VOLUME XV

IJFS

No. 1, 2003

CONTENTS

REVIEW

Present and Potential Applications of Membrane Processing in the Food Industry **3**
M. Moresi and S. Lo Presti

PAPERS

Eating Behavior and Risk Factors for Overweight and Obesity in School Children in Liguria (Italy) **35**
S. Ciangherotti, D. Risso, R. Rondinone, E. Ciangherotti, S. Menoni, S. Sensi and P.B. Lantieri

Analytical Methods for the Determination of the Geographic Origin of Emmental Cheese. Parameters of Proteolysis and Rheology **49**
L. Pillonel, B. Albrecht, R. Badertscher, U. Bütikofer, J.F. Chamba, R. Tabacchi and J.O. Bosset

The Effect of Mesophilic Starter Culture Composition on the Volatile Compounds of Raw Ewe Milk Cheese **63**
J.A. Centeno, P. Morales, E. Fernández-García, P. Gaya and M. Nuñez

Influence of Cheese Weight and Type of Rennet on Composition and Proteolysis of Canestrato Pugliese Cheese II. Chromatographic Characterization of Soluble Nitrogen **75**
M. Faccia, G. Gambacorta, V.A. Liuzzi, G. Alviti and A. Di Luccia

The Effect of Heat-Induced Structural Changes on the Prooxidant Activity of Myoglobin **85**
A. Berisha, E. Yasushi and K. Fujimoto

Microbiological Quality of Minced Beef from Butcher Shops and Supermarkets **95**
M. Kammenou, J. Metaxopoulos and E.H. Drosinos

Effect of Polydextrose and NaCl on the Initial Freezing Point of Chicken, Egg and Surimi **105**
D. Kovacevic, Z. Kurtanjek and D. Subaric

Inactivation of Microorganisms by Supercritical CO_2 in a Semi-Continuous Process **115**
S. Spilimbergo, N. Elvassore and A. Bertucco

SHORT COMMUNICATIONS

Chemical and Microbiological Characteristics of Ewe Milk Cheese (Los Pedroches) Made with a Powdered Vegetable Coagulant or Calf Rennet **125**
L. Tejada and J. Fernández-Salguero

Carotenoid Profile of Sweet Orange and Mandarin Essential Oils **133**
I. Bonaccorsi, A. Verzera, A. Trozzi, M. Zappalà, P. Dugo and L. Mondello

Decline Rates of Chlorpyrifos Residues in Green Beans Grown in Plastic Greenhouses **141**
A. Aguilera del Real, M. Rodríguez Sánchez and A. Valverde García

A GC/MS Method for the Routine Determination of Acrylamide in Food **149**
F. Tadeo and M. Bononi

FLAIR FLOW **153**
NEWS **158**
BOOKS **160**
GUIDE FOR AUTHORS **161**

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REVIEW

PRESENT AND POTENTIAL APPLICATIONS OF MEMBRANE PROCESSING IN THE FOOD INDUSTRY

APPLICAZIONI ATTUALI E POTENZIALI DEI PROCESSI A MEMBRANA NELL'INDUSTRIA ALIMENTARE

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ABSTRACT

This paper reviews the most recent innovations in membrane modules and/or processes that appear to affect the food (dairy products, fermented and alcoholic beverages, fruit juices, vegetable oils, etc.) and drinks (potable water and soft-drinks) industries in the short-medium term. Basic mass transport equations and the general relationship between solvent permeation flux and solute concentration are examined as a function of the separation range of membranes. Future perspectives for membrane processing in the food sector are also outlined.

RIASSUNTO

In questo lavoro si presenta una breve rassegna sulle più recenti innovazioni nei moduli e nei processi a membrana che appaiono a breve-medio termine di particolare interesse per il settore alimentare (lattiero-caseario, enologico, oleario, dei succhi di frutta, delle bevande analcoliche ed alcoliche, acqua potabile inclusa). Si esaminano anche le principali equazioni di trasporto dei soluti e del solvente e la generica correlazione tra il flusso di permeazione del solvente e la concentrazione del soluto in funzione del tipo di membrana utilizzata. Viene infine prospettato lo sviluppo di nuovi processi a membrana per il settore alimentare.

- Key words: classification, design parameters, food and drinks industry, membrane processing, modelling -

INTRODUCTION

Membrane separation processes, as developed starting from the pioneering research work by LOEB and SOURIRAJAN (1963), have only recently begun to be recognised as efficient, economical and reliable separation processes. For years membranes have existed as products in search of the right markets. Now the markets exist and range from water purification, ultra-pure chemicals, gas separation and electrochemistry to biotechnology and foods and cosmetics. The market is unevenly distributed with 75% of the share belonging to the USA, Japan and Western Europe.

In 1999 the world-wide membrane sales was of the order of US$2 billion (http://www.tifac.org.in/news/memb.htm). As shown in Table 1, membrane applications in medical devices and water treatment accounted for about 55 and 29% of the total membrane market, respectively, while as little as 9% was directed towards the food industry. Since membranes account for only 40% of the total investment for a membrane separation system, the total annual turnover for the membrane-based industry was estimated as close to US$5 billion (http://www.tifac.org.in/news/memb.htm). Moreover, according to the latest news release by the McIlvaine Company (Northbroke, IL, USA) membrane sales will rise from US$5.2 billion in 2002 to US$7 in 2006 with an average annual growth rate in the range of 6-7.8%. This is due to the growing market in seawater desalination and municipal water purification, despite the present depression in the semi-conductor and power market (http://www.mcilvainecompany.com/news%20releases/NR755.htm).

The advantages of membrane processes are multiple. Unlike other mechanical separations units (i.e. centrifugation, conventional filtration, screening, sedimentation with coagulants), membrane separation processes involve relatively low energy requirements, no additives, generally mild operating conditions and easy scaling-up rules. Membranes can be specifically tailored to match practically any end user requirement and this allows not only the generic solute to be recovered, but also a practically zero total organic carbon effluent to be formed and reused or disposed of without further treatment.

Despite all these advantages, membrane processing potentialities have not been completely exploited, probably because of the high specific membrane costs or short membrane lifetime (no longer than one year, especially if membrane separation plants have not been well designed or are not properly conducted).

Table 1 - Major applications and sales (in million US$ per year) of membrane processes in 1999 (http://www.tifac.org.in/news/memb.htm).

Application / Membrane Process	Water Treatment	Food Industry	Medical Devices	Chemical & Pharma	Total
Microfiltration	310	95	20	35	460
Ultrafiltration	60	44	130	15	249
Reverse Osmosis	120	15	-	10	145
Electrodialysis	60	15	-	20	95
Dialysis	-	-	900	-	900
Gas Separation	-	-	-	45	45
Pervaporation	-	-	-	5	5
Total	550	169	1050	130	1899

The aim of this paper was to review the most recent product and/or process innovations that appear to be of particular interest to the food and drinks sector in the short-medium term, as well as the basic mass transport equations that might help membrane module designing and engineering.

CLASSIFICATION OF MEMBRANE PROCESSES

As shown in Table 2, membrane processes can be distinguished as dialysis (D), electrodialysis (ED), gas separation (GS), pervaporation (PV), or filtration processes depending upon the driving force (i.e. chemical, electric voltage or pressure potential differences) used to affect species separation. Filtration processes are further classified as microfiltration (MF), ultrafiltration (UF), nanofiltration (NF), and reverse osmosis or hyperfiltration (RO) depending on membrane pore size, feed cross flow velocity, transmembrane pressure difference applied (ΔP) and permeation flux. For instance, MF is designed to retain suspended particles in the range 0.1-10 μm; UF macromolecules (proteins and polysaccharides) or particles larger than 1-20 nm; NF polyvalent ions, sugars and dissociated forms of several organic acids; while RO monovalent ions and undissociated acids (HO and SIRKAR, 1992; CHERYAN, 1998). Their main alternative conventional technologies are also given.

To characterise the filtration capability of a given membrane, especially with UF membranes, it is customary to refer to the nominal molecular weight cut-off (NMWCO), which represents the molecular weight of a test solute (generally a globular protein or a linear polysaccharide, such as albumin or dextran) that is 90% rejected by the membrane under standard conditions. The NMWCO rating should be regarded just as a rough guide, since it depends on the form and chemical structure of the macromolecules involved. At constant molecular weight, linear molecules will have a greater probability of permeating through the membrane than globular ones; on the contrary, molecules tending to fold will be more easily rejected.

In MF processes the filtration capability is generally expressed in terms of mean pore diameter, which ranges from 0.1-1.5 μm. Owing to the greater membrane pore size in MF, water permeation flux (500-1,000 $dm^3\ m^{-2}\ h^{-1}$) is generally by far greater than that in RO membranes (15-60 $dm^3\ m^{-2}\ h^{-1}$). Even the operating transmembrane pressure difference, ΔP, is quite different and is reduced from 40-100 bar in RO processes (to counteract the osmotic pressure of the solution undergoing concentration) to 0.3-4 bar in MF processes (Table 2).

The terminology used to describe pressure-driven membrane processes was reviewed by GEKAS (1988). The main operating variables are: feed flow rate (Q_F) and solute concentration (C_B), transmembrane pressure difference (ΔP), temperature (T), and pH, as well as suspended solid concentration (C_S). Any membrane process gives rise to two streams: the permeate and retentate (or concentrate), that is the feed fractions passing through or rejected by the membrane. A scheme of a typical membrane operation is shown in Fig. 1 and its performance can be assessed by accounting for some of the indicators listed in Table 3.

MEMBRANE MORPHOLOGY, MATERIALS, CONFIGURATION AND CLEANING PROCEDURES

Membrane structure can be isotropic or anisotropic (or asymmetric) with pores of uniform or changing size throughout the body of the membrane. The isotropic structure is characterised as cylindrical porous, porous or homogeneous, while

Table 2 - Characteristics of membrane processes.

Separation Process	Membrane Type	Driving Force	Mechanism for Transport	Application	Alternative Technologies
Dialysis D	Symmetrical microporous	Concentration Gradient	Diffusion	Salt & micro-solute separation	Liquid Extraction Adsorption
Electrodialysis ED	Anion- & cation- exchange membrane	Electrical Potential Gradient	Counter-ion transport	Ion separation	Ion-Exchange
Gas Separation GS	Asymmetrical Homogeneous Polymer	Pressure and Concentration Gradient	Solution- Diffusion	Gas Mixture Separation	Cryogenic Distillation
Pervaporation PV	Polymeric non-porous	Partial Pressure Gradient	Solution- Diffusion	Organic & Azeotropic Mixture Separation	Azeotropic or Extractive Distillation
Microfiltration MF	Symmetrical Asymmetrical 0.1-10 μm	Pressure Gradient 0.3-4 bar	Molecular Sieving	Suspended Particle Separation Sterile Filtration Clarification	Filtration Centrifugation Sedimentation Flotation
Ultrafiltration UF	Asymmetrical Microporous 1-10 nm	Pressure Gradient 0.5-10 bar	Molecular Sieving	Macromolecule & Virus Separation	Coagulation Flocculation Chromatographic Techniques
Nanofiltration NF	Asymmetrical	Pressure Gradient 10-40 bar	Solution- Diffusion	Polyvalent Ion, Dissociated Acid & Microsolute Separation	Multiple Effect Evaporation Mechanical Vapour Compression
Reverse Osmosis RO	Asymmetrical skin-type 0.5-1.5 nm	Pressure Gradient 40-100 bar	Solution- Diffusion	Monovalent Ion & Microsolute Separation	Multiple Effect Evaporation Mechanical Vapour Compression

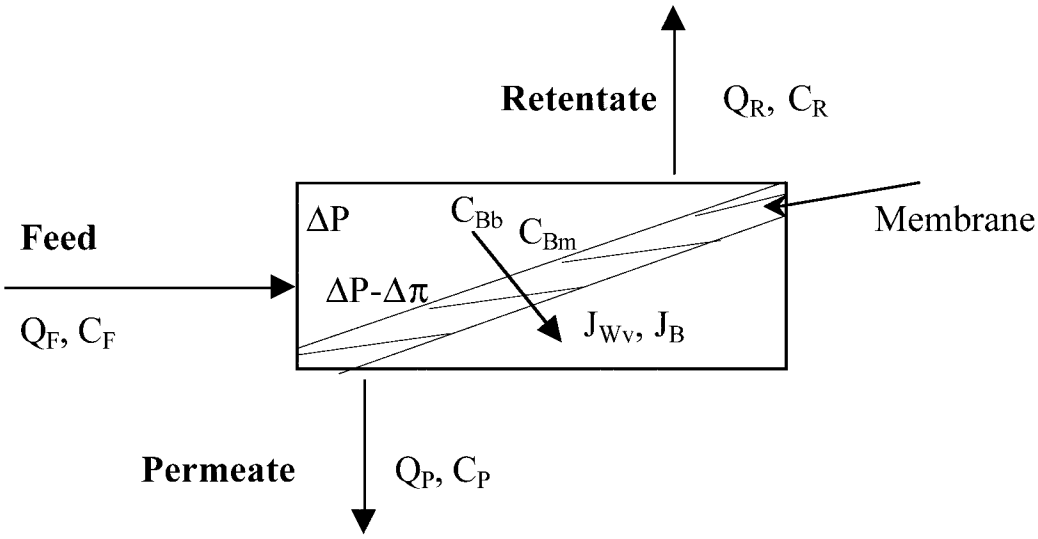


Fig. 1 - Membrane process scheme: all symbols are listed in the *Notation* section.

Table 3 - Main indicators used to describe the performance of membrane processes.

Parameter	Equation
Solute rejection (r)	$r = \frac{Q_R C_{BR}}{Q_F C_{BF}}$ { observed apparent solute rejection: $r_a = 1 - \frac{C_{BP}}{C_{BR}}$; intrinsic or true solute rejection: $r_t = 1 - \frac{C_{BP}}{C_{Bm}}$
Volumetric (J_{Wv}) permeation flux	$J_{Wv} = \frac{Q_P}{A_m}$
Solvent recovery (SR)	$SR_b = \frac{\sum J_{Wv} A_m \Delta t}{V_F}$ $\quad SR_c = \frac{J_{Wv} A_m}{Q_F}$
Volume (VCR) concentration ratio	$VCR = \frac{\text{Initial Feed Volume } (V_F)}{\text{Retentate Volume } (V_R)}$
Volume Permeate-to-Feed Ratio or Percent water removed	$VR = \frac{V_F - V_R}{V_F} \times 100 = \frac{V_P}{V_F} \times 100$

the asymmetric one consists of a very dense top layer (i.e. thin film composite, TFC, membranes) or skin (i.e gas separation membranes) with a thickness of 0.1-0.5 m, attached to a thicker 50-200 m porous sub-layer to confer adequate mechanical resistance to the membrane.

Membrane materials range from cellulose acetate (CA) to several polymers, such as polyamide (PA), used for hollow-fibres RO, UF and MF membranes; polysulfone (PS), widely used as a basic polymer for UF membranes and support for TFC RO and NF membranes; polyvinylidene fluoride (PVDF), a fluor-based polymer used for manufacturing UF and MF membranes more tolerant to chlorine than PS membranes; polytetrafluoroethylene (PTFE), mainly used for specific MF membranes; polypropylene (PP), mainly used as MF and GS membranes; polyacrylonitrile (PAN), polyesters (PE), etc. Homogeneous cellulose-based 10-100 μm thick membranes are used as dialysis membranes, while non-porous, usually homogeneous, polystyrene cross-linked with 100-500 μm thick divinylbenzene membranes are used as monopolar electrodialysis membranes.

Inorganic (or mineral or ceramic) membranes are mostly used for MF and UF applications and are formed by combining metals, such as aluminium, titanium or zirconium, with a non-metal in the form of an oxide, nitride or carbide.

Further details are given by CHERYAN (1998), DAUFIN *et al.* (1998), HO and SIRKAR (1992) and NIELSEN (2000).

Membranes can be assembled in more or less complex multiple structures using 4 different modules, the configuration of which can be tubular, hollow-fibre, spiral-wound, and plate-and-frame.

Tubular membrane systems may consist of one or more than one tubular membrane with internal diameters ranging from 6 to 25 mm, each encased in a fibreglass-reinforced epoxy porous support fixed to two terminal plates as in a classic shell-and-tube heat exchanger. A typical tubular membrane module consists of a bundle of 18 tubes, each with a diameter of 14.1 mm, encased in a larger cylindrical housing (144 mm in diameter and 1.3 m long) with a nominal membrane surface area of 1 m^2. These modules can be arranged in either series or parallel configuration in the case of low feed flow rates or high suspended solid concentrations, respectively. Removal of reversible fouling can be achieved by using several chemical or mechanical methods. The former generally consists of a series of sequential recirculation cycles of 30 min each with neutral (deionised water), alkaline (NaOH at pH 10-11) and acidic (HNO_3 at pH 2.4) solutions at ca. 50°C. On the contrary, the latter relies on the erosion action exerted by numerous sponge rubber balls, which are slightly larger than the tube diameter and are suspended in the cleaning liquid.

Being less prone to foul and easier to clean, these modules can process slurries containing high levels of suspended particles (i.e. tomato squash); however, they suffer from the disadvantages of high specific costs and replacement costs for membrane tubes. Actually, such a replacement cost should no longer be taken into account, since the porous supports can be returned to the manufacturer and reused by replacing any irreversibly fouled or broken membrane with a new one.

Hollow-fibre membrane systems differ from tubular ones for their smaller inner diameters that range from 0.2 to 1 mm. These modules are very densely packed and, being extremely sensitive to fouling and plugging, are recommended when the feed suspended solid concentration is less than 1% w/w. To compensate for reversible fouling, these systems may be periodically cleaned by backflushing. If they are fabricated from polyethersulfone (PES), hollow-fibres can be sterilised with water at about 100°C under pressure.

Spiral-wound elements are the most compact and inexpensive designs available today. They are produced by winding spirally two flat sheets (separated from each other by a thin, mesh-like polypropylene material, glued together on three sides) around a perforated central tube and inserting them into a cylindrical cartridge. These elements combine the advantages of tubular (high cross-flow velocities) and plate-and-frame ones (easy manufacture), while their disadvantage is that the entire filtering element is not suited for handling viscous solutions or thick slurries and has to be discarded once fouled.

Spiral-wound systems may consist of a single module or 2-6 modules assembled in series within cylindrical cartridges (50.8-304.8 mm diameter and 0.15-1.52 m long). Pressure drop through the single or multi-leaf module can be controlled up to a maximum of 3 bar by using 0.76-2.29 mm thick spacers and regulating the feed flow rate. In this way, it is possible to affect the state of turbulence within the feed channel and thus to control the thickness of the polarisation layer.

Nowadays, any module is equipped with antitelescoping devices to prevent the components of these systems from being pushed out in the direction of flow when the feed flow rate exceeds the recommended rate.

Plate-and-frame modules consist of a series of rigid, flat plates on which rectangular or disk-shaped flat sheet membranes are placed. A mesh-like material is placed between each membrane sheet and plate and, sometimes, between the membrane sheets themselves to provide channels for permeate and retentate flow, respectively. Despite the fact that De Danske Sukkerfabbriker (DDS) plate-and-frame systems had been widely used in the food and dairy industries in the 1970s and 1980s, they are no longer manufactured. The Pleiade® system developed by Orelis S.A. (Miribel, France: http://www.orelis.com) consists of rectangular ceramic plates with a membrane surface area of 0.35 m^2 each, which are assembled in a horizontal position like in a plate heat exchanger using sealing rings and gaskets to make the system work up to 6 bar (NIELSEN, 2000). The parallel leaf system launched by Dorr-Oliver (Milford, Connecticut, USA) consists of one to 12 cartridges with an overall membrane surface area of 15.6 or 25.2 m^2 depending on the channel height. Each cartridge is composed of several rigid, rectangular plastic plates having on both faces a membrane, a grid and a support (scrim) to allow the permeate to be discharged from a unique central collecting port (NIELSEN, 2000). Another system composed of ceramic membrane plates shaped as cassettes and set up between two metallic plates (KéRAM) was developed by TAMI Industries S.A. (Nyons, France: http://www.tami-industries.com; //www.najade.com), as described by GRANGEON and LESCOCHE (2000).

A more detailed description of these modules is reported in the technical literature (CHERYAN, 1998; DAUFIN *et al.*, 1998; HO and SIRKAR, 1992; NIELSEN, 2000), as well as in the Website of membrane manufacturers in Europe, Japan, USA and other countries: http://www.tcn.zaq.ne.jp/membrane/english/MembManufE.htm.

During the conventional mode of operation of MF and UF modules, the solvent permeation flux tends to be reduced owing to the accumulation of material deposited on the membrane surface (Fig. 2a). Periodic backwash (PBW) can be operated by pumping permeate back into the feed channel to lift deposited particles off the membrane surface (Fig. 2b). Permeate is generally compressed at 1-10 bar for 1-5 s at a frequency of one to ten per minute. Such in-use cleaning procedure has been found to be efficient in plate-and-frame modules operating at low cross-flow velocities, but useless in

tubular ones operating at cross-flow velocities greater than 4 m/s (LO PRESTI and MORESI, 2000a). Lately, new backwashing techniques based on compressed air sparging instead of permeate recycling have been set up to avoid significant reduction in the net permeation flux.

Co-current permeate flow (CPF) is another mode of operation aimed at minimising the formation of the secondary membrane of polarised particles by keeping the transmembrane pressure difference practically constant throughout the module and as low as possible to minimise compacting of the polarised layer (Fig. 2c). Such a mode of operation (termed Bactocatch by Alfal Laval) has been found to be quite efficient in the case of skim milk, the average water permeation flux being increased up to 500-900 $dm^3\ m^{-2}\ h^{-1}$ (CHERYAN, 1998).

In plate-and-frame modules, membrane fouling can be greatly limited by applying periodic oscillations of 19 to 32 mm peak to peak to the filter pack where the membranes are arrayed as parallel discs and separated by gaskets, as in the so-called Vibratory Shear-Enhanced Processing systems (New Logic International, Emeryville, CA, USA: http://www.vsep.com) and PallSep™ PS10 or 1000 Membrane systems (Pall Corp., East Hills, NY, USA: http://www.pall.com), or spinning the membrane configured disks near a series of stationary elements, as in the Cross Rotational Membrane Systems (ELIAS and van CLEEF, 1998) and Centrifugal Membrane Filtration systems (SpinTek Filtration Systems, Huntington

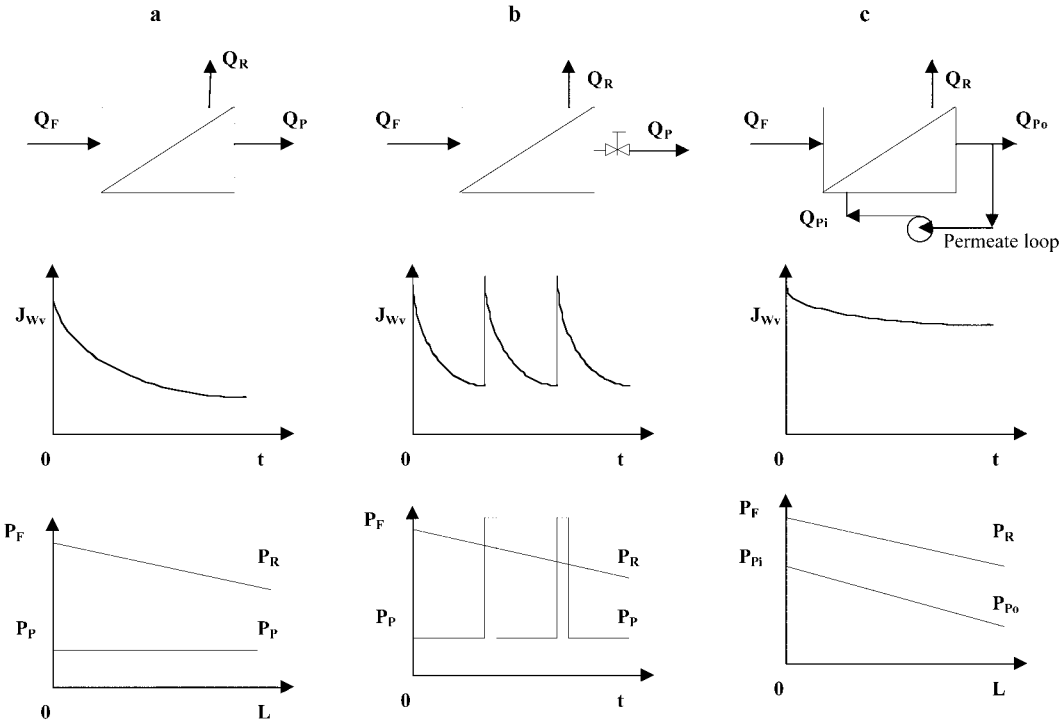


Fig. 2 - Time course of the solvent permeation flux (J_{Wv}) and effect of module length (L) or time (t) on the absolute pressure (P) at both sides of a membrane module operating in the conventional batch mode (**a**), under periodic back-wash (**b**) or co-current permeate flow (**c**), as extracted from CHERYAN (1998).

Beach, Ca, USA: http://www.spintek-systems. com). The latter can use polymeric and ceramic MF or UF membranes. In the case of ceramic membranes, they are rated to perform in a pH range of 1-14 and up to 90°C. In particular, they have been successfully used to recover 90% of whey protein from whey and thicken baker's yeast suspensions up to 35% (w/w) (http://www.spintek-systems.com).

TRANSPORT MODELLING

The numerous transport models cited in the literature (BHATTACHARYYA and WILLIAMS, 1992) can be divided into three types: non porous or homogeneous membrane models (i.e. solution-diffusion, extended solution-diffusion, and solution-diffusion-imperfection models), pore-based models (preferential sorption-capillary flow, finely porous, and surface force-pore flow models), and irreversible thermodynamics phenomenological models (such as Kedem-Katchalsky and Spiegler-Kedem models). For nanofiltration and electrodialysis, Donnan exclusion models and the extended Nernst-Planck model can be used to determine solute fluxes. Table 4 lists the models most widely used to describe solvent (J_{Wv}) and solute (J_B) permeation fluxes through semi-permeable membranes.

All these models are special cases of the statistical-mechanical model which was described by MASON and LONSDALE (1990). In such models permeate and rejection can be described by no more than 4 different parameters, which have constant values for any separation system involving the same solution, membrane, temperature and superficial flow velocity across the membrane surface. Temperature, as well as superficial flow velocity, is often constant in industrial membrane processes and only pressure and concentration are generally regarded as operating variables. Moreover, for the case of σ=1, that is, in absence of solute and solvent flow coupling, the Kedem-Katchalsky model reduces to the solution-diffusion model, which accounts for just two parameters A_D and B (Table 4). For B tending to zero, solute rejection tends to be unitary, thus allowing the membrane process under study to be described by a single parameter, that is, by only the membrane constant (A_D) for water transport by diffusion. NIEMI and PALOSAARI (1993) described a method to assess the only statistically significant transport parameters by performing a minimum number of experiments.

Despite the fact that MASON and LONSDALE (1990) retained that the traditional filtration theory is able to describe MF adequately, analysis of cross-flow microfiltration is complex and the interpretation of flux decline is complicated. In fact, the steady-state cake thickness and solvent permeation flux are governed by the concentration polarisation layer adjacent to the cake of rejected particles accumulated on the membrane surface. As the characteristic particle size increases from 0.1 to 10 μm and the tangential shear rate is increased, Brownian diffusion, shear-induced diffusion or inertial lift is regarded to be the dominant mechanism for particle back transport in the polarisation layer (DAVIES, 1992a). Nevertheless, both the short- and long-term flux declines (occurring over time scales of hours or days due to cake building or membrane fouling, respectively) may be described by Darcy's law with the two membrane resistance terms R_f and R_g (Table 4) being time and shear rate dependent (DAVIES, 1992b).

Finally, as far as modelling of transport of ions through electro-membranes is concerned, the Nernst-Planck relationship shown in Table 4 can be simplified by neglecting both ion diffusion and osmotic water transfer with respect to the ion flux through the membranes due to the applied electric field. Therefore, the

Table 4 - Main models used to describe solvent (J_{Wv}) and solute (J_B) permeation fluxes through semi-permeable membranes.

Model	Equation	Ref.
Solution-diffusion	$J_{Wv} = A_D (\Delta P - \Delta\pi)$ $J_B = B (C_{Bm} - C_{Bp})$ $r_t = \left[1 + \frac{B}{A_D} \frac{1}{\Delta P - \Delta\pi}\right]^{-1}$	LONSDALE *et al.* (1965)
Solution-diffusion-imperfection	$J_{Wv} = A_D (\Delta P - \Delta\pi) + A_P \Delta P$ $J_B = B (C_{Bm} - C_{Bp}) + A_P \Delta P C_{Bm}$ $r_t = \left[1 + \frac{B}{A_D} \frac{1}{\Delta P - \Delta\pi} + \frac{A_P}{A_D} \frac{\Delta P}{\Delta P - \Delta\pi}\right]^{-1}$	SHERWOOD *et al.* (1967)
Porous medium	$J_{Wv} = \frac{\varepsilon d_p^2 (\Delta P - \Delta\pi)}{32 \eta \Delta x}$	BIRD *et al.* (1960)
Finely porous model	$J_{Wv} = \frac{(\Delta P - \Delta\pi)}{A_1 C_{BP} + A_2 \eta}$ $r_t = 1 - \left[C_1 - (1 - C_1) e^{-\frac{C_2 J_{Wv}}{D_{SW}}}\right]^{-1}$	JONSSON and BOESEN (1975)
Membrane Resistance	$J_{Wv} = \frac{\Delta P - \Delta\pi}{\eta (R_m + R_f + R_g)}$	CHERYAN (1998)
Irreversible thermodynamics	$J_{Wv} = L_p (\Delta P - \sigma \Delta\pi)$ $J_B = B (C_{Bm} - C_{Bp}) + C'_{Bm} J_{Wv} (1 - \sigma)$ $C'_{Bm} = (C_{Bm} - C_{Bp}) / \ln(C_{Bm}/C_{Bp})$ $r_t = \frac{\sigma (e^{Pe} - 1)}{e^{Pe} - \sigma}$ $Pe = \frac{(1 - \sigma) J_{Wv}}{D_P}$	KEDEM and KATCHALSKY (1958); MASON and LONSDALE (1990); SPIEGLER and KEDEM (1966)
Nernst-Planck	$J_{\pm} = J_{Wv} c_{\pm} + z_j c_{\pm} D_{\pm} \frac{F E}{R T} - D_{\pm} \frac{dc_{\pm}}{dx}$	NEWMAN (1973)

anion or cation permeation flux through anion- or cation-exchange membranes can be regarded as simply proportional to the corresponding transport number in the membrane concerned as well as the applied current density (BONIARDI *et al.*, 1997).

Despite the fact that thus far no commercial membrane system in the food industry has been designed and installed on the basis of theoretical approaches, all experimental trials tested on the bench- or pilot-plant scale have been simulated by using one of the transport

models listed in Table 4, which are regarded as useful tools for membrane system designing and engineering.

In the case of RO membrane processes characterised by solute rejection coefficients not smaller than 0.99, the membrane resistance model (CHERYAN, 1998), as modified by replacing the viscosity of pure solvent with that of the retentate, was found to be the simplest and most accurate model to fit solvent flux against feed input pressure, temperature and solute concentration, as for instance as observed in the recovery of a few sodium salts of citric, gluconic, itaconic and lactic acids from aqueous model solutions (LO PRESTI and MORESI, 2000b).

In the case of NF membrane processes with solute rejection coefficients quite smaller than 0.95, the Kedem-Katchalsky model, as modified by SPIEGLER and KEDEM (1966), allowed a quite good simulation of solvent permeation flux and solute rejection during the recovery of ammonium fumarate from aqueous solutions (MORESI *et al.*, 2002).

In the case of tubular ceramic MF or UF membrane processes, the membrane resistance model (CHERYAN, 1998), characterised by a fouling resistance (R_f) varying as a power function of the feed superficial velocity, enabled the baker's yeast or sodium alginate recovery from model solutions to be accurately described (LO PRESTI and MORESI, 2002).

Finally, in the case of ED processes, the aforementioned simplified form of the Nerst-Planck model together with the experimental procedure outlined by FIDALEO (2002) to recover sodium lactate from aqueous solutions using cation- and anion- exchange membranes, can be recommended to determine the real independent process and design parameters (i.e. cation, anion, and water transport numbers in electro-membranes, as well as membrane electro-resistances) of an ED stack.

EFFECT OF THE MEMBRANE SEPARATION RANGE ON SOLVENT PERMEATION FLUX

Generally speaking, solvent permeation flux (J_{Wv}) varies according to the separation range of membranes (Fig. 3a). The linear relationship between J_{Wv} and ΔP with reverse osmosis becomes much more problematic either in the UF or MF

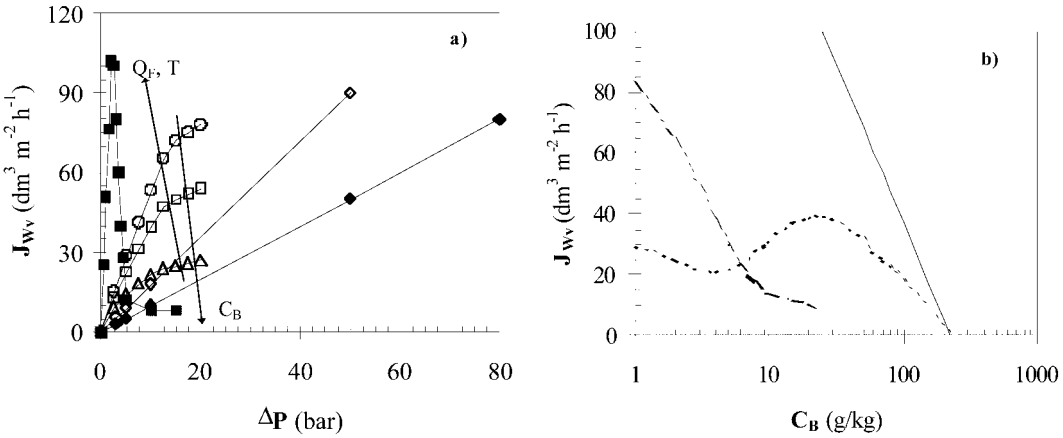


Fig. 3 - Solvent volumetric permeation flux (J_{Wv}) against (a) the transmembrane pressure difference (ΔP) or (b) the logarithm of solute concentration (CB) for RO (—, ♦), NF (◊), UF (---,—·—, Δ, □, ○), MF (■) membranes. For UF membranes only, the J_{Wv}-ΔP relationship was plotted as a function of feed flow rate (Q_F), C_B or temperature (T).

range. In the former, J_{Wv} is controlled by pressure for $\Delta P<6$ bar and by mass transfer for $\Delta P>6$-10 bar, this effect being counteracted by increasing feed flow rate (Q_F) or process temperature (T), as well as decreasing feed solute concentration (C_B). In the latter, J_{Wv} increases for $\Delta P<1$bar, but abruptly decreases for $\Delta P>1$-2 bar owing to the progressive consolidation of the particle or gel polarisation layer on the surface of the membrane.

Moreover, for RO systems the J_{Wv}-logC_B plot exhibits a typical, almost linear decreasing trend with J_{Wv} vanishing at C_B values corresponding to an osmotic pressure in the retentate equals to the feed input pressure, as shown by the continuous line in Fig. 3b, as extracted from LO PRESTI and MORESI (2000b). On the contrary, for the UF systems the J_{Wv}-logC_B plot does not go to zero, but it may reach a minimum value (definitively different from zero), which remains practically constant (see the dash-and-dot line in Fig. 3b), or increases up to a maximum value before finally decreasing as the solute concentration increases (see the broken line in Fig. 3b).

The former was observed in the case of pectin concentration in a tubular module (12.5 mm diameter, 65 kDa NMWCO membrane) under laminar flow at pectin concentrations ranging from 8 to 20 g/kg; whereas the latter was observed in the UF of xanthan dispersions in a flat-sheet module equipped with 250 kDa NMWCO membranes under laminar flow as xanthan concentrations ranged from 5 to 20 g/kg or from 20 to 250 g/kg (PRITCHARD *et al.*, 1995).

MAIN MEMBRANE APPLICATIONS

Table 5 lists the main applications of membrane processing in the food and beverage sector together with specific membrane type and configuration, range of solvent flux and solute rejection. A few selected examples will be briefly pointed out in the following sections.

Brackish and seawater desalination

The first (and still widest) application of reverse osmosis was brackish or seawater desalination. In 1992 RO processes yielded about 23% of the 11.5 million m^3 of drinking water produced daily worldwide by all desalination plants (WILLIAMS *et al.*, 1992). Presently, RO's share is fast approaching 50% of the 20 million m^3/day of water produced by about 10,000 seawater desalination plants (PARKINSON *et al.*, 1999). Originally, RO membranes found wide use in cleaning up brackish waters with salt contents of 1.5-3 kg m^{-3} in competition with ED membranes (STRATHMANN, 1992); both these processes are too expensive for seawater that has a salt level ranging from 35 kg m^{-3} (in major oceans) to 46 kg m^{-3} (in the Persian Gulf).

The development of new, more efficient and less expensive RO modules has made RO seawater desalination plants competitive with multistage flashing evaporation (MFE) and mechanical vapour compression (MVC) (PARKINSON *et al.*, 1999). In fact, the operating costs of a new RO desalination plant producing ca. 1900 m3/day of industrial water (with total dissolved solids less than 10 g m^{-3}) were about €0.9 per m^3 against a cost of €1.2 per m^3 in the case of MVC plants (PARKINSON *et al.*, 1999). Although MFE is still the process most widely used to yield drinking water, it is claimed that RO will tend to be competitive with distillation on any scale because of its main advantages, such as reduced energy consumption, lower investment and operating costs (owing to minimum steam requirement), room temperature operation, minimum corrosion problems, reduced lay-out, ease of construction, etc.

Fig. 4 shows a simplified scheme of a modern RO seawater desalination plant, as extracted from PARKINSON *et al.*

Table 5 - Main applications of membrane processes in the food sector.

Membrane process	Application	Example	Membrane module characteristics				
			Type	Material	Cut-off	J_{Wv} ($dm^3\ m^{-2}\ h^{-1}$)	r_t (%)
MF	Fractionation	Bacteria, yeast & mould recovery from culture media or beverages	T, PF	C, CA, PA, PP	0.1-1.5 µm	150-900	>99
	Clarification	Fermentation media before or after cell lysis	T	C	0.1-0.2 µm	50-500	>99
		Raw juices from sugar cane or beet	T, SW	C, PS, PES	0.1-5 µm	20-200	>99
	Concentration	Microbial starters	T, PF,HF	C, CA, PES	0.1-0.2 µm	30-200	>99
		High molecular weight polysaccharides (dextrans)	T, HF	C, PS	10-500 kDa	30-100	>99
UF	Fractionation	Milk or whey (protein from lactose and minerals)	PF, SW, T	C, PS, PES	10-100 kDa	5-100	70-97
		Oil fractions from oil-in-water emulsions	SW	TFC	8 kDa	10-13	70-90
	Clarification	Alcoholic juices and beverages	HF, SW, T	PAN, PS, PES	10-100 kDa	5-100	70-97
		Removal of colloids, pigments, low MW compounds	HF, T	PAN, PES	10-50 kDa	5-50	70-83
	Concentration	Albumin and proteins	PF, SW, T	C, PS, PES	10-100 kDa	5-30	70-83
		Polysaccharides (karragineen, xanthan)	HF	PS	500 kDa	5-10	-
NF/OI	Water treatment	Pre-treatment of boiler water	SW	CA, PES		5-25	60-95 (Na_2SO_4)
	Concentration	Juices (*i.e.* tomato, orange, apple, grape)	SW	PA, PES		5-30	60-95 (Na_2SO_4)
		Milk and whey	SW	PS		5-25	60-95 (Na_2SO_4)
	Fractionation	Pigments from dye-baths	SW	PA, PES		10-50	60-95 (Na_2SO_4)
		Antibiotics or organic acids from culture media	SW	PS		10-70	60-95 (Na_2SO_4)
		Demineralisation of cheese whey	SW	PA, PES		20-50	30-60 (Na_2SO_4)
		Removal of ethanol from wine or beer	SW	PA, PES		50-100	30-60 (Na_2SO_4)
		Recovery of acids and sugars from rinse waters	SW	PS, PA, PES		5-30	60-95 (Na_2SO_4)

T: Tubular; PF Plate-and-Frame; SW: Spiral-Wound; HF: Hollow-Fibre; C: Ceramic; CA: Cellulose Acetate; PA: Polyamide; PP: Polypropylene; PS: Polysulfone; PES: Polyethersulfone; PAN: Polyacrylonitrile; TFC: Thin Film Composite.

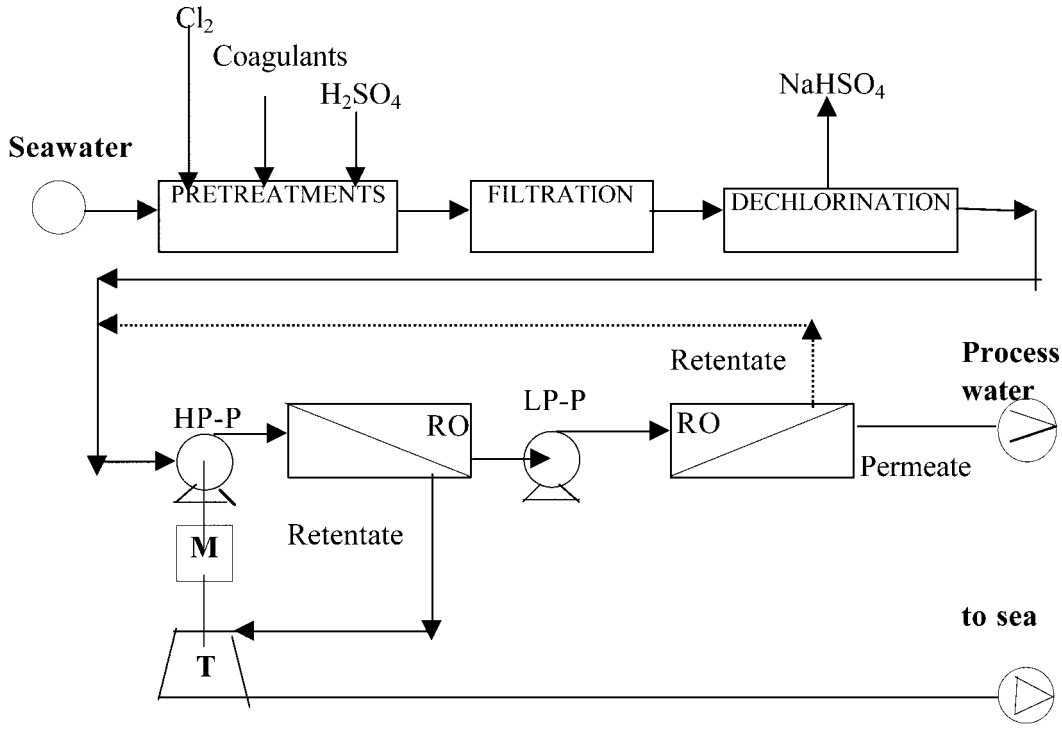


Fig. 4 - Simplified scheme of a two-stage RO seawater desalination plant, as extracted from PARKINSON *et al.* (1999): HP-P, high-pressure pump; LP-P, low-pressure pump; M, electric motor; T, turbine.

(1999). Such a plant requires more pretreatments than MFE or MVC methods. Chlorination, for biological control (micro-organisms, algae, etc.), is followed by dechlorination with sodium bisulphite, as chlorine would damage the membranes. Moreover, seawater must be accurately filtered to reduce turbidity and colloidal substances (silica), thus minimising fouling. The major operating cost refers to the high-pressure pump, since the feed has to be compressed up to 58-68 bar to overcome seawater osmotic pressure. Such a cost may be reduced by expanding the still-pressurised brine through a turbine, which can help drive the high-pressure pump. The first high-pressure RO stage allows 45 to 50% of the feed to be recovered as permeate, while the retentate is returned to the sea. This permeate has a total dissolved solids (TDS) content of 250-300 g m^{-3}, which meets drinking-water standards, but needs further treatment for industrial water use. By re-compressing the permeate to 12-14 bar and feeding it into a low-pressure RO module, it is possible to recover a permeate containing less than 10 g m^{-3} TDS with 85-90% recovery. The low-pressure RO retentate is recycled to the seawater feed line.

Membrane-based treatment systems, as developed by Osmonics Inc. (Minnetonka, MN, USA; http://www.osmonics.com), can be used to provide potable water to the beverage and bottled water industries. Such systems consistently remove a wide variety of inorganic salts instead of only reducing alkalinity and a few contaminants. Moreover, by operating the membrane systems before using a traditional activated carbon tower, it is pos-

sible to increase significantly the porous carbon effectiveness by removing the organic precursors, which ultimately may become potentially health-damaging trihalomethane (THM) precursors or haloacetic acid (HAA) compounds.

Ozone is presently preferred to chlorine to disinfect drinking water obtained from surface water, since it not only destroys bacteria faster than chlorine without forming carcinogenic trichloro-methanes, but also destroys viruses (SHANLEY, 1999). Moreover, UF membranes might improve drinking water quality by both removing pathogenic micro-organisms, such as *Cryptosporidium parvum* and *Giardia muris* cystis, and minimising Cl_2 addition. Finally, some commercial UF filters allow viruses larger than 50 nm (VireSolve NFR module, Millipore Corp., Bedford, Mass., USA: http://www.millipore.com) or 20 nm (Ultipor VF grade DV20 module, Pall Biopharmaceuticals, East Hills, N.Y., USA: http://www.pall.com) to be removed from biological solutions.

Dairy industry

Among the food industries, dairy applications probably account for the largest share of membrane applications (CHERYAN, 1998; DAUFIN *et al.*, 1998; NIELSEN, 2000).

In addition to the examples cited in Table 5, it is worth pointing out the following:

- sanitation of skim milk or whey by MF to remove bacteria and spores;
- milk or whey protein concentration or fractionation by UF to yield high value-added products like milk (MPC) or whey (WPC) protein concentrate, isolated milk (IMP) or whey protein (IWP), purified α-lactalbumin, β-lactoglobulin, etc.;
- milk pre- or full-concentration by UF in new UF-based cheesemaking processes;
- whey demineralisation by ED or NF;
- skim milk or whey concentration up to 20-22% total solids by RO;
- dairy wastewater treatment by UF.

Further details on these processes are given by CHERYAN (1998), DAUFIN *et al.* (1998), NIELSEN (2000), and ROSENBERG (1995). Specific commercial applications amenable to further diffusion in the short-term will be briefly described.

Membrane-based processes in milk processing

In Italy, in addition to the 3 categories of pasteurised milk (i.e. high-quality pasteurised milk, fresh pasteurised milk and pasteurised milk) allowed by Italian legislation (G.U., 1989), removal of bacteria from milk by MF has recently generated a new type of pasteurised milk, the so-called micro-filtered pasteurised fresh milk (G.U., 2002a, b). The shelf-life of micro-filtered milk at 8°C is prolonged from 4-5 days to more than 8 days (ANON., 2002) or to 12-18 days (ROSEMBERG, 1995) with no adverse effects on sensory quality; moreover, its whey protein content may be even greater than 14% of the total protein content (ANON., 2002), which is the legal constraint for a pasteurised milk to be marketed as fresh pasteurised milk.

Raw milk is centrifuged at 50°-60°C to yield a cream with 35-40% fat content and skim milk at 0.1% fat content. The latter is fed into 0.6-0.7 μm MF membrane modules, thus leading to a 99.8-99.9% reduction in total bacterial counts, a 99.95% reduction in *Bacillus cereus* counts, a reduction in anaerobic spore counts to less than 3 spores per litre, and almost 100% reduction in somatic cell counts (ROSEMBERG, 1995). The cell-rich retentate is mixed with the cream and submitted to high-temperature treatment (HTT at 115°-130°C for 4-6 s) to sterilise the fat-rich milk phase. Although very efficient in the removal of bacteria and spores MF processes cannot guarantee 100% removal of patho-

genic bacteria as required in milk pasteurisation. Therefore, once mixed and homogenised, HTT-treated cream and skim milk permeate are submitted to pasteurisation (at 72°-74°C for 15-25 s) to guarantee full destruction of pathogenic bacteria, this being assured by alkaline phosphatase negative test. Fig. 5 shows the flow sheet of the so-called Bactocatch process for milk treatment, as developed by Alfa Laval (Aarhus, Denmark).

Similar extended shelf-life (ESL) milk products have been recently marketed in France, Scandinavia and Canada. In France the ESL milk product, labelled Marguerite, is guaranteed to have a 15-day shelf-life, and in Canada the ESL product, labelled Purfiltre, seems to have conquered about 35% of the Canadian milk market (MAUBOIS, 1997).

Membrane-based processes in cheesemaking

Such processes have been recently applied in the manufacture of a variety of fresh, soft, hard and semi-hard cheeses (ROSEMBERG, 1995). A milk concentration ratio (VCR) of 1.2-2 allows not only natural variation in milk composition to be overcome (thus leading to milk standardisation), but also the protein content of milk to be increased up to 4.5-5% (w/v). The so-called low-concentrated retentate (LCR) has been used to prepare several cheese varieties, such as Cheddar, Cottage cheese, Mozzarella, Edam, and Quarg (ROSEMBERG, 1995).

A VCR of 2-6 gives rise to medium- or intermediate-concentrated UF retentates (MCR), which were converted into Cheddar, Gouda, Blue cheese, and Feta using unconventional cheesemaking equipment, as in the Cheddar cheese manufacture via the APV-SiroCurd process (Land O'Lakes, Perham, MN, USA) (ROSEMBERG, 1995).

If the composition of the UF retentate is increased up to that of drained curd, several fresh cheese varieties (i.e. Camembert, Quarg, Cream cheese, Mascarpone, Mozzarella, and Feta) can be manufactured using the so-called liquid precheese technology (ROSEMBERG, 1995).

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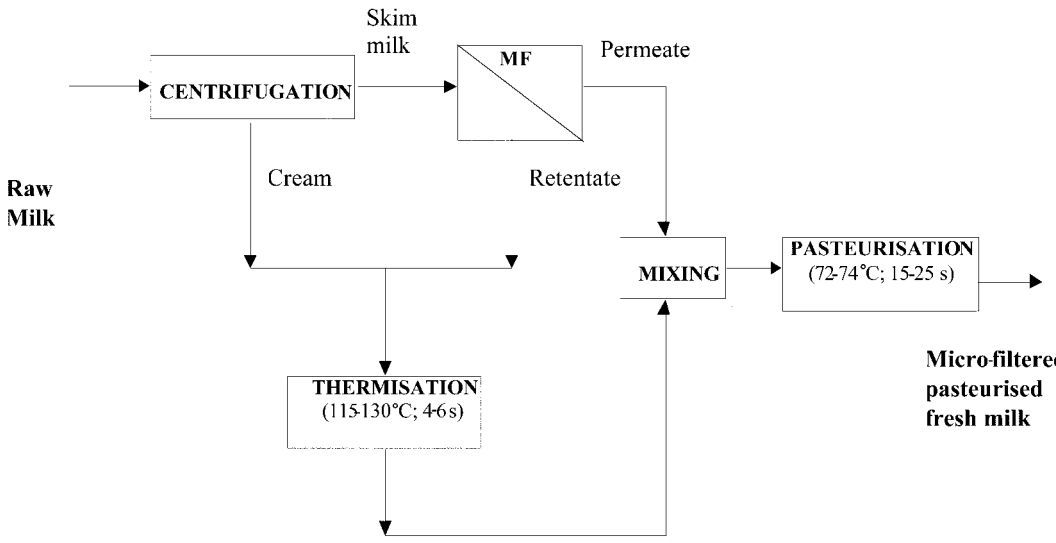


Fig. 5 - Flow sheet of the so-called Bactocatch process for milk treatment, as developed by Alfa Laval (Aarhus, Denmark).

the membrane-based production processes of cast Feta, Philadelphia cheese and Ricotta (NIELSEN, 2000). The cast Feta process involves the following steps: whole milk pasteurisation, concentration by UF to yield a retentate at 36-38% total solids (TS), which is thoroughly mixed with the starter culture, rennet, and salt before being directly cast into tins as final packaging material.

The Quarg and Cream cheese production processes can be subdivided into the following phases: fermentation of pasteurised milk or cream to a final pH of 4.5-4.6, heat treatment at 55°C for 3 min (thermisation), ultrafiltration, cooling and packaging.

Another specific UF application (using the UF P20F NADIR® membranes) (ZERBINATI, 2001) has so far gained a great deal of commercial success in the industrial production of Philadelphia, a soft, fresh-cultured cheese made by Kraft Foods Italia SpA (Milan, I); its hygienic safety and shelf-life have been significantly enhanced with no addition of antimicrobial agents. Skim milk is submitted to MF, thus removing about 99% of the initial microbial load. The retentate is mixed with cream and eventually other spices; it is then pasteurised and finally added to the MF permeate. In this way, it is possible to obtain a whole milk practically sterile with optimal coagulation properties, since only the fat fraction of the milk has been submitted to thermal treatment (CHERYAN, 1998).

Also the traditional production process of Ricotta, the typical Italian dairy product obtained by heating whey at 95°C, has been recently revised as follows: high-temperature pasteurisation of a mixture of milk and whey, ultrafiltration up to 30% TS, in-line addition of NaCl, and heating at 90°C. In-line acidification using concentrated lactic acid solutions in specially designed, APV-patented pressure vessels, followed by a sudden release of pressure, allows the characteristic Ricotta grains to be formed. Once the resulting suspension is cooled to 70°C and drained, it is filled into airtight containers, which are further cooled to 10°C in a cold room. In this way, the taste and texture of Ricotta is maintained, while reducing the risk of infection and extending the shelf-life of the product from 4-9 days to 20-40 days.

As in the Philadelphia cheese production process, the application of MF in hard-cheesemaking might provide a solution to the quality problems related to the presence of several bacteria, mainly propionic or butyric acid bacteria that are, respectively, responsible for eye formation and early blowing or late blowing in hard cheeses like Grana Padano and Parmigiano-Reggiano (Parmesan).

Finally, MF of the brine was found to control more efficiently microbial contamination of cheese during brining than other system involving heat and/or chemical treatments.

Membrane-based processes in whey processing

Besides the conventional UF and RO applications in whey processing, there is a near explosive growth in using whey protein concentrates (WPC) in high protein “sports” and “health” drinks. Direct UF of sweet whey allows the protein content to be increased from 12-15% to 65% TS. Dialysis can further increase the protein content to about 85% TS. The main disadvantage of this process is that fat globules, as well as denatured proteins, follow the protein into the final product. This affects not only the emulsifying, foaming and gelling characteristics of WPC products, but also off-flavour development in the final product.

A new radial flow chromatography (RFC) process (using chromatographic columns where the liquid undergoing fractionation flows along the radius rather than parallel to the axis of a bed of small bead resins to minimise pressure

drops and maximise resin separation efficiency) allows all proteins present in the whey to be adsorbed, thus discharging a practically de-proteinised whey. By washing the ion-exchange resin with acidic or alkaline water, a protein-rich solution is eluted, which can be further concentrated by UF and spray dried to yield an IWP at more than 90% protein content and less than 0.5 and 0.1% fat and lactose contents, respectively (NIELSEN, 2000). Alternatively, it might undergo further fractionation into its basic constituents, like α-lactoglobulin, β-lactalbumin, bovine serum albumin, immunoglobulin, and lactoferrin (ROSEMBERG, 1995; NIELSEN, 2000).

The so-called milk protein concentrate (MPC) and milk protein (MP), that contain 50-85% and more than 85% protein, respectively, are competitive products for WPCs and IWPs. They can be obtained via different combinations of UF, MF and dialysis membranes, as well as correct adjustment of pH and temperature conditions.

Despite the fact that whey demineralisation by ED has generally been regarded as the process of choice in the production of valuable raw materials for baby-foods (BATCHELDER, 1987), use of the new generation NF membranes allows whey to be concentrated up to 15-18% TS together with a de-ashing of 30-35% at no extra-cost. By using dialysis the de-ashing level can be increased to 40-42%.

Finally, it is worth mentioning a novel integrated membrane process, developed by PIZZICHINI *et al.* (1997) and applied by Cadelmonte S.r.l. (Roverè Veronese, VR). Its process flow sheet is shown in Fig. 6. This process allows not only the environmental impact of whey to be reduced to zero, but also all valuable whey components to be recovered. More specifically, whey protein as rejected by the UF module can be reused to formulate new enriched fermented milks or fresh soft cheeses. The lactose syrup as rejected by the NF module can be further concentrated under vacuum evaporation to yield lactose crystals of food or pharmaceutical grade. Finally, by feeding the NF permeate to the RO module, it is possible to split it into a permeate (desalted water), that can be used for final washing of process lines, and a retentate very rich in mineral salts, that can be recycled to formulate the brine used for hard- or semi-hard cheese ripening. Since food and pharmaceutical uses of lactose are limited, enzymatic hydrolysis of the lactose syrups resulting from NF might be suggested in order to yield hydrolysed lactose concentrated syrups usable, for instance, in ice-cream or fruit puree formulations.

Fermented and alcoholic beverage industry

As shown in Table 5, the most successful membrane applications in the beer industry refer to beer recovery from maturation tanks by use of 0.5-0.8 μm ceramic membranes. In this way, all microbial cells and haze are retained with no need for kieselguhr usage and disposal, while all the components giving beer its colour, flavour, and texture are permeated. MF is also recommended for the recovery of beer from spent yeast, as well as filtration of return and residual beer, since unlike centrifugation this process minimises yeast cell lysis, the degradation products of which increase the pH of the beer and affect its bitterness and aroma.

Owing to consumer interest in low-alcohol beer, beer dialysis at cellar temperature allows the ethanol content to be reduced from 4.2 to 0.5% (v/v) without affecting the product quality in terms of head retention, colour, taste and shelf-life. The alcohol in the dialysate can be distilled off and the residue reused in the process (DAUFIN *et al.*, 1998).

Concerning the wine industry, the technical feasibility of the following mem-

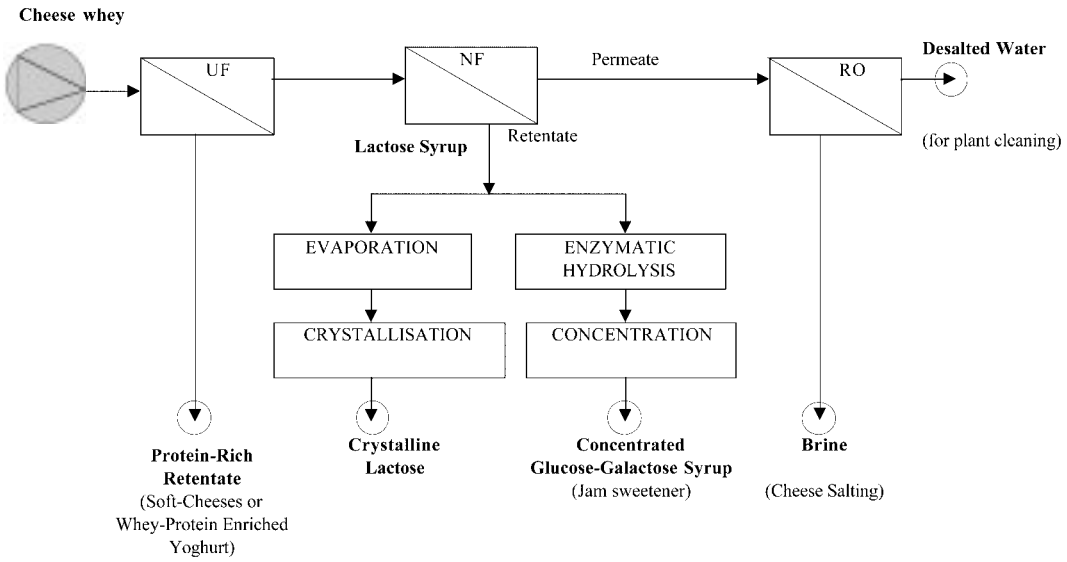


Fig. 6 - Flow sheet of a novel integrated membrane process for cheese whey upgrading (Cadelmonte S.r.l., Roverè Veronese), as modified by PIZZICHINI *et al.* (1997).

brane applications has been assessed so far:

- sanitation of grape musts and especially dessert wines by MF,
- clarification and stabilisation of grape musts and wines by UF,
- removal of polyphenols or other specific compounds (e.g. polyphenoloxidases and anthocyanases responsible for wine madeirisation and enzymatic browning, respectively) from grape musts or wines by UF,
- concentration of MF- or UF-treated grape musts or MF-treated wines by RO,
- tartaric stabilisation of wines by ED.

Despite the great contribution of SO_2 to the current wine technology for its high reducing and aseptic properties, there is an increasing demand for the so-called biological wines, their content of chemical additives (such as SO_2, filter aids, coagulants, etc.) being practically zero. Membrane processing might help limit the use of such additives, as well as avoid any pasteurisation treatment (generally resulting in loss of flavour compounds and sugar caramelization) or other finishing operations. By selecting accurately UF membranes, it would be possible to remove the protein fraction responsible for turbidity in clarified wines with practically no alteration of their initial amino acid profile.

The great potentialities of membrane processing have been confirmed by the extensive technical experience accumulated throughout the last decade starting from the pioneering work by PERI *et al.* (1988) and more recently by the large-scale experimental work carried out by PASTIS CNRSM Research Centre (Brindisi, Italy). Based on such tests the operating costs for hollow-fibre clarification and stabilisation would be about € 0.007 per litre of wine treated when referred to a plant capacity of 450 m^3 of wine per year (LA SALA *et al.*, 2000).

In France, Germany and Italy mobile membrane plants equipped with UF hollow-fibre modules are presently available for rent at € 0.002-0.003 per litre of bottled wine, thus allowing the SO_2 content to be reduced to a low value to fulfil the strict US legislation (ZERBINATI, 2001).

Fig. 7 shows a simplified flow sheet of a novel integrated membrane process for producing low-SO_2 wines stabilised without chemical additives. Sterile clarified grape must can be produced by MF, while the sugar content can be adjusted by RO. At the end of fermentation, raw wine can be clarified by centrifugation or MF, while its tartaric acid content can be stabilised by electrodialysis or by continuous flash-cooling to -4°C to induce precipitation of potassium bitartrate as minute crystals, that are finally removed by MF. While the tartaric stabilisation of wines by ED is based on the pioneering work carried out by INRA (Montpellier, France) in co-operation with Eurodia Industrie (Wissous, F) (AUDISON *et al.*, 1979; RIPONI, 1993), the latter is based on the research carried out by Velo SpA (Altivole, I) (BELLUSSI, 2001).

Also RO of wines has been tested to increase their initial alcoholic content by 0.5-1.5% (v/v) (DAUFIN *et al.*, 1998), as well as their polyphenol and anthocyanin content, even if a small loss in monomeric anthocyanins in young red wines was detected by BOSSO *et al.* (2001).

Finally, in the vinegar industry the complex and 3-6 month long clarifying process of the broth resulting from the acetic acid fermentation can be accelerated by UF clarification. Moreover, dialysis of the UF retentate allows the residual acetate in the dialysate to be recovered and used to dilute the UF permeate from 11-12% to 6% (v/v) acetic acid, thus standardising the vinegar and enhancing the overall product yield (http://www.kochmembrane.com).

Fruit juice and sugar industry

Raw or de-pectinised fruit, cane or beet sugar juices can be clarified by MF and/or UF to avoid all settling steps, as well as addition of several coagulants. In this way, processing times are reduced from 12-36 h to 2-4 h, while clarified juice extraction yields are increased from 80-90% to 95-99%. Permeation fluxes of about 35 or 300 $dm^3\ m^{-2}\ h^{-1}$ have been observed in the case of raw juices using 30-kDa MWCO membrane or de-pectinised juices using 0.2 µm membranes, respectively (CHERYAN, 1998). Similar-

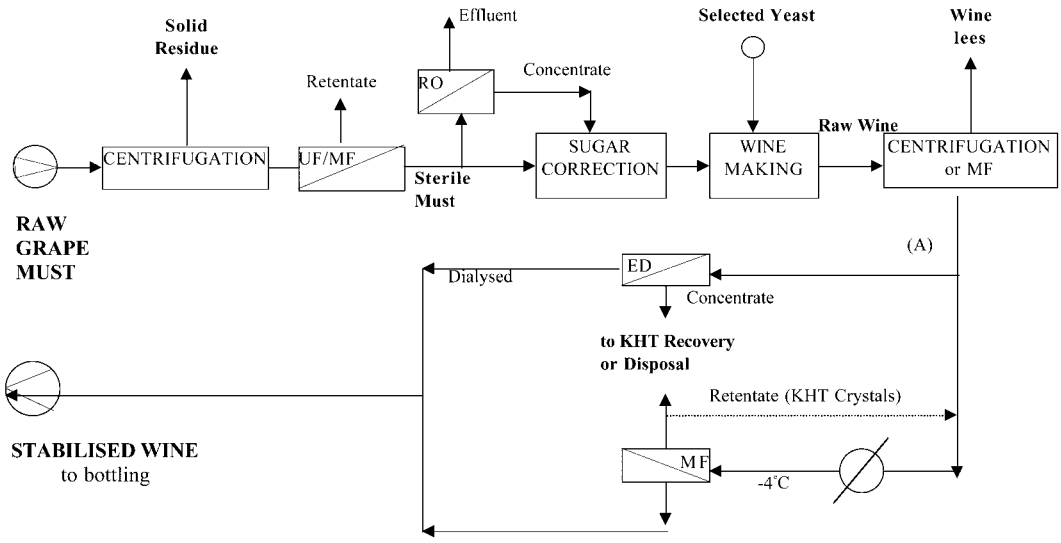


Fig. 7 - Flow sheet of a novel integrated membrane process for producing low-SO_2 wines stabilised without chemical additives KHT, potassium bitartrate.

ly, clear raw sugary syrups at 14°-15°Brix can be obtained, thus preventing depositing of their initial suspended matter (polysaccharides, colouring substances, microorganisms, etc.) onto the heat transfer surfaces of the evaporators. Dialysis of the MF retentate allows sugar loss to be reduced to as low as 0.1% of the feed sugar content. Finally, the MF permeate could be pre-concentrated up to 30°-35°Brix by NF or RO (DAUFIN *et al.*, 1998).

Hollow-fibre membrane treatment is also a prerequisite to debittering of fresh citrus juices. The permeate is fed into an adsorption column containing a macroreticular Amberlite XAD-16 resin to remove the main bitter components (limonin, naringin, hesperidin, etc.). By recombining the debittered juice with the pulpy UF retentate, in the case of orange juices it is possible to reduce the limonin content from 25 to 5 g m^{-3} (http://www.kochmembrane.com).

In the production of concentrated fruit juices, pre-concentration up to 25°-30°Brix by RO permits overall concentration costs to be minimised (MORESI, 1988), as well as thermal degradation phenomena and flavour loss.

Combined use of low- and high-retention membranes allows juice to be concentrated to 40°-42°Brix with feed input pressures of 40-55 bar (van WIJK *et al.*, 1984; GOSTOLI, 1994; WATANABE *et al.*, 1990). More specifically, a previously UF clarified juice can be pre-concentrated using a high-retention module, thus yielding a retentate which is fed to a low-retention module (NF) to achieve the aforementioned total sugar contents. The resulting permeate, being rich in solutes, is recycled to the high-retention module (RO) together with the feed.

Fruit juices can be partially de-acidified by NF or ED, but this operation has to be cautiously carried out. In fact, permeation of low molecular weight components (i.e. acids, salts, alcohols, hydrosoluble flavours) gives rise to semi-concentrated juices, which yield reconstituted juices with quite a different flavour from those obtained starting from multiple-effect evaporated fruit juices. For instance, acidic fruit juices submitted to membrane concentration are more appreciated due to their reduced acidic level, while juices of some low-acidic fruits such as peach and a few varieties of grape become less appealing as a result of a further increase in the sugar-to-acid ratio (DEMECZKY *et al.*, 1981).

Membrane distillation (MD) is a relatively new thermally-driven process, which is being investigated world-wide because of its potential benefits, i.e. almost 100% rejection of ions, macromolecules, colloids, cells and other non-volatiles, lower operating temperatures and pressures than conventional distillation and membrane filtration processes, respectively, and reduced chemical interaction between membrane and process solutions, etc. (LAWSON and LLOYDS, 1997). This has made MD attractive in the food industry where concentrated orange and apple juices can be prepared with better flavour and colour (CALABRÓ *et al.*, 1994; LAGANÀ *et al.*, 2000). In desalination, solvent permeation fluxes as high as 75 kg m^{-2} h^{-1} (which are competitive with the fluxes observed in RO) have been measured using direct contact membrane distillation (LAWSON and LLOYDS, 1997). However, in the production of concentrated apple juices using 100 µm thick polypropylene hollow-fibre modules such fluxes were significantly smaller, that is about 1 kg m^{-2} h^{-1} in the concentration range of 20°-50°Brix. Moreover, at 64°Brix the flux was reduced to as low as 0.6 kg m^{-2} h^{-1} due to temperature polarisation effects induced by the great increase in the apparent viscosity of the concentrated juices (LAGANÀ *et al.*, 2000). Therefore, before the food industry can accept MD as a viable alternative to RO, more must be learned about flux, long term operation and maintenance costs, as well as the spe-

cific costs of MD modules which need to be reduced significantly.

Egg processing industry

Because of the high sensitivity of egg proteins to mechanical shear and heat, membrane processing of egg white (EW) or whole egg (WE) seems to be the technology of choice. In fact, EW or WE can be UF pre-concentrated to dispose of some of the glucose and ash content, and then concentrated by RO from 12 or 25% (w/w) to 32 or 42% (w/w), respectively, prior to being used as such (EW or WE concentrate). Further dewatering by spray-drying gives rise to EW or WE powder (DAUFIN *et al.*, 1998).

Lysozyme extraction from EW can also be performed as described by CHERYAN (1998), DAUFIN *et al.* (1998), and NIELSEN (2000).

Soy bean processing industry

The current soy processing technologies are similar to those of milk processing and many of the products resemble dairy-based products like soy milk, traditional tofus, soy cheeses, etc. As a direct transfer of technology to this sector, a typical soy milk base (8% TS, 47% being protein matter), pre-heated to 65°-85°C, might be concentrated to 22-27% TS using ceramic UF membranes with the following advantages: formation of a retentate amenable to continuous thermal or acidic coagulation, increase in isolate yield by 5-10%, and disposal of a less polluting permeate (DAUFIN *et al.*, 1998).

Vegetable oil industry

The edible oil processing technologies, including oil extraction from oilseed flakes, expanded collets or press-cake using commercial "hexane", solvent distillation from oil miscellas at 25-30% oil content, and several refining steps (i.e. degumming, neutralisation, washing, drying, bleaching, filtration, and deodorisation), suffer from many drawbacks. These are mainly high energy requirement, large consumption of water and chemicals, disposal of highly polluting effluents and residues, and loss of neutral oil and nutrients (YOUNG *et al.*, 1994).

To minimise these disadvantages, there has been a great deal of interest in membrane applications in edible oil processing (SOURIRAJAN, 1964), especially in the removal of hexane from oil miscellas, as well as degumming or deacidification of vegetable oils. Recently, a five-year, $8.3 million research project has been started by Cargill and Osmonics, Inc. (Minnetonka, MN, USA) to develop commercially viable polymeric membranes and membrane systems for non-water liquid processing (http://www.cargill.com/today/releases/10081998a.htm).

The main bottleneck of membrane-based edible oil processing is that most of the membranes actually marketed are incompatible with hexane. In fact, in the presence of this solvent membranes tend to buckle in the holder, swell and close almost all of their pores, thus resulting in low solvent permeation fluxes and unacceptably low oil retention. Recently, BHANUSHALI *et al.* (2001) examined the performance of several hydrophilic and hydrophobic membrane materials for polar (methanol, ethanol, and isopropanol) and non-polar (pentane, hexane, octane) solvents. For instance, hexane and methanol permeation fluxes at about 13 bar through a hydrophobic silicone-based NF membrane or a hydrophilic polyamide-based RO membrane were about 22 and 11 $dm^3\ m^{-2}\ h^{-1}$ or 2.5 and 18 $dm^3\ m^{-2}\ h^{-1}$, respectively, thus indicating that for the transport of non-polar solvents the membrane material needs to be chosen accordingly. Low hexane permeation fluxes, as well as limited solvent-resistance of the

present commercial hydrophobic membranes, have thus far made such a process inapplicable on the production scale. In fact, interesting pilot-plant scale results in the removal of solvents from cottonseed oil miscellas have been obtained using only ethanol or iso-propanol as solvent for oilseed leaching (KÖSEOGLU *et al.*, 1990).

The bench-scale degumming process for hexane-oil miscellas via a commercial, hexane-resistant, non-aqueous UF thin-film polymer membrane (i.e. the DS-7 membrane module manufactured by Desalination Systems Inc., Escodido, CA, USA) appears to be more susceptible to industrial use, owing to an average flux of about 27 $dm^3\ m^{-2}\ h^{-1}$, a 99.6% rejection of phospholipids and a significant reduction in colouring pigments (LIN *et al.*, 1997).

Even in the case of removal of free fatty acids (FFA) from crude oils, the present membrane alternatives to conventional physical or chemical methods (based on distillation or conversion into soapstock to be washed out by water rinsing and centrifugation) are still too premature for being transferred to an industrial scale.

However, a new process based on a combination of high- and low-rejection NF membranes appears to be quite promising (RAMAN *et al.*, 1996). It makes use of methanol to separate selectively FFAs from triglycerides. The extract consisting of FFA, solvent and residual triglycerides is first decanted to yield a FFA-rich methanolic phase, that is then NF-split into a retentate at 35% (w/v) FFA and a permeate with less than 0.04% FFA. While the former may be further processed in an evaporator, the latter can be directly recycled to the extractor (RAMAN *et al.*, 1996). Acetone has also been found to be an effective solvent for oil deacidification (ZWIJNENBERG *et al.*, 1999).

Finally, membranes might help in releaving the environmental problems associated with the disposal of used frying oils by extending their life in virtue of the fact that composite membranes have been found to be effective in reducing soluble impurities and suspended matter, including oxidation products, polymers and colour compounds, with no undesirable changes to the oil (SUBRAMANIAN *et al.*, 2000). Even in this case, any commercial application would require greatly improved permeation fluxes.

Fermentation industry

The potential application of membrane processing in the fermentation industry is likely to play a significant role in redesigning classic fermentation and down-stream operations for relatively low energy requirements, no additives, generally mild operating conditions and easy scaling-up rules (CHERYAN, 1998; WANG, 2001). Regardless of the end product of the fermentation process concerned, that is microbial biomass or metabolite, the potential applications of membrane processes are outlined in Fig. 8.

The conventional batch procedures do not allow cell growth to exceed certain limits, due to the simultaneous excretion of inhibitory metabolites by the micro-organisms themselves that inhibit both cell growth and metabolism. Removal of toxic metabolites from the production media by MF is a prerequisite for maximising cell density with minimum mechanical damage. This gives rise to the so-called membrane recycle bioreactors (MBRs) (ENZMINGER and ASENJO, 1986; BUBBICO *et al.*, 1997), that combine the typical advantage (that is high cell density) of cell-immobilised fermentation systems with those (i.e. homogeneous, higher reactant diffusion rates, etc.) of free-cell ones. Moreover, they might help minimise the contribution of the cell growth phase to the overall bioproduct formation rate, as well as maximise bioproduct yield per unit of substrate consumed and achieve great-

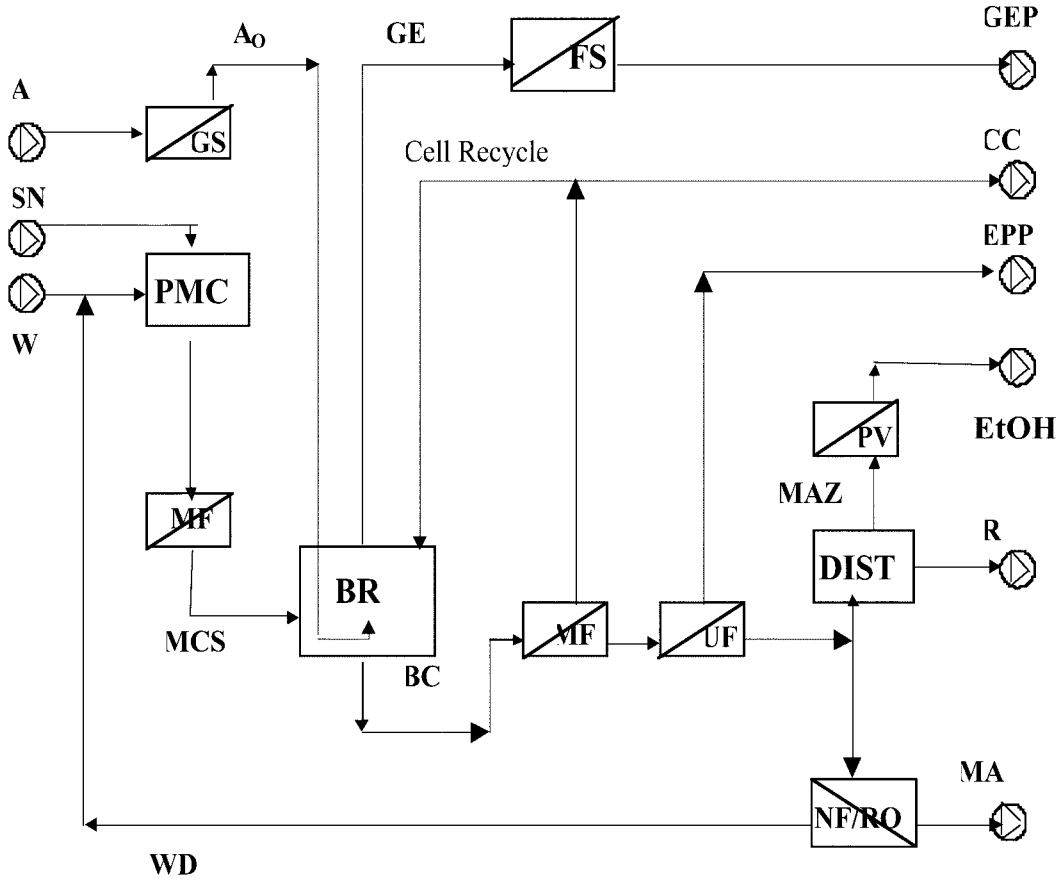


Fig. 8 - Scheme of the potential applications of membrane processes in the fermentation industry: A, air feed; A_O, oxygen-enriched air; BC, culture broth; BR, bioreactor; CC, cell concentrate; DIST, distillation unit; EPP, enzymes, protein or polysaccharides; EtOH, ethanol; FS, filtration unit; GE, exhausted gas; GEP, sterile exhausted gas; GS, gas separation unit; MA, acidic metabolites; MAZ, azeotropic mixture, MCS, sterile culture medium; MF, microfiltration unit; NF/RO, nanofiltration or reverse osmosis unit; PMC, culture medium preparation; PV, pervaporation unit; R, residue; UF, ultrafiltration unit; SN, carbon substrate and nutrients; W, process water; WD, desalted water.

er product formation rates than those pertaining to the conventional fermentation processes or synthetic alternative processes.

The present interest in the development of novel integrated fermentation processes using MBRs relies on the possibility of coupling MF membrane modules (to recover microbial cells from fermentation media and recycle them back into the bioreactor) to other membrane modules. In this way, it would be possible to fractionate different microbial metabolites from MF permeates, thus allowing, for instance, the recovery of enzymes (NIELSEN, 2000) or polysaccharides (LO *et al.*, 1997) by UF, ethanol (TOLEDO, 1984) by PV, and a few salts of several organic acids (LAUFENBERG *et al.*, 1996), mainly fumaric (MORESI *et al.*, 2002), itaconic (BAUER *et al.*, 1991; LO PRESTI and MORESI, 2000b), lactic (LO PRESTI and MORESI, 2000b; SCHLICHER and CHERYAN, 1990; TEJAYADI and

CHERYAN, 1995) or citric (LO PRESTI and MORESI, 1999, 2000b; MORESI and SAPPINO, 1998; 2000; SAPPINO *et al.*, 1996) acids by NF/RO or ED.

Fig. 9 shows a scheme of a novel membrane integrated process for citric acid production from glucose syrups by *Yarrowia lypolitica* ATCC 20346, based upon a prolonged fed-batch fermentation carried out in a stirred bioreactor coupled to a MF unit equipped with tubular ceramic membranes, and di-sodium citrate recovery from MF permeates using spiral-wound polysulfone RO modules (LO PRESTI and MORESI, 1999).

Fig. 10 illustrates a simplified flow sheet concerning the recovery of xanthan gum produced from glucose syrups by *Xanthomonas campestris* from the clarified fermentation medium using UF hollow-fibre, 500 kDa NMWCO modules (LO *et al.*, 1997). In this way, xanthan gum is thickened from about 3 to 7% (w/w), thus reducing the amount of either ethanol or live steam used to flocculate the biopolymer or re-distillate ethanol from the exhausted alcoholic media, respectively, with a reduction in the operating costs from US$2.7 to US$1.5 per kg of xanthan recovered.

Pure ethanol (EtOH) production or separation of several organic solvents by pervaporation is becoming an industrial reality. Since May, 2000 in the 150 m^3/day distillery of *Sucrerie Cooperative de Bezancourt* at Betheniville (F), a pervaporation unit has been installed to produce 99.8% (w/w) EtOH (ONDREY, 1999). This unit consists of plate-and-frame modules equipped with a film of hydrophilic, pol-

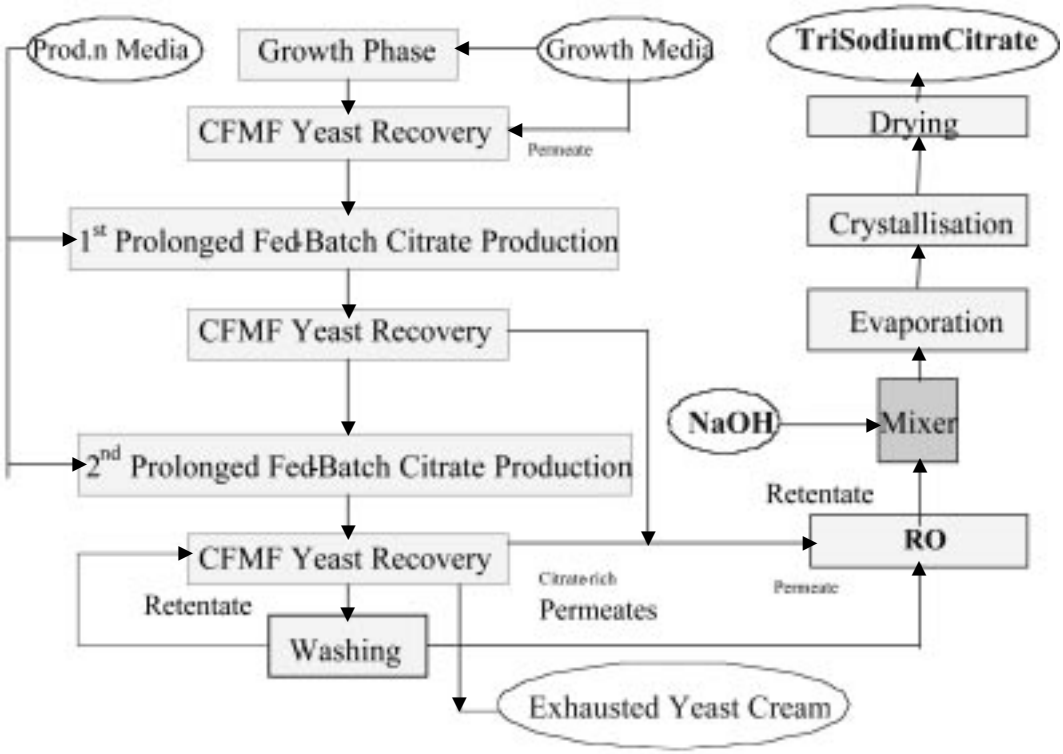


Fig. 9 - Flow sheet of a novel integrated membrane process for citric acid production from glucose syrups by *Yarrowia lipolytica*, as proposed by LO PRESTI and MORESI (1999).

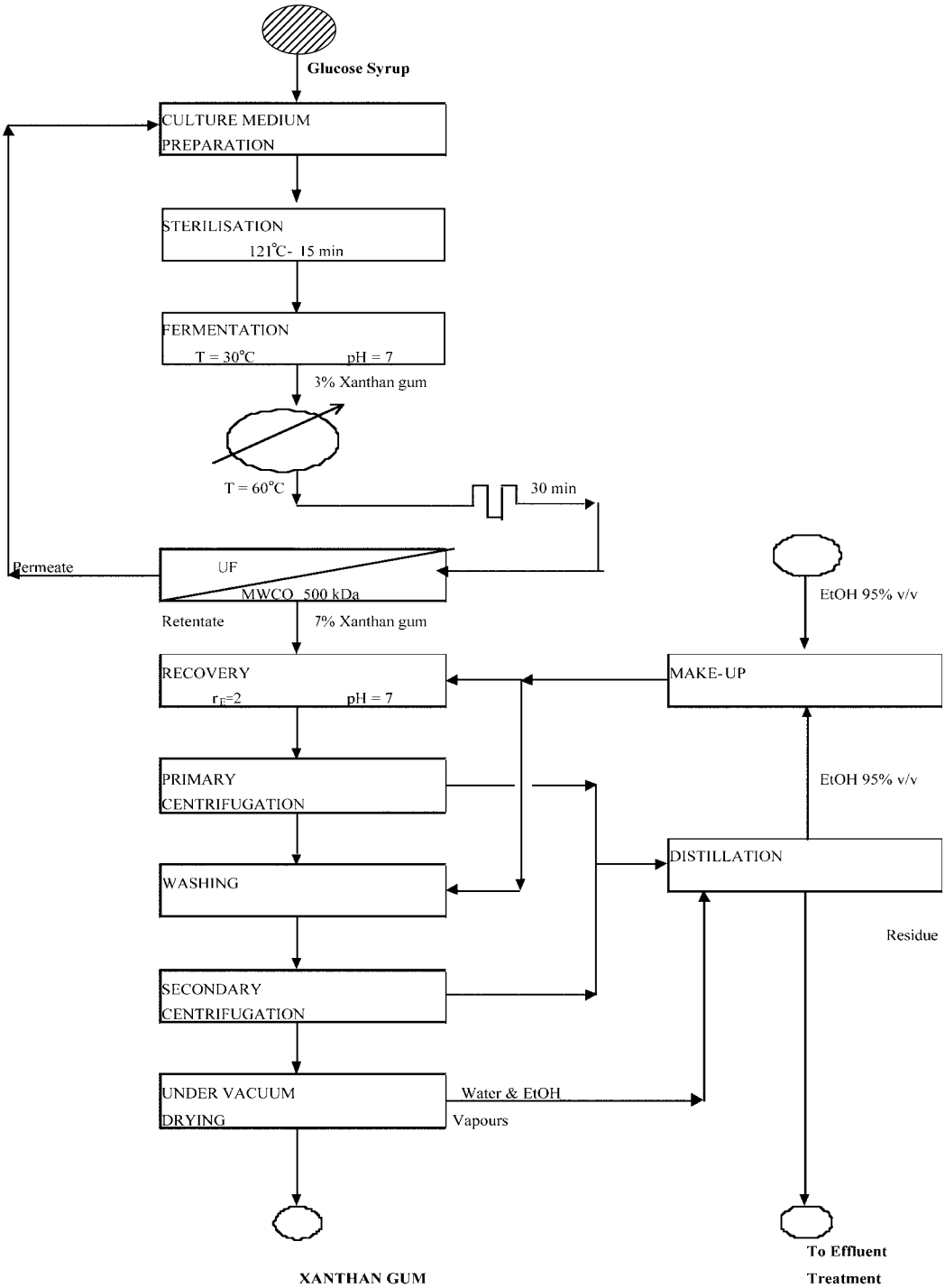


Fig. 10 - Flow sheet of a novel integrated membrane process for xanthan gum production by *Xanthomonas campestris*, as derived from LO *et al.* (1997).

yvinyl alcohol on a support that permits only water to permeate to the other side of the module, which is held under vacuum by continuous pumping. Despite a greater energy requirement (34.5 kWh m^{-3} against 20 kWh m^{-3} of EtOH distilled), live steam consumption is drastically reduced to 0.11 kg *versus* 1.1 kg dm^{-3} of EtOH distilled. Moreover, it avoids azeotropic or extractive distillation, as well as any entrainers, such as benzene and toluene, used to suppress the water-EtOH azeotrope. Alternatively, hollow-fibre, solvent-resistant polymeric membranes are marketed by Cascade Separations (Austin, TX, USA). Liquid feed is vaporised and fed to the inside of hollow-fibres, while nitrogen is fed to the external side to avoid operating under vacuum. It will be interesting to see which technological solution will be able to conquer such an important area of the food industry.

In the case of aerobic fermentation processes, ambient air is generally filter-sterilised using membrane filter modules (FS), but it might be oxygen-enriched using, for instance, the perfluorinated polymeric membranes supplied by Compact Membrane Systems, Wilmington, DE, USA (HAIRSTON, 2000). A US patent (# 57097732), filed by Praxair Technology Inc. (Danbury, CT, USA) and granted in 1998, describes an advanced membrane system to yield purified oxygen gas at 60-90% purity (http://www.tifac.org.in/news/memb.htm). For instance, such oxygen-enriched air might enhance the productivity of several microbial processes (such as those used in the production of baker's yeast, microbial biomass, citric acid, and amino acids), increase fuel efficiency or reduce greenhouse gas emissions from high-temperature furnaces.

The exhaust gases flowing out of the bioreactor can be filtered using membrane filters (FS) to isolate aseptically the vessel and simultaneously avoid spreading of industrially cultured micro-organisms in the environment.

Finally, due to the limited content of soluble solids the permeates from NF or RO membrane modules might be totally recycled to reformulate production media (thus minimising process water consumption) or to dispose of them without further treatment.

PRESENT PROBLEMS AND FUTURE PERSPECTIVES

Despite the fact that membrane processing potentialities are innumerable, membrane applications in the food industry are still very marginal. What are the reasons?

Technical progress in the food industry has generally proceeded quite slowly with a 20-30 year delay with respect to that of the chemical and pharmaceutical industries (CANTARELLI, 1987). For instance, it is worth noting that plate heat exchangers started to be used in Italy in the late 1950s in spite of the fact that they were industrially manufactured and patented by Seligman, founder and director of APV Co., in 1923 (MÜLLER-STEINHAGEN, 1997).

To counteract the typical misoneism of the Italian food industry, it is necessary to resort to appropriate scaling-up exercises on the pilot- or industrial plant scale to assess precisely the technical-economical feasibility of a given membrane processing alternative.

There are however a number of problems that undoubtedly limit growth in membrane sales, like membrane resistance to solvents, fouling problems, design considerations, cleanability, investment and membrane replacement costs and competing technologies.

Provided that there is full compatibility between the solvent and membrane material, it is the incomplete understanding of mass transfer mechanisms in membrane systems that makes the design of membrane plants difficult and, consequently, hampers their diffusion,

as clearly stated by DREAM (2000). This author proposed a unitary approach for all membrane filtration processes, where the effect of polarisation on J_{Wv} has to be initially neglected to simplify the task of membrane design and then accounted for by assessing whether the resulting overdesign of the required membrane surface area is within the common rules for a chemical plant design. Formation of a polarised layer onto the membrane surface, as well blocking of membrane surface pores or fouling of support materials, results in a more or less pronounced permeation flux decay, that is difficult to assess precisely at the design stage. This generally results in a somewhat lukewarm response from some process engineers, who feel more comfortable specifying traditional separation processes (such as distillation and solvent extraction).

To overcome such uncertainties, as well as lack of reliable design equations, long-term laboratory- and pilot-scale experiments are therefore needed to determine the effects of the main operating parameters (i.e. membrane constitution, porosity and configuration; cross-flow velocity; applied pressure; slurry density, pH and temperature) on membrane process performance and test the performance of novel membrane-based processes in terms of either specific production costs, intrinsic (sensorial, hygienic, nutritional) quality attributes or shelf-life of the final food product. Unfortunately, such trial tests are often refused by the final user, who in principle does not fully understand why rather expensive industrial membrane modules (costing about €1,300-2,600 each) may have to be discarded after only a few hours of operation or anyway more or less frequently replaced.

In spite of all these drawbacks, there is renewed interest in membrane processing, that can most probably be ascribed to:

- a 50% reduction in specific membrane costs (especially in the case of spiral-wound filtering elements),
- commercial availability of larger capacity multi-leaf, or multi-tubular modules,
- development of new ceramics and polymeric membrane materials rated to perform at pH levels from 1 to 14, to a maximum temperature of 110°C with fairly good chemical resistance and significantly longer lifetimes (up to 5 or 10 years in the case of polymeric or ceramic membranes, respectively),
- total compatibility between the food and inorganic membrane materials to assure the same degree of safety guaranteed to a food product packaged in glass containers rather than in epoxy-coated tin cans and no leaching of basic monomers in the food fractionated streams.

This will promote further applications on the industrial scale, such as filtration as well as make-up water in soft-drink and beverage manufacturing, sugar refining and concentration, high-fructose corn syrup production, recovery of starches and derivatives, concentration of egg and egg white (to recover lysozyme), clarification of vinegar, whisky (PIRIE, 2001) and fruit juices, removal of alcohol from wine or beer. For further details consult the following Web pages: http://www.kochmembrane.com; //www.osmonics. com; //www.celgard.-com.

In principle such membrane applications might be regarded as mature, since they were established in the 1970s, but they are still relatively new due to the fact that no membrane-based process is presently an established procedure for given production processes. This is true even in the case of brackish- and sea-water desalination, where the membrane technology is being competed with by under-vacuum multiple effect evaporators or mechanical vapour compression, especially at capacities greater than 2,000

m^3/day (PARKINSON *et al.*, 1999). In Italy membrane sales are actually increasing in the dairy sector and a great deal of concern towards micro-filtered pasteurised fresh milk is most likely to boost MF ceramic membrane sales. Further applications are likely to regard pure ethanol production by PV, clarification of raw beet sugar juices, fruit juices, beer and wines by UF, partial concentration of grape musts or wines by NF or RO, as well as microbial stabilisation of wines by MF.

If liquid-solid separations represent the present for membrane processes, their future lies in replacing other fundamental operations, such as distillation, and oil-in-water emulsion and cryogenic separation. Pure ethanol (EtOH) production, separation of several organic solvents or removal of trace organics from water by pervaporation is becoming an industrial reality and this sector is expected to experience a 17% growth rate per year (http://www.bccresearch.com/editors/RDMS97.html).

The final outcome of the Cargill and Osmonics research project on the development of commercially viable polymeric membranes and membrane systems for non-water liquid processing will in all probability have a profound impact on traditional vegetable oil processing technologies by reducing significantly the energy consumed per unit of solvent recovered by distillation, the amount of wastewater used in manufacturing, as well as air emissions.

Further diffusion is also expected for the thin-film UF membrane systems (GF-50: http://www.osmonics.com/products/Page303.htm), characterised by a molecular weight cut-off of 8 kDa on polyethylene glycol and capable of recovering 70-90% of emulsified oil from wastewater (i.e. olive oil wastewater) at permeation fluxes of 12 $dm^3\ m^{-2}\ h^{-1}$ with no sludge generation, no need for coagulants and filter aids and thus meeting discharge regulations.

Finally, concerning cryogenic gas supply, Air Products and Chemicals Inc. (Allentown, PA) estimated that 30-40% of this market is amenable to conversion to non-cryogenic membrane systems, especially because not all cryogenic gas applications require nitrogen purity greater than 99.9%, which can quite easily be reached with commercial membrane systems (HAIRSTON, 2000).

NOTATION

A_1 friction factor ($m\ s^{-1}$)

A_2 membrane constant (m^{-1})

A_D membrane constant for water transport by diffusion ($m\ Pa^{-1}\ s^{-1}$)

A_m membrane surface area (m^2)

A_p membrane constant for solute transport by pore flow ($m\ Pa^{-1}\ s^{-1}$)

B membrane constant for solute transport by diffusion ($m\ s^{-1}$)

$c_\pm$ anionic or cationic concentration in solution ($mol\ m^{-3}$)

C_1 maximum rejection factor (dimensionless)

C_2 effective thickness of the membrane (m)

C_B solute concentration ($kg\ m^{-3}$)

C'_{Bm} natural logarithmic difference of solute concentration at membrane surface ($kg\ m^{-3}$)

$D_\pm$ ionic diffusivity in solution ($m^2\ s^{-1}$)

d_p mean pore diameter (m)

D_p solute diffusivity permeability $\left[= \frac{(1-\sigma)\ J_{Wv}}{Pe}\right]$ ($m\ s^{-1}$)

E electric field ($V\ m^{-1}$)

F Faraday's constant (96,486 Coulomb/g-equiv.)

$J_\pm$ permeation flux of anionic or cationic species ($mol\ m^{-2}\ s^{-1}$)

J_B solute flux of solvent ($kg\ m^{-2}\ s^{-1}$)

J_{Wv} volumetric permeation flux of solvent ($dm^3\ m^{-2}\ h^{-1}$ or $m\ s^{-1}$)

L module length (m)

L_p hydraulic permeability of membrane ($m\ Pa^{-1}\ s^{-1}$)

Pe Péclet number (dimensionless)

P absolute pressure at any stream side (bar)

Q volumetric flow rate ($m^3\ s^{-1}$)

R gas-law constant (= 8.31 $J\ mol^{-1}\ K^{-1}$)

R_f membrane resistance due to fouling layer (m^{-1})

R_g membrane resistance due to polarisation layer (m^{-1})

R_m intrinsic membrane resistance (m^{-1})

r solute rejection (dimensionless)

r_a apparent solute rejection (dimensionless)

r_t intrinsic or true solute rejection (dimensionless)

SR solvent recovery (dimensionless)

t time (h)

T absolute temperature (K)

VCR volume concentration ratio (dimensionless)

VR volume permeate-to-feed ratio (dimensionless)

V volume of a generic stream (m^3)

x membrane thickness variable (m)

$z_{\pm}$ ionic valence (dimensionless)

Greek Symbols

$\Delta\pi$ osmotic pressure difference between the retentate and permeate sides (bar)

ΔP transmembrane pressure difference (Pa)

Δt time interval (s)

Δx membrane thickness (m)

ε surface porosity of membrane (dimensionless)

η viscosity of the fluid permeating the membrane (Pa s)

σ reflection coefficient (dimensionless)

Subscripts

B referred to any generic solute

b referred to batch operation or stream bulk

c referred to continuous operation

F referred to the feed

i input

m referred to the membrane surface

o output

P referred to the permeate

R referred to the retentate

W referred to water

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PAPER

EATING BEHAVIOR AND RISK FACTORS FOR OVERWEIGHT AND OBESITY IN SCHOOL CHILDREN IN LIGURIA (ITALY)

COMPORTAMENTO ALIMENTARE E FATTORI DI RISCHIO DI SOVRAPPESO ED OBESITÀ NEI BAMBINI DI ETÀ SCOLARE IN LIGURIA

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ABSTRACT

A study was carried out on a sample of 1,342 children of both sexes (mean age 8.5±0.4 years) representative of third-graders in Liguria, aimed at evaluating anthropometric characteristics, energy intake and nutrient consumption, lifestyle factors and the presence of overweight family members. The relationship between different behaviors as risk factors and the importance of body weight were evaluated. A prevalence of being overweight was detected in 36% of the males and 28% of the fe-

RIASSUNTO

È stata condotta un'analisi su di un campione di 1.342 bambini, ambosessi (età media 8,5±0,4 anni), rappresentativo della popolazione scolare ligure di terza elementare, mirata a valutare caratteri somatometrici, consumi energetici e nutrizionali, stile di vita e presenza di sovrappeso in famiglia. In relazione ai dati antropometrici è stata riscontrata una prevalenza di sovrappeso del 36% tra i maschi e del 28% tra le femmine. Il regime alimentare risulta nel complesso bilanciato anche se

- Key words: energy balance, lifestyle, logistic regression, overweight parents, school children anthropometry -

males. Overall the diet was balanced, even though in approximately 50% of the children the energy balance was positive by at least 1.3 MJ. The results indicate that a positive energy balance, a high-calorie diet, sedentary activity and overweight parents are decisive elements in the multifactorial mechanism of the increasing incidence of children being overweight and obese.

circa il 50% dei bambini presenta un bilancio energetico positivo di almeno 1,3 MJ. Sono state ricercate relazioni tra possibili fattori di rischio e sovrappeso: è emerso che bilancio energetico, regime ipercalorico, attività sedentaria e sovrappeso parentale costituiscono, elementi di un meccanismo plurifattoriale, nella prevalenza del soprappeso e dell'obesità infantile.

INTRODUCTION

The problem of being overweight during childhood and adolescence is increasing in western countries and a genetic and familial predisposition, as well as such factors as birthweight, maternal diabetes and socio-economic behavior can increase its incidence in prepubertal children (STETTLER *et al.*, 2000). Being overweight in childhood is associated with obesity in adulthood (FREEDMAN *et al.*, 1987) and obesity is associated with a higher incidence of cardiovascular and metabolic disorders such as high levels of blood lipids, high blood pressure, glucose intolerance, type II diabetes mellitus and a higher incidence of neoplasias correlated with chronic dystrophy.

The body mass index (BMI, weight/height^2) classifies adults as: underweight (<18.5), normal weight (18.5-24.9), overweight (25.0-29.9) and obese (>30.0) (W.H.O., 1998). Children and adolescents of both sexes are considered overweight if they have a BMI value that falls between the 85th and 95th percentiles, whereas they are considered obese if they are above the 95th percentile (MUST *et al.*, 1991; HIMES and DIETZ., 1994; WHITAKER *et al.*, 1997). Actually, the cut-off points between overweight and obesity varies from the 85th to 97th percentiles. The nomenclature can also vary; for instance, the 97th percentile of the BMI is called obesity in The Netherlands (SPERVAN DER WEKKES *et al.*, 1994; BRUGMAN *et al.*, 1995) and super-obesity in France, (ROLLAND-CACHERA *et al.*, 1987). Some other methods used to define obesity, include triceps skinfold >85th percentile (TANNER and WHITEHOUSE., 1975), percentage body fat (BF) >50% of that of reference children (FOMON *et al.*, 1982), weight/height, as well as weight/ideal weight, the latter with a cut-off limit of 120% (CREPALDI *et al.*, 1991). The variability in the prevalence of being overweight/obese reported in several studies could be due to the different methods and criteria used to define being overweight and obese.

The results of the NHANES III protocol for the period 1988-1994 in the United States showed that obesity was prevalent in approximately 35% of American adults, with peaks of 46.7 and 48.6% for the Hispanic and Black female populations, respectively (KUCZMARSKI *et al.*, 1997). Among American children approximately 22% were overweight (GORTMAKER *et al.*, 1987; TROIANO *et al.*, 1995; DANIELS *et al.*, 1999), while 10.9% were obese. These results indicate a considerable increase during the last few years (TROIANO *et al.*, 1995; FREEDMAN *et al.*, 1997).

Using data from the Italian National Health Survey for the entire Italian adult population, it was found that 31.6% of the adults were overweight and 6.5%

were obese (PAGANO and LAVECCHIA, 1994). In northeastern Italy, the prevalence of being overweight or obese was found to be similar in boys and girls (overweight, 15.7 and 11.0%, respectively; obese, 14.1 and 10.6%, respectively). The highest prevalence of obesity (23.4%) was observed in 10-year-old boys (MAFFEIS *et al.*, 1993). More recent data gathered in Liguria indicate that 35.8% of school-age children (3rd grade) are overweight (39.4 males and 32.1% females) (LANTIERI *et al.*, 1999).

Some of the habits that affect eating behavior, include present-day family habits (parents and children only eat together in the evening and on weekends), a predominantly sedentary lifestyle and an excessive intake of sweetened drinks and pre-packaged foods, especially in children. The media, especially television, seems to encourage the overconsumption of food (CIANGHEROTTI *et al.*, 1996).

In an effort to plan a preventive strategy against obesity and its complications, the aim of this study was to analyse anthropometric parameters, energy balance, eating and nutritional habits, as well as risk indicators, such as being overweight and the associated behavioral factors, in school children in the region of Liguria.

MATERIALS AND METHODS

Data were collected from a population of third-grade children in 1999-2000, stratified according to provinces [Genoa (GE), Imperia (IM), Savona (SV), and La Spezia (SP)], geographical location (coast, hinterland) and the socio-economic character of the area (residential, working class, industrial, rural).

Unit samples per class (cluster sampling) were used instead of individual pupils, because it was more convenient for data collection while still being statistically valid. The rationale for selecting third graders was based on a plan to monitor the children's eating behavior and anthropometric maturation in subsequent years. Moreover, within each population layer, the choice of classes was aimed at respecting the regional geography whose variability, even in terms of local traditions, could affect eating habits.

The final outcome was a population-stratified sample of 126 classes (GE 57, IM 22, SP 21, SV 26) with 1,714 pupils, or 17% of all third-graders in Ligurian schools. The number of students was planned in view of a predictable information loss due to the transfer or absence of some children, the refusal of some families to participate and unreliable answers, while ensuring results from at least 10% of the total third-grade population.

The study was conducted by means of diet questionnaires (a 24-h recall; a weekly food frequency; some family eating habits), biometric and behavioral questionnaires (collection of anthropometric characteristics of both children and their parent(s); physiological and pathological case history data, description of tasks carried out and physical activity, play activity and sports performed).

The questionnaires were designed and assessed by a team of nutritionists, dieticians, paediatricians, biostatisticians, school doctors and teachers. The questionnaires were assessed by the National Institute of Nutrition and were validated through a pilot study (CIANGHEROTTI *et al.*, 1996; 1999a). The questionnaires also included some information that was not used in this study.

The diet questionnaires, except the ones related to the family, were filled out by the pupils in collaboration with their teachers; the biometric and behavioral questionnaires were filled out by school doctors and/or healthcare assistants for the schools included in the sample. Teachers, school doctors and healthcare

assistants were trained in specially organized meetings in each province.

The following parameters were measured: height (m), body mass (kg), body mass index (BMI: kg/m^2) and relative body mass index (rBMI = BMI / desirable BMI). The desirable BMI according to age and sex was calculated by interpolating European data considered valid for the national milieu by the Italian Society of Human Nutrition (COMMISSION OF THE EUROPEAN COMMUNITIES, 1993; S.I.N.U, 1996). The overweight index was defined as a BMI value at least 10% higher than the desirable one (rBMI ≥ 1.10); the obesity index was defined as a BMI value at least 20% higher than desired (rBMI ≥ 1.20) and an underweight index was defined as a BMI value at least 20% less than desired (rBMI ≤ 0.80). The body mass of the parents was reported by the pupils using semi-quantitative description criteria (underweight, normal weight, overweight) and by the parents, themselves as height and weight.

To analyse the diet, a food composition database was designed (CARNOVALE *et al.*, 1996; HOLLAND *et al.*, 1991) which included over 200 dishes packaged according to traditional and/or local cooking criteria, as well as software to quantify 64 nutritional parameters.

Carbohydrates were expressed as simple carbohydrates, complex carbohydrates and dietary fibres; lipids were classified as saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA, n6 and n3); proteins were listed as essential and nonessential amino acids. Among the vitamins, some coenzymes of oxidative biochemical reactions (pro-oxidant vitamins: B_1, B_2, PP) and the vitamins anti-free radicals (antioxidant vitamins: A, C, E) were calculated. The energy distribution among the daily meals and among the nutrients, and the PUFA n6/n3 ratio, n6/EI and n3/EI ratios were evaluated.

The energy balance (EB) value was calculated as the difference between energy intake (EI) and energy expenditure (EE). The EE was estimated by the average daily costs for: basal metabolic rate, as a function of body weight and height (SCHOFIELD *et al.*, 1985), dietary induced thermogenesis, calculated from dietary composition, physical activity thermogenesis, including sedentary activity, and growth (2% EI) (BUTTE, 2000).

Sedentary activity refers to the time spent studying, watching and listening to media, using the computer or other interactive audiovisual systems in the course of a typical day; other sections included the time spent sleeping and eating. Physical activity was estimated according to the average daily EE values for specific physical activities considering both the commitment and duration of the training sessions in the case of competitive sports (FERRO-LUZZI, 1987; COMMISSION OF THE EUROPEAN COMMUNITIES, 1993).

All questionnaires were checked, and crosschecked, for rationality, accuracy and consistency, and were classified accordingly. Unlikely data were noted and, when possible, corrected. The questionnaires deemed unreliable were not processed and only the information considered correct was used.

The statistical analysis included:

- Chi-square test to compare the rate of qualitative variables.
- Student's t test or Mann-Whitney U test or multifactorial analysis of variance to compare mean values of quantitative variables.
- Multivariate linear regression analysis, stepwise regression analysis and multivariate logistic regression analysis to evaluate obesity risk factors and the probability of the risk of being overweight

$$P = 100 \cdot \frac{e^{\lambda_0+\lambda_1 \cdot x_1 + \ldots + \lambda_n \cdot x_n}}{1 + e^{\lambda_0+\lambda_1 \cdot x_1 + \ldots + \lambda_n \cdot x_n}}$$

where λ_i are logistic regression coefficients of x_i risk factors.

The significance level was taken as $\alpha = 0.05$.

RESULTS AND DISCUSSION

Diet questionnaires from 1,342 children (685 boys and 657 girls, mean age 8.5±0.4 years, range 7.5-11.0) were deemed reliable for statistical analysis and included the information required to define the anthropometric profile. This sample represented 13.3% of all third-graders in Ligurian schools and its strata proportions were not significantly different from the regional strata: the sample was statistically representative.

Anthropometric results

Height, weight and BMI, subdivided according to gender and geographical location (Table 1), were generally comparable to normal values for the reference population and with the results from a similar earlier study (LANTIERI *et al.*, 1999). BMI values tended to be higher in the hinterland (P = 0.041), for girls in the entire region and for boys in the provinces of Genoa and Imperia. No socio-economic differences were observed.

The results revealed that 31.9% of the children were overweight, with a significantly different rate (P = 0.001) between males (35.9%) and females (27.7%), and that 18.3% (20.7% males, 15.7% females; P = 0.020) were obese. Obesity was prevalent in the hinterland (21.4% vs. 16.4%, P = 0.028), and was statistically more evident in girls (20.5% vs. 13.2%; P = 0.015). Thirty-five percent (35.3%) of the subjects had at least one overweight parent. The results also showed that 2.5% of the subjects (1.8% males, 3.3% females; P = 0.081) were underweight.

Diet

The average daily intake of energy and nutrients and their distribution are shown in Tables 2 and 3, respectively. The EI for the Ligurian school children was significantly higher in males (P = 0.0001), with a mean value that increased by approximately 0.5 MJ / day for both sexes during the last five years. It also seemed to be higher in the province of Imperia, especially when compared to Savona and was significantly higher when compared to La Spezia (P = 0.020). These results confirmed the results of an earlier study (CIANGHEROTTI *et al.*, 1999b). No particular socio-economic differences were observed nor was there

Table 1 - Anthropometric values: mean and (95% confidence interval) subdivided according to gender and geographical location.

		Male			Female		
		Overall	Coast	Hinterland	Overall	Coast	Hinterland
Height	(cm)	132.8 (132.3-133.3)	132.7 (132.1-133.3)	132.9 (132.1-133.6)	131.9 (131.4-132.4)	131.4 (130.8-132.0)	132.8 (131.9-133.7)
Weight	(kg)	31.3 (30.8-31.9)	31.2 (30.5-31.9)	31.6 (30.7-32.5)	30.3 (29.8-30.8)	29.9 (29.3-30.5)	31.2 (30.4-32.1)
BMI [a]	(kg/m^2)	17.6 (17.4-17.9)	17.6 (17.3-17.8)	17.8 (17.4-18.2)	17.3 (17.1-17.5)	17.2 (16.9-17.4)	17.6 (17.3-18.0)

[a] Coast vs. Hinterland P = 0.041 (by multifactorial analysis of variance).

Table 2 - Nutritional composition of the diet: mean and (95% confidence interval) subdivided according to gender and geographical location.

		Male			Female		
		Overall	Coast	Hinterland	Overall	Coast	Hinterland
Energy	(MJ)	8.92 (8.71-9.13)	8.99 (8.71-9.28)	8.82 (8.51-9.13)	8.34 (8.14-8.54)	8.31 (8.07-8.56)	8.41 (8.06-8.75)
Carbohydrates	(g)	294 (286-301)	296 (287-306)	289 (279-300)	268 (261-275)	268 (259-276)	269 (257-281)
	(% MJ)	53.9 (53.4-54.3)	53.9 (53.4-54.5)	53.8 (53.1-54.5)	52.5 (52.1-53.0)	52.6 (52.0-53.2)	52.4 (51.6-53.3)
Complex	(g)	175.4 (170.7-180.2)	177.9 (171.5-184.3)	171.7 (164.7-178.7)	160.6 (156.2-165.0)	160.3 (154.9-165.6)	161.2 (153.7-168.8)
Soluble	(g)	98.4 (94.8-101.9)	98.3 (93.8-102.8)	98.4 (92.7-104.2)	89.4 (86.2-92.6)	89.0 (85.0-93.0)	90.1 (84.6-95.6)
Fibres	(g)	13.6 (13.2-14.0)	13.8 (13.3-14.3)	13.2 (12.6-13.9)	12.8 (12.4-13.2)	12.8 (12.4-13.3)	12.6 (12.0-13.3)
Proteins	(g)	74.7 (72.9-76.6)	75.7 (73.2-78.2)	73.3 (70.5-76.1)	71.3 (69.6-73.1)	71.1 (68.9-73.2)	71.9 (68.8-75.0)
	(% MJ)	14.5 (14.3-14.7)	14.6 (14.3-14.9)	14.4 (14.1-14.8)	14.9 (14.6-15.1)	14.9 (14.6-15.2)	14.9 (14.5-15.3)
Lipids	(g)	75.5 (73.4-77.6)	75.8 (73.0-78.5)	75.1 (71.9-78.4)	72.6 (70.6-74.6)	72.2 (69.7-74.6)	73.4 (69.9-77.0)
	(% MJ)	31.6 (31.3-32.0)	31.5 (31.0-32.0)	31.8 (31.2-32.4)	32.6 (32.2-33.0)	32.6 (32.1-33.0)	32.7 (32.1-33.4)
Vitamin B_1	(mg)	0.96 (0.83-1.09)	1.04 (0.86-1.21)	0.86 (0.68-1.03)	0.86 (0.75-0.98)	0.91 (0.75-1.06)	0.78 (0.63-0.93)
Vitamin B_2	(mg)	1.48 (1.44-1.52)	1.49 (1.43-1.54)	1.47 (1.41-1.54)	1.42 (1.38-1.46)	1.41 (1.36-1.45)	1.45 (1.37-1.52)
Vitamin PP	(mg)	16.0 (15.5-16.6)	16.3 (15.6-17.0)	15.6 (14.7-16.4)	15.0 (14.5-15.5)	14.9 (14.3-15.5)	15.2 (14.2-16.1)
Vitamin A	(μg)	607 (554-659)	594 (542-647)	625 (518-732)	588 (550-627)	573 (544-603)	619 (521-717)
Vitamin C	(mg)	58.1 (55.4-60.8)	59.1 (55.7-62.6)	56.6 (52.4-60.8)	57.0 (54.2-59.8)	57.6 (54.3-60.9)	55.7 (50.6-60.8)
Vitamin E	(mg)	7.1 (6.9-7.4)	7.2 (6.9-7.5)	7.1 (6.8-7.5)	6.9 (6.6-7.1)	6.9 (6.6-7.1)	6.9 (6.5-7.3)
Na	(mg)	1,160 (1,117-1,202)	1,161 (1,106-1,215)	1,159 (1,091-1,226)	1,118 (1,077-1,158)	1,107 (1,056-1,158)	1,139 (1,071-1,206)
K	(mg)	2,232 (2,167-2,298)	2,237 (2,154-2,320)	2,225 (2,119-2,331)	2,133 (2,066-2,199)	2,120 (2,043-2,198)	2,158 (2,032-2,284)
Ca	(mg)	712 (686-738)	704 (670-737)	725 (685-765)	698 (674-722)	690 (660-719)	714 (674-754)
P	(mg)	1,153 (1,125-1,181)	1,155 (1,118-1,192)	1,151 (1,108-1,193)	1,104 (1,077-1,132)	1,095 (1,062-1,128)	1,123 (1,075-1,172)
Fe	(mg)	8.86 (8.60-9.11)	8.81 (8.49-9.13)	8.93 (8.51-9.35)	8.37 (8.13-8.61)	8.22 (7.94-8.50)	8.68 (8.23-9.12)

Table 3 - Energy intake distribution in the six daily meals: MJ mean values and (percent of total 95% confidence interval) subdivided according to gender and geographical location.

	Male			Female		
	Overall	Coast	Hinterland	Overall	Coast	Hinterland
Breakfast	313 (14.2-15.5)	325 (14.5-16.2)	293 (13.0-15.1)	273 (13.4-14.6)	271 (13.1-14.6)	276 (13.1-15.5)
Midmorning break	184 (8.4-9.4)	177 (7.9-9.2)	194 (8.7-10.2)	171 (8.3-9.3)	172 (8.2-9.3)	169 (8.0-9.7)
Lunch	754 (35.0-36.7)	746 (34.1-36.2)	766 (35.5-38.3)	716 (35.6-37.2)	703 (35.3-37.3)	741 (35.3-38.0)
Afternoon snack	213 (9.3-10.4)	214 (9.0-10.4)	212 (9.1-10.9)	209 (9.8-10.8)	209 (9.6-11.0)	209 (9.3-11.3)
Dinner	627 (28.0-29.6)	650 (28.6-30.7)	591 (26.1-28.8)	587 (28.0-29.6)	596 (28.3-30.3)	569 (26.5-29.1)
Evening snack	41 (1.5-2.1)	35 (1.3-1.9)	51 (1.7-2.6)	38 (1.4-2.0)	34 (1.2-1.8)	44 (1.5-2.6)

disparity between the coast and hinterland. The EI was more than 10.5 MJ for 20.4% of the sample (23.5% of the males, 17.2% of the females; P = 0.004) and less than 6.3 MJ for 17.9%, with a prevalence of females (20.7% vs. 15.2%; P = 0.010). The rounded off sum of the mean values predicted by the basal metabolic rate, dietary thermogenesis and growth was 6.3 MJ and 10.5 MJ corresponds to the mean EI value plus 20%. In this sample, these values represent approximately the 20^{th} and 80^{th} percentiles in males and the 15^{th} and 85^{th} percentiles in females.

Macronutrient intake and distribution was adequate, i.e., they corresponded to the recommended levels for the age group of the Italian population examined (S.I.N.U, 1996). More than half the children (60.0%) consumed amounts of simple sugars that were higher than 15% of EI. This was probably linked to the consumption of snacks, sweets and sweet drinks; almost one-third of all sugar intake was in the form of simple sugars.

The average amount of daily dietary fibre was 13.2±5.4 g, corresponding to 6.5 g / 4.2 MJ, adequate for 8-9 year olds. This value was probably due to the regular consumption of whole-wheat foods (cereals) recorded in more than half of the families, and was, perhaps, associated with the low incidence of constipation reported by the children (10%). WILLIAMS *et al.* (1995) recommend that children and adolescents, between the ages of two and twenty years, should eat a daily amount of fibre equal to how old they are plus 5. Diets low in fibre content, with a low consumption of fruit and vegetables and a high consumption of red meat and milk products, correspond to common eating habits in western countries. When these habits persist from childhood to adulthood high incidences of colon cancer (WILLET *et al.*, 1990) and other neoplasias are likely in adulthood (JAIN, 1998).

The average fat content was 74.1±27.4 g/day, corresponding to 32.1% of the EI; the value is the same as that estimated for the Italian diet (CARNOVALE *et al.*, 1994). An upper limit of dietary fat of 30% of the EI for children more than two years old was fixed in 1995 by the Commission of Paediatricians of the American Academy (DGA, 1995) and is also the recommended level in Italy for children and adolescents (S.I.N.U, 1996). In our sample 66.8% of the pupils had

values higher than this level (69.9% females, 63.8% males; P = 0.020).

The dietary fatty acid composition revealed mean SFA and MUFA values of 40.3 and 48.9%, respectively, corresponding to 11.9 and 14.3% of the EI. MUFA-rich diets (olive oil consumption was preferred by 92% of the families for cooking and by 96% of the families for salads), compared to calorically equal SFA-rich diets, modify the lipid status in human plasma by reducing the cholesterol-LDL/HDL ratio, and by reducing the activity of cholesteryl ester transfer protein (CETP) (GROENER *et al.*, 1991).

PUFA accounted for 10.8% of the lipid fraction, equal to 3.1% of the total EI, presently considered optimal for satisfying growth requirements. For 7.9% of the pupils, however, the PUFA intake was below the lower optimal limit of 2% of the total EI.

The dietary n6/n3 ratio, an important determinant of the fatty acid pattern (HARRIS, 1989; PHILLIPSON *et al.*, 1985, SIRTORI *et al.*, 1997), was equal to 5.7. This value is considered adequate for growing children. On average, the daily consumption of cholesterol was found to be high (283 mg) and almost one-third of the pupils (31.7%) consumed more than 150 mg / 4.2 MJ, compared to an upper limit of 100 mg / 4.2 MJ.

The average protein intake (73.1±24.2 g/day, corresponding to 2.5 g/kg/day and to 35.9 g / 4.2 MJ) was much higher than the recommended level, 1.28 g/kg/day (S.I.N.U, 1996). The amount of essential amino acids was between 35 and 50% of the total amino acids giving an adequate total value in terms of biological value of the protein dietary mixture.

In some cases, although the EI was adequate, the dietary fat and/or protein load was excessive. This risk factor was present in 4.5% of the subjects for protein and in 25.9% of the subjects for fat. These values increased significantly ($P < 0.0001$) compared to 0.7 and 15%, respectively, which were recorded in a previous study (CIANGHEROTTI *et al.*, 1996). A high-protein intake (>20% EI) starting in the early years of life predisposes a child to obesity (CLEMMONS and UNDERWOOD, 1991; ZAPF *et al.*, 1981).

While the average values for vitamin intake were within the normal range, 17.7% of the school children did not take an adequate amount of at least one vitamin from the pro-oxidant group (especially vitamin B_1), 22.4% from the antioxidant group (especially vitamin A), and 9.4% from both groups. For the first group, the low vitamin intake could have been related to a reduced calorie intake observed in 17.7% of the subjects; for the second group, it could also have been related to inadequate consumption of fruit and vegetables, given that the subjects did not regularly consume, or explicitly refused fruit (31%), raw vegetables (27%), or both (14%).

Regarding inorganic micronutrients, the subjects consumed an average of 705 mg/day of calcium; this amount was only 70% of the recommended level (S.I.N.U., 1996) for achieving peak bone mass (MATKOVIC and ILICH, 1993). The reduced calcium intake, recorded in 39% of the pupils, increased to 49% among those who did not consume milk or cheese (9%) and reached 76% in those who refused milk altogether. Low milk consumption (only 37% of the pupils drank milk more than once a day) might have been due to reports of allergy and, for almost a hundred of them, to complaints of β-galactosidase deficiency symptoms (painful abdominal cramps, meteorism, flatulence, diarrhoea), though without an objective histological diagnosis. The results were consistent with the occurrence of lactose malabsorption observed in Liguria on a sample of preschool children of both sexes (ARRIGO *et al.*, 1980).

Iron intake (8.6 mg/day), which varied significantly between males and females (8.9 vs. 8.4; P = 0.012), was at the lower limit of the recommended level for that age (9 mg/day); about 10% of the subjects did not consume 5 mg/day. Some minor statistical differences were observed between children from the coast and the hinterland, and from the different provinces, but was of little importance.

Lifestyle and energy balance

The time spent in daily physical activity (sports and open-air activity) and the time for sedentary activities were 1.2±0.8 and 2.9±1.2 h, respectively. Sports were significantly less practiced in the hinterland in term of both frequency (P = 0.001) and time given to it (P = 0.002) and less by females than males, though the difference was slight and not significant.

Watching television and interactive videogames (TV/PC) made up more than two-thirds of the sedentary activity and so represent an index of sedentary behaviour. The EB value was positive by at least 1.3 MJ in half of the subjects, regardless of gender.

CONCLUSIONS

The EI did not seem to be positively correlated with the BMI. Conversely, a high-calorie diet (> 10.5 MJ) with an EI ≥ 10% of EE (EI ≥ 1.1 EE) seemed to favour the development of being overweight. The prevalence of being overweight in our sample was significantly higher (P < 0.0001), under these conditions, than in those subjects who, although they had a positive EB ≥ 10%, did not have a high-energy diet (30.0% vs. 12.1% females and 37.1% vs. 13.8% males).

OBARZANEC *et al.* (1994) indicated that physical activity was inversely correlated with the BMI, as was the percentage of body fat in a sample of black American girls aged 9-10 years. Some of these results, confirmed by recent studies (MAFFEIS *et al.*, 1996; MYERS *et al.*, 1996; GUILLAME *et al.*, 1997), emphasize the importance of physical exercise for the prevention of obesity (WOLF *et al.*, 1993). In the mid-to-long term the daily watching of TV/PC, especially for extended periods of time, may represent a risk factor for the onset of obesity (SHANNON *et al.*, 1991). Conversely, a reduction in time dedicated to TV/PC implies a decrease in being overweight (SCHONFELD-WARDEN and WARDEN, 1997). Our sample showed a moderate, though significant (P = 0.045), correlation between sedentary activity and being overweight. Children who spent at least three hours watching TV/PC had an average rBMI that was higher than the rBMI of those who spent less time watching TV/PC (1.08±0.19 vs. 1.05±0.18; P = 0.030). In terms of weight, this corresponded to a difference of almost 1 kg. This result confirms that children who spend more time listening to and watching TV/PC tend to be overweight (LANTIERI *et al.*, 1999).

Dietary fat intake seems to be associated with the incidence of obesity (SCHAEFER *et al.*, 1995). Recent studies confirm that high-fat diets can, in time, result in adipose cell hypertrophy, just as with high SFA and MUFA intake, whereas high PUFA intake does not seem to have any effect (DOUCET *et al.*, 1998). Other hypotheses have been proposed to support the relationship between dietary fat and body composition, such as "passive over consumption" (BLUNDELL and MACDIARMID, 1997) and a reduced thermogenetic response to fat ingestion (SCHUTZ *et al.*, 1989). Our results showed a non-significant relationship between fat intake and being overweight.

In the absence of an overweight parent, the rate of obesity was 14% in the males and 9% in the females, while in the presence of one overweight parent,

the rate of obesity was 26 and 21% and, in the presence of both overweight parents, it was 51 and 42%, respectively. The risk of obesity in adulthood is greater if the subject, as a child, lived in a family where at least one cohabitant parent was obese, regardless of the latter's sex and age, (GRILO and POGUE-GEILE, 1991; MAES *et al.*, 1997; WHITAKER *et al.*, 1997; CIANGHEROTTI *et al.*, 1999b).

The multivariate linear regression and stepwise regression analyses were applied by taking the children's rBMI as the dependent variable and the birthweight, parental BMI, EI, hours spent in physical activity, hours dedicated to TV/PC as an index of sedentary activity, carbohydrate, protein and fat energy intake as regressors. We found the variability in rBMI to be significantly associated with the parental BMI ($P < 0.0001$), especially maternal, TV/PC ($P = 0.0036$) and birthweight ($P = 0.0220$). If we consider only the subjects with a positive EB by at least 10% ($EI \geq 1.10$ EE), the regressions highlighted the parent being overweight (father $P = 0.0013$; mother $P < 0.0001$), the hours spent watching TV/PC ($P < 0.0001$) and the EI ($P < 0.0001$) as risk factors. This was confirmed by the logistic regression relative to the incidence of being overweight or not ($rBMI \geq 1.10$).

For a much simpler interpretation of the results, the number of overweight parents, EI >10.5 MJ or ≤10.5 MJ and hours spent watching TV/PC ≥ 3 h or < 3 h were entered in the logistic analysis. The logistic model confirmed that all these factors contributed significantly to being overweight and, except for sedentary activity, obese (Table 4). All other factors being equal, the approximate relative risk of being overweight was about double for each overweight parent (OR = 2.049; 95% CI 1.457-2.882) and with at least three hours spent watching TV/PC (OR = 1.722; 95% CI 1.047-2.830), and it tripled with an EI > 10.5 MJ (OR = 3.330; 95% CI 2.060-5.383). The relative risk of being obese was more than double for each overweight parent (OR = 2.339; 95% CI 1.557-3.513) and four times greater if the EI was high (OR = 4.531; 95% CI 2.508-8.186).

Logistic analysis estimated the risk that the factors might play if overweight or obese. As the number and level of significant factors increase, so does the probability of being overweight or obese. For all the significant factors, the estimated probability of being overweight was over 60% and that of being obese was about 40% (Fig. 1). The higher probability for overweight compared with the probability being obese is do to the fact that when the subject is found to be overweight efforts are made to prevent further increase in weight.

In conclusion, the prevalence of being overweight and obese in Ligurian chil-

Table 4 - Logistic Regression (subjects with Energy Intake ≥ 1.1 Energy Expenditure): coefficients λ, Odd Ratio (OR) and 95% confidence interval of OR.

	Coefficient λ	Std. Error	P-Value	OR	95% Lower	95% Upper
Overweight						
Constant	-2.575	0.244	<.0001	0.076	0.047	0.123
Overweight parent (n°)	0.717	0.174	<.0001	2.049	1.457	2.882
Energy Intake >10.5 MJ	1.203	0.245	<.0001	3.330	2.060	5.383
TV/PC ≥ 3h	0.543	0.254	0.0321	1.722	1.047	2.830
Obesity						
Constant	-3.679	0.313	<.0001	0.025	0.014	0.047
Overweight parent (n°)	0.850	0.208	<.0001	2.339	1.557	3.513
Energy Intake >10.5 MJ	1.511	0.302	<.0001	4.531	2.508	8.186

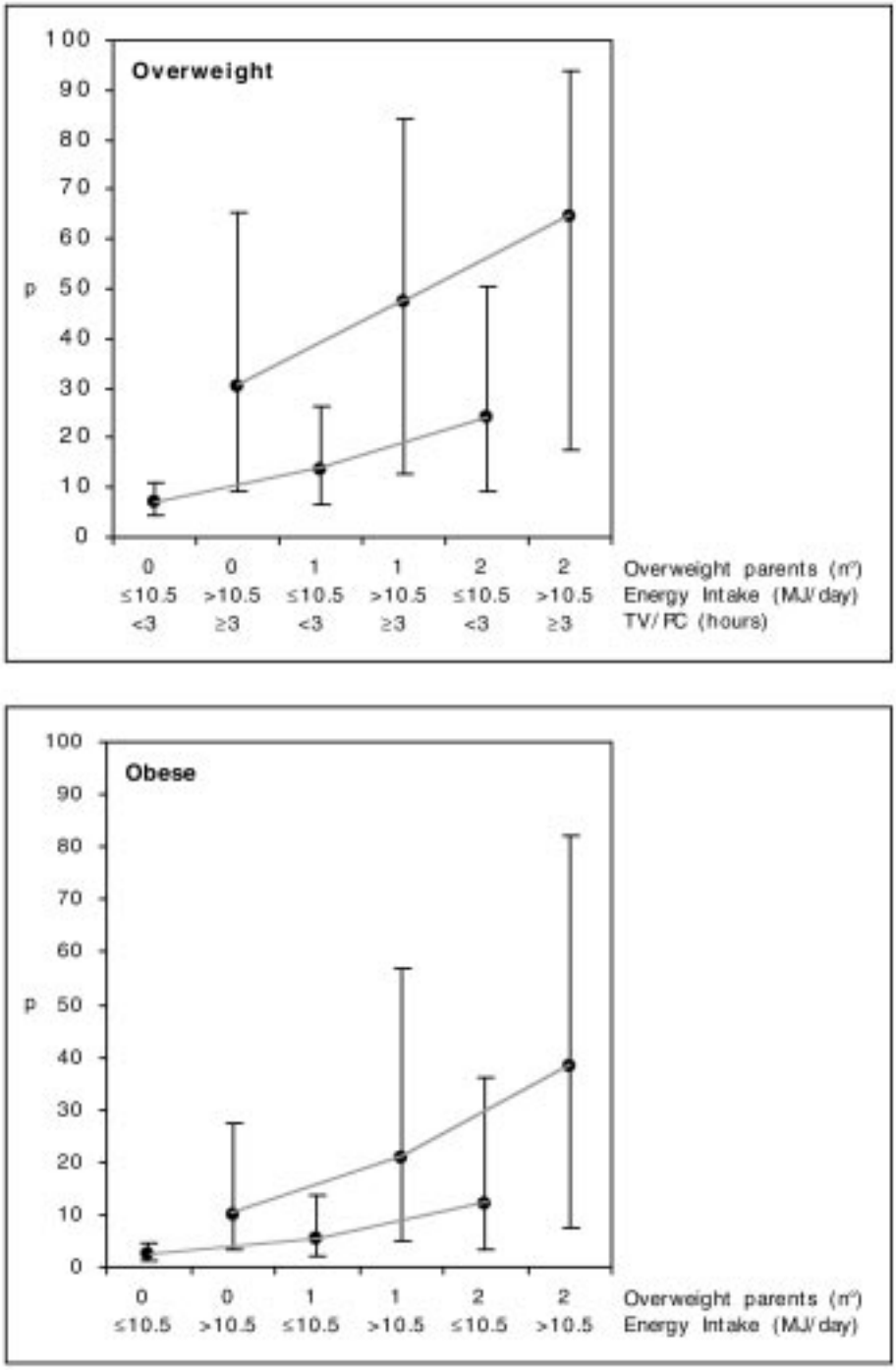


Fig. 1 - Probability of being overweight or obese (with 95% confidence interval) in pupils with positive Energy Balance (Energy Intake ≥ 1.1 Energy Expenditure) as related to overweight parents, Energy Intake and TV/PC (calculated by logistic regression coefficient λ).

dren has increased when the results of this study are compared with the results obtained in an earlier study (LANTIERI *et al.*, 1999). The number of overweight parents, overeating associated with a positive EB and sedentary activity played a part in defining eating habits and lifestyles.

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PAPER

ANALYTICAL METHODS FOR THE DETERMINATION OF THE GEOGRAPHIC ORIGIN OF EMMENTAL CHEESE. PARAMETERS OF PROTEOLYSIS AND RHEOLOGY

METODI ANALITICI PER LA DETERMINAZIONE DELL'ORIGINE GEOGRAFICA DEL FORMAGGIO EMMENTHAL. PARAMETRI DELLA PROTEOLISI E REOLOGIA

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ABSTRACT

Nitrogen fractions, biogenic amines, free amino acids, casein fractions, HPLC-peptide profiles and rheological properties were investigated in 20 Emmental cheeses from six European regions (Switzerland, Vorarlberg (A), Allgäu (D), Finland, Bretagne (F) and Savoie (F)). The ripening times of the samples differed, according to what is found on the market in each region. The non-protein nitrogen and the water-soluble nitrogen fractions showed significant inter-regional differences due

RIASSUNTO

Le frazioni azotate, le ammine biogene, gli amminoacidi liberi, le frazioni caseiniche, i profili dei peptidi-HPLC e le proprietà reologiche sono stati studiati in 20 formaggi Emmental provenienti da sei diverse regioni europee (Svizzera, Vorarlberg (A), Allgäu (D), Finlandia, Bretagne (F) e Savoie (F)). Le frazioni dell'azoto non proteico e dell'azoto solubile in acqua hanno mostrato differenze inter-regionali significative. "Allgäu" e "Savoie" hanno rivelato il più alto tasso di proteolisi mentre

- Key words: authenticity, Emmental cheese, free amino acid, peptide, proteolysis, traceability -

mostly to the different ripening times. "Allgäu" and "Savoie" showed the highest proteolysis rate and "Bretagne" had the lowest. Biogenic amines and rheological properties were of lesser significance for discriminating geographic origin. The relative amounts of five free amino acids allowed "Switzerland" to be separated from the others. The X3 (unknown structure) and α_{s1} casein fractions combined with one peak from the peptide profile allowed three distinct groups to be differentiated: "Finland", "Bretagne" and "Savoie".

"Bretagne" quello più basso. Le ammine di provenienza biologica e le proprietà reologiche si sono mostrate meno importanti nel determinare l'origine geografica dei formaggi. I contenuti relativi di cinque amminoacidi hanno permesso di separare "Switzerland" dagli altri. Le frazioni della caseina X3 (struttura sconosciuta) e α_{s1}, assieme a uno picco del profilo dei peptidi, hanno permesso di differenziare tre gruppi diversi: rispettivamente quello "Finland", quello "Bretagne" e quello "Savoie".

INTRODUCTION

The enzymatic degradation of casein into peptides and amino acids is a very complex process because of the various origins of the proteases and peptidases involved. They may come from the milk itself (plasmin, cathepsin), the coagulant (chymosin, pepsin and other fungal proteases), the starter, as well as non-starter micro-organisms, secondary cultures (e.g., *P. camemberti*) and exogeneous proteinases or peptidases. Proteolysis occurs to a great extent during ripening. The degree of protein breakdown can be expressed by factors such as the content of water soluble nitrogen (WSN), non-protein nitrogen (NPN) or total free amino acids. Proteolysis has been considered as the basis for classifying different cheese types (MARCOS *et al.*, 1979; SMITH and NAKAI, 1990; AISHIMA and NAKAI, 1987; McGOLDRICK and FOX, 1999; FOX, 1993). The evolution of proteolysis, based on peptide mapping at various stages of ripening, has already been studied for Emmental (BICAN and SPAHNI, 1993, BÜTIKOFER *et al.*, 1997) Gruyère, Appenzell (BICAN and SPAHNI, 1993), Parmigiano-Reggiano (ADDEO *et al.*, 1994), Blue cheese (GONZALEZ DE LLANO *et al.*, 1991) and for several artisanal cheeses (GONZALEZ DE LLANO *et al.*, 1995). The influence of specific Lactobacilli strains on the peptide profile has been studied in cheeses such as Emmental (BÜTIKOFER *et al.*, 1997; CHOPARD *et al.*, 2001) and Cheddar (McSWEENEY *et al.*, 1994; PRIPP *et al.*, 1999). Considerable differences were found between peptide profiles of Cheddar cheese made from raw or pasteurised milk (McSWEENEY *et al.*, 1993).

Further degradation of peptides by peptidases leads to small peptides and free amino acids (FAA). The total concentration of FAA is also an indicator of age. RESMINI *et al.* (1985, 1993) identified genuine Parmigiano-Reggiano and Grana Padano cheese by their free amino acid composition. BÜTIKOFER and FUCHS (1997) were able to correctly classify Emmental, Gruyère and Sbrinz with more than 93% accuracy from their relative amounts of free amino acids. Appenzeller and Tilsiter could be correctly classified to 70-75%.

The degradation of the free amino acids through enzymatic decarboxylation may lead to the formation of biogenic amines. In cheese, non-starter bacteria such as enterococci, salt tolerant lactobacilli and enterobacteriaceae may be responsible for the formation of biogenic amines (JOOSTEN and NORTHOLT,

1987). Some of these amines may provoke allergies or illness. The best known amines in cheese are histamine, tyramine, cadaverine, putrescine, tryptamine and β-phenylethylamine (SIEBER and LAVANCHY, 1990).

Rheological characteristics are often correlated with proteolysis (BOSSET *et al.*, 1993; EBERHARD, 1985). Large peptides are important for the development of the correct texture. PIRISI *et al.* (2000) described large differences in mechanical properties of similar PDO ewe's milk cheeses from three different countries. Cheeses from milk produced on mountain pastures exhibited different rheological properties than those produced with milk from valley pastures; they were less elastic and less deformable (BUGAUD *et al.*, 2001).

The present work is part of a broad screening test, which is the first step in a 3-year study on the authenticity of Emmental cheese and its geographic traceability (PILLONEL *et al.*, 2002a; BOSSET, 2001). To date, a large number of analytical methods have been tested for their discriminating potential (PILLONEL *et al.*, 2002a-b; PILLONEL *et al.* 2003a-c), while the number of cheese samples for each region has been limited. Obviously, the analytical results obtained from a modest number of cheese samples from a region can only give trends which will need to be confirmed later if they appear to be of value for discriminating cheeses produced in different countries. The objective of the present paper was to determine whether chemical and physical parameters linked to proteolysis, such as nitrogen fractions, biogenic amines, free amino acids, casein fractions, peptide pattern and rheological analyses make it possible to discriminate between the different geographic origins of Emmental cheese samples. The goal was not to build a detailed model for predicting the origin but rather to observe the trends. The cheese samples, with different ripening times, were chosen from what is sold by retailers in the corresponding regions.

MATERIALS AND METHODS

Origin and selection of the cheese samples

The main framework of this study and the sampling methods are described in detail in a previous work (PILLONEL *et al.*, 2002a). Table 1 summarises the origin, date of production and ripening time of the samples.

Analysis of the compounds produced by proteolysis

The samples were deep-frozen prior to the following analyses: total nitrogen (TN), water-soluble nitrogen (WSN) and non-protein nitrogen (NPN) according to Kjeldahl (COLLOMB *et al.*, 1990); free amino acids (FAA) using HPLC after pre-column derivatisation with o-phthalaldehyde and fluorenylmethyl chloroformate (FMOC) (BÜTIKOFER and ARDÖ,

Table 1 - Origin and ripening time of the 20 cheese samples investigated.

Abbreviation	Region (country)	Number of samples	Date of manufacture	Ripening time (months)
AL	Allgäu (D)	3	25.12.2000	4
BR	Bretagne (F)	3	20.02.2001	2.5
CH	Switzerland (CH)	6	26.12.2000	4
FI	Middle Finland (FI)	2	04.02.2001	3
SA	Savoie (F)	3	05.02.2001	3
VO	Vorarlberg (A)	3	02.02.2001	3

1999); biogenic amines with HPLC after precolumn derivatisation with dansylchloride (BÜTIKOFER *et al.*, 1990); o-phthalaldehyde-value (OPA) photometrically (FRISTER *et al.*, 1989). Casein fractions were determined by SDS electrophoresis (COLLIN *et al.*, 1987). The peptide pattern of the water-soluble peptide fraction was investigated by RP-HPLC (CHOPARD *et al.*, 2001).

Rheological analyses

The following three rheological properties were measured with a universal testing machine (Zwick 1435, Zwick GmbH, Germany) at 15±0.5°C according to the procedure of PESENTI and LUGINBUEHL (1999): i) the stress at 33% deformation, which characterises the elasticity of the body, ii) the strain at fracture, which is a measure of the consistency of the body and iii) the stress at fracture, which corresponds, more or less, to the sensation of hardness in the mouth. The measurements were carried out on a cylindrical, holeless cheese sample (diameter: 12 mm, height: 15 mm).

The penetration depth was determined using a constant force penetrometer (Petrotest PNR-10, Petrolab, USA) under the following conditions: force, 0.63 N; time, 5 s; standard needle.

Statistical analyses

The averages and standard deviations were calculated for each parameter (Table 2-6). Descriptive statistics, analysis of variance (ANOVA), pairwise comparisons of mean values with Fisher's LSD test, principal component analyses of the correlation matrix and correlation test were performed with Systat for Windows version 9.0 (SPSS Inc., Chicago, IL). The correlation coefficients were calculated using the individual values for cheeses from each region.

RESULTS AND DISCUSSIONS

Since the number of samples analysed was very limited, we restricted the discussion of the results to the tests of the mean value differences and untrained classification techniques, such as principal component analysis (PCA). Trained classification techniques were not used because they require larger data sets in order to be reliable.

Nitrogen fractions, OPA-values and biogenic amines

The results for the nitrogen fractions, the OPA-values and the biogenic amines are given in Table 2. The total nitrogen concentration values were only slightly different whereas the differences between the NPN and WSN values were highly significant. The NPN and the OPA-values were strongly correlated ($r = 0.94$). The OPA-values were less discriminating due to the higher relative standard deviations of the results. "Bretagne" had the lowest NPN and WSN values, as expected for a cheese with such a short ripening time. It was followed by "Finland". "Allgäu" and "Savoie", ripened 4 and 3.5 months, respectively, had the highest NPN and WSN values. The values for "Switzerland" fell in the middle. This Emmental, aged for 4 months, is still considered to be very young in Switzerland. In fact, the proteolysis of "Switzerland" is deliberately slowed down to allow a ripening time of up to 12 months, leading to a very characteristic and mature cheese. In the other regions, fast maturation is preferred for economic reasons. The two ratios, WSN/TN and NPN/WSN, are indices of protein and peptide hydrolyses, respectively. The WSN/TN ratio showed exactly the same trend as that of WSN. For the NPN/WSN ratio, the differences were less significant. A point of interest is that the peptide hydrolysis in "Finland" was significantly higher.

Table 2 - Nitrogen fractions and biogenic amines in the 20 Emmental cheese samples investigated.

Analytes	Nova	Region (n=)											
		AL (3)		BR (3)		CH (6)		FI (2)		SA (3)		VO (3)	
		x	s_x	x	s_x	x	s_x	x	s_x	x	s_x	x	s_x
TN (g/kg)	*	**44.1**^{AB}	1.7	**45.03**^{AB}	0.72	**44.42**^{AB}	0.85	**45.5**^{AB}	1.3	**46.20**^{A}	0.77	**42.9**^{B}	1.6
WSN (g/kg)	***	**11.2**^{A}	1.1	**6.4**^{C}	1.7	**9.35**^{B}	0.41	**8.31**^{BC}	0.51	**10.72**^{AB}	0.99	**8.77**^{B}	0.38
WSN / TN (%)	***	**25.5**^{A}	1.8	**14.2**^{C}	4.0	**21.1**^{B}	1.0	**18.3**^{BC}	0.6	**23.2**^{AB}	1.8	**20.4**^{B}	0.6
NPN (g/kg)	***	**7.37**^{A}	0.65	**3.98**^{D}	0.90	**5.86**^{BC}	0.55	**6.19**^{ABC}	0.58	**7.20**^{AB}	1.07	**5.42**^{CD}	0.37
NPN / WSN (%)	ns	**65.6**	0.8	**62.9**	8.2	**62.6**	4.3	**74.4**	2.4	**66.9**	4.3	**61.7**	2.6
OPA (mmol/kg)	*	**246**^{A}	63	**136**^{B}	39	**184**^{AB}	20	**222**^{AB}	34	**255**^{A}	61	**187**^{AB}	34
Cadaverine (mg/kg)	**	**44**^{A}	35	**5.6**^{B}	7.0	**0.22**^{B}	0.27	**25**^{AB}	15	**8.6**^{B}	6.7	**1.13**^{B}	0.75
Histamine (mg/kg)	*	**672**^{A}	376	**123**^{B}	149	**12.3**^{B}	7.4	**59**^{B}	26	**478**^{A}	434	**205**^{A}	105
Isopentylamine (mg/kg)	**	**1.80**^{AB}	0.66	**0.93**^{B}	0.55	**2.78**^{A}	1.12	**1.05**^{AB}	0.21	**0.53**^{B}	0.15	**0.17**^{B}	0.29
Putrescine (mg/kg)	*	**15**^{AB}	21	**2.3**^{AB}	1.2	**<2**^{B}	—	**<2**^{B}	—	**24**^{A}	19	**<2**^{B}	—
β-Phenylethylamine (mg/kg)	ns	**6.0**	4.0	**1.1**	1.9	**11**	16	**15**	14	**34**	27	**1.2**	2.1
Tryptamine (mg/kg)	ns	**<2**	—	**<2**	—	**<2**	—	**3.0**	2.2	**<2**	—	**<2**	—
Tyramine (mg/kg)	*	**178**^{AB}	121	**8.6**^{B}	8.4	**54**^{B}	123	**220**^{AB}	45	**403**^{A}	269	**117**^{AB}	91
Total biogenic amines (mg/kg)	*	**920**^{A}	420	**142**^{B}	165	**81**^{B}	134	**325**^{AB}	14	**948**^{A}	709	**325**^{AB}	195

Caption: x = mean value; s_x = standard deviation; ANOVA: ns = not significant, *) $p \leq 0.05$, **) $p \leq 0.01$, ***) $p \leq 0.001$.
Production sites: A>B>C>D (=significantly different contents $p \leq 0.01$) or AB = A and B overlap by using a univariate discriminant analysis.
AL = Allgäu, BR = Bretagne, CH = Switzerland, FI = Finland, SA = Savoie, VO = Vorarlberg.

The lowest total concentration of biogenic amines was found in "Switzerland" and the highest in "Allgäu" and "Savoie". These differences were largely due to the degree of proteolysis. Significant differences were found for cadaverine, histamine, isopentylamine, putrescine, tryptamine and tyramine. "Switzerland" had the lowest cadaverine, histamine and putrescine values and slightly higher isopentylamine values. Spermine and spermidine could not be detected in the samples. It should be noted that the biogenic amine concentrations determined did not present any danger to health.

The microbial flora was probably the main factor influencing the amount of biogenic amines. Propionibacterium and thermophilic *Lactobacillus* (the latter being added specifically for this experiment) may account for the formation of histamine in Maasdamer (JOOSTEN, 1987). The influence of Propionibacterium on the formation of histamine and tyramine in Emmental has been reported previously (ANTILA *et al.*, 1984). Non-starter lactic acid bacteria (lactobacilli, enterococci) may cause the formation of tyramine and histamine in Gouda (JOOSTEN and NORTHOLT, 1987). According to the latter authors, salt-tolerant lactobacilli account for a massive formation of putrescine and cadaverine.

Furthermore a concentration gradient was found in Gouda, with the central part of the block containing higher amounts of the investigated amines (tyramine, histamine, putrescine and cadaverine) than samples taken close to the rind (JOOSTEN and STADHOUDERS, 1987). Many chemical, physical and microbial factors also influence the formation of biogenic amines within a cheese variety (SIEBER and BILIC, 1992), leading to very scattered concentrations in samples which come from the same region of production. This was confirmed in "Switzerland" in earlier studies (SIEBER *et al.*, 1988, BOSSET *et al.*, 1992).

Free amino acids (FAA)

The sum of FAA showed the same trend as the WSN and the NPN values but with smaller significant differences between the groups. To produce a discrimination that was less age-dependant, relative amounts were considered (normalisation by the total free amino acids). Out of the 23 free amino acids analysed (Table 3), 16 had a significant difference between at least two groups. No phosphoserine was found. The highest significant differences were found for asparagine, glutamine, methionine, phenylalanine, proline and valine. "Bretagne" could easily be differentiated using the values of methionine and glutamine. With five parameters, asparagine, glycine, lysine, phenylalanine and proline (selection made with the help of a trained classification technique), it was possible to isolate "Switzerland" by PCA (Fig. 1). The other regions could not be clearly separated.

HPLC-peptide profile and casein fractions

Fig. 2 is a typical peptide chromatogram. The 18 peptide peaks which showed significant differences are listed in Table 4. The highest values for peaks 10.1, 11.3, 12.1, 14, 22 and 24 were recorded for "Switzerland". The use of *L. helveticus* in starter cultures is known to lead to a smaller peak 14 (CHOPARD *et al.*, 2001). These findings were confirmed in this study; the highest peak 14 values were found in "Switzerland", the only sample that did not contained *L. helveticus* (PILLONEL *et al.*, 2002a). Peaks 2 and 3 were only found in "Finland" and peaks 4, 7, 12 and 14.1 had much higher values in "Finland" than in cheeses from the other regions.

The relative proportions of α-, β-, and indirectly γ-caseins in milk are subject to genetic variations between cows within the same breed (VARNAM and SUTHERLAND, 1994). In cheese however, the differences between two samples are more likely to be due to different intensities of proteolysis than to the milk used. The results of the casein fractions are listed in Table 5. All casein fractions showed significant differences except for X1-2, X2-1, X2-2 and β degraded. The fractions coded with an "X" are of unknown structure. X1 and X1-2 migrate after β, X2-1 and X2-2 after αS and X3 before α_{s1}. The α_{s1} fraction was highest in "Bretagne" and "Finland" and lowest in "Switzerland", followed by "Allgäu". In soft cheeses, the α_{s1} fraction is typically degraded to α_{s1}-I by the rennet enzyme chymosin. In cooked cheeses such as Emmental, chymosin is extensively denatured during manufacturing and contributes little to ripening (COLLIN *et al.*, 1988; BOUDJELLAB *et al.*, 1994). It is more likely that the α_{s1} degradation is due to the enzymes of the lactobacilli present in the cheese (OLSON, 1990; CHAMBA, 2000). This type of proteolysis however is relatively slow. The low α_{s1} level in "Switzerland" and "Allgäu" can therefore be explained by the longer ripening time in these regions and/or by a higher protease activity of the lactobacilli. "Bretagne" showed the highest value because of the very short ripening time and probably because of the thermisation applied to the milk (PERREARD and

Table 3 - Free amino acid ratios in the 20 Emmental cheese samples investigated.

Analytes	Nova	Region (n=)											
		AL (3)		BR (3)		CH (6)		FI (2)		SA (3)		VO (3)	
		x	s_x	x	s_x	x	s_x	x	s_x	x	s_x	x	s_x
Total free amino acids (g/kg)	*	**28.2**^{A}	7.1	**17.2**^{B}	5.2	**19.8**^{AB}	3.0	**22.5**^{AB}	7.3	**28.7**^{A}	4.4	**19.3**^{AB}	2.0
Alanine (%)	ns	**3.08**	0.58	**3.84**	1.3	**3.34**	0.17	**3.71**	0.84	**3.26**	0.49	**3.03**	0.21
α-Amino butyric acid (%)	ns	**1.49**	2.10	**0.13**	0.01	**0.18**	0.12	**0.06**	0.02	**0.20**	0.11	**0.06**	0.06
Arginine (%)	ns	**0.10**	0.04	**0.04**	0.01	**0.84**	0.83	**0.86**	0.15	**0.08**	0.03	**0.08**	0.03
Asparagine (%)	***	**3.08**^{BC}	1.17	**4.63**^{AB}	1.95	**7.06**^{A}	0.62	**1.35**^{C}	0.11	**2.51**^{BC}	1.22	**4.77**^{AB}	1.8
Aspartic acid (%)	*	**2.91**^{A}	1.54	**1.27**^{AB}	0.80	**1.78**^{AB}	0.42	**0.16**^{B}	0.00	**0.96**^{B}	0.16	**1.35**^{AB}	1.00
Citrulline (%)	*	**0.89**^{B}	0.39	**2.14**^{AB}	0.52	**3.31**^{A}	0.83	**2.43**^{AB}	0.92	**2.10**^{AB}	1.10	**2.45**^{AB}	0.55
γ-Amino butyric acid (%)	*	**0.40**^{A}	0.34	**0.30**^{AB}	0.28	**0.05**^{B}	0.04	**0.01**^{AB}	0.01	**0.05**^{AB}	0.04	**0.03**^{AB}	0.02
Glutamine (%)	***	**2.30**^{B}	0.55	**4.25**^{A}	0.67	**1.94**^{B}	0.58	**3.20**^{AB}	0.74	**2.81**^{B}	0.26	**2.43**^{B}	0.52
Glutamic acid (%)	ns	**18.38**	0.54	**16.99**	0.24	**18.19**	1.1	**17.63**	0.62	**18.23**	0.41	**19.03**	0.64
Glycine (%)	*	**2.03**^{AB}	0.07	**2.03**^{AB}	0.14	**1.90**^{B}	0.15	**1.83**^{B}	0.01	**1.97**^{AB}	0.02	**2.16**^{A}	0.01
Histidine (%)	**	**0.35**^{B}	0.36	**2.81**^{A}	0.09	**1.84**^{A}	0.32	**3.24**^{A}	0.17	**2.20**^{A}	1.8	**1.87**^{AB}	0.41
Isoleucine (%)	**	**5.12**^{A}	0.72	**3.87**^{ABC}	0.65	**2.86**^{C}	0.28	**3.77**^{ABC}	0.66	**4.50**^{AB}	0.78	**3.53**^{BC}	0.73
Leucine (%)	**	**12.14**^{B}	1.2	**12.19**^{B}	0.84	**13.90**^{A}	0.53	**11.87**^{B}	1.3	**11.94**^{B}	0.59	**13.87**^{A}	0.55
Lysine (%)	*	**12.58**^{A}	0.34	**10.82**^{B}	1.6	**11.34**^{AB}	0.43	**12.69**^{A}	0.26	**12.69**^{A}	0.07	**11.60**^{AB}	0.35
Methionine (%)	***	**2.50**^{A}	0.11	**1.95**^{B}	0.09	**2.44**^{A}	0.07	**2.57**^{A}	0.03	**2.28**^{A}	0.22	**2.45**^{A}	0.09
Ornithine (%)	*	**5.09**^{A}	0.31	**3.63**^{AB}	0.49	**2.47**^{B}	1.0	**2.99**^{AB}	1.7	**4.33**^{AB}	1.8	**3.73**^{AB}	0.82
Phenyalanine (%)	***	**6.26**^{AB}	0.72	**5.33**^{C}	0.18	**6.96**^{A}	0.35	**6.06**^{BC}	0.34	**5.95**^{BC}	0.09	**6.53**^{AB}	0.29
Proline (%)	***	**6.28**^{BC}	2.74	**9.44**^{AB}	1.00	**4.05**^{C}	0.35	**9.98**^{AB}	0.78	**10.21**^{A}	0.74	**5.27**^{C}	2.4
Serine (%)	ns	**2.10**	0.10	**2.20**	0.47	**1.82**	0.20	**2.35**	0.38	**1.80**	0.37	**1.98**	0.28
Threonine (%)	ns	**2.90**	0.07	**2.57**	0.44	**2.77**	0.08	**3.16**	0.34	**2.83**	0.28	**2.97**	0.07
Tryptohphan (%)	*	**0.34**^{B}	0.05	**0.37**^{B}	0.10	**0.41**^{AB}	0.04	**0.53**^{A}	0.07	**0.45**^{AB}	0.09	**0.39**^{AB}	0.00
Tyrosine (%)	ns	**2.21**	0.65	**2.42**	0.37	**2.76**	1.0	**2.48**	0.99	**1.30**	0.57	**2.63**	0.72
Valine (%)	***	**7.49**^{AB}	0.20	**6.80**^{C}	0.16	**7.78**^{A}	0.19	**7.08**^{BC}	0.00	**7.34**^{AB}	0.40	**7.77**^{A}	0.13

Caption: x = mean value; s_x = standard deviation; ANOVA: ns = not significant, *) $p \leq 0.05$, **) $p \leq 0.01$, ***) $p \leq 0.001$.
Production sites: A>B>C>D (=significantly different contents $p \leq 0.01$) or AB = A and B overlap by using a univariate discriminant analysis.
AL = Allgäu, BR = Bretagne, CH = Switzerland, FI = Finland, SA = Savoie, VO = Vorarlberg.

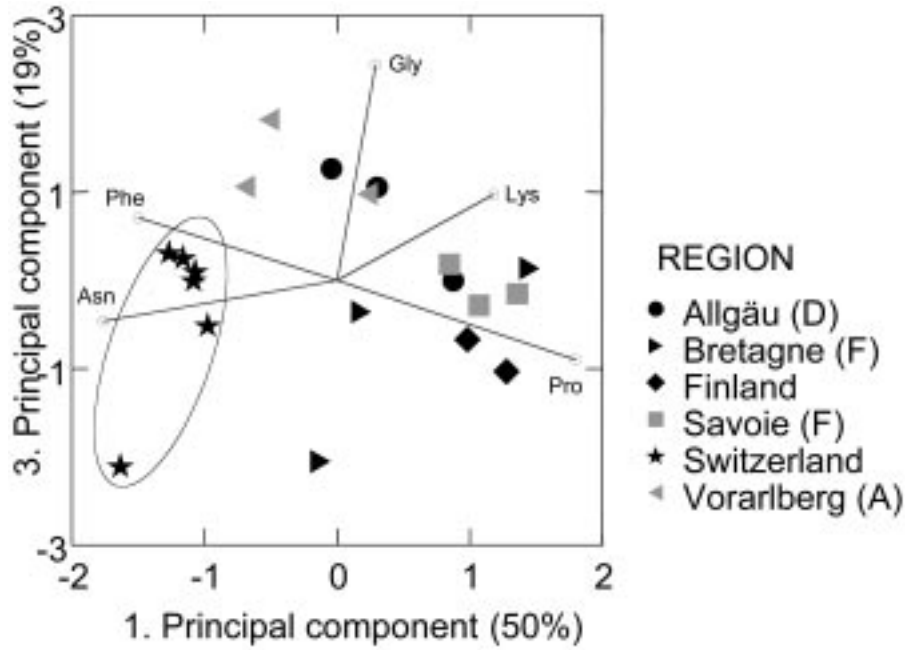


Fig. 1 - Principal component analysis using relative concentrations of asparagine, glycine, lysine, phenylalanine and proline. Separation of the region "Switzerland".

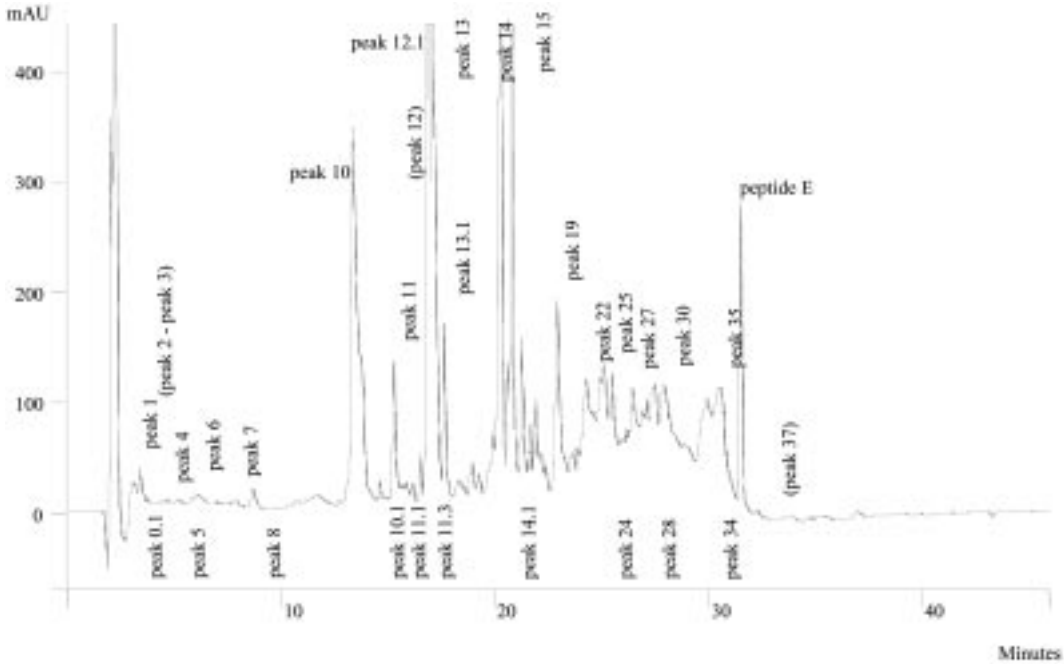


Fig. 2 - Peptide chromatogram of an Emmental sample from Switzerland. Peptide E is a synthetic standard added.

Table 4 - Peptide pattern of the 20 Emmental cheese samples investigated using HPLC (peak area, arbitrary units).

Peak number	Nova	Region (n=)											
		AL (3)		BR (3)		CH (6)		FI (2)		SA (3)		VO (3)	
		x	s_x	x	s_x	x	s_x	x	s_x	x	s_x	x	s_x
1	*	**158**^{AB}	138	**459**^{A}	59	**190**^{B}	51	**251**^{AB}	8	**262**^{AB}	58	**154**^{B}	176
2	***	**n.d.**^{B}	—	**n.d.**^{B}	—	**n.d.**^{B}	—	**107**^{A}	20	**n.d.**^{B}	—	**n.d.**^{B}	—
3	***	**n.d.**^{B}	—	**n.d.**^{B}	—	**n.d.**^{B}	—	**122**^{A}	18	**n.d.**^{B}	—	**n.d.**^{B}	—
4	*	**n.d.**^{B}	—	**26**^{B}	45	**29**^{B}	71	**193**^{A}	83	**32**^{B}	55	**n.d.**^{B}	—
7	***	**n.d.**^{B}	—	**n.d.**^{B}	—	**12**^{B}	29	**528**^{A}	32	**30**^{B}	53	**n.d.**^{B}	—
8	**	**212**^{B}	120	**269**^{B}	60	**434**^{A}	51	**466**^{A}	8	**279**^{B}	90	**351**^{AB}	38
10.1	**	**74B**^{C}	69	**27**^{C}	47	**184**^{A}	67	**177**^{AB}	13	**24**^{C}	42	**129**^{ABC}	18
11	*	**1,276**^{A}	73	**595**^{AB}	587	**1,144**^{A}	429	**554**^{AB}	297	**322**^{B}	398	**687**^{ABC}	248
11.1	*	**367**^{A}	227	**55**^{B}	96	**91**^{B}	115	**283**^{AB}	91	**31**^{B}	53	**278**^{AB}	206
11.3	**	**215**^{AB}	19	**79**^{BC}	138	**309**^{A}	110	**101**^{ABC}	143	**n.d.**^{C}	—	**145**^{ABC}	13
12	*	**n.d.**^{B}	—	**447**^{AB}	496	**n.d.**^{B}	—	**783**^{A}	696	**263**^{AB}	77	**n.d.**^{B}	—
12.1	***	**8,827**^{AB}	8,275	**1,537**^{BC}	1,347	**12,380**^{A}	2,285	**1,178**^{BC}	1,666	**n.d.**^{C}	—	**8,122**^{ABC}	667
13.1	*	**1,402**^{AB}	340	**908**^{B}	399	**1,745**^{AB}	379	**2,187**^{A}	249	**829**^{B}	823	**1,722**^{AB}	419
14	***	**5,707**^{BC}	3,774	**2,554**^{C}	1,246	**14,755**^{A}	3,873	**955**^{C}	585	**683**^{C}	58	**9,649**^{AB}	1,641
14.1	**	**268**^{B}	326	**558**^{B}	401	**202**^{B}	170	**2,020**^{A}	480	**1,043**^{AB}	866	**227**^{B}	242
22	*	**997**^{B}	818	**589**^{B}	300	**2,791**^{A}	1,069	**2,219**^{AB}	1,937	**536**^{B}	292	**1,135**^{AB}	387
24	*	**970**^{AB}	984	**234**^{AB}	17	**1,832**^{A}	816	**492**^{AB}	188	**98**^{B}	22	**819**^{AB}	709
37	*	**n.d.**^{B}	—	**53**^{A}	47	**n.d.**^{B}	—	**n.d.**^{B}	—	**n.d.**^{B}	—	**n.d.**^{B}	—
Total area	***	**70,092**^{A}	8,108	**37,076**^{B}	2,338	**81,370**^{A}	12,527	**73,532**^{A}	1,769	**35,576**^{B}	24,376	**68,974**^{A}	15,313

Caption: x = mean value; sx = standard deviation; ANOVA: ns = not significant, *) $p \leq 0.05$, **) $p \leq 0.01$, ***) $p \leq 0.001$.
Production sites: A>B>C>D (=significantly different contents $p \leq 0.01$) or AB = A and B overlap by using a univariate discriminant analysis.
AL = Allgäu, BR = Bretagne, CH = Switzerland, FI = Finland, SA = Savoie, VO = Vorarlberg.
n.d.: below the detection limit.

Table 5 - Casein fractions using SDS-PAGE-electrophoresis in the 20 Emmental cheese samples investigated (peak area, arbitrary units).

Fraction	Nova	Region (n=)											
		AL (3)		BR (3)		CH (6)		FI (2)		SA (3)		VO (3)	
		x	s_x	x	s_x	x	s_x	x	s_x	x	s_x	x	s_x
Para K	*	**8.87**^{A}	0.85	**6.60**^{B}	0.56	**8.02**^{AB}	0.83	**7.05**^{AB}	0.92	**7.33**^{AB}	0.58	**7.87**^{AB}	0.55
β degraded	ns	**3.40**	0.66	**1.93**	0.71	**2.72**	0.58	**1.85**	0.07	**3.87**	0.32	**3.8**	1.2
γ1	*	**12.40**^{A}	0.96	**8.4**^{B}	1.7	**11.1**^{AB}	1.4	**9.6**^{AB}	2.6	**10.13**^{AB}	0.87	**9.57**^{AB}	0.23
γ2	**	**7,1**^{A}	1.0	**3.73**^{B}	0.80	**6.53**^{A}	0.69	**6.0**^{A}	1.2	**5.83**^{A}	0.32	**5.93**^{A}	0.81
γ3	*	**3.46**^{ABC}	0.76	**2.00**^{C}	0.10	**4.23**^{A}	0.88	**3.05**^{ABC}	0.78	**3.93**^{AB}	0.72	**4.0**^{AB}	1.2
X1	ns	**3.53**	0.38	**2.40**	0.50	**3.50**	0.42	**3.00**	0.57	**3.37**	0.15	**2.87**	0.86
X1-2	ns	**n.d.**	—	**n.d.**	—	**0.40**	0.67	**n.d.**	—	**n.d.**	—	**n.d.**	—
β	**	**14.4**^{C}	1.4	**21.5**^{A}	1.6	**16.8**^{B}	1.5	**17.6**^{AB}	2.7	**17.1**^{B}	1.6	**18.1**^{AB}	1.4
X2-1	ns	**1.87**	0.57	**2.37**	0.72	**1.97**	0.27	**1.90**	0.85	**2.43**	0.25	**2.73**	0.90
X2-2	ns	**8.67**	0.83	**7.77**	1.21	**8.68**	0.37	**7.70**	0.28	**8.30**	0.92	**7.80**	0.61
α_{s2}	**	**n.d.**^{B}	—	**2.7**^{A}	2.4	**n.d.**^{B}	—	**n.d.**^{B}	—	**n.d.**^{B}	—	**n.d.**^{B}	—
α_{s1}	***	**8.7**^{BC}	1.5	**20.6**^{A}	1.4	**6.68**^{C}	0.96	**19.70**^{A}	0.28	**11.7**^{B}	1.8	**10.5**^{B}	1.7
α_{s1}-I	**	**19.5**^{ABC}	1.8	**15.7**^{C}	1.8	**22.3**^{A}	1.8	**16.9**^{BC}	2.2	**19.47**^{ABC}	0.92	**21.1**^{AB}	2.1
X3	***	**8.00**^{A}	0.66	**4.23**^{C}	0.60	**7.1**^{AB}	1.2	**5.70**^{BC}	0.14	**6.60**^{AB}	0.17	**5.53**^{BC}	0.23

Caption: x = mean value; sx = standard deviation; ANOVA: ns = not significant, *) $p \leq 0.05$, **) $p \leq 0.01$, ***) $p \leq 0.001$.

Production sites: A>B>C>D (=significantly different contents $p \leq 0.01$) or AB = A and B overlap by using a univariate discriminant analysis.

AL = Allgäu, BR = Bretagne, CH = Switzerland, FI = Finland, SA = Savoie, VO = Vorarlberg.

X1, X1-2: fractions with unknown constitution migrating after β.

X2-1, X2-2: fractions with unknown constitution migrating after α_{s1}.

X3: fractions with unknown constitution migrating before α_{s1}.

n.d.: below the detection limit.

CHAMBA, 1999). The significantly higher α_{s1} level in "Finland" compared with "Savoie" and "Vorarlberg", though all three were ripened for approximately three months, may be explained by the higher cooking temperature in the former (54°C vs. 52°-54°C) and by the milk thermisation process applied in "Finland" and not in the other two countries (information held by the producers).

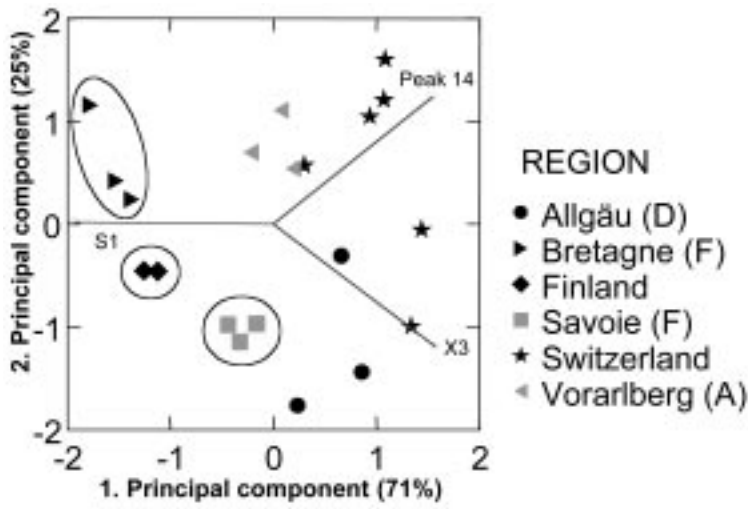


Fig. 3 - Principal component analysis using values of α_{s1}, X3 (unknown structure) and peak 14. Separation of the regions "Bretagne", "Finland" and "Savoie".

Unlike chymosin, the endogenous milk enzyme, plasmin, is not damaged by the high temperature used in cooked cheese. The plasmin activity even elevated in "high cooked" cheese varieties is due to thermal inactivation of inhibitors of plasminogen activators. This results in the increased conversion of plasminogen to the active form, plasmin (LU and NIELSEN, 1993; FARKYE and FOX, 1990). Plasmin degrades α_{s2} and β casein. α_{s2} commonly disappears in Emmental after 45 days of ripening (CHAILLET, 2002). In the current study, two samples from "Bretagne" still contained α_{s2}, probably due to the very short ripening time. The degradation of the β casein leads to the γ1 to γ3 fractions. The same tendency was observed in β as in α_{s1}. "Bretagne" had the highest value followed by "Finland", whereas "Allgäu" and "Switzerland" showed the lowest values. The likely explanation is once again the longer ripening time. The X1-2 fraction was only found in two Swiss samples. The parameters α_{s1} and X3, which gave the best discrimination, combined with peak 14 from the HPLC-peptide profile, gave an interesting separation profile (Fig. 3). The ratio of casein fractions to TN gave a similar separation profile.

Rheology

No significant differences were found between the cheeses from any of the regions except for the penetration depth (Table 6). "Switzerland" showed the highest penetration depth, while "Savoie", "Bretagne", and "Finland" had the lowest. The penetration depth is supposed to be correlated with the water and fat content (BOSSET *et al.*, 1993). This was not observed in this case with water and the correlation with the fat content was poor (r = 0.40). The penetration depth was negatively correlated with the stress at 33% deformation (r = -0.873), which corresponds to the firmness of the body. The stress at fracture was positively correlated with the strain at fracture (r = 0.704), which corresponds to the length of the body.

CONCLUSION

Compounds linked to proteolysis allowed some of the Emmental cheese samples to be separated according to

Table 6 - Rheological analyses of the 20 Emmental cheese samples investigated.

Parameters analysed	Nova	Region (n=)											
		AL (3)		BR (3)		CH (6)		FI (2)		SA (3)		VO (3)	
		x	s_x	x	s_x	x	s_x	x	s_x	x	s_x	x	s_x
Depth of penetration (mm)	*	**10.6^{AB}**	1.6	**9.4^{B}**	1.4	**12.0^{A}**	1.2	**9.5^{AB}**	1.3	**9.3^{B}**	0.77	**10.9^{AB}**	1.3
Stress at fracture (N)	ns	**68**	14	**74**	7.7	**74**	8.7	**65**	3.1	**64**	2.3	**71**	5.8
Strain at fracture (%)	ns	**56**	19	**104**	46	**68**	31	**79**	3.6	**56**	5.7	**60**	25
Stress at 33% deformation (N)	ns	**18**	5.4	**19**	4.8	**14**	1.4	**20**	3.1	**18**	3.7	**17**	2.4

Caption: x = mean value; sx = standard deviation; ANOVA: ns = not significant, *) $p \leq 0.05$, **) $p \leq 0.01$, ***) $p \leq 0.001$.
Production sites: A>B>C>D (=significantly different contents $p \leq 0.01$) or AB = A and B overlap by using a univariate discriminant analysis.
AL = Allgäu, BR = Bretagne, CH = Switzerland, FI = Finland, SA = Savoie, VO = Vorarlberg.

their geographic origin. The NPN, WSN and WSN/TN fractions showed highly significant differences which were partly due to differences in ripening times. These differences are useful because each region produces Emmental with a particular ripening time before being put on the market. The high standard deviations of the biogenic amine concentrations made them unusable as markers of origin. The relative amounts of the free amino acids asparagine, glycine, lysine, phenylalanine and proline made it possible to separate "Switzerland". A clear differentiation of "Bretagne" was achieved using the methionine and glutamine values. The casein fractions (α_{s1} and X3) and the peptide pattern (peak 14) provided a good separation of the "Finland", "Bretagne" and "Savoie" groups. The rheological results were of less interest for the geographic discrimination of Emmental cheese.

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PAPER

THE EFFECT OF MESOPHILIC STARTER CULTURE COMPOSITION ON THE VOLATILE COMPOUNDS OF RAW EWE MILK CHEESE

EFFETTO DELLA COMPOSIZIONE DI COLTURE STARTER MESOFILICHE SUI COMPOSTI VOLATILI DEL FORMAGGIO DI LATTE CRUDO DI PECORA

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ABSTRACT

The formation of volatile compounds in raw ewe milk cheese made using different mesophilic starter cultures was investigated in duplicate experiments. Each experiment consisted in five vats of cheese manufactured according to Manchego cheese technology and ripened for 240 days. Milk was inoculated with a mesophilic commercial starter culture alone, or combined with different amounts of *Lactococcus lactis* strains, some of which produce branched-chain volatile com-

RIASSUNTO

La formazione di composti volatili nel formaggio ovino prodotto con latte crudo inoculato con diversi fermenti lattici mesofili è stata studiata nelle sperimentazioni in doppio, ciascuna consistente in cinque caldaie di formaggio ottenuto con la tecnologia del formaggio Manchego e stagionato per 240 giorni. Il latte è stato inoculato con un fermento lattico commerciale, solo o combinato con diverse proporzioni di ceppi selezionati di *L. lactis*, generatori o non di composti volatili ramificati.

- Key words: branched-chain volatile compounds, cheese flavour, ewe milk cheese, *Lactococcus*, starter culture -

pounds, while others do not. Cheese proteolysis and pH values increased when large amounts of *L. lactis* strains that produce branched-chain volatile compounds were used. Concentrations of 2,3-heptanedione, 3-methyl butanal, 2-methyl propanol, 2-pentanol, 3-methyl butanol, isobutyl acetate, isoamyl acetate, isobutyl butyrate, isoamyl butyrate, methyl caproate and isoamyl caproate were influenced by starter culture composition. Milk inoculation with a large amount of *L. lactis* strains which produce branched-chain volatile compounds enhanced flavour intensity of ripe cheese without impairing flavour quality.

La proteolisi si è rilevata più rapida ed il pH più elevato nelle produzioni con proporzioni maggiori di ceppi di *L. lactis* generatori di composti volatili ramificati. La concentrazione di 2,3-heptanodiona, 3-metil butanal, 2-metil propanolo, 2-pentanolo, 3-metil butanolo, isobutil acetato, isoamil acetato, isobutil butirrato, isoamil butirrato, metil caproato ed isoamil caproato è stata influenzata dalle proporzioni dei ceppi selezionati. L'inoculo del latte con una proporzione maggiore di ceppi di *L. lactis* generatori di composti volatili ramificati ha incrementato l'intensità di sapore nel formaggio stagionato senza decrescerne la qualità.

INTRODUCTION

Nowadays, there is growing consumer demand for a greater diversity in the flavour of cheeses and other dairy products. The use of strains of lactic acid bacteria with particular physiological properties, which confer different, unusual sensory characteristics to dairy products, is a simple tool to satisfy this demand (AYAD *et al.*, 1999). Therefore, strains of lactic acid bacteria with novel properties are needed that can be used alone or in combination with conventional lactic cultures.

Certain flavour characteristics of raw milk cheeses are rarely found in pasteurized milk cheeses. Therefore it seems feasible to isolate strains of lactic acid bacteria from spontaneously fermented dairy products that can produce unusual aroma compounds, that are different from those formed by commercial starter cultures (WEERKAMP *et al.*, 1996; COGAN *et al.*, 1997). Wild strains of lactic acid bacteria probably harbour more amino acid convertases than commercial strains; this could explain their ability to produce unusual flavours in cheese. The wild strains of *Lactococcus lactis* which produce branched-chain volatile compounds, derived from the catabolism of branched-chain amino acids, could play an interesting role, even though those strains have usually been regarded as being undesirable in the dairy industry (URBACH, 1993). Compounds such as 3-methyl butanal and other methyl aldehydes and methyl alcohols have been identified in ripened cheeses, and it has been postulated that they may contribute positively to cheese flavour (BOSSET and GAUCH, 1993; BARBIERI *et al.*, 1994; URBACH, 1995; ENGELS *et al.*, 1997; FOX and WALLACE, 1997; McSWEENEY and SOUSA, 2000).

The objective of this work was to combine a commercial mesophilic starter culture with wild *L. lactis* strains with different physiological characteristics and study the effects on the volatile profile of raw ewe milk cheese and on its sensory characteristics.

MATERIALS AND METHODS

Cheese manufacture

Freeze-dried concentrated (FDC) starter culture MA 016 (Rhodia Iberia, Madrid, Spain), containing *Lactococcus lactis* strains, was inoculated directly into cheese milk. Viable counts of lactococci in the FDC were $3.4x10^{11}$ cfu/g, equivalent to $2.6x10^{11}$ cfu/unit. The FDC was used at 0.008 units/L alone, according to the manufacturer's instructions, or at lower rates in combination with a mixed milk culture of *L. lactis* subsp. *lactis* B7 and *L. lactis* subsp. *cremoris* R20, strains which produce branched-chain volatile compounds (BCP), and/or a mixed milk culture of *L. lactis* subsp. *lactis* B6 and *L. lactis* subsp. *cremoris* M21, strains which do not produce those compounds (BCN). The four *L. lactis* strains had been isolated in our laboratory and their technological characteristics had been determined. *L. lactis* strains B6, B7, M21 and R20 strains were incubated separately for 16 h at 25°C, in order to obtain fresh coagulated cultures containing 1.4-1.8 x 10^9 cfu/mL to be used in cheese-making.

In two experiments, carried out in consecutive weeks, cheeses were manufactured from industrial bulk raw ewe milk ripened overnight, with total viable counts that were close to 10^7 cfu/mL. In each experiment, five 35 L vats were used in which the following starter cultures were added: vat 1, 0.008 units/L of FDC; vat 2, 0.006 units/L of FDC + 1 mL/L of BCP + 1 mL/L of BCN; vat 3, 0.006 units/L of FDC + 2 mL/L of BCP; vat 4, 0.004 units/L of FDC + 2 mL/L of BCP + 2 mL/L of BCN; and vat 5, 0.004 units/L of FDC + 4 mL/L of BCP. Rennet (0.15 mL/L Maxiren, 1:15000 strength, Gist Brocades, Delft, The Netherlands) was added to the milk 20 min after lactic culture inoculation. Forty min after rennet addition, the curds were cut into 6-8 mm cubes, scalded at 37°C for 15 min, and put into cylindrical moulds. Three cheeses, each weighing approximately 2.2 kg, were obtained from each vat. Cheeses were pressed for 18 h at 20°C, salted in brine (150 g NaCl/L) at 12°C for 16 h, ripened at 12°C and 85% RH until day 120, and then kept at 4°C and 90% RH until day 240.

Microbiological and chemical analyses

Cheese samples (10 g) were homogenised in 90 mL of 20 g/L sodium citrate solution for microbiological analysis and decimal dilutions were prepared in sterile 1 g/L peptone solutions. Total viable counts were determined in duplicate on plate count agar (Difco, Detroit, MI), incubated at 30°C for 3 days and lactic acid bacteria counts, also in duplicate, were determined on MRS agar (Biolife, Milano, Italy) at pH 5.7, incubated at 30°C for 3 days.

Aminopeptidase activity in cheese was measured on duplicate samples with lysine *p*-nitroanilide (Lys *p*-NA) and leucine *p*-nitroanilide (Leu *p*-NA) as substrates (GARDE *et al.*, 1997). One activity unit corresponded to the amount of enzyme that produced 1 nmol *p*-nitroaniline/min per g of cheese at 37°C. The cheese pH value was measured in duplicate after homogenising 10 g of cheese with 20 mL of distilled water at 70°C. Proteolysis was determined in duplicate by the *o*-phthaldialdehyde method, with 50 µL of filtered cheese extract in the assay mixture (GARDE *et al.*, 1997).

Cheese pieces were wrapped in aluminium foil, vacuum packed and frozen at -40°C until analysed for volatile compounds. Prior to volatile extraction, frozen pieces were thawed overnight at 4°C, and held at 20°C for 90 min. Duplicate 15 g cheese samples were homogenised in an analytical grinder (IKA, Labortechnik, Staufen, Germany), with 20 g anhydrous sodium sulphate (Merck, Darmstadt, Germany) and 75 µL of an aque-

ous solution containing 0.5 mg/mL cyclohexanone (Fluka Chemie, Buchs, Switzerland) and 0.5 mg/mL camphor (Sigma-Aldrich Chemie, Steiheim, Germany) as internal standards. An aliquot (2 g) of this mixture was subjected to helium purge in a 25 mL glass sparger (Schmidlin CO, Neuheim, Switzerland). Extraction and analysis were carried out in duplicate as previously described (OUMER *et al.*, 2001), using an automatic dynamic headspace apparatus (HP 7695 Purge and Trap, Hewlett-Packard, Palo Alto, CA) with a Tenax Trap (Tekmar, Cincinnati, OH), connected to a gas chromatograph-mass spectrometer (HP 6890, Hewlett-Packard) with a HP-Innowax column (60 m long x 0.25 mm ID, 0.5 mm film thickness). Data were collected with the HP ChemStation program, and volatile compounds were identified by comparison of spectra with the Wiley 275 Library and by comparison of their retention times with authentic reference standards. The relative abundance of each particular compound was expressed in arbitrary units (a.u.), calculated as the percentage of its peak area with respect to the area of the cyclohexanone peak.

Sensory and statistical analyses

For each session five cheese slices were presented in closed individual Petri dishes to 15 trained panelists for sensory analysis. Flavour intensity and flavour quality of the cheeses were evaluated on a 10-point scale, as previously described (NUÑEZ *et al.*, 1991).

Analyses of variance with starter culture composition and cheese age as main effects were performed using the SPSS program Win version 9.0 (SPSS, Chicago, IL). At each cheese age, mean comparisons ($P < 0.05$) for each volatile compound and sensory characteristic were carried out using Tukey's test. Pearson correlation coefficients were also calculated (STEEL and TORRIE, 1980).

RESULTS AND DISCUSSION

Microorganisms

Total viable counts were in the range of 8.5-9.0 log cfu/g in the 60-day-old cheeses and decreased to 7.9-8.2 log cfu/g in the 120-day-old cheeses. Similarly, lactic acid bacteria decreased from 8.1-8.2 log cfu/g in the 60-day-old cheeses to 7.8-8.1 log cfu/g in the 120-day-old cheeses. Total viable counts and lactic acid bacteria were, on average, 0.24 log units higher in the 120-day-old cheeses made with BCP strains than in those made without BCP strains (Table 1). However, differences in microbial counts due to starter culture composition were non significant.

Aminopeptidase

Aminopeptidase activities on Leu *p*-NA and Lys *p*-NA in 60-day-old cheeses were not influenced by starter culture composition, and the significant differences observed in 120-day-old cheeses could not be correlated to the addition of BCP strains (Table 1). The aminopeptidase activity values in the raw milk cheeses were 3- to 5-times higher than those reported for pasteurized milk cheeses (OUMER *et al.*, 2001). Cheese pH did not differ between vats until day 240, when higher values were recorded for some of the cheeses made with BCP strains; a similar pattern was found for cheese proteolysis (Table 1). Differences in cheese proteolysis cannot be attributed to differences in the proteolytic activity of *L. lactis* strains used as starter cultures, because the activity was very similar. However, the higher counts of lactic acid bacteria in the 240-day-old cheeses made with BCP strains indicates that BCP strains have a longer viability than the FDC culture strains, which could be partially responsible for the differences in proteolysis.

Table 1 - Bacterial counts, aminopeptidase activity, pH value and proteolysis in cheeses made with a freeze-dried concentrated culture (FDC, in units/L) and strains of *Lactococcus lactis* that produce branched-chain volatile compounds (BCP) and those that do not (BCN).

	Days	0.008 FDC	0.006 FDC 1 mL/L BCP 1 mL/L BCN	0.006 FDC 2 mL/L BCP	0.004 FDC 2 mL/L BCP 2 mL/L BCN	0.004 FDC 4 mL/L BCP
Total viable counts (Log)	60	8.52 a	9.01 a	9.01 a	8.80 a	8.51 a
	120	7.85 a	8.07 a	8.16 a	8.13 a	8.00 a
Lactic acid bacteria (Log)	60	8.11 a	8.21 a	8.24 a	8.17 a	8.12 a
	120	7.78 a	7.96 a	8.03 a	8.07 a	8.04 a
Leu-aminopeptidase	60	5.88 a	6.41 a	6.13 a	5.95 a	6.39 a
	120	8.90 ab	7.86 a	9.31 b	7.96 ab	8.76 ab
Lys-aminopeptidase	60	3.28 a	3.49 a	3.05 a	2.97 a	3.18 a
	120	5.44 bc	4.69 ab	6.20 c	3.59 a	4.09 a
pH value	60	5.22 a	5.24 a	5.25 a	5.26 a	5.25 a
	120	5.43 a	5.46 a	5.48 a	5.49 a	5.54 a
	240	5.47 a	5.57 ab	5.52 a	5.77 b	5.61 ab
Proteolysis (OPA)	60	0.56 a	0.60 a	0.60 a	0.62 a	0.60 a
	120	1.12 a	1.14 a	1.27 a	1.21 a	1.21 a
	240	1.23 a	1.32 ab	1.46 b	1.36 ab	1.48 b

Means in a row followed by the same letter do not differ ($P < 0.05$) according to Tukey's test.

Cheese volatile profile

Ketones, aldehydes, alcohols, esters, hydrocarbons, sulphur compounds, benzenic compounds and acids were among the 68 volatile compounds identified in the cheeses, but most of them did not depend on the composition of the starter culture. The concentrations of only 11 volatile compounds (2,3-heptanedione, 3-methyl butanal, 2-methyl propanol, 3-methyl butanol, 2-pentanol, isobutyl acetate, isobutyl butyrate, isoamyl acetate, isoamyl butyrate, methyl caproate and isoamyl caproate) were influenced by adding *L. lactis* BCP strains to the starter culture (Tables 2 and 3). The marked formation of volatile compounds in the cheeses, reaching levels that exceeded those usually found in raw milk Manchego cheese using the same analytical methodology (FERNANDEZ-GARCIA *et al.*, 2002a,b), could be traced back to the high bacterial counts in the raw milk. The effect of adding lactic acid bacteria to the starter culture on the production of volatile compounds in the cheese might be partially masked by the potent volatile profile background due to the native microbiota.

Ketones

Among the eight ketones detected, only 2,3-heptanedione was influenced by starter culture composition. Although it was present in greater amounts in cheeses made using BCP strains (Table 2), its low concentration with respect to the other ketones probably minimizes its role in the overall cheese flavour. Ketones are important contributors to the flavour of mould- ripened cheeses (MOLIMARD and

Table 2 - Relative abundance of ketones, aldehydes and alcohols influenced by starter culture composition in cheeses made with a freeze-dried concentrated culture (FDC, in units/L) and strains of *Lactococcus lactis* that produce branched-chain volatile compounds (BCP) and those that do not (BCN).

	Days	0.008 FDC	0.006 FDC 1 mL/L BCP 1 mL/L BCN	0.006 FDC 2 mL/L BCP	0.004 FDC 2 mL/L BCP 2 mL/L BCN	0.004 FDC 4 mL/L BCP
2,3 Heptanedione	60	0.00 a	0.06 a	0.07 a	0.03 a	0.21 b
	120	0.00 a	0.35 a	0.76 ab	0.88 ab	1.42 b
	240	0.11 a	0.74 ab	1.79 c	1.04 bc	1.29 bc
3-Methyl butanal	60	2.70 b	2.39 ab	2.60 ab	2.09 a	2.14 ab
	120	2.66 a	3.04 ab	3.00 ab	2.47 a	4.29 b
	240	5.34 a	5.60 a	6.91 a	6.58 a	7.68 a
2-Methyl propanol	60	7.54 a	20.93 b	31.62 c	38.16 c	60.57 d
	120	7.22 a	18.99 b	25.53 c	30.61 c	47.69 d
	240	8.11 a	20.45 b	28.12 c	17.03 b	50.69 d
3-Methyl butanol	60	45.56 a	86.77 b	133.1 c	139.9 c	222.5 d
	120	40.89 a	87.43 b	120.3 c	120.4 c	193.6 d
	240	52.19 a	100.1 b	126.8 c	98.61 b	189.9 d
2-Pentanol	60	14.72 a	12.27 a	18.16 ab	24.30 b	21.13 ab
	120	42.10 a	50.98 a	35.84 a	35.20 a	42.82 a
	240	61.55 a	61.21 a	45.32 a	50.43 a	57.43 a

Means in a row followed by the same letter do not differ ($P < 0.05$) according to Tukey's test.

Table 3 - Relative abundance of esters influenced by starter culture composition in cheeses made with a freeze-dried concentrated culture (FDC, in units/L) and strains of *Lactococcus lactis* that produce branched-chain volatile compounds (BCP) and those that do not (BCN).

	Days	0.008 FDC	0.006 FDC 1 mL/L BCP 1 mL/L BCN	0.006 FDC 2 mL/L BCP	0.004 FDC 2 mL/L BCP 2 mL/L BCN	0.004 FDC 4 mL/L BCP
Isobutyl acetate	60	0.00 a	0.00 a	0.00 a	0.11 b	0.26 c
	120	0.00 a	0.10 a	0.26 ab	0.29 bc	0.46 c
	240	0.09 a	0.40 b	0.61 b	0.59 b	0.97 c
Isobutyl butyrate	60	0.00 a	0.06 a	0.16 b	0.22 b	0.33 c
	120	0.10 a	0.31 b	0.45 c	0.77 d	1.11 e
	240	0.19 a	0.62 b	0.81 b	0.82 b	1.55 c
Isoamyl acetate	60	0.99 a	2.04 ab	3.10 bc	3.67 c	5.80 d
	120	2.35 a	5.67 b	7.71 b	7.28 b	12.68 c
	240	3.62 a	9.09 ab	12.67 b	12.70 b	20.40 c
Isoamyl butyrate	60	0.24 a	0.39 ab	0.57 bc	0.64 c	1.05 d
	120	0.50 a	0.99 ab	1.49 ab	2.15 bc	3.20 c
	240	0.90 a	1.75 b	2.31 b	3.05 c	4.26 d
Methyl caproate	60	0.37 a	0.40 a	0.36 a	0.44 a	0.40 a
	120	0.83 a	1.04 a	1.04 a	1.06 a	1.04 a
	240	1.61 a	2.36 ab	1.90 ab	2.94 b	2.54 ab
Isoamyl caproate	60	0.00 a	0.00 a	0.00 a	0.00 a	0.00 a
	120	0.00 a	0.00 a	0.00 a	0.00 a	0.00 a
	240	0.00 a	0.00 a	0.04 ab	0.16 b	0.03 ab

Means in a row followed by the same letter do not differ ($P < 0.05$) according to Tukey's test.

SPINNLER, 1996; ENGELS *et al.*, 1997; MOIO *et al.*, 2000).

The relative abundances of ketones that were not influenced by starter culture composition were in the following ranges for 240-day-old cheeses: acetone (24.1-55.8 a.u.), butanone (368-662 a.u.), 2,3-butanedione (18.6-30.7 a.u.), 2-pentanone (65.3-142.4 a.u.), 2-hexanone (1.57-3.64 a.u.), 2-heptanone (18.6-60.8 a.u.), and nonanone (0.48-4.13 a.u.). Butanone had the highest relative abundance among all the volatile compounds determined in the present study. All ketones increased as the cheeses aged, with the exception of 2-hexanone which did not change significantly during ripening.

Aldehydes

The only aldehyde affected by starter culture composition was 3-methyl butanal (Table 2). While the addition of *L. lactis* BCP strains in the starter culture was not a condition for the formation of this compound, higher levels of 3-methyl butanal were generally found in 120- and 240-day-old cheeses made with high levels of BCP strains. This aldehyde is derived from the amino acid leucine (MORGAN *et al.*, 1966; SHELDON *et al.*, 1971). A higher activity of BCP lactococci in converting leucine to 3-methyl butanal rather than converting isoleucine to 2-methyl butanal, as previously reported for *Streptococcus lactis* var. *maltigenes* (TUCKER and MORGAN, 1967), could explain the higher levels of 3-methyl butanal found in the present work, and may also apply to the conversion of valine to 2-methyl propanal. Small amounts of 3-methyl butanal are usually found in cheese; in some varieties the levels may be higher and 3-methyl butanal may even be a major volatile constituent (BOSSET and GAUCH, 1993; PREININGER *et al.*, 1996; ENGELS *et al.*, 1997).

The relative abundances of aldehydes not influenced by starter culture composition were in the following ranges for 240-day-old cheeses: acetaldehyde (0.43-0.65 a.u.), propanal (0.18-0.34 a.u.), propenal (1.85-3.31 a.u.), 2-methyl propanal (0.02-0.04 a.u.), butanal (0.23-0.55 a.u.), 2-methyl butanal (0.41-0.60 a.u.), hexanal (0.47-1.10 a.u.), nonanal (0.29-0.34 a.u.), benzaldehyde (0.27-0.83 a.u.), and phenylacetaldehyde (0.14-0.21 a.u.). Acetaldehyde, 2-methyl butanal, 3-methyl butanal, nonanal and benzaldehyde increased as the cheeses aged, 2-methyl propanal decreased, and the other aldehydes did not vary significantly with cheese age.

Alcohols

Three alcohols, 2-methyl propanol, 3-methyl butanol and 2-pentanol, were significantly influenced by starter culture composition (Table 2). A clear relationship was found between the level of *L. lactis* BCP strains in the starter culture and the relative abundances of 2-methyl propanol (r values of 0.976, 0.974 and 0.930 on days 60, 120 and 240, respectively) or 3-methyl butanol (r values of 0.980, 975 and 0.954 on days 60, 120 and 240, respectively). In 60-day-old cheeses, the addition of 4 mL/L of BCP strains in the starter culture resulted in an 8.0-fold increase of 2-methyl propanol and a 4.9-fold increase of 3-methyl butanol.

Methyl alcohols are formed as the reduction products of aldehydes derived from branched-chain amino acids (MORGAN *et al.*, 1966; SHELDON *et al.*, 1971). The conversion of 3-methyl butanal to the alcohol takes place mostly during cheese ripening (SHELDON *et al.*, 1971; DUNN and LINDSAY, 1985; AYAD *et al.*, 2000). However, in fresh milk cultures of some wild lactococcal strains, 3-methyl butanol was the dominant compound (WEERKAMP *et al.*, 1996). In the reductive environment of cheese, the equilibrium of the aldehyde reduction

reaction is shifted to the alcohol species, a fact that might explain the higher amounts of branched-chain alcohols compared with the branched-chain aldehydes observed in the present work and which resulted in greater differences between cheeses with respect to alcohols rather than aldehydes.

Branched-chain alcohols have a considerably higher perception threshold (about 50-times greater in skim milk) than the corresponding aldehydes (SHELDON *et al.*, 1971; MORGAN, 1976), and are probably of less significance for flavour. Branched-chain aldehydes and alcohols have generally been considered as off-flavours or flavour defects in milk and cheeses (MORGAN, 1976; DUNN and LINDSAY, 1985). However, in some cheese varieties these volatile compounds are considered to be key flavour compounds (BOSSET and GAUCH, 1993; BARBIERI *et al.*, 1994; ENGELS *et al.*, 1997).

Relative abundances of alcohols not influenced by starter culture composition were in the following ranges for 240-day-old cheeses: ethanol (11.0-41.8 a.u.), 1-propanol (5.4-29.9 a.u.), 2-propanol (38.9-83.1 a.u.), 2-propen-1-ol (0.88-3.66 a.u.), 1-butanol (9.2-47.0 a.u.), 2-butanol (120-248 a.u.), 1-pentanol + 2-methyl-3-buten-1-ol (0.65-1.34 a.u.), 3-methyl-2-buten-1-ol (0.06-0.18 a.u.), 1-hexanol (0.43-3.06 a.u.), 2-hexanol (0.28-1.08 a.u.) and 2-heptanol (2.5-11.4 a.u.). Three alcohols (2-propanol, 2-pentanol and 2-heptanol) increased as the cheeses aged, three others decreased (ethanol, 2-butanol and 1-pentanol + 2-methyl-3-buten-1-ol), and the other alcohols did not change significantly as the cheeses aged. Ethanol is produced from lactose by lactococci and other lactic acid bacteria (COGAN, 1995), while other alcohols derive from amino acid catabolism. Decreases in the levels of ethanol and other alcohols during ripening was most probably due to esterification by fatty acids.

Esters

The relative abundances of six esters were influenced by starter culture composition (Table 3), with high correlations between the level of BCP strains and the concentration of isobutyl acetate (r = 0.876, 0.956 and 0.938 on days 60, 120 and 240), isobutyl butyrate (r = 0.945, 0.885 and 0.968 on days 60, 120 and 240), isoamyl acetate (r = 0.927, 0.927 and 0.920 on days 60, 120 and 240), and isoamyl butyrate (r = 0.937, 0.858 and 0.960 on days 60, 120 and 240). In 240-day-old cheeses, the inclusion of 4 mL/L of BCP strains in the starter culture resulted in 11.0-, 5.6-, 8.2-, 4.7- and 1.6-fold increases in isobutyl acetate, isoamyl acetate, isobutyl butyrate, isoamyl butyrate and methyl caproate, respectively. Isoamyl caproate was not detected until day 240, and only in cheeses made with 2 or 4 mL/L of BCP strains.

Enzymatic or chemical reactions of short- to medium-chain fatty acids with alcohols are responsible for the formation of esters in cheese (BARBIERI *et al.*, 1994). Oxidative deamination of amino acids produces acetic acid and branched-chain fatty acids such as isobutyric acid (FOX and WALLACE, 1997). Native milk lipase plays an important role in the release of free fatty acids in raw milk cheeses. Esterases of some lactic acid bacteria may also contribute to the release of free fatty acids during ripening (MORGAN, 1976; HARPER *et al.*, 1980). Free fatty acids are found in relatively high amounts in ripe raw milk cheeses, since the alcohol concentration is the limiting factor in ester formation. In the present work, the high concentrations of branched-chain alcohols in the cheeses made with the higher levels of BCP strains resulted in the abundant formation of branched-chain alkyl esters.

Ethyl acetate, ethyl butyrate, ethyl isovalerate, ethyl caproate, isoamyl acetate or isoamyl isobutyrate have been found in milk cultures of wild *L. lactis* strains

(WEERKAMP *et al.*, 1996; AYAD *et al.*, 1999). Most of these esters were detected in the cheeses analyzed in the present work. Fruity flavours, considered as off-flavours in some cheeses, are due to the presence of esters (BARBIERI *et al.*, 1994; MOLIMARD and SPINNLER, 1996). Fruity notes have also been found in milk cultures and cheeses made with wild *L. lactis* strains, and attributed to the presence of ethyl esters (AYAD *et al.*, 1999, 2000).

The relative abundances of esters not affected by starter culture composition were in the following ranges for 240-day-old cheeses: ethyl acetate (3.6-9.7 a.u.), ethyl propionate (0.41-1.54 a.u.), ethyl butyrate (7.0-11.0 a.u.), ethyl pentanoate (0.20-0.44 a.u.), ethyl caproate (9.9-14.2 a.u.), ethyl caprylate (0.45-0.73 a.u.), ethyl decanoate (0.03-0.07 a.u.), propyl acetate (41-103 a.u.), propyl propionate (1.1-7.0 a.u.), propyl butyrate (18.4-26.2 a.u.), 2-propenyl butyrate (0.04-0.10 a.u.), propyl caproate (0.69-0.86 a.u.), butyl acetate (8.1-9.0 a.u.), 2-butyl acetate + methyl butyrate (7.6-20.2 a.u.), butyl butyrate (2.3-3.1 a.u.), 2-butyl butyrate (11.6-15.0 a.u.), butyl caproate (0.08-0.13 a.u.) and 2-butyl caproate (1.9-2.4 a.u.). The esters that increased with cheese age were methyl caproate, ethyl caprylate, propyl acetate, propyl butyrate, butyl acetate, butyl isobutyrate, isobutyl acetate, isobutyl butyrate, isoamyl acetate, isoamyl butyrate and 2-butyl acetate + methyl butyrate. The other esters did not change significantly during ripening.

Miscellaneous volatile compounds

Other volatile compounds detected in the present work, that were not influenced by starter culture composition, were in the following concentration ranges in 240-day-old cheeses: heptane (0.4-3.7 a.u.), octane (0.87-1.37 a.u.), 3-methyl-1-heptene (0.79-1.05 a.u.), α-pinene (1.7-2.4 a.u.), toluene (0.88-1.09 a.u.), dimethyl sulphide (0.22-0.30 a.u.), dimethyl disulphide (0.00-0.60 a.u.), acetic acid (0.98-1.39 a.u.) and butyric acid (0.14-0.23 a.u.). None of these compounds were significantly influenced by cheese age.

Sensory characteristics

The flavour intensities of cheeses made with different starter cultures were similar on days 60 and 120, but after 240 days of ripening the intensity tended to be stronger in cheeses made with BCP strains of *L. lactis* (Table 4).

Table 4 - Sensory characteristics of cheeses made with a freeze-dried concentrated culture (FDC, in units/L) and strains of *Lactococcus lactis* that produce branched-chain volatile compounds (BCP) and those that do not (BCN).

	Days	0.008 FDC	0.006 FDC 1 mL/L BCP 1 mL/L BCN	0.006 FDC 2 mL/L BCP	0.004 FDC 2 mL/L BCP 2 mL/L BCN	0.004 FDC 4 mL/L BCP
Flavour intensity	60	7.10 a	6.82 a	6.60 a	7.06 a	7.19 a
	120	7.68 a	7.69 a	7.85 a	7.67 a	7.62 a
	240	7.98 a	8.28 bc	8.46 c	8.09 ab	8.12 ab
Flavour quality	60	6.19 a	5.88 a	6.49 a	6.31 a	5.89 a
	120	6.93 b	6.96 b	6.14 ab	6.13 ab	6.02 a
	240	6.99 ab	6.74 a	6.98 ab	7.21 b	7.16 ab

Means in a row followed by the same letter do not differ ($P < 0.05$) according to Tukey's test.

Flavour quality was not affected by starter culture composition in 60-day-old cheeses, it decreased in 120-day-old cheeses as the amount of BCP strains increased, and was slightly higher in 240-day-old cheeses when a large amount of BCP strains had been added to the milk. Cheeses in this study had potent volatile profiles with a strong flavour, which could be partially attributable to the raw milk microbiota, which minimised the effect of starter culture composition on the sensory characteristics. In general there were few differences in the sensory scores, which resulted in low correlation coefficients between the sensory scores and the relative abundances of the particular volatile compounds.

CONCLUSIONS

Starter culture composition influenced the volatile profile of raw ewe milk cheeses and had a significant effect on the concentrations of 11 compounds: one ketone, one aldehyde, three alcohols and six esters. It also influenced the sensory characteristics of the cheese. Milk inoculation with large amounts of BCP strains of *L. lactis* enhanced the flavour intensity of 240-day-old cheese without impairing its flavour quality. While the effect of starter culture composition on the profile of the volatile compounds and on the sensory characteristics of the cheese was significant, its influence was partially masked by a notable formation of volatile compounds by the abundant native microbiota in the raw milk used in these experiments.

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PAPER

INFLUENCE OF CHEESE WEIGHT AND TYPE OF RENNET ON COMPOSITION AND PROTEOLYSIS OF CANESTRATO PUGLIESE CHEESE II. CHROMATOGRAPHIC CHARACTERIZATION OF SOLUBLE NITROGEN

INFLUENZA DELLA PEZZATURA DELLA FORMA E DEL TIPO DI CAGLIO SULLA COMPOSIZIONE E PROTEOLISI DEL FORMAGGIO CANESTRATO PUGLIESE II. CARATTERIZZAZIONE CROMATOGRAFICA DELL'AZOTO SOLUBILE

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ABSTRACT

A chromatographic study, based on reversed-phase, size exclusion and hydrophobic interaction chromatography, was performed to evaluate the influence of weight of the cheese and type of rennet used on the evolution of soluble nitrogen in "Canestrato Pugliese", a Protected Designation of Origin (PDO) hard cheese produced in Southern Italy from

RIASSUNTO

È stato condotto uno studio cromatografico basato sulla cromatografia a fase inversa, ad esclusione molecolare e ad interazione idrofobica, per valutare l'influenza della pezzatura della forma e del tipo di caglio sull'evoluzione dell'azoto solubile nel "Canestrato Pugliese", formaggio a pasta dura a Denominazione di Origine Protetta prodot-

- Key words: Canestrato Pugliese, ewe's cheese, rennet, soluble nitrogen -

sheep milk. The weight of the cheese had only a quantitative influence, with higher amounts of soluble nitrogen in the smaller cheeses. The influence of the rennet was both quantitative and qualitative, probably due to the different chymosin/pepsin ratios. More soluble nitrogen was found in cheeses obtained with lamb rennet and significant differences were observed among the chromatographic profiles. Size exclusion chromatography revealed a significant accumulation of medium- molecular-weight peptides during ripening in the lamb rennet cheeses, whereas a faster formation of low-molecular-weight peptides was found in those produced with bovine rennet. The most distinctive peptide fraction (about 4 KD), recovered and analysed by hydrophobic interaction chromatography, revealed the presence of further qualitative differences, which were confirmed by reversed phase chromatography and free amino acid analysis. Such findings indicate a different evolution of secondary proteolysis among the cheeses. Further study is needed to better understand the mechanism of pepsin retention in the curd and its residual activity during ripening.

to in Italia da latte ovino. Il peso della forma ha mostrato una influenza essenzialmente quantitativa, con maggiore accumulo di azoto solubile nelle forme più piccole. L'influenza del tipo di caglio è risultata, invece, sia quantitativa che qualitativa, probabilmente a causa del differente rapporto chimosina/pepsina: nel formaggio ottenuto con caglio di agnello sono state riscontrate maggiori concentrazioni di azoto solubile, inoltre, i profili cromatografici sono risultati molto diversi tra i lotti di forme. La cromatografia ad esclusione dimensionale ha rivelato una prevalente formazione di frazioni peptidiche a medio peso molecolare, nel corso della stagionatura, per i formaggi da caglio di agnello; l'impiego di caglio bovino ha comportato una più rapida formazione di frazioni a basso peso molecolare. La frazione peptidica maggiormente caratterizzante (circa 4 KD), recuperata ed analizzata mediante cromatografia ad interazione idrofobica, ha rivelato la presenza di ulteriori differenze tra i formaggi, che sono state confermate dai risultati della cromatografia a fase inversa e dall'analisi degli aminoacidi liberi. I risultati ottenuti dimostrano una diversa evoluzione della proteolisi secondaria e suggeriscono la necessità di ulteriori indagini, per approfondire le modalità della ritenzione della pepsina nella cagliata e della sua attività residua nel corso della stagionatura.

INTRODUCTION

Curd-retained rennet plays a key role during the first stages of casein degradation in cheese, inducing the formation of high- and medium-molecular-weight peptides which are successively involved in the degradation steps known as secondary proteolysis (FOX, 1989; FOX *et al.*, 1993; GRAPPIN *et al.*, 1985).

The production protocols of the Protected Designation of Origin (PDO) cheeses usually indicate the type of rennet to be used for cheesemaking; in some cases the origin is also specified (ASSOLATTE, 1996). For "Canestrato Pugliese", an Italian PDO cheese made from sheep milk, the protocol only indicates the use of "animal rennet" (C.D., 1996). A previous study on this cheese suggested that rennets derived from different animal species could affect the

evolution of proteolysis differently, giving rise to compositional and organoleptic differences, due to their different enzymatic profiles, namely the chymosin/pepsin ratio. The weight of the cheese (available in different sizes ranging from 3 to 15 kg) has also been proven to influence the rate of casein degradation and ripening time (SANTORO and FACCIA, 1998). Such findings require further investigation, especially in view of the growing demand for the characterization of little-known PDO products, traditionally directed to the local markets. The present study was undertaken to clarify the effect of the type of rennet used and of the weight of the cheese on the ripening process of "Canestrato Pugliese", particularly on the qualitative evolution of soluble nitrogen.

MATERIALS AND METHODS

Experimental design

The cheeses were manufactured from a stock of pasteurised sheep milk at a dairy located in the geographic area indicated in the EC official protocol (Caseificio Pugliese of Corato, Puglia, Italy). Two replicates were made, each consisting of two milk vats processed according to the same technology except for the type of rennet used and the weight of the cheese. The rennets used were lamb (70/30 chymosin/pepsin) and bovine (40/60 chymosin/pepsin); the enzyme concentrations, assessed according to the method of CORRADINI *et al.* (1993), were 0.38/0.16 mg mL^{-1} and 0.21/0.32 mg mL^{-1}, respectively. After pasteurisation, the milk was put into 500 L vats at 37°C and a liquid lactic starter was added (10^8 cfu mL^{-1} of a mixed culture of *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactobacillus helveticus* and *Streptococcus thermophilus*, ChemiFerm, Livraga, MI, Italy). After 15 min, it was coagulated with 0.3 mL L^{-1} liquid rennet, 1:10,000 Soxhlet units strength. Cheeses weighing 3 or 7 kg obtained from each vat were kept at room temperature for 24 h and, then, brine salted. The choice of producing the cheeses of different weights from the same vat derived from the necessity of operating on identical curds.

Four lots of cheese were produced from each replicate, labelled as B (bovine rennet, 3 and 7 kg) and L (lamb rennet, 3 and 7 kg). Sampling was done at 40, 120 and 240 days of ripening.

Cheese analysis and water soluble nitrogen (WSN) fractionation

The gross composition of the cheese was determined according to the Italian Official Analysis Methods (M. D., 1986) except for the WSN, which was extracted according to the procedure reported by KUCHROO and FOX (1982). Fractionation was performed by High Performance Liquid Chromatography (HPLC), using a Waters apparatus composed of a 600E pump, a 996 Diode Array detector and a computer work station for data acquisition (Waters Assoc., Milford, MA, USA). A multi-step procedure was adopted, in which different chromatographic techniques were used:

a) Reversed Phase Chromatography (RPC). The WSN samples were ultrafiltered through disposable filters with a 10 KD cut-off (Amicon Inc., Beverly, MA, USA) and the recovered permeates were chromatographed through a Delta-Pak C18 column (250x3.9 mm, Waters Assoc., USA) using a linear gradient from 0 to 70% acetonitrile in water, both containing 0.1% TFA, in 50 min. Flow rate 0.8 mL/min; λ = 220 nm.

b) Size Exclusion Chromatography (SEC). A Pro-Gel TSK G2000 SW column (600x7.5 mm, Supelco Inc., Bellefonte, PA, USA) was used, calibrated with Bovine Serum Albumin (molecular weight 60 KD), carbonic anhydrase (29 KD),

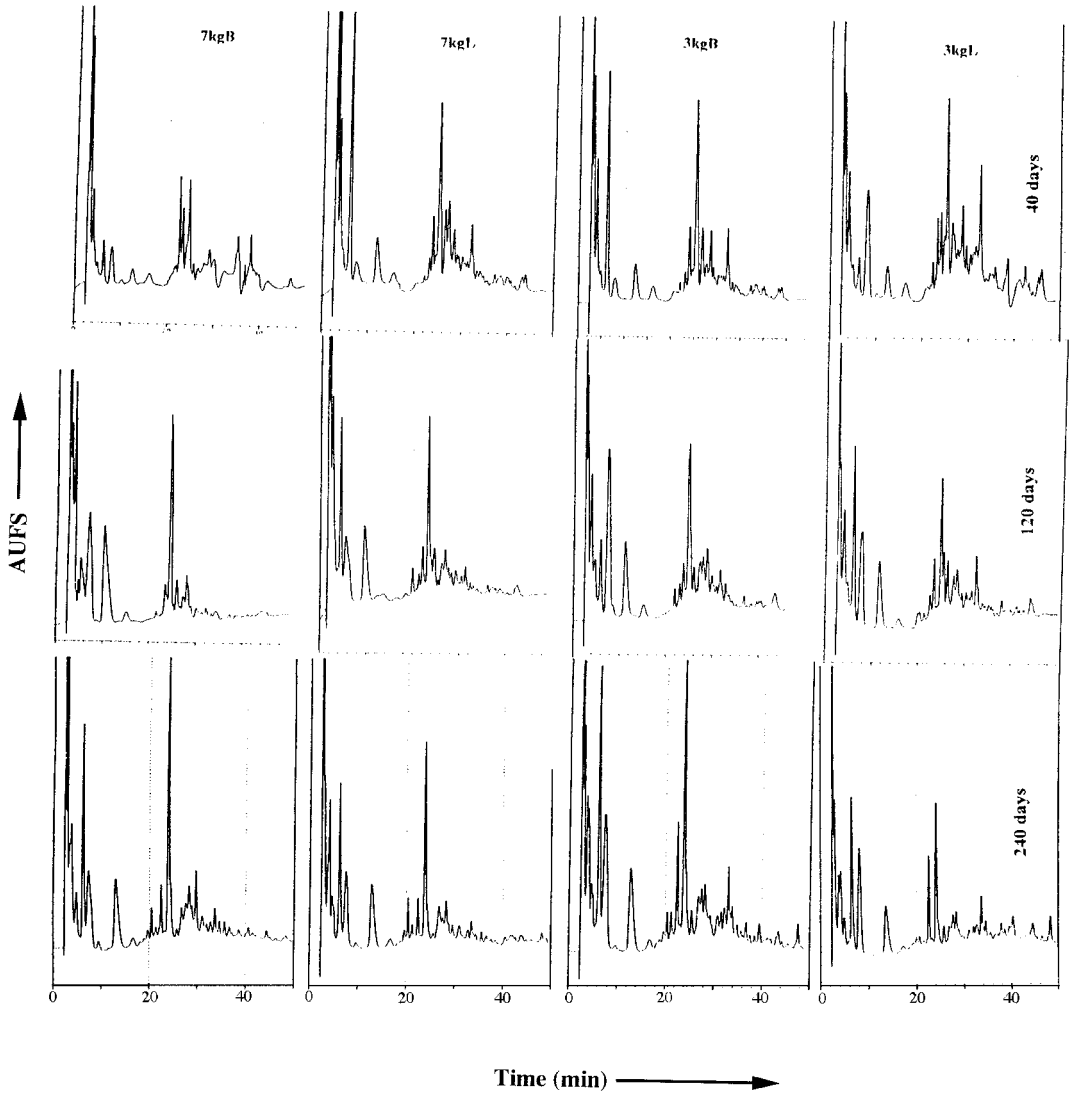


Fig. 1 - Reverse-phase chromatograms of ultrafiltered soluble nitrogen. B = bovine rennet; L = lamb rennet.

cytochrome C (12.4 KD), aprotinin (6,5 KD) and esaglycine (0,36 KD), all purchased from SIGMA (St. Louis, MO, USA). The operating conditions were: mobile phase phosphate buffer 10 mM, pH 6.8, flow rate 0.5 mL/min, λ = 220 nm.

c) Hydrophobic Interaction Chromatography (HIC). This technique was used to characterize the most distinctive peptide fraction, as assessed by the SEC analysis. The fraction was recovered, concentrated and analysed using a Spherisorb S5C1 column (250x4.6 mm, Waters Ass., USA) using the following operating conditions: linear gradient

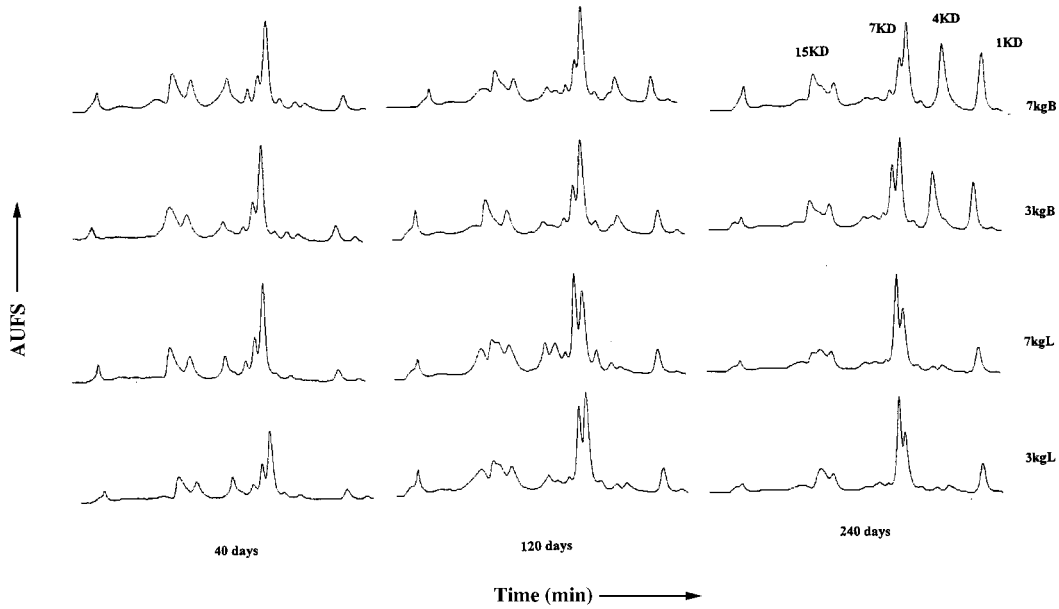


Fig. 2 - Size exclusion chromatograms of soluble nitrogen. B = bovine rennet; L = lamb rennet.

from 0 to 10% acetonitrile in water, both containing 0.1% TFA, in 14 min. Flow rate 0.6 mL/min; λ = 220 nm.

Free amino acid analysis

Free amino acids were extracted according to the method reported by RESMINI *et al.* (1988), involving cheese extraction with citrate buffer (pH 2.2), protein precipitation with sulphosalicylic acid and final pH adjustment. A sample clean-up was also performed to remove small peptides, by ultrafiltering the final extract through 1 KD cut-off membrane (Amicon Inc., Beverly, MA, USA). Quantitative analysis was performed by HPLC, using the Pico-Tag method (COHEN *et al.*, 1982).

Statistical analysis

Data were subjected to analysis of variance (ANOVA). Each source of variability was statistically tested by an F Fisher evaluation.

RESULTS

The main compositional differences among the cheeses regarded the fat/protein ratios and soluble nitrogen values. The highest values occurred at the end of ripening in the 3 kg cheeses in those obtained with lamb rennet (Table 1). Statistical analysis (Table 2) revealed that soluble nitrogen was significantly related to both weight and rennet whereas the other chemical parameters were influenced differently. The weight of the cheese was correlated with dry matter, fat and pH, but only at the end of ripening and at different levels of significance, whereas the type of rennet, as expected, exerted a strong influence on fat and protein retention in the cheese. Total free amino acid content was similar for all the cheeses within the first 40 days of ripening, after which the bovine rennet lots showed a faster accumulation of amino acids, with the highest levels in the 3 kg samples. This

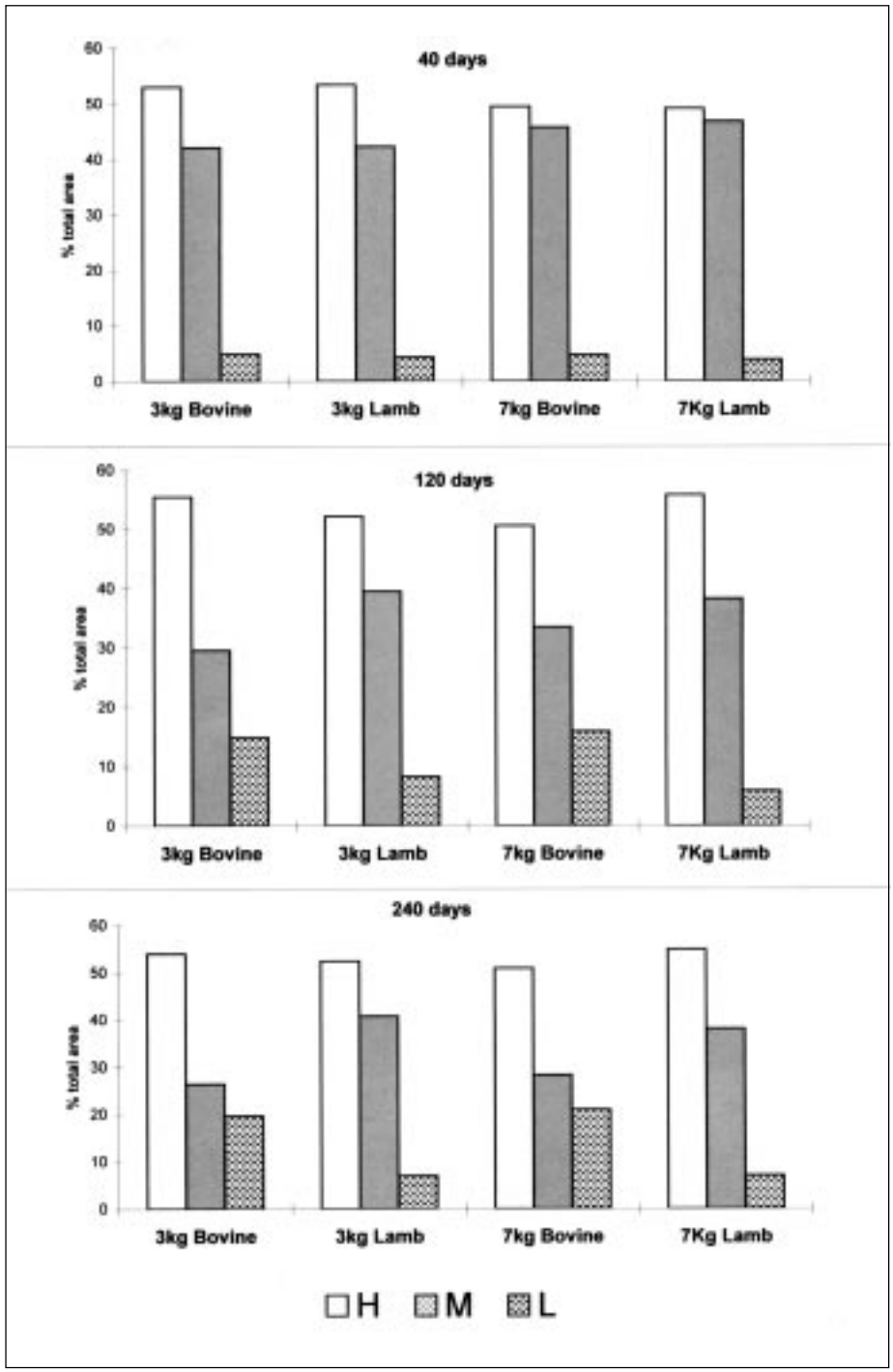


Fig. 3 - Molecular weight (m.w.) profiles of peptides obtained by size exclusion chromatograms. H = high-m.w. peptides (> 10 KD); M = medium-m.w. peptides (5-10 KD); L = low-m.w. peptides (< 5 KD).

Table 1 - Gross composition of cheese during ripening (% dry matter). B = bovine rennet; L = lamb rennet.

Days of ripening	40				120				240			
	3B	3L	7B	7L	3B	3L	7B	7L	3B	3L	7B	7L
Dry matter	61.5	62.5	60.9	61.8	63.4	64.4	62.8	63.3	66.9	66.8	65.3	65.7
Fat	39.0	41.1	38.8	41.8	39.6	41.7	38.9	41.5	38.6	41.2	39.9	42.0
Protein (Nx6.38)	47.1	44.0	46.9	43.3	46.5	44.0	47.8	44.0	47.4	44.7	47.0	45.3
Salt in moisture	5.9	5.9	5.8	5.8	6.3	6.6	6.2	6.4	7.5	7.8	7.6	7.3
pH	5.3	5.2	5.3	5.3	5.3	5.4	5.3	5.3	5.6	5.6	5.5	5.5
Fat/protein	0.83	0.93	0.83	0.97	0.85	0.95	0.81	0.94	0.81	0.92	0.85	0.93
WSN (% Total N)	9.8	9.9	8.3	8.2	17.8	19.9	15.9	16.9	24.4	26.7	19.4	20.4
Total amino acids (% of WSN)	3.0	2.9	2.8	2.7	6.2	5.0	6.0	4.9	11.1	7.9	8.8	7.6

chemical parameter was strongly related to cheese weight and the rennet used and also to their interaction. Only at the end of ripening did the amino acid patterns show some significant differences (Tables 3 and 4), His, Arg and Tyr were more abundant in the lamb rennet cheeses, whereas the bovine cheeses where characterized by higher amounts of Asp, Thr and Phe. In this case the influence of cheese weight was not statistically significant.

Reversed phase chromatography of WSN revealed the prevalence of high and intermediate polarity compounds after the first stages of ripening (Fig.

Table 2 - Statistical significance of weight of the cheese and type of rennet and their interactions with respect to composition. **** = significant at the $P < 0.0001$ level; *** = significant at the $P < 0.001$ level; ** = significant at the $P < 0.01$ level; * = significant at the $P < 0.05$ level; ns = not significant.

Factors		Weight	Rennet	Weight x rennet
Dry matter	40 d	ns	ns	ns
	120 d	ns	ns	ns
	240 d	*	ns	ns
Fat	40 d	ns	***	ns
	120 d	ns	**	ns
	240 d	*	**	ns
Protein	40 d	ns	***	ns
	120 d	ns	***	ns
	240 d	ns	**	ns
WSN	40 d	**	ns	ns
	120 d	**	**	ns
	240 d	***	**	ns
pH	40 d	ns	ns	ns
	120 d	ns	ns	ns
	240 d	***	ns	ns
Total free amino acids	40 d	ns	ns	ns
	120 d	ns	***	ns
	240 d	****	****	***

Table 3 - Free amino acid patterns at the end of ripening (% of total amounts).

	3B	3L	7B	7L
Asp	6.3	5.1	5.9	4.9
Glu	12.8	12.6	12.2	11.9
Asn	3.0	2.7	2.8	3.1
Gln	4.0	3.5	3.8	3.5
Ser	3.7	4.1	3.9	3.8
His	3.0	5.1	3.0	5.0
Arg	3.4	5.0	3.5	5.1
Gly	2.7	2.8	2.9	2.6
Thr	2.3	0.6	2.8	0.7
Ala	5.0	3.9	4.1	4.2
Tyr	3.0	4.4	2.8	4.5
Met	5.8	6.3	5.7	6.0
Val	9.9	9.8	10.1	9.9
Phe	7.9	7.0	7.8	6.8
Ile	6.0	6.3	6.7	6.5
Leu	8.9	9.3	9.6	9.7
Lys	10.1	9.8	10.9	9.9

1). The high polarity compounds were eluted at acetonitrile concentrations less than 10% and increased steadily with ripening time, whereas the intermediate polarity compounds, eluted at acetonitrile concentrations from 20 to 45%, showed heterogeneous behaviour, some tended to increase, while others decreased. The most abundant peak, with a retention time (RT) of about 24 min, corresponded to the phenylalanine/lysine amino acid pair.

A comparison of the chromatograms of the four cheese lots allowed the following differences to be noted:

- the B lots showed a faster increase in almost all the peaks;
- most of the intermediate polarity compounds decreased during ripening for the L lots;
- evolution of the peak with RT 24 min seemed to be directly related to the kind of clotting enzyme: for the B cheeses, it increased with the ripening time, whereas only slight variations were detectable for L cheeses.

SEC fractionation showed that at the early stages of ripening all the cheeses had similar profiles, characterized by peaks with apparent molecular weights (m.w.) ranging from 7 to 15 KD. As ripening proceeded the chromatograms became more complex, due to the formation of the lowest m.w. fractions, and significant differences among the cheeses were observed. The B lots were mainly characterized by an increasing peak of about 4 KD, which was only detected in traces in the L lots, while the most characterizing peak for the L lots remained that of about 7 KD (Figs. 2 and 3). The 4 KD peak, recovered and further analysed by HIC, was composed of several peptides, that were not fully resolved in our experimental conditions. However, the chromatograms showed substantial differences among the samples and, in particular, a more complex profile was found for the B cheese (Fig. 4).

DISCUSSION AND CONCLUSIONS

The results of this study confirm the general view that both composition and proteolysis in "Canestrato Pugliese" cheese are closely related to the type of rennet used in the cheesemaking process, whereas the influence exerted by the weight of the cheese is essentially quantitative. The chymosin/pepsin ratio seems to be responsible for the different amounts of milk fat incorporated into the cheeses, which is probably a consequence of a different state of aggregation of the casein micelles in the curd. Specificity of chymosin and pepsin and their residual activities in the cheese, which are influenced differently by the manufacturing conditions (FOX, 1969; ALAIS, 1984), are probably responsible for the qualitative differences detected in the WSN composition. Lamb rennet induced a higher primary proteolysis, which is in agreement with the results reported by others (GREEN, 1977; BINES *et al.*, 1989; PSZCZOLA, 1989), whereas in bovine rennet cheeses secondary pro-

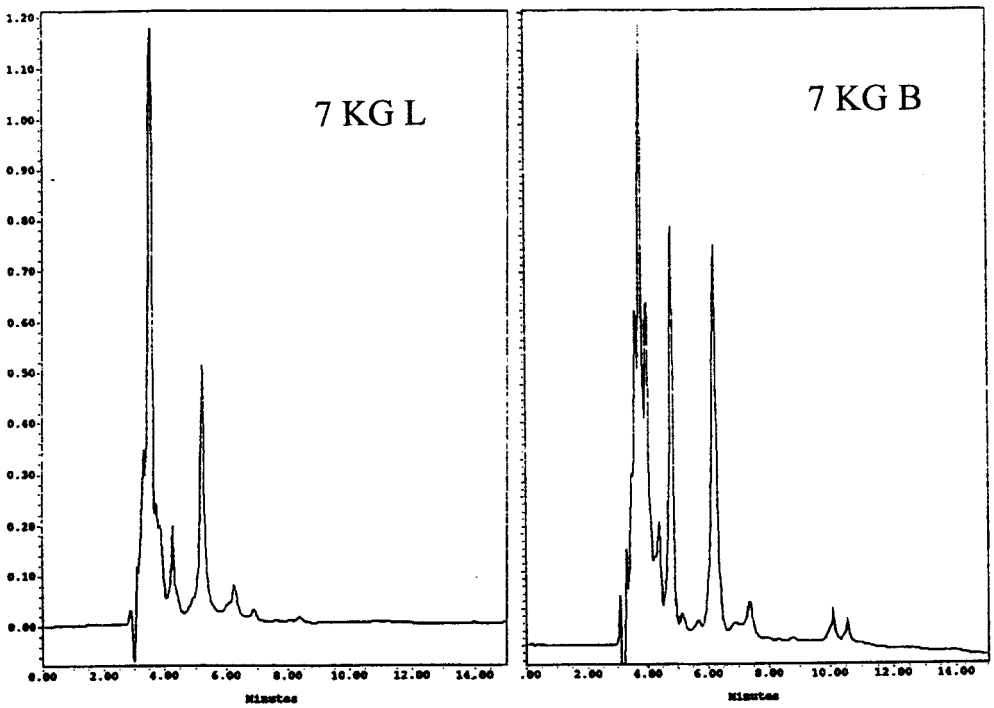


Fig. 4 - Hydrophobic interaction chromatography of the 4 KD peptide fractions recovered from the size exclusion chromatography of 7 kg cheeses at the end of ripening. B = bovine rennet; L = lamb rennet.

teolysis seemed to be faster. The higher concentration of lower m.w. peptides, which are easily degraded by bacterial amino- and carboxypeptidases, could account for the higher formation of free amino acids in the B cheeses. Their formation could be related to the broad specificity of pepsin, but the increase occurs mainly in the last stages of ripening, when the contribution of rennet to proteolysis is considered to be negligible and the microflora enzymes are the main proteolytic agent (O'KEEFE *et al.*, 1978; VISSER, 1993). Since information about the residual activity of pepsin in cheese is limited and, sometimes, conflicting (EMMONS *et al.*, 1976; HOLMES *et al.*, 1977; NOOMEN, 1978; ANDREN *et al.*, 1983), further study is needed to better evaluate the role of this enzyme in secondary proteolysis and its relationship to the microbial activity.

The chromatographic procedure used in this work allowed the main composi-

Table 4 - Statistical significance of weight of the cheese and type of rennet and their interactions with respect to free amino acids. ** = significant at the $P < 0.01$ level; * = significant at the $P < 0.05$ level; ns = not significant

Factors	Asp	Glu	Asn	Gln	Ser	His	Arg	Gly	Thr	Ala	Tyr	Met	Val	Phe	Ile	Leu	Lys
Weight	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Rennet	*	ns	ns	ns	ns	**	*	ns	**	*	**	ns	ns	*	ns	ns	ns
Weight x rennet	ns	ns	ns	ns	ns	ns	ns	ns	ns	**	ns	ns	ns	ns	ns	ns	ns

tional differences in WSN composition to be characterized quickly, giving detailed information about the whole proteolytic process. High performance SEC was an effective tool for discriminating the cheeses obtained with the different rennets. With this technique, it should be noted that the molecular weights calculated for the large- and medium-sized peptides could be overestimated, due to the abnormal chromatographic behaviour of these casein fragments, which can elute more slowly than the globular proteins used to calibrate the column. This behaviour is related to their molecular conformation (NOELKEN *et al.*, 1981). Some indications about the characterization of the most distinctive peptide fraction was obtained with HIC. However, it is necessary to obtain better resolution of its compounds, whose high polarity did not allow conventional RP-columns to be used. A further investigation is currently underway to isolate the single peptides and to evaluate the presence of molecular markers of the different cheese-making processes.

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PAPER

THE EFFECT OF HEAT-INDUCED STRUCTURAL CHANGES ON THE PROOXIDANT ACTIVITY OF MYOGLOBIN

EFFETTO DEI CAMBIAMENTI STRUTTURALI INDOTTI DAL CALORE SULL'ATTIVITÀ PROOSSIDANTE DELLA MIOGLOBINA

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ABSTRACT

To observe the effect of heat-induced structural changes on the prooxidant activity of myoglobin, horse heart myoglobin was heated at 74° or 100°C for 30 min. UV spectroscopy, circular dichroism (CD) spectra, hydrophobicity, ^{1}H-NMR were used to observe the structural alterations of the heat-denatured myoglobin. The release of iron from the heat-denatured myoglobin and its role in the lipid peroxidation processes were assessed after dialysis of the heat-denatured myoglobin. Both UV and CD

RIASSUNTO

Per esaminare gli effetti dei cambiamenti strutturali indotti dal calore sulla attività proossidante della mioglobina, la mioglobina di cuore di cavallo è stata riscaldata a 74° o 100°C per 30 min. Per osservare le alterazioni strutturali della mioglobina denaturata con calore, sono stati usati metodi basati sull'assorbimento UV, sulla spettroscopia indicroismo circolare (CD), sull'idrofobicità e sulla risonanza magnetica nucleare (^{1}H-NMR). Il rilascio di ferro dalla mioglobina denaturata e il ruolo

- Key words: NMR, thermal treatment of myoglobin, myoglobin, absorbance spectra, CD spectra -

spectra at the Soret band suggested that heating of myoglobin at 74°C for 30 min resulted in minor structural changes in the vicinity of heme, while, heating it at 100°C markedly reduced absorbance, suggesting drastic changes in the myoglobin structure. ^{1}H-NMR spectra of the heat-denatured myoglobin did not show any changes in the interaction between heme and the globin residue at 74°C, whereas, at 100°C drastic structural changes were observed with the disappearance of the ^{1}H-NMR peaks. In contrast to the spectral results, the hydrophobicity of the heat-denatured myoglobin increased when myoglobin was heated at 100°C. Dialysis of the heated myoglobin revealed that the released iron made up only a negligible part of the total iron of the myoglobin and had only minor prooxidant activity.

del ferro libero nei processi di perossidazione lipidica sono stati misurati dopo dialisi della mioglobina denaturata I metodi UV e CD e la banda di Soret hanno indicato che il riscaldamento della mioglobina a 74°C per 30 min ha causato cambiamenti strutturali minori in vicinanza dell'eme, mentre il riscaldamento della mioglobina a 100°C ha dato luogo ad una riduzione significativa dell'assorbanza, permettendo di ipotizzare cambiamenti importanti nella struttura. Il metodo ^{1}H-NMR non ha indicato cambiamenti a livello delle interazioni tra eme e residuo globinico sulla mioglobina denaturate a 74°C. Il riscaldamento a 100°C ha invece prodotto drastici cambiamenti strutturali evidenziati dalla scomparsa dei picchi ^{1}H-NMR. In contrasto con i risultati spettrali, l'idrofobicità della mioglobina al calore è aumentata quando la mioglobina veniva riscaldata a 100°C. La dialisi della mioglobina riscaldata ha rilevato che il ferro libero era solo una insignificante parte del ferro totale e che la sua attività proossidante era minima.

INTRODUCTION

Traditionally, lipid peroxidation in meat is attributed to heme catalysts such as myoglobin and hemoglobin. Althought there have been numerous studies on lipid peroxidation, the relative importance of nonheme iron versus heme pigments in oxidation is still unclear (KRISTENSEN and ANDERSEN., 1997; HAN *et al.*, 1995). ERIKSSON *et al.* (1991) proposed that the heme pigments are the major catalysts in the lipid peroxidation of meat. Irrespective of whether the lipid is refined back fat or phospolipid- rich intramuscular fat, it seems that the heme pigments at the levels present in meat are more powerful catalysts of oxidation than inorganic iron (JOHN *et al.*, 1989). When meat is heated, any heat-induced changes which increase the exposure of heme to the surrounding environment will enhance lipid peroxidation in the meat and its products (KRISTENSEN *et al.*, 1997). Some authors have proposed that non-heme iron plays a major role in lipid oxidation of cooked meat (APTE and MORRISSEY., 1987a, b; LOVE and PEARSON., 1974). According to them, cooking of meat may destroy the myoglobin structure, releasing a considerable amount of non-heme iron which accelerates lipid peroxidation (TICHIVANGANA and MORRISSEY., 1985).

In our previous paper (BERISHA *et al.*, 2000), we reported that the prooxi-

dant and hydroperoxide decomposition activity of heat-denatured myoglobin increased as the denaturation temperature increased up to 74°C, while, further heating at 100°C resulted in the opposite effect. The proposed role of the temperature-induced structural changes on the prooxidant activity of myoglobin was based on those results.

In an attempt to explain the mechanism of the temperature-induced structural changes on the prooxidant activity of myogobin, this paper focuses on the following questions; First, why was the prooxidant activity of the heated myoglobin lower at 100°C than at 74°C? How does the heat-induced structural changes affect the proxidant activity of heat-denatured myoglobin? Second, does the heating of myoglobin facilitate the release of free iron or does the iron remain inside the heat-denatured myoglobin? If the former occurs, how important is the role of this released iron in the prooxidant processes?

To address these questions the following approaches were used to study the heat-induced structural changes of myoglobin and the results were compared with the prooxidant activity of heat-denatured myoglobin (BERISHA *et al.*, 2000).

1. Polarized absorption spectra of the heme-proteins were used to follow the temperature induced-changes in the contact between heme and distal histidine as a swinging door for the entrance of lipids into the heme pocket (EATON and HOFRICHTER, 1981; GERACI., and PARKOUSE., 1981: MATSUOKA *et al.*, 1992).

2. Hydrophobicity of the heat-denatured myoglobin was used to observe the structural alterations where the charged amino acids are located (UCHIDA *et al.*, 1997).

3. Dialysis of the heat-denatured myoglobin was used to assess the prooxidant role of the released iron in the lipid peroxidation, assessed by measurement of the thiobarbituric acid (TBA) value.

4. ^{1}H-NMR was used to investigate the relationship between the structural alterations and the prooxidant activity of the heat-denatured myoglobin as a function of the heating temperature.

MATERIALS AND METHODS

Heat denaturation of myoglobin

Three milliliters of 0.24 mM myoglobin (horse heart, Sigma, St. Lewis, MO) in 0.2 M phosphate buffer at pH 7, were heated at 74° or 100°C for 30 min in a test tube. Heating time and temperatures were based on the results of BERISHA *et al.*, (2000). In order to obtain a clear solution (not turbid) for UV and CD spectra, heat-denatured myoglobin solutions were further diluted with the same phosphate buffer.

Linoleic acid preparation

Preparation of the purified linoleic acid hydroperoxides was performed as previously reported (BERISHA *et al.*, 2000).

Ultraviolet and visible absorption spectra

Ultraviolet and visible absorption spectra of the heated myoglobin (24.0 μM in 0.2 M phosphate buffer at pH 7.0) were monitored in the range of 350-750 nm (EATON and HOFRICHTER., 1981) on a JASCO U best-35 (JASCO, Tokyo, Japan).

Circular dichroism spectra

Circular dichroism spectra (CD-Spectra) of heated myoglobin (0.6 μM in 0.2 M phosphate buffer at pH 7.0) was monitored in the range of 390-430 nm (GERACI and PARKURST., 1981) on a JASCO J-720 Spectropolarimeter (JASCO, Tokyo, Japan).

Dialyses of the heated myoglobin

Three milliliters of 0.24 mM heated myoglobin solutions were dialyzed twice against 100 mL of distilled water for 24 h at 5°C in seamless cellulose tubes (Sanko, Tokyo, Japan), principally based on the method of BOWEN (1948). After dialysis, the inner solution was brought up to 10 mL and the outer solution was carefully reduced to 10 mL under vacuum at low temperature. An aliquot containing 1 mL of inner solution (myoglobin) or 1 mL of the outer solution (low molecular fractions) was incubated with 9 mL linoleic acid (5 mM in 0.2 M phosphate buffer at pH 7.0 containing 10% ethanol) to assess the prooxidant activity. The samples were incubated at 5°C for 48 h and the prooxidant activity was evaluated using the thiobarbituric acid values (BERISHA *et al.*, 2000; OHKAWA *et al.*, 1978).

To determine the amount of released iron, 0.5 mL of the outer solution was incubated with 2.5 mL of the coloring reagent for 10 min at room temperature (SCHRICKER *et al.*, 1982) and the iron was determined by reading the absorbance at 535 nm. The iron standard was prepared using ammonium iron (II) sulphate hexahydrate (Wako Pure Chemicals, Osaka, Japan) in 1.5% hydroxylamine hydrochloride in 0.2 M acetate buffer at pH 4.5 (PANTER, 1994).

Hydrophobicity of heated myoglobin

Two milliliters of 0.12 μM heated myoglobin in 0.2 M phosphate buffer (pH 7.0) were incubated with 10 μL of 8 mM ANS (8-aniline-1-naphthalene-sulfonic acid; Wako Pure Chemicals, Osaka, Japan) at 20°C for 1 h.

The fluorescence intensity (Ex at 390 nm and Em at 470 nm) was measured on a JASCO FP Spectrofluorometer (JASCO, TOKYO, JAPAN) calibrated with a standard solution of the 1 μg quinine sulfate / mL of 0.1 N H_2SO_4 (TAKAGI *et al.*, 1979).

^{1}H NMR of the heated myoglobin

Myoglobin (0.24 mM in 0.2 M phosphate buffer, 100% D_2O, pD 7.0) was heated at 74° or 100°C for 30 min. Myoglobin solutions were concentrated to 1.1 mM using a Millipore filter (05.000NMWL, Nihon Millipore, Yonezawa, Japan).

To assign the two exchangeable resonances for the His64 and His93 (as the important stabilizers of the heme plane within the globin pocket), which cannot be observed in 100% D_2O solutions, myoglobin samples were heated and handled as described above. However, prior to ^{1}H-NMR measurements, the myoglobin samples were spin suppressed with 10 mM KCN (LLOYD *et al.*, 1996).

The ^{1}H NMR spectra of the heated myoglobin were monitored at 25°C on a JEOLJNM-GX 400 Spectrometer (JEOL, Tokyo, Japan) operating in quadrate mode at 200 MHz by collecting 20,000 transients with 65,536 data points, frequency at 100,000.0 Hz, and acquisition time of 0.328 sec.

RESULTS AND DISCUSSIONS

The UV absorption spectrum at the Soret band of the myoglobin heated at 74°C was very similar to that of the native one. However, the absorbance at 410 nm of the myoglobin heated at 100°C was weak (Fig. 1). The absorbance of the heated myoglobin at 410 nm showed a positive correlation with the hydroperoxide decomposition activity (R^2 = 0.87) and with the lipid peroxidation activity assessed by TBA values, which reflects the overall progress of the lipid peroxidation (R^2 = 0.85), both previously reported by BERISHA *et al.*, (2000).

The absorbance of CD spectra (Fig. 2) was in agreement with the changes of the absorption spectra at the Soret band.

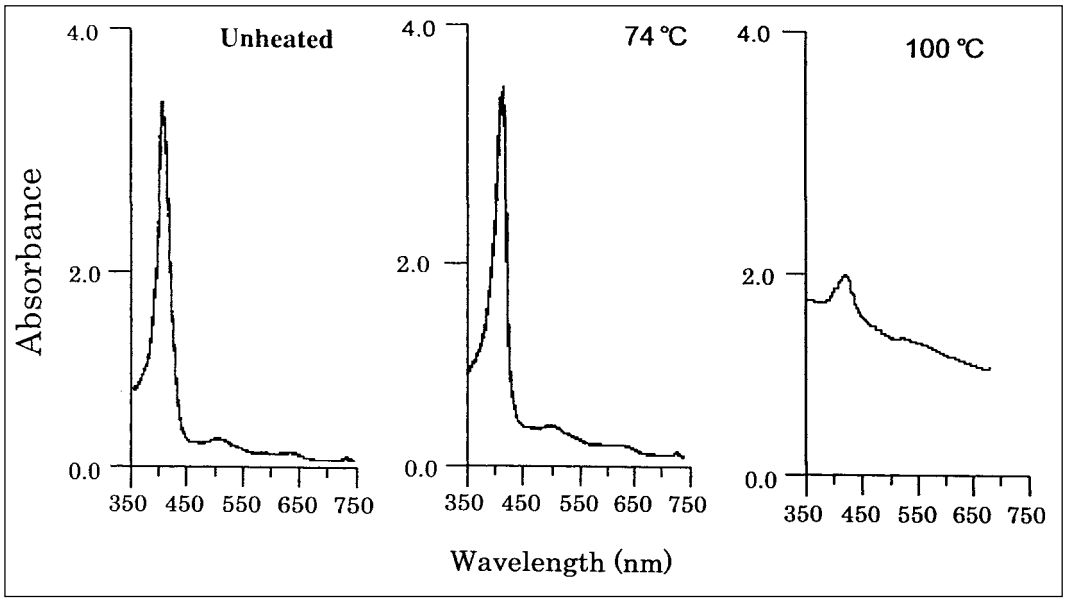


Fig. 1 - Visible absorption spectra of the heat-denatured myoglobin solutions heated at different temperatures for 30 min in a phosphate buffer at pH 7.0. The results are the means of 4 determinations.

Heating at 74°C caused little change in the CD absorbance of the myoglobin. However, myoglobin heated at 100°C showed very weak absorption. Similar to the absorption spectrum at the Soret band, the absorbance showed a very good correlation with hydroperoxide decomposition activity ($R^2 = 0.97$) and with the previously reported TBA values ($R^2 = 0.99$; BERISHA *et al.*, 2000).

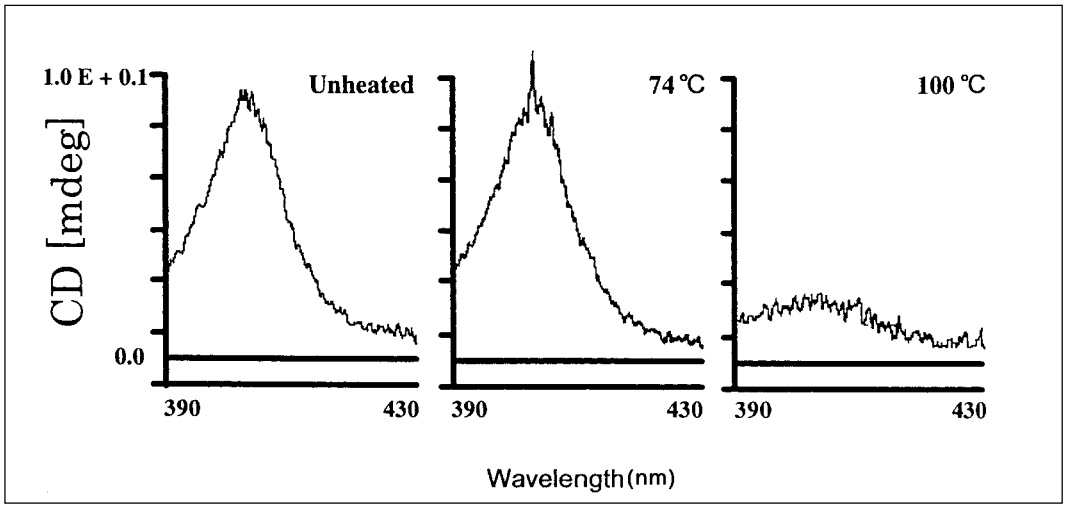


Fig. 2 - Circular dichroism spectra of heat-denatured myoglobin solutions heated at different temperatures for 30 min in a phosphate buffer at pH 7.0. The results are the means of 4 determinations.

In the initial phase of heating, some disturbances in the region of the heme group occur due to minor local alterations in the tertiary structure. During this phase, the position of tryptophan (as well as that of tyrosine and phenylalanine) remain essentially unaltered and no changes occur in the heme-ligand binding (AWAD and DERANLEAU., 1968).

Heating causes relatively limited increase in the prooxidant activity of myoglobin (BERISHA *et al.*, 2000) due to a more favorable exposure of the heme group. This was correlated with an increase of the Soret and CD absorbances, suggesting an increase in the cooperation between heme and distal histidine (EATON and HOFRICHTER., 1981; ERACI and PARKHOUSE., 1981; MATSUOKA *et al.*, 1992). The highest temperature of transition (determined to be 74°C) coincided with an increase in the absorbance at 540 nm and a reduction of the absorbance at 410 nm (Soret region). Above this temperature, the catalytic heme group becomes less exposed to its surroundings (KRSITENSEN and ANDERSEN., 1997), resulting in the decreased prooxidant activity of myoglobin at 100°C (BERISHA *et al.*, 200).

The hydrophobicity of the heated myoglobin expressed by the fluorescence intensity is shown in Fig. 3. No measurable change was observed in the hydrophobicity of the myoglobin heated at 74°C. However, over this temperature, the hydrophobicity increased as the heating temperature increased. Changes in the hydrophobicity of the heated myoglobin (columns) did not correlate with the previously reported TBA values (line; BERISHA *et al.*, 2000).

These data suggested that the myoglobin structure was not changed significantly by heating at 74°C. However,

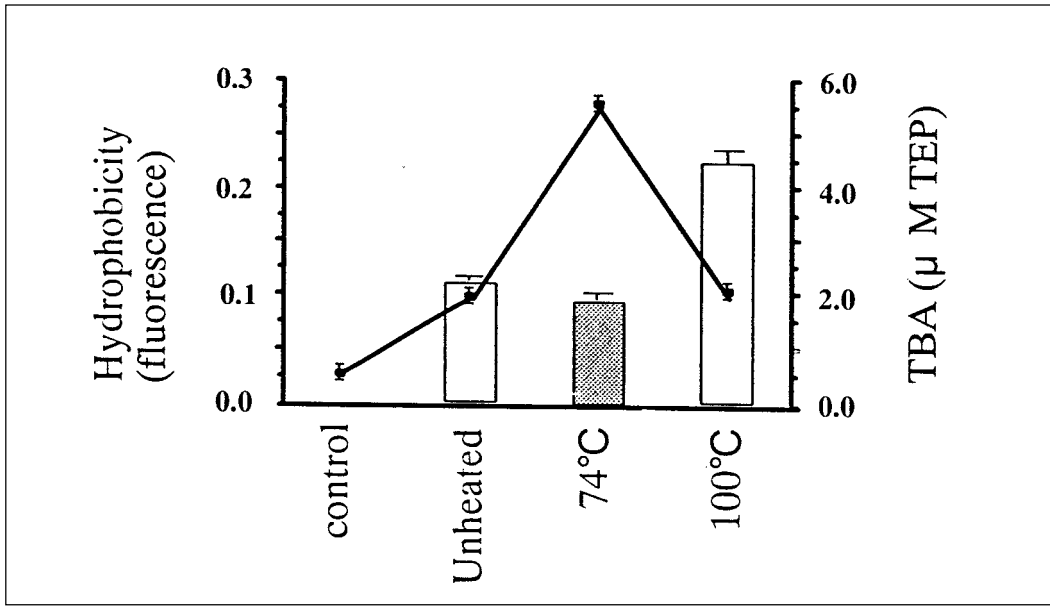


Fig. 3 - Hydrophobicity (expressed as fluorescence intensity) of the heat-denatured myoglobin solutions heated for 30 min at different temperatures. Hydrophobicity results (in columns) are plotted against the prooxidant activity (line) of the heated myoglobin (expressed as TBA values; BERISHA *et al.*, 2000). The results and the standard deviations are the means of 4 determinations.

heating at higher temperatures increased the hydrophobicity which was negatively correlated with the absorbance at the Soret band, CD spectra, prooxidant and hydroperoxide decomposition activity of the heated myoglobin. Heating at high temperatures may result in the loss and precipitation of hemin due to extensive structural alterations in the secondary and tertiary structure of the globin molecule resulting in the reduction of the prooxidant and hydroperoxide decomposition activity of the heat-denatured myoglobin (BERISHA *et al.*, 2000). This loss and precipitation of hemin might hinder the hydrophobic amino acids of the heme-pockets from reacting with ANS. However, the greatest increase of hydrophobicity may be due to the unfolding of the myoglobin, exposing hydrophobic amino acids, other than those located in the heme pocket (UCHIDA *et al.*, 1997).

The dialysis experiments showed that the prooxidant activity of the outer solution (non-myoglobin iron) was increased by heating at 74°C, but, heating at 100°C decreased the activity. Regardless of the heating temperature, the amount of the iron released from the myoglobin was low (Fig 4a). The maximum amount of the released iron was about 1 μM from about 70.5 μM total present in 70.5 μM dialyzed myoglobin (about 1.42% of the total iron). The iron released from myoglobin played only a minor role in the prooxidant activity (Fig 4b) as noted by TBA values that were remarkably lower than those of myoglobin iron (inner solution; Fig. 4b). Under our experimental conditions, heating of myoglobin at higher temperatures did not accelerate the release of iron from the myoglobin, but rather encapsulated it within the molecule.

Regardless of the heating temperature, the released iron is not important in the lipid peroxidation processes compared with myoglobin itself because the free iron is responsible for only 20% of the

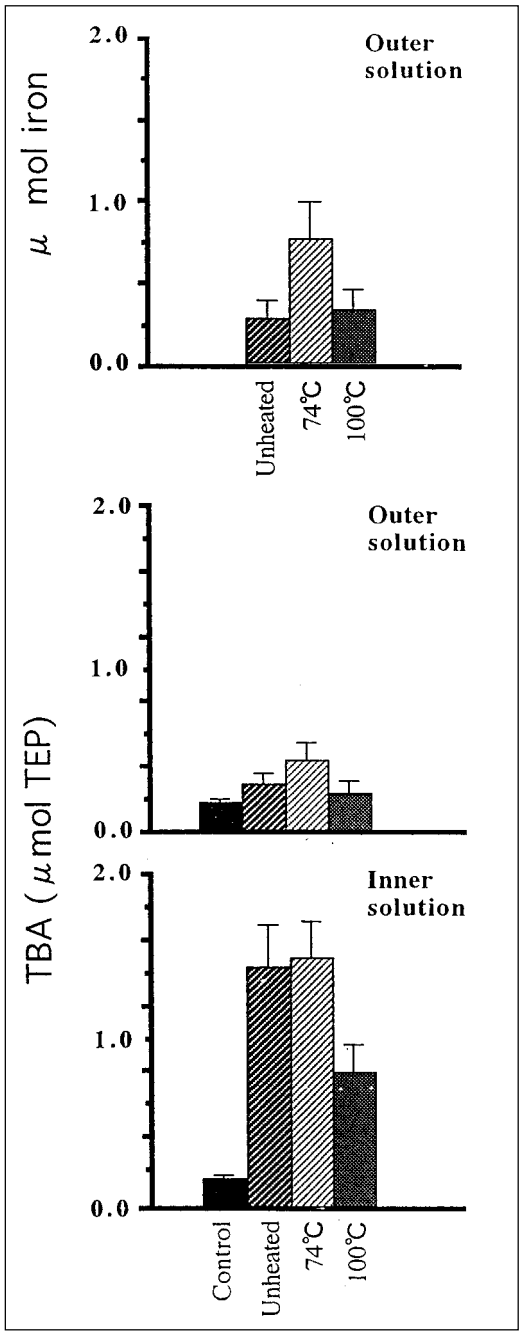


Fig. 4 - Iron released during 24 h of dialysis of the heat-denatured myoglobin. The prooxidant activity of the released iron (outer solution) and the heat-denatured myoglobin (inner solution) were assessed by TBA values. The result and the standard deviations are the means of 4 determinations.

prooxidant activity as exhibited by the TBA values.

Fig. 5 shows the down field region of ^{1}H-NMR spectra of heated myoglobin. Among assigned protons, peaks c, j, and l are based on the structural consideration in close proximity to the heme which forms the hydrogen bonds between pyrrole A propionate and the charged amino acids located in the globin of the native myoglobin and the rest of the peaks are assigned as hyperfine shifted resonance of the heme methyl protons (LLOYD *et al.*, 1996). The unpaired spin distribution of heme was not substantially changed when heating at 74°C (Fig. 5). This was indicated by the almost identical chemical shifts clearly exhibited by their similar positions and composition when compared with the unheated myoglobin. However, heating at 100°C resulted in a very different pattern, i.e. all the specific ^{1}H-NMR peaks were eliminated.

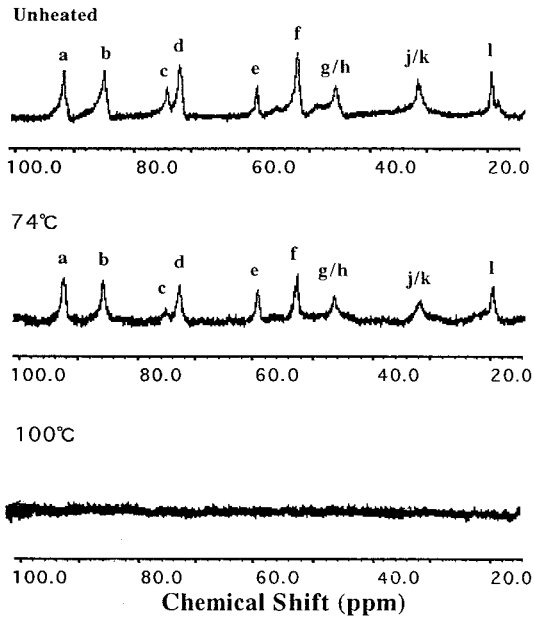


Fig. 5 - The down field region of the 200 MHz ^{1}H-NMR spectra of the horse heart myoglobin heated at different temperatures prior to the NMR analysis. Peak assignments and interpretations were made according to LLOYD *et al.* (1996).

The ^{1}H-NMR spectrum (Fig. 6) for the cyanide form of myoglobin is based on the exchangeable resonances assigned to His64 and His93 (peaks b and c). These peaks were not observed in 100% D_2O (LLOYD *et al.*, 1996). Heating of myoglobin at 74°C did not result in any significant alteration of the KCN spin-suppressed myoglobin ^{1}H-NMR spectra. However, heating at 100°C resulted in a drastic change where all the peaks were eliminated. Furthermore, for the myoglobin-CN derivates, the ratio between the major and minor orientations of the porphyrin group in the heme pocket of the myoglobin heated at 74°C (DECATUR and BOXER., 1995; HUNTER *et al.*, 1997) was similar to the unheated one. This fact was exhibited by peak "a" which remained the same at this temperature (Fig. 6). The elimination of the peaks (in free or cyanide myoglobin) is correlated with the reduction of the prooxidant of the myoglobin heated at 100°C (BERISHA *et al.*, 2000), the reduction of the UV absorption and CD spectra and the increase in hydrophobicity.

During ^{1}H-NMR spectral analysis, two group of peaks were observed. The first group of peaks was connected with the porphyrin shell that is not dependant on the position and the hydrogen bonds even when the hydrogen bonds were eliminated by the amino acid mutations of the globin (peaks c, j, l in Fig. 5 and peaks b, c in Fig. 6; HILDERBRANT *et al.*, 1995; DECATUR and BOXER., 1996). The second group of peaks was dependent on the hydrogen that stabilizes the heme in the globin pocket (the other peaks in Fig. 5 and 6; LLOYD *et al.*, 1996).

Heating at 74°C changed nothing in all the peaks, especially peaks c, j, l in Fig. 5 and peaks b, c in Fig. 6 which correspond to the resonances of bonds which stabilize heme in the myoglobin. These results suggest that no/or very insignificant changes occurred in the

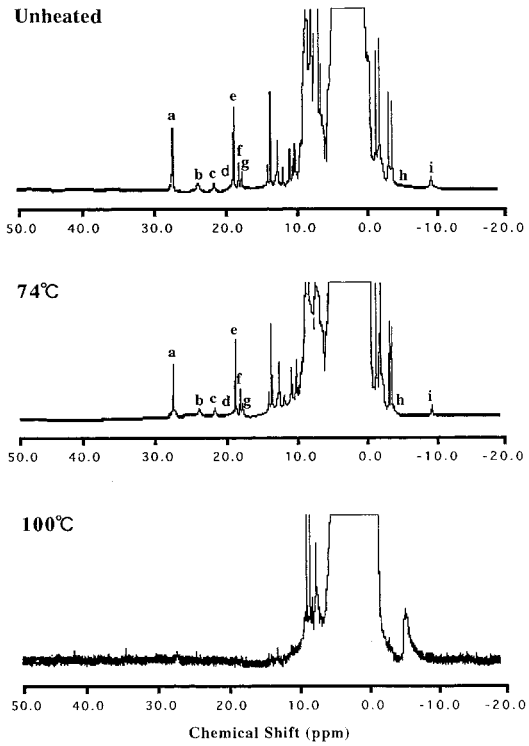


Fig. 6 - The down field region of the 200 MHz ^{1}H-NMR spectra of the horse heart myoglobin heated at different temperatures. Myoglobin solutions were spin suppressed with KCN prior to the NMR analysis. Peak assignments and interpretations were made according to LLOYD *et al.* (1996).

myoglobin structure and no changes occurred in the heme orientation (peak "a" in Fig. 6; HUNTER *et al.*, 1997) when heated at 74°C. However, further heating to 100°C resulted in an entirely different spectrum. No peaks associated with heme or with hydrogen bonds between the heme and globin remained. When the myoglobin was heated at 100°C, the NMR peaks disappeared. The Soret absorbance and CD were drastically reduced and the prooxidant activity was reduced. If we combine these results with those of LLOYD *et al.* (1996) where changes in the hydrogen bonds between heme and amino acids in globin did not have any impact on the peaks connected only with heme, we can propose that:

Heating (APTE and MORRISSEY, 1987) destroys the hydrogen bonds between porphyrin and globin which was shown by the disappearance of the corresponding peaks in ^{1}H-NMR. In contrast, even the ^{1}H-NMR peaks, which are exclusively derived only from the interactions within the porphyrin group, disappeared in the spectra. Based on these two observations, we can suggest that the precipitation of hemin within the interior of the heat-coaguled myoglobin will hinder it from the ^{1}H-NMR magnetic field. This could explain the decreased prooxidant activity of the myoglobin heated at 100°C (BERISHA *et al.*, 2000).

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PAPER

MICROBIOLOGICAL QUALITY OF MINCED BEEF FROM BUTCHER SHOPS AND SUPERMARKETS

VALUTAZIONE DELLA QUALITÀ MICROBIOLOGICA DELLA CARNE TRITATA NELLE MACELLERIE E NEI SUPERMERCATI

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ABSTRACT

The microbiological quality of 230 samples of minced beef obtained from butcher shops and supermarkets in eight areas of Athens was examined. The aerobic plate count (APC), the number of *Enterobacteriaceae*, *Brochothrix thermosphacta* and *Pseudomonas* spp. were determined. Average APC $\log_{10}$ ranged from 6.20 to 6.84, while for the *Enterobacteriaceae*, *B. thermosphacta* and *Pseudomonas* spp., the counts ranged from 3.15 to 4.50, 4.82 to 6.33 and 4.98 to 6.13 cfu g^{-1}, respectively. The influ-

RIASSUNTO

È stata valutata la qualità microbiologica di 230 campioni di carne tritata prelevata nelle macellerie e nei supermercati di otto quartieri di Atene. Sono stati determinati la conta microbica aerobica (APC) e il numero delle *Enterobacteriaceae*, delle *Brochothrix thermosphacta* e delle specie di *Pseudomonas*. I valori logaritmici di ACP erano compresi tra 6,20 e 6,84, mentre la conta per le *Enterobacteriaceae*, *Brochothrix thermosphacta* e *Pseudomonas* spp. era compresa rispettivamente tra 3,15 e 4,50, 4,82 e 6,33 e 4,98 e 6,13 ufc g^{-1}.

- Key words: microbiological quality, minced meat, shelf life -

ence of geographical area, type of shop and month in which the samples were purchased was investigated. The area of purchase significantly influenced (P<0.001) the counts of *Enterobacteriaceae* and *B. thermosphacta* but not the counts of *Pseudomonas* spp. (P=0.059). Moreover, there were significant differences in the bacterial counts in all the categories of microorganisms due to the type of shop (P<0.005). The correlation coefficients (r) of *Enterobacteriaceae*, *B. thermosphacta* and *Pseudomonas* spp. with respect to the aerobic plate count were 0.616, 0.678 and 0.605, respectively.

È stata inoltre valutata l'influenza del quartiere cittadina, del negozio e del mese di prelievo. È stato verificato che la conta dei *Enterobacteriaceae*e di *Brochothrix thermosphacta* era influenzata significativamente (P<0.001) dal quartiere di prelievo. Non c'era invece alcuna relazione per quelle relative a *Pseudomonas* spp. (P<0,059). Inoltre, differenze significative sono emerse per la conta batterica di tutte le categorie in funzione del tipo di luogo di prelievo (macellerie e supermercati) (P<0,005). Il coefficiente di relazione (r) della conta microbica aerobica di *Enterobacteriaceae*, *Brochothrix thermosphacta* e *Pseudomonas* spp. era rispettivamente di 0,616, 0,678 e 0,605.

INTRODUCTION

The microbiological quality of fresh minced meat depends on the conditions that prevail during slaughtering, butchering, and processing (MOSSEL and STRUIJK, 1992). The main sources of contamination are the animal and the environment. Mince is a product with a short shelf life compared to whole meat cuts. Microorganisms on the surface are spread throughout the mass and microbial growth takes place throughout the whole mass (NYCHAS *et al.*, 1988).

Studies on the microbiological quality of mince have shown a correlation between the day of the week and the packaging atmosphere. Intrinsic parameters such as glucose concentration have also been correlated with the microbiological quality (NYCHAS *et al.*, 1991; DROSINOS and BOARD, 1995a, b). In a survey of retail pork DUFFY *et al.* (2001) concluded that handling and processing were correlated with the microbiological quality. EISEL *et al.*, (1997) found that the microbial contamination of the incoming meat was the decisive factor that determined the quality of the mince.

The objective of the present study was to correlate the microbiological quality of minced beef with the area in which it was purchased, the type of shop and time of purchase and to provide reference values for planning quality management systems.

MATERIALS AND METHODS

Mince samples

Samples of minced beef prepared in butcher shops and supermarkets were bought in eight areas within the metropolitan area of Athens over a three-month period, July to September 2000 (Table 1). Immediately upon arrival at the laboratory, the samples were stored at chill temperature (4°C) and taken separately, in a random sequence for sampling. Analysis was started within two hours.

Microbiological analyses

A sample (25 g) was transferred to a stomacher bag and 225 mL of sterile

Table 1 - Sampling area and number of samples analyzed in the various months at butcher shops (B) or supermarkets (S).

Area	Total (N)	July		August		September	
		B	S	B	S	B	S
1	39	7	6	7	6	7	6
2	36	7	5	7	5	7	5
3	28	8	6	8	6	-[b]	-
4	36	5	7	5	7	5	7
5	33	5	6	5	6	5	6
6	22	5	6	-	-	5	6
7	18	5	1	5	1	5	1
8	18	5	4	-	-	5	4

-: *Samples n*ot available.

0.1% (w/v) bacteriological peptone were added (Oxoid, Unipath, Basingstone, UK). The sample was homogenized at room temperature with a stomacher (Stomacher 400, Seward) for 1 min at low speed, and for 30 s at high speed, and serial decimal dilutions in quarter-strength Ringer solution (Oxoid) were prepared. For the enumeration of aerobic plate count (APC), *B. thermosphacta* and pseudomonads, 0.1 mL was spread on Plate Count Agar (PCA) plates (Merck, Darmstadt, Germany), streptomycin sulphate – thallous acetate – cycloheximide (actidione) (STAA) agar plates (GARDNER, 1966) and cetrimide – fucidin – cephaloridine (CFC) agar plates (MEAD and ADAMS, 1977), respectively. The PCA, STAA and CFC plates were incubated at 30°C for 3 d, at 25°C for 3 d and at 20°C for 2 d, respectively. For the enumeration of *Enterobacteriaceae*, 1.0 mL was poured in Violet Red Bile Dextrose (VRBD) Agar (Merck). After gelling, a 10 mL overlay of molten medium was added and incubated at 37°C for 24 h. All plates were examined visually for typical colony types and morphological characteristics associated with each growth medium. Performance of CFC, STAA and VRBD agars were checked by Gram-staining and microscopic examination of isolates. Confirmation of the STAA colonies was done by catalase test and Gram strain (GARDNER, 1981). Oxidase reaction was tested on about 10% of the colonies grown on VRBD agar and CFC agar. Oxidase negative isolates in VRBD agar and oxidase positive isolates on CFC were counted.

Statistical analysis

The microbiological counts were transformed to $\log_{10}$. The transformed counts were tested for normality with the Kolmogorov-Smirnov test and analyzed by multivariate analysis (three way analysis of variance) to examine the influence of area, shop and month of purchase on the microbiological quality of the mince. Pearson correlations were conducted among the counts. Descriptive statistics of the bacterial counts and analysis were performed with the SPSS 8.0 statistical programme.

RESULTS

Fig. 1 shows the average microbial counts of the aerobic plate count (APC). These ranged from $\log_{10}$ 6.20 cfu g^{-1} in area 8 to $\log_{10}$ 6.84 cfu g^{-1} in area 7. Samples obtained from traditional butcher shops and modern supermarkets had an average APC of 6.34 and 6.78, respectively. The average APC related to survey time ranged from 6.45 to 6.61 in July and August, respectively. The highest count (8.14) was found in a mince sample from a supermarket purchased in September, while the lowest microbiological count (3.78) was recorded in mince obtained from a traditional shop purchased in July (data not shown).

The average counts of *Enterobacteriaceae* are shown in Fig. 2. They ranged from $\log_{10}$ 3.15 cfu g^{-1} in area 2 to $\log_{10}$ 4.50 cfu g^{-1} in area 3. Samples obtained from traditional shops showed an average count of $\log_{10}$ 3.21 cfu g^{-1}, while those

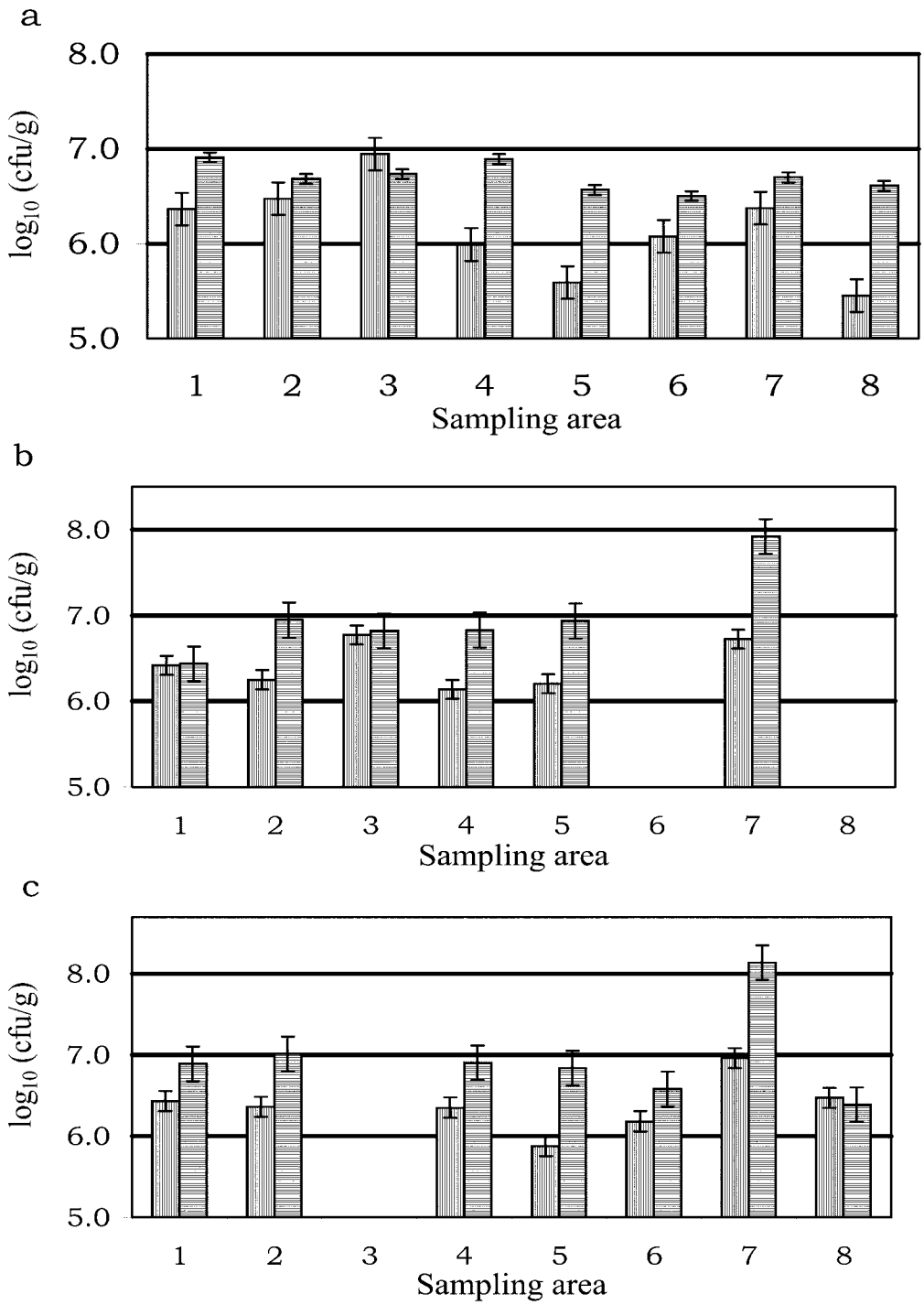


Fig. 1 - Aerobic plate counts of minced beef obtained from butcher shops (vertical lines in bars) and supermarkets (horizontal lines in bars) (a: July, b: August and c: September).

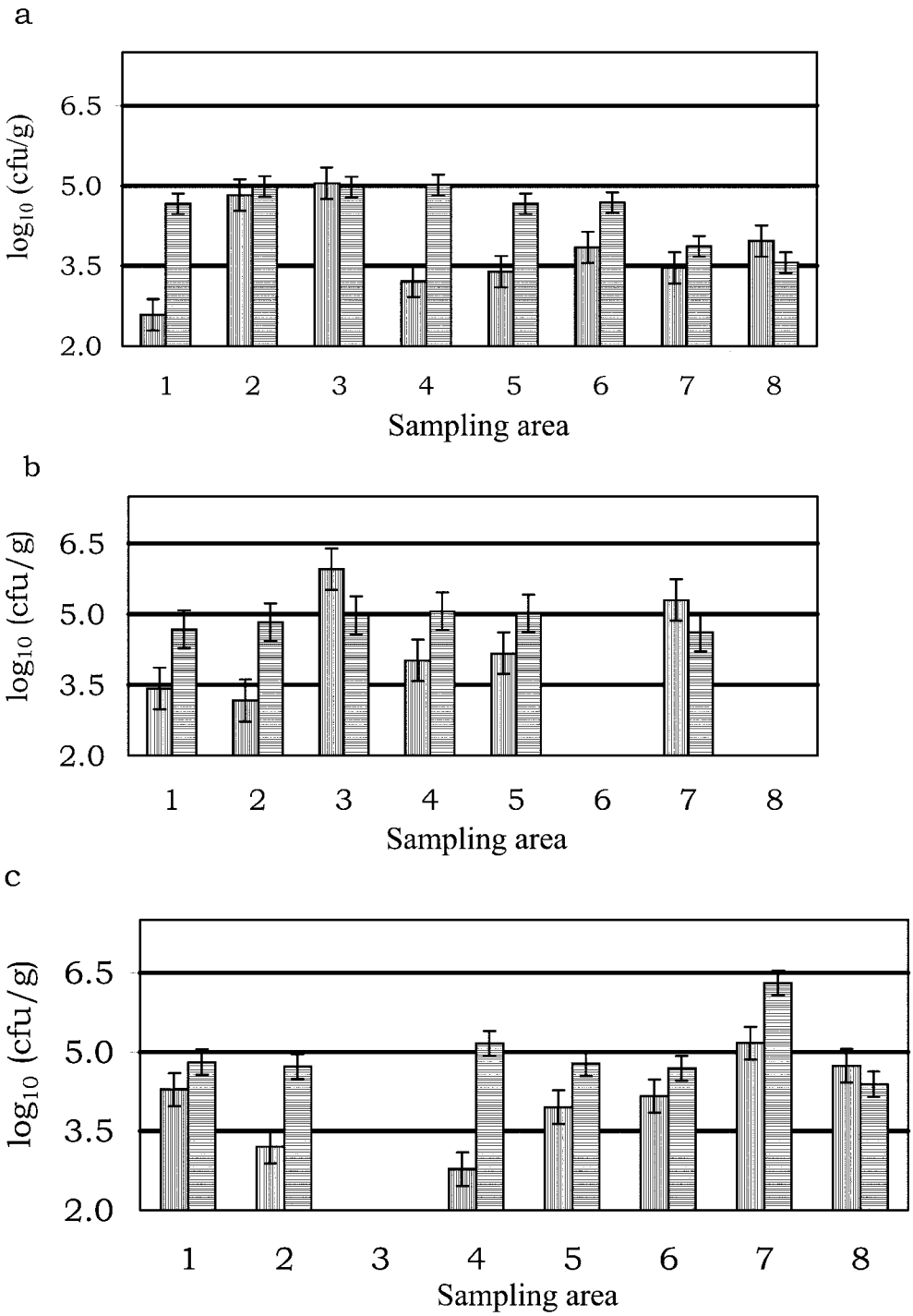


Fig. 2 - *Enterobacteriaceae* counts of minced beef obtained from butcher shops (vertical lines in bars) and supermarkets (horizontal lines in bars) (a: July, b: August and c: September).

from supermarkets averaged 4.28. The average count related to the month of purchase ranged from $\log_{10}$ 3.69 cfu g^{-1} to $\log_{10}$ 3.86 cfu g^{-1}. The lowest count (<10 cfu g^{-1}) was recorded in samples purchased from traditional shops in area 2 during the whole survey period (data not shown). The highest counts (6.30) were recorded in samples obtained from a supermarket in area 7.

The average count of *B. thermosphacta* related to the purchase area ranged from $\log_{10}$ 4.82 cfu g^{-1} to $\log_{10}$ 6.33 cfu g^{-1}. Samples obtained from traditional shops had a count of 5.41, while those from supermarkets had a count of $\log_{10}$ 5.82 cfu g^{-1}. The average count related to the month of purchase ranged from 5.56 in September to 5.64 in August (Fig. 3). The lowest count (2.00) was noted in samples from traditional outlets in area 1 during the whole survey period (data not shown), while the highest (7.20) counts were recorded in the samples obtained from a supermarket in area 7.

Pseudomonas counts related to the purchase area ranged from $\log_{10}$ 4.98 cfu g^{-1} to $\log_{10}$ 6.13 cfu g^{-1}. Samples obtained from traditional shops had counts of $\log_{10}$ 5.48 cfu g^{-1}, while the counts for those from supermarkets were $\log_{10}$ 5.90 cfu g^{-1}. The average count related to the month of purchase ranged from $\log_{10}$ 5.63 cfu g^{-1} to $\log_{10}$ 5.72 cfu g^{-1} (Fig. 4). The lowest count (3.00) was noted in traditional and modern outlets in several areas during the whole survey period (data not shown). The highest count (7.94) was observed in the samples obtained from a supermarket in area 7.

The interaction (three-way analysis of variance) of the three factors 8 (area) x 2 (type of outlet) x 3 (purchase month) on each microbiological variable is shown in Table 2. There were significant differences ($P<0.001$) for the microbial counts of *Enterobacteriaceae* and *B. thermosphacta* between the different areas, but no significant difference for pseudomonads ($P=0.059$). A similar significance ($P<0.001$) was noted for APC between the types of shops. The purchase month did not have a significant influence on microbial counts. An interaction was noted only for *Enterobacteriaceae* counts between the area and type of outlet ($P=0.030$) and area and purchase month ($P=0.037$). A slight interaction was noted between the area and type of outlet ($P=0.074$) for the *B. thermosphacta* counts. Pearson correlation analysis between counts of APC and pseudomonads, *Enterobacteriaceae* and *B. thermosphacta* was significant at $P<0.01$. Correlation coefficients for pseudomonads, *B. thermosphacta* and *Enterobacteriaceae* were 0.605, 0.678 and 0.616, respectively.

CONCLUSIONS

The results of the present study show a significant difference between the areas where the beef mince was purchased with respect to the microbiological variables, except for pseudomonads. In a similar study in Louisiana (LAW *et al.*, 1971), the city was divided into four areas based on the income of the citizens. In the present study the city of Athens was divided into eight areas based on geographical criteria that are also linked with income. LAW *et al.* (1971) did not conduct a statistical analysis between the four areas, and the microbial counts ranged from $1.0x10^6$ to $4.6x10^8$ cfu g^{-1}. In the present study the bacterial population ranged from 10^4 to 10^8 cfu g^{-1} and the average value in all areas ranged from 10^6 to 10^7 cfu g^{-1}. The samples from area 8 had the lowest bacterial counts and samples from area 7 had the highest counts.

In Finland, SKRÖKKI (1997) studied the hygienic quality of commercial minced meat. They found that 80% of the beef samples were of good hygienic quality (<10^7 cfu g^{-1}), 20% of tolerable quality (10^7-10^8 cfu g^{-1}) and only one was

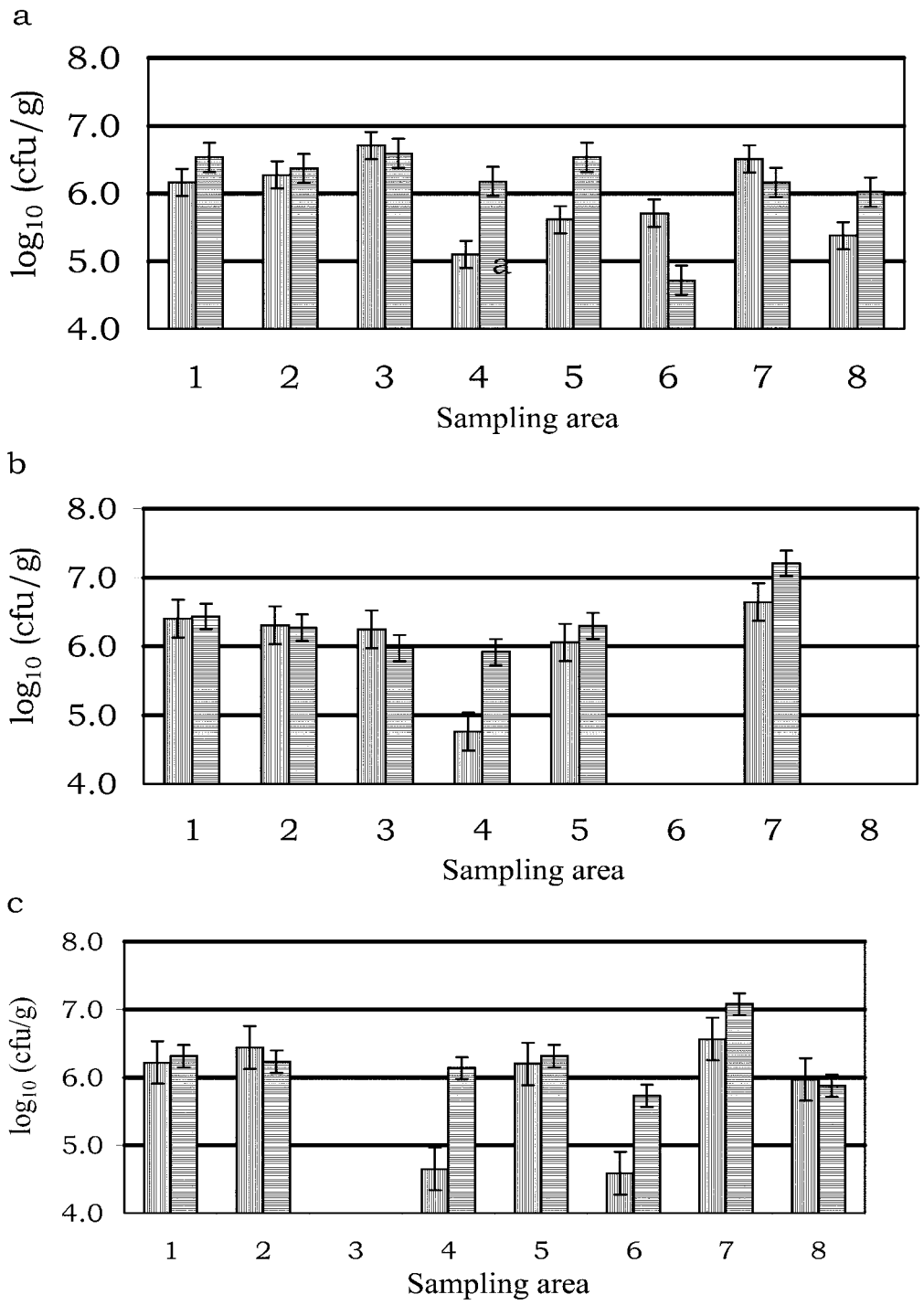


Fig. 3 - *Brochothrix thermosphacta* counts of minced beef obtained from butcher shops (vertical lines in bars) and supermarkets (horizontal lines in bars) (a: July, b: August and c: September).

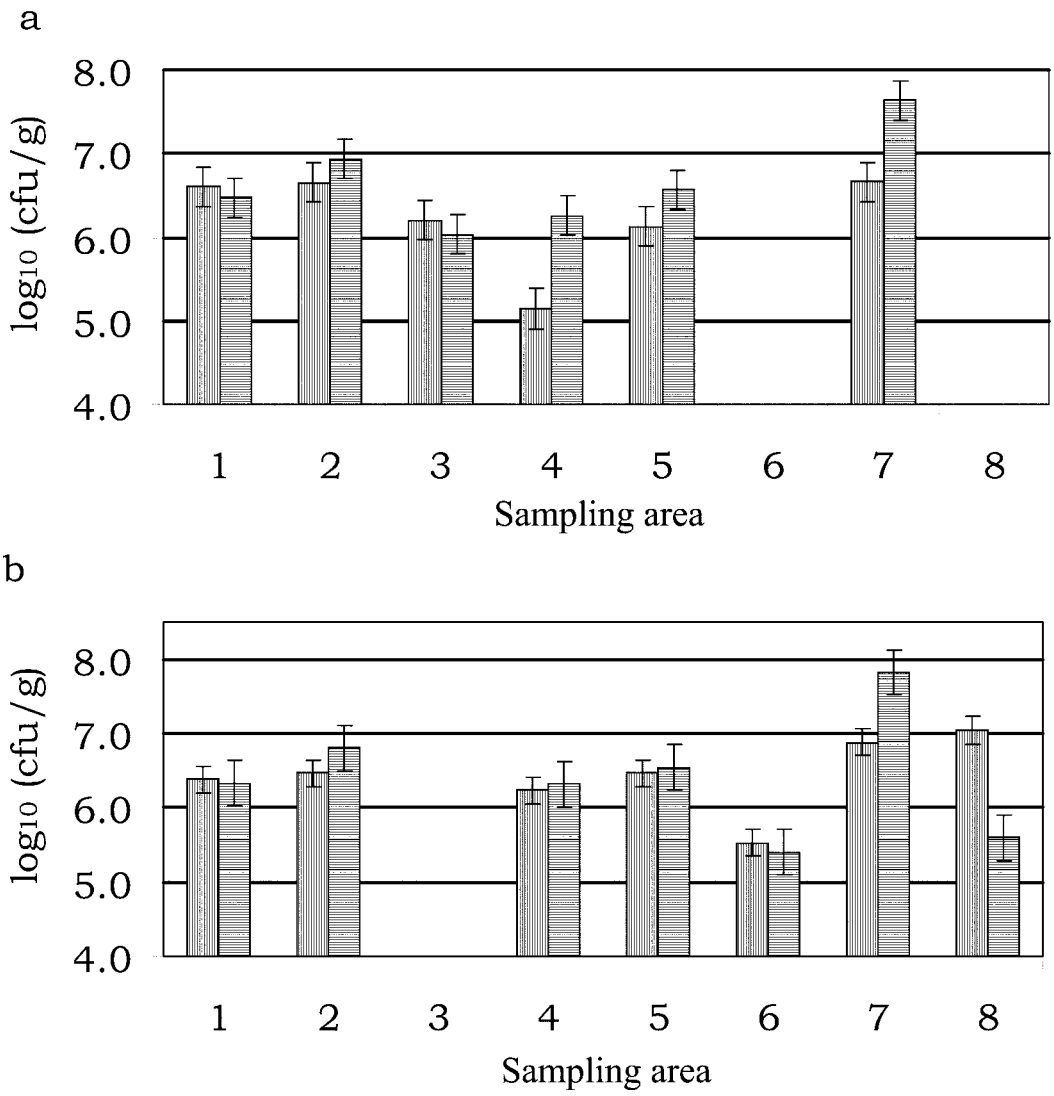


Fig. 4 - Pseudomonad counts of minced beef obtained from butcher shops (vertical lines in bars) and supermarkets (horizontal lines in bars) (a: August and b: September).

Table 2 - Analysis of variance P.

Factor	Aerobic Plate count	*Enterobacteriaceae*	*Brochothrix thermosphacta*	*Pseudomonas* spp.
Area x Shop	0.261	0.030	0.074	0.285
Area x Month	0.842	0.037	0.630	0.885
Shop x Month	0.983	0.489	0.682	0.996
Area x Shop x Month	0.826	0.845	0.234	0.949

of bad quality, with an aerobic microbial flora of $3.1x10^8$. In Berlin (Germany) KLEIN and LOUWERS (1994) investigated 295 samples of minced meat. They found that the critical point for the microbiological quality of the product was the quality of the processed meat, and pseudomonad counts increased after the third day of storage at 2°C.

ROBERTS *et al.* (1980) studied the bacteriological quality of minced beef in the U.K. They reported that the bacterial count of minced beef was not influenced by the type of shop. However, NYCHAS *et al.* (1991) in the city of Bath (England), ROGERS and McCLESKEY (1957) in Louisiana (USA) and SHOUP and OBLINGER in Florida (USA) (1976) observed that the type of shop had an effect on the microbial population of the meat. The type of retail shop correlated with lower microbial counts differed between the studies. ROGERS and McCLESKEY (1957) observed a significant difference in the total microbial count that was dependent on the size of the outlet. SHOUP and OBLINGER (1976) noted that mince produced in a centralised processing operation had a better microbiological quality than that prepared traditionally. In Maryland (USA), FIELD *et al.* (1977) did not support this view as they noted that mince produced centrally and distributed in retail outlets had low bacterial counts at the time of production and packaging, but the conditions during distribution had a marked influence on its final microbiological quality. In the present study the results are in agreement with NYCHAS *et al.* (1991), who noted a significant difference between mince made in butcher shops and supermarkets with respect to certain microbiological variables. They noted a correlation between the type of outlet and the population of *B. thermosphacta*, yeasts and lactic acid bacteria. The results of the present study confirm the view of FIELD *et al.* (1977) that the microbiological quality of mince obtained from butcher shops was better than that from supermarkets. A possible explanation is that mince is prepared in butcher shops upon a purchase order. In supermarkets mince is prepared in advance and this may account for the higher microbiological counts. ROGERS and McCLESKEY (1957) and ROBERTS *et al.* (1980) did not observe a seasonal effect on the microbiological quality of mince but NYCHAS *et al.* (1991) observed that the climatic conditions during summer (July-August) and winter (November-December) had an effect on the initial microbial contamination with higher counts in the summer months. In the present study, the purchase month did not have a significant influence on the microbiological quality.

In England, HINTON *et al.* (1998) reported a low correlation ($r<0.20$) between the different microbial populations. In Canada, AL-DELAIMY and STILES (1975) noted a high correlation between the psychrotrophic populations and the total microbial flora ($r=0.95$), while the correlation between coliforms and *Staphylococcus* with total viable count, was 0.70 and 0.69, respectively.

In the present study there was a positive correlation of the total population (APC) with its components. It is generally recognized that under aerobic conditions at chill temperatures pseudomonads dominate the microbial population. In the present study the bacterial counts contributed equally to the APC, with a slightly higher count of *B. thermosphacta*. In other words, the samples of the present survey did not have climax populations but rather they had non-homogeneous flora. This may have occurred because the mince was analyzed immediately after purchase before a spoilage association had been established. The subtle dominance of *B. thermosphacta* is in agreement with the observations of DROSINOS and BOARD (1995a, b) who found that the *B. thermosphacta* population was high in minced lamb under

aerobic storage conditions. GILL and NEWTON (1977) also noted that the population of *B. thermosphacta* was due to initial contamination in which the pseudomonads became the dominant ones under aerobic conditions. The correlation of *Enterobacteriaceae* with APC is an index of inadequate hygienic conditions at the retail outlets. In terms of hygiene and quality assurance the results of the present survey provide a realistic basis for discussing critical control limits. With the compulsory application of HACCP systems, the training of personnel, the application of modified atmosphere packaging and the trend to apply quality management systems, the situation should be changed from practices in past decades.

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PAPER

EFFECT OF POLYDEXTROSE AND NaCl ON THE INITIAL FREEZING POINT OF CHICKEN, EGG AND SURIMI

EFFETTO DEL POLIDESTROSIO E DEL CLORURO DI SODIO SUL PUNTO INIZIALE DI CONGELAMENTO DI UOVA E CARNI DI POLLO E DEL SURIMI

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ABSTRACT

Initial freezing points (T_i) of chicken, egg (mixture of egg yolk and white, egg yolk, egg white) and surimi mixed with different mass fractions of polydextrose (w = 1-10%) and NaCl (w = 1-10%) were determined by using differential thermal analysis (DTA). Relationships between the decrease of T_i as a function of mass fractions (w) of the added substances were determined by linear regression analysis. The greatest effect on freezing point depression was exhibited by egg yolk mixed with NaCl (from -

RIASSUNTO

Il punto iniziale di congelamento (T_i) di carne e di uova (intere o frazionate) di pollo, e di surimi, quando mescolate con diverse quantità di polidestrosio (w = 1-10%) e di NaCl (w =1-10%) è stato misurato per mezzo dell'analisi termica differenziale (DTA). Utilizzando l'analisi di regressione lineare, sono state valutate le interrelazioni tra la diminuzione di T_i e la frazione di massa (w) delle sostanze aggiunte. L'effetto più importante sul punto di congelamento è stato verificato sul rosso d'uovo me-

- Key words: chicken, egg, initial freezing point, NaCl, polydexstrose, surimi -

0.59° to -18.77°C), and the least effect was exhibited by egg mixture with polydextrose (from -0.40° to -0.49°C). Two multivariable regression models based on mass fractions of protein and fat content and the added substances predicted T_i with coefficients of determination (R^2) of 0.96 and 0.98. There were differences in the T_i values for samples of chicken, surimi, egg yolk, egg white, egg mixture and water solution of polydextrose, as a function of the mass fraction of polydextrose calculated on the total mass of water. These findings support the assumption that polydextrose interacts with proteins in egg, chicken and surimi, resulting in an increase in the mass fraction of bound water, which depresses T_i. The negligible interaction of NaCl and protein can be explained by the same argument. The results are compared and discussed with the Pham model for prediction of T_i.

scolato con NaCl (da -0,59° a -18,77°C), mentre il meno importante è stato quello sull'uovo intero con polidestrosio (da -0,40° a -0,49°C). Due modelli di regressione multivariabile, basati sulla frazione di massa del contenuto in proteina, in grasso e sulla sostanza aggiunta, hanno portato alla previsione di coefficienti di determinazione (R^2) di 0,96 e 0,98. Sono state notate differenze, tra i valori di T_i di tutti i campioni, legate alla frazione di massa del polidestrosio, se calcolata sulla massa totale di acqua. Queste evidenze rafforzano l'ipotesi che il polidestrosio interreagisce con le proteine dell'uovo, della carne di pollo e del surimi, dando luogo ad un aumento della frazione di massa di acqua legata, ciò deprime il valore di T_i. La trascurabile interazione del NaCl e delle proteine può essere giustificata nello stesso modo. I risultati sono stati messi a confronto e discussi con il modello di Pham per la predizione di T_i.

INTRODUCTION

Thermal properties of food are important for determining process parameters, designing process units and developing technology, and for the efficient performance of unit operations with heat transfer. Most mathematical models for predicting thermal properties of frozen food are based on the equation for freezing point depression, T_i (HELDMAN, 1982; SCHWARTZBERG, 1976; CHANG and TAO, 1981; CHEN, 1985; 1986, FIKIIN, 1998, MILES *et al.*, 1997; PHAM, 1996) which states that, $\Delta T = K_k \cdot \gamma$, where ΔT is the temperature decrease, K_k is the cryoscopic constant, and γ is molality. Since colligative properties depend upon the number of particles in solution, a one molal solution of a strong electrolyte (NaCl), which dissociates completely in water, lowers the freezing point more than a one molal solution of a non-electrolyte (sucrose). The freezing point of a one molal solution of NaCl is actually -3.37°C, only 1.81 times that of a non-electrolyte, not the -3.62°C that would be expected if NaCl were completely dissociated (HOLTZCLAW *et al.*, 1980).

The initial freezing point is one of the related properties which also includes: boiling point, osmotic pressure and water activity. The lower the initial freezing point, the more microbiologically stable the food, the lower the water activity, the higher its boiling point and the slower the ice content increases as the temperature is lowered (MILES *et al.*, 1997). The experimental methods most frequently used to determine T_i are differential scanning calorimetry (DSC) and differential thermal analysis (DTA)

which are methods based on changes of apparent enthalpy (FINDLAY and BARBUT, 1990).

To protect miofibilar proteins from freeze-denaturation during frozen storage, cryoprotectants are generally added (PARK *et al.*, 1996). The most effective cryoprotectants for myofibrillar proteins are carbohydrates, such a sucrose, sorbitol, maltodextrins and polydextrose (TOMANIAK *et al.*, 1998). The use of non-sweet bulking additives and low sweet additives as a cryoprotectant in muscle foods (e.g. palatinit, lactitol and polydextrose) have been patented (LANIER and AKAHANE, 1986). Polydextrose is a highly branched polysaccharide prepared by the thermal polymerization of glucose. The cryoprotective effects of polydextrose may be attributed to the numerous hydroxyl groups available for hydrogen bonding with proteins, leading to increased protein hydration, reduced surface tension of water and decreased aggregation (denaturation) (SMOLINSKA *et al.*, 1995; PARK *et al.*, 1993). Other possible mechanisms such as hydrophobic interaction effects reduction of the quantity of frozen water (increase mass fractions bound or unfreezable water) and reduction of solute concentration might also be implicated (SYCH *et al.*, 1990). WANG and KOLBE (1990, 1991) also showed that an increase in the mass fraction of the cryoprotectant (mixture of sorbitol and sucrose) increased the mass fraction of bound (unfreezable) water and lowered the initial freezing point, T_i, which decreased the thermal conductivity and increased the apparent specific enthalpy.

In this work, laboratory design of DTA apparatus was applied in order to determine the relative effects of different mass fractions of polydextrose and NaCl on the T_i of samples of chicken, egg (mixture of egg yolk and white, egg yolk, egg white) and surimi. Samples were selected that had globular (egg) and miofibilar (surimi) protein structures in order to experimentally determine their effects on freezing point depression.

MATERIALS AND METHODS

Sample preparation and treatment

Samples of surimi were prepared in the laboratory from Adriatic pilchard (*Sardina pilchardus*) according to the technique by LEE (1984). The samples were divided into two groups and each group was mixed, with polydextrose and NaCl in different mass ratios. The mass fractions, ranged from 0 to 10%, determined as percent of total mass. The moisture and ash content values were determined according to the official method of AOAC (1980) for meat products before NaCl and polydextrose were added. Total protein was determined on 1 g samples by the Kjeldahl method; (Kjeltec System, model 1002 Distilling Unit, Tecator Inc., Boulder, CO, U.S.A.). Samples were packaged in polyethylene bags and quickly frozen in liquid nitrogen and stored at -25°C. Average storage time was 1 week before DTA experiments.

Samples of egg were prepared from two-day-old chicken eggs produced on an industrial farm. The samples were divided into three groups (egg yolk, egg white, mixture of egg yolk and white), and each was mixed with polydextrose and NaCl in different mass ratios. The mass fractions ranged from 0 to 10%, determined as percent of total mass. The moisture and ash content values were determined according to the official method of AOAC (1980) for egg and egg products before NaCl and polydextrose were added. The samples were packaged in polyethylene bags and quickly frozen in liquid nitrogen and stored at -25°C. Average storage time was 1 day before DTA experiments.

Samples of chicken were prepared

from 7-week-old chickens produced on an industrial farm. The samples, prepared from skinless, manually deboned chicken breast were mechanically minced twice through a 3 mm plate of a grinding machine. The samples were divided into two groups, one group was mixed with polydextrose and the other with NaCl in different mass ratios. The mass fractions ranged from 0 to 10%, determined as percent of total mass. The moisture and ash content values were determined as described for surimi above before the addition of polydextrose and NaCl. The samples were packaged in polyethylene bags and quickly frozen in liquid nitrogen and stored at -25°C. Average storage time was 2 weeks before DTA experiments. Two groups of water solution samples were prepared: a) water solutions with polydextrose, and b) water solutions with NaCl. The mass fractions ranged from 0 to 15%, determined as percent of total mass.

Thermal analysis

The DTA measurements of the water solution samples with polydextrose and NaCl, and the samples of chicken, surimi, egg mixture, egg white, and egg yolk mixed with polydextrose and NaCl were conducted in a temperature range of -25° to 5°C. The DTA apparatus was constructed in the laboratory (KOVAČEVIĆ and KURTANJEK, 1993) and was used to measure the initial freezing point, T_i. Thermocouples were made from Alumel-Chromel wire (0.07 mm diameter). The thermocouples were calibrated using a standard platinum resistance thermometer, Pt-100 (RIDDLE *et al.*, 1976) in the temperature range of -30° to 25°C. The instruments were interfaced with a standard PC and a sampling rate of 3.5 kHz was used. All data were prefiltered with a $\pm 3\sigma$ rule for noise rejection prior to data analysis (MENDENHALL and SINCHIC, 1988). From statistical analysis of the measurement signal, the calibration error of 50 mK and sensitivity of 10 mK were estimated. An aqueous solution of $CaCl_2$, $w(CaCl_2) = 30\%$, was used as reference substance for DTA measurement. Distilled water was used as calibration substance for the static correction of the initial freezing point. Data were statistically evaluated by application software STATISTICA v. 6.0, StatSoft, Inc., Tulsa, OK, USA.

RESULTS AND DISCUSSION

The moisture, ash and total protein content values of the chicken, surimi and egg samples are presented in Table 1. The results of DTA for surimi samples mixed with NaCl are presented in Fig. 1. Systematic shifts were observed in the initial freezing points toward lower temperatures in samples with increased concentrations of NaCl. Similar trends were observed with other samples. The DTA curves have a low level of noise, which is the result of filtering the statistical data, rejecting of outliers by the $\pm 3\sigma$ rule (MENDENHALL and SINCHIC, 1988), and recording a large number of data at a high frequency of data sampling. Drift from the base line in the temperature range of 0° to 0.1°C was due to small differences in the thermal properties of the samples and the reference substance. In the DTA diagrams, the peak

Table 1 - Moisture, ash and total protein content of the samples.

Samples	Mass fraction *w* (%)		
	Moisture content	Total protein	Ash
Surimi	80.90	17.10	0.45
Egg white	87.99	11.01	0.71
Egg yolk	52.56	17.60	1.30
Egg mixture	65.82	12.11	0.95
Chicken	74.56	22.76	1.07

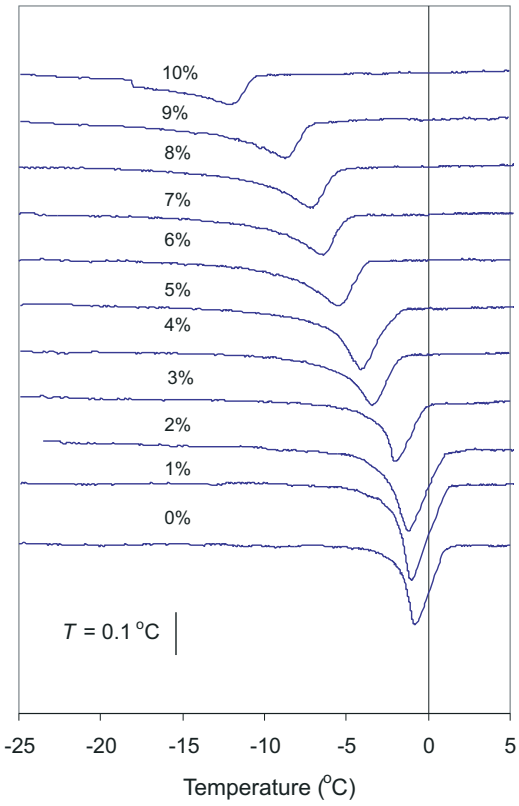


Fig. 1 - DTA curves of surimi as a function of the mass fraction of NaCl. ΔT is the temperature difference between sample and reference substance.

points were read as the initial freezing points. Data for T_i are given in Table 2.

Using the method of linear correlation of T_i with the mass fraction of the added substances, the parameters, a and b, of the following regression equation were determined:

$$T_i = a + b \cdot w \qquad (1)$$

Parameters of the regression models (eq. 1) are given in Table 3. The difference in the slopes of the lines is in accordance with the equation of the freezing point depression which is inversely proportional to the molecular mass of dissolved substances. The greatest effect of freezing point depression was exhibited by the samples with NaCl.

Comparison of T_i values of different samples with the same mass fraction of NaCl showed decreasing freezing point depression values in the following order: egg yolk, surimi, chicken, egg mixture, and egg white. The polydextrose freezing point depression decreased in the following order: chicken, surimi, egg yolk, egg white and egg mixture. The results confirm the assumption that T_i is not just a function of mass fractions of dry matter. It can be assumed that T_i is determined

Table 2 - Initial freezing point, T_i, of chicken, egg white, egg yolk, egg mixture and surimi.

Mass fractions of added substances w (%)	T_i (°C)									
	Chicken + NaCl	Chicken + polydextrose	Egg white + NaCl	Egg white + polydextrose	Egg yolk + NaCl	Egg yolk + polydextrose	Egg mixture + NaCl	Egg mixture + polydextrose	Surimi + NaCl	Surimi + polydextrose
0	- 0.99	-0.99	-0.51	-0.51	-0.52	-0.52	-0.40	-0.40	-0.19	-0.28
1	-1.26	-1.34	-1.57	-0.52	-1.00	-0.55	-1.94	-0.42	-1.02	-0.34
2	-3.18	-1.14	-2.00	-0.49	-3.01	-0.55	-2.66	-0.44	-2.01	-0.36
3	-4.16	-1.23	-3.20	-0.49	-5.04	-0.53	-4.50	-0.41	-2.52	-0.38
4	-4.10	-1.24	-4.10	-0.51	-6.80	-0.55	-4.18	-0.43	-3.48	-0.39
5	-5.56	-1.27	-4.20	-0.51	-8.13	-0.57	-4.80	-0.44	-4.08	-0.49
6	-6.56	-1.33	-4.85	-0.53	-7.65	-0.56	-5.30	-0.44	-5.45	-0.50
7	-7.96	-1.44	-5.40	-0.53	-11.54	-0.57	-6.46	-0.44	-6.40	-0.51
8	-9.04	-1.66	-5.87	-0.54	-12.01	-0.58	-7.22	-0.46	-7.05	-0.58
9	-9.23	-1.62	-6.79	-0.57	-14.96	-0.60	-7.98	-0.45	-8.68	-0.69
10	-9.62	-1.72	-7.37	-0.56	-18.77	-0.57	-8.74	-0.49	-11.11	-0.67

Table 3 - Parameters for the regression model (eq. 1) for initial freezing point, T_i.

Samples	Added substances (w = 1-10%)	*a*	*b*	*e(T)*	R^2
Surimi	NaCl	0.2725	-1.0007	0.0591	0.97
	polydextrose	-0.2824	-0.0367	0.00243	0.95
Egg white	NaCl	-0.8991	-0.6540	0.02719	0.98
	polydextrose	-0.4809	-0.0078	0.00130	0.80
Egg yolk	NaCl	0.4127	-1.7086	0.10985	0.96
	polydextrose	-0.0057	-0.5305	0.00122	0.71
Egg mixture	NaCl	-1.0809	-0.7689	0.04362	0.97
	polydextrose	-0.4041	-0.0066	0.00108	0.81
Chicken	NaCl	-0.9386	-0.9334	0.04608	0.98
	polydextrose	-1.0509	-0.06218	0.00976	0.81
Water	NaCl	0.0574	-0.67373	0.009744	0.99
	polydextrose	0.0109	-0.0391	0.001318	0.98

by mass fraction, the types of added substances and proteins, and the intensity of the interactions between water and NaCl (polydextrose) and proteins. NaCl decreases the initial freezing point more strongly than polydextrose, which is a consequence of the colligative properties

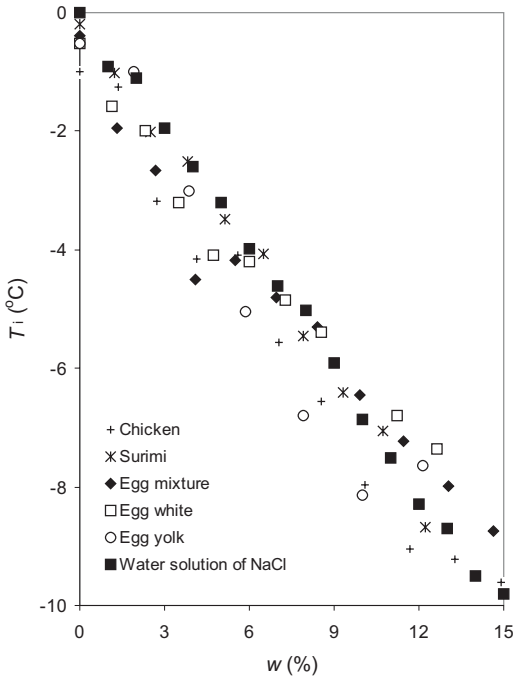


Fig. 2 - Dependency of T_i for samples, and water solution of NaCl on the mass fraction, w, of NaCl calculated on total mass of water.

(small molecular mass and the effect of dissociation) of NaCl and corresponds to the equation for T_i, $\Delta T = K_k \cdot \gamma$, (HOLTZCLAW *et al.*, 1980).

The dependency of T_i for chicken, surimi, egg yolk, egg white, egg mixture, and water solution of NaCl on the mass fraction of NaCl, calculated on total mass of water, is presented in Fig. 2. The good correlation for T_i and water can be attributed to a weak interaction between water and NaCl and the dry matter of chicken, surimi and egg. Statistical evaluation of the results gave an adjusted coefficient of correlation (R^2) of 0.942, standard error of predicted variable 0.267°C and Fisher ratio (F) of 1105.5 with degrees of freedom (1.67).

The dependency of T_i for chicken, surimi, egg yolk, egg white, egg mixture, and water solution of polydextrose on the mass fraction, w, of polydextrose, calculated on total mass of water, is presented in Fig. 3. Deviations of initial freezing points T_i confirm that polydextrose acts in accordance with the cryoprotecting mechanism and interacts with protein in chicken meat, surimi and egg, and therefore increases the amount of bound water and decreases the initial freezing point.

The results of multivariate linear regression models based on mass fractions of protein (w_P), fat (w_F), polydextrose

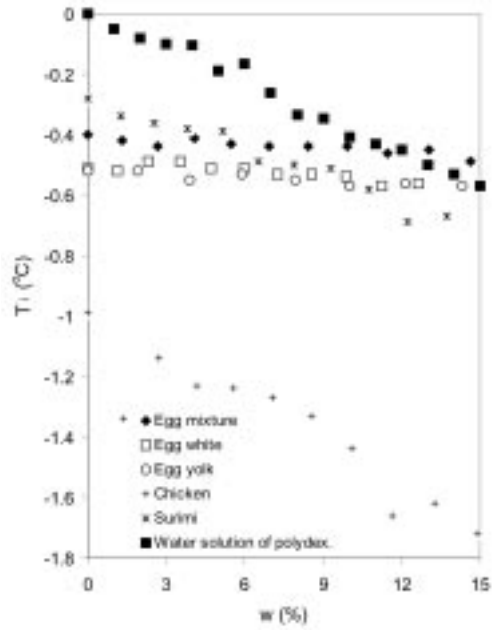


Fig. 3 - Dependency of T_i for samples, and water solution of polydextrose on the mass fraction, w, of NaCl calculated on total mass of water.

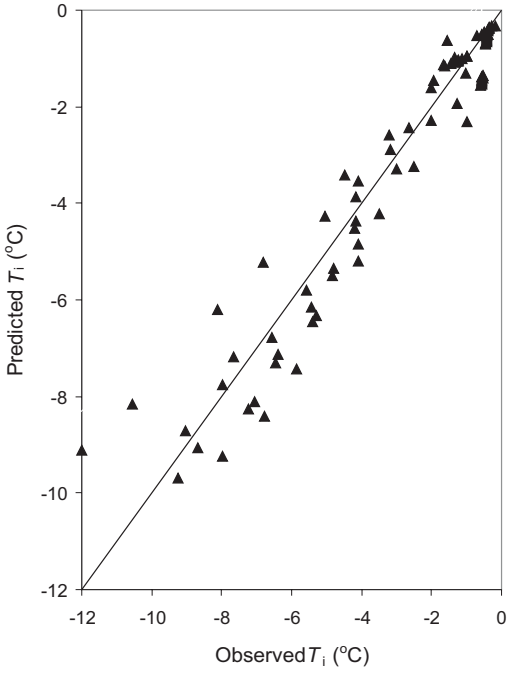


Fig. 4 - Multivariable linear regression model (eq. 2) for predicting T_i based on mass fractions of protein, NaCl and polydextrose.

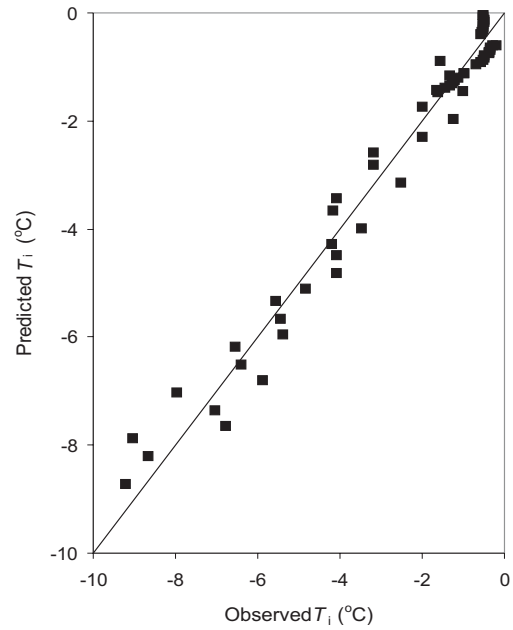


Fig. 5 - Multivariable linear regression model (eq. 3) for predicting T_i based on mass fractions of protein, NaCl, polydextrose and fat.

(w_{PD}), and NaCl (w_{NaCl}) are presented in Figs. 4 and 5. The multivariate regression is based on 100 different samples; the data are given in Table 2. For the samples with negligible fat (surimi, egg white, chicken) the following multivariable regression model was obtained:

$$T_i = b_0 + b_1 \cdot w_P + b_2 \cdot w_{NaCl} + b_3 \cdot w_{PD} \quad (2)$$

while for the samples with a considerable amount of fat, the following regression model was used:

$$T_i = c_0 + c_1 \cdot w_P + c_2 \cdot w_{NaCl} + c_3 \cdot w_{PD} + c_4 \cdot w_F \quad (3)$$

where b_0, b_1, b_2, b_3, c_0, c_1, c_2, c_3, and c_4 are coefficients in the multivariable regression model. The content of fat in the egg yolk samples was 27.1% and was determined experimentally according to AOAC (1980) for egg and egg products before polydextrose and NaCl were added. The respective coefficients of deter-

Table 4 - Parameters for the regression models eq. 2 and eq. 3 for initial freezing point, T_i.

Samples with negligible fat (eq. 2)					
b_0	b_1	b_2	b_3	$e(T_i)$	R^2
1.1	-0.0963	-0.9203	-0.06428	0.17	0.98
Samples with considerable amount of fat (eq. 3)					
c_0	c_1	c_2	c_3	c_4	$e(T_i)$	R^2
1.713	-0.115	-1.065	-0.055	-0.034	0.41	0.96

mination for the two models are R^2 = 0.98 and 0.96. The parameter and error estimates for regression models (2) and (3) are given in Table 4. The effects of the individual components (protein, NaCl, polydextrose, fat) on T_i of the samples are determined from the values of the corresponding model parameters. The most pronounced effect was noted in samples with NaCl; a 1% increase in the mass fraction decreased T_i by -1.065°C (Table 4).

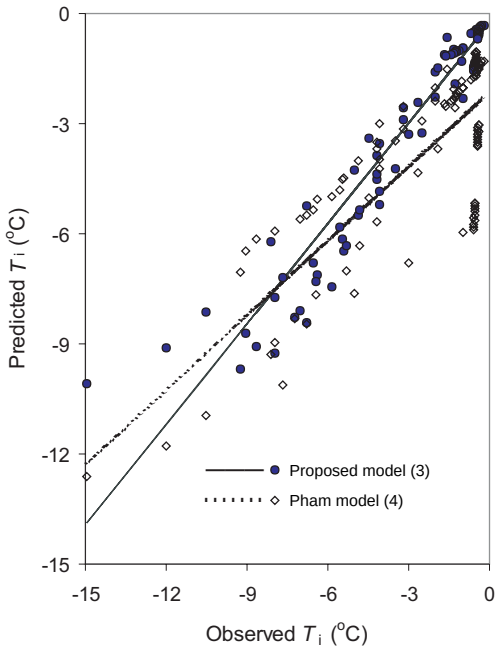


Fig. 6 - Comparison of the observed T_i values and those predicted by the Pham model (4) and the proposed multivariabile regression model (eq. 3).

The observed values of T_i are presented in Fig. 6 along with the values predicted by the Pham model (PHAM, 1994), given by the regression equation with respect to the mass fraction of water (w_w), ash (w_a), and other components (w_o):

$$T_i = -4.66\ w_o / w_w - 46.4\ w_a / w_w \quad (4)$$

The results predicted by model (3), proposed in this work, are also presented in Fig. 6. The two models can be statistically compared based on the values of the adjusted coefficients of determination. The Pham model yields R^2 = 0.90, while the proposed model gives more accurate predictions with R^2 = 0.96. One advantage of the Pham model is its simplicity; it requires only data for the water, ash and other mass fraction components of the sample. In the proposed and more accurate model, detailed data about the analytical composition, of the food is required.

CONCLUSIONS

Freezing point depression of chicken, surimi, and egg (egg yolk, egg white, egg mixture) is a linear function of the increased mass fraction of NaCl and polydextrose. For samples with equal mass fractions of added substances the greatest depression of T_i occurred when NaCl was added.

The maximum decrease of the initial freezing point was observed with egg yolk mixed with NaCl (from -0.59° to -18.77°C), while the least effect was observed with the egg mixture with polydextrose (from -0.40° to -0.49°C).

The T_i values for samples of chicken, surimi, egg yolk, egg white, egg mixture, and water solution of polydextrose as a function the mass fraction of polydextrose calculated on total mass of water were different for all the samples. The results support the assumption that polydextrose interacts with the protein

of egg, chicken and surimi, increases the mass fraction of bound water and lowers T_i. The NaCl and protein interaction is negligible and can be explained by the same argument.

A comparison of the Pham model for T_i with the model proposed in this work, favors the applicability of the Pham model for samples (surimi, chicken and egg) with added NaCl and polydextrose. The proposed model gives more accurate predictions, but it requires more detailed data about the analytical composition of the food.

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PAPER

INACTIVATION OF MICROORGANISMS BY SUPERCRITICAL CO_2 IN A SEMI-CONTINUOUS PROCESS

INATTIVAZIONE MICROBICA MEDIANTE CO_2 SUPERCRITICA IN UN PROCESSO SEMI-CONTINUO

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ABSTRACT

The anti-microbial effect of CO_2 in a semi-continuous experimental set-up was investigated and different values of temperature, pressure and treatment time were applied. Microorganisms were suspended in a phosphate buffer solution and inactivated under a continuous flow of supercritical CO_2 (SC-CO_2). Three microorganisms with different features were treated as models: *S. cerevisiae*, *B. subtilis* and *S. marcescens*. Exposure of the cells to SC-CO_2 was investigated and the mass transfer and

RIASSUNTO

In questo lavoro è stato studiato l'effetto della CO_2 supercritica (SC-CO_2) sull'inattivazione microbica in un processo semi-continuo per diversi valori di temperatura, pressione e tempo di trattamento. Tre specie microbiche (*S. cerevisiae*, *B. subtilis* e *S. marcescens*) sono state sospese in una soluzione tampone e inattivate mediante un flusso continuo di SC-CO_2. È stato preso in considerazione e approfondito lo studio del trasporto di materia e della termodinamica del sistema acqua-CO_2 nel

- Key words: bacteria, high pressure, pasteurization, supercritical fluid, yeast -

thermodynamics of a CO_2-water system were studied by theoretical modeling. Results confirmed that the microorganisms considered were sensitive to the SC-CO_2 treatment at mild temperatures and, showed that the semi-continuous experimental set-up is much more efficient than a batch one. The development of SC-CO_2 pasteurization units for heat-sensitive foodstuff at the industrial level is proposed.

processo semicontinuo. I risultati, sia sperimentali sia teorici, confermano che già a bassa temperatura i microrganismi considerati sono sensibili alla SC-CO_2 e dimostrano che un processo semicontinuo risulta più efficiente di un processo batch. Pertanto, la tecnica proposta in questo lavoro si presta molto bene allo sviluppo industriale della pastorizzazione di sostanze termosensibili mediate SC-CO_2.

INTRODUCTION

Ultra high temperature (UHT) treatment is still the most widely used technique in the food industry to microbiologically stabilize foodstuffs. However, it has some disadvantages related to the high temperatures used, such as the thermal denaturation of sensitive substances and the formation of toxic compounds as by-products.

Among the alternative non-thermal processes, high-pressure treatment can play a fundamental role as a novel food pasteurization technique. High hydrostatic pressure treatment (HPT) has been studied the most (JOHNSON and ZOBELL, 1949; CLOUSTON and WILLIS, 1970; LUDWIG *et al.*, 1994; SPILIMBERGO and BERTUCCO, 2001) and a number of processing units using HPT have already been marketed. The main limitation of this technology is that it is very expensive because of the extremely high pressure used.

Recently, an alternative high pressure technique based on the use of supercritical CO_2 (SC-CO_2) has been proposed (SPILIMBERGO and BERTUCCO, 2001). The effect of SC-CO_2 on microorganisms has been known for some time (QUIRIN, 1988). The first specific study of inactivating microorganisms by CO_2 treatment was published by KAMIHIRA *et al.* (1987). Supercritical or near critical CO_2 to reduce microbial activity has been used with promising results (BALLESTRA *et al.*, 1996; ENOMOTO *et al.*, 1997; ISENSCHMID *et al.*, 1995). ISHIKAWA *et al.* (1995) applied CO_2 in a semi-continuous reactor (the so-called "micro-bubble" method) and achieved greater efficiency than in the batch device. SMELT and RIJKE (1992) demonstrated that the effect of CO_2 at supercritical conditions on microbial inactivation assures microbiologically safe and stable food preservation at pressures much lower than those employed by the traditional HPT method. Moreover, the microbial activity at near-ambient temperatures is reduced, thereby preserving the organoleptic features of the treated materials.

The importance of SC-CO_2 pasteurization can be noted in the increased number of industrial applications and patents granted, concerning the efficiency of CO_2 in treating materials (DILLOW *et al.*, 2000) or use of supercritical CO_2 for viral inactivation (CASTOR *et al.*, 1999). More recently, ERKMEN (2000a, b, c) has published a number of papers dealing with the antimicrobial effect of pressurized CO_2 in a batch system on a wide range of bacteria. Kinetic studies have clearly shown that the rate of reduction in all the species treated was sensitive to pressure, temperature and exposure time (ERKMEN 2001a; ERKMEN

and KARAMAN 2001). HONG *et al.* (1999) found similar results and showed that the efficiency of microbial inactivation was mainly due to the transfer rate of CO_2 into the cells. Mathematical modeling of *E. coli* and *L. monocytogenes* inactivation under high-pressure allowed the microbial inactivation due to exposure to different CO_2 pressures and temperatures to be predicted (ERKMEN 2000d; 2001b). The influence of the dissolved CO_2 concentration on the death kinetics of *S. cerevisiae* was investigated by SHIMODA *et al.* (2001) using a plug-flow system.

Although many papers can be found in the literature, the exact mechanism of CO_2 antimicrobial effect is not yet fully understood. It is clear that bacterial inactivation is made possible due to the CO_2 dissolution in the liquid phase; the experimental set-up and the operation mode are therefore key parameters for the successful development of this technique.

The aim of this study was to confirm the enhanced CO_2 mass transfer in the liquid phase in a semi-continuous device compared to a batch system. The microbial reduction of a suspension of Baker's yeast (*S. cerevisiae*), *S. marcescens* and the vegetative form of *B. subtilis* was investigated in a semi-continuous process. In addition, the influence of the operating variables in both processes (batch and semi-continuous) was studied using a simplified model of the CO_2-water system, addressing both thermodynamics and mass transfer issues. The experimental and theoretical results confirm the enormous efficiency of SC-CO_2 as a tool for reducing microbial activity by treating different microorganisms at suitable operating parameters in a semi-continuous apparatus. Even if the inactivation mechanisms are not fully understood, the results reported in this work support the industrial application of this technique for treating foodstuff.

MATERIALS AND METHODS

Bacterial cultures

A sourdough yeast strain, *Saccharomyces cerevisiae* ATCC (American Type Culture Collection) 8100, and two bacterial species *Serratia marcescens*, ATCC 9763 and *Bacillus subtilis* ATCC 6633 were used as test microorganisms. Starter cultures of each strain were initiated by inoculating a single strain in a specific medium at its optimum incubation conditions: *S. cerevisiae* was grown in yeast peptone dextrose (YPD), a water solution of: 10 g/L yeast extract [Fluka, Milan, Italy], 10 g/L peptone [Oxoid, Basingstoke, Hampshire, UK], 20 g/L dextrose [R.P. Normapur AR-Prolabo, Fontenays S/Bois, France] at 28°C for 48 h, while *S. marcescens* and *B. subtilis* were grown in 37 g/L of Heart Brain Infusion (HBI) [Sigma, Milan, Italy] at 37°C for 24 h. The cultures were then stored in a refrigerator and refreshed at least 12 h before carrying out each experiment; this procedure assures that the cells are in their stationary growth phase, which has been demonstrated to be the most resistant to inactivation. Before the experiment, the cultures were suspended in a sterile phosphate buffer solution (PBS), at pH 7.4, consisting of 8 g/L NaCl [R.P. Normapur, AR-Prolabo, Fontenays S/Bois, France], 0.2 g/L KCl, 1 g/L Na_2HPO_4 [J.T. Baker, Deventer, Holland], 0.6 g/L KH_2PO_4 [Carlo Erba, Milan, Italy], which was diluted to reach about 10^7 CFU/mL for bacteria and 10^5 CFU/mL for yeasts.

If not indicated otherwise all the above-mentioned chemicals were purchased from Sigma [Milan, Italy].

Pasteurization equipment

The experiments were performed in the semi-continuous apparatus shown in Fig. 1. The liquid CO_2 (99.95% purity, Air Liquid, Italy) is continuously fed

into a high-pressure vessel by a HPLC pump (Rainin Dynamax, mod SD-200) at a maximum flow rate of 25 mL/min. The high-pressure vessel (V) consists of a 30 mL cylinder with a 5 µm porous metallic filter (frit) and two screwed taps. They are equipped with one cavity where a small amount of bacteriological cotton is placed in order to avoid contamination by microorganisms in the environment. The frit allows the atomization of the CO_2 flow in micro-bubbles but it does not allow the liquid to drain.

The thermally insulated vessel is equipped with a resistance temperature

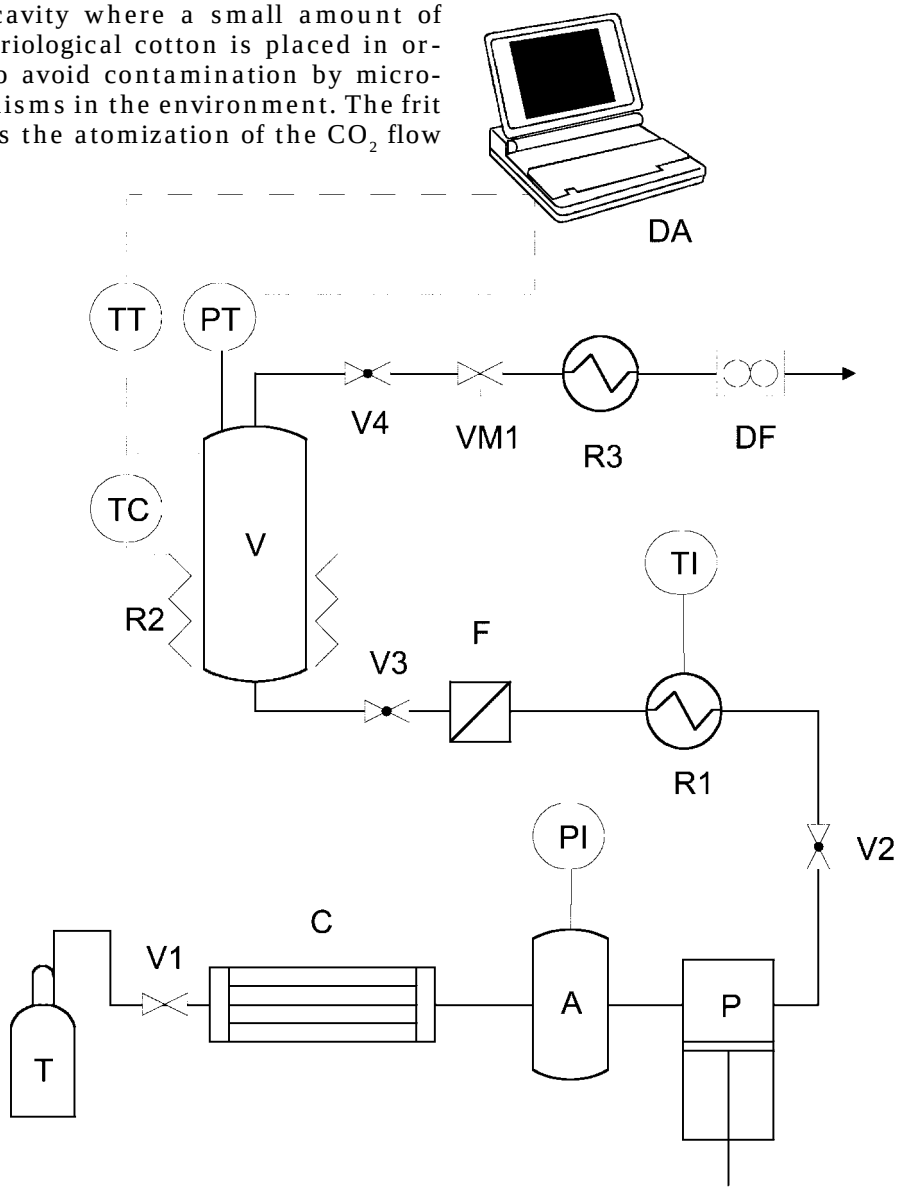


Fig. 1 - Scheme of the semi-continuous apparatus: T, CO_2 tank; C, condenser; A, CO_2 reservoir; P, CO_2 pump; F, micrometric filter; V, sterilization vessel; DF, dry flowmeter; V1, V2, V3, V4, on-off valves; VM1, micrometric valve; R1, R2, R3, electrical resistances; TT, temperature transmitter; PT pressure transmitter; TC, temperature controller; DA, data acquisition system.

indicator, Pt 100Ω, located inside, a temperature on-off control and an electric resistance (R2). The outlet micrometric valve VM1, that regulates the CO_2 pressure in the autoclave, is heated by another resistance (R3) to prevent freezing during expansion. Pressure and temperature are continuously recorded by a real time acquisition data system (NASAQ, National Instruments) and monitored by specific software (LabView™).

Pasteurization procedure

In the high-pressure vessel 25 mL of suspended solution were always used. The process involved three steps: pressurization of the vessel with CO_2 up to the operating pressure *P* and temperature *T*, treatment at these steady conditions for a selected time and depressurization of CO_2. The temperature was set at 38°-40°C, which was above the critical temperature of CO_2, but below the value that leads to protein or vitamin denaturation; the pressure was set between 5.5 and 8.0 MPa. A treatment time ranging from 0-30 min was chosen to achieve complete pasteurization of the initial microbial counts. After the CO_2 treatment was completed, the sample was collected from the vessel at atmospheric pressure and the residual viable microorganisms were counted.

Plate counting

All samples were successively diluted in the PBS and plated on Petri dishes containing an appropriate nutrient agar, Heart Brain Agar (HBA) [Sigma, Milan, Italy] (same composition as the starter cultures described above but with 15 g/L of agar added). Before counting the colonies according to standard plate procedures (SPECK, 1976) the plates were incubated at their optimal conditions (*S. cerevisiae* at 28°C for 48 h, *S. marcescens* and *B. subtilis* at 37°C for 24 h). The microbial counts were checked to see if the counts were the same after 24 h as after one week of incubation time. The results are expressed as N/N_o versus time, where N_o is the initial microbial count in the control sample and N is the microbial count in the CO_2-treated sample. All the values are the arithmetic means of N/N_o among three different runs.

RESULTS AND DISCUSSION

Since the parameters that influence the efficiency of microbial inactivation in CO_2 treatment are mainly pressure, temperature and processing time, pressure and time were varied to measure the differences in the penetration of CO_2 into the cells. Pressure is considered the most relevant parameter in this study as it controls both the mass transfer of CO_2 and its solubility in the suspending medium.

Table 1 gives a summary of the results. Data marked with 0 mean that no colony forming units (CFU) were detected at the corresponding conditions. From the data it is clear that complete pasteurization was obtained for all the microorganisms investigated, at suitable pressures, temperatures and exposure times.

Under a pressure of 7.4 MPa, *S. cerevisiae* was inactivated in 10 min, while inactivation was not yet complete after 7.5 min. Under a pressure of 8.0 MPa, 7.5 min were sufficient to inactivate the yeast completely.

As regards the bacterial species, the gram-positive bacteria *B. subtilis*, as well the gram-negative bacteria *S. marcescens* were completely inactivated at an operating pressure of 7.4 MPa and at an exposure time in the steady-state of 2.5 and 0 min, respectively. Note that 0 min means that the vessel is pressurized to the operating pressure and immediately depressurized. Normally, 2 min are re-

Table 1 - *S. cerevisiae*, *B. subtilis* and *S. marcescens* reduction at 38°C as a function of time and pressure in a semi-continuous process. Note that the treatment time, *t*, starts to be counted when the operating pressure, *P*, is reached.

S. cerevisiae				*B. subtilis*				*S. marcescens*			
P (MPa)	*t* (min)	$[N_0]$	$[N/N_0]$	*P* (MPa)	*t* (min)	$[N_0]$	$[N/N_0]$	*P* (MPa)	*t* (min)	$[N_0]$	$[N/N_0]$
5.5	30	$5x10^5$	0	5.8	30	$2x10^7$	0	5.8	30	$5x10^7$	0
7.4	15	$6x10^5$	0	7.4	30	$2x10^7$	0	7.4	15	$3x10^7$	0
7.4	10	$6x10^5$	0	7.4	15	$2x10^7$	0	7.4	10	$5x10^7$	0
7.4	7.5	$6x10^5$	$6.3x10^{-4}$	7.4	7.5	$2x10^7$	0	7.4	7.5	$3x10^7$	0
8.0	15	$5x10^5$	0	7.4	2.5	$4x10^7$	0	7.4	0*	$2x10^7$	0
8.0	10	$5x10^5$	0	7.4	0*	$4x10^7$	$4.0x10^{-4}$				
8.07.5	$5x10^5$	0									

Valve means that no colonies were counted after the treatment. N/N_0 0 minute value of the treatment time, *t*, means that the vessel was pressurized up to the operating pressure and immediately depressurized.

quired to reach the desired operating pressure.

The results reported in Table 1 show that the two bacteria behaved differently: the gram-negative one has a thinner cell wall and therefore the CO_2 penetrated more easily than in the gram-positive bacteria, which showed a higher resistance to penetration. The results are in agreement with the fact that the main cause of microbial death is the CO_2 concentration in the liquid phase, which is higher at higher pressures and longer processing times.

The present results also clearly confirm that a semi-continuous device is more efficient than the batch one. The treatment times needed to reach complete inactivation with a batch set-up are on the order of 40-60 min for a wide range of bacteria (DILLOW *et al.*, 2000). Moreover, the results from the batch device for the vegetative form of *B. subtilis* at a temperature of 40°C and a pressure of 8.8 MPa show that complete inactivation by SC-CO_2 was reached after 30 min (ROCCHI, 2002).

In this respect, the correct experimental set-up is essential for increasing the process efficiency. The time needed for the CO_2 to reach equilibrium in a semi-continuous device can be estimated by modeling the CO_2 bubbling in the liquid phase as a function of time. The equations governing this have been written for a system of bubbles of pure CO_2 in a completely mixed aqueous liquid phase (PAULINE et al., 1995).

The CO_2 mass-transfer rate per unit volume of liquid phase ($kg\ s^{-1}\ m^3$) is expressed as:

$$F = k_l \cdot a \cdot (c_s - c) \quad (1)$$

where k_l is the CO_2 mass transfer coefficient in the liquid phase, a is the interface area per unit volume and $(c_s - c)$ the driving force decreasing with time.

In the unsteady-state condition, F is expressed as dc/dt, thus we obtain:

$$dc/(c_s - c) = k_l \cdot a \cdot dt \quad (2)$$

By integrating Eq. (2) we have:

$$c/c_s = 1 - \exp(-k_l \cdot a \cdot t) \quad (3)$$

a is calculated as:

$$a = S_b \cdot n_{bubble}/V \quad (4)$$

where S_b is the CO_2 bubble surface area, n_{bubble} is the instantaneous number of bubbles in the reactor and V is the vol-

ume of liquid in the reactor (25 mL). The diameter of a bubble has been estimated to be 0.054 cm by a dimensionless correlation (PERRY, 1984) used for bubble formation at a single orifice and in a single-bubble regime. So, S_b = 0.00916 cm^2. The residence time is defined as:

$$\tau = h / v_z \qquad (5)$$

where h is the height of the liquid phase in the reactor, while v_z is the rate of a single bubble through the liquid phase; v_z was calculated by applying the force balance on the bubble under the hypothesis of cylindrical symmetry (v_x=v_y=0 and v_z is constant through the reactor). Its value was estimated as 0.114 m s^{-1}, so that, with h = 2 cm, τ = 0.1754 s. The n_{bubble} can be determined from:

$$n_{bubble} = G \cdot \tau / v_b \qquad (6)$$

where G is the CO_2 volumetric flow rate and v_b the bubble volume. We found n_{bubble} = 887, therefore a value of 0.325 cm^{-1} was obtained for a, calculated from Eq. (4).

The mass-transfer coefficient k_l was evaluated from the Calderbank dimensionless correlation (CARBERRY and VARMA, 1987), which gives k_l = 0.0146 $cm \cdot s^{-1}$; therefore $k_l a$ = 0.0048 s^{-1}. From the integration of Eq. (1) up to c = 0.9 c_s, the time required by the gas to reach 90% of the equilibrium concentration is t = 8 min.

On the other hand, in a batch device the transport of CO_2 in the liquid phase is usually governed by diffusion only; so, the time required to reach equilibrium in the CO_2-water system is a function of the distance z from the liquid-gas surface. Assuming that the gas is diffusing through a semi-infinitive region and that the concentration c_s at the surface is constant, the differential equation of the transport model is (WALAS, 1986):

$$\partial c / \partial t = D_l \cdot \partial^2 c / \partial z^2 \qquad (7)$$

Eq. (7) provides an analytical solution:

$$c(z,t) / c_z = 1\text{-}erf(z / \sqrt{4 \cdot D_l \cdot t}) \qquad (8)$$

Several profiles of CO_2 in the liquid phase as a function of time according to Eq. (8) are reported in Fig. 2. The time required to reach equilibrium in a batch device, even at a distance of 1 cm from the interface, is in the order of hours.

Therefore, under the given assumption the dissolution of CO_2 in the liquid phase in a semi-continuous apparatus requires a contact time that is at least one order of magnitude less than in a batch device.

The pasteurization mechanism clearly involves the ability of SC-CO_2 to diffuse readily into the cell and irreversibly alters its regular metabolism and physiological equilibrium. Once CO_2 penetrates the cell, it supposedly reacts with water and forms carbonic acid that leads to a sudden decrease in the intracellular pH which probably affects the biological activities of the microbial cell (HONG and PYUN, 1999).

SC-CO_2 however, does not cause macroscopic modifications of the microbial cell. Examples of scanning electron microscope (SEM) photographs of *S. marcescens* (Figs. 3a and 3b) illustrate that the cell walls remain largely

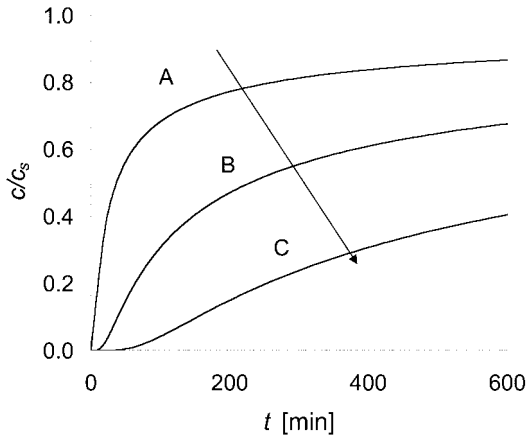


Fig. 2 - CO_2 profiles in the liquid phase, as a function of time in the batch device. Distance from the liquid-gas interface: A=0.2 cm, B=0.5 cm, C=1 cm.

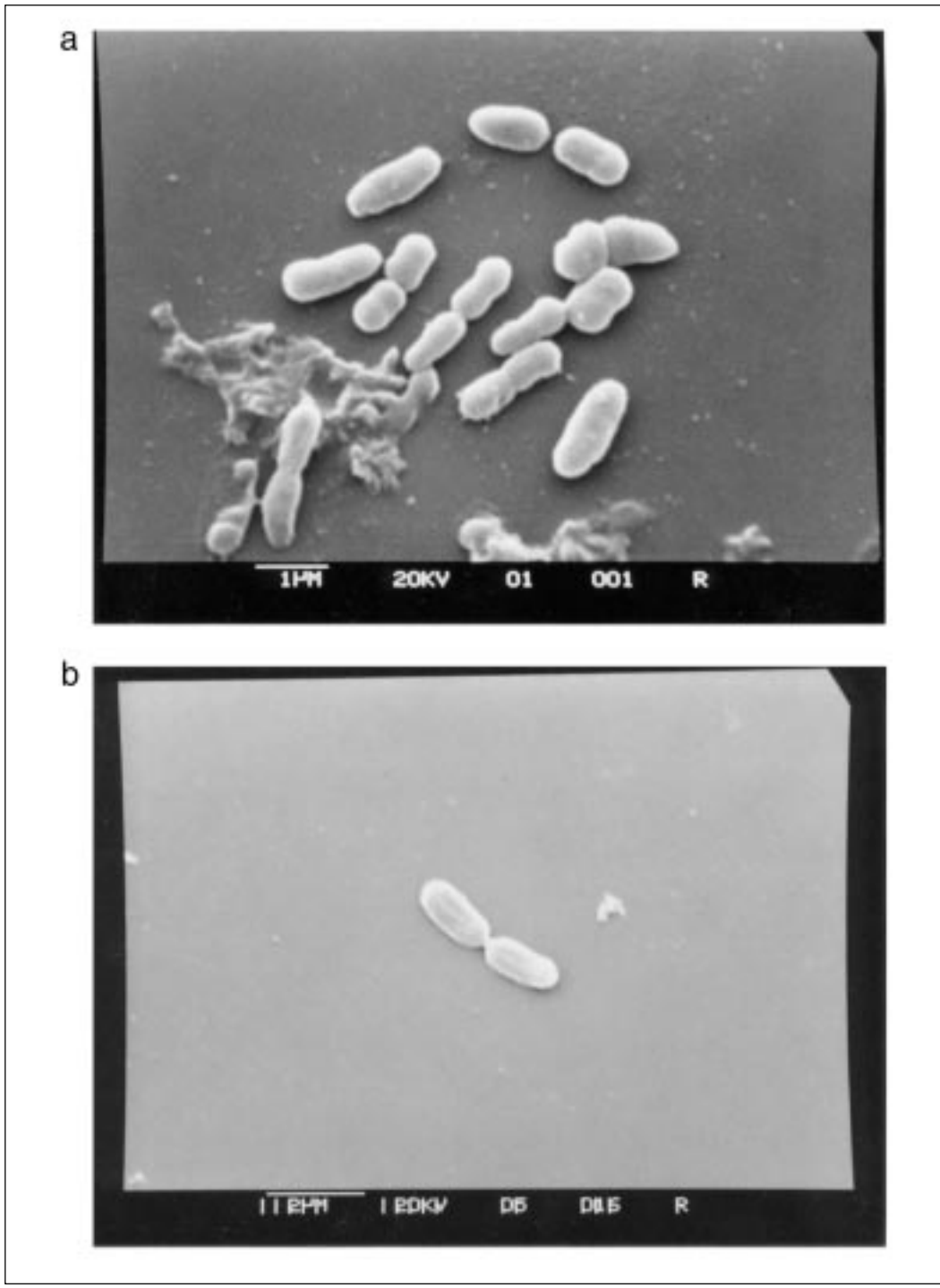


Fig. 3 - SEM micrographs (10,000x) of *S. marcescens* (a) and after (b) SC-CO_2 treatment.

unchanged after treatment with SC-CO_2. Similar results were observed for other microorganisms tested. However, HONG and PYUN (2001) reported microscopic cellular damage with the leakage of cell components into the environment that may occur during the SC-CO_2 pasteurization processes. High concentrations of CO_2 may dissolve the phospholipid bilayer of the membrane (SPILIMBERGO *et al.* 2002), greatly modifying its structure, so that it loses its integrity and disrupts its biological activities. Both pH modification and membrane damage are caused by the penetration of CO_2 into the cells, the effectiveness of which can be improved by enhancing the CO_2 mass transfer.

CONCLUSIONS

It was found that the bacteria *B. subtilis* and *S. marcescens* and the yeast *S. cerevisiae* suspended in water were completely inactivated by supercritical CO_2 when processed in a semi-continuous system under a pressure as low as 7.4 MPa with a contact time in the range of minutes.

These results are consistent with the hypothesis that the driving force of this process is the concentration of CO_2 in the liquid phase; they support the development of a simple, safe and inexpensive method for pasteurization of microorganisms in foodstuffs.

The experimental set-up proposed, appears to be suitable for processing heat-sensitive foodstuffs and for developing a SC-CO_2 pasteurization unit at the industrial level. Since longer treatment times are required to obtain a high inactivation in complex suspensions compared to simple ones (ERKMEN, 2000a), additional studies may be necessary to confirm and extend the efficiency of the proposed method to inactivate microorganisms in different substrates. Furthermore, additional experiments are needed regarding the dependence of the CO_2 solubility rate on the liquid phase and the rate of extra- and intra-cellular pH decrease in an attempt to elucidate the inactivation mechanism.

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SHORT COMMUNICATION

CHEMICAL AND MICROBIOLOGICAL CHARACTERISTICS OF EWE MILK CHEESE (LOS PEDROCHES) MADE WITH A POWDERED VEGETABLE COAGULANT OR CALF RENNET

CARATTERISTICHE CHIMICHE E MICROBIOLOGICHE
DEL FORMAGGIO "LES PEDROCHES"
OTTENUTO CON CAGLIO VEGETALE E CON CAGLIO DI VITELLO

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ABSTRACT

A powdered vegetable coagulant obtained from the flowers of cardoon (*Cynara cardunculus*) was compared with calf rennet in batches of Los Pedroches cheese, by determining different chemical, biochemical, microbiological and sensory parameters, which were monitored over 3 months of ripening. For most chemical parameters (moisture, fat, protein, lactic acid, NaCl and pH), a_w and the main groups of micro-organism (total viable, *Enterobacteriaceae*, coliforms, *E. coli*, lactic acid bacteria, moulds and yeasts) studied, no differ-

RIASSUNTO

Un caglio vegetale in polvere ottenuta dal cardo (*Cynara cardunculus*) è stato comparato con caglio del vitello in lotti di formaggio di "Los Pedroches", determinando diversi parametri analitici (chimico, biochimico, microbiologico e sensoriale) nel corso di 3 mesi di maturazione. Per la maggior parte dei parametri chimici (umidità, grasso, proteina, acido lattico, NaCl e pH), a_w e per i principali gruppi di microrganismi (conta totale, *Enterobacteriaceae*, coliformi, *E. coli*, batteri lattici, muffe e lieviti) non sono state osservate differen-

- Key words: cheese, microbiology, proteolysis, vegetable coagulant -

ences were observed between the two types of coagulants. However, higher casein hydrolysis was observed after 2 days of ripening in cheeses produced with the vegetable coagulant compared to those made with rennet. All nitrogen components (SN, NPN, AAN and NH_3N) and flavour and aroma scores were significantly higher ($P<0.05$) in the cheese obtained with powdered vegetable coagulant.

ze significative tra i due tipi di caglio. Comunque, una maggior idrolisi della caseina è stata osservata dopo 2 giorni di maturazione di formaggi ottenuti con il caglio vegetale rispetto a quelli ottenuti con caglio di vitello. Tutti i componenti azotati (SN, NPN, AAN e NH_3N) ed i punteggi sensoriali hanno dato valori significativamente più alti ($P<0.05$) nel formaggio ottenuto con caglio vegetale in polvere.

INTRODUCTION

Dried wild thistle flowers from various species of the genus *Cynara* L. are traditionally used for cheese-making in some Mediterranean countries (CARR, 1981). Their aspartic proteinases are among the few plant enzymes that have been used for this purpose (VERISSIMO *et al.*, 1996). *Cynara cardunculus* L. is used chiefly in the making of various Spanish and Portuguese cheeses (FERNÁNDEZ-SALGUERO and SANJUÁN, 1999; MACEDO *et al.*, 1993). Some cheeses from Portugal and Spain that have the Appellation d'Origine Controllée status, are produced from raw ewes' milk with only a crude aqueous extract of *C. cardunculus* flowers as coagulant. The use of these plant proteinases as milk coagulants is also of interest for producing cheeses destined for the vegetarian market (GÓMEZ *et al.*, 2001).

The proteinases can be easily extracted from the cardoon flowers, using plain water and light maceration. However, the picking, storage, handling and marketing of these cardoon flowers is not yet subject to the legal regulations governing food additives. Flowers gathered by different pickers may be a mixture of *C. cardunculus* and *C. humilis* or may even be contaminated by other cardoon species (VIOQUE *et al.*, 2000). In addition, crude aqueous extracts from *C. cardunculus* flowers were recently reported to be of poor microbiological quality (FERNÁNDEZ-SALGUERO *et al.*, 1999; GÓMEZ *et al.*, 2001; CANADA, 2001), with viable total and *Enterobacteriaceae* counts of over 5.0 log cfu/mL and with coliform counts of more than 3.5 log cfu/mL.

As an alternative to using the crude aqueous extract (CAE) from *C. cardunculus*, a powdered coagulant has been developed from CAE which is substantially free of micro-organisms. This powdered coagulant has been patented (FERNÁNDEZ-SALGUERO *et al.*, 2000). The aim of this study was to compare changes in the physicochemical, biochemical, microbiological and sensory characteristics of Los Pedroches cheese [a traditional Spanish fatty and semihard variety made from ewes' milk (FERNÁNDEZ-SALGUERO and SANJUÁN, 1999)] during ripening when manufactured with either animal rennet (chymosin) or the above-mentioned powdered vegetable coagulant.

MATERIALS AND METHODS

Cheesemaking procedure and sampling

The powdered vegetable coagulant was prepared from aqueous extracts of *C. cardunculus* by macerating *ca*. 150 g of dried flowers in 1 L of water for 24 h.

After freezing the extracts at -32°C, samples were lyophilized at a pressure between 4 and 13 Pa for roughly 24 h, as described previously (FERNÁNDEZ-SALGUERO *et al.*, 2000; TEJADA, 2001). On three different days, the ewe's milk obtained during one milking day was split into two batches; one batch was coagulated with powdered vegetable coagulant (PVC) by adding about 20 g/100 L milk and the other batch was coagulated with calf rennet by adding about 2.5 g/100 L milk (commercial calf rennet powder from Chr. Hansen; Denmark). A commercial starter culture EZAL® from Larbus S.A. (Madrid, Spain) containing a mixture of *Lactococcus lactis* subsp. *lactis*, *L. lactis* subsp. *cremoris* and *L. lactis* subsp. *lactis* var. *diacetylactis* and *Streptococcus thermophilus* was added to the milk. Each of the six experimental batches (three of them coagulated with PVC and three coagulated with rennet) was made into 5 wheels of cheese. The coagulation time was about 60 min at 29±1°C. After pressing and salting on brine, the cheeses were ripened at 11°C and 85% relative humidity and analysed at 2, 15, 30, 60, and 90 days of ripening.

Determination of chemical components

Moisture, fat, protein and lactic acid were determined by AOAC (1980) methods. Water activity (a_w), pH and nitrogen fractions, viz. soluble nitrogen (SN) at pH 4.6, non-protein nitrogen (NPN) in 12% TCA and ammonia nitrogen (NH_3N) were determined as described elsewhere (CARMONA *et al.*, 1999; VIOQUE *et al.*, 2000). Amino acid nitrogen (AAN) was determined as described by (FOLKERTSMA and FOX, 1992).

Microbiological analyses

Milk, curd and cheese samples, were analysed for different microbial groups according to APHA (1985) as follows: total viable counts were determined on plate count agar (PCA, Oxoid, Unipath Ltd. Basingstoke, UK) and incubated at 30°C for 72 h, *Enterobacteriaceae* (Gram negative and cytochromo-oxidase negative) on violet red bile glucose agar (VRBG, Oxoid) and incubated at 37°C for 24-48 h, coliforms on violet red bile agar (VRBA, Oxoid) and incubated at 37°C for 24 h, lactic acid bacteria on MRS agar in anaerobiosis and incubated a 37°C for 24 h and molds and yeasts on potato dextrose agar (PDA, Oxoid) and incubated at 26°C for 96 h. *E. coli* was analyzed as MPN according DeMAN (1983). All determinations were made in duplicate and expressed as log colony-forming units (cfu)/g sample.

Sensory evaluation

The flavour and aroma of the cheeses were assessed by 12 trained panelists on a 0-10 point scale with anchor points (from extremely mild to extremely strong). The sensory evaluation of the cheeses was carried out after 60 and 90 days of ripening.

Statistical treatment

The results obtained at the different ripening stages were subjected to an analysis of variance (ANOVA) using the SAS 6.09 program (SAS, 1990).

RESULTS AND DISCUSSION

The mean values and standard deviations for moisture, fat, protein, lactic acid and NaCl (as g/100 g cheese), different nitrogen fractions [SN, NPN, AAN and NH_3N; as g/100 g of total nitrogen (TN)] as well as pH and a_w throughout ripening (at 2, 15, 30, 60 and 90 days) of cheeses obtained with powdered vegetable coagulant (PVC) or animal rennet (AR) are shown in Table 1. Most of the chem-

ical parameters, pH and a_w were very similar in the two types of coagulants studied and in both cases most of the parameters changed significantly during ripening ($P<0.05$). Only the pH values showed no significant differences throughout ripening. The nitrogen fraction values differed markedly between the cheeses produced using the two types of coagulants. After 2 days of ripening the SN values for the cheeses made using powdered vegetable coagulant were double those of samples produced with animal rennet (Table 1). High levels of SN during the early stages of ripening, also reported by other authors for different varieties of ewes' milk cheese made with vegetable coagulant (NUÑEZ *et al.*, 1991; FERNÁNDEZ-SALGUERO and SANJUÁN, 1999; VIOQUE *et al.*, 2000), are the result of the intense proteolytic action of the enzymes in the plant coagulant, which exhibited virtually maximum activity at the pH studied (HEIMGARTNER *et al.*, 1990). At the end of ripening, the mean SN values were more than 34% of TN in cheeses made with vegetable coagulant while those made with animal rennet were only 21.6% TN ($P<0.01$). In cheese made from ewes' milk from the Serra de la Estrela region (Portugal) using animal rennet and *C. cardunculus* (SOUSA and MALCATA, 1997) the water-soluble N (WSN) levels were similar to ours; however, these authors found NPN values that were lower than ours and

Table 1 - Average values and standard deviations of chemical components (g/100 g cheese), pH, a_w and nitrogen fractions (SN, NPN, AAN and NH_3N; as g/100 g of total nitrogen) of the cheeses obtained with powdered vegetable coagulant (PVC) or animal rennet (AR) during the ripening period.

		Days of ripening				
	Batch	2	15	30	60	90
Moisture	PVC	48.42±2.08^{e}	45.62±1.80^{d}	42.63±1.25^{c}	38.34±2.50^{b}	35.41±2.01^{a}
Fat		27.55±1.11^{a}	29.08±1.33^{bc}	30.25±1.04^{cd}	32.09±2.59^{fg}	33.30±3.15^{g}
Protein		19.13±0.68^{a}	20.01±0.78^{ab}	21.21±0.88^{ab}	23.29±0.33^{c}	24.19±0.62^{de}
Lactic acid		0.86±0.04^{ab}	1.11±0.10^{c}	1.23±0.15^{d}	1.51±0.31^{e}	1.68±0.33^{f}
NaCl		0.64±0.11^{a}	1.27±0.18^{c}	1.34±0.11^{cd}	1.35±0.05^{cd}	1.56±0.10^{ef}
PH		5.10±0.03^{a}	5.09±0.03^{a}	4.99±0.13^{a}	5.03±0.18^{a}	5.02±0.14^{a}
a_w		0.990±0.003^{a}	0.978±0.002^{b}	0.971±0.002^{c}	0.959±0.004^{d}	0.947±0.003^{e}
SN		12.44±1.51^{c}	19.98±2.12^{e}	23.28±2.64^{f}	26.63±0.91^{g}	34.11±3.50^{h}
NPN		5.75±0.33^{b}	9.67±0.53^{c}	14.99±2.17^{e}	16.06±1.62^{e}	20.70± 0.84^{f}
AAN		0.43±0.09^{a}	0.81±0.05^{bc}	1.41±0.50^{d}	2.27±0.22^{e}	3.54±0.42^{g}
NH_3N		0.58± 0.05^{a}	0.91±0.16^{b}	1.17±0.22^{c}	1.27± 0.19^{d}	1.47± 0.22^{e}
Moisture	AR	47.88±2.08^{ab}	46.01±2.13^{bc}	42.87±1.41^{d}	38.76±1.18^{e}	36.41±1.41^{fg}
Fat		27.50±1.98^{a}	29.04±2.88^{ab}	30.33±2.21^{bc}	31.64±2.15^{cd}	33.77±1.86^{d}
Protein		19.40±0.41^{a}	20.27±0.69^{ab}	21.49±1.71^{bc}	22.88±0.92^{cd}	25.09±0.50^{e}
Lactic acid		0.74±0.07^{a}	0.87±0.13^{b}	1.05±0.21^{bc}	1.20±0.25^{d}	1.29±0.28d^{e}
NaCl		0.75±0.05^{b}	1.30±0.10^{c}	1.33±0.07^{cd}	1.52±0.07^{e}	1.65±0.19^{f}
pH		5.14±0.07^{a}	5.11±0.03^{a}	5.02±0.16^{a}	5.07±0.22^{a}	5.15±0.21^{a}
a_w		0.992±0.003^{a}	0.981±0.001^{b}	0.9740.003^{c}	0.970±0.004^{c}	0.953±0.004^{de}
SN		6.23±0.44^{a}	9.09±1.29^{b}	13.65±1.78^{cd}	15.58±0.51^{d}	21.57±2.75^{ef}
NPN		3.60±0.32^{a}	6.42±0.49^{b}	9.34±1.70^{c}	12.84±0.24^{d}	15.78±0.58^{e}
AAN		0.27±0.06^{a}	0.45±0.23^{ab}	1.16±0.12^{cd}	2.00±0.36^{e}	3.04±0.51^{f}
NH_3N		0.44± 0.07^{a}	0.67± 0.14^{ab}	0.87± 0.30^{b}	0.88± 0.30^{b}	1.12± 0.29^{c}

$^{a-g}$ Means of the same parameter in the same row without a common superscript differ significantly ($P<0.05$).

Table 2 - Log counts of different microbial groups in raw initial milk, curd and cheeses manufactured with powdered vegetable coagulant (PVC) or animal rennet (AR) during the ripening period.

	Days of ripening Milk	Curd	2	15	30	60	90
Total viable							
PVC	5.50±0.53^{a}	7.09±0.36^{b}	9.09±0.21^{de}	9.06±0.30^{de}	8.85±0.45^{de}	8.81±0.49^{de}	8.63±0.28^{c}
AR	5.50±0.53^{a}	7.38±0.06^{b}	8.98±0.30^{de}	9.18±0.21^{e}	8.76±0.37^{de}	8.78±0.49^{de}	8.67±0.39^{cd}
Enterobacteriaceae							
PVC	4.48±0.35^{c}	6.28±0.55^{de}	6.88±0.45e	6.08±0.95^{de}	5.27±0.91^{d}	3.47±0.46^{b}	2.37±0.83^{a}
AR	4.48±0.35^{c}	6.31±0.53^{de}	6.79±0.31^{de}	5.97±1.10^{de}	5.26±0.95^{d}	3.68±0.66^{b}	3.16±0.61^{ab}
Coliforms							
PVC	4.48±0.20^{c}	5.98±0.57^{efg}	6.79±0.57^{fg}	5.79±0.82^{def}	5.19±1.00^{d}	3.39±0.42^{ab}	2.63±0.21^{a}
AR	4.48±0.20^{c}	6.19±0.63^{efg}	6.83±0.52^{g}	5.90±1.15^{def}	5.16±1.02^{d}	3.76±0.65^{b}	2.69±0.28^{a}
E. coli							
PVC	2.11±0.21^{c}	4.90±0.76^{ef}	5.57±1.12^{f}	3.85±0.97^{e}	2.76±0.98^{cd}	2.18±1.69^{b}	0.00±0.00^{a}
AR	2.11±0.21^{c}	5.01±0.93^{ef}	5.59±1.10^{f}	4.01±1.08^{de}	2.79±1.02^{cd}	1.94±1.57^{b}	0.00±0.00^{a}
Lactic acid bacteria							
PVC	5.74±0.44^{a}	7.39±0.49^{b}	9.02±0.29^{def}	9.25 0.08^{f}	8.42±0.42^{c}	8.76±0.34^{cde}	8.71±0.46^{de}
AR	5.74±0.44^{a}	7.22±0.43^{b}	9.10±0.43^{ef}	9.27±0.12^{f}	8.68±0.11^{cd}	8.56±0.26^{c}	8.66±0.52^{cd}
Moulds							
PVC	1.45±0.26^{de}	1.500.10^{de}	1.44±0.18^{de}	1.52±0.18^{de}	1.25±0.30^{d}	0.62±0.48^{c}	0.04±0.09^{a}
AR	1.45±0.26^{de}	1.40±0.12^{d}	1.38±0.19^{d}	1.85±0.44^{e}	1.39±0.59^{d}	0.51±0.30^{bc}	0.19±0.34^{ab}
Yeasts							
PVC	4.98±0.19^{f}	2.70±0.23^{c}	4.07±0.40^{e}	3.99±1.25^{e}	2.72±0.44^{c}	1.51±0.20^{b}	0.50±0.25^{a}
AR	4.98±0.19^{f}	2.71±0.17^{c}	4.00±0.41^{e}	4.14±1.09^{e}	2.67±0.47^{c}	1.84±0.31^{b}	0.77±0.45^{a}

$^{a-g}$ Means of the same parameter in the same row without a common superscript differ significantly ($P<0.05$).

those of La Serena Cheese (NÚÑEZ *et al.* 1991).

The proportion of total soluble nitrogen (SN) has traditionally been regarded as a "ripening index" for cheese because it reflects the extent of proteolysis. The strong proteolytic activity of the enzymes (cyprosins) in *Cynara cardunculus* L. flowers compared to chymosin in the formation of soluble nitrogen makes it suitable for use as a proteinase system for accelerated ripening in some ewes' milk cheese varieties involving the addition of enzymes (LAW and WIGMORE, 1982). Some alternatives could be a cyprosin + peptidase combination or mixtures of cyprosins and attenuated cultures.

The other nitrogen fractions (NPN, AAN and NH_3N) increased steadily and significantly during cheese ripening; at most sampling times, significant differences were recorded between the cheeses from the two types of coagulants (Table 1). NPN (containing mainly small peptides of 2 and 20 residues and free amino acids), has traditionally been regarded as an index of "ripening depth". Although lactic bacteria and other enzymes (MCSWEENEY and FOX, 1993) are the principal agents for the production of NPN, higher proteolytic activity in the breakdown of caseins and their first breakdown products in cheeses obtained with *C. cardunculus* enzymes suggests that cheeses obtained with the powdered vegetable coagulant contain more substrates (casein polypeptides) for producing higher amounts of low molecular weight nitrogen than those obtained with animal rennet (SN values in Table 1).

The average values (log cfu g^{-1} cheese) and standard deviations for some micro-

Table 3 - Flavour and aroma[1] of the cheeses obtained with powdered vegetable coagulant (PVC) or animal rennet (AR), at 60 and 90 days of ripening.

Characteristics	Days after manufacture	Type of coagulant	
		PVC	AR
Flavour	60	4.71±1.19[b]	4.15±1.31[a]
	90	5.88±1.12[c]	4.57±1.05[ab]
Aroma	60	5.11±1.33[b]	4.70±1.63[a]
	90	6.60±0.89[d]	5.35±1.12[bc]

[1] Mean values from 12 panelists;
[a-d] Means of the same parameter in the same row without a common superscript differ significantly (P<0.05).

bial groups determined in the initial milk, curd and cheeses during ripening of the two products, made with powdered vegetable coagulant from *C. cardunculus* or animal rennet, are reported in Table 2. The microbial counts in the milk used for cheese-making were high in most of the groups analysed. The mean microbial counts were higher in the curd than in the initial milk (except for yeasts) in both cheese trials. This is partly due to microbial growth during the milk-clotting stage at around 29°C and the physical entrapment of bacteria in the curd (TATINI *et al.*, 1971). The values were similar for all the microbial groups analysed on the different days in the curd and cheeses obtained with rennet and with powdered vegetable coagulant. This may be due to the fact that, like commercial rennet, the powdered coagulant assayed was free of viable micro-organisms (TEJADA, 2001) which also avoids the problem of poor microbiological quality reported with the use of crude extracts from *C. cardunculus* flowers (FERNÁNDEZ-SALGUERO *et al.*, 1999; GÓMEZ *et al.*, 2001).

The average scores and standard deviations for flavour and aroma at 60 and 90 days of ripening for cheeses manufactured with both the coagulants assayed are reported (Table 3). Flavour and aroma increased with the age of the cheeses and were significantly higher (P<0.05) in cheeses produced with PVC than in those obtained with rennet.

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SHORT COMMUNICATION

CAROTENOID PROFILE OF SWEET ORANGE AND MANDARIN ESSENTIAL OILS

I CAROTENOIDI DEGLI OLI ESSENZIALI DI ARANCIA DOLCE E MANDARINO

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ABSTRACT

This study was carried out to determine the carotenoid composition of blond and pigmented sweet orange and mandarin essential oils. The oils were analysed after alkaline hydrolysis by RP-HPLC/PDA. Fourteen compounds, carotenes and xanthophylls, were identified. Sweet orange and mandarin oils have characteristic carotenoid profiles that could be used to detect the addition of sweet orange to mandarin essential oil.

RIASSUNTO

È stato intrapreso uno studio sulla composizione carotenica degli oli di arancia dolce e mandarino. Gli oli sono stati analizzati dopo idrolisi alcalina tramite RP-HPLC/PDA. Sono stati identificati, complessivamente, quattordici carotenoidi, caroteni e xantofille. Gli oli di arancia dolce e di mandarino analizzati presentano un profilo cromatografico della frazione carotenica che permette di caratterizzarli e di evidenziare l'eventuale addizione di olio di arancia dolce a quello di mandarino

- Key words: carotenes, citrus essential oils, HPLC/PDA, xanthophylls -

INTRODUCTION

Mandarin and sweet orange essential oils have been extensively studied. Particular attention has been given to the volatile compounds (DUGO *et al.*, 1994; VERZERA *et al.*, 1997) and to the oxygen heterocyclic compounds (DUGO *et al.*, 1996), while no information has been reported on the carotenoids present in the non-volatile residue. Carotenoids present in citrus juices have also been studied, particularly those present in orange juice (PERFETTI *et al.*, 1988; PHILIP *et al.*, 1989; ROUSEFF *et al.*, 1996; MOULY *et al.*, 1999a;b); however, there have been few studies on the carotenoids present in the peel (NOGA and LENZ, 1983; FISHMAN and CHIKOVANI, 1997).

Carotenoids, some of the most diffuse pigments in nature, are typical of fruits, vegetables and flowers, but are also in birds and fish. Carotenoids are made up of eight isoprenoid units which have various end groups on the 40-carbon (C-40) skeletons. They are classified into two major groups, carotenes (hydrocarbons) and xanthophylls (oxygenated carotenes). Carotenoids are characterized by typical absorbance profiles, with maximum values at wavelengths that range from the UV to the visible region. There is a wide range of polarity indices for the different classes of compounds found in this fraction (mono- and poly-alcohols, epoxides, furanoid and hydrocarbons), and within each class these indices are very similar (STEWARD, 1973; GROSS, 1987).

Due to the widespread distribution of carotenoids in nature, and their many different biological functions, carotenoids have been the subject of studies in many different scientific disciplines such as plant physiology, food science, food chemistry, photochemistry, biotechnology and medical science. Some carotenoids act as precursors of vitamin A and are therefore essential in the human diet; they also function as antioxidants, scavenging free radicals in biological systems protecting cells, tissues and DNA from damage and preventing the induction of cancer, heart disease and degenerative eye diseases. Lutein, cantaxanthin, β-carotene, α-carotene, and lycopene can decrease the risk of cancer. Lycopene, in particular, can decrease the risk of prostate, breast, cervix, and stomach cancer. Carotenoids are largely used as colorants in food and beverages (ROBERFROID, 1995; AGARWAL and RAO, 1988; KHACHIK *et al.*, 1988; KRINSKY, 1998).

Based on the characterisation of citrus essential oils, this paper reports the carotenoid profiles, of carotenes and xanthophylls of sweet orange and mandarin oils after alkaline hydrolysis by RP-HPLC using a Photodiode Array Detector (PDA). The purpose of this preliminary study was to develop a simple method for determining the carotenoid profile of these oils in order to characterise them by this fraction.

MATERIALS AND METHODS

The analyses were carried out on the essential oils of five samples of blond sweet orange, five samples of pigmented sweet orange and four samples of mandarin. The samples were selected from among genuine Sicilian industrially produced essential oils extracted by the FMC technology, during the 2000-2001 production season. The authenticity of the oils analysed was tested by evaluating the composition of the volatile fraction as previously reported (DUGO *et al.*, 1994; VERZERA *et al.*, 1997). One sample of commercial mandarin oil was also analysed.

Each sample was analysed after alkaline hydrolysis: 5 g of essential oil, stabilized with 0.1% BHT, were treated with 5 mL of methanolic potassium hydroxide (2 N) under nitrogen. The mixture was stirred for 16 h at room temperature

(22°C) in the dark. The free carotenoids were extracted with three aliquots of 5 mL each of diethyl ether. The organic extracts were combined and washed several times with water until neutral pH. The solvent was evaporated under vacuum at 30°C and the residue was dissolved in THF to a final volume of 5 mL for chromatographic analysis.

HPLC analysis

Each sample was analysed by reversed phase HPLC under the following conditions:

Instrumentation, Waters Associates (Division of Millipore, Milford, MA, USA): quaternary pump, Waters 519; gradient controller, Waters 600E; Rheodyne injector 9125 with a 20 µL loop; photodiode array detector, Waters 996; acquisition range 250-550 nm; data acquisition program, Millenium 2010.

HPLC column, Ultra C18, 200 x 4.6 mm, 5 µm p.s. (Restek Corp. Bellefonte, PA, USA). Solvents: A acetonitrile; B acetonitrile/dichloromethane/ethyl acetate (40:25:35); C water. Gradient: 4 min C 90%, A 10%; linear gradient in 6 min to A 80%, B 10%, C 10%; then in 5 min to A 80%, B 20% isocratic under this composition for 10 min, then linear gradient to B 100% and isocratic for 25 min. Flow 1.0 mL/min.

Carotenoids were identified according to their chromatographic pattern on RP-HPLC and UV-Vis absorption spectra, by comparing both their retention times and absorbance spectra with those of authentic carotenoids, when available, or with data found in the literature (PERFETTI *et al.*, 1988; ROUSEFF *et al.*, 1996).

RESULTS AND DISCUSSION

Figs. 1 and 2 report, respectively, the HPLC chromatograms of hydrolysed blond and pigmented sweet orange and mandarin essential oils plotted at 470, 400 and 286 nm. Since no differences were noted in the carotenoid profiles, one chromatogram for each variety is sufficient to be used to describe the results obtained.

In both blond and pigmented sweet orange essential oils (Fig. 1) the main xanthophyll compounds were violaxanthin, antheroxanthin, with carotenes, β-carotene, phytoene and phytofluene, present, but at lower levels. The pigmented orange essential oil did not differ from blond orange oil with respect to the qualitative composition of the carotenoid fractions.

In mandarin essential oil (Fig. 2) β-cryptoxanthin was the main carotenoid, while phytoene was the most representative hydrocarbon. Auroxanthin, lutein and α- and β-cryptoxanthin, were the xanthophylls identified; ζ-carotene, phytofluene, β-carotene and phytoene were the carotenes detected.

Fig. 3 reports the HPLC chromatograms of commercial mandarin oil after alkaline hydrolysis. Comparing this chromatogram with that reported in Fig. 2, a few compounds extraneous to mandarin oil, but typical of orange oil, were present, namely, antheroxanthin, violaxanthin, zeaxanthin. Moreover, the ratios of violaxanthin/β-cryptoxanthin and β-carotene/β-cryptoxanthin were different from those of the genuine oils. The differences between the commercial mandarin oil and the genuine samples, could be indicative of the addition of sweet orange oil residue.

The xanthophylls and carotenes identified in the oils are reported in Table 1 along with the maximum absorbance wavelengths, retention times, Chemical Abstracts Service (CAS) number and structural information. Fourteen carotenoids were identified.

Using the HPLC method described here, it was possible to characterise the carotenoid profile of mandarin, sweet blond and pigmented orange essential oils, which provides a fingerprint of their

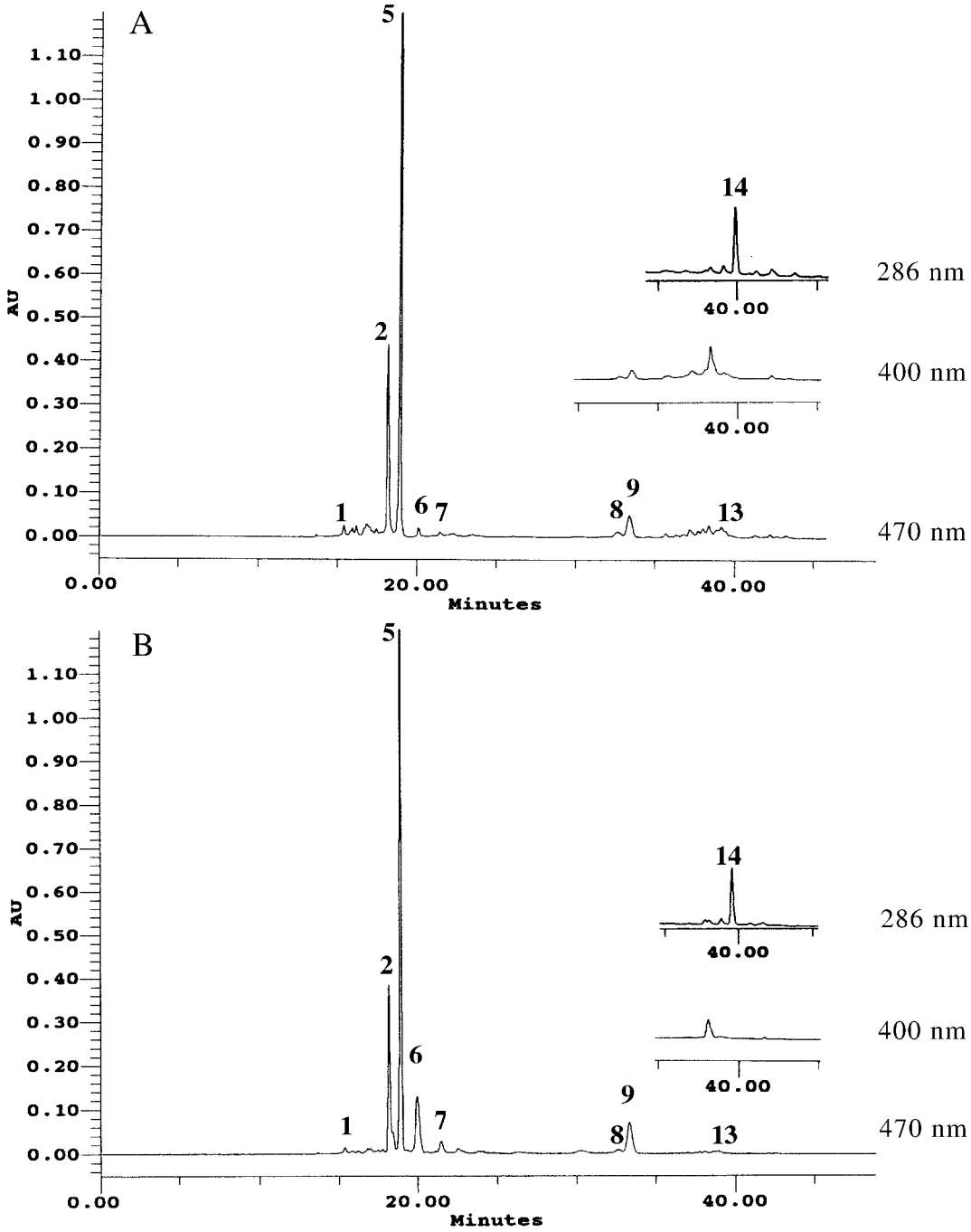


Fig. 1 - HPLC chromatograms of sweet blond orange (A) and pigmented (B) essential oils after alkaline hydrolysis. For compound identification see Table 1.

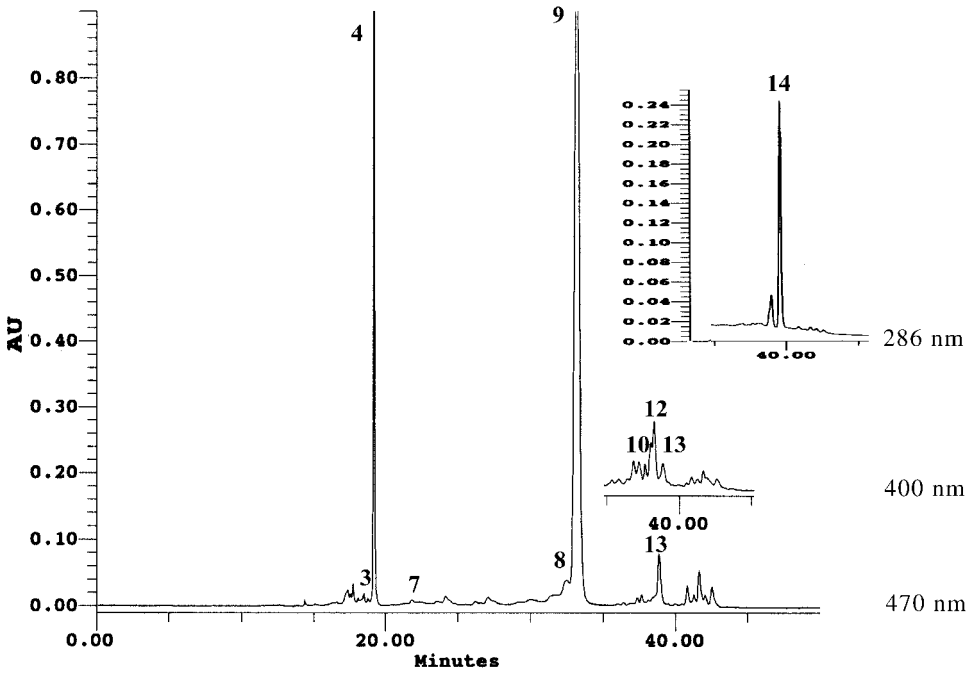


Fig. 2 - HPLC chromatogram of a genuine mandarin essential oil after alkaline hydrolysis. For the compound identification see Table 1.

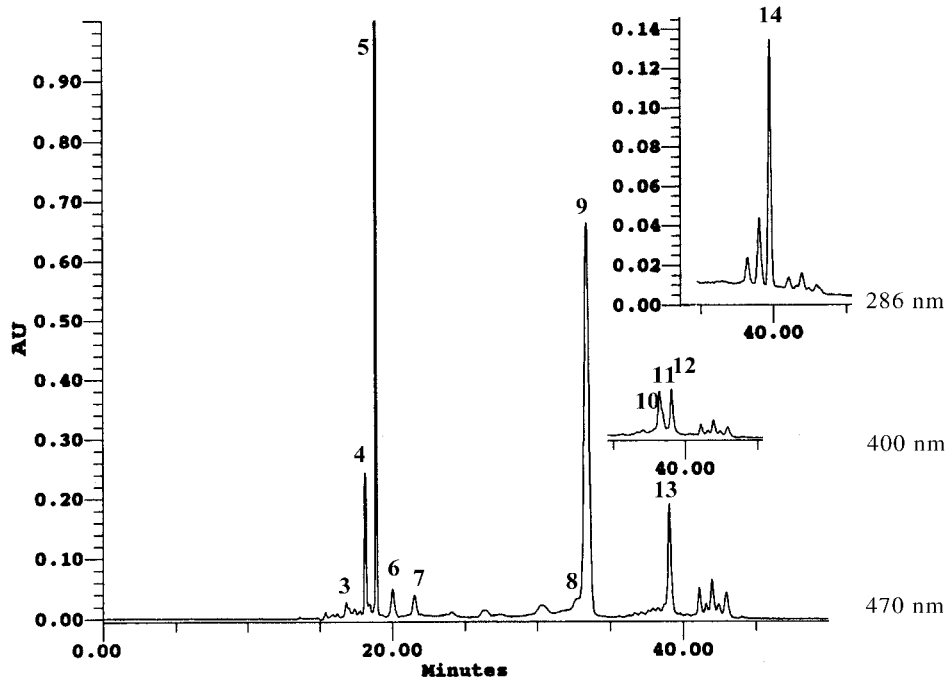


Fig. 3 - HPLC chromatogram of a commercial mandarin essential oil after alkaline hydrolysis. For compound identification see Table 1.

Table 1 - Carotenoids identified in the samples analyzed, their retention times (min) and relative UV maximum wavelengths.

Compounds [CAS N°]	λ^1 nm	λ^2 nm	λ^3 nm	RT	S.O.	M.	C. M.
1) trollichrome [25548-02-1] (6',7'-didehydro-5,8-epoxy-4',5,5',8'-tetrahydro-3, 3,5-trihydroxy-β,ε-carotene)	397.5	421.5	447.5	16.54	X	-	-
2) antheraxanthin [640-03-9] (5,6-epoxy-5,6-dihydro-3,3'-dihydroxy-β,β- carotene)	417.2	441.0	470.2	18.08	X	-	X
3) luteoxanthin [1912-50-1] (5,8,5',8'-diepoxy-5,6',5',8'-tetrahydro-3,3'-dihydroxy-β,β-carotene)	398.0	421.0	443.2	18.78	-	X	X
4) auroxanthin [27785-15-5] (5,8,5',8'-diepoxy-5,5',8,6'-tetrahydro-3,3'-dihydroxy-β,β-carotene)	402.1	424.2	441.6	19.15	-	X	X
5) violaxanthin [126-29-4] (5,6,5',6'-diepoxy-5,5',6,6'-tetrahydro-3,3'-dihydroxy-β,β-carotene)	s413.0	436.0	465.0	19.20	X	-	X
6) zeaxanthin [144-68-3] (3,3'- dihydroxy -β,β-carotene)	s427.5	449.5	476.5	20.02	X	-	X
7) luteina [7481-64-3] (3,3'- dihydroxy -β,ε-carotene)	-	445.0	472.2	21.23	X	X	X
8) α-cryptoxanthin [24480-38-4] (3-hydroxy -β,ε-carotene)	-	447.4	475.0	32.70	X	X	X
9) β-cryptoxanthin [472-70-8] (3-hydroxy -β,β-carotene)	-	453.6	479.2	33.15	X	X	X
10) ζ-carotene [72746-33-9] (7,7',8,8'-tetrahydro-9-cis,9'-cis-ψ,ψ-carotene)	s379.6	400.6	425.4	38.12	X	X	X
11) ξ-carotene [13587-06-9] (7,7',8,8'-tetrahydro-ψ,ψ-carotene)	s381.0	402.2	427.8	38.33	-	-	X
12) phytofluene [27664-65-9] (7,7',8,8',11,12-hexahydro-15-cis-ψ,ψ-carotene)	s333.4	349.8	369.0	38.39	X	X	X
13) β-carotene [7235-40-7] (β,β-carotene)	-	452.9	479.5	38.71	X	X	X
14) phytoene [540-04-5] (7,7',8,8',11,11',12,12'-octahydro-ψ,ψ-carotene)	-	286.0	-	39.83	X	X	X

RT = retention time; S.O. = sweet orange; M. = mandarin; C. M. = commercial mandarin. s = shoulder.

carotenoid fractions. In particular, the major differences in the qualitative composition of the carotenoid fractions allow mandarin oil to be distinguished from sweet orange oil. The profiles of the blond and pigmented sweet orange essential oils were similar, so it was difficult to differentiate these two sweet orange oils based on the carotenoid profiles.

Considering that mandarin essential oil can be adulterated by adding sweet orange oil or a fraction of it, the major differences in the carotenoid profiles of these oils allow such addition to be detected. The example here reported proves that the analysis of the carotenoid fraction and the composition of the volatile fraction and non-volatile residue could be used to determine the genuineness and define the quality parameters for sweet orange and mandarin essential oils.

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SHORT COMMUNICATION

DECLINE RATES OF CHLORPYRIFOS RESIDUES IN GREEN BEANS GROWN IN PLASTIC GREENHOUSES

DEGRADAZIONE DEL CHLORPYRIFOS IN FAGIOLINI COLTIVATI IN SERRA

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ABSTRACT

Residue levels and decline rates of chlorpyrifos residues in green beans sprayed with Dursban 48 at single and double dose and grown in a greenhouse were studied. Chlorpyrifos residue levels were determined by applying the ethyl acetate based extraction method and analyzing extracts by GC-NPD. Statistical analysis confirmed that decline of chlorpyrifos residue levels can be described as a pseudo-first-order reaction. Half-life periods determined were 3.26 days and 3.17 days for single and double dose treatments, respectively,

RIASSUNTO

Sono stati studiati i residui di chlorpyrifos in fagiolini coltivati in serra dopo trattamento con Dursban 48 in dose singola e doppia. I residui dei chlorpyrifos sono stati valutati mediante GC-NPD dopo estrazione con acetato di etile. La degradazione del chlorpyrifos segue una cinetica vicina quella di primo ordine. Il tempo di vita del principio attivo era di 3,26 e 3,17 giorni, rispettivamente, per la dose singola e doppia, mentre i corrispondenti valori iniziali erano 2, 68 e 11, 66 mg/kg, rispettivamente. I residui di Chlorpyri-

- Key words: chlorpyrifos, double treatments, half-life periods, residue decline curves -

whereas the corresponding initial residue values were 2.68 and 11.66 mg/kg, respectively. Chlorpyrifos residues calculated after 21 days were 0.03 (single dose) and 0.12 (double dose) mg/kg. The value for the double dose is higher than the maximum residue level (0.05 mg/kg) established by the European Union for chlorpyrifos in green beans.

fos presenti nei fagiolini 21 giorni dopo il trattamento erano mediamente 0,03 e 0,12 mg/kg, rispettivamente, per dose singola e dose doppia. Questo ultimo valore era maggiore del limite legale (0,05 mg/kg) fissato dall'UE per i fagiolini.

INTRODUCTION

Pesticides are necessary and essential in agricultural production, but along with their use is the risk of residues remaining in food. For this reason, governments and international organizations are working on establishing regulations for the use, manufacture, handling and marketing of pesticides and have published lists of pesticides and their tolerance levels or maximum residue limits (MRLs) in food. Information about the persistence of pesticides in crops is essential for these purposes, and therefore, it is important to carry out studies on different crops, application rates and climatic conditions for new pesticides as well as to re-evaluate those pesticides, like chlorpyrifos, included in periodic review programs of international organizations (FAO, 2001).

Chlorpyrifos (O,O-diethyl O-(3,5, 6-trichloro-2-pyridinyl)- phosphorothioate) is a non systemic organophosphorous insecticide-acaricide, effective primarily by contact, but may also kill target pests by ingestion and vapour (TOMLIN, 2000). Like other organophosphates, its insecticidal action is due to the inhibition of the enzyme acetylcholinesterase resulting in the accumulation of the neurotransmitter, acetylcholine, at the nerve endings which causes excessive transmission of nerve impulses and eventually mortality of the target pest. Introduced in 1965 and first described by KENAGA *et al.* (1965), it is recommended for control of *Dociostaurus* sp., aphids, white fly and other insects in a wide range of crops, including cereals, fruits and vegetables (LIÑAN and VICENTE, 2002).

Chlorpyrifos is widely used all over the world on a great number of important agricultural crops. It also has many urban uses mainly as a termiticide (LEMUS and ABDELGHANI, 2000; MURRAY *et al.*, 2001). Its residues have been commonly found in fruits and vegetables in the pesticide residue monitoring programs and total diet studies carried out in many countries (FERNANDEZ *et al.*, 2001; TAHIR *et al.*, 2001; FDA, 2000; NFA, 2001).

The objectives of this work were to evaluate the decline behaviour and residue levels of chlorpyrifos in green beans grown in a plastic greenhouse after single and double dose treatments, under the typical climatic and growing conditions of Almería.

MATERIALS AND METHODS

Chemicals and apparatus

Chlorpyrifos standard was supplied by Riedel de Haën (Seeelze, Germany). All solvents and anhydrous sodium sulfate 12-60 mesh (99.5%) were obtained from Merck (Darmstadt, Germany).

The gas chromatograph was a Perkin Elmer Model 8700 (Norwalk, CT)

equipped with an HP1 wide-bore fused capillary column (30 m x 0.53 mm i.d. x 2.65 µm film thickness) attached to a nitrogen-phosphorus detector (NPD). The chromatographic conditions used for the analysis of chlorpyrifos residues were as follows: detector temperature 250°C; injector temperature 250°C; oven temperature program 1 min at 100°C, 30°C/min to 150°C, hold for 2 min, 20°C/min to 220°C, hold for 5 min; carrier gas (nitrogen) flow rate, 20 mL/min; air flow rate, 150-160 mL/min; hydrogen flow rate, 1-3 mL/min; injection volume 2 µL, in a splitless mode.

Decline study

Decline experiments were conducted in an experimental greenhouse located at the "La Mojonera Agricultural Research Centre" in Almería, Spain. The study was carried out on a crop of green beans (Helda variety, spring cycle) in a 400 m^2 (20x20) "parral" type greenhouse, orientated south-west and divided into two zones (north and south), with a planting density of 60,000 plants/ha.

Green bean plants, grown under routine horticultural practices, were sprayed with a single dose (1.5 mL/L) and double dose (3 mL/L) of Dursban 48 (Chlorpyrifos 48%) at a spray volume of 2,000 L/ha. In both cases, this represents application rates of 1.4 and 2.8 kg of chlorpyrifos (a.i.) per ha for single and double treatments, respectively. These treatments were done when the green beans were of commercial size. The average daily maximum/minimum temperatures outside and inside the greenhouse throughout the study were 25°/18° and 31°/16°C, respectively, whereas the maximum/minimum absolute temperatures outside and inside the greenhouse were 29°/14° and 35°/9°C, respectively.

Three samples per treatment were collected at random from 200 m^2 plots at 0, 1, 3, 7, 14 and 21 days after application; each sample consisted of 500 g of green beans. Immediately after picking, the samples were put into polyethylene bags and transported to the laboratory, where they were chopped and thoroughly mixed. The samples were kept deep-frozen until analysis. In all cases, <90 min passed between harvest and storage in the freezer and analyses were always carried out 24 to 48 hours after samples were stored in the freezer. Stability of chlorpyrifos in the matrices during homogenization and storage procedures was previously tested on spiked samples.

Extraction of chlorpyrifos residues from green beans was carried out according to the LEARY (1971) procedure followed by a short-column Florisil clean-up step. A brief description of the procedure is as follows. Fifty grams of chopped sample were weighed into a high-speed blender jar; 100 mL of ethyl acetate and 80 g of anhydrous sodium sulfate were added and then blended for 5 min. The mixture was decanted and filtered through a 12 cm Büchner funnel with 20 g of anhydrous sodium sulfate. Two successive 100 mL portions of ethyl acetate were added to the solid residue, blended for 2 min, and filtered through anhydrous sodium sulfate. The combined extracts were evaporated to dryness using a rotatory evaporator (40°C water bath) and diluted to exactly 10 mL with light petroleum ether in a 10 mL volumetric flask. After that, 5 mL of extract were passed through a 20 mm i.d. glass column filled with a 1 cm layer of Florisil and then eluted with 50 mL of a mixture of ethyl-ether/petroleum ether (30:70) at a flow of 5 mL/min. The eluate was evaporated in a rotatory evaporator to 1 mL, and then diluted to 10 mL with petroleum ether. Chlorpyrifos residues in the light petroleum ether extracts were determined by GC-NPD using the operating conditions described above. The method was validated at 0.52, 0.10 and 0.01 mg/kg fortification levels, resulting in an overall recovery of 89.1%

with coefficients of variation less than 10%. The estimated limit of quantification was 0.01 mg/kg.

RESULTS AND DISCUSSION

The residue data, including mean values and standard deviations, obtained in the decline study after single and double dose treatments of chlorpyrifos are summarized in Table 1.

Chlorpyrifos residue levels in green bean samples after the single treatment were higher than 0.05 mg/kg, the maximum residue limit established by the EU (EUROPEAN COMMUNITIES, 1998) and which also corresponds to the limit of determination. The only exception was for day 21. For green beans treated with the double dose, residue levels at the beginning of the study were around two hundred times the MRL, and the residue level was still over two times the MRL 21 days after treatment.

Residue data were subjected to statistical analysis to evaluate the decline of chlorpyrifos in green beans as a function of time. This analysis was carried out according to the models proposed by TIMME *et al.* (1986) to study the behavior of pesticide residues on crops prior to harvest. These models were successfully applied on endosulfan and methamidophos by AGUILERA del REAL *et al.* (1997; 1999). To obtain a measure of the quality of fit, the modified coefficient of determination r^2 proposed by FREHSE and WALTER (1995) was calculated. The value of r^2 was tested with the aid of the test quantity D described by TIMME and FREHSE (1980). If the calculated test quantity is greater than nil, the correlation is confirmed. If the modified coefficient of determination becomes negative, the fit is automatically regarded as not assured. Thus, the residue values and/or time were transformed using the six models described by these authors. Table 2 reports the values calculated for r^2 and D. As can be seen correlation was only assured for the first order function since D>0.

Table 2 - Modified coefficient of determination (r^2) and test quantity (D) for correlation values determined from the six functions proposed by TIMME *et al.* (1986).

Function	r^2 (D)	
	Single dose	Double dose
1st	0.971 (0.174)	0.989 (0.183)
1.5th	-81.841	-175.25
2nd	-1.683	-1.594
1st RF *	0.278 (-0.283)	-0.233
1.5th RF	0.307 (-0.257)	-2.271
2nd RF	-7.685	-7.024

* RF= Root Function.

Table 1 - Residue levels (R, mg/kg) of chlorpyrifos in the green bean samples with time after single and double treatments with Dursban. Values are the average of three determinations.

Time (days)	Single Dose		Double Dose	
	R	Mean±S.D.	R	Mean±S.D.
0	2.65/3.16/2.92	2.90±0.20	10.92/11.52/10.18	10.87±0.67
1	2.35/2.19/2.25	2.26±0.08	8.50/9.52/10.69	9.57±1.10
3	0.95/1.10/1.17	1.07±0.11	6.74/7.22/6.23	6.73±0.50
7	0.69/0.7/0.76	0.72±0.04	2.31/2.48/2.42	2.40±0.09
14	0.11/0.16/0.15	0.14±0.03	0.55/0.49/0.59	0.54±0.05
21	0.03/0.03/0.02	0.03±0.01	0.13/0.10/0.12	0.12±0.02

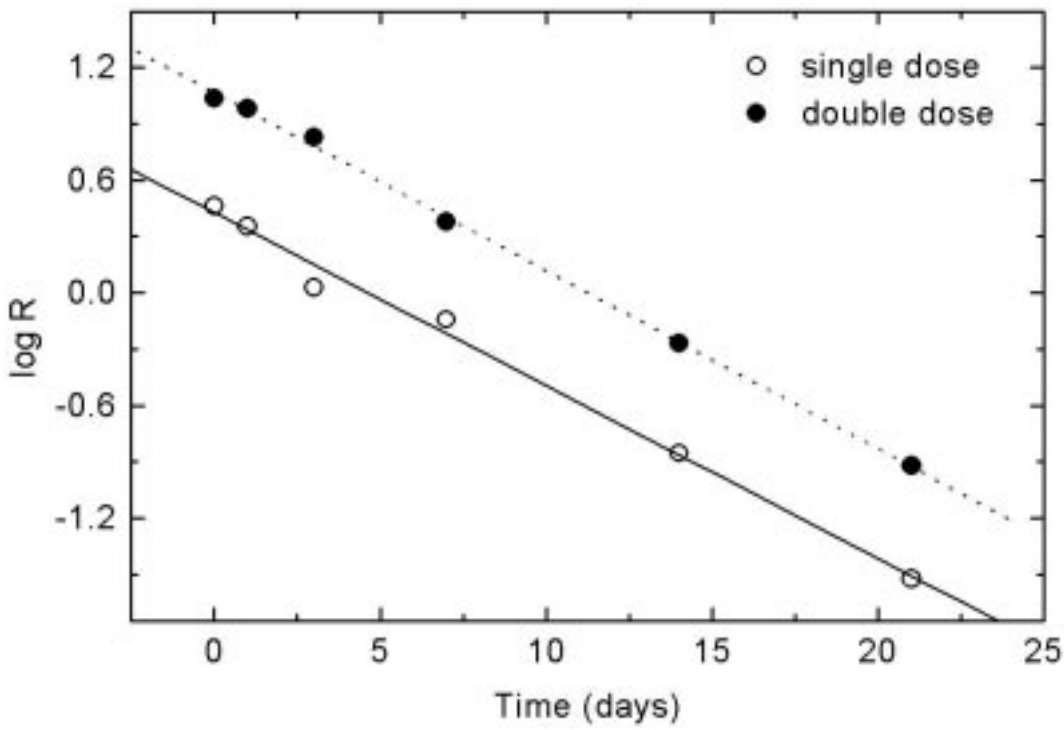


Fig. 1 - Residue decline curves (log R vs time) of chlorpyrifos in green beans after single and double dose treatments.

The first order function was the only one that fit the data for both single and double treatments. This indicates that the decline behavior of chlorpyrifos residues in green beans can be described as a pseudo-first order reaction. This is the approach usually assumed for interpretation of residue decline experiments (MURRAY *et al.*, 2001, BOUAID *et al.*, 2001, MUKHERJEE and GOPAL, 1992).

In Fig. 1 the decimal logarithms of the residue are plotted versus time. The corresponding straight lines were computed by regression analysis, and the statistical parameters were calculated.

Half and tenth life times ($t_{1/2}$ and $t_{1/10}$) of chlorpyrifos, first order decline constants (K), initial residue (R_0) and residues after 21 days (R_{21}) determined from this model are given in Table 3 for both doses. The initial residue level of chlorpyrifos in green beans treated with the double dose (11.66 mg/kg) was four times that of the single dose (2.68 mg/kg). However K, $t_{1/2}$ and $t_{1/10}$ values were

Table 3 - Statistical parameters calculated for the decline of chlorpyrifos in green beans.

Parameter	Mean (±C.I.)*	
	Single dose	Double dose
K($days^{-1}$)	0.21 (±0.02)	0.22 (±0.01)
R_0(mg/kg)	2.68 (+0.86/-0.65)	11.66 (+1.40/-1.24)
$t_{1/2}$ (days)	3.26 (±0.40)	3.17 (±0.15)
$t_{1/10}$ (days)	10.84 (±1.32)	10.54 (±0.50)
R_{21}(mg/kg)	0.03 (+0.02/-0.01)	0.12 (+0.02/-0.02)

* Confidence intervals at a level of significance of 95% (p = 0.05).

very similar for both treatments which confirms the goodness of adjustments to a pseudo-first order reaction. $t_{1/2}$ values calculated for single and double dose (3.26 and 3.17 days, respectively) were very similar to those obtained by MARTINEZ VIDAL *et al.* (1998) for tomatoes and green beans grown in the greenhouse and by NAVARRO *et al.* (2001) for grapes. However, the $t_{1/2}$ values obtained by MONTEMURRO *et al.* (2002) for chlorpyrifos for oranges ranged from 25 to 43 days.

The $t_{1/2}$ values obtained in this work can be compared with the $t_{1/2}$ values reported by other authors for different pesticides in green beans. For example, HERNANDEZ TORRES *et al.* (2002) obtained $t_{1/2}$ values of 2-3 days for methiocarb, whereas the $t_{1/2}$ values reported by EGEA GONZÁLEZ *et al.* (1998) for methamidophos and by CASTILLO SANCHEZ *et al.* (2000) for procymidone were 7 days and 11 days, respectively.

Finally, chlorpyrifos residue levels calculated after 21 days were 0.03±0.02 mg/kg (single dose) and 0.12±0.02 mg/kg (double dose). For the single dose this value is less than the MRL established by the European Union, but for the double dose it is 200% of the MRL.

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SHORT COMMUNICATION

A GC/MS METHOD FOR THE ROUTINE DETERMINATION OF ACRYLAMIDE IN FOOD

METODO GC/MS PER LA DETERMINAZIONE ROUTINARIA DI ACRILAMMIDE NEGLI ALIMENTI

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ABSTRACT

A simplified analytical method to determine the amount of acrylamide in food is described. Routine analysis is not possible using previously published procedures (CASTLE, 1993; ROSEN and HELLENÄS, 2002; TAREKE *et al.*, 2002; GERTZ and KLOSTERMANN, 2002; SFOPH, 2002). The method consists in a preliminary sample defatting step followed by extraction with methanol and concentration. The acrylamide concentration is evaluated by GC/MS without derivatization.

RIASSUNTO

Il lavoro propone un metodo atto alla determinazione di acrilammide negli alimenti, consentendo semplificazioni utili ad analisi di carattere routinario per matrici di varia natura. I criteri di estrazione adottati, che prevedono l'impiego di metanolo come solvente, si differenziano da quelli proposti fino ad oggi nelle altre metodiche (CASTLE, 1993; ROSEN and HELLENÄS, 2002; TAREKE et al., 2002; GERTZ and KLOSTERMANN, 2002; SFOPH, 2002).

Il livello di acrilammide viene valutato attraverso analisi GC/MS che non prevedono procedimenti di derivatizzazione.

MATERIALS AND METHODS

A sample of a baked good was finely ground, mixed with an electric blender and defatted in a Soxhlet extractor with hexane (Merck, Darmstadt). The residual solvent was removed under vacuum. Fifty millilitres of methanol (Merck, Darmstadt) were added to 10 g of the defatted sample in a sealed flask which was then stirred for 15 min followed by shaking for 1 min in an ultrasonic bath. The methanol phase was put into a sealed tube and centrifuged at 2,500 rpm for 5 min, then the recovered clear fraction (about 35 mL) was concentrated in a rotary evaporator to less than 2 mL. The residue was carefully put in a graduated 5 mL vial, diluted to 2 mL and then fast filtered. One microlitre was injected into the GC/MS (splitless mode 1 min, using an autosampler). For food samples containing more than 800 µg/kg of acrylamide, the sample solution was diluted to 4 mL. If the sample is high in sugar or other methanol-soluble compounds, it is suggested that it be concentrated to a total volume of ca. 4-5 mL. Precipitation was aided by cooling and the liquid fraction was withdrawn with a pasteur pipette and transferred to a graduated 5 mL vial. The precipitate was washed with a small volume of methanol, and the washing fraction was added to the first one and then diluted to volume.

A GC/MS QP2010 (Shimadzu; Milan, Italy) in EI mode was used. The column was a Supelcowax (30 m x 0.25 mm i.d., 0.20 µm film thickness; Supelco; Milan, Italy) and the temperature program was: isothermal for 1 min at 60°C, incr. at 10°C/min to 240°C. The acquisition mode was a MID (Multiple Ion Detector) monitoring ions *m/z* 27, 55 and 71. The relative abundances in the acrylamide mass spectrum were, respectively, 85, 74, 100. The calibration curve was made by meas-

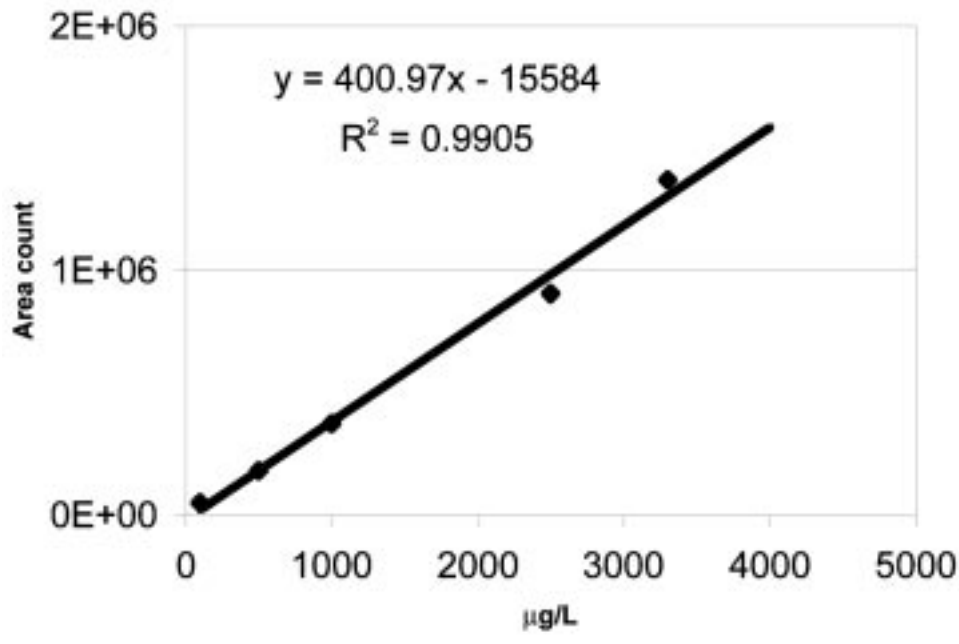


Fig. 1 - Calibration curve for acrylamide, GC/MS detection.

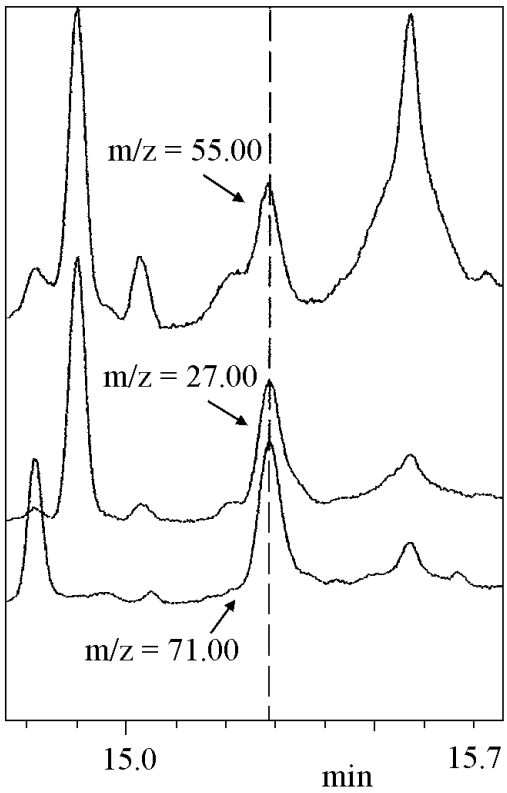


Fig. 2 - GC/MS detection (MID mode) of 20 µg/kg of acrylamide in a baked good.

uring the SIM area of the *m/z* 71 peak (molecular ion).

The external standard method was used: the calibration curve was made from standard solutions of acrylamide (99%, Sigma, Milan, Italy) ranging from 100 to 4,000 µg/L (ppb) in methanol, injecting 1 µL into the GC/MS (splitless mode 1 min, using an autosampler).

RESULTS AND DISCUSSION

The calibration curve was linear (R^2 = 0.9905) in the range considered (Fig. 1). The detection limit was 2 µg/kg of sample and the quantitation limit was calculated as 10 µg/kg, i.e. 5 times the detection limit. The amount of acrylamide detected needs to be first corrected, multiplying by $50/V_r$, where V_r is the volume of methanol recovered from the extraction. Adopting the operating conditions suggested for the extraction and quantitation procedure, the level of acrylamide in a food sample is the value taken from the calibration curve divided by 5 (or 2.5 in case of dilution to 4 mL). Recovery ranged from 68.0 to 75.4% (acrylamide concentration added = 100-800 µg/kg; CV% between 4.5 and 1.8). Fig. 2 shows the GC/MS spectrum of a solution containing 100 µg/L of acrylamide, corresponding to 100/5 = 20 µg/kg in the baked good sample.

ACKNOWLEDGEMENT

Thanks are due to A. Fossati, M. Todeschini and A. Rosa for technical collaboration.

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FLAIR-FLOW EUROPE



EUROPEAN INNOVATION WORKSHOPS

FFE 540/02/SME54

The European Commission, Quality of Life and Management of Living Resources Programme, Key Action 1 on Food, Nutrition and Health is now further pushing food innovation and exploitation of new scientific results in the food industry. The approach is to organise 16-18 Innovation Workshops throughout Europe, emphasizing major research areas with promising research results, e.g. within functional foods and nutrition, on-line food quality control and traceability, food safety, biotechnology and genetic modified foods, low allergy foods, and new food processing.

At each Innovation Workshop, major scientific findings and the market opportunities are presented and discussed in the language of the country in question and the workshop management intends to initiate and support new industrial innovation groups working on commercialisation of the scientific findings together with the inventing scientists.

Innovation and exploitation of new scientific findings is difficult and requires more than technology transfer. Close collaboration between all relevant stakeholders, the industrialists (in particular SME-representatives) and the scientists in smaller innovation groups as well as in depth market analysis may promote new science based products and technologies, says Dr. Achim Boenke from the EC, Research Directorate-General in Brussels.

All European food, ingredients and technology industries are welcome to participate and the workshop plans, programmes and registration / contact information may be obtained from the organisers (Flair-Flow Europe) web-site www.Flair-Flow.com

Contract No: QLK1-CT-2002-30137

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

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ANTIOXIDANTS AGAINST AGEING

FFE 544/02/CG52

Scientists are now trying to resolve the mystery of ageing and to define the possible role of antioxidants in preventing age-related diseases and changes occurring in the human body.

The ageing process triggers heart disease, cancer, blindness and diseases of the neurological system like dementia. Although we know that our family background influences the onset of these through our genes, the importance of our lifestyle and environmental factors such as diet are of increasing interest and relevance. A diet rich in plant foods is known to reduce the risks of developing certain age-related diseases. Naturally occurring compounds in cereals, fruit and vegetables may reduce the risk of these ageing-associated diseases.

The mechanism underpinning ageing and the initiation of age-related diseases is thought to be due to the production of highly reactive molecules that oxidise or damage components of cells, causing these to malfunction. Antioxidants are a group of substances that have the potential to stop these harmful reactions.

In an on-going European project called "EUROFEDA" participants are reviewing the evidence that antioxidants in the diet are beneficial and what intakes are associated with any beneficial effects. They have looked into:

– Those substances that are naturally occurring antioxidants and those substances that ensure antioxidant defences in the body are maximised.

– The effectiveness of present methods to estimate the damage caused by oxidants in body tissues.

– The information that exists on the biological effects of antioxidants in the body and

– Evaluating what is known about the distribution of antioxidants in the body, particularly at sites where oxidative damage has the potential to cause severe illness.

A conference is being held on 25th to 28th September 2002, organised by the coordinators of the project in Cambridge, UK, on "Antioxidants: Benefits and Risks", where the latest advances in the understanding of the effects of dietary antioxidants on health and age-related disease are being presented. For more details of either meeting please visit the project website or contact the co-ordinator.

Project No: QLK1-1999-00179 (EUROFEDA) http://www.ifrn.bbsrc.ac.uk/EUROFEDA/

Project Co-ordinator: Dr Siân Astley - Institute of Food Research - Norwich Research Park, Colney, Norwich, NR4 7UA, UK - Tel. +44 (0)1603 255000; Fax+44 (0)1603 507723 - e-mail: sian.astley@bbsrc.ac.uk

This one-pager was written by Ms. Marika Lyly, VTT Biotechnology, Finland, in September, 2002.

FRUIT SENSORS FIT LIKE A GLOVE

FFE 549/02/SME57

Fruit growers or handling staff may now determine the quality of fruit and classify it by just picking it up. Scientists have recently designed and set up a glove-

shaped apparatus equipped with various miniaturised sensors which provide information on fruit quality parameters like sugar content, maturity, mechanical properties (firmness, stiffness) and internal colour.

Sugar content and internal colour are measured by a miniaturised spectrometer coupled with optical fibres. A sound sensor measures the mechanical properties. The size of the fruit is measured by a potentiometer placed at the hand aperture. These sensors are coupled to a microcomputer that delivers processed pieces of information about fruit characteristics. The output is the sugar content index, firmness, size index, stiffness index and a maturity index based on the internal colour.

These parameters can be used in various ways:

– as individual values (mean and standard deviation is given for each parameter);

– as an assigned class for each fruit, where each class has been defined by previously set thresholds or by previous learning process.

The scientists constructed and field-tested a prototype consisting of a glove weighing 400 g and a 1 kg rucksack containing the electronics and linked to a computer. They tested different varieties of nectarines and apples and determined the precision of each quality parameter. For further details you may contact the coordinator of the project.

Contract No: FAIR-CT97-3399 (GLOVE)

http://www.montpellier.cemagref.fr/teap/projets/caporal/projetglove.htm

Co-ordinator: Michel Crochon - CEMAGREF - Sensors and Information Engineering for Food Quality and Agriculture Division - BP 5095, 361 rue J.F. Breton - F-34033 Montpellier cedex 1, France - Tel. +33 (0)4 67046323; Fax +33 (0)4 67635795 - http://www.montpellier.cemagref.fr/giqual

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

HEART DISEASE AND CANCER: IS GARLIC THE ANSWER?

FFE 552/02/HP57

An EU-funded research project is now underway to explore the impact on atherosclerosis and colorectal cancer of bio-active sulphur-containing compounds, found in garlic.

Garlic is well known for its alleged therapeutic role in prevention of cancer and cardiovascular disease. These diseases are by far the leading causes of death in the EU. Increasing garlic consumption and increasing the content of the bio-active sulphur-containing components in garlic may, therefore, have the potential to prevent these diseases.

Clinical trials suggest that garlic significantly reduces serum cholesterol and triglyceride levels, it reduces blood pressure and increases fibrinolytic activity (*i.e.* it affects blood clotting). Epidemiological and animal studies have also suggested the antitumour potential of garlic. However, despite these reputed therapeutic effects, scientific studies of the mechanisms, with well-defined garlic preparations and compounds, are lacking.

The new project has two primary scientific objectives:

– To understand and improve the production of bio-active garlic compounds by sophisticated plant breeding techniques

– To improve understanding of the role of garlic in the diet and as a therapeutic agent in promoting and sustaining health, in particular its potential in preventing cancer and cardiovascular disease processes like atherosclerosis.

The project specifically aims to identify the mechanisms by which garlic interferes with the inflammatory processes of atherosclerosis and the development of cancer in cells, animals and humans. The project will also provide an insight into the synthesis of beneficial garlic constituents and their mechanisms of action. Further details about this project and results are available from the co-ordinator or by visiting the project website.

Project Reference: QLK1-1999-00498 (G&H) http://www.plant.wageningen-ur.nl/projects/garlicandhealth/

Project contact: Dr Chris Kik - Plant Research International - Wageningen University & Research Center, P.O. Box 16 - 6700 AA Wageningen, The Netherlands - Tel. +31 317 477278/477001; Fax +31 317 418094 - e-mail: c.kik@plant.wag-ur.nl - URL: http://www.plant.wageningen-ur.nl/expertise/alliumresearch/

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

HELP - A NEW UNIT OPERATION IN FOOD PROCESSING

FFE 556/02/SME58

HELP is a new emerging and non-thermal technology using High Intensity Electric Pulses, which rupture the membranes of, in particular, vegetative (microbial)cells, or make these membranes irr eversibly permeable and cause cell death. Electric field pulses of more than 10 kV/cm are used and the pulses used are in the range of micro-seconds.

High electric field pulses (HELP) may kill pathogens and other microorganisms in foods without any detrimental influence on food nutritional or sensory quality.

This new emerging food processing technology is a non-thermal "pasteurisation" operation, which lives up to the concept of Minimal Processing (MP). This concept is based on consumer demands of safe and fresh foods without traditional preservatives. It implies that many traditional thermal treatments are excluded, while others like high temperature short time techniques (HTST), aseptic processing and sous-vide processing may be considered MP treatments. Other examples of non-thermal MP processing are: high pressure technology, modified atmosphere packaging, food irradiation, pulsed light treatments, natural preservatives and any combination of these and moderate thermal treatments in order to introduce efficient hurdles to microbial growth.

The HELP project has recently been completed and results are published in "Trends in Food Science & Technology (2002), 12, no. 3-4, p. 93-144". Major outcomes are:

1) Unlike vegetative cells, most food enzymes are unaffected by HELP, proteins are not denatured and emulsions did not change;
2) HELP may provide a preservation technology for liquid foods, retaining food quality;
3) HELP may inactivate many microorganisms, in particular yeasts but also gram-positive and negative bacteria, while bacterial spores are more resistant;
4) HELP has a softening effect on the texture of raw fish and meat;
5) HELP can be effectively used to aid mass transfer in subsequent processes such as drying, extraction and expression.

Project No: FAIR-CT97-3044 (HELP) http://www.tu-berlin.de/~foodtech/html/body_hsi.html

Contact Details: Prof Dietrich Knorr - Head of Department of Food Biotechnology and Process Engineering - Berlin University of Technology - Koenigin-Luise-Str. 22 - D-14195 Berlin, Germany - Tel. +49 30 31471250; Fax +49 39 8327662 - E-mail: dietrich.knorr@tu-berlin.de - URL: http://www.tu-berlin.de/~foodtech

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

IAFP 2003

The International Association for Food Protection has announced preliminary topics for the 2003 Annual Meeting, August 10-13 at the Hilton New Orleans Riverside in New Orleans, Louisiana.

Food safety topics include: Investigation Techniques in an Age of Biosecurity; Global Update on Trends in the Food Safety Regulatory Structures; Dairy Regulations; Cost of Food Safety; Intervention Strategies for Ready to Eat Meats; Emerging Issues in Water Quality; Current Issues in Food Toxicology; Risk Assessment in the Fresh Produce Industry; *Campylobacter*: A Pathogen in Need of Resolution; Food Worker Hygiene Management; Impact of Bioinformatics: Safety-based Shelf-life Dating; Risk Communication; Food Allergen Control; Aquaculture; and many others.

Registration information is available on the Association Web site, www.foodprotection.org and the preliminary program will be available in March 2003.

Announcement of competition

"PREMIO DEL MUSEO" EUROPEAN MUSEUM PRIZE XII EDITION 2003

Section for journalism

The Board of Scientific consultants of the National Museum of Pasta, the only one of its kind in the world, will award a Prize for journalism in 2003.

The Prize aims to promote knowledge and appreciation of Italian pasta food, education in learning the nutritional value of this typical national food, which can contribute to alleviate the world-wide problem of hunger. The theme for 2003 is "Patria e regioni unite dalla pasta, formula vincente di devoluzione".

For more information: Piazza Scanderbeg 114-120, 00187 Roma, Italy, Tel. +39 06 6991119-6991120 - Fax +39 06 6991109.

12TH WORLD CONGRESS OF FOOD SCIENCE AND TECHNOLOGY
July 16-20, 2003 - Chicago, IL (USA)

For over 40 years, the World congress of Food Science and Technology has fostered the international cooperation and exchange of information among scientists, food technologists and specialists in order to advance current knowledge and technologies in the areas of food processing, manufacturing, preservation, storage and distribution.

The World Congress will present a full spectrum of approximately 700 poster topics available for viewing and discussion with poster abstract authors throughout the course of the week. The 18 selected poster sessions will augment the plenary sessions and symposia under the general theme "Feeding the World ... Opportunities without Boundaries" and will include such categories as:

Alternative Technology for Food Preservation; Aquaculture: Health and Technology; Biotechnology Applications; Childhood and Adolescent Feeding Issues; Consumer Surveys: Meth-

ods and Evaluation; Culinary Art and Food Science; Emerging Food Microbiology; Emerging Packaging Technology; Environmental Impact of food Technology - Opportunities and Threats; Ergogenic Aids and Sports Foods; Fermentation Technology - Multicultural Foods; Food Allergens; Food Chemistry - New Understanding; Food Composition Data: an International Effort; Food Flavors - International Palate; Food Fortification and Public Health; Food Laws and Regulations: Trade Barriers or Opportunities?; Food Science Education; Functional Foods; International Food Marketing; International Innovations in Food Science; Men's Health Issues; Natural Colorants; Non-thermal Processing; Obesity and Malnutrition - Over and Under Nutrition?; Phytochemicals and Health; Post Harvest Technology; Probiotics and Prebiotics; Proteins & Protein Fractions: Bioactives; Quality Assurance Management; Toxicology and Food Safety; Urban and Rural Issues in Agriculture; Viticulture and Enology; Waste Management; Water Security for Production and Processing; Women's Health Initiative, World Food Security.

Information/questions: Tel. +1 708 4860715 - e-mail: IUFoST@compusystems.com - Mail to: 12th World Congress Registration c/o CompuSystems - P.O. Box 552 - Brookfield, IL 60513-0522 - USA - Online: www.WorldFoodScience.org/worldcongress - Credit Card Registrations Only

BOOKS

FRONTIERS IN COMPUTATIONAL GENOMICS

By M.Y. Galperin and E.V. Koonin, National Center for Biotechnology Information, Bethesda

January 2003, c. X+348 pages

ISBN: 0954246446

Hbk £90 or $180

Publisher: Horizon Scientific Press - HSP, 32 Hewitts Lane, Wymondham, Norfolk NR18 0JA, UK - Fax +44 1953 603068 - e-mail: orders@horizonpress.com - order online at: www.horizonpress.com/order

Computational biology and bioinformatics form a major, integral part of genomics. Major new programs, software and internet facilities have evolved recently that facilitate computational analysis and more novel technologies are currently being developed. Written by experts in the field, this volume highlights research on the emerging, perhaps somewhat controversial, topics in genomics and shows the feedback between the new developments and the established methods of computational biology. An essential book for anyone involved in genomic science or bioinformatics.

GENOMICS OF GC-RICH GRAM-POSITIVE BACTERIA

By A. Danchin, HKU-Pasteur Research Centre, Hong Kong

September 2002, VIII+178 pages

ISBN: 0954246438

Hbk £75 or $150

Publisher: Horizon Scientific Press - HSP, 32 Hewitts Lane, Wymondham, Norfolk NR18 0JA, UK - Fax +44 1953 603068 - e-mail: orders@horizonpress.com - order online at: www.horizonpress.com/order

Eminent scientists have contributed comprehensive reviews on all aspects of the genomics of *Rhodococcus*, *Mycobacteria*, *Streptomyces* and other organisms. The authors provide an insight into the many facets of large-scale functional investigation in a variety of bacteria with the explicit goal to find common rules in the dynamic and structural organisation of these organisms.

This volume is the definitive guide to current research in this increasingly important area. Aimed at microbiologists, biotechnologists, and genome scientists.

GUIDE FOR AUTHORS

ITALIAN JOURNAL OF FOOD SCIENCE - IJFS

1. Manuscript Preparation

(1) Manuscripts must be typed, double-spaced and **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 should seek help from a fluent person before the final version is typed. The Assistant Editor reserves the right to make literary corrections and to make suggestions to improve brevity, but **the paper should be revised by a native English speaker** before submission.

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

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

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

Abstract. Clearly state the objective of the study, give a concise description of experiment(s), observations, results and conclusions. No references should be cited. DO NOT EXCEED 100 WORDS. An abstract and title in Italian must also be included. DO NOT EXCEED 200 WORDS.

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

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

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

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

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