 5 kg each) of RR soybeans was obtained from a producer who certified the lot as 100% RR. Three test samples of 500g each, were randomly taken from each bag, thoroughly slurried with water (1:1) and 4 test portions (300 mg each) were analysed in triplicate by the "Real-Time" PCR simplex method based on copy number calibration (BERDAL *et al.*, 2001) for verifying the zygosity of the kernels. The homozygosity of RR soybean kernels was confirmed by the mean percentage of RR soybeans obtained (102.6 ± 9.8%).

Lots C, D, E: lots A and B were used to prepare 35 kg lots of RR soybean at concentrations of 1%, 10% and 5% RR soybean percentages as follows:

4 lots of 1% RR soybean (C_1-C_4): 0.35 kg of 100% RR soybean were added to 34.65 kg of RR-free soybean.

2 lots of 10% RR soybean, (D_1-D_2): 3.5 kg of 100% RR soybean were added to 31.5 kg of RR-free soybean.

1 lot of 5% RR soybean (E): 1.75 kg of 100% RR soybean was added to 33.25 kg of RR-free soybean.

Each lot (N=7) was prepared by stratifying layers of GM and non-GM materials in order to simulate the heterogeneous conditions potentially found in realistic shipping conditions. The stratification method adopted for the preparation of the lots at different GMO percentages was performed on the basis of the hypothetical condition in which shipped lots can be found, that is, from the loading of lots from GMO and non-GMO farms without any form of mixing and homogenisation with the formation of layers of different origin and nature.

Sampling strategy

In order to obtain information about the best reliability (of the sub-sampling technique) attributable to the correlation between the different numbers and sizes of increment samples and the distribution curves of the single samples, the bulk sample n of 14.23 kg was obtained by performing four different sub-sampling procedures corresponding to the withdrawal of 1, 10, 20 and 50 increment samples. This procedure was carried out only at the 1% GMO level. On the basis of the results obtained from the 1% lots, 10% and 5% GMO levels were successively performed.

The rationale for the withdrawal of increment samples (a sample of material from one location in the lot) from the lots and the formation of test samples (a portion of the increment sample to be analysed) was based upon the following scheme:

- Lot 1%, (C_1-C_4): a different number of increment samples was taken from each lot:

Lot C_1 One increment sample of 14.23 kg

Lot C_2 Ten increment samples of 1.423 kg each

Lot C_3 Twenty increment samples of 0.711 kg each

Lot C_4 Fifty increment samples of 0.286 kg each

- Lot 10%, (D_1-D_2): two different number of increment samples were taken:

Lot D_1 Ten increment samples of 1.423 kg each

Lot D_2 Twenty increment samples of 0.711 kg each

- Lot 5% (E): ten increment samples of 1.423 kg each were withdrawn from the lot.

All the increment samples were randomly withdrawn. In all the procedures, each increment sample was taken using a probe (PBI international, Milan, Italy) and ground with water to form a homogeneous aqueous slurry with an Ultraturrax (T80KT) (IKA® Werke GmbH & Co. KG, Staufen, Germany) for the 14.23 kg increment sample and with a blender for the remaining samples.

After homogenisation with water, each increment sample from all the lots (except the 14.23 kg increment sample from lot C_1) was split into two samples. One half sample was analysed individually, while the remaining halves were combined and analysed as a whole test sample. The total number of samples was as follows:

- 4 Lots 1%, (C_1-C_4): one 14.23 kg test sample, 3 combined test samples and 80 single test samples.

- 2 Lots 10%, (D_1-D_2): two combined test samples and 30 single test samples.

- 1 Lot 5%, (E): one combined test sample and 10 single test samples.

Sample analysis

A sample of 300 mg was taken from each slurried sample, extracted and analysed in triplicate by Multiplex "Real-Time PCR" to quantify the RR soybeans (for Lots C_1, C_2, D_1 and E, three different samples were analysed in triplicate, for Lots C_3, C_4 and D_2, two different samples were analysed in triplicate). Analyses of the lots were performed with the multiplex method optimised by FOTI *et al.* (2005), which is currently used for national control activities.

Two primer pairs and fluorogenic probes for the detection of RR soybean and of non GM-soybean (endogenous PCR system) were chosen according to HUBNER *et al.* (2001) and purchased from Applera, Milan, Italy. The endogenous PCR system refers to the *lectin* gene (Le 1), while the transgenic PCR system amplifies a part of the synthetic construct CP4 EPSPS.

PCR reactions were carried out by the same analyst with reagents of the same lot, in a MicroAmp® 96-well reaction optical plate, mixing the TaqMan Universal Mastermix (Applera, Milan, Italy) (including: AmpliTaqGold DNA polymerase, dATP, dCTP, dGTP and dUTP MgCl2, 2x TaqMan buffer, AmpErase uracil N-glycosilase) with endogenous and transgenic probes (200 nM), and with transgenic primers (100 nM), endogenous primers (40 nM) and 5 µL of extracted DNA (20 ng/µL). The final total volume was 50 µL. The "Multiplex" TaqMan assays were performed with the ABI PRISM® 7700 Sequence Detection System (SDS). PCR reactions were run using the universal thermal cycling parameters as follows: initial step of decontamination to activate UNG (2 min at 50°C), a second step to activate DNA polymerase and denature DNA template (10 min at 95°C) and 50 cycles (15 s at 95°C and 1 min at 60°C). No degradation of the DNA occurred during the procedure of slurring, since the electrophoretic gels did not show any smear, in accord with similar results obtained by TRAPMANN *et al.* (2002).

Rationale for the lot size and bulk sample

The design of the trial was to calculate the bulk sample size (*n*, expressed in kg) to be drawn as a representative sample of the lot. To calculate the size, the following equation was used (MARBACH, 1992):

$$\max n = \frac{N}{(N-1)\theta^2 + 1} \text{ x } 0.00015 \quad (1)$$

where

n = bulk sample size expressed in kg

N = lot size expressed as number of kernels corresponding to lot size expressed in kg

θ = maximum acceptable error.

Considering that the average weight of a single soybean kernel is 0.00015 kg, a lot of 35 kg corresponds to 233.333 kernels. This was considered as a representative population for laboratory simulation. Based on equation (1), for a laboratory lot size (N) of 35 kg, and an acceptable error (θ) of 0.25%, a weight of n = 14.23 kg was calculated for the bulk sample.

Furthermore, to correlate the laboratory situation to real conditions (ships, lorries, silos, etc.), the lot size N should be as large as possible. Therefore, by making it infinity, equation (1) becomes:

$$\lim_{N \to \infty} \max n = \frac{1}{\theta^2} \text{ x } 0.00015 \quad (2)$$

For an acceptable error of 0.25%, the maximum bulk sample size (n) was determined to be 24 kg.

From the approach described, it should be noted that the high sampling rate (50%) of the entire lot is a consequence of the Marback equation only for the simulation trial, while for the real lots, the same equation leads to a sampling rate of less than 1%.

RESULTS AND DISCUSSION

GM levels in lots at 1% concentration

The results of the analyses of the combined and single half-test samples are shown in Table 1. The comparison between the combined half-test sample concentrations and the theoretical value (1%) provides an estimate of the most reliable sampling scheme. In particular, in the case of 10- and 20-increment samples, mean values of 1.00% and 1.15%, respectively, were obtained. The former was coincident with the theoretical value, while the latter was potentially overestimated. The percentage in the case of the 50-increment samples was even less accurate (1.32%).

Figures 1a,b, and c illustrate the distribution of RR soybean percentages in the single half-test samples for each series of increment samples (10, 20, and 50, respectively). The wide spread of the concentration levels associated with the single test samples is relevant. As far as the 10-increment sample scheme is concerned, the test sample concentration ranged from 4.50% to 0.23% with 60% of the results below the theoretical value. In the case of the 20-increment samples, the range was rather narrow (1.82-0.20%), with 50% of the results being below the real value (1%). The range of the concentration for the 50 test samples was between 2.30 and 0.42%, with almost 50% below the real value. These results could be attributable to the correlation existing between the degree of homogenisation of the large and small increment sample sizes. In fact, a large increment sample size could correspond to greater heterogeneity between the GM and non-GM materials as a result of a larger GM kernel/non-GM kernel ratio. In fact, in the 10-increment sample trial, almost 80% of the results fell close to the reference value with 60% of the values below, while in the 20- and 50-incre-

Table 1 - Mean values of GM RR soybeans in the single half test samples and of the combined half test samples of lots of 1, 10 and 5% RR soybeans with coefficient of variation (CV%).

Lot	N. Increment samples	Weight of increment samples (kg)	GM RR soybean (%)			
			Single half-test samples		Combined half-test samples	
			mean	CV%	mean	CV%
1%						
1	1	14.23			1.50±0.05	3.30
2	10	1.423	1.27±1.24	97.69	1.00±0.05	5.00
3	20	0.711	1.07±0.50	47.00	1.15±0.01	1.30
4	50	0.286	1.08±0.57	52.72	1.32±0.04	3.03
10%						
2	10	1.423	9.41±0.58	6.1	9.90±0.05	0.50
3	20	0.711	10.90±1.72	15.6	12.03±0.15	1.24
5%						
2	10	1.423	5.13 ±2.1	42.69	4.99±0.012	0.24

ment sample trials, only 50% of the results were close to the reference value.

Furthermore, the procedure of combining the half-test samples and analysing them as a whole after slurring is very important. In fact, looking at the coefficient of variance (CV) in Table 1 there is a dramatic reduction of the variance associated with the analysis of the whole sample in comparison with that of the single slurried test samples.

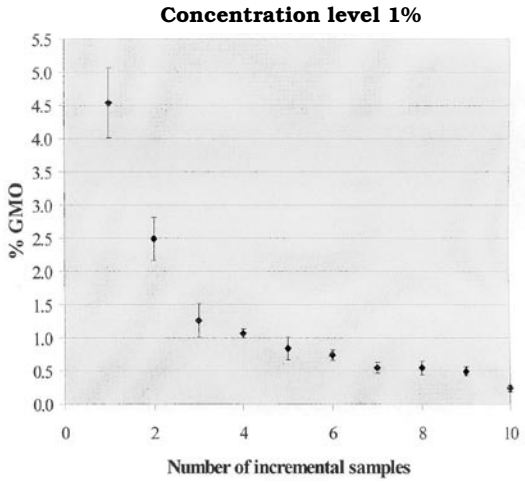
GM levels in lots at 10% concentration

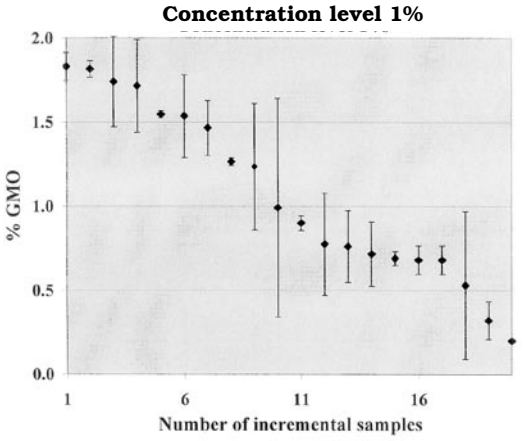
In the case of the two lots of 10% RR soybean, only the sampling schemes that were the most accurate in the case of the 1% lots (10- and 20-increment samples) were taken into consideration. The results of the analyses for the combined and single half-test samples are reported in Table 1. The results show that the same considerations as those made for the 1% lots can be applied to the 10% lots. The sampling throughout the 10-increment samples provided the most accurate mean value in the combined test sample, with an average of 9.9% and a standard deviation of 0.05. Figures 2a and 2b show the distribution curves of the 10% RR soybean percentages in the single half-test samples in the 10- and 20- increment samples, respectively. The distribution of results was similar to the 1% experiment with 70% and 60% of values below the theoretical value in the 10-increment and 20-increment samples, respectively.

GM levels in lots at 5% concentration

To verify the reliability of the sampling scheme of drawing 10-increment samples, since this was the best scheme from the previous observations, a single lot at 5% concentration was therefore prepared and sampled following this procedure. Table 1 and Fig. 3 show the results and the distribution curve of the single increment sample analysis, respectively. The results confirmed the reliability of the procedure of drawing of 10-increment samples; the GM concentration was very close to the reference value and had a low standard deviation.

Based on these results, the sampling





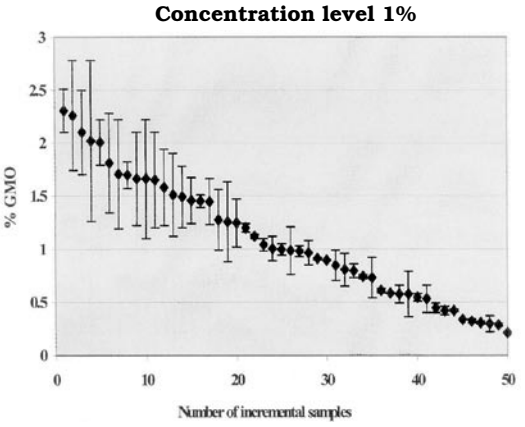
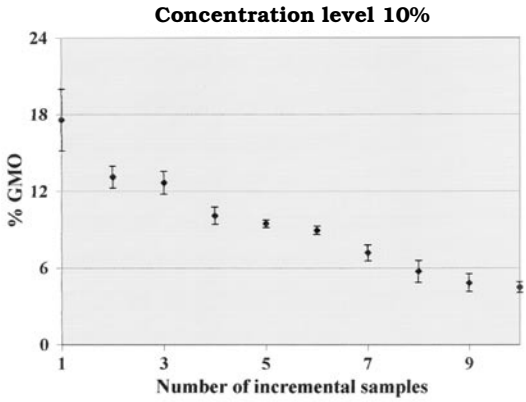


Fig. 1a,b,c - Percentage of GM material in 1% concentration of RR soybean for single half-test samples: (a) 10 incremental samples; (b) 20 incremental samples; (c) 50 incremental samples.



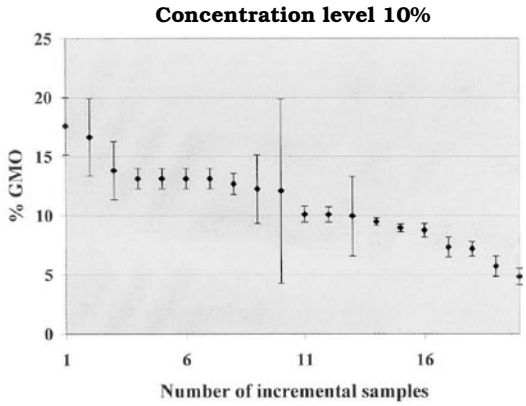


Fig. 2a,b - Percentage of GM material in 10% concentration of RR soybean for single half-test samples: (a) 10 incremental samples; (b) 20 incremental samples.

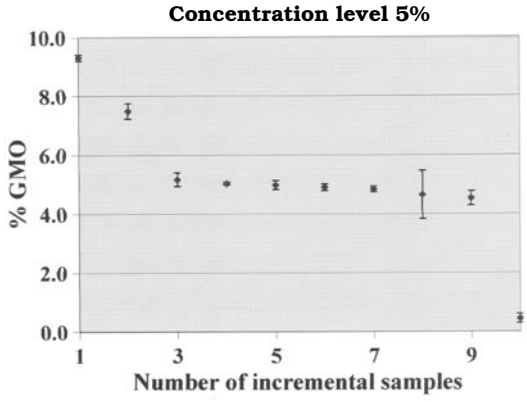


Fig. 3 - Percentage of GM material in 5% concentration of RR soybean for single half-test samples: 10 incremental samples.

Table 2 - Recommended sampling scheme for use in control and research activities (10-increment samples with a tolerance of 0.25%).

Lot size kg	Bulk and Laboratory sample, kg	Increment sample size, kg
30	13.33	1.333
60	17.14	1.714
90	18.94	1.894
120	20.00	2.000
300	22.00	2.200
600	23.00	2.300
N>600 kg corresponding to 4.000.000 soybean kernels	23.00	2.300
All samples were slurried with water (ratio 1:1) before DNA quantification.		

scheme of drawing 10-increment samples, as shown in Table 2, is proposed for official control and research activities.

CONCLUSIONS

The most reliable and accurate procedure was the withdrawal of 10-increment samples of the size derived from equations (1) and (2). The sampling variance was dramatically reduced by performing a slurry technique with water prior to the analysis. In addition, the variance was reduced even more when the single half-test samples were combined into one whole sample.

The results of this study may provide a starting point for the implementation of a sampling scheme that is both reliable and feasible, especially considering its potential application in activities to check imported goods at ports of entry. Furthermore, the availability of this sampling plan may supply a basis for further comparison with other existing methodologies at the international level.

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Revised paper received July 30, 2004 Accepted December 20, 2004

PAPER

POD CHARACTERISTICS OF TWO *CERATONIA SILIQUA* L. VARIETIES FROM JORDAN

CARATTERISTICHE DEI FRUTTI DI DUE VARIETÀ DI *CERATONIA SILIQUA* L. DELLA GIORDANIA

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ABSTRACT

Pods and seeds of *Ceratonia siliqua* L. varieties *(siliqua* and *macrocarpa*) from Jordan were investigated for their physical properties, proximate analysis, sugar and mineral contents. The *macrocarpa* variety had higher values than the *siliqua* variety for pod width, thickness, volume, weight and length as well as fat and carbohydrate contents. The *siliqua* variety had higher protein, fiber and ash contents than the *macrocarpa* variety. *Macrocarpa* pods had higher glucose (42.2%), fructose (18.9%) and

RIASSUNTO

I frutti e i semi di due varietà di *Ceratonia siliqua* L., originarie della Giordania (var. *siliqua* e *macrocarpa*), sono posti a confronto considerando le loro proprietà fisiche, i risultati di analisi chimiche routinarie e il contenuto in zuccheri e sali minerali. I frutti della var. *macrocarpa* possiedono maggior larghezza, lunghezza, spessore, volume e peso rispetto a quelli della var. *siliqua,* nonché un maggior quantitativo di lipidi e carboidrati. Al contrario, la var. *siliqua* contiene più proteine, fibre e cene-

- Key words: carob; *Ceratonia siliqua* L.; macrocarpa; pods; seeds; *siliqua* -

sucrose (38.9%) than the *siliqua* variety that had values of 33.7, 29.6 and 36.6%, respectively. A similar trend was observed for the chemical composition of the seeds. The mineral contents in carob seeds varied significantly for all the minerals except Mn. *Siliqua* pods had higher Zn, Na, K, Ca, and P contents than *macrocarpa* pods. The correlation values between ash and fiber content of the pod (r = 0.75), fiber and protein (r = 0.91), sucrose and carbohydrate (r = 0.88), fructose and sucrose (r = 0.91) and carbohydrate and fructose (r = 0.88) were significant.

ri. Per quanto riguarda i carboidrati, i frutti della var. *macrocarpa* sono risultati quasi sempre più ricchi in glucosio (42.2%), fruttosio (18.9%) e saccarosio (38.9%) rispetto a quelli della var. *siliqua* che ne presentano rispettivamente il 33.7, 29.6 e il 36.6%. Un trend simile si è osservato per la composizione chimica dei semi. Ad eccezione del Mn, il contenuto di tutti gli altri minerali varia significativamente nei semi di carruba. I frutti della var. *siliqua* contengono maggiori quantitativi di Zn, Na, K, Ca e P rispetto all'altra varietà analizzata. Sono risultati statisticamente significativi i seguenti valori di correlazione (r): r = 0.75 tra ceneri e contenuto in fibre delle silique; r = 0.91 tra fibre e proteine; r = 0.88 tra saccarosio e carboidrati; r= 0.91 tra fruttosio e saccarosio e r= 0.88 tra carboidrati e fruttosio.

INTRODUCTION

Ceratonia siliqua L., carob, is an evergreen sclerophyllous tree, native to Syria and northern Africa (DE CANDOLLE, 1883), but also grows in warm regions along the Mediterranean coast (DIAPOULIS, 1950) and in other countries such as India, Rhodesia, Mexico and the United States (IMRIR and VLITOS, 1975; ESBENSHADE and WILSON, 1986). There are over 50 varieties of *Ceratonia* (ESBENSHADE and WILSON, 1986). Carob fruit from Greece (GAITIS *et al.*, 1994), Spain (CAJA *et al.*, 1987), Portugal (WURSCH, 1987), Cyprus (ORPHANOS and PAPACONSTANTINOU, 1969), California and Arizona (COIT, 1967; THOMSON, 1971) has been studied from different points of view (MARAKIS, 1996; GAITIS *et al.*, 1994). Many investigators have studied the carob trees, pods (beans) and their seeds (BRITO DE CARVALHO, 1987; CHARALAMBOUS and PAPACONSTANTINOU, 1966; THOMSON, 1971; MARAKIS, 1980; MARAKIS *et al.*, 1993 a, b) and recently, investigators isolated and identified the major polyphenols in carob fibers (OWEN *et al.*, 2003; PAPAGIANNOPOULOS *et al.*, 2004).

Carob trees abundant in Jordanian forests and have a vast economic potential (MARAKIS, 1996). The two main carob varieties in Jordan are *macrocarpa* which is edible and consumed by humans for its sweet taste and *siliqua* which is used for livestock feed. The *siliqua* variety usually has smaller pods and is not as sweet as the *macrocarpa* variety.

El-SHATNAWI and EREIFEJ (2001) investigated the chemical composition of livestock ingestion of carob seeds. They used pods and seeds as animal feed and studied the influence of scarification on the germination of carob seeds, chemical composition and the influence of ingestion on seed coat dormancy.

The aim of the present study is to re-

port the physical properties and chemical composition of the pods and seeds of Jordanian *siliqua* and *macrocarpa* carob varieties.

MATERIALS AND METHODS

Carob samples: One to two kg of mature pods were collected from *siliqua* and *macrocarpa* carob trees grown in Ajloun and Anjara in northern Jordan. A total of seventy trees (35 trees of each variety) were included in the study.

Physical measurements: Pod width (cm), thickness (cm), volume (mL), number of seeds per pod, pod weight (g), specific gravity (g/mL) and pod length (cm) were measured. The size index was calculated by dividing the length of the pod by its width. Specific gravity was calculated using the pod weight and volume. The pod was cut open and the number of seeds was recorded. The deseeded pods were then ground to a powder using a hammer mill, to pass through a 0.5 mm sieve. The pod powder was then saved for chemical analysis. The seeds from each variety were also crushed and saved for chemical analysis.

Chemical analyses: Moisture, protein (Nx6.25), fat, crude fiber and ash were determined in pods and seeds according to AOAC (1984) procedures. Carbohydrate was calculated by difference; glucose, fructose, and sucrose were analyzed according to the procedure of DUBOIS *et al.* (1956).

Mineral analyses: The concentrations of Mn, Fe, Cu, Zn, Na, K, Mg, and Ca were determined in pods and seeds with an atomic absorption spectrophotometer (Unicam, model SP9, UK) using the wet digestion method with a mixture of nitric, sulfuric and perchloric acids (10:1:4). The phosphorus content in pods and seeds was determined according to the method of WATANABE and OLSEN (1965). All chemical values are reported on a dry weight basis.

Statistical analysis: All measured traits were subjected to analysis of variance using the MSTATC program. Means were separated and compared using the least significant difference (LSD) values according to STEEL and TORRIE (1980).

RESULTS AND DISCUSSION

The physical properties of *siliqua* and *macrocarpa* carob variety pods are presented in Table 1. As expected the pods varied significantly in all the physical properties except specific gravity. The pods of the *macrocarpa* variety had higher pod width, volume and pod weight than the *siliqua* variety. These results agree very well with the description of the pods of ungrafted carob trees reported by MARAKIS (1996) in which the fruits were about 9 cm in length, thinner, shorter and woodier than grafted carob. The variation in the physical properties of the pods could be due to varietal and environmental factors (GAITIS *et al.*, 1994; MARAKIS *et al.*, 1987).

The pod weight values agree with those reported by MARAKIS (1996) and MITRAKOS (1987) for some Greek carob. In some varieties, the pod is 25 cm long, 4 cm wide and weighs 5-30 g. The

Table 1 - Mean values of the physical measurements of pods of the two carob varieties.

Trait	var. *siliqua*	var. *macrocarpa*
Pod width (cm)	2.00±0.04	2.50±0.04
Pod thickness (cm)	0.40±0.02	0.60±0.03
Pod volume (mL)	5.90±0.34	12.20±0.83
No. seeds/pod	6.60±0.33	5.50±0.31
Pod weight (g)	6.20±0.38	14.10±0.83
Specific gravity (g/mL)	1.10±0.04	1.20±0.09
Pod length (cm)	8.50±0.30	9.30±0.32
Size index*	4.30±0.17	3.70±0.11

Values are the average of two replications ± standard error of the mean.
* pod length by the width.

characteristics of the *macrocarpa* and *siliqua* carob varieties are reported for the first time and are in agreement with data reported for other varieties. The size index, which is the ratio between pod length and width, can be used to differentiate the pods of different carob varieties.

The chemical composition and sugar analysis data are given in Table 2. The pods of the *siliqua* variety had higher protein content than *macrocarpa*. These values are higher than the protein content reported for carob from Greece (MARAKIS *et al.*, 1987), Spain (SAURA-CALIXTO and CAÑELLAS 1982), Portugal (AMARAL-COLLACO *et al.*, 1987), Cyprus (ORPHANOS and PAPACONSTANTINOU, 1969) and California (COIT, 1967). These values agree very well with the values reported by AVALLONE *et al.* (1997).

Table 2 - Mean value and standard error of the chemical composition (%) of deseeded pods and seeds.

Traits	var. *siliqua*	var. *macrocarpa*
	Pods	
Protein	7.6±0.01	5.9±0.06 **
Fat	1.0±0.02	1.3±0.04 **
Fiber	10.8±0.04	7.6±0.05 **
Ash	3.4±0.02	2.7±0.04 **
Carbohydrates	77.3±0.05	82.4±0.15 **
Glucose	33.7±0.05	42.2±0.67 **
Fructose	29.6±0.20	18.9±0.22 **
Sucrose	36.6±0.32	38.9±0.15 **
	Seeds	
Protein	18.9±0.02	14.4±0.17 **
Fat	3.1±0.02	2.8±0.02 **
Fiber	11.3±0.03	11.8±0.07 **
Ash	0.4±0.07	0.4±0.06
Carbohydrates	66.3±0.08	76.6±0.19 **
Glucose	9.5±0.04	22.6±0.07 **
Fructose	8.1±0.03	15.0±0.07 **
Sucrose	10.3±0.03	24.0±0.11 **

* = Significant at P≤0.05. ** = Significant at P≤0.01. Values are the average of two replications and are calculated on weight basis. ± standard error of the mean.

The crude fat content in the *macrocarpa* variety was higher than that in the *siliqua* variety. These values agree very well with those reported by other investigators regarding European and American carob; SAURA-CALIXTO and CAÑELLAS (1982) reported a higher fat content (1.5-2.3%) in Spanish carob.

The fiber content of these varieties ranged from 7.6 (*macrocarpa*) to 10.8% (*siliqua*). These values are lower than the fiber content reported by MARAKIS (1996) and MARAKIS *et al.* (1987; 1993 a, b), who reported a range of 3.7-15.9% in Greek carob cultivars. Comparing the fiber content of the Jordanian carob varieties with values reported by MARAKIS (1996) in European and American carob, Jordanian *siliqua* and *macrocarpa* varieties have the lowest fiber content, whereas Portuguese carob (AMARAL-COLLACO *et al.*, 1987) has the highest fiber content (27.6-35.2%).

The ash content of the *macrocarpa* variety was slightly less than the *siliqua* variety. These values are in agreement with values reported by MARAKIS (1996) in pods from various countries.

Sugar contents varied significantly with the *macrocarpa* variety having higher sucrose and glucose, than *siliqua*. The variation in carob sugars has been reported by several investigators and shows significant variation (RHIZOPOULOU *et al.*, 1989). It is clearly indicated that carob pods contain sucrose, fructose and glucose regardless of the variety and the country of origin.

The results of the chemical analyses of the seed are also shown in Table 2. The protein, fat, fiber, carbohydrate, glucose, fructose and sucrose contents varied significantly. The *siliqua* variety had higher protein and fat values, whereas *macrocarpa* seeds had higher fiber, carbohydrate, glucose, fructose and sucrose values following the same trend as that for chemical composition of the pods.

The mineral content of the two carob

Table 3 - Mineral analysis of deseeded pods and seeds.

Element (mg/100 g)	Pod		Seed	
	var. *siliqua*	var. *macrocarpa*	var. *siliqua*	var. *macrocarpa*
Mn	0.99±0.01	1.23±0.03 **	2.63±0.10	2.50±0.05
Fe	4.55±0.08	24.26±0.37 **	26.70±1.01	11.96±0.20 **
Cu	0.99±0.01	1.28±0.03 **	1.88±0.04	2.44±0.06 **
Zn	2.75±0.06	2.08±0.05 **	3.34±0.07	5.44±0.08 **
Na	60.83±0.68	44.48±0.28 **	64.46±0.50	101.160±1.43 **
K	1463.57±18.15	1169.18±15.47 **	1199.64±17.80	1420.94 ±23.53 **
Mg	120.82±1.79	137.83±2.78 **	155.290±2.24	252.820±2.98 **
Ca	553.65±3.53	432.400±4.08 **	548.60±3.60	798.890±7.17 **
P	236.230±140.0	55.68±0.09 **	65.41±0.78	185.060±1.54 **

* = Significant at P≤0.05. ** = Significant at P≤0.01.
Values are the average of two replications and are calculated on dry weight basis.
± standard error of the mean.

Table 4 - Correlation coefficients between the physical characteristics of the pods.

	2	3	4	5	6	7	8
1 Pod width	0.33 **	0.99 **	0.11	0.71 **	0.15	0.34 **	- 0.34 **
2 Pod thickness		0.43 **	0.17 *	0.53 **	0.03	0.30 **	0.06
3 Pod volume			0.37 **	0.79 **	- 0.14	0.57 **	0.15
4 No. Seed/pod				0.47 **	0.06	0.82 **	0.71 **
5 Pod wt. (g)					0.13	0.74 **	0.21 *
6 Sp.gravity						0.12	0.03
7 Pod length (cm)							0.73 **
8 Size index							

* = Significant at P≤0.05
** = Significant at P≤0. 01

variety seeds varied significantly for all the elements except Mn (Table 3). *Siliqua* pods had higher Zn, Na, K, Ca, and P contents than *macrocarpa* pods, while *macrocarpa* seeds had higher Na, K, Mg, Ca, and P contents than *siliqua*. The mineral contents of both *siliqua* and *macrocarpa* are higher than values reported for carob pods from Cyprus (CHARALAMBOUS and PAPACONSTANTINOU, 1966) and Spain (PUCHADES *et al.*, 1987) which may be due to varietal and environmental effects.

Data on correlation coefficients are presented in Tables 4, 5 and 6. Table 4 shows the correlation coefficient between the physical characteristics of carob pods. Significant correlation values were found between pod weight and pod volume (r = 0.99), and pod length and number of seeds/ pod (r = 0.82). In addition, the correlation between size index and pod length (r = 0.73) is highly significant.

Table 5 shows the correlation coefficients between the chemical analysis of the carob pods and seeds. The correlations between ash and fiber con-

Table 5 - Correlation coefficients of the chemical composition (%) of the carob pods and seeds.

Traits	2	3	4	5	6	7	8
			Pods				
1 Ash	0.75 **	0.74 **	- 0.38 **	- 0.78 **	- 0.68 **	- 0.55 **	- 0.70 **
2 Fiber		0.91 **	- 0.55 **	- 0.95 **	- 0.92 **	- 0.92 **	- 0.93 **
3 Protein			- 0.49 **	- 0.90 **	- 0.88 **	0.74 **	- 0.87 **
4 Fat				0.47 **	0.55 **	0.45 **	0.58 **
5 Carbohydrate					0.88 **	0.69 **	0.88 **
6 Sucrose						0.77 **	0.91 **
7 Glucose							0.68 **
8 Fructose							
			Seeds				
1 Ash	- 0.14	- 0.01	- 0.03	- 0.78 **	- 0.02	0.00	- 0.01
2 Fat		0.63 **	- 0.43 **	0.47 **	- 0.67 **	- 0.67 **	- 0.67 **
3 Protein			- 0.05 **	- 0.90 **	- 0.91 **	- 0.91 **	- 0.91 **
4 Fiber				- 0.95 **	0.54 *	0.53 **	0.53 **
5 Carbohydrate					0.69 **	0.88 **	0.69 **
6 Glucose						0.99 **	0.99 **
7 Fructose							0.99 **
8 Sucrose							

* = Significant at P≤0.05
** = Significant at P≤0.01.

Table 6 - Correlation coefficients of the mineral content of the pods and seeds.

Element (mg/100 g)	2	3	4	5	6	7	8	9
				Pods				
1 Mn	0.48 **	0.33 **	- 0.40 **	- 0.46 **	- 0.35 **	0.26 **	- 0.48 **	- 0.09
2 Fe		0.57 **	- 0.52 **	- 0.86 **	- 0.71 **	0.43 **	- 0.88 **	- 0.11
3 Cu			- 0.24 **	- 0.48 **	- 0.28 **	0.33 **	- 0.51 **	- 0.05
4 Zn				0.51 **	0.52 **	- 0.10	0.53 **	0.16
5 Na						- 0.36 **	0.80 **	0.18
6 K						- 0.15	0.67 **	0.08
7 Mg								- 0.08
8 Ca								0.10
9 P								
				Seeds				
1 Mn	0.08	0.08	- 0.10	- 0.08	- 0.02	- 0.07	-0.08	- 0.09
2 Fe		- 0.36 **	- 0.63 **	- 0.69 **	- 0.43 **	- 0.68 **	- 0.71 **	- 0.76 **
3 Cu			0.47 **	0.52 **	0.26 **	0.52 **	0.52 **	0.55 **
4 Zn				0.81 **	0.51 **	0.83 **	0.80 **	0.86 **
5 Na					0.57 **	0.85 **	0.82 **	0.89 **
6 K						0.52 **	0.51 **	0.51 **
7 Mg							0.85 **	0.91 **
8 Ca								0.92 **
9 P								

* = Significant at P≤0.05
** = Significant at P≤0.01.

tent of the pod (r = 0.75), fiber and protein (r = 0.91), sucrose and carbohydrate (r = 0.88), fructose and sucrose (r = 0.91) and carbohydrate and fructose (r = 0.88) are positive as are the correlation coefficients for fructose and sucrose (r = 0.99), glucose and fructose (r = 0.99), sucrose and glucose (r = 0.99) and protein and fat (r = 0.63). These values could be due to genetic variation in the pods.

Table 6 shows the correlation coefficients between the mineral contents in the pods and seeds. The correlation coefficients between Ca and Na (r = 0.80), Ca and K (r = 0.67) and Mn and Fe pod content (r = 0.48) are highly significant. Seeds showed more significant correlation coefficients. The correlation coefficients between P and Ca contents (r = 0.92), P and Mg (r = 0.91), Ca and Zn (r = 0.80), and Mg and Na (r = 0.85) are highly significant.

The correlation coefficient might be a useful evaluation tool for plant breeders when breeding for a single trait.

CONCLUSIONS

The results of this study show that the pods of the *macrocarpa* variety are heavier, longer, and wider and contain more carbohydrates than pods of the *siliqua* variety. The results also show that fructose makes up the lowest percentage of carbohydrate in both varieties.

Mineral analysis of pods and seeds showed that both varieties contain appreciable amounts of nutritive elements including K and P in the pod and Mg and Ca in the seed.

ACKNOWLEDGMENTS

The authors would like to thank Dr. Rami Owies for his technical assistance. Support from the Deanship of Scientific Research is also acknowledged.

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Revised paper received July 23, 2004 Accepted October 29, 2004

PAPER

LEVELS OF PHENOLIC ANTIOXIDANTS IN VIRGIN OLIVE OIL PURCHASED IN BULK

LIVELLI DI FENOLI CON ATTIVITÀ ANTIOSSIDANTE NELL'OLIO D'OLIVA VERGINE ACQUISTATO ALL'INGROSSO

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ABSTRACT

The total polar phenol (TPP) and α-tocopherol (α-T) contents were determined in 120 virgin olive oil samples bought in bulk in 16 L containers, obtained directly from urban households throughout Greece. TPP content was determined colorimetrically and α-T content was determined using normal phase HPLC. Samples were categorized as edible (extra virgin and virgin) and non-edible. The levels of both classes of antioxidants were generally lower than those reported for high quality

RIASSUNTO

Nel presente lavoro è stato determinato il contenuto di fenoli polari totali (FPT) e di α-tocoferolo (α-T) in 120 campioni di olio d'oliva vergine prelevati direttamente da famiglie che vivono in città di tutta la Grecia e che acquistano grandi quantitativi di olio d'oliva, generalmente in contenitori da 16 L. I FPT sono stati dosati per via colorimetrica, mentre per l'α-T si è ricorso all'analisi cromatografica con HPLC in fase normale. I campioni sono stati classificati come ido-

- Keywords: α-tocopherol, bulk virgin olive oil, nutritional value, total polar phenols, traditional consumers -

Greek virgin olive oil. Sixty-five percent of the extra virgin olive oil samples contained 100-150 mg/kg of TPP expressed as caffeic acid, while ~80% of the virgin olive oil samples contained < 100 mg/kg. α-tocopherol levels were higher than 200 mg/kg for 1/3 of the extra virgin and 1/4 of the virgin samples, respectively. Non-edible samples had even lower levels of the antioxidants. These findings illustrate the need to educate olive oil packers, distributors and consumers as to how the nutritional value of virgin olive oil can be maintained from field to table. The recent EC regulation that has set the maximum packaging capacity for the final consumer at 5 L is expected to help maintain the nutritional value of virgin olive oil.

nei al consumo alimentare (oli vergini d'oliva ed extra-vergini) e non idonei. I livelli trovati, per entrambe le classi di antiossidanti, sono risultati generalmente più bassi di quelli riscontrati per il VOO greco di alta qualità. Il 65% dei campioni di olio extra vergine d'oliva conteneva 100-150 mg/kg di FPT, espresso come acido caffeico, mentre circa l'80% dei campioni di olio vergine ne conteneva meno di 100 mg/kg. I livelli di α-T sono risultati maggiori di 200 mg/kg rispettivamente per un terzo dei campioni di olio extra vergine e per un quarto degli oli vergini. I campioni di olio non idonei al consumo umano hanno presentano inoltre un quantitativo di antiossidanti minore. I risultati hanno messo in evidenza la necessità di istruire i confezionatori, commercianti e consumatori di olio su come preservare il valore nutrizionale dell'olio vergine d'oliva dal campo alla tavola. Il recente regolamento comunitario, stabilendo che la capacità massima delle confezioni di olio d'oliva vergine vendibili al consumatore deve essere di 5 L, può in questo senso migliorare le attuali pratiche produttive.

INTRODUCTION

As stated in the introduction of the Commission Regulation N. 1019/2002 (EC, 2002) "*Olive oil has certain properties, in particular organoleptic and nutritional properties, which, taking into account its production costs, allow it access to a relatively high-price market compared with most other vegetable fats*". However, virgin olive oil (VOO) consumers, even traditional ones, such as Greeks, are familiar with the organoleptic characteristics of the product, but have not been properly educated with regard to the nutritional and functional properties of this unique fruit oil.

The nutritional value of VOO is partially due to the presence of nutrient and non-nutrient phenolic antioxidants. Among the latter, the most important are polar phenols, especially oleuropein derivatives and hydroxytyrosol. The biological activity of these phenols, tested *in vitro* and *in vivo*, is related to their antioxidant, antiplatelet and anti-inflammatory effects (MANNA *et al.*, 1997; MARTÍNEZ-DOMÍNGUEZ *et al.*, 2001; VISIOLI *et al.*, 2002). On the other hand, the main nutrient antioxidant present in

olive oil is α-tocopherol, which is known to possess the highest vitamin E activity among all tocopherol homologues (EITENMILLER and LANDEN, 1999). Considering that the daily requirement for α-tocopherol depends on the daily unsaturated fatty acid intake (BELITZ and GROSCH, 1999), high quality virgin olive oil consumption may cover ~50% of the daily requirements of traditional consumers (PSOMIADOU *et al.*, 2000). While both categories of phenolic antioxidants contribute to the stability of the oil in the dark, tocopherols seem to play a very important role when the oil is exposed to light (PSOMIADOU and TSIMIDOU, 2002a, b) and polar phenols contribute to the bitter taste of the oil (MORALES and TSIMIDOU, 2000).

The *per capita* annual consumption of olive oil in Greece is ~18 kg (FAO, 2004). Approximately 40% of the oil consumed is bought in bulk (TSIMIS and KARAKASIDES, 2002), either directly from the producers, or through distributors, who are not trained in how to maintain the high nutritional level of olive oil throughout the sales period. At various establishments such as oil mills, cellars, warehouses, etc., large quantities of olive oil are kept in various types of containers, usually in 16 L ones, that are not always suitable for maintaining oil quality. These practices have a great impact on the nutritional value of the oil bought by the consumer.

The aim of this study was to evaluate the nutritional value of VOO consumed in Greek households. The levels of non-nutrient and nutrient phenolic antioxidants were evaluated in olive oil samples obtained directly from urban households throughout Greece that had been bought in bulk. The samples (n=120), obtained from sealed containers, were examined for their total polar phenols (TPP) and α-tocopherol (α-T) contents. The analytical data are discussed in relationship to information obtained from questionnaires filled in by the consumers during sampling.

MATERIALS AND METHODS

Samples

Olive oil samples (n=120) that had been purchased in bulk were collected from households throughout Greece according to a certain protocol, during a campaign sponsored by SEVITEL (Greek Association of Industries and Processors of Olive Oil, Athens, Greece) within the framework of a European project to promote olive oil consumption. Sampling was from sealed, 16 L, tinplate containers. Samples were collected from urban areas [Athens, code no. 001-090; Thessaloniki, 101-130; Larissa, 131-140; Komotini, 141-150 and Alexandroupolis, 151-160]. Consumers were asked to fill in a questionnaire during sampling. On the basis of EEC Regulation N. 1989/2003 (EC, 2003), samples were grouped as extra virgin, virgin, or non-edible olive oils, by the Food Industrial Research and Technological Development Company (ETAT) S.A. (Athens, Greece).

Reagents and standards

All chemicals were of the appropriate purity from various suppliers. HPLC grade solvents were used without further purification. *n*-Hexane 95% (UV, IR, HPLC), 2-propanol (UV-IR-HPLC-HPLC preparative) and methanol (UV-IR-HPLC-HPLC preparative) were purchased from Panreac Quimica SA (Barcelona, Spain). dl-α-tocopherol (≥ 98% for HPLC) was from Fluka Chemie GmbH (Buchs, Switzerland) and caffeic acid from Riedel-de Haën (Seelze, Germany). Folin-Ciocalteau reagent was from PANREAC QUIMICA SA and sodium carbonate from Riedel-de Haën.

Apparatus

The solvent delivery system consist-

ed of two Marathon IV series HPLC pumps (Rigas laboratories, Thessaloniki, Greece) and a Rheodyne injection valve (model 7125) with a 20 µL fixed loop (Rheodyne, Cotati, CA). The liquid chromatograph was equipped with an SSI 502 programmable fluorescence detector (Scientific Systems Inc., State College, PA), set at 294 nm (excitation) and 330 nm (emission). The data were stored and processed with the chromatographic software EZChrom (Scientific Software, Inc., San Ramon, CA). Absorbance was measured with a Hitachi U-2000 spectrophotometer (Hitachi Ltd., Tokyo, Japan) in 1 cm quartz cells.

Colorimetric determination of total polar phenol content

Olive oil (2.5 g) dissolved in 5 mL of hexane was extracted with 5 mL of a mixture of MeOH/H_2O (60:40, v/v). The mixture was shaken vigorously by means of a mechanical shaker (Vortex) and centrifuged at 3500 rpm for 10 min. An aliquot of the polar fraction (0.4 mL) was transferred to a 10 mL volumetric flask and, subsequently, water (4.8 mL) and the Folin-Ciocalteau reagent (0.5 mL) were added. Three minutes after the addition of the reagent, 1 mL of a sodium carbonate (Na_2CO_3) saturated solution was added to the reaction mixture. The solution was diluted with water to 10 mL and after one hour the absorbance at 725 nm was measured against a blank solution. Calibration curves were constructed using standard solutions of caffeic acid within the range 0.2-3.2 mg/L. Extraction repeatability was checked for eight replicates (CV% = 6.2%). Then, one extract was prepared for each sample. Two measurements were obtained for each of these extracts. The measurement repeatability ranged from 1.4 to 5.8% for the caffeic acid solutions (12-32 µg/10 mL).

HPLC determination of α-tocopherol

The elution system was a mixture of *n*-hexane/2-propanol (98:2, v/v). Separation was achieved on a LiChrospher 100 Si 5 µm column (4.0 mm i.d. x 250 mm) (Hewlett Packard) at a flow rate of 1.0 mL/min. The injection volume was 10 µL. α-Tocopherol standards and samples (4% w/v) were prepared in *n*-hexane. Oil sample solutions were filtered through a 0.45 µm membrane filter (Schleicher & Schuell, Dassel, Germany) just before HPLC analysis. Tocopherols were detected using a fluorescence detector, set at $\lambda_{excitation}$ = 294 nm and $\lambda_{emission}$ = 330 nm. The calibration curve was constructed using standard solutions of α-tocopherol within the range 2.5-50 mg/L. The detection limit (mean sample blank +3s) was 4.8 ng, (n=10). The coefficient of variation (CV %) of the injection volume on the same day was 4.4% ($\underline{n}$=5). Each sample was analyzed once.

RESULTS AND DISCUSSION

Only one third of the 120 samples were found to belong to the two edible commercial categories permitted in the EU. Eighty-one (81) of the samples were categorized as non-edible, because the value of at least one analytical parameter related to quality deviated from that required for extra virgin or virgin olive oil. Polar phenol and tocopherol contents are not included among the quality parameters listed in the international legislation. Therefore, information about the actual levels of these compounds in commercial samples (bottled or bulk) is rather limited (e.g. PSOMIADOU *et al.*, 2000; GARCÍA *et al.*, 2003). The present study focuses on oils purchased in bulk by consumers who live in selected urban areas of Greece. These consumers use olive oil almost exclusively for culinary purposes and usually buy large quantities directly from pro-

ducers or through distributors. Based on the answers given in the questionnaire, all of the consumers claimed that the oil they consumed was "virgin olive oil of the highest quality".

Edible VOO samples

Tables 1 and 2 present the total polar phenol and α-tocopherol contents of extra virgin and virgin olive oil samples, respectively.

Total polar phenol content: The total polar phenol content varied from 65 to 218 mg/kg in the extra virgin olive oil samples, (mean value 117 mg/kg, n=17). Frequency distribution analysis showed that 65% of the samples contained 100-150 mg/kg, whereas 29% of the samples had less than 100 mg/kg. These results are in contrast with our previous findings for extra virgin olive oils according to which 39% of the samples directly obtained from olive mills had >150 mg/kg TPP content (BLEKAS *et al.*, 2002). As shown in Table 2 the TPP content of virgin olive oil samples was generally lower and ranged from 22 to 187 mg/kg (mean value 82 mg/kg, n=22). Frequency distribution analysis revealed that the majority of the samples (82%) contained < 100 mg caffeic acid/kg oil.

High TPP levels were recently reported for some commercially bottled Spanish VOO (~400 mg/kg) (GARCÍA *et al.*, 2003). The high levels can be attributed to the analytical method used (HPLC instead of colorimetric determination). Considering that the TPP content of Greek extra virgin olive oil rarely exceeds 250 mg/kg as caffeic acid, it is obvious that the Greek olive oil sector has to do more to preserve satisfactory TPP levels in commercial samples. Since it is not clear whether the Greek cultivars yield low or high levels of phenolic compounds, the processing, packing and distribution sectors have to take every precaution to maintain the highest TPP levels possible.

α-Tocopherol content: The overall range of α-tocopherol content recorded for the extra virgin olive oil samples was 116-283 mg/kg (mean value 188 mg/kg, n=17) (Table 1). Frequency distribution analysis showed that 47% of the samples contained 150-200 mg/kg and 35% of the samples had more than 200 mg/kg. These values are lower than those reported by PSOMIADOU *et al.* (2000) for extra virgin olive oil

Table 1 - Total Polar Phenol (TPP) and α-tocopherol (α-T) content of extra virgin olive oil samples.

Sample Code	TPP [a] (mg/kg)	α-T (mg/kg)	Sample code	TPP [a] (mg/kg)	α-T (mg/kg)
006	85.9	283	*121*	217.5	147
026	80.1	250	*123*	134.3	179
033	92.9	212	*124*	106.7	186
075	135.1	173	*135*	127.7	170
076	111.2	214	*144*	138.8	153
089	102.5	155	*147*	107.6	158
090	128.3	263	*156*	137.2	147
103	64.5	235	*159*	95.4	116
110	118.2	155			
Range	64.5-217.5	116-283	*Average*	116.7	188

[a] TPP content is expressed as mg caffeic acid /kg oil.

Table 2 - Total Polar Phenol (TPP) and α-tocopherol (α-T) content of virgin olive oil samples.

Sample Code	TPP [a] (mg/kg)	α-T (mg/kg)	Sample code	TPP [a] (mg/kg)	α-T (mg/kg)
003	29.8	167	*118*	71.6	211
018	58.0	163	*122*	61.6	230
034	98.8	179	*127*	55.3	196
037	154.0	158	*129*	53.6	170
044	57.7	145	*137*	98.8	305
050	94.1	194	*138*	85.1	186
051	46.6	237	*146*	187.3	153
057	63.4	195	*148*	150.8	213
077	67.9	156	*149*	22.3	96
082	69.6	191	*151*	57.4	74
088	70.5	195	*153*	140.5	58
Range	22.3-187.3	58-305	*Average*	81.6	176

[a] TPP content is expressed as mg caffeic acid /kg oil.

samples obtained from the production line. In that study, more than 60% of the samples contained >200 mg/kg of α-tocopherol. A wider range in α-T content was noted for the virgin olive oil samples (58-305 mg/kg, mean value 176 mg/kg, *n*=22) (Table 2). The majority of the samples, 59%, contained 150-200 mg/kg of α-T, while 23% of the samples had > 200 mg/kg.

Greek virgin olive oils have been reported to contain higher levels of α-tocopherol than Italian or Spanish ones [PSOMIADOU *et al.*, 2000 (98-370 mg/kg, *n*=90); LO CURTO *et al.*, 2001 (36-314 mg/kg, *n*=183); SALVADOR *et al.*, 2001 (55-315 mg/kg, *n*=152)]. The lower values observed in the present study could be attributed to inappropriate conditions along the olive oil distribution chain and/or during domestic storage (e.g. urban apartments without cellars) (PSOMIADOU *et al.* 2000; PSOMIADOU and TSIMIDOU, 1998, 2002a).

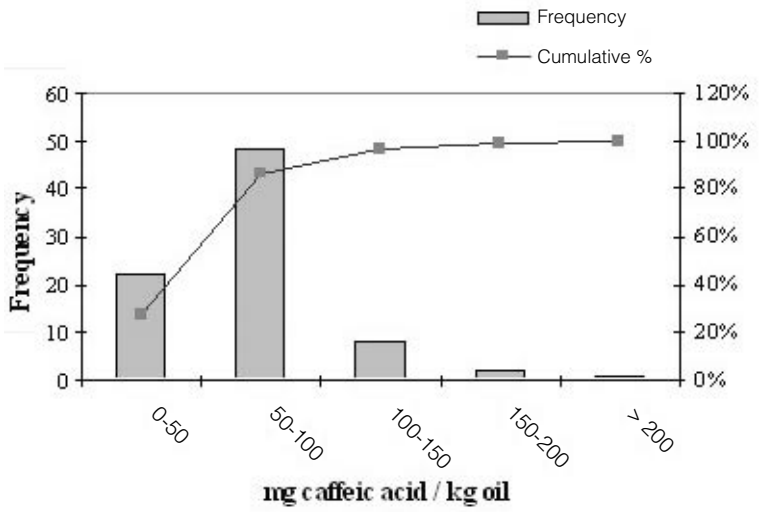
Non-edible VOO samples

Table 3 shows the ranges of acidity, peroxide value, K_{232} and K_{270} for the non-edible samples (*n*=81). The TPP and α-T contents of the samples are given by means of frequency distribution analysis (Fig. 1). The distribution pattern of TPP content is similar to that found for the virgin oil samples (86% contained <100 mg/kg) but the α-T levels were lower than in either edible category (68% contained <150 mg/kg of α-T). Thus, the samples that were characterized as non-edible in terms of current legislation, were also inferior from the nutritional point of view.

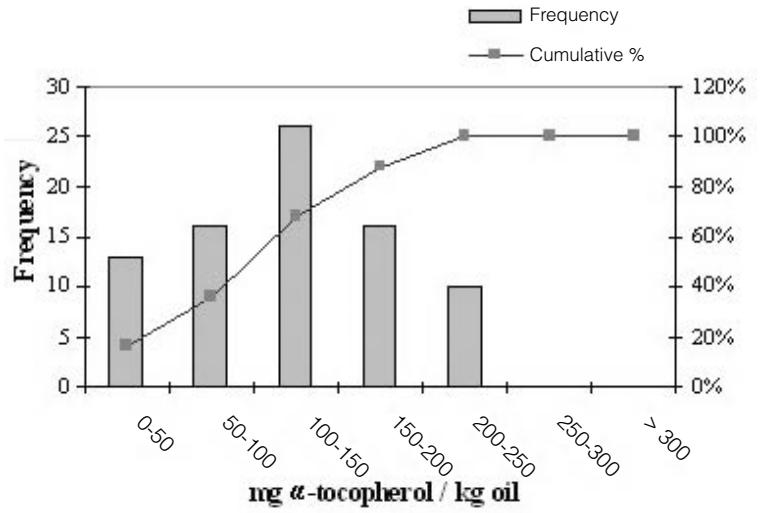
Table 3 - Ranges of acidity, peroxide value, K_{232} and K_{270} for virgin olive oil samples that exceeded legal limits for one or more of the quality parameters set by EEC Regulation N. 1989/2003.

Quality parameter	*Range*
Acidity (% as oleic acid)	0.2-4.6
Peroxide value (meq O_2/kg of oil)	19-65
K_{232}	1.72-7.46
K_{270}	0.13-0.54

Since polar phenols and α-tocopherol are easily oxidized, large container packaging (e.g. 16 L, tinplate contain-



a)



b)

Fig. 1 - Frequency histograms of (a) total polar phenol and (b) α-tocopherol content of virgin olive oil samples that exceeded legal limits for one or more of the quality parameters set by the EEC Regulation N. 1989/2003.

ers) does not help maintain VOO nutritional properties. EEC Regulation N. 1019/2002 (EC, 2002), which states that the maximum capacity of packaging to final consumers is 5 L, is expected to help improve current practices. In addition producers, packers, retailers and consumers should be properly informed and educated about the nutritional properties of the oil and ways to maintain product quality through proper handling and storage.

ACKNOWLEDGEMENTS

The work was partially funded by SEVITEL (Athens, Greece) within the framework of the project: "Virgin olive oil. Study of the levels of minor constituents with nutritional value in virgin olive oils consumed in Greek households".

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Paper received October 5, 204 Accepted December 17, 2004

PAPER

AN ELECTRONIC NOSE TO EVALUATE OLIVE OIL OXIDATION DURING STORAGE

DETERMINAZIONE MEDIANTE NASO ELETTRONICO DELLO STATO D'OSSIDAZIONE DEGLI OLI DA OLIVA DURANTE LA CONSERVAZIONE

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ABSTRACT

An electronic nose based on an array of 12 Metal Oxide Semiconductor (MOS) sensors and 10 Metal Oxide Semiconductor Field–Effect Transistor (MOSFET) sensors was investigated to determine if it could be used to monitor olive oil oxidation during storage. Samples of extra virgin olive oil, olive oil and olive-pomace oil were stored at 40°C in the dark for 64 days. At established times, the three types of olive oil were evaluated for volatile compounds by the electronic nose and for peroxide value. The

RIASSUNTO

Nel presente lavoro è stata valutata la capacità di un naso elettronico, composto da un array di 12 sensori tipo MOS (Metal Oxide Semiconductor) e di 10 sensori tipo MOSFET (Metal Oxide Semiconductor Field–Effect Transistor), di monitorare l'ossidazione degli oli da oliva durante la conservazione. Un olio extravergine d'oliva, un olio d'oliva ed un olio di sansa d'oliva sono stati conservati per 64 giorni a 40°C al buio. A tempi prestabiliti, per ciascun olio è stato determinato il numero di perossidi e va-

- Key words: electronic nose, oil oxidation, olive oils, peroxide value, storage time -

electronic nose data and the peroxide values were correlated and, on the basis of the correlation coefficients, a small number of sensors were selected. The responses of the selected sensors were analysed by Principal Component Analysis. The first principal component was able to discriminate olive oil samples on the basis of aging time. These results suggest that the electronic nose may be suitable for measuring changes in volatile compounds associated with olive oil oxidation during storage.

lutata la componente volatile mediante naso elettronico. Lo studio delle correlazioni tra i dati derivanti dal naso elettronico e dal valore dei perossidi, ci hanno permesso di selezionare solo i sensori più rappresentativi. Le risposte derivanti dai sensori selezionati sono state analizzate mediante Principal Component Analysis. La prima componente principale è risultata importante nel discriminare i campioni di oli da oliva sulla base del tempo di conservazione. Questi risultati dimostrano la capacità del naso elettronico di registrare i cambiamenti della componente volatile degli oli da oliva associati con i fenomeni ossidativi che insorgono durante la conservazione.

INTRODUCTION

Oxidation, one of the most critical factors affecting the shelf-life of olive oil, produces some unstable compounds (hydroperoxides) and volatile compounds (mainly aldehydes and ketones) that are responsible for modifying the sensory and nutritional characteristics of the oil (KIRITSAKIS, 1998; NAWAR, 1985). Current practices for evaluating olive oil quality during storage are based on sensory analysis and chemical indices such as acidity and peroxide value (BONASTRE *et al.*, 2004). While sensory analysis is complex, time-consuming and expensive, chemical indices are destructive, manual and unsuitable for routine analysis. Therefore, there is a need to develop objective, rapid and non-destructive analytical methods to discriminate "fresh" and "oxidized" oils.

In recent years there has been considerable effort devoted to the development of the so-called "electronic nose", an instrument made up of an array of sensors capable of recognizing simple and complex odours. They are an array of slightly specific to broad spectrum chemical sensors that are intended to mimic the human olfactory system and convert sensor signals into data that can be analysed with an appropriate pattern recognition system (PERSAUD and DODD, 1982). Such characteristics greatly facilitate the application of the electronic nose to rapidly monitor volatile components of food, providing real-time information about various characteristics of the food under study (SCHALLER *et al.*, 1998; BARTLETT *et al.*, 1997).

Several examples have been reported on the use of an electronic nose to characterize vegetable oils (GONZALEZ MARTIN *et al.*, 2001; GUADARRAMA *et al.*, 2001) and for the quality control of olive oil aroma (GUADARRAMA *et al.*, 2000; CERRATO OLIVEROS *et al.*, 2002). This device has also been used to determine frying oil quality (INNAWONG *et al.*, 2004) and to investigate the quality of heated cooking oil (ALI *et al.*, 2001). Information about the use of an electronic nose to predict the shelf-life of vegetable oils or to monitor oil oxidation is very limited (SHEN *et al.*, 2001).

The aim of the present study was to investigate the use of an electronic nose to monitor olive oil oxidation during storage. For this purpose, a commercial electronic nose was used to evaluate the head-space of extra virgin olive oil, olive oil and olive-pomace oil during storage at 40°C.

MATERIALS AND METHODS

Samples

Extra virgin olive oil, olive oil and olive-pomace oil were purchased from a local grocery store. Extra virgin olive oil is produced only with pressed olives; olive oil is a mixture of refined olive oil and virgin olive oil; olive-pomace oil is a mixture of refined oil extracted from olive pomace (flesh and pits) and virgin olive oil.

The three oil categories were divided into 20 aliquots and stored in 20 mL vials at 40°C in the dark for 64 days. Every three or four days a vial of each oil was removed for chemical and electronic nose analyses.

Electronic nose

Apparatus

Analyses were performed with a commercial electronic nose (model 3320 Applied Sensor Lab Emission Analyser; Applied Sensor Co., Linkoping, Sweden), which consisted of three parts: an automatic sampling apparatus, a detector unit containing the array of sensors, and pattern recognition software. The automatic sampling system had a carousel of 12 sites for loading the samples that were kept under controlled temperature.

The sensor array contained 23 different sensors. Ten sensors were Metal Oxide Semiconductor Field Effect Transistors (MOSFET) and twelve were Taguchi type sensors (Metal Oxide Semiconductors - MOS). The MOSFET sensors were divided into two arrays of five sensors each, one array operating at 140°C and the other at 170°C, while the MOS, mounted in a separate chamber, were kept at 400°-500°C during all the process phases.

Operating procedure

Two grams of each sample were placed in 40 mL Pirex® vials having a pierceable Silicon/Teflon disk in the cap. The sample was incubated at 40°C for 10 min before injection. The oil headspace, sampled with an automatic syringe, was then pumped over the sensor surfaces for 60 s during which the sensor signals were recorded. The sensors were then exposed to filtered air at a constant flow rate (60 mL/min) in order to obtain the baseline. Each sample was evaluated 3 times and the average of the results was used for statistical analysis.

Determination of the peroxide value

The peroxide value, a measure of the amount (meq. O_2/kg) of hydroperoxides formed through oxidation during storage, was determined according to Regulation 2568/91, Annex III of the official method (EEC, 1991).

Data processing

The correlation analysis and the Principal Component Analysis (PCA) were performed using SCAN software (Minitab Inc, PA, USA). PCA is a procedure that permits useful information about the data structure, the relationship between objects and the global correlation of the variables to be extracted from the data (BEEBE *et al.*, 1998).

RESULTS AND DISCUSSION

Determination of experimental conditions

To determine the best operating conditions for the electronic nose, the sam-

ple composition, injection time and incubation temperature and time were studied and optimised in order to obtain signals of suitable intensity and good reproducibility.

The sample incubation temperature and time are particularly important since they guarantee a constant headspace generation. The electronic nose used allowed the samples to be kept under controlled temperature before injection. The temperature was increased from 30° to 45°C, in 5°C increments, and a small increase in the signal intensity occurred. A temperature of 40±0.5°C for 10 min was chosen because under these conditions signals were sufficiently intense and a constant headspace was generated.

The injection time, the time during which the signals of the sensors are recorded, must be long enough to record the signals at their maximum value. This time was varied between 30 and 150 s with 30 s increments. An injection time of 60 s was sufficient for recording the useful zone of the signal.

The flow rate, the variable that influences the signals at most because it affects the speed with which the volatiles pass over the surface of the sensors, was varied between 30 to 100 mL/min at increments of 10 mL/min. A flow rate of 60 mL/min was chosen because at low flow rates the signal intensity was low, while at high flow rates the reproducibility of the measurements worsened.

Evaluation of olive oils during storage

Once the experimental conditions of the electronic nose were optimised, peroxide values (PV) of the three types of olive oils were analysed during storage at 40°C. The PV is routinely used to determine lipid oxidation, especially at the beginning of storage. It increases as lipid oxidation progresses and, then reaches a plateau after which it decreases. Fig. 1 shows the PV evolution of extra virgin olive oil, olive oil and olive-pomace oil during storage. The PV maximum according to regulation n. 2568/91 Annex I (EEC, 1991) is 20 meq O_2/kg for extra virgin olive oil and 15 meq O_2/kg for olive and olive-pomace oil. After 64 days of storage only the extra virgin olive oil exceeded the maximum value, while the olive oil and olive-pomace oil were below the legal limit. The PV of extra virgin olive oil

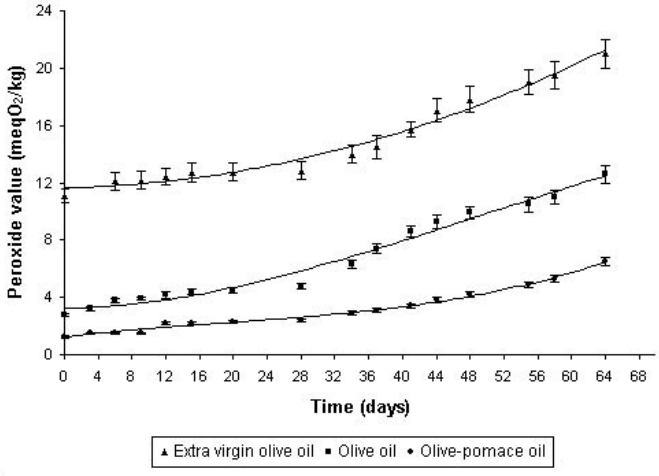


Fig. 1 - Evolution of the peroxide value of olive oils during storage at 40°C in the dark.

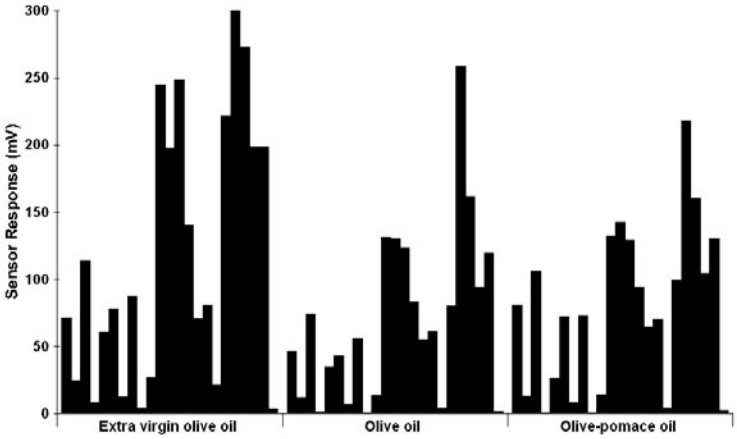


Fig. 2 - Typical sensor responses of olive oil samples at time 0.

increased after 34-37 days of storage, that of olive oil increased after 28 days of storage, while the PV of olive-pomace oil increased very little beginning after 34-37 days of storage.

The electronic nose was used to evaluate the oil headspace. Typical signal patterns of the three types of olive oils at time 0, are shown in Fig. 2. The bars within a sample (e.g. extra virgin olive oil) represent the signals of the 22 different sensors. The differences in the signal patterns are clearly visible. These differences could be explained by the kinds of oils used: extra virgin olive oil has more volatiles because it has not been refined; olive oil and olive-pomace oil are a mixture of refined oil and virgin olive oil so they have a lower content of volatiles.

The sensor responses were collected and elaborated along with the peroxide values. The relationship between the electronic nose and the PV was evaluated by taking into account the correlation coefficients calculated using the whole data set. The correlation coefficients between each sensor of the electronic nose and the peroxide value are listed in Table 1. For each type of olive oil, the highest absolute values are reported in bold. On the basis of the correlation coefficients, a small subset of electronic nose variables were selected in order to reduce the number of data used in the subsequent multivariate statistical analysis.

Three sensors, MOSFET 104A, MOSFET 104B and MOS 113, were selected for the extra virgin olive oil, while for the olive oil and the olive-pomace oil, MOSFET 104A, MOS 114 and MOS 116 were selected. The sensor responses were analysed using PCA. The score plot of extra virgin olive oil (Fig. 3) clearly separates the samples into two groups in the space defined by the first two principal component (97.7% of the total variability). The first group in the positive part of the first principal component is made up of samples stored for less than 37 days. None of these samples were oxidised (see the peroxide values in Fig. 1) and can be considered "fresh"; the second group in the negative part of the first principal component is made up of samples "in oxidation". The sample corresponding to 37 days of storage, located in the centre of the plot between the two groups, indicates the beginning of aging. As shown in Fig. 4, similar considerations can be made for the olive oil samples. The score plot in the space defined by the first two

Table 1 - Correlation coefficient matrix of the sensor responses and peroxide values.

		Peroxide value		
		Extra virgin olive oil	Olive oil	Olive-pomace oil
Sensors	FE101A	-0.147	-0.473	-0.457
	FE102A	0.445	0.858	0.451
	FE103A	-0.190	0.561	-0.302
	FE104A	**0.904**	**0.884**	**0.798**
	FE105A	0.373	0.769	-0.377
	FE101B	-0.208	-0.490	-0.530
	FE102B	-0.074	0.858	-0.692
	FE103B	-0.153	0.810	0.174
	FE104B	**0.769**	0.834	0.746
	FE105B	0.588	0.833	0.104
	MOS101	0.198	0.841	0.650
	MOS102	0.679	0.826	0.539
	MOS104	-0.052	0.822	0.711
	MOS110	0.419	0.821	-0.073
	MOS111	0.216	-0.306	-0.480
	MOS112	0.344	-0.160	-0.456
	MOS113	**-0.881**	0.702	0.705
	MOS114	-0.555	**0.871**	**0.760**
	MOS115	-0.099	0.857	0.690
	MOS116	0.496	**0.859**	**0.833**
	MOS117	0.106	0.821	0.234
	MOS118	0.672	0.852	0.271

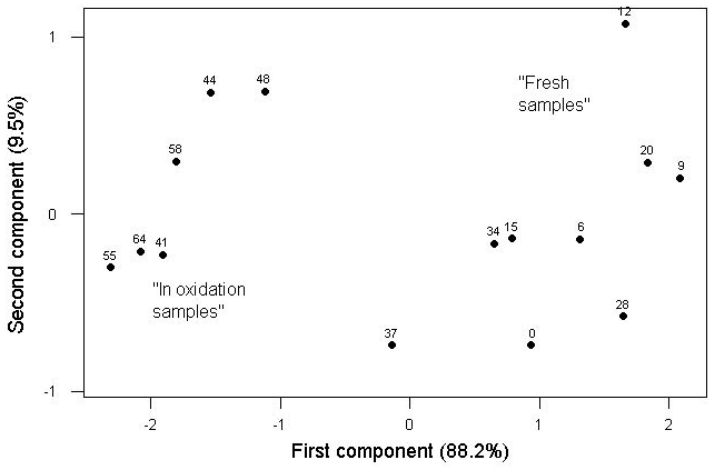


Fig. 3 - Score plot of the first two principal components for extra virgin olive oil at different storage times. Numbers close to the symbols refer to storage time.

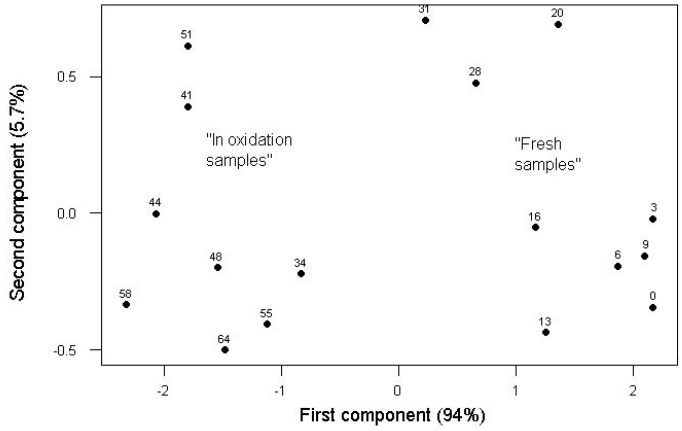


Fig. 4 - Score plot of the first two principal components for olive oil at different storage times. Numbers close to the symbols refer to storage time.

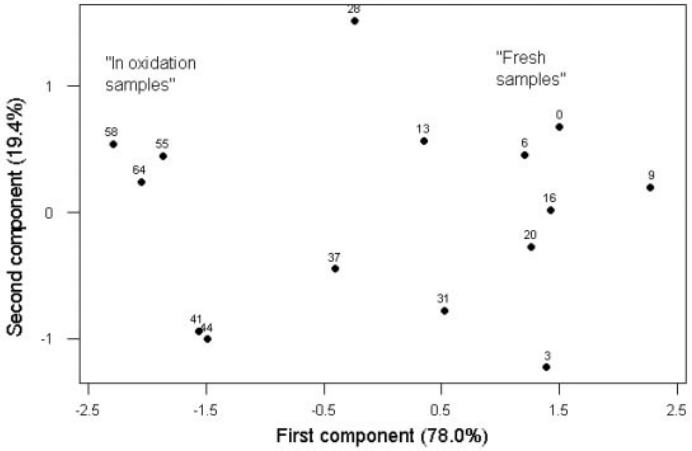


Fig. 5 - Score plot of the first two principal components for olive-pomace oil at different storage times. Numbers close to the symbols refer to storage time.

principal components (99.7% of the total variability) shows a clear separation between "fresh" samples, stored for less than 31 days, and "in oxidation" samples grouped in the negative part of the first principal component.

The groups of olive-pomace oil samples (Fig. 5) are probably less defined because the increase in the PV was very little and the oil oxidation was at an early stage. However, in this case, too, the samples are distributed along the first principal component, from positive to negative, according to storage time.

CONCLUSION

The results clearly show that a small number of selected sensors from the electronic nose can be used to monitor olive oil oxidation during storage and that the first principal component is important for discriminating "fresh"

and "in oxidation" olive oil samples. The proposed method is simple and rapid in comparison with traditional analytical techniques and is non-destructive. Work is in progress to test this new approach on a larger number of samples and to determine if the electronic nose can be used to identify olive oil samples of unknown storage times.

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Paper received June 25, 2004 Accepted November 22, 2004

SHORT COMMUNICATION

BIOGENIC AMINE CONCENTRATION IN "LARDELLATO" SALAMI, A TRADITIONAL PRODUCT OF CENTRAL ITALY, DURING RIPENING

CONTENUTO DI AMINE BIOGENE NEL CORSO DELLA STAGIONATURA NEL SALAME LARDELLATO, PRODOTTO TRADIZIONALE DELL'ITALIA CENTRALE

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ABSTRACT

The presence of biogenic amines (BAs) in food is widely reported, especially in fermented products. The evolution of some physical and chemical features was studied throughout the manufacturing process, in particular the concentration of some BAs (β-phenylethylamine, putrescine, cadaverine, histamine, tyramine, spermidine and spermine) of a tradition-

RIASSUNTO

La presenza di amine biogene (AB) negli alimenti è ampiamente documentata, in particolare nei prodotti sottoposti a processi fermentativi. Nel presente lavoro è stata studiata l'evoluzione nel corso del processo di produzione di alcune caratteristiche fisiche e chimiche di un prodotto carneo insaccato a lunga stagionatura inserito nell'elenco dei prodotti tradizionali della regione Mar-

- Key words: biogenic amines, fermented sausages, "lardellato" salami, RP-HPLC, traditional products -

al Italian long ripened fermented sausage, the "lardellato" salami, produced in the Marche region of central Italy, was studied. The BA levels ranged from 56.040 mg/kg in the initial mixture to 919.255 mg/kg after 90 days of ripening. Low levels of histamine (the highest value was 41.450 mg/kg in samples at 120 days) were observed, as well as an expected progressive increase in total amines during ripening, which also went beyond the usual ageing time of 90 days.

che, il salame lardellato, con particolare riferimento alla concentrazione di alcune AB (β-feniletilamina, putrescina, cadaverina, istamina, tiramina, spermidina e spermina). Sono stati riscontrati livelli di amine compresi fra 56,040 mg/kg nell'impasto iniziale e 919,255 mg/kg nel prodotto dopo 90 giorni di stagionatura. Nei campioni analizzati sono state rilevate basse concentrazioni di istamina (il valore medio più elevato è stato di 41,450 mg/kg nei campioni a 120 giorni), così come un aumento progressivo, peraltro atteso, del contenuto totale di AB nel corso della maturazione, che è stata protratta anche oltre 90 giorni, normale tempo di commercializzazione del prodotto.

INTRODUCTION

Biogenic amines (BAs) are formed by the enzymatic decarboxylation of specific amino acids of bacterial and/or tissue origin and are present in different concentrations in food depending on their type and source. The most important BAs occurring in food are histamine, putrescine, cadaverine, tyramine, tryptamine, β-phenylethylamine, spermidine and spermine, which originate from histidine, ornithine or arginine (via agmatine), lysine, tyrosine, tryptophane, phenylalanine, arginine (via putrescine) and spermidine, respectively. They may have toxic effects and cause disease in humans and animals (STRATTON *et al.*, 1991; TREVIÑO *et al.*, 1997; VINCI and ANTONELLI, 2002), but being natural components of many foods, they generally pose no health risk to consumers if present at relatively low concentrations.

Histamine and tyramine are the most dangerous BAs for human health; histamine is more abundant but less toxic than tyramine. Other amines like phenylethylamine and tryptamine are not always detected in food and have an action similar to tyramine. Furthermore, they may interact with the detoxification mechanisms of the body, as do many other amines like putrescine and cadaverine, and act as potentiators of histamine and tyramine, thus hampering their oxidation mechanisms (Monoamine Oxidase or MAO, Diamine Oxidase or DAO and Histamine Methyltransferase or HMT) (HUI and TAYLOR, 1985; STRATTON *et al.*, 1991; SHALABY, 1996; SILLA SANTOS, 1996; HERNÀNDEZ-JOVER *et al.*, 1997). People who are sensitive due to a deficiency or lower activity of natural detoxifying enzymes caused by inhibiting factors such as drug and/or alcohol intake, certain medical treatments, gastrointestinal disorders, etc., may be negatively affected even by low doses of BAs (LEUSCHNER and HAMMES, 1998).

Virtually all food containing proteins and/or free amino acids may contain different amounts of BAs, especially food subjected to conditions that give rise to

microbial or biochemical activity. Consequently, fermented food is considered to contain higher amounts of these compounds (SILLA SANTOS, 1996), particularly those which have undergone lactic acid fermentation (BRINK *et al.*, 1990; NOUT, 1994). Fermented meat products make up an important part of such foods. During normal sausage fermentation, proteolysis occurs and causes an increase in the concentration of non-protein nitrogen compounds (including BAs), which sometimes reach high levels (BLOM *et al.*, 1996). Therefore, attention should not be focused only on a particular amine when evaluating the potentially toxic levels of BAs in food. The type and amount of products eaten in a meal (e.g. various fermented food like wine, cheese, sausages and vegetables) could result in the simultaneous presence and cumulative intake of many BAs, which could result in poisoning, whereas the consumption of each of these foods alone would not cause a problem (BRINK *et al.*, 1990). While some very high concentrations of BAs, particularly histamine, have been detected in retail fermented sausages (HERNÀNDEZ-JOVER *et al.*, 1997), the recorded incidences of food-borne poisoning by these substances are exclusively related to fish or cheese consumption (BRINK *et al.*, 1990).

Fermented sausages, an important part of Italian processed meat production, are prepared in a wide variety of ways, including many homemade products that are processed according to local practices and traditions. Few studies have been carried out on the BA content of traditional Italian fermented sausages (CANTONI, 1995; PARENTE *et al.*, 2001; GARDINI *et al.*, 2002; COÏSSON *et al.*, 2004). The "lardellato" salami, a traditional fermented raw sausage of central Italy, is listed among the traditional products of the Marche region contained in the Ministerial Decree of 22 July 2004 (M.D., 2004). The aim of the present work was to determine some physical and chemical features of this product during maturation, with particular attention given to the content of BAs. Among the physical features, water activity (A_w) and pH are important non-microbial intrinsic factors that affect the enzymatic activity responsible for BA production. Among the chemical features, moisture, indicative of the extent of drying, allows the amount of the determined substances to be reported with respect to dry matter (D.M.). The total volatile basic nitrogen (TVBN) value is indicative of the proteolytic activity within the product. These chemical and physical parameters, together with the processing and storage conditions, determine the micro- and macro-environmental conditions that may influence microbial and tissue enzymatic activity and thus proteolysis, which involves the release of free amino acids and BA formation in the product. The evolution of BA concentration in "lardellato salami" during ripening was evaluated over a time period longer than the usual marketing time (90 days). The salamis were kept between 90 and 120 days in the factory's retail store in order to determine the BA content in overstored products. This was done to simulate the very frequent cases of delayed consumption due to a prolonged time behind the counter or at home.

MATERIALS AND METHODS

Sample preparation

"Lardellato" salamis were prepared according to traditional procedures at a plant in the province of Macerata in the Marche region in central Italy, located within the original area of production. The mixture was prepared from pork only, using refrigerated meat from shoulders (66%) and scraps from thigh, neck and loin processing (33%). After grinding, loin lard (20% of meat weight) cut

into small cubes, salt (3%), spices (0.3%), antioxidants (ascorbic acid and sodium ascorbate, as needed) and preservatives (potassium nitrate 0.02%) were added to the mixture. No starter cultures were used. The mixture was kept at 0°-4°C for 1 day and then stuffed into natural casings. The salamis were dried for 4 days in a drying room at a temperature progressively decreasing from 25° to 17°C and relative humidity (RH) progressively increasing from 40 to 80%. The salamis were then ripened in conditioned rooms at 13°-15°C and 75-80% RH until the usual time of marketing (90 days). The marketed products were about 600 g in weight, 40 cm in length and 4.5 cm in diameter. The overstored products were kept under retail sale conditions (in the factory's retail store) from the 90th to the 120th day. Samples, consisting of 4 salamis for each sampling day, were taken at 0 (mixture after 1 day of storage at 0°C before stuffing), 15, 25, 40, 60, 90 (usual end of ripening) and 120 days (overstored products) and quickly prepared for the analyses. For each salami, a slice (1 cm thick) was cut from the middle of the product length, minced twice in a grinder and then analysed.

Physical and chemical analyses

All samples were subjected to the following analyses in duplicate: moisture according to the A.O.A.C. (1990) method; pH according to BENDALL (1975); activity water (A_w) using a BT RS1 Rotronic Hygroskop (PBI International, Milano, Italy); total volatile basic nitrogen (TVBN) according to the Luke and Geidel technique modified by PEARSON (1973) and further arranged for auto-titration using a Kjeltec 2300 apparatus (Foss Analytical, Hillerød, DK), through distillation under steam flow of samples suspended in double-distilled water and titration of collected distillates with 0.1 N HCl after addition of receiver solution (1% boric acid and bromocresol green/methyl red as indicators) to the receiver flask.

Biogenic amine analysis

BA concentration was determined according to the a slightly modified RP-HPLC method described by ŞENÖZ *et al.* (2000). Ten grams of sample were homogenized in 35 mL of 5% trichloroacetic acid for 5 min with an Ultraturrax (IKA Werken, Staufen, GE). The homogenate was centrifuged at 3,000 rpm for 10 min and the supernatant filtered into a 100 mL flask. The extraction was repeated once under the same conditions. Working solutions of β-phenylethylamine, cadaverine, histamine, putrescine, spermine, spermidine and tyramine containing 10 mg/mL of each amine in distilled water were prepared as standards, using Sigma (St. Louis, MO, USA) chemicals. A water solution of 1,7-diaminoheptane (Sigma, USA) at the same concentration was used as internal standard (I.S.) and added (960 µL) to the sample homogenates during the extraction step. All sample extracts and standard solutions were derivatized by dansyl chloride (Dns-Cl) (Sigma, USA) before injection onto the HPLC. RP-HPLC analyses were carried out on an HP 1090 Liquid Chromatograph (Hewlett Packard, Avondale, PA, USA) equipped with an HP diode array detector (DAD). The HPLC column was an HP Spherisorb C18 ODS 2 (250x4 mm, particle size 5 µm) mounted with a guard column LiChroCART 4-4 LiChroSPHER 100 RP-18 (particle size 5 µm) (Merck, Darmstad, GE). The elution system was 0.1 M ammonium acetate as solvent A and acetonitrile as solvent B in isocratic conditions (25 and 75%, respectively). The flow rate was 1 mL/min and 20 µL of each sample were injected onto the column. BAs, dansylated according to ŞENÖZ *et al.* (2000), were detected by DAD using the following acquisition parameters (wavelength-band width): signals 254-4 nm, 230-4 nm and

275-4 nm, for evaluating peak purity; reference 550nm-100nm; spectra from 225nm to 600nm, to confirm the eluted peaks. The peaks were detected at the following mean retention times (min): 5.70 for β-phenylethylamine, 6.10 for putrescine, 6.80 for cadaverine, 7.80 for histamine, 9.30 for 1,7 diaminoheptane (I.S.), 13.00 for tyramine, 15.30 for spermidine and 42.90 for spermine. Quantitative determinations were made using the ratio of each BA area to I.S. area of the same sample and the values were then reported on the calibration curve of standards. To perform the BA recovery of the method, 500 µL of each standard amine working solution were added to some samples of salami. The percentage of recovery was determined comparing the amount of each amine detected in the sample extract without standards to the amount of the corresponding amine in another extract from the same sample added to a known amount of standards, as follows:

$$R(\%) = (BA_2\text{-}BA_1)/BA_3$$

where

BA_1= BA content before addition;
BA_2= BA content after addition;
BA_3= BA added.

The recovery analysis was repeated at three different times during the experiment.

Statistical analysis

Microsoft Excel software (Microsoft Co., Redmond, WA, U.S.A.) was used to perform the statistical analysis and all data are expressed as means ± standard deviation (SD).

RESULTS AND DISCUSSION

Water activity (A_w), pH, moisture and total volatile basic nitrogen (TVBN) values of "lardellato" salami samples are listed in Table 1 which shows the evolution of these parameters during ripening. The values show a progressive decrease in pH up to the 60th day of ageing when the minimum was reached (pH 5.11). This was followed by an increase as high as 5.56 at the usual time of marketing (90 days) and a further increase (pH 5.87) in overaged products (120 days). TVBN increased from 15.466 mg/100 g in the initial mixture to 52.371 mg/100 g at the usual end of ripening (90 days). It increased further during the last 30 days of storage to 58.128 mg/100 g in the overaged product. The constant increase of TVBN was also confirmed by reporting the values as dry matter (D.M.), that ranged from 32.617 at 0 days to 73.339 mg/100 g in the overstored products. The A_w value progressively dropped from 0.944 to 0.792 until the usual end of ripening and continued to decrease to 0.748 at 120 days as ageing progressed. Moisture decreased progressively from 52.58% in the initial mixture to 20.74% in the overstored product.

The values of BAs in the samples are listed in Table 2. The total amount of BAs ranged from 56.040 mg/kg at 0 days to 919.255 mg/kg at 90 days of ripening followed by a slight decrease between 90 and 120 days to 897.815 mg/kg. As expected, the total BAs progressively increased over the ripening period with a marked increase especially between 0 and 15 days. In the initial mixture, the BAs included spermine (22.259 mg/kg), cadaverine (18.162 mg/kg), putrescine (11.095 mg/kg) and spermidine (4.524 mg/kg). Putrescine, cadaverine, histamine and tyramine values increased throughout the experiment. Tyramine was not detected in samples at 0 days, but levels reached 180.288 mg/kg after 15 days of ripening, increasing up to 297.282 mg/kg at 60 days of ripening and then stabilizing. There was a progressive increase of spermidine (from 4.524 to 7.162 mg/kg), whereas spermine increased up to 40 days (from

Table 1 - Some physical and chemical features of "lardellato" salami at different ageing times.

Ageing (days)	Moisture (%)	pH	A_w	TVBN* (mg/100 g)
0	52.58±2.24	6.00±0.01	0.944±0.001	15.466±0.43
15	41.82±0.37	5.35±0.01	0.907±0.010	31.441±1.77
25	36.23±1.02	5.28±0.06	0.886±0.010	39.832±0.83
40	30.90±1.61	5.22±0.07	0.856±0.005	46.006±0.07
60	28.40±1.90	5.11±0.04	0.812±0.001	48.398±1.57
90	23.89±1.62	5.56±0.04	0.792±0.005	52.371±3.15
120	20.74±0.34	5.87±0.01	0.748±0.004	58.128±2.02

* Total volatile basic nitrogen;
Each value is the average of 4 determinations and the standard deviation.

22.259 to 42.501 mg/kg) and then decreased slightly until the end of the experiment (from 42.501 to 34.404 mg/kg). β-phenylethylamine was not detected in the initial mixture, but increased slowly and progressively to 19.300 mg/kg at 120 days.

The recovery of BAs varied between 88.1% for β-phenylethylamine and 109.6% for histamine, with intermediate values for spermine (96%), spermidine (97.4%), tyramine (98.8%), putrescine (99.8%) and cadaverine (102.9%).

The trend of pH was nearly in accord with that reported by LEISTNER (1992), who reported a decrease to 5.2 followed by an increase to 5.8-6.0 (final values) in Italian salamis and in long ripened salamis in general, which are also characterized by low acidity like "lardellato". Among the basic compounds responsible for the normally observed increase in pH in the later maturation stages, TVBN probably plays an important role because it is associated with the intensity of proteolysis. Furthermore, it has been observed that TVBN is related to the levels of some BAs (putrescine, cadaverine and histamine) (SHALABY, 1996). The increased TVBN levels up to the end of ripening have also been described by DIAZ *et al.* (1997) and FANCO *et al.* (2002) in Spanish dry fermented sausages, where variable values were detected. The stability of such low acid salamis is assured especially by A_w, which usually drops progressively by the end of ripening. In long-ripened Italian salamis, the A_w values drop below 0.88 at the end of ripening (INCZE, 1993). The moisture values decreased progressively until the usual time of marketing; the values continued to decrease even under retail store conditions, making the overstored product undesirably tough.

The levels of BAs recovered were satisfactory and were higher than almost all those obtained by VINCI and ANTONELLI (2002) and ŞENÖZ *et al.* (2000) for histamine, using a similar analytical procedure. The presence of some of these substances (putrescine and cadaverine) in the initial mixture was probably due to the 1-day storage it underwent before stuffing. The marked increase in the level of total BAs between 0 and 15 days was especially due to the noticeable increase of tyramine (from 0 to 180.288 mg/kg), as well as putrescine (from 11.095 to 152.845 mg/kg) and cadaverine (from 18.162 to 166.755 mg/kg). As expected, the values of the total BAs increased progressively, but calculating the values with respect to dry matter (D.M.), the increase was constant up to 60 days (from 118.180 mg/kg to 1247.201 mg/kg) and

Table 2 - Content of biogenic amines (mg/kg) in "lardellato" salami at different ageing times.

Ageing (days)	β-phenylethylamine	Putrescine	Cadaverine	Histamine	Tyramine	Spermidine	Spermine	Total
0	0.000±0.00	11.095±0.001	18.162±2.52	0.000±0.00	0.000±0.00	4.524±0.001	22.259±0.64	56.040
15	15.818±8.12	152.845±2.22	166.755±0.25	10.598±5.15	180.288±8.77	6.597±0.27	29.231±2.86	562.130
25	19.752±0.001	172,198±5.42	218.485±1.26	15.097±4.85	213.346±11.69	6.408±1.07	28.782±1.59	674.069
40	19.752±8.95	241.591±1.48	244.173±16.90	21.310±2.73	268.615±0.73	6.408±0.53	42.501±1.91	844.350
60	24.727±5.76	240.196±26.14	252.556±18.67	32.452±0.91	297.282±5.48	6.031±0.001	39.802±0.001	893.047
90	16.134±1.28	253.796±12.82	264.865±9.84	38.665±12.12	300.898±5.48	6.220±0.27	38.678±2.86	919.255
120	19.300±1.92	253.447±14.79	243.994±27.25	41.450±10.00	298.057±0.73	7.162±1.07	34.404±3.82	897.815

Each value is the average of 4 determinations and the standard deviation.

then decreased slightly but progressively until 120 days (1132.751 mg/kg).

The continuous increase in the levels of putrescine, cadaverine, histamine and tyramine is in accord with the data reported in the literature (MAIJALA *et al.*, 1995; AYHAN *et al.*, 1999; BOVER-CID *et al.*, 2000a, 2001; PARENTE *et al.*, 2001). Reporting the values of these substances as D.M., a continuous increase was confirmed for histamine, but putrescine, cadaverine and tyramine showed a progressive increase up to 40, 25 and 60 days, respectively, when the highest concentrations were reached (349.636, 357.874 and 415.175 mg/kg of D.M., respectively), followed by a slight but progressive decrease until the end of the experiment (319.768, 307.841 and 376.051 mg/kg D.M., respectively).

While the level of histamine clearly increased by the end of the experiment, the maximum value of 41.450 mg/kg (52.297 mg/kg D.M.) was far from the 100 mg/kg of total matter considered by law in most countries (at present only for some fishery products) as the limit for a product to be deemed safe. According to the literature, doses of histamine between 100 and 180 mg are considered to be potentially toxic. This amount corresponds to 1.6-3 mg/kg bodyweight, assuming the average weight of a person to be 60 kg (LEHANE and OLLEY, 1999). The observed behaviour of histamine is in accordance with SILLA SANTOS (1996), who reported at least a tenfold increase of this amine during the first days of maturation in long-ripened dry sausages. However, a slight increase in the histamine concentration has been consistently observed in fermented sausages by almost all authors. While STRATTON *et al.* (1991) reported that histamine production occurs mostly during the first 2-4 weeks of ripening, a constant increase with a doubling in concentration between 40 and 120 days was observed in the present study. PARENTE *et al.* (2001) reported an increased histamine content during

ripening in some samples of both industrial and artisanal Italian "soppressata", and in a few cases the concentration was rather high at the end of ripening. They did not find this amine in any of the Italian "salsiccia" samples tested. CANTONI (1995) detected increasing levels of histamine in other Italian fermented sausages, reaching a mean value of 31.35 mg/kg after 4 weeks of ripening. Very high concentrations of histamine (from 143.1 to 357.1 mg/kg) were reported by HERNÀNDEZ-JOVER *et al.* (1997) in different Spanish fermented sausages.

As reported by most authors, tyramine is the prevailing amine in this kind of product (HERNÀNDEZ-JOVER *et al.*, 1997; TREVIÑO *et al.*, 1997; AYHAN *et al.*, 1999; BOVER-CID *et al.*, 2000b, 2001; ŞENÖZ *et al.*, 2000). PARENTE *et al.* (2001) reported rather high values of tyramine in Italian "soppressata" (556.88 mg/kg) and "salsiccia" (338.85 mg/kg) both with and without added starters. CANTONI (1995) reported low levels of tyramine in Italian fermented sausages made without starter cultures after 4 weeks of ripening.

The trends for spermidine and spermine over the ripening period described in the present paper and the BA concentration were similar to those reported by CANTONI (1995) in mixtures of Italian raw dry fermented sausages without starters, who also reported appreciable levels of spermine in the raw material. PARENTE *et al.* (2001) found spermidine and spermine in Italian "salsiccia" and "soppressata" at 0 days and observed that both of these substances progressively increased in Italian "soppressata" during ripening. The presence of spermidine and spermine in raw material is normal, as they are considered physiological constituents of fresh meat (BRINK *et al.*, 1990; SILLA SANTOS, 1996; VINCI and ANTONELLI, 2002).

While attempts are often made to find β-phenylethylamine in food, it is not always detected or has sometimes been detected in trace amounts in fermented sausages even after 30 days of ripening (DIAZ *et al.*, 1997; AYHAN *et al.*, 1999). It was found by PARENTE *et al.* (2001) in samples of artisanal Italian "soppressata" after 7-10 days of ripening (17.83 mg/kg) and in industrial samples after 20-40 days of ripening (10.56 mg/kg). HERNÀNDEZ-JOVER *et al.* (1997) reported the presence of β-phenylethylamine in the majority of Spanish ripened fermented sausages. SUZZI and GARDINI (2003) reported the occurrence of β-phenylethylamine when high levels of tyramine were present, due to the non-specific activity of tyrosine decarboxylase. Not much is known about the toxic doses of amines other than histamine, but values of 100-800 mg/kg for tyramine and 30 mg/kg for β-phenylethylamine, levels never reached in "lardellato" salami, have been suggested (BRINK *et al.*, 1990; NOUT, 1994). SHALABY (1996) also reported that these same values were acceptable from the point of view of Good Manufacturing Practices.

CONCLUSIONS

Chemical and physical parameters observed in "lardellato" salami resemble the typical evolution described in other traditional Italian long ripened salami. An expected an increasing trend of total BAs was observed, that was not confirmed when the values were reported as dry matter: the absolute BA content increased up to 60 days of ripening and then decreased by the end of the experiment. A similar trend was observed for putrescine, cadaverine, tyramine and spermidine. From a health viewpoint, the progressive increase of total amines in total matter assumes greater importance than as D.M., because it reflects the amount of such substances that can be eaten in a meal. In this respect, there were no differences between samples at the usual time of marketing and those from overstored products. Actually, total

BAs at 120 days were slightly less concentrated than at 90 days. This observation, together with the physical and chemical parameters investigated, may be indicative of the stability of this product even when overstored. It is understood however that the optimum end of ripening and time of consumption is at 90 days as can be justified by tradition, since "lardellato" may become excessively tough in overaged products.

Information regarding the whole amine profile of each product rather than just the level of the better-known amines like histamine and tyramine should be useful for determining its real potential toxic level. The data on the final total amount of BAs detected in the present study resembled the data reported for similar products; the dose levels were far below the potentially toxic levels. These amounts however could be high enough to affect susceptible consumers, particularly when considering the synergistic effect of eating several fermented products at one meal. BA formation could be limited by using defined starter cultures. In this respect, it is important to consider the many traditional and typical products found in most European countries, particularly in Italy. Actually, in most of these products, some of which are protected under European law with the Protected Designation of Origin (PDO) and the Protected Geographical Indication (PGI), the use of starter cultures is not authorized and not even considered. Therefore, the raw materials and production hygiene must absolutely be taken into account when preparing food, especially fermented products, particularly if no starter cultures are added.

ACKNOWLEDGMENTS

This study was financially supported by the Italian Ministry of Education, University and Research (Ministero dell'Istruzione, Università e Ricerca) during the period 2001-2002.

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Revised paper received September 24, 2004 Accepted October 25, 2004

SHORT COMMUNICATION

EFFECT OF THREE *SACCHAROMYCES CEREVISIAE* STRAINS ON THE VOLATILE COMPOSITION OF ALBARIÑO WINES

INFLUENZA DI TRE CEPPI DI *SACCHAROMYCES CEREVISIAE* SULLA COMPONENTE VOLATILE DI VINI ALBARIÑO

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ABSTRACT

Must obtained from *Vitis vinifera* L. cv Albariño grapes was inoculated with three strains of *Saccharomyces cerevisiae* (Asln1, Asln2 and Asln20) and fermented under identical conditions. Standard chemical analyses were carried out on the final wines and volatile compounds were analysed by gas chromatography. Statistical analysis of the data obtained by the latter technique showed that the wines produced differed depending on the yeast strain used. The formation of alcohols,

RIASSUNTO

Tre ceppi di *Saccharomyces cerevisiae* (Asln1, Asln2 and Asln20) sono stati inoculati su mosto ottenuto da uve di *Vitis vinifera* L., cv. Albariño, e successivamente fatti fermentare nelle medesime condizioni. Sui vini finiti sono state condotte analisi chimiche routinarie e la componente volatile è stata determinata tramite gas cromatografia. Le analisi statistiche dei dati, ottenuti seguendo le tecniche sopradescritte, hanno messo in evidenza che la differenza riscontrata nei vini prodotti è dovuta al

- Key words: Albariño wine, alcoholic fermentation, *Saccharomyces cerevisiae*, volatile composition -

esters and isoamyl acetate by Asln1, Asln2 and Asln20 differed significantly (P<0.05). The probability associated with the differences for isobutanol and isoamyl alcohol was 99.9%.

ceppo di lievito usato. La formazione di alcoli, esteri e isoamil acetato differisce in modo statisticamente significativo (P<0,05) a seconda dei lieviti impiegati (Asln1, Asln2 and Asln20). La probabilità statistica associata alle differenze riscontrate nei diversi vini per quanto riguarda l'isobutanolo e l'alcool isoamilico è risultata del 99,9%.

INTRODUCTION

Yeasts, key microorganisms in winemaking, conduct alcoholic fermentation. The selection of the yeast strain and fermentation conditions are claimed to be the most important factors influencing the volatile compounds in wine. The aroma profile of wine is derived from the grape through changes in grape flavor precursors caused by yeast. The composition and quality of wine are closely related to the yeast (FLEET and HEARD, 1993; GIL *et al.*, 1996) which produces volatile compounds as secondary products during fermentation. While the volatile metabolites contribute to the fermentation bouquet ubiquitous to all young wines, the amounts of these by-products vary and are yeast-strain specific (LAMBRECHTS and PRETORIUS, 2000).

The main groups of compounds that form the fermentation bouquet are higher alcohols and esters (RAPP and VERSINI, 1991). When present in excess, some fermentation bouquet compounds, such as acetaldehyde, acetic acid, ethyl acetate, higher alcohols and diacetyl, may also be regarded as undesirable (LAMBRECHTS and PRETORIUS, 2000). It is known that volatile compounds of wines vary little during alcoholic fermentation (DUBOURDIEU *et al.*, 1988; DARRIET *et al.*, 1995; MURAT *et al.*, 2001) since the glycosidases of *Saccharomyces cerevisiae* have little effect on terpenic glycosides at the pH of the must.

Studies in which starter cultures and indigenous yeasts have been investigated have shown that there are significant differences in the chemical composition of the resulting wines (EGLI *et al.*, 1998; HENICK-KLING *et al.*, 1998; ESTÉVEZ *et al.*, 2004). PATEL and SHIBAMOTO (2002, 2003) showed that the formation and concentration of the volatile compounds found in Symphony, Napa Gamay and Petite Sirah wines were dependent on the strain of *S. cerevisiae* used.

Furthermore, it has been discovered that during alcoholic fermentation Sauvignon blanc varietal precursors are transformed into aroma varietal compounds by *S. cerevisiae* (DARRIET *et al.*, 1995)

The effect of *S. cerevisiae* yeast strains on the liberation of the volatile compounds was studied in Palomino (ESTÉVEZ *et al.*, 2004), Emir (NURGEL *et al.*, 2002) and Sauvignon blanc (MURAT *et al.*, 2001) wines.

Albariño, a grape variety of *Vitis vinifera L.*, is a typical white variety from Galicia in northwestern Spain, mostly on the Atlantic coast (Appellation Controlée Rías Baixas) and in northern Portugal (VQPRD Vinhos verdes). It is characterised by a high intensity of floral aromas. Free monoterpenes are responsible for these floral notes (CARBALLEIRA *et al.*, 2001). Young white wines prepared from Albariño grapes are dominated by fruity and floral aromas (CARBALLEIRA *et al.*, 2001; FALQUÉ *et al.*, 2001). The high

amount of monoterpenes could explain the floral aroma in Albariño wines.

The objective of this study was to determine the role of the selected *S. cerevisiae* strains on the volatile composition of single varietal wines produced from Albariño grapes. Multivariate data analysis techniques were used for these comparisons.

MATERIALS AND METHODS

Yeast

The *S. cerevisiae* yeast strains used in this study (ASln1, ASln2 and ASln20) were isolated from musts obtained from grapes harvested in the Rías Baixas region (Spain) from a winery which had never used commercial yeast starters. The karyotypes of the three yeasts used (PFGE) are shown in Fig. 1. Chromosomal DNA was prepared in agarose plugs following the method of BELLIS *et al.* (1987). Electrophoresis was performed with a Pharmacia-LKB (Pulsaphor) apparatus based on the contour homogeneous electric field (CHEF) principle (DUBOURDIEU and FREZIER, 1990) under the following conditions: 0.8% agarose gel, migration at 10°C, TBE 1X (Tris Sigma 7-9 90 mM, boric acid 90mM, EDTA 2mM pH 8) as the migration buffer, 165 v, pulsed time program: 90 s-20h, 100 s-12 h, 120 s-12 h, 30 s-4 h.

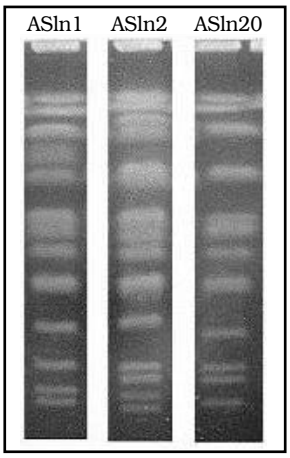


Fig. 1 - Pulsed Field Gel Electrophoresis (PFGE) of *S. cerevisiae* yeast strains (ASln1, ASln2 and ASln20).

Fermentations

Must from Albariño grapes was collected from a winery in the Rías Baixas region before the addition of sulfur dioxide. The must was transported to the laboratory at 8°C and then stored at -20°C. Prior to fermentation, it was centrifuged (10,000 x g for 20 min) and sterilized by membrane filtration through a Millipore system (0.45 µm membrane); sulfur dioxide was then added (50 mg/L). The initial sugar concentration was 190 g/L and the pH was 3.1.

Fermentations were performed in 16 L flasks containing 14 L of Albariño must. Yeast cells were cultured in liquid YEPD media, washed twice with sterile water and suspended in the must at a density of 10^6 cells/mL. Fermentations were conducted at 18°C for 15 days. Samples of 10 mL were periodically collected from the flasks and used to test strain dominance by Pulsed Field Gel Electrophoresis (PFGE).

All fermentations were performed in triplicate. The data obtained on cell growth, as well as the analytical results, are means of three replicates. At the end of fermentation, the wines were centrifuged and sulfur dioxide was added (50 mg/L). The wines were bottled and 1L of each wine was analyzed after 30 days.

Chemical analysis

The following measurements were made to compare the effect of each yeast according to Office International de la Vigne et du vin (OIV, 1990) official methods: pH (measured with a pH meter), ethanol content (by distillation of wine made

with a suspension of calcium hydroxide), total acidity (by titration with bromothymol blue as an indicator), volatile acidity (by titration of the volatile acids separated from the wine by steam distillation and titration of the distillate), total dry extract (by measurement with a densitometer), tartaric acid (gravimetric method), malic acid (enzymatic method) and reducing sugar content (by determination of glucose and fructose using an enzymatic method. Determinations were made in triplicate.

Volatile compounds

The contents of the most relevant aromatic fermentation compounds (higher alcohols, esters and acetates) of the final wines were determined by gas chromatography (GC).

The analyses were carried out using a Hewlett Packard 5890 Series II Gas-Chromatograph equipped with a Flame Ionisation Detector (Hewlett Packard, EEUU) The compounds were separated on a Chrompack CP-Wax 57CB (polyethylene glycol stationary phase; 50 mx0.25 mm id with 0.25 µm film thickness) fused-silica capillary column (Varian, The Netherlands). Instrumental conditions were: injector temperature, 250°C; detector temperature, 260°C; carrier gas, helium at 1.07 mL/min; make-up gas, nitrogen 30 mL/min. The detector gas flow rates were: hydrogen, 40 mL/min and air, 400 mL/min.

Due to the high concentrations of methanol and higher alcohols in the wines, 1 mL of an internal standard solution (1 g of 4-methyl-2-pentanol per 1 L of ethanol) was added to 10 mL of the sample prior to analysis. A 1-µL aliquot of this sample was injected directly and split 1:1. The temperature program was: held at 60°C for 15 min and then increased at 3°C/min to 200°C.

Extraction of esters and acetates was carried out according to the method described by BERTRAND (1981): 2 mL of 3-octanol (50 mg/L) as internal standard and 1 mL of sulphuric acid (1/3) were added to 50 mL of wine. Each sample was extracted three times with 4, 2 and 2 mL of diethyl ether-hexane (1:1, v/v). 1 µL of the organic extract was injected into the chromatograph in splitless mode (30 s). The temperature program was: held at 55°C 15 min and then increased at 3°C/min to 200°C.

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

Internal standards were used to quantify concentrations of individual compounds.

Statistical analyses

Significant differences among wines for each of the compounds were assessed with one-way analysis of variance (ANOVA) using the procedure of the SAS statistical package (SAS Institute Inc., Cary, N.C., USA).

RESULTS AND DISCUSSION

Chemical composition

The basic wine composition is shown in Table 1. The results were within the normal range of values expected. The wine made with *S. cerevisiae* ASln20 had the highest ethanol content (11.15% v/v) and extract (26.4 g/L). All wines were fermented to dryness; the concentration of reducing sugar (which determines the capacity of different strains to complete fermentation) was less than 1 g/L. Total acidity ranged from 9.65 to 10.15 g/L; Albariño wines from Galicia are always acidic. Malic and tartaric acid were

Table 1 - Chemical analyses of cv. Albariño wines.

Parameter	Samples		
	ASln1	ASln2	ASln20
Ethanol (%v/v)	10.15±0.212	10.35±0.212	11.15±0.221
Total acidity (g/L)	10.15±0.017	9.65±0.919	10.1±1.131
Volatile acidity (g/L)	0.4±0.141	0.375±0.035	0.48±0.028
Reducing sugar (g/L)	<1±0.000	<1±0.000	<1±0.000
Density (20ºC)	0.998±0.000	0.998±0.000	0.996±0.001
pH	3.2±0.106	3.25±0.071	3.35±0.163
Extract (g/L)	25±0.000	25.15±0.212	26.4±0.424
Tartaric acid (g/L)	2.5±0.000	2.55±0.071	2.35±0.071
Malic acid (g/L)	7.4±0.707	7.4±0.566	7.05±0.919

Means ± SD. No significant differences were found among the three wines.

present in similar concentrations in all wines produced. No significant differences were found between the three wines.

Volatile composition

The volatile composition of a wine depends on the grape variety, fermentation conditions and the yeast involved. Higher alcohols and esters are the main aroma constituents of wine that are measurable by GC. These compounds are produced mainly by yeast metabolism during fermentation (NURGEL *et al.*, 2002).

The production of major volatiles differed among the three strains (Table 2). A total of 13 free volatile compounds were identified in Albariño wines fermented with the three strains. Strain ASln2 produced the highest quantities of volatile compounds.

Alcohol

Higher alcohol formation seems to depend on the yeast strain used, the use of amino acids and total nitrogen demand (JINAREK *et al.*, 1991). Small amounts of higher alcohols contribute positively to wine quality, but excessive amounts may impair it (LAMBRECHTS and PRETORIUS, 2000).

The amount of total alcohols in the wines ranged from 109.07 to 227.8 mg/L; the highest amounts were in wines fermented with ASln2, which also had the highest concentration of methanol (Table 2). The highest concentrations of isoamyl alcohol and isobutanol were obtained with ASln2, with significant differences among all the wines produced. However, neither of these alcohols were present at levels above the threshold of perception (40 and 60 mg/L respectively) (SIMPSON, 1979; LÓPEZ *et al.*, 1999). The wine with the lowest isoamyl alcohol (14.31mg/L) and methanol concentrations (52.50 mg/L) was obtained with ASln20.

Esters

Esters are a group of volatile compounds that impart a pleasant smell and contribute fruity and flowery notes to the overall aroma (LAMBRECHTS and PRETORIUS, 2000). Total ester concentrations in the present wines ranged from 3.51 to 5.93 mg/L (Table 2). Differences were mainly due to ethyl octanoate; ASln1 and ASln20 produced the highest amounts of ethyl octanoate, ethyl hexanoate and ethyl decanoate, that are responsible for floral and fruity odours.

Table 2 - Volatile compounds produced by three *S. cerevisiae* strains during fermentation of Albariño musts.

Compound	Descriptors for chemical compounds (CLIFF *et al.*, 2002)	Samples			
		ASln1	ASln2	ASln20	Sig.
Alcohol					
1-Propanol	Floral, fruity, sweet	51.75±6.02	34.47±2.35	27.05±0.70	**
Isobutanol	Fusel	39.75±3.51	68.85±4.60	15.22±1.65	***
Isoamyl alcohol	Marzipan	27.21±3.02	53.93±1.05	14.31±0.25	***
Methanol	Alcohol	52.93±1.02	70.50±2.68	52.50±1.14	*
Total alcohols		171.64	227.8	109.07	
Esters					
Ethyl butyrate	Fruity	0.70±0.18	0.31±0.08	0.45±0.01	*
Ethyl hexanoate	Apple, banana, violets	1.23±0.21	0.86±0.04	1.11±0.03	*
Ethyl octanoate	Pineapple, pear, floral	2.32±0.22	1.12±0.14	1.74±0.07	**
Ethyl decanoate	Floral, sweet	0.94±0.06	0.33±0.01	0.60±0.03	*
Diethyl succinate	Fruity	0.45±0.05	0.80±0.05	0.63±0.03	*
Ethyl laurate	Fruity	0.29±0.10	0.09±0.01	0.50±0.04	*
Total esters		5.93	3.51	5.03	
Acetate					
Isoamyl acetate	Fruity, banana, pear	6.41±0.78	3.82±0.52	1.73±0.05	**
Hexyl acetate	Fruity	0.49±0.02	0.30±0.02	0.29±0.018	ns
Ethyl acetate	Varnish, fruity	87.23±4.27	66.32±3.98	65.33±3.24	ns
Total acetates		94.13	70.44	67.35	

Concentrations are reported in mg/L. The data are mean values of triplicates; Sig.: significance at which means differ as shown by analysis of variance: *, **, *** denote significance at $p<0.05$, $p<0.01$, $p<0.001$, respectively. ns: not significant.

Acetates

The isoamyl acetate concentrations in the wines differed, but were always well above the perception threshold (1 mg/L) (ETIEVANT, 1991). Strains ASln1 and ASln2 produced the greatest amounts of total acetates (94.13 and 70.44 mg/L, respectively). ASln20 produced the least (67.35 mg/L).

Ethyl acetate, the most abundant ester in the volatile fraction of wines, contributes significantly to "acetic nose" defect (LAMBRECHTS and PRETORIUS, 2000). The wine fermented with ASln20 had the lowest ethyl acetate content, while that fermented with Asln1 had the highest. The ethyl acetate concentrations in all the wines were above the threshold of perception (50 mg/L).

Significant differences were found among the three wines for all the variables (95% probability) except for hexyl acetate and ethyl acetate. The probability associated with the differences between isobutanol and isoamyl alcohol was 99.9%.

CONCLUSIONS

Musts from Albariño grapes were fermented to dryness with three *S. cerevisiae* strains isolated in the Rías Baixas region. ASln2 produced the highest amounts of alcohol. Marked differences in the volatile composition of the fer-

mented musts, determined by GC were dependent upon the yeast strain used. *S. cerevisiae* ASln1 and ASln20 produced the greatest quantities of esters, (ethyl butyrate, ethyl hexanoate, ethyl octanoate and ethyl decanoate) which can give a wine a fruity aroma. These results suggest that the production level of these compounds is characteristic of the individual yeast strains, which highlights the importance of characterising yeast strains for industrial use.

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Revised paper received October 20, 2004 Accepted December 21, 2004

IDF NEWS

DAIRY INDUSTRY PUSHES DEVELOPMENTS IN FRESH DAIRY PRODUCTS

Advanced developments in fresh dairy products mean greater choice for the consumer. Innovations in dairy technology make this possible. Convenience, good health and treating yourself are the key market concepts driving the development of fresh products. A product delivering all three of these benefits stands a high chance of success.

Dairy products delivering convenience include flavoured milks, coffee, tea beverages and ready-to-eat desserts. Indulgence products are those with high fat content such as cream, clotted cream and alcoholic creams whilst healthy dairy products are low fat, fat free or low carbohydrate. Well-being products also fall into the healthy category offering additional minerals and nutrients including probiotics, omega-3 fatty acids, conjugated linoleic acids and peptides.

Changes in consumer needs and demands open up new markets for developing alternative dairy products in order to cater for specific requirements. One example of a special market need is intolerance to lactose especially in some countries and thus dairy products can be modified through technological advancements, a process known as enzymatic hydrolysis of lactose.

For further information please contact: Ken Burgess of Dairy Crest on 07831 255 208 or Ramsay Smith of Media House on 0207 397 8460 or 07788 414856.

Alternatively please contact: Marylène Tucci at IDF on +32 2 706 86 44.

27th IDF WORLD DAIRY CONGRESS
October 20-23, 2006
Shanghai, China

Placed under the auspices of the International Dairy Federation, the 27th IDF World Dairy Congress is the global dairy sector's most important meeting. Dairy producers, dairy companies, dairy academia and government departments will be attracted in large numbers. The programme will also appeal to the participants from South East Asian region.

It is predicted that 3,000 people from 80 countries will attend this important meeting. A major dairy exhibition will be held at the same time as the Congress. 1.3 billion Chinese people extend a warm invitation to you to join us.

For more information please log on the Congress website: www.idf2006shcn.com.

IDF SYMPOSIUM ON ENZYMES IN MILK

Potential through diversity was the message that emerged from last week's first ever IDF Symposium on Indigenous Enzymes in Milk at University College Cork in Ireland. International experts revealed the wide range of functions these enzymes have: some of these

milk enzymes attack pathogenic bacteria and have other health-influencing effects; some improve digestion in the newborn human child; others are responsible for the development of characteristic flavours in cheeses and other dairy products; yet more affect the shelf-life of products. Over 120 scientists, industry representatives and postgraduate students, from over 30 countries, enjoyed three days of overview presentation and research updates on almost all aspects of milk enzymes, from phosphatases to proteinases, in contexts from mastitis to cheese-making.

For further information please contact Marylène Tucci at IDF on +32 2 706 86 44.

FIRST IDF BULLETIN IN ELECTRONIC FORM

The Bulletin of the IDF n. 393/2005 "Trans-fatty Acids: Scientific Progress and Labelling" is the first IDF Bulletin available in electronic form. The International Dairy Federation is moving into electronic publishing. This decision is in line with our policy of improving service to the dairy sector by using modern communication tools.

From 28 February 2005, the IDF will start distributing publications in electronic form, which will be obtainable on the Internet with a login and password. This move towards electronic publishing has been initiated in response the numerous advantages that electronic publishing offers in terms of speed, accessibility and cost compared with traditional paper publishing. Moreover, any article will henceforth be obtainable individually and as soon as it is ready for release.

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

Visit the range of IDF Publications on the IDF website at

http://www.fil-idf.org/content/default.asp?PageID=311.

FINAL PROGRAM FOR IAFP 2005

The International Association for Food Protection has set the final program for IAFP 2005 – the Association's 92nd Annual Meeting, August 14-17, 2005 at the Baltimore Marriott Waterfront Hotel in Baltimore, Maryland. The meeting has earned recognition as the leading food safety conference. Online registration and program information is available on the Association Web site at www.foodprotection.org.

There will be three days of diversified sessions with over 500 presentations including 27 half-day symposia, 80 technical and 278 poster presentations. A sample of symposium topics includes: Laboratory Response to Food Bioterrorism, Update on Foodborne Disease Outbreaks, Managing the Risk of *Listeria monocytogenes* at Retail and Restaurants, Microbiological Predictive Models, Safety of Raw Milk Cheeses, Risk and Control of *Salmonella* in Raw Nuts, Produce Packinghouse Sanitation, Microarray Technology, Food Allergens, Global Water Quality Concerns, Food Chemical Contaminants, Oceans and Human Health, International Food Safety Opportunities and many others.

Attendance is expected to exceed 1,600 of the top industry, academic and government food safety professionals. The meeting will also showcase more than 100 companies demonstrating the latest products and technologies in the exhibit hall.

For further information contact: Bev Brannen, Public Relations, 515-276-3344, fax 515-276-8655, e-mail:bbrannen@foodproteciton.org, 6200 Aurora Avenue, Suite 200W, Des-Moines, Iowa 50322-2864.

10th EUROPEAN MEETING ON SUPERCRITICAL FLUIDS
December 12, 13, 14, 2005
Strasbourg, France
First Announcement

Supercritical fluid processes open promising opportunities to manufacture new products from natural or synthetic sources, to "design" new materials with specific properties or to improve classical materials, or to propose new reactions and waste management routes.

The purpose of this meeting is to reflect recent progresses in areas of great scientific interest as well as new industrial development.

Sessions will be organized according to all topics related to Supercritical Fluids, both on fundamental aspects and on applications in various industries (food, pharmaceuticals, nutraceuticals, cosmetics, electronics, textile, fine chemistry, waste management, etc.) including industrial scale-up and economical evaluation.

The program will comprise three plenary lectures (45 minutes) presented by senior scientists, oral communications (20 minutes) and two poster sessions. An abstract of 200-300 words should be sent before July 15, 2005 to:

Francoise Brionne, e-mail: brionne@ensic.inpl-nancy.fr.

Or by mail to ISASF – ENSIC, I Rue Grandville, BP 451, F-54001 Nancy, France.

SEVENTH INTERNATIONAL GRADUATE COURSE
On Production and Use of Food Composition Data in Nutrition
FoodComp 2005 Wageningen
31 October – 16 November 2005
Wageningen, The Netherlands

Objectives of the course

The aim of FoodComp 2005 is to show how the quality and usefulness of food composition data is determined by the quality of the production of analytical data for nutrients in foods, and the compilation of these and other data into food composition tables and nutritional databases. The course will be based on the philosophy that the preparation of nutritional databases requires close understanding of the needs of the users by both compilers and producers of analytical data. The course will show how this understanding can be achieved and the benefits that flow from the collaboration of users, analysts and compilers.

Course content

The major elements of the course, which will comprise lectures, seminars and group work, will be:

- use of compositional databases at international, national, household and individual levels and for nutrition labelling;
- steps in establishing a food composition database including advocacy issues;
- national and international co-operation in food composition issues;
- selecting and establishing priorities for including foods, nutrients and other food components in databases;
- food nomenclature and coding;
- reviewing existing data;
- designing protocols for sampling foods;
- methods of analysis: critical evaluation and choice of methods;
- assuring and documenting the quality of analytical data;
- taking bioavailability into account with respect to food composition data;
- component identification and expression;
- compilation of data;
- computerisation of nutrient data and selection of appropriate software;
- dissemination of data including copyright issues;
- strengths and limitations of food composition data.

Organisation and registration

Ms L.A. Duym-Brookman, Secretariat Foodcompo2005

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3rd INTERNATIONAL CONGRESS FLOUR-BREAD '05 October 26-29, 2005 Opatija, Croatia First Circular

The main objective of the Congress is to bring together scientists and researchers from Croatia and worldwide to exchange their knowledge and experience and present their latest achievements in the field of cereal science. The Organising Committee wishes to extend a warm invitation to take part in this event.

Congress topics

- Innovative analytical and rheological methods;
- Cereals, ingredients and product quality;
- Technological achievements of flour production;
- Bread and baked products;
- Breakfast cereals and snacks;
- Extrusion and pasta production;
- Cookies and waffle production;
- Nutritional aspects of cereal based products;
- Others.

Call for Papers

Abstracts (not more than 200 words) for oral and poster presentation are to be sent preferably by e-mail to the Congress Secretariat before July 1, 2005. The Scientific Committee will decide on acceptance and on the way of presentation.

Congress Secretariat

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All information can be found on the website: http://www.ptfos.hr/brasnokruh/.

NEW DIRECTOR GENERAL FOR THE INTERNATIONAL DAIRY FEDERATION

Brussels, June 2 - The International Dairy Federation announced today the appointment of Mr Christian Robert as its new Director General.

Mr Robert has more than 20 years' experience of the international dairy industry and has held management positions at the highest level. He served 12 years in the senior management team of Nestle and most recently was Managing Director, Europe with the Bulmer's Group. Mr Robert also has extensive experience of trade associations and representative organisations.

Mr Robert, is a Belgian who is married with four children. He will take up his post on September 1. He succeeds Mr Edward Hopkin who is retiring as Director General after 25 years of service with the IDF.

BOOKS

Air quality standards in food production areas

Air is a potential source of food contaminants, but help is at hand to minimize the risk of contamination via this route. A new edition of CCFRA's well-established "*Guidelines on air quality standards for the food industry*" provides extensive practical guidance for food and construction companies on the installation, monitoring and maintenance of air quality systems to help prevent food contamination and assure product safety.

Devised by experts drawn from the food, air handling and research communities, the new edition spans the complete air handling chain from identifying the design and type of system most appropriate to particular food production operations through construction and validation to maintenance, cleaning, monitoring and assessing environmental impact.

The guide is one of a series to help technical personnel in the food and construction industries with hygienic aspects of building or refurbishment of food production facilities. The other titles in the series are: *Guidelines for the hygienic design, construction and layout of food processing factories; Guidelines for the design and construction of floors for food production areas* (second edition); and *Guidelines on the design and construction of walls, ceilings and services for food production areas* (second edition).

Further information or copies of this document can be obtained from Mrs. Sue Hocking, CCFRA, Chipping Campden, Glos. GL55 6LD (UK) - Tel. +44(0)1386 842225 - Fax +44(0)1386 842100 - e-mail: pubs@campden.co.uk

IDF Research highlights need for global standards

The increase in international trade must be matched by an increasing commitment to the implementation of uniform standards in milk and milk products, according to the International Dairy Federation.

The IDF has published *"Method Standardization in the Third Millennium"* a comprehensive research document, which presents the latest scientific thinking on performance improvement.

The symposium organized during the IDF/ISO/AOACInternational Analytical Week 2004, in May in Parma (Italy), covered the performance of methods, the reliability of the results and the never ceasing quest for improvement. This bulletin features presentations delivered at the Symposium.

The publication *"Method Standardization in the Third Millennium"* is the new Bulletin of IDF n° 395/2005. It can be ordered in electronic form via the IDF Website at 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 **two** copies submitted along **with** the computer disk. There should be liberal margins on top, bottom and sides (2.5 cm). English is the official language. Authors who are not fluent in written English must seek help from a person fluent in scientific English. The Assistant Editor reserves the right to make literary corrections and to make suggestions to improve brevity, but **the paper must be revised by a native English speaker** before submission.

Pages and lines on all pages, including those pages for "References" and figure legends, **must be electronically numbered in the left margin**, beginning with number one at the top of the page.

The paper must also be submitted by e-mail or on a digital support (cd-rom or floppy disk). Indicate which word processor was used to generate the file and save the file also in format "Text only", DCA-RTF or ASCII, if you do not have programs for Macintosh; **graphics, pictures and diagrams must be saved at 300 dpi in TIF, JPEG, EPS or PICT formats** (not included in MsWord documents).

(2) Every paper should be divided under the following headings in this order:

Title. Informative of the content of the article (<50 characters + spaces). Author(s). Initials and Surname, omit professional and official titles. The Institute and address where the research was carried out and the current address of each author should be given as a footnote on the title page.

Abstract. Clearly state the objective of the study, give a concise description of experiment(s), observations, results and conclusions. No references should be cited. DO NOT EXCEED 100 WORDS. An abstract and title in Italian (corresponding to the English) must also be included.

Keywords. Up to six words, in alphabetical order, which describe the document must be given to aid data retrieval and indexing.

Introduction. Review pertinent previous work and cite appropriate references. State the purpose of the investigation.

Materials and Methods. Indicate apparatus, instruments, reagents, etc., giving sufficient detail to allow the work to be repeated.

Results and Conclusions. Results and Conclusions may be presented together or separately. Concisely present results using tables and figures to help justify conclusions (do not present the same information in both forms). Use statistical analysis when appropriate. Unsupported hypotheses should be avoided. Conclusions should point out the significance of the findings and, if possible, relate the new findings to some problem in Food Science and Technology.

Acknowledgments. Acknowledgments of assistance are appropriate provided they are not related to analyses, or other services performed for a fee. Financial support, thanks for assistance, article number or thesis fulfillment may be included.

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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

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

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CONTRIBUTORS

Gratitude is expressed to the following entities for contributing to the realization of the Journal by being supporting subscribers for 2005.

Si ringraziano i seguenti Enti, Ditte ed Istituti per aver voluto contribuire fattivamente alla realizzazione della Rivista, sottoscrivendo un abbonamento sostenitore per il 2005.

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ITALIAN JOURNAL OF FOOD SCIENCE
Rivista Italiana di Scienza degli Alimenti
DIRETTORE RESPONSABILE: Giovanni Chiriotti
AUTORIZZAZIONE: n. 3/89 in data 31/1/1989
del Tribunale di Perugia
Proprietà dell'Università di Perugia
TIPOGRAFIA Giuseppini - Pinerolo
Una copia € 6.00

ISSN 1120-1770 © 2005

CHIRIOTTI EDITORI sas - 10064 Pinerolo - Italy

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