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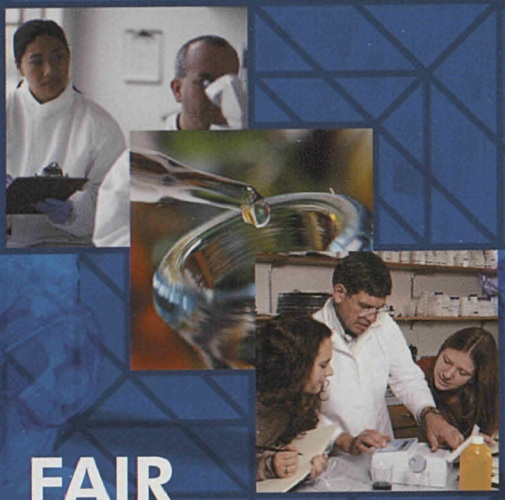

FAIR

Marie Curie Research Training Grants (1994-1998)

Agro-Industrial Research

QUALITY OF LIFE AND MANAGEMENT OF LIVING RESOURCES

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European Commission
Research Directorate-General
Quality of Life programme

FAIR
Agriculture and Fisheries
(including Agro-Industry, Food Technologies, Forestry, Aquaculture and Rural Development)

Marie Curie Research Training Grants
(1994-1998)
Agro-Industrial Research

Edited by A. Luchetti

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Table of Contents

Introduction

This publication contains information on the majority of training grants relevant to the agro-industrial sector and funded under the FAIR programme. This programme of research and technological development in the field of Agriculture and Fisheries including Agro-Industry, Food Technologies, Forestry, Aquaculture and Rural Development was adopted on 26 April 1994 as part of the Community's Fourth Framework Programme. It concerned all of agriculture, horticulture, forestry, fishery, aquaculture, and related food and non-food industries and was jointly managed by three Commission services, DG VI-FII-3: Agricultural Research, DG XII-E-2: Agro-Industrial Research, and DG XI V-C-2: Fisheries Research. The FAIR programme represented a natural evolution of the previous CAMAR, BIOMASS, ECLAIR, FLAIR, FOREST and FAR programmes adopted under the Second Framework Programme and of AIR adopted under the Third Framework Programme.

This directory focuses on the grants funded under the first three areas of the FAIR programme i.e. agro-industrial research. They are divided first by scientific and technical area and then by contract number. They are managed by the Health, Food and Environment Unit (formerly the Agro-Industrial Research Unit of the Directorate General for Research (DG XII-E-2)).

The ultimate objective of the FAIR programme was to contribute to securing a better match between production of land- and water-based biological resources and their use by consumers and industry through pre-competitive research, technological development and demonstration.

The FAIR programme was organised into five distinct scientific and technical areas:
Area 1. Integrated production and processing chains. This area addressed the use of raw plant materials, such as timber, fibres, carbohydrates, oils, proteins and speciality chemicals contained in new and traditional crops and trees. The extraction and processing of higher value-added materials from animal and crop agro-industrial wastes were also concerned. This area was broken into the following three sub-areas:

Sub-area 1.1	The biomass and bioenergy chain
Sub-area 1.2	The "green" chemical and polymer chain
Sub-area 1.3	The forestry-wood chain

Area 2. Scaling-up and processing methodologies. This area was closely aligned to the development of the non-food industry and had links with the bioenergy, chemicals and forest production chains of Area 1. Scale-up is intrinsically a process for designing and operating a larger scale system on the basis of the results of experiments with small scale or laboratory models thus permitting a better evaluation of both the technical feasibility and costs. There were three main sub-areas where scale-up problems were addressed:

Sub-area 2.1	Chemical and physical processes
Sub-area 2.2	Bioprocessing
Sub-area 2.3	Control systems

Area 3. Generic science and advanced technologies for nutritious foods. Research in this sector had the major objective to improve the competitive position of the food industry, which is composed of leading multinationals and a wide range of specialist food SMEs throughout Europe. The following were the main areas in which Food Science was addressed:

Sub-area 3.1.	**Consumer nutrition and well-being**
Sub-area 3.2	**New and optimised food materials and nutritious food products**
Sub-area 3.3	**Advanced and optimised technologies and processes**
Sub-area 3.4	**Generic food science**

Area 4. Agriculture, forestry and rural development. This area had six objectives. It aimed to adjust agricultural production methods and systems to the new conditions arising from the reform of the Common Agricultural Policy (CAP); to encourage the development of quality products; to increase efforts to diversify agricultural products and activities; to improve plant and animal health and animal well-being; to support protection, development and exploitation of European forests and the Union' commitment to sustained multifunctional management of forests; to accompany the considerably increased effort in favour of rural development.

Area 5. Fisheries and aquaculture. The overall objective was to provide a sound scientific basis for the balanced, sustainable exploitation of the fisheries resources of the Community and the further controlled development of aquaculture. This was to be achieved by a better knowledge and understanding of the aquatic ecosystem, including the interactions between fishing activities, aquaculture and the environment. Socio-economic considerations were recognised as an integral part of the programme, together with the associated requirement to develop appropriate methodologies for evaluating fisheries and aquaculture policies.

For research centres and researchers, a continuous exchange of know-how and expertise is very important. However, the current lack of mobility across national boundaries is widely recognised as one of the factors hampering the full development of new technologies based in agro-industry. Greater mobility would undoubtedly benefit European companies too, which are always seeking highly skilled personnel. Given that the harmonisation of study curricula and qualifications in Europe can only be achieved in the long run, the effective exchange of young researchers is seen to be vital to the acquisition and/or exchange of competencies across national boundaries.

In this respect, approximately 5% of the resources allocated to the FAIR programme were earmarked for the training and mobility of scientists. Twice a year, candidates were selected with a programme of work falling within the scientific area defined by this programme. By means of training and mobility of researchers the FAIR programme aimed to promote the exchange of scientific and technological know-how between the participating countries and economic and institutional sectors involved in the Programme. Support for training relevant to Community policies and directed at providing the technical skills and for ensuring technology transfer and cohesion among public institutions, industry and primary producers was particularly encouraged.

The training activities could not be completed in the applicant's country of citizenship or in the country in which he or she normally resides. Nevertheless, the choice of the host laboratory was left to the applicant and based on scientific excellence criteria.

This programme offered research grants at **post graduate** (category 20) and **post doctoral** (category 30) level with an emphasis on post doctoral researchers. It also offered grants to **experienced researchers** requiring specific training in a field other than their own, or wishing to carry out specific experiments using scientific facilities or techniques unavailable in their own country or wishing to join a research team in a less-favoured region (category 40). In order to strengthen cohesion and in synergy with the Training and Mobility of Researchers Programme, special grants were available to enable researchers to **return** to a less-favoured region (category R) after a post doctoral grant from the Programme.

The duration of a grant varied from 6 to 24 months for categories 20 and 30 (exceptionally, up to 36 months for category 20) and from 3 to 12 months for category 40 and for up to 12 months for category R.

Research grants are continuing under the Fifth Framework Programme (FP5) for Community activities in research, technological development and demonstration (1998 - 2002) and offered by a number of specific research programmes with different scientific aims such as the Quality of Life programme.

Further information on these activities and necessary application forms and deadlines for submission are all available from: **http://www.cordis.lu/improving/**

These presentations offer useful and direct examples of how the training grants influenced the beneficiaries' scientific careers. Moreover, they constitute a detailed visiting card for each individual, opening up the possibility of new professional contacts and opportunities. To this end, the directory will be widely circulated in both academic and industrial circles throughout the European Union.

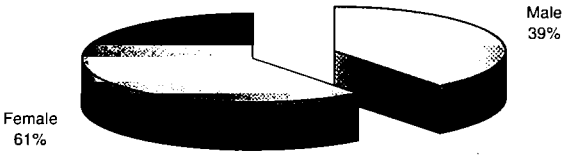
While every effort to ensure that the information contained in this directory is correct, the contact details of some of the supervisors may have changed since going to press. The supervisors contained in this directory correspond to the supervisors named at the time of signing the training grants contract. Moreover, for some grants, the results are only an indication of progress to date, given it was the only information available at the time of compilation.

Statistics on trainees under the FAIR programme

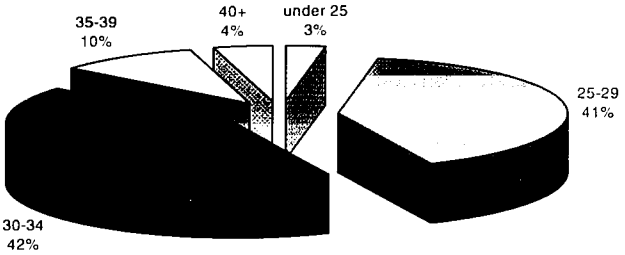
Almost 900 proposals for training grants were submitted to the five areas of the FAIR programme and 315 awarded in total out of which 161 were awarded in the agro-industrial sector.One hundred and fifty fellowships are described in this catalogue and awarded to 144 trainees under the agro-industrial areas of the FAIR programme. Six trainees received follow-on grants to continue their research.

What follows below is some summary statistics about the training grants awarded, broken down by gender, age, nationality, host institution and area of specialism.

Distribution by gender



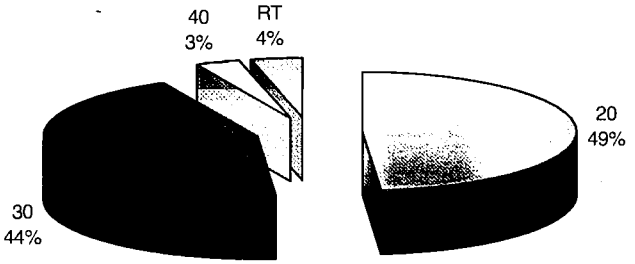
Distribution by age (at start of training)



Nearly two-thirds of the trainees were female. The youngest group of trainees, the under 25s, were very much in a minority (3%). The majority were either 25-29 (41%) or between 30-34 (42%). A small proportion of grant holders were over 40 (4%).

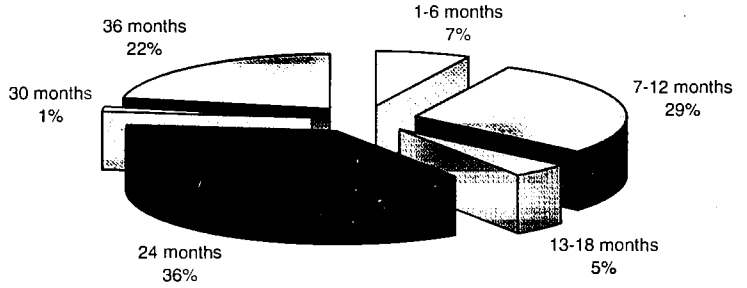
Distribution by category

Nearly one half of all grant holders were awarded either a category 20 (post-graduate) (49%) training grant or category 30 (post-doctoral) (44%). The rest were divided between those given a category 40 (experienced researcher) grant (3%) or a return grant (4%).

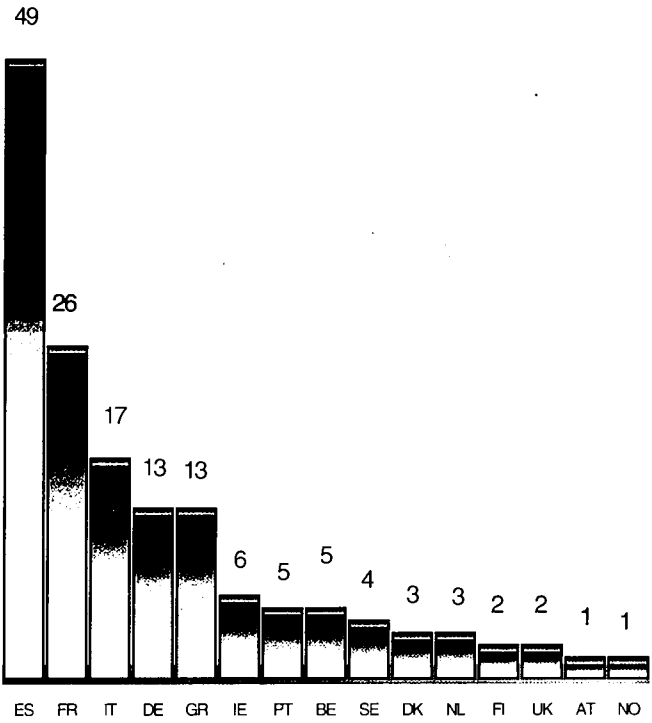


Distribution by duration

Only 7% of grant holders held a grant for between 1-6 months. Over a quarter (29%) were awarded a grant for between 7-12 months with another 5% holding a grant for 13-18 months. By far, the greatest proportion of trainees had a grant for 24 months (36%). The remainder either were awarded a grant for 30 months (1%) or 36 months (22%).

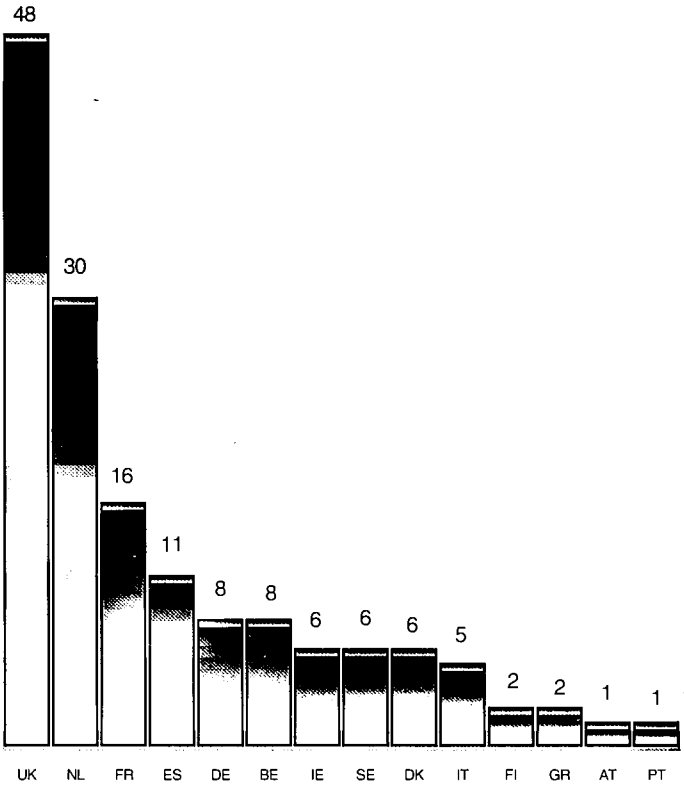


Distribution by nationality of grant holders



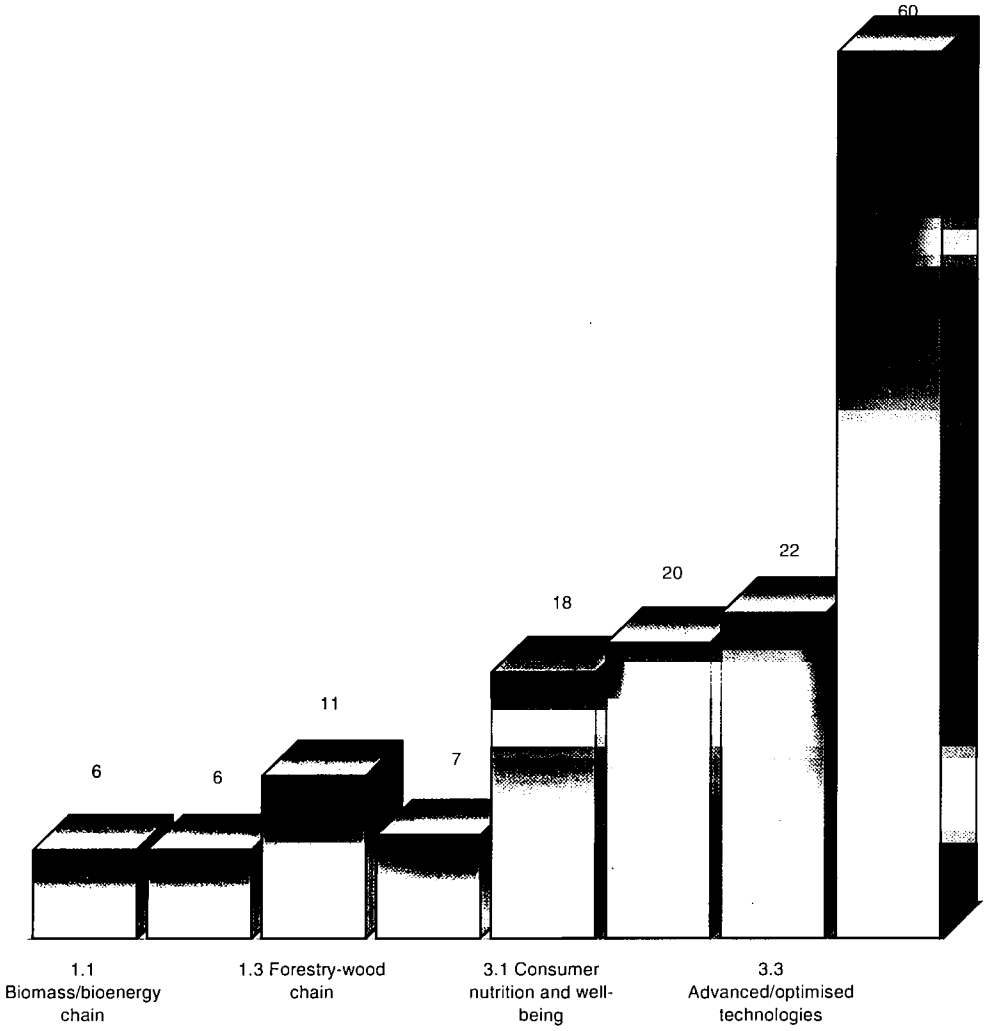
Two Member States, Spain and France, provided the greatest number of trainees with 49 and 26 respectively. The next highest countries where (in decreasing order) Italy, Germany and Greece who had less than half of those from Spain. The remaining countries where spread fairly evenly from 5 to 2 grant holders.

Location of Host Laboratories



The United Kingdom proved to be, by far, the most popular Member State with nearly 32% of all grant holders undertaking their research in UK laboratories. The second most popular country was The Netherlands where almost a fifth chose to conduct their research. France, Germany and Spain had roughly 10% of trainees with Finland, Greece, Austria and Portugal all having a relatively low number of trainees attending host laboratories.

Distribution of grants by scientific sector



The most popular area was area 3, Generic science and advanced technologies for nutritious foods, with 117 grant holders in total. Area 1, Integrated production and processing chains, and area 2, Scaling-up and processing methodologies had 28 and 5 grant holders in total respectively. Of the different sub-areas generic food science (3.4), was the most favoured.

Individual presentations of FAIR trainees

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01/05/1967, French

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Scientific supervisor
GIDLEY, Mike

Fellowship data

Category:	30
Starting date:	17/06/96
Duration:	24 months
Contract number:	FAIR-CT96-5006

Construction of plant cell wall analogues based on *Acetobacter* cellulose polymer interactions and mechanical properties

Objectives:
In order to get a better understanding of the links between pectin structure, cell wall architecture and mechanical properties, the approach of re-constructing *in vitro* composite networks from isolated cell wall polymers has been used.

Summary of achievements:
The composites were based on cellulose synthesised by the bacterium *Acetobacter xylinus*. Cell wall deposition phenomena were mimicked by making pectins available at the point of cellulose synthesis, and the fermentation yield was sufficient to produce enough material to carry out analytical and mechanical tests. The role of ionic and covalent cross-links were studied. In contrast with glucan-binding polymers, pectic substances did not demonstrate a driving force for molecular association with cellulose. A pre-formed pectic network was required for the formation of a composite of the two polymers. The best incorporation was obtained with pectin of intermediate gel strength in the presence of calcium, or a very low number of covalent cross-links.

Though no covalent bonds were present between the two kind of polymers, extensive microstructural interactions were observed. Solid state NMR indicated a large proportion of pectins immobilised by the cellulose network. On the other hand, the presence of pectins modified greatly the cellulose fibril interactions, leading to a drastic increase of the composite uni-axial extensibility. This effect was not directly clue to pectin presence in composites, but was due to pectin effect on cellulose network formation. This suggests that the composition of the matrix in which cellulose is deposited during cell wall synthesis could have a previously unsuspected importance in determining the architecture and subsequently the mechanical properties of the cell wall.

A biaxial extension testing device has been constructed to mimic the effect of turgor pressure on a cell wall. The preliminary results obtained on *Acetobacter* cellulose composites with this device indicated that whilst cellulose remains the main load bearing component the soluble polysaccharides (pectins, xyloglucans) play a very different mechanical role than under uni-axial extension. This demonstrates that cell wall analogues respond in characteristic ways to different applied stresses, and has lead to the development of models linking molecular interactions through microstructural organisation to mechanical properties.

Keywords:
Pectin, Acetobacter, cell wall, cellulose, polymer, xyloglucan

Main Publications/Patents/Participation in conferences:

E. Chanliaud, J.E Brigham, A.H. Darke and M.J Gidley (1997) Plant cell wall analogues: composites of *Acetobacter xylinum* cellulose and pectic substances. In *Plant Biomechanics 1997* (Eds.: G. Jeronimidis and J.F.V Vincent), ISBN 0704912414, Ashford Colour Press, Gosport.

E. Chanliaud, K.M Burrows, G. Jeronimidis and M.J. Gigley (1998) Mechanical properties of primary cell wall analogues submitted to uni-axial and biaxial tensile deformations. Poster presented at the 8t^{h} International Cell Wall meeting, Norwich (UK), 1-5 September 1998.

Plant Biomechanics, University of Reading, UK, 7-12 September 1997

Ferulate 98, IFR, Norwich, UK 8-11 July 1998.

8th International Cell Wall Meeting, John Innes Centre, Norwich, UK, 1-5 September 1998.

VENTURI, Piero
16/02/1964, Italian

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Scientific supervisor
HUISMAN, Willem

Fellowship data

Category:	30
Contract signed:	31/01/1996
Duration:	24 months
Contract number:	FAIR-CT96-5011

Modelling the logistics of harvest of *Miscanthus giganteus*

Objectives:
The largest costs relating to *Miscanthus* production concerns farm machinery, conservation systems and transport and are dependent on such criteria as crop and soil characteristics, and local weather. For accurate cost calculations or optimizations the various costs factors as they depend on workability are needed. Since no data on long-term experience is available, many of these parameters have to be calculated by models. A general method for energy and cost calculation has been developed as a simulation model.

The objective of the research was to select chains for harvest and storage of *Miscanthus* as a biomass crop for energy.

Summary of achievements:
This research can be split up in two main periods: initially data was collected through laboratory and field tests and literature research. Later an optimisation model was built. The first part of the research paid particular attention to the behaviour of machines in the field with the target to select the most interesting chains and to calculate their costs. Furthermore some tests on crop behaviour were carried out, mainly with respect to shoot regrowth and moisture content trend during the harvest season.

Literature investigations focused mainly on the research of energy data sources.

The second part of the research project concerned the design of the model. This model, called APROCHOPS, Agricultural PROduction CHain Optimization Simulation model, is developed and applied first for *Miscanthus* but can also be modified for other crops. It can be applied to different regions so the constraints due to workability, and its effects on cost and energy input variations can be showed as well as a method for the optimization of costs of harvest, storage and transport for various applications. In future the model can be modified and applied also to other crops (hemp, sorghum, etc.) and will become an important tool for long term planning.

It includes various submodels about drying in the field and in storage, soil conditions and effect of soil damage, regrowth of shoots, loss of leaves or stem tops in the course of the spring, decrease of yield of the next year crop due to the removal of shoots, workability and costs. The model was programmed in Delphi. At present the model is working but in some specific situations the outputs do not match real data. For this reason further studies must be carried out.

Keywords:
Model, simulation, harvest, *Miscanthus giganteus,* weather, cost, forecast

Main Publications/Patents/Participation in conferences:

P. Venturi, W., Huisman, J. Molenaar (1996). Cost calculations of production chains of *Miscanthus giganteus,* Internal Report A559 -94211, Dept. of Agricultural Engineering and Physics, Wageningen Agricultural University, The Netherlands

W. Huisman, P. Venturi. J. Molenaar (1996) Volledig loonwerk geeft bij Miscanthus een lagere grondstofprijs (Contractors work in *Miscanthus* production given a lower price of raw material), Landboumechanisatie, 9, 18 sept 1996

W. Huisman, P. Venturi, J. Molenaar (1997) Costs of supply chains of *Miscanthus giganteus.* Industrial Crops and Products, vol. 6, nos. 3,4, pp 353-367.

P. Venturi, W. Huisman, J. Molenaar (1998) Mechanization and costs of primary production chains for *Miscanthus x giganteus* in The Netherlands, Accepted for of Agricultural Engineering Research of March 1998

W. Huisman, P. Venturi, J. Molenaar (1996) Costs of production chains of *Miscanthus giganteus.* presented at "Industrial Crops and Products Congress", Reims, France, 22-24 April

P. Venturi, W. Huisman and J. Molenaar, (1996) Mechanisation of *Miscanthus x giganteus* in Northern Europe. Paper 96D-005, "CIGR/Ag Eng congress", Madrid 23-26 September

FAIVRE-RAMPANT, Odile
27/04/1972, French

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Fellowship data

Category:	20
Starting date:	01/10/1996
Duration:	36 months
Contract number:	FAIR-CT96-5034

Fundamental and technological aspects of rooting in micropropagation: relationship with quality *ex vitro*

Objectives:
This project studied auxin receptors. It aimed to investigate shoot growth limitation and rooting recalcitrance in the *rac* tobacco mutant by hyperauxiny causing a saturation of the auxin-binding sites.

Summary of achievements:
Previous preliminary hormonal characterisation was performed on the *rac* mutant tobacco to better understand its recalcitrance to rooting in comparison with its wild-type homologue. This included ethylene production and accumulation, peroxidase and IAA-oxidase activities, auxin protection, polyamine metabolism.

This hormonal characterisation was completed first by a study of the endogenous auxin contents of the whole shoots and the basal parts of the stems of the two genotypes of tobacco during the growth cycle. More free and conjugated auxins were measured in the *rac* shoots. A maximum of free auxins occurred in both types of shoots: it was visible at day 14 in whole shoots and earlier (day 7) in the basal parts of the wild-type stems. A similar picture was found with the results of the conjugated auxins.

The cytokinin levels were determined at the end of the growth cycle in the whole shoots of the wild-type and the *rac* mutant. At this stage, the auxins to cytokinins ratio was the same for the two types of tobacco.

In order to check the "auxin-resistance" of the *rac* mutant, the wild-type and the mutant shoots have been treated by increasing concentrations of IBA and NAA included in the culture media for three subcultures. The wild-type shoots responded to both auxins showing

increased growth rates at concentrations below 10 µM and growth inhibition at higher concentrations. As expected, the *rac* shoot reactions were very low or inexistent.

Further characterisation of the phenolic pattern in the shoots of the two types of tobacco was conducted by studying the phenylammonia-lyase (PAL) activity on the one hand and the soluble phenolic contents on the other during the course of their multiplication cycle. The *rac* shoots contained more phenolic compounds, due to a higher PAL activity, with a maximum at day 14 in both tobacco. These phenolics were then determined by HPLC: chlorogenic acid was the major phenolic found in both shoots and is known for its auxin protector role. The *rac* shoots contained also more rutine (a flavonoid) which has been shown to be associated with the absence of root formation. Two another phenolics (which had the spectrum of hydroxycinnamic esters) have been found in higher amounts in the *rac* mutant.

In order to check the assumption of the association of the peroxidase minima and putrescine maxima with rooting induction in both tobaccos shoots, and because the wild-type tobacco roots automatically (without auxinic treatment) in the multiplication medium used, the latter medium was modified in such a way that even the wild-type shoots did not root, except after auxin treatment. In the non-rooting (without auxin) conditions, the peroxidase activity hardly changed in both tobaccos. The auxin treatment induced a peroxidase decrease with a minimum occurring after three or four hours in the wild-type and the *rac* shoots respectively.

Keywords:
Rooting, micropropagation, quality, *ex vitro*, auxin, receptor, shoot, growth, tobacco

Main Publications/Patents/Participation in conferences:

O. Faivre-Rampant, C. Kevers, and T. Gaspar (2000) "IAA-oxidase activity and auxin protectors in non-rooting, *rac*, mutant shoots of tabacco *in vitro*". *Plant Science*, in press.
O. Faivre-Rampant, C. Kevers, J. Dommes and T. Gaspar (2000) "Modified hormonal balance in rooting-recalcitrant *rac* mutant tobacco shoots". *Plant Biosystems*. Accepted
O. Faivre-Rampant, C. Kevers, and T. Gaspar (1999). "Endogenous auxin and cytokinin levels in shoots of a non-rooting tobacco mutant". *Biol. Plant.* (Suppl.) S 47.
O. Faivre-Rampant, C. Kevers and T. Gaspar (1998) "Hormonal characterisation of a nonrooting mutant of tobacco" Fourth Meeting of Working Group 5 of Cost 822, Melle, Belgium, 9-13 December 1998.
O. Faivre-Rampant, C. Kevers and T. Gaspar (1999) "Endogenous auxin and cytokinin levels in shoots of a nonrooting tobacco mutant". International Symposium on Auxins and Cytokinins in Plant Development, Prague, Czech Republic, 26-30 July 1999. Poster no. S 4.13.
O. Faivre-Rampant, C. Kevers J.P. Charpentier, C. Jay-Allemand, H. Van Onckelen, T. Gaspar (1999) "Auxin, auxin protector and phenolic levels and IAA-oxidase activity in shoots of a nonvoting, *rac*, mutant *in vitro*" Fifth Meeting of a Working Group 5 of Cost 822, Ancona, Italy, 29 September- 3 October 1999.

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Fellowship data

Category:	30
Starting date:	01/11/1996
Duration:	21 months
Contract number:	FAIR-CT96-5055

The effects of differential compartmentation of the antioxidant defence system on the sensitivity of the bundle sheath and mesophyll cells of maize to low temperature.

Objectives:
The aim of this work is to analyse the distribution of glutathione reductase (GR) between bundle sheath and mesophyll cells of maize leaves from plants grown at optimal and sub-optimal temperatures.

Summary of achievements:
Glutathione reductase was assayed in bundle sheath (BS) and mesophyll (M) cells of maize from plants grown at 20°C, 18°C and 15°C. The purity of each fraction was determined by measuring the compartment-specific marker enzymes, ribulose-1, 5-bisphosphate carboxylase/oxygenase and phosphoenolpyruvate carboxylase, respectively.

GR activity and the abundance of GR protein and mRNA increased in plants growing at 15°C and 18°C compared to those grown at 20°C. In all cases GR activity was found only in the mesophyll fractions of the leaves with no GR activity detectable in the bundle sheath extracts. Immunogold labelling with antibodies specific to GR showed that GR protein was exclusively localised in the mesophyll cells of leaves at all growth temperatures, whereas GR transcripts determined by *in situ* hybridisation techniques were observed in both cell types at equal abundance.

These results indicate that post-transcriptional regulation prevents GR accumulation in the BS cells of maize leaves. The limitation on the capacity for regeneration of GSH in this compartment that this engenders may contribute to the extreme chilling sensitivity of maize leaves.

1.1 The biomass and bioenergy chain

Keywords:
differential compartmentation, bundle sheath, mesophyll, cells, maize, temperature, glutathione reductase

Main Publications/Patents/Participation in conferences:
C. H. Foyer, A. H. Kingston-Smith, C. Pastori, J. Harbinson - Proceedings of the XI Int. Congress on Photosynthesis, Budapest, (1999) (in press – Kluwer Academic Publishers).
C. Pastori, P. Mullineáux, C. H. Foyer (1999) The Plant Journal (submitted).
C. Pastori, C. H. Foyer, P. Mullineáux (1999) The Plant Physiology (submitted).
3rd International Conference on Oxygen Free Radicals & Environmental Stress in Plants, Pisa, Italy.
The Society for Experimental Biology Congress, York, UK.
XI International Conference on Photosynthesis, Budapest, Hungary.
4th International Conference on Oxygen Free Radicals & Environmental Stress in Plants, Granada, Spain.

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STRUIK, Paul

Fellowship data

Category:	30
Starting date:	01/12/1998
Duration:	24 months
Contract number:	FAIR-CT98-5063

Multi-use industrial crops for quality raw material

Objectives:
The renewed world-wide interest in hemp as a source for a variation of industrial products coincides with a breakdown of European subsidies for agricultural primary production. The objective of this project was to provide a preliminary framework for fine-tuning hemp production.

A model to predict and evaluate the effects of environmental factors, agrotechnic and genotype on yield and quality of raw materials from biomass will be created. A holistic approach will be chosen; evaluation of yield and quality parameters will be matched with an overall and integrated assessment of the environmental impact of the production of biomass crops. A further aim of the project is the implementation of the aforementioned model in a Geographical Information System (GIS) to extrapolate the model on a regional base. GIS will be used on a European regional level to map areas particularly suitable for biomass production. Biomass productions will be characterised qualitatively - this will be useful to create an informative base for normative purposes but also to find various applications for the same biomass crop according to its peculiar quality and to specific market requirements.

Summary of achievements:
The first twelve months of research has centred on different aspects of hemp modelling and hemp fibre quality. An extensive bibliographic search on the subject of natural fibres (emphasis on hemp) has been undertaken. To increase the amount of data available on hemp production and hemp fibre quality, a field experiment was set up where two different hemp cultivars were compared over five plant densities, in order to assess the effect of density, genotype and harvest time on yield and quality. During plant growth a series of samplings was carried out in order to determine the dynamics of stem dry matter production and quality.

Preliminary results of the experiment confirm that hemp production is not affected by plant population when the crop density ranges from 30 to 270 plants per m^2. Extended plant and fibre analysis is currently being undertaken. The data collected during the field experiment will be used to validate a hemp growth model.

Keywords:
hemp, fibre, density, genotype, harvest time, yield, quality

Main Publications/Patents/Participation in conferences:

S. Amaducci, M. T. Amaducci, R. Benati, G. Venturi, "*Crop yield and quality parameters of four annual fibre crops (hemp, kenaf, maize and sorghum) in the north of Italy*" – Proceedings of 6th symposium on Renewable Resources for the Chemical Industry together with the 4th European symposium on Industrial Crops and Products, 23-25th March 1999, Bonn, Germany.

P. C. Struik, S. Amaducci, M. J. Bullard, N. C. Stutterheim, G. Venturi, H. T. H. Cromack. (1999) "*Agronomy of fibre hemp (Cannabis sativa L.)*" – Industrial Crops and Products, in press.

N. C. Stutterheim, S. Amaducci, G. Gorchs Altarriba, H. Sankari, "*Quantified framework for Hemp (Cannabis sativa L) production throughout Europe as a tool to fine-tune crop component quantity and quality*" – Proceedings of 6th symposium on Renewable Resources for the Chemical Industry together with the 4th European symposium on Industrial Crops and Products, 23-25th March 1999, Bonn, Germany.

P. C. Struik, S. Amaducci, M. J. Bullard, N. C. Stutterheim, G. Venturi, H. T. H. Cromack, "*Agronomy of fibre hemp (Cannabis sativa L.)*" – Proceedings of 6th symposium on Renewable Resources for the Chemical Industry together with the 4th European symposium on Industrial Crops and Products, 23-25th March 1999, Bonn, Germany.

Natural Fibres Performance Forum (Conference: Plant fibre products, essential for the future), 27-28th May 1999, Copenhagen, Denmark.

Alternative Crops for Sustainable Agriculture, 13-15th June 1999, Turku, Finland.

33 Convegno annuale della Societa Italiana di Agronomia - Le colture "non alimentari", 20-23rd September 1999, Padova, Italy.

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Fellowship data

Category:	20
Starting date:	02/10/1998
Duration:	36 months
Contract number:	FAIR-CT98-5064

Role of pectinases in determining the quality of flax fibres

Objectives:
Most hemi(cellulose) degrading enzymes have a two-domain structure consisting of a catalytic domain and a cellulose-binding domain (CBD). The domains form well-defined units which are separated by a distinct linker region. When introduced in plants, bacterial CBD genes have been shown to alter growth properties, plant architecture or synthetic mechanisms within the plants. Transformed plants should acquire greater biomass, primarily through the acceleration of the process of cellulose synthesis.

This proposal aims to identify the role of cell wall modifying enzymes, more specific pectinases in determining the quality of cellulose flax fibres. This will be accomplished by isolating endogenous flax genes encoding pectic enzymes which will be studied both *in vitro* and *in vivo.*

The objectives are:

- To study the role of flax pectinases by *in vitro* production systems;
- To define the role of specific pectic isoenzymes (such as pectin methyl esterases) during the development of flax plants.

During phase 1, *in vitro* studies will focus on the role of flax-specific pectinase enzymes by isolating full length cDNA sequences and protein production using a suitable eukaryotic expression system. The produced enzymes will be analysed for functionality using a broad range of commercially-available substrates. During phase 2, isolated DNA sequences and *in vitro* produced pectinases will be using to characterise *in situ* during flax development. These

studies will elucidate the role of specific isoforms during fibre development both at a molecular and a biochemical level.

Summary of achievements:
The expression of the CBD in *E. coli* using the pQE60 expression system (Qiagene) was not successful. Even as a 6xhis tagged protein, the product (mw 3,58 Kda) could not be detected. As short peptides (<5Kda) are sometimes unstable in *E. coli*, the CBD had to be expressed as a fusion protein. The sequence coding for the CBD was amplified using new primers and the product was then inserted in the pGEX-4T-1 plasmid (pharmacia) which allowed the production of a GST (gluthation S transferase) fusion protein. This GST, exhibiting a molecular weight of 25 Kda, can be easily expressed in *E. coli*. Cleavage of the protein of interest from the fusion protein can be achieved using a specific protease site. The recombinant plasmid has been introduced in *E. coli* M15 and the expression will be checked by SDS-PAGE.

Once the expression conditions are optimised in *E. coli*, a large-scale production of the CBD will be achieved. Purified peptides will first be purified by affinity chromatography, using the CBD property to bind cellulose. The purified peptide will then be tested for its binding capacities on different (hemi)cellulose substrates.

CBD genes will be inserted first in a plant model (*Arabidopsis thaliana*) to study the cell wall modifications, and then in flax (*Linum usitatissimum*) to modify cellulosic fibres production.

Recombinant protein including a CBD and a pectinase (such as pectate lyase) will be engineered, produced in *E. coli* and then tested *in vitro* to modify fibre surface.

Keywords:
cellulose, cellulose-binding domain, protein, fusion protein, peptide, pectinase, flax

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Category:	20
Starting date:	01/10/1996
Duration:	36 months
Contract number:	FAIR-CT96-5036

The improvement of industrial corn gluten for non-food applications

Objectives:
Maize is one of the major cereal crops in the world and one of its main uses is as animal feed. However, maize is also processed, principally by wet milling. The main purpose of wet milling is for starch production. Maize gluten and zeins are insolubles protein obtained from starch production produced by wet milling. Maize gluten is mainly used as animal feed. It does not possess any functional properties essential for food and non-food applications (coatings, adhesives, surfactants, disposables, binders for paint and ink). Based on its biochemical structure such as high hydrophobicity, low price and high abundance, maize gluten is a potentially interesting source for these applications. This project focused on the improvement of the functional properties by modification. Additionally it aimed to study the effect of the industrial processing of the proteins in the maize gluten, especially the functional properties.

Summary of achievements:
There are two steps in the industrial process that can have an effect on the functionality of the proteins: steeping and the drying of the end product. During steeping the maize kernels are soaked in a dilute sulphuric acid solution for 49-53°C for 36-46 hrs. The effect of steeping temperature, steeping time and amount of metabisulfite was studied. The protein loss increased with steeping time - up to 18.5% of the total protein in the kernel was lost after 48 hrs. The steeping temperature and amount of metabisulfite did not have any significant effect. Surprisingly a high loss of the zeins was found during steeping.

The biochemical properties of the maize gluten dried industrially and under milder conditions (vacuum drying) were also studied. The project concluded that drying did not have an effect on the gluten.

1.2 The "green" chemical and polymer chain

Another part of the project was to improve the functionality of maize gluten. The first step in this task was to make maize gluten more soluble by modification. Several types of modification were screened as physical modification (using additives) and chemical modification (deamidation, hydrolysis and use of glyoxylic acid). When the maize gluten was physically modified in a mixture of guanidine and 1,4 dithioreitol it resulted in a nice waxy film. Unfortunately this film did not have any mechanical strength. Chemical modification by deamidation and reaction with glyoxylic acid did not improve the functionality. However, hydrolysis gave promising results which was optimised according to pH and reaction time. Maize gluten was hydrolysed at different pH from 1-14 for 1, 3, 6 and 12 hrs. The functionality of the hydrolysed samples was characterised according to molecular weight distribution and functional properties such as emulsifying, film and foam forming properties. No effect on the maize gluten was observed at pHs lower than 12. When maize gluten was hydrolysed at pH 12 for 12 hours, the functionality increased, resulting in film forming properties (low strength) and good emulsifying properties. When the maize gluten was hydrolysed at pH 14 for 1, 3, 6 and 12 hrs the samples had good emulsifying and also film forming properties. The emulsifying properties were better than for casein which are considered one of the best protein emulsifiers. Hydrolysing the samples at pH 14 for 12 hrs also produced a foam with high expansion and reasonable good stability.

Keywords:
Corn, gluten, non-food applications, maize, wet milling, emulsifiers

Main Publications/Patents/Participation in conferences:
Effect of steeping on maize recovery and quality. 1999. Poster presentation at Sixth Symposium on Renewable resources for the Chemical Industry, Bonn (Germany) 23-25 March 1999.

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Category:	20
Starting date:	01/03/1997
Duration:	12 months
Contract number:	FAIR-CT96-5059

Pharmaceuticals from *Phillyrea latifolia L*: A new crop in the future of Mediterranean less-favoured areas

Objectives:
Phillyrea latifolia L. represents a characteristic species of the Mediterranean bush (Italian "macchia") and is well known through its long use in traditional medicines for people from Southern Europe. The aims of this project are to develop processes to isolate and identify the constituents responsible for certain properties of *Phillyrea latifolia L*, to investigate the occurrence of possible new chemical structures and to enhance the agro-industrial interest for the utilisation of a potential crop in less-favoured Mediterranean rural areas.

Summary of achievements:
Pharmacological tests on total raw methanolic extracts and on middle polar fraction of *Phillyrea* showed a remarkable aptitude to modulate classical pathway of the human complement system.

Photochemical investigations of these fractions permitted the isolation and identification, through means of sophisticated techniques, of some phenolic compounds: apigenin and luteolin derivatives and the propanoidglycoside verbascoside. In particular a relative consistent amount of the unusual 4'-O-substituted flavon *Luteolin-4'-O-β -glucoside* was isolated. Most of these were never found before in the Genus of *Phillyrea*. All the pure obtained compounds were pharmacologically tested and were shown to have interesting anti-inflammatory properties thus confirming the uses of *Phillyrea* decoctions in the Mediterranean ethnomedicine and the properties of the whole extracts of the species.

Clinical and toxicological tests are proposed, in order to evaluate the possible utilisation of *Phillyrea latifolia L.* leaves in forms of macerates and/or Galenic preparations or in smooth anti-inflammatory pharmaceutical natural products. A cultivation of the plants in the less

favoured areas of Southern Europe could represent an interesting economic activity and may result in a possible way to recuperate degraded semi-arid territories.

Keywords:
Phillyrea latifolia, traditional medicine, phenolic, anti-inflammatory

Main Publications/Patents/Participation in conferences:
A. Pieroni, Y. Huang, A. J. Vlietinck, D. Heimler (1998) Anticomplementary activity of extractives from *Phillyrea latifolia* L. leaf" – Fitoterapia, Milan, Italy (accepted).
A. Pieroni, D. Heimler, Y. Huang (1998) A TLC method to separate and identify flavons from flavon glycosides and from biflavons in vegetal extracts – Journal of Planar Chromatography, 11, 230-232, Budakalász, Hungary,.

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Category:	30
Starting date:	13/06/1997
Duration:	24 months
Contract number:	FAIR-CT96-5068

Molecular farming of therapeutic antibodies in plants

Objectives:
The enormous potential of antibodies in human health has created demand for large amounts of functionally active forms of recombinant antibodies (rAbs) produced at low cost with no risk to the patient during therapeutic applications. In this project, the tumour specific antibody T84.66 will be studied because it recognises a major target of colon, breast and lung adenocarcinomas, the carcinoembryonic antigen (CEA), and is suitable for use in diagnosis and therapy.

An aim of the project is to generate fusion proteins comprising a single chain antibody scFvT84.66, derived from T84.66, genetically fused to the biological response modifier Interleukin 2 or Pseudomonas exotoxin. These fusion proteins will then be expressed in tobacco. In addition, genetic linkage of the tumour specific scFvT84.66 to another scFv with specificity for cytotoxic T-cell markers will be carried out to potentially enhance the tumour killing properties of the rAb.

Summary of achievements:
Eight different plant expression constructs have been obtained by cloning the scFv84.66 gene using a combination of the following elements: a 5'UTR (CHS or Omega), a leader sequence (LPH or LPL – codon optimised leader peptides of the heavy chain and light chain genes of mAb24), a 3' tag (His6 or a KDEL EER retrieval signal), and the 3' UTR of tobacco mosaic virus. These eight constructs have been cloned into the pSS plant expression vector. Functional expression of scFv84.66 using these constructs has been tested by *Agrobacterium* mediated transient expression in tobacco leaves. Plant expressed scFvT84.66 completed against the mAb T84.66 for binding to CEA/NA3 protein in competition ELISA, indicating that the recombinant protein is functionally expressed. Sufficient quantities of the proteins were generated for affinity purification of scFvT84.66, coupled to a 6-histidine tag, and its characterisation. Tobacco plants have been stably transformed with the constructs and the

antibody is functionally expressed up to the currently analysed T2 generation. To develop a direct assay for detecting scFvT84.66 expression, chicken IgY antibodies have been raised using plant expressed or bacterial scFv84.66.

Fusion proteins between IL2 and scFvT84.66 have been generated and tested by transient expression in tobacco leaves, where the antibody domain of the fusion protein is functional and the activity of the Il2 domain is under investigation. Stably transformed tobacco plants expressing this fusion protein are currently being generated and fusions of scFvT84.66 with Pseudomonas exotoxin and CD8 are in progress.

Keywords:
Carcinoembryonic, antigen, antibody, engineering, plant expression, single chain antibodies

Main Publications/Patents/Participation in conferences:
IXth International Plant and Cell Tissue Culture Congress – 14-19th June 1998, Jerusalem.
R. Fischer, C. Vaquero, M. Sack, J. Drossard, N. Emans, U. Commandeur, (1999) Toward Molecular Farming in the future: Transient protein expression in plants – Biotechnology and Applied Biochemistry (in press).
C. Vaquero, M. Sack, J. Chandler, J. Drossard, F. Schuster, M. Monnecke, S. Schillberg, R. Fischer (1999) Transient expression of a tumour specific single chain fragment and a chimeric antibody in tobacco leaves – PNAS (in press).

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Category:	20
Starting date:	01/10/1997
Duration:	36 months
Contract number:	FAIR-CT97-5008

Protein engineering of a xylanase to improve its agricultural uses

Objectives:
The overall objective of this research project is to use knowledge of the structure/function relationship of xylanases to (a) improve the thermostability of industrially important hemicellulases and (b) alter the catalytic residues in the active site of xylanases, and increase their resistance to organic solvents so that these enzymes can be used to synthesise molecules containing polysaccharides which will be of considerable use in the food industry.

Summary of achievements:
The first year of the training programme focussed on the modification of active site residues that have the potential to alter the catalytic activity of the model enzyme, xylanase A (XYLA) from *Pseudomonas*. Previous studies in the laboratory have shown that XYLA can accommodate seven xylose residues in its active site. The region of the enzyme that binds one xylose moiety is known as a sub site. Cleavage of the substrate occurs between subsites -1 and +1, and the number assigned to the other subsites indicates how close it is to the site of bond cleavage. In the first year of the project site-directed mutagenesis has been used to substitute several active residues with alanine.

The data obtained can be summarised as follows:

- Disruption of either subsite -2 or +1 by mutations E43A and F181A, respectively, reduced the activity of XYLA against xylooligosaccharides with a dp>7 50-100 fold. In contrast these mutants retained full catalytic activity against xylan.
- The generation of xylanases whose end-products are xylooligosaccharides, rather than xylose and xylobiose could have important implications in the agriculture and food industries where oligosaccharides are playing an increasingly important role.
- In addition to modify the reaction products produced by XYLA, the importance of Trp-313 and Asp-248, which are located at the +1 subsite, has also been probed. These residues were substituted for alanine and the biochemical properties of the mutant enzymes were evaluated. Both W313A and D248 A were 100-fold less active against xylan_than native XYLA. Interestingly both the *kcat* and *K*m of these enzymes against

substrates such as dinitrophenyl-ß-cellobioside, which have excellent leaving groups, were reduced 100 fold suggesting that the mutations were influencing the position and/or ionisation state of the catalytic nucleophile, Glu-127, rather than the catalytic nucleophile.

Keywords:
Xylanases, Xylan, nucleophile, protein engineering, agricultural use, active site, catalytic site, enzyme, *Pseudomonas*

Main Publications/Patents/Participation in conferences:
S.J. Charnock, T.D. Spurway, H.F. Xie, M.H. Beylot, R. Virden, R.A.J. Warren, G.P. Hazlewood, and H.J. Gilbert (1998) The topology of the substate binding clefts of glycosyl hydrolase family 10 xylanases are not conserved. Journal of Biological Chemistry 273: 32187-32199.

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Category:	20
Contract signed:	18/03/1998
Duration:	36 months
Contract number:	FAIR-CT98-5014

Development of starch-based nanoparticles: structure, colloidal and rheological properties

Objectives:
Not stated

Summary of achievements:
In a previous study, it was found that surface charges should contribute largely to the rheological behaviour of starch-based particles (cross-linked with epichlorohydrine) in salt-free aqueous suspensions.

Further research continued with performing rheological experiments on dilute and semi-dilute suspensions, and varying the ionic strength, the cross-linking degree of the particles, and the type of the cross-linking agent (using trisodium trimetaphosphate). Supplemental experiments have been also performed on charged, rigid silica spheres in order to establish a parallel between the rheological behaviour of particles with a well-defined structure and starch-based hydrogels.

It has been found that starch-based particles behave like polyelectrolytes in aqueous suspensions, most likely due to the dissociation of hydroxyl groups carried on the glucose units. Charged rigid spheres also produce the polyelectrolyte effect (decrease of the reduced viscosity versus the particle concentration) at very dilute regimes.

Two important conclusions can thus be put forward: on one hand, the polyelectrolyte effect is a one-particle problem, involving only the number density of surface charges contributing to the Debye length and thereby the effective volume of the particles. On the other hand, the polyelectrolyte effect is a generic phenomenon characterising charged particles in suspensions.

Keywords:
starch, nanoparticle, structure, colloid, rheology, suspension

Main Publications/Patents/Participation in conferences:

Y. Dziechciarek, J.J.G. van Soest, A.P. Philipse. 2000 Viscosity of starch-based sub-micron hydrogels and charged, rigid silica spheres. A comparative study of the "polyelectrolyte effect", Chemische Wetenschappen Dagen (vloeistof en grensvlakken), Lunteren, The Netherlands, 28-29 February 2000

J.J.G. van Soest, E. Westerweele, Y. Dziechciarek. 2000 Starch Microparticles or Microgels: A New Way for Adjusting Rheology and Thickening in Food and Cosmetic Application Systems. Polymerix 2000, Rennes, France, 7-9 June 2000

Y. Dziechciarek, J.J.G. van Soest, A.P. Philipse, Chemische Wetenschappen Dagen (vloeistof en grensvlakken), 28-29 February 2000, Lunteren, The Netherlands

J.J.G. van Soest, E. Westerweele, Y. Dziechciarek. Polymerix 2000, 7-9 June 2000, Rennes, France

J.J.G. van Soest, Y. Dziechciarek, A.P. Philipse, IXr" International Starch Convention, 13–16 June Cracow, Poland

Y. Dziechciarek, J.J.G. van Soest, A.P. Philipse, 10 International Association for Colloid and Interface Science Conference, 23-28 July 2000, Bristol, UK

J.J.G. van Soest, Y.Dziechciarek, Plant Polysaccharides, 23-25 August 2000, Wageningen, The Netherlands

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Category:	20
Contract signed:	24/07/1998
Duration:	24 months
Contract number:	FAIR-CT98-5057

The chemical composition of non-textile flax fibres

Objectives:
The aim of the project is to: (1) characterise the hemicellulose and lignin fraction of flax fibres; (2) develop a strategy for determining the chemical composition of flax fibres; (3) investigate the chemical structure of flax fibres depending on variety (linseed/textile) at different harvesting times; (4) investigate the effect of different pre-processing routes (retting and decorticating) on surface chemistry and chemical composition (key parameters).

Lignin and hemicellulose characterisation
Lignin will be isolated from mature fibres by acid hydrolyses and as Bjorkman lignin. It will be characterised by elemental analysis, HPLC, UV and IR/FTIR spectra and compared with lignin *in situ* and with wood lignin. Non-lignin, aromatic substances will be determined by HPLC. The acetyl bromide method for determining true lignin content will be adjusted and developed for flax lignin. Hemicelluloses are isolated by a multiple-step sequential extraction. The fractions will be analysed for sugar content and uronic acids using HPLC and GLC of the derivatives. The fractions will be further analysed using GPC and/or NMR. A strategy for determining hemicellulose content in flax fibres will be developed.

Chemical characterisation of fibres at different harvest times
The total chemical composition for the different raw materials will be determined using the techniques developed in the project combined with established methods for wood chemistry analysis of extracts, ash and cellulose.

The effect of pre-processing on fibre chemical characteristics
The identified key parameters for characterisation of flax fibre chemistry are used to characterise differences in chemical composition due to different processing schemes (combinations of retting degree and mechanical decorticating). Furthermore, the cellulose DP/levelling-off DP will be determined to identify excess cellulose degradation. The effect of

retting will be followed using CEC, NIR and FTIR analysis. The surface chemistry characteristics of fibres will be determined using established methods. Surface energy will be determined using wetting analysis. Microscope FTIR, ESCA and CLSM will be also used. An attempt will be made to relate the different surface chemistry characteristics to the degree of removal of chemical components as an effect of the different processing routes.

Summary of achievements:
To date, no information available

Keywords:
Composition, non-textile, flax, fibres, lignin, retting, cellulose, processing, decorticating

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Fellowship data

Category:	30
Starting date:	15/04/96
Duration:	12 months
Contract number:	FAIR-CT96-5003

Transcriptional regulation and tissue specificity of walnut shoot chalcone synthase genes

Objectives:
The rooting of walnut shoots is hampered by trees ageing. This is an obstacle to amelioration and mass propagation programmes. Flavonoids are good markers of plant development and it has been shown that two flavonols, namely myricitrin and quercitrin, and condensed tannins accumulate during the growth period in aged walnut shoots. It has also been shown that the addition of myricitrin to *in vitro* culture medium diminishes rooting ability.

Flavonois accumulate mainly in leaves, buds and phloem. An enzymatic study has shown that phenylalanine ammonia lyase (PAL), the first enzyme of the phenylpropanoid pathway, does not control flavonoid accumulation but is linked to the lignification process. On the other hand, chalcone synthase (CHS), the first enzyme of the flavonoid pathway regulates the accumulation of flavonoid in phloem, buds and leaves.

Consequently, this research aims to improve knowledge of walnut ageing and related consequences on rooting ability through the regulation level of CHS expression in the different tissues of a walnut shoot.

Summary of achievements:
Two full length chalcone synthase cDNAs, about 98% identical, at the nucleic acid level were characterised from leaves of the adult walnut tree. The open reading frame sequences coded for two 389 amino acid polypeptides, 99.5% identical. The two deduced amino acid sequences exhibited the typical CHS consensus in the middle of the sequences and the essential amino acids for CHS activity. Additionally, they showed a high degree of identity to CHS of *Pyrus malus* (91%) and *Pinus sylvestris* (85%). As with CHS from other trees, some

parts of the predicted protein sequences from the walnut CHS are similar to the stilbene synthase protein sequences (between 70 and 76% identity).

Southern blot analysis indicated that CHS in walnut is encoded by a small gene family consisting of maximum three members. In addition a CHS promoter of 1.7 kb length has been isolated, which can be sequenced now.

The expression of CHS during growth of adult and rejuvenated walnut shoots was investigated. A 1.5 kb CHS transcript was detected in leaves, buds, fibre and bark, whereas no transcript was detected in wood and medulla. Growth of walnut shoots resulted in a transient increase of leaf and liber CHS transcripts which was much more pronounced in adult than in rejuvenated tree shoots These data, correlating with CHS enzyme activity and flavonoid accumulation, indicate a tissue specific influence of age on flavonoid biosynthesis at the CHS mRNA level.

Keywords:
Walnut, shoot, chalcone synthase, flavonoid, myricitrin, quercitrin

Main Publications/Patents/Participation in conferences:
A-C. Claudot, D. Ernst, H. Sandermann, A. Drouet (1997) Chalcone synthase activity and polyphenolic compounds of shoot tissues from adult and rejuvenated walnut trees. Planta, in press

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Category:	30
Contract signed:	03/04/1996
Duration:	24 months
Contract number:	FAIR-CT96-5004

Genetic fingerprinting of hardwood trees and application of molecular techniques to characterise gene expression and physiological states

Objectives:
The initial aim of the project was to produce arbitrary molecular markers (RAPDs) for five species of broadleaved trees (*Quercus petreae*, *Q. robur*, *Fraxinus excelsior*, *Prunus avium* and *Acer pseudoplatanus*) by the use of the PCR method and arbitrary oligonucleotide primers. The technique although useful for a great number of species has been widely criticised for the lack of reproducibility from laboratory to laboratory and from user to user. It was also judged inappropriate as a tool for evaluating biodiversity.

At the start of the project, the general trend in plant genetics was a move to the characterisation and the use of microsatellite loci as molecular markers. It was said that these microsatellite polymorphisms displayed all the required criteria for being the best markers available.

A molecular marker must be highly polymorphic, display a co-dominant inheritance allowing homo- and heterozygotic states in diploid organisms to be identified, occur frequently, be evenly distributed in the genome and display a selectively neutral behaviour. It also must be of easy access, quickly assayed, be highly reproducible and must allow easy exchange of data between laboratories. All these qualities are displayed by the microsatellite loci.

The emphasis shifted slightly from a project entirely based on RAPD to a new approach involving the analysis of microsatellites. These reasons behind this change were: the first sequences of microsatellite regions for *Quercus robur* and *Quercus macrocarpa* were published or available in GenBank; the development and the accessibility of the more recent method of DNA library enrichment in microsatellites; and the recent availability of a new sequencing device with precast sequencing gels.

1.3 The forestry-wood chain

Summary of achievements:
The elite collections for different trees, made available by the Irish forestry company, Coillte, enabled the analysis of a greater genetic diversity which could be improved and optimised in the future for the benefit of new forests management, with the use of these microsatellite markers.

The choice of microsatellites against RAPD has proved to be worthy and very rewarding in terms of produced knowledge.

These results, particularly the *Fraxinus* microsatellite sequences and amplifying primers, have been made available as quickly as possible for the benefit of the scientific community through publication in GenBank and the publication of primers in TreeGenes Database. This new knowledge will be very useful for genetic structure, pollen dispersal, parentage analysis and taxonomy studies in a broad range of *Fraxinus* species and other *Oleaceae* species in which some of these microsatellites markers of *F. excelsior* have been shown to amplify.

These microsatellites are already being used by one French team and one Greek team. Three other European teams have expressed intention of using these sequences.

Keywords:
Genetic fingerprinting, hardwood trees, molecular markers, gene expression, physiological, diversity

Main Publications/Patents/Participation in conferences:
G.C. Douglas, J. McNamara, F. Lefort & C. Barren (1997) Ways to accelerate the genetic improvement of our broadleaved species. Irish Timber and Forestry September/October 1997, pp38-39 (ISSN 0791 7422)
F. Lefort (1997) Survey of different commercial DNA extraction kits and microscale methods for DNA extraction from oak, ash tree, sycamore and cherry tree leaves. Molecular Screening News 10:103-104.
F. Lefort and G.C. Douglas (1997) A simplified method to purify a pharmaceutical grade wax for use in the polymerase chain reaction (PCR). Biologie 52(6):803-806.
C. Barret, F. Lefort and G.C. Douglas, (1997) Characterisation of oaks by RAPD and microsatellite PCR. Seedlings, shoots, epicormic shoots crown shoots and in vitro cultures from mature trees. Scientia Horticulture, 70(4):269-366.
F. Lefort and G.C. Douglas (1997) An efficient method of DNA isolation for microsatellite analysis from mature leaves of four hardwood tree species. Annales des Sciences Forestieres.

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Category:	30
Starting date:	03/04/1996
Duration:	24 months
Contract number:	FAIR-CT96-5007

Induction of morphogenesis (somatic embryos and/or adventitious shoots) from thin cell layers of cork oak (*Quercus suber*) and chestnut (*Castanea sativa*)

Objectives:
The main objective of this work was to establish reliable micropropagation and rooting protocols for chestnut and of cork oak. These protocols will be useful for breeding programmes and, in particular, for genetic transformation experiments.

Summary of achievements:
Shoot formation was obtained from internodal explants (longitudinal sections and longitudinal segments) of chestnut. The presence of a higher cut surface area for bacterial contamination in these explants may improve the results obtained in genetic transformation of chestnut.

From sections isolated at the proximity of the nodal region, shoot regeneration and these shoots were induced to root *in vitro*. These results are important as with this method the number of shoots regenerated can be increased and induced to root *in vitro*. This would enable *in vitro* plants to be transferred to soil and allow further experimentation in this field, essential for breeding and reforestation programs of chestnut.

As for the cork oak, structures resembling floral buds in shoots regenerated from *in vitro* material or from in-field growing material were obtained. Flower induction of cork oak under *in vitro* conditions may be valuable in breeding programmes. However, histological studies remain to be earned out to identify the morphological and anatomical structure of these buds. Nevertheless these preliminary results are interesting as the precocious development of axillary floral buds from juvenile material of cork oak were obtained. Further experimentation is necessary before these results can be confirmed.

In addition somatic embryo formation was induced from *in vitro* cork oak shoots maintained in culture for four years. This experiment has to be repeated to obtain shoot regeneration from

the embryo. These results once achieved may be useful for genetic transformation experiments.

Keywords:
Micropropagation, cork, oak, chestnut, explants, *Quercus suber*, *Castanea sativa*, regeneration

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Category:	RT
Starting date:	29/01/1998
Duration:	12 months
Contract number:	FAIR-CT96-5041

Valorisation du sorgho papetier dans l'industrie papetière à l'aide de la technologie d'extrusion Bi-Vis. Etude des carateristiques papetières

Objectives:
Broom sorghum contains 10 - 20 % of pith in the stalk. The pith is responsible for problems of lower quality of pulp and lower amount of pulp requiring more processing. Processing broom sorghum by extracting pith using the Bi-Vis method developed by the Centre Technique du Papier, allowed the production of pulp that could be used for cardboard. Current solutions require the mechanical extraction of pith using Pallman machines to produce good quality cardboard.

During this study a Bi-Vis extrudeur BC 45 was used, allowing for the processing of the entire sorgho resolving the difficulty arising from the extraction of pith.

Summary of achievements:
The results delivered by the work produced within Athens Papermill, reached the objectives. It demonstrated that the entire plant can be used for the fabrication of cardboard paper without the need for extracting pith. Pulp obtained after refining with Sprout-Waldron, is characterised by a similar mechanical resistance as that obtained through the C.T.P. pith extraction method.

The economic advantages are considerable compared with existing extraction methods.
- The output of fibre matter through Bi-Vis is close to 85 %,
- Energy and the consumption of reagents is lower than with classic methods,
- Recycling of the extract could minimise the production of unwanted components and reduce the need for reagents

Keywords:
Pulp, cardboard, paper, broom, sorghum, extraction, pith

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Category:	20
Starting date:	06/02/1997
Duration:	36 months
Contract number:	FAIR-CT96-5050

Molecular genetic analysis and tagging of specific genes controlling wood quality in walnut (*Juglans* spp)

Objectives:
The main objective of this project is to tag genes controlling wood quality in walnut *(Juglans spp)*. Wood quality is an important trait and the formation of heartwood is a key process as it affects colour and durability of wood. While some biochemical mechanisms of wood colouring are already described, no information is available on gene expression during heartwood formation in walnut. In this work, genes involved in heartwood formation will be tagged as genes potentially related to wood quality. This strategy is based on two approaches:

- RT-PCR and gene expression study for candidate genes controlling phenolics metabolism;
- Construction and screening of cDNA libraries. The selected cDNA clones will provide homologous probes and sequence information to be used in mapping programmes, as well as in wood physiology studies.

Summary of achievements:
The formation of heartwood has a strong influence on the quality of walnut wood. Nevertheless, very little information is available on gene expression during heartwood formation. The research activity has focused primarily on gaining a deeper knowledge of the molecular basis of this process by cloning genes expressed in aged sapwood (transition zone) transforming into heartwood.

The application of molecular analyses to aged wood tissues has required a special approach for the set up of suitable techniques. Taking into account the chemical composition of wood extractives, the key enzymes of phenylpropanoid pathway and flavonoids biosynthesis have been targeted as candidate genes, that could potentially control wood quality. The expression of specific genes was investigated by Reverse Transcriptase-PCR (RT-PCR) in xylem tissues of black walnut *(Juglans nigra* L.) trees. By this approach it was possible to detect the

expression of genes encoding 4-Coumarate:CoA ligase (4CL), Chalcone synthase (CHS), Flavanone 3-hydroxylase (F3H), and Dihydroflavonol-4-reductase (DFR) in the transition zone of *Juglans nigra.*

The RT-PCR products were cloned and sequenced and the obtained sequences confirmed the identity of the cloned cDNA fragments. These results represent a technical breakthrough as far as this particular tissue is concerned. This is the first report showing the activation of phenylpropanoid metabolism at the transcription level in the transition zone.

In order to describe the temporal dynamics of heartwood formation, walnut trees were cut and collected for analyses every 6 weeks along the year. According to the results of chemical analyses and anatomy observations, the study of gene expression was focused on *Juglans nigra* trees collected in July and January. Indeed, these periods should represent highest and lowest peaks in the accumulation of wood extractives. Flavonoids compounds play an important role as wood extractives. Thus the expression of genes specific of flavonoids biosynthesis (CHS, F3H, DFR) is being studied by RT-PCR in young and aged wood tissues. The expression of these genes was detected in all tissues analysed. By differential cleaving of RT-PCR products with restriction enzymes it was possible to show that only two members of CHS multigene family are expressed in walnut wood tissues. These analyses are now extended to F3H and DFR. A quantitative analysis of gene expression is being carried out on the same samples by Northern Blotting hybridisation. A severe limitation for this type of analysis is the very low yield of RNA extracted from aged wood tissues (1500 to 3000 folds lower than in young tissues).

These results may be a starting point for promising perspectives. The cloned cDNA fragments provide sequence information and homologous cDNA probes. Future evolution of the present work will be the construction of a cDNA library from transition zone. This library will be screened to tag genes differentially expressed in this tissue. Selected cDNA clones will be used as Expressed Sequence Tags (EST) in studies of genetic variability, in mapping programmes, as well as in wood physiology studies.

Keywords:
Heartwood, walnut, *Juglans* spp, gene expression, phenolics, metabolism, cDNA, enzymes, flavonoids

Main Publications/Patents/Participation in conferences:
I. Beritognolo, C. Jay-Allemand and C. Breton (1998) Study of gene expression during heartwood formation in walnut (*Juglans* spp). Quatrième rencontre de biologie moleculaire des ligneux. Bordeaux, France, March 25-26, 1998 (oral presentation)
P. Label, I. Beritognolo, C. Breton, J.P. Charpentier, and C. Jay-Allemand (1999) First results of the study of cambial activity and xylem differentiation in walnut SEB Annual Meeting. Edinburgh, UK, March 22-26, 1999 (oral presentation)

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Category:	20
Starting date:	01/01/1998
Duration:	24 months
Contract number:	FAIR-CT97-5022

Stem modelling and simulation of sawmill outputs in *Pinus pinaster* for optimising the wood conversion chain

Objectives:
The project deals with the technological quality of pinewood (*Pinus pinaster* Ait.), using material with known silvicultural management, by modelling stem wood quality and simulation for the optimisation of sawmill outputs. The project will involve the conversion of real stems into computer models and the use of simulation software for converting stems into end products. The project, will use a simulation software developed by the host institution (VTT - Simulog) and apply it to the *Pinus pinaster* Ait., the most important species in Portugal for timber production.

Summary of achievements:
The application of image processing techniques for Maritime pine (*Pinus pinaster* Ait.) wood resulted in an accurate mathematical 3D model for the studied logs. The WOODCIM® sawing simulation process has shown potential to optimise the operating instructions in the sawing process of this wood species and to clarify the influence of different production variables on the recovery of sawn timber.

The model allowed the internal defect distribution and features for Maritime pine to be studied. The analysis clearly shows that the studied material has a large proportion of defect (knot) free wood. The average volume percentage of knots compared to the total volume of the log varies from 0.06% for butt logs to 1.7% for top logs. The results show that correct management of Maritime pine forests can provide Portugal with high quality wood, ensuring competitiveness of the Portuguese wood industry through value added production.

1.3 The forestry-wood chain

Keywords:
Pinus pinaster, sawmill, optimisation, simulations, wood, pine

Main Publications/Patents/Participation in conferences:
I. Pinto, H. Pereira, A. Usenius (2000) Characterisation of *Pinus pinaster* Ait. stems – In preparation.

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Category:	20
Starting date:	10/11/1997
Duration:	36 months
Contract number:	FAIR-CT97-5023

Roles of polyphenol oxidases and peroxidases in wood colour quality of walnut (*Juglans* spp)

Objectives:
Concerning the production of high quality timber of walnut (*Juglans* sp.) in Europe. The general objective of the research concerns one of the main characters of the walnut wood quality, namely its colour.

Heartwood formation is related to the process of ageing of xylem cells. The ultimate stage of this process is the death of the last living sapwood cells (parenchyma cells). The coloured compounds produced during this stage are the result of a series of oxidations and polymerizations by the way of chemical and enzymatic reactions. The choice of studying peroxidases and polyphenoloxidases (PPO) is linked to the role of these enzymes in different browning processes of tissues in particular physiological conditions (defense against pathogens, fruit maturation, etc.).

There are three scientific objectives to understand the role of peroxidases and PPO in the browning of wood. These objectives are:
(i) the determination of which oxidase and which substrate of this enzyme plays a role in browning;
(ii) the precise localisation of oxidase activities;
(iii) to understand the mechanisms of interaction between enzymes and substrates leading to the colouring of wood.

Summary of achievements:
With respect to the localisation of peroxidases and polyphenoloxidases activity, the project found that classic histology and immunohistolocalisation yielded detailed information about the localisation of two families of oxidative enzymes.

Due to where they were found, PPOs do not seem to have a direct function in the process of heartwood formation. On the other hand, the localisation of peroxidase activity and appearance of colouring at the sapwood/heartwood interface seems highly significant. It was also shown that the same xylem band is made up of different "phenol cells " and "peroxidase cells". This result is only slightly surprising given the strong inhibiting capacity of many phenolic compounds, in particular with respect to peroxidase activity.

At the cellular level (banded parenchyma, paratracheal parenchyma), the peroxidase gaïacol-dependent activity appeared to be confined to the vacuole, although the 3,3`-diaminobenzidine test reveals more that one cytoplasmic form. The isoelectrofocalisation gels revealed the presence of many peroxidase activity bands which were detected in the transition zone, including one band indicating high activity with an isoelectric point of 7.7. This band is also present in the cambial zone of *Juglans nigra*, but with even higher activity.

Keywords:
Xylem cells, enzyme, colouration, wood, walnut, *Juglans*, substrate, peroxidase, polyphenoloxidase

Main Publications/Patents/Participation in conferences:
Peroxidases and walnut (*Juglans nigra* L) Wood colouring. *Acta Horticulturae* (*in prep.*)
Histolocalisation and purification of peroxidases in relation with heartwood colour. In Second General Meeting European project FAIR CT 96-1887-Sorrento, Italy, 14–17 April 1999, Oral Communication
Peroxidases and walnut (*Juglans nigra L)* Wood colouring in the Fourth International Walnut Symposium-Bordeaux, France, 12-16 September 1999, Poster

Links with EC projects:
PL 96-1887
This project is part of the European FAIR project n° PL 96-1887 (W-Brains)

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Category:	30
Starting date:	01/06/1998
Duration:	18 months
Contract number:	FAIR-CT98-5009

Methods to improve the selectivity of lignin degradation in biopulping processes

Objectives:
The goal of this research is to develop new methods to improve the selectivity of biopulping processes. The strategies considered were based on removing nitrogen from wood chips by both incubation with sap-staining fungi and pre-treatment with proteases. The investigation was based on three major research tasks:

- Wood pre-treatment with sap-staining fungi that reduce the nitrogen availability for subsequent biopulping.
- Wood pre-treatment with proteases and washing of liberated amino acids to reduce the nitrogen availability in subsequent biopulping.
- Optimise the biopulping of nitrogen depleted wood chips.

Summary of achievements:
The effectiveness of reducing the N content in lignocellulosic materials as a means to enhance the selectivity of lignin degradation in biopulping processes was evaluated.

To remove the N from woody materials they used pre-inoculation with a sap-stain fungus *(Ophiostoma piliferum)*, solvent extraction and enzymatic pre-treatment with proteases. The best results were obtained with the protease treatment that reduced up to 68% of the original N in hemp stem wood and by water extraction that decreased the N content by 39%.

A rapid screening method to assess wood delignification selectivity by different white-rot fungal strains was developed and optimised. *Bjerkandera* sp. showed the highest selectivity in the degradation of Klason lignin in both hemp stem and poplar wood.

Hemp stem wood containing different levels of N (from 32% to 500% of the initial N content in fresh material) was inoculated with *Bjerkandera* sp. The results obtained showed a strong positive correlation between the level of N in hemp and the selectivity of lignin degradation.

The highest selectivity index (i.e. ratio of percent lignin removal to percent holocellulose removal by the fungus) was determined in the treatment corresponding to the lowest N content in the substrate (hemp pre-treated with the protease). A sharp decrease in the selectivity of Klason lignin degradation was observed with increasing N content in hemp. High N contents also contributed to enhance the overall degradation of the lignocellulosic material. Significant differences were also found in the ligninolytic (MnP, LiP, MlP) as well as the cellulolytic activities, regarding the different N levels in the substrate tested. The highest ligninolytic enzyme activity was detected after the water extraction treatment and the lowest cellulase activity was found for the treatment with the lowest N level.

Finally, several experiments were carried out in order to confirm the key role played by N in the biopulping of other lignocellulosic feedstocks commonly used by the pulp and paper industry. The same tendency shown in hemp wood was observed for the rest of species, with the exception of *Miscanthus*. These results indicate the potential of wood pre-treatment methods geared to reduce the N content in wood chips as a mean to enhance the selectivity of lignin degradation in biopulping.

Keywords:
biopulping, lignin, hemp, cellulase, degradation, wood chips, wood

Main Publications/Patents/Participation in conferences:

J. Dorado, T. A. v. Beek, F. W. Claassen, R. Sierra-Alvarez (1999) Degradation of pitch constituents in various wood species by the white-rot fungus *Bjerkandera* sp. strain BOS55 – 30th Annual Meeting of the International Research Group on Wood Preservation, 6-11th June 1999, Rosenheim, Germany.

J. Dorado, T. A. v. Beek, F. W. Claassen, J. B. P. A. Wijnberg, G. Lenon, R. Sierra-Alvarez (1999) Biotreatment of softwood chips to reduce pitch problems – 27th EUCEPA Conference – Crossing the Millennium Frontier: Emerging Technical and Scientific Challenges, 12-14th October 1999, Grenoble, France.

S. Bosch-González, J. Dorado, F. W. Claassen, G. Lenon, R. Sierra-Alvarez (1999) Effect of biomechanical pulping on effluent treatability – IAWQ International Specialised Conference of Chemical Industry Group, 14-18th November 1999, Mérida, Yucatán, Mexico.

J. Dorado, R. Sierra-Alvarez, J. A. Field (2000) Biopulping of non-woody plant species – International Conference & Exhibition on Sustainable and Renewable Raw Materials (Green-Tech® 2000), 3-5th April 2000, Utrecht, The Netherlands.

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Category:	30
Starting date:	19/03/1998
Duration:	12 months
Contract number:	FAIR-CT98-5023

Biological aspects of chemical wood modification

Objectives:
Acetylation of solid wood to enhance anti-shrink-efficiency and resistance against fungal decay is a fairly new approach in wood technology. In the near future acetylation of solid wood will be carried out on an industrial scale. Nevertheless some questions concerning the effects of acetylation on wood structure and biological degradation remain unanswered.

Summary of achievements:
Wood samples of Scots pine, Norway spruce and beech treated in different acetylation processes were investigated using several techniques. These include: light microscopy, electron microscopy, confocal laser scanning microscopy together with image analysis, UV-spectroscopy and fluorescence spectroscopy. Furthermore decay patterns in non-treated and acetylated wood under soil exposure were studied using different microscope techniques.

It was found that the optimised processes designed for industrial acetylation do not cause any damages to wood structure. Thin walled parenchyma tissue as well as pit membranes were unaffected. A bulking effect due to acetylation led to a higher cell wall/lumen relation in the early-wood of spruce: The wood becomes denser. Only sub-optimised processes with uncontrolled temperature might cause damages such as fibre detachment and dissolution of pit membranes.

Acetylated spruce revealed a lower permeability for waterborne solutions. The impregnation deepness was measured using image analysis and revealed significantly lower impregnation depth in the axial direction. UV-spectroscopy of acetylated spruce could prove that even a high acetylation degree does not affect the aromatic structures of lignin, implicating a possible side chain reaction. A change in the fluorescence characteristic of lignin was found using fluorescence spectroscopy. Intensity of lignin fluorescence in softwoods was significantly correlated with the degree of acetylation.

Microscopy of acetylated wood from soil exposure tests was not finished at the end of the project. The preliminary results, however, showed that a high degree of acetylation did not suppress colonisation of wood by fungi, but biological degradation was severely reduced or completely missing.

Keywords:
Wood, acetylation, Scots pine, Norway spruce, beech, microscopy, resistance, anti-shrink, decay

Main Publications/Patents/Participation in conferences:
C. Sander, E.P.J. Beckers, H. Militz, W. van Veenendaal Analysis of acetylated wood by electron microscopy. (in preparation)
C. Sander, M. Willemse, E.P.J. Beckers, H. Militz Effects of wood modifying treatments on the autofluorescence of lignin. (in preparation)
C. Sander, E.P.J. Beckers, H. Militz Swelling and anisotropy of wood due to acetylation. (in preparation)
C. Sander, E.P.J. Beckers, H. Militz Decay pattern of acetylated and CC-treated timber after five years soil exposure. (in preparation)

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Category:	30
Starting date:	22/07/1998
Duration:	24 months
Contract number:	FAIR-CT98-5038

The influence of the site factors "wind exposure" and "slope" on wood quality

Objectives:
A detailed experiment to investigate the effect of wind exposure on Sitka spruce wood quality was established. This is an important consideration for British timber production and is the first work on this subject in this country.

Summary of achievements:
A major effort was made at the beginning of the project to identify suitable sites for the work. It was quickly realised that finding forest sites on which everything but the wind exposure remained the same would be extremely difficult, if not impossible. Therefore, it was decided that a single site would be used, which had a variation in wind exposure. The most suitable possibility was a stand with a clearly defined westerly edge in which the wind exposure would be a function of the distance of trees from the edge. This removes worries about variations in temperature, rainfall, soil nutrition etc. The requirement that the trees would be large enough to pass through a conventional sawmill and provide sawn pieces on the inside and outside of the trunk restricted the search to stands planted at least 40 years ago. A rigorous search for such a site was conducted throughout Britain by contacting Forestry Commission offices and Forest Research field stations. Potential sites were visited to determine their suitability and the final site chosen was near Lochgilphead, Argyll.

Prior to beginning the tree mechanical measurements the characteristics of the trees at different distances from the forest edge were obtained. Statistical analysis was carried out to determine the number of trees required to be measured to provide a statistically robust data set and to fit in with standard British mensurational protocol. From this a detailed experimental protocol was drawn up prior to the beginning of any formal work. The experimental work in the field was extremely thorough and established the basic physical characteristics of the 15 trees in each of four rows as well as the competition from neighbouring trees. The mechanical

measurements consisted of attaching strain gauges at eight heights on each tree and pulling the tree with known loads to establish the Modulus of Elasticity (MOE) as a function of height. In addition sway tests were conducted with the strain gauges attached to the stem in order to determine the resonant frequency and damping of the trees. All data were logged to portable computer and the system was specifically designed for the project. The fieldwork was very intensive and conducted over many weeks.

Following the mechanical measurements the trees were felled and 4m logs cut from the base, centre and top of the trunk. Discs were cut from the ends of each section. The stem diameter was measured at 1m intervals and the branches from each metre section weighed. The sections were transported to a sawmill and battens of approximately 100 x 50-mm cut from the base and top logs. The battens are now being measured at BRE to determine the green timber value of MOE and then they will be dried to determine the level of distortion.

By following each piece of timber from the forest through the sawmill and to final analysis it will be possible to determine the effects of wind exposure on the properties of industrially prepared timber. This link between research and the industrial sector is a key part of the work. Furthermore, every effort has been made to integrate the work with other work on timber quality in the Forestry Commission in order to establish an integrated database on the timber properties of British softwoods. This has required taking some additional measurements not required for the actual project but which enhance the value of the data obtained.

Keywords:
Sitka, spruce, wind, exposure, slope, growth, mechanical properties, wood quality

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Category:	RT
Starting date:	01/10/1998
Duration:	12 months
Contract number:	FAIR-CT98-5056

Regeneration and micropropagation of transgenic *"Eucalyptus globulus"* plants

Objectives:
In the framework of plant biotechnology, the project was a continuation of a previous project started in 1996, the purpose of which was to use genetic engineering techniques for improving *Eucalyptus globulus* – a woody species extensively grown in southern Europe for paper pulp production. The main objective of this study was the final optimisation of an *in planta* procedure for transferring genes into *Eucalyptus globulus* adult clonal material and to study the regeneration of the transformed plants.

Summary of achievements:
Results indicated that it was possible to obtain (first time) stable transformation efficiency of up to 40% for the selected clone when the following parameters were established:

- Explant preculture of three weeks in the R7 medium with dark/photoperiod combination;
- Agrobacterium culture in liquid LB medium in the presence of acetosyringone and proline;
- Agrobacterium resuspension in liquid MS medium with 2,4-D in the presence also of acetosyringone and proline;
- Transformation by wounding/infection technique of sonication 60 s followed by infiltration five minutes under electric pump;
- The use of a solid co-culture R7 medium in presence of acetosyringone.

The histochemical analysis showed that leaves, shoots and meristems expressed uidA gene at transient level, whereas mostly leaves expressed the gene 45 days after transformation. In order to be able to regenerate plants through adventitious organogenesis from the transformed tissues, 18 different media, containing higher cytokinins (BA, CPPU, TDZ) and lower auxins (NAA) concentrations, were tested initially on non-transformed leaves. From successive experiments, it was possible to establish a regeneration medium that, containing TDZ at 1 mg/l and BAP at 2 mg/l, obtained the lowest necrosed explants, the highest percentage of

explants producing buds and the highest percentage of buds producing shoots. The new shoots were able to develop normally and grow from the new buds when micropropagation conditions were established.

The results indicated the availability of the selected material to be transformed and regenerated by using the developed procedures, thus successfully satisfying the main objective of the project.

Keywords:
Eucalyptus globulus, regeneration, transformation, explant, micropropagation.

Main Publications/Patents/Participation in conferences:
B. Villar, J. J. Oller, C. Teulieres, A. M. Boudet, P. P. Gallego (2000) *In planta* transformation of adult clones of *Eucalyptus globulus* using a hypervirulent *Agrobacterium* strain – Applications of biotechnology to forest genetics, 297-306.
B. Villar, J. J. Oller, C. Teulieres, A. M. Boudet, P. P. Gallego (1999) Optimisation of a genetic transformation method for *Eucalyptus globulus* by *Agrobacterium tumefaciens*" – International Symposium on Plant genetic Engineering, La Habana, Cuba.

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Contract signed:	02/02/1996
Duration:	36 months
Contract number:	FAIR-CT96-5014

Product formation in tissue cultures of neem (*Azadirachta indica*) for the control of insect pests

Objectives:
This project evaluates the potential of neem tissue cultures to produce azadirachtin and other bioactive compounds on a year round basis for the control of insect pests.

Summary of achievements:
Characterisation and growth and product formation in scaled up cultures of neem hairy roots has been successfully achieved. Thus in 21 flasks, the exponential growth phase lasted three weeks with a doubling biomass time of 0.95 weeks. This is faster than for the cultures grown in 250 ml flasks where td was 1.02 weeks. Azadirachtin peaked at the end of the exponential growth of the cultures which occurred at three weeks of culture with a yield of 0.0043 mg per g of dry weight (mg/gDW). This yield of azadirachtin per unit biomass is lower than in 250 ml flasks but if the rate of growth is taken into consideration in terms of the increased biomass and the decreased culture time in the 2l. flasks then the resultant yield is higher in the scaled up culture system than the 250 ml flasks system (0.0095 mg/gDW/week and 0.007 mg/gDW/week respectively).

The development of improved high performance liquid chromatographic (HPLC) methods (water and acetonitrile as system solvents gradient) allowed good separation of the different compounds throughout the chromatographic run except for azadirachtin and 3-tigloylazadirachtol which co-eluted. To overcome this problem isocratic HPLC conditions were applied with water and acetonitrile as solvent eluents at 57% water enabling efficient quantification of both azadirachtin and 3-tigloylazadirachtol.

Six week old hairy root cultures (grown in 250 ml flasks) were fractionated using HPLC with fractions ranging from polar (F 1) to non polar compounds (F5) and individual fractions were tested for antifeedancy against the desert locust. High antifeedancy levels were observed in fractions (F) 2, 3 and 4 whilst F 1 and F5 were not significantly antifeedant. Interestingly F3

did not contain any of the well known neem chemicals while F2 contained azadirachtin and 3-tigloylazadiractol and F4 nimbin and salannin. Further fractionation of the 173 indicated that the bioactive molecules were eluted during the second and third minutes of the five minute F3.

Studies of the effects of exogenously supplied auxins into the culture medium and C/N concentration ratio on culture growth and azadirachtin production by the cultures are presently undergoing extraction prior to HPLC analysis. In order to speed-up analysis an additional HPLC system is being used which has the ability for continuous analysis.

Hairy root extracts were also tested for their potential to control the peach potato aphid on oilseed rape plants in greenhouse experiments and data are presently subject to analysis.

Keywords:
azadirachtin, Nimbin, biomass, antifeedant

Main Publications/Patents/Participation in conferences:
Neem (*Azadirachta indica*) hairy root cultures for the production of environmentally sound bioactive compounds with insecticidal properties. Participation and oral presentation of a talk at the Phytochemical Society of Europe Symposium of Future Trends in Phytochemistry, Rolduc (The Netherlands) 10-13 May 1998.
Bioactive compounds from neem tissue cultures and screening against insects. Participation and poster presentation at the 9th International Congress of Pesticide Chemistry, London (UK) 2-7 August 1998.
A.K Zounos, E.J Allan and A.J.Mordue (Luntz), Bioactive compounds from neem tissue cultures and screening against insects, Pesticide Science, *In Press*.
Participation and poster presentation at the Zoology Research Day, Aberdeen (UK). 14th December 1998.

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Category:	30
Starting date:	18/03/96
Duration:	12 months
Contract number:	FAIR-CT96-5016

Potential for upgrading lignocellulosic material by treatment with enzyme demand from rumen bacterial genes

Objectives:
The utilisation of lignocellulosic material in agricultural and industrial processes can be enhanced by treatment with plant cell wall degrading enzymes. Cloned genes potentially allow the production of specific enzymes with desirable properties economically and in large quantities. Rumen bacteria are a source of plant cell wall degrading enzymes that could prove particularly valuable for improving the digestibility of plant material by farm animals. Many genes coding for these enzymes have been isolated from rumen bacteria in the host laboratory. This project aims to examine the functional properties and potential applications of an unique bifunctional hemicellulase, XynD, from *Ruminococcus flavefaciens* and to enhance expression of the cloned XynD gene by introducing it into lactic acid bacteria. It is also proposed to isolate further valuable genes that lie next to XynD in the bacterial chromosome.

Summary of achievements:
Initial work established that both bacteria produced inducible esterases when grown with xylans as the energy source. Clones that express activity against model esterase substrates (α-napthyl acetate) were isolated from a *R. flavefaciens* 17 DNA library. One clone has subsequently been shown to encode an enzyme that can deacetylate acetylated plant xylans.

A second component of the project involved sequence analysis of a region of DNA downstream from the *xynD* gene in *R. flavefaciens*, known to encode α-arabinosidase activity, in order to locate genes coding for additional debranching activities. The gene most likely to account for the α-arabinosidase activity is a family 3 glycoside hydrolase that may function primarily as a β-(1,4)-xylosidase, although an adjacent ORF is also present that does match a known database entry. The region downstream of *xynD* has since been shown to encode a gene cluster responsible for the utilisation of xylan breakdown products.

Keywords:
Rumen, *Ruminococcus*, esterase, xylan, glycoside hydrolase, lignocellulose

Main Publications/Patents/Participation in conferences:
V. Aurilia, S. Ekinci, J., Martin, J. Kirby and H.J. Flint (1996) Organisation and expression of plant cell wall polysaccharidase genes from the rumen cellulolytic bacterium *Ruminococcus flavefaciens 12th European Meeting on Bacterial Gene Transfer and Expression* Sienna, Italy, 4-7 September 1996
V. Aurilia, J. Kirby, J. Martin, S. Ekinci and H.J. Flint (1997) Organisation of genes involved in xylan utilisation and xylan debranching in the rumen cellulolytic bacterium *Ruminococcus flavefaciens* International Meeting on *Evolution of the Rumen Microbial Ecosystem* Aberdeen, Scotland, 20-21 March 1997.
V. Aurilia, J. Kirby, J. Martin and H.J. Flint (1996) Molecular Biology of plant cell wall degrading enzyme systems in *Ruminococcus flavefaciens 137th Meeting SGM-Society for General Microbiology* Edinburgh 24-27 March 1997.

Links with EC projects:
FAIR-CT97-5007 (p. 49)

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Category:	30
Starting date:	13/05/1996
Duration:	24 months
Contract number:	FAIR-CT96-5025

Modification of the specificity of starch-degrading enzymes by genetic fusions with the starch binding domain of glucoamylase

Objectives:
Starch from cereal seeds is the raw material for industries that produce ethanol and mono-and oligosaccharide syrups and a myriad of derived products. Starch comprises two major components: amylose (mainly α14-linked D-glucose residues) and amylopectin (containing both α1,4- and α16-linked D-glucose residues). The relative content of amylose and amylopectin in starch varies with the source. Due to the importance of starch, its breakdown sugars and subsequently derived products, cereal seeds are some of the most important agriculture products in the world and starch degrading enzymes are among the most important enzymes.

Glucoamylase and α-amylase are widely used in industrial applications, e.g. for the production of glucose and various alcoholic beverages. The glucoamylase has a strong ability to digest raw starch and the α-amylase synergistically enhances the digestion with glucoamylase.

The aim of this project is to enhance activity and binding of barley α-amylase on insoluble starch by fusion of the glucoamylase starch binding domain (SBD) from *A. niger* to the C-terminal end of the protein. This work involves the construction of the AMY1-SBD gene by genetic engineering, cloning of the fusion gene as well as the AMY1 cDNA in an expression vector and transformation in a heterologous organism, production and purification of the corresponding recombinant proteins and biochemical characterisation of the purified enzymes.

Summary of achievements:
An in-frame fusion of the entire barley α-amylase 1 isozyme (AMY1) encoding sequence to the 3' end of the *A. niger* glucoamylase region coding for the starch binding domain via the

full length glucoamylase linker was made by overlap extension PCR. The resulting fusion gene as well as the cDNA encoding AMY1 isozyme have been expressed in *A. niger* under the control of the *A. nidulans* glyceraldehyde-3-phosphate dehydrogenase promoter.

Both recombinant AMY1 and AMY1-SBD were effectively secreted in *A. niger* when using the AMY1 native signal sequence. Polyclonal antibodies, raised against barley α-amylase 2 isozyme and cross-reacting with AMY1, recognised the recombinant AMY1 as well as the AMY1-SBD fusion protein. The N-terminal sequence of recombinant AMY1 and AMY1-SBD, His-Gln-Val-Leu-Phe, was identical to that of AMY1 from malt, indicating native-like processing of AMY1 signal sequence in *A. niger*.

Both recombinant enzymes were then purified to homogeneity from the culture supernatant by a two-step process involving affinity chromatography followed by ion exchange chromatography, allowing further biochemical characterisation.

The purified recombinant AMY1 is structurally identical to the mature malt protein according to the following criteria N-terminal amino acid sequence, electrophoretic mobility, immunoreactivity, ESMS. Furthermore the recombinant and native barley α-amylases closely- resembled each other in terms of enzymatic activity on insoluble and soluble starch as well as on 2-chloro-4-nitrophenol β-D-maltoheptaoside. The purified recombinant fusion protein and reAMY1 displays the same specific activity on pNPG7 and potato soluble starch.

Genetic engineering allowed the production of an active fusion protein between barley α-amylase and the starch binding domain of *A. niger* glucoamylase. Further characterisation of the fusion protein in terms of activity and binding oil insoluble corn starch is under investigation.

Keywords:
Starch, glucoamylase, amylase, cereals, genetic engineering

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Contract signed:	31/01/1996
Duration:	6 months
Contract number:	FAIR-CT96-5027

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Simulation of oxygen gradients in a poorly mixed ethanol fermentation: modelling and experimental design of scale-up through a series of STRs

Objectives:
A thermotolerant yeast from soil samples obtained from a distillery in India has been isolated. The yeast strain designated *Kluyveromyces marxianus* var. *marxianus* IMB3 was capable of growth and ethanol production at temperatures between 25-48°C. *K. marxianus* is a facultative anaerobe i.e. it can metabolise glucose in the absence of oxygen to ethanol and carbon dioxide. It has been classified as a respiratory yeast as under aerobic conditions less than 30% of the glucose metabolised is converted to ethanol. This is compared with fermentative yeast i.e. *Saccharomyces* spp. in which over 90% of glucose is converted to ethanol. Anaerobic conditions are therefore required for a high specific ethanol production, however growth can not be sustained under anaerobic conditions. This led to a low biomass concentration in the fermenter during continuous fermentation and consequently a low ethanol productivity (13 ml/l.hour).

The objectives of the project were to:
- Confirm the results obtained elsewhere, in particular, to check carbon balances and by-product formation with analytical equipment available at the research institution.
- The effect of scale-up on oxygen distribution in poorly mixed fermentation vessels.
- The effect of changes in oxygen transfer rates on ethanol productivity, growth and ethanol yield.
- Simulation of plug flow through poorly mixed fermentation vessels using a series of small STRs.

Summary of achievements:
The results obtained showed that high specific ethanol productivity of *Kluyveromyces marxianus* can be achieved during aerobic growth by intermittently switching the specific oxygen uptake, qOpX levels to zero. This switch did not significantly affect the growth rate. Ethanol production is also seen at high specific growth rates under aerobic conditions.

Using STRs in series to simulate plug flow through the reactor yielded some interesting results but the method in itself is complicated due to a number of factors. These include the requirement to incorporate cell recycle in order to simulate back-mixing and the increased complexity of measurement and analysis of data.

It was also found that glycerol was the major by-product during aerobic and anaerobic conditions. The carbon balance also suggested that a second or more by-product was also formed.

Keywords:
Kluyveromyces marxianus, yeast, ethanol, fermentation, oxygen, simulation

Main Publications/Patents/Participation in conferences:
C.J. Hack, M.P. Keogh, D. Singh, and R. Marchant (1995) Fermentation characteristics of a thermotolerant yeast *Kluyveromyces marxianus* var. *marxianus.* J. Tech. Biotech.
C.J. Hack, W.S. McClean and R. Marchant (1994) The use of oxygen uptake rates to optimise air feed rate to a continuous ethanol fermentation. Prehrambeno-technol. Biotechnol. Rev. 32(4) 187-190.
C.J. Hack, I.M. Banat and R. Marchant (1994) Ethanol production by a strain of *Kluyveromyces marxianus* var. *marxianus* at elevated temperatures in various bioreactor configurations. Proceedings of Conference on Fermentation Physiology 2nd UK Congress of Biotechnology 7-9.

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Category:	30
Starting date:	04/08/1997
Duration:	12 months
Contract number:	FAIR-CT97-5007

Roles and potential uses of genes from rumen bacteria concerned with the degradation and utilisation of lignocellulose

Objectives:
This project set out to isolate genes encoding xylan debranching activities from the rumen bacteria *Ruminococcus flavefaciens* and *Prevotella ruminicola.*

Summary of achievements:
The utilisation of lignocellulosic material in agricultural processes can be enhanced by appropriate treatment with plant cell wall degrading enzymes. Rumen bacteria are a source of plant cell wall degrading enzymes that could prove particularly valuable for improving the digestibility of plant material by farm animals.

Cloned genes potentially allow the production of specific enzymes with desirable properties economically and in large quantities, while also providing important basic information on the degradative enzyme systems of these bacteria. Many genes coding for xylan degrading activities have now been isolated from rumen bacteria in the host laboratory, including putative xylan debranching activities.

This proposal aims first to establish the role and potential for application of an acetyl esterase gene from the rumen anaerobe *Ruminococcus flavefaciens* while also screening for genes for further debranching activities. Secondly it is proposed to investigate the structure, regulation and strain distribution of an unique gene cluster concerned with xylan degradation and utilisation in *R. flavefaciens.*

Keywords:
Rumen; *Ruminococcus;* esterase; xylan, glycoside hydrolase, lignocellulose

Main Publications/Patents/Participation in conferences:

V. Aurilia, S. Ekinci, J., Martin, J. Kirby and H.J. Flint (1996) Organisation and expression of plant cell wall polysaccharidase genes from the rumen cellulolytic bacterium *Ruminococcus flavefaciens 12th European Meeting on Bacterial Gene Transfer and Expression* Sienna, Italy, 4-7 September 1996

V. Aurilia, J. Kirby, J. Martin, S. Ekinci and H.J. Flint (1997) Organisation of genes involved in xylan utilisation and xylan debranching in the rumen cellulolytic bacterium *Ruminococcus flavefaciens* International Meeting on *Evolution of the Rumen Microbial Ecosystem* Aberdeen, Scotland, 20-21 March 1997.

V. Aurilia, J. Kirby, J. Martin and H.J. Flint (1997) Molecular Biology of plant cell wall degrading enzyme systems in *Ruminococcus flavefaciens 137 'Meeting SGM-Society for General Microbiology* Edinburgh 24-27 March 1997.

Links with EC projects:

FAIR-CT96-5016 (p. 55)

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Category:	20
Starting date:	01/10/1996
Duration:	36 months
Contract number:	FAIR-CT96-5047

Genetic modification of *Clostridium acetobutylicum* to improve ABE fermentation

Objectives:
ABE fermentation stands for the anaerobic conversion of carbohydrates by strains of *Clostridium* spp. into acetone, butanol and ethanol (ABE). This process was one of the first large-scale industrial fermentations to be developed, but was stopped in the 1960s due to its inability to compete economically with the chemical synthesis of these solvents. However, interest in the use of renewable resources as substrates for the production of chemicals and developments in the field of biotechnology have revived interest in this process. The main factor that hampers the economic viability of the ABE fermentation is the high price of the carbohydrate substrates needed. Genetic research aimed at the development of strains able to use cheap cellulosic substrates (i.e. agricultural wastes) is seen as a way to make this process economically viable. The main enzymes involved in microbial cellulose degradation can be classified as exo- and endo-glucanases. Most of the soventogenic clostridial strains show production of endoglucanases, but cannot grow on cellulose as sole carbon source, indicating a possible lack in exoglucanase enzymes. Within this project, the use of lignocellulosic substrates for ABE fermentation and the cloning and expression of exo-glucanase genes from cellulolytic organisms in selected clostridial strains is being studied.

In the work programme, detailed in the grant proposal, the task for the first year was to extend the substrate range of solventogenic Clostridia by transforming the strains with cellulase genes from other organisms and study expression of these genes. During the second year of the project, attention should be given to the suppression of degenerative changes and autolysis in solventogenic clostridia.

Summary of achievements:
The growth and solvent production of clostridial strains on Domestic Organic Waste (DOW) has been studied. The DOW was collected from a number of houses in Wageningen (The

Netherlands). The collected material was treated by extrusion in order expand the (hemi)cellulose fibres present so they are more accessible to the attack of cellulolytic enzymes. The extruded material was analysed for solvent- and hot water extractives, lignin, pectin, sugars and ash composition. A solution of 10% (w/v) DOW in demineralised water was used as medium for fermentation. All strains tested grew and produced solvents on this medium. The utilisation of the polymeric sugars present in the DOW by the bacteria has been determined.

In order to optimise a transformation method for strains different vectors have been tested. Best results were obtained with the pAMβ 1 derivative plasmid pIL.253. The above mentioned strains have been transformed with the plasmids pGh9 and pGh9:ISS1, which contain a thermo-sensitive replicon derived from the one of plasmid pGK12.

The cellulase gene *celD* from the fungus *Neocallimastix patriciarum* has been cloned in an expression secretion cassette containing the promoter and signal peptide sequence of the *eglA* gene from C. *acetobutylicum* NCP262. C. *beijerinckii* NCIMB 8052 has been transformed with this construct and the transformants obtained analysed.

Recently, the genome of the strain ATCC 824 has been sequenced. Surprisingly, in the chromosome of this strain the whole set of genes coding for enzymes involved in cellulose degradation appears to be present. In particular, an operon containing two putative cellulose binding protein genes, four putative endo-glucanase genes and two putative exo-glucanase genes are present. It is not known if this operon is expressed or if the cellulase genes present in it code for active enzymes. Therefore, one of the putative endo-glucanase genes and one gene coding for a putative exo-glucanase have been cloned in *E. coli*, and their activity on different cellulosic substrates has been tested.

Keywords:
Genetic modification, *Clostridium acetobutylicum*, ABE fermentation, acetone, butanol, ethanol, carbohydrate, endo-glucanase, exo-glucanase

Main Publications/Patents/Participation in conferences:
A.M. Lopez-Contreras A.M., P.A.M. Claassen, H. Mooibroek and W.M. De Vos (1998) Improvement of the ABE (Acetone-Butanol-Ethanol) Fermentation. 7th Netherlands Biotechnology Congress. De Reehorst Congress Centre, Ede, The Netherlands. March 12-13.
A.M. Lopez-Contreras, P.A.M. Claassen, H. Mooibroek and W.M. De Vos (1998) Improvement of the Cellulolytic Properties of Solventogenic Clostridia. Clostridium V: Fifth International Workshop on the Regulation, Genetics and Development of the Solvent- and Acid -forming Clostridia. Toulouse, France, June 25-27.

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Category:	20
Contract signed:	04/08/1998
Duration:	36 months
Contract number:	FAIR-CT98-5051

Biofinishing and enzymatic modification of bast fibres

Objectives:
The objectives of this project are twofold:

- To gain a better understanding of the composition and juxtaposition of specific components of flax fibres, i.e. pectins and hemicelluloses;
- To treat flax fibres enzymatically in order to optimise isolation of pure cellulosic fibres.

Summary of achievements:
The main conclusions from the project are:

- The sequential extractions of retted and non-retted fibres demonstrated differences in their content of rhamnogalacturonans, arabinans and xylans.
- Analysis of molecular weights of the extracts demonstrated the most significant differences were in the pectin extraction and in the xylan containing 1M KOH extraction.
- Initial low dose enzyme treatments of the fibres demonstrated that:
  - endoglucanases solubilised the most material including pectins;
  - mannanase treatment was specific for galactoglucomannans;
  - xylanase treatment solubilised small amounts of xylan; and
  - mannanase treatment of processed fibres solubilised approximately 10% of mannan and 20% of glactose.
- Enzyme action on fibres is a surface phenomenon and access to components beyond the surface is limited without any prior opening or loosening of the fibre structure.
- More enzyme treatments are needed on processed fibres in order to optimise conditions for the selective removal of non-cellulosic polymers.

Keywords:
Biofinishing, flax, textile fibres, hemp, pectin, oxidative enzymes, mannanase, hydrolytic enzymes, treatment, xylanase, dyeability, crease resistance

Main Publications/Patents/Participation in conferences:

Mooney, C.A., Mansfield, S.D., Tuohy, M.G., Saddler, J.N. 1998. The effect of initial pore volume and lignin content on the enzymatic hydrolysis of softwoods. Bioresource Technology 64: 113-119

Mooney, C.A., Mansfield, S.D., Beatson, R.P., Saddler, J.N. The effect of fibre characteristics on hydrolysis and cellulase accessibility to softwood substrates. Enzyme and Microbial Technology (*submitted*)

Mooney, C.A., Mansfield, S.D., Saddler, J.N. Substrate and enzyme characteristics that limit hydrolysis – a review. Biotechnology progress (*submitted*)

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Category:	20
Starting date:	01/02/96
Duration:	24 months
Contract number:	FAIR-CT96-5015

Ways of decreasing symptoms of lactose intolerance

Objectives:
Lactose intolerance concerns the majority of the world's adult population and may cause a severe health hazard for children, especially in the third world. The objective was to explore different possibilities of modifying the biochemical properties, texture and composition of milk products, in order to develop products suitable for lactose maldigesters. Knowledge concerning the properties of milk and milk products is vital to the dairy industry in order to develop products for groups of consumers with specific needs.

Summary of achievements:
The research assessed the effects of varying 1) bacterial ß-galactosidase of fermented milk 2) viscosity of milk, and 3) energy content of milk in lactose digestion and tolerance.

Digestion and tolerance of 18g lactose, ingested in the form of yoghurt with high bacterial ß-galactosidase content and in two fermented milks with low and moderate bacterial ß-galactosidase was studied in 15 lactose maldigesters. The products were well tolerated and lactose digestion did not vary between the products. Lactose maldigestion, calculated from the area under the curve breath H_2 excretion compared with lactulose, was 18% for yoghurt and 21% for the two other milks. The good digestion and tolerance of these products was suggested to be due to their semi-solid consistency which slows down gastric emptying.

To study the effect of milk consistency on lactose digestion and the possibility of improving the digestibility of therapeutic milk used in nutrition rehabilitation in developing countries, the viscosity of the therapeutic milk was increased with rice starch, and the gastric emptying of 18 g lactose and its digestion and tolerance in 13 lactose maldigesters was assessed. Despite a remarkable increase in the milk viscosity, gastric emptying and digestion and tolerance of lactose were not altered. The milk chosen for this study contained twice as much

energy as regular milk, and it was hypothesised that the high energy content resulted in slow gastric emptying, which could not be slowed down any further with viscosity.

To examine the role of energy content of milk on digestion and tolerance of lactose, gastric emptying and digestion of 18 g lactose from high-energy milk and regular half skimmed milk in 11 lactose maldigesters was compared. Gastric emptying was significantly slower after half skimmed milk than after' high energy milk, but there was only a trend towards a better digestion of lactose, determined from the area under the curve breath H_2 excretion, with no difference in the tolerance between milks.

The results of these three works indicate that neither the viscosity nor the β-galactosidase content of milk is of concern to lactose maldigesters, and the weak improvement of lactose digestion from high energy milk does not support the hypothetical benefit of replacing low-fat milk by full fat milk for lactose intolerant subjects.

Keywords:
Maldigesters, Lactose, Digestion, Gastric Emptying

Main Publications/Patents/Participation in conferences:
T.H. Vesa, P. Marteau, S. Zidi, F. Briet, P. Pochart, J.C. Rambaud (*In press)* Digestion and tolerance of lactose from yoghurt and different semi-solid fermented dairy products containing Lactobacillus acidophilus and bifidobacteria in lactose maldigesters. Am J Clin Nutr.
T.H. Vesa, P. Marteau, F.B. Briet, B. Flourié, A. Briend, J.C. Rambaud (*In press)* Effects of milk viscosity on gastric emptying and lactose tolerance in lactose maldigesters. Am J Clin Nutr.

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Category:	20
Starting date:	31/06/1996
Duration:	36 months
Contract number:	FAIR-CT96-5022

Novel technological approaches to functional sweeteners

Objectives:
The project will attempt to establish an enzyme technology-based approach to the synthesis of dextran syrups from starch. The project will use immobilised amylase and dextrin dextranase in bioreactors to continuously prepare the syrups. The performance of these reactors will be characterised and optimised. Once produced, these syrups will be characterised for their probiotic effects on intestinal microflora. Their physical properties relating to sweet taste will also be investigated.

Summary of achievements:
Following the work of the first year, stock culture suspensions were prepared by mixing three appropriate cell cultures and dispensing the mix in glycerol, followed by storage without freezing at -20°C for later use as inocula. The cell cultures were generated from the master *G. oxydans* NCIB 4943 culture, utilising complex media consisting of 0.2% glucose and 0.5% yeast extract, in order to generate a "healthier" biomass. The cultures were harvested at 24h when a maximum average OD_{620} of 0.857 was obtained, with an average cell concentration of $3.6x\ 10^8$ cfu/ml.

Two substrates were screened for their suitability regarding dextran production and ease of separation from the dextran. Application of ultrafiltration methods for separation of residual substrate from the dextran polysaccharide was considered as a promising approach compared with the traditional ethanol precipitation techniques, which were also used in this study. A commercial spray dried maltodextrin (DE 20) was found to be an appropriate substrate. In addition, rheological measurements of various culture broths of *G. oxydans* grown on the above substrate at 2, 5 and 10% (w/v) concentrations generated interesting preliminary data for the use of rheometry in the investigation regarding the amount and type of polysaccharide produced.

Until a suitable procedure for dextran production is established, industrial grade dextran has been employed to investigate the rapes of oligodextrans that could be generated via a biochemical depolymerisation process using endo-dextranase and membrane reactors. The first stage of this investigation using a stirred cell membrane reactor has been successfully accomplished and three types of oligodextrans have been produced. The process has been modelled statistically using response surface methodology, and empirical models have been generated that relate product characteristics to enzyme concentration, substrate concentration and transmembrane pressures. In addition, the above three types of oligodextrans have been tested for their potential as prebiotics in collaboration with the Institute of Food Research. These screening results indicated a close similarity to the results obtained from fructo-oligosaccharides (FOS), used as controls in the investigation.

Keywords:
Sweeteners, cell cultures, substrates

Main Publications/Patents/Participation in conferences:
K.C. Mountzouris, S.G. Gilmour, A.S. Grandison, R.A. and Rastall (1998) Modelling of oligodextran production in an ultafiltration stirred cell membrane reactor. Enzyme Microbial Technology, 1/98.

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Category:	20
Contract signed:	12/07/1996
Duration:	36 months
Contract number:	FAIR-CT96-5033

A study on age-related eye disease and hyperhomocysteinaemia in British elderly

Objectives:
The project aimed to study age-related eye disease and hyperhomocysteinaemia (an indicator of increased heart disease risk) in British elederly. The British National Dietary and Nutrition Survey in Elderly (NDNS) will provide a wide range of information on the diet, nutrition and biomedical status of a representative sample of British elderly.

This information will be linked to the results of additional measurements of visual acuity, cataract and macular degeneration and plasma homocysteine levels. The prevalence of degenerative eye disease and hyperhomocysteinaemia, and their potential relationship with diet and biochemical status was studied. The NDNS in combination with the added measurements will provide data of great value for further study of two common health problems of elderly in Europe, which have not yet been investigated to a satisfactory extent.

Summary of achievements:
All activities carried out for The Survey of Nutrition and the Eye in British Elderly (SNEBE) aimed at collecting information on the eye status of participants of the National Diet and Nutrition Survey: People Aged 65 Years Or Over (NDNS), and the linkage of SNEBE and NDNS data for analysis.

At the start of the SNEBE study, all NDNS data and subject details were made available. Applications for ethical approval and the organisation of eye examinations was carried out at the MRC Dunn Nutrition Unit. A total of 61 applications to Local Research Ethics Committee's were carried out. Activities related to subject participation included: updating of subject details, informing GPs and head of institution, invitation of and making appointments with subjects and reporting of examination findings to the subjects.

Visual acuity was measured in the NDNS participants during a visit by a nurse at the subjects' home. Visual acuity was measured at three metres distance by using the Glasgow Acuity Card method. To ensure refractive correction, measurements were made with and without glasses (if normally worn) and with and without pin-hole occluder. Results of these measurements were linked with nutritional indices available from the survey, showing significant relationships between visual acuity and plasma levels of vitamin C and zinc and dietary fibre intake, after correction for confounding variables in a multivariate model.

Total plasma homocysteine (tHCY) was measured in 972 participants of the NDNS. Cross-sectional relationships between tHcy and diet and health were investigated. Relationships were identified between tHcy and plasma nutrient levels and acute phase indicators, folate intake, vascular disease indicators, age, domicile and geographical area.

Keywords:
eye disease, hyperhomocysteinaemia, elderly, United Kingdom, homocysteine, diet, survey

Main Publications/Patents/Participation in conferences:
Bates, C.J., Mansoor, M.A.., van der Pols, J., Prentice, A., Finch, S. 1997 Plasma total homocysteine in a representative sample of 972 British men and women aged 65 and over. European Journal of Clinical Nutrition. 51: 691-697.
Two presentations given at the British Nutrition Society Summer Meeting, Newcastle, 1997

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Fellowship data

Category:	20
Starting date:	15/05/1997
Duration:	36 months
Contract number:	FAIR-CT96-5058

Effect of dietary nucleotides on the modulation of genetic expression of fibrogenesis markers

Objectives:
Liver stellate cells (LSC) play a key role in hepatic fibrogenesis. Under the influence of inflammatory stimuli, they are transformed to myofibroblast-like cells. This process is mediated by cytokines and growth factors, such as TGF-b and PDGF.

The group has recently reported that deprivation of dietary nucleotides results in a transient decrease in acid soluble nucleotides and RNA content in rat liver as well as in a protein decreased synthesis rate. In addition, dietary nucleotides improve liver structural recovery and binuclearity in experimental cirrhosis induced by thioacetamide. In that model, dietary nucleotides led to a lower number of stellate cells to a lower collagen deposition. But very little is known about the possible role of nucleosides on the modulation of cells growth and proliferative response of LSC under culture conditions.

In this work, the effect of some nucleosides (individual and mixtures) and/or some growth factors (EGF, TGFa, TGFb, PDGF) on the proliferative processes of the LSC and the potential effect of cytotoxicity was studied. We have cultured the cells in the presence and absence of nucleosides and/or growth factors, in two different experimental designs. We have analysed the protein, DNA and RNA contents, the synthesis of DNA and the cellular cytotoxicity/viability.

Summary of achievements:
With the experimental design A, using 10% FBS, a culture condition of LSC the most similar to the one *in vivo* was established. The results obtained could indicate that the nucleosides and the growth factors did not induce significant changes on the proliferation markers of LSC analysed. However, the proliferation results, using the experimental design B, showed that the DNA synthesis was significantly modulated by the different growth factors. The different results found in both experimental designs suggest that the high concentration of fetal bovine

serum (100) in the culture medium (experimental design A) may minimise the potential effect of the growth factors and the nucleosides on the proliferation processes of LSC.

Preliminary assays by Northern blot and Western blot showed that the nucleosides maybe involved in the regulation of the mRNA expression of some components of the extracellular matrix such as fibronectin, laminin, collagen I and III and TIMPs.

In conclusion, the nucleosides may not modulate the proliferation processes of the LSC, but could regulate the expression of some proteins linked to fibrogenesis.

Keywords:
Nucleosides, fibrogenesis, hepatic stellate cells, hepatocytes, proliferation, differentiation

Main Publications/Patents/Participation in conferences:
19th Espen Congress, Amsterdam The Netherlands, 21 August – 3 September 1997
XV Congreso Nacional de la Sociedad Española de Nutrición Parenteral y Enteral, Alicante, Spain, 13 –15 June 1998
FEBS'98, Copenhagen, Denmark, 5 –10 July 1998

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Category:	30
Contract signed:	18/12/1996
Duration:	15 months
Contract number:	FAIR-CT96-5074

Kinetic investigation of folate absorption using stable isotopes

Objectives:
The aim of this project was to assist in the establishment of a dual-label stable isotope technique to investigate the kinetics of folate absorption and clearance from plasma in humans.

Summary of achievements:
The following were optimised: blood-sampling times, does of stable isotopes and analytical methodology (GC-MS/MS method for determining various labels in plasma and urine samples).

The results from this project were used to design a larger study where the relative absorption of folic acid given as a supplement, and in two fortified foods, was measured in a group of women. The results from this work will provide a more reliable basis for the formulation of dietary recommendations for folate requirements.

Keywords:
folates, folic acid, food, absorption, metabolism, humans, stable isotopes, mass spectrometry

Main Publications/Patents/Participation in conferences:
M. L. Ovaskainen, L. Vahteristo (1997) Folate data in Finnish food composition tables – COST 99: Food Consumption and Composition, Workshop on Folic Acid Data in Food Tables, 18-20th April 1997, Norwich, UK (invited talk).
C. Witthoft, P. M. Finglas, L. Vahteristo et al. (1997) Bioavailability of folates using stable isotopes – Bioavailability 97, 25-28th May 1997, Wageningen, Netherlands (invited talk).
P. Maunder, P. M. Finglas, A. I. Mallet, F. Mellon, M. A. Razzaque, B. Ridge, L. Vahteristo, C. Witthoft The synthesis of folic multiply labelled with stable isotopes for bioavailability studies in human nutrition – Chemistry and Biology of Pteridines and Folates, Blackwell Science, pp. 77-80 (poster presentation).

S. Ruggeri, L. Vahteristo, P. M. Finglas et al. (1997) HPLC determination of folate vitamers in food and Italian reference diet – Poster Presentation at 22nd Congress of Chromatography, September 1997, Rome, Italy.
Folic acid – A new protective vitamin with functional food potentials – 11th March 1998, Agricultural University of Uppsala, Sweden (invited talk).

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Category:	30
Contract signed:	18/12/1996
Duration:	9 months
Contract number:	FAIR-CT96-5077

In-vitro antioxidant activity of selected foods

Objectives:
There is considerable evidence for a role for the antioxidant constituents of fruit, vegetables, beverages and grains namely, vitamin E, vitamin C and the carotenoids in the maintenance of health and protection from chronic diseases, especially coronary heart disease, certain cancers and age-related cataracts.

Recent epidemiological observations have demonstrated that the dietary polyphenols, especially flavonoids, that occur ubiquitously in plants, could contribute to the protective effect. Flavonoids, because of their structure and favourable reduction potential of their phenoxyl radicals, may act as efficient antioxidants. Even though the structure-antioxidant activity relationships of pure substances has been determined, information on the total antioxidant activity of more complex matrices such as beverages, fruit and vegetables is still lacking.

The aim of this project was to establish procedures and to adapt current methods in order to evaluate the total antioxidant capacity of fruit, vegetables and beverages as they occur in the human diet. In particular, the research project involved the assessment of antioxidant activity (both in aqueous and lipophilic-phases) of extracts obtained from different foods.

The project was essentially divided into two components. The first was the establishment and validation of a new method for assessing the total antioxidant status of fruit, vegetables, beverages and oils. The applicability of the method, in comparison with previous and other established methods, has been validated in relation to a variety of antioxidants - especially, flavonoids, hydroxycinnamates and phenolics, rich constituents of the Mediterranean diet, and in relation to plasma antioxidants.

The second component was applying this method to screening the relative antioxidant activities of a range of olive oils in relation to their vitamin E and phenolic contents.

Summary of achievements:
Results demonstrate that the decolourisation of the $ABTS^{\bullet+}$ radical cation is an efficient, accurate assay for screening the antioxidant activities of lipophilic substances and food extracts. The inhibitory response of the radical cation is proportional to the antioxidant concentration, and the time points selected 92.5 min) for the analysis demonstrates that this can be an ideal measuring point when the reaction is complete. Results for the carotenoids are of the same order as previously reported data using alternative systems for generating the $ABTS^{\bullet+}$ radical cation.

Interestingly, the antioxidant activity of the aqueous methanolic extract in this variety of tomatoes, rich in flavonoids and phenolics, is three times that of the lipophilic extract to which the carotenoids contribute. Clearly the relative proportions as well as the individual contributions will vary for different varieties and different origins.

Keywords:
antioxidant, food, vegetable, beverage, grains, vitamin E, vitamin C, carotenoid, coronary heart disease, cancer, age-related cataracts

Main Publications/Patents/Participation in conferences:
N. Pellegrini, R. Re, M. Yang, C. Rice-Evans (1999) Screening of dietary carotenoids and carotenoid-rich fruit extracts for antioxidant activities applying 2,2'-azinobis(3-ethylenebenzothiazoline-6-sulfonic acid radical decolourisation assay. Meth in Enzymol 299:379-389
16th International Congress of Nutrition. 27 July –1 August 1999, Montreal Canada.

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Category:	30
Starting date:	01/03/1997
Duration:	12 months
Contract number:	FAIR-CT96-5080

Antioxidant capacity in selected vegetable foods

Objectives:
Research objectives and content Conventional epidemiology has shown an inverse relationship between consumption of fruit and vegetables (as in a typical "Mediterranean diet") and incidence of heart disease and cancer. The protective effect has been attributed to the antioxidant compounds present in the diet such as polyphenols, vitamin C, vitamin E and carotenoids that are crucial in aetiology of this disease. These compounds inactive reactive oxygen species (ROS) responsible for cellular oxidative damage. After harvesting, fresh fruits and vegetables are subjected to nutritive losses on ageing.

Refrigeration is the most common used procedure for extending the shelf life of fruit and vegetables. This storage could have an effect on the active compounds of foods destroying/improving their antioxidant properties.

The main objectives of the proposed work were:

- to evaluate the effect of different time and temperature of the conservation in the content of selected phenols, antioxidant vitamins and carotenoids relevant to human health present in selected fresh vegetables.
- to evaluate the effect of the different time and temperature of storage on the total antioxidant capacity of selected vegetable foods.

Summary of achievements:
The activity developed during this period has been:

1. Identification of foods to be tested.

2. Optimisation of the analytical procedure for the extraction and quantification of single antioxidant compounds: polyphenols, carotenoids, and ascorbic acid.

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3. *In vitro* measurement of the total antioxidant capacity (TRAP) of water- and liposoluble food extracts.

4. Study of the effect of temperature on the antioxidant content and the antioxidant activity of selected vegetables.

Keywords:
Antioxidant, capacity, vegetable, food, polyphenols, carotenoids, ascorbic acid, temperature

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Category:	20
Starting date:	01/01/1997
Duration:	12 months
Contract number:	FAIR-CT96-5081

Cholesterol oxides in meat and meat products

Objectives:
The project aimed to study the effect of prooxidants removal from the chickens' diet on cholesterol and lipid oxidative stability in the raw and cooked chicken meat.

Summary of achievements:
The removal of iron and copper (prooxidants) from the chickens' diet did not increase the cholesterol and lipid oxidative stability of meat. On the contrary, a decreased oxidative stability was noted for the meat proceeding from the broilers deprived of iron, copper and of both metals. Indeed, higher levels of oxysterols and TBARS were observed for these latter groups when compared to the control group. The higher production of oxysterols and TBARS was also corroborated by a higher selenium glutathione peroxidase activity which was found to be a good indicator of the oxidative stress prevailing in the muscle tissue.

Regarding the production of oxysterols in meat products, oxysterols were detected in dry cured ham. The levels depended on the duration of the processing. No traces of oxysterols were detected in dry sausages. These findings suggest that further investigation should be carried out on meat products in order to evaluate the contribution of oxysterols to the diet since these compounds are thought to be implicated in the etiology of artherosclerosis.

Keywords:
cholesterol, oxides, meat, prooxidants, lipid, oxidative, oxysterols

Main Publications/Patents/Participation in conferences:
C. Maraschiello, C. Sárraga, J. A. Garcia-Regueiro (1996) GSH-PX activity in chicken fed supplemented diets – Poster presentation at the 8th Biennial Meeting International Society for Free Radical Research, 3-5th October 1996, Barcelona, Spain.

J. A. Garcia-Regueiro, C. Maraschiello (1997) Procedure for the determination of eight cholesterol oxides in poultry meat using on-column and solvent venting capillary gas chromatography – Journal of Chromatography, A 764, 279-293, 1997.
C. Maraschiello, J. A. Garcia-Regueiro, E. Esteve (1997) La oxidación del colesterol y su influencia en la calidad de la carne y productos derivados – Eurocarne 53, 67-74, 1997.
C. Maraschiello, I. Diaz, J. A. Garcia-Regueiro (1997) Determinación de colesterol en gras y musculo de cerdo mediante HPLC y chroatografia de gases con columna capilar e inyección con eliminación de solvente – Técnicas de Laboratorio 220, 207-211, 1997.
C. Maraschiello, E. Esteve, J. A. Garcia-Regueiro (1998) Cholesterol oxidation in meat from chicken fed α-tocopherol and β-carotene supplemented diets with different unsaturation grades – Lipids, 1998 (in press).

Links with EC projects:
AIR1-CT94-5025
The results of this project were compared to those obtained with broilers (period from 01/09/1994 to 31/08/1996, contract AIR1-CT-94-5025) and within the framework of the DietOx project (PL921577).

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Category:	20
Starting date:	01/06/97
Duration:	12 months
Contract number:	FAIR-CT97-5001

People's intention to reduce red meat consumption or to become a vegetarian; an application of the Theory of Planned Behaviour

Objectives:
There is evidence that the consumption of some types of meat is decreasing and that vegetarianism is increasing. It is argued by some that vegetarian diets (or those low in meat) may be beneficial for nutrition and health. The reasons for people's consumption of vegetarian diets, or moves towards reducing meat consumption, are not well understood and these forms of behaviour are likely to be influenced by many interrelating factors.

Within this project people's intention to reduce red meat consumption and to become vegetarian will be explored with regards to be able to better explain the step towards becoming a vegetarian, but also to predict particularly people's intention towards the mentioned behaviours. This will follow the theory of planned behaviour and address the influences of attitudes, perceived social pressure and perceived control, in addition to availability and information concerning such foods. It will explore the barriers to increased consumption of such foods and means for overcoming such barriers.

Summary of achievements:
In summary, attitude, subjective norm, moral obligation, perceived difficulty and self identity have strong associations with intention but also they have a strong inter correlation with each other. Within the regression however, the attitude construct is the greatest predictor. People perceive a lot of difficulties towards the mentioned behaviour which does not play such an important predictive role as their attitude does.

This may allow the prediction of people's intention if asked whether they feel morally obliged to reduce red meat consumption, or if there are individuals close to them who share the same belief.

Looking more closer at behavioral belief reveals a strong concern with health issues such as their benefits, cholesterol intake, the risk of getting cancer and the BSE scare. People may have strong worries concerning the appropriate intake of protein, iron and vitamins. As the high beta value proves, it may be possible to predict people's attitude rather well.

Attitude is further determined by a strong disapproval with the way in which animals are treated. As one may have expected, mainly people close to the individual such as family members, parents or friends, influence their thoughts towards reducing red meat consumption.

People are further concerned with a wide range of issues regarding their perceived difficulty towards reducing red meat consumption. The expressed obstacles refer firstly to the taste of meals without red meat or to the taste of meat alternatives. Regarding the latter, people think about the availability of meat alternatives and the expense of purchasing them. One of the most striking issues, however, is that people feel they would have to think more careful about dieting, if they were to reduce red meat consumption. This includes daily practical manners such as planning shopping, preparing meals more carefully and paying more attention to eating healthily. Another pressing matter is that people may still remember how much they enjoyed red meat. When everything else is taken into account, then these two latter issues enable the model to predict people's perceived difficulty rather well.

Keywords:
Vegetarianism, red meat, consumption, Theory of Planned Behaviour, issues, prediction, health scares, behaviour

Main Publications/Patents/Participation in conferences:
Attended BNF-seminar "Meat, more or less?" held on September 1997 in London.

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Category:	30
Starting date:	01/09/97
Duration:	12 months
Contract number:	FAIR-CT97-5005

Training in new biomarker methodologies to assess the role of diet in preventing oxidative damage to proteins and peptides

Objectives:
The techniques to determine oxidised and reduced glutathione in red cells (a sensitive index of oxidative stress) were mastered and then applied to a human intervention study on the effects of vitamin E supplementation on exercise induced muscle damage. The aim of this study was to assess whether any exercise-induced changes in oxidative damage could be ameliorated by increase intakes of the lipid soluble antioxidant nutrient, vitamin E. Consequently, healthy volunteers, half of whom consumed 160 mg vitamin E/day (as dα-tocopherol acetate for 9 ± 1 week undertook 70 eccentric contractions of the biceps brachii and brachialis muscles of the non-dominant arm using a weight stack attachment and pulley employing calibrated maximal resistance. Blood was removed at intervals before and after the exercise from the anticubital vein into EDTA-lined vacuatiners. Blood was separated into plasma, red cells and lymphocytes and analysed for glutathione and DNA damage.

Summary of achievements:
At various time points post exercise, there were increases in muscle soreness, muscle damage and lipid peroxidation and a decrease in grip strength. However there were no exercised induced changes in reduced or oxidised glutathione application of the Comet assay indicated that a transient decrease in lymphocyte DNA strand breakage in the placebo group did not occur in the vitamin E supplemented group. Moreover there was a transient increase in oxipyrimidines but not purines associated with the vitamin E supplementation. Consequently, supplementation with vitamin E did not markedly protect against aspects of exercise induced oxidative damage.

Keywords:
Glutathione, oxipyrimidine, lymphocyte, biomarker, diet, oxidative damage, proteins, peptides, vitamin E

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Category:	30
Starting date:	01/04/1998
Duration:	24 months
Contract number:	FAIR-CT98-5001

Determination of neurotoxins present in commercially available seed sprouts devoted to human food. A potential hazard for consumers

Objectives:
The research focused on free amino acids and particularly on some toxic free nonprotein amino acids and trigonelline (a plant hormone) in seeds and seedlings sold on the European market for human consumption. The aim of this project was to provide information for a potential proposition of regulation of production and sale of germinated seeds on European markets.

Summary of achievements:
Several sprouts sold in the market and sprouts germinated in the laboratory under different conditions were studied. The seedlings studied were garden pea, chickpea, mungbean, soybean, pinto bean, adzuki bean, lentils, wheat (kamut), leek and red cabbage. The determination of these amino acids was completed by the determination of total protein amino acids in lentils after acidic hydrolysis of proteins. Lentil is the prevalent legume seeds around the world and is consumed by all populations. This new parameter gives more complete information on the nutritional importance of these seedlings in comparison to the seeds.

After germination the majority of the seedlings produce trigonelline and free nonprotein amino acids and some of them are known to be rather toxic: β-(isoxazolin-5-on-2yl)-alanine (BIA) and α-aminoadipic acid. The toxicity of the other components such as trigonelline, γ-glutamyl-D-alanine, γ-glutamyl-BIA, homoserine, 2-carboxymethyl-isoxazolin-5-one (CMI) and isowillardiine are still unknown.

The seedlings that produce the highest amount of non-protein amino acids are lentil and garden pea seedlings. A study on lentil *(Lens culinaris)*, bean *(Phaseolus vulgaris)* and pea *(Pisum sativum* var Esla) seedlings was performed in collaboration with a Spanish laboratory. The germination was followed from 0 to 6 days in darkness/light conditions. For each variety the free amino acid content increased after 2, 4 and 6 days of germination. This increase is

most dramatic for asparagine in lentil growing in light and homoserine in peas after six days growing in darkness (homoserine is absent from ungerminated seeds).

The protein hydrolysis was performed on five species of lentil seeds and seedlings: *L. culinaris, L. orientalis, L. ervoides, L. nigricans* and L. *odemensis. L. culinaris* is the major consumed species. For the nutritional evaluation, the knowledge of total amino acids content of the five species is fundamental because of the many breeding programmes worldwide on lentil. The content in free protein amino acids increased dramatically after germination as well as the total protein amino acid content. Considering that also the content of fibres increased after germination, and that lentil seedlings are commercialised as a healthy food, this study of the five lentil seedlings is of nutritional importance.

Another part of the study was to use the capillary zone electrophoresis (CZE) to quantify the UV-absorbing nonprotein amino acids content in seedlings. Different experiments were done to determine the optimal conditions of use of CZE and to determine the reproducibility, the detection limit and the linear range for each nonprotein amino acids. Lentil and garden pea seedlings were analysed and the results confirmed the presence and concentration of isoxazolinones and uracil derivative compounds. It was found that lentil seedlings contain CMI that was never detected before by the classical HPLC.

Keywords:
neurotoxin, commercial, seed, sprout, human, food, hazard, consumer, lentil, nonprotein

Main Publications/Patents/Participation in conferences:
6th International Congress on Amino Acids, Bonn Germany, 3-7 August 1999 Oral Communication
5de Vlaams Jongerencongres van de Chimie, Brussels, Belgium, 11 April 2000. Poster

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Category:	30
Contract signed:	18/03/1998
Duration:	12 months
Contract number:	FAIR-CT98-5012

An evaluation of the use of British Household Budget Surveys (HBS) for epidemiological purposes. A contribution to the DAFNE initiative

Objectives:
Household Budget Surveys (HBS) have been used for the extraction of nutritional information, through the projects DAFNE I and II (EC funded). Such surveys have many advantages in comparison to other methods for obtaining information on food availability:

- Conducted at regular time intervals.
- Collect data from samples representative of each countries' population .
- Collect socio-demographic data.

The main object of this project is the validation of the nutritional data extracted from the HBS, through the comparison between Great Britain's National Food Survey (NFS) and Great Britain's National Dietary and Nutrition Survey (NDNS). Additionally, it will develop transferable methodologies for similar validation exercises in other countries. Finally, it will develop a more sophisticated model for estimating age-sex distribution of foods and nutrients intake within households through the use of the data provided by the NFS.

Summary of achievements:
To date, no information available

Keywords:
British Household Budget Survey, HBS, epidemiology, DAFNE, National Food Survey

Main Publications/Patents/Participation in conferences:
Presentation of 'Food aggregation problems and decisions' and 'UK: Progress of work' in the DAFNE II (Data Food Networking for Pan European Food Data Bank based on Household Budget Surveys funded by the EU by the Agriculture and Agro-industry, including Fisheries, Programme) meeting, Brussels, 4th October 1996.

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Presentation of 'From Foods to Nutrients' for the DAFNE project in the COST Action 99 Food Composition and Consumption 6th Management Committee and Associated Workshops, Norwich, UK 18-20 April 1997.
Presentation of 'Validating the Conversion of Food Expenditure to Food Quantities' in the DAFNE II meeting, Brussels, 23rd May 1997.

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Contract signed:	26/02/1998
Duration:	24 months
Contract number:	FAIR-CT98-5017

Development and evaluation of an immunoassay for a novel marker of free radical status

Objectives:
Considerable evidence has implicated reactive free radicals in many types of tissue injury, including cardiovascular disease. Excess exposure to free radicals from environmental or other sources (e.g. radiation, smoking and other pollutants, trauma, certain drugs and toxins) results in oxidative stress, which is defined as a disturbance of the pro-oxidant/antioxidant balance in favour of the former. While peroxidation of lipids is a well recognised consequence of oxidative stress and is postulated to be an early event in atherosclerosis, the lack of a convenient and reliable method to assess lipid peroxidation *in vivo* has greatly hampered research in this area.

An easy and quick method to determine free radical formation *in vivo* will be developed. So far, developed methods are either time consuming or expensive. In order to establish this goal a monoclonal antibody against 8-iso-PGF2 alpha will be raised, which has shown to be increased in subjects which are under oxidative stress (i.e. show a high free radical formation). Once the antibody has been raised, an enzyme immunoassay will be developed to determine 8-iso-PGF2 alpha in urine and thus determine free radical formation. Ideally the method will be quick and easy so that a large number of urine samples can be screened (both from controls and subjects that are under oxidative stress). The developed method will also be compared to already established methods (e.g. MDA assay).

Summary of achievements:
The first stage of the work involved evaluation of a monoclonal antibody (1D11) that had already been developed against 8-iso-prostagiandin F2α (8epi), which has a potential for use as a urinary marker for oxidative stress and free radical status.

A competitive enzyme immunoassay was established a solid phase antibody and an 8epi-HRP enzyme conjugate. The assay could measure in the range of 1-100 ng/ml (ED_{50} 24 ng/ml)

which was not sensitive enough for measurement of 8epi in urine (concentration in pg/ml range).

Work is continuing in two directions aimed at the development of a more sensitive assay for 8epi. Firstly, a range of different 8epi immmogen conjugates were prepared, aimed at improving the immune response to 8epi. Both BSA and KLH were used as carrier proteins; different hapten:protein ratios were used and conjugations were carried out in the presence and absence of adjuvant peptide. Immune responses were obtained and some fusions carried out. To date, one monoclonal which is more sensitive than the existing antibody OED_{50} 9 ng/ml) has been produced and further fusions are planned.

The second approach taken aimed at development of an immunometric assay for 8epi. This reagent excess assay configuration has potential to be more sensitive, specific and more robust than competitive assays. Two antibodies are needed for this type of assay, the first antibody is directed against the analyte (8epi), while the second antibody is directed against the primary antibody-analyte complex. Monoclonal antibody. ID 11, has been purified and potentially useful responses against this antibody have been obtained in mice. This work is ongoing.

Keywords:
Protein purification, conjugation methods, monoclonal antibody production, assay development

Main Publications/Patents/Participation in conferences:
Work presented at BioResearch Ireland Annual Conference at Athlone, Ireland, February 1999.

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Category:	30
Starting date:	20/03/1999
Duration:	12 months
Contract number:	FAIR-CT98-5024

Presence of high concern chemical contaminants in selected biological matrices and the impact on human health

Objectives:
Highly toxic compounds such as polychlorinated biphenyls (PCBs), organochlorinated pesticides (OCPs), polycyclic aromatic hydrocarbons (PAHs), polychlorodibenzodioxins (PCDDs) and dibenzofurans (PCDFs) are commonly found in the environment due to human influences. They are very persistent, widely distributed and have a high propensity for accumulating in living tissue. This project aimed to estimate the extent of contamination by these compounds in edible marine species. Different fishing harbours along the coast of the Adriatic Sea were sampled. Using this information, the level of human exposure to these contaminants through these fish products would be evaluated.

Summary of achievements:
Edible marine species from the Adriatic Sea were analysed for their content in persistent organic pollutants (POPs). PCB cumulative findings in the selected marine species revealed that a remarkable difference of contamination levels seems to characterise species obtained from the northern, central, and southern sampling sites.

The greatest PCB concentrations were found in mackerel. DDE concentration levels varied from 0.7 to 32.4 ng/g fw. The highest levels of contamination were found in mackerel, red mullet and anchovy.

On the whole, PCDD/F contamination levels were low. In general, I-TE findings were greater for those species at higher levels in the trophic web (mackerel > red mullet > anchovy). Contamination levels were within 0.23 and 1.07 pg TE/g fw in the species above, while all remaining species exhibited contamination levels ranging from approximately 0.07 to 0.25 pgTE/g fw. Besides, I-TE cumulative findings in species from the northern area were in

general higher than those from the central and southern areas (with the questionable exception of mackerel).

In general, contamination levels of PAHs in the edible marine species were low, often below the limit of determination. Although low, the highest levels were determined in mussel and clam. Results of the Ames test revealed that, under the established experimental conditions, northern mussel extracts were not mutagenic in the tested bacterial species *(Salmonella typhimurium* TA100).

Daily intakes of POPS with consumption of Adriatic seafood were estimated in 707.0 ng of PCBs/person/day in the southern area to 1813.8 ng/day in the northern one; from 127.7 to 183.5 ng of DDE/person/day; and average daily intake of PCDD/F was estimated in approximately 7 pgTE/person/day. Although it may be inferred from these results that analysed fish is an important source of human exposure to PCBs, estimated daily intakes of POPS are far below the Tolerable Daily Intakes (TDIs) set by the FAO/WHO or established in different countries.

In conclusion, marine species appear to show, as expected, a trend towards higher contamination levels with increasing anthropic impact. However, contamination levels determined in this study give no indication of particular health risks associated with consumption of these products.

Keywords:
persistent organic pollutants, POPs, Tolerable Daily Intakes, *Salmonella typhimurium*, seafood, PCB, contamination

Main Publications/Patents/Participation in conferences:

S. Bayarri, L. Turrio Baldassarri, N. Iacovella, F. Rodriguez, C. La Rocca, A. di Domenico (1999) Levels of PCBs, PCDDs, and PCDFs in selected marine species from the Adriatic Sea: preliminary data. Communication presented at the Fifth European Conference on Ecotoxicology and Environmental Safety - SECOTOX 99. Neuherberg/Munich, Germany.

S. Bayarri, L. Turrio-Baldassarri, N. Iacovella, F. Rodriguez, A. di Domenico (1999) Toxic organic microcontaminants in edible marine species from the Adriatic Sea. *Organohalogen Compounds* (in review process for the DIOXIN'99).

L. Turrio-Baldassarri, S. Bayarri, A. di Domenico, A., Fulgenzi, A., La Rocca, C., Iacovella, N. (1998) Supercritical Fluid Extraction-HRGC-HRMS Determination of PCB in Lyophilized Samples of Antarctic Krill. *Organohalogen Compounds, Vol. 35,* 187-190 (DIOXIN'98).

L. Turrio-Baldassarri, S. Bayarri, A. di Domenico, N. Iacovella, C. La Rocca (1999) Supercritical Fluid Extraction of mollusk samples for simultaneous GC-MS determination of Polychlorobiphenyls and Polycyclic Aromatic Hydrocarbons. *Int. J. Environ. Anal. Chem.* (in press).

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Category:	40
Starting date:	2/11/1998
Duration:	12 months
Contract number:	FAIR-CT98-5033

Evaluation of the anticarcinogenic potential of recently isolated, pure naturally occurring polyphenols

Objectives:
There is strong epidemiological evidence that the consumption of diets rich in vegetables and fruit is associated with reduced cancer incidence at specific sites as well as in the overall cancer incidence. However, the plant components responsible for this chemo-preventative effect remain unidentified and their underlying mechanism elusive.

Polyphenolic compounds are believed to be responsible for the low incidence of cancer incidence. Tea is one such substance rich in polyphenolics.

Therefore the objectives of the current project were to:
- Set-up the ^{32}P-postlabelling procedure for measurement of DNA adducts following their separation by HPLC.
- Establish whether IQ, a carcinogen, can generate DNA adducts *in vitro* following incubation with appropriate activation systems.
- Evaluate different enrichment techniques for the detection of IQ-DNA adducts.
- Investigate the *in vivo* formation of IQ-DNA adducts in the liver of rats.
- Investigate the formation of IQ-DNA adducts in extrahepatic tissues.
- Determine the effect of tea consumption on the hepatic concentration of IQ-DNA adducts.
- Investigate the use of liver slices in studying IQ-DNA adduct formation and how this may be modulated by naturally occurring polyphenolics.

Summary of achievements:
A method has been developed for the determination of the binding of the food carcinogen 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) to DNA. The formation of IQ-DNA adducts was determined using the ^{32}P-postlabelling procedure, following the separation of the DNA adducts by HPLC. The detection limit of IQ-DNA adducts could not be improved by enrichment procedures such as butanol extraction and solid phase extraction.

Initial investigations conducted *in vitro* revealed that photoactivated azido-IQ generated four adducts with DNA, of which three were major. When administered to rats by gastric intubation, IQ produced four DNA adducts in the liver as early as two hours after the administration of the carcinogen. Formation of IQ-DNA adducts in the liver appeared to be dose-dependent. DNA adducts were detected also in the heart and, to a lesser extent, in the kidney. On the basis of retention times, the same IQ-DNA adducts appear to be produced in all the tissues studied. Exposure of rats to green tea, as their sole drinking liquid, for four weeks failed to influence the extent of binding of IQ to hepatic DNA, 16 hours after the administration of the carcinogen. However, the same treatment with black tea or decaffeinated black tea appeared to increase the binding if IQ to hepatic DNA in rats.

Keywords:
Anticarcinogenic, polyphenols, rats, IQ-DNA, carcinogen, liver, cancer

Main Publications/Patents/Participation in conferences:
Participation in two conferences as part of the European Union Project PLO653 (CPT and Health).

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Category:	20
Starting date:	06/10/1998
Duration:	24 months
Contract number:	FAIR-CT98-5043

Contribution of individual wheats and their blends to processing behaviour and quality of prepared consumer foods

Objectives:
Three different European flours were investigated on their own, as 50/50 blends and with gluten addition. Their compositional, rheological and baking properties were assessed to determine suitability for use in bakery products.

Summary of achievements:
German and English flour samples had lower gluten quality in comparison to the Irish flour as measured by a gluten index test. When blended with Irish flour the final gluten quality improved, resulting in better bread-making potential.

The bread from Irish flour had the lowest volume and darkest crumb colour. In consequence, when it was blended with German flour the final volume increased and the crumb colour improved. Adding gluten also improved bread volume, although it slightly darkened the breadcrumbs.

Sensory panels were carried out to evaluate the consumer acceptability of pan bread and bread rolls. Breads resulting from blended flours were found to be softer than breads from individual flours; differences in flavour were not found. On the other hand, breads from flours with the addition of gluten were least preferred in flavour and were firmer in texture.

An instrument texture profile analysis performed on the different breads indicated that Irish bread was less chewy than the German (which had the highest chewiness); however, when the flours were blended the chewiness improved.

In conclusion, blending improved the compositional, rheological and baking quality (except in flavour) of individual flours. In addition, adding gluten had a negative effect on the crumb colour, but improved the bread volume.

Keywords:
flour, gluten, rheology, baking

Main Publications/Patents/Participation in conferences:
29th Annual Food Science & Technology Research Conference, September 1999, Cork, Ireland.

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Category:	30
Starting date:	01/01/1999
Duration:	24 months
Contract number:	FAIR-CT98-5061

The content and bioavailability of folates in processed foods

Objectives:
Health benefits of folates regarding their prevention of neural tube defects in babies and occlusive vascular diseases caused by elevated plasma homocysteine, their link to mental fitness and possibly certain forms of cancer have already been recognised. However, food folate analysis is still tedious due to a lack of validated methods. Therefore the assessment of folate losses through industrial and household food processing is still incomplete as well as knowledge on folate bioavailability in humans.

This project's objective was to evaluate various methods of industrial food processing for their impact on folate contents in food.

Summary of achievements:
Contacts to industry and co-operating research partners were established, most analytical methods were established and under validation. Sampling of food samples and investigation of the impact of processing on folate contents is currently ongoing. Preparations for the human study are in the final stage, enabling soon a start of the human bioavailability work on ileostomists. Ethical approval for the study has already been received.

Keywords:
Health, folates, neural tube, defects, baby, occlusive vascular disease, disease

Main Publications/Patents/Participation in conferences:
C.M. Witthöft, J. Jagerstad, I. Bitsch (1999) "Folate content and bioavailability in food using HPLC-methods and a human model". In Schubert R, Flachowsky G., Bitsch R., Jahreis, G. (ed.): 7th Symposium Micronutrients 1999, Vitamine und Zuzatzstoffe in der Ernährung von Mensch und Tier. 22-23 September 1999, Jena, Thüringen, Germany, pp. 109-114.

"The content and human bioavailability of folates in food using HPLC-methods", Third International Food Data Conference. Rome FAO Headquarters, 5-7 July 1999.
Representative for Prof. M. Jägerstad, The Swedish University of Agricultural Sciences, Uppsala, Sweden: Cost Action 99, Eurofoods Meeting, Rome FAO Headquarters, 4&8 July 1999: Poster.
"Folate content and bioavailability in food using HPLC-methods and a human model", A talk at the 7th Symposium Vitamins and Additives in the Nutrition of Man and Animal, Jena (Thüringen), Germany, 22-23 September 1999.

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Category:	20
Starting date:	01/02/1999
Duration:	30 months
Contract number:	FAIR-CT98-5071

Conjugated Linoleic Acid (CLA): Is the nutritional benefit mediated by an interaction with eicosanoid synthesis?

Objectives:
Conjugated linoleic acid (CLA) is a mixture of positional and geometrical isomers of linoleic acid, which have two conjugated double bonds. In the last decade it was reported that CLA might have beneficial effects on human health. Several animal experiments have shown anticarcinogenic and antiatherogenic properties as well as an influence on body fat and energy metabolism. Since like linoleic acid, CLA are metabolised by desaturation and elongation in C20:3 conjugated fatty acids, it has been suggested that CLA influences the eicosanoid pathways and may modulate the inflammatory process. The research objectives of this project are:

- To examine the influence of food processing technologies (preparation of Emmental cheese) on the CLA content and the composition in different CLA isomers;
- To synthesise stereoselectivity the metabolities of CLA, the (8Z, 11Z, 13E) – eicosatrienoate (C20:3 c8c11t13) and the (6Z, 9Z, 11E) octadecatrienoate (C18:3 c6c9t11) in multistep synthesis to obtain pure isomers; and
- To study the effects of desaturation and elongation products of CLA (C18:3 and C20:3 conjugated fatty acids) on eicosanoid production.

Summary of achievements:
In the first year, the work focused on synthesis of (8Z, 11Z, 13E)-eicosatrienoate (C20:3 c8c11t13) as it is found as the metabolite of the major CLA-isomer in dairy products, the (9Z, 11E)-octadecadienoate.

A common intermediate in the normal synthesis of these three molecules, ((E)-Non-2-enyl) triphenylphosphonium bromide (A), was obtained in three steps. The synthesis used 2-nonynol (commercial product) as starting material, which was reduced selectively to the (E) 2-nonenol by using Sodium bis(methoxyethoxy)-aluminiumhydride (Red-Al). Replacement of

the hydroxy-group to a bromine atom was accomplished by triphenylphosphine dibromide. The resulting bromide was transformed to the phosphonium salt under standard conditions. By using this intermediate, the (8Z, 11Z, 13E)-eicosatrienoate (C20:3 c8c11t13) can be obtained in seven steps. At first, a second intermediate (3, 3-di-ethoxypropyl) triphenylphosphonium iodide (C) was prepared from acrolein in a two step synthesis.

The acetalester (B1) was obtained by a cross-coupling reaction of the Grignard reagent prepared from the commercial 2-(2-bromoethyl)-1, 3-dioxalane and the ethyl-5-bromovalerate in presence of dilithium chlorocupate. After selective hydrolysis of the acetal moiety of B1, a Wittig condensation between B2 and C under cis-olefination conditions furnished the ethyl (Z)-11, 11-diethoxyundec-8-enate (B3). The aldehyde function was then deprotected by an acidic hydrolysis of the acetal function using trifluoroaecetic acid (B4). A second stereoselective Wittig-condensation between the common intermediate A and B4 in the presence of HMPA will provide the ethyl (8Z, 11Z, 13E)-eicosatrienoate. The corresponding free fatty acid was obtained by alkaline saponification at room temperature.

The synthesis of the molecule with a conjugated double bond was found to be more difficult than for formerly described synthesis of nonconjugated polyunsaturated fatty acids, because of several by-reactions. The conjugated double bond implies intermediate products with one double bond in an allylic position to another functional group which causes the elimination process. Likewise the conjugated double bond has preferably formed to reduce the possible isomerisation risk.

The radiolabelled [1-^{14}C] molecule of the (8Z, 11Z, 13E)-eicosatrienoate, the (9Z, 11E)-octadecadienoate and the (6Z, 9Z, 11E) Octadecatrienoate will be carried out at the beginning of the second year using a similar reaction pathway.

An analysis of the CLA content during the Emmental cheese production was carried out. Samples were taken at different processing stages to determine a change in CLA-content and CLA-isomer composition depending on processing conditions. The cooking of the cheese was carried out at three different temperatures, to look at the possible influences of this parameter on CLA-content. Different fermenting cultures were also used. The analysis of the samples was optimised. The composition in different CLA-isomers was examined using Silver-Ion-HPLC with four columns in series. This method permits the separation of 16 different CLA isomers.

Keywords:
conjugated linoleic acid (CLA), eicosanoid synthesis, Emmental, cheese

Main Publications/Patents/Participation in conferences:
J. L. Sébédio, S. Gnädig, J. M. Chardigny (1999) Recent advances in conjugated linoleic acid research – Curr. Opin. Clin. Nutr. Metab. Care, 2, 499-506.

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Category:	30
Contract signed:	31/01/1996
Duration:	36 months
Contract number:	FAIR-CT96-5002

Molecular approaches against mushroom browning

Objectives:
The edible, cultivated mushroom, *Agaricus bisporus*, is prone to a brown discolouration. This impacts on quality causing subsequent economic losses. Recent research has tackled the mechanisms behind regulation of the genes that code for the enzymes that cause this browning.

Tyrosinase, is the main polyphenol oxidase enzyme, involved in the oxidation of phenolic substrates into quinones that result in discolouration. It exists in different isoforms.

The heterokaryotic organisation of the nuclei in *A. bisporus*, with more than one copy of the parental genetic information per cell, means that there is little genetic variation between mushroom strains. Such complex heterokaryotic systems can be studied in greater detail through transformations whereby donor DNA can be integrated into the genome. Similarly, a combination of gene disruption and antisense inhibition molecular techniques could be used to down-regulate the genes encoding enzyme activity.

The ultimate aim of this research was to accumulate enough knowledge so as to modify a strain that will improve the quality aspects associated with diminished levels of browning through manipulation of the genes that are ultimately responsible for the discolouration.

Summary of achievements:
One antisense tyrosinase transformant from *A. bisporus* has been isolated which in contrast to wild-type (commercial) strain shows lower tyrosinase (EC 1.14.18.1) activity. Genetic analysis has demonstrated that transformation of an adenine-auxotropic strain *of A. bisporus*, based on selection with the hygromycin B *(hpt)* gene, is well established and reproducible.

Included in the transgenic genome are the partial fragments of two tyrosinase genes *(AbPP01* and *AbPP02,* which have been shown to be involved in the browning process of *A. bisporus)* cloned in the antisense direction. Tyrosinase-mediated browning (the polyphenoloxidase activity of main importance for brown discoloration) was selected as a model to study possibilities for gene silencing in *A. bisporus.* Various co-transforming constructs were applied in order to achieve gene disruption or antisense inhibition. Molecular analysis of recombinant DNA by DNA hybridisation reveals stable integration of the donor DNA sequences yielding various banding patterns. The expression of these sequences was demonstrated using Northern blot analysis showing short novel transcripts of approximately 600 nt length. This work is the first example of attempts to modify commercially important properties of the edible mushroom, *A. bisporus* through genetic engineering. Although in some transformants tyrosinase mRNA pools were strongly diminished or absent, no complete inhibition of all tyrosinase isoforms was observed.

Keywords:
Agaricus bisporus, tyrosinase, antisense, mushroom browning, recombinant DNA

Main Publications/Patents/Participation in conferences:
A.C. Möller, C. Soler Rivas, H. Mooibroek and H. Wichers (1997) Antisense tyrosinase inhibition to study bacterial blotch browning in the cultivated mushroom, *Agaricus bisporus.* Acta Botanica Neerlandica. 46 p. 429-430
A.C. Möller, M.D. van de Rhee, O. Mendes, M.W.T. Werten, D. Donkers, H. Wichers and H. Mooibroek (1996) Molecular approaches against mushroom browning. Acta Botanica Neerlandica. 45 (4) 1996, p.574-575
H. Mooibroek, M.D. van de Rhee, A. Möller and H. Wichers (1996) Transformation of the cultivated mushroom *Agaricus bisporus* to prevent browning? Symposium on Fungal Biotechnology. November 1996, Quest, Weesp, The Netherlands.

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Category:	30
Starting date:	01/06/1996
Duration:	24 months
Contract number:	FAIR-CT96-5009

Molecular and somatic cell techniques for legume improvement

Objectives:
New techniques of molecular biology and genetic engineering now make it possible to identify and transfer individual genes, thus speeding up the process of plant breeding, and also enable the introduction of genes not previously present in a particular species. It has also proved possible to achieve beneficial modifications by inactivating existing genes, for example by antisense technology.

The objectives of this research project were:

- to explore a standard plant regeneration system in elite breeding lines of *Phaseolus vulgaris* and landraces of *P. coccineus*, which allows the identification of genotypes that are competent for *in vitro* culture and that could produce highly responsive hybrids. The development of germplasm with enhanced levels of *in vitro* ability would result in significant progress in plant biotechnology and breeding technologies,
- to estimate the genetic diversity among *Vigna* landraces and the relatedness between cultivated with wild and weedy forms using RAPD markers, which would contribute to a better understanding of the genus, and to save misdirected efforts on the part of plant breeders dealing with this crop.
- to analyse the function of a CTR clone homologue tCTR, which was isolated from a tomato fruit cDNA library. Antisense technology is used to down-regulate tCTR in tomato fruit, and the effect on fruit ripening was to be studied. The possibility of manipulating ripening of tomato fruits and also controlling processes such as abscission and senescence of leaves and flowers was also addressed.

Summary of achievements:
In vitro studies of *Phaseolus*
In vitro culture response and regeneration capacities varied significantly between species and amongst genotypes; although no significant differences were observed amongst genotypes for the number of buds per explant and the rooting efficiency of shoots. *P. coccineus* produced

more buds and shoots per explant, with a higher rooting efficiency of shoots than *P. vulgaris*, although *P. vulgaris* had a short response for the development of buds but presented a long response for the elongation and rooting of shoots compared to *P. coccineus*. All bean genotypes were more or less responsive to tissue. Differences between genotypes for in vitro response have been previously reported in other crops, such as *Arabidopsis*, maize, and wheat, which indicates that a genotype-specific regeneration ability may be presented in this work, and that the genotype used is of major importance for the plant tissue culture. Indeed; the intraspecific but also interspecific variation observed for regeneration capacity could be exploited in breeding programs aimed at constructing regeneration-competent genotypes.

RAPD studies in Vigna

Sixty primers were used to amplify the genomic DNA of the 22 Vigna landraces. The multivariate techniques show that germplasm collected on India presented by far the widest range of variation compared to that from the other countries. Landraces from India were found in most of the clusters obtained. India has been considered to be the region of first domestication of mungbean and contains a very wide diversity of cultivated forms and weedy types. This result emphasise the importance to carry on exploration of the regions in India in which the mungbean species originated and where it was first domesticated, in contrast to that available from other countries. The fact that landraces from China, Korea, Bangladesh, Thailand and Madagascar all show similar restricted variation supports the view that they could have a common origin. Closest to these mungbean accessions on the dendogram was the wild relative *V. radiata* var. *sublobata* collected on China. It is found all through tropical Asia, Southeast Asia, Indonesia and Northern Australia. The wild form *V. radiata* var. *sublobata is* of immense practical value to plant breeders since a yellow mosaic virus resistant type and bruchids resistant has been reported, and there are indications that wild types can be used to widen the genetic variability of the cultivated ones.

tCTR antisense experiments

Two constructs have been prepared to test the effect of down-regulation of tCTR. There was some inconsistency in this delayed ripening phenotype within individual transformants. The individuals producing fruit showing a delay also produced other fruit that ripened normally. It was quite difficult to regenerate plants using this construct, which could confirm that this gene has an important function.

Keywords:
Legume, antisense technology, breeding lines, *Vigna*, races, *Phaseolus*, genes, hybrid

Links with EC projects:
FAIR-CT98-5026 (p.134)

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Category:	30
Starting date:	01/08/1996
Duration:	24 months
Contract number:	FAIR-CT96-5021

Mapping Quantitative Trait Loci (QTL) associated with potato tuber quality: special reference to reducing sugars during storage

Objectives:
The general aim of the research was the identification in potato of Quantitative Trait Loci (QTLs) associated with cold sweetening, that are stable across different genetic backgrounds and environments. The project aimed at identifying molecular markers associated with low content of reducing sugars after cold storage to be used in potato breeding programmes.

Summary of achievements:
The accumulation of the reducing sugars glucose and fructose in potato tubers stored at low temperatures, is of significant commercial importance for the potato industry because of its effect on processing quality.

This research identified ten genomic regions that contain putative QTLs for reducing sugar content, of which three are conserved among environments and one across populations.

This research has the potential to identify diagnostic markers for sugar content after storage for a range of potatoes with different genetic backgrounds, and environmental origins. These markers have been tentatively identified based on results from chromosomes VII and VIII. They should be further tested at tetraploid level to asses their value as useful tools in marker-assisted potato breeding programmes. The identification of potential candidate genes will help elucidate the role of these genes in the mechanisms involved in sugar accumulation and their relative contribution to genetic variation of the trait. Analysis of the correlation with other important traits like starch and yield or maturity, will; be very useful to confirm associations of traits that have been observed by breeders but whose genetic basis have not been elucidated as the result of linkage in selected clones, or the result of pleiotropy.

Keywords:
Mapping, Quantitative Trait Loci, QTL, potato, tuber, quality, reducing sugars, storage, breeding programmes, glucose, fructose

Main Publications/Patents/Participation in Conferences:
Plant and Animal Genome VI Jan 18-22, 1998. San Diego, CA, USA.
5th International Symposium on the Molecular Biology of the Potato. Aug 2-8, 199?, Bogensee, Germany

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Category:	20
Starting date:	01/01/1996
Duration:	12 months
Contract number:	FAIR-CT96-5028

Characterisation of resistance to broomrape (*Orobanche cernua* LOEFL.) of sunflower

Objectives:
Six cultivated sunflower (*H. annuus* L.) sources from different origins, resistant to *O. cernua* Loefl., one carrying the gene of resistance Or_5 and two susceptible lines, along with the F_1 crosses (resistant x susceptible and resistant x resistant) and segregating generations (F_2 and BC_1F_1) were studied to characterise the gene number and mode of inheritance of resistance to broomrape.

Summary of achievements:
Segregation ratios of F_2 and backcross generations indicate one major dominant gene controlling resistance. The lack of segregation in the F_2 and BC_1F_1 generation between resistant lines indicate that the gene for resistance in the different resistant lines is allelic to or closely linked to Or_5 gene. Further evaluations of all the resistant lines of this study to other new more virulent inoculum, which overcome resistance given by Or_5 gene were carry out. Two of the resistant lines used in this study, JB-2 and Kavk showed resistance to the new population (SE296). These results suggest that these lines may have an allele or gene different from the Or_5 with was not possible to distinguish with inoculum SE194. This resistance to the new virulent broomrape population SE296, is an important finding since resistance of current sunflower cultivars is based on Or_5 gene.

The results of segregating generations of an interspecific hybrid of a wild annual sunflower resistant species, *H. exilis* and a cultivated susceptible line suggested that two genes could be involved in the resistance of the species and that they are different from Or_5. This was an interesting possibility since the resistance of Or_5 was starting to be overcome by new virulent races: Interspecific hybrids and backcross generations between the resistant wild perennial species *Helianthus resinosus* Small, *H. pauciflorus* Nutt., *H laevigatus* T.&G., *H. nuttallii*

nuttallii T.&G. and *H. giganteus* L., *H. hirsutus*, *H. californicus* and *H. gracilentus* and the broomrape susceptible *H annuus* L. cultivated inbred line HA89 were made to study the behaviour of hybrids and backcrosses on the transmission and expression of resistance to this parasite. The resistance of the F, plants between the wild perennial species and HA89 indicated that the resistance is dominant in this species, thus facilitating its transfer in backcross programmes. In the case of the diploid *H. giganteus* and *H. nuttallii*, resistant plants with 34 chromosomes, the diploid number of cultivated sunflower, were obtained whereas in the case of BC,Ft of hexaploid perennials x *H. annuus*, the resistant plants had 51 chromosomes. Segregation for susceptible and resistant individuals indicated that the transfer of resistance found in these species with different ploidy levels into cultivated sunflower was feasible.

Studies on the effect of temperature on disease reactions of sunflower plant material to infection by *O. cernua* using three different virulent populations of the parasitic and four lines of sunflower (KA-41, J-8281, HA-89 and RHA-273) were carried out in growth chambers set at four different temperatures (from 15 to 27°C). The three populations of broomrape were able to infect sunflower plants at a wide range of temperatures (IS-23 C), whereas 27°C restricted the level of infection in all cases. These results were confirmed with field observations. Our results suggest that the effect of temperature on the host-parasite relationship is complex, a linear effect being shown for line HA-89. Therefore, the establishment of differential lines should consider temperature. Recommendations for sunflower planting dates should include results for specific lines. Evaluations of early plantings should take into account the possibility of "hidden" infections by broomrape plants that do not emerge but impair the host crop.

Keywords:
Characterisation, resistance, broomrape, *Orobanche cernua,* sunflower, wild species, inheritance, interspecific hybridisation, *Helianthus*

Main Publications/Patents/Participation in conferences:
S. Sukno, C.C. Jan, J.M. Melero Vara, J. Fernandez-Martinez (1998) Reproductive behaviour and broomrape resistance in interspecific hybrids of sunflower. Plant Breeding 117: 279-285
S. Sukno, J.M. Melero Vara, J. Fernandez-Martinez (1998) Genetic analysis of resistance to *Orobanche cernua* in several lines of cultivated sunflower. Crop Science. *In press*
S. Sukno, J. Ruso, C.C. Jan, J.M. Melero Vara, J. Fernandez-Martinez (1998) Interspecific hybridisation between sunflower and wild perennial species. Euphytica. *In press*

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Category:	30
Contract signed:	12/07/1996
Duration:	18
Contract number:	FAIR-CT96-5039

Enhancement of the carotene content of tomatoes

Objectives:
The objective of the project was to increase the β-carotene content of tomato fruit by genetic transformation in order to produce a fruit crop containing high dietary levels of this carotenoid.

The high-beta variety of tomato was chosen to be transformed with a bacterial phytoene synthase gene, coupled to a fruit specific promoter (the polygalacturonase promoter) and the leader sequence of the tomato phytoene synthase gene.

Summary of achievements:
The aim of this project was originally to improve the β-carotene content in tomatoes by transforming plants with the phytoene synthase gene. The results obtained, however, showed no significant modifications of the carotenoid levels in these fruits, using the phytoene synthase gene.

The strategy was therefore altered by modifying the expression of another carotene enzyme, lycopene cyclase. This enzyme is responsible for the conversion of lycopene to β-carotene by catalysing the formation of two β-rings at each end of the carotene.

The first step involved cloning the full-length cDNA corresponding to this enzyme in the tomato. This cDNA, was isolated by RT-PCR using total RNA extracted from fruit, and specific oligonucleotides to amplify the cDNA.

This DNA fragment was 1.2 bp long and was coupled to a ripening specific promoter and incorporated to the binary transformation vector. After being transferred to *Agrobacterium* this construct was used for transforming cotyledons of the tomato variety Ailsa Craig.

A second fragment was generated in order to prepare a second construct with a antisense orientation. Both constructs directed to up and down-regulate the expression of this enzyme are now in progress.

In addition, a study on the mechanisms of regulating carotenogenesis in fruit was undertaken, using RT-PCR to analyse the expression of mRNA lycopene cyclase (Lcy).

To determine the level of transcript of Lcy gene in fruit, total RNA was extracted at mature green and ripe stages, and subjected to Northern blot analysis. No expression was detected by this method, indicating the low level expression for the transcript corresponding to this enzyme. For that, the amount of Lcy mRNA was measured by reverse-transcription followed by polymerase chain reaction (RT-PCR) of total RNA. The results show Lcy mRNA decreases through ripening. These results demonstrate that the expression of Lcy is down-regulated during ripening, in contrast with other enzymes involved in earlier steps in the pathway.

Keywords:
β-carotene, carotenoid, phytoene synthase gene, lycopene cyclase, cDNA, carotenogenesis, Emit, mRNA, lycopene cyclase, Lcy mRNA

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Category:	20
Starting date:	08/11/1996
Duration:	24 months
Contract number:	FAIR-CT96-5052

Biochemical and molecular characterisation degradation in transgenic melons expressing an antisense acc oxidase gene

Objectives:
The plant hormone ethylene affects a diverse array of plant growth and developmental processes, including germination, fruit ripening, senescence, abscission, flowering, and stress response. Mutagenesis is a powerful approach to the identification of genes through the lass of or change in their function. Mutants can be generated by chemical and physical means, but insertional mutagenesis, using known DNA sequences as insertion elements, has the added advantage of greatly facilitated gene cloning.

Etiolated *Arabidopsis* seedlings treated with ethylene show the triple response which in seedlings constitutes an exagerated curvature of the apical hook, inhibition of hypocotyl and root elongation and radial swelling of the hypocotyl. Alterations in the triple response to ethylene of dark and light-grown seedlings were used to isolate mutants implicated in ethylene biosynthesis and response. T-DNA-transformed *Arabidopsis thaliana* (ecotype Wassilewskija) populations were generated by vacuum infiltration of flowering plants immersed in a high-density suspension of *Agrobacterium tumefaciens* C58C1 (pMP90), containing the binary vector pGKB5. This vector carries a *nptII* gene, conferring kanamycin resistance, a bar gene, conferring resistance to the herbicide Basta (phosphotricin) and a promoterless GUS gene, at the right border of the T-DNA for promoter trapping. The plants were then returned to the greenhouse and allowed to set seed, and the seed was then screened for transformants.

Summary of achievements:
The Cantaloupe Chanterais Melon (*Cucumis mela* var. *cantaloupensis*, Naud) is a climateric fruit exhibiting poor keeping quality that severely limits its commercial development. Beside its poor storage capability, the Chanterais melon has remarkable organoleptic traits such as accumulation of large amounts of sugars and aroma volatiles. Because of these specific traits it represents a valuable target for improving shelf life through genetic engineering. Transgenic melons had been generated that harboured an antisense ACC oxidase gene encoding the enzyme of last step of ethylene biosynthesis. In these melons ethylene synthesis was inhibited by more than 99% and several ripening pathways were blocked (de-greening of the rind, softening...). However, the normal ripening phenotype could be reversed by exogenous

ethylene treatment. These transgenic melons represent a very good model for discriminating between the biochemical events that are dependent and independent from ethylene.

The activity of cell wall degrading enzymes in transgenic and control melons were compared. Electron microscopy studies were carried out on the structure of the rind and on the accumulation of calcium in water-soaked areas (a physiological disorder which impairs fruit quality).

Keywords:
cell wall degrading enzyme, melon, electron microscopy, cantaloupe, characterisation, degradation, transgenic, antisense, oxidase, gene

Main Publications/Patents/Participation in conferences:
C. du Chatenet, E. Olmos, A. Latché, J.C. Pech, B. Ranty, R. Ranjeva, A. Graziana, C. Balague (1997) Spatio-temporal variation of tissues structure, gene expression and protein accumulation during watersoaking development in melon fruit. In: 3e Colloque Général de la Société Française de Physiologie Végétale. Plant Science. Toulouse 1,2,3 December 1997. Edited by: Pech J.C., Latché A. and Bouzayen M. pp151-152.
E. Olmos, A. Latché, J.C. Pech, N. Bechtold, G. Pelletier, M. Bouzayen (1997) Isolation of *Arabidopsis thaliana* T-DNA tagged mutants with a altered ethylene response. In: 3e Colloque Général de la Société Française de Physiologie Végétale. Plant Science. Toulouse 1,2,3 December 1997. Edited by: Pech J.C., Latché A. and Bouzayen M. pp65-66.
S. Ramassamy, E. Olmos, M. Bouzayen, J.C. Pech, A. Latché (1998) 1-aminocyclopropane-1-carboxylate oxidase of apple fruit is attached to the external face of the plasma membrane. Journal of Experimental Botany. 49:1090-1915

Links with EC projects:
FAIR-CT98-5044 (p.272)

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Category:	20
Starting date:	01/02/1997
Duration:	12 months
Contract number:	FAIR-CT96-5062

Molecular aspects of bacterial blotch disease of *Agaricus bisporus*

Objectives:

- Testing of the "White line test" that normally is used to identify the pathogenic pathotype of *P. tolaasii* as preventing or retarding reaction for the mushroom discolouration. Study of using the compound WLIP as chemical to avoid the bacterial brown blotch.
- Establishment of the effect of *P. tolaasii* or its toxin on genetically modified mushroom with anti-sense tyrosinase constructors.
- Study of possible biological control agents such as antagonistic *Pseudomonas* strains and the non-pathogenic form of *P. tolaasii*. Molecular effects of the application of antagonistic agents.
- Investigation on possible biochemical control by addition of chelating agents in combination with anti-bacterial agents for which *Pseudomonas* may be sensitive. Use of specific enzymes as control of the disease.
- Study test whether the activation of tyrosinase is specific for the *P. tolaasii* infection or it is a general answer of the mushroom to any disease.

Summary of achievements:
Discolouration due to senescence differs from blotch-related discolouration, which might indicate differential mechanisms being operational. In samples infected with bacteria or with a partially purified toxin extract (PT), a higher degradation of total tyrosinase than in senescening mushrooms was found. Simultaneously, the active tyrosinase was increasing resulting in an increase in percentage of active tyrosinase. Phenolic substrates of the active tyrosinase were being oxidised, proportionally to the damage detectable on the mushroom cap. GDHB was degraded first, followed by GHB and later tyrosine. The amount of melanin that was synthesised was larger than the sum of oxidation of the phenols measured. PCA explained 84 % of the variance in the symptoms, and it demonstrated the phenol oxidation and active tyrosinase level as the most important parameters for the browning induced by bacteria or a tolaasin preparation treatment.

In *A. bisporus* treated with *P. tolaasii*, or with a partially purified tolaasin extract or with pure tolaasin, induction of a specific mRNA encoding tyrosinase was found. The induction was not correlated to the amount of tolaasin applied.

Tyrosinase isoforms were visualised after SDS-PAGE electrophoresis, western blotting and staining with polyclonal tyrosinase-specific antibodies in samples infected with several concentrations of a PT extract. A common band at 67 kDa is observed, which may be the latent form of tyrosinase. A polypeptide of 43 kDa, whose intensity increased with increasing toxin concentration, was detected. This band might represent an active tyrosinase form.
The discolouration occurring in *A. bisporus* after infection by various pathogens such as *P. tolaasii, P. gingeri, P. agarici, P. reactans, V. fungicola* and *T. harzianum* can be distinguished by chromametric measurements. All pathogens were able to provoke degradation of total tyrosinase but only *P. tolaasii* infection induced activation of tyrosinase until 7%.

The lipodepsipeptide (WLIP) produced by *Pseudomonas reactans*, responsible for the white line test, could inhibit the browning produced by *P. tolaasii*, bacterium responsible of the brown blotch disease. Mushroom caps treated with several concentrations of WLIP and later inoculated with different *P. tolaasii* concentrations did not develop the symptoms of the disease. The inhibition of the browning was also effective when incubating at low temperatures and during four days.

Treatment of the mushroom caps with lysozyme also protected them against the bacterial brown blotch disease if the infection is not very severe.

Keywords:
Molecular, bacteria, blotch disease, *Agaricus bisporuss, Pseudomonas,* electrophoresis, senescence, mushroom

Main Publications/Patents/Participation in conferences:
H. Mooibroek, M. Van de Rhee, C. Soler-Rivas, O. Mendes, M. Werten, H. Huizing, H.J. Wichers (1996) Progress in transformation of common mushroom, *Agaricus bisporus.* Mushroom biology and mushroom products. Proc. 2nd International Conference. Ed. D.J. Royse. Penn State University, Pennsylvania, 37-46.
C. Soler-Rivas, N. Arpin, J.M. Olivier, H.J. Wichers (1997) Activation of tyrosinase in *Agaricus bisporus* strains following infection by *Pseudomonas tolaasii* or treatment with tolaasin. Mycological Research 101, 375-382.
C. Soler-Rivas, S. Jolivet, D. Yuksel, N. Arpin, J.M. Olivier, H.J. Wichers (In press) Analysis of *Agaricus bisporus* tyrosinase activation and phenolics utilisation during *Pseudomonas tolaasii*- or tolaasin- induced discolouration. Mycological research.
A. Moller, C. Soler-Rivas, H. Mooibroek, H.J. Wichers (1997) Antisense tyrosinase inhibition to study bacterial blotch browning in the cultivated mushroom, *Agaricus bisporus* Nederlandse Vereniging voor Plantecel- en -weefselkweek (NVPW) voorjaarssymposium. March 97: Wageningen (The Netherlands).

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Category:	30
Starting date:	24/03/1997
Duration:	12 months
Contract number:	FAIR-CT96-5063

Comparison of manganese oxidizing peroxidases from *Pleurotus* with extracellular peroxidases from other ligninolytic fungi

Objectives:
The recent studies on the diverse activities and substrate specificities of the peroxidases of different *Pleurotus* and other ligninolytic fungi demonstrate the high potential of degradative forces, but also the difficulties to analyse and control the multitude of possible reactions. Further more interactions and chain reactions by radicals formed by peroxidases, by other enzymes or by autoxidation of other components make it difficult to understand the real impact of the Mn-oxidizing peroxidases in the complex system of lignin degradation. This project aims to add to the current knowledge of this topic.

Summary of achievements:
The ligninolytic fungus *Pleurotus pulmonarius* secrets high levels of Mn-oxidizing peroxidase (MnP), exhibiting additional Mn-independent activity on aromatic compounds. During enzyme production in a glucose peptone medium the main activity peak is very ephemeral due to enzyme instability, causing up to 80% of enzyme loss within 15 h. In culture filtrate MnP inactivation is even faster with a total loss of enzyme activity observed within a few hours.

Using different inhibitors it was found that proteases are not responsible for the MnP decline. MnP instability, however, coincided with increased H_2O_2 concentration in cultures, attaining up to 200 µM when culture filtrates were incubated at room temperature for several hours. MnP stabilisation was observed after the addition of catalase, Mn^{2+} or some phenols, or after dialysis of the culture filtrate. This suggests that the extracellular H_2O_2 produced by the fungus is responsible for MnP inactivation and that the enzyme is protected by reducing substrates.

Keywords:
protein, chromatography, spectroscopy, interaction, macromolecules, aroma, β-lactoglobulin, ligand, complexation, biopolymers

Main Publications/Patents/Participation in conferences:

M. Lübke, E. Guichard, J. L. Le Quéré (1999) Infrared spectroscopic study of interactions of aroma compounds with β-lactoglobulin – Poster presentation at MADGELAS, 17-18th May 1999, Ayr, Scotland.
M. Lübke, E. Guichard, J. L. Le Quéré (1999) Infrared spectroscopic study of interactions of aroma compounds with β-lactoglobulin – Oral presentation at COST Action 96: Interactions of Food Matrix with Small Ligands, 20-21st May 1999, Oslo, Norway.
M. Lübke, E. Guichard, J. L. Le Quéré (1999) The study of interactions between food macromolecules and small ligands, and how infrared spectroscopy can contribute to it – Oral presentation at the American Chemical Society Annual Meeting 1999: Flavour Release Symposium, 22-26th August 1999, New Orleans, USA.
M. Lübke, E. Guichard, J. L. Le Quéré (2000) Infrared spectroscopic study of β-lactoglobulin interactions with flavour compounds – Flavour release, D. Roberts and A. Taylor (eds.), ACS Symposium Series, American Chemical Society, Washington DC, 2000 (in press).

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Category:	30
Starting date:	01/04/1998
Duration:	18 months
Contract number:	FAIR-CT96-5069

Expression of ethylene biosynthesis and signal transduction genes during pollination induced senescence and abscission of tomato flowers

Objectives:
The objectives of this project were to study the expression of ACC synthase. ACC oxidase and ethylene receptor genes in tomato flowers.

Summary of achievements:
Pollination of many flowers induces a rise in ethylene production that coordinates post-pollination events. Our results indicate that ethylene production by stigma and style of tomato flowers within 4 h after pollination and production continues to increase up to 48 h after pollination. The analysis of the expression of genes involved in ethylene biosynthesis indicates that pollination induces an increase in the expression of the ACC synthase genes designated as *LEACSIA* and *LEACS6*. The expression of the other ACC synthase genes is not affected by pollination. To investigate the role of ethylene in regulating the expression of *LEA CSIA* and *LEACS6* we utilised the *Never-ripe (Nr)* mutant of tomato, which is deficient in ethylene perception. The *Nr* mutation did not prevent the accumulation of transcripts of these genes after pollination, indicating that the expression of *LEACSIA* and *LEACSIS* is ethylene-independent. All four ACC oxidase genes were expressed in tomato pistils and showed different regulation in response to pollination. *LEACOI* mRNA was not detected in pistils of non-pollinated flowers but accumulated dramatically after pollination. In contrast with *LEACO1*, the mRNAs corresponding to *LEAC02*, *LEAC03* and *LEAC04* were present in pistils at anthesis and they show a constant level of expression in non pollinated pistils. However, the expression of these genes was modified by pollination. A transient increase of *LEAC03* mRNA was observed following pollination whereas the level of *LEAC02* transcripts gradually decreased. The expression of *LEAC04* was constant during the first hours after pollination but clearly decreased after 24 h. Analysis of the expression of *LEACO* genes in pistils from Nr flowers indicated the involvement of ethylene in modulating the response of *LEACOl*, *LEAC02* and *LEAC03* genes to pollination. In contrast, the pattern of expression of

LEAC04 gene in Nr flowers was similar to that observed in dl flowers, indicating that ethylene is not responsible for the pattern of expression of *LEAC04* in pollinated pistils.
The abundance of three ethylene receptor transcripts *(LeETRI, LeETR2* and *LeETR3)* has been analysed in pistils in response to pollination. No changes in their levels of expression have been observed following pollination.

The expression of ethylene biosynthesis and signal transduction genes was also examined in petals. *LEA CSIA* and *LEA CS6* were present in petals the day of anthesis and showed basal level of expression in non-senescing petals. The expression of these genes was up-regulated in senescing petals. *LEACS2* and 3 were not detected in non-senescing petals but were induced at the onset of senescence. The analysis of the expression of four *LEA CO* genes in petals showed that they are subjected to a different regulatory control: while the abundance of the *LEAC02* gene was constant in all samples examined, the expression of *LEACOI, LEAC03* and *LEAC04* was up-regulated in association with petal senescence. The expression of the ethylene receptors *LeETRI, LeETR2* and *LeETR3* did not change significantly in senescing petals.

Keywords:
Ethylene biosynthesis, pollination, signal transduction, genes

Main Publications/Patents/Participation in conferences:
The Molecular Basis of the Signal Transduction in Plants. Royal Society, London. February 1998.
Biology and Technology of the Plant Hormone Ethylene II. Santorini (Greece). September 1998.
Genetic and Environmental Manipulation of Horticultural Crops. Wellesbourne (UK). October 1998.

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Category:	40
Starting date:	03/01/1997
Duration:	3 months
Contract number:	FAIR-CT96-5071

Studies on ripening-specific gene expression in apple using the biolistic PDS-1000/HE device

Objectives:
Transgene expression in apple plants is currently driven by constitutive promoters. Therefore, expression occurs in all tissues in an un-targeted manner. For the production of genetically improved apples, it will be vital to target the effects of transgene activity by the use of tissue-specific promoters. Two ripening-specific promoters have recently been isolated.

Transgenic apple plants are being produced in which these promoters drive a marker gene (*gus*) to permit analysis of gene expression in the fruit. Because of the time it will take before the plants bear fruit, it is planned to use particle bombardment to directly introduce promoter constructive DNA into apple fruit tissues to produce transient *gus* expression. A biolistic device has recently been obtained for this purpose.

It is intended that this study will be used to demonstrate that particle bombardment is a suitable method for examining promoter activity in apple fruit tissues and to optimise parameters to achieve the highest levels of transient gene expression. Once the methodology has been established, this will permit later dissection of fruit-specific promoters to identify minimal promoter cassettes for use in genetically improved apple plants of the future.

Summary of achievements:
Apple fruit tissues seem not to be amenable to the study of transient expression using particle bombardment. In the cortex, cells are large and have a higher water content which appears to make them fragile and more susceptible to lysis caused by bombardment. Furthermore, the cells appear to be damaged by the vacuum conditions required for use of the biolistic device. The large cell size is also likely to reduce the probability that microprojectiles penetrate the nucleus where exogenous genes can be expressed.

Keywords:
biolistics, promoters, transformation, genetic engineering, apple, transgene

Publications/Patents/Participation in conferences:
J. R. Gittens, E. R. Hiles, T. K. Pellny, S. Biricolti, D. J. James (submitted) Use of heterologous ribulose-1-5-biphosphate carboxylase/oxygenease small sub-unit gene promoters to drive transgene expression in the vegetable tissues of apple.
J. R. Gittens, E. R. Hiles, T. K. Pellny, S. Biricolti, D. J. James (submitted) Use of the *Brassica napus* extA promoter to drive transgene expression in the vegetative tissues of apple.
J. R. Gittens, E. R. Hiles, T. K. Pellny, S. Biricolti, D. J. James (submitted) Transgene expression in the vegetative tissues of apple driven by the vascular-specific promoters rolCP and CoYMVP.

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Category:	20
Starting date:	01/01/1996
Duration:	12 months
Contract number:	FAIR-CT96-5079

Regulation of APX gene family under stress conditions

Objectives:
The project is a first approach to understanding the different roles that each APX isoform could play as H_2O_2 scavengers in plants. *Arabidopsis thaliana* plants were selected in this study because it is a fast growing plant and because five members of the APX gene family were already identified and sequenced. The main objective was centred around the analysis of the early regulation of the APX gene family members identified under different stress conditions.

Summary of achievements:
The differential regulation of the known members of the APX gene family in *Arabidopsis thaliana* encoding cytosolic isoforms (APX1, APX2) and putative stromal (APX4) and thylakoid (APX5) isoforms was investigated under stress conditions. This study may provide some clues on the early regulation of APX genes. Chemicals that were expected to alter H202 levels provoked a dramatic accumulation of transcripts encoding both cytosolic APX isoforms. However, stresses expected to alter the oxidative-state of glutathione pool in the plant did not produce major changes. APX genes encoding plastidal isoforms were not differently responsive to the stresses applied, however, wounding or senescence signals might be involved in their regulation.

Keywords:
APX genes, stress conditions, isoform, *Arabidopsis thaliana*, regulation.

Main Publications/Patents/Participation in conferences:
C. Escobar, S. Karpinski, M. Santos, G. Creissen, P. Mullineaux (1996) A novel form of ascorbate peroxidase has been identified in *Arabidopsis thaliana.* Poster presentation, VIII Biennial Meeting, International Society of Free Radical Research, October 1996, Barcelona, Spain.

S. Karpinski, C. Escobar, B. Karpinska, G. Creissen, P. Mullineaux (1997) Photosynthetic electron transport regulates the expression of cytosolic ascorbate peroxidase genes in *Arabidopsis* during excess light stress. Plant Cell, 9:627-640, 1997.
P. Mullineaux, C. Escobar, G. Pastori, A. Jimenez, H. Reynolds, D. Kular, N. Joyce, G. Creissen (1998) Antioxidant metabolism in senescence. Oral presentation, Annual Meeting of the Society for Experimental Biology, March 1998, York, UK.
P. Mullineaux, S. Karpinski, C. Escobar, B. Karpinska, H. Reynolds, G. Creissen, (1996) Glutathione and the regulation of the response of plants to photooxidative stress. Oral presentation, Annual Meeting of the Society for Experimental Biology, March 1998, York, UK.
The nucleotide sequences of APX genes have been submitted to the GeneBank™/EMBL data Bank with accession numbers X98276 (APX1) and X98275 (APX2).

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Category:	20
Starting date:	12/01/1998
Duration:	36 months
Contract number:	FAIR-CT97-5010

Structural and functional analysis of yeast squalene synthase

Objectives:
The isoprenoid biosynthetic pathway has been subject of intense research over the past several decades. The importance of this biochemical route lies not only in the vital role played by isoprenoids in diverse cellular functions but also in the commercial potential of these compounds. Rubber, terpenoids and carotenoids represent some clear examples of compounds widely used for commercial applications. Similarly, inhibitors of the sterol branch of the pathway are used today by the agrotechnological and pharmaceutical industries to fight fungal infections and some other diverse diseases such as hypercholesterolemia and arteriosclerosis. Squalene synthase (farnesyl diphosphate: farnesyl diphosphate farnesyl transferase, EC2.5.1.21) catalyses the first committed step in sterol biosynthesis: the reductive condensation of two molecules of farnesyl diphosphate (FPP), in the presence of divalent cations and reduced pyridine nucleotides, into squalene.

Yeasts have proven to be perfect organisms to study sterol biosynthesis. For the current project three different yeast species, *Saccharomyces cerevisiae*, *Yarrowia lipolytica* and *Cryptoccus curvatus* were initially selected on the basis of several properties that made them ideal targets for the production of isoprenoids.

As a first step in the use of yeast for the production of isoprenoids, this project aimed at the inhibition of the enzyme SQS by a novel approach using recombinant antibodies. This approach may yield a new system and a model for gene silencing which cannot be accomplished by conventional methods.

3.2 New and optimised food materials and nutritious food products

Summary of achievements:
Synthetic peptides based on the amino acid sequence of the protein of two of the test organisms *(Saccharomyces cerevisiae* and *Yarrowia lipolytica)* have been used for the production of anti-peptide antibodies. The selection of the peptides was based on antigenicity prediction. Several physico-chemical parameters that have been frequently correlated with the location of epitopes (antigenic determinants) were used for this prediction, namely hydrophilicity/inverted hydrophobicity, flexibility, accessibility, antigenicity and turn location.

Among all the sequences with good antigenic properties, the ones close to the putative active sites of the protein were selected since antibodies raised against these regions are more likely to have an effect on enzymatic activity. These sequences (three for each micro-organism) were chemically synthesised and have been used for immunisation purposes (after conjugation to a carrier-protein). PCR amplification of the variable regions obtained in this way from the antibodies and the construction of a phage display library will be addressed. Those antibodies (variable regions) cross-reacting with the protein will be selected and their inhibitory activity tested.

A PCR approach for the cloning of the squalene synthase gene in *Cryptococcus curvatus* has been attempted. On the basis of a sequence comparison between the squalene synthase genes from different organisms and the information derived from site directed mutagenesis studies, oligonucleotides from the most conserved regions have been selected to be used as primers. So far no positive result has been obtained but the work on this specific point will carry on.

Keywords:
antigenicity, PCR, amplification, yeast, squalene synthase

Main Publications/Patents/Participation in conferences:
Merkulov, S.; van Assema, F.; Springer, J.; Fernández del Carmen, A. & Mooibroek, H. (2000) Cloning and characterisation of the *Yarrowia lipolytica* squalene synthase (*SQS1*) gene and functional complementation of the *Saccharomyces cerevisiae erg9* mutation. Yeast 16: 197-206.

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Category:	20
Starting date:	01/10/1998
Duration:	36 months
Contract number:	FAIR-CT97-5021

Elucidation of the physiological role of ascorbate oxidase in melon development, ripening and ascorbic acid degradation

Objectives:
Melon ascorbate oxidase (MAO) is a multigene family with at least four members. Four genomic clones have been isolated and three of them (MAO1, MAO2 and MAO3) were characterised while MAO4 is under characterisation. The study of the sequences of all AO genes revealed that it is very difficult to direct a single antisense RNA against all AO genes.

This project objectives can be divided into three main tasks:

1. Location of the most promising sequence of the clones in which the catalytic antisense RNAs will be most effective. Since AO is encoded by a multigene family, the located sequence will be one conserved in the multigene family, based on the knowledge of the melon and other species AO so it will be possible to silence all AO. Construction of the antisense RNA against AO.

2. Construction of the cassette containing the constitutive promoter (35S promoter of a CaMV) and the antisense RNA against the AO gene. Construction of the cassette containing the constitutive promoter (35S promoter of a CaMV) and the cDNA of cucumber AO. Transformation of *A. tumefaciens* with each of the two constructs.

3. Transformation of the melon plants using leaves and cotyledons. Melon plant regeneration, PCR-screening of transformed plants. Southern, northern and western blotting

Summary of achievements:
Two full-length cDNAs of ascorbate oxidase, MAO1 and MAO4 corresponding to the CMAO1 and CMAO4 genes respectively were successfully cloned. In addition transgenic melon plants over-expressing and antisensing ascorbate oxidase were produced.

Keywords:
Transgenic, Agrobacterium, Melon Ascorbate Oxidase, antisense, melon, multigene, cDNA

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Fellowship data

Category:	30
Starting date:	07/01/1998
Duration:	24 months
Contract number:	FAIR-CT97-5024

Isolation of genes involved in programmed cell death during post harvest sensescence of horticultural crops

Objectives:
Programmed cell death (PCD) is a genetically controlled process involving a number of regulatory pathways that ultimately lead to the selective removal of cells that are no longer needed or can be dangerous for the organism as a whole. In animal systems, research in PCD has led to essential advances in relevant fields such as cancer immunology and AIDS. Research undertaken in plant PCD runs far behind its equivalent in animals and thus the understanding about regulation of this fundamental mechanism in plants is still very poor. This project aims to:

- Improve the depth of knowledge and understanding of PCD in plants;
- Identify and characterise genes and proteins implicated in plant PCD.

Summary of achievements:
A differential display technique for the isolation of differentially regulated genes during the process of chemically induced death was developed. In this approach, the drug camptothecine was used as a trigger for PCD in a tomato cell suspension system. As a result of the differential display analysis, 5 PCD-repressed (CTD1 to 5) and 2 PCD-induced (CTU1 and CTU2) cDNA fragments were isolated. CTD1 is highly similar to Aux/IAA early-auxin responsive genes. CTD2 corresponds to the tomato RSI-1 gene, CTD3 may be an amino-acylase and CTD5 shows limited homology with a proline-rich protein from maize. CTU1 shows homology to various glutathione-S-transferases and CTU2 has been identified as a pirin gene from tomato.

In animal systems pirin proteins are believed to act as a bridge in the molecular interaction between anti-apoptotic regulators such as Bc1-3 and /or NFkB and the basic transcription machinery. Full-length cDNA from tomato pirin cDNA was isolated and used for further characterisation of its expression patterns. Northern analysis showed that tomato pirin

induction takes place only in final stages of the PCD process. Stress inducing treatments do not activate pirin mRNA production, and only chemicals that significantly increase the percentage of cell death in the culture are capable of triggering the expression of tomato pirin.

The identification of pirin as a plant gene potentially implicated in the regulation of programmed cell death opens the possibility of using this new knowledge as a starting point for further developments in the understanding of PCD in plants. The understanding of mechanisms regulating the balance between cell death and cell survival in plants will undoubtedly produce benefits to agriculture-related industries.

Keywords:
programmed cell death, PCD, genes, plants, tomato, pirin

Main Publications/Patents/Participation in conferences:
F. A. Hoeberichts, D. V. Orzaez, L. H. W. van der Plas, E. J. Woltering (submitted) Changes in gene expression during programmed cell death in tomato cell suspensions. Plant Molecular Biology.
FWO meeting on Plant growth regulators – January 8th 1999, Antwerp, Belgium.

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Category:	20
Starting date:	08/04/1998
Duration:	36 months
Contract number:	FAIR-CT98-5002

Engineering carotenoid content in plant cells: modification of carotenoid deposition by over-expressing heterologous structural proteins in transgenic tomato plants

Objectives:
Chromoplasts are a type of plant organelle characterised by a large accumulation of carotenoids and by the formation of new structures. The objective of this project is to study alternative ways of modifying carotenoid content other than over-expressing biosynthetic enzymes – a strategy currently used in industrial applications. The study will examine the influence on carotenoid accumulation and deposition in tomato fruit of a protein called fibrillin. Additional effects on plant development caused by the over-expression of fibrillin will also be investigated. Finally, the project will evaluate other genes that may influence indirectly carotenoid accumulation in tomato fruit.

Summary of achievements:
Due to difficulties in obtaining large numbers of transformants and the recent finding that the fibrillin promoter can be induced under stress conditions it was decided to further characterise the regulation of the fibrillin promoter under different conditions in different tissues. Specifically work was carried out to analyse the activity of the promoter during the transformation protocol. Results indicated a strong induction of the fibrillin promoter in callus tissue at an early stage of transformation. Using this information the transformation protocol was optimised in an attempt to minimise the apparent adverse effect of early expression of fibrillin. Currently transformants are growing in several pre-greenhouse and greenhouse stages and further analysis of these plants will continue. The development of a second fibrillin construct is under way using CCS promoter.

On the first series of plants we analysed transgene expression by RT-PCR and protein accumulation by Western blot and have carried out carotenoid analysis on transgenic fruit.

The results show an accumulation of fibrillin mRNA and a corresponding increase in protein in tomato pericarp during fruit development, showing successful over-expression of the transgene. However, preliminary carotenoid analysis has shown little influence on carotenoid content of transgenic fruit. Currently the project is in the process of carrying out electron-microscopy on transgenic fruit and flowers to determine if the expression of fibrillin causes any structural cellular changes within chromoplasts.

Preliminary investigations into stress resistance of the transgenic plants appears to indicate that they are no more resistant to salt/dehydration stress than control plants.

The Comparative RT-PCR protocol developed during the first year was used to study the expression of several key carotenoid biosynthetic genes, namely *psy*, *pds*, and *zds*, as well as *ptox* and *fibrillin* in both tomato and pepper. The expression data show co-induction of these biosynthetic genes with *ptox*, suggesting that rising expression of all these genes is necessary to obtain an up-regulation of the carotenoid pathway.

Keywords:
carotenoid, tomato, biosynthetic, fibrillin, over-expression, chromoplasts.

Main Publications/Patents/Participation in conferences:
E. M. Josse, A. J. Simkin, J. Gaffé, A. M. Labouré, M. Kuntz, P. Carol (2000) A plastid terminal oxidase associated with carotenoid desaturation during chromoplast differentiation. Plant Physiol.
New Frontiers in Plant Science and Plant Biotechnology, 5-9th March 2000, Toulouse, France.

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Category:	20
Starting date:	05/04/1998
Duration:	12 months
Contract number:	FAIR-CT98-5008

Role of thermostable peroxidases in determining the processing quality and pathogen resistance of potatoes

Objectives:
Peroxidases (POD) are distributed ubiquitously in plants. They are very important for the plant physiology since they are involved in many physiological processes such as ripening, lignification, and defence mechanisms.

PODs are related to food quality, and can contribute to deterioration of stored fruits and vegetables by changes in flavour, texture, and colour unless they are inactivated. The texture of processed fruit and vegetables is an important quality aspect and is directly related to the mechanical properties of the cell wall. At this tissue level, both the cell wall strength and the cell-cell adhesion play a role in determining the plant texture. PODs are able to cross-link cell wall glycoproteins like extensin in the presence of H_2O_2 leading to the deformation of the cell wall without breaking. Researchers deduced from their work on extensin POD of tomato that cross-linking was not a property of plant POD in general but involved specific enzymes. Thus, the texture could be controlled by modifying the action of enzymes like PODs.

Genetic engineering of PODs in plants could lead to changes in texture. Depending on the goals (persistence of firmness during processing, softening during ripening...), characteristics of PODs could be exploited Moreover POD are well known as thermostable enzymes, and able to recover after heat treatment. Therefore, thermostable PODs are an interesting source of investigation for food applications like modification of texture, improvement of fruit, vegetable shelf-life, food processing, or for non-food applications like cross-linking of economically interesting materials, decontamination of water etc.

This work will complete the biochemical study carried out on characterisation of PODs of potato sprouts, and will focus on a molecular biological study of PODs of potato sprouts.

The purpose of the present study was to purify and characterise a thermostable cationic isoPOD from potato sprouts, and to isolate its full-length POD clone in order to over-express it in potato tubers. The outcome of this study aimed to give insight into the potential of genetic engineering in modifying vegetable crops with respect to processing quality and pathogen resistance.

Summary of achievements:
A thermostable cationic isoPOD from potato sprouts with, a molecular weight of ca. 38 kD was purified by chromatography techniques. POD activity was also analysed for potato plant tissues such as leaves, stems, and roots from 1 month-old and 2 month-old plants. PODs from roots contained the highest enzyme activity for all POD fractions (up to ca. 13000 µkat/mg protein) whereas leaves contained a low POD activity (up to ca. 500 µkat/mg protein). Moreover PODs from roots appeared to be the most thermostable still showing about 20% of POD activity after 10 min at 90°C.

Concurrently, a putative full-length POD cDNA clone of 993 by encoding for 331 amino acids was isolated in order to be over-expressed in potato tubers.

Keywords:
Peroxidases, enzymes, quality, pathogen, resistance, potatoes, genetic engineering

Main Publications/Patents/Participation in conferences:
C. Boucoiran, J.W. Kijne, K. Recourt (1998) Isolation and partial characterization of thermostable isoperoxidases from potato (*Solanum tuberosum* L.) tuber sprouts. J. Agric. Food Chem.
K. Recourt, M. Ebbelaar, C. Boucoiran, C. van Dijk (1998) Cell wall biotechnology in relation to the potato processing quality. Poster. 5th International Symposium on the Molecular Biology of the Potato. August 2 - 6, 1998, Bogensee, Germany.
T. Sholle-Smits, C. Boucoiran, M. Ebbelaar, K. Recourt (1999) Biotechnologie brengt staring van aardappeltextuar dichterbij. Aardappelwereld magazine, 3: 32-33.

Links with EC projects:
FAIR-CT96-5001 (p.178)

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Category:	30
Starting date:	01/07/1998
Duration:	24 months
Contract number:	FAIR-CT98-5015

Engineering alkaloid pathways through expression of recombinant antibodies

Objectives:
In this project, the goal was to use metabolic engineering to optimise the production of the therapeutic anti-cancer agent vinblastine in *Catharanthus roseus*. Key enzymes that regulate bottlenecks in the synthesis of these alkaloids have been identified. The project intends to manipulate the steps these enzymes catalyse to increase the production of the therapeutic alkaloids. This will be achieved using two complimentary approaches. In the first, the activity of alkaloid synthesising enzymes will be optimised by targeting them to membranes and subcellular compartments where their function could be stabilised and enhanced (cytosol, vacuole, ER, chloroplast). In the second, enzymes involved in branch pathways of alkaloid biosynthesis will be targeted and inhibited by specific recombinant antibodies. These antibodies will be expressed in transgenic plants to block the activity of two enzymes, cathenamine reductase and tetrahydroalstonine synthase, that produce end products that are therapeutically ineffective against tumours. These approaches will increase the synthesis of therapeutic alkaloids in *C. roseus*.

Summary of achievements:
In the initial phase of the project, two genes encoding enzymes involved in vinblastine synthesis, strictosidine synthase (STRI) and tryptophan decarboxylase (TDC), have been cloned into plant expression vectors. Within the vectors, the enzymes have been tagged with a range of signal peptides. The signals target the proteins to the cytosol, vacuole, chloroplast and endoplasmic reticulum as soluble or membrane anchored proteins. To test that the enzymes are still functional in these compartments and orientations, the enzymes will be transiently expressed in tobacco leaves using vacuum infiltration of recombinant *Agrobacteria*. The transient expression studies are underway and constructs that enhance the levels of expression or the activity of the synthetic enzymes will be identified. These constructs will then be used to generate stably transformed tobacco and *C. roseus* plants. The

ability of the targeted enzymes to increase vinblastine biosynthesis will then be assayed. Antibodies blocking cathenamine reductase and tetrahydroalstonine synthase are being raised and after testing their activity in transient expression, will be used for the generation of stably transformed transgenic plants. In combination with increasing the levels of bottleneck synthetic enzymes, these antibodies will synergistically increase the levels of vinblas5ne by decreasing products made in metabolic branch pathways.

Keywords:
Metabolic engineering, antibodies, vinblastine, *Catharanthus roseus,* cathenamine reductase, tetrahydroalstonine synthase, strictosidine synthase, tryptophan decarboxylase

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Category:	30
Starting date:	01/06/1998
Duration:	24 months
Contract number:	FAIR-CT98-5021

Functional foods and physical activity effects on cognitive performance psychomotor skills, appetite control and energy balance

Objectives:
The project will develop a methodology to jointly examine different but related effects of "functional" foods. Innovative aspects of this project are represented by the simultaneous and combined use of psychological and physiological procedures including:

- evaluation of cognitive performance (including real life assessment in the "Leeds Driving Simulator").
- assessments of consumer behaviour, eating style, eating patterns and sensory testing.
- measures of Basal Metabolic Rate, respiration, energy balance, heart rate, food-induced changes in metabolism.

This project could be divided into four different parts. It aimed to look at:

- The effects of moderate alcohol consumption on cognitive performance: sensitivity analysis
- The effects of habitual diet and eating style on cognitive performance
- The effects of acute exercise on cognitive performance and mood
- The acute effects of dietary fat on mood, appetite control and cognitive performance in high fat and low fat consumers

This strategy is designed to provide a comprehensive understanding of the effects of new or novel foods.

Summary of achievements:
The effects of four drinks varying in alcohol content (0 to 6.33% vol.) on cognitive performance were assessed in 16 young adult male subjects. A battery of cognitive tests was administered before lunch and during the afternoon, just before and after the consumption of 330 ml of the drinks. Alcohol, in the concentrations administered in this study, produced small shifts in arousal which affected simple processes (i.e. reaction time), but did not affect more complex cognitive processes (i.e. vigilance).

A study investigating the impact of habitual diet (e.g. dietary fat) and eating style (e.g. dietary restraint) on cognitive performance was conducted. Low fat consumers had higher Body Mass Index and higher psychometric test scores on factors of Restraint and Hunger. This coupled with lower reported energy intake. Low fat consumers became slower over trials while high fat consumers became faster. No effect of fat group was found on verbal and spatial tests. Habitual high-fat diet and dietary restraint do not seem to have a strong effect on cognitive performance. Effects are small such that large samples size and/or large manipulations are needed to detect them.

The results showed that exercise facilitated performance on a tapping task in all participants and reaction time in males only. On the complex cognitive tasks, sex and time mediated the effects of exercise. In both males and females, exercise increased positive mood states (happiness and energy), whereas rest increased negative mood states (irritability and lethargy). In conclusion, exercise positive effects on mood in all participants whereas the effects on cognitive function were task and/or gender specific.

The acute responses to nutrient manipulations (fat content of the diet at breakfast, lunch and dinner) on cognitive performance, mental states and appetite control are under investigation in a group of high and low fat consumers, at a fixed level of low physical activity.

Keywords:
Functional foods, physical activity, cognitive performance, psychomotor skills, appetite control, energy balance, diet, eating style

Main Publications/Patents/Participation in conferences:
N.A. King, A. Lluch, C. Ruxton, D. Hughes, J. Stubbs, J.E. Blundell (1999) Effects of 4 weeks of exercise on body weight hunger, mood and taste perception in lean and overweight females. Int J Obes 23, suppl 3:75.
A. Lluch, N.A. King, F.C. Smith, C. Ruxton, D. Hughes, J. Stubbs, J.E. Blundell (1999) Improvement in appetite control but no compensatory increase in food intake following a 4 week exercise intervention in lean and overweight females. Int J Obes, suppl 5:94.
A. Lluch, N.A. King, J.E. Blundell (1999) Energy compensation at the meal following exercise and diet manipulations, in dietary restrained and unrestrained females, Int J Obes 23, suppl 3:75
N.A. King, A. Lluch, C. Ruxton, D. Hughes, J. Stubbs, J.E. Blundell (1999) Effects of 4 weeks of exercise on body weight hunger, mood and taste perception in lean and overweight females, J Obes 23, suppl 3:75.
A. Lluch, N.A. King, F.C. Smith, C. Ruxton, D. Hughes, J. Stubbs, J.E. Blundell (1999) Improvement in appetite control but no compensatory increase in food intake following a 4 week exercise intervention in lean and overweight females. Int J Obes 23, suppl 5:94.

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Category:	40
Starting date:	01/09/1998
Duration:	6 months
Contract number:	FAIR-CT98-5025

Study of the interactions between protein and endogenous bioactive seed components that affect the nutritional value of legumes

Objectives:
The major objective of the research project was the setting up of novel *in vivo* model systems of wide application in the identification of specific interactions between proteins and endogenous food compounds with consequence on the bioavailability of essential amino acids and other bioactive components (minerals, phenolic compounds).

Summary of achievements:
A short-term *in vivo* (rat) digestibility method was successfully applied to either complex food matrix (raw or cooked legumes and milk), or purified proteins (7S and 11S globulins, BSA, casein, lactalbumin). Rats were intubated or fed with a single dose of protein and killed after 1 hour. Stomach and small intestine content was washed out and analysed for: protein content (Lowry method), protein/peptide MW (SDS-PAGE, HPLC, MALDI-MS), presence of aggregated species (native PAGE, FPLC, MALDI-MS), amino acid composition (HPLC), copper, iron, zinc, manganese, calcium, magnesium and phosphorus content (atomic absorption spectrophotometry).

A higher protein and mineral bioavailablity was found from animal foods (milk) than from plant foods (legumes). Differences in the mechanism of heat-induced aggregation between 7S and 11S proteins with consequence on nutrient (protein, mineral) bioavailability were demonstrated. Absolute values of protein and mineral absorption were in good agreement with those of other *in vivo* methods.

In the same period, a rapid method for the evaluation of polyphenol bioavailability has been worked out using segments of small intestine of rat. Following 5 minutes incubation of tannic acid, catechin, quercetin or rutin solutions at different concentrations with segments of small

intestine, amounts of compound remaining free in the lumen and those that crossed the gut wall were assayed by measuring absorption at UV/VIS maxima. The results indicated a concentration-dependent disappearance of all compounds from the small intestine of rats, although significant absorption was observed only with catechin and quercetin. Binding of part of polyphenols by endogenous proteins in the intestinal lumen was also demonstrated. Differences in both kinetic of interaction and extent of absorption between quercetin and rutin (quercetin glycoside) were established. The results were confirmed by *in vivo* (rat) experiments where the kinetic of appearance of quercetin and rutin in the plasma during 24 h was followed in single-dose experiments after administration of different amounts of flavonoids.

Keywords:
protein, legumes, bioactive, amino acid

Main Publications/Patents/Participation in conferences:
M. Carbonaro, G. Grant, A. Pusztai (1999) A novel method for the estimation of polyphenol absorption from rat intestine. Proceedings 9th Workshop COST 98 Concerted Action Effect of antinutrients on the nutritional value of legume diets, Tromso, Norway. Krogdahl, A., Pryme, I., Eds.; Luxembourg: Office for Official Publications of the European Communities, 1999, in press.
M. Carbonaro, G. Grant, A. Aguzzi, A. Pusztai (1999) Alternative strategies for the investigation of the mechanisms governing mineral and protein bioavailability from legumes. Proceedings of 10th Workshop COST 98 Concerted Action Effect of antinutrients on the nutritional value of legume diets. Lund, Sweden. Pierzynowski, S., Ed.; Luxembourg: Official Office for Official Publications of the European Communities, 1999, in press.

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Category:	RETOUR
Starting date:	01/06/1997
Duration:	12 months
Contract number:	FAIR-CT98-5026

Molecular marker technology for legume improvement

Objectives:
Phaseolus bean is also known as the green, dry, dwarf, haricot, french bean etc. More sustainable and more economic *Phaseolus* production in Europe, leading to high quality products, would contribute to the diversification of agricultural systems and the development of more environmentally- friendly agricultural practices. Molecular studies are contributing to the improvement of beans by means of a better knowledge of relationships among genetic pools and development of molecular markers for genes of interest.

This research project aimed to:
1. Identify significant associations between polymorphic markers and seed size, that could be useful in marker assisted selection programmes.
2. Undertake variability studies of morphological, seed protein, phaseolin and allozyme markers in the bean landraces and pure lines maintained at tile MBG-CSIC. This research would allow the identification of the different genetic relationships among the land-races, and identity useful markers for the crossing programmes.
3. Study BCMV-resistance population development. This research would allow the introduction of resistance to BCMV in autochthonous common bean germplasm, and to identify resistant local lines. Markers will be introduced to speed up the selection procedure.

Summary of achievements:
Sixteen intra-racial and inter-racial crosses were obtained from 29 acceptable quality pure lines in a previous project and family selection arise up to F_6 F_2 and F_3 families were analysed for allozymes, seed protein, phaseolin and seed size, and the data were used for Quantitative Trait Loci (QTL) analysis. In addition, bean populations from Portugal and Galicia maintained in the collection of the MBG-CSIC were analysed for seed protein, phaseolin and

allozyme markers. Moreover, numerous bean accessions were screened for resistance to BCMV, and at the present no pure lines were identified as resistant to a local strain of BCMV isolated at Pontevedra. Hybridisation experiments were carried out between acceptable quality lines and introduced BCMV-resistant lines.

Keywords:
Phaseolus, bean, marker, Quantitative Trait Loci analysis, selection

Main Publications/Patents/Participation in conferences:
M. Santalla, A.P. Rodiño, A.M. de Ron, A. Abelleira, P. (1998) Mansilla Estudio preliminar de la incidencia del virus del mosaico común en variedades autóctonas de judía común. Actas de Horticultura 22: 228-232
M. Santalla, M.A. Fueyo, A.P. Rodiño, I Montero, A.M. de Ron (In press) Breeding for culinary and nutritional quality in intercropping systems of common bean (*Phaseolus vulgaris* L.) and maize (*Zea mays* L.) Biotechnology, Agronomy, Society and Environment.
Second *Phaselieu* Workshop. Lecture: Breeding for culinary and nutritional quality in intercropping systems of common bean and maize. Place: Faculté des Sciences Agronomiques de L'Etat. UER Phytotechnie des Regions Chaudes. Gembloux (Belgium). 1998.

Links with EC projects:
FAIR-CT96-5009 (p.98)

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Category:	20
Starting date:	01/09/1996
Duration:	36 months
Contract number:	FAIR-CT96-5012

Meat demand in the European Union: Dynamic approach and future prospects

Objectives:
The objective was to predict meat consumption per individual for France and where possible for other EU countries. The difficulty presented by other countries is the lack of data per individual as they tend to publish only aggregated data.

For that reason, the study mainly focused on France, for which no equivalent study was available yet. A prediction model was produced which analysed change in consumer behaviour.

Summary of achievements:
Since the 70s consumption of fresh meat stagnated mainly due to saturation of demand. Change of demand structure and the evolution of meat prices due to a productivity gain in agriculture were expressed as increased demand for more elaborate products. Demand for "Delicatessen" type products doubled in the last three decades while consumption of pre-prepared meat-based and fish meals grew by 167% over the same period while consumption of fresh meat only grew by 7.6 %.

Spending on meat based products grew by 40 % between the beginning of the 70s and the end of the 80s. However growth in consumption of beef meat grew only moderately while veal pork and poultry consumption grew considerably more. Younger generations, middle and lower income, families of single mothers and those where the mothers worked were mainly responsible for the change. Studies show that price variations greatly affect consumption especially for beef meat. Beef seems to still be considered as a luxury meat by French consumers which is not true of poultry. The occurrence of mad cow disease did affect consumption.

Although the study would have benefited from more detailed data of meat consumption regarding particular parts of the animal or modes of preparation of the meat, such research requesting data produced by panel interviews was too costly to be included in this work.

3.3 Advanced and optimised technologies and processes

Keywords:
Meat, Demand, European Union, France, consumption, beef, pork, poultry

Main Publications/Patents/Participation in conferences:
Modelisation de la demande des consommateurs. Application au cas de la viande en France 1999. In *Défis à l'économie rurale* (Ed. Garant) pp 203-210.

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Contract signed:	01/02/1996
Duration:	24 months
Contract number:	FAIR-CT96-5029

Improving quality in dessert wines through understanding maturation processes during storage in wood

Objectives:
Young red port is deep purple-red in colour, and astringent and harsh in flavour from grape tannins and yeast-derived congeners. After years of storage in wood, wines assume characteristic tawny colours with softening of astringent and fiery characters. Complex nutty and other flavour notes associated with wood extraction are also acquired during maturations. The chemistry and biochemistry of port maturation and nature of changes in molecular structures of grape- and wood- derived components is not well understood. This study is to determine how a number of key maturation processes proceed in fortified wines. Recent studies of changes in new distillates during storage have given new insights into wood maturation processes. Similar studies were performed examining the maturation processes in port wines with characterisation of changes in compounds unique to wines. Information obtained would be of general applicability to improvement of quality and marketability of dessert wines produced in Southern Europe.

Summary of achievements:
A primary aim was to study the key 18-month initial period of wood maturation of port wines. Four batches of new immature ports were obtained immediately following inhibition of the primary fermentation by the addition of grape spirit. A study of quality required the development of a sensory vocabulary meaningful to consumers and methodology for relevant composition analyses. A total of 118 descriptors were obtained and clustered into: appearance; aroma; taste; mouth feel and aftertaste. The consensus product space showed differentiation of ports on the basis of type and length of wood maturation.

Twenty-four commercial ports were then assessed for sensory quality employing a panel of 12 trained assessors. The consensus product space showed differentiation of ports on the basis of style and length of wood maturation.

The four immature ports were sampled on a 2-3 months interval and a control sample kept in glass with no wood contact. Samples were assessed for appearance and flavour. The consensus product space showed differentiation by grape variety. Assessors showed more confidence and ability in differentiating ports by appearance then by flavour.

Compositional analyses were effected by the following parameters: headspace volatiles, using solid phase microextraction (SPME), followed by high resolution gas chromatography (HRGC); total volatiles by HRGC following sorbent extraction; non-volatiles by high pressure liquid chromatography (HPLC) after sorbent extraction. Principal component analysis was used to reveal underlying structures.

Although Free Choice Profiling provided valuable data on how consumers perceived character in the 12 ports, only one significant dimension was obtained. The unique sets of descriptors were used to develop a formal vocabulary that could be used for conventional profiling of retailed ports and the maturing wine. It was clear that appearance was an important sensory factor to the assessors and this may explain the distinct relationship between wine non-volatile components and sensory data. However the obvious relationship between headspace volatiles and sensory data indicated that the sensory panel had provided information of value in understanding port quality in terms of flavour. Tawny and late-bottled wood matured (LBV) ports were differentiated and clustered on the basis of sensory data, total and headspace volatile components and non-volatiles. Aroma components (headspace volatiles), important for aged tawny character, included: isaomyl alcohol, hexanol, diethyl succinate, acetic acid and ethyl lactate. Components important in LBV ports included 2 phenylethanol and octanoic acid.

In summary, ports were differentiated by style; appearance is an important factor in port differentiation; and conventional vocabulary is successful in establishing meaningful relationships between ports. Also, partial least squares modelling techniques support hypotheses that changes in port non-volatile phenolics influence flavour character in the wine. There appears to be relationships between headspace concentrations of flavour components and non-volatile phenol compounds. The relationship may be explained as oxidation of wine compounds during the port maturation, resulting in retention of volatile flavour compounds within the liquid.

Keywords:
Dessert, wine, maturation, storage, quality, assessment, volatiles

Main Publications/Patents/Participation in conferences:
E. Cristovam, J.M. Conner, A. Paterson, and J.R. Piggott (1997) Relationship between aroma volatiles and sensory character in Port. Proceedings of the First Symposium In Vino Analytical Scientia, June, Bordeaux.

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Category:	20
Starting date:	20/11/1996
Duration:	6 months
Contract number:	FAIR-CT96-5031

Interactions between wood and flavour molecules as a mechanism for improving the quality and sensory attributes of white wines

Objectives:
The primary aroma of wine is due to compounds derived from grapes or arising during fermentation. A maturation period is also required during which hydrolysis and oxidation reactions involving fusel alcohols, terpenes, acetates and ethyl esters lead to the final flavour and aroma. Maturation in wood, releasing phenolic compounds including vanillin and tannins, impart a particular flavour to the wine. Storage on yeast lees, due to absorbive phenomena and remaining active enzymes, leads to a reduction in these phenolic compounds. Recent studies on spirits have reported interactions occurring between wood derived materials and flavour compounds in alcoholic systems, causing a reduction in the volatility of the flavour compounds. This work was carried out as a preliminary investigation of the effect of wood on the flavour attributes of white wines, comparing three different styles of products (barrel fermented, tank aged and wood aged). The concentrations of volatile compounds in solution and in the headspace were compared to study the effects on volatility of wood contact. In parallel the attributes of wines were investigate by sensory analysis to investigate the possibility that wine flavour could be modelled in terms of headspace composition.

Summary of achievements:
White wines were selected from the Friuli region of Italy to represent wood-aged, barrel-fermented, and tank-aged types, each from four different years. Wines were evaluated by Free Choice Profiling (FCP) and the data were submitted to Generalised Procrustes Analysis. Wood derived and other non-volatile compounds were analysed by HPLC after sample preparation by solid phase extraction. Volatile compounds in solution were determined by gas chromatography after concentration by solid phase extraction. Volatile compounds in the headspace were determined by gas chromatography after absorption by solid phase micro extraction. Compounds were identified by comparison of mass spectra and retention times with those of authentic compounds. Data matrices were examined by principal component

analysis. Partial least squares regression analysis (PLS) was used to study the relationship between chemical and sensory data. FCP differentiated the twelve wines in respect of aroma and flavour according to their different styles and processing, but not by year. Higher acidity and optical density were found for the wood treated wines, increasing with age. There were significant effects of grape type and wood exposure for principal components 1,2, and 3 of the chemical data, and of the year for principal components, 1,3, and 5. PLS regression using two components successfully predicted the scores on the first two sensory dimensions from the chemical data.

Keywords:
white wine, flavour, quality, sensory, tannin, volatile, wine

Main Publications/Patents/Participation in conferences:
The role of sensory analysis in new product development and quality assurance seminar. The Institute of Food Science and Technology, UK, Heriot-Watt University, Edinburgh, UK, 27 November 1996.

AMANATIDOU, Athina
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Category:	20
Starting date:	01/01/1997
Duration:	24 months
Contract number:	FAIR-CT96-5038

Development and application of new combined processes to improve the safety and natural quality of food products

Objectives:
The spoilage and poisoning of foods by microorganisms is a problem that is not yet under adequate control, despite the range of preservation techniques (e.g. refrigeration, freezing, blanching, pasteurising, canning) employed. The current consumer demand for more natural and fresh-like foods may make this problem even greater, because food manufacturers have to use very mild preservation techniques (e.g. refrigeration, modified atmosphere packaging, biopreservation) that individually may not control microbial growth adequately. Thus, for the benefit of food manufacturers there is a strong need for new or improved mild preservation techniques that allow for the production of stable and safe foods, but that still better preserve its natural quality than more traditional preservation techniques. The concept of Combined Processes (hurdle Technology was developed recently as a suitable strategy for the development of such new mild preservation techniques.

The concept of Combined Processes advocates the deliberate combination of different preservation techniques in order to establish a series of preservative factors (hurdles which undesired microorganisms present in a food should not be able to overcome). These hurdles may be temperature, water activity (aW), pH, redox potential, (bio-)preservatives, HTST or high pressure treatment, etc. Due to the concerted, sometimes synergistic, effect on the growth of the target microorganism(s), the individual hurdles may be set at lower intensities than would be required if only a single hurdle would be used. Individual preservative factors target at specific or aspecific cellular sites of microorganisms (e.g. cell membrane, DNA, enzyme systems, protein synthesis or homeostasis systems for pH, Eh or aW), due to which they will not multiply but remain inactive or even die. The most optimal food preservation effect is achieved by hitting undesired microorganisms at different cellular targets simultaneously. This approach often is more effective than single-targeting and may allow the use of hurdles of lower intensity, and thereby has less of an effect on the natural product quality. The multi-target approach using combinations of synergistic preservative factors, is the basic strategy that will improve the safety and natural quality of foods.

The focus will be on food products of vegetable and fruit origin and on the control of relevant psychotrophic food pathogens (i.e. *Listeria monocytogenes*, *Escerichia coli*, *Aeromonas hydrophila*), mesophilic food pathogens (i.e. *Salmonella typhimurium*, *Bacillus cereus*) and food spoilage organisms (i.e. enterobacteria, pseudomonads, yeasts). In that respect combinations of the mild preservation techniques were tested and the most promising were studied in more details.

Summary of achievements:

Modified Atmosphere Packaging (MAP) using relatively high levels of oxygen or carbon dioxide is common practice to extend the shelf-life of meat products. High carbon dioxide levels (over 60%) reduce microbial spoilage in fish, poultry and white meat types. High oxygen levels (over 80%) are used with red meats where they maintain the bright red colour.

With MAP of fruits and vegetables, carbon dioxide levels (up to 10-20%) can be employed due to the sensitivity of the tissue to more extreme levels. Oxygen levels are generally also kept below 5-10% in order to minimise microbial spoilage.

In line with the current interest in the use of combined processes ("hurdle technology") as a means to achieve a prolonged shelf-live or keeping quality of minimally processed foods, it was thought relevant to investigate the effect of novel gas compositions on the growth microorganisms. In particular, compositions that would enhance the antimicrobial effect of carbon dioxide should be of interest for MAP of vegetables. Further enhancement of the antimicrobial activity of gases can be achieved by combining MAP with other techniques like e.g. with high pressure. The concept of hurdle technology is supported during the terms of this project with results on *in vitro* growth of microorganisms (pathogenic and spoilage) as well as *in vivo* studies (on products like carrots, potatoes, mixed salads, salmon).

Keywords:

Safety, natural quality, food products, microorganisms, Modified Atmosphere Packaging, preservation techniques

Main Publications/Patents/Participation in conferences:

A. Amanatidou, M.H.J. Bennik, L.M.M. Tijskens, L.G.M. Gorris (1997) Growth of spoilage microorganisms on vegetables in a high oxygen modified atmosphere system in relation with keeping quality. COPERNICUS workshop Shelf-life Prediction for Safety and Quality of Food, Wageningen, The Netherlands, November, 27-28.

A. Amanatidou, E.J. Smid, L.G.M. Gorris (1999) Effect of elevated oxygen and carbon dioxide on the surface growth of vegetable associated microorganisms. J. Appl. Bacteriology

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Category:	20
Starting date:	21/10/1996
Duration:	24 months
Contract number:	FAIR-CT96-5048

The identification of chemical markers in order to predict and evaluate meat quality early post-mortem

Objectives:
To detect and quantify important quality indicators of meat by means of novel chemical markers at the early post-mortem period. These chemical markers will be used to predict a number of sensory attributes and technological properties. The detection and quantification by capillary electrophoresis and SDS polyacrilamide electrophoresis, of proteins, peptides and proteolytic fragments should allow an accurate prediction of the ultimate tenderness of the meat for the end user and the consumer. The relative significance of the various components of the protease systems to quality attributes will be detected applying FPLC techniques. Subtle changes in the solubility of proteins will be examined and related to meat quality, by SDS-polyacrilamide electrophoresis. These results will be used for the development of rapid tests kits for detecting and quantifying both chemical markers and protease activity, and will entail an essential contribution towards the establishment of the fundamental basis of meat quality and its accurate measurement.

Summary of achievements:
Evidence suggests that the early post-mortem measurement of troponin-T in beef muscles may help us assess important final quality characteristics of meat i.e. water holding capacity (WHC) and tenderness. Results also suggest that early post-mortem (24h post-mortem) enzymatic activity of calpain I in pork as potentially useful indicators of organoleptic variables. In bovine muscle it was found that calpain II activity at 24 hour post mortem could provide information on the tenderness and WHC of the meat. The activities of both enzymes could be found to be related to WHC again at still earlier post-mortem (at 6h). In conclusion, the results of this project indicate that pH measurements, enzymatic activity and levels of troponin-T are biochemical parameters that accurately measure the ultimate quality of the meat in early post-mortem. The combination of a number of these indicators has predication

potential, but none of them is conclusive enough when utilised in isolation. In addition, the slight discrepancies between the types of meat investigated suggests that it may be necessary to further investigate the differences changes in early post-mortem muscle for different species.

Keywords:
Chemical markers, meat, quality, pork, beef, post-mortem

Main Publications/Patents/Participation in conferences:

M. Vidal, A.M. Mullen, D.J. Troy, D.J. Buckley (1998) Early post-mortem pH measurements as indicators of meat quality. Proceedings of the 44th International Congress of Meat Science and Technology, Barcelona, Spain

M. Vidal, A.M. Mullen, D.J. Troy, D.J. Buckley (1998) The calpain/capastatin enzyme system as a potential indicator of pork meat quality. Irish Journal of Agriculture and Food Research. Abstract. (*in press*).

M. Vidal, A.M. Mullen, D.J. Troy, D.J. Buckley (1997) Biochemical indicators of pork meat quality. Irish Journal of Agriculture and Food Research. Abstract. Vol. 36, No.2, 257.

M. Vidal, A.M. Mullen, D.J. Troy, D.J. Buckley (1998) Early post-mortem pH measurements as indicators of meat quality. Poster presentation. 44th International Congress of Meat Science and Technology, Barcelona, Spain.

M. Vidal (1997) Biochemical indicators of pork meat quality. 27th Annual Food Science & Technology Research Conference, University College Cork. Oral Presentation.

M. Vidal (1997) Biochemical indicators of pork meat quality. NFC Annual Research Seminars. Oral Presentation.

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Category:	20
Starting date:	08/11/96
Duration:	24 months
Contract number:	FAIR-CT96-5053

Rapid authentication of virgin olive oil by Fourier Transform mid-infrared (FT-Mir) and fourier transform raman spectroscopy (FT-Raman)

Objectives:
The study concerned the application of the FT-Raman and infrared spectroscopy in food chemistry and quality control. The research mainly concerned the study of the potential of Raman spectroscopy and the comparison with the results achieved in infrared spectroscopy. The discrimination of virgin olive oil from other edible oils, and the detection and quantification of virgin olive oil adulteration have been experimented with this new technique of fast and non-destructive analysis.

Summary of achievements:
This study has allowed the establishment of a complete protocol for the collection and normalisation of the FT-Raman spectra from edible oils. FT-Raman spectroscopy could be useful in detecting adulteration beyond the limits achieved by techniques suggested by the current regulations. The potential of this technique was demonstrated for adulterated oils rich in LLL (soyabean, corn) as well as for oils poor in LLL (pomace).

It is possible to clearly discriminate between genuine and 1% spiked samples using the information contained in the Raman spectra. This observation attested to the potential of FT-Raman spectroscopy in low levels of adulteration. The results of the discrimination between the three groups (1, 5, 10%) of adulterated samples indicate that the technique could also be useful in the quantification of olive oil adulteration.

Keywords:
Virgin, olive oil, infrared spectroscopy, quality control,
FT-Raman spectroscopy

Main Publications/Patents/Participation in conferences:

R. Aparicio, V. Baeten (1998) Fats and oils authentication by FT-Raman. Oléagineaux Corps gras Lipides, 4, (5), 293-295.

V. Baeten, M.T. Morales, R. Aparicio (1998) Oil and fat classification by FT-Raman spectroscopy. J Agric. Food Chem, 46, 2638-2646.

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V. Baeten, R. Aparicio (1997) Possibilities Offered by Infrared and Raman Spectroscopic Techniques in Virgin Olive Oil Authentication. *Olivae*, 69, 38-43.

V. Baeten, M. Meurens, M.T. Morales, R. Aparicio (1996) Detection of Virgin Olive Oil Adulteration by Fournier Transform Raman Spectroscopy. J. Agric. Food Chem, 44, 2225-2230.

V. Baeten, M.T. Morales, R. Aparicio (1998) Oil and Fat analysis by FT- Raman spectroscopy. Oral presentation at the 13th International Symposium on Plant Lipids. July 5th-10th. Seville, Spain.

R. Aparicio, M.T. Morales, V. Baeten (1996) "Virgin Olive Oil Authentication". Proc. 87th American Oil Chemists' Society Annual Meeting, Indianapolis IN

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Category:	30
Starting date:	16/08/1996
Duration:	12
Contract number:	FAIR-CT96-5054

Functional barrier studies of aroma compounds in fibre polymer laminates for food packaging with consideration on recycled fibres

Objectives:
This project aimed to investigate volatile odorous compounds on recycled fibres and their transfer behaviour through different types of polymer layers acting as functional barriers. The effectiveness of these barriers was studied by measuring the migration of odorous compounds from the fibre into food stimulants. In addition, the kinetic data of migration was collected.

Summary of achievements:
The project found that the use of waste paper-based corrugated boards for direct food contact has to be treated with caution if the contamination of sensitive food, either through transfer of substances or real migration, is to be avoided. In addition it was found that waste paper contained substances not present in free virgin fibres, and that these did not therefore meet the recommendations set out in positive lists. The deterioration of food depended very greatly on its structural qualities, but also upon the type of barrier used.

Low density polyethylene/polyethylene terephthalate (LDPE/PET) layers were found to be the best barrier. Barriers made of cold applicable water borne polymer dispersions were less effective. However, barriers made of LDPE have only a delaying effect at room temperature. Functional barriers made of polymer dispersions might form a sufficient barrier for low concentration contaminants, but their contribution to volatile substance emissions must be taken into account given that, at least for the tested ones, they were higher compared than from PET and LDPE.

Keywords:
Polymer, fibre, recycle, waste, aroma, packaging

Main Publications/Patents/Participation in conferences:
National Swedish conference on the use recycled fibres in food packaging.

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Category:	20
Starting date:	01/03/1997
Duration:	12 months
Contract number:	FAIR-CT96-5061

Influence of novel food processes in food safety and quality

Objectives:
Novel food processes are processes involving the application of industrial techniques like irradiation, microwaving, very high pressures and others, which has not hitherto been used for food production or processing. In determining whether a significant change has been made to the composition of the product after the process, adverse effects, specially in the biochemical and the toxicological domains, must be evaluated.

The aim of the proposal was to investigate the effects of the so-called Novel Food Processes on an adequate biochemical model -including representative sugars, lipids, proteins, liposoluble and hydrosoluble vitamins-, helped by biochemical (mainly HPLC, but also GC and others) and toxicological (Microadenomes test) analysis, after and before some processes like microwaving, very high pressures, irradiation. They also were compared to the classical processes, like pasteurisation, sterilisation and others. With this strategy a real approach to the effects of the food processes should be performed, avoiding inadequate toxicological tests and dietary intakes with laboratory animals, generally recognised as far away from active biochemical modifications of food substrates.

This project is involved in general assessment of nutritional and safety aspects of Novel Foods. In order to contribute to develop new methods, especially applied to the assessment of new food processes, the proposed program wants to validate a protocol based on the analytical approach from model matrices or model systems prior to toxicological testing.

Summary of achievements:
As a first step, some biochemical model matrices were selected. Under several conditions (i.e., water content, rheological parameters), proteins (especially milk proteins like casein), lipids, glucides (lactose), liposoluble (A, E) and hydrosoluble (Bx, C) vitamins were tested and studied.

The second step consisted of developing of analytical techniques with the aim to evaluate the changes eventually induced by the food processes, including a) chemical techniques, usually applicable in the industrial environment, like high performance liquid chromatography (HPLC) and gas chromatography (GC). The approach at this stage was based on the optimisation of existing techniques. This allowed the modifications of different food molecules, like the formation of Maillard products or pyrolytic intermediates, the oxidation of vitamin C or structural changes in proteins and enzymatic methods to be studied. Indeed, this approach resulted in several biochemical markers, capable of monitoring the effect of the process on the quality of the food matrix.

An additional step, also of great importance and now under development, should be coupling a toxicological evaluation of the treated food fractions by means of a) *in vitro* tests, like the Ames test, and b) *in vitro* tests, such as a test particularly adapted to assess the safety of food components, as aberrant crypt foci (ACF) induction in rodent colon tissues.

This general protocol has been validated by comparative testing with different existing or developing food processes (oven heating, sterilisation, pasteurisation, microwaves, ultra high hydrostatic pressures, ionisation or irradiation).

Keywords:
Food, quality, evaluation protocol, industrial processes

Main Publications/Patents/Participation in conferences:
Fat-soluble vitamins as chemical markers of food processes (1996) International Journal of Vitamin and Nutrition Research 66 (3): 274-275.
Effet du proc d'ionisation sur la conservation des lipides des ovoproduits. "La Conservation des Aliments" Tec-Doc Lavoisier, pp. 301-307, Paris 1997.
Effect of ultra-high hydrostatic pressure on hydrosoluble vitamins (1998) Journal of Food Engineering, *In press*

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Category:	20
Starting date:	20/01/1997
Duration:	36 Months
Contract number:	FAIR-CT96-5065

High quality foods by high pressure processing: case study of liquid egg products and fruit juices

Objectives:
The general objective of the project was to make a comparative study of the potential of high pressure-temperature combined processes for the preservation of an acidic versus non-acidic food. High hydrostatic pressure is an emerging technology which can be used to inactivate microorganisms and quality-deteriorating enzymes in foods allowing better retention of the original flavor and taste than with thermal treatment.

Summary of achievements:
The study showed that when evaluating the microbial safety of pressure processed foods, studies of the product itself are essential. To predict and guarantee safe process, a number of representative strains of the most relevant and resistant pathogens and spoilage micro-organisms had to be studied and to be used as safety indicators. In practice, inactivation levels should be established for appropriate products and categories of food.

Keywords:
Quality, egg, fruit, juice, pathogens, high pressure processing

Main Publications/Patents/Participation in conferences:
B. Masschalck, C. Garcia- Graells, C.E. Van Haver, C.W. Michiels (2000) Inactivation of high pressure resistant *Escherichia coli* by lysozyme and nisin under high pressure. *Innovative Food Science and emerging technologies*, In press.
C. Garcia-Gaealls, B. Masschalck, C.W. Michiels (1999) Inactivation of *Escherichia coli* in milk by high-hydrostatic-pressure treatment in combination with antimicrobial peptides. *J. Food Prot.* 62 : 1248-1254.

C. Garcia-Graells, K.J.A Hauben, C. W. Michiels (1998) High- Pressure inactivation and sub-lethal injury of pressure resistant *Escherichia coli* mutants in fruit juices. Appl. Environ. Microbiol. 64: 1666-1568.

C. Garcia-Graells K. Hauben, C. Michiels (1997) High-pressure inactivation and survival of pressure resistant *Escherichia coli* mutants in fruit juices. Poster presentation. 35th meeting of the European High Pressure, Food Science, Bioscience and chemistry, September 1997, Reading, UK. P. 304-309. In. Isaacs N (ed.), High pressure food science, bioscience and chemistry. The Royal Society of Chemistry, Cambridge, U.K.

C. Garcia- Graells, C. Valckx, C. Michiels (1999) Inactivation of *E. coli* and *L. innocua* by HHP treatment combined with the Lactoperoxidase system. Oral communication in the 17th International Symposium of the International Committee on Food Microbiology and Hygiene (ICFMH), 13-17 September 1999, Veldhoven (Holland). P 234-238. In Tuijtelaars A.C. (ed.). Food Microbiology and Food safety into the next millenium. Ponsen en Looyen, Wageningen, The Netherlands.

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Category:	30
Starting date:	01/03/1997
Duration:	7 months
Contract number:	FAIR-CT96-5070

Improving starch-based films for food packaging: effect of controlled changes in structure during processing on permeability and mechanical properties

Objectives:
The general aim of this project was to establish a relationship among processing parameters, structural characteristics, and oxygen diffusivity and mechanical properties of starch-based films. During these months, most of the time was spent establishing the procedures to study the relationship between molecular structure and oxygen diffusivity.

Summary of achievements:
The films were created by casting and drying. Drying kinetics as well as ratio amylose:amylopectine of the potato starch source were used to generate different structures in the starch films. Important points of the film making procedure were: i) no plasticisers were needed to make intact films; ii) constant shear stress was applied to the starch solution while gelatinisation of the starch granules took place; iii) solutions were degassed under vacuum after gelatinisation; iv) casting was done on flat plates made of high density polyethylene.

The structure of the films was analysed by different apparatus: i) crystallinity and crystal type by X-ray diffraction; ii) location of glass transition temperature by differential scanning - calorimeter; iii) molecular conformation by Fourier-transform infrared spectrometry and iv) phase separation between amylose and amylopectin by light microscopy.

Furthermore, oxygen transmission through the films was measured using an Ox-trap apparatus (Modem Control Inc.). Both steady and unsteady-state values from the transmissions were used to obtained oxygen permeability and oxygen diffusivity of the different films.

The preliminary experiments conducted at ATO-DLO seem to indicate that:

3.3 Advanced and optimised technologies and processes

i) Both oxygen permeability and oxygen diffusivity were found to be much lower than the values found in the literature.

ii) Temperature during drying, seems to be a very critical factor affecting structure collapse and film compactness and as a consequence, the barrier properties of the films.

Keywords:
Starch, films, food packaging, structure, processing, permeability, mechanical, Temperature

Main Publications/Patents/Participation in conferences:
International Congress of Engineering and Food, Brighton, UK, April 1997

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Category:	30
Starting date:	01/04/1997
Duration:	14 months
Contract number:	FAIR-CT96-5076

Lipolysis and Cholesterol Oxidation in Dry-cured Ham

Objectives:
Two sub-projects were undertaken to provide new knowledge in the field of lipolysis and cholesterol oxidation. They aimed to 1) investigate the development of compounds resulting from cholesterol degradation and 2) search for inter-relations between variables believed to influence lipolysis.

Summary of achievements:
Rather low levels of cholesterol oxides were found in the hams analysed. It appears that their presence in dry-cured ham is a minor problem. It is however interesting to note that the specific compounds 7-ketocholesterol and Cholestane-3β,5α,3β-triol are strongly interrelated and that they are the most frequently occurring always are the cholesterol oxides. This could lead to a future application in which a more simple analysis is developed for one or both of these compounds, permitting for rapid and extensive evaluation of a broad range of meat products. The present method for detection of cholesterol oxides is rather complex and very labour costly.

The concentrations of COPS found in dry-cured ham in this study are generally at a level which does not give rise to concern considering that the lean edible part was almost unaffected by oxidative problems. It is evident that some COPS form in the subcutaneous fatty tissue of DCH at such a level as has been typically reported in other meat products. Surprisingly, the mechanism does not seem to be related to two of the most common indices of oxidative changes in foods, TBARs and peroxide value, nor to the time of ageing. It seems likely that other factors, such as naturally occurring anti-oxidative properties of the meat or processing factors, might be major determinants in the onset of the oxidative process in dry-cured ham (DCH).

The fact that 7β-hydroxycholesterol correlated rather well with most other COPS assayed, and that it was present in relatively large amounts might enable replacing the checking of meat products for all COPS, by a simple analysis for this compound.

Results for this part of the project show a large variability in all lipolytic activities measured and an increase during the mid-period of dry-curing process.

Keywords:
Lipolysis, cholesterol-oxidation, dry-curing process, ham, meat

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Category:	20
Starting date:	01/04/1997
Duration:	6 months
Contract number:	FAIR-CT96-5078

Development of a novel methodology to validate optimal sterilisation conditions

Objectives:
The objective of this research work was to design a new methodology to validate experimentally optimal conditions, for maximising final volume average and surface quality retention of particular in-pack food products considering still and end-over-end rotary processes in a water cascading retort. The objective was to make progress in modelling, characterising heat transfer for the products and to combine this with relevant kinetic data.

Summary of achievements:
Optimal thermal processes were designed for green and white beans in glass jars heated in a still and end-over-end rotary pilot water cascading retort. For this purpose, kinetics of thermal degradation of hardness of white beans were conducted and evaluated using a tenderometer and a texterometer. The fractional conversion model was applied in both cases to model the texture degradation. The Arrhenius equation was used to describe the temperature dependence of the reaction rate constant. Also the 1st order kinetics was used as a simplifying model to be further compared in optimisation procedures. The thermal degradation of colour of green beans as well as the sensory analysis model of hardness of white beans were taken from literature research.

The process value (F_0) and the heat penetration parameters (f_h and j_h) were determined from 100 containers for static processes for both green and white beans. Also the rotations (end-over-end) of 4, 7, 10 and 15 rpm were performed using white beans. These experiments were conducted at the coldest part of the containers. Heat penetration curves obtained were log-linear and both products heat by convection. Increasing the rotational speed, the heat penetration on white beans was improved. However, over 10 rpm this influence became smaller due to increase leakage of starch. This means, standard deviations and coefficients of variation of the heat penetration parameters and process values were determined. Also

statistical tests for normality and for run-to-run variability were discussed. The largest f_h and corresponding j_h were selected as the worst case for each condition. Not all the parameters followed normal distribution and the run-to-run variability found was mainly due to product variability.

Theoretical optimum temperatures were calculated using a computer program previously developed for conduction heating foods. This program required information about the thermal degradation kinetics for quality attributes under consideration, characterisation of the heat transfer into the product, processing conditions and the desired sterilisation value. Maximisation of volume average and surface quality retention was used as objective functions. Experimental validation of the calculated results was conducted using a colorimeter to measure the colour of green beans and a tenderometer for hardness evaluation of white beans. Optimum temperatures theoretical and experimental were of the same order of magnitude for average retention of colour of green beans and in the case of white beans considering the fractional conversion model for hardness degradation. Applying the 1st order model for the tenderometer data and for sensory analysis evaluation instead of the fractional conversion to describe the hardness degradation of white beans, completely deviating optimal temperatures were found. Despite the success obtained in validation of calculated optimal temperatures, large differences in theoretical and experimental profiles of retention of quality as a function of temperature were found for both products. Simplifying assumptions made in the heat transfer model used are the reason for these results. Infinite heat transfer coefficient at the product surface was assumed but glass jars were used instead of metal cans. The heat transfer considered was by conduction, but products in study heat by convection. We can conclude that the theoretical approach was successfully applied to convective and mixed heating mode products. The use of the correct kinetic model and its associated parameters is crucial in calculating optimal processing conditions. However, implementation of novel processes should follow all the important steps referred to as each new design is only valid for the considered products, containers, fill weights, retort, heat medium and processing conditions.

Keywords:
methodology, optimisation, sterilisation, thermal process, beans, hardness, tenderometer, texterometer, normal distribution

Main Publications/Patents/Participation in conferences:
I. M. L. B. Avila, C. L. M. Silva (1997) Experimental validation of optimal sterilisation conditions for maximising quality of carotenoids and colour in canned peach puree. Oral presentation at Institute of Food Technologists Annual Meeting, 14-18th June 1997, Orlando, USA.

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Category:	20
Starting date:	01/09/1997
Duration:	36 months
Contract number:	FAIR-CT97-5003

Sensory characterisation studies on warmed-over flavour in meat

Objectives:
The overall aim of the project is to develop a more in-depth understanding of mechanisms involved in the formation of Warmed-Over Flavour (WOF) from a sensory perspective and to determine the variations occurring within *WOF* in different meats.

Summary of achievements:
In the first year comprehensive sensory descriptive vocabularies have been developed for *WOF* in pork and chicken meat with pre-slaughter stress and cooking temperature differences, respectively included as added sensory dimensions. In addition, descriptive sensory profiling has been carried out on pork and chicken meats utilizing these developed vocabularies. From these sensory profiles the results have been analysed by multivariate statistical methods. In the near future GC-MS analysis will be carried out on the chicken for subsequent correlation with the sensory data.

Keywords:
Sensory, characterisation, warmed-over, flavour, meat, slaughter

Main Publications/Patents/Participation in conferences:
D.V. Byrne, L.S. Bak, W.L.P. Bredie, G. Bertelsen, M. Martens (1999) Development of a sensory vocabulary for warmed-over flavour: Part I in porcine meat. Journal of Sensory studies 14: 47-67
D.V. Byrne, W.L.P. Bredie, M. Martens (1999) Development of a sensory vocabulary for warmed-over flavour: Part II in chicken meat. Journal of Sensory studies 14: 67-78
J. Brundøm, D.V. Byrne, L.S. Bak, G. Bertelsen, S.B. Engelsen (2000) Warmed-over flavour in porcine meat-a combined spectroscopic, sensory and chemometric study. Meat Science 54: 47-67

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D.V. Byrne, W.L.P. Bredie, M. Martens (1998) Sensory characterisation studies on warmed-over flavour in meat. In The Abstracts of the Centre for Advanced Food Research (LMC) Congress, pp. 93, Danish Technical University, Lyngby, Denmark. 29-30 January 1998.
D.V. Byrne, W.L.P. Bredie, M. Martens (1998) Reliability in sensory profiling of a complex meat material. The Royal Veterinary and Agricultural University, Copenhagen, Denmark. 6-8 August 1998.
D.V. Byrne, W.L.P. Bredie, M. Martens (1998) Reliability in sensory profiling of a complex meat material. In The Conference Programme Abstracts of "Sense and Sensibility" the 3rd Pangborn Sensory Science Symposium, pp. 63, Ålesund, Norway. 9-13 August 1998.

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Category:	30
Starting date:	29/01/1998
Duration:	24 months
Contract number:	FAIR-CT97-5011

Measurement of interactions between macromolecules and aroma compounds by high performance liquid chromatography and spectroscopic methods: determination of thermodynamic data

Objectives:
In recent years a considerable number of traditional foods have been reformulated because of dietary or health considerations. In order to maintain a satisfactory organoleptic quality, it is currently necessary to empirically change the formulation. The aim of a certain number of studies has been to show the existence of interactions between aroma compounds and other food components. The nature of these interactions, even in simple models, has not been addressed in detail. The main aim of this project was to establish novel chromatographic and spectroscopic methods for the study of interactions between food macromolecules and aroma compounds.

Summary of achievements:
The chosen model macromolecule, β-lactoglobulin, allowed the project to build upon the expertise already available at INRA Dijon. It became clear at an early stage that an HPLC-based technique called Hummel & Dreyer was of limited applicability only, due to problems with the solubility of the tested compounds in water and with their UV detection.

This led to the adoption of affinity chromatography on β-lactoglobulin-boded silica phases. The technique of preparing these phases is now well mastered and a certain number of aroma compounds were tested and their binding constants measured. This allowed a unique insight into the relative strength of interactions between β-lactoglobulin and aroma compounds within a given class of compounds and across chemical classes.

The next stage involved studying these interactions by infrared spectroscopy. The sample preparation, data acquisition and data processing techniques have been optimised according to

the requirements for in-depth analysis of spectra of water-soluble proteins. The method has been developed to a stage where routine analysis of complexes of further aroma compounds can be carried out easily.

Spectra of aqueous solutions of β-lactoglobulin recorded in the presence and absence of aroma compounds acting as ligands show differences indicating that the protein changes its secondary structure on complexation. A more detailed analysis revealed the structural features involved in this process, and made it possible to determine where, depending on the nature of the ligand, complexation on the protein molecule takes place.

In the last part of the project, nuclear magnetic resonance spectroscopy was used to examine selected complexes in further detail. Two complementary techniques were used and the results provide a deeper insight in the structure of these complexes, compared to infrared spectroscopy. Amino acid residues of β-lactoglobulin participating in the interactions were identified, which allows the determination of their repartition on the protein structure as an image of the ligand's position relative to protein.

β-lactoglobulin is amongst the most popular model proteins in food science with large technological importance. The present project has not only led to the establishment of new generic methods for the study of interactions between biopolymers and aroma compounds, but has also been a significant step forward in the knowledge of this protein.

Keywords:
protein, chromatography, spectroscopy, interaction, macromolecules, aroma, β-lactoglobulin, ligand, complexation, biopolymers

Main Publications/Patents/Participation in conferences:
M. Lübke, E. Guichard, J. L. Le Quéré (1999) Infrared spectroscopic study of interactions of aroma compounds with β-lactoglobulin. Poster presentation at MADGELAS, 17-18th May 1999, Ayr, Scotland.
M. Lübke, E. Guichard, J. L. Le Quéré (1999) Infrared spectroscopic study of interactions of aroma compounds with β-lactoglobulin. Oral presentation at COST Action 96: Interactions of Food Matrix with Small Ligands, 20-21st May 1999, Oslo, Norway.
M. Lübke, E. Guichard, J. L. Le Quéré (1999) The study of interactions between food macromolecules and small ligands, and how infrared spectroscopy can contribute to it. Oral presentation at the American Chemical Society Annual Meeting 1999: Flavour Release Symposium, 22-26th August 1999, New Orleans, USA.
M. Lübke, E. Guichard, J. L. Le Quéré (1999) Infrared spectroscopic study of β-lactoglobulin interactions with flavour compounds – Flavour release, D. Roberts and A. Taylor (eds.), ACS Symposium Series, American Chemical Society, Washington DC, 2000 (in press).

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Category:	30
Starting date:	01/10/97
Duration:	12 months
Contract number:	FAIR-CT97-5016

Isolation, quantification and identification of major oxidized fatty acids formed during food processing and storage

Objectives:
During the course of food processing which affects fats and oils, oxidation is the main route for the formation of new alteration compounds. Although there have been a number of studies on the altered non-polar fatty acids in triglyceride molecules, namely, *trans* fatty acids, cyclic fatty acids, conjugated fatty acids, there has been very little work on the levels and structures of oxidized fatty acids present in dietary fats and oils. Undoubtedly, knowledge of such analytical aspects of the major oxidized fatty acids formed during food processing is essential to determine their occurrence in the diet and establish realistically their nutritional relevance. However, the complexity and multiplicity of the compounds (hydroperoxyacids, epoxyacids, hydroxyacids, ketoacids and compounds with various oxygenated functions) have limited investigations in this area.

Therefore the general objective of this study was to gain knowledge about the amounts and structures of new compounds formed from fats and oils as a consequence of food processing. The studies have focused on three main aspects:
1. The structures of oxidized fatty acid monomers
2. Their formation and evolution
3. A quantification of the amount of oils and fats being ingested

Summary of achievements:
Studies have been carried out with to deepen our knowledge of the structure of oxidation compounds formed in fats and oils heated at the high temperatures of processes like baking and frying. Due to the complex mixture of the new compounds, experiments have been carried out on three different levels:
1. Model systems of fatty acid methyl esters (FAME), i.e., methyl oleate and linoleate, and monoacid triacylglycerols, i.e. triolein and trilinolein, heated at high temperatures under well controlled conditions. These experiments allowed identification of the main new compounds by means of GLC-MS and to select the most adequate derivatisation techniques for accurate quantitation.

2. Oils of different degree of unsaturation subjected to thermoxidative treatment for different periods of time. In these samples formation and evolution of the oxidation compounds was studied.
3. Used frying fats and oils from different origins, collected by Food Inspection Services. This last step was essential to know the importance of the oxidation compounds in fats and oils being consumed.

Characterisation, quantification and evolution of major aldehydic FAME and short-chain FAME formed by hydroperoxide decomposition, as well as monoepoxy FAME are among the main new results obtained.

Keywords:
Oxidation, lipid, triacylglycerols, frying, fats, oils, food processing

Main Publications/Patents/Participation in conferences:
C. Dobarganes, G. Marques-Ruiz, O. Berdeaux, J. Espejo (In press) Determination of oxidised compounds and oligomers by chromatographic techniques. In Frying of foods: Chemistry and Nutrition. Eds. D. Boskou and I. Elmadfa. Technomic Publishing Co. Lancaster PA (USA).
2nd meeting of the European Section of AOCS Cagliari, Italy, 1-4 October 1998
Quantitation of Major Low-Molecular-Weight Compounds Linked to the Glyceridic Backbone Formed in Triacylglycerols Heated at High Temperature. Oral presentation

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Category:	20
Starting date:	28/07/1997
Duration:	36 months
Contract number:	FAIR-CT97-5018

Improvement of some aspects of brewing and processing: research on feruloyl esterases from barley and malt

Objectives:
A recent collaborative study between BRF International (UK) and IFR (Norwich, UK) has shown that some microbial ferulic acid esterases are capable of solubilising beta-glucans from barley insoluble beta-glucan preparations.

These results suggest that either ester linkages involving ferulic acid hold beta-glucans and arabinoxylans together in the endosperm cell walls (although only ferulic acid arabinoxylan linkages have been reported to occur in barley up to date) or disruption of ferulic acid-arabinoxylan ester linkages possibly disrupts arabinoxylans directly from the cell wall and, due to the organization of the polysaccharides in the wall, subsequently brings into solution beta-glucans which are physically associated with arabinoxylans. In any case, it does appear that efficient degradation of beta-glucans during malting and brewing may be enhanced by feruloyl esterase activity.

Preliminary work carried out at IFR (Norwich, UK) has shown that an extract from malted barley exhibited ferulic activity. This semi-purified malt extract was not only able to release ferulic acid from its methyl ester and from a feruloylated polysaccharide (FAXX), but also from spent grain (inactivated malt material rich in cell walls). This finding brings up another brewing aspect related to FAEs. The aim of this project is to investigate the presence of feruloyl esterases in barley/malt and the role of these enzymes in brewing.

- task 1. Purification and characterization of ferulic acid esterase/s from malt.
- task 2. Monitoring of ferulic acid esterase activity during malting and mashing.
- task 3. Scanning of barley varieties.
- task 4. Determination of the effect of malt ferulic acid esterase on beta-glucan solubilisation.

3.3 Advanced and optimised technologies and processes

Summary of achievements:
The development of a sensitive assay to detect ferulic acid esterase activity in barley was achieved. Ferulic acid was found to be endogenous to both stored and germinating barley grain and did not arise from surface contamination by micro-organisms.

Kinetic data on the specificity of the crude barley extract was determined for both synthetically methyl esterified hydroxycinnamic acids and plant cell wall derived ferulolyated oligosaccharides.

The project determined the effect of pH, temperature, concentration and time on enzyme activity.

Variations in ferulic acid esterase activities due to barley variety was investigated using malting and feed grade single varieties from Spain and the United Kingdom.

Keywords:
Barley, beer, esterase, ferulic acid, xylanase, inhibitor

Main Publications/Patents/Participation in conferences:
A.I. Sancho, C.B. Faulds, B. Bartolomé, G. Williamson Characterisation of feruloyl esterase activity in barley. Journal of the Science of Food and Agriculture.
A.I. Sancho, C.B. Faulds, B. Bartolomé, G. Williamson (1998) Ferulic acid esterases from barley. Abstract for Ferulate 98, Institute of Food Research, Norwich, UK, 8-11 July 1998.
A.I. Sancho, C.B. Faulds, B. Bartolomé, G. Williamson (1998) Detection of cinnamoyl esterases in barley grain. Abstract for 8th International Cell Wall Meeting, John Innes Centre, Norwich, UK, 1-5 September 1998.
A.I. Sancho, C.B. Faulds, B. Bartolomé, G. Williamson (1999) Detection of an inhibitor of barley xylanases. Abstract for European Brewery Convention, 27th International Congress, Cannes, France, 29 May-3 June 1999.

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Category:	30
Starting date:	20/03/1998
Duration:	24 months
Contract number:	FAIR-CT98-5003

Application of advanced technologies to model flavour release for improvement of sensory quality and consumer preference in new hard cheese varieties

Objectives:
This project focused on the relationships between the cheese matrix composition, as fat and protein content, pH, moisture, and the sensory characteristics of the cheese products in attempt to model flavour release for improvement of sensory quality and consumer preference in new hard cheese varieties.

Summary of achievements:
First the functionality of the sensory panel and its performance to describe cheese odours and flavours was evaluated and improved. A vocabulary was developed aided by a multivariate statistical procedure. Then the functionality of enzyme-modified cheese was evaluated as a model cheese to predict the flavour release of natural cheeses. The sensory characteristics of fifteen commercially manufactured Cheddar-type Enzyme Modified Cheeses (EMC) (Cheddar-type) were determined using Quantitative Descriptive Analysis. These EMC sensory profiles were compared to the natural Cheddar cheeses. Only five EMCs have sensory quality closed to natural Cheddar cheese ones. Some other EMCs were described as having a mouldy and strong flavour as mould-type cheese. Relationships between EMC sensory data and their compositional data revealed a high correlation for pH, fat content and protein content. Six of the EMCs studied described as having a cheddary character were positively correlated with the protein content, whereas some other EMCs were described as having a mouldy and strong character.

In parallel, the sensory characteristics that determined consumer preference for ten speciality cheeses were investigated. Descriptive analysis was carried out and 198 consumers rated their preferences for these ten cheeses. Internal preference mapping illustrated individual consumers preferences for the cheeses. Hierarchical cluster analysis showed that the sampled consumer population was heterogeneous by identifying seven clusters of consumers with

different preferences. Gruyere was the preferred cheese of three clusters of consumers, and blue Shropshire, the preferred cheese of two clusters of consumers (but the least liked cheese overall).

Keywords:
Cheese, flavour, cheese matrix composition, odorous key compounds, sensory analysis, enzyme modified cheese, model cheese

Main Publications/Patents/Participation in conferences:
C.N. Raynaud, S. Hulin-Bertaud, C. Delahunty (1999) Electronic nose: a useful tool for the selection of discriminant variables from EMC sensory profile. Flavour consortium postgraduate symposium, Reading, UK, 21-23 July 1999.
S. Hulin-Bertaud, E.M. Sheehan, C. Delahunty (1999) Development and selection of a sensory vocabulary for quantitative descriptive analysis on hard cheese. Flavour consortium postgraduate symposium, Reading, UK, 21-23 July 1999.

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Category:	20
Starting date:	01/01/1999
Duration:	24 months
Contract number:	FAIR-CT98-5031

Effects of high pressure processing on health-promoting substances of fruit and vegetable juices

Objectives:
High pressure is a promising technique for liquid food processing and several European companies are interested in its application to manufacture fruit and vegetable juices which must keep the nutritional properties intact and have an optimum fresh like product with an extended shelf-life. Therefore researchers are interested in the possible modifications in activity occurring on health protective substances (dietary fibres, vitamin C and polyphenols) induced by combined high pressure/thermal treatments of self-processed fruit juices. These substances are related to the prevention of cancer and cardiovascular diseases, and the improvement of the diet of diabetics or obesities. The main objective of this work is to accumulate knowledge about the less destructive treatments, in terms of preserving the health protective qualities of the juice.

Summary of achievements:
Antioxidant capacity of the water soluble fraction of vegetable and fruit juices during three weeks cold storage

- The antioxidant capacity of the water soluble fraction of orange juices was only slightly influenced by high pressure treatments.
- Pressurized ACE juice has shown an important loss of antioxidant capacity during storage, probably meaning the presence of higher amounts of diluted pectin.
- Antioxidant capacity of apple puree high pressure treated in aerobic conditions has been slightly improved although chilled storage has caused a loss approximating results to controls. Regardless better maintenance was arisen when high intensity pressure was applied. Apple puree treated in anaerobic conditions has initially preserved antioxidant capacity, but losses during storage caused comparable results with aerobic samples.
- Tomato and carrot puree high pressure treated have exhibited a largely improved antioxidant capacity during storage, encouraging these results extended studies about the properties of these matrixes.

- Tomato juice antioxidant capacity was not modified by high pressure treatments, suggesting that changes found in tomato puree could be due to enzymes associated to the pulpy fraction.

Effects of high pressure on non-water soluble antioxidants of tomato

- No quantitative changes in the concentration of carotenoids caused by high pressure application (600MPa, 20 °C), and also not by high temperatures (95 °C), after one hour treatment. Very important result as high pressure is a candidate for tomato puree processing.
- Alterations in the extractability of carotenoids with petroleum ether after high pressure application (600MPa, 20 °C, 60 min) suggests a change in the ultrastructure of tomato puree, leading possibly to variations in viscosity and colour described in the literature.
- Changes in water activity or tissue reorganisation are confirmed by experiments as water retention was higher in high pressure treated tomato puree. Further investigation is required to discard one of the two possibilities. Nature of alterations in temperature treated samples was different to pressurised tomato.
- Differences in extractability of carotenoids manifested with petroleum ether have not conducted to a proportional diminution of total antioxidant capacity, indicating the possibility of synergistic interactions between different fractions in the emulsion.

Glucose retardation index

- Negligible differences in diffusion of glucose from dialysis bags have been caused by temperature processing of pectin with different methylation degrees. Pressure itself has not contributed to changes in the glucose retardation index.
- Pressure/temperature combinations have caused a slight decrease in the viscosity of pectin of different methylation degrees which have not lead to alterations in the glucose retardation index.
- Significant differences in the glucose retardation index have been detected when tomato puree was high pressure treated. Pressurised tomato puree has significantly delayed the diffusion of glucose from dialysis bags compared with controls and temperature treated samples.

Keywords:
high pressure processing, health-promotion, fruit, vegetable, juice, glucose, carotenoid, antioxidant

Main Publications/Patents/Participation in conferences:

A. Fernandez Garcia, P. Butz, B. Tauscher, "Does the antioxidant potential of high pressure treated apple juice change during storage?"

A. Fernandez Garcia, P. Butz, B. Tauscher, "Effects of high pressure treatment on fermentation processes during ripening of Gouda cheese".

(Both articles are to be published in the proceedings of the XXXVII EHPRG Meeting (Montpellier, Sept. 1999).

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Category:	30
Starting date:	15/07/1998
Duration:	24 months
Contract number:	FAIR-CT98-5040

High pressure treatment of soya protein

Objectives:
Use of high pressures to modify the functional properties of soya proteins in a consumer-friendly, "green" way to lead to products of added value. Application of rheological analysis and chemical techniques to give a fuller and more fundamental understanding of the different textured of the new products. Study of the influence of the parameters involved in the high pressures procedures on the texture of new soya products.

Summary of achievements:
Characterisation of soya protein isolate by SDS-PAGE and differential scanning calorimetry. Soya protein isolate (SPI) was characterised by SDS-PAGE. Band assignment was by comparison to the patterns of SPI in the literature, major bands corresponding to lypoxygenase (LOX) (94 kDa); β-conglycinin (7S) subunits α' and α (80 kDa) and β (55 kDa); acidic glycinin (A-11 S) polypeptidic chains A_3 (43 kDa) and A_1 A_2 and A_4 (37 kDa); and basic subunits of glycinin (B-11S) (22 kDa). Aggregates were found in the high pressure (HP) treated samples compared to the native at pH 5.5. Differential scanning calorimetry (DSC) can provide fundamental information on the denaturation of proteins. The thermograms of 20% SPI dispersion showed endothermic peaks at 75 °C and 95 °C corresponding to 7S and 11 S respectively, in agreement with literature values.

Keywords:
High pressure, soya, protein, rheology, texture, protein denaturation, gels, protein aggregation

Main Publications/Patents/Participation in conferences:
E. Molina, D.A. Ledward (in prep.) Influence of the high pressure in the emulsifying properties of soy protein isolate and its major constituents.

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Category:	20
Starting date:	09/11/1998
Duration:	6 months
Contract number:	FAIR-CT98-5055

Assessment of diterpenoids content in sage, oregano and other officinal plants. Evaluation of their anti-oxidant activity and their influence on the shelf-life of meat products

Objectives:
From the point of view of the quality and shelf-life of meat products, preservation of their lipidic fraction from oxidative deterioration represents an important aim. It is indeed well known that the products derived from lipid oxidation give rise to a decay of organoleptic quality (presence of off-flavours which reduce significantly the shelf-life) and a reduction of the nutritional properties and safety of this food. In order to limit these degradative effects, various means can be used. Apart from physical processes such as oxygen removing and refrigeration, the use of substances which slow down these oxidation processes, play a very important role. Many officinal plants, especially species of the families *Apiaceae* and *Lamiaceae* show significant antioxidative properties. Among *Lamiaceae* species, sage and oregano have been widely studied for their antioxidant activities, due to their content of phenolic compounds. In recent years, extracts of officinal plants have been used to stabilise fat and fat-containing foods. Besides, the use of officinal plants in the form of feed additives is continuously increasing, since they offer an alternative for various feed additive categories, among these, antioxidants.

For that reason analytical methods for evaluation of the efficiency of these treatments should be established.

In this work an analytical method to examine the aforementioned treatments is proposed, based on the determination of the antioxidant activity, measured by photochemiluminescence (PCL), in animal fat samples in the presence of methanolic extracts of sage or oregano.

Summary of achievements:
From the point of view of meat products quality and shelf-life, preservation of their lipidic fraction from oxidative deterioration represents an important aim. The use of substances which slow down these oxidation processes, plays a very important role. Many officinal

plants, especially species of the families *Apiaceae* and *Lamiaceae* show significant anti-oxidative properties.

Therefore, essential oils and extracts from these plants can represent a health alternative as food and feed additives. In recent years, extracts of officinal plants have been used to stabilise fat and fat-containing foods. Besides, the use of officinal plants in the form of feed additives is continuously increasing, since they offer an alternative for various feed additive categories, among these, antioxidants.

In this work an analytical method to estimate aforesaid treatments is proposed, based on the determination of the antioxidant activity, measured by photochemiluminescence (PCL), in animal fat samples in presence of methanolic extracts of sage or oregano. The sensitivity of this assay lies within nmol quantities of substances.

Before photochemiluminescence analysis a step of purification of the fat sample is required, in order to separate and concentrate the phenolics from lipidic substances. Recovery rate and repeatability of the method were calculated and the results were acceptable.

In addition, fat samples deriving from sage and oregano plants-fed swines and oregano essential oil-fed chickens were analysed in order to investigate the effect of this treatment on the durability of the product.

In contrast to what is observed in fat samples added with sage and oregano extracts, fat samples deriving from swines fed with the same plants do not show a higher antioxidant activity.

Results of analysis of fat from oregano essential oil-fed poultry show a significant influence of this treatment on the antioxidant activity of the fat samples.

It is possible to suppose that a treatment of fat or meat products directly with sage and oregano extracts would have a more effective anti-oxidative effect than those resulting by feeding the animals with entire plants.

Keywords:
diterpenoids, officinal, anti-oxidant, shelf-life, meat, health, feed, additives

Main Publications/Patents/Participation in conferences:
V. Stefania, K. Zitterl-Eglseer, F. Chlodwig (1998) Determination of the Antioxidant Activity in Animal Fat in Presence of Sage and Oregano Extracts by Photochemiluminescence Analysis. Zeitschrift für Lebensmitteluntersuchung und Forschung.
"Gewürze in Lebensmitteln", Vienna, 23rd April 1998.

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Category:	20
Starting date:	14/09/1998
Duration:	36 months
Contract number:	FAIR-CT98-5070

Concentration of fruit juices using membrane technology

Objectives:
The aims of this project were:
- To improve the quality of the concentrated product (in comparison to conventional methods);
- To extend the degree of concentration achieved by other workers using reverse osmosis (RO);
- To develop an economically viable process for future industrial application.

Summary of achievements:
The direct osmosis concentration method of concentrating tomato juice has been assessed and is not considered to be economically viable. Consequently attention has focused on the use of reverse osmosis (RO). The literature on reverse osmosis concentration of tomato juice has been reviewed.

Many workers have investigated the use of RO for concentrating tomato juice. However, there have been few viable industrial applications due to the difficulty in achieving high degrees of concentration combined with sufficient productivity. Water fluxes of 15 l/m^2 h are considered to be economically feasible. Presently RO has been applied successfully to industrial production of tomato semi-concentrates only in Italy and Japan. In all cases the RO concentrates showed better sensory characteristics in terms of colour, taste, flavour, overall preference and, in some studies, consistency, when compared to the conventional evaporation concentrates. The most important factors that limit the degree of concentration achievable with RO are high osmotic pressure, high pulp content and high viscosity in the tomato juice. Therefore a high operating pressure is required to achieve high degrees of concentration.

The membrane solvent (water) permeability and resistance of the membranes have been determined by measuring the water flux at different operating pressures and feed (water) flow

rates. The maximum membrane water permeability obtained was 1.4l/m^2 h bar.

Model solutions have been used to investigate membrane behaviour and predict the maximum degree of concentration/permeate fluxes achievable. Glucose solutions, which model the osmotic pressure of tomato juice, were concentrated up to 32 wt % at constant operating pressures of 60, 90 and 100 bar respectively. The maximum permeate flux obtained was 89.4 l/m^2h at a pressure of 100 bar. Permeate flux measured as a function of time for glucose solutions of constant composition at constant pressure (90 bar) remained constant. This data will be used as a benchmark to measure the degree of fouling obtained with tomato concentrates.

Different methods of separating suspended solids and serum prior to concentration have been investigated, including centrifugation, rotary vacuum filtration and combinations of the two. Centrifugation followed by rotary vacuum filtration (using diatomaceous earth as a filter aid) produced a highly purified serum.

Keywords:
Fruit, juices, reverse osmosis, tomato, concentrate, membrane

Main Publications/Patents/Participation in conferences:
Hodur, Szabo, Godek, Fiorenza, Morris and Smith (In press) Energy saving concentration of fruit and vegetable juices. Elelmezes ipar (Hungarian Food Industry).

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Category:	30
Starting date:	17/06/1999
Duration:	24 months
Contract number:	FAIR-CT98-5074

Development of a mathematical model for predicting migration/sorption processes in food packaging systems

Objectives:
The aim of the work is to generate an advanced scientific understanding and modeling of the physico-chemical behavior of chemical contaminants in recycled plastics layers buried by functional barrier polymers as a basis for safety evaluation. The criteria for the appropriate functional barrier protection design against recycled plastic for food packaging will be defined.

The goal was to select a list of representative surrogates in order to cover a wide range of worst-case contaminants and the development of analytical procedures. The selected surrogates were benzophenone, uvitex, trichloroethane, toluene, chlorobenzene, phenylcyclohexane, metylmargarinate and DMSO. The list of selected surrogates is composed of components of the ILS1 Guidelines "Recycling of Plastics for Food Contact Use" (May, 1998) and some additional components with specific properties. Initially, DMSO was chosen as an extremely polar component, but due to analytical problems this surrogate will only be used in special cases (polar polymers). Methylmargarinate gave less blank problems than methylstearate that was used in the ILSI Guidelines.

Summary of achievements:
Two analytical methods, one GC/MS-method and one HPLC-method, to determine the surrogate concentrations in polymers were developed and validated. The goal was to evaluate the barrier properties on the bases of the diffusion coefficient in order to determine which polymers are suitable to be used as a Functional Barrier. The first step was to make an inventory of methods to determine diffusion coefficients of both volatile and non-volatile components and to evaluate some of these methods experimentally. The second step was to

select the most suitable methods in order to determine the diffusion coefficients of a number of possible functional barrier polymers (e.g. PP, HDPE, PS, PET, PEN, PAN, PC, EVOH).

In this experiment, the diffusion coefficients of Uvitex and Benzophenone in LDPE-films were determined.

It was not possible to calculate the diffusion coefficient of benzophenone due to the fact that equilibrium had already been reached throughout the stack during the applied incubation time. The theoretical diffusion curve can only be applied in the case that the stack can be considered as a semi-infinite plane sheet. As it appeared that the concentration at the end of the stack was not zero, it was not a correct approximation to consider the stack as a semi-infinite plane sheet.

In the case of uvitex concentration at the end of the stack was zero and the experimental concentration curve was, as expected, decreasing with the diffusion distance. It was therefore possible to calculate the diffusion coefficient using the "Moisan equation". The similarity between the results validates the correct performance of the Moisan method at the different laboratories.

The Moisan experiment with pure Uvitex-powder as source was performed. From this experiment it can be concluded that it is not suitable to use the component as a pure powder instead of incorporated in a PE-WAX source.

The diffusion coefficient of uvitex, benzophenone, methylmaragarinate and phenylcyclohexane have been determined in PP at 40 °C. In order to check the effect of the mutual influence of surrogates in the diffusion process the test was performed in two different ways. The results obtained seem to show that the presence of more than one surrogate cause some kind of effect in the diffusion of the biggest surrogate (uvitex) but this has to be confirmed with further experiments.

Keywords:
mathematical model, prediction, migration, sorption, food packaging, Moisan, Uvitex, benzophenone, contaminants , recycle, plastics

Main Publications/Patents/Participation in conferences:
2nd International Symposium on Food Packaging, Ensuring the safety and quality of foods. Vienna, Austria, 8-10 November 2000. Poster

Links with EC projects:
FAIR-CT98-4318

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Category:	20
Starting date:	01/03/96
Duration:	24 months
Contract number:	FAIR-CT96-5001

Peroxidases in relation to the quality of beans and potatoes

Objectives:
The texture of processed fruit and vegetables is an important quality aspect and is directly related to the mechanical properties of the cell wall. At a tissue level, both the cell wall strength and the cell-cell adhesion play a role in determining the plant texture. For potato, high levels of cell separation correlate with the so-called "mealiness" of cooked potato tissue.

The interplay between cell wall and middle lamellae is shown for cooking of (canned) vegetables. Excessive cell separation leads to oversoftening while some vegetables like Chinese water chestnut exhibit a firm and crunchy texture due to strong intercellular bindings.

At a chemical level, it has been shown that mostly pectins determine the intercellular binding. During processing pectic regions are released affecting the final texture strength. In addition, processing can result in the activation of some cell wall enzymes such as: Pectin methylesterase (PME) and peroxidase (POD; EC. 1.11.1.7). Both enzymes can increase the strength of the cell tissue by the ionic or covalent binding of cell wall components. POD is able to cross-link cell wall glycoproteins like extensin as well as ferulic acid in presence of H_2O_2 leading to the deformation of the cell wall without breaking while PME is able to catalyze the de-esterification of pectic compounds in the cell wall.

Earlier research deduced that cross-linking was not a property of plant POD in general but involved specific enzymes. Thus, the texture could be controlled by modifying the action of enzymes like POD. Genetic engineering of POD in plant could lead to changes in texture. Depending on the goals (persistence of firmness during processing, softening during ripening etc.), characteristics of POD could be exploited.

The purpose of this study was to purify the thermostable isoPOD of potato sprouts and to isolate its partial cDNA clone.

Summary of achievements:
In relation to processing, thermostable aspects of endogenous PODS are of crucial importance. For potato processing, pre-heating treatments are applied to inactivate endogenous enzymes. As a start of a study on the molecular basis of POD thermostability, soluble and bound PODS were investigated using tubers and sprouts from potatoes *(S. tuberosum L)* of cv. Agria. Total POD was fractionated in soluble, ionically, and covalently bound POD. Activity measurements (using ABTS and H_2O_2 as substrates) and isoelectricfocusing (followed by activity staining) showed that the ionically bound POD fraction of potato sprouts was the most thermostable. It still contained POD activity (4%) after a heat treatment of 10 min at 90°C. Preheated ionically bound POD fractions (90°C) appeared to regain up to ca. 17% of enzyme activity. This thermostable fraction was established to be cationic with a pH of about 10. To identify this isoform, it will be further purified and sequenced N-terminally.

Keywords:
Peroxidases, pectins, transgenic, suberin, POD, potato, tomato, processed food

Main Publications/Patents/Participation in conferences:
C. Boucoiran, J.W. Kijne, K. Recourt (1997) Characterisation of thermostable potato peroxidases. Poster. Plant Molecular Cell Biology Symposium, Lunteren. The Netherlands, April 1.
C. Boucoiran, J.W. Kijne, K. Recourt (1997) Characterisation of thermostable potato peroxidases. 5th International symposium on "Plant Peroxidases: Peroxidase Biochemistry and Physiology". St Petersburg, Russia, June 1997.

Links with EC projects:
FAIR-CT98-5008 (p. 126)

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Category:	30
Starting date:	01/03/1996
Duration:	24 months
Contract number:	FAIR-CT96-5010

Involvement of the kinetic characteristics of lipoxygenase in the quality and processing of tomato

Objectives:
The oxidation of lipids is a process of major importance for food quality as it gives rise to different compounds involved in the flavour, taste and nutritional value of foods. Plant lipoxygenases (LOX) account for a substantial part of lipid oxidation in raw food sources. LOXs catalyse the oxidation of polyunsaturated fatty acids containing a cis, cis 1,4 pentadiene system to the corresponding hydroperoxides derivatives. These compounds can be transformed by enzymes such as hydroperoxide lyase in other products responsible for the flavour of vegetables.

The main objective of this project was the purification and detailed study of the kinetic characteristics of tomato lipoxygenase and the influence of its reaction products in the quality and processing of tomato. This objective was subdivided into several steps:

1. Purification and stabilisation of membrane bound and soluble tomato LOX;
2. Characterisation of the reaction products of both enzymes using different endogenous substrates;
3. Influence of different physico-chemical parameters such as pH, viscosity, water activity and water soluble cosolvents in the regio-specificity of this enzyme;
4. Kinetic study of both enzymes.

Summary of achievements:
During the first period of the project, tomato LOX was purified and its kinetic properties and the regio- and stereo- specificity of the enzyme studied using free fatty acids as substrates. To check whether the specificity of tomato LOX changed when the fatty acid was esterified, the oxidation of dilinoleoyl phosphatidylcholine by lipoxygenase was studied. Preliminary results showed a very low conversion rate of phosphatidylcholine with tomato LOX.

When soybean LOX-1 is incubated with DL-PC in the presence of 10 mM sodium deoxycholate, an increase in absorbance at 234 nm is observed. In addition, the increase in absorbance at 234 nm is more specific for lipoxygenase than the polarographic method and permits the detection of autoxidation products of dilinoleoyl phosphatidylcholine (DL-PC) before the enzymatic reaction starts. The difference spectra of this reaction shows a lack of absorbance in the 270-280 nm region, thus indicating that, under experimental conditions, no oxodienes are formed. This reaction proceeds till the depletion of the substrate.

Contrary to the results of other authors, the present results, when carried out using DL-PC and tomato LOX, indicate that lipoxgenase is unable to act on phospholipids.

The project has developed a method to purify to homogeneity soluble tomato-LOX without interference of other proteins - useful for further research. Their work with hydrogen peroxidase also clarifies its role as an inhibitor which will contribute to the interpretation of results obtained when H_2O_2 is used in LOX containing extracts.

Keywords:
Lipoxygenase, tomato, fatty acids, enzymes, kinetics

Main Publications/Patents/Participation in conferences:
M. Perez Gilabert, G.A. Veldink, J.F.G. Vliegenthart (1996) Protection by different agents against inactivation of lipoxygenase by *hydrogen peroxide*. Lipids, vol 31 (12), 1245-1250.
M. Perez Gilabert, G.A. Veldink, J.F.G. Vliegenthart (1998) Oxidation of dilinoleoyl phosphatidylcholine by lipoxygenase 1 from soybeans. Archives Biochem. Biophys.

Links with EC projects:
FAIR-CT98-5027 (p.257)

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Category:	30
Starting date:	01/04/1996
Duration:	6 months
Contract number:	FAIR-CT96-5018

Prediction and manipulation of the outgrowth potential of microorganisms in foods

Objectives:
The present study focused on the responses of foodborne bacteria to combined inactivation treatments and on the correlation of subpopulation distributions to outgrowth properties. To be able to interfere with survival strategies after processing and to assess the outgrowth potential, the resulting heterogenic responses at the physiological and structural level had to be analysed by a flow cytometry-based system. *Lactobacillus plantarum* served as a model organism, while the antimicrobial peptide nisin and mild heat acted as stress conditions. The fluorochromes under investigation were mainly PI, CFDA and CFSE. Cell analysis was carried out by fluorometry and flow cytometry (FCM). The latter allowed simultaneous assessment of several physiological and structural parameters on a single-cell basis.

Summary of achievements:
1. Survival of bacteria could be predicted by post-treatment subpopulation distributions and validated by fluorescence activated cell sorting.

2. Underlying mechanisms of (combinations of) preservation treatments could be determined. The results facilitated the selection of efficient treatment combinations and levels, e.g. FCM studies on sequential application of heat and nisin treatments revealed synergistic effects, which were due to amplification of nisin-induced membrane damage by heat.

3. CFSE allowed tracking of cell divisions as well as determination of lag times and internal pH after inactivation treatments.

4. FCM in conjunction with cell sorting revealed heterogeneities in lag times before and after processing even in pure cultures, indicating the importance of culture history for the susceptibility to inactivation steps.

Keywords:
Microorganisms, *Lactobacillus plantarum*, Survival, foodborne

Main Publications/Patents/Participation in conferences:
J.E. Ueckert, G. Nabs von-Caron, A.P. Bos, P.F. ter Steeg (1997) Flow cytometric analysis of *Lactobacillus plantarum* to determine lag times, cell divisions and injury. Letters in *Applied Microbiology*

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Category:	20
Starting date:	01/04/96
Duration:	24 months
Contract number:	FAIR-CT96-5019

Natural isotopic fractionation in fatty acids and triglycerides: an application to the authentication of vegetable oils

Objectives:
The distribution of naturally occurring. non-radioactive isotopes in natural substances is non-random. Such "isotopic fingerprints" can be used to show whether a product is natural or not and can often provide further information about the origin of a sample. Isotopic distribution patterns of deuterium ($^{2}H/^{1}H$) and carbon-13 ($^{13}C/^{12}C$) are assessed by determining the site-Specific Natural Isotopic Fractionation by Nuclear Magnetic Resonance (SNIF-NMR) and Isotope Ratio Mass Spectrometry (IRMS). These techniques have proved to be powerful tools in the authentication of food samples and in the detection of fraudulent practices.

In the present study, the technique has been applied to triglycerides (TGs), which are composed of glycerol esterified with fatty acids. The objective was to assess the optimal isotopic parameters for applying this methodology to the authentication of plant oils and animal fats. Adulteration of the former is reputedly common for expensive oils. In addition, glycerol from different sources has been added as it is an important commodity, too. In order to investigate the isotopic correlation and fractionation between food and triglycerides produced by animals, labeling experiments were carried out with chicken. As glycerol is produced via the glycolysis pathways in the living organism, the deuterium filiation mechanism and the isotopic effects during its production through fermentation with *Saccharomyces cerevisiae* were studied by isotope labelling technique. These studies are essential for understanding isotopic filiation processes and for interpretation of isotopic data obtained from glycerol samples.

Summary of achievements:
Thirty-three samples have been analysed and it has been established that oils from peanut, olive, maize and sunflower can be distinguished solely on the basis of their isotopic

composition. Analysis of the samples comprised: ^{2}H SNIF-NMR of derived glycerol (triacetate), ^{13}-IRMS of glycerol and of the related oil. A little more than one third of the samples were extra virgin olive oil examined in detail as this commodity as it has a long history of adulteration. Trangenic sunflower seed oil, which has a fatty acid composition similar to olive oil, was examined as this constitutes a potential fraud ingredient. This oil can readily be distinguished as of sunflower origin and it can easily be distinguished from virgin olive oil on the basis of its isotopic composition.

To achieve a higher precision in the ^{2}H NMR experiment, new solvents, derivates and new NMR parameters were tried. Measurements are now performed in acetonitrile instead of chloroform and at higher temperatures. The derivatisation procedure was modified and glycerol triacetate can now reproducibly be synthesised with high purity and yield, rendering additional gas chromatographic measurement unnecessary. For all the following measurements, the analytical error margin in the ^{2}H measurements could be reduced by a factor of two.

Keywords:
Isotopes, triglycerides, glycerol, vegetable, oil, animal, fat

Main Publications/Patents/Participation in conferences:
B.L. Zhang, S. Buddrus, M. Trierweiler, G.J. Martin (1998) Charactarisation of glycerol from different origin by $_{2}H$ and $_{13}C$ NMR studies of site-specific Natural Isotope Fractionation J. Agric. Food Chemistry, 46, 4, 1374-1380, 1998.
S. Buddrus, B.L. Zhang, M. Lees, F. Mabon, G.J. Martin (1996) Authentification de l'huile d'olive et des huiles végétales par leur isotopes. 2ème Forum de la Recherche, Sept. 1996, Nantes, France.
B.L. Zhang, V. Bordage, S. Buddrus, G.J. Martin (1996) Glycerol: A new probe compound for the characterisation of food origins by NMR. 3rd International Conference on Applications of Magnetic Resonance in Food Science, Sept 1996, Nantes, France.
S. Buddrus, B.L. Zhang, G.J. Martin (1997) Study of the Origin of Triglycerides and Glycerols from Diverse Sources by $_{2}H$-SNIF-NMR and $_{13}C$-IRMS, 4th European symposium on Food Authenticity, June 1997, La Baule, France
B.L. Zhang, M. Trierwieler, S. Buddrus, G.J. Martin (1997) SNIF-NMR applied to the determination of ($_{13}C/_{12}C$) of glycerol. 4th European symposium on Food Authenticity, June 1997, La Baule, France.
S. Buddrus, B.L. Zhang, G.J. Martin (1997) Study of the Origin of Triglycerides and Glycerols from Different Sources by $_{2}H$-SNIF-NMR and $_{13}C$-IRMS, 20th annual meeting of the Stabgle Isotope Working Group, Technical University of Munich, October 1997, Munich, Germany

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Category:	20
Contract signed:	31/01/1996
Duration:	24 months
Contract number:	FAIR-CT96-5020

Biotransformation of phospholipids

Objectives:
Lecithin sludge is a low value by product in vegetable oil production. It consists mainly of a mixture of different phospholipids. These phospholipids can be purified from the sludge and be converted enzymatically into high prize fine chemicals. The present research deals with the synthesis of phosphatidylcholine with a defined molecular structure with the help of hydrolytic enzymes (1-3 specific lipases and phospholipase Δ2).

Summary of achievements:
The work dealt mainly with the factors that determine the yield in the enzymatic modification reactions. Some of these factors are water activity, fatty acid concentration and the degree of acyl migration (an unwanted side reaction). The modification reactions in the sn-1 position (catalysed by a 1-3 specific lipase) turned out to be thermodynamically much more favourable compared to the modification reactions in the sn-2 position (catalyzed by phospholipase Δ2).

An HPLC method for the analysis of phosphatidylcholine, 1 -acyl lysophosphatidylcholine and 2-acyl lysophosphatidylcholine was developed. This method is very well suited to study enzymatic modification reactions on phosphatidylcholine. Moreover it is also suitable to study acyl migration in lysophosphatidylcholine. This subject has hardy been studied, mainly due to the fact, that expensive NMR equipment was required.

Most of the reactions studied have been carried out on synthetic phosphatidylcholine. The next aim is to utilise the knowledge obtained for the modification of phosphatidylcholine derived from lecithin sludge. The first results have already been promising.

The most promising applications of phosphatidylcholine with a defined molecular structure are (i) phosphatidylcholine containing polyunsaturated fatty acids (from fish oil) as an additive in functional food and (ii) dipalmitoyl phosphatidylcholine as a lung surfactant for prematurely born infants (iii) phosphatidylcholine with defined fatty acid composition for medical and physico-chemical research.

Keywords:
biotransformation, phospholipids, HPLC, Lecithin, phosphatidylcholine, lipase, enzyme

Main Publications/Patents/Participation in conferences:
D. Adlercreutz, E. Wehtje (Submitted) A simple HPLC method for the analysis of phosphatidylcholine and its partial hydrolysis products 1-acyl lysophosphatidylcholine and 2-acyl lysophosphatidylcholine. BBA.

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Category:	30
Contract signed:	31/01/1996
Duration:	24 months
Contract number:	FAIR-CT96-5024

Biotechnology for production of functional food ingredients. A new method for the enzymatic synthesis of sugar-based food emulsifiers

Objectives:
The main objective of this project was to develop a novel enzymatic method for the synthesis of new sugar based surfactants as an alternative to some-existing products such as ethoxylated sugar alcohol esters. It was further intended to exploit the well-known ability of sugars to form eutectic mixtures and supersaturated solutions as a medium for enzymatic reactions.

Summary of achievements:
In the course of this work, the feasibility of enzymatic transformations in supersaturated (supercooled) solutions of carbohydrate substrates was established. Several partial diagrams for ternary systems containing sugar/water and other plasticisers were also constructed to characterise the physical chemistry of the system and identify the regions which are most suitable for biotransformations. It was confirmed that the glass transition in this system occurred below the reaction temperature at any given composition of the constituent components. The synthetic utility of the system was demonstrated by the preparative enzymatic synthesis of several new ethoxylated glycoside sugar fatty esters. The enzymes used displayed excellent selectivity with no by-product detected in the reaction mixture.

Keywords:
Food, ingredients, enzyme, emulsifier, sugar, fatty acid, ester, supersaturated supercooled solution

Main Publications/Patents/Participation in conferences:
A. Millqvist-Fureby *et al.* (1998) Enzymatic synthesis of ethoxylated glycoside esters using glycosides in supersaturated solutions and lipases in organic solvents. Biotechnol. Bioeng. 59, 747-753.
A. Millqvist-Fureby *et al.* (1998) Enzymatic transformations in supersaturated solutions: part I. Biotechnol. Bioeng. 60, 190-196.
A. Millqvist-Fureby *et al.* (1998) Enzymatic transformations in supersaturated solutions: part II. Biotechnol. Bioeng. 60, 197-203.

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Category:	20
Starting date:	01/02/1996
Duration:	6 months
Contract number:	FAIR-CT96-5026

Characterisation of molecular mobility in low moisture systems to understand modifications during storage in relation to the glass transition

Objectives:
This study proposed to elucidate the molecular mobility mechanisms related to the glass-rubber transition in model systems consisting of sucrose-water mixtures and in systems containing biomacromolecules, i.e. Dextran (MW 40000) in order to control the evolution in food products at low water content or in the frozen state during storage.

Summary of achievements:
For this purpose, spin probe electron spin resonance (ESR) spectroscopy was applied. A very broad range of rotational correlation time τc, was covered: from 10^{-11}s to 10^{-7}s with conventional ESR and up to 10^{-3}s with saturation transfer ESR (ST-ESR).

The mobility of two spin probes, Tempol and 4-Maleimido-Tempo, was studied to examine the influence of the size of diffusing molecules on the transport process in the vicinity of the glass transition temperature (Tg). The spin probe behaviour in pure glycerol was used as a reference to calculate the rotation correlation time as a function of the spectral line shape because the viscosity of glycerol is well-known over a wide temperature range. This type of calibration can be applied for Tempol spin probe. However, it was not applicable for the 4-Maleimido-Tempo spin probe, because this spin probe does not have the same motional behaviour in the glycerol reference system, as compared to the carbohydrate-water samples. Nevertheless, a comparison of the spectra of the two probes provides information about their relative mobility.

Two concentrated sucrose-water systems were studied: one freeze concentrated and the other with an initial low water content.

Below the glass transition temperature of the maximally freeze concentrated phase (Tg_{DSC}'), the results for the frozen systems were not repeatable due to the difficulty to form the ice. Nonetheless the position of Tb was well indicated by a change of slope in the decrease of rotational correlation time as the temperature increased. For the system without ice at low temperatures, the position of Tg_{DSC} was not so easy to define because the mobility was very low and out of the limit of the ST-ESR method.

Ageing experiments were carried out in concentrated sucrose systems (without ice) over a wide range of time from ten hours to around two months at 5°C below Tg_{DSC}. After ten hours, no change of rotational correlation times was observed; after two months, a slight decrease in mobility could be found but the difference was so small that it was difficult to make conclusions in term of relaxation process.

In samples with Dextran (Mw 40000), the shape of ST-ESR spectra was quite different from those from the reference sets making it impossible to quantify the rotational correlation time. A qualitative comparison of the evolution of the spectra suggested that the mobility of the small molecule was less influenced by temperature in a polymeric system.

This study shows some of the difficulties to obtain quantitative values of molecular motion in the vicinity of the glass transition of model food samples. However, ESR and ST-ESR still enable the behaviour of small molecules in a qualitative way to be studied.

Keywords:
molecular mobility, low moisture, storage, glass transition, electron spin resonance

Main Publications/Patents/Participation in conferences:
Amorphous Polyhydroxy compounds: a discussion conference, 25-27 September 1996. Cambridge, United Kingdom.
Biological spectroscopy. 22-25 April 1996. Wageningen, The Netherlands

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Category:	30
Starting date:	01/04/1996
Duration:	24 months
Contract number:	FAIR-CT96-5030

Substrate recognition properties of the oligopeptide transport system of *Lactococcus lactis*

Objectives:
The breakdown of milk proteins by lactic acid bacteria is one of the major biotechnological processes under worldwide investigation. Lactic acid bacteria require multiple amino acids and are weakly proteolytic. For degradation of proteins (caseins), the lactococci bacteria possess a cell wall-bound proteinase. Some of the peptides formed by the action of the proteinase are transported to the inside of the cell via the oligopeptide transport system (Opp). Once inside the cells, these peptides are degraded further to free amino acids by the action of several peptidases.

The analysis of intracellular and extracellular amino acid and peptide pools of cells grown on β-casein has revealed that all essential and growth-stimulating amino acids are taken up via Opp in the form of peptides that range in size from five to ten residues. Remarkably, amino acids did not accumulate inside the cell when the Opp system was inactivated, indicating that this is the only system that transports casein breakdown products into the cell.

The aim of this project was to analyse the substrate recognition properties of the oligopeptide transport system of *Lactococcus lactis*. Two food-grade Δ*opp*A strains were constructed, using the wild type strain MG1363 and the four-fold peptidase mutant IM15 as parent. These strains no longer express the peptide binding protein, but still carry the genes for the integral membrane components OppD, OppF OppB and OppC. They were used to achieve the expression of wild type and mutant OppA proteins independent of the membrane components. For that purpose, the *opp*A gene was cloned in plasmids under the control of the constitutive P32 promoter. A 6-His-tag was engineered at the carboxyl-terminus to facilitate purification of the protein.

Summary of achievements:
On the basis of the 3-D structure of the OppA protein of *S. typhimurium* and the alignment of the primary sequence of homologous OppA, a number of putative active-site mutants were constructed by site-directed mutagenesis. This work has led to at least one mutant with enhanced affinity for peptides, as shown in transport assay studies *in vivo*. It provides the first clues towards the nature of the peptide binding pocket in OppA.

The wild type and mutant OppA proteins were purified from solubilised membrane vesicles by metal affinity chromatography and the binding of several peptides was followed by fluorescence measurements. These measurements should provide indications as to whether the uptake of particular peptides is limited by the binding to OppA or translocation via the integral membrane components of Opp.

The genes encoding the membrane components of the oligopeptide transport system (two ATPase domains and two integral membrane domains) were cloned under the control of the inducible nisin promoter of *L. lactis* and a 6-His-tag was engineered to the carboxyl-terminus of OppC. Amplified expression of at least two of the components (OppD and OppC) was confirmed by immunoblotting. This construct is to be used to isolate and purify the membrane components, which will enable us to study the transport of peptides without interference from other cellular processes.

Keywords:
oligopeptide transport system, substrate, membrane, amino acid, peptide, *Lactococcus*

Main Publications/Patents/Participation in conferences:
V Lactic Bacteria Meeting (Veldhoven, The Netherlands) September 1996
25th International Dairy Congress (Aarhus, Denmark) September 1998

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Category:	30
Starting date:	01/04/1996
Duration:	12 months
Contract number:	FAIR-CT96-5032

Effect of starter cultures on biogenic amines, N-Nitrosoamines and lipid peroxides formation in meat products

Objectives:
During the ripening of fermented sausages, biogenic amines can be formed by the action of bacterial decarboxylases of microorganisms originating from raw materials. Tyramine, histamine and phenylethylamine have vasoactive properties and high concentrations of these amines in these sausages can be a potential risk for the most sensitive of consumers. The existence of those amines, together with e.g. putrescine and cadaverine is always a sign of either a poor quality raw material or contamination during processing or storage. Starter cultures may inhibit the growth of either proteolytic or decarboxylating microorganisms

This study had four main aims:
a) to develop, or validate a number of chemical methods, including: biogenic amines; nitrosamines, liquid chromatography-mass spectrometric method; malonaldehyde, spectrophotometric and liquid chromatography method; free aldehydes, liquid chromatography method. The validation of methods included evaluation of the following parameters: detection and determination limits, reproducibility, recovery of fortified samples, linearity and working areas;
b) to determine the biogenic amine levels of Finnish type dry sausages and to compare these results to other surveys from different types of fermented sausages;
c) to determine the effect of starter cultures on the formation of biogenic amines;
d) to determine the effect of slicing and vacuum packaging on the formation of biogenic amines and lipid peroxidation products.

Summary of achievements:
The use of starter culture, although reducing the growing of contaminant microorganisms and the formation of some biogenic amines on the raw materials, is not enough to prevent the formation of histamine and tyramine. Good manufacture practices and good quality raw materials, free of amine-positive must be employed. Control of the presence of these LAB

should be systematically applied to the raw materials in the meat industries. The study also demonstrated that contamination during the slicing and packaging may increase the formation of biogenic amines.

Keywords:
starter cultures, biogenic amines, n-nitrosoamines, lipid peroxide, meat, products

Main Publications/Patents/Participation in conferences:
S. Eerola, A.X. Roig-Sagues (1996) The influence of a starter culture on the amine formation capability of an amine-positive lactobacillus strain. In: Proceedings of the 42nd International Congress of Meat Science and Technology, Lillehammer (Norway) 1-6 September, pp.550-551
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S. Eerola, A.X. Roig-Sagues, T. Hirvi (Accepted) Biogenic amines in Finnish dry sausages. Journal of Science Agriculture and Food Chemistry
S. Eerola, A.X. Roig-Sagues, L. Lilleberg, H. Aalto (Accepted) Biogenic amines in dry sausages during shelf-life storage. Zeitschrift fur Lebensmittel-Untersuchung and -Forschung
42nd International Congress of Meat Science and Technology, Lillhammer, Norway, 1-6 September, 1996.

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Category:	30
Starting date:	01/10/1996
Duration:	18 months
Contract number:	FAIR-CT96-5035

Multi-element and multi-compound stable isotope distribution patterns of Spanish wines and musts for authenticity characterisation and origin assignment

Objectives:
Over the last few years isotopic distribution methods based on isotope ratio mass spectrometry (IRMS) and deuterium magnetic resonance (2H NMR) have been successfully developed and have been shown to be a powerful tool for natural origin recognition and authentication studies of wines, and for the detection of wine adulteration. However, the knowledge of the isotopic characteristics of European wines and, particularly Spanish wines, is still underdeveloped. Moreover, efforts to improve the current isotopic methodologies for the assessment of wine genuineness, for the assignment of the geographical origin and for the detection of eventual adulterations are being encouraged by the EU Commission. At present, the most innovative approaches to the question of authenticity and origin evaluation of European wines are being developed at the Lehrstuhl fur Allgemeine Chemie und Biochemie of the Technische Universitat München by Prof. Dr. H.-L. Schmidt's group on the basis of multielement and multicompound stable isotope abundance investigations. Accordingly, the object of the proposed research project is the application of the IRMS methods developed at the TU München for the isotopic characterisation of Spanish wines from four relevant wine-producing areas (Rioja, Catalonia, Castilla-La Mancha and Valencia).

Subfractions and single natural products will be isolated from Spanish wines (sugars, amino acids, organic acids, glycerol). Stable carbon and nitrogen isotope ratio determination will be performed by IRMS. Multielement and multicompound isotopic patterns will be statistically evaluated, and inter- and intramolecular isotopic correlations will be looked for which may tackle the question of authenticity and origin Characterisation, and which may provide a more reliable proof for the detection of adulterations.

Thus, comparison of results between the four European most relevant winegrowing countries will be able.

Summary of achievements:
An in-depth characterisation study of Spanish wines has been carried out by application of innovative stable isotope methodologies of food authentication based on isotope ratio mass spectrometry (^{13}C- and ^{15}N-IRMS) and deuterium nuclear magnetic resonance (^{2}H-NMR). The feasibility of the multi-element and multi-compound stable isotope analysis for the assessment of authenticity and for the geographical origin assignment of Spanish wines has been demonstrated. Multi-compound intermolecular isotopic correlations between defined grape must and wine fractions and/or single compounds have been shown to provide an improved proof for the detection of eventual adulterations and for the quality certification of Spanish wines in regard to regional origin.

The project has also added to previous results from the Technische Universität München for the stable isotope investigation of European wines, allowing for the comparison of results and for the estimation of the environmental (climatic) effects on the interregional variation of the stable isotope parameters over a number of relevant wine-growing areas. In this respect, results arising out of this research have in particular contributed to updating the knowledge of the multi-element stable isotope characteristics of Spanish wines, which were until now underdeveloped.

Keywords:
Stable hydrogen, carbon, nitrogen, isotope ratio, mass spectrometry, IRMS, wine, authentication, multi-element, multi-compound, origin assignment

Main Publications/Patents/Participation in conferences:
J.E. Giménez-Miralles, A. Roßmann, H.-L. Schmidt (1997) Regional origin assessment of Spanish wines by multielement and multicompound stable isotope ratio analysis. Presentation at 4th European symposium on food authenticity and FAIM Euroconference, La Baule (France), June 4-6, 1997.
J.E. Giménez-Miralles, A. Roßmann, H.-L. Schmidt (1997) Intermolecular stable carbon isotope ratio correlations in the authenticity assessment of Spanish D.O. Rioja and D.O. Penèdes wines. Presentation at 20. Jahrestasgung der Arbeitsgemeinschaft Stabile Isotope, Freising (Germany), October 6-8, 1997.

Links with EC projects:
AIR3-ST92-005
FAIR-CT98-5068 (p.195)

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Category:	20
Contract signed:	12/07/1996
Duration:	12 months
Contract number:	FAIR-CT96-5037

The effects of processing on saponins in legumes of importance to Spain. A preliminary study into the relationship between saponin structure and rat gut permeabilising activity

Objectives:
Saponins are a complex and chemically diverse group of compounds. Among the naturally-occurring compounds of grain legumes, saponins are attracting considerable interest as a result of their diverse properties, both deleterious and beneficial. Fungitoxic, haemolytic, and membranolytic activities have been ascribed to saponins. Conversely, a beneficial lowering of plasma cholesterol levels in humans has also been attributed to saponins whilst some have been reported to exhibit anticancer activity and inhibitory effect on the infectivity of HIV *in vitro*. However, all these behavioral properties are related to certain saponin structures rather than to all members of this family.

Legumes, normally used in human nutrition, need to be processed prior to consumption to reduce their levels of antinutritional factors. Soaking, cooking and germination are the most traditional domestic methods of legume processing.

A method for the quantitative determination of intact saponins in lupin seed by high performance liquid chromatography (HPLC) which enables quantitative studies to be made on the effect of processing has been developed, which, in turn, allows the fate of individual saponins to be determined. Since some of the biological properties reported for saponins are now recognised to be dependent on their particular chemical structures, this type of information allows further understanding of not only the relationship between chemical structure and bioactivity but also the impact of processing on that bioactivity in relation to both human and animal health. Therefore the objectives of the project were to study:

- the effect of soaking using distilled water, citric acid and bicarbonate solutions,
- the effect of cooking, after the seeds were presoaked in distilled water,

- the effect of germination in the dark on both the saponin content and the composition of two cultivars of both chickpeas and lentils.
- the ability of extracts from both raw and processed chickpeas and lentils with known levels of different saponins to depolarise and permeabilise *in vitro* the small intestinal mucosal epithelium.

Summary of achievements:
Neither soaking, regardless of the pH of the soaking solution, nor germination modified the saponin content or composition of chickpeas and lentils.

The native saponin, soyasaponin VI was partially degraded during cooking into soyasaponin I, and both of these saponins leached into the cooking solution. An overall loss of saponin content was found for lentil, but none was observed for chickpea. This may be attributed to the fact that in the case of lentils the seed structure was highly disrupted, releasing more saponins into the cooking solution. The saponins might then be more liable to suffer damage without the protective environment supplied by the seed. This investigation has proved that the important physiological properties ascribed to DDMP saponins in relation to the prevention of biomolecular damage due to free radical attack are dependent on the type of processing used on these legumes.

On the other hand, one of the better-known biological effects of saponins is their capacity to permeabilise the small intestinal mucosal epithelium and there is a clear relationship between the stereochemistry of saponins and their permeabilising activity. An *in vitro* bioassay, which measures changes in the Na^+-glucose-dependent transmittal potential across an everted segment of rat proximal jejunum and in the permeability of the gut wall has been used in a preliminary study to assay the ability of extracts from both raw and cooked chickpeas and lentils with known levels of different saponins to influence gut transport. However, this preliminary assay resulted to be unsuccessful probably due to the fact that a further purification of the legumes extracts, eliminating the high concentration of sugars, is required in order to achieve depolarisation of the small intestinal mucosal epithelium, which is an indicator of increased permeability.

Keywords:
Saponins, soyasaponin, 2,3-dihydro-2,5-dihydroxy-6-methyl-4H-pyran-4-one, DDMP, high performance liquid chromatography, HPLC

Main Publications/Patents/Participation in conferences:
R.G. Ruiz, K.R. Price, A.E. Arthur, M.E. Rose, M.J.C. Rhodes, R.G. Fenwick (1996) Effect of soaking and cooking on the saponin content and composition of chickpeas (*Cicer arietinum*) and lentils (*Lens culinaris*). J. Agric. Food Chem. 44: 1526-1530.
R.G. Ruiz, K.R. Price, M.E. Rose, M.J.C. Rhodes, R.G. Fenwick (1996) A preliminary study on the effect of germination on saponin content and composition of lentils and chickpeas. Z. Lebensm. Unters. Forsch. 203: 366-369.

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Category:	20
Starting date:	01/07/1996
Duration:	24
Contract number:	FAIR-96-5040

Role of γ-glutamyltransferase in mushroom discolouration

Objectives:
Brown discolouration of mushrooms (*Agaricus bisporus*), is an important quality aspect as it leads to a decreased consumer appreciation. Discolouration is the result of the action of tyrosinases on phenolic substrates endogenously present in mushrooms. The most important substrates for discolouration in *A. bisporus* are γ-glutaminyl-4-hydroxybenzene (GHB) and its oxidative products. The aim of this project is to provide information about the enzyme directly responsible for GHB synthesis, γ-glutamyl transferase (GGT). GGT occurs in other fungi but also in plants, bacteria and mammalians where it has been extensively studied because of its role in the glutathion metabolism. In fungi and plants GGT catalyses the synthesis of secondary metabolites involved in many kinds of process such as ripening (in tomato fruits) or synthesis of flavour related compounds (in Shiitake mushrooms or onions). In *A. bisporus* GGT is almost unknown and, to their knowledge, the last study carried out on the enzyme was in 1964. The first part of the work consists in the isolation of the *A. bisporus* GGT gene. The second part includes the study of the distribution of GGT activity within mushroom tissues during ageing and in the purification of the enzyme and determination of its kinetic parameters.

Summary of achievements:
Many kinds of γ-glutamyl transferase (GGT) have been isolated from mammalians, bacteria, plants and fungus. Based on the homology of sequence between these GGT, degenerated primers were designed in order to isolate the coding sequence of GGT in *Agaricus bisporus*. The PCR products amplified were cloned in *E. coli* and sequenced. Unfortunately none of the amplified sequences did present enough homology with the ones from the literature to confirm their belonging to the mushroom GGT gene. Therefore, another approach was investigated consisted in first purifying the *A. bisporus* enzyme and then digesting it with endonucleases or chemicals. The N-terminal amino acid sequence of the digested peptides

will be determined and used to design new primers. These primers will be used for PCR amplification. The *A. bisporus* GGT is still being purified.

In the meanwhile spatial distribution of *A. bisporus* GGT was studied in fruiting bodies from stage 1 to 7. Glutamyl-*p*-nitroaniline was used as substrate. In the presence of GGT glutamyl-*p*-nitroaniline was hydrolyzed to form *p*-nitroaniline measured at 410nm. GGT activity was mainly present in gills and skin. It increased in the gills with mushroom development; rather low at stage 2 (~30 U/mg DM), the highest activity was found at stage 7 (~400 U/mg DM). In the skin the activity decreased with mushroom maturation. The highest values were measured in young stages (~200 U/mg DM) in stages 2-3; and they decreased slightly until stage 7. Cap, stipe base and stipe were almost devoid of GGT especially at young stages. The activity increased slightly with development. Space-time distribution of GGT activity was very similar to the one of glutamate/glutamine and GHB content. These results confirmed the role of the enzyme in the biosynthesis of GHB occurring during sporulation.

Keywords:
discolouration, tyrosinases, phenolic, bisporus are γ-glutaminyl-4-hydroxybenzene, GHB, γ-glutamyl transferase, GGT

Main Publications/Patents/Participation in conferences:
S. Jolivet, H. Mooibroek, H. Wichers (1998) Space-time distribution of γ-glutamyl transferase in *Agaricus bisporus FEMS MicrobiologyLetters 163,* 263-267.
J.C. Espin, S. Jolivet, H. Wichers (1998) Inhibition of Mushroom polyphenol oxidase by agartine. Journal of Agricultural and Food Chemistry, 46 (8), 1998, 2976-2980.
J.C. Espin, S. Jolivet, A. Overeem, H. Wichers (Accepted) Agaritine from Agaricus bisporus is capable of preventing melanin formation. Phytochemistry.

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Category:	20
Contract signed:	12/07/96
Duration:	12 months
Contract number:	FAIR-CT96-5042

Research of antioxidants in citrus by-products

Objectives:
The objective of this project was to measure the antioxidant activity of the methanol extracts of some other citrus by-products (mandarin, sweet orange, pummelo and lime seeds) and of some of the hydrolysed extracts (thus containing the bound phenolics) and to identify their main phenolic constituents. The project endeavoured to analyse the relationship between the antioxidant power and the composition of the extracts, and the synergistic and antagonistic effects that occur in the extracts between the different components.

All the results concerning the antioxidant activity measurement had to be compared with those found in another model-system used in the laboratory, the DPPH test.

Summary of achievements:
During an earlier contract an accelerated test based on citronellal oxidation was developed in order to measure the antioxidant activity of phenolic compounds. The extraction of free and bound phenolic compounds from citrus by-products (peels and seeds) was also worked out.

In this part of the research programme, using the citronellal test, the structure-activity relationships of several phenolic compounds (flavanones, flavonols, flavones, phenolic acids) was studied.

The antioxidant and antiradicalar power of 11 citrus extracts containing the free phenolic compounds and of four extracts containing the bound phenolics was then measured. In the first case, the most active samples were found to be mandarin and sweet orange seeds and sour orange peel; in the second one, sour orange peel.

The composition of the extracts was studied by HPLC and the main phenolic compounds (flavanones and phenolic acids) were quantified.

Finally, the antioxidant activity of every extract was compared with its composition. It was impossible to define a clear relationship between the two data, especially in the case of seeds. Flavanones and phenolic acids explain only a small part of the efficiency of the extracts. Other compounds must then be responsible for most of the activity.

Keywords:
antioxidant, citronella, phenolic, glycosylated flavonoids, flavanones

Main Publications/Patents/Participation in conferences:
A. Bocco, M.E. Cuvelier, H. Richard, C. Berezet The antioxidant activity of various phenolic compounds measured by an citronellal oxidation. Sciences de Aliments

Links with EC projects:
AIR1-CT94-8860

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Category:	40
Starting date:	06/07/1996
Duration:	12
Contract number:	FAIR-96-5044

Reduction of the urea content of wines by engineering of a *Saccharomyces cerevisiae* strain secreting an acid urease from *Lactobacillus fermentum*

Objectives:
Urea is reported to be the main precursor of ethyl carbamate, which is suspected to be a carcinogen, in fermented beverages and foods. In order to minimize or eliminate production of urea in wine, a *Saccharomyces cerevisiae* strain secreting acid urease from *Lactobacillus fermentum* will be constructed. The urease genes will be cloned on yeast expression plasmids containing different promoters (PGK, ADH I,...) and signal peptides for secretion (alpha factor, invertase). The urease genes will be expressed first in a model strain. The efficiency of the system will also be assess in industrial strains and natural musts after transformation with constructions containing a positive marker. The effect of urease secretion on the final urea content of wine will be evaluated.

Summary of achievements:
Urea was reported to be the main precursor of ethyl carbamate, which is suspected to be a carcinogen, in fermented beverages and foods. In order to minimise or eliminate production of urea in wine, the *Saccharomyces cerevisiae* DUR1,2 gene was cloned, encoding the ATP-urea amidolyase which performs its degradation in ammonia and $C0_2$. The DUR1,2 gene was PCR amplified and cloned into the yeast expression vector pvT100-U. The effect of DUR1,2 overexpression was been assessed in an enological derived haploid tester strain of *Saccharomyces cerevisiae* and propagated in enological fermentations conditions. It was observed that cells overexpressing the DUR1,2 gene from the yeast alcohol dehydrogenase (ADH 1) promoter on multicopy plasmids notably decreased the urea content in fermented medium. The decrease in urea excretion was correlated with an increase in ATP-urea amidolyase activity mainly in the last part of the fermentation. The efficiency of the urea reduction was assessed with varying concentrations in arginine, main precursor of urea.

In order to assess the effect of DUR1,2 overexpression in an enological strain which produces high levels of urea the DUR1,2 was overexpressed in the UCD522 strain. The strain was transformed with the plasmid YEP-DUR1,2 containing the DUR1,2 gene and the 6418 selection system. Fermentations were carried out in synthetic must (MS 400 containing 0.372 g/l arginine). In such conditions the 522/YEP-DUR1,2 strain displayed higher $C0_2$ production rate and a slightly shorter fermentation time while the total biomass formed was lower than in the control strain. The urea content in the fermented medium after 161 hours was reduced to about 10% of the urea content of the control strain.

The interesting results obtained here may be of a great interest for the enology or other industry of fermented beverages, in order to reduce the ethyl carbamate. After some complementary experiments this approach will be patented. From another point of view, it is important to mention that this project is one of the rare applications of genetic engineering to enological yeast, which is motivated by a healthy objective. Such engineered strain could be of paramount importance for the consumer acceptance of genetically engineered yeasts in fermented beverages.

Keywords:
Urea, ethyl carbamate, ATP-urea amidolyase, *Saccharomyces cerevisiae* DUR1,2, enological, fermentations

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Category:	20
Starting date:	01/10/1996
Duration:	36 months
Contract number:	FAIR-CT96-5045

Etherification and esterification of polyols over solid catalysts. Example of glycerol-uses for detergents

Objectives:
Glycerol is mainly a natural product issued from the methanolysis of vegetable oils. In Europe, due to the increasing use of methyl esters as fuel additives, one can expect an increase of glycerol production, which could become a cheaper raw material for chemistry. For example, polyglycerols and specially polyglycerols-esters (PGEs) are gaining prominence in new products for tensioactive, lubricants, cosmetics, foods additives etc. Indeed PGEs exhibit multifunctional properties and a wide range of formulating options, if it is possible to control: i) the length of the polyglycerols chain, ii) the degree of esterification and iii) the fatty acid molecular weight. These reactions are quite interesting goals for shape-selective catalytic processes.

Previous work has shown that the selectivity of the first step is not really controlled and that a mixture of di- to hexa-glycerol (linear or cyclic) is obtained. It is difficult to get a well-defined product and to predict the Hydrophilic Lipophilic Balance (HLB) after esterification. In the laboratory it was shown that the esterification of glycerol could be selective to a monoglycerides over cationic resins. Nevertheless polyglycerols and polyglycerol esters as well as acroleine were obtained as main by-products. Moreover it was observed that the modification of the pseudo-pore size of these materials improved the selectivity to (PG + PGEs) but acroleine is always obtained over these acid catalysts. In order to obtain a selective formation of di-, tri-, tetra- or poly-glycerols, the selective etherification of glycerol over solid bases were investigated and compared with the results to those obtained with Na_2C0_3 catalysts.

The main objective of this work was to prepare selectively diglycerol or a mixture of (di-+tri-) glycerol by direct etherification of glycerol without formation of acroleine and without the use of solvent. In order to achieve this goal, new mesoporous basic solids were prepared and modified.

Summary of achievements:
The main part of this study consisted in the synthesis and the modification, by different techniques, of mesoporous solids in order to make them active, selective and stable for the target reaction. The catalytic results obtained show that the impregnation method gives the most important activity, which must be correlated to a more important active species incorporation. The grafting method also produces solids with relatively high activity regarding to the low number of active sites created.

Concerning the selectivity of the modified mesoporous catalysts, the best value to (di+ tri-) glycerols are obtained over solids prepared by the impregnating or the grafting methods. The re-use of the caesium impregnated catalysts does not affect the selectivity to the (di- + tri-) glycerol fraction. In the presence of lanthanum or magnesium containing catalysts, the glycerol dehydration to acrolein is very important whereas this unwanted product is not formed when caesium is used as promoter. Moreover, compared to homogeneous catalysts, the mesoporous solids could also induce a different chemioselectivity and the formation of 2-(1,2-dihydroxypropoxy)-propane-1,3-diol.

Finally, as far as the catalyst leaching and stability is concerned, the most demonstrative results are obtained with the grafted solids which keep their structure and their specific area after the promoter addition or incorporation. Such property is not observed over impregnated catalysts.

Keywords:
etherification, esterification, glycerol, polyglycerols-esters, PGEs, Hydrophilic Lipophilic Balance, HLB, acroleine, mesoporous, impregnation

Main Publications/Patents/Participation in conferences:
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J. Barrault, Y. Poilloux, C. Vanhove, S. Cottin, S. Abro, J-M. Clacens (1998) Selective esterification and selective etherification of glycerol over solid catalysts. Catalysis in organic reactions Ed. F.E. Herkes 13-24
J. Barrault, Y. Poilloux, J-M. Clacens (1998) Réaction d'etherification du glycérol en présence de catalyseurs solides mésoporeux. Enveloppe Soleau no. 8566, 03/03/1998
J-M. Clacens, Y. Poilloux, J. Barrault, C. (1999) Procédé catalytique de production de polglycérols à partir de glycérol sur catalyseurs solides mésoporeux modifiés. Patent November 1999

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Category:	30
Contract signed:	31/07/1996
Duration:	24 months
Contract number:	FAIR-CT96-5046

A comparison of new product development and new product introduction strategies for branded and non-branded nutritious products

Objectives:
Greece and the UK, although both members of the unified European market, are two extremes in the area of food markets. The food industry is large in the UK, medium to small in Greece. There are high percentages of own label products in the UK, whilst own label products in Greece are almost unknown. What will happen in the future? Will the Greek supply chain (farmers, manufacturers, distributors, retailers) for food products follow the British pathway? What will be the implications for each participant in the chain? Some of those questions are going to be answered through this research opportunity.

The present study is divided into two main chapters. Firstly the environment from the perspective of the consumer, retailer and food industry is examined. This environment is one where new food products will be introduced by manufacturers or retailers in both branded and own label form. Case studies, from both the UK and Greece, illustrate the marketing strategies followed in order to introduce new products.

Summary of achievements:
The question asked at the beginning is how the Greek retail shopping industry will evolve. Presently, the retail chain stores are some of the fastest growing businesses in the Greek economy, especially the larger chains.

When one looks at the history of the UK supermarkets, isolated shops in villages was the norm, with individual grocers and butchers, much like the Greek market used to be. However this rapidly changed, spurred by the revolution in the transport industry, and so lessening the problems of distance.

In Greece, the change was slower, but as the continent becomes more international, so the supermarket structures will slowly become more homogeneous. The first structures of supermarket chains are already taking place, each with around 10 locations added to which, small traditional food stores (pantopolia) are either transferring their business into small supermarkets (minimarkets) or closing down. Already, the Greek retailing market is highly concentrated. With the top 14 retailers own 52% of the market share.

A critical factor for concentration to arise is through acquisitions or horizontal alliances in the food retailer market, and the structure of vertical alliances between different players in the food chain. With the present generation growing up in a more international environment, will tradition fall by the wayside? Many of these alliances are built on trust and a sharing of knowledge, which the Greeks, traditionally up-to-now, have not been happy to share.

Another critical factor for success is own labeling. Retailers are now more interested in selling their own labels rather than the brands. Greek consumers are brand loyal. Most of the own label products in Greece are at the stage of imitation of well-known branded products, and their main characteristic is low price. Another characteristic is the fact that instead of the retailer's brand, the name of manufacturer is sometimes written on the package of the product. However, it is true that own labels give strength to the big players that have the advantage of intensive advertising, and are causing problems to the small and medium suppliers. Will own labels in Greece gain strength? And will consumers be able to attach quality to own label products. Marks and Spencer have already been a great success in Greece, the epitome of own label product quality. Surely this is a sign that the multinational retailers, together with their own label products will succeed?

Keywords:
new product development, introduction strategy, branded, non-branded, food, supermarkets, own label, Greece, UK, retail

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Category:	20
Starting date:	01/08/1996
Duration:	12 months
Contract number:	FAIR-CT96-5051

A theoretical and experimental study of the flow properties of food slurries in pipes and manifolds

Objectives:
Many food processes involve the conveying of solid-liquid mixtures. The particles can be large (of diameter similar to that of the process equipment), fragile and the carrier fluid of complex and non-Newtonian viscosity. The technical problems in handling this kind of material are significant: the process equipment is commonly over designed. For example, in order to ensure that particles have been processed it is common to deliver a significant overcook in terms of time and temperature. It is also unclear how to design ways of processing to ensure that particulate integrity is maintained through processes, and how particles flow through process plant. The latter problem is both technically complex and of industrial importance - the dynamics of two-phase flows are a subject of considerable academic interest, whilst industry is concerned with the design of flow equipment which would permit whole particles to be processed and conveyed.

The purpose of this project was to study both theoretically and experimentally the flow of particulate fluids through an impacting T-junction. The objectives were:

- to carry out a systematic study of how particle-liquid food mixtures behave in flow;
- to develop an experimental system which could be used to study food flows and identify particulate behaviour;
- to develop a computational model which could be used to predict particle trajectories in flow.

Summary of achievements:
The first section of the work involved the construction of a rig which could be used to quantify the flow of food mixtures. A simple T-junction geometry was chosen as representative of real equipment whilst being simple enough to study in academic detail. The rig was built from perspex to allow video recordings of particle trajectories to be made:

particle flows could be quantified as a function of overall flow rate, flow in the two side arms of the T -junction, particle position and loading and fluid and particle properties.

Preliminary experimental work was necessary to establish that food flows followed the principles established in experimental work on other systems. Little published work exists in this area. Although there is an extensive literature on two-phase flows in T -junctions, most is for air-water or coal/sand-water flows, where the density ratio between the phases is much higher than in this case.

The Single Particle Tracking experimental work was accomplished, and the respective numerical modelling experiments finalised. Experimental data was analysed and particle trajectories and velocity profiles were obtained for different flow conditions, as aimed. This set of experiments was a very innovative piece of work which has never done before. Simulated data were compared with the experimental data and a very good agreement was found to exist.

Keywords:
food, flow, particle trajectories, T-junctions

Main Publications/Patents/Participation in conferences:
S.A. Silva, P.J. Fryer, L. Zhang, L. Barratt (1997) Models and measurements of particles flowing in a T-junction. Proc. Seventh Internat. Congr. on Eng. and Food, April 1997, Brighton, UK.

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Category:	30
Starting date:	20/11/1996
Duration:	12 months
Contract number:	FAIR-CT96-5056

A multi-disciplinary approach to modelling the biochemical reaction pathways in tea fermentation systems

Objectives:
The main objectives of this project were to use a predictive computer model to investigate the key reaction pathways of tea fermentation and to study the interactions between heat transfer and biochemical reactions during tea fermentation.

Summary of achievements:
Fermentation is a key process of black tea manufacture and is crucial to the development of colour and taste of black tea infusion. The colour and flavour changes during tea fermentation are due mainly to the oxidation of flavonoids into theaflavins (dimers) and thearubigins (polymers). The biochemical reactions during tea fermentation are exceedingly complex and involve many compounds and pathways.

Within the fellowship, a predictive computer model recently developed at Colworth has been applied to investigate the key reaction pathways of tea fermentation. The interactions between heat transfer and biochemical reactions during tea fermentation have also been studied.

It has been found that heat has been generated during the early stage of tea fermentation, resulting in a temperature rise in the packed bed. Effect of airflow and depth of packed bed on temperature rise has been predicted by the computer model and validated by experimental data. The reaction rates for various pathways have also been estimated. Using the computer model, contour plots of theaflavin accumulation and temperature rise during tea fermentation have been produced to aid the process control and optimisation.

Keywords:
Tea, fermentation, computer modelling, biochemical reaction, theaflavin, thearubigin, heat transfer.

Main Publications/Patents/Participation in conferences:
R. Tomas, G. Lian, D. Davies Modelling the biochemical reaction pathways during black tea fermentation. Research Report.

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Category:	30
Starting date:	01/04/1997
Duration:	11 months
Contract number:	FAIR-CT96-5066

The role of Quorum sensing and RSMA in RPOS mediated resistance of attached *Pseudomonas aeruginosa*

Objectives:
The project was initiated to determine the significance of RpoS for the resistance of *Pseudomonas aeruginosa* to various stresses. Whether the resistance of cells can be manipulated via RpoS by adding various N-AHLs to *P. aeruginosa* was also investigated. In order to study the possible role of CsrA and the interaction between CsrA and RpoS, a *csr4* mutant was constructed. To determine the role of quorum sensing, CsrA and RpoS in the biofilm formation of *P. aeruginosa* collaboration was set up with Dr. Hilary Lappin-Scott and the biofilm group at the Hatherly laboratory at Exeter University. Within this group preliminary biofilm studies have been initiated.

Summary of achievements:
In *P. aeruginosa* diffusible chemical signals (*N*-acyl homoserine factories; N-AHLs) regulate the production of virulence determinants and secondary metabolites in a cell density dependent manner also known as quorum sensing. Two quorum sensing regulons have been identified in which the LuxR homologues LasR and VsmR are activated by *N*-(3-oxododecanoyl)-L-homoserine lactone (OdDHL) and *N*-butanoyl-L-homoserine lactone (BHL) respectively. *Erwinia* spp. possesses a CsrA homologue (also known as RsmA) which has been identified as a negative regulator of quorum sensing. A homologue of Csr4 was recently identified in *P. aeruginosa* at the host laboratory. In *P. aeruginosa rpoS*, a gene known to affect stress tolerance in other bacterial species, is controlled through quorum sensing via VsmR suggesting that the regulation of virulence is linked to adaptation and survival in stationary phase.

An RpoS-negative *P. aeruginosa* mutant was 2-3-fold more sensitive to oxidative stress, heat, low pH, ethanol and osmotic stress compared to the wild type strain. The addition of BHL or OdDHL during exponential growth to wild type cells had no effect on heat or oxidative stress

tolerance. This suggests that the resistant stationary phase phenotype cannot develop by attempting to induce RpoS during exponential growth through the addition of cell density-dependent signal molecules.

In order to study the role N-AHLs in the biofilm development of *P. aeruginosa*, a biofilm model was set up. As flow conditions may modulate the effect of N-AHLs due to their freely diffusible nature, biofilms were characterised under both high flow (turbulent) and low flow (laminar) conditions. The biofilms formed were viewed using bright field microscopy and assessed by percent surface cover and cell cluster thickness. Surface coverage increased initially at a higher rate in the wild type strain than in two quorum sensing mutants. The maximum percent surface covered by biofilm was higher in the wild type compared to the BHL-deficient PAN067 mutant and the OdDHL-deficient *lasR lasI* mutant after seven days of culture. However, the biofilm formed by the *lasR lasI* mutant could reach a similar degree of surface coverage as observed in the wild type biofilm when cultured for up to 12 days. Cell cluster thickness was also initially higher in the wild type than in the mutants but on day 12 cluster thickness was similar in the *lasR lasI* mutant biofilm compared to the maximum cell cluster thickness observed in the wild type biofilm. In a recent study on the role of cell signalling in *P. aeruginosa* biofilms OdDHL was required for the development of the complex structure formed by wild type cells. These experiments were conducted in a continuous-culture once-flow-through system with a glass substratum, similar to the set-up used in the present study. However, the flow rate was much lower than in this set-up under laminar conditions. Taken together these results suggest that the relative contribution of cell signalling to the overall structure of biofilms growing in aqueous environments will be determined, to a significant extent, by the hydrodynamic conditions.

A *P. aeruginosa csrA* mutant was constructed during the course of this work and the resulting phenotype in respect to biofilm development and possible effect on RpoS expression is currently being investigated. Antibodies against RpoS and CsrA were raised as part of this study and will provide the basis for Western blot analysis studies aiming to investigate the role *of* CsrA and quorum sensing in RpoS-mediated resistance *of Ps. aeruginosa*.

Keywords:
Quorum sensing, *Pseudomonas aeruginosa*, RSMA, RPOS, resistance

Main Publications/Patents/Participation in conferences:
M.K. Winson, S. Swift, L. Fish, J. Throup, F. Jorgensen, S.R. Chhabra, B.W. Bycroft, P. Williams, G.S.A.B. Stewart (1998) Construction and analysis of *luxCDABE*-based plasmid sensors for investigating N-acyl-homoserine lactone-mediated quorum sensing. *FEMS Microbiology Letters* 163, 185-192
Society for General Microbiology 137th Ordinary Meeting, March 1997, Heriot-Watt University, Edinburgh.

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Category:	20
Starting date:	01/04/1997
Duration:	6 months
Contract number:	FAIR-CT96-5067

Effect of fat source and vitamin E level on lipid composition and lipid oxidation of pig meat

Objectives:
Lipid peroxidation is one of the primary mechanisms of quality deterioration in stored foods, particularly in raw and cooked muscle tissues. It is believed to be initiated in the highly unsaturated phospholipid fraction in subcellular membranes.

In muscle foods, the effect of lipid oxidation is manifested as a loss in quality characteristics such as, colour, texture, nutritive value, and flavour. It also results in the formation of potentially toxic compounds such as oxidized cholesterol derivatives that have been shown to be related to the development of cardiovascular and other degenerative diseases.

The rate of lipid oxidation in muscle foods is dependent on a number of factors including the degree of lipid unsaturation and the presence of antioxidants.

In recent years there has been considerable emphasis placed on the modification of fatty acid composition of animal tissues. Fat included in monogastric diets is partially incorporated into various tissue lipids (neutral lipids and phospholipids), thus leading to different lipid compositional characteristics. The beneficial effect of consumption of polyunsaturated fatty acids (PUFA) of the n-3 series in reducing the risk of coronary heart disease has been widely studied, however, increasing the degree of unsaturation of the muscle membranes reduces oxidative stability in pig meat. The use of vegetable oils rich in monounsaturated fatty acid increases the lipid stability to oxidation, decreasing the undesirable flavour development associated with polyunsaturated fatty acids.

On the other hand, dietary supplementation with α-tocopheryl acetate is known to be an effective method in increasing muscle α-tocopherol levels, thereby, improving oxidative stability post slaughter.

- To evaluate the effect of dietary α-tocopheryl acetate supplementation in diets enriched with different kinds of fat on the oxidative stability of muscle tissue and membrane structure.
- To evaluate the influence of added fat, high in n-6 fatty acids (sunflower oil), n-9 fatty acids (olive oil) and n-3 fatty acids (linseed oil) with respect to diets containing no added fat on lipid composition and on lipid oxidation of muscle tissue and on membrane structure.

Summary of achievements:

1. Rearing, slaughtering and analysis of feed.

Fatty acid profiles of meat lipids reflected dietary fatty acid composition. The consumption of olive, sunflower and linseed oils altered fatty acid composition of meat lipids by increasing the levels of n-9, n-0 or n-3 fatty acids, respectively. Dietary fat enriched with n-6 fatty acids (sunflower oil) reduced n-3 fatty acid concentration, increasing the n-6/n-3 ratio in the phospholipids.

2. Analysis of longissimus dorsi muscle: chemical composition, α-tocopherol concentration and fatty acid composition of intramuscular fat.

Pigs receiving enriched diets containing n-3 fatty acids (linseed oil) had a higher rate of lipid oxidation than other dietary groups. Groups enriched with n-6 fatty acids (sunflower oil) showed higher values of oxidation than diets enriched with n-9 fatty acids (olive oil).

3.Storage studies: water holding capacity, measurement of TBARS and color changes during refrigerated storage for fresh and cooked meat.

Dietary α-tocopheryl acetate supplementation (200 mg/kg feed), resulted in higher concentrations of α-tocopherol in pork muscles compared to pigs receiving a basal diet.

4. Measurement of cholesterol oxidation products.

Supplementation of diets containing oils with α-tocopheryl acetate resulted in a reduction of lipid oxidation in M. longissimus dorsi and microsomal fraction and significantly lower total COPS values.

5. Measurement of lipid oxidation in microsomal fraction.

Pigs receiving a diet deficient in fat showed intermediate values of oxidation and had higher drip losses than other dietary groups. Meat tissue from these pigs had intermediate levels of n-3 fatty acids, a lower concentration of α-tocopherol and n-6 fatty acids and a higher concentration of saturated fatty acids than animals receiving a diet containing fat.

Keywords:

Fat, vitamin E, lipid, composition, oxidation, pig, meat

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Category:	30
Contract signed:	20/12/1996
Duration:	12 months
Contract number:	FAIR-CT96-5072

Measurement of diffusion coefficients in starch gels using holographic laser interferometry

Objectives:
The aim of the project was to develop the Holographic Laser Interferometry (HLI) technique further for the application on the study of diffusion of macromolecules in gels.

Summary of achievements:
HLI was applied to the measurement of the diffusion coefficient of polymers in agarose gel. A diffusion cell was especially designed and built for these kinds of experiments. It was shown that the method was well adapted to study different polymer probes such as proteins and polysaccharides by using bovine serum albumin and pullulans. The error on D was in the range 2-10% with an average value of 6%. In the case of BSA in the 0.1M NaCl, different concentrations of agarose gel (from 2% up to 6%) allowed us to find an experimental correlation which compared well with what was expected from scaling theory. In another set of experiments using pullulans with molar masses in the range $5.9x10^3$ g/mol – $1.1x10^5$ g/mol as probes allowed us to follow the influence of molar mass on D/D_0. In a 5% concentration agarose gel, D/D_0 was not dependent on molar mass from $5.9x10^3$ g/mol up to $2.3x10^4$ g/mol then D/D_0 decreased for the last two highest molar masses of pullulan. The restriction in diffusion observed above the critical molar mass M_0 is in line with what is expected from the reptation model.

The research thus shows that the HLI-technique can be used for the study of the diffusion characteristics of transparent gels with macromolecules as proteins and pullulan as diffusants.

Keywords:
diffusion coefficients, starch gels, holographic laser interferometry, agarose gel, reptation, model

Main Publications/Patents/Participation in conferences:

C. Mattisson, P. Roger, A. Axelsson, G. Zacchi (1997) Diffusion measurements of proteins in agarose gels using holographic laser interferometry – Presentation at Conference on Bioseparation, 27-28th August 1997, Lund, Sweden.

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Category:	20
Starting date:	01/05/1997
Duration:	36 months
Contract number:	FAIR-CT96-5073

Phase separation, glass transition and crystallisation in low water content gelatin sugar mixtures

Objectives:
Biopolymer sugar mixtures at low water contents are of significant importance in many food systems particularly in baked and some confectionery product; so far, it is well understood only the behaviour of biopolymers at low sugar contents.

In this project the behaviour of biopolymer sugar mixtures at high sugar contents will be studied, because the only evidence that exists is that as the sugar content is raised there is a phase separation into two separate domains. Therefore, the objectives of this project will be: to obtain a phase diagram for gelatin-sugar-water at low water contents (dry solid basis), to determine the domain sizes in the phase separate region and finally monitor the extent and kinetics of order for the two components (sugar-gelatin).

Summary of achievements:
The findings are of considerable fundamental and applied importance to food manufacturing but also to the use of biopolymers as edible/biodegradable packaging. These could be summarised as follows:

- Vibrational spectroscopy in the microspectroscopy mode (particularly FTIR in this study) represents the only viable technique for the imaging of the distribution of sugars in polysaccharide matrices.
- The hypothesis of the importance of the unequal partitioning of water between the constituents of phase separated biopolymer blends has been confirmed in a comprehensive investigation on various gelatin-amylopectin blends studied over a range of water contents. This investigation also demonstrated that the mechanical properties of these blends, as studied over a range of temperatures using Dynamic Mechanical Thermal Analysis (DMTA) could only be predicted if the selective partitioning of plasticisers, which was successfully evaluated from in the case of water from vapour

sorption/desorption isotherms, was accounted for. The finding will be published in an extensive paper which was submitted and accepted for publication in the scientific journal POLYMER.

- The idea of unequal partitioning of plasticisers is now being extended to: (i) amylopectin-gelatin systems where the recrystallisation of the polysaccharide takes place on storage and (ii) amylopectin-water systems in which the polysaccharide has been recrystallised in different polymorphic forms depending on the water content and the temperature of storage. The preliminary results of these studies are very promising and are in line with the theory proposed in the project. The findings are anticipated to have a major relevance to the understanding of the phenomena occurring during the processing and storage of foods in the rubbery state such as baked goods.

Keywords:
Crystallisation, gelatin, glass transition

Main Publications/Patents/Participation in conferences:
Z. Mousia, I.A. Farhat, J.F. Blachot, J.R. (In press) Mitchell Effect of water partitioning on the glass-transition behaviour of phase separated amylopectin-gelatin mixtures *Polymer*
Workshop on Biopolymer Science, Food and Non Food Applications, Montpellier, France, (28-30 September 1998). Presented a poster entitled" *The Glass-Transition Behaviour of Phase Separated Starch-Gelatin Systems"*
Molecular Mobility in Foods, Camogli, Italy, (April 6-7, 1999). Presented a poster entitled *"The GlassTransition Behaviour of Phase Separated Starch-Gelatin systems"*

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Category:	20
Starting date:	01/02/1997
Duration:	36 months
Contract number:	FAIR-CT96-5075

Structure and function of lactose synthase

Objectives:
Lactose synthase is the enzyme that catalyses the biosynthesis of lactose in the lactating mammary gland. Lactose synthase (LS) is a complex of two protein moieties: α-lactalbumin (LA) and galactosyltransferase. Each of these components has different structural and functional features and it is only during milk secretion that they come together to form LS complex. The aim of this project is to understand the structure and function of lactose synthase.

Summary of achievements:
LA is known as a "specifier protein" because of its ability to modulate galactosyltransferase's (GT) affinity for glucose. During lactation the substrate specificity of the transferase is altered from N-acetylglucosamine to glucose, the binding of which is enhanced 1000-fold due to the synergistic binding of LA. The modulatory role of LA to GT's substrate specificity is mediated by the functional sites of the molecule. An important feature of LA is that its 3D structure is similar to that of c-type lysozyme (LZ) and the two proteins are homologous with divergent functions. The overall structure of LA is very similar to that of LZ and is divided into two subdomains (lobes) by a deep cleft, which is the corresponding active site of LZ.

LA binds calcium strongly and specifically whereas in the LZs there are two subgroups, representing paralogous gene lines, of which only one binds the Ca^{2+} ion. High affinity Ca^{2+} binding to LA stabilises the native structure and is required for the efficient generation of native protein with correct disulphide bonds from the reduced denatured state. At ambient temperature and low ionic strength the apo-protein assumes a molten globule state. Calcium greatly accelerates folding by binding to rate limiting intermediaries in the folding process. To investigate the role of calcium in the LA structure with the goal of obtaining high-resolution data relevant to its role in folding and stability, X-ray structures at 2.2 Å resolution have been determined for crystals of the apo- and holo-forms of bovine LA. Although Ca^{2+} removal has little effect on protein structure in the metal binding site, a significant structural change was

observed in the LA cleft region at the periphery of the hydrophobic box in the region around Tyr-103 of the helical lobe and Gln-54 of the beta lobe. This change results in a more open cleft structure in the apo-protein and appears to reflect an effect of calcium binding on buried solvent molecules which in turn affects interactions between the lobes.

In the absence of a 3D structure for the LS complex, bovine LA had been used as a model system for the design of a series of LA variants at regions proposed to be directly involved in LA action in LS complex in order to follow a "structure-based" approach. A detailed structural investigation was performed for three LA variants: Ala109-Pro, Tyr103-Pro and Trp118-His while crystallisation conditions for additional mutants: Phe31-Tyr, His32-Tyr, Lys114-Asn were also established. Most of the LA variants exhibited "merohedral twinning" which is an inherent property of the molecule when crystallised in the hexagonal forms.

The crystal structure of apo-LA could be set as a template for further binding studies of metal ions in LA molecules. Recent studies concerning the effect of pH on apo-LA showed that apo-LA crystallises in different pHs. It is hoped that structure determination of the molecule under these conditions might reveal the intermediate stages during the folding process.

Keywords:
lactose synthase, enzyme, protein, α-lactalbumin, galactosyltransferase, binding, folding process, merohedral twinning

Main Publications/Patents/Participation in conferences:
E. D. Chrysina, K. Brew, K. R. Acharya (1999) Structural studies on bovine α-lactalbumin. Poster presentation at XVIII IUCγ Congress and General Assembly, 4-8th July 1999, Glasgow, Scotland.

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Category:	30
Starting date:	01/09/1997
Duration:	24 months
Contract number:	FAIR-CT97-5002

The interaction between the proteolytic activity and growth of *Streptococcus thermophilus* and its consequential effect on various fermented dairy products

Objectives:

- To investigate the degree of proteolysis in milk following the growth of a number of *Streptococcus thermophilus* strains, using urea-PAGE, SDS-PAGE and RP-HPLC analysis of the free amino acids.
- To compare the proteolytic activity *S. thermophilus* with other lactic acid bacteria (LAB).
- To determine proteolysis in milk following the combined growth of *S. thermophilus* and *Lactobacillus lactis* subsp. *lactis*.
- To measure the proteolysis of a number of commercial cultures formulated from *S. thermophilus* and *L. lactis* strains.
- To characterise by polymerase chain reaction (PCR) the cell envelope proteinase of *S. thermophilus* and *L. lactis*.

Summary of achievements:

The proteolysis of a number of commercial Chr. Hansen A/S *S. thermophilus* strains was evaluated following acidification of ten reconstituted skim milk by directly measuring changes to the nitrogen components (caseins, peptides and free amino acids).

The analytical techniques employed were, urea-PAGE (separation by charge differences), SDS-PAGE, free amino acid analysis (ion exchange HPLC) and peptide fingerprinting (reverse phase HPLC). The urea-PAGE electrophoretograms showed the appearance of two new bands following the growth of four *S. thermophilus* strains, all fast acid producers; however, the level of proteolysis was very limited, with more than 95 % of the casein remaining intact. SDS-PAGE was not a suitably sensitive technique, for this analysis and was consequently abandoned. The HPLC free amino acid analysis was a particularly discriminating technique which demonstrated both quantitative and qualitative differences

between the strains. Most individual free amino acid levels increased following growth, especially phenylalanine, histidine, tyrosine and leucine. Interestingly, two *S. thermophilus* strains actually consumed arginine. For some *S. thermophilus* strains the glutamate level of the milk was significantly reduced, while for others strains it remained at a constant level. The HPLC peptide fingerprinting indicated that during milk acidification there appears to be a net reduction in the level of peptides present, with most of the decrease due to the consumption of hydrophobic peptides.

Proteolysis in milk was also measured following acidification by four commercial cultures, known as RST cultures (such cultures contain a *S. thermophilus* and a *L. lactis* component). It was clearly observed that the free amino acids produced by the *L. lactis* culture component were consumed by the *S. thermophilus* culture component, when they were grown together as a RST culture. The differences observed between the four RST cultures were not significant with respect to their HPLC peptide fingerprints and urea-PAGE electrophoretogams. However, the free amino acid analysis showed moderate differences between the four RST cultures, especially in relation to the levels of tyrosine, lysine and arginine in the milk following fermentation.

The detailed classification of the cell envelope-associated proteinase of *L. lactis* strains (a, b, c, d, e, f, g and h) was performed by PCR amplification of the subtilisin-like binding region and the C-terminally located remote region. This PCR technique is a useful alternative and provides greater information than the traditional classification (PI, PIII or PI/III) of the cell envelope associated proteinase of *L. lactis* strains based on the hydrolysis of specific chromogenic substrates. Five *S. thermophilus* strains were also studied using the same methodology; no PCR products were detected with the *L. lactis* primers, indicating that the cell envelope-associated proteinase of *S. thermophilus* is different from that of *L. lactis*. Another set of primers based on the average homology between prtP of *L. lactis* subsp. *cremoris* SKI 1 and prtB of *L. delbrueckii* subsp. *bulgaricus* was also tested, but none of the PCR products sequenced coded for proteinases. It was therefore established from these results that the cell envelope-associated proteinase of *S. thermophilus* strains regardless of their acidification rate is distinctly different from the cell envelope-associated proteins of either *L. lactic* or *L. delbrueckii* subsp. *bulgaricus*.

Keywords:
proteolytic activity, growth, *Streptococcus thermophilus*, fermentation, dairy products, electrophoresis, PCR, HPLC, milk

Main Publications/Patents/Participation in conferences:
E. Johansen, C.M. Henriksne, E. Brokman, E. Hoeier, F.P. Rattray, T. Jansen (1999) The production application and action of lactic cheese starter cultures. The technology of cheesemaking, Sheffield Academic Press, UK

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Category:	30
Starting date:	01/01/1998
Duration:	24 months
Contract number:	FAIR-CT97-5004

A kinetic and structural model form (mushroom) tyrosinase

Objectives:
The objectives of this project are:

- To establish the relationship between pro-forms of tyrosinase, latent forms and corresponding active forms at a level of molecular changes;
- To isolate specific latent isoforms of tyrosinase from mushrooms;
- To study the effect of various activators on these latent isoforms to determine the relationship between latent isoforms, activation mechanism and active isoforms;
- To kinetically describe the behaviour of various active isoforms towards specific natural substrates;
- To establish a maturation and activation model for mushroom tyrosinase and to evaluate the possibility to exploit such models in designing rapid assays that can predict the eventual degree of discolouration.

Summary of achievements:
The research has yielded the following main results:

- The activation process for latent mushroom tyrosinase has been kinetically characterised in the presence of different activators: serine-proteases, SDS and endogenous volatile compounds (benzyl alcohol, 1-octen-2-ol, 3-octanone etc., which are responsible for the characteristic mushroom-like aroma).
- The use of protease inhibitors has been proposed as an alternative and promising tool to prevent enzymatic browning in mushrooms by preventing the activation of latent tyrosinase.
- The oxidation of the main phenolic compounds in mushrooms (GHB, GDHB, L-Tyr and L-DOPA) catalysed by active tyrosinase.

- The inhibition of active mushroom tyrosinase by agartine (a very abundant compound in mushrooms of the *Agaricus* genus).
- The prevention of melanin formation by using the depigmenting power of agaritine.
- The inhibition of active mushroom tyrosinase in a non-classical manner by tropolone.
- The possible role of active tyrosinase on the antioxidant capacity of foodstuff. Oxidation of ascorbic acid and resveratrol catalysed by tyrosinase from various sources (pear, grape and mushroom, respectively).
- The possible implication of tyrosinase in the oxidation pathway of the phytoalexins stilbenes.
- The kinetic characterisation of the free radical scavenger (antioxidant) capacity of foodstuffs (oils, fruits, vegetables, wines, coffees, teas, etc).
- The kinetic characterisation of the antioxidant capacity of natural colourant extracts from various sources (black chokeberry, blackthorn, strawberry, elderberry and others) in order to offer to the food industry alternative additives more compatible with (or attractive to) human consumption.
- The inhibition of tyrosinase by the anti-hypertensive drug "captopril" *in vitro*. Possible inhibition of tyrosinase *in vivo* as results of long-term treatment with this drug?
- The protease subtilisin proved to be a useful tool to purify active mushroom tyrosinase to homogeneity. This will be approached in order to determine the crystal structure of mushroom tyrosinase in the near future for the first time.

Keywords:
tyrosinase, mushroom, isoforms, agartine

Main Publications/Patents/Participation in conferences:

J.C. Espín, S. Jolivet, H.J. Wichers (1998) Inhibition of mushroom polyphenol oxidase by agaritine. J. Agric. Food Chem. 46, 2976-2980.
J.C. Espín, S. Jolivet, A. Overeem, H.J. Wichers (1999) Agartine from Agaricus bisporus is capable of preventing melanin formation. Phytochemistry 50, 555-563.
J.C. Espín, H.J. Wichers (1999) Slow-binding inhibition of mushroom tyrosinase isoforms by tropolone. J. Agric. Food Chem. 47, 2638-2644.
J.C. Espín, S. Jolivet, H.J. Wichers (1999) Kinetic study of the oxidation of γ-glutamyl-4-hyrdoxybenzene catalysed by Agaricus bisporus tyrosinase. J. Agric. Food Chem. 47, 3495-3502.
J.C. Espín, H.J. Wichers (1999) Kinetics of activation of a latent mushroom tyrosinase by benzyl alcohol. J. Agric. Food Chem. 47, 3503-3508.
J.C. Espín, J. Van Leeuwen, H.J. Wichers (1999) Kinetic study of the activation process of latent mushroom tyrosinase by serine proteases. J. Agric. Food Chem. 47, 3509-3517.
J.C. Espín, H.J. Wichers (1999) Activation of a latent mushroom tyrosinase by sodium dodecyl sulfate (SDS). Kinetic properties of the SDS activated isoform. J. Agric. Food Chem. 47, 3518-3525.

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Category:	20
Starting date:	01/09/97
Duration:	12 months
Contract number:	FAIR-CT97-5012

Interaction between myoglobin species and peroxides in relation to oxidative stability of meat and meat products

Objectives:
Initiation of lipid oxidation in muscle foods has been attributed to free metals ions and to haeme proteins such as myoglobin. However, it has been suggested that hydroperoxides may be necessary for myoglobin to be an effective catalyst initiating lipid oxidation in muscle foods. Intensive research on the interaction between haeme proteins and peroxides has shown that this proceeds through the formation of a transient haeme protein radical or perferryl species which is subsequently reduced to ferryl species. Both perferryl and ferryl are known to be strong prooxidant in many biological systems and in meat systems. However, the exact nature of the mechanisms involving haeme protein as an initiator of lipid oxidation in muscle foods and resulting in rancidity has not been completely understood.

In contrast to most haeme proteins, the colour-imparting compound in nitrite cured meats, nitrosylmyoglobin which is formed during the reaction between myoglobin and nitrite under reducing conditions possesses antioxidative activity in model systems and in meat systems. The mechanisms behind its antioxidative properties are far from completely understood. Moreover, the reaction between nitrosylmyoglobin and different peroxides has shown that nitrosylmyoglobin interacts with peroxides without formation of perferryl and ferryl species unlike other myoglobin derivatives. The latter phenomenon may contribute to the oxidative stability of cured meat products and deserves further investigation.

The objectives of the project were:
i) to provide information regarding the reactivity of haeme proteins towards peroxides
ii) obtain a better understanding of the mechanisms responsible for rancidity in muscle foods
iii) gain knowledge about muscle foods preservation with emphasis on oxidative processes in order to control and minimise oxidative deterioration

iv) set up guideline for the food industries regarding handling, storage and display of meat and meat products.

Summary of achievements:
The antioxidative activity of MbFe(II)NO was attributed to different interacting mechanisms. It was demonstrated that, in contrast to most haeme proteins, the presence of peroxides did not convert MbFe(II)NO to a higher oxidation state (perferrylmyoglobin and ferrylmyoglobin). Instead, MbFe(II)NO was found to neutralise hydrogen peroxide and *tert*-butylhydroperoxide resulting in the formation of metmyoglobin and nitrite. At high concentrations of hydrogen peroxide, metmyoglobin was re-activated to a high oxidation state myoglobin species, a process, which was not observed with excess *tert*-butylhydroperoxide. It is suggested that interactions between nitrosylmyoglobin and *tert*-butylhydroperoxide result in modification and inactivation of the haeme protein, preventing subsequent reactivation to a higher oxidation state. Furthermore, formation of nitrite, known to act as an antioxidant, can further contribute to stabilise the antioxidative potential of cured meat products. Additionally, MbFe(II)NO was found to reduce ferrylmyoglobin.

Keywords:
Lipid oxidation, catalyst, nitrosylmyoglobin, myoglobin, haeme, meat, meat product

Main Publications/Patents/Participation in conferences:
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C.P. Baron, L.H. Skibsted, H.J. Andersen (1997) Prooxidative Activity of Myoglobin Species in Linoleic Acid Emulsion J.Agric. Food Chem. 45: 1704-1710
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Links with EC projects:
AIR1-CT94-7657

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Category:	20
Starting date:	01/10/1997
Duration:	36 months
Contract number:	FAIR-CT97-5013

Microbiological and technological characterisation of novel bacteriocins produced by enterococci

Objectives:
Recently, interest in antimicrobial activities of enterococcal bacteriocins against pathogenic bacteria such as listeria has been growing. Enterococci could hence be used as starter or coculture in food fermentation processes. *Enterococcus faecalis* and *E. faecium* are indeed found as natural starter cultures in the manufacture of Mozzarella and other Mediterranean cheeses, where they play an important role in the final organoleptic characteristics of these cheeses.

Summary of achievements:
Thirty-one enterococci bacteria were investigated as to their capacity to produce antimicrobials. The final selection included 11 enterococci with antimicrobial activity towards *Listeria innocua* and/or *Clostridium tyrobutyricum*: five food isolates, one feed isolate, two veterinary isolates, two industrial strains, and one of unknown origin. All selected enterococci were compared on the basis of their *Sma*I pulsed-field gel electrophoresis (PFGE) patterns. It was found that *E. faecium* FAIR E-178 and *E. faecium* LMG 11423^T are the same strain. In previous work it was shown that the selected enterococci were vancomycin-sensitive and γ-haemolytic. The absence of the genes involved in the resistance to vancomycin and the production of cytolysin was confirmed by PCR. The latter properties were investigated in view of the possible use of the selected enterococci as starter or cocultures in cheese production.

The enterocins of different enterococci were isolated by ammonium sulphate precipitation and partially purified by chloroform/methanol extraction, resulting in an increase of the specific activity of more than 100-fold in some cases. The temperature and pH stability of the partially purified products was tested. All enterocins were thermostable and stable in a wide range of pHs (from 2.5 to 9.0). The mode of action was performed using *L. innocua* LMG 13568 as indicator strain. The enterocins displayed a bactericidal activity, except in the case of enterocin INRA 1 that showed a bacteriolytic activity. However, a population of around 105 of *L. innocua* LMG 13568 survived with all the enterocins.

The molecular mass of the partially purified enterocins was. It averaged from 3.8 to 4.0 kDa.

The presence of the genes involved in the production of known enterocins was tested by PCR. It was found that only *E. faecium* SF 68 could be producer of a novel enterocin.

Finally, different methods were performed for the purification to homogeneity of the enterocin produced by *E. faecium* RZS C5. When the captured bacteriocin was eluted, 10 % of the initial enterocin activity was recovered, a value that corresponded to an activity of 6800 AU/ml and a specific activity of 108727 AU/mg. 2) Extraction with organic solvents. The specific activity increased more than 100-fold. The last step was performed by reversed-phase fast performance liquid chromatography (FPLC). MALDI mass spectrometry revealed a compound with a molecular mass of 5457 Da.

Keywords:
Enterococcus faecalis, *E. faecium*, enterococci, *Listeria innocua*, LMG

Main Publications/Patents/Participation in conferences:
M.R. Foulquie Moreno, L. De Vuyst (1999) Characterisation of novel enterocins. Sixth symposium on Lactic Acid Bacteria, C71.
R. Callewaert, M. Zemfir, M.R. Foulquie Moreno L. De Vuyst (1999) Are bacteriocins involved in intestinal ecology? Host microflora interface in health and disease, M10.
Third Meeting of the European Project FAIR-CT97-3227, Parma, Italy, October 22-23, 1998
Fourth Meeting of the European Project FAIR-CT97-3078, Brussels, Belgium, March 29-30, 1999
Sixth symposium on Lactic Acid Bacteria. Netherlands Society for Microbiology, Veldhoven, The Netherlands, September 19-23, 1999

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Contract signed:	31/07/1997
Duration:	24 months
Contract number:	FAIR-CT97-5014

Physico-chemical characteristics of spore dormancy and resuscitation

Objectives:
The project aimed to investigate bacterial spore structure in the dormant and resuscitated state by applying non-invasive physico-chemical techniques. Spores from *Bacillus subtilis* 357 lux+ and CMCC 604, *B. cereus* T and of outer and inner coatless mutants of *B. subtilis* 322 and 325 were used. The techniques included nuclear magnetic resonance spectroscopy (NMR), differential scanning calorimetry (DSC), environmental scanning electron microscopy (ESEM), scanning transmission electron microscopy (STEM), bright field, phase and interference contrast light microscopy combined with image analysis systems, laser light scattering (Mastersizer). Conventional transmission electron microscopy (TEM) was also used. The techniques revealed results on a microscopic, supramolecular and molecular level of the structure of dormant and resuscitated spores.

Summary of achievements:
1.) A "spore website" was established on the Internet which structured publications related to the project (http://chw708l.ch.ul890.unilever.com/spore/).
2.) Characterisation of *Bacillus* spore suspensions was necessary to obtain knowledge about the homogeneity of a spore crop to provide large homogeneous sample batches. Good agreement between the average individual spore dimensions determined by electron microscopy and those obtained by laser diffraction was evident. Homogeneity of *B. subtilis* spore crops was improved, and vegetative cells were separated from spores by centrifugation.
3.) Hydration studies were carried out using freeze dried spores, rehydrated spores and spores in excess water (spore slurry). The results revealed reversible water diffusion between the spore centre and the environment. There was no complete phosphorus mobilisation due to the addition of excess water.
4.) Heat activation studies were carried out with *B. subtilis* and *B. cereus*. This low temperature endotherm was studied along with its relevance for heat activation. It seems to be a melting event with minor importance in the sequence of events during heating which led to

a higher germination rate of spores. It occurs at a temperature below those generally applied for heat activation and is terminated after approximately 1 minute, whereas heat activation continues to produce higher germination rates at longer heating times (20 - 30 min).
5.) Single spore germination was examined using phase-contrast microscopy and by determining the increase in greyness with colour image analysis software. Heat activation led to an increase in synchrony of germination and reduced the initial lag time prior to a phase change of individual spores. An optimal germination temperature (37 °C) resulted in faster and more homogenous germination than a suboptimal temperature of 20 °C.
6.) Potential of NMR and DSC for structural analysis of spore dormancy and resuscitation was further investigated. DNA had a reversible peak which was not observed in spores. Transmission electron microscopy (TEM) revealed detailed structural difference in spores resulting from *B. cereus, B. subtilis* and the coat mutants of *B. subtilis*. NMR reflected these structural differences well. *B. cereus* had a thinner coat than *B. subtilis* which resulted in less intense protein signals and the coat mutants lacking the inner or outer coat displayed similar reduced signal intensities. Germinated *B. subtilis* spores revealed higher molecular mobilities than the dormant counter part particularly in the region where carbohydrates resonated which would be compatible with cortex hydration. DPA in spores of *B. cereus* showed higher solid-state molecular order (possibly crystalline) than in spores of *B. subtilis*.

Keywords:
Spore, dormancy, resuscitation, spectroscopy, electron microscopy, heat activation, germination

Main Publications/Patents/Participation in conferences:
R.G.K. Leuschner, A. Darke, P.J. Lillford (1998) Impact of bacterial spores on food safety. Proceedings for the poster session (part 2) of the 3rd Karlsruhe Nutrition Symposium, European Research towards Safer and Better Food, pp. 10-15, 18-20 October, Karlsruhe, Germany
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R.G.K. Leuschner, R.B. Leslie (1999) Panel Discussion: Research and Training Needs of the Food Industry. In: Water Management in the Design and Distribution of Quality Foods. Y.H. Roos, R.B. Leslie, P.J. Lillford (eds.). pp. 561-567. Technomic Publishing Company, Pennsylvania, USA.
R.G.K. Leuschner, A.T. Weaver, P.J. Lillford (1999) Characterisation of *Bacillus* spore suspensions. Colloid and Surfaces, B: Biointerfaces, 13:47-57.

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Category:	30
Starting date:	22/09/1997
Duration:	6 months
Contract number:	FAIR-CT97-5015

Confocal laser scanning microscopy and image analysis on polysaccharides-based biopolymer mixtures

Objectives:
Biopolymers are often used in the food industry because they impart desirable properties (textural, organoleptic, nutritional) to foodstuffs. For health reasons, consumers tend towards a reduction of the fat content in the food products.

Biopolymer mixtures are often used in low-fat products, because they achieve similar properties to fat. Both polysaccharides/polysaccharides and polysaccharides/protein mixtures are used, and as thermodynamic incompatibility between different species is a general behaviour, leading to phase separation of the biopolymers, some stability problems may easily occur. Indeed, the stability of products is very dependent on parameters such as the intrinsic characteristics of polymers, their concentration, temperature and nature of the solvent.

A lot of studies on biopolymer mixtures have been performed at equilibrium conditions. But, in the food industry, the process conditions are such that equilibrium conditions are never reached and phase separation phenomena occur under dynamic events.

The general aim of this project is then to better understand the dynamic aspects of phase separation in biopolymer mixtures, in order to obtain better control of the stability of foodstuffs and to develop new textural properties in food products. This project focused on the microstructure of two protein/polysaccharide mixtures (gelatin/maltodextrin and gelatin/κ-carrageenan).

Summary of achievements:
A staining method for polysaccharides was developed which allows observation by Confocal Laser Scanning Microscopy.

The influence of the cooling rate has been determined in the case of the gelatin/maltodextrin system. In all cases, phase separation occurred, with the formation of maltodextrin inclusions in a gelatin continuous phase. The temperature of structuration decreased when the cooling rate increased but it was always under the gelation temperature of gelatin alone. Image analysis was performed on the pictures. As the temperature decreased, an increase in the fluorescence intensity of the droplets was observed while their size remained roughly the same. From these observations, it was concluded that phase separation in this system was probably due to a spinodal decomposition mechanism rather than to a nucleation and growth one.

The structuration of gelatin/κ-carrageenan systems has been studied in different ionic environments (NaCl, NaCl + KCl, NaCl + $CaCl_2$) during a cooling process at 1°C/min. Except when KCl was present, κ-carrageenan particles were formed at temperatures higher or lower than the gelation temperature of gelatin alone. This depended on concentrations and on the solvent. In NaCl + KCl, it seemed that a fluorescent κ-carrageenan network formed under cooling.

Keywords:
Polysaccharides, biopolymers, foodstuffs, carrageenan, gelatin

Links with EC projects:
FAIR-CT96-1015

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Category:	40
Contract signed:	05/08/1997
Duration:	12 months
Contract number:	FAIR-CT97-5017

Investigation of the physiological mechanisms modulating fat-induced satiation and satiety in man

Objectives:
The project aimed to investigate the physiological role of the gastrointestinal hormones CCK, GPL-1 and GIP on determining gastric emptying, motility, satiety and induction of satiety in response to nutrient ingestion in man by clinical studies combining the simultaneous measurement of circulating hormones and metabolites, gastric emptying, motility and satiety following a series of test meals varying in energy, fat type and nutrients.

Gastric emptying and motility will be studied by Electrical Impedance Epigastrography (EIE) developed in the Physics Department, University of Surrey and collaborators.

Summary of achievements:
It was possible to improve the technique of EIE for studying gastric function and to test and modify a new model of the epigastrograph, which is used to measure the electrical impedance over the abdominal area covering the stomach. The new model, Mark IV, is based on the same principles as the preceding instruments but is built using technological developments and components, such as optical fibres and battery power operation, making it a light and completely portable system. It has the capability of using different input current values and a multiplex electrode system which permits the monitoring of electrical impedance at three points on the abdomen compared to two previously, thus allowing better stomach localization. This is also done at a higher sampling rate (5Hz instead of 1 Hz) and it applies an AC current of 32 kHz and 1-4 mA rms.

Our experimental results on normal healthy volunteers using different meals have shown that:

i) the meal size (total energy) and carbohydrate content affect gastric emptying, the secretion of gastric inhibitory peptide (GIP), glucagon-like peptide 1 (GLP-1) secretion as well as satiety, with statistical significance. Similar results were shown, also, with meals varying in fat content;

ii) the intravenous infusion of GLP-1 induces a statistically significant elongation of half-emptying time (T50) using epigastrography with a 400 ml water load (meal).

This goes from 7.0 min to 11.8 min. Increased satiety under GLP-1 infusion was not so clear at the end of the test but later on after a buffet meal was given, the hunger rating (self assessment) was found to be lower;

iii) the motility pattern of the stomach was illustrated by using running power spectral analysis, based on Fast Fourier transformation. The frequency of the stomach contractions was found to be in the range 1.5 cycles per minute to 4.3 cycles per minute, in agreement with values found in the literature and other techniques. However, there is need for further work and analysis, in order to relate the power of the contractions to the meal size (total energy) and its composition. Other gastrointestinal hormones and satiety inducing supplements are currently being investigated.

Keywords:
Electrical Impedance Epigastrography, satiety, fat, human, meal

Main Publications/Patents/Participation in conferences:

S. Long, A. Sutton, A. Kesidis, B. Amaee, N.M. Spyrou, P. Rogers, L. Morgan (1998) Does GLP-1 act as a short-term regulator of food intake in man? A124, Abstracts from Proceedings for the 12th International Symposium on Regulatory Peptides, Dig.Dis. and Sci., 43(8)

S. Long, B. Amaee, A. Kesidis, A. Sutton, N.M. Spyrou, P. Rogers, L. Morgan (1998) Effect of meal size and carbohydrate content on GLP-1 secretion, gastric emptying and satiety. A125 Abstracts from the proceedings of the 12th International Symposium on Regulatory Peptides, Dig. Dis and Sci., 43(8).

S. Long, A. Sutton, A. Kesidis, B. Amaee, N.M Spyrou, P. Rogers, L. Morgan (1998) Effect of glucagon-like peptide-1 infusion on gastric emptying, energy intake and satiety in man. Abstracts from the Proceedings of the Nutrition Society Meeting, University of Surrey, Guilford, Great Britain, June 1998, *in press*.

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S. Long, A. Sutton, B. Amaee, A. Kesidis, N.M Spyrou, P. Rogers, L.Morgan (1999) Is GLP-1 a short-term regulator in man? Effects of GLP-1 infusion on gastric emptying, energy intake and satiety. British Journal of Nutrition, *in press*.

Nutrition Society meeting, University of Surrey, Guilford, Surrey, Great Britain, 29 June-2 July 1998.

Achieving faster phase 1 trials, IIR, Hilton, Park Lane, London, 21 September 1998.

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Category:	30
Starting date:	01/08/1997
Duration:	12 months
Contract number:	FAIR-CT97-5019

The relationship between (progress of) lipid oxidation and antioxidant content in vegetables

Objectives:
The objective of the project was to test the hypothesis that lipid oxidation is, in part, responsible for the post-harvest loss in antioxidant content in peas.

This was conducted by (1) the identification of selected enzymes responsible for lipid and ascorbate degradation and (2) the confirmation of a link between lipid oxidation and the loss of ascorbate in peas.

Summary of achievements:
The effect of antioxidants on lipoxygenase activity and associated degradation of ascorbate was examined in pea (*Pisum sativum*) extracts using standard peas (*lox*2+) and peas lacking the lipoxygenase-2 isoenzyme (*lox*2-). Addition of antioxidants, ascorbate and α-tocopherol, showed different effects on lipoxygenase activity present in the extracts. Ascorbate could be shown to inhibit lipid oxidation whereas α-tocopherol stimulated the reaction. The inhibition and stimulation effect of these antioxidants was dependent on the maturity and type of pea (standard and *lox*2-) and presumably reflected the presence of different lipoxygenase isoenzymes. (Total lipoxygenase activity increased during seed development in both *lox*2+ and *lox*2- pea extracts with the ratio of the formation of oxodienes to hydroperoxides also changing.) In standard peas the inhibitory effect of ascorbate decreased and the stimulatory effect of α-tocopherol increased with maturity. In contrast to this, the inhibitory effect of ascorbate increased with maturity in *lox*2- pea extracts and the stimulating effect of α-tocopherol was not as dramatic as seen in the more mature standard peas. Activation of lipoxygenases by α-tocopherol in *lox*2- peas was only two-fold in seeds 15 days after anthesis, and even lower in seeds 22 and 28 days after anthesis, compared to 28 day *lox*2+ pea extracts where a 6.5-fold activation was seen.

Ascorbate degradation was determined during lipid oxidation and greatly increased with lipoxygenase activity in standard peas. However, ascorbate degradation was constant and independent of lipoxygenase activity in *lox*2- pea extracts. Superoxide anion production and 13-linoleic acid hydroperoxides were not responsible for ascorbate degradation. This indicates that the link between lipid oxidation and ascorbate degradation is co-oxidation, which must be a property of the lipoxygenase-2 present in standard peas, not present in *lox*2- peas.

Lipid hydrolysis and oxidation in peas were examined with standard peas which contain two major lipoxygenase isoenzymes (LOX-2 and LOX-3) and in peas lacking the LOX-2 isoenzyme. (i.e. LOX-3 is the only major lipoxygenase in *lox*2- peas). Lipoxygenase activity in standard peas showed a different pH optimum compared with *lox*2- peas. Extracts from both pea types catalysed the oxidation of free fatty acid (linoleic acid) and the monoglyceride (monolinolein), with LOX-2 showing an apparent preference for the monoglyceride. Linoleic acid esterified in 1,3 diglyceride, triglyceride, or in the polar lipids, phosphatidyl choline and phosphatidic acid, did not act as substrates in either case, but oxidation did occur after a lag period, presumably reflecting release of the free- or mono-linolein by (phospho)-lipase driven hydrolysis.

Preliminary evidence indicates that pea seed lipoxygenase LOX-2 is a stress related enzyme. Pea seeds deficient in LOX-2 (*lox*2- seeds) display a different response to stress with respect to expression of the stress related enzymes guaiacol peroxidase and ascorbate oxidase when compared to standard (*lox*2+) pea seeds. Release of choline from phospholipids and subsequent release of free fatty acid also appear to be different.

Keywords:
Lipid, oxidation, antioxidant, vegetables, ascorbate, lipoxygenase, α-tocopherol

Main Publications/Patents/Participation in conferences:
H. Dornenburg, C. Davies, (1998) The relationship between (progress of) lipid oxidation and antioxidant content in vegetables. Inf. Rev. Food Technol.
H. Dornenburg, K.J. Hunter, C. Davies (1998) Evidence that pea seed lipoxygenase-2 (Pisum sativum) is a stress related enzyme. Plant Physiol.
H. Dornenburg, K.J. Hunter, C. Davies (1998) Antioxidants and lipoxygenase activity in cell free extracts of standard and *lox*2- peas. J. Agric. Food Chem.

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Category:	30
Starting date:	01/04/1998
Duration:	24 months
Contract number:	FAIR-CT98-5005

Design of peptide inhibitors of lipoxygenase for use as food ingredients

Objectives:
The purpose of the research was to evaluate the effects of various types of bovine casein on the activity of lipoxygenase *in vitro* and then to identify the domain within the protein responsible for the lipoxygenase inhibitory activity. Research was also needed to elucidate the properties of caseins and casein-derived peptides.

Summary of achievements:
Casein and casein-derived peptides inhibit enzymatic and non-enzymatic lipid peroxidation, most probably by being a preferred target for fatty acid free radical intermediates. The structure of several casein-derived peptides has been elucidated by means of mass spectrometry.

Indirect evidence led to the suggestion that the peptides can be oxidised during the process, according to a site- or sequence-specific mechanism. The use of proteins and peptides as antioxidants should therefore be evaluated on the basis of both lipid and protein oxidation. It can be supposed that proteins and peptides can, together with the fatty acid, be the target of oxidative degradations, and either initiate further unwanted radical-mediated side reactions, or terminate chain reactions depending on the stability of the resulting protein/peptide radical.

Keywords:
casein, lipoxygenase, peptides

Main Publications/Patents/Participation in conferences:
S.G. Rival, S. Fornaroli, C.G. Boeriu, H.J. Wichers, (Submitted) Antioxidant properties of proteins and protein hydrolyzate – 1. Caseins and casein-derived peptides inhibit lipoxygenase-catalysed oxidation of linoleate. J. Agric. Food Chemistry, 2000.

S.G. Rival, C.G. Boeriu, H.J. Wichers (Submitted) Antioxidant properties of proteins and protein hydrolyzate – 1. Caseins and casein-derived peptides inhibit lipid oxidation, probably by scavenging free radicals. J. Agric. Food Chemistry.

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Category:	30
Starting date:	18/03/1998
Duration:	12 months
Contract number:	FAIR-CT98-5006

The biochemistry of melanoidins formed in aqueous sugar-amino acid model processed food systems heated with and without lipid

Objectives:
The Maillard reaction is one of the most important and widely studied reactions in food chemistry. The Maillard reaction occurs when components containing a free carbonyl group *(e.g.* reducing sugars) can react with a component containing a free amino group.

It is responsible for the formation of the flavour and colour that develop during the thermal treatment of many foods. Many processes of the food industry are based on the development of a certain degree of browning, e.g. in brewing, in coffee or in chocolate production, etc. Understanding the parameters involved in these reactions is very important, in order to control and optimize the quality of the product. The majority of the studies about the colour development have been done using two components systems, usually an amino acid and a reducing sugar. Studies involving three component systems, i.e., sugar, amino acid and lipid, are less common, and they have been focused on flavour rather than colour generation.

Although some progress has been made in recent years, very little is known about the structure of coloured compounds formed during the Maillard reaction. In addition, no studies have been reported concerning the effect of lipid or lipid degradation products on colour formation in model Maillard systems.

The objectives of this project were:
1) To find out if the addition of a lipid or a lipid degradation product to an aqueous sugar/amino acid led to a change in colour development, and which lipid was more effective.

2) To compare the chromatographic profile of the formed melanoidins, in the presence or in the absence of a lipid.
3) To extract one or more of the coloured compounds in order to establish their chemical structures.

Summary of achievements:
Aqueous glucose/phenylalanine (0.1 M with respect to each reactant) systems were heated in an autoclave for 30 min at 140°C, in the presence of hexanal (0.04, 0.1 and 0.2 M) or $FeCl_2$ (0.01 M). Results show that hexanal significantly inhibited color development at pH 5 and 6, and led to an increase of 5-HMF. Iron addition had similar effects at pH 5, bill only small effects at pH 6. The reaction routes proposed give key roles to the α-dicarbonyl compounds, to the Strecker aldehyde and to the Schiff base formed by the reaction between hexanal and phenylalanine.

Keywords:
Maillard Reaction, amino acid, lipid, melanoidin, flavour, sugar, processed food, colour

Main Publications/Patents/Participation in conferences:
B. Fallico, J.M Ames (1999) The effect of hexanal and iron on colour development in a glucose/phenylalanine model system. J. Agric. Food Chem. (JF981191G).
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Category:	20
Starting date:	19/04/1998
Duration:	36 months
Contract number:	FAIR-CT98-5007

Improving the functional properties of durum wheat gluten by transformation

Objectives:
Low molecular weight glutenin subunits (LMW-GS) are endosperm proteins- encoded by multigene families at the Glu-3 loci.

The members of this family are highly polymorphic and the proteins they encode have been demonstrated to play a primary role in determining the viscoelastic properties of durum wheat gluten.

In wheat dough the LMW-GS participate in the formation of the extensive disulphide-linked polymers of the endosperm, in association with another class of wheat prolamins, the high molecular weight glutenin subunits (HMW-GS). At the molecular level, two characteristics of LMW polypeptides are believed to be important in influencing dough quality: the number and the position of the cysteine residues necessary for the formation of the disulphide bonds and the length and the regularity in structure of the central repetitive domain, composed of short peptide motifs repeated in tandem series.

The superior performance of the semolina of some durum wheat cultivars has also been correlated with a higher quantity of specific LMW-GS encoded, at the Glu-B3 locus.

The transformation protocols now available to transform wheat with glutenins can allow, through the overexpression of particular LMW-GS genes, better definition of the correlation between the molecular structure of LMW-GS and the functional properties of gluten.

Summary of achievements:
Three different constructs to be utilised in the transformation step have been prepared by insertion of the coding region of three different LMW-GS genes into the vector $pLRPT_{DX5T}$. This is a pUC18-based vector containing 1.3 kb of the promoter region of the gene encoding the HMW-GS 1Dx5and 650 by of the 3' untranslated region of the same gene.

The LMW-GS genes have been inserted between the promoter and the terminator, using the restriction sites *Sal*I and *Xba*I.

The plasmid pLRPTnxsTA3* contains the coding region of the gene lmWIA3, cloned by PCR from the durum wheat cv. Langdon using primers specific for the A genome.

The plasmid pLRPTux5TIB* contains the coding region of the gene lmw1B, cloned by PCR from the genomic DNA of durum wheat cv. Langdon using primers specific for the B genome.

The plasmid pLRPTnx5TIB-* contains a mutant form of the gene lmw1B. The mutation has occurred during specific genomic amplification of the gene and caused the substitution of the first cysteine of the polypeptide chain with an arginine.

An epitope-tag (c-myc) recognised by a specific monoclonal antibody (9.E10) has also been added at the 3'ends of the genes in order to facilitate the analysis of the transgenic plants.

Since the presence of the epitope tag may influence the expression or the functionality of the transgenes, three further constructs, identical to the ones described above but lacking the epitope tag, have been also prepared.

The cereal transformation procedure utilised was developed at IACR-Rothamsted based on particle bombardment of cultures derived from immature inflorescences or scutellum tissues. In this process, the DNA to be transferred is precipitated onto microscopic gold particles which are accelerated into the target tissues by high-pressure helium gas.

Co-transformation is carried out, using a plasmid containing selectable marker genes together with the plasmid containing the gene of interest. Several bombardments have been made so far using the constructs described above and immature explants of two different cultivars of durum wheat. The regeneration and selection phases of the transformation procedure are currently in progress.

Keywords:
durum wheat, low-molecular-weight, glutenin, genes, PCR; wheat, storage proteins, transformation, biolistic, epitope tag

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Category:	20
Starting date:	01/04/1998
Duration:	36 months
Contract number:	FAIR-CT98-5013

Behaviour, rheology and physical stability of food emulsions containing proteins, commercial emulsifiers and hydrocolloids

Objectives:
Oil-in-water emulsions form a major category of consumer goods. They are found in products of the food, cosmetics and pesticide industries as well as in a number of other applications such as drug delivery. During the project, attention will be directed towards the influence of emulsifier composition on the stability and rheology of fine oil-in-water emulsions prepared with a mixture of sodium caseinate and small-molecule emulsifier.

Summary of achievements:
The rheology of concentrated emulsions based on milk protein sodium caseinate and the commercial surfactant Tween 20 has so far been investigated. Oscillation measurements were used to study the effect of changing ratios of caseinate/Tween 20 to the rheology of such systems. The complex moduli were found to increase in higher ratios. Additionally, greater caseinate content was found to increase the overall viscosity of the emulsions in a wide range of stresses, without changing the viscoelastic character of the systems.

The contribution of controlled calcium chloride-induced flocculation to the rheology of these concentrated (caseinate-only) systems has also been examined. It was found that the complex moduli of those systems lower with the temperature increase in a reversible fashion. Additionally, higher levels of calcium chloride were found to change the shear-thinning character of such systems to Newtonian, the trend being independent of caseinate concentration.

Keywords:
rheology, caseinate, emulsion, hydrocolloids, protein

Main Publications/Patents/Participation in conferences:

E. Dickinson, C. Ritzoulis, M.J.W. Povey (1999) Stability of emulsions containing sodium caseinate and Tween 20. Journal of Colloids and Interface Science, 212, 466-473.

E. Dickinson and C. Ritzoulis (2000) Creaming and rheology of oil-in-water emulsions containing sodium dodecyl sulphate and sodium caseinate. Journal of Colloids and Interface Science, 224, 148-154.

13th Conference of the European Colloid and Interface Society, 12-17th September 1999, Dublin, Ireland.

Innovation in the Biosciences, Joint MCFA and Unilever workshop, 21-22nd February 2000, Bedford, UK.

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Starting date:	01/04/1998
Duration:	27 months
Contract number:	FAIR-CT98-5016

Interactions between sugar surfactants and proteins in food colloids

Objectives:
The project aims to investigate in detail the surface behaviour of sugar esters and examine their interactions with the commonly occurring milk proteins β-lactoglobulin and β-casein. In the first steps of our approach the basic surface characteristics of several esterified sugars (xylose, lactose, galactose, sucrose, etc.) were determined by the means of surface tension measurements. At a later stage, the method of the Langmuir balance was used to determine the static and dynamic properties of sugar ester monolayers at the air/water interface. Preliminary results have shown possible existence of interactions between sugar esters of different headgroup. A parallel study has been carried out for β-lactoglobulin monolayers. The data collected agree with the current idea of protein conformational change at interfaces with time. The introduction of sugar surfactants in the protein monolayer system has been seen to alter the apparent kinetics of the protein denaturation in a degree dependant on the surfactant to protein ratio. Current research is expanding our investigation to model-oil/water interfaces.

Summary of achievements:
The dynamic behaviour of monolayers of stearate esters of sucrose, lactose, glucose and galactose, using a Langmuir trough technique, has been studied in detail. Qualitatively, the esters of sucrose, lactose and glucose behaved similarly. Galactose stearate, however, exhibited behaviour equivalent to that of a solid film with low compressibility. In terms of the dilatational elastic modulus measured, sucrose, lactose and glucose stearate monolayers gave values of 50-80mNm^{-1} for surface pressures over 20mNm^{-1}, whereas galactose stearate had a dilatational modulus of >150mN m^{-1} for a similar surface pressure range. This difference in behaviour at the air-water interface was also confirmed by Brewster angle microscopy.

A number of insoluble monolayer experiments have also been undertaken, where the change in dilatational rheology of a β-lactoglobulin film in the presence of small amounts of sugar esters was monitored. It was found that all surfactants could disrupt the protein network

resulting in lower values of the dilatational modulus. The monosaccharide esters were more potent in lowering the dilatational modulus and the effect of the added surfactant was more pronounced at the higher surface pressures.

In addition, the dilatational and foaming properties of a series of pure, chemically synthesised, water soluble sucrose esters of various hydrophobicities was examined. At a concentration of 0.0125 wt. %, the dilatational modulus varied significantly with the tail chain length. The foamability was maximum for the laurate sample. The case where β-lactoglobulin is present (0.050 wt. %) was also investigated. The dilatational rheology studies suggested that, in cases of the more hydrophobic surfactants, the air-water interface was dominated by the surfactant. Additional data, however, indicates that the protein can affect the dilatational behaviour of the interface at low frequencies of deformation. By performing foaming tests, it was found that the presence of the protein can aid foam formation; however, the stability of the resulting foams is currently believed to be dependent on the composition of the interface soon after the foam formation.

Future research includes (a) the test of the foaming properties of the enzymatically synthesised sugar esters and the emulsifying performance of the enzymatically and chemically synthesised sugar surfactants, and (b) a further investigation of the morphology of sugar ester films at the air-water interface using Brewster angle microscopy.

Keywords:
monolayers, esters, sucrose, lactose, glucose, galactose, rheology, β-lactoglobulin, foaming

Main Publications/Patents/Participation in conferences:

G. Garofalakis and B.S. Murray (1999) Effect of film ageing on the surface properties of β-lactoglobulin and β-lactoglobulin + sucrose stearate monolayers. Colloids and Surfaces B: Biointerfaces, 12 231.
G. Garofalakis and B.S. Murray (1999) Interactions between β-lactoglobulin and sucrose stearate at the air-water interface. Food Science and Technology Today, 13 151.
G. Garafalakis and B.S. Murray Dynamic Behaviour of β-lactoglobulin films: The effect of sugar surfactants. *Poster presentation*, Interfaces and Colloidal Systems, Aghia Pelagia, 18-23rd September 1999, Crete, Greece.
G. Garafalakis and B.S. Murray. Dynamic properties of β-lactoglobulin films at the presence of Sucrose Monoesters. *Poster presentation*, Food Colloids 2000, Potsdam, 2-6th April 2000, Germany.

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Category:	30
Starting date:	15/06/1998
Duration:	24 months
Contract number:	FAIR-CT98-5018

Biogenic amines production by lactic acid bacteria in wines

Objectives:
The most significant amines encountered in wine are histamine, tyramine, diaminobutane (putrescine) and diaminopentane (cadaverine). In the laboratory, the study on the enzyme involved in histamine production was carried out. The enzymes tyrosine decarboxylase and ornithine decarboxylase, are also investigated.

Summary of achievements:
Tyrosine decarboxylase (TDC) activity has been shown in a strain of *Lactobacillus brevis* of the American Type Culture Collection (*L. brevis* ATCC ATCC 367). The characterisation of the enzyme and the influence of several compounds present in wine (citric acid, lactic acid and ethanol) was considered. The study was carried out on cell suspensions and cell-free, extracts. For biogenic amines analysis an HPLC was used. The enzymatic activity TDC is determined with a specific $C0_2$ electrode. A pH optimum of 5.5 has been shown for both cell suspensions and cell-free extracts. At this pH, the TDC activity exhibited a Michaelis-Menten kinetic. Tyramine, the final product of the reaction, acted as a competitive inhibitor of the enzymatic activity. When the substrate specificity of the TDC from *L. brevis* 367 was tested with different potential amino acids precursors of the others biogenic amines, none of these compounds have been decarboxyled in these conditions. Citric acid and lactic acid had an inhibitory effect on cell suspensions and cell-free extract TDC activities. The results obtained with ethanol showed a higher inhibition on cell suspension than on cell-free extracts.

Another objective of this work is the isolation of suitable lactic acid bacteria strains producing biogenic amines in wines. For this purpose, samples of wines throughout the vinification process were taken for HPLC analysis. A strain of *Lactobacillus brevis* (*Lb. Brevis* IOEB 9809) able to produce tyramine was isolated from a Bordeaux wine. The TDC enzymatic

study of this strain will be carried out. The purification of the enzyme, and the cloning and sequencing of the TDC gene will be considered.

Keywords:
biogenic amines, wine, tyrosine decarboxylase, ornithine decarboxylase, lactic acid, bacteria

Main Publications/Patents/Participation in conferences:
V. Morreno-Arribas, S. Torlois, A. Joyeux A. Lonvaud-Funel Tyramine production by wine lactic acid bacteria. Study of the tyrosine decarboxylase activity of *Lactobacillus brevis* ATCC 367 and *Lactobacillus brevis* IOEB 9809 isolated from wine. *Actualités Enologiques 1999, VIième Symposium International d'Oenologie Bordeaux, 10-12 June 1999.*
S. Torlois, V. Morreno-Arribas, A. Bertrand, A. Lonvaud-Funel Production d'amines biogènes dans les vins par les bactéries lactiques. Etude biochimique et physiologique de la production de la putrescine. *Actualités Enologiques 1999, VIième Symposium International d'Oenologie Bordeaux, 10-12 June 1999.*

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Category:	30
Contract signed:	19/03/1998
Duration:	24 months
Contract number:	FAIR-CT98-5019

Development of a biomarker for exposure to dietary glucosinolates

Objectives:
This project aims to carry out *in vitro* studies on the absorption and transport of glucosinolates in human epithelial cells. Further work will concentrate on the production of the appropriate nitrile as the second possible degradation product of glucoraphanin and the effects on cells. It will be important to identify possible metabolites and the effect of the food matrix on uptake and metabolism.

Summary of achievements:
A framework for carrying out the main experiments for the "Development of a Biomarker for Exposure to Dietary Glucosinolates" was developed. It was:

- Biosynthesis of labelled intact glucoraphanin by feeding the appropriate labelled precursor to living plants.
- Development of a method for the extraction and purification of glucosinolates from the plant material using flash chromatography
- Method for the analysis of intact and desulphoglucosinolates
- Chemical synthesis of sulphoraphane which is one of the main, and in terms of health benefits, most interesting, breakdown products.
- Handling of human cell cultures as an *in vitro* method for investigation of absorption and transport of drugs on a cellular level
- Literature search for evaluation of different *in vitro* systems to assist in choosing an appropriate method

Keywords:
biomarker, exposure, diet, glucosinolate, glucoraphanin, absorption, transport, human epithelial cells

Main Publications/Patents/Participation in conferences:
4th Plenary Meeting of the Biomarkers Concerted Action (4-6/02/99)
Biomarkers. Diet and Health 99 (20-22/06/99, Jena, Germany)
Food and Cancer Prevention III (5-8/09/99, Norwich, UK)
4th Karlsruhe Nutrition Symposium (10-12/10/99, Karlsruhe, Germany)

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Category:	BRT
Starting date:	01/06/1998
Duration:	12 months
Contract number:	FAIR-CT98-5020

Study on the enzymatic systems involved in the activated oxygen metabolism in plants subjected to salt stress

Objectives:
Salinity is a major factor in reducing plant growth and productivity in the world. There is increased evidence concerning the involvement of active oxygen species (AOS) in salt-mediated damage in plants. The differences in plant protection mechanisms determine their tolerance to stress conditions associated with AOS toxicity. The steady-state level of AOS in the cell is determined by the activity of the antioxidant system. Augmentation of the antioxidant defences plays a pivotal role in preventing oxidative stress in plants. The levels of low molecular mass antioxidants such as glutathione and ascorbate together with the activities of antioxidant enzymes are generally increased in plants under stressful conditions and correlate with enhanced tolerance.

H_2O_2 and glutathione make important contributions to the redox state of plant cells and are implicated in the activation of genes that lead to acclimation, stress tolerance and other defence responses. Since H_2O_2 is an endogenous oxidant which accumulates in many stress situations, a central role for this metabolite as a diffusible signal for selective inductions of defense genes has been envisaged. Furthermore, H_2O_2 causes an increase in cytosolic calcium, indicating a role for calcium in the signal transduction processes.

There are no reports on the effect of NO on plant peroxidases, which are haeme containing enzymes and, since - a NO-synthase-like activity has been putatively localised in the vascular bundles of the root of *Lupinus albus,* the effect of some NO-releasing compounds, and of NO itself, on xylem peroxidase from *Zinnia elegans* was studied. In addition, the effect of these compounds on H_2O_2 production by the lignifying xylem was also studied.

Summary of achievements:
Nitric oxide (NO), which is a synchronising chemical messenger in animals and an air pollutant, has been studied in order to ascertain whether NO has any effect on coniferyl

alcohol peroxidase and on H_2O_2 production by the lignifying xylem of *Zinnia elegans*. The results obtained showed that NO-releasing compounds such as sodium nitroprusside (SNP) and S-nitroso-N-acetyl-penicillamine (SNAP) provoke an inhibition in the mM concentration range of coniferyl alcohol peroxidase activity of a basic peroxidase isoenzyme present in the intercellular washing fluid of *Z. elegans*. However, SNP at 5 mM, does not have any effect on H_2O_2 production by the xylem of *Z. elegans*. These results suggested a differential effect on NO in lignification, in which this compound inhibits peroxidase without affecting the enzymatic system which delivers the H_2O_2 used by the xylem peroxidases for the synthesis of lignins.

Compound III (COM) of peroxidase decay into ferriperoxidase most likely involves the dissociation of a ferric-$O_2^{\cdot-}$ complex to yield the ferric form of the enzyme and $O_2^{\cdot-}$. Diphenylene iodonium chloride (DPI), at 50 µM, enhances the stability of CoIII of peroxidase, reducing the k_{decay} from $8.6 \pm 3.9\ 10^{-3}\ s^{-1}$ to $4.6 \pm 2.0\ 10^{-3}\ s^{-1}$. This observation could explain the inhibitory effect of DPI on the $O_2^{\cdot-}$ generating activity of this haeme protein.

Keywords:
oxygen, haeme, metabolism, salt, stress, antioxidant, lignify, xylem

Main Publications/Patents/Participation in conferences:
M.A. Ferrer, A. Ros, Barceló (In press) Differential effects of nitric oxide on peroxidase and H_2O_2 of *Zinnia elegans*. Plant, Cell and Environment (1999) 22.
A. Ros Barceló, M.A. Ferrer Efecto del óxido nítrico sobre la actividad peroxidasa y la producción del H_2O_2 por el xilema de *Z. elegans*. V Reunión del Grupo Español de Radicales Libres. Granada 15-16th December 1998.

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Category:	30
Starting date:	20/03/1998
Duration:	24 months
Contract number:	FAIR-CT98-5022

Optimisation of the release of ferulic acid from sugar-beet pulp: studies on the enzymic activities and associations involved

Objectives:
The main objectives of this project are:

- To further elucidate the enzymic activities and associations required for quantitative de-feruloylation of sugar-beet pulp;
- Building on this basic knowledge, to improve the efficiency of the enzymic saccharification of this co-product, a critical step shown previously to be the economical bottleneck in large-scale production of *natural* pectin constituents (including ferulic acid) with high potential added value.

Summary of achievements:
Hydroxycinnamates (HCA) such as caffeic, ferulic and coumaric acid are commonly found as ester conjugates in dietary plant materials. Free HCA have antioxidant properties, and the ferulic acid content of wheat bran is essential for its proposed anticarcinogenic properties.

This research focused on the isolation of micro-organisms accounting for the cinnamoyl esterase (CE) activity previously detected in the human large intestine, where ingested ester-linked HCA arrive essentially unaltered.

The CE activity of human faecal samples proved to be essentially cell-associated and was enhanced when chlorogenic acid (ChlA = caffeoyl-quinic acid) was used as specific substrate in liquid cultures. Thirty-five isolates were recovered after overnight incubation of the gut microflora in a ChlA-based medium (11 mM). Ethyl ferulate-hydrolysing species were identified as *E. coli*, *Bifidobacterium lactis* and *Lactobacillus gasseri*. Esterase activity on ChlA was also found in the latter bacteria and was essentially intracellular. *L. gasseri* produced at least two types of cell-associated cinnamoýl esterases after growth in a glucose-

based medium. Both were completely inhibited by the serine-specific protease inhibitor aminoethylbenzenesulphonylfluoride (1mM). The purified chlorogenic acid esterase was mainly active on methyl caffeate, methyl *p*-coumarate and ChlA (0.1 mM) with lower but appreciable levels of activity on feruloylated oligosaccharides from wheat bran (FAXX) and sugar-beet pulp (Ara_2F).

The results show that a variety of bacteria, including some already recognised as potentially health-promoting, are involved in the release of bioactive hydroxycinnamates in the human colon. The present work on gut microbial cinnamoyl esterases will allow further studies to be completed, with possible exploitation in bioconversion processes such as the production of ferulic acid from agricultural residues (cereal brans, sugar-beet pulp) for high added value applications, including a flavour precursor.

Keywords:
ferulic acid, sugar-beet, pulp, enzymic activities, enzymic associations, hydroxycinnamates, cinnamoyl esterase, bacteria

Main Publications/Patents/Participation in conferences:
D. Couteau, A.L. McCartney, C.B. Faulds, G. Williamson Isolation of human colonic bacteria with cinnamoyl esterase activity. Oral presentation at the International Conference on Diet and Prevention of Cancer, 28th May to 2nd June 1999, Tampere, Finland.

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Category:	RT
Starting date:	15/12/1998
Duration:	12 months
Contract number:	FAIR-CT98-5027

Purification and characterisation of two enzymes involved in the production of colour and flavour in eggplant: lipoxygenase and polyphenol oxidase

Objectives:
Oxidative and reductive processes play a key role in foods. They influence not only its taste, but also the colour, texture and nutritional value of food products. Two oxidases, lipoxygenase (LOX) and polyphenol oxidase (PPO) play an important role in some of these processes.

Eggplant is a crop of economical importance for Mediterranean countries. Adverse physical and biochemical changes in the fruit tissue can lower its acceptability and nutritional value. The knowledge of the characteristics of both enzymes (LOX and PPO) in eggplant is still very limited. The main objective of this project is the purification and characterisation of eggplant lipoxygenase and polyphenol oxidase

Summary of achievements:
The results obtained in this project cannot be applied immediately to the industry, but can be used as a starting point for future research.

The project has established a method to purify to homogeneity and to characterise a novel chloroplastic-LOX, an enzyme probably involved in fruit ripening. This provides the starting point for further research on this enzyme. The method to determine the regiospecificity of lipoxygenase products facilitates the analysis of a large number of samples that can be processed in a short period of time.

The purification and characterisation of PPO from eggplant fruit and, specifically, the results obtained with inhibitors is useful to control enzymatic browning in vegetables.

Keywords:
lipoxygenase, polyphenoloxidase, purification, characterisation, eggplant, vegetable, enzyme

Main Publications/Patents/Participation in conferences:
J.M. López Nicolás, M. Pérez Gilabert, F. García Carmona (1999) Rapid reversed-phase high-performance liquid chromatographic determination of the regiospecificity of lipoxygenase products on linoleic acid. Journal of Chromatography, 859, 107-111.
J.M. López Nicolás, M. Pérez Gilabert, F. García Carmona Eggplant lipoxygenase (Solanum melongena L, cv Belleza negra): Effect of physicochemical properties of linoleic acid in the enzymatic activity and product characterisation European Journal of Biochemistry.
M. Pérez Gilabert, F. García Carmona (In press) Characterisation of catecholase and cresolase activities of eggplant polyphenol oxidase. Journal of Agricultural and Food Chemistry (2000).
M. Pérez Gilabert, J.M. López Nicolás, F. García Carmona Purification of a novel lipoxygenase from eggplant (*Solanum melongena* L, cv Belleza negra) fruit chloroplasts. Physiologia Plantarum.

Links with EC projects:
FAIR-CT96-5010 (p.180)

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Category:	30
Starting date:	18/03/1998
Duration:	24 months
Contract number:	FAIR-CT98-5029

Biological activity of peptides derived from milk proteins

Objectives:
Milk is not only a source of nutrients, but also a carrier of various forms of specific factors influencing bacterial growth, which may have significant influences on suckling neonates. In fact, the intestinal flora of the newborn mainly depends on the source of the ingested milk. Milk-derived immunoglobulins, for example, have long been known to play a significant role in protecting infants from intestinal infections. Other milk proteins which are supposed to be responsible for this bacterial growth modulation are lysozyme, lactoperoxidase and lactoferrin.

More recent studies have revealed that milk contains a number of bioactive peptides which may play a role in regulating postnatal gut development in suckling neonates. Antibacterial peptides have been derived from the minor whey protein lactoferrin. The finding of an antibacterial sequence near the N-terminus of lactoferrin, in a region distinct from its iron-binding sites, has provided a new antibacterial mechanism for lactoferrin, more complex than reflecting a simple binding of iron.

Later on, a heat- and mild acid-stable antibacterial peptide has been isolated after acid treatment of bovine milk. Further characterisation revealed that it was a 39 amino acid-containing fragment derived from α_{s2}-casein. Recently, three different fragments with bactericidal properties have been isolated from various digests of α-lactalbumin.

One potential limitation for the useful application of antimicrobial peptides is the cost of production. Peptides derived from longer proteins can be produced *in vitro* by enzymatic hydrolysis of the precursor protein, but the procedure is expensive and labour-intensive since it comprises, first, the purification of the precursor protein and, in a second step, the isolation of the active peptide from a generally complex hydrolysate. An alternative strategy is the

production of cationic antibacterial peptides as fusion proteins in bacteria. By this procedure, the cationic peptide is produced in a longer, inactive form (normally with an extra anionic segment) to stabilise the cationic peptide and to prevent both antibiotic activity against the host bacterium and proteolysis during recombinant protein production. Therefore, it is important to develop cost-effective production methods for cationic peptides starting from polypeptides or proteins which may be present in complex mixtures.

The objective of this project was to prepare and purify cationic peptide fractions of some milk proteins and test these fractions for antimicrobial activity. Fractions of interest were further purified and the structure of these bioactive peptides was determined in order to study the structure-function relationship.

Summary of achievements:
Two distinct domains with antibacterial activity were isolated from a peptic hydrolysate of bovine α_{s2}-casein. The digested α_{s2}-casein was fractionated by cation-exchange chromatography, after which the peptides in the two active fractions obtained were separated by high performance liquid chromatography and sequenced by electrospray-ionisation tandem mass spectrometry.

A new method for the isolation of a fraction enriched in cationic peptides with antibacterial activity from biological fluids, such as milk and whey, were developed.

Keywords:
biological activity, peptides, milk, proteins, whey, chromatography

Main Publications/Patents/Participation in conferences:
I. Recio, S. Visser (1999) Identification of two distinct antibacterial domains within the sequence of bovine α_{s2}-casein Biochimica et Biophysica Acta 1428: 314-326.
S. Visser and I. Recio. Process for producing cationic peptides from biological fluids European Patent Application No. 98203107.2-2110.
I. Recio, C.J. Slangen, S. Visser. Isolation and mass spectrometric analysis of antibacterial domains present within the sequence of bovine α_{s2}-casein. 1st International Symposium on Enzymatic Protein Processing. December 2-4, 1998, Noordwijkerhout, The Netherlands. Poster.
I. Recio, C.J. Slangen, S. Visser. Production of antibacterial peptides from biological fluids by membrane ion-exchange chromatography. IDF-FIL Seminar on New Applications of Membrane Technology in the Dairy Industry. June 7-10 1999, Saint Malo, France. Oral communication.

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Category:	20
Starting date:	15/01/1999
Duration:	36 months
Contract number:	FAIR-CT98-5030

Functional properties of sunflower proteins for food applications

Objectives:
The goal of the project is to determine the intrinsic properties of Sunflower Proteins (SFP).

Summary of achievements:
During isolation care has to be taken to avoid damaging (unfolding) the proteins. The gentleness of the isolation procedure was tested by using protein solubility as the main reference for protein denaturation. Differential Scanning Calorimetry and Native electrophoresis have also been used for this purpose.

In the first year of the project SFP isolation was aimed at. The first step in isolation is defatting. Several methods were tested for defatting the milled, dehulled sunflower seeds. Hexane was the solvent selected for defatting the meal; the defatted meal has a protein content of 50 %.

Then the removal of CGA was aimed at. Therefore a HPLC method was set up-to determine CGA content. UV-Spectrophotometry was also used. The defatted meal contains about 2.5 % CGA and less than 0.1 % CA. Other non-identified polyphenols absorbing at 324 nm are also present. CGA removal has been performed by washing the defatted meal with solvents. Adsorption of CGA to several solid substances such as PVP, Dowex, Charcoal, etc was also performed. Methanol 80% seems to be the best for CGA removal and denaturation of proteins have not been observed so far. After methanol extraction, the protein content increases to 60% due to removal of soluble methanol compounds such as carbohydrates and phenolic compounds.

At present, ultrafiltration is being optimised for obtaining the final sunflower protein isolate.

Keywords:
Functional properties, sunflower, proteins, food applications, electrophoresis, calorimetry

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Category:	20
Starting date:	01/10/1998
Duration:	30 months
Contract number:	FAIR-CT98-5034

Evaluation of microbial safety of Italian dairy and meat products with regard to enterococci

Objectives:
This is one of the first approaches to investigate the microbial safety of Italian dairy and meat products. Several cheese and meat samples were chosen from geographically well spread locations, in order to record the distribution of vanomycin resistant Enterococci. This was to be achieved through both classical methods for both the identification and establishment of the *van*A gene and modern techniques such as species-specific PCR, RAPD-PCR and a specific PCR for the detection of the *van*A gene.

Summary of achievements:
In the first year of the project, 109 strains of Enterococci have been used in several tests and trials. A division can be made as follows: 60 originated from cheese, 35 were isolated from meat products, 7 from other foods (sandwich, pizza dough etc) and 7 came from fish. Furthermore, the type strains of *Enterococcus casseliflavus*, *E. durans*, *E. faecalis*, *E. faecium*, *E. gallinarum*, *E. hirae*, *Clostridium tyrobutyricumum*, *Listeria innocua* and *Propioinibacterium freudeureichii* subsp. *shermanii* and a reference strain of *Listeria innocua* were available for this project.

All strains were Gram positive catalase negative cocci. No motility was observed. All strains grew in the presence of 2, 4 and 6.5% of NaCl and at pH9.6. None produced CO_2, from glucose. All grew at 45°C and at 10°C. All strains grew on KAA and KF agar and showed typical enterococcal growth. All strains could hydrolyze Arginine. When tested for resistance against vancomycin and teicoplanin, 13 strains (all from meat samples) showed a high resistance to both antibiotics (i.e. > 64 $\mu g\ ml^{-1}$) and were suspected to be harbouring the vanA gene. This was confirmed by a specific PCR, which revealed the presence of this gene. Using the disc diffusion method the non-meat strains were also tested for resistance against the following antibiotics: Erythromycin, Trimethoprim, Ampicillin, Piperacillin, Penicillin,

Norfloxacin and Chloramphenicol. Only two strains were not resistant against trimethoprim. three strains were resistant against Norfloxacin and three strains were resistant against Erythromycin.

All but the strains isolated from meat were identified using the RAPID ID 32 STREP test and by the laboratory for microbiology in Gent by means of SDS-PAGE. In case of a doubtful identification by the RAPID ID 32 STREP test, a species specific PCR was performed. The original protocol was described by Dutka-Malen, but modifications were made in order to obtain reliable results with the equipment and reagents present. In 9 cases there was a discrepancy between the two methods, since the phenotypic test problems recognizing the less common species of Enterococcus, i.e. *E. gallinarum*, *E. casseflavus* and *E. hirae*.

The results of the SDS-PAGE confirmed the results obtained with the species-specific PCR, no discrepancies were found. The strains isolated from meat were identified using the species-specific PCR 20 were identified as E. *faecium*, 15 as *E. faecalis*. Only the non-meat strains were tested in and on milk and no strain showed a strong acidifying activity in milk. After six hours the lowest pH was 5.54, after 16 hours, the lowest pH was 4.48. 16 strains had coagulated milk after a six hours incubation at 37°C. After 16 hours, 22 strains coagulated milk. When grown on plates containing milk, 24 showed to have proteolytic activities. Of the 16 strains which coagulated milk after 6 hours, 15 had a proteolytic activity. Most coagulating strains did so at a fairly high pH (around 6), suggesting that coagulation is not only connected to a low pH, but also to an enzymatic activity. This might need further investigation. Of the non-meat strains, the inhibitory activity against: *Clostridium tyrobutyricum*, *Listeria innocua* and *Propioinibacterium freudeurreichii* subsp. *shermanii* was tested, as well as the haemolytic activity on human blood. Several strains were found to inhibit growth of one or more target strains. Only one strain was found to be α-haemolytic.

A start was made with the genetic identification of the strains. After several trials in which 7 different primers (1253, CAND, COC 1, D8635, Hpy 1, M14 and Opal 1) and amplification programs were tested two primers were found to give reproducible and reliable profiles which contained enough information to identify the strains used for the testing: D8653 and 1253. DNA was extracted from all strains and for apart of the strains, a genetic profile has already been obtained with both primers, but work is still in progress and results are not available at this point.

Keywords:
microbial safety, Italy, dairy, meat, enterococci, PCR

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Category:	20
Starting date:	01/11/1998
Duration:	30 months
Contract number:	FAIR-CT98-5035

Characterisation of the stress response of coryneform bacteria on red smear cheese using high-resolution two-dimensional gel electrophoresis (Proteom Analysis)

Objectives:
A major risk in red smear cheese production is the development of a faulty smear surface. Fast development of a closed smear surface inhibits growth of spoilage and pathogen micro-organisms and, therefore, contributes to safer products. Moreover, rapidly growing surface cultures exhibit enhanced tolerance towards retarded acidification and deviation in the salting process.

Unfortunately, red smear bacterial cultures often show a delayed growth rate, when they are transferred from liquid culture to the surface of the young cheese. Due to the changing environmental conditions, red smear bacteria are subjected to strong abiotic stress factors, such as pH, salt concentration and low ripening temperature. An optimal stress response of these organisms is decisive for a rapid and faultless development of the smear. That red smear bacteria are able to survive these harsh conditions on the cheese surface, implicates the existence of special mechanisms that resist the injury process caused by stress. But the molecular basis of stress response of coryneform red smear bacteria is yet unknown.

The aim of this study is therefore to achieve a better understanding of the behaviour of coryneform bacteria in the complex food system of red smear cheeses by investigating their stress resistance mechanisms. Understanding the stress response on a molecular basis should make it possible to manipulate red smear cultures in such a way that they are more tolerant against specific stress factors. This knowledge allows the development of more stress tolerant and, therefore, more appropriate red smear cultures in future.

Summary of achievements:
The effect of stress factors on microbial growth was studied. Growth rates on cheese-agar medium and in liquid culture were determined under defined stress conditions. The

characterisation of the intracellular stress response was done by Two-Dimensional-Gel Electrophoresis with immobilised pH gradients. Specific proteins and the corresponding metabolic pathways were identified.

The study required the development of a solid surface to grow red smear cheese bacteria in a way which is as similar as possible to the surface of red smear cheese. This so-called cheese agar medium was produced and tested. Results show that it is possible to grow red smear bacteria on the medium until they show a smeary surface. However, it was not possible to maintain a constant pH value over at least five days on the surface of the medium, without influencing the whole cheese environment. Therefore investigations to grow the bacteria on filters on the cheese medium surface were done. This technique enables rapid transfer of the bacteria from one habituate to another (e. g. changing pH conditions), makes it possible to get enough cell material for protein analysis and prohibits the input of cheese proteins in the protein analysis. Comparisons between bacterial counts on cheese agar medium grown with and without filters showed no difference.

As the induced stresses should reflect the conditions bacteria face on the cheese surface, one step in this project is to determine the conditions that provoke stress in bacteria. Therefore bacteria are cultured in liquid medium as well as on cheese-agar medium with different salt concentrations, temperatures, pH values and atmospheres. Growth rates are determined under these stress conditions.

Stress response of coryneform bacteria is characterised by proteom analysis. After the analysis of the constitutive proteome, changes of the proteome expression, introduced for instance by different stress factors can be studied in different development phases. Only with two-dimensional (2-D) gel electrophoresis it is possible to obtain a snapshot of the entire protein spectrum of a cell in a certain defined stadium.

Keywords:
Stress, coryneform bacteria, red smear cheese, gel electrophoresis, proteom analysis

Main Publications/Patents/Participation in conferences:
Pathogenic *Yersinia* species carry a novel, cold-inducible major cold shock protein in tandem gene duplication producing both bicistronic and monocistronic mRNA. October 1999. Journal of Bacteriology

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Category:	20
Starting date:	22/07/1998
Duration:	36 months
Contract number:	FAIR-CT98-5036

Simultaneous introduction of bacterial genes mediating polyester biosynthesis by particle bombardment of potato and pea

Objectives:
Due to their biodegradability, bacterial polyesters known as poly(3-hydroxyalkanoates) (PHAs) are of interest as valuable alternatives to fossil oil-derived synthetic polymers. The characteristics of the final product depend on the nature (chain-length) of the hydroxy-fatty acids and their individual ratio when fed to the fermentation medium.

Although PHAs have already been produced in a number of Pseudomonad bacteria and also in transgenic *Arabidopsis thaliana* and *Brassica napus*, the application of starch-storing crop plants is considered more suitable for large-scale accumulation of PHAs. In these crops the metabolic flux of carbon in the plastid will have to be re-directed from starch to *de novo* fatty acid biosynthesis. Transgenic potato lines have been produced that express the *P. oleovorans* PHA polymerase (*phaC*) gene in microtubers.

For precursor feeding studies, *in vitro* cell cultures have been prepared that still express the *phaC* gene. In order to provide a pool of suitable precursors, additional steps of transgenesis will be required. Three approaches are being investigated: retransformation under secondary bialaphos selection, co-infection with *Agrobacterium tumefaciens* and particle co-bombardment. The latter proved successful for soybean, more recently for sugarcane, and resulted in our case with separate constructs in GUS-positive, kanamycin-resistant potato shoots. Applications that have been developed from bacterially produced PHAs can be found in very different areas and cover packaging, hygienic, agricultural and biomedical products. Recent application developments based on medium chain length PHAs range from high solid alkyd-like paints to pressure-sensitive adhesives, biodegradable cheese coatings and biodegradable rubbers

Summary of achievements:
The fellowship was initiated with the conventional *Agrobacterium tumefaciens*-mediated transformation of axenically grown stem segments of potato diploid and amylose-free genotype *1024-2 (amf)*. The binary vector system was used with the NPTII kanamycin-resistance gene (driven by the nopaline synthase [NOS] promoter), the 0-glucuronidase (GUS) reporter gene (driven by the Cauliflower Mosaic Virus 35S [CaMV35S] promoter) or the *Pseudomonas oleovorans* PHA polymerase *(phaC2)* gene (driven by the granule bound starch synthase [GBSS] promoter). Transformants were generated with the expected high efficiency and expressing either type of transgene introduced. In parallel performed particle bombardment-mediated transformation experiments of potato leaf material has resulted in blue-stained spots after introduction of the GUS gene and subsequent X-glue treatment, indicating that particle bombardment is a suitable technique for (transient) transformation of potato leaf material. Simultaneous transfer of pUC-based vectors either containing the GUS cassette, the NPTH cassette or a bialaphos resistance *(bar)* gene cassette are in progress in order to assess the applicability of this system to generate stable kanamycin- or bialalaphos-resistant plants and to perform co-transformations.

Keywords:
Potato, pea, particle bombardment, co-transformation, reporter genes, poly-(3-hydroxyalkanoates), PHA, biosynthesis, polymerase genes, precursor genes

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Category:	20
Starting date:	07/10/1998
Duration:	24 months
Contract number:	FAIR-CT98-5037

Controlled release systems containing antimicrobial peptides for application in food and food products

Objectives:
Problems accompanying the use of chemical antimicrobial additives in the preservation of food and food products include possible negative effects on human health and cause for environmental contamination.

This project aims at overcoming these drawbacks by the development of a controlled release (CR) system, based on low priced biodegradable bulk proteins or phospholipids, containing antimicrobial peptides. These peptides are studied within the framework of a recently granted project on the application of antimicrobial peptides in food and food products. CR-systems can provide protection of the enclosed peptides against destabilizing factors such as oxygen, light, and enzymatic degradation. In addition, the enclosed peptides can be released into food products in a controlled manner, i.e. slow or triggered release. The main advantage of controlled release of peptides in food is the initial lower dose of peptides which can be added to the food products. Both the fundamental aspects involved in peptide encapsulation (encapsulation efficiency, biological activity of the peptide etc.) and the performance of the developed CR-systems during processing in food products are the subject of research.

In conclusion, enclosure of antimicrobial peptides in CR-systems based on cheap components will substantially contribute to their feasibility for application in food and food products.

Summary of achievements:
In the first year, a Controlled Release (CR)-system was developed both with and without a model antimicrobial peptide. For the initial studies of the CR-system, protein based microspheres were prepared. Whey protein-based microspheres were prepared by an emulsion stabilisation method. By this method, first a water/oil emulsion is prepared using the whey protein as emulsifying agent. Then, the droplets in the emulsion are stabilised by heat treatment, enzymatic cross-linking (transglutaminase (TGA)) or chemical cross-linking

(tannic acid). The obtained microspheres are then washed with acetone to remove the oil and air-dried.

Different cross-linking methods were compared. From initial experiments it appeared that the microspheres linked chemically (tannic acid) were not stable in water. All micropshere samples showed an initial swelling behaviour followed by a decrease in particle size. The decrease in particle size was not accompanied by an increase in the free protein concentration, indicating that the particles were not redissolving. Thermally cross-linking produced microspheres that were small, quite closely linked and had a porous surface (from morphology, (SEM and light microscopy)). In comparison, the samples linked with TGA revealed microspheres that were small compacted together and had no pores.

Nisin, a FDA-approved antimicrobial peptide was studied as a model peptide for microencapsulation. Encapsulation of nisin was done both during (dissolving in the protein solution) and after (via swelling) microsphere preparation. Encapsulation of nisin in whey protein-based microspheres during preparation caused a rougher surface of the micropsheres. In initial antimicrobial bioassays, no growth inhibition was observed (after 24 hrs). These results indicated either that nisin was not encapsulated or inactivated, or that insufficient time was allowed for the release of nisin. In changing the format of the bioassay, a longer time period of 14 days was used. With this bioassay, the inhibition of bacteria growth due to release of nisin from the microspheres reached to a maximum at -6-8 days before slowly decreasing in effect. Further optimisation and characterisation of this peptide-containing CR-system will be investigated.

Keywords:
Controlled release systems, antimicrobial, peptides, food, food products, nisin, microsphere

Main Publications/Patents/Participation in conferences:
S. Spillane, M.H. Vingerhoeds, A. van der Bent, A. van Amerongen (1999) Controlled release of antimicrobial peptides for food application. Proceedings of 26th International Symposium on Controlled Release of Bioactive Materials, 20-23 June 1999, Boston, USA.

Links with EC projects:
FAIR-CT97-3135

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Category:	30
Starting date:	22/07/1998
Duration:	24 months
Contract number:	FAIR-CT98-5039

Potential transfer of maize-derived transgenes to microorganisms in the avian gastrointestinal tract.

Objectives:
Genetically modified (GM) plants are increasingly being used in animal feeds. This has raised serious concerns as to whether GM plants represent a health hazard. The primary potential risk accompanying the use of GM plants in animal feeds is the transfer of plant-derived transgenes to gastrointestinal (GI) microorganisms. Such gene transfer events could represent a serious risk to animal and human health through the generation of persistent antibiotic resistant pathogenic microorganisms.

The overall objective of this project is to develop methodology designed to evaluate whether there is a significant risk that transgenes within GM plant can be transferred to microorganisms in the avian GI tract following two specific objectives:

1) Establish whether transgenes can be excised from GM plants as vector containing circular DNA molecules, that are potential targets for transformation events, or are only present as linear DNA fragments.

2) Investigate whether GM plant DNA can survive in the avian GI tract and whether the survival of the DNA is influenced by the age of the birds and specific components in the diet.

Summary of achievements:
The first year of the project has focussed on investigating the rate at which soya and maize DNA is degraded *in vitro* using intestinal simulations. These simulations consisted of an initial acid treatment at pH 2.5 for 3 hours in the presence of 1 mg/ml pepsin. The mixture was then neutralised with sodium carbonate, fresh small intestinal contents were then added and the incubation was continued for up to 16 hours. At regular time intervals an aliquot was removed. DNA was extracted using the Promega Wizard kit, and the DNA was subjected to competitive PCR. The primers employed in the PCR, amplified the BT gene in Soya and a

herbicide resistance gene in Maize. The competing DNA consisted of plasmid DNA containing the appropriate transgene in which a 100 by sequence of DNA has been inserted so that amplified competitive DNA can be distinguished from the plant derived transgenes.

The experimental protocol outlined above was performed on both whole maize and soya transgenic material and naked DNA containing the transgenes. The data from these experiments can be summarised as follows. In the first series of experiments the sensitivity of the competitive PCR experiments was evaluated. The data showed that using maize or soya that comprises 5% transgenic material, could detect the transgenes at a level of 0.01% of the starting material i.e.0.0005% of a sample that is 100% transgenic. Having satisfied the sensitivity of the system, the stability of the DNA in gastro-intestinal simulations was then looked at. Although one might predict that the acid conditions in the stomach simulations would cause extensive depurination of the DNA, the data showed that the nucleic acid remained intact. Thus, there was plenty of high molecular weight DNA present after these acid conditions, and competitive PCR indicated that the amount of transgenic DNA did not significantly decrease. Incubation of the naked DNA with deal contents caused rapid degradation of the DNA, and after 30 min there was no transgene DNA detected i.e. > 99.9% of the DNA had been destroyed. In contrast the transgene DNA in the whole maize and soya material was considerably more stable, and approximately 10% of the transgenes were still detectable after three hours of incubation with ileal contents. These data show that there is a continuous release of transgenic DNA as material passes down the intestines, and the DNA is definitely not degraded as soon as it reaches the small intestines.

Keywords:
Maize, transgenes, microorganisms, avian, gastrointestinal tract

Main Publications/Patents/Participation in conferences:
3rd Carbohydrate Bioengineering Conference, Newcastle, April 1999

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Category:	RT
Starting date:	01/01/1999
Duration:	12 months
Contract number:	FAIR-CT98-5044

Effect of different environmental conditions *in vitro* on ethylene metabolism: its application for a quality control in plant micropropagation

Objectives:
The loss of plants during the last stages of conventional micropropagation and its associated raised costs often complicate commercial large-scale application of this technology for plant production. The high relative humidity as well as the accumulation of ethylene in the culture container cause some of these inconveniences.

Using shoot cultures of carnation and tobacco as the plant material, several modifications of the standard *in vitro* conditions have been tested to:

- prevent physiological alterations *in vitro*;
- normalise the *in vitro* environment;
- study their impact on the physiology of ethylene metabolism.

Summary of achievements:
Normalisation of the environmental conditions *in vitro* by the application of bottom cooling coupled to a double layer system, has improved the quality of micropropagated plants in relation to ethylene metabolism and hyperhydricity by the promotion of positive physiological changes, in particular, a delay in the senescence of the micropropagated plants. The reduction of the level of humidity in the aerial part of the cultures brought about a significant decrease in the level of ethylene in the culture atmosphere and a clear reduction in the appearance of hyperhydricity. Under controlled normalised environmental conditions, micropropagated plantlets improved their quality, biomass production, vegetable development and survival to *ex vitro* acclimatisation.

The results derived from this research can be applied to the improvement and quality control of current micropropagation protocols. A normalised ecophysiology *in vitro* can provide a better environment for the micropropagated plants with a positive impact on the final productivity of commercial plant production by micropropagation. In addition, an easy-to-measure parameter such as ethylene concentration in the atmosphere of cultures can be used to assess the physiological status of the cultures and to modify the time course of the culture conditions in a more efficient way.

Keywords:
micropropagation, ethylene, hyperhydricity, environment, physiological

Main Publications/Patents/Participation in conferences:
XIX Reunión Bienal de la Sociedad Española de Microscopía Electrónica, 28-30th April 1999, Murcia, Spain.
XII Reunión de la Sociedad Española de Fisiologia Vegetal, 19-22nd September 1999, Sevilla, Spain.

Links with EC projects:
FAIR-CT96-5052 (p.106)

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Category:	30
Starting date:	29/01/1998
Duration:	24 months
Contract number:	FAIR-CT98-5045

Water exchange in gluten over mesoscopic length scales during kneading of a wheat dough

Objectives:
In breadmaking, the behaviour of dough (flour and water) depends strongly on the wheat storage proteins (gluten). However, the amount of added water is critical to form an "optimum developed dough". Although there are many studies on the identification of the proteins involved in the formation of gluten network, little is known about the hydration of gluten and the exchange of water in dough during kneading. The understanding of water movement during kneading was improved. The osmotic pressure in dough depending on the stress applied to the dough, the role of the unfolding of gluten proteins on water exchange by physical methods and the influence of gluten quality on the water absorption was studied.

Summary of achievements:
To date, the results have been the following:

- gluten extensions in water and gluten extensions coupled with NMR have been developed to observe water exchange. No observation of water exchange in gluten under deformation was observed probably due to the insensitivity of the first method and also due to the rapidity of the water exchange compared to time needed to perform NMR experiments
- using infrared analysis, gluten showed different conformations during kneading with D_2O. This implies that after hydration and a first change in conformation, the gluten is stretched by the kneading deformations and showed a second change in structure by an increase of the extended β-sheet.
- effect of temperatures below zero on the gluten structure. Preliminary rheology results (uniaxial and planar extensions) showed that the frozen gluten stored for long time required more stress to be deform at a certain strain than the fresh gluten. The gluten seems to concentrate in time at low temperatures. This concentration speed increases when the temperature is higher than the glass transition temperature of the gluten. Thus,

in this model system (water and gluten), the ice crystals, formed at low temperatures, does not seem to damage the gluten film in time by their growing.

Keywords:
Water exchange, gluten, kneading, wheat, dough

Main Publications/Patents/Participation in conferences:
American Association of Cereal Chemistry. Annual Meeting. Seattle, USA. 31 October – 3 November 1999.
Symposium on Food Rheology and Structure. Zurich, Switzerland. 12 – 16 March 2000.
Gluten 2000 workshop. Bristol, United Kingdom. 2-6 April 2000.

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Category:	20
Contract signed:	07/09/1998
Duration:	24 months
Contract number:	FAIR-CT98-5047

The identification of chemical markers in order to predict and evaluate meat quality at the early post mortem period

Objectives:
A large variation exists in the rate and extent of tenderisation which is responsible for variation in meat tenderness at the consumer level. As consumers consider tenderness to be the single most important component of meat quality, solving the problem of inconsistent meat tenderness is of high priority to the industry.

Unpredictable variability in meat tenderness is a major problem for both the meat industry and the consumer. These difficulties would be reduced if carcasses showing inferior quality could be identified at the early post mortem period, enabling them to be handled and marketed separately from high quality carcasses. The ability to deal with a great number of samples in a short time and at a very early stage of production are two useful features that would go towards determining meeting this aim.

This project therefore aims to isolate and identify proteins and proteolytic fragments from pork muscle tissue and to evaluate their potential as predictors of meat quality.

Summary of achievements:
Soluble fragments of myofibrillar proteins produced during the ageing process can be isolated and identified using high performance liquid chromatography (HPLC). The enhanced appearance of such proteolytic fragments with ageing suggests that they may be useful indicators of meat quality. However, further studies showed this not to be the case. A calibration curve created using the synthesised peptide APPPPAEVPEVHEEVH did not appear to be useful in estimating the low concentrations of this fraction in the pork soluble extracts.

Due to the reduced solubility of the freeze dried extracts and the extra time penalties imposed by inclusion of freeze drying, it was found to be unnecessary. Consistent comparative results can be obtained by the analysis of frozen extracts alone.

Keywords:
Chemical marker, quality, freeze-drying, meat, post-mortem, pork

Main Publications/Patents/Participation in conferences:
S. Stoeva, C.E. Bryne, A.M. Mullen, D.T. Troy, W. Voelter (In press) Isolation and identification of proteolytic fragments form soluble extracts of bovine *M. longissimus dorsi.* Food Chemistry. 1999.

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Category:	30
Contract signed:	15/07/1998
Duration:	12 months
Contract number:	FAIR-CT98-5048

Digestibility of raw and extruded legume proteins and biological effects in the small intestine of the rat

Objectives:
In recent years, extrusion cooking has been used increasingly in the production of foods and food ingredients. As a hydrothermal-mechanical process, extrusion is able to improve the nutritive value and textural properties of seeds, to allow their use as fodder for calves, pigs and poultry or as instant porridges and weaning foods, vegetable purees, moist baby foods, texturised vegetable proteins and snacks for humans.

Previous studies have shown that extrusion treatment greatly improved the nutrient quality and availability of kidney beans for animals. However, these improvements were not evident or statistically significant when either peas or field beans were extrusion-treated. The main objective of this project is to study the reasons for the lower-than-expected nutritional value of extruded proteins.

Summary of achievements:
Faba bean globulins, which comprise the bulk of the protein, were moderately digestible in *in vitro* and *in vivo* assays. Extrusion treatment of the beans improved overall nutrient availability from faba beans. However, it also adversely affected the digestibility of the globulin proteins.

The major globulin proteins were purified [11S (legumin) and 7S (vicilin)]. The 7S proteins were much less digestible that the 11S globulins both *in vitro* and *in vivo*.

Rats appeared to be able to adapt to the inclusion of 7S protein in their diet. Thus, after 10 days, the digestibility of 7S proteins was increased and was similar to that of the 11S proteins.

Studies are continuing on the effects of thermal treatment on the properties of the 7S and 11S faba bean globulins and on the nature of the adaptation that occurs in rats fed dietary 7S proteins.

Keywords:
extrusion, protein, globulin, digestibility, faba bean, nutrient

Main Publications/Patents/Participation in conferences:
R. Alonso, G. Grant, A. Pusztai (In press) Digestibility of faba bean proteins *in vitro* and *in vivo*. Proceedings of the 11th Workshop COST 98 Concerted Action: Effect of antinutrients on the nutritional value of legume diets, 2000, Budapest, Hungary. Luxembourg: Office for Official Publications of the European Communities.
G. Grant, R. Alonso, J.E. Edwards, S. Murray (In press) Dietary soyabean and kidney bean stimulate secretion of CCK and pancreatic digestive enzymes in 400 day-old rats but only soyabean induces growth of the pancreas. Pancreas, 2000.

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Category:	30
Starting date:	01/09/1998
Duration:	24 months
Contract number:	FAIR-CT98-5050

Valorising agro-food products: from quality control of natural products to biotechnological synthesis of flavours

Objectives:
For about ten years the "Unite de Brasserie et des Industries Alimentaires" has focused on specific volatile compounds derived from original nectar sources, in all likelihood responsible for the characteristic aroma of monofloral honeys. These volatile compounds have proved adequate for authenticating floral origin.

A few odourant markers of eucalyptus, chestnut, lime tree, lavender, and heather honeys have proved reliable as references for establishing floral origin. In this framework, this research project aims to:

- confirm this new authentication procedure based on the aroma composition of honeys. First the influence of geographic origin on heather honeys from different European countries will be investigated. In the case of lavender samples, whether the species origin can be distinguished by means of this methodology shall be examined. Eventually, we how weather conditions during the harvest year affect eucalyptus, chestnut, and lime tree honeys will be determined;
- synthesise a few unexplored potent odourants by biotechnology, after identification of their precursors. The aroma profile of honeys, nectars, and essential oils to identify suspected precursors will therefore be examined.

Summary of achievements:
It is now possible to authenticate fir, rape, orange blossom, rosemary, sunflower, lavender, eucalyptus, chestnut and lime tree honeys on the basis of a few odour markers. To determine the limits of the methodology, further comparisons with heather honeys from the same family (Ericaceae), but from different species (*Erica arborea* and *Calluna vulgaris*) were made.

The floral markers concentrations of heather honeys may be influenced by the geographic origin, when the climatic conditions are very different. Lower contents in some tracers seem to be characteristic of heather honeys from southern Europe. However, discrimination between family or species remains possible in all cases.

Authentication of monofloral lavender honeys
According to the results obtained with heather honeys, the research was extended to lavender samples (*Labiatae* family). Aroma profiles of *Lavandula angustifolia* and *Lavandula anguslifolia x latifolia* honeys were compared. In both cases, larger amounts of hexanal, heptanal, n-hexanol, phenylacetaldehyde and coumarin were detected compared to other unifloral species. From a qualitative point of view, no discrimination was possible between both species. Only quantitative differences in phenylacetaldehyde and heptanoic acid were evidenced by a discriminant analysis. Other analyses must still be achieved with *Lavandula sloechas* honeys.

Aroma profile comparison of monofloral honeys harvested during successive years
In the case of chestnut honeys, the concentrations of the five markers do not significantly vary from years to years. Fifteen floral origin markers were evidenced in the case of lime tree honeys, but only the 8-*p*-menthen-1,2-diol is significantly affected by the weather conditions. Finally, among the seven floral markers of *Eucalyptus* honeys, only dimethyldisulfide varied from years to years.

Weather conditions have a negligible impact on most of floral origin markers of lime tree, chestnut and *Eucalyptus* honeys. Therefore, such compounds are proved reliable indicators of the floral origin.

Keywords:
Heather, lavender, honey, quality, aroma, flavour, weather

Main Publications/Patents/Participation in conferences:
C. Guyot, A. Bouseta, V. Scheirman, S. Collin (1998) Floral origin markers of chestnut and lime tree honeys. Journal of Agricultural and Food Chemistry, 46, 625-633.
C. Guyot, V. Seheirman, S. Collin (1999) Floral origin markers of heather honeys: *Calluna culgaris* and *Erica arborea.* Food Chemistry, 64, 3-11.
L. Gijs, C. Guyot, S. Collin (In press) Lipophilicity and sensorial properties of aroma libraries: application to sulfur and carotenoid-derived compounds. COST Action 96: Interactions of food matrix with small ligands. Oslo, Sweden, May 1999

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Starting date:	04/08/1998
Duration:	6 months
Contract number:	FAIR-CT98-5052

Study of synthetic media for the development of a semiautomatic TLC video scanning system for *Fusarium*

Objectives:
Due to the importance of mycotoxins production by different species of *Fusarium*, further studies must be taken in this area. This genus has a high frequency of presentation in different foods and feeds, mainly in cereals and by-products, which are still the base of human nutrition. Mycotoxins have high toxicity effects and most of them are carcinogens or potential carcinogens. An important number of human intoxications have been reported to be related to the consumption of contaminated cereals with different species of *Fusarium*. Also important economical loses have been reported in animal production in relation to contaminated cereals used for their feeding.

The correct identification of *Fusarium* species is very important, as mycotoxin production varies from one species to another. The main objective of the project focuses on the utilisation of the mycotoxigenic pattern for identification of *Fusarium* species. More concretely in the optimising of a Semiautomatic Thin Layer Chromatography (TLC) Video Scanning System for the genus *Fusarium*.

This system has been developed for the detection of metabolites and species identification for the genus *Penicillium* in the Department of Biotechnology (Technical University of Denmark, Lyngby).

The first step to perfecting the Semiautomatic TLC video scanning system was to find an appropriate synthetic media for the detection of mycotoxins. Two complex media were used frequently to compare the results of the *Fusarium* studies: PSA (Potato Sucrose Agar) and YES (Yeast Extract Agar). Four synthetic media were also tested: Borrows media, MOSS media, MOSS modified media (Doubled Carbon and Nitrogen sources) and Raulin-Thorn modified media (pH modified).

The colony characteristics (diameter and colour of the colony) were studied at the same time. Further HPLC analysis was performed on some of the species to complete the conclusions obtained.

Summary of achievements:
The optimisation of a Semiautomatic TLC video scanning system requires different parameters to be tested. The TLC technique needs to use developing solvents. In the study nine different combinations of solvents were tested. In conclusion TEF (Toluene : Ethyl acetate : 90% Formic acid; 5:4:1) and CAP (Chloroform : Acetone isoPropanol; 85:15:20) were the ones which gave a better pattern of mycotoxins. Interestingly, a new combination of TEF 5:3:2 gave a clearer pattern with thinner spots and metabolites not seen in the known TEF. A system of Sprays was used to visualise the mycotoxins in the TLC technique. The Sprays used more frequently were AIC13 and Anysaldehyde. In the study four different Sprays were also tested, but without much success, although they provided new information.

After establishing the system conditions a computer database was created comprising 30 Standard mycotoxins of *Fusarium*. Mycotoxigenic TLC patterns from different strains of 11 *Fusarium* species were also saved onto the database. It is now possible to introduce a new sample and the system will compare the metabolites with the information on the database. It is also possible to identify a sample is between a range of species. As there are difficulties with *Fusarium* identification, the Semiautomatic TLC video scanning system will be of great use.

In order to generalise all the conditions around the System, a synthetic media was required for the incubation of the strains studied. Four different synthetic media were tested in comparison with two complex media (PSA and YES). The synthetic media used were Borrows media: MOSS and Doubled MOSS media and Raulin-Thom media. In Borrows media a poor mycotoxigenic pattern was obtained. The Doubled MOSS and Raulin-thom gave better information regarding the variation of mycotoxins production and the differentiation of *Fusarium* species. Further studies were carried out on these two media and in comparison with PSA and YES using the analytical technique HPLC (High Performance Liquid Chromatography). In general, the patterns obtained in the media Raulin-Thom were most similar to the ones in PSA and the patterns obtained in Doubled MOSS were most similar to the ones obtained in YES. Even though interesting results were obtained in these two media, more studies are required in this area, due to the difficulty of finding one synthetic media, which will enable us to identify all the *Fusarium* species. The same standard mycotoxins analysed in TLC were also analysed in HPLC and a manual database of spectra was obtained. In the different *Fusarium* strains analysed an important number of unidentified metabolites were detected and also a manual database of its spectra was created.

Keywords:
mycotoxins, *Fusarium*, carcinogens, mycotoxigenic, Semiautomatic Thin Layer Chromatography, TLC, Video Scanning System

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Starting date:	02/11/1998
Duration:	36 months
Contract number:	FAIR-CT98-5054

Compartmentalisation in plant cells: The role of the chromoplast in lipid oxidation: A tomato model

Objectives:
In plant cells, particularly in fruits, chromoplasts are key organelles impacting on the appearance and flavour of the final product. This is due to the presence of oxidoreductase enzymes, particularly lipoxygenase/hydroperoxide lyase. Disruption of this organelle during cellular damage (first stage of fruit processing) initiates a rapid chain of reactions due to the local concentration of substrates (phospholipids and pigments).

A study of isolated chromoplasts, providing a simplified system, should clarify the role of this key organelle in ripe fruit in the absence and presence of induced stress. It could also provide routes to control the degradation of lipid and terpenoids in the direction of volatile flavour formation.

In order to start the study of the compartmentalisation of the enzymes involved in the metabolism of the major volatile compounds within the tomato cell, protoplasts were first chosen as a simplified system from which to derive knowledge of the whole fruit.

The work of research was divided into two parts. The first one, consisted of setting up a method to isolate protoplasts and the second one concerned a means to determine a way to initiate the wound response from isolated protoplasts, in order to monitor the release of volatile derived from the lipid oxidation, mainly hexanal and hexenals.

Summary of achievements:
A technique to isolate protoplasts from the pericarp of red ripe tomatoes was set up using a two step procedure. First, the cell walls were removed by the action of enzymes. Then, protoplasts were purified from the digestion medium.

The choice of the mixture of cellulase and pectinase was based on the work of other researchers. Depol40L, a commercial enzyme mixture, was also tested as it contained a mixture of cellulases, hemicellulases and pectinases. After the breakdown of the cell walls, different methods of purification were tested. The technique that gave the best yield of protoplasts was the one using three centrifugation and resuspension steps. An additional filtration through cotton wool was also found beneficial for the increase of the final yield.

The yield of protoplasts was measured using a modified haemocytometer (0.2 mm depth), and the viability of the protoplasts was determined using a fluorescing technique with fluorescein diacetate.

Results showed the best viability (87%) when using a mixture of 1% cellulase RS, 1% cellulase R10 and 2% pectinase.

Then, to initiate a wound response from the protoplasts, plasma membranes were disrupted using sonication technique. The energy of sound waves transmitted through the suspension caused disruption of the protoplasts. Volatiles released were monitored in the headspace above sonicated samples using Atmospheric Pressure Chemical Ionisation-Mass Spectrometry (APCI-MS) technique. Observations were focused on the following ions: m/z 101 and 99 (M+1 ions of the major lipid oxidation derived volatile, hexanal and hexenals; M= molecular weight), and their corresponding fragments, respectively m/z 83 and 81.

First results show a slight increase in the response of the ions of interest after disruption of the protoplasts. Conditions of experiment and sensitivity of the APCI-MS still need to be optimised to monitor the changes from a small amount of protoplasts. Nevertheless, those promising results showed that the use of protoplasts to mimic the behaviour of a whole tomato fruit is possible.

Keywords:
Compartmentalisation, plant, cells, chromoplast, lipid, oxidation, tomato, model

Main Publications/Patents/Participation in conferences:
"Science of Quality: Improving vegetables and fruit" at Horticulture Research International. Wellsbourne, UK. 16th March 1999.
Universities Flavour Consortium Postgraduate Symposium (presentation of a poster) at the Department of Food Science and Technology – the University of Reading, UK. 21st-23rd July 1999.

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Category:	30
Starting date:	01/09/1998
Duration:	24 months
Contract number:	FAIR-CT98-5059

Role of cholecystokinin and leptin in the anorexia induced by simmondsin extracted from the jojoba plant

Objectives:
The jojoba plant (*Simmondsia chinensis*) is a shrub cultivated in arid and semi arid regions for its oil containing seeds. Jojoba oil is used as a heat-resistant lubricant and in cosmetics. The by-product of the oil industry, jojoba meal, can not be marketed as animal feed because supplementation with jojoba meal results in reduced food intake and emaciation in several animal species. Experiments with jojoba meal were performed in rats, mice, sheep, cattle, dogs, rabbits and chickens with identical results, namely reduced growth or food intake reduction, although ruminants seemed to be less sensitive to jojoba meal than non ruminants. The major food intake-reduction agents in jojoba meal are simmondsin (a cyanomethylene glucoside) and its derivatives. Simmondsin produces a satiating activity in rats and has similar effects as cholecystokinin (CCK). The aim of the research project is to study the effect of simmondsin in rat and to elucidate the working mechanism of the food intake reducing activity of simmondsin.

Summary of achievements:
The physiological effects of simmondsin and cholecystokinin (CCK) are compared in order to confirm the hypothesis that simmondsin produces a satiating activity by a stimulation of the endogenous cholecystokinin system. 1) In a first experiment, the effect of simmondsin on food intake was compared to the effect of CCK in obese and lean Zucker rats (with or without vagotomy). As during other experiments with simmondsin, an anaemic reaction was observed in Wistar rats. A second experiment was conducted in order to study the nature of this anemia and the effect of purified simmondsin on the structure and function of the internal organs in rats.

Vagatomy has no significant influence on short term food intake reduction caused by Simmondsin, this in fatty as well as in lean Zucker rats. food intake reduction caused by CCK is influenced by vagotomy.

In the short term, lean Zucker rats are a little bit more sensitive to the food intake reducing effect of simmondsin, but in the long term, fatty Zucker rats are more sensitive to simmondsin. From the experiment results the hypothesis that the long term influence of simmondsin on food intake in vagotomised and Sham operated Zucker fatty, simmondsin acts through the activation of CCK receptors situated on the vagal nerve could not be confirmed.

Keywords:
Jojoba, simmondsin, Zucker rats, cholecystokinin, vagotomy, HPLC, high performance liquid chromatography

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Category:	20
Contract signed:	22/07/1998
Duration:	36 months
Contract number:	FAIR-CT98-5062

Biochemical basis of mushroom texture

Objectives:
The mushroom is a fragile product as it can lose its texture very rapidly, for example during storage. It is possible to modify the texture of the mushroom (i.e. increasing firmness) by altering the growing conditions. This project aims to look at the level of the cell wall (CW), the basis of mushroom texture, and how the texture can be improved by the growth conditions.

Summary of achievements:
The analysis of the cell wall content was done on mushrooms from the three flushes of a crop and on mushrooms having different maturity stages. In total there were six sample of mushroom CW.

On each sample the percentage of Cell Wall (CW) was determined from the fresh weight, the amount of alkali-soluble and alkali-insoluble fractions, the glycan content, chitin content, protein content and glucuronic acid content. The proportion of monosaccharides was determined from the alkali-soluble glycan and alkali-insoluble glycan.

The percentage of CW recovered from the fresh mushroom tissue is on average 1.8%. There was not much variation between the different flushes and the maturity of the mushrooms. On average fresh mushrooms contain about 10% of dry material, thus about 18% of the dry material represents the cell walls.

The mushroom cell walls were fractionated in alkali solution. The alkali-insoluble fraction obtained was about 75-80% of the CW. Only one sample, from the second flush and mushrooms from maturity stage 5, had less insoluble fraction (about 67%).

The alkali-soluble fraction recovered, after precipitation with ethanol, was 10-15%. No significant difference was detected between the samples.

On average, 90% of material after alkali fractionation was recovered. About 10% of the cell wall content were lost in the alkali supernatant – this fraction is likely to have contained proteins and small molecules.

In the insoluble fraction, the amount of glycans and chitin was determined by hydrolysis with H_2SO_4 or 6N HCl respectively. On average the insoluble fraction contained 28-37% glycan and 2-30% chitin. No significant difference was found between samples. The soluble fraction was found to contain about 5-7.5% glycans from hydrolysis with H_2SO_4. The ratios of monosaccharides present in the soluble and insoluble-glycans were also determined.

Keywords:
mushroom, cell wall, texture, alkali-soluble, alkali-insoluble, fractions, glycan, chitin, protein, glucuronic, monosaccharides

Main Publications/Patents/Participation in conferences:
Poster: "The content and human bioavailability of folates in food using HPLC-methods", Third International Food Data Conference. Rome FAO Headquarters, 5-7 July 1999.
Representative for Prof. M. Jägerstad, The Swedish University of Agricultural Sciences, Uppsala, Sweden: Cost Action 99, Eurofoods Meeting, Rome FAO Headquarters, 4&8 July 1999.
"Folate content and bioavailability in food using HPLC-methods and a human model", A talk at the 7th Symposium Vitamins and Additives in the Nutrition of Man and Animal, Jena (Thüringen), Germany, 22-23 September 1999.

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Category:	30
Starting date:	01/09/1998
Duration:	6 months
Contract number:	FAIR-CT98-5068

$^{18}O/^{16}O$-ratio determination in organic compounds from fruit juices and wines: investigation of isotopic correlations

Objectives:
Research work, integrated within the framework of an ongoing project (SMT4-CT98-2219), has been concerned with the development, harmonisation and application of online CF-P-IRMS and cGC-C-IRMS methods coupled to pyrolysis (P) or combustion (C) for the determination of $^{18}O/^{16}O$ and $^{13}C/^{12}C$ isotope ratios in organic compounds from fruit juice (glucose, fructose, sucrose) and wine (ethanol, higher alcohols, glycerol), respectively.

Summary of achievements:
Methodologies for fruit sugar isolation (ion exchange chromatography, HPLC), and for extraction of ethanol, higher alcohols and glycerol from wine were developed and improved. Subsequently, research tasks were undertaken concerning the optimization of CF-P-IRMS and cGC-C-IRMS methods for the measurement of $^{18}O/^{16}O$ and $^{13}C/^{12}C$ isotope ratios in organic compounds, and for the development of routine applications for authenticity control. The $\delta^{18}O$ values of fruit juice sucrose determined by CF-P-IRMS were found to be well correlated to the 700 value of the related fruit water. This may provide an improved criterium for authentication of fruit juices and concentrates (where determination of the $\delta^{18}O$ value of original water is not possible) in relation to regional provenance and sugar addition. However, glucose and fructose were shown to be less confident probes because of the broader variability ranges of $\delta^{18}O$ values potentially due to $\delta^{18}O$ shifts through oxygen exchange at the anomeric group and/or oxidation. On the other hand, a novel 7 cGC-C(P)-IRMS method coupled to on-line combustion (C) or pyrolysis (P) has been developed for the one-step measurement of $^{13}C/^{12}C$ or $^{18}O/^{16}O$ isotope ratios (respectively) on various wine components. This has been first demonstrated for the cGC-C-IRMS determination of the $\delta^{13}C$ values of ethanol, higher alcohols and glycerol, and relevant isotopic correlations have been identified.

Feasibility of extension towards analogous *c*GC-P-IRMS determination of the corresponding $^{18}O/^{16}O$ values and correlations has been initially demonstrated, further development being needed.

Keywords:
organic compounds, fruit juice, wine, isotope, ^{18}O, ^{16}O

Main Publications/Patents/Participation in conferences:
*c*GC-C-IRMS monitoring of $\delta^{13}C$ profiles of higher alcohols and glycerol in wine. EU Thematic network Food Analysis Using Isotropic Techniques (FIT). Final Meeting, Norwich, UK, 2-4 December 1998.

Links with EC projects:
SMT4-CT98-2219
FAIR-CT96-5035 (p.290)

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Category:	30
Starting date:	18/01/1999
Duration:	24 months
Contract number:	FAIR-CT98-5069

Towards a new understanding of the texture of fresh and processed asparagus

Objectives:
Asparagus Cell Wall Material (CWM) has been investigated for changes in phenolic chemistry and carbohydrate composition during post-harvest storage of asparagus spears.

Summary of achievements:
The main changes of CWM composition took place in the last portion of the spear and were:

The main sugars in the cell wall material of asparagus were glucose, galactose, arabinose, xylose and uronic acids. Glucose and xylose increased and galactose decreased during storage.

Phenolics compounds, including monomers and dimmers have been detected in asparagus CWM. Coumaric acid was the most abundant phenolic component in the CWM, comprising 42% of the total phenolic complement. Fifty five percent of the CA was in the *p*-coumaric acid, 45% was in the cis-coumaric acid. Ferulic acid (FA) comprised 17% of the total. The bulk of the remaining phenolic components consisted of FA dehydrodimers. These dimers comprise a very high percent (60%) of the total fetulic complement. The presence of such dimers indicates that FA is likely to act as inter-polymeric cross-links. During storage of asparagus, all of phenolic compounds increase, mainly ferulic acid and the proportion of dimers. The comparison of different tissue types from the bottom section of the spears showed that these changes are more important in the external tissue than in the internal one.

Lignin has been also characterised and quantified from the residue of the cell wall material after sequential extraction of phenolic compounds. These experiments have allowed to confirm that asparagus lignin consist mainly in coniferyl (70%) and sinapyl (30%) units, and the majority of these are present as monoacetylated and diacetylated monolignols. The monoacetylated compounds comprised 67% of the total coniferyl and 77% of the sinapyl complement. Lignin from dicot plants, such as beet root, has also been characterised and coniferyl and sinapyl units showed to be the most prominent components. But, in this case the

percent of sinapyl derivatives was higher (43%) than in asparagus samples; and both of coniferyl and sinapyl acetates are mainly diacetylated monolignols (81% of the total coniferyl and 51% of the sinapyl complement).

- In parallel, mechanical properties of asparagus spears have been studied. Three points on the spear were tested using three different methods of cutting, compression and puncture; and fresh asparagus were compared before and after cooking the spears. After statistical analysis of all data, the results showed that puncture method was the best test to establish differences between fresh and cooked samples and also among top, middle and bottom section of each spear.

To study mechanical property changes of asparagus during shelf-life, fresh and cooked samples were assessed for mechanical properties, using two different methods: 1. Puncture test, for measurements of texture from whole spear. A Texture Analyser model TA-XT2, fitted with a pin, was used to measure the Strength (N/mm^2) required to "cut" through uncooked and cooked asparagus spears. 2. Tensile test, for measurements of texture from two different tissue types. The separation of stem tissues was performed from middle and bottom sections. Two different tissue types were obtained from each section: *white tissue,* from central region of the stem and *green tissue,* from the outside of the stems.

Results from puncture test allowed to confirm that strength decrease during cooking of asparagus and increase during post-harvest storage. This tendency was found in each section of the spear, but the changes were higher in the last portion of the spear (bottom). Tensile test showed that the decrease of texture during cooking takes place in the external tissues from the middle and bottom sections, while the strength of the internal tissues increases slightly. During storage, the value of strength increased only in the bottom section, mainly in the outside of the spear.

Concluding it maybe suggested that the external tissues of green asparagus seem to be more implicated than the internal tissues in changes of mechanical properties during storage. The *complete characterisation of* cell wall material, including extraction of polysaccharides, and phenolic and lignin studies will allow to establish relationship between those changes of mechanical properties and cell wall composition modifications.

Keywords:
asparagus, spear, Cell Wall Material, phenolic , carbohydrate composition, post-harvest storage

Main Publications/Patents/Participation in conferences:
R. Rodriguez, A.J. Parr, G. Wende, A.C. Smith, K.W. Waldron (to be submitted to Phytochemical Analysis) Analysis of lignin composition by HPLC.
R. Rodriguez, A.C. Smith, K.W. Waldron (to be submitted to J. Texture Studies) Mechanical properties of green asparagus tissues.
R. Rodriguez, A.C. Smith, K.W. (to be submitted to Physiologia Plantarum) Effect of storage on wall-bound phenolics in green asparagus.

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Starting date:	27/09/1999
Duration:	36 months
Contract number:	FAIR-CT98-5072

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The function of lipase in baking

Objectives:
Lipases can be defined as carboxyl-esterases, which catalyse the hydrolysis of long chain acylglycerols resulting in glycerol and free fatty acids. They are well known enzymes, and have shown very interesting effects in bakery applications.

This project can be divided into three parts:
- The effect of lipase and the extraction of lipids on the rheological properties of dough and gluten,
- The influence of lipase in the dough system studied with the scanning electron microscopy
- Study of Gluten Fractions and Dough using a Diode Array Spectrometer

Summary of achievements:
The nonpolar solvent diethyl ether extracted substantially fewer total lipids than the more polar solvents (chloroform, ethanol). The lipase released some of the lipids, which were not possible to extract. The action of lipase increased and released a large amount of free fatty acids (FFA) on all the lipids that can be extracted with these three solvents. According to the lipids extracted, the polarity of chloroform affects the extraction. In the case of diethyl ether lower amounts of lipids were extracted.

Thin layer chromatography (TLC) indicated that lipids extracted with the nonpolar solvents diethyl ether, contained less of the polar lipids digalactosyl diglycerides and phosphatidyl choline than the lipids extracted with chloroform, ethanol. TLC shows that it is possible to extract about the same lipids from flour and dough to which lipase had been added. It seems like lipase releases some of the bound lipids in dough, which otherwise would not be able to extract. The amount of free extractable lipids in dough with/without lipase is significantly lower than that in flour.

The microstructure of the dough is characterised by large gas cells, thin gas cell walls (containing small pores), and an overall gluten-protein matrix that is relatively fine and even. It has been considered that dough has a foam structure in which individual gas cells are completely separated by a continuous starch-gluten matrix. Discontinuities were apparent in the starch-gluten matrix surrounding the gas cells in the dough with and without the addition of lipase (in both levels). The "holes" observed in the gas cell walls may appear to be due to the rupture of the dough phase during sample preparation. During the course of dough expansion, portions of the gas cell, surface contain only the thin, liquid lamella or film, unprotected by the solid dough phase. These, areas would be less stable and would rupture more easily during sample separation. Wheat lipids, which are found to be closely associated with the liquid phase of the dough, could assist in the foamability of dough by forming a lipid monolayer at the gas/liquid interface.

In the third part of the project it was found that DA 7000 can be used to distinguish between normal and defatted flour and dough and also between dough fractions. It is not possible to conclude if the DA 7000 spectrometer may distinguish between gluten fractions depending on the amount of water added to the dough or the mixing time. Samples measured through the optic glass are spectrally different than the samples measured directly through a test tube.

Keywords:
Lipase, baking, dough, Thin layer chromatography, scanning electron microscopy, gluten

Main Publications/Patents/Participation in conferences:
The effect of extraction of lipids and lipase on the rheological properties of dough and gluten. Poster presentation at the European meeting in Lipase and Lipid interface.

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